TECHNISCHE UNIVERSITÄT MÜNCHEN

FAKULTÄT
WISSENSCHAFTSZENTRUM WEIHENSTEPHAN
FÜR ERNÄHRUNG, LANDNUTZUNG UND UMWELT

Lehrstuhl für Technische Mikrobiologie

EFFECT OF HIGH HYDROSTATIC PRESSURE ON *CLOSTRIDIUM BOTULINUM* TYPE E ENDOSPORES

Christian A. Lenz

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender der Dissertation: Univ.-Prof. Dr. H.-C. Langowski

Prüfer der Dissertation:
1. Univ.-Prof. Dr. R. F. Vogel
2. Univ.-Prof. Dr. W. Liebl
3. Univ.-Prof. Dr. W. Schwab

Die Dissertation wurde am 28.09.2016 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 11.01.2017 angenommen.

ZUSAMMENFASSUNG

Hochdruck-Temperatur-(HDT)-Verfahren zur Sterilisation von Lebensmitteln bringen, im Vergleich zu konventionellen thermischen Verfahren, einige Vorteile mit sich, besonders den Erhalt sensorischer Produktqualität und wertgebender Inhaltsstoffe. Solche Verfahren werden allerdings noch nicht im industriellen Maßstab eingesetzt. Einer der Hauptgründe hierfür ist der lückenhafte Wissensstand bezüglich der druckvermittelten Inaktivierung von bakteriellen Endosporen einschließlich Sporen von lebensmittelsicherheitsrelevanten Organsimen wie z.B. *Clostridium* (*C.*) *botulinum*. Das Ziel dieser Arbeit war es, auf der Basis von Untersuchungen zur HDT-vermittelte Inaktivierung von *C. botulinum* Typ E Sporen, zu der Schließung dieser Wissenslücken beizutragen.

Um die Effizienz von HDT-Verfahren zur Inaktivierung von *C. botulinum* Typ E Sporen, und damit deren Beitrag zur Lebensmittelsicherheit, möglichst genau beurteilen zu können, wurden zunächst mögliche Einflussfaktoren auf das Inaktivierungsergebnis identifiziert. Zu den identifizierten Faktoren mit signifikantem Einfluss zählen vor allem intrinsische und extrinsische Faktoren während der Sporulation, genauer, die Zusammensetzung des Sporulationsmediums und die Sporulationstemperatur. Da die Nährstoffverfügbarkeit, sowie die Temperatur im natürlichen Habitat von *C. botulinum* Typ E sehr wahrscheinlich erheblichen Schwankungen ausgesetzt sind, sollten diese Einflussfaktoren bei der Evaluierung von HDT-Verfahren zur Inaktivierung *C. botulinum* Typ E Sporen bedacht werden. Während einige andere, mögliche Faktoren vernachlässigt werden konnten, hatten subletale Hitzebehandlungen während, bzw. zum Zwecke der Sporenaufreinigung einen deutlichen Einfluss auf die HDT-Resistenz von *C. botulinum* Typ E Sporen, und sollten somit vermieden werden. Unter Berücksichtigung der bestimmten Einflussfaktoren, wurden detaillierte Kinetiken zur Sporeninaktivierung, zur Entstehung hitze- oder lysozymempfindlicher Fraktionen und zum Austritt von Dipicolinsäure aus dem Sporenkern bestimmt. Hierbei konnten nur relativ kleine stammspezifische Unterschied festgestellt werden. Die so erhaltenen Daten liefern Anhaltspunkte dafür, dass es neben großen Unterschieden auch gewisse Gemeinsamkeiten zwischen Inaktivierungsmechanismen bei *C. botulinum* Typ E und *Bacillus subtilis* Sporen gibt, obwohl sich beide Organismen gravierend unterscheiden. Generell werden für eine schnelle Inaktivierung von *C. botulinum* Typ E Sporen > 500 MPa bei > 60 – 70 °C benötigt. Bemerkenswerter Weise können Drücke ≤ 300 MPa bei Raumtemperatur die durchschnittliche Zeit reduzieren, welche eine Spore braucht um anzuwachsen, was das Risiko der Toxinbildung während eine bestimmten Lagerzeit (z.B. bis zur Mindesthaltbarkeit) erhöhen kann. Abschließend wurden Gründe für die beschriebenen Einflussfaktoren untersucht. Die Ergebnisse legen nahe, dass die vorhandene Konzentration und Art von Kationen im Sporulationsmedium die HDT-Resistenz von *C. botulinum* Typ E Sporen stark beeinflusst und, dass kationen- und temperaturvermittelte Effekte in Verbindung miteinander stehen. Außerdem scheint die Sporenhülle eine Rolle für die HDT-Resistenz von *C. botulinum* Typ E Sporen zu spielen, wobei zwei Proteine identifiziert werden konnten, die wichtig für diese Rolle sein könnten.

Die erhaltenen Daten können eine wertvolle Grundlage für künftige Inaktivierungsstudien, die Interpretation von Ergebnissen für andere *Clostridium* Sporen und künftige Überlegungen zur Lebensmittelsicherheit darstellen, sowie eine industrielle Implementierung von HDT-Prozessen erleichtern.

ABSTRACT

High pressure thermal (HPT) sterilization processes have several advantages in comparison with conventional (thermal) retorting, particularly, less detrimental effects on organoleptic and nutritional food quality. However, in contrast to high pressure processing at or below room temperature, HPT processes are not yet applied on the industrial level. One major reason for this can be found in the lack of knowledge on basic mechanisms for the inactivation of pathogenic spore-forming organisms such as *Clostridium* (*C.*) *botulinum*. The aim of this study was to contribute to closing the knowledge gap regarding the HPT-mediated inactivation of spores from *C. botulinum* type E, which primarily presents a threat for the safety of refrigerated processed food of extended durability (REPFED) from aquatic environments.

To be able to reliably assess the efficiency of HPT treatments to inactivate *C. botulinum* type E spores, the first part of this study comprised the identification of factors influencing inactivation results. Especially intrinsic and extrinsic factors during sporulation, i.e., the sporulation medium composition and the sporulation temperature had significant effects on the inherent HPT resistance of *C. botulinum* type E spores. Since nutrient availability and temperature are factors that likely fluctuate in the natural habitat of *C. botulinum* type E, these results should be considered in the evaluation of food safety. While other possible influence factors related to the experimental setup had negligible effects, a sublethal heat treatment applied during spore suspension purification prior to an HPT treatment significantly lowered the HPT resistance of *C. botulinum* type E spores and, thus, should be avoided during inactivation studies. Once critical influence factors were identified, detailed kinetics for the inactivation of viable spores, the development of heat- and lysozyme-susceptible spore fractions, and dipicolinic acid (DPA) release were determined. Strain-dependent differences in the HPT resistance of *C. botulinum* type E spores were relatively small. Furthermore, results indicate that some commonalties, but also significant differences exist between the HPT-mediated inactivation of *C. botulinum* type E and mechanisms describe for the extensively studied model organism *B. subtilis*. For rapid inactivation of *C. botulinum* type E spores, > 500 MPa combined with > 60 – 70 °C are required. Notably, pressure levels ≤ 300 MPa at ambient temperatures can reduce the mean detection time for growth from individual *C. botulinum* type E spores and narrow the associated distribution, which could enhance the chance for spores to grow out and produce toxin within a district period. Furthermore, results indicate that the amount and type of cations present in a sporulation medium can (at least partially) account for the medium-dependent effects on the HPT resistance of *C. botulinum* type E spores observed in the beginning of this study. Additionally, effects exerted by cations in the sporulation medium are interconnected with sporulation temperature-mediated effects on HPT resistance. Finally, there is some evidence that the coat of *C. botulinum* type E spores might be involved in their HPT resistance, and two coat-associated proteins were identified as possible common targets altered by sporulation medium cation contents and temperature, which can be speculated to be involved in effects on HPT resistance. The generated data can be valuable for the design of future spore inactivation studies, for the interpretation of results for other *Clostridium* spores, for future considerations about food safety, and, finally, contribute to the establishment of HPT food processing at the industrial level.

ACKNOWLEDGMENTS

Mein besonderer Dank gilt meinem Doktorvater Prof. Dr. Rudi Vogel für die Möglichkeit diese Arbeit an seinem Lehrstuhl durchführen zu können, für die Diskussionsfreude, inspirierende Gespräche, wertvolle Ratschläge, für den großen Gestaltungspielraum bei Projektplanung und -durchführung und das entgegengebrachte Vertrauen.
Ebenfalls besonders bedanken möchte ich mich bei Dr. Jürgen Behr für seine außerordentliche Hilfsbereitschaft und seine wertvollen Ratschläge, besonders in der Anfangszeit dieser Arbeit.

Des Weiteren bedanke ich mich herzlich bei

- Prof. Dr. Wolgang Liebl und Prof. Dr. Wilfried Schwab für die Begutachtung dieser Arbeit und Prof. Dr. Horst-Christian Langowski für die Übernahme des Prüfungsvorsitzes.
- Prof. Dr. Manfred Gareis und Dr. Sonja Lick (ehemals MRI, Kulmbach) sowie Dr. Ute Messelhäußer und Dr. Ulrich Busch (ehemals LGL, Oberschleißheim) für das Überlassen der *Clostridium botulinum* Stämme ohne die diese Arbeit nicht möglich gewesen wäre.
- Dr. Kai Reineke für die Unterstützung bei den Hochdruckexperimenten an der TU Berlin und bei der Modellierung des Datenwaldes, sowie Prof. Dr. Dietrich Knorr für die Ermöglichung der Hochdruckexperimente in Berlin.
- Prof. Dr. Jürgen Geist und Jörg Steinhilber vom Lehrstuhl für Aquatische Systembiologie (TUM) für ihre Hilfsbereitschaft und die regelmäßige Versorgung mit frischen Forellen für Versuchszwecke.
- Prof. Dr. Matthias Ehrmann für sein stets offenes Ohr und hilfreiche Diskussionen.
- Prof. Dr. Ludwig Niessen für die hervorragende Gewährleistung der Laborsicherheit.
- meiner ehemaligen Hochdruckmitstreiterin Dr. Juliane Schnabel (geb. Dschulie) für die nette Zeit am Lehrstuhl.
- der aktuellen Hochdrucktruppe Tomas van Nassau, Thomas Kafka, Dominik Reitermayer und Maximilian Maier für ihren Einsatz und die hervorragenden Zusammenarbeit.
- allen betreuten Bachelor- und Masterstudenten für ihr Engagement und die gute Zusammenarbeit, besonders den externen Diplomanden und Doktoranden Rita Paralta (Universidade de Aveiro, Portugal), Sergio Ramos (Centro Nacional de Tecnología y Seguridad Alimentaria, San Adrian, Navarra, Spain) und Katherine Evert (Arriagada, Universitat Autònoma de Barcelona, Spain).
- allen langjährigen Mitarbeiterinnen am Lehrstuhl, besonders Monika Hadek und Angela Seppeur für ihre zuvorkommende Art und ihre logistische Unterstützung.
- den ehemaligen Doktoranden Simone Freiding, Angela Lindenstrauß, Georg Lutterschmid, Matthias Stübner, Patrick Preissler, Jasmin Stadie, Carola Kern, Benjamin Schurr, Katja Minenko, Anna Gulitz, Frank Jakob und Claudia Stecker, sowie den aktuellen Doktoranden Lisa Vogt, Marion Fraunhofer, Veronika Kupfer, Ann-Sophie Schott, Meike Kliche, Alexander Lauterbach und Maik Hilgarth, für die schöne Zeit.

Abschließend gilt mein besonderer Dank meiner Familie Walter, Claudia, Holger und Verena für deren unbedingte Unterstützung und deren Zuspruch, sowie Ju für ihre Hilfe, Zuneigung und wahnsinnige Geduld während der arbeitsintensiven Zeit. Danke!

Teile dieser Arbeit entstanden im Rahmen des Teilprojekts 05 des Verbundprojekts zum “Einsatz der Hochdrucktechnologie in Kombination mit einer neuen Verpackung zur Herstellung sicherer, qualitätsoptimierter Frischeprodukte mit verlängerter Haltbarkeit” gefördert durch das Bundesministerium für Ernährung und Landwirtschaft (Träger: Bundesanstalt für Landwirtschaft und Ernährung (BLE); Förderkennzeichen: 2816302407).

TABLE OF CONTENTS

ABBREVIATIONS and SYMBOLS

General abbreviations:

2D, 3D, 4D	two-dimensional, three-dimensional, four-dimensional
3PGA	3-phosphoglyceric acid
6D, 12D	Six or twelve log cycles inactivation of the initial number of CFU
A.	*Alicyclobacillus*
ASME	American Society of Mechanical Engineers
B.	*Bacillus*
B.a.	*Bacillus anthracis*
B.s.	*Bacillus subtilis*
BLAST	Basic Local Alignment Search Tool (registered trademark of the National Library of Medicine, National Institutes Of Health, U.S. Department of Health & Human Services)
bp	Base pair(s)
C.	*Clostridium*
C.p.	*Clostridium perfringens*
CDC	Centers for Disease Control and Prevention, Atlanta, Georgia, USA
cDNA	Complementary DNA (dsDNA synthesized from mRNA catalyzed by RT)
CFD	computational fluid dynamics
CFSPH	The Center for Food Security and Public Health, Iowa State University, USA
CFU	Colony forming units
CS pathway	Csp–SleC cortex hydrolysis pathway/system
D.	*Desulfotomaculum*
E.	Escherichia
e.g.	For example
ECDC	European Centre for Disease Prevention and Control, Solna, Sweden
FDA	U.S. Food & Drug Administration, Silver Spring, Maryland, USA
FS	Forespore
G.	*Geobacillus*
G+C content	guanine+cytosine content of, e.g., a gene sequence
HACCP	Hazard Analysis and Critical Control Points
HP, HPP	High pressure, high pressure processing in general
HHP	High hydrostatic pressure - commonly refers to pressure treatments at or below room temperature
HPT	High pressure thermal… (commonly: HP treatments at > room temperature)
HP	High pressure (generally means high hydrostatic pressure)
HPT	High pressure thermal (pressure treatments at elevated temperatures)
i.e.	That is
IAPWS	International Association for the Properties of Water and Steam
MAP	Modified atmosphere packed
MC	Mother cell
mRNA	Messenger RNA
n/a	Not available, insufficient data
NCBI	National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland, USA
NIST	National Institute of Standards and Technology under the U.S. Department of Commerce (Maryland, USA)
NSF	N-ethylmaleimide-sensitive fusion protein

o/n	Over night
OD_x	Optical density at wavelength x
PCR	Polymerase chain reaction
PTF	Pressure-transmitting fluid
qPCR	Real-time quantitative PCR
REPFED	Refrigerated processed food of extended durability
RKI	Robert-Koch-Institut, Berlin, Germany
RT	Room temperature (or reverse transcriptase)
RTE	Ready-to-eat
T.	*Thermoanaerobacterium*
TMW	Technische Mikrobiologie Weihenstephan
VP	Vacuum packed
YSCQ pathway	YpeB–SleB–CwlJ–GerQ cortex hydrolysis pathway/system

Media, buffer solutions, and additives:

ACES	N-(2-Acetamido)-2-aminoethane sulfonic acid
CDM	Chemically defined medium
CMM	Cooked meat medium
DEPC	Diethylpyrocarbonate
DMM	Defined minimal medium
EDTA	Ethylendiamine tetraacetate
Em	Erythromycin
EtOH	Ethanol
$H_2O_{dest/bidest/DEPC}$	Distilled/bidistilled/DEPC-treated water
HCl	Hydrochloric acid
IB	Imidazole buffer
Imidazole	1,3-Diaza-2,4-cyclopentadiene (originally glyoxaline)
IPB	Imidazole Phosphate Buffer
MDM	Modified defined minimal medium
NaOH	Sodium hydroxide
PW	Peptone Water
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
TBE	Tris borate EDTA Buffer
TE	Tris EDTA Buffer
THB	TRIS-HIS (TRIS-HCl, Histidine-HCl) Buffer
Tm	Thiamphenicol
TPY	Tryptone, peptone, yeast extract medium
TPYC	Tryptone, peptone, yeast extract medium including a sugar mix
TPYG	Tryptone, peptone, yeast extract, glucose medium
TRIS	Tris(hydroxymethyl)aminomethan
TS+	Tryptone, Sodium Chloride plus Antifoam B dilution medium

Proteins/Enzymes, domains, and biomolecules

~P	Phosphorylated (usually activated) protein
Ach	Neurotransmitter acetylcholine, 2-Acetoxy-*N*, *N*, *N* -trimethylethanaminium
AgrX	Accessory gene regulator X
BD	Binding domain of the botulinum toxin complex (= H_C)

BoNT/x	Botulinum neurotoxin of the toxinotype x, e.g., BoNT/E
CD	Catalytically active domain of the botulinum toxin complex (= LC)
CLE	Cortex lytic enzyme
Csp	Germination specific serine proteases activating SleC
CwlJ	Cortex lytic enzyme (CLE)
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotid phosphate
DPA	Dipicolinic acid (pyridine-2,6-dicarboxylic acid)
dsDNA	Double stranded DNA
GerQ	Involved in the establishment of proper CwlJ function
GerX	Germination receptor protein X
GPR	Germination protease
GT_{1b}	Binding domain., high-density, low-affinity gangliosides
H	Heavy chain of the botulinum toxin complex
HA	Haemagglutinin protein encoded by the botulinum locus
H_C	C-terminal domain of the heavy chain of the botulinum toxin complex (BD)
H_N	N-terminal end of the heavy chain of the botulinum toxin complex (= TD)
KinX	Senor kinase X
LC	Light chain of the botulinum toxin complex (= CD)
NTNH	Non-toxic, non-haemagglutinin protein encoded by the botulinum locus
OrfX1,2,3	Proteins encoded by open reading frames 1 through 3 in the botulinum locus
P27	27 kDa protein encoded by the botulinum locus
PG	Peptidoglycan
PGM	Phosphoglycerate mutase
PhrX	Peptide X as phosphatase regulators (part of Phr-Rap system)
RapX	Receptor aspartyl phosphatase X (part of Phr-Rap system)
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT	Reverse transcriptase (or room temperature)
SASP	α-/β-type small acid soluble protein
SleB	Cortex lytic enzyme (CLE)
SleC	Cortex lytic enzyme (CLE), exo-acting lytic transglycosylase
SNAP-25	Synaptosomal-associated protein, 25 kDa
SNARE	Complex, soluble NSF-attachment protein receptor complex
SpoXY	Sporulation protein Y primarily associated with sporualtion stage X, e.g., Sporulation master regulator Spo0A
SytII	Specific protein receptor synaptotagmin
Taq polymerase	DNA polymerase from *Thermus aquaticus*
TD	Translocation domain of the botulinum toxin complex (=H_N)
Tgl	Transglutaminase
YpeB	Coat protein required for proper SleB localization

Symbols and physical units

c_p	Isobaric specific heat capacity [J/(kg K)]
A^-	Conjugate base
d	Day(s)
d	Differential (in equations)

Da	Dalton
f	Function
g	Gram(s)
h	Hour(s)
H	Enthalpy [kJ kg^{-1}]
H_2O	Water
H_3O^+	Hydroxonium ion
HA	Proton donator/acid
K	Kelvin
k_C	Compression heating coefficient [Pa-1]
L	Liter
M	Molar (mol per liter); in front of physical unit: Mega (10^6)
m	Meter; in front of physical unit: milli (10^{-3})
min	Minute(s)
mL	Milliliter(s)
mol	Amount of chemical substance
n	Number of samples investigated (in surveys)
N(t)	Viable cell count at time t
N_0	Initial viable cell count at time 0
N_i	Number of particle in a thermodynamic system of a specific type i
p or *p*	Pressure [Pa]
Pa	Pascal
Q	Heat energy [J]
s	Second(s)
S	Entropy of a thermodynamic system [J K^{-1}]
T or *T*	Temperature [°C or K]
T_m	Primer Melting Temperature (50% of primer and complement hybridized)
U	Internal energy of a system [J]
V	Volume [in mL or L] or Volt
v	Specific Volume [m^3 kg^{-1}]
v/v	Volume/volume
W	Work [J]
w/v	Weight/volume
ΔV	Reaction Volume [mL/mol]

Greek symbols

$\propto_{\boldsymbol{p}}$	Isobaric thermal expansion coefficient [K^{-1}]
$\boldsymbol{\partial}$	Partial differential (in equations)
Δ	Difference
μ	In front of physical unit: micro (10^{-6})
μ_i	Chemical potential or partial molar free energy of particles of a specific type i in a thermodynamic system
$\boldsymbol{\rho}$	Density [kg m^{-3}]
$\boldsymbol{\vartheta}$	Temperature [°C or K]
σ^H, σ^F, σ^G, σ^E, σ^K	Sporulation sigma factors H trough K
γ_i	Activity coefficient (= correction factor) for the concentration of a molecule of type i for the calculation of equilibrium constants

LIST OF FIGURES

LIST OF TABLES

1 Background

This first section aims at providing background information that is necessary to understand the approaches selected and results obtained for the high pressure thermal inactivation of *Clostridium botulinum* type E endospores. Beyond that, this literature review may be helpful to others generally engaged in high pressure research, working with bacterial endospores, or considering safety aspects of fishery products. For example, section 1.2 contains and compares available information on both *Bacillus* and *Clostridium* spores, section 1.4 highlights some important points related to HPT process control, and section 1.3 contains detailed data on the environmental prevalence of *C. botulinum* type E and its toxin.

1.1 History of Spores and Pressure Treatments

In addition to salting, drying, smoking or fermenting food, the application of heat treatments has a long history in the preservation of food and dates back to not later than the last decade of the 17th century (in: (Gould, 2006)). More than 100 years later, a method to thermally sterilize food using hermetically sealed containers was described by Appert (1810). This was long before the microbiological origin of food spoilage was identified (Pasteur, 1866) and bacterial endospores were studied. Although the presence of refractile bodies in bacterial cells was already reported in the first half of the 19th century (Ehrenberg, 1838), it took more than 30 years until the term “spores” appeared within the context of “permanent” cells of bacteria (*Bacillus subtilis*, (Cohn, 1872)), which was followed by first studies on bacterial spores conducted by Cohn (Cohn, 1876) and Koch (*Bacillus anthracis*, (Koch, 1876)). Although there exist other cell forms that are commonly also called “spores” (e.g., fungal spores), the term spores exclusively refers to bacterial endospores throughout this manuscript.

The application of high hydrostatic pressure (HHP) treatments to preserve food was first described only 23 years later. In 1899, Hite tried to find alternative preservation methods for heat sensitive food and was able to demonstrate that HHP treatments at approx. 700 MPa can significantly increase the shelf-life of milk with less detrimental effects on sensorial properties than heat treatments (Hite, 1899). However, relatively soon it became clear that bacterial spores are highly resistant to HHP treatments (Chlopin and Tammann, 1903) and even pressures levels of about 1200 MPa are not sufficient to achieve an effective inactivation (*B. subtilis*, (Larson et al., 1918)). This clearly indicated already back then that such treatments are not suitable for an application in food sterilization processes. In the late 1960s some attempts were made to utilize HHP-triggered germination at low pressure levels to establish a “tyndallization”-like two-step HHP process, in which germinated, i.e. heat sensitive spores were subsequently killed by mild heat treatments (Clouston and Wills, 1969; Gould and Sale, 1970). However, the great heterogeneity in germination requirements and pressure sensitivity of spores from different species and single spores within a population resulting in non-log-linear germination kinetics made such approaches too unreliable for an application in commercial food sterilization processes (Gould, 2006).

In the 1980s, technological progress and the increasing consumer demand for fresh-like food with increased shelf-life resulted in first commercial applications of HHP pasteurization processes for the production of acidic (pH < 4.5) food where spores from pathogenic species are not able to initiate growth. Thereafter, the number of pressure treated, chilled, low-acid

food products (pH ≥ 4.5) steadily increased. This group of foods, nowadays, presents the majority of pressure treated products on the market. Commonly, pressure levels of up to 600 MPa and initial temperatures below (or at) room temperature are used in the production of meat products and vegetables each separately accounting for approx. 27 % of HHP-treated food, followed by (fruit) juices and beverages (approx. 14 %), and seafood and fish products (approx. 13 %) (Tonello-Samson, 2014).

However, such commonly applied processes are not sufficient to effectively inactivate spores. Spore inactivation by HHP can be enhanced by applying two-step processes (optimized processes similar to that tested back in the 1960s/1970s) or by adding synergistically acting substances to food. Both approaches have some disadvantages and were not yet shown to be capable of inactivation spores completely in a broad range of food products. An effective way to inactivate spores using HHP-based processes requires a combination of HHP and heat. For such processes, many different acronyms can be found across literature including PATP ("pressure-assisted thermal processing", e.g., (Park et al. 2014)), HP/HT ("high pressure/high temperature", e.g., (Shpigelman et al., 2014)), HPHT (e.g., (Kebede et al., 2014)), HPS ("high-pressure sterilisation treatments combined with elevated starting temperatures", e.g., (Krebbers et al., 2003)), HPTP ("high pressure–thermal processing", e.g., (Devi et al., 2015)), HPTS ("high pressure thermal sterilization", e.g., (Reineke et al., 2013b)), and THP ("combined thermal-high pressure processing", e.g., (Fraeye et al., 2010)). Throughout this manuscript, HPT (standing for "high pressure thermal"…, e.g., (Delgado et al., 2013; García-Parra et al., 2014; Knoerzer and Chapman, 2011)) is used whenever, high pressure is combined with temperatures above ambient temperatures. Additionally, the acronym PATS (high pressure thermal sterilization) is used occasionally to describe processes, where the adiabatic heat of compression is be exploited to reach the sterilization temperature of 121.1 °C in a preheated product, the HPT process that was established and certified by the U.S. FDA in 2009. Generally the use of the acronym HPT has the advantages that it is relatively short, can be used to describe treatments / processing / inactivation / resistance, does not predetermine the desired inactivation result (such as sterilization), and does not prejudge the process with respect to the contribution of pressure and temperature to the inactivation result (such as PATP).

Properly designed HPT processes can be used to achieve required sterilization results, i.e., enable the production of stable and safe food, while nutritionally valuable molecules (e.g., vitamins), the appearance (color, texture), and the taste are affected to a lower extent than it would be provoked conventional retorting (Heinz and Buckow, 2010; Knorr et al., 2011).

However, HPT treatments are not yet applied in an industrial scale, which is presumably due to two major reasons: (i) Technical constraints including the deficiency in robustness and increased fatigue of industrial HHP equipment at elevated temperatures impede commercial application. (ii) Despite of the discovery of bacterial spores and the first experiments on high pressure treatment of food more than 100 years ago and the wealth of knowledge that has been gained since then, some basic mechanisms including the HHP/HPT resistance of spores still evade complete understanding.

Due to continuous technical progress, increasing consumer demands for minimally processed, healthy food with long shelf-life, and considerable efforts made by legal authorities to decrease the levels of traditional food preservatives (e.g., salt), HHP-based

sterilization, i.e., HPT processes increasingly appear to present a promising alternative to thermal processing.

1.2 Bacterial Endospores

1.2.1 Important Spore-Forming Species

Industrially and Clinically Relevant Species

Many members of the orders *Bacillales* and *Clostridiales* can produce spores. The ubiquitous presence of bacterial spores in nature, their dormancy, their extreme resistance to chemical and physical stress conditions, and their ability to grow out under favorable conditions prevalent in many (mainly low-acid) food products makes them a key problem in food industry.

Various genera such as *Alicyclobacillus* (*A.*), *Desulfotomaculum* (*D.*), *Thermoanaerobacterium* (*T.*), and *Geobacillus* (*G.*) include certain species that are associated with food spoilage, e.g., *A. acidoterrestris* (Steyn et al., 2011), *D. nigrificans*, *T.* (formally *Clostridium* (*C.*)) *thermosaccharolyticum*, and *G.* (formally *Bacillus* (*B.*)) *stearothermophilus*. The genera, which include species that are most frequently associated with problems in food preservation (spoilage and safety), are *Bacillus* and *Clostridium*. Typical spoilage organisms can be found in the species *B. coagulans* (flat-sour), *B. thermoacidurans* (acid spoilage), and, occasionally, *B. licheniformis*, *B. pumilus*, and *B. subtilis* (Oomes et al., 2007). Additionally, various *Clostridium* species can be involved in spoilage including *C. bifermentans* (sulfide spoilage, rotten egg odor, black color), *C. sporogenes* (putrefactive fermentation), *C. butyricum*, *C. tertium* (butyric odor), and various psychrophilic and psychrotrophic *Clostridium* species primarily associated with spoilage of chilled red meat (Adam et al., 2010).

Major spore-forming, food-associated, pathogenic species include *B. cereus* (emetic toxin cereulide and diarrheal toxins), *C. perfringens* (intoxication, diarrhea/vomiting, enterotoxin; infection, gas gangrene, alpha toxin), and *C. botulinum* (highly potent botulinum neurotoxin (BoNT), intoxication, flaccid paralysis; infection, infant botulism) (Scallan et al., 2011). Additionally, *C. difficile* (infection, diarrhea, enterotoxin A, cytotoxin B) can present a problem for food safety.

In addition to species of significance in food, the genera *Bacillus* and *Clostridium* also comprise clinically important species (e.g., *B. anthracis* (anthrax) and *C. tetani* (tetanus)) and strains of importance in industrial biotechnology (e.g., *B. amyloliquefaciens* (alpha amylase, BamH1 restriction enzyme), *B. thuringiensis* (insecticidal toxin), *C. phytofermentans*, *C. thermocellum* (cellulose degradation), *C. acetobutylicum*, and *C. beijerinckii* (solvent production)).

Spore-forming Model Organisms

Although *C. botulinum*, due to its capability of forming the highly potent BoNT, presents one of the most important threats to food safety, knowledge on basic mechanisms such as the sporulation cascade, resistance, and germination mechanisms, is scarce compared to that gathered for other spore-forming species.

In the past decades, *B. subtilis* served as model organism for studying such basic mechanisms. Although this organism can be occasionally involved in food spoilage, it does

certainly play no important role in food spoilage and does not present any threat to food safety. Reasons for this choice may be found in its non-pathogenic nature, its natural transferability and the early availability of genetic and molecular biological data, probably most importantly, its complete genome sequence. Although basic mechanisms are still not understood completely, a wealth of knowledge has been obtained on *B. subtilis* spores (Setlow and Johnson, 2013), which includes insights into HHP- and HPT-mediated inactivation mechanisms (Reineke et al., 2013a).

For *Clostridium* species, *C. acetobutylicum*, has been suggested to present a suitable model organism studying basic sporulation and germination mechanisms due to its interesting solventogenic properties, its non-pathogenicity, and a high degree in genetic homology compared to many other *Clostridium* species (Paredes et al., 2005). Additionally, a considerable amount of research on basic mechanisms has been conducted using *C. perfringens* (Paredes-Sabja et al., 2011). However, there are still huge differences in the state of knowledge on *B. subtilis* compared to other spore-forming bacteria.

Although considerable variations in some key proteins and signal transduction pathways can impede the direct transferability of results, basic elements involved in sporulation (de Hoon et al., 2010; Li and McClane, 2010; Paredes et al., 2005; Wörner et al., 2006) and germination (Paredes-Sabja et al., 2011) are highly conserved among various spore-forming species. Additionally, the basic structure and composition of spores of various species appear to be very similar (Setlow and Johnson, 2013). Thus, available data for model organisms present a valuable basis for understanding basic mechanisms in other spore formers.

1.2.2 The spore cycle

Knowledge on the developmental program leading to spore formation, i.e., sporulation, presents an important basis for understanding the development of the extremely high resistance of spores to physical treatments such as HHP or HPT processing. Furthermore, a detailed picture of molecular events during spore germination facilitates an in-depth understanding of how spores can lose their resistance properties acquired during sporulation, plays a crucial role in HHP-mediated spore inactivation. Therefore, the following paragraphs provide an overview of the spore cycle and describe events during sporulation and germination. Since available data for *Clostridium* in general and specifically for *C. botulinum* spores is limited, this section includes data for *Bacillus* spores, which can build a valuable basis for understanding basic mechanisms. Moreover, major differences in germination pathways between the genera *Bacillus* and *Clostridium* can directly explain some major genus-specific difference in HHP- and HPT-mediated spore inactivation mechanisms. Before going into detail on what is happening on the molecular level during sporulation and germination, this section provides a short summary on what can cause a cell to enter sporulation, alternative pathways instead of entering sporulation, and major steps occurring during the spore cycle.

1.2.2.1 Decision to Enter the Spore Cycle

In response to unfavorable conditions, which can be typically found during the stationary growth phase, cells from spore-forming species can initiate sporulation. Notably, the entry into stationary phase does not obligatory lead to immediate sporulation. Typical stationary phase events, which are not necessarily required for but often regulated by factors involved in sporulation, include that cells upregulate genes associated with nutrient transport and metabolism (carbohydrates, amino acids and inorganic ions), try to make additional nutrient sources accessible, and to evade or somehow to adapt to the new situation. The induction of alternative metabolic substrate pathways can occur in response to nutrient depletion (e.g. arginine depletion, arginine biosynthesis), but does not necessarily require the complete depletion all nutrients (e.g., >40% of initial glucose remaining, *C. acetobutylicum*, (Jones et al., 2008)). Strategies to improve nutrient accessibility include the secretion of degradative enzymes such as amylases and proteases (e.g.: *B. subtilis*, (Phillips and Strauch, 2002) and harming/killing of other organism including sister cells (e.g.: *B. subtilis*, (Gonzalez-Pastor et al., 2003)), the competitive microbiota (antibiotics) (e.g.: *B. subtilis*, (Irigul-Sonmez et al., 2014)), or higher organisms such as insects (e.g., *B. thuringensis*) or animals (protein toxins) (e.g.: *C. botulinum*, *C. tetani*, (Connan et al., 2013)). Motility and chemotaxis can present a stationary phase response to evade the nutrient-poor environment (e.g.: *C. difficile*, (Saujet et al., 2011)) with the underlying machinery being well conserved in *Bacillus* and *Clostridium* species and closely linked to sporulation (e.g., *B. subtilis*, (Aizawa et al., 2002); *C. acetobutylicum* (Alsaker and Papoutsakis, 2005; Tomas et al., 2004; Zhao et al., 2005)). In contrast, competence development to adapt to the new situation presents a relatively uncommon stationary phase response (e.g., *B. subtilis*, (Stiegelmeyer and Giddings, 2013)). Finally, cells can initiate sporulation. The decision for a single cell within a population to form a spore depends on the success of the strategies mentioned above, starvation conditions (Chubukov and Sauer, 2014), and extracellular signals (e.g.: cell density-dependent (Bischofs et al., 2009; Lazazzera et al., 1999; Lopez and Kolter, 2010; Schultz et al., 2009)).

Quorum-sensing (QS) mechanisms mediated by signal peptides are thought to play an important role in both *Bacillus* and *Clostridium* species. Prominent examples are the Phr-Rap system in *Bacillus* species (peptides as phosphatase regulators-receptor aspartyl phosphatases; *B. subtilis* (Bischofs et al., 2009)) and the Agr-like system in *Clostridium* species (Accessory gene regulator, (Myers et al., 2006); *C. perfringens*, (Li et al., 2011); *C. sporogenes*, *C. botulinum* type A, (Cooksley et al., 2010)). Although various environmental factors, which push a cell towards or delay the decision to initiate sporulation have been identified and the mechanism of sporulation induction is quite well characterized (at least in *B. subtilis*; see section 1.2.2.3.2), single cell fate in the context of the entire population is largely unexplored. However, it seems likely that differences in the fate of single cells at this stage influence the heterogeneity of a spore population, which has a significant impact on the success of HHP-based inactivation strategies.

1.2.2.2 Leaving the Spore Cycle

Mature spores are metabolically dormant and differ significantly from vegetative cells in their composition, structure, and resistance properties enabling spore-forming organisms to survive long periods under harsh environmental conditions without the availability of nutrients. Under certain environmental conditions (e.g., in the presence of nutrient germinants) spores can germinate, grow out, and convert back to a growing cell.

The spore cycle of *B. subtilis* has been most extensively studied (Setlow and Johnson, 2013). A simplified scheme of the spore cycle of such a rod shaped bacterium without terminal swelling of the forespore compartment or elongation of the mother cell is depicted in Fig. 1-1. Important morphological events during sporulation, i.e. sporulation stages as indicated in this figure, are explained in more detail in sections 1.2.2.3 and 1.2.2.4.

Generally, the spore cycle in *Clostridium* species is presumed to include essentially the same morphological steps with some minor differences, e.g., that the sporangium swells during the course of sporulation. Based on genomic information, *Bacillus* and *Clostridium* sporulation programs show many parallels and many components of the spore germination machinery are highly conserved among spore-forming members of the *Bacillales* and *Clostridiales* orders. However, although orchestration of differentiation programs is less understood in *Clostridium* species and far from being completely elucidated at the molecular level, studies on the transcriptional level have contributed to better understanding of molecular events that underlie sporulation and suggest that there are some major differences between *Bacillus* and *Clostridium* sporulation programs (see section 1.2.2.3) (Jones et al., 2008; Paredes et al., 2005). Additionally, both proteins and signal transduction pathways involved in germination are substantially different between *Bacillus* and *Clostridium* species (see section 1.2.2.4) (Paredes-Sabja et al., 2011).

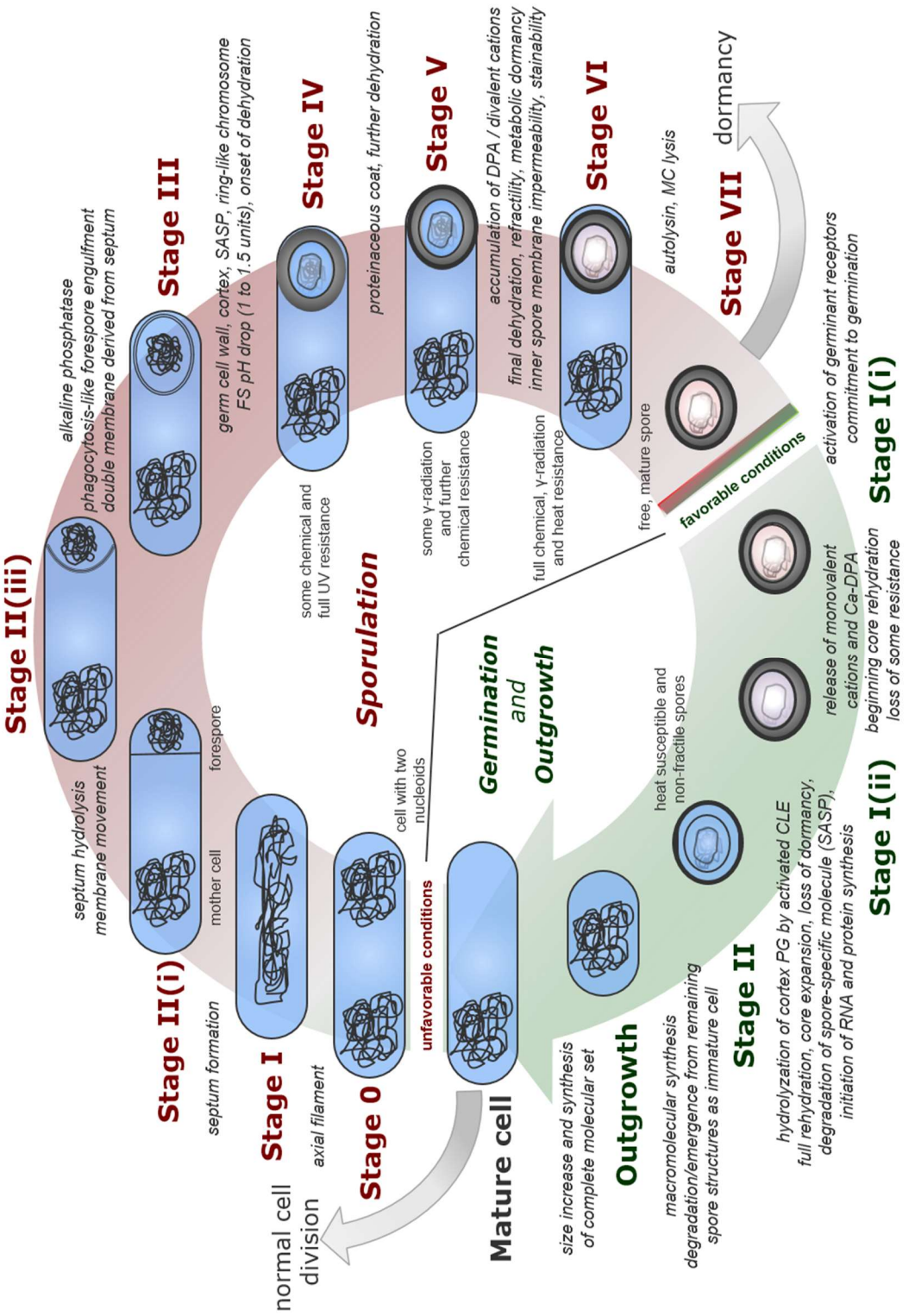


Fig. 1-1: General scheme of the spore cycle.
Stages 0 through VII indicate major morphological stages during sporulation. Stages I(i) through II indicate germination stages. Major events between the stages (italics) and important spore properties at a specific stage (plain text inside of the circle) are described. Black coils in the oval cells indicate DNA. Spore layers: thin black line = inner membrane; thick gray layer = cortex; outer black layer = coat. Notably, spore-formers are not always rod-shaped, the sporangium can swell during sporulation, spore formation does not necessarily occur at a terminal position, and spores can contain a large exosporium as outermost layer, which is not depicted. MC = mother cell, SASP = small acid soluble protein, FS = forespore, DPA = dipicolinic acid, PG = peptidoglycan, CLE = cortex lytic enzymes. Figure from (Lenz and Vogel, 2015) adapted from Setlow and Johnson (2013) and Setlow (2003).

1.2.2.3 Sporulation

1.2.2.3.1 Morphological Changes during Sporulation

Morphological changes during sporulation putatively occur in a similar manner in *Bacillus* and *Clostridium* species and lead to a stepwise development of the extreme resistance of spores. Although sporulation is a continuous process and intermediate forms do not present discrete entities in which a sporulating cell remains, this developmental process is commonly divided into eight stages (stage 0 through VII; red part of the spore cycle in Fig. 1-1) based on observable morphological changes and according to the description of mutant strains (*spo* mutants), in which sporulation is blocked in a particular stage.

In a vegetative, growing cell, two nucleotides are formed (stage 0) that align in an axial filament (stage I). Transition to stage II is characterized by typical stationary phase responses (e.g., secretion of degradative enzymes) as mentioned above (section 1.2.2). In stage II, the first distinct morphological changes become visible. A septum is formed leading to an asymmetric division that separates the sporulating cell into two unequal cellular compartments, i.e., a large mother cell (MC) and a smaller forespore (FS) compartment (stage II(i)). This is followed by septum hydrolysis, membrane movement and curvature (stage II(iii)). At this point, first scaffold proteins crucial to later coat assembly are attached to the outer surface of the membrane that encases the forespore compartment. A phagocytosis-like process leads to the complete engulfment of the forespore, which now presents an intracellular protoplast surrounded by a double membrane, i.e., two membranes with opposite polarities (stage III). The transition to the next stage is characterized by significant changes in the forespore core region including a decrease in volume and pH, the onset of dehydration, and synthesis of small acid soluble proteins (SASP), which are involved in arranging the chromosome in ring-like structure. Additionally, the germ cell wall (essentially identical peptidoglycan (PG) structure compared to growing cells) and the cortex (similar components but unique PG structure) are formed between the inner and the outer forespore membranes (stage IV). The assembly of unique coat proteins, which is a continuative process beginning shortly after asymmetric cell division, is then intensified. This leads to the encasement of the spore by several proteinaceous coat layers (stage V). A large amount of pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) is synthesized in the mother cell and accumulated in the spore compartment. This is paralleled by the uptake of divalent cations, i.e., predominately Ca^{2+}, but also high amounts of Mg^{2+} and Mn^{2+}. Final dehydration of the forespore interior is crucial to the development of metabolic dormancy and causes increased refractility resulting in the typical phase-bright appearance of spores when examined by phase-contrast microscopy. The permeability of the inner spore membrane decreases, which impedes the access of substances commonly used to stain vegetative cells to the spore interior. Spore encasement by the outermost layers (coat and exosporium) is completed, which marks the end of the maturation process (stage VI). Finally, the mother cell lyses and releases the mature spore into the environment (stage VII) (Eichenberger et al., 2004; Hoch, 1993; Paredes et al., 2005; Piggot and Hilbert, 2004; Setlow and Johnson, 2013; Sonenshein, 2000).

1.2.2.3.2 Developmental Program Underlying Sporulation

This paragraph contains an overview of mechanisms regulating the initiation of sporulation and the developmental program, which ensures the timely and spatially ordered stepwise development of morphological spore characteristics considering differences between *Bacillus* and *Clostridium* species (commonalities highlighted in green in Fig. 1-2).

Prerequisites for the Initiation of Sporulation

Prerequisites for the initiation of sporulation are a complete chromosome replication (Sonenshein, 2000), DNA damage repair (Rowland et al., 2004), and the induction of tricarboxylic acid cycle enzymes (Phillips and Strauch, 2002). Sporulation triggers during stationary phase can include nutrient limitation (starvation from carbon and/or nitrogen) (*B. subtilis*, (Setlow and Johnson, 2013)), a decrease in (intracellular) pH (*C. acetobutylicum*, (Jones and Woods, 1986)), and increased levels of intracellular reduction energy (NAD(P)H) (*C. acetobutylicum*, (Meyer and Papoutsakis, 1989)). Furthermore, the addition of various substances can promote sporulation, e.g., decoyinine (inhibitor of guanine nucleotide synthesis, *B. subtilis*, (Setlow and Johnson, 2013)), carboxylic acids (membrane uncouplers, *C. acetobutylicum*, (Husemann and Papoutsakis, 1988)), high carbon source and ATP levels (mimicking phosphate or nitrogen limitation, *C. acetobutylicum*, (Meyer and Papoutsakis, 1989)), butyrate, and carbon monoxide (inhibitors of H_2 formation, (Bahl et al., 1995; Girbal and Soucaille, 1998; Jones and Woods, 1986; Woods, 1995)).

Master Regulator Spo0A as Bottleneck

The master regulator Spo0A presents a highly sensitive, self-reinforcing switch determining whether a single cell initiates sporulation or not. Once Spo0A levels in its activated, i.e., phosphorylated form exceed a certain threshold level, sporulation is initiated and the sporulation cascade proceeds (Fig. 1-2). Although a direct relationship has not yet been proven, especially the entry into sporulation via the activation of the sporulation master regulator Spo0A is likely to present a feature that plays an important role in the development of heterogeneous spore populations comprising spores different properties including their resistance to physical (e.g., HPT) treatments.

Spo0A Synthesis

The transcription of *spo0A* already begins during the transition between the exponential and stationary growth phase (*B. subtilis*, (Piggot and Hilbert, 2004); *C. acetobutylicum*, (Jones et al., 2008)) and is aided by the earliest sporulation sigma factor, σ^H. Despite of some differences in the time-dependent expression pattern (Durre and Hollergschwandner, 2004; Weir et al., 1991), σ^H is highly conserved among different spore formers (e.g. *B. subtilis*, (Stragier and Losick, 1996); *C. acetobutylicum*, (Jones et al., 2008)). At the onset of sporulation, a burst of Spo0A synthesis has been observed in various spore formers (e.g., *C. acetobutylicum*, (Harris et al., 2002); *C. perfringens*, (Huang et al., 2004); *C. beijerinckii*, (Wilkinson et al., 1995); *C. cellulolyticum*, (Ravagnani et al., 2000)). This is thought to occur due to a just-in-time control by multiple regulatory mechanisms (*B. subtilis*, (Chastanet and Losick, 2011)). Whereas the Spo0A amino acid sequence is highly conserved in *Bacillus* and *Clostridium* species (Stephenson and Lewis, 2005), signaling used is different between spore-forming species (Durre and Hollergschwandner, 2004; Stephenson and Hoch, 2002). For example, Spo0A of a proteolytic *C. botulinum* type A strain can repress growth genes in *B. subtilis* but fails to induce sporulation (Wörner et al., 2006), and *C. acetobutylicum* or *C.*

tetani but not *C. botulinum* or *C. difficile* Spo0A can complement a *C. perfringens spo0A* mutant (low transcription instead of general incompatibility is possibly involved in the latter effect) (Sarker and Huang, 2006).

Spo0A Activation

Initiation of sporulation requires Spo0A activation, i.e., phosphorylation, which presents a highly conserved mechanism among various spore formers. In both *Bacillus* and *Clostridium* species, the first step towards Spo0A phosphorylation is the activation of sensor kinases. Although the Spo0A activation mechanism is barely understood in many organisms, spore-forming species are thought to generally possess multiple orphan kinases allowing initiation of sporulation in response to a variety of environmental conditions (Brunsing et al., 2005), e.g., two major (KinA, B) and three minor kinases (KinC, D, E) in *B. subtilis* (Jiang et al., 2000; Stephenson and Hoch, 2002). In addition to various candidates, which have not yet been proven to lead to Spo0A activation, several sensor histidine kinases can directly phosphorylate Spo0A in *Clostridium* species, e.g., CBO1120 in *C. botulinum* (Wörner et al., 2006), HK CD2492 in *C. difficile* (Underwood et al., 2009), and CAC0323, CAC2730, and CAC0903 in *C. acetobutylicum* (Alsaker and Papoutsakis, 2005; Jones et al., 2008; Steiner et al., 2011). However, similar to the strong species dependency of extracellular signals that drive sporulation, the functionality of such kinases can vary considerably between different species, which is thought to reflect the adaptation to different environmental niches.

A major difference between *Bacillus* and *Clostridium* species can be found in intermediate steps leading to Spo0A activation. In *Bacillus* species, activated kinases phosphorylate the sporulation initiation phosphotransferase, Spo0F, followed by the single-domain response regulator / phosphotransferase, Spo0B, which activates Spo0A (*B. subtilis* KinA can also activate Spo0A directly with low efficiency (Trach and Hoch, 1993)). In contrast, Spo0A is directly activated by sensor kinases in *Clostridium* species, which generally lack Spo0F and Spo0B phosphotransferases (Sebaihia et al., 2007).

Regulation of Spo0A Activation

Spo0A~P modulates the expression of over 100 genes directly or indirectly by binding to sites upstream of several key genes (Fujita et al., 2005). Since a threshold level of Spo0A~P needs to be exceeded for the initiation of sporulation (Fujita and Losick, 2005), (i) a decrease in *spo0A* expression levels, (ii) inhibition of sensor kinases, or (iii) dephosphorylation of Spo0A (or one of the phosphotransferases Spo0F in *Bacillus* species) represses the initiation of sporulation. Indeed, a complex regulatory network employing such mechanisms (of which some are well characterized in *B. subtilis*) regulates and fine-tunes the initiation of sporulation in response to various intracellular and extracellular conditions (Fujita et al., 2005; Fujita and Losick, 2005; Jiang et al., 2000; Piggot and Hilbert, 2004).

(i) An example for a direct repressor of *spo0A* is *sinR*, which directly binds to the promoter sequences of the *spo0A* operon (also to *sigF* and *sigE*, *B. subtilis*, (Mandicmulec et al., 1995)) and putatively plays a similar role in some *Clostridium* species (*C. acetobutylicum*, *C. tetani*, (Jones et al., 2008; Paredes et al., 2005; Scotcher et al., 2005)). In contrast the SinR repressor, SinI (stimulated by Spo0A~P and probably repressed by AbrB and Hpr) is absent in *Clostridium* species (Mandicmulec et al., 1995; Paredes et al., 2005). A conserved (Jones et al., 2008; Mandicmulec et al., 1995; Nolling et al., 2001; Scotcher and Bennett, 2005) indirect mechanism regulating *spo0A* expression levels is the transition-state regulator AbrB, which negatively controls σ^H leading to a decrease in *spo0A* transcription and activates other

repressors. Near the onset of sporulation, high Spo0A~P levels decrease intracellular AbrB levels caused by the high affinity of the gene *abrB* for Spo0A~P (*B. subtilis*, (Mandicmulec et al., 1995)).

(ii) Kinase inhibitors are well characterized in *B. subtilis,* e.g., Sda inhibits KinA in response to impaired DNA replication or DNA damage (Rowland et al., 2004) or CodY (with its corepressor GTP), which is involved in the regulation of Spo0A~P levels, putatively via inhibiting KinB (Molle et al., 2003; Ratnayake-Lecamwasam et al., 2001). Although kinase inhibitors are likely to be also present in other spore-formers, knowledge on such inhibitors is scarce, which is putatively related to their kinase-specificity and the low degree of conservation of certain kinases among spore formers.

(iii) Sporulation repressors acting antagonistic to the histidine kinases by dephosphorylating Spo0A~P identified in *B. subtilis* include the aspartyl phosphate phosphatases Spo0E (Diaz et al., 2008) and YnzD and YisI (highly homologous to Spo0E) (Perego, 2001). In contrast to YnzD and YisI, Spo0E-like aspartyl phosphate phosphatases are conserved in spore-forming *Bacillus* species (e.g., *B. subtilis*, (Diaz et al., 2008); *B. anthracis*, (Grenha et al., 2006)) and some *Clostridium* species (Paredes et al., 2005). Additionally, Spo0F~P dephosphorylation (e.g., by RapA, RapB) plays a role in modulating the phosphorylation state of Spo0A in *B. subtilis*. However, both Spo0F and Rap phosphatases are absent in *Clostridium* species (Sebaihia et al., 2007).

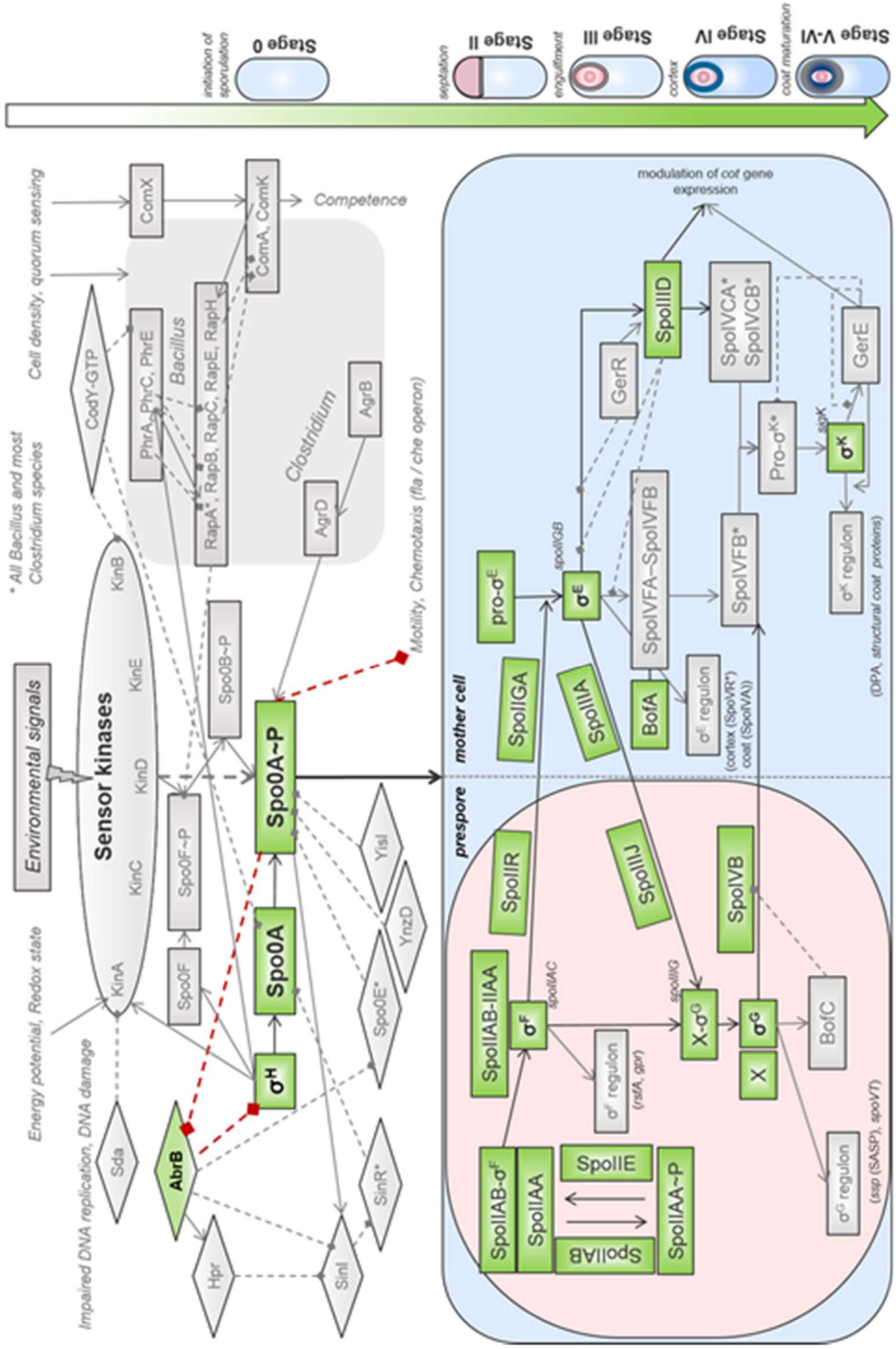


Fig. 1-2: Sporulation cascade.
Top: initiation of sporulation; Bottom left: forespore-specific regulation; Bottom right: mother cell-specific regulation; Arrow on the right: approximate time line of corresponding morphological events during sporulation; Diamond shape of fields: stage 0 repressors of sporulation; Dashed lines connect repressors and targets (targets marked by small diamond at the end of this line); Straight arrows: activation process pointing on the activated target; Green boxes: parts presumably conserved in *B. subtilis* (*Bacillus* species) and *Clostridium* species; Asterisk: parts conserved in *Bacillus* and only a few *Clostridium* species; Gray dashed lines, arrows, and boxes: *B. subtilis* mechanisms not conserved in *Clostridium* species (except the *Clostridium* Agr system, labeled explicitly). Figure from (Lenz and Vogel, 2015) adapted from Paredes et al. (2005) and modified according to Jones et al. (2008), and Setlow and Johnson (2013). Note that there are some more sporulation genes conserved among *Bacillus* and *Clostridium* species (Galperin et al., 2012), which might be not directly involved in the cascade or play uncertain roles.

The Sporulation Cascade

Sporulation is regulated by a complex network, in which gene expression is temporally and spatially (mother cell / forespore) different, and highly ordered (Fig. 1-2). In this process, increased levels of σ^H and Spo0A~P activate the expression of several key sporulation genes, most importantly four sporulation-specific sigma factors. Associated with RNA polymerase, these sigma factors drive different patterns of gene expression in a compartment-specific manner, i.e., the early and the late sigma factors σ^F and σ^G in the prespore, and the early and the late sigma factors σ^E and σ^K in the mother cell. An extensive 'cross-talk' between the mother cell and the forespore compartment and developmental checkpoints ensure that gene expression remains temporally coordinated in the two compartments. Many target genes (i.e., sigma factor regulons) described in detail in *B. subtilis* are different or absent in *Clostridium* species (Paredes et al., 2005). However, the four sigma factors appear to be highly conserved in *Bacillus* (Stragier and Losick, 1996) and several *Clostridium* species (Jones et al., 2008). This and the high degree of conservation of other key sporulation genes suggests that in addition to the basic sporulation stages, the genetic background of the sporulation cascade is similar in various spore-forming organisms.

Stage II Prespore Sigma F

The forespore chromosome is transported across the sporulation septum, i.e., from the mother cell to the forespore compartment, via a SpoIIIE-mediated channel formation (*B. subtilis*, (Becker and Pogliano, 2007; Fleming et al., 2010); SpoIIIE conserved, (Galperin et al., 2012)), which is followed by septal membrane fission, i.e., the final step in unequal cell division.

In *B. subtilis*, activated Spo0A leads to an upregulation of the *spoIIA* operon coding for SpoAA, SpoAB, and the first forespore-specific sigma factor, σ^F (Schmidt et al., 1990). Before septum formation is completed, σ^F is present in an inactive form σ^F-SpoIIAB (promoted by a high ATP/ADP ratio). After septum formation, the anti-anti-σ factor SpoIIAA is dephosphorylated by the phosphatase SpoIIE present in the sporulation septum (Clarkson et al., 2004; Yudkin and Clarkson, 2005), SpoIIAA interacts with the anti-σ factor SpoIIAB and releases σ^F in its active form (Hilbert and Piggot, 2004). Genes involved (Paredes et al., 2005) and the mechanism of σ^F activation (*C. acetobutylicum*, (Bi et al., 2011; Jones et al., 2008; Jones et al., 2011)) are thought to be conserved in the *Clostridium* species.

In *B. subtilis*, $E\sigma^F$ transcribes numerous genes in the forespore including genes coding for the DNA binding protein RsfA (modifies $E\sigma^F$ specificity), SpoIIR (compartmental cross-talk necessary for σ^E activation), Gpr (protease acting on SASP in the early germination process), and the late forespore-specific sigma factor σ^G (*spoIIIG*) (Wang et al., 2006). Although *spoIIR* along with other sporulation related genes (such as *gpr*, *spoIIP*, *sigG*, *lonB*) are conserved in *Clostridium* species (Galperin et al., 2012; Paredes et al., 2005) they are likely not controlled by σ^F in such organisms (*C. acetobutylicum*, (Jones et al., 2011)).

Stage II Mother Cell Sigma E

In the mother cell compartment, the *spoIIG* operon, coding for both the protease SpoIIGA and the first mother cell specific sigma factor σ^E, is transcribed early by $E\sigma^A$ (*B. subtilis*, (Piggot and Hilbert, 2004)). Activation of σ^E from its inactive precursor, pro-σ^E, requires septation and the physical interaction between SpoIIGA and the σ^F-regulated SpoIIR (Labell et al., 1987).

Active σ^E has been reported to control the expression of over 250 genes in the mother cell (Eichenberger et al., 2004) including SpoIIIA (involved in compartmental cross talk and activation of the late forespore specific σ^G), genes required for cortex formation (e.g., *pdaA*) (Fukushima et al., 2002), and some genes involved in coat biosynthesis and assembly (e.g., *cot* genes) (Eichenberger et al., 2004). Additionally, σ^E-containing RNA polymerase is responsible for the transcription of *spoIIID* (transcriptional regulator modulating $E\sigma^E$ action), *gerR* (requiring SpoIIID; directly controlling late sporulation genes *cotB*, *cotU* and *spoVIF* (Cangiano et al., 2010)), the *spoIVF* operon (required for activation of the late mother cell-associated sigma factor σ^K), and the *spoIVC* operon (precursor protein for σ^K).
Genes coding for σ^E and SpoIIGA (Paredes et al., 2005) and the mechanism of σ^E activation are conserved in other spore-forming organisms (*C. acetobutylicum*, (Jones et al., 2011)). Additionally, σ^E-dependent operons *spoIIIAA-AH* (required for the activation of σ^G), *spoVR* (involved in cortex synthesis), and *spoIVA* (involved in cortex formation and spore coat assembly) are similar in *Bacillus* and *Clostridium* species (Jones et al., 2008). GerR, the SpoIVF and the SpoIVC operon are not conserved in *Clostridium* species (Paredes et al., 2005).

Stage III-V Prespore Sigma G

Activation of the late, sporulation stage III-associated (Cutting et al., 1990) forespore specific sigma factor σ^G (*spoIIIG*) requires the products of the $E\sigma^E$-transcribed *spoIIIA* operon in the mother cell and the action of SpoIIIJ in the prespore compartment (*B. subtilis*, (Eichenberger et al., 2004)). However, regulation of σ^G activity appears to be multilayered and macromolecular synthesis and σ^G-directed gene activation in the forespore depends on mother cell-forespore channels (possibly gap junction-like feeding tubes) through which the mother cell nurtures the developing spore by providing small molecules needed for biosynthetic activity (Camp and Losick, 2009). SpoIIIAH is targeted specifically to the membrane surrounding the forespore, through an interaction of its C-terminal extracellular domain with the C-terminal extracellular domain of the forespore membrane protein SpoIIQ and forms part (maybe by forming a ring structure) of such a channel that is required for the activation of σ^G (Meisner et al., 2008).
The genes coding for SpoIIIA, SpoIIIJ, and σ^G are conserved in *Clostridium* species (Paredes et al., 2005). In contrast to *B. subtilis*, adjacent *sigE* and *sigG* do not have a σ^F promoter between them (Paredes et al., 2004) and are expressed as a single transcript *spoIIGA-sigE-sigG* (*spoIIGA-sigE* and *sigG* transcripts also detected (Harris et al., 2002)).
σ^G controls the expression of over 110 genes in the forespore (*B. subtilis*, (Steil et al., 2005)) including *spoIVB* (required for the expression of the late mother cell-specific sigma factor, σ^K (Piggot and Hilbert, 2004)), the gene coding for BofC (SpoIVB inhibitor), *ssp* genes (coding for SASP, (Setlow, 1994, 1995)), and *spoVT* (coding for the DNA binding protein modulating transcription of genes by $E\sigma^G$, (Wang et al., 2006); modulating transcription of nutrient germination receptor genes, (Ramirez-Peralta et al., 2012a)).
In *Clostridium* species, genes under the control of σ^G also include those coding for SpoIVB, SpoVT and SASP (*C. acetobutylicum*, (Jones et al., 2008)), and σ^G is also required for the expression of the late mother cell-specific σ factor σ^K (*C. difficile*, (Piggot and Hilbert, 2004)). In contrast, BofC is not conserved in *Clostridium* species (Paredes et al., 2004).
Stage III-V mother cell sigma K

In *B. subtilis*, σ^K is formed by splicing together two genes (*spoIVCB* and *spoIIIC*), both under the control of σ^E and SpoIIID, separated by a skin element (Stragier et al., 1989). Synthesized in an inactive form (pro-σ^K) activation requires the expression of *spoIVB*, which is under the control of the forespore specific σ^G and the σ^E-controlled *spoIVF* operon (protease processing pro-σ^K to σ^K, SpoIVFB and its inhibitor, SpoIVFA) in the mother cell.
In conjunction with the σ^E controlled SpoIIID, Eσ^K transcribes genes responsible for DPA synthesis, *cot* genes, and *gerE* (transcriptional activator modulating Eσ^K action, leading to altered *cot* gene expression patterns (Cangiano et al., 2010)). Among the last genes transcribed by Eσ^K are genes involved in mother cell lysis (e.g., *cwlC*) (Piggot and Hilbert, 2004).
Interruption of the *sigK* gene and the requirement of the excision of a skin (*sigK* intervening sequence) element is very uncommon among spore-formers and was only found in *B. subtilis* and *C. difficile* (Haraldsen and Sonenshein, 2003). In other spore-formers there is a single gene encoding σ^K (e.g., *C. acetobutylicum*, (Nolling et al., 2001; Sauer et al., 1994)) likely under the control of *spoIIID* and σ^E. The genes coding for σ^K and SpoIIID but not GerE, SpoIVF, and SpoIVC are conserved in *Clostridium* species (Paredes et al., 2005). However, σ^K but not its important role has been proposed to be conserved among *Bacillus* and *Clostridium* species (Jones et al., 2008; Jones et al., 2011).

Temporal Orchestration

The sporulation sigma factors σ^F, σ^E and σ^G, and σ^K controlled genes are primarily (but not exclusively) involved in early, middle, and late sporulation, respectively. However, there are some exceptions, e.g., the σ^K-controlled phosphatase *yisI* acting on Spo0A~P or late sporulation genes (*yusW* and *yhbA*) under the control of the early sporulation sigma factor σ^F (*B. subtilis*, (Oomes et al., 2009)). Generally, the *Bacillus* model describing the temporal orchestration of known sporulation-related transcription factors with the cascade progressing in the order σ^H, Spo0A, σ^F, σ^E, and σ^G appears to hold true in *Clostridium* species. Additionally, the key sigma factors and major activating/processing proteins involved in sigma factor activation in *B. subtilis* are likely to play a similar role in *C. acetobutylicum* (Jones et al., 2008; Jones et al., 2011). Perturbations in the ordered course of the sporulation phosphorelay can cause problems in proper spore assembly and alterations in structural features of spores (Veening et al., 2006). This putatively plays a major role in altered sporulation conditions affecting resistance properties of spores from both genera.
In addition to these general commonalities there are major differences in the time span required for sporulation (e.g., 2–3 hours in *B. subtilis* and 8–20 hours between sporulation initiation and initial forespore formation in solventogenic *Clostridium* species, (Long et al., 1984; Santangelo et al., 1998)) and in the regulons of the specific sporulation sigma factors in *Bacillus* and *Clostridium* species. These major differences during sporulation, i.e., where spore properties determining their resistance, dormancy, and germinability are developed, certainly contribute to difficulties in transferring HPT inactivation strategies between these genera.

1.2.2.4 Germination and Outgrowth

Various environmental conditions can cause the conversion of a dormant spore back to an actively growing and doubling cell. Since the required apparatus is already present in mature dormant spores, germination is an essentially biophysical process, which does not require any new macromolecule synthesis (Moir, 2006). The complete process can be divided into three main events, i.e., germination, outgrowth, and doubling. The times a spore requires to pass through each of the individual stages (i) vary considerably (e.g., 11%, 71%, and 16% of the total lag time for non-proteolytic *C. botulinum* type B spores (Stringer et al., 2011)), (ii) represent independent events (e.g., it is impossible to predict the total lag from germination times), (iii) are not correlated for single spores within a population, (iv) are differentially affected by environmental sporulation and recovery conditions (e.g., presence of NaCl or temperature), and (v) can be dramatically changed by physical treatments such as heat and HHP (Stringer et al., 2011; Webb et al., 2007). The high heterogeneity in the time span a single spore within a population requires from the presence of suitable conditions to the first cell doubling, i.e., the total lag time, together with the high dependence of this time span on environmental factors and the history of a spore has major implications for food safety risk assessment (Augustin, 2011). Additionally, the heterogeneity in germination times in particular presents a major reason impeding a successful implementation of (two-step) spore inactivation processes relying on spore germination and aiming at the inactivation of an entire spore population.

Generally, germination can be triggered by the activation of germinant receptors responding to the presence of nutrients, directly by exogenous enzymes, or under certain physical conditions (e.g., HHP/HPT). However, pathways involved can vary considerably depending on the germination trigger and on the spore-forming species.

In addition to the importance of germination for food safety considerations (e.g., *C. botulinum* spores need to germinate and grow in a food product to produce the highly potent BoNT), germination and the concomitant loss of resistance properties plays a crucial role in several food preservation/sterilization strategies. Against this backdrop, this section aims at providing some helpful information on germinant receptor-dependent (Fig. 1-3) and -independent germination pathways considering differences between *Bacillus* and *Clostridium* spores.

1.2.2.4.1 Germinant Receptor-Mediated Germination

<u>Major Morphological Events</u>

Nutrient germinant receptors (nGRs) are highly conserved among spore-forming species (Paredes-Sabja et al., 2011). Although this class of receptors can also respond to non-nutrient low-molecular-weight germinants and be activated by external physical conditions, the term nGRs is used throughout this manuscript.

The nGR-mediated germination process comprises two major stages (Setlow et al., 2001; Setlow, 2003; Setlow and Johnson, 2013). In the beginning of stage I (stage I(i) in Fig. 1-3), specific chemical compounds, i.e., nutrient or non-nutrient germinants, have to penetrate outer spore layers (exosporium, coat, and cortex), bind to and activate receptor proteins, i.e., different substrate-specific nGRs localized in the inner spore membrane. This leads to the commitment of spores to germination. The time required for this first step, i.e., the germination initiation time, represents a major variable in kinetics of nGR-triggered spore germination and, at least in *B. subtilis*, largely accounts for the superdormancy of spores (Zhang et al.,

2012). After commitment, removal of the germinant or reversal of germinant binding can no longer interrupt germination, which indicates that a generally irreversible cascade of degradative processes is initiated downstream of the first germination stage. Later in stage I (stage I(ii) in Fig. 1-3), monovalent cations are released from the spore core (approx. 80% of Na^+ and K^+ in *B. megaterium* (Rode and Foster, 1966)) via an energy-independent process (Setlow, 2003). The release of H^+ elevates the core pH from ~ 6.5 to 7.7, which is essential for the initiation of spore metabolism later in germination. Furthermore, activated nGRs trigger the release of over 90% of the spore core's large depot of DPA present in the core as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (> 90% Ca release, *B. megaterium*, (Rode and Foster, 1966)) (Paidhungat and Setlow, 2000, 2001; Wang et al., 2011). The rapid loss of Ca-DPA marks the beginning of partial core rehydration (from approx. 35 to 45% water in *B. subtilis* (Setlow et al., 2001)), putatively requiring an increase of the inner spore membrane permeability. Partial rehydration at this point is not sufficient for protein mobility or enzyme activity (Cowan et al., 2003), but already causes some decrease in spore wet-heat resistance. This and the initiation of further degradative steps are the major reasons for Ca-DPA release being a crucial and rate-liming step in the inactivation of *Bacillus* spores by HHP and HPT treatments.

In germination stage II (Fig. 1-3), cortex lytic enzymes (CLEs), activated by the DPA release from the core, hydrolyze the cortex peptidoglycan (Setlow et al., 2001), which presents a prerequisite for complete core rehydration. Since spores contain preformed enzyme-substrate pairs with the substrates protected from degradation by regulatory mechanisms in the developing forespore and by dehydration in the resting spore, rehydration enables the rapid resumption of enzyme activity in the spore protoplast. For example, the PGM / 3PGA (phosphoglycerate mutase / 3-phosphoglyceric acid) and the GPR / SASP (germination protease / α-/β-type SASP) substrate / enzyme pairs, which are well characterized in *B. subtilis*, where they are stable for up to years, but interact with each other in the first 15 to 30 min after rehydration (Setlow, 1994). The core expands and rehydration allows for to the initiation of RNA and protein synthesis. Germinated spores completely lost spore-specific properties including dormancy, refractility, and their extreme resistance to physical stress including HHP/HPT treatments.

During outgrowth the fully germinated spore emerges from remaining spore structures (i.e., the coat layers) and turns into an immature cell. This is followed by a maturation process, in which the complete macromolecular set of a normal vegetative cell is restored, finally, leading to active cell division. Interestingly, the time required from this point to the first cell division after outgrowth can largely differ from the time that a normal cell needs for doubling and significantly contributes to the total lag time between nGR activation and growth (Stringer et al., 2011).

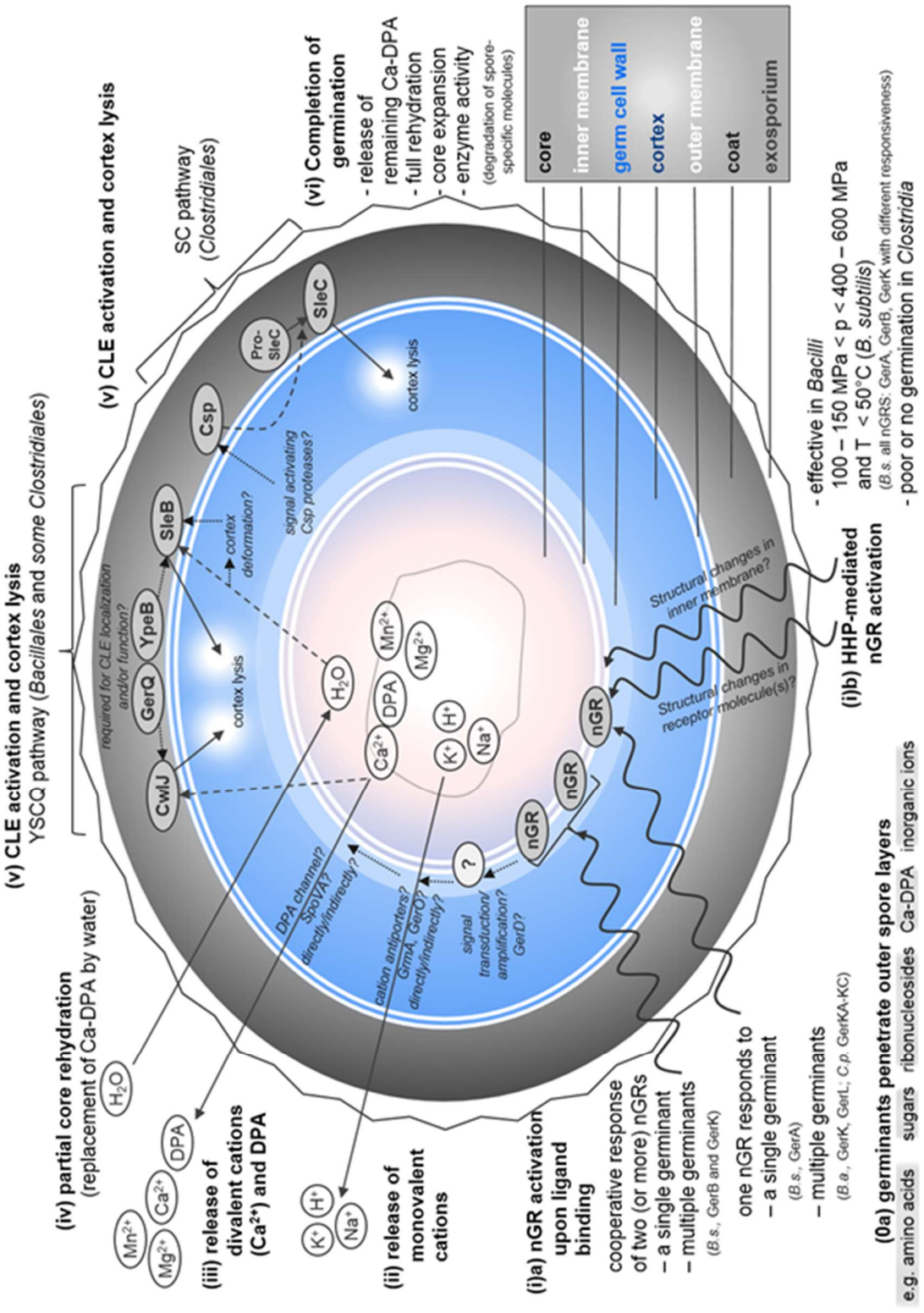


Fig. 1-3: Nutrient germinant receptor (nGR)-dependent germination
Pathways in *Bacillus* and *Clostridium* spores. Different spore compartments are labeled on the right. Note that the actual size of spore layers in relation to each other can vary considerably. The coat comprises multiple (up to four) distinct layers. Spores of some species possess no, some a very large exosporium. The position of the proteins involved in germination might not be depicted precisely and some of them are additionally present in other layers. Germination steps, which are not yet completely understood are described in italics. Curled arrows = exogenous activation events initiating germination; straight solid arrows = efflux or influx of molecules and enzymatic action on the cortex; dashed arrows = activation within the degradative cascade; dotted arrows = interaction mechanism not completely clear. *B.s.* = *B. subtilis*; *B.a.* = *B. anthracis*; *C.p.* = *C. perfringens*. Figure from (Lenz and Vogel, 2015) adapted from Paredes-Sabja et al. (2011).

Substances Acting as Nutrient Germinants

The nGR-mediated germination pathway (Fig. 1-3) can be triggered by numerous nutrients and non-nutrient agents. Nutrient germinants include a wide variety of amino acids (most prominently, L-alanine, effective in *Bacillus* and *Clostridium* spores (Peck, 2009)), sugars (e.g., glucose and fructose), and purine ribonucleosides (inosine and adenosine). Non-nutrient germinants that are capable of inducing physiologic germination via the activation of nGRs include exogenous Ca-DPA or inorganic ions (e.g., K^+).

Generally, a single compound can be sufficient to trigger germination, e.g., L-alanine in *B. subtilis* (Moir and Smith, 1990) and *B. anthracis* (Luu et al., 2011), L-cysteine and L-threonine in *B. cereus* (Hornstra et al., 2006b), L-Asparagine, KCl, or Ca-DPA in *C. perfringens* (Paredes-Sabja et al., 2008c), glucose in proteolytic *C. botulinum* (Chaibi et al., 1996; Foegeding and Busta, 1983), inosine in *B. cereus* (Abel-Santos and Dodatko, 2007), and salts such as KBr in *B. megaterium* (Setlow and Setlow, 1977)). Additionally, combinations of germinants can be required for the induction of germination presenting either an alternative to germination induced by single components, e.g., inosine together with amino acids in *B. anthracis* (Fisher and Hanna, 2005) and the AGFK mixture (L-asparagine, glucose, fructose, and potassium ions) in *B. subtilis* (Setlow, 2003), or the only way to induce germination, e.g., amino acids together with L-lactate (or other substances) in non-proteolytic *C. botulinum* (Plowman and Peck, 2002).

The presence of co-germinants, i.e., substance that are not essential for the induction of germination but can stimulate the activity of another co-germinant or germinant, can influence (enhance) germination (e.g., sodium bicarbonate or sodium thioglycolate in non-proteolytic *C. botulinum*, (Plowman and Peck, 2002); inorganic phosphate and sodium ions in *C. perfringens*, (Paredes-Sabja et al., 2009c); inorganic ions (Ca^{2+}) assist in low level inosine germination, *B. cereus* T, (Shibata et al., 1993)). On the other hand, different nutrient combinations can result in a negative effect on the induction of germination, e.g., L-methionine can impede L-serine- and L-valine-mediated germination in *B. anthracis* (Luu et al., 2011). For the same organism, further addition of L-histidine can counteract the negative effect on L-serine-, but not L-valine-mediated germination, which suggests that there can exist a complex interplay between the effects of different germinants (Luu et al., 2011).

In addition to co-germinants, a low environmental pH, contact with reducing agents, and, most prominently, a sublethal heat shock can activate spores, i.e., facilitate subsequent nutrient germination. Although heat shock requirements (effective activation temperatures) are species-specific, sublethal heat appears to be a common activator of both *Bacillus* and *Clostridium* spores (Garcia et al., 2010; Plowman and Peck, 2002). This effect might be related to changes in nGR proteins or their immediate vicinity altering receptor responsiveness or a facilitated access of germinants to the receptors (Alimova et al., 2006). Although it was demonstrated that a sublethal heat shock leads to the denaturation of some spore proteins, which appears to be a largely reversible process, the underlying mechanism is still not completely clear (Zhang et al., 2009).

The striking differences in the ability of bacterial spores to sense different nutrients appears to reflect their adaptation to different environmental niches (Paredes-Sabja et al., 2009c). In addition to the species-dependence, germination requirements can significantly vary among different strains of a species (e.g., *B. cereus*, (van der Voort et al., 2010)) and between individual spores within a population (Ghosh et al., 2009). Whereas the latter might be the

result of a stochastic process (Ghosh and Setlow, 2009), organism-dependent differences in the specificity for germinants initiating germination via the activation of germinants receptors is due to the differences in such highly specific receptors.

Germinant Receptor Synthesis and Properties

Nutrient GRs are thought to be synthesized in the forespore compartment of the developing spore (in *B. subtilis* transcribed under the control of the late sporulation sigma factor σ^G and regulated by transcription factors SpoVT and YlyA). Nutrient GRs are commonly incorporated into the inner spore membrane (e.g., *C. perfringens*, (Banawas et al., 2013); *C. botulinum*, (Alberto et al., 2005)) (Fig. 1-3) in low numbers, where they potentially co-localize (Setlow and Johnson, 2013) and associate with other small proteins modulating their function (Ramirez-Peralta et al., 2013).

Orthologues of the well-characterized *B. subtilis* nGR, GerA are highly conserved among spore-forming species (except *C. difficile*, (Paredes-Sabja et al., 2011; Ramirez et al., 2010)). However, the organization of genes encoding nGR subunits is heterogeneous in different spore-formers, where mono- through quattrocistronic *gerA* family operons can be found (approx. 50% tricistronic operons as in *B. subtilis*) (Paredes-Sabja et al., 2011). Although individual nGR-specificities for nutrients can broadly overlap (Luu et al., 2011), nGRs are generally highly substrate-specific, e.g., inosine analogs fail to induce germination and even small modifications of nucleobase or sugar moieties of inosine impede the inosine-triggered initiation of germination of *B. cereus* spores (Abel-Santos and Dodatko, 2007). Substrate-specificity frequently even includes stereospecificity (e.g., L-alanine might trigger but D-alanine can inhibit germination), which is determined by only a few amino acids within a germination protein (*B. subtilis*, (Paidhungat and Setlow, 1999)). This substrate-specificity of nGRs considerably varies between different species and strains, which is a major reason for differences in germination requirements.

Despite of the specificity of nGRs, the effectiveness of a certain germinant or mixture of germinants, i.e., germination rate (time required for germination) and germination efficiency (spore fraction within a population, which will germinate) depends on several other factors. Such factors include the germinant concentration, the number of functional nGRs in a spore, and their accessibility, i.e., permeability of outer spore layers for germinants. The passage of germinants through outer layers might be facilitated by specific coat proteins (e.g., GerP, *B. subtilis* and *B. cereus*, (Behravan et al., 2000); *B. anthracis*, (Carr et al., 2010)). Additionally, the presence of specific enzymes in the outer spore layers might be involved in modulating germination, i.e., converting substrates to effective or ineffective germinant molecules. For example, purine-specific nucleoside hydrolase present in the outer layers of various *Bacillus* spores (*B. cereus*, (Hornstra et al., 2006a); *B. anthracis*, (Redmond et al., 2004) was reported to suppress inosine- or adenosine-induced germination of *B. thuringiensis* spores (Liang et al., 2008), and spore-specific alanine racemase catalyzing the interconversion of D- and L-alanine can modulate L-alanine-induced germination in *B anthracis* (Chesnokova et al., 2009).

Generally, one nGR can respond to a single germinant (e.g., *B. subtilis* GerA, L-alanine, (Moir and Smith, 1990)) or multiple germinants (e.g., *C. perfringens* GerKA–GerKC, L-asparagine, KCl, Ca-DPA, (Paredes-Sabja et al., 2008c)). Additionally, multiple nGRs can respond cooperatively to single or multiple germinants (e.g., *B. subtilis* GerB and GerK, AGFK (L-asparagine, glucose, fructose and K^+ ions, (Setlow, 2003)). In addition to nutrient-triggered

germination, nGRs, appear to be also required for outgrowth in some organisms (e.g., *C. perfringens* GerKA–KC and GerKB, (Paredes-Sabja et al., 2011; Paredes-Sabja et al., 2008c)). However, the underlying mechanism is not clear.

Signal Transduction in the Germination Cascade

The exact process of nGR activation and the nature of signal transduction after their activation, i.e., the spores' commitment to germination, both remain to be elucidated even in well-characterized model organisms. The lipoprotein GerD, which is localized in the inner membrane of *Bacillus* spores (Pelczar et al., 2007; Pelczar and Setlow, 2008) in discrete clusters with nGRs (*B. subtilis*, (Griffiths et al., 2011)), but absent in *Clostridiales* species, has been discussed as being involved in signal transduction form activated nGRs to proteins acting downstream in the germination process (Chen et al., 2014; Paredes-Sabja et al., 2011) (Fig. 1-3). For the release of monovalent cations, cation antiporters could play a role (e.g., GrmA in *B. megaterium*, (Tani et al., 1996); GerO (and GerQ) in *C. perfringens*, (Paredes-Sabja et al., 2009a)). However, this role is questionable as it might be only indirect (during sporulation) and appears to be species- and even strain-dependent (*B. megaterium*, (Christie and Lowe, 2007)), and not involved in germination triggered by different germinants (inosine but not L-alanine germination in *B. cereus*, (Senior and Moir, 2008)). Additionally, proteins encoded by the *spoVA* operon that are involved in the uptake of Ca-DPA into the developing spore (Li et al., 2012) can putatively physically interact with nGRs and might be involved in the release of Ca-DPA (e.g., by DPA channel formation) at least in some *Bacillus* species (Paredes-Sabja et al., 2011). However, the exact mechanism and if SpoVA proteins are directly involved in germination or contribute indirectly via its role during sporulation is not clear.

Cortex Hydrolysis

Cortex lytic enzymes (CLEs) are synthesized during sporulation in the forespore, the mother cell, or both compartments, localized in proximity to the inner and outer cortex surfaces can have different binding-specificities but typically recognize and cleave cortex PG exclusively, which is thought to be due to the presence of cortex PG-specific muramic acid δ-lactam (Makino and Moriyama, 2002) (Fig. 1-3).

Two distinct pathways for cortex hydrolysis are highly conserved in *Bacillales* and *Clostridiales* species, i.e., the YpeB–SleB–CwlJ–GerQ (YSCQ) and the Csp–SleC (CS) pathway. *Bacillales* species commonly rely on the YSCQ pathway, while the CS system appears to be generally absent. In contrast, *Clostridiales* can utilize either one or both pathways (Paredes-Sabja et al., 2011). Additionally, other CLEs can be present in spore-forming species, which might be not essential for germination (e.g., SleM present in *B. weihenstephanensis* and some *Clostridium* species (Paredes-Sabja et al., 2011)). In a few species lacking obvious orthologues of the proteins involved in cortex hydrolysis via the YSCQ or CS pathway various different potential CLEs are present, which might be involved in different mechanisms of cortex hydrolysis (Paredes-Sabja et al., 2011). This, however, remains to be elucidated.

In the YSCQ pathway, either one of the two CLEs SleB and CwlJ is sufficient to complete germination. CwlJ is thought to be primarily located in the spore coat possibly associated with other coat proteins (Bagyan and Setlow, 2002) and requires GerQ, which is cross-linked into high-molecular-mass complexes in the spore coat by a transglutaminase (Tgl) (Monroe and Setlow, 2006). Cortex hydrolysis by CwlJ can be triggered by both Ca-DPA released from

the spore core during germination and by exogenous Ca-DPA. SleB is thought to be localized in the coat, the cortex and putatively also in the inner membrane (*B. subtilis*, (Chirakkal et al., 2002). Proper localization has been demonstrated to require the interaction between the SleB peptidoglycan-binding motif and the δ-lactam structure of the cortex (Masayama et al., 2006). Additionally, YpeB, which can be found in the same locations as SleB (Chirakkal et al., 2002), is required for proper SleB localization, stabilization, activation, and/or function. SleB activation is putatively triggered by partial core rehydration and the accompanying deformation of the cortex (Fig. 1-3).
In the Csp–SleC (CS) pathway, the CLE SleC is essential for cortex hydrolysis during spore germination (Paredes-Sabja et al., 2009b). SleC is an exo-acting lytic transglycosylase possessing a peptidoglycan-binding domain (Gutelius et al., 2014), which might be localized in the spore similar to SleB. In contrast to SleB and CwlJ, SleC is synthesized as a precursor and exists as an inactive zymogen (pro-SleC) in dormant spores. Thus, SleC requires activation by germination specific serine proteases (Csp proteases, e.g., encoded by the *cspABC* operon in *C. difficile* (Adams et al., 2013)) to initiate cortex hydrolysis (Paredes-Sabja et al., 2011). However, the CS germination pathway is not activated upon Ca-DPA release (as it is the case for CwlJ) or partial core rehydration and cortex deformation (as it is the case for SleB) and it is not clear how the activity of Csp proteases is regulated in nGR-mediated germination.

1.2.2.4.2 Germinant Receptor-Independent Germination

In addition to the initiation of germination via the activation of nGRs in response to favorable nutrient conditions, specific nGR-independent germination pathways allow spores to respond to the presence of host-specific factors and to sense germination and vegetative growth occurring in their environment (Fig. 1-4).
For example, in addition to germination via the activation of nGRs (e.g., in *C. perfringens* (Paredes-Sabja et al., 2011)), exogenous Ca-DPA can directly activate the CLE CwlJ in some species resulting in cortex hydrolysis and complete germination (e.g., *B. subtilis*, (Setlow, 2003)). Although concentrations of Ca-DPA released by naturally occurring spore populations might be low and possibly too low to efficiently trigger germination of spores, exogenous Ca-DPA could play a role in sensing spore germination in their environment. An example for the response to host-specific factors can be found in the germination-specific protease CspC, which can act as germinant receptor and is activated in *C. difficile* spores in the presence of bile acids (Francis et al., 2013). This leads to the proteolytic activation of the cortex hydrolase SleC and enables *C. difficile* spores, in which orthologues to common nGRs have not yet been identified (Paredes-Sabja et al., 2011), to initiate germination specifically in mammal intestines (Adams et al., 2013). Furthermore, enzymes with peptidoglycan hydrolyzing activity, e.g., host serum germination factor (lysozyme) can trigger sporulation by directly degrading the PG of spores (*B. anthracis*, (Giebel et al., 2009; Giebel et al., 2012)). Although many spores are well protected against cortex lysis by lysozyme due to the impermeability of their proteinaceous coat layers, lysozyme can be active in initiating germination when outer spore layers are severely damaged (Peck and Fernandez, 1995). Since Ca-DPA release triggered by a lysozyme treatment of decoated *B. subtilis* spores requires SpoVA proteins, it has been speculated, that Ca-DPA channel opening presents a step in lysozyme-induced germination (Vepachedu and Setlow, 2007).

A highly conserved mechanism (including non-spore formers) (Paredes-Sabja et al., 2011), which is (putatively) completely independent from nGRs and proteins involved in the nGR-mediated initiation of germination, involves PrkC as germinant receptor. PrkC is an eukaryotic-like protein kinase, which is localized in the inner spore membrane, phosphorylates serine/threonine residues, and enables spores to sense growth of cells of the same or closely related species via the detection of PG fragments from growing but not from lysed cells (Shah et al., 2008). Such muropeptides functioning as germinants are thought to activate PrkC by physically binding to the extracellular region of PrkC through interactions mediated by their mesodiaminopimelic acid (DAP) moiety (Squeglia et al., 2011). Divalent cations (Zn^{2+}) were shown to influence the activity of PrkC and its cognate phosphatase PrpC in *B. anthracis* (Arora et al., 2013) and possible substrates phosphorylated by PrkC have been identified including the ribosomal GTPases EF-G and CpgA, and the elongation factor EF-Tu (Shah and Dworkin, 2010). However, the exact mechanism by which PrkC triggers spore germination remains to be elucidated.
Finally, germination can also be induced by cationic surfactants such as dodecylamine (Rode and Foster, 1960). The role of surfactants as non-nutrient germinants in spore germination in the environment is not clear. Dodecylamine bypasses nGRs to trigger spore germination, most likely by causing DPA release in some fashion (Setlow et al. 2003). In this process it has been speculated that either dodecylamine directly opens Ca-DPA channels, where SpoVA might participate in channel formation, or alters inner membrane properties resulting in the release of Ca-DPA, (*B. subtilis*, (Vepachedu and Setlow, 2007). Ca-DPA release then triggers subsequent germination events similar to the nGR-mediated germination pathway.

In addition to responding to various nutrient and non-nutrient germinants as described above, spore germination can also be triggered by physical treatments. It has been reported that violent agitation in the presence of glass beads, i.e., mechanical abrasion, can trigger germination of *B. subtilis* spores, which might occur due to physical damage of the cortex structure inducing CLE activation, i.e., either CwlJ or SleB (Jones et al., 2005)).
As indicated in Fig. 1-3 and Fig. 1-4 and more relevant to food processing and the results of this study, HHP and HPT can trigger the germination of spores from various species via nGR-dependent (at least in *Bacillus* species; Fig. 1-3) and -independent (Fig. 1-4) pathways, which plays an important role in HP spore inactivation strategies.

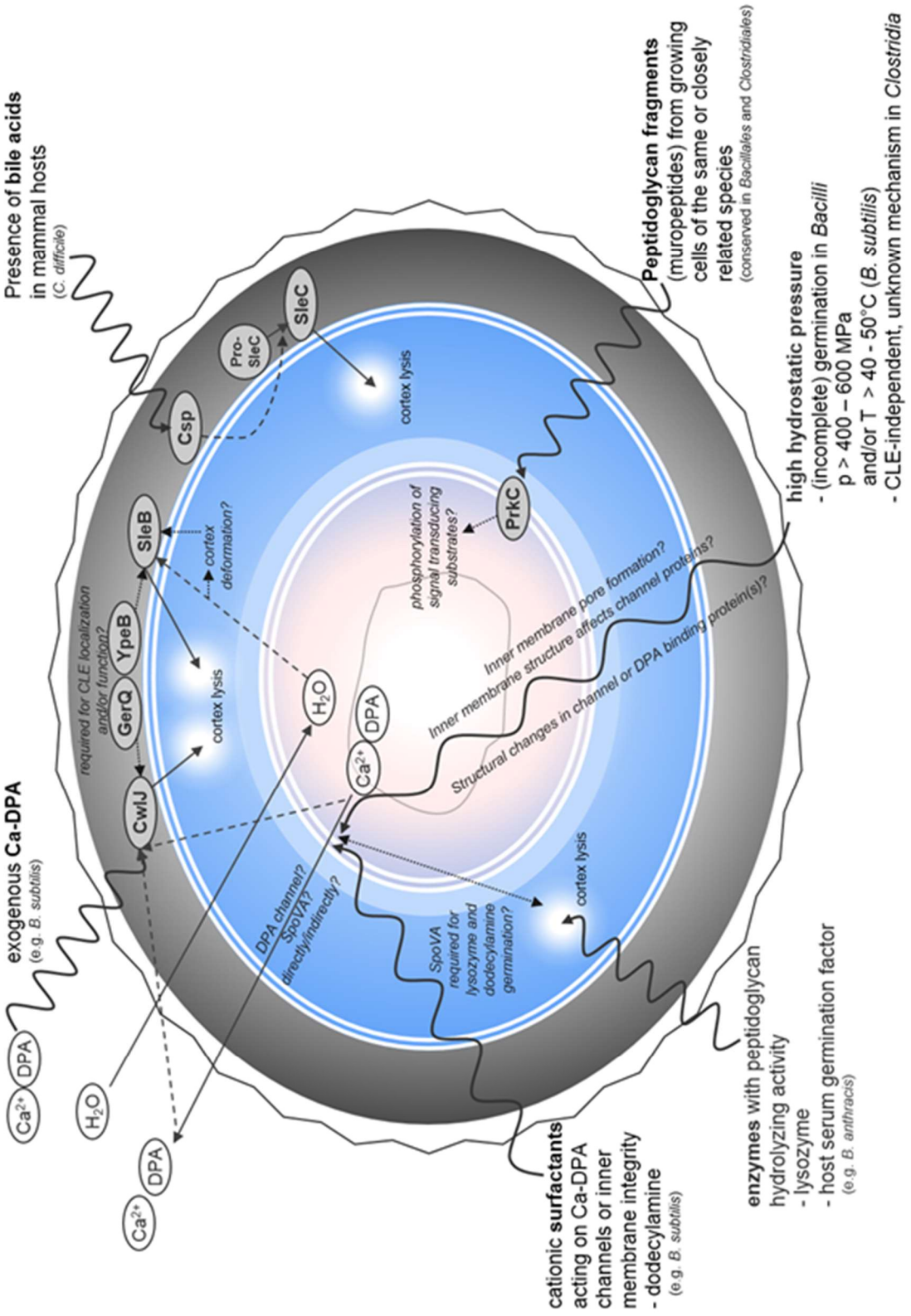


Fig. 1-4: Nutrient germinant receptor (nGR)-independent germination
Pathways in *Bacillus* and *Clostridium* spores. The different spore compartments are identical to those described in Fig. 23.3. The position of the proteins involved in germination might not be depicted precisely. Events, which are not yet completely understood, are described in italics. Curled arrows = exogenous activation events initiating germination; straight solid arrows = efflux or influx of molecules and enzymatic action on the cortex; dashed arrows = activation within the degradative cascade; dotted arrows = interaction mechanism not completely clear. Figure from (Lenz and Vogel, 2015). Data for figure adapted from Paredes-Sabja et al. (2011) and: (Adams et al., 2013; Chen et al., 2014; Francis et al., 2013; Francis et al., 2015).

1.2.3 Spore Structure

In addition to understanding sporulation and germination processes, i.e., to understand processes by which spores gain and lose their dormancy and their high resistance to various type of stresses, a closer look at the spore structure and the properties of its different compartments can help to identify factors making spores this resistant.
As already indicated in Fig. 1-3, the spore structure generally consists of three major compartments separated by the inner and outer membrane, i.e., the **core**, the inner membrane, a **peptidoglycan layer** (i.e., germ cell wall and cortex), the outer membrane, and an **outer layer** consisting of the coat and the exosporium (the latter layer may be absent or is sometimes referred to as part of the coat). The composition and the most important properties of each of the different spore compartments are described below. In case no specific information on the species or genera is stated, described findings refer to the model organism *B. subtilis*, for which the overwhelming majority of data was generated in the past.

1.2.3.1 Spore Core

In the transition between sporulation stages III and IV, there are dramatic alterations in the inner forespore compartment (Fig. 1-1). The pH of the forespore drops around 1 – 1.5 units, dehydration begins, and SASP (small acid soluble proteins) are formed, which bind to the forespore chromosome arranging it in a ring-liked shape. This relatively early event was reported to already lead to resistance to UV light and some chemical agents. Dehydration of the forespore interior continues during stages V and VI. In the transition between these two stages, the forespore also accumulates high levels of divalent cations and dipicolinic acid (DPA). The spore core contains DNA, ribosomes, enzymes required for metabolism reactivation, a large SASP pool, DPA, and high amounts of various divalent cations. Lower pH, dehydration, SASP accumulation, divalent cations, and DPA were reported to contribute to the dormancy and resistance properties of bacterial endospores (primarily shown for wet heat resistance of *Bacillus* spores, (Setlow and Johnson, 2007)).

1.2.3.1.1 Dehydration and Low pH

Water was shown to be distributed unequally within fully hydrated dormant spores, i.e., the relatively dehydrated protoplast is surrounded by an integument containing significantly more water. Reported protoplast water content values range from about 20 or 30 to approx. 50% wet weight of a spore (*B. subtilis*, (Gould, 1999; Setlow and Johnson, 2007)). However, higher core water contents were also reported and the actual core water amount depends on numerous factors such as sporulation conditions (e.g., temperature, cations) as well as strain and species. Values of 28 – 61 % core water content were reported for various *Bacillus* species (*B. megaterium*, (Beaman et al., 1984); *B. stearothermophilus*, *B. subtilis*, *B. cereus*, *B. thuringiensis*, (Beaman and Gerhardt, 1986; Nakashio and Gerhardt, 1985); *B. coagulans*, *B. caldolyticus*, *B. macquariensis*, (Beaman and Gerhardt, 1986); *B. sphaericus*, (Beaman et al., 1989). In addition to the total water content, the physical state of water in the different spore compartments is thought to be important for spore dormancy and resistance properties. The small amount of core water was shown to be relatively mobile at ambient temperatures (Nguyen Thi Minh et al., 2010). However, proteins are largely immobilized preventing irreversible aggregation after denaturation (Cowan et al., 2003; Moir, 2003; Sunde et al., 2009). In addition to the low water content, the core also has a relatively low pH compared to

vegetative cells. In *B. cereus* (Setlow and Setlow, 1980), *B. megaterium* (Magill et al., 1994; Setlow and Setlow, 1980), and *B. subtilis*, it was demonstrated that the pH of the forespore compartment drops during sporulation irrespective of the external pH and that values of vegetative cells and spores are around 7.6 – 8.1 and 6.3 – 6.9, respectively. The low pH, which rises again during germination, was reported to regulate the activity of the enzymes such as phosphoglycerate mutase involved in 3-phosphoglyceric acid accumulation during sporulation (Magill et al., 1994) and to be an important factor that contributes to the metabolic dormancy of spores (Setlow and Setlow, 1980).

1.2.3.1.2 Protective and Metabolism-Related Molecules

DPA

Dipicolinic acid (pyridine-2,6-carboxylic acid; DPA) is a characteristic component of bacterial spores, present in the core as a 1:1 chelate with divalent cations (predominantly Ca^{2+}), and involved in the establishment of the low core water content, spore dormancy, wet-heat resistance, and germination (Paidhungat et al., 2000).

In *B. subtilis*, other *Bacillus* and some *Clostridium* species, the precursor dihydroxydipicolinic acid (DHDPA) is produced by DHDPA synthase within the lysine biosynthesis pathway and oxidized to DPA by products of the *spoVF* operon (Daniel and Errington, 1993). In pathogenic *Clostridium* species including *C. botulinum*, *C. perfringens*, and *C. tetani*, no *spoVF* orthologues can be found. However, experiments using *C. perfringens* showed that it is likely that the electron transfer flavoprotein EtfA is in charge of the oxidizing step form DHDPA to DPA, which might be a conserved mechanism in other *Clostridium* species lacking *spoVF* (Popham et al., 2010). DPA is putatively co-imported with calcium from the mother cell compartment during sporulation (Hintze and Nicholson, 2010) and released as Ca-DPA during the first minutes of germination (Dring and Gould, 1971). Proteins involved in DPA transport are putatively encoded by the *spoVA* operon (Tovar-Rojo et al., 2002) such as the putative DPA binding protein SpoVAD (Li et al., 2012). Reported DPA contents of *B. subtilis* spores, which were shown to be largely strain-dependent, range from 0.365 (Hindle and Hall, 1999) up to 0.43 – 0.85 fmol DPA/spore (Kort et al., 2005). However, the presence of DPA in a range between 5 – 20% of the dry weight of the core makes DPA a major component of this innermost spore region (Popham et al., 2010; Setlow and Johnson, 2007).

Cations

In addition to the important role of calcium complexed with DPA, the mineral content of spores was reported to be important for spore resistance and dormancy. An early spectrochemical analysis of twelve aerobic spore formers including *B. cereus*, *B. megaterium*, *B. subtilis*, *B. macerans*, *B. cohaerens*, and *B. albolactis* showed that spores have markedly higher Ca and slightly higher Mn and Cu contents, whereas K and P contents tend to be lower in spores than in the respective vegetative cells. The uptake of Mg, Fe, and Al was reported to be strain-dependent with no big difference between vegetative forms and spores (Curran et al., 1943). Levels of Ca, Mg, and Mn in spores were reported to range between 1.0 – 2.7 %, 0.3 – 1.0 %, and 0.003 – 0.011 % of their dry weight, respectively (Curran et al., 1943). High levels of such minerals in spores were confirmed in later studies, where, in spores from *B. cereus*, *B. megaterium*, and *B. subtilis*, the average contents of the putatively most important cations, Ca^{2+}, Mg^{2+}, and Mn^{2+}, were reported to range between 1.52 – 3.67 % (380 – 916 µmol), 0.21 – 0.29 % (86 – 120 µmol), and 0.15 – 0.31 % (27 – 56 µmol) per g dry weight,

respectively (Loshon and Setlow, 1993; Setlow, 1994; Setlow and Johnson, 2007). In both intact and sectioned spores, calcium was distributed throughout the spore, similarly to carbon, and concentrated mainly in a central region corresponding to the spore protoplast (*B. cereus*, *B. megaterium*, (Scherrer and Gerhardt, 1972); *B. megaterium*, (Nishihara et al., 1982)). Consistently, electron micrographs of cryosectioned *B. cereus* T spores indicated that almost all of the Ca, Mg, and Mn and most of the phosphorus is located in the spore protoplast and only a small amount can be found in the integuments (Stewart et al., 1980). Interestingly, it was also found that spores with lower DPA levels contain granules with high concentrations of Ca, Mn, and phosphorus (Stewart et al., 1980).

SASP

Small, acid-soluble proteins (SASP) are synthesized in the forespore during sporulation stage III (σ^G-dependent) and account for approx. 10% (7–20% (Hayes et al., 2001)) of the total spore protein (Setlow and Johnson, 2007). *B. subtilis* spores contain a large SASP pool consisting of α-/β-type SASP (60 – 75 aa, 3 – 5% of spore protein, associated with and protecting DNA), γ-type SASP (75 – 105 aa, 5% of spore protein, being degraded and functioning as amino acid source during outgrowth, absent in *Clostridium* spores), and minor SASP (34 – 71 aa, unknown function). The α-/β-type SASP are conserved across species, are able to interact (primarily) with the DNA sugar phosphate backbone, and, thus, bind to double stranded DNA (not to RNA or single stranded DNA) in a sequence-unspecific manner, but with high affinity to G-C rich DNA (*B. subtilis*, (Setlow et al., 1992)). The latter fact is largely dependent on the charge of the first few N-terminal residues of the SASP (Hayes et al., 2001). The SASP-saturated chromosome adopts a ring-like toroidal structure. SASP protect DNA from physical stress (e.g., UV radiation or heat), cleavage by hydroxyl radicals or orthophenanthroline-Cu^{2+}, and digestion by DNase or restriction enzymes. Spore core DNA is usually saturated with SASP, i.e., protein/DNA ratio of 4:1 to 5:1 (wt/wt) or 1 SASP per 4 bp (Setlow et al., 1992). The other way round, SASP bound to DNA are protected against protease digestion (Setlow and Setlow, 1995). Although the binding of SASP to DNA is strong, these molecules have to be rapidly degraded to amino acids early in germination, i.e., too strong binding would lead to perturbed outgrowth due to SASP interfering with transcription (Hayes and Setlow, 2001). In addition to SASP, the spore nucleotide contains the major protein associated with vegetative cells, HBsu, which was shown to modulate effects of α-/β-type SASP on spore DNA properties (Ross and Setlow, 2000).

Other molecules

In addition to small protective molecules, spores possess some metabolism-related molecules either exclusively or in largely different amounts compared with their respective vegetative cells. For example, levels of total acetyl coenzyme A (CoA) are lower in spores than in the respective vegetative cells, but CoA in disulfide linkage to another CoA or other proteins was reported to accumulate exclusively in developing spores during late sporulation, i.e., at about the same time when dipicolinic acid is accumulated. During germination, CoA-protein linkages are cleaved by NADH-dependent CoA-disulfide reductase (*B. megaterium*, *B. cereus*, and *C. bifermentans*, (Setlow and Setlow, 1977)). Additionally, spores accumulate a large depot of 3-phosphoglyceric acid (3PGA, (Setlow and Johnson, 2007)). On the other hand, spores and cells presumably have almost identical contents of DNA, rRNA, tRNA, and many enzymes including those important for RNA/protein synthesis, DNA repair systems, amino acid catabolism, and carbohydrate catabolism (Setlow and Johnson, 2007).

Vegetative cell molecules that were shown to be absent in spores include amino acid and nucleotide synthesis enzymes and common highly energetic compounds (ATP, ADP, deoxynucleotides, NADH, NAD, NADPH, NADP, Acyl-CoA, (Setlow and Johnson, 2007)).

1.2.3.2 Inner Membrane

The inner spore membrane is formed very early in sporulation as a result of the phagocytose-like engulfment of the forespore in the transitions between sporulation stages II and III. It simply constitutes the inner membrane of the double membrane, which is derived from the septum formed in sporulation stage II (Piggot and Hilbert, 2004). Thus, the inner forespore membrane is a functional membrane with a phospholipid content similar to that of growing cells (Cowan et al., 2004). However, the volume surrounded by this membrane is smaller than it would be expected from the phospholipid content and largely expends during germination without membrane synthesis, which indicates that this membrane is densely packed (Cowan et al., 2004).

The inner membrane has two major properties involved in spore resistance and dormancy. It presents an extremely strong permeability barrier for almost all molecules and therefore confers spore resistance to chemicals that damage DNA (Cortezzo and Setlow, 2005) (Westphal et al., 2003). Additionally, the high degree of lipid immobilization in this membrane leads to a low permeability even to water, which has been described as being at least 2 orders of magnitude lower than for model membranes and protects the core from dehydration important for spore dormancy (Sunde et al., 2009). Secondly, the inner membrane harbors important proteins such as proteins involved in germination (e.g., nutrient germinant receptors GerA, B and K (Setlow, 2003), and GerD, a predicted lipoprotein required for efficient l-alanine and amino acids plus sugars spore germination in *B. subtilis* (Mongkolthanaruk et al., 2009)) and proteins essential for the uptake of the 1:1 CA-DPA into developing spores (e.g. SpoVA proteins in *B. subtilis* such as the Ca-DPA and DPA binding SpoVAD (Li et al., 2012)).

1.2.3.3 Germ Cell Wall

The primordial germ cell wall is assembled at the surface of the inner (pre)spore membrane in the transition between sporulation stages III and IV, represents a thin layer of peptidoglycan with an almost identical composition compared to the vegetative cell wall (Warth, 1978) and serves as primordial wall of vegetative cells after spore germination and template for the peptidoglycan of the outgrowing new vegetative cell wall (Tipper and Linnett, 1976) (Popham, 2002). Additionally, this peptidoglycan layer has been suggested to harbor germination associated proteins such as GerD, as it has been detected in coat-defective spores and the cell wall fraction of outgrowing cells. However, GerD can be also found in the inner membrane and it is not known, which of the multiple locations is important for its function (Mongkolthanaruk et al., 2009). Spore peptidoglycan is comprised of two distinct though contiguous layers, the germ cell wall and the cortex (Popham et al., 1996a).

1.2.3.4 Cortex

The spore cortex is a thick peptidoglycan layer located between the germ cell wall and the outer spore membrane and crucial for the establishment and the maintenance of spore core dehydration, mineralization and dormancy (Driks, 1999) (Henriques and Moran, 2000). It is composed of the same amino acids and sugar constituents as found in vegetative cell wall

peptidoglycan but is more negatively charged and has a chemically distinct structure (Fig. 1-5).

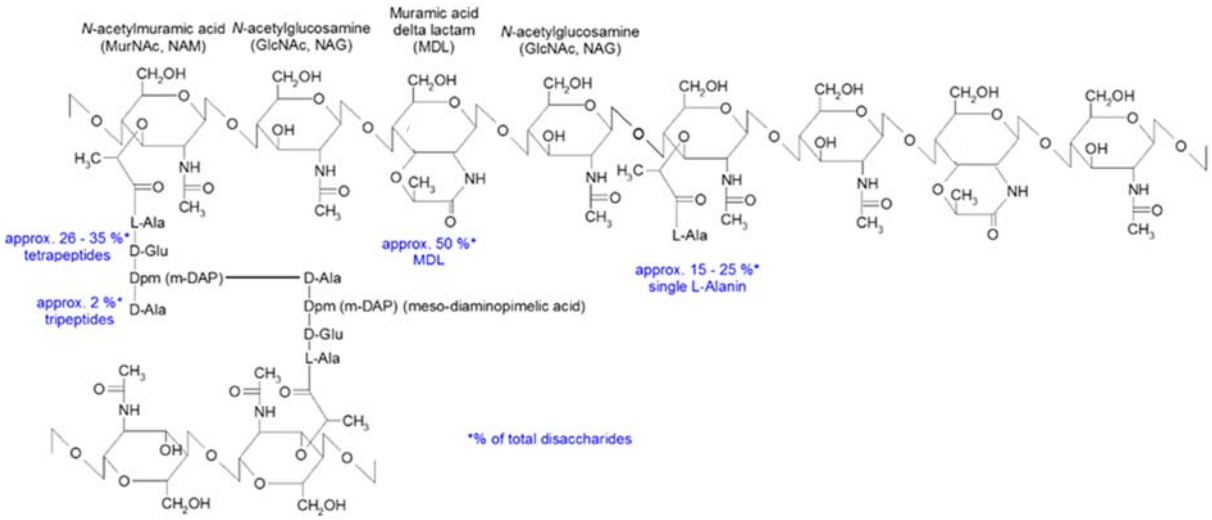


Fig. 1-5: Cortex peptidoglycan structure
The general structure of spore cortex peptidoglycan is depicted. Values in blue indicate the abundance of a specific disaccharide type in % of the total disaccharide content within the cortex layer of *B. subtilis* spores.

The degree of cortex cross-linking is reduced by partial cleavage or complete removal of approx. 75% of the peptide side chains from the glycan strands (Popham et al., 1999a), which results in only one cross-linked peptide per 35 disaccharide units corresponding to approx. 3 % (Atrih et al., 1996) (Popham et al., 1999a) up to 9 % (Warth, 1978) cross-links in *B. subtilis* spore peptidoglycan as compared to approx. 33% (Forrest et al., 1991) in vegetative cells. This low degree of cross-linking has been suggested to contribute to the flexibility of this layer, which is might play a role in attainment and maintenance of core dehydration (Lewis et al., 1960) (Popham et al., 1999a). In contrast to the cell wall peptidoglycan every alternate disaccharide (about 50% of all disaccharides) are substituted with muramic acid δ -lactam residues (Warth and Stroming.Jl, 1972) (Atrih et al., 1996) (Horsburgh et al., 2003) (Popham et al., 1996b), which represents a unique feature of the dormant spore cortex and their presence is crucial for substrate recognition by the lytic enzymes responsible for cortex degradation in *B. subtilis* (i.e., SleB, CwlJ) during germination (Atrih et al., 1998). Remaining residues have been reported to be substituted with a tetrapeptide (L-alanine–D-glutamic acid–meso-diaminopimelic acid–D-alanine) (26 to 35%) (Horsburgh et al., 2003), single L-alanine substituent with a free carboxyl group (15 to 23% (Warth, 1978); about 20% (Horsburgh et al., 2003); about 25% (Atrih et al., 1996; Popham et al., 1996a)) or tripeptide residues (up to 2%) (Atrih et al., 1996) in *B. subtilis*.

The basic molecular structure of spore peptidoglycan has been shown to be highly conserved among different species including *B. cereus*, *B. megaterium*, *B. subtilis*, and *C. botulinum* with the only major difference being the level of de-*N*-acetylation of an amino sugar, most likely glucosamine, which can modulate the access of lytic enzymes to their peptidoglycan substrate such as lysozyme or cortex lytic enzymes involved in the germination process (Atrih and Foster, 2001a).

Assembly begins at the inner surface of the outer membrane in the transition between sporulation stages III and IV, is predominantly accomplished by the mother cell and depends

on the successful initiation of coat assembly, which is probably controlled via a developmental checkpoint involving SpoVM and CmpA where the σ^E and SpoIIID controlled CmpA is removed by a post-translational mechanism after the successful initiation of coat formation (Ebmeier et al., 2012). Cortex synthesis in sporulating *B. subtilis* cells has been reported to be triggered by the activation of the sporulation-specific, membrane-bound, high molecular weight, class B penicillin-binding protein SpoVD via the breakage of a disulphide bond in SpoVD. This is putatively catalyzed by the membrane-bound thioredoxin-like protein StoA (SpoIVH), which is relate to thiol-disulphide oxidoreductases previously shown to be capable of catalyzing disulphide bond formation or breakage to control the red-ox status of a variety of proteins (Liu et al., 2010) (Eichenberger, 2010).

Peptidoglycan polymerization is done by penicillin-binding proteins (PBP) (review: (Ghuysen, 1991)). High-molecular-weight PBP (approx. 60kDa) with transglycosylase and transpeptidase activities are involved in polymerization and cross-linking of the glycan strands, whereas low-molecular-weight PBP with D,D-carboxypeptidase activity modify the peptide side chains and, thus, prevent the side chain from serving as a peptide cross-link donor. Low-molecular weight PBP identified in *B. subtilis* include *dacA* ((Todd et al., 1986) = PBP5 (Lawrence and Stroming.JI, 1970), low expression during sporulation (Sowell and Buchanan, 1983), cleaves peptide side chains from tetra- to tripeptide (Popham et al., 1996a), no effect on growth, sporulation, spore characteristics or spore germination (Todd et al., 1986) (Buchanan and Gustafson, 1992)), *dacB* ((Buchanan and Ling, 1992) = PBP5* (Todd et al., 1985), mother cell σ^E controlled (Buchanan and Ling, 1992) (Simpson et al., 1994), (Sowell and Buchanan, 1983), increases tetrapeptide substituted muramic acid residues in glycan backbone (Atrih et al., 1996), prevents peptidoglycan cross-links (Atrih et al., 1996) slight effect on dehydration and resistance (Popham et al., 1995a) (Popham et al., 1999b)), *dacC* ((Pedersen et al., 1998) early stationary phase σ^H controlled no D,D-carboxypeptidase activity) and *dacF* (Wu et al., 1992), forespore σ^F controlled no D,D-carboxypeptidase activity but regulates degree of cross-linking together with DacB (Popham et al., 1999a)).

While the time course of cortex biosynthesis has been determined in detail (Popham, 2002), the distribution of cross-links throughout the cortex is not known in detail. However, a cross-link gradient model has been proposed (Popham et al., 1999a) with the innermost layers being loosely and the outer layers being highly cross-linked, which has been speculated to be dependent on the time and/or location resolved activity of PBP5* (*dacB*) and DacF, maybe due to the association of DacF with the inner forespore membrane, decrease in its enzymatic activity and/or decreased expression in combination with effects decreasing the relative activity of PBP5* (*dacB*) such as expansion of the surface area over which cortex synthesis is taking place, protein instability and/or reduced expression (Simpson et al., 1994) (Popham et al., 1999a).

Additional genes that have been reported to be involved in *B. subtilis* cortex formation include *ybaN* and *ytrH* (cortex formation blocked in mutants (Silvaggi et al., 2004)), *ytrI* and *ytrHI* (cortex degraded in mutants (Silvaggi et al., 2004)), *cwlD* and *pdaA* (CwlD = sporulation specific amidase and putative peptidoglycan hydrolase (Atrih et al., 1996), PdaA = polysaccharide deacetylase, both involved in formation of δ-lactam muramic, spore formation but no cortex hydrolysis and outgrowth in mutants (Popham et al., 1996b) (Fukushima et al., 2002)), *gerJ* (possibly by influencing *dacB* expression (Warburg et al., 1986)), *lytH* (L-Ala–D-

Glu endopeptidase, formerly YunA, production of the single L-Ala side chains by cleaving nascent non-cross-linked tetrapeptide side chains, σ^K controlled, δ-lactam independent (Horsburgh et al., 2003)).
Although not all of the enzymes are conserved among different spore formers, especially the percentages of muramic acid delta lactam substituted disaccharides and the resulting low degree of cross-linking have been reported to be similar in cortex peptidoglycan of spores of other species (e.g., *C. perfringens* muramic acid delta lactam : tetrapeptide : tripeptide : dipeptide : no peptide = 49.9 : 42.9 : 2.9 : 2.1 : 2.2% with a crosslinking degree of 1.5% (Orsburn et al., 2008)).

1.2.3.5 Outer membrane

Like the inner spore membrane, the outer spore membrane is formed very early in sporulation as a result of the phagocytose-like engulfment of the forespore in the transitions between sporulation stages II and III. It simply constitutes the outer membrane of the double membrane, which is derived from the septum formed in sporulation stage II (Piggot and Hilbert, 2004). However, it is important in serving as assembly site of cortex and coat structures with crucial morphogenetic coat proteins being anchored to this membrane (Ramamurthi et al., 2006).

1.2.3.6 Coat

The spore is surrounded by the spore coat, a multilayered proteinaceous shell, which plays an important role in spore resistance, dormancy, and germination. Various studies using electron microscopic analyses revealed strong variations in the morphology of all classes of external spore structure (Driks, 2007) including the number of coat layers and the presence, size, and structure of the exosporium layer (Warth et al., 1963) (Aronson and Fitz-James, 1976) (Lawley et al., 2009). Similar to the spore compartments described above, the majority of data on the coat structure is available for the model organism *B. subtilis*. This, especially, applies to the characterization of dependencies of individual coat proteins and the time-dependent assembly of the different coat layers. Where this was possible, data for *B. subtilis* is compared with data for other spore-forming organisms. In case no specific information on the species or genera is stated, described findings refer to the model organism *B. subtilis*.

1.2.3.6.1 Coat Characteristics

Synthesis of the coat is mainly associated with the transition between sporulation stages IV and V but already starts around the time when the cortex is formed. It is deposited at the surface of the outer spore membrane and its assembly has for long been thought to be a function exclusively of the mother cell. However, proper encasement of the spore with coat protein requires not only proper gene expression in the mother cell, but also in the forespore as the forespore protein SpoIIQ has been shown recently to be required for proper coat assembly. SpoIIQ and the mother cell protein SpoIIIAH represent protein components of a transmembrane channel linking the mother cell and the forespore involved in the cross-compartmental talk between these two compartments during sporulation (McKenney and Eichenberger, 2012) (Setlow, 2012).

The spore coat was reported to contribute to the extreme physical and chemical stress resistance of spores, is structurally flexible (Driks, 2003) and can expand or retract in response to environmental parameters such as humidity (Westphal et al., 2003). Such structural properties might represent important sensorial features of the spore and suggests that the coat layer might be a main modulator of spore interaction with its environment. Additionally, biochemical traits of the coat might play a role in the spore-environment relationship as the coat harbors enzymes that are involved in spore protection, can modulate germination, and putatively affect the spores environment including the behavior of present organisms (Driks, 1999) (Nicholson et al., 2000).

Until recently, spores of *B. subtilis* were described as being composed of an amorphous undercoat region between the cortex and inner coat layers, which is putatively involved in adherence of the two neighboring layers, the inner coat and the outer coat ((Henriques and Moran, 2007) and references therein). However, more recent studies describe the coat as being organized into four distinct layers: a basement layer closely apposed to the membrane, the inner coat, outer coat and crust. Assembly of each coat layer has been reported to be controlled by one major morphogenetic protein i.e. SpoIVA (Ramamurthi and Losick, 2008), SafA (Ozin et al., 2001), CotE (Little and Driks, 2001) and CotY/ CotZ (Krajcikova et al., 2009), respectively. The sequential nature of encasement driven by these morphogenetic proteins is supposed to be a mechanism crucial for spore coat organization (McKenney and Eichenberger, 2012). As these proteins have been shown to multimerize in vitro, a possible mode of assembly for each layer may be polymerization of a basement layer consisting of a respective morphogenetic protein and localization of other coat proteins guided by that scaffold (McKenney et al., 2010) (Setlow, 2012). Spatial distribution of *B. subtilis* major coat proteins has been studied extensively and a distance-weighted interaction map based on the examination of green fluorescent protein labeled coat proteins allows a detailed view on the composition of mature *B. subtilis* spores (McKenney et al., 2010). Results from this study indicate that SpoVM, SpoIVA, SpoVID, and YhaX form an *cotE*- and *safA*-independent innermost coat layer that is spatially distinct from the *safA*-dependent proteins CotD, CotT, YaaH, and YuzC, and that two spatially distinct layers within the outer coat are comprised of an cotE-dependent outer coat layer with CotA, CotS, CotO, CotM, and the, also cotE-dependent, crust with YtxO and CotG, CotW, and CotZ. However, particularly proteins with extended domains might reside in multiple layers (McKenney et al., 2010).

In *B. subtilis*, the proteinaceous coat layers consist of more than 70 different proteins (Kim et al., 2006) and harbor approximately 30% of the total spore protein (Driks, 1999) (Henriques and Moran, 2000). The soluble fraction of the inner and most of the outer coat contains about 6% carbohydrates and the majority of coat proteins of which at least two are glycosylated (Jenkinson et al., 1981) (Pandey and Aronson, 1979). An insoluble fraction was reported to contain highly cross-linked material harboring about 30 % of the total coat proteins (Pandey and Aronson, 1979) (Aronson and Fitzjame.Pc, 1971) (Goldman and Tipper, 1978) including cysteine-rich polypeptides encoded by the *cotVWXYZ* gene cluster (Zhang et al., 1993) recently described as a distinct spore coat layer, the crust (McKenney et al., 2010) (Setlow, 2012). However, despite of a few morphogenetic proteins, the absence of most coat proteins (> 65) has little effect on known coat properties (Setlow, 2012).

1.2.3.6.2 Basement

The attachment of the coat to the forespore outer membrane represents the first stage in coat assembly. The *B. subtilis* spore coat basement layer contains the morphogenetically important proteins SpoVM, SpoIVA, SpoVID as well as YhaX, LipC, YheD, YjzB and YppG.

SpoVM

In *B. subtilis*, the σ^E-controlled, very small (26 aa) sporulation protein, SpoVM, forms a amphipathic α-helix with its hydrophobic residues being buried in the lipid bilayer leading to an orientation parallel to the outer prespore membrane (Levin et al., 1993) (van Ooij and Losick, 2003) (Ramamurthi et al., 2006). Its amphipathic structure has been shown to be crucial for its proper localization, which, in turn, is essential for proper spore formation (Levin et al., 1993) (Ramamurthi et al., 2006). SpoVM is produced in the mother cell, where it embeds in the membrane that surrounds the developing forespore but not in the cytoplasmic membrane of the mother cell putatively by discriminating between positive and negative curvature of the membrane surrounding the forespore and the mother cell (cytoplasmic membrane), respectively (Ramamurthi et al., 2009). SpoVM localized to positively curved membrane surfaces putatively directly interacts with SpoIVA, the major morphogenetic protein of the basement layer (Ramamurthi et al., 2009).

B. subtilis spoVM mutants have been reported to have a thinner, loosely attached coat and to show improper cortex formation (Levin et al., 1993). It has been proposed that SpoVM, together with CmpA (37aa), is involved in a developmental checkpoint control mechanism ensuring proper orchestration of coat and cortex morphogenesis, where the σ^E and SpoIIID controlled CmpA is removed by a post-translational mechanism after the successful initiation of coat formation by correct assembly of SpoVM. This hypothesis is supported by the findings that CmpA absence leads to more rapid sporulation, whereas its overproduction leads to sporulation delay and a failure in cortex assembly (Ebmeier et al., 2012).

However, SpoVM was reported to be absent in many species including some Bacillus and most Clostridium species (Henriques and Moran, 2007), except, for example, *C. taeniosporum* (Walker et al., 2007) (Henriques and Moran, 2007).

SpoIVA

In *B. subtilis*, SpoVM together with SpoIVA are reported to be codependent for their localization (Ramamurthi et al., 2006), which has been speculated to be due to a direct directly interaction between the C-terminal end of SpoIVA with the N-terminal end of SpoVM (Ramamurthi et al., 2006). Both SpoVM and SpoIVA play a critical role in to the earliest stages of coat morphogenesis ((Ramamurthi and Losick, 2008) (Ramamurthi et al., 2009)) and are required for proper spore cortex and coat assembly (Roels et al., 1992) (Stevens et al., 1992) in *B. subtilis*.

SpoIVA proteins have been shown to be capable of forming large oligomers (Mullerova et al., 2009) and, in addition to direct interactions with SpoVM, SpoIVA putatively directly interacts with SpoVID (Wang et al., 2009) (Mullerova et al., 2009) and establishes weak, direct, noncovalent, low-affinity interactions with SafA (Mullerova et al., 2009) (Qiao et al., 2012).

The localization of SpoIVA marks the outer membrane of the engulfed forespore as site for both cortex synthesis and coat assembly, while it was reported that these two events are linked and inseparable on the genetic level in *B. subtilis* (Catalano et al., 2001). SpoIVA harbors a Walker A box that is required for the proper deployment of the protein to the surface

of the developing spore and proper assembly of the entire coat. It both binds and hydrolyzes ATP and self-assembles into a cable-like structure in an ATP-dependent manner (Ramamurthi and Losick, 2008). It is produced in the mother cell under the control of σ^E, already localizes at the mother cell site of the cell division septum early in sporulation (Pogliano et al., 1995) and entirely surrounds the prespore after engulfment (Driks et al., 1994) (Price and Losick, 1999). The importance of the presence and correct localization of SpoIVA has been demonstrated in several studies where it has been shown that correct localization of SpoIVA is essential for the assembly of the major outer coat morphogenetic protein, CotE (Driks et al., 1994) (Webb et al., 1995) (Zheng et al., 1988). In spores where the absence of functional SpoIVA leads to failure in the assembly of CotE, (Driks et al., 1994) partially structured coat material has been observed to be present in the mother cell cytoplasm, which fails to attach to the forespore surface (Roels et al., 1992) (Stevens et al., 1992). This suggests that SpoIVA represents the major morphogenetic protein for the assembly of the coat basement layer (Setlow, 2012), anchors this layer to the spore surface and is crucial for the initial recruitment of proteins to the spore surface (de Francesco et al., 2012). In contrast to SpoVM, SpoIVA was reported to be present in all related spore-formers throughout Bacillus and Clostridium species, and the fact that its C-terminal region is highly conserved among SpoIVA orthologues from other species lead to the suggestion that the SpoIVA-dependent mechanism for coat attachment to the outer forespore membrane is a common step in coat assembly in various spore-forming bacteria.

SpoVID

In *B. subtilis*, another σ^E-controlled, highly acidic, morphogenetic protein, SpoVID, requires SpoIVA for its localization to the forespore surface (Beall et al., 1993) (Driks et al., 1994) (Ozin et al., 2000).

Like SpoIVA, SpoVID proteins have been shown to be capable of forming large oligomers (Mullerova et al., 2009). SpoVID (575 aa) possesses a C-terminal LysM domain (50 aa), which is capable of binding to peptidoglycan (Beall et al., 1993) (Ozin et al., 2000) and important for its function (Beall et al., 1993) but not the only factor determining its SpoIVA-dependent localization to the outer forespore membrane (Driks et al., 1994) (Ozin et al., 2001). Thus, it has been speculated that other structural properties play a role in the SpoIVA-SpoVID interaction and localization at the outer forespore membrane (Ozin et al., 2000) (Ozin et al., 2001).

Indeed SpoVID has been shown too directly interact not only with SpoIVA (Wang et al., 2009) but also with the important morphogenetic proteins SafA (Ozin et al., 2001) and CotE (de Francesco et al., 2012). It has been shown that the SpoVID C-terminus is necessary for the interaction with SpoIVA (Wang et al., 2009). Furthermore, SpoVID correctly localized at the membrane, guides SafA to the spore coat (Ozin et al., 2000) via the establishment of direct, noncovalent, low-affinity interactions (Qiao et al., 2012). Residues involved in the SpoVID-SafA interaction can be found within the first 200 residues (N-terminus) of SpoVID contacting residues in region A of SafA, which has been shown to be is essential for SafA localization and function. Additionally, a motif located in the middle of the SafA is thought to interact with SpoVID (Costa et al., 2006) (Ozin et al., 2000) (Ozin et al., 2001). As the phenotype of *spoVID* mutants is more drastic than that of *safA* spores interactions of SpoVID with other coat protein have been presumed (Beall et al., 1993) (Ozin et al., 2000) (Takamatsu et al., 1999a). More recently it has been shown that SpoVID also directly interacts with the major outer coat

morphogenetic protein CotE and that specific residues in the N-terminal domain of SpoVID other than those important for its interaction with SafA are essential for this interaction (de Francesco et al., 2012).
Similar to *spoIVA* deletion, the absence of SpoVID leads to the formation of partially structured coat fragments in the mother cell cytoplasm, which fail to attach to the forespore surface (Beall et al., 1993). The fact that swirls of partially assembled coat material can be detected in the mother cell cytoplasm where SpoIVA or SpoVID are absent shows that these two proteins are crucial for coat attachment and that the structural coat proteins have a certain capacity for self-assembly (Aronson et al., 1992) (Goldman and Tipper, 1978). The absence of functional SpoVID leads to initially correct localization of CotE but a collapse of the CotE layer together with the rest of the partially assembled coat once the forespore is fully engulfed (Driks et al., 1994). This and the finding that in *spoVID* mutant cells, most coat proteins assembled into a cap at one side of the developing spore but failed to migrate around and encase it (Wang et al., 2009) indicates (1) that SpoVID controls deposition of some structural proteins in the precoat but its interactions (de Francesco et al., 2012) with CotE are not necessary for correct CotE assembly and only become important for the coat stability once the forespore is completely engulfed and (2) that, whereas SpoIVA is mainly responsible for the initial recruitment of proteins to the spore surface, SpoVID promotes the encasement (i.e. migration of the coat proteins around the circumference of the spore in successive waves) by establishing direct protein-protein interactions with other coat morphogenetic proteins (de Francesco et al., 2012).

1.2.3.6.3 Inner Coat

The inner coat of *B. subtilis* contains the major morphogenetic protein SafA as well as CotD, CotP, CotT, Yaah, YhjR, YisY, YjqC, YmaG, YsnD, YsxE, YutH, YuzC, YYbI, OxdD, Yeek and YxeE (Setlow, 2012). Additionally, CotF and GerQ have been reported to be present in the inner coat (Imamura et al., 2010).

SafA

SafA was independently found to be required for proper coat assembly (Ozin et al., 2000) (Takamatsu et al., 2000a). However, although SafA independently adheres to the septum center early during sporulation, interaction with SpoVID is required to migrate along the spore surface until it fully encircles the forespore after engulfment (Costa et al., 2006) (Ozin et al., 2001) (van Ooij et al., 2004). Like SpoVID, SafA is under the control of σ^E. It has a high proline content and its C-terminal regions show sequence similarity to inner coat proteins such as CotT (Bourne et al., 1991), CotD (structural protein, required for efficient germination) (Zheng et al., 1988) (Donovan et al., 1987), and CotJA (Henriques et al., 1995). Like SpoVID, SafA also has a LysM domain (here N-terminal instead of C-terminal in SpoVID), which might be responsible for initial targeting of SafA to the forespore outer membrane and its final localization at the cortex/coat interface in mature spores (Ozin et al., 2000). In addition to SpoVID and SafA, other coat proteins were shown to possess an LysM domain (Kodama et al., 1999) (Kodama et al., 2000), which might play a role in the determination how tightly cortex and coat are attached (Costa et al., 2006). As SafA contains a predicted ATP-binding domain (Ozin et al., 2000) (Costa et al., 2006) and multimerizes in vitro (Ozin et al., 2001) it

is thought to create a scaffold for the localization of other inner coat proteins (McKenney et al., 2010).

This is in accordance with findings from mutation studies, where the importance of SafA for inner coat assembly has been demonstrated. Deletion of *safA* has been shown to lead to the misassembly of the inner coat (Ozin et al., 2000) visible in a loosely adhered spore surface and a reduction of the number of inner coat layers (Takamatsu et al., 1999a). As 16 of a total of 40 coat proteins examined have been shown to be impaired in localization in *safA* mutants it was suggested that SafA functions as an interaction hub protein for the inner coat (McKenney et al., 2010). Among the proteins that fail to assemble to the coat are also proteins primarily associate with the outer coat or crust such as CotG (Kim et al., 2006) (Sacco et al., 1995). This has led to the suggestion that SafA extends radially from the forespore outer membrane, connects spore cortex and coat with its N-terminal part binding to SpoVID and its C-terminal part binding to structural coat proteins and reaches the outer layer of the coat directly influencing the composition of this layer (Ozin et al., 2000) (Ozin et al., 2001). However, SafA is primarily associated with inner coat assembly and contributions to the outer coat structure might be due to its essential role in inner coat assembly and data obtained from GFP-fusion studies clearly indicates that SafA represents the major morphogenetic protein for the assembly of the inner coat (Setlow, 2012).

The immature coat region between SpoIVA and CotE has been designated as matrix/precoat in earlier studies (Driks et al., 1994) (Kim et al., 2006) representing the scaffold for the basement and inner coat layer in mature spores (Setlow, 2012). This region presumably first contains early synthesized, σ^E-controlled, SpoIVA (also SpoVID and SafA) recruited, but CotE independent proteins (Henriques et al., 1995) (Kim et al., 2006) (Seyler et al., 1997), which are not essential for proper coat assembly. It differs from the mature basement and inner spore coat layers in the absence of structural features provoked by the presence of σ^K-controlled proteins incorporated into that layer (Driks, 1999) (Henriques and Moran, 2000) in later kinetic waves (Setlow, 2012). The precoat region (i.e. early basement and inner coat layers) has been reported to contain enzymes such as CotJA,B,C (CotJC hexameric Mn^{2+}-dependent catalase) (Henriques et al., 1995) (Seyler et al., 1997) and other structural proteins such as SafA-N21, SafA-C30, YaaH, YdhD ((Henriques and Moran, 2007) and references therein).

The activation of the mother cell specific sigma factor σ^K from its inactive form has been reported to mark the beginning of the conversion of the precoat into the basement and inner coat and the assembly of outer coat layers (Driks, 1999) (Henriques and Moran, 2000). Changes in the precoat include modifications of the present proteins and recruitment of additional, σ^K-controlled structural proteins such as CotT (Bourne et al., 1991), YhjR, YmaG, YodI, YppG, YsnD, YsxE, YxeE and CotN as well as enzymes such as YisY, which were all described as being assembled in a SpoIVA-dependent but CotE-independent manner (Kim et al., 2006) (Henriques and Moran, 2007).

SafA and its region A have been reported to have a degree of conservation among Bacillus species, which suggests that the interaction with SpoVID is also conserved (Costa et al., 2006). However, this morphogenetic protein is not conserved in Clostridia (Henriques and Moran, 2007). As homologues of SpoIVA but not SpoVM, SpoVID and SafA can be found in Clostridia, it has been speculated that, if any, only the first part of the *B. subtilis* localization

cascade involving SpoIVA localization at the outer forespore membrane is conserved in these species (Henriques and Moran, 2007).

1.2.3.6.4 Outer Coat

The outer coat of *B. subtilis* contains the major morphogenetic protein CotE as well as CotA, CotB, CotG, CotH, CotM, CotO, CotQ, CotS, CotU, CotW, CotZ, Tgl, YknT, YlbD, YncD and YtxO, which are classified as outer coat proteins due to their localization dependency on CotE (Kim et al., 2006). Additionally, CotC has been described as outer coat protein (Imamura et al., 2010) forming heterodimers with CotU (Isticato et al., 2010). Interestingly, CotB has been reported to be located at the middle of the spore as a ring- or spiral-like structure, which indicates that the coat assembled in multilayers also exhibits uneven spatial distributions of particular proteins (Imamura et al., 2010).

CotE

In contrast to morphogenetic proteins of the basement and inner coat layers, which are all under the control of σ^E, the production of CotE is dependent on two promoters, a σ^E recognized one for early production and second promoter that additionally requires SpoIIID and remains under the control of σ^K to be repressed in the last stages of coat assembly by GerE (Eichenberger et al., 2004) (Zheng et al., 1988). After asymmetric cell division, CotE has been reported to localize in approximately 75 nm distance to the outer forespore membrane at the edge of the precoat with its C-terminal end, which has been suggested to harbor regions important for the localization of several prominent outer coat proteins (Bauer et al., 1999) (Little and Driks, 2001) projecting out of this layer. CotE has been reported to entirely encircle the forespore after engulfment is completed (Driks et al., 1994) (Webb et al., 1995). It has been reported to represent the nucleation site for outer coat assembly remaining in this position until the very end of sporulation (Driks et al., 1994). Assembly of about 40% of the total coat proteins (mainly outer coat proteins) is directly or indirectly CotE-dependent (Kim et al., 2006). The assembly of these proteins involves the cooperation of CotE with additional morphogenetic proteins, such as CotH and CotO, which regulate the assembly of a partially overlapping protein subset but do not have a specific role in coat attachment (McPherson et al., 2005) (Naclerio et al., 1996) (Zilhao et al., 1999) putatively reflecting interactions between outer coat proteins during their assembly (Kim et al., 2006).

CotE has been suggested to contribute to the attachment of the coat to the spore surface (Aronson and Fitz-James, 1976) (Costa et al., 2007) (Serrano et al., 1999) and, additionally, to some extent to the structure of the inner coat layer (Zheng et al., 1988). This may be related to postulated interactions with inner coat proteins such as CotT (Chada et al., 2003) and morphogenetic dependencies from CotE of inner coat proteins such as Yeek, YxeE and OxdD (McKenney and Eichenberger, 2012) (OxdD (oxalate decarboxylase) previously described as assembled CotE in an independent manner but does not persist in *cotE* mutant spores (Costa et al., 2004)). The fact that proteins such as OxdD are assembled but do not persist in the inner coat (Costa et al., 2004) suggests a role of CotE important to retain proteins in the inner coat layer of mature spores, which has been speculated to play a role in the lysozyme sensitive phenotype of *cotE* mutant spores (Costa et al., 2007). However, CotE is not responsible for the overall lamellar pattern of the inner coat but essential for outer coat structure formation (Zheng et al., 1988). In indirect role of CotE in spore dormancy has also

been suggested as it directs assembly of GerQ, which, in turn, controls assembly of the inner coat protein and cortex lytic enzyme CwlJ (Takamatsu et al., 1998) (Takamatsu et al., 1999b), which can be activated by core DPA release or environmental Ca^{2+}-DPA. This interaction cascade may partly explain why *cotE* spores germinate poorly in response to exogenous Ca^{2+}-DPA (Bagyan and Setlow, 2002).

As CotE has been shown to multimerize in vitro (Little and Driks, 2001) (Little and Driks, 2001) (Krajcikova et al., 2009) and deletion of *cotE* primarily causes misassembly of the outer coat (Driks et al., 1994) it has been reported to mainly function as an interaction hub protein (McKenney et al., 2010) and to represent the major morphogenetic protein for the outer coat layer (Setlow, 2012).

CotH/CotO

In addition to the major morphogenetic protein CotE, two other proteins, CotH and CotO, have been previously assigned as outer coat morphogenetic proteins based on studies mainly using SDS-page. Both, CotH (Naclerio et al., 1996) (Zilhao et al., 1999) and CotO (McPherson et al., 2005), have been suggested to direct the assembly of a subset of outer coat proteins and to play a role in germination and lysozyme resistance and, thus, potentially also influencing inner coat assembly. One suggested feature of CotH is its function as a protease inhibitor in the mother cell cytoplasm stabilizing the outer coat protein CotC (and the crust component CotG), which was, for CotC, shown in a *cotE* mutant strain (Isticato et al., 2004) (Zilhao et al., 2004) (Isticato et al., 2008). CotB and CotC (Isticato et al., 2004) have been reported to be among the most abundant coat proteins, which are primarily associated with outer coat layer (Donovan et al., 1987) and suggested to assembled in a CotE/CotH-dependent manner, where CotB assembly is additionally dependent on CotO (SDS page studies).

CotC-CotU

In addition to controlling the assembly of outer coat proteins, CotE has been reported to contact other outer coat components such as CotC (12-kDa protein forming multimers with itself and CotU (Isticato et al., 2004)) and CotU (almost identical to CotC (Isticato et al., 2004)), and that CotE is required and sufficient to allow formation of the CotC-CotU (coat component of 23 kDa) heterodimer in a heterologous host (Isticato et al., 2010).

CotB

CotB (389 aa) contains four repeats of a serine-rich motif in its C-terminal half (residues 252 through 330) (Zilhao et al., 2004). CotB, like CotG, undergoes multimerization with an abundant 64 kDa species presumably resulting from direct interaction with CotG (Zilhao et al., 2004). It has been suggested that of the ordered assembly and the striation pattern of the outer spore coat may result from the repetitive structure of CotG and CotB (Henriques et al., 1998) (Zilhao et al., 2004), which is supported by findings that the outer coat of *B. cereus* spores, where CotB is shorter (149 residues, 19 kDa) compared to *B. subtilis* CotB and has no serine-rich repeats, lacks a striation pattern (Lai et al., 2003).

In contrast to *B. subtilis*, CotE of *B. anthracis* was reported to guide the assembly of the exosporium but plays a minor role in outer coat assembly (Giorno et al., 2007). CotH homologues can be found in some, but not all Bacilli (e.g. reported to function directing coat protein assembly and as germination repressor in *B. anthracis* (Giorno et al., 2007)) and some, but not all Clostridia including *C. perfringens*, *C. cellulolyticum*, *C. difficile* and *C.*

thermocellum (Henriques and Moran, 2007). In the latter the protein CseP is similar to *B. subtilis* CotH and represents a non-enzymatic component of the cellulosome with yet unknown function (Zverlov et al., 2003).

1.2.3.6.5 Crust

Spores of *B. subtilis* do not possess an exosporium and have the crust as outermost layer (McKenney et al., 2010). A gap between the crust layer (previously also denoted as glycoprotein nap visible only when stained with ruthenium red (Waller et al., 2004)) and the outer coat surface is readily apparent. This spore crust is likely to be composed of protein in addition to polysaccharide. The protein fraction is presumably comprised of cross-linked products of the *cotVWXYZ* gene cluster as spores of *cotX*, *cotYZ* or *cotXYZ* deletion mutants show drastically altered surface properties (clumping), altered outer coat size (30 % reduction of the coat insoluble fraction in *cotX* mutant spores) and more rapid response/ increased accessibility to germinants. Additionally, heat and lysozyme resistance was increased in such mutant spores (Zhang et al., 1993).

This outermost coat layer of *B. subtilis* contains its major morphogenetic protein, CotZ, as well as CotG and CotW (Setlow, 2012). Other reported crust proteins include CotV, CotY, CotX and CgeA with the localization of CotY being depended on CotZ, CotY and CotZ described as being depended on each other for spore assembly, CotW affecting the assembly of CotV and CotX affecting the assembly of both CotV and CgeA (Imamura et al., 2011) (Imamura, 2012). Interestingly, CotZ and CgeA have been reported to be more abundant at the mother cell proximal pole, whereas CotA and CotC are more abundant at the mother cell distal pole of the forespore and mature, released spores (Imamura et al., 2010). In addition to the putative major morphogenetic protein CotZ, CotY has been reported to play a critical role in crust formation (Imamura, 2012).

It has been suggested that a structure equivalent to the *B. subtilis* crust with similar spore surface properties (Chen et al., 2010a) may be present in spores harboring an exosporium such as *B. anthracis* (Ball et al., 2008), where the CotZ homolog, ExsY (Redmond et al., 2004), is either a component of the exosporium or localizes to the coat surface like in *B. subtilis* (McKenney et al., 2010).

On the surface of *cotXYZ* mutant spores, the crust layer is absent and only similar structures present as misassembled material can be detected in the surrounding medium. It has been suggested that CotZ and possibly CotX and CotY play a morphogenetic role in the assembly of the crust around the spore. Among the crust proteins it has been shown that CotZ and CotY multimerize in vitro (Krajcikova et al., 2009), CotZ and CotY can interact strongly and CotV and CotW can interact relatively weak, yet significant (Krajcikova et al., 2009). As deletions of *cotZ* causes dramatic misassembly of the crust (McKenney et al., 2010) (Imamura et al., 2011) and the important crust component CotW is unable to localize in *cotXYZ* mutant spores, CotZ has been designated as major morphogenetic protein (Setlow, 2012).

Additionally, the spore crust contains one of the most abundant coat proteins is the crust component CotG (Kim et al., 2005) (Sacco et al., 1995). Its assembly is dependent on CotE (GFP-fusion studies, (McKenney and Eichenberger, 2012)) and putatively on CotH and CotO (SDS page studies). One suggested feature of CotH is its function as a protease inhibitor in

the mother cell cytoplasm stabilizing the crust component CotG (and the outer coat protein CotC) (Isticato et al., 2004) (Zilhao et al., 2004). The central region of CotG (residues 35 to 155) contains nine tandem repeats of a lysine-, serine-, and arginine-rich sequence (Sacco et al., 1995). The major form of CotG in the coat has a size of 36 kDa, and, after the release from the mother cell, this protein extensively forms multimers (Zilhao et al., 2005) forming a major compound of the crust/outer coat region, which is reduced in size in *cotG* mutants (Henriques et al., 1998). The *cotG* gene is adjacent to *cotH* on the chromosome and divergently transcribed by σ^K. CotG is present only in *B. subtilis*, *B. amyloliquefaciens*, and *B. atrophaeus* and in two Geobacillus strains (Giglio et al., 2011).

1.2.3.6.6 Dynamics of Spore Coat Assembly

In addition to the major morphogenetic proteins (SpoIVA, SafA, CotE, CotZ) and mother cell -specific transcription factors (SpoIIID, σ^K (McKenney and Eichenberger, 2012), GerE, SpoVIF (Kuwana et al., 2004), GerR (Cangiano et al., 2010)), SpoVM and SpoVID are important for proper coat protein encasement (Wang et al., 2009). Additionally, SpoIIQ and SpoIIIAH (transmembrane channel) have been reported to be required for the assembly of coat proteins from kinetic classes II to VI (McKenney and Eichenberger, 2012).

Generally, an exact time schedule for the expression of coat protein genes (e.g. *cotE* (Costa et al., 2007)) is crucial for correct coat morphology as the coat assembly process is not uniform and several proteins follow complex patterns of deposition along the spore surface. Sequential activation of σ^E and σ^K in the mother cell compartment results in early and late waves of gene expression (Cutting et al., 1990). Additionally, subsets of the σ^E and the σ^K regulon are regulated by SpoIIID (Halberg and Kroos, 1994) and GerE (Zheng et al., 1992), respectively, acting both as repressor and activator and generating distinct pulses of gene expression (Eichenberger et al., 2004).

Genes coding for coat components are expressed throughout sporulation under the control of the early mother cell-specific sigma factor σ^E and the late mother cell-specific sigma factor σ^K (Eichenberger et al., 2004). The localization of spore coat proteins begins shortly after asymmetric division (Webb et al., 1995) and continues throughout sporulation. According to the time span of assembly, spore coat proteins can be assigned to six kinetic waves of localization (McKenney and Eichenberger, 2012), which correlate with the regulation of spore coat gene promoters during sporulation (Eichenberger et al., 2004).

Initiation of coat formation

Initially, coat proteins localize to the forespore mother cell pole (MCP) shortly after asymmetric division of the sporangium, when the asymmetric division septum begins to exhibit the curvature associated with forespore engulfment (Webb et al., 1995) under the genetic control of *spoVM* (Ramamurthi et al., 2006) and *spoIVA* (Roels et al., 1992). Solely controlled by σ^E, the spore coat initially forms an organized multilayered scaffold on the mother cell proximal (MCP) pole with at least 19 spore coat proteins from all four coat layers i.e. basement, inner coat, outer coat and crust, including the major morphogenetic proteins, contributing to the formation of this early spore coat scaffold.

This initial assembly occurs apparently simultaneously in a small focus adjacent to the MCP and includes about 50% of individual coat proteins examined so far (McKenney and Eichenberger, 2012). The fact that some coat proteins initially target to the septum and need

other coat components to migrate along the spore surface indicates that the spore pole close to the mother cell midpoint presumably contains topological signals for specific protein targeting (Driks, 2004) (van Ooij et al., 2004).

Kinetic wave I

Wave I follows the engulfing mother cell membrane and results in encasement of the spore protoplast in a rudimentary basement structure, while a few inner coat proteins are assembled as well. More detailed, as curvature proceeds, the basement focus, a set of at least six proteins including SpoVM, SpoIVA, SpoVID (kinetic class I), tracks with the engulfing mother cell membrane and encase the forespore with a basement layer, while foci of proteins from other layers expand somewhat, which also occurs in a σ^E-dependent manner. The spatial distribution of proteins in all four expanding layers is essentially the same as in the mature spore, which has led to the suggestion that assembly at the initial focus already creates a scaffold for the assembly of the mature spore coat (McKenney and Eichenberger, 2012) (Setlow, 2012). As SafA directly interacts with SpoVID (Ozin et al., 2001) (Costa et al., 2006) and some SafA-dependent coat proteins are members of kinetic class I it has been speculate that SafA also belongs to that class (McKenney and Eichenberger, 2012).

Kinetic wave II

Once the basement fully encases the forespore (soon after engulfment is completed) and foci of proteins from the other layers are somewhat extended the second wave (kinetic class II) begins with the nucleation of inner and outer coat protein foci on the mother cell-distal pole (MDP), which is putatively controlled by σ^E and SpoIIID. Kinetic class II proteins include CotE as well as proteins from the basement, inner and outer coat layer and encase the forespore (McKenney and Eichenberger, 2012). The mother cell-distal pole serving as second nucleation site (McKenney and Eichenberger, 2012) requires the presence of the inner forespore membrane protein SpoIIQ (McKenney and Eichenberger, 2012), which directly interacts with SpoIIIAH via a domain that is localized to the inter-membrane space between the mother cell and forespore compartments (Blaylock et al., 2004) (Doan et al., 2005) (Jiang et al., 2005). It has been shown that *spoIIQ* deletion results in mislocalization of many mother cell expressed proteins (Doan et al., 2009), SpoIVFA and SpoIVFB involved in σ^K processing (Doan et al., 2005) (Campo and Rudner, 2006), SpoIID/ SpoIIM/SpoIIP engulfment proteins (Aung et al., 2007) and SpoIIIAG essential for σ^G activation in the forespore (Guillot and Moran, 2007). Such deletion mutants complete engulfment but are structurally unstable and tend to collapse (Doan et al., 2009). This and evidence that spore polarity appears to be independent of membrane fission (Sun et al., 2000) led to the speculation that *spoIIQ* possibly synergistically with proteins of the engulfment machinery (e.g. SpoIVFA, SpoIVFB, BofA, SpoIIIAH, SpoIIIE) might be responsible for proper assembly of CotE at the mother cell distal pole defining spore polarity and priming this site for nucleation of the mother cell distal-pole spore coat cap (McKenney and Eichenberger, 2012). The involvement in coat morphogenesis in an spore pole dependent manner and the observation that vegetative cells from germinating *B. anthracis* spores grow exclusively out of sporulation mother cell distal pole (Steichen et al., 2007) has led to the suggestion that SpoIIQ and SpoIIIAH may also be involved in the co-ordination of the assembly of a site that is easily broken down and cracked open during germination (McKenney and Eichenberger, 2012). In addition to σ^E, SpoIIID is necessary for encasement waves II and III. As most class II and all class III proteins appear to form a small cap at the mother cell-distal pole in *spoIIID* mutant cells, SpoIIID has been

suggested to affect expansion of the mother cell-distal pole cap (McKenney and Eichenberger, 2012).

Kinetic wave III

Wave three of encasement begins after spores turn phase dark and is characterized by nucleation of crust components on the mother cell distal pole including CotZ, but contains proteins from all four layers (McKenney and Eichenberger, 2012). Wave three is characterized by σ^K-controlled extention of foci of proteins from inner and outer coat from the mother cell-distal pole, while assembly of a focus of proteins from the crust begins at the mother cell-distal pole. Further extention of inner coat, outer coat and crust layer loci until the forespore is fully encased is controlled by σ^K and GerE (McKenney and Eichenberger, 2012) (Setlow, 2012). Kinetic encasement classes I–III are characterized by protein assembly leading to the transition from a polar scaffold to concentric layers.

Kinetic waves IV–VI

Kinetic classes IV–VI include proteins from all four coat layers, which are assembled into the existing scaffold resulting in full prespore encasement. Some coat proteins have been reported to be assembled on the spore surface in a spot dependent manner (Costa et al., 2004) (van Ooij et al., 2004) (Imamura et al., 2010). Such localization patterns have been speculated to occur due to a complex web of protein interactions within the spore coat. However, this is not yet fully understood and does not appear to correlate with classes of genetic dependencies, transcriptional regulation or localization kinetics (McKenney and Eichenberger, 2012).

An overview of the assignment of coat proteins to different kinetic waves and detected inter dependencies of various proteins in the coat is provided in Fig. 1-6.

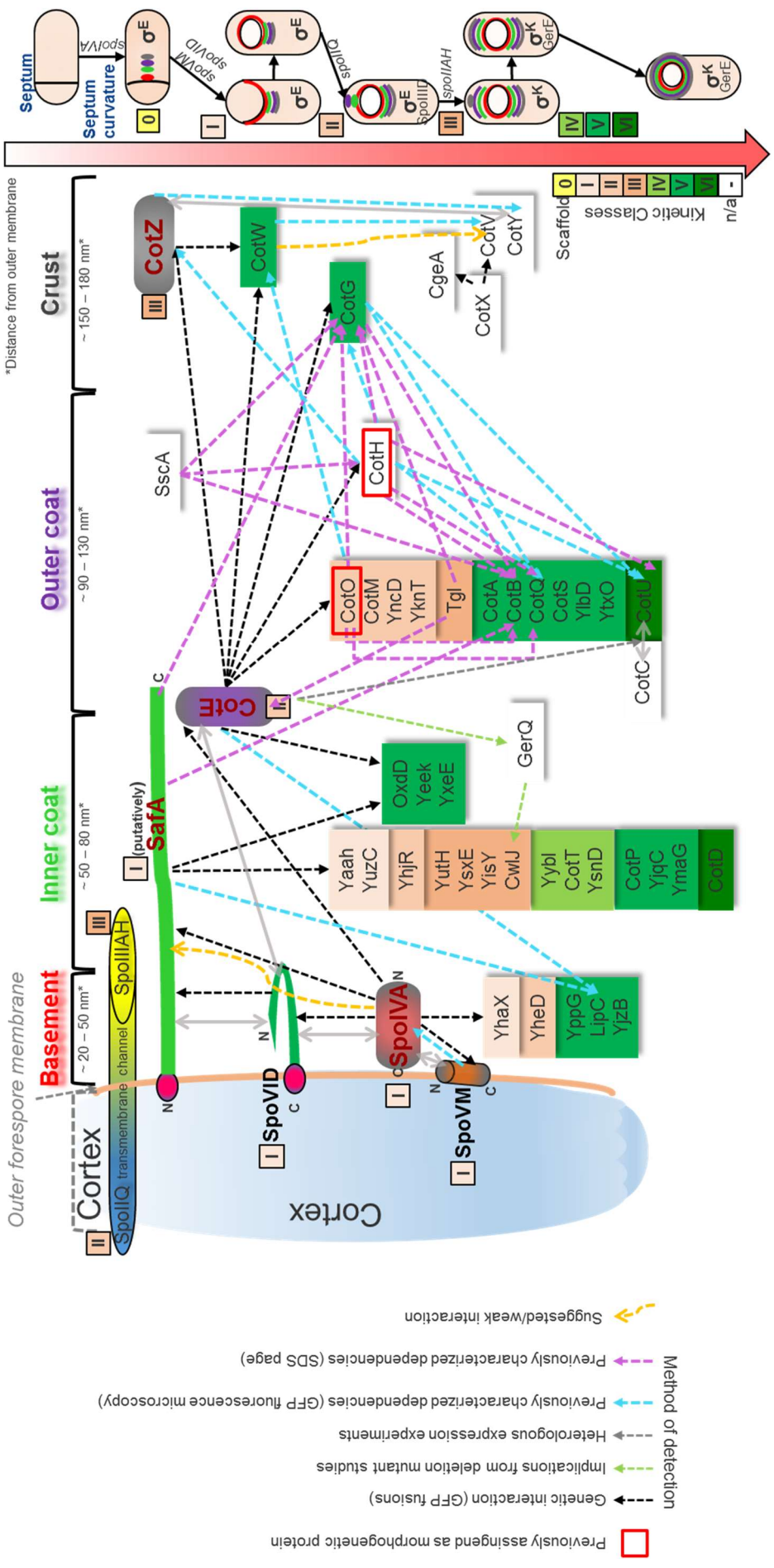

Septum
Septum curvature
spoIVA
σE
spoVM
spoVID
spoIIQ
spoIIIAH
σK GerE
Kinetic Classes
Scaffold
n/a
*Distance from outer membrane
Crust
Outer coat
Inner coat
Basement
~ 150 – 180 nm*
~ 90 – 130 nm*
~ 50 – 80 nm*
~ 20 – 50 nm*
Outer forespore membrane
Cortex
SpoIIQ transmembrane channel
SpoIIIAH
(putatively) SafA
SpoVID
SpoIVA
SpoVM
CotZ
CotW
CotG
CotH
CotO CotM YncD YknT
Tgl
CotA CotB CotQ CotS YlbD YtxO
CotU
CotC
CotE
SscA
CgeA
CotX
CotV
CotY
GerQ
OxdD Yeek YxeE
Yaah YuzC
YhjR
YutH YsxE YisY CwlJ
YybI CotT YsnD
CotP YjqC YmaG
CotD
YhaX
YheD
YppG LipC YjzB
Previously assigned as morphogenetic protein
Genetic interaction (GFP fusions)
Implications from deletion mutant studies
Heterologous expression experiments
Previously characterized dependencies (GFP fluorescence microscopy)
Previously characterized dependencies (SDS page)
Suggested/weak interaction
Method of detection

Fig. 1-6: *B. subtilis* spore coat structure.
The four proposed layers of *B. subtilis* spore coats and their average distances from the outer spore membrane are indicated on top of the graph, i.e., basement (red), inner coat (bright green), outer coat (purple), crust (black). Major morphogenetic coat proteins are indicated by bold, dark red text (SpoIVA, SafA, CotE, and CotZ). Other coat proteins were placed in color boxes. The color of each box indicates the putative primary assembly time, i.e., light brown through dark green correspond to kinetic classes I – VI, as described in the previous section. This timely schedule is also depicted on the right side of the graph, where the evolvement of the spore coat from scaffold to a complete layer is indicated. Colored arrows indicated described dependencies of the assembly of coat proteins. Different colors indicate different experimental approaches as listed in the legend above. Data for graph summarized from: (Giglio et al., 2011; Imamura, 2012; Imamura et al., 2010; Liu et al., 2016a; Liu et al., 2016b; McKenney et al., 2010; McKenney and Eichenberger, 2012; Qiao et al., 2012; Setlow, 2012) and references therein.

1.2.3.6.7 Physical coat properties

Permeability

As early-expressed proteins localize to the outer coat (CotE, CotO, and CotM) and the crust (CotW and CotZ) and late-expressed coat proteins are part of the inner coat (CotD and CotT) suggests a large amount of simultaneous assembly of all coat layers and implies that the inner layer is accessible to molecules as large as GFP-fusion proteins until quite late in coat morphogenesis (McKenney et al., 2010). Especially in kinetic waves IV–VI, added coat proteins appear to permeate through and assemble on the coat scaffold formed in previous waves and include proteins from all four coat layers. This has led to the suggestion that the maturing coat must be permeable to large proteins (50–70 kDa) in order to allow assembly of coat proteins expressed late in sporulation. However, the degree of permeability, however, is likely to change during coat maturation as protein permeability of mature coats is obviously decreased resulting in impermeability to proteins > 15 kDa such as lysozyme (McKenney and Eichenberger, 2012) (Setlow, 2012). On the other hand, permeability has to be maintained to some extent, which represents an important feature of the coat as germinants must be able to reach germination receptors localized at the inner forespore membrane (Griffiths et al., 2011).

Flexibility

The spore surface appears flexible as spores can respond to dehydration (Plomp et al., 2005a, b; Plomp et al., 2005c) or changes in relative humidity (Westphal et al., 2003) by the reduction of spore size and the reversible formation of ridges and smaller wrinkles on the surface presumably caused by contraction or expansion of the spore cortex and/or core, without changing its surface area (Driks, 2003) (Westphal et al., 2003). The flexibility of coat layers can be also observed during germination when folds tend to disappear as the spore core is rehydrated und, thus, gains volume. It was even speculated that some mutations affecting coat assembly affect germination by the inability of the coat to accommodate volume changes, which exacerbates full coat rehydration and, thus, proper germination (Henriques and Moran, 2007). Coat flexibility has been speculated to be derived from an adaptive mechanical response where, during sporulation, spores harness mechanical instabilities to fold into a wrinkled pattern, which persists during spore dormancy and allow the spore to accommodate changes in volume without compromising structural and biochemical integrity (Sahin et al., 2012).

1.2.3.6.8 The role of the coat in germination

The coat is crucial for maintaining spore dormancy and suppressing spontaneous germination and spores with incomplete coats are unstable and germinate readily and uncontrolled (Jenkinson et al., 1981). Correct coat assembly is important for germination as it mediates proper recognition and access of nutrients to specific receptors found on the inner spore membrane (Setlow, 2003) and harbors cell wall hydrolases that are required during germination for degradation of the spore peptidoglycan (Lambert and Popham, 2008).

gerP

A number of coat proteins have been reported to be essential for proper germination. Genes identified to be important for germination of *B. subtilis* and *B. cereus* spores include those of the hexacistronic *gerP* operon (*yisH* to *yisC*). GerP proteins have been found to be involved in germinant receptor-independent Ca-DPA (Butzin et al., 2012) an germinant receptor (GerA, GerB, GerK in *B. subtilis* situated in the inner membrane) dependent nutrient (Behravan et al., 2000) triggered germination. It has been reported that *gerP* mutant spores of *B. subtilis* (i) are defective in germinant receptors triggered nutrient germination visible in an impaired initiation of cortex hydrolysis and spore rehydration (Moir, 2003) (Setlow, 2003) (ii) have a longer lag time between germinant addition and DPA release (iii) germinate better than wild-type spores with germinant receptor-independent dodecylamine and (iv) show germination identical mediated by high hydrostatic pressure directly activating the germinant receptors as compared to the wild type (Butzin et al., 2012). Additionally it has been found that defects in germinant receptor triggered nutrient germination (v) can be bypassed when spores have severe spore coat defect and the coat undergoes permeabilization either provoked by extraction or the absence of functional *cotE* (Behravan et al., 2000) and (vi) can be partly overcome by the overexpression of germinant receptors or the excessive addition of germinants (Butzin et al., 2012). In *B. anthracis*, similar to *B. subtilis*, wild-type-like germination characteristics could have been restored upon the creation of coat defects i.e. the physical removal of the spore coat and, *gerP* mutants exhibit a severe defect in Ca-DPA dependent germination (Carr et al., 2010). However, in *B. anthracis*, germination in *gerP* mutants has been reported to be delayed in response to nutrient germinants but to reach a germination level similar to wild-type spores. Therefore, it has been suggested that an additional entry point for nutrient germinants may exist in such spores. Additionally, GerP proteins A – E have been reported to be essential for proper sporulation although the absence of functional GerPF has can be compensated by the presence of two GerPF-like orthologues present in *B. anthracis* (Carr et al., 2010). All these findings have led to the suggestion that *gerP*-encoded proteins are involved in the permeabilization of the coat and facilitate access of small hydrophilic germinants such as L-alanine or inosine to their cognate germinant receptors situated in the spores' inner membrane (Behravan et al., 2000) (Butzin et al., 2012). Interestingly, the expression genes of the *gerP* operon, which is switched on after 3 h of sporulation (Behravan et al., 2000), can be influenced by sporulation conditions i.e. upregulated in a high calcium environment (Oomes et al., 2009).

cotD

Findings for the *gerP* dependency of germination also imply that the coat structure plays an important role in germination, which is in accordance with reports that structural coat proteins

such as the σ^K-controlled structural inner coat protein CotD is required for efficient germination (Donovan et al., 1987) (Zheng et al., 1988).

Cortex lytic enzymes

Cortex lytic enzymes are responsible for cortex lysis during germination and commonly associated with the coat. Hydrolysis of the cortex during germination of spores of several different species has been examined previously and structural characterization of peptidoglycan fragments allowed prediction of enzymatic activities of cortex lytic enzymes. In B. subtilis (Atrih and Foster, 2001b), (Atrih et al., 1996) and *B. megaterium* (Atrih et al., 1999) glucosaminidase and putative muramic-δ-lactam epimerase products have been identified. Additionally, *B. subtilis* has been reported to possess an active lytic transglycosylase (Atrih and Foster, 2001b), (Atrih et al., 1996). In *C. perfringens*, enzymatic products of muramidase, lytic transglycosylase, and amidase have been detected (Chen et al., 1997), (Kumazawa et al., 2007). However, some but not all enzymes cleaving peptidoglycan into the respective products have yet been identified and, the other way round, the activity of enzymes reported to be involved in cortex lysis is not known for all of such enzymes.

CwlJ and Sle proteins

The two major cortex lytic enzymes in *B. subtilis*, CwlJ and SleB (or Sle proteins in general), were already mentioned in section 1.2.2.4. CwlJ orthologues can be found in various spore formers including *Bacillus* and *Clostridia* species (Henriques and Moran, 2007), but its precise enzymatic activity is not fully elucidated in several species. In addition to SleB, which is well characterized in *B. subtilis*, there can be several other putative cortex lytic enzymes found in other species and in *B. subtilis* itself. Although SleL (alternatively YaaH) has been reported to have a putative epimerase activity in *B. subtilis* (Chirakkal et al., 2002), this enzyme was shown to have a N-acetylglucosaminidase activity specific for muramic-delta-lactam, which is well characterized in *B. anthracis* (Lambert et al., 2012) and suggested for *B. cereus* (Chen et al., 2000). Additionally, enzymatic activities have been directly demonstrated for SleC and SleM of *C. perfringens*, which do not appear to have orthologues in *Bacillus* species, and reported to have a bifunctional lytic transglycosylase/amidase and a muramidase, respectively (Chen et al., 1997) (Kumazawa et al., 2007).

Other coat proteins involved in germination

Another coat protein involved in germination is the lipolytic enzyme LipC (YcsK), a phospholipase B (Masayama et al., 2010), involved in L-alanine-stimulated germination (Masayama et al., 2007). Another spore protein, SscA, has been reported to be associated with spore germination in *B. subtilis* and also in the assembly of several components of the spore coat, including CotB, CotG, and CotH (Kodama et al., 2011). However, its exact role remains to be elucidated.

Quorum sensing

The coat has been also reported to be involved in quorum-sensing as the coat-associated alanine racemase (Alr), situated in the coat-insoluble fraction in *B. subtilis* (Kanda-Nambu et al., 2000) and the exosporium in *B. cereus*, appears to be involved in a mechanism that links germination to the spore count present in a nutrient-limited or other environmental conditions (Yasuda et al., 1993). Additionally, a second alanine racemase, YncD, may also have a function in buffering the environment within the developing sporangium and around the released spore (Pierce et al., 2008). Another example for coat components involved in

interaction with the spore's environment includes the protein Cot43 from *B. anthracis*. In *B. anthracis* it has been shown that the coat contains a plasmid-encoded, tetratricopeptide repeat domain protein, Cot43, which is related to those which function as phosphatases in the sporulation phosphorelay and as regulators of competence and pathogenic factors. Interestingly, it has been demonstrated that not the synthesis but the assembly of this protein in the coat is sporulation medium dependent. Furthermore, it has been proposed that it is involved in a mechanism mediating germination as its presence is associated with a possibly L-histidine triggered germination delay in macrophages putatively enhancing spore survival and thus increasing the chances for a successful infection (Aronson and Hu, 2008).

1.2.3.6.9 Coat maturation

Coat proteins can be divided in several groups according to their function. In *B. subtilis*, at least 20 coat proteins were identified that putatively function as enzymes involved in coat assembly by the posttranslational modification of other surface proteins or directly in spore protection (Henriques and Moran, 2007). The coat of spores recently released by the mother cell contains all proteins required for full maturation but is still unfinished and the maturation process driven by the activity of the coat cross-linking, e.g. the extent of cysteine cross-linking of the spore proteins, enzymes can be influenced by the environment of the released spore (Zhang et al., 1993). This allows the adaptation of spores to environmental conditions, which could also affect spore properties such as permeability to germinants. In addition to cross-linking activities by enzymes such as Tgl and cleavage of coat proteins to their mature forms by proteases (Driks, 1999) (Henriques and Moran, 2000) (Isticato et al., 2004; Zilhao et al., 2004) (Kim et al., 2006) (Wang et al., 2009), events speculated to play a role during spore maturation include disulfide bond formation, possibly facilitated by enzymes, the activity of membrane-embedded thiol-disulfide oxidoreductases such as CcdA and StoA (SpoIVH) (Schiott and Hederstedt, 2000) (Erlendsson and Hederstedt, 2002) (Moller and Hederstedt, 2006) and cycling of proteins into and out of the coat compartment.

Tgl, (GerQ), CwlJ

The *B. subtilis* spore coat was reported to be irreversibly cross-linked by two types of covalent bonds. The first type of cross-link consists of *o*,*o*-dityrosine bonds (Driks, 1999) (Pandey and Aronson, 1979), which are putatively catalyzed by peroxidases although such an enzyme activity was up to date only found in the forespore envelopes of *B. cereus* (Ishida et al., 1987). The second type of cross-link consists of protease resistant (Lorand and Graham, 2003) ε-(γ-glutamyl)-lysil isopeptide bonds (Kobayashi et al., 1996), which are thought to play an important role in the development of rigidity and physical and chemical stability of biological tissues such as the spore coat (Lorand and Graham, 2003). The formation of such bonds is putatively catalyzed by transglutaminases such as the coat specific transglutaminase Tgl (Kobayashi et al., 1998) (Suzuki et al., 2000b) requiring cysteine residues in the coat to form crosslinks (Zilhao et al., 2005). Tgl has been reported to be σ^K-controlled, GerE-repressed (Eichenberger et al., 2004) (Kuwana et al., 2006) (Steil et al., 2005) and controls the extractability of several coat proteins. Tgl is promptly assembled (Zilhao et al., 2005), but the complete cross-linking of the Tgl dependent polypeptides occurs many hours later (Ragkousi and Setlow, 2004) (Zilhao et al., 2005), after mother cell lysis, which putatively functions as a checkpoint for the final stages in spore surface maturation (Monroe and Setlow, 2006). Tgl

is reported to assemble independently from its function or the presence of an intact inner or outer coat and to be important for proper assembly, structural integrity, and functional properties of the coat (Zilhao et al., 2005). Increased assembly of Tgl reduces the extractability of several coat proteins, affects the structure of the spore surface and reduces the efficiency of germination (Zilhao et al., 2005), which might be related to increased partitioning of proteins into the coat-insoluble fraction similar to the observable effect at elevated temperatures (e.g., CotA, (Melly et al., 2002).

However, to date only GerQ (20 kDa, previously YwdL)) in the outer coat has been identified as substrate (Kim et al., 2006) (Ragkousi et al., 2003) (Ragkousi and Setlow, 2004) (Zilhao et al., 2005), which is putatively cross-linked by Tgl using N-terminal lysine residues of GerQ as lysine donors with Tgl being involved in the formation of a preformed GerQ protein scaffold contributing to its stabilization (Lorand and Graham, 2003; Monroe and Setlow, 2006) (Lorand and Graham, 2003) and leading to the development of various high-molecular-mass forms cross-linked with yet unidentified molecules (Monroe and Setlow, 2006) (Ragkousi and Setlow, 2004). GerQ, in turn is essential for the presence of the cortex lytic enzyme CwlJ.

GerQ recruits the cortex lytic enzyme CwlJ to the coat, but CwlJ function is independent from Tgl (Monroe and Setlow, 2006) (Ragkousi and Setlow, 2004).

As the N-terminal lysine residues of GerQ are not conserved among GerQ homologues in other Bacillus species, it may there act mainly as acceptor substrate. Neither GerQ, nor Tgl are conserved in Clostridia, and, thus, recruitment of the cortex lytic enzyme CwlJ to the coat has to work differently (Henriques and Moran, 2007).

YabG

Tgl has been also reported to cooperate with the YabG protease and possibly other cross-linking enzymes at the spore surface. YabG putatively generates substrates from protein precursors for or facilitates access of Tgl and other cross-linking enzymes (Kuwana et al., 2006). The protease YabG is crucial for normal coat protein composition and involved in the proteolysis and maturation of the spore coat proteins SpoIVA, YrbA (Takamatsu et al., 2000b) (Takamatsu et al., 2000a) and YeeK (18-kDa, σ^K and GerE dependent expression, not important for vegetative growth, spore resistance or germination, only detectable in *yabG* mutant spores, guided into the spore coat by CotE, SafA, and SpoVID) (Takamatsu et al., 2009). The fact that increased temperature compensates for the lack of functional YabG might be due to increased Tgl activity (Suzuki et al., 2000b) or the altered conformation of Tgl substrates at higher temperatures (Kuwana et al., 2006).

CotG

Similar to Tgl, the activity of the cross-linking enzyme CotG might be prevented until all the components of the coat are in place, which is when mother cell lysis takes place putatively functioning as a checkpoint for the final stages in spore surface maturation (Monroe and Setlow, 2006).

CotA

The intrinsically highly thermostable, copper-dependent laccase CotA (oxidoreductase) is capable of binding and reducing dioxygen (via the oxidation of phenolic compounds) providing spore protection (Hullo et al., 2001) (Martins et al., 2002) (Chen et al., 2010b)).

SodA

Other important coat associated enzymes are involved in cross-linking or maturation of other coat proteins or have been speculated to be directly involved in spore resistance. The Mn-dependent superoxide dismutase SodA is putatively responsible for the covalent cross-link of tyrosine-rich outer coat protein CotG into the insoluble matrix and the striated appearance of the outer coat layer (Henriques et al., 1998).

YtaA (CotI)

The spore coat has also been reported to harbor bacterial spore kinases (BSKs) such as YtaA (CotI) from *B. subtilis*, which are widely distributed in spore-forming Bacillus and Clostridium species, show substantial structural similarity to CAKs (CDK(Cyclin-dependent kinase)-activating kinases). Although the function of BSKs is not known in detail they are assumed to bind and phosphorylate distinct targets. However, as several classes of BSKs have apparently independently lost catalytic activity to become pseudokinases a major non-catalytic function of this group has also been proposed (Scheeff et al., 2010)

1.2.3.6.10 Coat proteins in other spore formers

B. subtilis spore coat consists of at least 70 different proteins are all produced in the mother cell under the control of the σ^E and σ^K factors. Orthologues of many spore coat proteins can only be found in some other Bacilli and some are present in *B. subtilis* exclusively (about 16 proteins) (Henriques and Moran, 2007). Although spore coats of *B. cereus* and *B. anthracis* appear to be thinner and more compact, *B. subtilis*, *B. cereus* and *B. anthracis* were reported to share a complex coat protein pattern (Lai et al., 2003) (Henriques and Moran, 2007), which appears to be not conserved and simpler in other Bacillus species including *B. pumilus*, *B. clausii*, and *B. megaterium* (Henriques and Moran, 2007). In Clostridia, only about 20 of the *B. subtilis* coat proteins were reported to be present, which is also partially species dependent. Generally, the genes of the σ^E regulon show a higher degree of conservation among *B. subtilis* and Clostridia species than genes of the σ^K regulon. This might be due to the presence of many genes coding for spore surface proteins in the latter regulon, which are likely to undergo the severest evolutionary changes in species adaptation processes to a certain habitat (Eichenberger et al., 2004) (Henriques and Moran, 2007). There are differences in the appearance and thickness of the coat layers among spores of various strains or species. In *B. thuringiensis*, the inner coat is laminated but consists of a patchwork of striated packets, appearing either stacked or comb-like, and the outer coat is granular. Spores of *B. popilliae* show a lamellar inner layer much like that of *B. subtilis*, but a thick and dense outer coat. In *B. cereus*, *B. anthracis*, *B. pumilus*, *B. megaterium*, and *B. clausii* the coat appears compact ((Henriques and Moran, 2007) and references therein). Thin section preparation of *C. botulinum* type E vegetative cells revealed a 30 nm to 40 nm thick (depending on the age of the culture), multilayered cell wall made up of several electrondense layers alternating with less dense ones (Ohyama et al., 1979). In contrast, *C. botulinum* type E spores, like all bacterial endospores, contain a large encasement composed of several layers. Early electron microscope studies on ultrathin sections of *C. botulinum* type E spores revealed that such spores are, similar to that of *C. butyricum*, composed of an outer spore coat of three layered structure, a less dense intermediate space, an inner spore coat, a relatively less dense portion of cortex, and the dense core containing nuclear elements of

lower density (Takagi et al., 1960). Two distinct coat layers can also be found in electron micrographs of the model organism *B. subtilis* ((Henriques and Moran, 2007) and references therein). Detailed studies in *B. subtilis* revealed that these two main layers are composed of lamellae and electron-dense striations, respectively, aligned along the spore periphery. However, determinants of surface topology have been reported to differ from those that establish the lamellar or striated pattern of the underlying layers of the coat (Henriques et al., 1998) (Zilhao et al., 1999) (Chada et al., 2003) and factors that contribute to the lamellar or striated pattern of the inner and outer coat layers were speculated to include the overlay of coat proteins by other proteins during coat assembly (Jenkinson et al., 1981) and physical properties of the coat components (Henriques and Moran, 2007).

In general, data about the spore coat composition or the interaction network in spores from the genus Clostridium is scarce. However, it has been shown that *Clostridium* spore coats harbor additional proteins, which cannot be found in Bacilli. The outer coat layers of *C. difficile* spores have been shown to harbor CotA, CotB, the putative manganese catalases CotCB, CotD and CotE (named after their coat association and their detection, not related to the identically named *B. subtilis* proteins), which has been described to function as a bifunctional protein with peroxiredoxin activity at its amino terminus and chitinase activity at its carboxy terminus (Permpoonpattana et al., 2011).

Possible functions of outer coat or exosporium proteins CotBC, CotD and CotE include the reduction of cellular toxicity of H_2O_2 converting it to oxygen and water. However, as H2O2 may play a key role in spore coat synthesis and could serve as a substrate for the oxidative cross-linking of spore coat monomers (Henriques et al., 1998), it has been suggested that the *C. difficile* CotE (with 1-Cys-peroxiredoxin activity) might be, similar to SodA involved in cross-linking of tyrosine-rich spore coat proteins and, thus, speculated to play a role in coat assembly (Permpoonpattana et al., 2011). More recently, it has been shown that *C. difficile cotA* mutants have a major structural defect in spore assembly with a clear miss-assembly of the outermost layers of the spore coat. This has led to the assumption that CotA is probably subject to post-translational modification and could play a key role in stabilizing the spore coat. In contrast, the structural coat integrity of *cotD*, *cotE*, and *sodA* mutants led to the suggestion that these enzymes are located in the exosporium involved in coat polymerization and detoxification of H_2O_2 or that they are structurally redundant. Two additional proteins, CotF (a tyrosine rich protein and potential substrate for SodA) and CotG (a putative manganese catalase) were shown to be located to the spore surface (Permpoonpattana et al., 2013).

However, there still exists a huge gap of knowledge regarding spore coat structures from organisms other than *B. subtilis*, which might be simple in compositions and/or possess other, yet unidentified proteins that play a significant role. To illustrate such differences, the same graph as shown in Fig. 1-6 for *B. subtilis* is shown in Fig. 1-7 considering only proteins present in *C. botulinum* type E that have at least some similarity to that identified in *B. subtilis*. Two lists containing information on important *B. subtilis* coat proteins and one with *B. subtilis* proteins sorted by their putative function and indication the degree of conservation in *Bacillus* and *Clostridium* species including *C. botulinum* type E are provided in the appendix section.

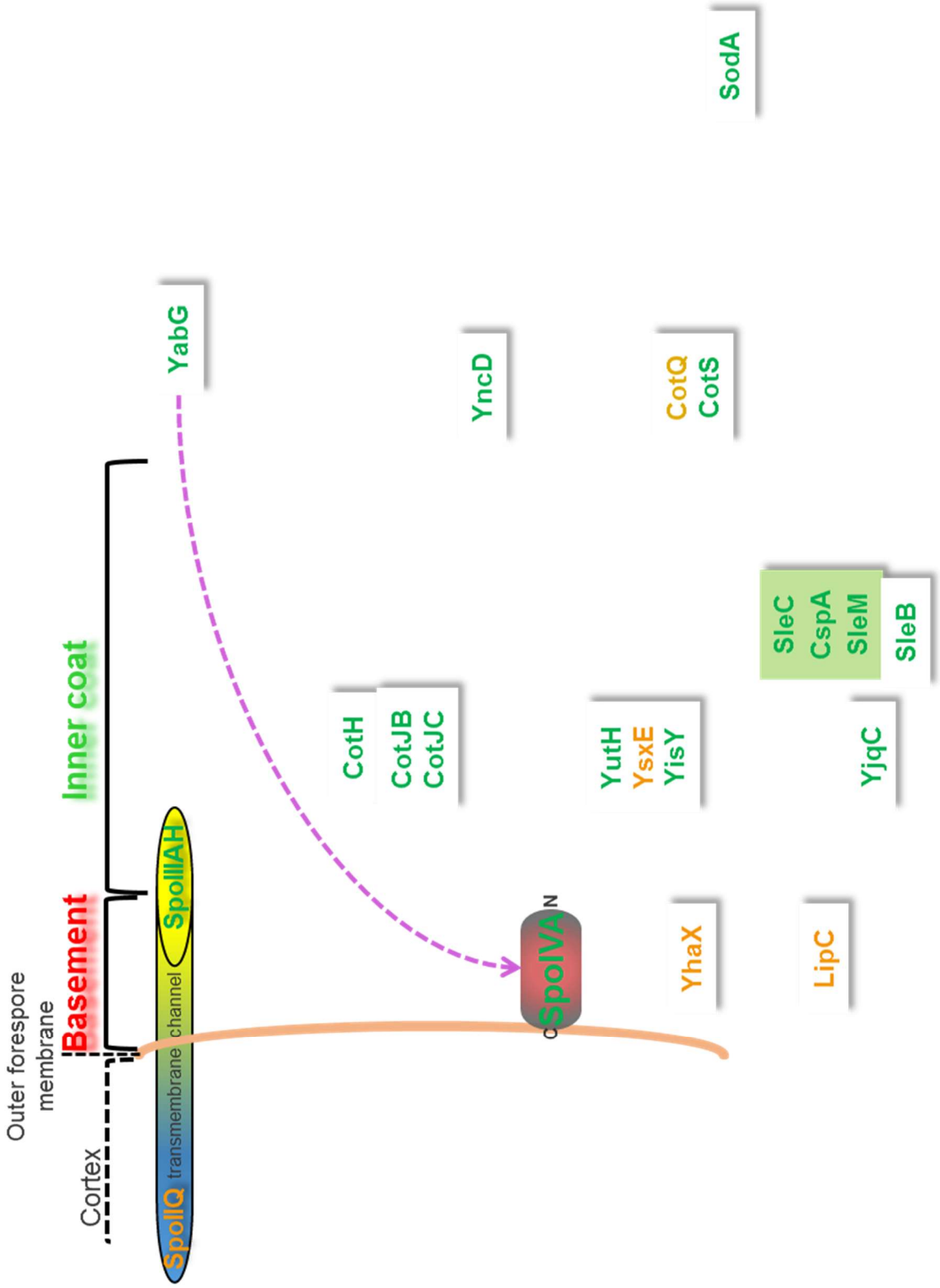


Fig. 1-7: Spore structure of *C. botulinum* type E???
Question marks indicate that this figure is just derived from homologies of *C. botulinum* proteins to characterized coat proteins in *B. subtilis*. The coat of *C. botulinum* might have less layers than that of *B. subtilis*, which, however, does impede a complex interplay between several, probably yet unidentified coat proteins in this species.

1.2.4 Factors Influencing Spore Properties

Generally, events during sporulation and, consequently, the properties of mature spores can be influence by numerous extrinsic (e.g., temperature and gas atmosphere) and intrinsic (e.g., sporulation medium composition, pH, water activity) factors. The best studied factor is certainly the sporulation temperature, which was one of the factors investigated in this study. Since there an enormous wealth of knowledge on effects of this extrinsic factor influencing sporulation and spore properties has been gained in the past, which can make it difficult to keep track of findings made, literature on how sporulation temperature could affect events during sporulation and properties of mature spores is summarized in the paragraphs below.

1.2.4.1 Sporulation Temperature

The sporulation temperature is one of the most important and certainly the best studies influence factors on spore properties.

1.2.4.1.1 Sensing cold/heat stress

To date, there are different mechanisms known how an organism senses and responds to altered temperature. Such mechanisms, which are likely to be involved in sensing temperature changes include (i) the stability of regulatory RNA elements (RNA thermometers) (Chowdhury et al., 2006), (ii) mRNA stability (Horn et al., 2007) (iii) phase state and microdomain organization of membranes activating a signal transduction systems composed of a sensor kinase and response regulator (Aguilar et al., 2001). (i) RNA thermometers have been suggested to undergo temperature mediated conformational changes and operate at the post-transcriptional level to transduce a signal to the translation machinery (Chowdhury et al., 2006). For example, a, normally occluded, ribosome binding site of the ROSE (repressor of heat-shock gene expression) element of small heat-shock genes is released at 42°C (vs. 30°C), via the destabilization weak hydrogen bonds within the RNA helix (Chowdhury et al., 2006). (ii) The stability of mRNA (e.g. *csp* mRNA) has been shown to increase at low temperatures, which, independent of the transcription rate, influences the amount protein produced (Horn et al., 2007). (iii) Temperature changes can be putatively sensed by the phase state and microdomain organization of membranes leading to the activation of a two-component signal transduction systems (input-sensing domain (histidine kinase), often membrane proteins responding to environmentally signals and output effector domain (response regulator)), which are extensively used by bacteria for signal transduction across the cell membrane (Hoch, 2000). Such a two-component signal transduction system (sensor kinase DesK and response regulator DesR) has been identified in *B. subtilis* and is responsible for cold induction of the *des* gene, which codes for Δ5-lipid desaturase producing unsaturated fatty acids, which, in turn, act as negative signaling molecules of *des* transcription (Aguilar et al., 2001) and might be present in various organisms (Vigh et al., 1998) (Hoppe et al., 2000) (Suzuki et al., 2000a).

Such sensing mechanism are responsible for cold and heat shock inducing a variety of, partially overlapping but often specific, alterations in gene expression in growing cells. Heat shock proteins commonly include chaperones required for protein folding/repair and peptidases (Yura et al., 2000), whereas, cold shock typically induces proteins involved in cellular functions e.g. general metabolism, transcription, translation and recombination

(Phadtare et al., 2000). Accordingly, changes in the structure and properties of resulting spores grown at low or high temperatures differ from each other.

1.2.4.1.2 Cold stress response

Typical cold stress induced proteins

Commonly microorganisms respond to environmental changes such as decreased temperature by modulating their metabolism, structure (Ermolenko and Makhatadze, 2002) and gene expression pattern enhancing the transcription cold tolerance genes (Brandi et al., 1994) (La Teana et al., 1991), and repressing the transcription of non-cold inducible genes after an acclimation period (Hofweber et al., 2005). Numerous studies investigated the cold shock response at different temperatures of various Bacilli on the protein level (Graumann et al., 1997), (Lottering and Streips, 1995), (Schindelin et al., 1992) (Beckering et al., 2002) (Graumann and Marahiel, 1999b) (Weber and Marahiel, 2002). The over 50 proteins induced by cold shock generally include those functioning in cellular metabolism as well as uniquely cold stress induced proteins (Lottering and Streips, 1995). Whereas other stresses such as heat, ethanol, and puromycin stress provoke the production of similar stress proteins the cold stress response appears to be very specific (Movahedi and Waites, 2002), partially even to a particular cold shock temperature (Graumann et al., 1997). Generally, cold stress has been shown to lead to an adaption/increased resistance to lower temperatures (e.g., *C. perfringens*, (Villarreal et al., 2002)).

Major Cold shock proteins (Csps)

Among the proteins that are typically induced by cold shock there is the family of nucleic acid binding cold shock domain (CSD)-containing, small (~7.4 kDa) cold shock proteins (Csps) (Wistow, 1990) (Graumann and Marahiel, 1998) (Mihailovich et al., 2010). It has been suggested that the cold induction of such proteins occurs, at least partially, due to an increased *csp* mRNA stability at low temperatures and not exclusively due to an overexpression of *csp* genes (Horn et al., 2007). Csps have been reported to function as RNA-chaperones, which are involved in the post-transcriptional regulation via their effect on RNA stability e.g. destabilizing undesired cold-derived secondary structures (Jiang et al., 1997).

Csps have been demonstrated to be involved in the regulation of a variety of stress response proteins (Phadtare and Inouye, 2001) (Graumann et al., 1997) rather than exclusively in the cold stress response. They facilitate the initiation of translation under low and optimal temperatures and are also implicated in cellular growth (Graumann et al., 1997), nutrient starvation (Graumann et al., 1996) (Graumann et al., 1997) (Phadtare and Inouye, 2004) (Yamanaka et al., 1998), oxidative stress (Loepfe et al., 2010) and stationary phase response (Graumann and Marahiel, 1998).

Mutations in *cspB* or *cspC*, but not *cspA* (possibly due to compensation by the other Csps), have been reported to result in a cold-sensitive phenotype of *C. botulinum* type A (Soderholm et al., 2011). CspB and CspC have been reported to be the major stationary phase-induced protein in B. subtilis (Graumann and Marahiel, 1999a), whereas CspB has been suggested be the major cold shock protein / cold shock regulon modulator in *B. subtilis* (Graumann et al., 1997) and *C. botulinum* (Soderholm et al., 2011).

However, the findings that

(i) the heat resistance of spores is not affected by mutations in the major cold shock proteins CspB, -C, and -D (*B. subtilis*, (Graumann et al., 1997))
(ii) cold shock induced genes are negatively regulated in the late-log and stationary phase (proteolytic *C. botulinum* (Derman et al., 2013)) / stress proteins induced by cold shock disappear later in sporulation (*B. subtilis*, (Movahedi and Waites, 2000)) and
(iii) sporulation temperature but not the growth temperature prior to sporulation significantly influences the heat resistance of spores (*B. weihenstephanensis* (Baril et al., 2011)) indicate that the induction of cold stress response does not influence the heat resistance of spores e.g. protecting cell structures during germination and outgrowth (Movahedi and Waites, 2002). Although a negative effect on sporal heat resistance in Csp mutants might be compensated by upregulation of other proteins (Graumann et al., 1997) available data implies that cold stress mediated changes e.g. in the spore structure rather than the Csp repair system is responsible for alterations in heat resistance. This is certainly true for *C. botulinum* type E as the cold shock genes *cspABCD* are conserved among various Bacilli and Clostridia including mesophilic *C. botulinum* strains (physiological groups I and III) but absent in *C. botulinum* type E (group II).

Interconnection of cold stress and sporulation

Cold stress has been reported to highly induce the master transcription factors governing general stress response (σ^B) (van Schaik and Abee, 2005) (Mendez et al., 2004) during logarithmic growth of *B. subtilis*. σ^B directly or indirectly controls over 200 genes, is important for growth and survival under low temperatures (Brigulla et al., 2003), the resistance of vegetative cells to multiple stresses (Hecker and Volker, 1998) (Petersohn et al., 2001) including heat (*B. subtilis*, (Holtmann et al., 2004); *B. cereus*, (van Schaik et al., 2004)) and antibiotics (*B. subtilis*, rifampin, (Bandow et al., 2002)).
It has been previously suggested that the regulons of cold stress and sporulation overlap (Lottering and Streips, 1995). The finding that σ^B also plays a role in sporulation at low temperature (*B. subtilis*, (Mendez et al., 2004)) and influences sporulation onset and spore maturation (*B. cereus*, (van Schaik and Abee, 2005)) indicates that stress resistance and sporulation are interconnected to some degree. This is supported by the fact that cold stress also highly induces sporulation sigma factors such as the sporulation sigma factor σ^G (Garcia et al., 2010) and the master transcription factor, Spo0A (Mendez et al., 2004) with the latter also playing a direct role in adaptation to and survival in the cold independently from its crucial role in sporulation (*B. subtilis*, (Mendez et al., 2004)). Although σ^B is absent in Gram-positive low GC bacteria including Clostridia, genes coding for the master regulator Spo0A and other sporulation sigma factors such as σ^H or σ^G are well conserved among spore formers (Paredes et al., 2005). Thus, it has been speculated that sporulation and stress response might be also interconnected in such organisms allowing a flexible response to environmental changes (Paredes et al., 2005).
However, available data summarized in Fig. 1-8 indicates that there is no generally valid linear relationship between sporulation rate or efficiency and cold stress. Indeed, sub-optimal sporulation temperatures have been reported exert no significant or a negative effect on sporulation rate, which might be related to slower protein synthesis at low temperatures (Kunclova et al., 1995). On the other hand, sporulation efficiency seems to be affected in a temperature- and species-dependent manner with temperatures slightly below the optimum growth temperature being effective in enhancing sporulation efficiency of some species.

1.2.4.1.3 Heat stress response

Heat stress has been shown to induce over 60 proteins in sporulating *B. subtilis* cells including DnaK, GroEL, enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and flagellin (Miller et al., 1991) with only a minority (eleven) designated as specific heat shock proteins (HSPs) (de novo synthesis or not induced by cold shock or glucose starvation). Thus cold and heat stress responses overlap partially. Many of the HSPs that are not inducible by cold stress have been reported to be chaparonins such as DnaK and GroEL, which are putatively involved in repairing heat damaged cell components / misfolded proteins, which are also induced by heat shock in vegetative cells (*B. subtilis*, (Lottering and Streips, 1995) (Movahedi and Waites, 2000)).

Heat shock has been frequently reported to increase the thermal resistance of vegetative cells (*C. perfringens* (Heredia et al., 1997)), which might at least partially be mediated by extracellular factors e.g. proteins with thermoprotective activity (Heredia et al., 2009). Similar to cold shock, heat stress induces the master transcription factors governing general stress response (σ^B), which has been shown to be also induced by osmotic and ethanol stress but not ATP depletion and to play a role in the adaptive response of vegetative cells to heat stress (*B. cereus* (van Schaik et al., 2004)). The activation of the general stress (σ^B) regulon at elevated temperatures has been reported to be controlled by regulators such as the anti-sigma factor, RsbW, the antagonist protein, RsbV and the environmental stress-responsive phosphatase RsbU but not the metabolic stress-responsive phosphatase RsbP (Holtmann et al., 2004). Interestingly, this control mechanism has been reported to be heat shock temperature dependent e.g. loss of RsbV (alone or in combination with RsbU) triggers a hyperactivation of the general stress regulon exclusively at high temperatures detrimental for cell growth (Holtmann et al., 2004).

It has been previously found that a *B. subtilis* heat shock protein mutant strain produces spores with decreased heat resistance (Khoury et al., 1990), which led to the assumption that thermal adaption plays a role in the increased heat resistance of spores grown at elevated temperatures.

Accordingly, it has been presumed that the biosynthesis of HSPs with intrinsic properties that stabilize tertiary and quaternary macromolecule structures at elevated temperatures, could affect spore heat resistance as cortex structure, mineral content and core dehydration have been suggested to be not responsible for increased heat resistance (Atrih and Foster, 2001a). However, in accordance to findings for cold stress proteins, the fact that heat shock increases heat resistance of formed spores independently of the presence of functional *sigB* leading to a lack of a number HSPs (*B. subtilis*, (Movahedi and Waites, 2000)) and that heat shock proteins formed in response to a short time heat exposure of sporulating cells disappear later in sporulation, has led to the suggestion that an increase in spore heat resistance occurs due to alterations in the spore structure rather than by repairing heat damage during germination and outgrowth (Melly and Setlow, 2001) (Nicholson et al., 2000) (Setlow, 2006) (Setlow and Johnson, 2007).

1.2.4.1.4 Spore components affected by the sporulation temperature

The sporulation temperatures was shown to affect a large variety of spore properties of both *Bacillus* and *Clostridium* spores. In spores of different *Bacillus* species, sporulation

temperature has been reported to negatively correlate with (i.e., low temperatures increase) surface roughness (Lindsay et al., 1990), spore size (Baweja et al., 2008; Garcia et al., 2010), wet density (Lindsay et al., 1990), the percentage of L-alanine substitution in the cortex peptidoglycan (at least slightly, (Atrih and Foster, 2001a; Melly et al., 2002)), the level of at least one coat protein (Melly et al., 2002), and the overall size and integrity of the exosporium (Faille et al., 2007). For both *Bacillus* and *Clostridium* species, it was shown that spore properties negatively correlating with the sporulation temperature include the core water content (Beaman and Gerhardt, 1986) (Melly et al., 2002; Paredes-Sabja et al., 2008a; Popham et al., 1995b) (not significantly, (Atrih and Foster, 2001a)), and the degree of unsaturation of the inner membrane (Aguilar et al., 1998; Evans et al., 1998; Gaughran, 1947; Peck et al., 1995a) (not significant (Planchon et al., 2011)).
Furthermore, sporulation temperature has been reported to positively correlate (i.e., low temperatures decrease) with the core/(core+cortex) volume ratio (Lindsay et al., 1990), the DPA content (no linear relationship, (Lindsay et al., 1990)) (Planchon et al., 2011) (not significant, (Melly et al., 2002)) (only at elevated temperatures, (Baweja et al., 2008)), the mineral contents (not linear, (Igura et al., 2003)) (Lindsay et al., 1990) (not significant, (Atrih and Foster, 2001a)), and cross-linked muramic acid, tripeptide side chains and tetrapeptides in the cortex (slight and sometimes not significant, (Atrih and Foster, 2001a; Melly et al., 2002)). Additionally, a decrease in the sporulation temperature was shown to decrease the average acyl chain length in the inner membrane of *Clostridium* spores (Evans et al., 1998; Peck et al., 1995a).
In contrast, α/β-type SASP levels putatively remain unaffected by the sporulation temperature (Melly et al., 2002; Movahedi and Waites, 2000) and the degree of δ-lactam residues in the cortex tend to peak at the optimum growth temperature (Atrih and Foster, 2001a; Melly et al., 2002). Such effects together with their putative (!) importance of spore resistance to high hydrostatic pressure are summarized in Tab. 7-4.

1.2.4.1.5 Effect of sporulation temperature on resistance to various stresses

In addition to heat and HHP stress, the sporulation temperature has been shown to modify the resistance of spores to various decontaminants, chemicals and physical stress including:
- hypochlorite, chlorine dioxide (positive correlation at elevated sporulation temperatures in *B. subtilis*; (Young and Setlow, 2003, 2004); hypochlorite resistance not affected in *B. cereus*, (Planchon et al., 2011))
- formaldehyde, nitrous oxide (positive correlation at elevated sporulation temperatures in *B. subtilis*, (Cortezzo and Setlow, 2005) (Melly et al., 2002), formaldehyde resistance not affected in *B. cereus*, (Planchon et al., 2011) and *B. anthracis* (Baweja et al., 2008))
- H_2O_2 (*B. subtilis*; (Melly et al., 2002) (Cortezzo and Setlow, 2005); *B. anthracis*; (Baweja et al., 2008)
- NaOH (positive correlation, *B. cereus*, (Planchon et al., 2011) and *B. anthracis,* (Baweja et al., 2008))
- HCl, (positive correlation at elevated temperatures, *B. anthracis*; (Baweja et al., 2008)),
- UV light (positive correlation at elevated sporulation temperatures in *B. cereus*, (Planchon et al., 2011); largely unaffected in *B. subtilis* (Melly et al., 2002); no significant effect on low dose UV irradiation resistance, decreased sporulation temperature lowers higher dose UV resistance, *B. anthracis* (Baweja et al., 2008)).

- although it has been reported early that increased sporulation temperature increases the resistance of spores from thermophilic and thermotolerant bacteria to dry heat (37 °C vs. 56 and 62 °C (Sames, 1900)), in contrast to wet heat, the sporulation temperature has been reported to have no significant influence on dry heat resistance of *B. subtilis* (Melly et al., 2002).

1.2.4.1.6 Sporulation Temperature and Sporulation Characteristics

Generally, available data is heterogeneous to some extent but indicates that sporulation rate and efficiency are negatively influenced when a sporulation temperature far below or above the optimum growth temperature is used. However, altered sporulation temperatures within certain organism-specific limits have also been frequently reported to have no significant influence on sporulation rate or efficiency. In a minority of studies, it has been shown that an increase in sporulation temperature leads to an increased sporulation rate and that lower sporulation temperatures enhance sporulation efficiency. A positive effect on sporulation rate mediated by a decrease of sporulation temperature as well as a positive effect on the sporulation efficiency mediated by an increase in the sporulation temperature have not yet been reported. For non-proteolytic *C. botulinum* types B and E it has been reported that sporulation at elevated temperatures (35 and 37°C) leads a significantly decreased sporulation efficiency, a decrease of the sporulation temperature to 25°C leads to an increased and unaltered sporulation efficiency, respectively. A further decrease to 20°C has been reported to result in a reduced sporulation efficiency for type E.

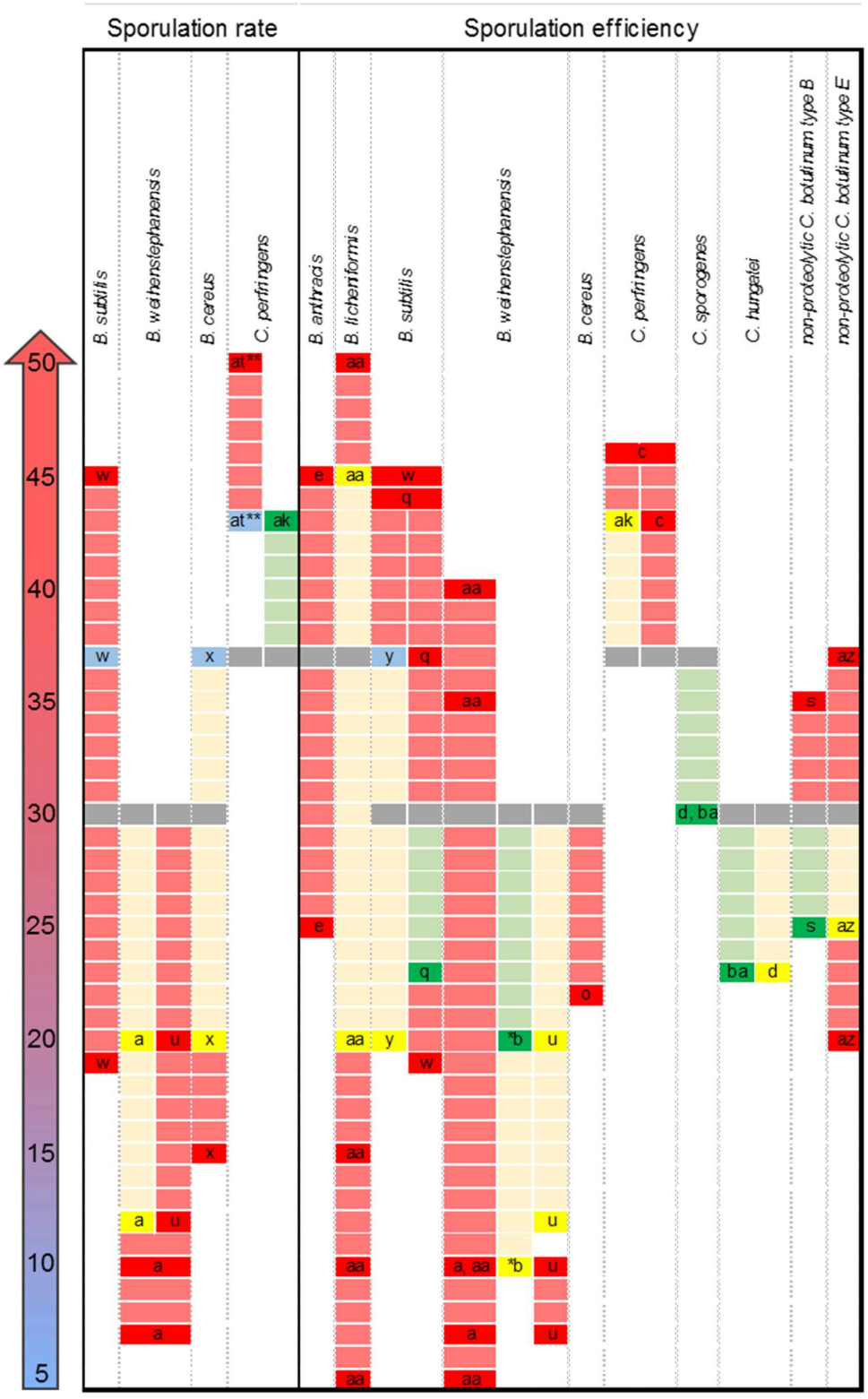


Fig. 1-8: Sporulation temperature and sporulation rate and efficiency.
Summary of data available on sporulation characteristics of spores from different species grown at different temperatures. The sporulation temperature is depicted on the left. Dark red fields indicate negative effects, dark yellow fields indicate no significant effects and dark green fields indicate positive effects compared to the standard temperature used in the respective study. Light red, yellow and green fields indicate extrapolated effects at temperatures between the sporulation temperature and the standard temperatures used in the studies. Gray fields represent the assumed standard temperature for an organism. Whenever a different standard temperature was used, this is indicated by light blue fields containing the letter code for the respective reference.

References for Fig. 1-8, Fig. 1-9, and Fig. 1-10:
a) (Garcia et al., 2010); b) (Baril et al., 2011); c) (Garcia-Alvarado et al., 1992b); d) (Ponce et al., 2009); e) (Baweja et al., 2008); f) (Movahedi and Waites, 2000); g) (Movahedi and Waites, 2002); h) (Sedlak et al., 1993); i) (Beaman and Gerhardt, 1986); j) (Gonzalez et al., 1999); k) (Sala et al., 1995); l) (Raso et al., 1998a); m) (Feig and Stersky, 1981); n) (Elbisi and Ordal, 1956); o) (Ryu et al., 2005); p) (Melly et al., 2002); q) (Cortezzo and Setlow, 2005); r) (Evans et al., 1997); s) (Peck et al., 1995a); t) (Igura et al., 2003); u) (Garcia et al., 2010); v) (Planchon et al., 2011); w) (Nguyen Thi Minh et al., 2011); x) (Gounina-Allouane et al., 2008); y) (Mendez et al., 2004); z) (Atrih and Foster, 2001a); aa) (Baril et al., 2012a); ab) (Baril et al., 2012b); ac) (Leguerinel et al., 2007); ad) (Condon et al., 1992); ae) (Raso et al., 1995); af) (Khoury et al., 1987); ag) (Condón et al., 1996); ah) (Palop et al., 1996); ai) (Rey et al., 1975); aj) (Palop et al., 1999); ak) (Garcia-Alvarado et al., 1992a); al) (Black et al., 2005); am) (Black et al., 2007b); an) (Margosch et al., 2004b); ao) (Lechowich and Ordal, 1962); ap) (Williams and Robertson, 1954); aq) (Williams, 1929); ar) (Weil, 1899); as) (Heredia et al., 1997) at) (Heredia et al., 1998) au) (Khoury et al., 1990) av) (Sugiyama, 1951) aw) (Lindsay et al., 1990) ax) (Paredes-Sabja et al., 2008a) ay) (Olivier et al., 2012) az) (Ohye and Scott, 1957) ba) (Yang et al., 2009)

1.2.4.1.7 Sporulation Temperature and Heat Resistance

The overwhelming majority of the studies reports on negative and positive effects on the heat resistance of spores grown at elevated and low temperatures, respectively. Thus, it has been proposed that sporulation temperature and heat resistance (logarithm of D values) frequently show an approximately linear relationship in various Bacillus species (Leguerinel et al., 2007). However, results from some studies indicate that elevated sporulation temperatures do not necessarily correlate with increased heat resistance as the positive effect is not further increased (*B. subtilis*, (Condon et al., 1992)) or even can turn into a negative one (e.g. *B. coagulans*, (Feig and Stersky, 1981); *B. subtilis* A, (Lindsay et al., 1990); *B. licheniformis*, (Baril et al., 2012b)) when sporulation temperatures are increased above certain species-specific values. However, some studies also reported on no significant alterations in the heat resistance of spores grown elevated or lower temperatures.

The major trend observed for *Bacillus* spores, i.e. positive correlation between sporulation temperature and heat resistance (at least up to certain organism specific temperatures) has been also demonstrated for *C. perfringens* (Paredes-Sabja et al., 2008a) (Garcia-Alvarado et al., 1992a). Also according to the majority of findings for *Bacillus* species, decreased sporulation temperatures (24 or 29 vs. 37°C) have been shown to reduce the heat resistance of proteolytic *C. botulinum* spores (Sugiyama, 1951). However, in contrast to *Bacillus* species, it has been reported that even a slight increase in the sporulation temperature (41 vs. 37°C) can reduce the heat resistance of proteolytic *C. botulinum* spores (Sugiyama, 1951). For non-proteolytic type B spores no significant differences in the heat resistance of spores produced at 25, 30 and 35°C has been reported (Peck et al., 1995a). Although data on the effect of the sporulation temperature on the heat resistance of *C. botulinum* spores is limited, available data indicates that, in *C. botulinum*, this effect does not necessarily follow the major trends observed for various Bacilli.

Additionally, it has to be considered that the effect of the sporulation temperature on sporal resistance can vary with intrinsic properties of the surrounding matrix such as the pH of the heating menstruum (Sala et al., 1995) and even the acid used to adjust the pH (Palop et al., 1996) as well as the intensity of the treatment, i.e., heating temperature applied (Sala et al., 1995) (Palop et al., 1996).

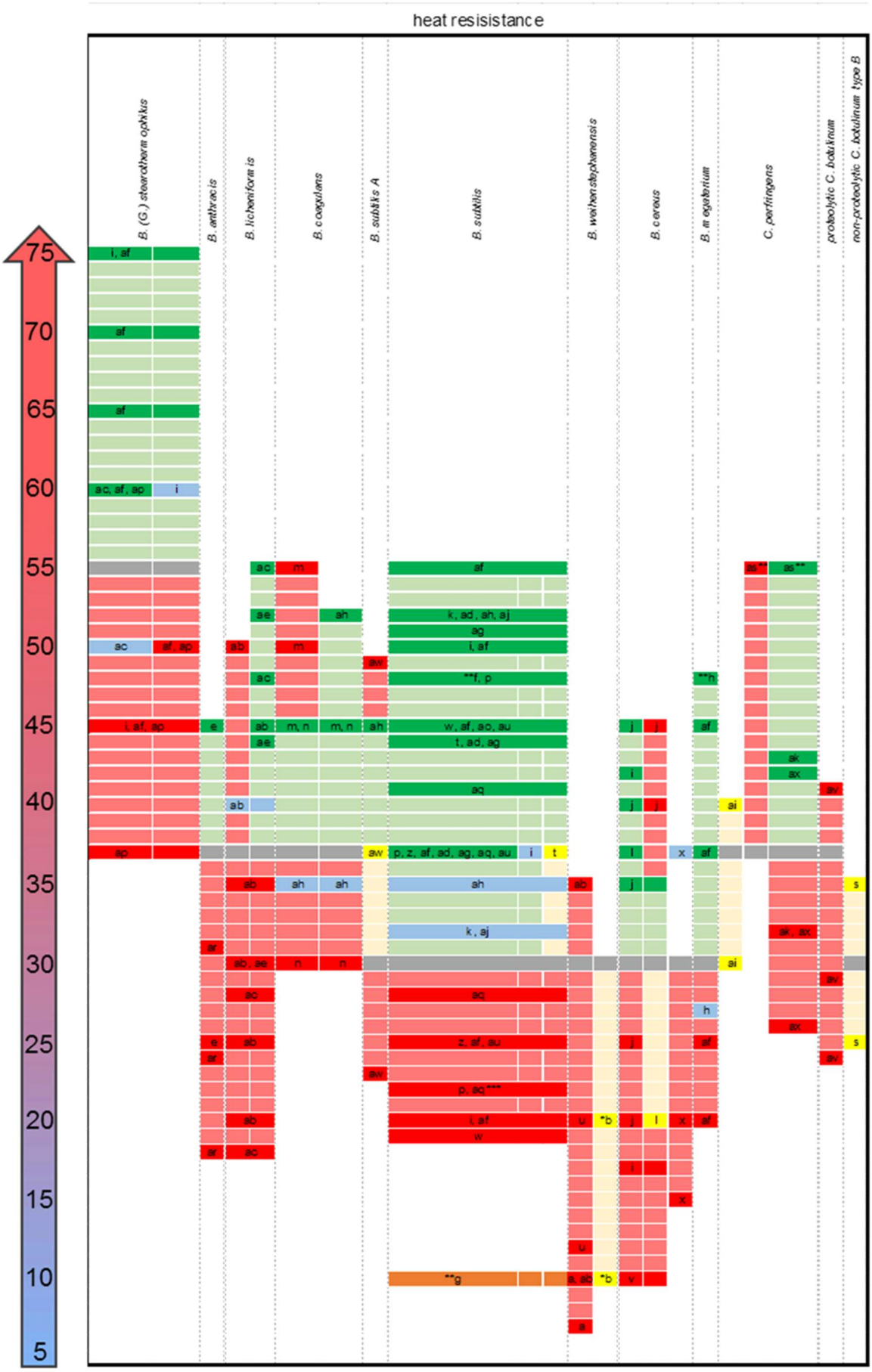

heat resisistance
B. (G.) stearothermophilus
B. anthracis
B. licheniformis
B. coagulans
B. subtilis A
B. subtilis
B. weihenstephanensis
B. cereus
B. megaterium
C. perfringens
proteolytic C. botulinum
non-proteolytic C. botulinum type B
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5

Fig. 1-9: Sporulation temperature and heat resistance.
Summary of data available on heat resistance of spores from different species grown at different temperatures. The sporulation temperature is depicted on the left. Dark red fields indicate negative effects, dark yellow fields indicate no significant effects and dark green fields indicate positive effects compared to the standard temperature used in the respective study. Light red, yellow and green fields indicate extrapolated effects at temperatures between the sporulation temperature and the standard temperatures used in the studies. Gray fields represent the assumed standard temperature for an organism. Whenever a different standard temperature was has been used, this is indicated by light blue fields containing the respective reference. Orange fields are used when a differential effect has been reported (here: effect on resistance dependent on heating temperature applied i.e. increased heat resistance for 85 and 90°C, decreased heat resistance for 95 and 100 °C treatments). *incubation temperature in nutrient broth prior to sporulation in buffer; **short time incubation at elevated or lower temperature (30 min)
[Additional remarks: ab) heat resistance peak at around 45 °C; aw) *B. subtilis* A: optimum growth temperature at 45 °C, biphasic survivor curves slight increase from 30 to 37°C in second i.e. heat resistant fraction; ad) no further increase in heat resistance when sporulation temperature is increased from 44 to 52 °C; aq) room temperature used (21 - 23°C); as) effect of heat shock on heat resistance dependent on the time point of heat shock - heat shock late in sporulation decreases heat resistance]

1.2.4.1.8 Sporulation Temperature, Germination, and Pressure Resistance

Spore components involved in germination are thought to include DPA, membrane proteins, cortex and coat layers (Setlow, 2003) (Moir, 2006) (Magge et al., 2008). The findings of several studies that sporulation at sub-optimal temperatures leads to increased germination efficiency, i.e., the ability to germinate negatively correlates with the sporulation temperature (Planchon et al., 2011) (Gounina-Allouane et al., 2008) (Cortezzo and Setlow, 2005) is in accordance with reports where it has been shown that cold stress induces σ^B (Mendez et al., 2004), which contributes to proper germination (*sigB* deletion mutant strain germinate less efficiently, *B. cereus*, (van Schaik and Abee, 2005) and references therein)). Additionally, germinant receptor operons have been reported to be activated by cold stress (Garcia et al., 2010). Accordingly, slower germination of *B. subtilis* spores prepared at lower temperatures (23 vs. 37°C) has been reported to be mainly due to altered levels of germinant receptors (GerAA,AB,AC) and, possibly to a lower extent, due to changes in GerD (essential for rapid germinant receptor-dependent spore germination) levels (Ramirez-Peralta et al., 2012b). On the other hand, levels of other proteins involved in germination i.e. SpoVA, CLEs (cortex lytic enzyme, at least CwlJ) (Paidhungat and Setlow, 2001) and the spore coat GerP proteins (required for normal nutrient germinant access to germinant receptors in the inner membrane (Carr et al., 2010)), have been suggested to be unlikely to be responsible for sporulation temperature-mediated changes in the germination properties of *B. subtilis* spores (Ramirez-Peralta et al., 2012b).
However, it was reported that the effect of sporulation temperature on germination is not necessarily identical for different nutrient germinants (e.g. different effect on L-alanine or inosine triggered germination in *B. cereus*; (Planchon et al., 2011)) and, from existing data, it is difficult to derive a major trend. The heterogeneity of described effects is likely to be due to differences in the species and germinants tested. For non-proteolytic, non-toxigenic *C. botulinum* it has been reported that both, an increase and a decrease of the sporulation temperature (20 or 37 vs. 30°C) results in better germination in a medium containing different germinants including alanine, lactate and glucose. However, this effect has been shown to be not linear as spores grown at lower temperatures (10 vs. 20 or 30°C) showed significantly lower germination (Evans et al., 1997). Although the same germination medium was used,

germination properties of non-proteolytic *C. botulinum* type B strains have been reported to remain largely unaltered irrespectively of the sporulation temperature (25, 30 or 35°C) (Peck et al., 1995a).

Fig. 1-10: Sporulation temperature, germination and HPT resistance.
Summary of data available on germination characteristics and HHP resistance of spores from different species grown at different temperatures. The sporulation temperature is depicted on the left. Dark red fields indicate negative effects, dark yellow fields indicate no significant effects and dark green fields indicate positive effects compared to the standard temperature used in the respective study. Light red, yellow and green fields indicate extrapolated effects at temperatures between the sporulation temperature and the standard temperatures used in the studies. Gray fields represent the assumed standard temperature for an organism. Whenever a different standard temperature was has been used, this is indicated by light blue fields containing the respective reference.
[Additional remarks: w) germination at low aw; u) L-alanine germination; v) different effects on L-alanine and inosine germination; x) inosine and L-alanine, strain dependent max. and min. sporulation temp., but effect on germination similar]

1.3 *Clostridium botulinum*

1.3.1 The Genus *Clostridium*

Clostridia are non-sulphur-reducing, rod-shaped prokaryotes that include strains of importance to human and animal physiology, cellulose degradation (e.g., *C. phytofermentans* and *C. thermocellum*), solvent production such as butanol, acetone and ethanol (e.g., *C. acetobutylicum* and *C. beijerinckii*) and bioremediation (Jones et al., 2008). Important pathogens within this genus include *C. tetani*, *C. perfringens*, *C. difficile*, and *C. botulinum*. Generally, Clostridia have four common phenotypic properties, i.e., they commonly have (i) a Gram-positive cell wall structure in their vegetative cell forms, (ii) an anaerobic, fermentative metabolism, (iii) a low G+C (guanine+cytosine) content (26–32% for toxigenic species (Cato et al., 1986; Hippe et al., 1999)), and (iv) the capability of forming dormant, non-reproductive endospores under unfavorable conditions, which are highly resistant to many environmental stresses including heat, pressure, UV radiation, and desiccation. Clostridia are widely distributed in the environment and typical habitats include soil and sea sediment as well as animal intestines.

A rough subdivision of the genus *Clostridium* into 19 clusters has been previously proposed (Paredes et al., 2005). The number of putative orthologous genes between *C. botulinum* (ATCC 3502), *C. difficile* (strain 630), *C. acetobutylicum* (ATCC 824), *C. tetani* (asporogeneous strain E88), and *C. perfringens* (non-motile strain 13) that is generally lower than among different *Bacillus* species points towards a high heterogeneity of the genus *Clostridium* (Paredes et al., 2005). Interestingly, *C. botulinum* has been shown to share the highest number of putative orthologues with any other of the *Clostridium* species analyzed having the highest number of putative orthologous pairs (Paredes et al., 2005).

1.3.2 The Species *Clostridium botulinum*

Clostridium botulinum strains have a relatively low G+C content in common (26–28%) (Cato et al., 1986). However, not only the genus *Clostridium*, but also the species *C. botulinum* itself is very heterogeneous. This is mainly due to their classification as *C. botulinum* upon the capability of producing botulinum neurotoxins (BoNTs).

Consistent with an early classification, BoNT-producing organisms belonging to the group I of 23S rRNA homology (Johnson and Francis, 1975) can be subdivided into four distinct groups (I – IV) with different physiological characteristics (Smith, 1975). Additionally, BoNT-producing organisms can be distinguished depending on the toxin type formed (BoNT types A – G). Group I contains proteolytic *C. botulinum* strains producing BoNT types A, B, or F, group II contains non-proteolytic *C. botulinum* strains producing BoNT types B, E, or F, group III contains *C. botulinum* strains producing BoNT types C or D, and group IV is assigned to *C. argentinense* (*C. botulinum* type G) producing BoNT type G. Notably, although all *C. botulinum* and *C. argentinense* strains are capable of producing one or more BoNTs, this is not an exclusive trait of this species, since particular strains of other *Clostridium* species (e.g., *C. butyricum* and *C. baratii* (Suen et al., 1988), sometimes referred to as groups V and VI (Johnson, 2007)) are capable of producing BoNT.

The phylogenetic interrelationship based on the comparative analysis of 16S rRNA sequences of some members of the genus *Clostridium* is depicted in Fig. 1-11 (adopted from

(Hutson et al., 1993)) demonstrating the high heterogeneity of the species *C. botulinum*. There are more detailed phylogenetic trees available, which are based on the sequences of more *C. botulinum* strains and strains from related non-toxic *Clostridium* species (e.g., (Hill et al., 2007; Hill et al., 2009)). Additionally, results from more comprehensive and detailed comparative genomic analyses demonstrating the broad diversity of BoNT-producing *Clostridium* species were published recently (e.g., (Williamson et al., 2016)). However, Fig. 1-11 is sufficient to clearly illustrate the presence of four phylogenetically distinct lineages corresponding the four groups within *C. botulinum* that have been previously defined on the basis of phenotypic criteria (Smith, 1975), i.e., group I, closely related to *C. sporogenes*, non-proteolytic group II with no apparent non-toxigenic relative, group III, closely related to *C. novyi*, and group IV, closely related to *C. subterminale* (Hutson et al., 1993).

The fact that this phylogenetic tree does not contain a non-toxigenic organism closely related to group II, to which *C. botulinum* type E is belonging, reflects that no 'common' *Clostridium* species exhibits a high homology to this group II. However, this comparative analysis of 16S rRNA sequences is certainly not complete and other, less well characterized *Clostridium* species might be closely related to either one of the four *C. botulinum* groups. For example, besides of its non-toxigenicity and differences in the spore morphology (ribbon-like spore appendages), *C. taeniosporum* isolated from Crimean lake silt has been reported to be physiologically and genotypically closely related to organisms from the non-proteolytic *C. botulinum* group II (Iyer et al., 2008).

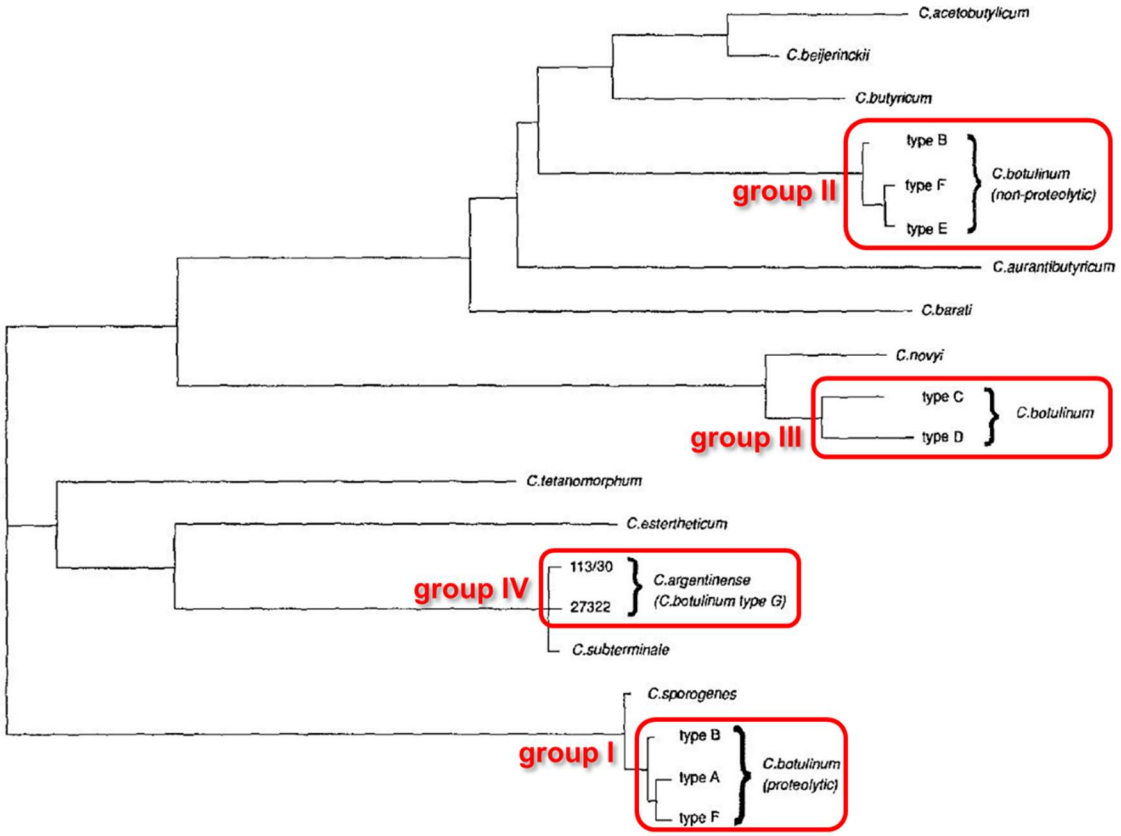


Fig. 1-11: Phylogenetic interrelationships among *C. botulinum* species.
Unrooted phylogenetic dendrogram showing evolutionary distances given by the sum of the horizontal lengths (physiological *C .botulinum* groups indicated in figure adopted from (Hutson et al., 1993)).

A summary of the different *C. botulinum* groups and their differences in physiological characteristics is provided in Tab. 1-1. The physiological groups I to IV corresponding to the four distinct clusters based on 16S RNA gene sequence comparison and DNA/DNA homology vary greatly with respect to conditions supporting growth, spore resistance properties and the capability of metabolizing various substrates.

Tab. 1-1: Physiological properties of BoNT-producing organisms
According to Setlow and Johnson (2007)[1], Johnson (2007)[2], Lindström et al. (2009), (Stringer et al., 2013)[3], and references therein. n/a insufficient data found; + positive; - negative; +/- weak or variable; asterisk* demarks brine values, i.e., NaCl concentration in water.

Neurotoxin producing *Clostridium*	Group I	II	III	IV (*C. argentinense*)	*C. butyricum*	*C. baratii*
Related non-BoNT producing *Clostridium*	*C. sporogenes*	n/a (putatively *C. taeniosporum*)	*C. novii* *C. haemolyticum*	*C. subterminale* *C. proteolyticus* *C. schimacherense*	*C. butyricum*	*C. baratii*
Toxin types	A, B, F	B, E, F	C, D	G	E	F
Physiologic properties	Highly thermo-resistant spores	Growth at 3°C	Optimum growth around 40°C	n/a	n/a	n/a
Growth temp. [°C]						
minimum	10	(2.5[3]) 3 (3.3[1])	15	12	10	20
optimum	35 – 40	18 – 25	35 – 40	35 – 40	30 – 37	30 – 40
maximum	48 (50[1])	45	n/a	45	~40	n/a
Minimum pH	4.6	5.0	n/a	n/a	~3.6	n/a
Inhibitory a_w	0.94	0.97	n/a	n/a	n/a	n/a
Inhibitory NaCl conc.*	10%	5%	3%	>3%	n/a	5%
Heat res. D values [min]			n/a	n/a	$D_{80°C}$ 4 – 5[1]	n/a
$D_{100°C}$	~25[2]	<0.1			$D_{85°C}$ 0.4 – 0.8[1]	
$D_{121°C}$	0.21[2]	<0.005				
max. redox potential (E_h), critical O_2 level [%]	+200 mV, 1 – 2%	n/a	n/a	n/a	n/a	n/a
Proteolysis	+	-	-	+	-	-
Saccharolysis	-	+	-	-	n/a	n/a
Lipase production	+	+	+	-	-	-
Metabolism						
Glucose	+	+	+	-	+	+
Fructose	+/-	+	+/-	-	+	+
Sucrose	+/-	+/-	+/-	-	+	+
Maltose	+/-	+/-	+/-	-	+/-	+
Lactose	-	-	-	-	+/-	+
Metabolism						
Gelatin	+	+	+	+	-	-
Milk	+	-	+/-	+	-	-
Meat	+	-	-	+/-	-	-
Lecithinase	-	-	-	-	+	-

1.3.3 Botulinum Neurotoxins (BoNTs)

1.3.3.1 Subtypes of BoNTs

Based on gene sequence comparison, the different BoNT main types (A – G) can be divided into subtypes, which show different binding affinity to monoclonal antibodies (Smith et al., 2005) and toxinotype-dependent nucleotide / amino acid identities of around 84 – 99% [A (92 – 95% / 84 – 90%), B (96 – 98% / 94 – 96%), E (99% / 97 – 98%), *C. botulinum* type E vs. *C. butyricum* type E (97 – 98% / 95 – 96%) (Hill et al., 2007)]. Such subtypes are not strictly, but tend to be source and geography dependent (e.g., A1 USA/foodborne; A2 Europe/foodborne or infant botulism) and, thus, potentially evolved separately in different genomic backgrounds (Hill et al., 2007). On overview of various subtypes according to Raffestin et al. (2009) is provided in Tab. 1-2.

Most frequently, one strain produces one toxin type and the botulinum locus is present in a single copy on the genome. However, there are exceptions and the localization of BoNT gene(s) (chromosome, plasmid (Marshall et al., 2007), bacteriophage encoded (Raffestin et al., 2009)) appears to be toxinotype- and can be strain-dependent (Tab. 1-2). Additionally, some strains can carry two different types of toxin, which is usually indicated by a lower case letter for the second toxin with lower production levels, e.g., Ba strains can produce 10-times higher amounts of type B toxin (Tab. 1-2). Smaller case letters in brackets indicate silent genes, e.g., A(b) silent toxin B (Tab. 1-2) (Hutson et al., 1996). A slash, e.g., C/D, indicates mosaic genes between BoNT genes types C and D, which can be distinguished from classical types C and D (Tab. 1-2) (Hill et al., 2007).

Tab. 1-2: Toxin types and subtypes of BoNT-producing organisms.
According to Hill et al. (2007), Raffestin et al. (2009) and references therein. See (Lindström et al., 2009)[1] and references therein.

Neurotoxin producing *Clostridium*	Group					
	I	II	III	IV (*C. argentinense*)	*C. butyricum*	*C. baratii*
Toxin Type	A proteolytic B F	E non-proteol. B F	C, D	G	E	F
Subtype	A1, A2, A3, A4, B1, B2, B3, bivalent B (Ba, Bf, Ab), proteolytic F	E1, E2, E3, E6, non-proteolyt. B, F	C, D, C/D	G	E4, E5	*baratii* F
Genomic localization of toxin gene	Chromosome (Subtype A3 and A4 found on plasmid)	Chromosome (Subtype B found on plasmid)	Bacteriophage	Plasmid	Chromosome	n/a
Target SNARE protein cleaved	A: SNAP-25 B and F: Synaptobrevin	E: SNAP-25 B and F: Synaptobrevin	C: SNAP-25 and Syntaxin D: Synaptobrevin	Synaptobrevin	SNAP-25	Synaptobrevin
Botulism	Human	Human (animal)	Animal	None or very rarely[1]	Human, animal?	Human, animal?

1.3.3.2 The Botulinum Locus

Genes coding for BoNTs and associated non-toxic proteins are clustered in close vicinity and constitute the BoNT locus. In addition to the BoNT structure and the localization of the *bont* gene cluster (chromosome, plasmid, phage), the composition of this cluster and flanking regions can differ greatly among different toxinotypes and slightly among subtypes. As an example, the *bont* gene cluster organization in two non-proteolytic *C. botulinum* type E strains Alaska (toxin subtype E3) and Beluga (toxin subtype E1; one of the strains used in this study) and that in the non-proteolytic *C. botulinum* type B strain Eklund 17B (toxin type non-proteolytic B; npB) are depicted in Fig. 1-12 (detail of figure from (Hill et al., 2009)). The organization of the botulinum locus is highly conserved in the 3' end containing *bont* (botulinum neurotoxin) genes that are preceded by *ntnh* (non-toxic-non-haemagglutinin) genes both transcribed in the same orientation (Hill et al., 2009). In contrast, the 5' part differs among BoNT-producing *Clostridium* species, where either *ha* (haemagglutinin, (*ha33*, *ha17*, *ha70*, type G no *ha33*)) genes or *orf* (*orfX1*, *orfX2*, *orfX3*, *orfX3* missing in some A2 clusters) genes can be located upstream of *ntnh-bont*. In the non-haemagglutinating toxinotypes including in the *C. botulinum* type E strains Alaska and Beluga, *p47* (encoding a 47 kDa protein) is located immediately upstream of *ntnh* and transcribed in the same orientation (Fig. 1-12). Additionally, the BoNT/E locus is the only one known to date that is lacking *botR* (previously referred to as *orf221* or *orf22*). BotR is a 21-22 kDa protein that harbors features of a regulatory protein and was suggested to present a main regulatory factor in toxin production. However, environmental triggers for this alternative sigma factor specific to the transition phase (exponential to stationary growth) are not completely known and the fact that toxin production kinetics and relative expression profiles of *bont* genes (and *p47*) are similar in *C. botulinum* type A and type E (Couesnon et al., 2006), makes it likely that other regulatory genes (at least in *C. botulinum* type E) are involved in the cascade regulating toxin formation (Hill et al., 2009).

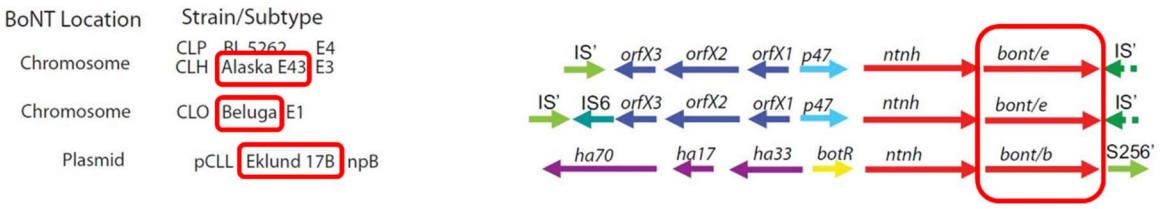


Fig. 1-12: The *bont* gene cluster organization.
Gene coding for BoNT type E: *bont/e*; non-toxic-non-haemagglutinin gene: *ntnh*; Botulinum toxin regulator: *botR*; 47 kDa protein: *p47*; open reading frames: *orfX123*; Possible flanking regions: IS elements, flagellin (*fla*; not depicted), *lycA* (not depicted), or hypothetical (hypo) proteins (not depicted) (detail of figure from (Hill et al., 2009); strains and *bont* genes highlighted).

1.3.3.3 *Bont/E* Expression

The relative expression of *bont/E* peaks during the transition between the exponential and stationary growth phases (Artin et al., 2008; Couesnon et al., 2006) However, BoNT/E can be already detected at a low level during the entry into the exponential growth phase (Couesnon et al., 2006). The maximum BoNT/E concentration is typically reached during the stationary phase around the time point when maximum cell numbers are reached (Couesnon et al., 2006).

Thus, the question whether toxin is formed or not generally depends on environmental factors affecting the growth of *C. botulinum*, i.e., growth limiting factors (e.g., metabolic substrates, temperature; Tab. 1-1) are also limiting factors for toxin production. However, besides of this general dependency, some factors can influence the amount of and time to toxin production independently from their effect on the growth rate. For example, high carbon dioxide concentrations (70% vs. 10% (v/v) headspace CO_2) were shown to increase the lag time and decrease the maximum growth rate, but lead to a two-fold increase in *bont/E* expression and formation of extracellular BoNT/E (Artin et al., 2008). Although it has been reported that headspace oxygen (up to 100%) or film transmission rate, respectively, have little and no significant influence on the time to toxigenesis or spoilage caused by *C. botulinum* type E (rainbow trout fillets; toxicity within 5 days at 12 °C storage) (Dufresne et al., 2000), it seems reasonable that food packaging material-dependent gas transmission rates can influence this time span. Accordingly, oxygen transmission rates were later reported to have an influence (raw, refrigerated, 100% CO_2-packaged flounder fillets; (Arritt et al., 2007)). Such seemingly contradicting results might differences in food characteristics (e.g., effect of food components on the local redox potential) or simply reflect different localization of the spores in the samples. Additional factors that can affect toxin production in various *C. botulinum* strains include glucose, the availability of nitrogen and carbon sources, arginine (represses BoNT/A production in minimal medium), and protein availability (casein supplementation increases toxin production in minimal medium) ((Raffestin et al., 2009) and references therein).
Thus, food composition, packaging atmosphere and material, and storage temperature are factors that can influence toxin production in food products. Additionally, the *C. botulinum* contamination level, its presence as vegetative cells or spores (probably even the degree dormancy of the spore population), the microenvironment of a spore (localization in the food), and co-existing microflora can affect toxin production. The number and possible interplay between these factors can make it difficult to predict the time required for and amount of toxin production in complex food products without hurdles preventing *C. botulinum* growth.
Factors that can affect the growth, generally, also affect the time of onset of detectable spoilage, i.e., the production of gas (CO_2, H_2) and typical volatile compounds (e.g., 1-butanol, butyric acid) caused by *C. botulinum* type E in food (Moschonas et al., 2010). However, toxigenesis may precede organoleptic spoilage (Baker et al., 1990), e.g., toxin formation on day 9 and detectable spoilage on day 15 (raw, refrigerated, 100% CO_2-packaged flounder fillets; (Arritt et al., 2007)). Similarly, several studies showed that, under suitable conditions, non-proteolytic *C. botulinum* can form toxin within 10 days at 8 °C, which is around the average shelf-life (approx. 10 days) of commercial short shelf-life chilled-stored foods (Peck et al., 2008).

1.3.3.4 BoNT/E Properties

1.3.3.4.1 The Botulinum Complex

Botulinum NTs are synthesized as inactive / weakly active precursor proteins (approx. 150 kDa), which are possibly released from the bacterium by a cell wall exfoliation mechanism. In the case of *C. botulinum* type E, BoNT/E is produced as RNA–toxin complex composed of the neurotoxin (15-kDa, 7.3S), the NTNH non-toxic protein (15-kDa, 7.3S), and RNA (23 S, 16 S rRNA or both). The latter component in this complex has been speculated to be involved in toxin biosynthesis via its role in the process of the secretion (release) from the bacterial

cell, since BoNT/E is released as inactive single chain without RNA (Kitamura, 2002). Released precursor BoNTs generally consist of a light chain (LC), which is the catalytically active domain (CD), and a heavy chain (H). The N-terminal end of the heavy chain (H_N) represents the translocation domain (TD), whereas the C-terminal domain of the heavy chain (H_C) represents the binding domain (BD) of the toxin.
Depending on the toxinotype, the botulinum complex can additionally contain multiple HA (haemagglutinin) proteins (e.g., in a ration of HA33:HA17:HA70 of 1:1:2 for type A). Such complexes might, in rare cases (type A), also form dimers (Sharma et al., 2003). In the non-haemagglutinating toxinotypes such as type E, it has bee reported that only BoNT-NTNH complexes can be found (Kitamura, 2002; Oguma et al., 1999). Additionally, non-toxic proteins OrfX1 and neurotoxin-binding protein (NBP) (but not OrfX2, OrfX3, or P47) have been detected in type E progenitor toxin complexes by mass spectrometry. However, they were speculated to primarily have a transcriptional regulatory function (Hines et al., 2005). The upper left part of Fig. 1-13 illustrating a typical BoNT/E intoxication route contains a cartoon of possible type E botulinum complexes.

1.3.3.4.2 Toxicity and Stability

With an LD_{50} in the range of 0.1 – 1 ng/kg body weight, i.e., theoretically, 10^{-15} – 10^{-16} M, if equally distributed in the body fluids (Schiavo et al., 1994), BoNTs belong to the most potent toxins known. Such amounts of toxin, sufficient to cause illness (botulism) and even death, could, potentially, be present in just a few micrograms of food (Lund and Peck, 2000). The toxinotypes that are most important as causative agents of botulism in humans are BoNT/A, B and E (produced by *C. botulinum* group I or II organisms) (Tab. 1-2). Some properties are similar between BoNT/E and the probably best studied BoNT, BoNT/A, which include extracellular receptor components, pH profiles for membrane association, channel/gating properties, and substrate specificity (SNAP-25) (Keller et al., 2004). However, in addition to the differences in the botulinum locus coding for these BoNTs, and associated non-toxic proteins (section 1.3.3), they differ in their place of activation, the botulinum complexes formed, as well as their potency, speed of action, and stability.
The BoNT/A is the most potent BoNT surviving remarkably longer in nerve terminals than BoNT/E, which presents a main reason for BoNT/A being used as a medical therapeutic for treating muscle disorders (Keller et al., 2004). However, BoNT/E acts faster than any other BoNT, which might be due to its unique domain organization resulting in a more globular protein structure compared to the more linear ones of BoNT/A and B and leading to faster internalization and translocation (Kumaran et al., 2009; Wang et al., 2008).
The botulinum complex consisting of BoNT/E and associated non-toxic proteins is functionally more stable against temperature than the BoNT/A complex and retains significant activity (42% of the optimum enzyme activity) even at 55 °C (30 min) and residual activity at temperatures up to 65 °C (Kukreja and Singh, 2007). Accordingly, normal cooking procedures should be sufficient to inactivate all types of BoNT/E (and a fortiori BoNT/A or B) (Licciardello et al., 1967). However, the toxin stability can be largely pH- (maximal BoNT/E stability at pH 5.5, decreasing stability upon increasing pH (Licciardello et al., 1967)) and food matrix-dependent (e.g., BoNT/B not inactivated by pasteurization in milk (Rasooly and Do, 2010)), which could potentially lead to residual activity of BoNTs present in food that is not

adequately heated before its consumption. Nonetheless, the major food safety issue naturally pertains to ready-to-eat (RTE) food products.

1.3.4 Botulism

1.3.4.1 BoNT/E Intoxication Route

A typical intoxication route and what typically happens to a BoNT/E complex during this route is summarized in Fig. 1-13.

In the case of foodborne botulism, BoNTs are **(i) preformed in food**, i.e., formed during *C. botulinum* growth (section 1.3.3.3) and released as complexes (section 1.3.3.4) in the surrounding food matrix. The preformed toxin is then **(ii) ingested** together with it. Precursors of BoNTs are often proteolytically activated (cleavage between CD and TD, which are then only held together by a disulfide bond) in the extracellular medium by specific proteases produced by neurotoxigenic strains (e.g., type A). However, in the case of BoNT/E this needs to be done by exogenous proteases, which can be naturally found in mammal intestines (e.g., trypsin). The formation of complexes of BoNTs with their respective NTNH protein, is thought to protect BoNTs against degradation in the host intestinal tract (Gu et al., 2012). On their way from the digestive tract to the circulation, BoNT complexes disassemble in an pH-dependent manner (near neutral pH) (Gu et al., 2012). This enables BoNTs to cross the epithelial barrier and **(iii) enter the circulation**. HA proteins have been reported to play a role in crossing the epithelial barrier of the gastrointestinal tract opening intercellular tight and adherence junctions and, thus, facilitate the entry into the systemic circulation of a host by passive diffusion (Sugawara and Fujinaga, 2011). However, are absent BoNT/E complexes and thought to present pathogenicity factors (increasing toxicity) rather than being essential for intoxication (Matsumura et al., 2008; Sugawara and Fujinaga, 2011). Simultaneous to and/or as alternative to crossing the mucosal barrier with the aid of HA proteins, transcytosis mechanisms (i.e., vesicle transport without altering epithelial cells) have been proposed previously (Couesnon et al., 2008). The toxin is transported to nerve endings via the hosts blood and/or lymphatic circulation, recognizes and **(iv) binds to specific receptors** on unmyelinated areas of the presynaptic membrane (i.e., at axon terminals), e.g., a ganglioside of G_{1b} series (G_{D1b}, G_{T1b}) and a glycoprotein such as synaptotagmin I or II (double receptor concept (Rummel et al., 2007)). In the case of BoNT/E the double receptor concept involves binding to charged, high-density, low-affinity gangliosides (GT_{1b}) and binding to the specific protein receptor synaptotagmin (SytII). Toxins are internalized into the nerve ending by receptor-mediated **(v) endocytosis** in a clathrin coated vesicle, where the special domain organization of BoNT/E is putatively responsible for the high translocation speed and the fast action of this toxin (faster than other BoNTs) (Kumaran et al., 2009). The vesicle is acidified putatively leading to conformational changes of the toxin and exposure of its hydrophobic domains at the surface. This results in pH-dependent channel formation by the H_N domain allowing the translocation of the light chain (LC), i.e., **(vi) catalytic domain (CD) into the cytoplasm** of nerve end cells.

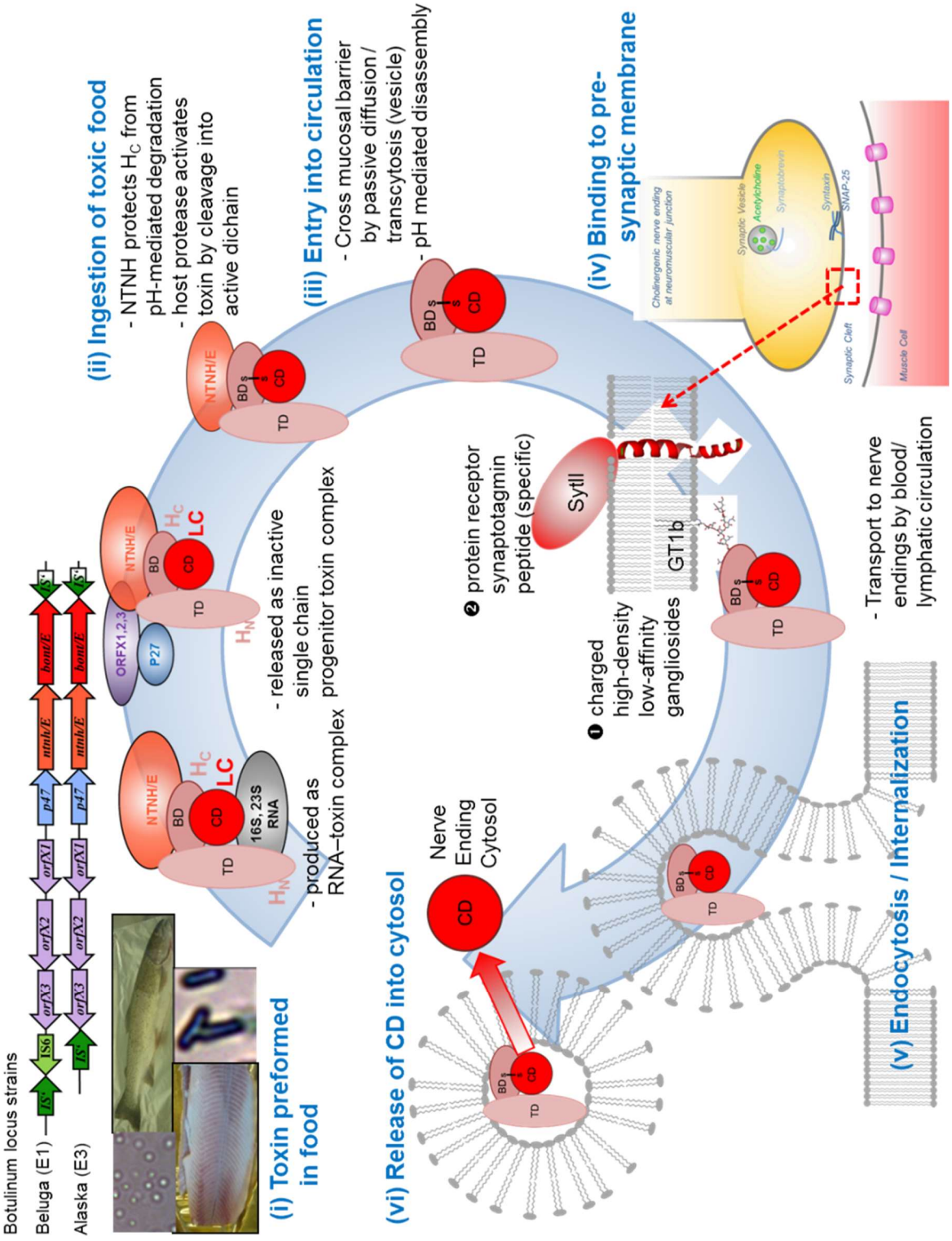


Fig. 1-13: The fate of BoNT/E during intoxication.
Typical intoxication route from the food to the nerve ending of the host. P27, 27 kDa protein; OrfX1,2,3, proteins encoded by open reading frames 1 through 3; NTNH, non-toxic, non-haemagglutinin protein; LC, light chain = CD, catalytically active domain; H, heavy chain; H_N, N-terminal end of the heavy chain = TD, translocation domain; H_C, C-terminal domain of the heavy chain = BD, binding domain. GT_{1b}, high-density, low-affinity gangliosides; SytII, specific protein receptor synaptotagmin. Data for figure from references cited in the text.

The mode of action of the CD of BoNT/E after its release into the cytoplasm are illustrated in Fig. 1-14. Depending on the toxinotype, different **(vii) target molecules are cleaved** (also see Tab. 1-2) mediated by a conserved zinc-dependent proteolytic site in the middle of the CD. These target molecules have in common that they are responsible for the formation of the SNARE (soluble NSF-attachment protein receptor, NSF = N-ethylmaleimide-sensitive fusion protein) complex. Formation of the SNARE complex is crucial for the attachment and fusion of synaptic vesicles with the synaptic membrane. In the case of BoNT/E, this target molecule is SNAP-25 (synaptosomal-associated protein, 25 kDa). Failure of the synaptic vesicles, which carry the neurotransmitter acetylcholine (Ach), to fuse with the membrane results in a blockade of Ach-release into the synaptic cleft. As no Ach can be released anymore, Ach-binding receptors at the muscle cell can no more trigger signal transduction, which leads to flaccid paralysis of muscle fibers. The specificity of cell surface receptor in addition to the intracellular target present major reasons for the different sensitivity of different mammals to different BoNTs and the blockade varies in intensity and duration depending on the neurotoxin type (Meunier et al., 2002).

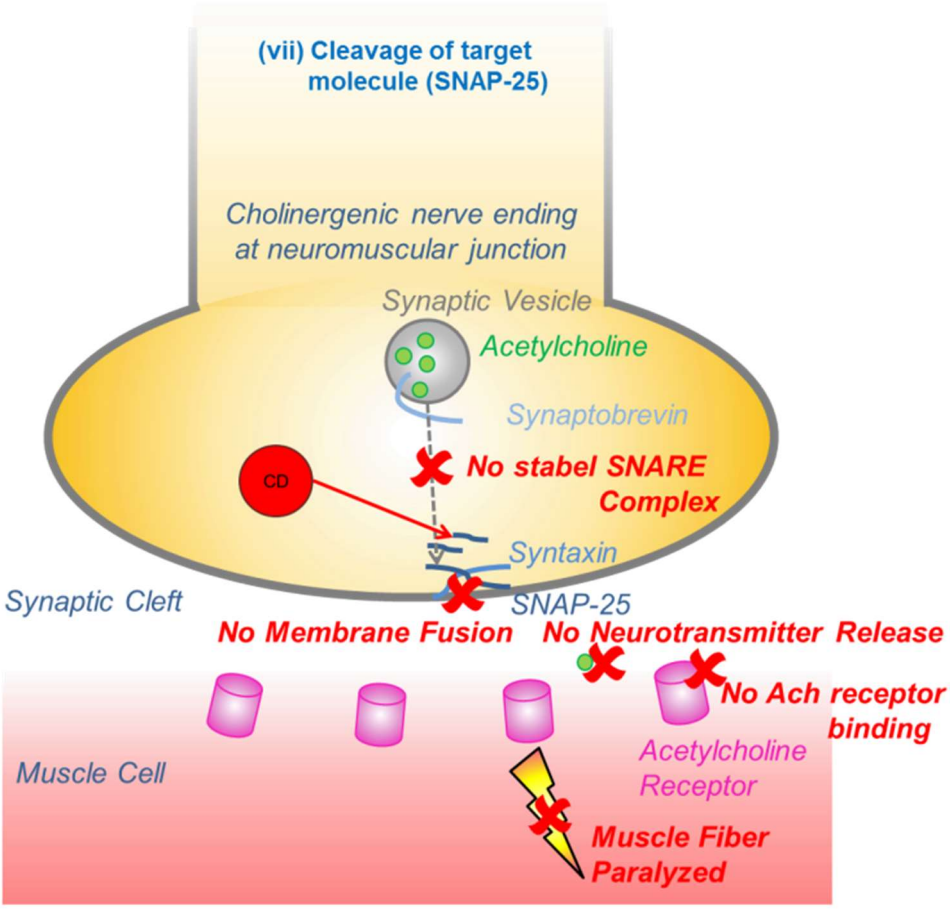


Fig. 1-14: BoNT/E action in the cytoplasm of a nerve terminus.
The catalytic domain of BoNT/E (CD, red) released into the nerve ending cytosol (yellow) cleaves SNAP-25 (dark blue) leading to unstable SNARE complex formation, no fusion of the synaptic vesicle (gray) with the membrane, no Acetylcholine (green) release, no Ach receptor (pink) binding and paralysis of the muscle fiber (light red). SNARE complex, soluble NSF-attachment protein receptor complex; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP-25, synaptosomal-associated protein, 25 kDa; Ach, neurotransmitter acetylcholine. Data for figure is cited in the text.

1.3.4.2 Botulism Symptoms and Mortality

Botulism symptoms usually appear within 12 to 36 hours (minimum 4 h, maximum 8 d) after exposure. In contrast to other BoNTs, toxin types E and B can cause nausea and vomiting. However, the main botulism symptoms are caused by interrupted signal transduction at neuromuscular junctions (Fig. 1-14) causing progressive flaccid muscle paralysis. Muscles supplied by cranial nerves, i.e., muscles controlling eye, facial, chewing, and swallowing movements, are usually affected first. Thus, first symptoms are double vision, drooping of eyelids, loss of facial expression, and problems to swallow or talk. Muscle paralysis typically then spreads to the arms (from shoulder to forearms) and legs (from thighs to feet). In addition to affecting the voluntary muscles, the intoxication can also cause disruptions in the autonomic nervous system leading to symptoms such as a dry mouth and throat, decreased blood pressure on standing, lightheadedness, blackouts, and constipation. In severe cases of botulism muscle paralysis can lead to respiratory failure resulting in coma and death (Sobel, 2005).

Due to advances in food processing / food safety regulations, botulism is a rare disease (see 1.3.4.3). Additionally, the case fatality rate for foodborne botulism decreased from 60 – 70% (before the 1950s) to approximately 5-10% in developed countries nowadays (CFSPH, 2010; WHO, 2002), which is related to an increased quality of supportive care and the comprehensive availability of antitoxins in many countries. Interestingly, first in 2010, the CDC released a heptavalent botulinum antitoxin (H-BAT) also covering BoNT/E replacing a bivalent antitoxin as approved/preferable treatment for food-borne botulism in the USA (Horowitz, 2010).

Nonetheless, *C. botulinum* still presents a major threat to food safety. This is due to the fact that it still presents a severe illness with a potentially high mortality rate if treatment is not immediate and proper (WHO, 2002). If botulism is not proper treated, the time of onset of the first symptoms, which depends on the amount of toxin ingested, is decisive for the course of this disease. For example, data for early type E botulism cases demonstrate that, without prompt antitoxin administration, the case fatality is more than 50% for patients developing symptoms within the first 18 h after exposure ((1960s, Japan), (Nishiura, 2007)). In addition to the amount of toxin ingested and the time point of treatment, toxin type (faster BoNT/E, more potent BoNT/A; also see 1.3.3.4) and host sensitivity to the toxin / host condition in general (e.g., higher case fatality rate for elderly people) influence the severity of botulism (WHO, 2002).

1.3.4.3 Botulism Incidence

Almost all cases of human botulism are caused by BoNT types A, B, or E (Tab. 1-2). *Clostridium botulinum* type E is primarily associated with foodborne botulism. In rare cases, this organism can colonize skin lesions causing wound botulism mediated by the *in vivo* production of BoNT/E (Artin et al., 2007). Type E botulism cases where BoNT/E is produced in the lumen of the intestinal tract, i.e., infant or adults intestinal botulism, is relatively rare and more likely associated with BoNT/E producing *C. butyricum* strains rather than *Clostridium botulinum* type E (Fenicia et al., 2007; Fenicia et al., 1999). Additionally, *C. botulinum* type E can cause botulism symptoms in fish (Getchell et al., 2006), which can represent a vector for transfer of BoNT/E to fish-eating birds (Lafrancois et al., 2011; Yule et al., 2006).

The yearly incidence of botulism in OECD countries has been reported to range between 0.01 and 1.6 cases per 100,000 population (Rocourt et al., 2003). In developed countries, the incidence of botulism is generally low, e.g., with an average of around 0.01 – 0.02 (RKI, 2016), 0.02 – 0.04 (ECDC, 2014), and 0.03 – 0.04 (CDC, 2013) cases per 100,000 population per year in Germany, the European Union, and the United States of America, respectively. The confirmed number of case and the incidence of botulism that have been reported for Germany and the EU in the years between 2000 and 2015 are summarized in Fig. 1-15. The number of botulism cases in the EU is somewhat country-specific. Interestingly, Romania, Poland, Italy, and France are constantly among the countries with the highest case numbers (e.g., in 2012: Romania (19 cases, 15 confirmed, 0.08 per 100,000); Italy (20 cases, 20 confirmed, 0.03 per 100,000); Poland (22 cases, 9 confirmed, 0.02 per 100,000); France (10 cases, 6 confirmed, 0.01 per 100,000)). This potentially reflects that home-curing is more widespread in some regions of these countries than in other countries.
Since authorities collecting data for cases of this reportable disease do not record data on the type of *C. botulinum*/BoNT type causing the illness (in some cases this might not even been known), it is impossible to draw conclusions from these numbers on how many cases were provoked by *C. botulinum* type E. However, it can be assumed that this organisms is responsible for only a fraction of the already small number of botulism cases.

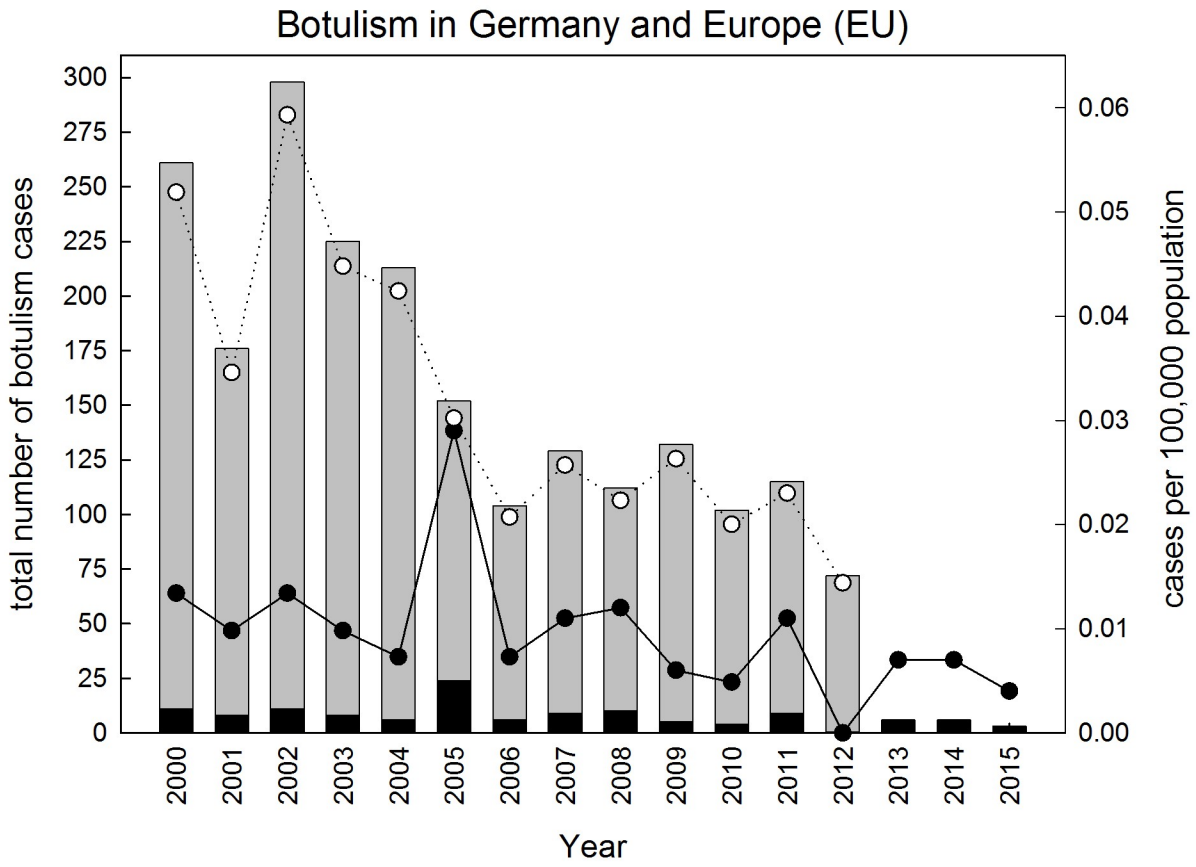


Fig. 1-15: Botulism in Germany and the European Union.
The reported number of botulism cases for Germany is indicated by black bars (left y-axis) (data source: (RKI, 2016)). The confirmed number of botulism cases in the EU is indicated by gray bars (left y-axis) (data source: (ECDC, 2014), data for 2013 – 2015 were not available). The incidence is expressed as cases per 100,000 population and indicated by line plots (right y-axis) (black: Germany; white: EU).

1.3.5 *Clostridium botulinum* Type E Prevalence

1.3.5.1 Environment

Generally, *C. botulinum* type E is associated with aquatic environments. Available data on the prevalence of this organism suggests a region-dependent distribution. Exact reasons for this region-dependent distribution are unknown, but it has been reported that the overall prevalence and spore counts in aquatic sediments tend to correlated with offshore bottom oxygen content, depth, and bioturbation activity, but not with the bottom water temperature (Hielm et al., 1998b). However, a higher sediment/water temperature together with anoxic layers near the sediment, decreased redox potential and pH, and low mean annual water levels have been reported to favor avian botulism outbreaks caused by *C. botulinum* type E (Lafrancois et al., 2011; Perez-Fuentetaja et al., 2011).

A number of surveys have been conducted in the past determining the prevalence of *C. botulinum* in soil and sediment samples from various places around the world, many of them in the Scandinavian region in Europe, which appears to be a common habitat for *C. botulinum* type E. Available data on the prevalence of *C. botulinum* type E including the percentage of positive sample and, if reported, average contamination levels in European environmental and food samples are summarized in Fig. 1-16.

Europe

In European environmental samples, high prevalence of *C. botulinum* type E has been reported for:

● marine sediment from Denmark (Huss and Eskildsen, 1974) (n = 212, 92% *C. botulinum*, exclusively type E, (Huss, 1980))

● Danish open sea (n = 21), Tidal area (n = 10), closed fjord (n = 60), bay and strait (n = 86), natural lake (n = 52) (sampling locality-dependent up to 100% positive for *C. botulinum*, almost exclusively type E, (Huss, 1980))

● Danish freshwater sediment (n = 87, 86% *C. botulinum*, mainly type E, sporadically type B, some sample serotypes not specified, (Huss, 1980))

● Danish fish farms (Huss and Eskildsen, 1974)

● the Sound region between Denmark and Sweden (~60% *C. botulinum* type E, (Johannsen, 1963))

● Sweden and adjacent waters (Johannsen, 1963)

● the Baltic Sea (81%, n = 110, N = 940 CFU/kg, exclusively type E) (Hielm et al., 1998a; Hielm et al., 1998b)

● Finnish mainland freshwater (61%, n = 110, N = 370 CFU/kg, exclusively type E) (Hielm et al., 1998a; Hielm et al., 1998b)

● Finnish trout farm sediment (highly sampling locality-dependent, two trout farms with 100% and 0% *C. botulinum* type E contamination (Ala-Huikku et al., 1977)) (68%, n = 333, N = 2020 CFU/kg, exclusively type E, (Hielm et al., 1998a; Hielm et al., 1998b))

● marine samples from the North Sea, British islands, and adjacent waters (Skovgaard, 1979)

● Greenland marine sediment (86%, n= 21, positive for *C. botulinum*, mainly type E, some sample serotypes not specified, (Huss, 1980)).

In contrast, *C. botulinum* type E has been found less frequently in other European regions, which indicates a high variability of *C. botulinum* type E environmental prevalence even in Europe. Such regions include:

● marine and freshwater sediments of the Faroe Islands and Iceland (0 – 7%) (Huss, 1980)
● terrestrial samples from Denmark and Iceland (almost exclusively non-proteolytic *C. botulinum* type B, (Huss, 1980)).
● river sediments from the south of France (Grand Rhone and Petit Rhone some positive sample for *C. botulinum* types B and E) (Smith and Moryson, 1977)
● mud from lakes, marshes, and waterways in the south of France (Camargue region, 4 – 5% positive, exclusively type E (Smith and Moryson, 1977)).
● northern France (Atlantic coast, n = 25, 4% positive, exclusively type E, average MPN counts: 1 – 2 spores/kg, (Fach et al., 2002))
● British sediments (type B predominant serotype (Dodds, 1993))
● trout farms in Scotland (n = 44, 20% positive, exclusively non-proteolytic type B (Burns and Williams, 1975)).

Worldwide

Similar to northern Europe, high prevalence of *C. botulinum* type E has been demonstrated in samples from:
● rivers in Japan (n = 98, 33 – 82% positive for type E, 7% type B, 9%type C (Yamakawa and Nakamura, 1992)).
● northern USA/southern Canada, e.g., Lake Erie (Perez-Fuentetaja et al., 2011).

Although reports for other regions of the world are scarce, which impedes drawing of meaningful conclusions, *C. botulinum* type E seems to less prevalent in other, e.g., tropical regions than in temperate regions of the northern hemisphere. For example, in aquatic environments of the tropical Indian subcontinent (sediment from brackish water, fresh water farms, and seashore, n= 71, 18% type C and D, 3% type A (Lalitha and Gopakumar, 2000)).

1.3.5.2 Raw food

The presence of *C. botulinum* spores in marine sediments can lead to contamination of fish and other seafood. Logically consistent, the prevalence of *C. botulinum* type E in fish and fishery products is region-dependent, i.e., it is generally more likely to be found in regions with a high prevalence of this organism in sediment.

Europe

Type E has been reported to be the predominant (or only) *C. botulinum* serotype contaminating raw fish in various studies, mainly form the Scandinavian region (unless explicitly stated % values refer to samples positive for *C. botulinum* type E):
● wild fish from Danish fjords (average 36.2%), the Baltic Sea (average 21.8%), , Kattegat (average 18.7%), the North Sea (average 4.8%), and the North Atlantic (average 0.4%) (herring, mackerel, salmon, cod, eel, and flatfish, n = 1407, (Huss and Pedersen, 1979))
● trout from fish farms in Denmark, with some samples being heavily contaminated (5300 spore/kg) ((Huss and Eskildsen, 1974) cited in (Hyytiä-Trees, 1999)).
● wild raw fish from Finland ((Rainbow trout, Burbot, Whitefish, Vendace, and Baltic herring, n = 438, average 18.7%, 180 spores/kg) and fish roe (n = 208, 4 – 14%, average 7.8%, 60 spores/kg). (Values for wild rainbow trout: total: n= 168, 16.7%, 175 spores/kg; intestinal samples: n = 117, 15%, 240 spores/kg; skin samples: n= 51, 20%, 110 spores/kg; trout roe: n = 55, 5%, 50 spores/kg) (Hyytia et al., 1998)

● fish caught in the German part of the Baltic Sea (7.1%, (Baumgart, 1972))
● wild river lampreys from Finland (n= 67, 1.5%, 100 spores/kg; exclusively type E, (Merivirta et al., 2006))
● trout from Finnish fish farms (intestinal: 4 – 10%, (Ala-Huikku et al., 1977)) (intestinal: n = 165, 15%, 166 spores/kg, skin: n = 43, 5%, 310 spores/kg, exclusively type E, (Hielm et al., 1998a))
● farmed fish (n = 87, 33.3% *C. botulinum*, predominantly type E, 265 spores/kg) and wild freshwater fish (river Lech, n = 10, 0%) from the south of Germany (Bavaria) (Hyytia-Trees et al., 1999) (fish species-dependent: Rainbow trout intestine n = 55, 3.6%, 115 spores/kg; Bream surface: n = 4, 100%, 435 spores/kg).

While type E is the most frequent type and almost exclusively found in environmental and fish samples from Scandinavian countries, it might not be the predominant serotype in fish from other European regions or even absent. For example, 4% type A, 12% type B and only 1.1% type E have been detected in seawater fish from northern France (n = 175, 3.5 – 7 spores/kg, (Fach et al., 2002)), and exclusively non-proteolytic *C. botulinum* type B has been found in fish samples from Scottish trout farms (n = 69, 1.4% type B, (Burns and Williams, 1975)).

Worldwide

Similar to Europe the prevalence *C. botulinum* type E in fish in other regions of the world is highly variable. Type E seems to be present and often the predominant serotype in many fish samples from temperate regions of the northern hemisphere including:
● intestinal fish samples from the Great Lakes, USA (16.5%, (Bott et al., 1966))
● whitefish chub from Lake Michigan, USA (n = 1071, freshly caught and eviscerated: 13 – 14%, at brining step: 20%, prior to the smoking 6 – 14%, (Pace et al., 1967))
● salmon gill samples from Alaska, Washington, and Oregon, USA (n = 733, 2.2% (Houghtby and Kaysner, 1969))
● fish samples from Canada and Alaska (serotype E present, (Boyer et al., 2001))
● fish samples from Russia (serotype E present, (Chulkova et al., 1976))
● fish samples from the Caspian Sea (predominant serotype E)
● fish samples from northern Japan (frequently type E, (Dodds, 1993))
● raw menhaden surimi (n = 565, 1.2%, (Rhodehamel et al., 1991))
● fish samples from northern China (not predominant but present (Gao et al., 1990)).

In rare cases *C. botulinum* type E has also been found in warmer regions but is generally reported to play a minor role in subtropical and even less in tropical regions:
● fish from the US Atlantic and Gulf coast (present, but not predominant, (Ward et al., 1967a; Ward et al., 1967b)),
● fish samples from Indonesia (detected but very rare, (Mortojudo et al., 1973))
● shrimps from Nigeria (BoNT/E contaminated, shipped to France, (Boyer et al., 2001))
● fish samples from the west and east coast of India (n =129, no type E, types C and D predominant, (Lalitha and Gopakumar, 2000)).

Additional worldwide prevalence data of non-proteolytic *C. botulinum* types in various raw foods in summarized in (Lindström et al., 2006).

Although *C. botulinum* type E has rarely also been detected in other animals, e.g., pig feces (Sweden, 62% type B, less than 1.3% types E and F, (Dahlenborg et al., 2003)) or food honey (BoNT/E detected, possibility of production by *C. butyricum* not addressed in this study, (Nevas et al., 2005)), overall results from surveys conducted to examine the presence of *C. botulinum* in raw food material indicate that *C. botulinum* type E is primarily associated with fish and seafood. In addition to regional differences, the contamination of raw fish samples can largely depend on the fish species (Hyytia-Trees et al., 1999), and the part of the fish (e.g., intestine vs. skin, (Hielm et al., 1998a)), i.e., highest contamination levels of demersal fish (e.g., cod and flatfish) can be expected in the guts, whereas pelagic fishes (e.g. herring) are mainly contaminated on outer surfaces and gills (Huss and Pedersen, 1979). Furthermore, the contamination level can be affected by preprocessing stages of food production (e.g., cleaning/brining, (Pace et al., 1967)).

1.3.5.3 Processed food

The detection of *C. botulinum* type E in processed food requires that (spores of) this organism survived food processing. Indeed, the presence of *C. botulinum* type E in a variety of fishery products has been previously reported in several studies.

Europe

Some studies from Scandinavia reported on the presence of *C. botulinum* type E even in products at retail level, whereas low or no contamination has been reported for some British fishery products:

- Swedish hot-smoked Baltic herring (n = 144, 4.2%, (Johannsen, 1965))
- Swedish hot-smoked eel (n = 10, 20%, smoking: 2 h at 55°C and 0.5 h at 60°C, (Abrahamsson, 1967))
- Danish hot-smoked trout (5%, immediately after processing, (Huss et al., 1974))
- Finnish fishery products at retail level (n = 337, 4.9 %, (Hyytia et al., 1998)) (in detail: vacuum-packed fishery products (n = 214, 5%), air-packed fishery products (n = 123, 3%) consisting of: raw pickled rainbow trout (n = 50, 2%, 40 spores/kg), cold-smoked rainbow trout (n = 64, 3%, 160 spores/kg), hot-smoked rainbow trout (n = 50, 4%, 30 spores/kg), hot-smoked whitefish (n = 50, 10%, 40 spores/kg), hot-smoked, air-packed vendace (n = 50, 6%, 30 spores/kg), hot-smoked river lamprey (n = 23, 4%, 60 spores/kg), hot-smoked Baltic herring (n = 50, 0%))
- fish farms sediments, a fishery product manufacturing plant in northern Germany, raw and hot-smoked trout (n = 366 in total, 3.8%, (Bach et al., 1971))
- British vacuum-packaged cold-smoked herring (n = 646, 0.8%, (Cann et al., 1966))
- various British vacuum-packaged fish samples (n = 200, 0%, (Hobbs et al., 1965))
- British hot-smoked trout (n = 42, 0%) and mackerel (n = 40, 0%) from various retail markets (Gibbs et al., 1994)

Worldwide

Data for the presence/absence of *C. botulinum* type E in processed fish from other regions worldwide is scarce and most studies focus on fishery products from North America:

- smoked fish products from small manufacturing facilities in the Pacific Northwest of the USA (n= 240, 4.6%, (Hayes et al., 1970))

● freshly hot-smoked whitefish chubs from the Milwaukee area, USA (n = 858, 1.1% type E, 0.1% type B, hot-smoking: 82°C (180°F) internal loin muscle temperature for 30 min, (Pace et al., 1967))
● cold- and hot-smoked fish at retail level in the USA (n = 201, 0%, (Heinitz and Johnson, 1998))
● cold- and hot-smoked fish at retail level in Canada (n = 100, 0%, (Dodds et al., 1992)).

An overview of the European prevalence data of *C. botulinum* type E in environmental samples, raw and processed food is provided in Fig. 1-16. The summarized data in this figure illustrates that prevalence and contamination levels (i) tend to decrease in the order sediment > raw fish > fish product, (ii) can differ greatly depending on the region of origin (even within Europe), (iii) depend on the fish species and (iv) the fish part examined (i.e., gut or surface), and may be high enough to enable some spores to (v) survive hot-smoking processes.

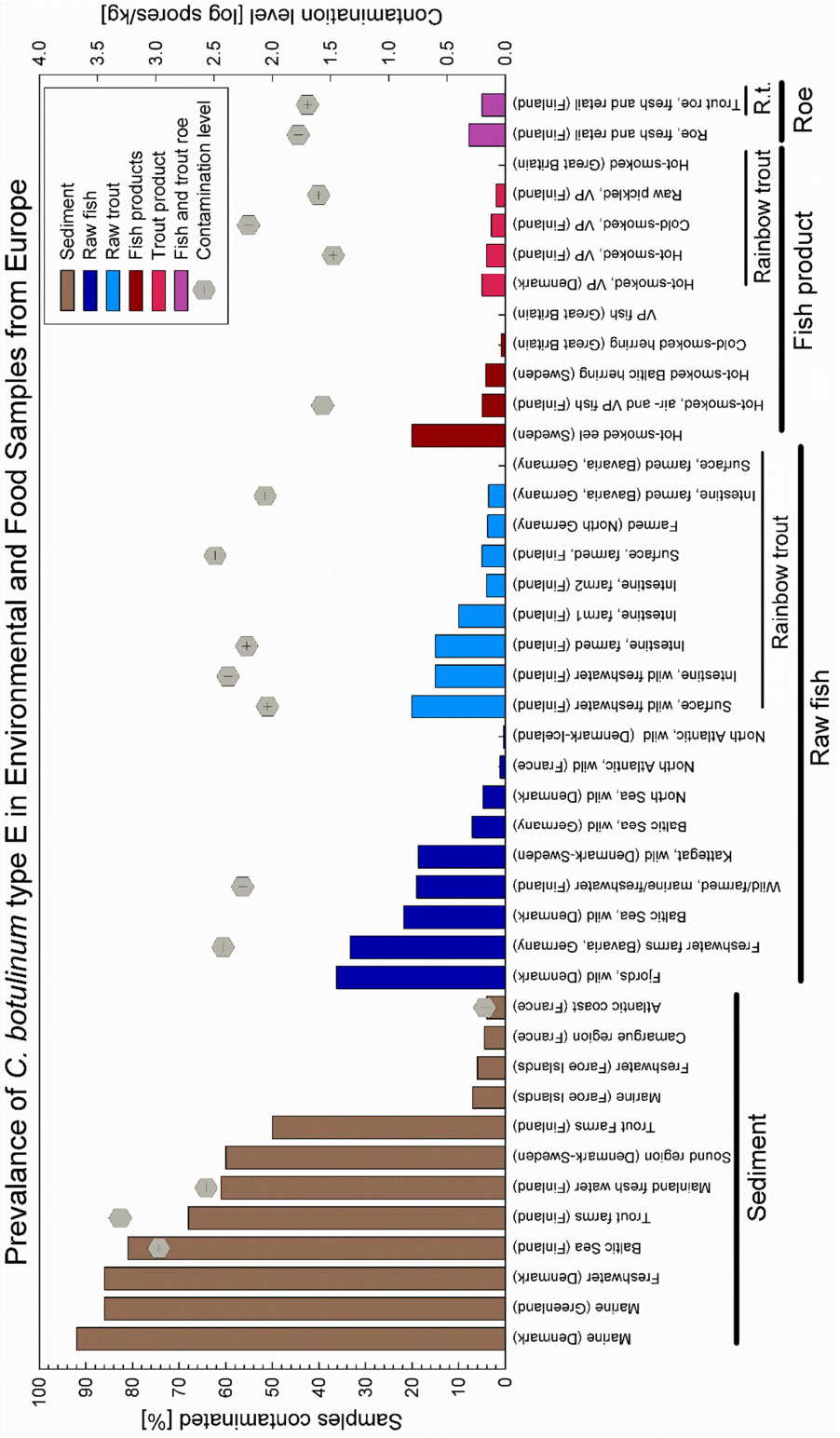


Fig. 1-16: *Clostridium botulinum* type E in environmental and food samples.
Average contamination levels, if reported, are indicated as gray dots. References: Sediment data (brown bars): Bars 1 – 3, 9, 10 (Huss, 1980), bars 4 – 6 (Hielm et al., 1998b), bar 7 (Johannsen, 1963), bar 8 (Ala-Huikku et al., 1977), bar 11 (Smith and Moryson, 1977), bar 12 (Fach et al., 2002); Average raw fish and specific trout data (dark and light blue bars): Bars 1, 3, 5, 7, 9 (Huss and Pedersen, 1979), bars 2, 17, 18 (Hyytia-Trees et al., 1999), bars 4, 10, 11 (Hyytia et al., 1998), bar 6 (Baumgart, 1972), bar 8 (Fach et al., 2002), bars 12, 15 (Hielm et al., 1998a), bars 13, 14 (Ala-Huikku et al., 1977), bar 16 (Bach et al., 1971); General fish product, specific trout product and fish roe data (dark red, light red and purple bars, respectively): Bar 1 (Abrahamsson, 1967), bars 2, 7 – 9, 11, 12 (Hyytia et al., 1998), bar 3 (Johannsen, 1965), bar 4 (Cann et al., 1966), bar 5 (Hobbs et al., 1965), bar 6 (Huss and Eskildsen, 1974), bar 10 (Gibbs et al., 1994).

1.3.6 *Clostridium botulinum* type E and Food Safety

1.3.6.1 Critical Food Products

Clostridium botulinum type E presents a primary safety determinant for **REPFED** primarily from specific **aquatic environments** that are packed under **anaerobic conditions** and have a **composition allowing** the **growth** of this organism, i.e.,

- **Re**frigerated – in comparison with proteolytic *C. botulinum* types relevant to food safety (group I), *C. botulinum* type E strains (group II) are less resistant to physical stress (Tab. 1-1) and grow less readily under harsh environmental conditions. However, its capability of growing at refrigerated temperatures (Tab. 1-1; Fig. 1-17) together with the fact that toxigenesis can, under certain conditions, precede organoleptic spoilage (section 1.3.3.3) represent major factors making *C. botulinum* type E a threat to food safety.
- **P**rocessed **F**oods – in the sense of foods that are (i) processed at conditions that are insufficient to completely eliminate *C. botulinum* type E spores (potentially including commercial hot-smoking processes; section 1.3.5.3) and (ii) ready-to-eat or not heated above pasteurization temperatures (BoNT destruction; section 1.3.3.4.2) immediately before its consumption (BoNT destruction would not necessarily prevent infant or adults intestinal botulism).
- of **E**xtended **D**urability – foods might be either contaminated primarily with spores or both spores and vegetative cells, but vegetative cells are more likely inactivated even at mild processing conditions. Spores generally need some time to germinate, grow out (section 1.2.2.4), and reach growth phases where they produce their BoNT (section 1.3.3.3).
- primarily from **aquatic environments**– specifically from temperate regions of the northern hemisphere as delineated in section 1.3.5. The high prevalence of *C. botulinum* type E in some regions can make it considerably difficult to completely avoid the contamination of seafood with spores from this organism.
- that are packed under **anaerobic conditions** –vacuum packed (VP) or modified atmosphere packed (MAP) foods are common ways to pack chilled foods in order to restrict the growth of aerobic bacteria and extend product shelf-life (Peck, 2006). This however, allows for the growth of anaerobic pathogens such as *C. botulinum*. Additionally, the possibility that local variations in the redox potential and anaerobic conditions inside of inhomogeneous food products might be sufficient to promote *C. botulinum* growth should be considered.

- that have a **composition allowing** the **growth** of *C. botulinum* type E – most important hurdles (suppositional the absence of added preservatives such as nitrite) for the growth of proteolytic and non-proteolytic *C. botulinum* strains are summarized in including the minimum growth temperature, minimum pH, minimum water activity, and maximum salt contents are summarized in Fig. 1-17 (details in Tab. 1-1).

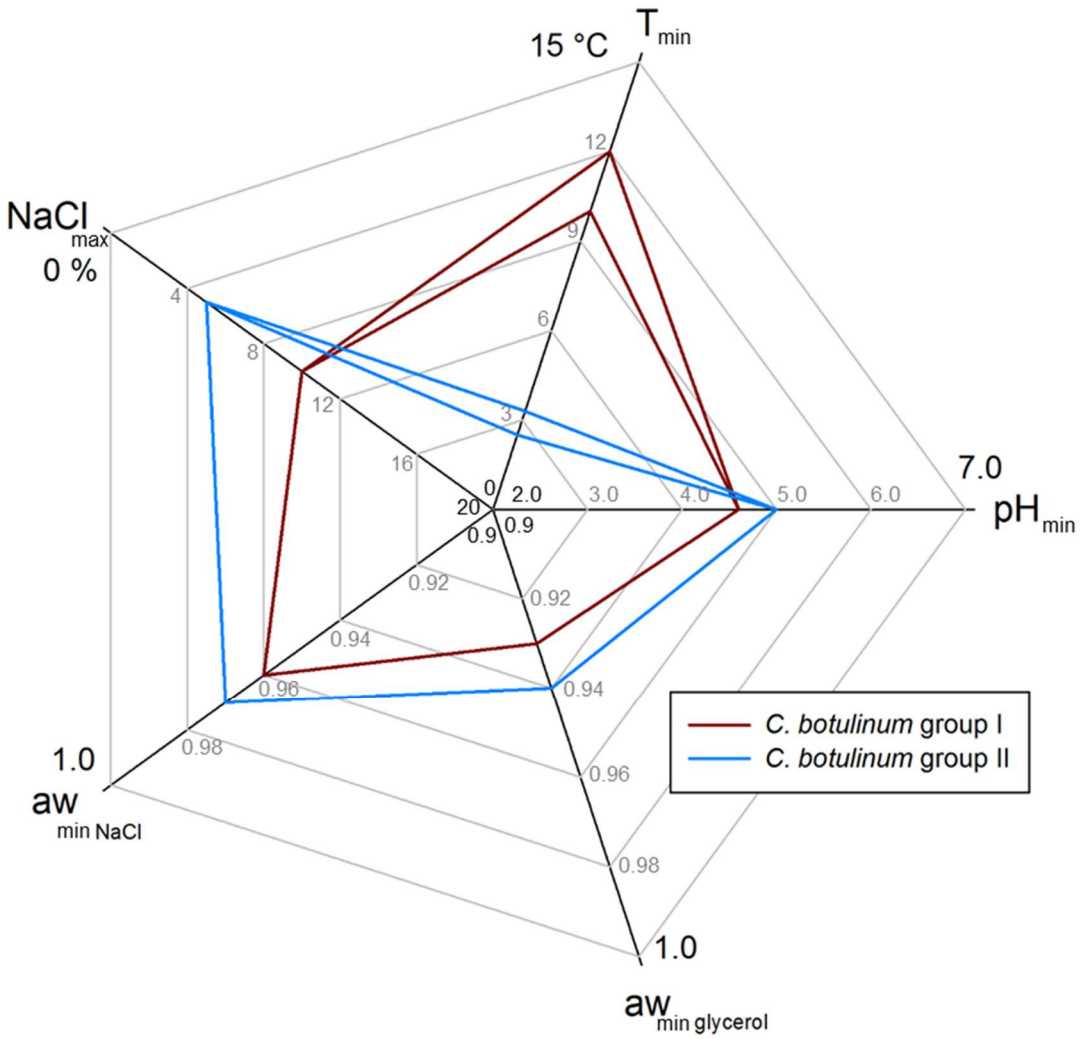


Fig. 1-17: Growth limits of *C. botulinum* groups I and II.
Most important hurdles (salt content, water activity adjusted with salt or glycerol, pH value, and temperature) for the growth of *C. botulinum* strains relevant to food safety (botulism in humans), i.e., strains from the proteolytic *C. botulinum* group I (types A or B) (red) and the non-proteolytic *C. botulinum* group II (types B, E, or F) (blue). Inner areas of the web represent harsh conditions, outer areas represent favorable conditions for growth. Two data points for T_{min} indicate differences reported for the minimum growth temperature. Detailed numbers and references are provided in Tab. 1-1.

1.3.6.2 Food Safety Measures – Hurdle Concept

Although *C. botulinum* type E is highly prevalent in environmental and food samples (section 1.3.5), the incidence of foodborne botulism is generally low (section 1.3.4.3). Furthermore, a look at data provided in single case reports, suggests that from the already low number of type E botulism cases/outbreaks, a large number is associated with traditionally produced fermented or raw fishery products (Horowitz, 2010), whereas cases associated with the

consumption of industrially produced food can occur but, fortunately, appear to be relatively rare (e.g., hot-smoked whitefish, (King et al., 2009; Korkeala et al., 1998)).
Reasons for this discrepancy between environmental prevalence and botulism incidence can be found in good hygiene and manufacturing practices (Autio et al., 2004), i.e., control measures to reduce the initial contamination levels, to inactivate spores, and to inhibit growth of *C. botulinum* type E in food products.
Measures to keep preprocessing contamination levels low can/should already start during fish farming, e.g., the use of sediment suction devices in traditional freshwater ponds and marine net cages (Hielm et al., 1998a). Once caught, careful handling, cleaning, and eviscerating can reduced/avoid the contamination of the final food product (depending on the fish species and the parts of the fish; section 1.3.5.2).
Since the high prevalence of *C. botulinum* type E can make it difficult to completely avoid the contamination of seafood with spores from this organism (section 1.3.5), food safety measures to inhibit growth from or inactivate non-proteolytic *C. botulinum* spores are essential. Widely acknowledged hurdles, i.e., measures to provide safety with respect to *C. botulinum* type E (Peck et al., 2008) include:

- Cold storage conditions
  - below 3 °C or
  - at maximum 8 °C when the shelf-life is maximum 10 d (‘’10 day rule’’).
- Adverse conditions throughout the food, always combined with chilled storage, i.e.,
  - either a maximum pH 5.0,
  - or a salt concentration of minimum 3.5%,
  - or an a_w of maximum 0.97.
- A heat treatment sufficient to result in a minimum 6-log reduction of non-proteolytic *C. botulinum* spores (e.g., 90 °C/10 min, 85 °C/36 min, 80 °C/129 min) combined with chilled storage. This is the category, which might apply to possible future applications, where the heat treatment could be replaced by a HPT treatment leading to a 6-log reduction.
- Combinations of a heat treatment and other preservative factors that together consistently prevent growth and toxin production combined with chilled storage.

1.3.6.3 Food Safety Influence Factors

To, finally, guarantee food safety, it is important to ensure that all of the mentioned requirements are satisfied constantly throughout the food product and over the entire period of storage (limited by the product shelf-life), for which several factors depending on the hurdle need to considered.
For refrigerated storage as preservation factor, it is important that the cold chain is not interrupted. Although, at a first glance this does not sound very challenging, it means that, theoretically every part of every product constantly stays in the specified temperature range, i.e., not too warm, but also not too cold, which might cause the product quality to suffer from humidity loss by sublimation. This can be problematic especially at retail level, where large temperature fluctuations depending on the construction type of refrigerated shelfs, the

number of thawing phases per day, and the ambient temperature near the shelf can be observed (Sabrowski, 2000). Additionally, the location of products, especially in open refrigerated shelfs (front/back, bottom/top) can significantly influence their temperature profile (Sabrowski, 2000). Such temperature fluctuations can result in the exceedance of the maximum allowed storage temperature for specific products (especially those in top and front positions in the shelf) as demonstrated for the core temperature of vacuum-packed smoked trout fillets in an open refrigerated shelf at retail level (Figure adopted from (Sabrowski, 2000).

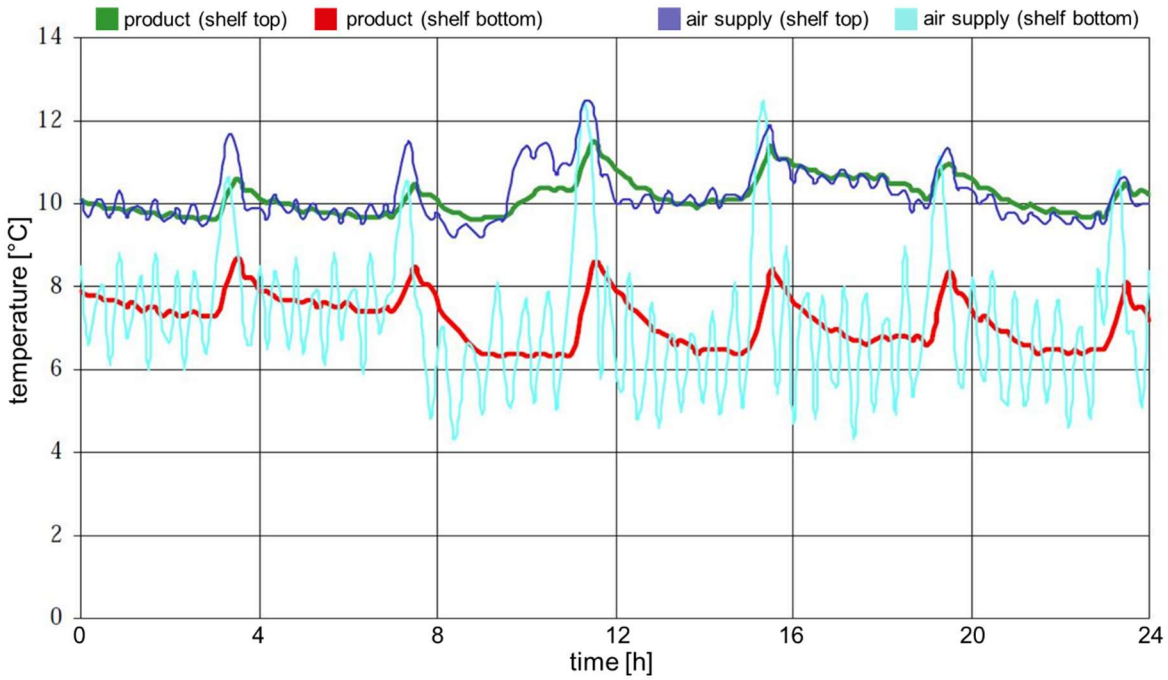


Fig. 1-18: Temperature profile of trout fillets in a retail shelf.
Temperature profiles of VP smoked trout fillets in an open refrigerated shelf with six thawing cycles in 24 h; green: core temperature product located near the top of the shelf; red: core temperature of product located near the bottom of the shelf; dark blue: temperature profile of air supply at the top of the shelf; light blue: temperature profile of air supply at the bottom of the shelf (Figure adopted from (Sabrowski, 2000).

For adverse conditions as preservation factor, this can be problematic for inhomogeneous foods with large local variations in their composition (e.g., a higher pH, lower salt concentration, or a high a_w) or foods with their local composition changing over time (e.g., creaming of emulsions, chemical reactions, or local microbial growth).
For heat treamtents as preservation factor, it has to considered that the success of thermal food processing to eliminate *C. botulinum* type E spores can depend on several factors including initial contamination levels and fitness/dormancy state of spores, processes temperature, holding time, and relative humidity (low RH can lower effective heat transfer), and, product size and composition (e.g., whole fishes need longer than fillets to reach a specific target core temperature; heat transfer inside the fish is composition-dependent). Such factors and regionally different products and manufacturing traditions likely determine whether even the relatively low, naturally occurring *C. botulinum* type E contamination levels are high enough to permit the survival of some spores during heat treatments such as industrial hot-smoking, which has been occasionally observed (e.g., (Hyytia et al., 1998; Pace et al., 1967); section 1.3.5.3). Another factor that can play a role in safety considerations

regarding thermal treatments as preservation method is that heat is likely to severely damages or eliminates vegetative cells of the competing microflora. This together with the possibility that heat can contribute to the activation dormant spores, indicates that hot-smoking procedures have the theoretical potential to even enhance the risk originating from *C. botulinum* type E. This highlights the importance of careful process design and control in addition to attempts to reduce *C. botulinum* type E contamination levels of raw fishery products.

1.4 High Pressure Processing

1.4.1 Basic Thermodynamic Principles

Laws of Thermodynamics

The zeroth, first, and second law of thermodynamics express fundamental relationships of the thermodynamic changes of state during HPT processing.

One of the commonly stated versions of the zeroth law of thermodynamics is: If two systems are both in equilibrium with a third system then they are in equilibrium with each other. This, for example, means that systems in thermal equilibrium with each other tend to have the same temperature after time.

The first law of thermodynamics states that the increase in internal energy (U), i.e., the energy contained within a closed system, is equal to the total of the energy added to the system in the form of work (W), heat (Q), or chemical potential (μ), i.e., the potential energy that can be absorbed or released during chemical reactions by a number of *N* particles of *i* different types present in the system, i.e., the sum chemical potential or sum partial molar free energy (Job and Herrmann, 2006).

$$dU = dW + dQ + \sum_{i=1}^{j} \mu_i dN_i$$

(Eq. 1-1)

In the case of HPT processes, *dW* represents the amount of volumetric work performed operating the high pressure pump/intensifier part of the high pressure unit, which can be expressed as:

$$dW = -pdV$$

(Eq. 1-2)

The amount of heat energy added to the system is represented by *dQ*, which is generally positive. The sum chemical potential depends on how many ("reactive") particles of a certain type with a certain chemical potential are present in the system. This potential can change during phase transition. At a constant pressure, the chemical potential ideally equals the partial molar Gibbs free energy. Chemical equilibrium (e.g., in the simplest case that only one type of particle is present (j =1)) and phase equilibrium are states where the sum chemical potential can equal zero.

The second law of thermodynamics generally describes the irreversibility of natural processes tending to reach homogeneity of matter and energy, i.e., thermodynamic equilibrium over time. This means that systems tend to reach the state with the maximum degree of disorder, i.e., maximum entropy (S). Accordingly, the degree of (molecular)

disorder within an isolated (macroscopic) system stays zero or increases, but never decreases. In the case of HPT processes, the intensive thermodynamic properties, heat (over time) and pressure (practically instantaneously), equalize between the high pressure vessel wall, the pressure transmitting fluid (PTF), and the food product tending to adapt the same level over time (for temperature: provided that the vessel wall is isolated against environmental temperature changes or its temperature is held constant). In a theoretical reversible heat transfer, the amount of transferred heat, ∂Q, can be expressed as the temperature (T) both of the system and space where the heat comes from or goes to multiplied by the increment of the entropy (*dS*).

$$dQ = TdS$$

(Eq. 1-3)

For situations where the sum chemical potential can be neglected, the equations according to the first and the second law of thermodynamics yield an equation expressing the functional relationship of the inner energy of a system with pressure, volume, temperature, and entropy.

$$dU = -pdV + TdS$$

(Eq. 1-4)

Additional General Principles

In addition to the laws of thermodynamics, there are three further general principles that govern/are helpful to explain observable pressure-induced physicochemical changes (Daryaei and Balasubramaniam, 2012; Reineke, 2013).

● Le Châtelier's principle, i.e., the equilibrium law discovered independently by Le Châtelier and Braun (late 19th century), states that chemical equilibria exposed to a change shift in the direction minimizing effects of this change. In the case increasing pressure, free volume is decreased, which forces a system to adapt a state with lower volume. This means that any processes (e.g., dissociation reaction, phase transition, or conformation and arrangement of macromolecules) that are accompanied by a decrease in volume is favored, whereas processes leading to an increase in the total volume are retarded upon pressurization. Notably, changes imposed by an increase in pressure can be different depending on the temperature. For example, the rate constant, k, of a chemical reaction (and therewith the rate of the chemical reaction) depends on the pressure, p, the pressure-dependent activation volume, $\Delta V^{\#}$ [cm^3 mol^{-1}], and the temperature, T (R = gas constant = 8.3145 J K^{-1} mol^{-1}; (Eyring, 1935)):

$$\left(\frac{\delta \ln k}{\delta p}\right)_T = -\left(\frac{\Delta V^{\#}(p)}{RT}\right)$$

(Eq. 1-5)

This means that, for example, a reaction could be speeded up due an increase in pressure at a constant temperature, but is slowed down at increased temperatures. In the case of HPT treatments, where generally both thermodynamic parameters are increased, a reaction rate, thus, could be similar to that at ambient pressure and temperature.

● The microscopic ordering principle states that an increased pressure leads to a high degree of ordering of molecules of a substance (provided that the temperature is constant). However,

pressure and temperature can act antagonistically on the degree of ordering on the molecular level, which can, similar to the example stated above, lead to molecular structures at specific p/T-combinations that are similar to native structures at ambient pressure and temperature.

● The isostatic principle states that pressure is transmitted (quasi-)instantaneously and throughout all substances in a pressure vessel regardless of their shape or size. This principle is generally applicable (although pressure levels in very large food products might vary slightly (Minerich and Labuza, 2003)), and explains phenomena such as the macroscopically simultaneous heating regardless of the location in a product and why HHP-treated products are macroscopically not damaged/usually retain their shape (at least products with high water and low air contents).

1.4.2 Physicochemical Changes under Pressure

Pressurization induces several physicochemical changes in aqueous systems, which are important to be considered for the application of high pressure processing. Important changes provoked by alterations in the pressure level include effects on the temperature (section 1.4.2.1), phase transition (section 1.4.2.2), dissociation equilibrium (section 1.4.2.3), and the structure and/or arrangement of macromolecules (section 1.4.2.4) in pressurized material.

1.4.2.1 Adiabatic Heating

Compressive work against intermolecular forces induces an increase in temperature during pressurization. This effect, which can be deduced from the first law of thermodynamics (Kessler, 2002), is commonly known as adiabatic compression heating or adiabatic heat of compression. The term adiabatic heating is used throughout this manuscript to describe heating effects during pressurization even though no ideal adiabatic conditions, i.e., the complete absence of thermal transfer between a sample and its environment, were present during the experiments conducted, which is, in practice, not achievable.

1.4.2.1.1 Principles for the Calculation of Adiabatic Heating Rates

From the laws of thermodynamics, basic equations can be derived that facilitate the description of adiabatic heating effects.

With the theoretical assumption that there are no thermal losses, the temperature reached during pressurization can be derived from the variation in entropy dS as a function of pressure p and temperature T (Knoerzer et al., 2010):

$$dS = \left(\frac{\partial S}{\partial T}\right)_p dT + \left(\frac{\partial S}{\partial p}\right)_T dp$$

(Eq. 1-6)

With the assumption that the process is completely reversible, the total entropy change is zero.

Rearrangement of the equation and the use of the Maxwell's relation

$$\left(\frac{\partial S}{\partial p}\right)_T = -\left(\frac{\partial V}{\partial T}\right)_p$$

(Eq. 1-7)

the expressions of the specific volume as the inverse of the density

$$v = f(p,T) = \frac{1}{\rho}$$

(Eq. 1-8)

the definition of the isobaric heat capacity according to the first fundamental theorem of thermodynamics as

$$c_p\,(p,T) = \left(\frac{\partial H}{\partial T}\right)_p = T\left(\frac{\partial S}{\partial T}\right)_p$$

(Eq. 1-9)

the definition of the isobaric thermal expansion coefficient according to Bridgman (Bridgman, 1912) as

$$\propto_p (p,T) = \frac{1}{v}\left(\frac{\partial v}{\partial T}\right)_p$$

(Eq. 1-10)

and, finally, the definition of the compression heating coefficient as used by Knoerzer et al. (Knoerzer et al., 2010) as

$$k_C = f(p,T) = \frac{\alpha_p}{\rho \cdot c_p}$$

(Eq. 1-11)

finally results in an equation expressing the compression heating rate (the temperature increase upon pressurization under adiabatic, isentropic conditions) as follows:

$$\frac{dT}{dp} = -\frac{\left(\frac{\partial S}{\partial p}\right)_T}{\left(\frac{\partial S}{\partial T}\right)_p} = \frac{v\left(\frac{1}{v}\left(\frac{\partial v}{\partial T}\right)_p\right)}{\frac{1}{T}\left(T\left(\frac{\partial S}{\partial p}\right)_p\right)} = \frac{\alpha_p}{\rho \cdot c_p} \cdot T = k_C \cdot T$$

(Eq. 1-12)

This equation expresses that the temperature change of materials upon physical compression under adiabatic (no heat exchange), isentropic (reversible adiabatic process at constant entropy) conditions depends on their compressibility (thermal expansion), their specific heat capacity, and their density, which are all pressure/temperature-dependent and can be summarized as compression heating coefficient k_C.

1.4.2.1.2 Determination of p/T-Dependent Adiabatic Heating Rates

Pressure/temperature-dependent adiabatic heating rates of water-based solutions (e.g. sugar solutions) and, putatively, various water-based dispersions can be derived using available data for water (Fig. 1-19, (NIST, 2002)), mixture rules for water soluble components, and correction factors to compensate for inaccuracies in mixture rules under high pressure conditions (Ardia et al., 2004b).

For complex mixtures and compressible material, the p/T-dependence of the compression heating coefficient (k_C) (Eq. 1-12) can be calculated on the basis of empirically data determined under (close to) adiabatic conditions (Knoerzer et al., 2010). This approach presents an accurate and reproducible way to describe/predict maximum adiabatic heating that can occur during pressurization. Additionally, such data is necessary for CFD modelling of high pressure processes (Juliano et al., 2009; Knoerzer and Chapman, 2011; Knoerzer et al., 2007).

Although such maximum adiabatic heating temperatures determined under (nearly) ideal adiabatic conditions are unlikely reached in practice due to improper insulation and

consequent heat transfer between a sample and its environment, information about the p/T-dependence of the compression heating coefficient (k_C) is valuable in estimating maximum possible process temperatures and, thus, can build the basis for HPT process design.

1.4.2.1.3 Adiabatic Heating of Water and Food Components

Water

As mentioned above, data for the p/T-dependent ideal adiabatic heating rates of pure water are available in a broad range of pressure and temperature levels (NIST, 2002), which can build the basis for various modeling approaches (Ardia et al., 2004b). Exemplarily, adiabatic heating curves for a range of starting temperatures between 20 and 80 °C and pressurization up to 800 MPa are depicted in Fig. 1-19, which clearly demonstrates the non-linearity and p/T-dependence of adiabatic heating.

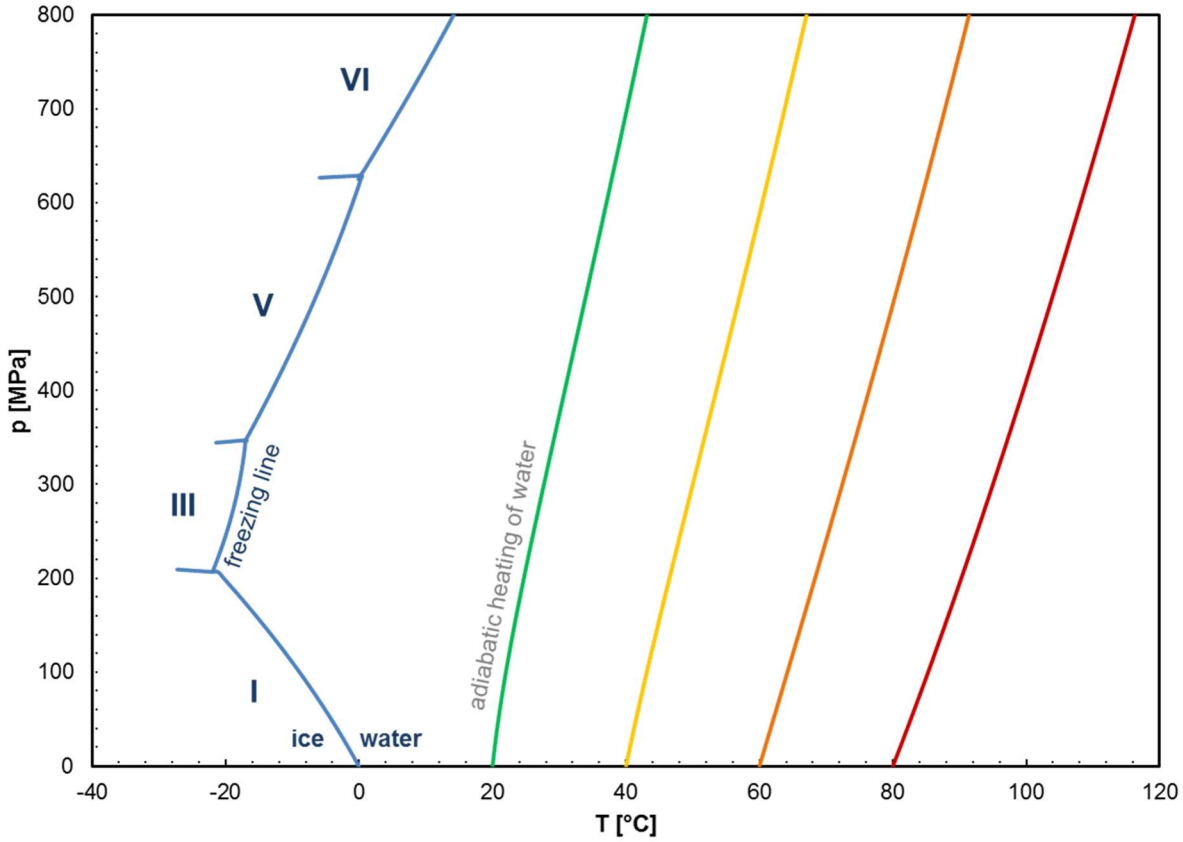


Fig. 1-19: Pressure/temperature-dependent adiabatic heating of water.
Colored lines indicate ideal p/T-dependent adiabatic heating data of pure water from NIST database (NIST, 2002). The blue line marks phase transition conditions.

Adiabatic Heating of Food

Due to the lack of thermodynamic data for real food under pressure, modeling of p/T-dependent adiabatic heating rates is difficult (Toepfl et al., 2006). The determination of the compression heating coefficient (k_C) and its p/T-dependency as described above is possible for food materials (Knoerzer et al., 2010), but has not yet been conducted for a broad range of products.

However, at least constants for heating rates at different pressure and initial temperature combinations have been determined previously in various studies. Such rates for various HPT process relevant materials including many food products and PTFs, which can largely

differ depending on the matrix pressurized, are listed in Tab. 1-3 (according to (Toepfl et al., 2006) (Gupta and Balasubramaniam, 2012) and references therein).
Since the listed adiabatic heating rates do not contain detailed information about compression heating properties as a function of pressure and temperature the values provided in the table might be not precise enough for accurate process design, but can be useful to roughly estimate adiabatic heating effects that can be expected to occur upon pressurization of a certain type of food product.
The fact that this table frequently contains ranges of adiabatic heating rates reflects the commonly non-linear p/T-dependence of adiabatic heating rates, which can be already observed for pure water (Fig. 1-19) and is generally likely to be stronger for food materials. For the foods listed in this table, the temperature increase per 100 MPa pressure increase is higher at elevated pressure levels than at lower pressure levels, i.e., lower numbers of ranges in the table refer to the average adiabatic heating rates at elevated pressure levels.

Tab. 1-3: Adiabatic heating of food.
Temperature increase in food material and common PTL components upon pressurization according to (Gupta and Balasubramaniam, 2012). When ranges are indicated, the lower numbers commonly refer to a lower temperature increase per 100 MPa, i.e., less adiabatic heating at higher pressure levels.

Initial temperature ϑ = 25 °C	**Temperature increase ϑ [°C] / 100 MPa**
Orange juice Tomato puree Milk (2 % fat) Other watery foods	2.6 – 3.0
Carbohydrates	2.6 – 3.3
Proteins	2.7 – 3.3
Water Egg albumin Mashed potatoes Beef sauce	~3.0
Tofu Yoghurt Chicken breast	~ 3.1
Whole milk Salmon Honey Minced beef meat	~ 3.2
Whole egg	~ 3.3
Avocado pulp	3.7 – 4.1
Water/Glycol (50/50)	3.7 – 4.8
Egg white	4.3 – 4.5
Raw beef fat	4.4
Chicken fat	4.5
Cheese cream	4.7 – 4.9

Mayonnaise	5.0 – 7.2
Propanediol	5.1 – 5.8
Linoleic acid	5.9 – 9.0
Soy oil	6.2 – 9.1
Extracted beef fat	6.3 – 8.3
Olive oil	6.3 – 8.7
Ethanol	6.8 – 10.6

1.4.2.2 Phase Transition under Pressure

Since water presents the main component of many food products, its properties under pressure are of special interest.

According to Le Châtelier's principle, equilibria under pressure are shifted towards a lower total volume. Since water and its various ice states can differ in their total volume, pressure modulates the temperature at which phase transition from liquid water to solid ice occurs. Pressure-dependent phase transition temperatures for pure water are indicated together with the adiabatic heating data (mentioned above) in Fig. 1-19. Notably, the designated ices I, III, V, and VI in this figure only present a rough indication of major states of water under pressure, while there can exist a variety of (partially metastable) crystalline and amorphous ices (Zheligovskaya and Malenkov, 2005, 2006). Interestingly, the phase transition from water to ice 1 is the only one depicted in Fig. 1-19 that has a positive ΔV, which is reflected by the phase transition line following a diagonal course from bottom right to top left in the respective area of the p/T-diagram.

1.4.2.3 pH under Pressure

1.4.2.3.1 Basic Principles of Dissociation Equilibrium and pH

Another property of aqueous systems under pressure is more important for HHP or HPT processing as it can significantly affect microbial inactivation under pressure, i.e., the shift in dissociation equilibrium and concomitant changes in the pH value.

Basically, a dissociation equilibrium in an aqueous system can be described as:

$$HA + H_2O \leftrightarrow A^- + H_3O^+$$

(Eq. 1-13)

with a proton donor/acid (HA), water, a conjugate base (A^-), and a hydroxonium ion.

To characterize such a chemical equilibrium, the equilibrium constant K depending on the concentration of every molecule in the equilibrium and correction factors for such concentrations, i.e., activity coefficients, γ, can be used (law of mass action):

$$K = \frac{\gamma_{A^-} \cdot \gamma_{H_3O^+}}{\gamma_{HA} \cdot \gamma_{H_2O}} \cdot \frac{[A^-] \cdot [H_3O^+]}{[HA] \cdot [H_2O]}$$

(Eq. 1-14)

Due to the high concentration of water in relation with that of the other molecules, it can be assumed that this concentration is not largely affected by dissociation and might be

neglected. This leads to the definition of the acid dissociation or acidity constant K_a characterizing the extent of dissociation of hydroxonium from an acid as follows:

$$K_a = \frac{\gamma_{A^-} \cdot \gamma_{H_3O^+}}{\gamma_{HA}} \cdot \frac{[A^-] \cdot [H_3O^+]}{[HA]}$$

(Eq. 1-15)

Depending on the nature of the proton donor, this acid dissociation constant K_a can be a very small or very high number, which led to the frequent use of the common logarithm of K_a (more exactly, the additive inverse of the common logarithm) rather than K_a itself, i.e., pK_a:

$$pK_a = -log_{10}(K_a)$$

(Eq. 1-16)

For the same reason, the H_3O^+ ion concentration is commonly expressed as the additive inverse of its common logarithm in a dimensionless form (divided by 1 mol/L) , i.e., the most prominent quantity to describe acidity, the pH value:

$$pH = -log_{10}\left(\gamma_{H_3O^+} \cdot [H_3O^+] \cdot \mathrm{mol}^{-1}\mathrm{L}\right)$$

(Eq. 1-17)

1.4.2.3.2 Dissociation Equilibrium and Its p/T-dependence

The dissociation equilibrium and, therewith, the pH value of aqueous solutions, is pressure- and temperature-dependent. Similar to the driving factor for shifts in the phase transition line and according to Le Châtelier's principle, reactions resulting in a lower total volume are favored under pressure. A basic dependency of the equilibrium constant, K, from the pressure, p, and the absolute temperature, T [K] has been already established more than 100 years ago by Planck (1887):

$$\left(\frac{\delta \ln K}{\delta p}\right)_T = -\left(\frac{\Delta V(p)}{RT}\right)$$

(Eq. 1-18)

with the gas constant R (= 8.314 J K^{-1} mol^{-1}) and the volume change ΔV [m^3 mol^{-1}], i.e., the difference between the partial molal volumes of products and reactants in an theoretically infinitesimally diluted solution. This equation commonly still presents the starting point for the calculation/determination of p/T-dependent changes of the equilibrium constant.

Converted and integrated it results in another basic equation describing pressure- and temperature-dependent changes in the acid equilibrium constant:

$$pK_a = {pK_a}^0 + \frac{\lg e}{RT} \int_{p^0}^{p} \Delta V(p) dp$$

(Eq. 1-19)

Although pH values are the way more common entity used to describe the acidity of food products or the acid tolerance of microbes, p/T-dependent pK_a values that consider changes in the concentration of all dissociation equilibrium reaction partners of water (Eq. 1-15), and

not hydroxonium ions alone, have been reported to be more suitable to describe dissociation equilibrium shifts (Mathys, 2008).

1.4.2.3.3 Determination of pH under Pressure

The p/T-dependency of dissociation equilibria in the context of basic thermodynamic principles (as described above) have been the matter of extensive research in the past (e.g., (Mathys, 2008; Stippl, 2005)). However, an easy, widely applicable way to determination pH or pK_a values under pressure has not yet been found.

Pressure- and temperature-dependent pK_a values aqueous systems can be calculated from models based on existing thermodynamic data for water (Mathys, 2008). However, this approach is limited to water and simple buffer solutions. Early attempts include the development of glass pH electrodes withstanding > 100 MPa (1500 kg cm^{-2}) to measure pH values in the deep-sea (Disteche, 1959). More recently, different optical methods for the in situ measurement of pH values have been developed (Hayert et al., 1999; Molina-Gutierrez et al., 2002; Quinlan and Reinhart, 2005; Stippl, 2005; Stippl et al., 2002). However, these methods have limits regarding their maximum pressure level (generally not higher than 450 MPa reported) and the pH range to be measured, which primarily depends on properties of the dyes and the optical HHP cells used. Most importantly, these methods are unsuitable for the accurate determination of pH values when high pressure is combined with high temperatures and are completely unsuitable for measurements in solid matrices.

1.4.2.4 Effect of Pressure on Macromolecules

Effects on macromolecules during pressurization are important with respect to both food quality and microbial inactivation. Effects on biological (microbial) macromolecules as possible targets for the HPT-mediated inactivation of bacterial endospores are discussed explicitly later in this manuscript.

Generally, in response to the decrease of free volume during pressurization, reactions that lead to a decrease in total volume will be favored (Le Châtelier). However, pressure-mediated changes in the macromolecular structure are highly dependent on the type of molecule.

Covalent bonds are highly stable under pressure even above 1 GPa, which is the reason why primary structures of molecules with a low molecular weight, including peptides, sugars, vitamins, and lipids remain largely unaffected upon pressurization ((Oey et al., 2008a; Oey et al., 2008b) and references therein).

Although the primary structure of lipids is not affected, the degree of molecular ordering of oil can change upon pressurization, which can, finally, result in crystallization under pressure (Ferstl, 2010). For carbohydrate macromolecules such as starch, the effect of pressure on intermolecular bonds can lead to gelatinization, which differs from thermally induced gel formation (Stute et al., 1996) and has a potential of being exploited in pressure-assisted food structure engineering. Both gelatinization and crystallization effects are dependent on the pressure level, holding time, temperature, and type of starch or oil.

Pressure-mediated changes in protein structures are diverse, since any formation or disruption of non-covalent bonds or conformational change leading to a decrease in total volume will be favored in response to the decrease of free volume during pressurization (Le Châtelier). Especially for proteins, it is important to consider not only the molecule itself, but also its environment, in the first instance, water. Thus, pressure favors a decrease in the total

volume of the protein–water systems (rather than looking at the protein alone). This can be achieved either by changes in the degree of hydration via filling the protein's void volumes when they become accessible to solvent upon dissociation, solvent-exposure of charged groups that have been involved in stabilization of protein assemblies (e.g., salt bridges; electrostriction effect), or, potentially, hydration of hydrophobic residues (Winter and Dzwolak, 2005). The consideration of complex protein–water systems rather than looking at isolated protein molecules alone, is important to be able to explain pressure-denatured states of proteins, which seem to have a greater volume than the native protein when protein hydration and the contribution of water molecules to the total volume change are neglected.
As stated above, covalent bonds determining the primary structure are highly pressure-stable. Disruption/rearrangement (HHP favors hydrogen bond formation) of the secondary structure of proteins typically occurs at pressure levels between 300 and 700 MPa (usually irreversibly). Effects on higher protein structures are often related to pressure being unfavorable for hydrophobic interactions. The tertiary structure is even more sensitive to pressure. Pressure levels of 200 MPa and above can result in complex unfolding effects resulting in the formation various possible denatured conformational states of a protein. Such conformations of proteins denatured by pressure often resemble compact 'molten globule' type structures (Winter and Dzwolak, 2005). Pressure-mediated effects on the quaternary structure can already be observed above 50 – 200 MPa. Protein oligomers can dissociate, paving the way for aggregation of protein subunits and precipitation (Kessler, 2002; Meersman et al., 2002; Smeller et al., 2008).
Generally, pressure levels where proteins denature are highly temperature-dependent and vary depending on the type of protein examined. Additionally, the effect of HHP/HPT on proteins largely depends on protein properties and the presence of molecules capable of interacting with water, particularly, salts / osmolytes (e.g., sucrose, sorbitol, and glycerol).

1.4.3 Advantages and Disadvantages of HPT Processing

Advantages of HPT processing over conventional retorting are primarily related to adiabatic heating effects (also see section 1.4.2.1).
Due to the isostatic principle of pressure transmission (section 1.4.1), all substances in a pressure vessel are subjected to the same pressure (small differences might be present in large solid products (Minerich and Labuza, 2003)). This means that, unlike via convection heating in conventional thermal sterilization processes, all material inside of a pressure vessel is heated simultaneously during pressurization. This effect can be exploited in HPT sterilization processes (or PATS, i.e., HPT treatments at the sterilization temperature of 121.1 °C), to achieve rapid (depending on the technically feasible pressure build-up rate) and homogenous heating and cooling of (largely homogenous) food products to and from a target temperature, where relevant microorganisms (i.e., bacterial endospores) can be inactivated. The sketch in Fig. 1-20 shows a possible exploitation of adiabatic heating effects comparing theoretic temperature profiles of a conventional retorting process relying on convection heating (orange line in Fig. 1-20) and a HPT process, where the product is preheated (orange line) before pressurization (green line). Rapid heating (depending on the technically feasible pressure build-up rate) can reduce the total process time (depending on the time needed for loading/unloading operations of the pressure vessel) and the thermal load on a food product

at temperatures that are deteriorating food quality (green shaded (HPT) versus yellow shaded (heat) areas).
Additionally, local excess heating, i.e., over-processing of a food product, which can occur depending on the target temperature, the design of heat sterilization processes (e.g., heating rate, equipment), and food product properties (e.g., composition and size, (de Heij et al., 2003)), can be prevented.
Nutritionally valuable low molecular weight molecules such as vitamins are relatively temperature-susceptible, but stable at pressure levels far beyond those technically feasible in industrial applications (> 1GPa, (Oey et al., 2008a; Oey et al., 2008b)). Thus, both the reduction of the thermal load and the prevention of overprocessing, which can be achieved even when the target temperatures of both processes are identical (Fig. 1-20), can enable the production of safe foods with less detrimental effects on their sensory and nutritional quality (Jaeger et al., 2010; Knorr et al., 2011).

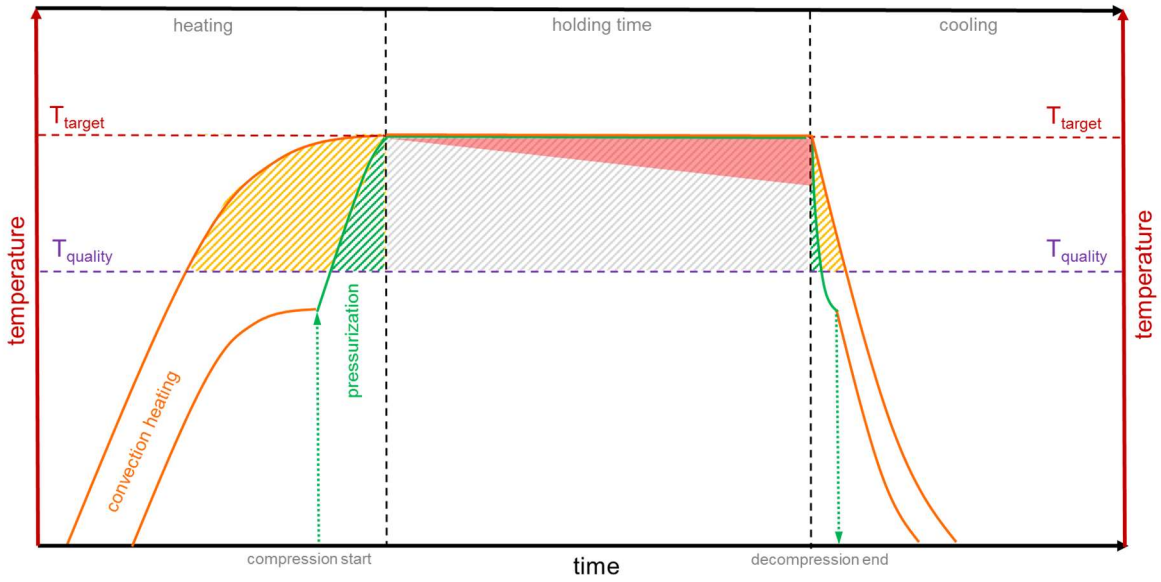


Fig. 1-20: Benefits from adiabatic heating.
Fictitious temperature profiles of a conventional sterilization process relying on convection heating (continuous orange line) and a HPT process (green line) with convection heating and cooling (orange lines) before compression (left green arrow) and after decompression (right green arrow). Both processes with an identical arbitrary target temperature during holding time (red dotted line). Yellow areas (heating and cooling phases of the heat process), green areas (heating and cooling phases of the HPT process), and gray area (during holding time) designate the thermal load on a product above an arbitrary threshold temperature (purple dotted line) above which detrimental effects on food quality occur. The red area indicates possible divergence of temperature profiles during non-adiabatic holding times, which might occur depending on the process design (colder PTL and no complete insulation of the product carrier). Note: Differences in temperature profiles of sample center and edges, which are likely to occur during convection heating and non-adiabatic holding times are not incorporated in the graph.

In addition to the reduction of process time and thermal load even at identical target temperatures (Fig. 1-20), synergistic effects of heat and pressure enhancing microbial inactivation could result in target temperature requirements for an effective inactivation lower than those when heat would be applied alone. This can allow a further reduction of the

thermal load leading to higher food quality. At best, the required target temperature lies below the range where food quality is affected, which might be rarely possible in practice.
Generally, pressure treatments can also have other advantages related to their effect on the phase transition behavior of water (also see section 1.4.2.2) or their effects on macromolecules (also see section 1.4.2.4). For example, shifted phase transition temperatures at elevated pressure levels (Fig. 1-19) can be exploited in pressure-assisted freezing/thawing processes. However, (unless this is desired and having a specifically adjusted process design) phase transition is unlikely to occur in commercial HHP or HPT processing of food (pressure too low (max. 600 MPa), holding times to short (no complete equilibration at environmental temperature), vessels not externally refrigerated, and depressurization too slow). Other examples include the exploitation of pressure-specific effects on macromolecules (food structure engineering) such beneficial structural properties of HHP-gelatinized starch or HHP-induced crystallization of, which might have the potential to be useful in technical processes. However, both effects primarily occur during pressure treatments at low/ambient temperatures, i.e., not or less during HPT processing (p/T/t-dependent). Finally, HHP-denatured proteins can exhibit better digestibility and HHP treatments have the potential to inactivate food allergens (Mackie et al., 2010). However, these effects are relatively unexplored (especially for HPT treatments, i.e., at elevated temperatures) and putatively highly p/T/t- and food dependent.
Although the occurrence of some pressure-specific effects on macromolecules (gelatinization/crystallization) can also be undesired in some foods deteriorating the desired sensory quality, such effects are currently not recognized as potential serious problems in HPT sterilization processes. Thus, major disadvantages of HPT compared with heat sterilization processes might be related to relatively high costs (acquisition and, especially, maintenance high fatigue of HHP equipment) and simply that fact that this technology is not on the market yet, which might be primarily due to the lack of suitable HPT equipment and probably (at least partially) due to the lack of knowledge regarding the inactivation of spores from food safety-relevant organisms such as *C. botulinum*. However, this study aims at contributing to the removal at least of the latter hurdle. Finally, factors that are not 100% clear, but could be seen as unlikely but potential disadvantages are the legal situation (PATS at 121.1 °C, but not HPT at lower temperatures certified by the U.S. FDA) and unsolved questions regarding the sustainability of HPT processes (e.g., energy consumption).

1.4.4 Design of High Pressure Thermal Processes

1.4.4.1 Process Temperature Control

Although the exploitation of adiabatic heating effects generally presents a major advantage of HPT over thermal sterilization processes (section 1.4.3), such effects generally complicate the precise parameter control during lab-scale and industrial HPT processes.

Adiabatic Heating in Lab-Scale HPT Experiments

While the critical process parameters pressure level and treatment time can be usually held constant easily, the control treatment temperatures is far more challenging. Accordingly, practical challenges in the design of HHP or HPT processes and the control of process parameters are mainly related to an inhomogeneous spatial and time-dependent temperature

distribution during the treatment. Fluctuations in the treatment temperature play an important role, especially when high pressure levels are combined with high temperatures, i.e., in HPT processes to achieve an effective inactivation of bacterial endospores (Rajan et al., 2006b). Therefore, several factors influencing the treatment temperature should be considered designing lab-scale and industrial HPT processes.

(i) Compression and decompression rates (Ratphitagsanti et al., 2009; Syed et al., 2012) can obviously influence the total process time and, thereby, the total “pressure and thermal load” a sample is subjected to. Since PTF temperatures and, especially vessel wall temperatures frequently lower than food sample temperatures, and a 100%-insulation is not possible, longer pressure come-up times are likely to result in lower maximum .food product temperatures. Both factors can have an influence on spore inactivation.

(ii) The type of high pressure vessel / the design of HPT unit used (e.g., vessel volume and geometry (Smith et al., 2014), insulating properties of sample containers, presence of thermostating jackets) can influence time-dependent temperature profiles and spatial temperature inhomogeneities inside of the vessel.

(iii) The type (thermodynamic properties) of pressure transmitting fluid used (Ardia, 2004; Balasubramaniam and Balasubramanian, 2003; Patazca et al., 2013; Scurrah et al., 2006).

(iii) The vessel design (material properties and shape) can influence the temperature distribution in HPT systems (applies mainly to larger or industrial HHP units) and heat transfer between vessel wall and PTF and (Juliano et al., 2009; Knoerzer and Chapman, 2011; Koutchma et al., 2010)).

(iv) Additionally, the position of monitoring process temperatures is important since thermocouples somewhere in the HPT vessel without an accurate process control and detailed knowledge about actual temperature distributions in the food products treated are useless. Even when sensors record temperature profiles directly in a food or dummy product, appropriate positions have to be selected (in terms of fulfilling HACCP requirements).

However, although numerous studies were conducted in the past evaluating the effectives of HHP or HPT treatments to inactivate all sorts of vegetative cells and bacterial endospores, a number of reports lack information on actual sample temperature profiles. This makes it often difficult to compare the obtained data with that from other studies.

To avoid this problem, relatively fast compression and decompression and the determination (exclusion) of inactivation effects occurring during these phases are advantageous. This way, only inactivation effects occurring during the holding time (i.e. inactivation after total treatment time minus inactivation during compression and decompression (0 or 1 s treatments)) can be determined, which can considerably facilitate the comparability of inactivation data. Additionally, a PTF with fast temperature response (conductive heat transfer) but low compression heating, relatively small and precisely tempered pressure vessels, monitoring the process temperature inside of the sample are advantageous.

Most importantly, the occurrence of adiabatic temperature peaks during the holding time should be avoided. This can be achieved setting the sample starting temperature to a suitable level, which can be roughly estimated from ideal p/T-dependent adiabatic compression heating profiles (if known) and corrected by a empirically determined HPT system-specific correction factor. The exclusion of effects occurring during pressure build-up and release and (close to) isobaric, isothermal holding times can drastically enhance data transferability between different HPT systems (Reineke et al., 2012). This principle is illustrated in Fig. 1-21.

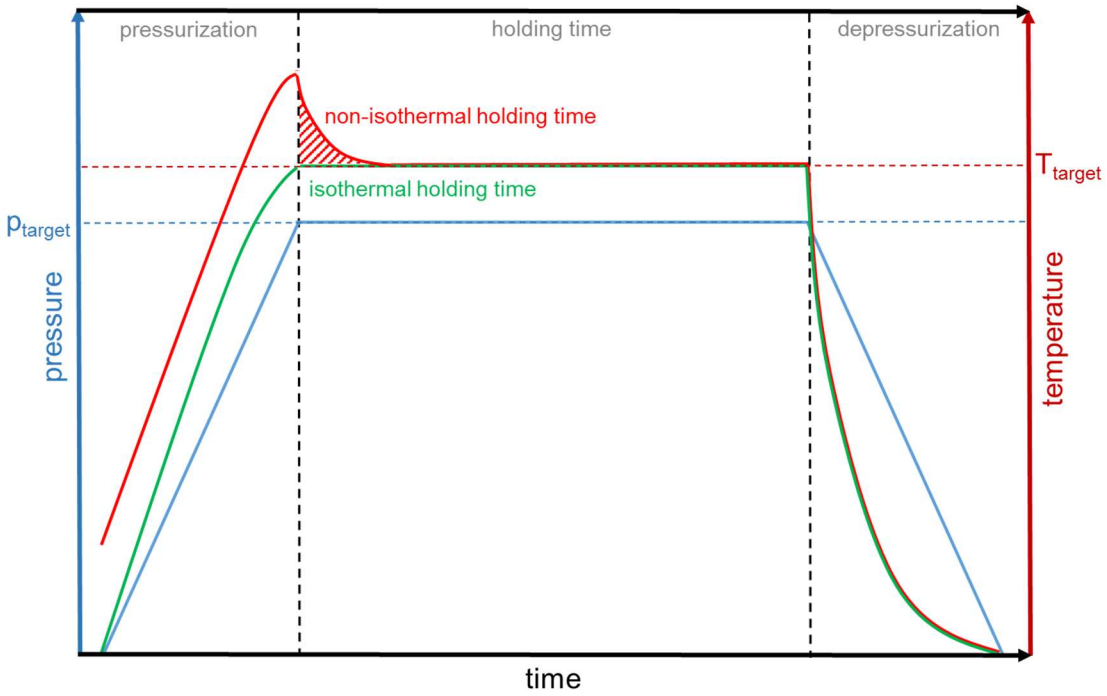


Fig. 1-21: Principle of isothermal isobaric holding times.
The temperature profile during a desired realization of an isothermal holding time is indicated in green. Under non-isothermal conditions (light red curve), thermal equilibrium occurs during dwell time. The light red shaded area indicates an adiabatic peak, i.e., additional thermal load that can interfere with the proper and comparable determination of spore inactivation.

Adiabatic Heating in Industrial Applications

Careful control of the process temperature presents a critical point in the industrial application of HPT processes. Factors influencing the temperature profile during lab-scale HPT treatments are identical with those playing a role in lab-scale experimental design, i.e., temperature profiles largely depend on the adiabatic heating properties of all materials inside of the pressure chamber, i.e. food product, packaging material, product carrier, and PTF, the starting temperature, the compression rate, and the target pressure of the pressurization process.

However, influence factors likely differ in their importance. For example, the vessel geometry, which can favor the development of horizontal temperature gradients in large industrial vessels, is usually no important factor influencing temperature profiles in the commonly small lab-scale HPT vessels. Heat convection resulting in a vertical temperature gradient can occur, which have to be considered especially when large (high) vessels and extended pressure dwell times are used. Additionally, industrial HHP systems are usually not temperature controlled, which increases the importance of heat transfer processes between the warm product, the colder PTF, and the even colder wall of the pressure vessel. This is generally a function of heat transfer properties of the different material involved and the surface available for heat transfer. Adiabatic heating and insulating properties of the product carrier and packaging material can slow down this process (Knoerzer and Chapman, 2011). Finally, in contrast to the small size of frequently homogenized samples in lab-scale-experiments, larger size and inhomogeneity of food products can cause difficulties. A temperature increase of approx. 6 up to almost 9 °C per 100 MPa (Rasanayagam et al.,

2003) in large fat-rich areas of the food may be significantly higher compared to water-rich areas. Furthermore, there exists the possibility of a matrix-related effect, which may occur during HHP processing of large solid food resulting in pressure non-uniformity. For example, the pressure in the center of 1.4 – 2.2 kg pieces of ham treated at 400 – 600 MPa, 7 – 24°C, was reported to be up to 9 MPa lower compared to the pressure in the pressure transmitting fluid (Minerich and Labuza, 2003). This effect, however, could be frequently neglected due to a smaller size and higher compressibility of many other food products. However, this effect is drastically smaller compared to other effects affecting spore inactivation such as temperature inhomogeneity.

1.4.4.2 Control of the pH Value

Since the pH value presents one of the most important factors influencing microbial inactivation (low pH value typically act synergistically with heat and pressure to inactivate vegetative cells and spores), shifted dissociation equilibria (section 1.4.2.3) can largely influence the outcome of microbial inactivation studies.

To ensure the comparability of results from lab-scale studies on microbial inactivation by HPT treatments, it is essential that model (buffer) suspensions are used that are highly pressure and temperature stable with respect to changes in their pH value (small dpKa/dT and dpKa/dp) (Mathys, 2008; Reineke, 2013; Stippl, 2005).

For lab-scale or industrial HPT treatments of complex/solid food products, a determination of p/T-dependent changes in the dissociation equilibrium is, up to now, not possible. Unfortunately, it is even considerably difficult to only estimate pH changes occurring upon pressurization in such samples. Thus, although alterations in pH levels might significantly contribute to observe inactive effects of vegetative cells and spores in food products, which should be kept in mind, a directed process design and control regarding the parameter, pH, is not possible.

1.5 HPT Inactivation of Bacterial Endospores

1.5.1 Possible Molecular Targets of Pressure

Generally, possible molecular targets in spores that are affected by pressure can be deduced from the general effects, pressure has on macromolecules (section 1.4.2.4). However, spore-specific microenvironment and state of macromolecules have to be considered in an attempt to estimate changes occurring upon pressurization. This section provides a short overview on macromolecules possibly affected by pressure, their putative behavior, and their potential roles in pressure-mediated spore inactivation.

1.5.1.1.1 DNA

Pressure generally stabilizes DNA hydrogen bonds, i.e., the melting temperature for duplex–single strand transition increases upon pressurization. Although HHP-mediated destabilization of DNA molecules might occur (depending on their melting temperature), DNA helices are thought to be stabilized by pressure in most cases (Winter and Dzwolak, 2005). Accordingly, DNA was shown to be stable at up to 1 GPa in vegetative cells (Cheftel, 1995). Since DNA of spores is present in the highly dehydrated environment within the spore core, where it is, additionally, protected against denaturation by physical stress (particularly heat)

by spore-specific molecules (e.g., SASP), it is unlikely that DNA presents a primary target in spore inactivation by HHP or HPT processes.

1.5.1.1.2 Lipid systems

In contrast to DNA, lipid systems such as biological membranes are way more sensitive to HHP and easily undergo phase transformations upon alterations in environmental conditions, which is dependent on their molecular structure, their microenvironment (water content, pH, and ionic strength), temperature, and pressure (Winter and Dzwolak, 2005; Winter and Jeworrek, 2009). Accordingly, membranes present a major target in the HHP-mediated inactivation of vegetative cells (Gänzle and Vogel, 2001). In spores, the outer spore membrane plays a role primarily during sporulation, whereas the inner spore membrane is additionally important for spore dormancy and resistance, which is due to its unique structure (density) and composition (membrane proteins). The inner membrane of the developing forespore is a functional membrane with a phospholipid content similar to that of growing cells, but the volume surrounded by this membrane in mature spores is smaller than it would be expected from the phospholipid content and largely expends during germination without membrane synthesis, which indicates that this membrane is densely packed (Cowan et al., 2004). This enables the inner membrane to present an extremely strong permeability barrier for almost all molecules including water. This barrier is one of the most important spore parts as it confers resistance to DNA damaging chemicals (Cortezzo and Setlow, 2005; Westphal et al., 2003) and protect the core from rehydration, which is crucial to spore dormancy (Sunde et al., 2009). Since the ability to retain DPA in the spore core presents an important factor for HHP and HPT resistance (Reineke et al., 2013b), the barrier function of the inner membrane is likely to be important for the resistance against such treatments.

Generally, the phase transformation behavior of membranes in response to HHP is highly organism-specific (extreme case: piezophilic deep-sea organisms (Abe, 2013)) and commonly adjusted by organisms in response to altered environmental conditions via modulating chain length, chain unsaturation, and head group structure (homeoviscous adaptation) (Winter and Jeworrek, 2009). Thus, differences in the HHP resistance observed between different species or in response to different sporulation conditions (e.g., incubation temperature), might be, at least partially, related to differences in the composition of their inner membrane. Although much work has been conducted to elucidate the phase behavior of model and vegetative cell membranes, knowledge on the exact effect of HHP on the inner spore membrane is scarce and further research is necessary to elucidate the role of this spore layer in HHP inactivation.

1.5.1.1.3 Proteins

In addition to its basic structure consisting of a lipid bilayer, the inner membrane harbors various proteins including some that are important for germination (e.g., nGRs (Setlow, 2003) and SpoVA (Li et al., 2012)). Although large integral and peripheral proteins can dissociate, and their interactions with the lipid phase can be weakened by HHP, membrane proteins can be very pressure-stable (no unfolding up to 1 GPa) (Winter and Jeworrek, 2009). However, the membrane structure and p/T-dependent phase behavior is influenced by membrane proteins and, in turn, protein conformation (and function) can be influenced by the lipid environment (Ulmer et al., 2002; Winter and Jeworrek, 2009). Thus, HHP, can generally act on proteins or protein complexes directly to alter their properties and function or act via

changing the structure of the surrounding lipid phase. Considering that large parts of the germination machinery are localized in the inner membrane and that germination is the first step in the HHP-mediated inactivation of spores from various species, this layer becomes even more likely to present a molecular target for HHP-mediated inactivation.
In addition to membrane proteins, damage to proteins in the core (e.g., preformed enzymes, translation machinery) would putatively have consequences during late germination, outgrowth, and protein *de novo* synthesis rather than directly observable implications such as DPA release, core rehydration, and germination. Although proteins in the spore core are thought to be highly stabilized especially by the extreme dehydration in this area, the germination protease Gpr, which is responsible for degrading α-/β-type SASP during late germination, appears to be not functional after HPT treatments at high pressure levels combined with moderate to high temperatures (Wuytack et al., 1998), which might be due to its HPT-mediated inactivation (Reineke et al., 2013a). Thus, inactivation of other important core proteins might play a role in spore inactivation by HPT processing.
Finally, the spore coat presents a thick proteinaceous layer, where proteins as target molecules affected by HPT treatments might be found. However, although this layer has been mentioned in the context of HPP resistance (Reddy et al., 2006), it seems to play no role in heat inactivation (Setlow, 2006), and its potential role in the pressure-mediated inactivation of spores is far from clear.
Notably, similar to heat inactivation, it is generally difficult to spot a single target for inactivation of microorganisms by HPT treatments as inactivation and resistance are likely to depend on a composition of different engaged processes. Once spores are germinated (no matter if triggered by high pressure or the presence of nutrient…), molecular targets become similar to that for the inactivation of vegetative cells, which also under continuous discussion, but way more explored.

1.5.2 Morphology of Pressurized Spores

Pressure treatments have been reported to be capable of inducing visible morphological changes in spore structures. For example, pressurization of *B. stearothermophilus* (Hayakawa et al., 1994) and *B. cereus* spores (Gola et al., 1996) was reported to result in lengthening / flattening of spores when treated at 300 MPa (20 °C, 5 min) and “breaking” of spores when treated at 500 MPa (20 °C, 5 min) (Gola et al., 1996). Accordingly, pressure treatments of *B. anthracis* spores were reported to result in physicochemical protein modifications, increased membrane permeability, and flattening and lengthening of *B. anthracis* spores (Clery-Barraud et al., 2004). Physicochemical alterations of spore components might be resposible for the repoted morphological changes. However, exact events on the molecular level are unknown and it cannot be excluded, that the initiation of germination might play a role in some of the observable morphological changes after HHP treatments.

1.5.3 Mechanisms of Pressure-Mediated Spore Inactivation

In contrast to direct inactivation by heat, an indirect mechanism (via germination) is presumed for high pressure-mediated inactivation of bacterial endospores from *Bacillus* species. Unfortunately, there exists a significant gap between available data on effects of pressure and underlying mechanisms in *Bacillus* and *Clostridium* spores. The wealth of knowledge

gained on pressure-mediated inactivation of *Bacillus* spores (predominantly for the model organism *B. subtilis*) can build a valuable basis for understanding effects in *Clostridium* spores. However, the existence of major differences in *Bacillus* and *Clostridium* spores, for example, but not exclusively in their germination pathways (section 1.2.2.4) (Sarker et al., 2013), makes it difficult to draw direct conclusions from mechanisms found in *Bacillus* on those in *Clostridium* spores, i.e., inevitable to consider differences, which was tried to be done in this section whenever applicable.

1.5.3.1 HPT Inactivation of *Bacillus* Spores

Generally, spore germination is thought to be the first step in high pressure-mediated spore inactivation of *Bacillus* spores (Margosch et al., 2006; Mathys et al., 2007a; Reineke et al., 2012; Wuytack et al., 1998), where the release of DPA presents an early and rate-limiting step of inactivation (Reineke et al., 2013b). Consequently, the ability of spores to retain DPA largely determines their resistance to high pressure treatments. This putatively accounts for the different resistance properties of spores to HPT treatments and heat alone (Margosch et al., 2004b) and the fact that high wet heat resistance does not necessarily correlate with high pressure resistance (Margosch et al., 2004a; Olivier et al., 2011). Generally, the effectiveness and the underlying mechanism of HPT inactivation of bacterial endospores is greatly dependent on the temperature and pressure applied. According to the results from a number of studies, which investigated the pressure-mediated inactivation of *Bacillus*, predominantly *B. subtilis* spores, the underlying mechanism of pressure-mediated inactivation varies among three more or less concrete ranges of pressure-temperature combinations: (i) Moderate high pressure (approx. 100 up to 600 MPa) combined with moderate temperatures (up to 50 °C), (ii) moderate high pressure (up to 500 - 600 MPa) combined with elevated temperatures (above 50 °C), (iii) very high pressure (above 600 MPa) (Reineke et al., 2012; Reineke et al., 2013b) (Fig. 1-22).

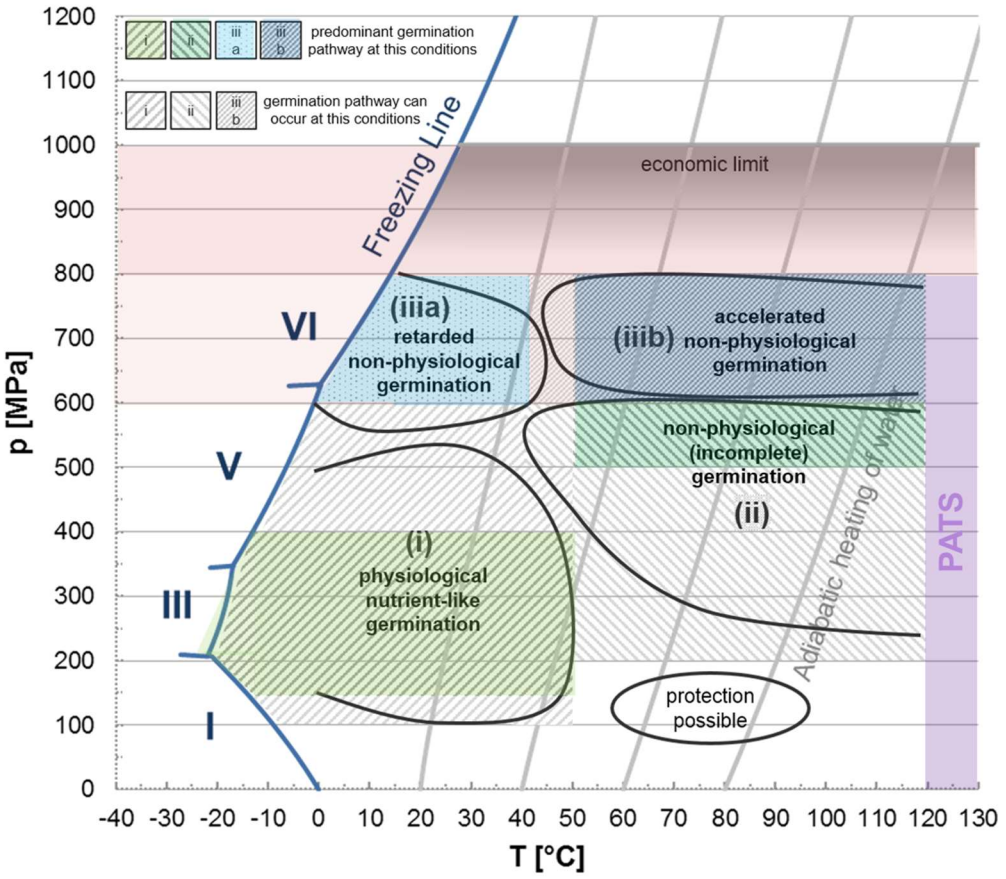


Fig. 1-22: p/T-dependent germination pathways in *B. subtilis*.
Colored areas mark core regions where a certain germination pathway is thought to dominate. Light green (i), dark green (ii), light blue (iiia), dark blue (iiib). See text for a description of the different pathways. Light gray shaded areas mark p/T combinations, where a certain pathway can potentially occur. Purple: pressure treatments at/above 121°C (PATS). Red area: rough indication of pressure-related economic limits beginning at around 600 MPa. Note: The different areas do not present strict prerequisites for a specific pathway to occur and can in fact significantly overlap. Therefore, black solid lines roughly indicate areas, where different pathways may be likely to be induced. Data for figure primarily from: (Black et al., 2005; Black et al., 2007b; Reineke et al., 2010; Reineke et al., 2012; Reineke et al., 2013a).

In the three major ranges of pressure-temperature combinations indicated in Fig. 1-22 following germination pathways are thought to dominate:

(i) Moderate High Pressure / Moderate Temperatures

Moderate p/T-combinations can trigger events that are frequently referred to as physiological, nutrient-like germination since nGRs are thought to be involved (as already indicated in Fig. 1-3). Notably, the designation as “physiological” germination might be somewhat misleading, since there are other “physiological”, but nGR-independent conditions that can trigger spore germination (e.g., bile salts in *C. difficile*, Fig. 1-4). Pressure levels that are sufficient to trigger germination already begin around 100 MPa (Margosch et al., 2006; Paidhungat et al., 2002; Torres and Velazquez, 2005). Typically, pressures ranging between approx. 150 and 400 MPa can effectively trigger germination in different *Bacillus* spores in the absence of nutrients and in a temperature and pH-dependent manner (Gould and Sale, 1970; Reineke et al., 2012). Notably, a short (30 s) HHP pulse at 150 MPa can be sufficient to commit *B. subtilis* spores to germination, although this commitment by a pressure pulse is, in contrast to nutrient

germination, more variable among individual spores and frequently reversible at ambient pressure (Kong et al., 2014).

Commitment to germination by pressure is thought to occur due to the activation of nGRs, putatively of any of the nGRs (GerA, GerB, GerK) present in *B. subtilis* (Black et al., 2005) and *B. cereus* (Wei et al., 2009), but with nGR-specific responsiveness to pressure (at least in *B. subtilis*, (Doona et al., 2014)). Requirements for a low pressure-mediated (e.g.,150 MPa) activation of *B. subtilis* nGRs were reported to be similar, though not identical to requirements for nutrient activation of these receptors (Black et al., 2005). For example, all *B. subtilis* nGRs require diacylglycerylation to induce nutrient germination, whereas this appears to be the case only for GerA to induce germination triggered by low pressures (Black et al., 2005; Wuytack et al., 1998). However, the exact mechanism of nGR activation is known neither for nutrient- nor for HHP-triggered germination. For the latter, structural changes of the receptor molecules and/or structural changes in the inner membrane, where nGRs are situated, seem to be possible (Black et al., 2007a). Although there is some evidence that non-nutrient germination can occur at pressures as low as 200 MPa (30 – 60 °C) presumably via direct Ca-DPA-release (*B. subtilis* Δ*gerABK*, Δ*sleB* spores, (Reineke et al., 2012)), nGR-mediated germination appears to be clearly dominating at moderate pressures and temperatures and essentially follows the nutrient-triggered germination pathway (Ca-DPA release, partial core rehydration, CLE activation, cortex hydrolysis, full germination (Setlow, 2003) (Black et al., 2005).

Similar to nutrient-induced germination, HHP-mediated activation of nGRs triggers a relatively slow release of Ca-DPA. Results from measured DPA-release and flow cytometry experiments indicate that *B. subtilis* spore germination via nGR-activation can be triggered by up to 600 MPa (threshold pressure) and up to 50 °C (Reineke et al., 2013b). Consistent with these findings, pressure-induced germination of *B. subtilis* at up to 600 MPa was suggested earlier to follow the physiological germination pathway, since inhibitors of nutrient-induced germination ($HgCl_2$ and N_α-*p*-Tosyl-L-arginine methyl ester (TAME)) also impeded pressure-induced germination at 600 MPa (Wuytack et al., 1998). Consistent with the proposed germination mechanism, nGR levels are the major factor determining *B. subtilis* germination rates at low pressure levels (150 MPa), while other germination related proteins play a minor role (Doona et al., 2014).

Since nGRs are highly species-specific proteins, which respond to a variety of different nutrients with different efficiency, it is reasonable that various nGRs from different species have different properties including heat stability and their responsiveness to pressure. Thus, it is not surprising that pressure-induced germination in the discussed range (100 – 150 MPa up to 400 – 600 MPa) does not occur with exactly the same efficiency in other spore-formers as it does in *B. subtilis*, especially when considering that events downstream of nGR activation also influence the germination efficiency. For example, the optimal temperature for the completion of spore germination at 150 MPa is around 40 and 65 °C for *B. subtilis* (Black et al., 2007a) and *B. cereus* spores (Wei et al., 2009), respectively. However, nGR-mediated germination triggered under such p/T-conditions is thought to be a common pathway in *Bacillus* spores, and might occur in a very similar fashion, especially in species with high similarity in *ger* operon organization and nGR structure.

(ii) Moderate High Pressure / Elevated Temperatures

In the same pressure range as discussed above (up to 600 MPa), higher inactivation levels of *B. subtilis* spores can be achieved by increasing the treatment temperature from below 40 – 50 °C to above 40 – 50 °C. In contrast to treatments at lower temperatures in the same pressure range, such parameter combinations lead to a rapid DPA release (up to 90% in less than 5 min), which does not continue after pressurization (Reineke et al., 2013b). This is indicative of a non-physiological release of DPA or non-physiological (incomplete) germination (Reineke et al., 2013b) (as already indicated in Fig. 1-4). Again the term physiological, or non-physiological in this case, can be commonly found in literature and is, therefore, used in this section. However, these terms might be not the best choices to indicate whether triggered events are thought to occur dependent or independent from the activation of nGRs. Suitable synonyms for the term non-physiological might be non-nutrient-like or nGR-independent. Pressure levels at which nGR-independent germination primarily occurs have been reported in some studies to begin around 550 MPa or slightly lower (Margosch et al., 2006; Torres and Velazquez, 2005). However, there is some evidence that such a mechanism can readily start to occur at pressure levels as low as 200 MPa and temperatures at or even below 50 °C in *B. subtilis* spores (Reineke et al., 2012).

The exact mechanism of a non-physiologic DPA release triggered by HHP is not clear. Effects on the solubility of DPA seem unlikely to be involved since the DPA concentration the core exceeds the solubility limit by far. Thus, the formation of pores due to inner membrane damage might be a possible mechanism. Additionally, DPA-channel opening (Paidhungat et al., 2002; Wilson et al., 2008) due to changes in inner membrane organization or alterations in the structure of channel proteins (Black et al., 2005) or associated DPA binding proteins (SpoVAD, (Li et al., 2012)) might be involved. The direct and rapid DPA-release under such p/T-conditions can occur independently from the presence of functional nGRs, is not limited by the activity of CLEs, is followed by partial core rehydration, but leads to an incomplete germination process in *B. subtilis* (Black et al., 2007b; Paidhungat et al., 2002; Reineke et al., 2012; Reineke et al., 2011; Reineke et al., 2013b; Setlow, 2003; Wuytack et al., 1998). In comparison to pressure treatments at a low pressure (100 MPa), elevated pressure levels (500 MPa, 40 °C) (Wuytack et al., 1998) also trigger Ca-DPA release and cortex degradation, but retain more resistance against a second HHP treatment, UV light, and hydrogen peroxide, which was suggested to be due to 500 MPa treated spores failing in degrading their SASPs (Wuytack et al., 1998). Additionally, 500 MPa-treated spores fail to induce ATP generation indicating that germination is arrested at some stage (Wuytack et al., 1998).

Inactivation via this incomplete germination has been previously described as occurring due to a two-stage mechanism. In the first stage, the HPT treatment generates sub-lethally injured, DPA free, and phase bright spores. In the second stage, these spores are inactivated by moderate heat, which putatively occurs largely independent of the pressure level (Margosch et al., 2004a; Margosch et al., 2004b). Later, the general description of this process has been extended based on results for pressure-treated *G. stearothermophilus* spores (600 MPa at 77 °C), where four distinct spore populations were detected (Mathys et al., 2007a). Thus, an unknown step occurring in between a germination step (following hydrolysis of the spore cortex) and the final inactivation step (where the spore's inner membrane is physically compromised) was introduced, which facilitated modeling the obtained *G. stearothermophilus* inactivation data (Mathys et al., 2007a).

(iii) Elevated High Pressure Levels / Moderate or Elevated Temperatures

Studies comparing the heat and the combined high pressure and heat resistances of bacterial spores showed that pressure and heat frequently act synergistically to deliver spore inactivation (Ahn et al., 2007; Bull et al., 2009). However, above pressure levels of 600 MPa the synergism between pressure and temperature appears to diminish and DPA release and subsequent inactivation is governed by the treatment temperature as major influence factor (Reineke et al., 2013b). Depending on the treatment temperature this has two major consequences:

(iiia) At 550 MPa, SpoVA protein levels rather than nGR levels in a spore influence germination rates, which indicates that pressure-induced germination primarily occurs via a non-nutrient, i.e., largely nGR-independent pathway (Doona et al., 2014). However, germination under such increased pressure levels (over 400 – 500 MPa) combined with ambient temperatures (up to 40 °C) is retarded. Although Ca-DPA-release from the spore core can be detected, its presence in the core appears to be not required for resistance under such p/T-conditions (550 MPa and 37 °C, *B. subtilis*, (Reineke et al., 2011)). This indicates that genetic material and essential enzymes inside a partially hydrated spore core lacking DPA are not damaged by such a treatment (Reineke et al., 2011). Similarly, it has been reported in various other studies that such elevated high pressure / moderate temperature combinations result in low or no inactivation, e.g., of *B. subtilis* (Reineke et al., 2012; Wuytack et al., 1998), *G. (B.) stearothermophilus* (Ardia, 2004; Mathys et al., 2009), and *B. amyloliquefaciens* spores (Rajan et al., 2006a).

(iiib) When pressure levels above 400 – 600 MPa are combined with elevated temperatures above 50 – 60 °C, an acceleration of both germination and inactivation rates can be observed, which indicates the important role of high treatment temperatures and has been suggested to be useful if germination should be induced in a relatively short process time (Reineke et al., 2012; Reineke et al., 2011). Since the temperature is the governing factor for spore inactivation it is likely that, at some point, germination is bypassed completely or at least plays a diminished role and a direct spore inactivation occurs similar to heat inactivation (de Heij et al., 2003).

The mechanism of direct, i.e., nGR-independent Ca-DPA release appears to be a common response of *Bacillus* spores to elevated pressure and/or temperature (areas (ii), (iiia), (iiib) in in Fig. 1-22). Since CLEs are commonly involved in the subsequent germination nGR-independent pathway, and the cortex lytic machinery is highly conserved among Bacillus species (Paredes-Sabja et al., 2011), it is likely that mechanisms underlying spore germination and inactivation of *Bacillus* spores under such conditions ((ii), (iiia), (iiib)) are similar to that described for *B. subtilis*. Obvious differences in the HPT resistance of different *Bacillus* spores, which of course exist, might occur due to differences in composition and structure of any spore components influencing DPA-release or events downstream of the release of DPA in the germination cascade.

Areas of Spore Stabilization

Interestingly, there are few reports that describe that specific p/T-combinations can lead to a stabilization of *Bacillus* spores, i.e., lower inactivation levels under pressure than at ambient pressure (0.1 MPa). This has been reported for very low and very high pressure levels combined with high temperatures.

For example, 61 MPa and combined with 93 °C, have been found to lead to less inactivation of *B. subtilis* than at 93 °C at ambient pressure (Johnson and Zobell, 1949). The reason for this is not clear, but there are several factors that might be involved. The activity of nGRs is likely to decrease as temperature increases (Paidhungat et al., 2002), which means that the temperature is likely too high to cause nGR-dependent pressure-induced germination. Additionally, the pressure applied is likely too low to cause significant damage of possible molecular targets for high pressure inactivation (section 1.5.1). Finally, this phenomenon could (purely speculative) be related to the fact that pressure and temperature can exert antagonistic effects on macromolecules (section 1.4.2.4). Thus, a pressure-mediated stabilization of molecular targets for heat inactivation could lead to lower inactivation at 61 than at 0.1 MPa.

More strikingly, although increasing pressure levels commonly accelerate spore inactivation strongly and steadily, a protective effect of extremely high pressure levels combined with high temperatures was also reported. For example, treatments at 800 – 1200 MPa and 120 °C resulted in a lower inactivation of *B. amyloliquefaciens* spores as compared to heat alone (Margosch et al., 2006). A pronounced pressure-dependent tailing effect even led to a small spore fraction surviving conditions at up to 120 °C and 1.4 GPa in isothermal treatments (Margosch et al., 2006). Although such pressure levels exceed the limit of industrially available HHP units, the possibility of spore stabilization of spores at very high pressure levels in combination with high temperatures should be kept in mind, e.g., when it should be planned to apply PATS processes at very high pressure levels.

1.5.3.2 HPT Inactivation of *Clostridium* Spores

The mechanism of pressure-mediated inactivation of *Clostridium* spores is largely unknown. However, molecules and pathways that are known to play a significant role in the HPT inactivation mechsnism in *Bacillus* spores exhibit some striking differences those present in *Clostridium* spores, which impedes the transferability of results obtained for *B. subtilis*.

Moderate high pressure / moderate temperatures

The majority of *Bacillus* and *Clostridium* spores have considerable differences in nutrient germination requirements, which is putatively related to the different genetic architecture and conformation of their nGRs (Paredes-Sabja et al., 2011). Hence, it is not surprising that *Clostridium* nGRs also harbor a different responsiveness to pressure and that pressure levels triggering nGR-like germination of *B. subtilis* spores do not provoke identical effects in *Clostridium* spores. Indeed, although there exists a huge gap of knowledge and it cannot be excluded that low pressure levels might be effective in inducing nGR-like germination in spores of some *Clostridium* species, available data indicate that low pressure levels at moderate temperatures have no or a very small effect on *Clostridium* spores. For example, 100 – 200 MPa, 7 min treatments do not induce germination in *C. perfringens* spores within 60 min after pressure treatment (Akhtar et al., 2009) and pressure cycling (60 MPa followed by 400 MPa at 60 °C) reduces *C. sporogenes* viable spore counts by less than 3 log cycles (Mills et al., 1998). Consistent with the slow pressure-induced germination rate of spores from various *Clostridium* species, the DPA release of pressure-resistant, proteolytic *C. botulinum* type B (YpeB negative) was observed quantitatively only at conditions where 5 log inactivation was reached (high pressure/high temperature, (Margosch, 2004; Margosch et

al., 2004a). This indicates that DPA is released by a physicochemical rather than a physiological (nGR-dependent) process and that the ability to retain DPA represents a crucial factor for the HPT resistance also of *Clostridium* spores.

Elevated high pressure / elevated temperatures

In addition to nGRs, CLEs, which are thought to play a major role in pressure-induced germination via the non-nutrient pathway in *Bacillus* spores are different in *Clostridium* spores. Many *Clostridium* species rely on the CS (Csp and SleC) machinery for spore cortex lysis during nutrient-triggered germination, which is completely absent in *Bacillus* spores (section 0) (Paredes-Sabja et al., 2011). Unfortunately, for this machinery, even the exact activation mechanism during nGR-mediated germination is not completely clear (Paredes-Sabja et al., 2011), which makes it difficult to speculate about its role in pressure-mediated germination.

The majority of *Clostridium* species lacks the CLE CwlJ (Paredes-Sabja et al., 2011), which is thought be activated by endogenous or exogenous DPA, it is unlikely that the direct release of Ca-DPA caused by treatments at elevate pressure levels triggers germination as it is proposed to occur in *Bacillus* spores.

Additionally, spores of many *Clostridium* species possess orthologues of SleB, which is thought to be activated by core rehydration and cortex deformation that are cause by the Ca-DPA release during germination and play a role in nGR-independent pressure-induced germination of *Bacillus* spores. However, YpeB, which is required for proper localization and/or function of SleB in *Bacillus* spores, is absent in the majority of *Clostridium* species (Paredes-Sabja et al., 2011). At least results obtained for *C. difficile* suggest that the requirement of YpeB for functional SleB might be similar in *Clostridium* spores, since such spores rely solely on their CS system, whereas SleB is present but not functional, possibly due to the absence of YpeB (Burns et al., 2010; Cartman and Minton, 2010). Provided that this is a broadly valid interrelation, the absence of YpeB in many *Clostridium* spores makes it unlikely that the activation of SleB by partial core rehydration and cortex deformation provoked by a pressure-mediated release of Ca-DPA plays a role in the HPT inactivation mechanism in *Clostridium* spores. Notably, there are some *Clostridium* species that possess the complete YpeB-SleB cortex lytic machinery such as *C. sporogenes* and at least one *C. botulinum* type A strain (Paredes-Sabja et al., 2011). Consistently, it has been speculated that only these species can germinate effectively via a pathway involving Ca-DPA release, SleB activation, and cortex lysis in response to HPT treatments at elevated temperatures (Sarker et al., 2013).

However, this absence/non-functionality of the YSCQ machinery in many *Clostridium* species brings us back to the CS system of which the exact activation mechanism is not known (as mentioned in the beginning of this paragraph). What is known, is that, *C. perfringens* spores, which possess the CS system (and SleB but not YpeB), are stable and germinate normally even when they lack DPA (Paredes-Sabja et al., 2008b). This makes it unlikely, that the CS cortex lytic machinery, if it is activated by pressure at all, is activated through a DPA-dependent pathway. Furthermore, it has be reported that SleC, CspB, and the Ca-DPA release process present possible targets for damage by wet heat (*C. perfringens*, (Wang et al., 2012)). This makes it even more unlikely, especially for HPT treatments at high temperatures, to trigger germination via SleC activated by Ca-DPA release.

In comparison with various *Bacillus* spores, HPT-induced germination appears to be less effective in spores of various *Clostridium* species, e.g., max. 52% germinated *C. perfringens* spores after 483 MPa, 50 °C, 5 min treatments (heat susceptible fraction 24 h after pressure treatment, (Kalchayanand et al., 2004)). In the same study, it has been shown that *C. sporogenes* germinates slightly better (82%) at 483 MPa, 50 °C (Kalchayanand et al., 2004), which is also not very effective (potentially related to the absence of GerQ-CwlJ), but more effective than in *C. perfringens* (potentially related to the presence/functionality of YpeB-SleB, not specified in this study). A further prolongation of the dwell time at 483 MPa, 50 °C (15 – 120 min) is necessary for an effective pressure-induced germination, which then occurs at an optimum pressure of around 200 MPa combined with mild heat (40 – 70 °C) (Ishimori et al., 2012).

In summary, although the exact mechanism of inactivation of *Clostridium* spores is not known, available data suggest that germination induced by low pressure and moderate temperatures via the activation of nGRs, occurs only poorly or very slowly in comparison to *Bacillus* spores. Since nGRs are highly species- and sometimes strain-specific molecules, HHP-triggered germination via the activation of nGRs could potentially work effectively in some *Clostridium* species or strains, which are not examined so far. Furthermore, the release of Ca-DPA at elevated pressures and temperatures also appears to occur slower than in *Bacillus* spores and different mechanisms are likely to be involved. Unlike in *Bacillus* spores, the activation of the cortex lytic machinery triggered by the release of DPA appears to play no role in *Clostridium* spores (maybe except of some (YpeB-SleB positive species/strains)). Thus, it seems that in *Clostridium* spores either the CS cortex lytic machinery (putatively the only CLE machinery in many *Clostridium* spores) is activated in a DPA-independent fashion or DPA-release is an exclusively physicochemical processes without any parts of the germination machinery being involved.

1.5.4 HPT Spore Inactivation Kinetics

High pressure inactivation kinetics of bacterial endospores can follow log linear, i.e., first order reaction kinetics. However, available data suggests that this is organism- and sometimes also treatment intensity-dependent. An initial lag phase in inactivation kinetics, i.e., shoulder, and flattening of survivor curves at longer treatment times, i.e., tailing, can be frequently observed. Especially HHP-treatments at moderate temperatures can result in a pronounced shoulder formation, i.e., an initially increased in the cell count from treated compared to untreated spore samples (at least reported for some *Bacillus* spores, e.g., (Reineke et al., 2011)). For example, an initial approx. 0.3 log (N_0 = 8 log) increase has been reported for *B. subtilis* spores treated below 600 MPa at 30 and 60 °C (Reineke et al., 2012). There are two main reasons discussed to account for shoulder formation including the pressure-induced germination of superdormant spores (e.g., *B. cereus*, *B. subtilis*, (Wei et al., 2010)) and the disassembly of spore agglomerates (e.g., *G.* (*B.) stearothermophilus*, (Mathys et al., 2007b)). Additionally, tailing of inactivation curves was frequently described and is putatively related to the heterogeneity of spore populations with respect to various resistance properties and dormancy. Notably, a pronounced tailing effect was observed for proteolytic *C. botulinum* spores where a highly resistant fraction within the spore population survived even at extreme pressure-temperature combination (1.4 GPa, 120 °C, (Margosch et al., 2006)).

1.5.5 Spore Population Heterogeneity

Spore populations are commonly heterogeneous, which, however, does not always lead to such a pronounced tailing effect as described in the previous paragraph (1.5.5). Population heterogeneity is likely a result of the highly regulated and timely differently proceeding sporulation program in single spores within a population (for details see 1.2.2.3.2).
For pressure-treated *Bacillus* spores, four distinct physiological states of pressure treated spores have been previously described, i.e., spores in a dormant, germinated, an unknown physiology state (after germination), and an inactivated state (inner membrane disrupted) (*B. licheniformis*, (Mathys et al., 2007a)). In addition to the differences between these physiological states, especially the still dormant spore fraction is likely to be highly heterogeneous within itself, since it may contain spores with various degrees of damage, e.g., damage of the germination and/or outgrowth machinery caused by a HPT treatment. Severity of such damage can be examined monitoring growth from single spores. For example, low pressure levels at ambient temperatures (100 MPa, 20 °C) left the detection times of *B. licheniformis* largely unaltered (Margosch et al., 2004b), whereas 200 MPa, 70 °C treatments (16 min, *B. subtilis*) slightly and 800 MPa, 70 °C treatments (4 min, *B. licheniformis*) strongly prolonged detection times of single *Bacillus* spores and widened associated distributions (Margosch et al., 2004b). These results illustrate the treatment intensity-dependent damage of spores within a population. The observed heterogeneity might be related to the presence of spores that are differently equipped, e.g., with functional nGRs, before and after pressure treatments. Notably, variations in the expression levels of nGRs were shown to be not the primary factor that controls spore germination heterogeneity in untreated *B. subtilis* spores (Zhang et al., 2013) indicating that other spore components modulating the germination and/or outgrowth are important.
The possible increase of the heterogeneity in spore populations after HPT treatments has two major practical implications: Extended times, which spores need to induce growth can lead to an overestimation of spore lethality in inactivation studies when incubation/recovery times after HPT treatments are too short. Secondly, reduced times and narrowed distributions at low pressures and ambient temperatures might lead to an enhanced chance of spores to grow out and, e.g., in the case of *C. botulinum*, produce toxin within a distinct period of time (e.g., product shelf life).

1.5.6 HPT Inactivation and Surrogate Organisms

Differences in the proposed inactivation pathways discussed above are likely to account for large differences observable in the p/T-dependent HPT resistance of *Bacillus* and *Clostridium* spores. Both species- and treatment intensity-dependence of inactivation levels are putatively related to differences in inactivation pathways, i.e., the inactivation pathways followed by two different organism at a specific p/T combination are not necessarily identical. This has important consequences on the possibility to predict the behavior of a certain organism in response to HPT treatments from existing data for other organisms, especially when species form different genera are compared. Thus, it seems obvious that spores of a *Bacillus* species can never be true surrogates for *Clostridium* spores for the purpose of evaluating the effectiveness of HPT processes.

Since treatment intensity-dependent differences in the inactivation can also vary considerably between species of the same genus, it might be impossible to find suitable surrogate organisms for HPT inactivation studies even within the same genus. There might be some exceptions, e.g., non-toxigenic *C. sporogenes* as surrogate organism for proteolytic *C. botulinum* type A, which are genetically and morphologically very similar including their germination machineries (Paredes-Sabja et al., 2011). However, at present, the lack of detailed inactivation data for several strains of both organisms makes it impossible to predict whether these two organism show similar enough behavior in response to HPT treatments in a wide range of conditions. Naturally, there can also exist huge differences in the HPT resistance between different strains within a species. However, such strains are likely to follow a similar inactivation pathway at a certain p/T combination, i.e., inactivation isorate curves are likely to be shifted but might have a similar shape. These general points might be considered when discussing about surrogate organisms and their use in the evaluation of food safety provided by HPT processing.

1.5.7 Influence factors on HHP-mediated spore inactivation

In addition to species- and strain-specific differences in the requirements for pressure-induced germination and HPT resistance, there exists a variety of factors that can influence the outcome of spore inactivation studies. Such influence factors can either affect the resistance properties of spores (e.g., sporulation conditions), act synergistically or antagonistically with the HPT treatment (e.g., the matrix surrounding spores), or influence the recovery and detection of treated spores (e.g., germination conditions). Additionally, the design of HPT experiments (equipment, parameter control) can significantly alter the outcome of spore inactivation studies. Different factors that might play a role are summarized in Fig. 1-23. With the exception of influence factors related to the control of HPT process, which are discussed in detail in section 1.4.4, the following paragraphs describe possible effects of sporulation conditions (1.5.7.1), germination conditions (1.5.7.2), matrix (1.5.7.3), and experimental design (1.5.7.4) highlighting putative major influence factors.

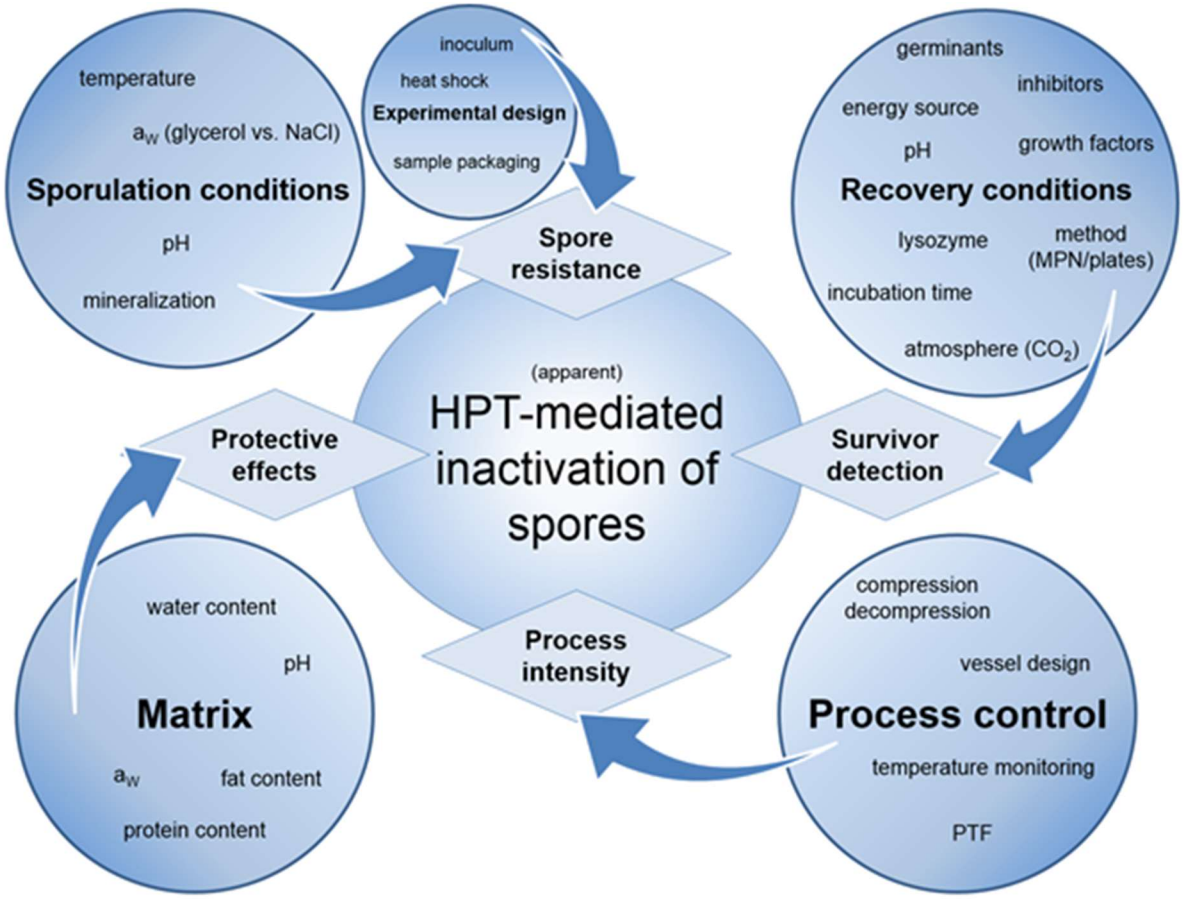


Fig. 1-23: Influence factors on spore inactivation results.
Overview of factors that can influence the outcome of HPT spore inactivation studies via their effect on spore resistance properties, the detection of survivors from HPT treatments, the intensity of the HPT process, and the protection of spores from their inactivation by HPT treatments.

1.5.7.1 Sporulation conditions

Two major factors affecting the resistance of spores to HPT treatments include the sporulation medium composition in general and the mineral content of spores in particular as well as the temperature at which spores are produced. Alterations either one of these factors can provoke a multitude of changes in the structure and resistance properties of spore. A detailed picture on alterations provoked by the sporulation temperature is provided in section 1.2.4.1. Since the effects of both sporulation temperature and medium mineral content on the HPT resistance of *C. botulinum* type E spores were investigated in this study, further details can also be found in the introduction and discussion sections of this manuscript.

In addition to sporulation temperature and medium, possibly any other factors during sporulation, which affect the germination machinery, spore layers where parts of this machinery are situated, or, generally, the ability of spores to retain Ca-DPA in the core appear likely to influence the HPT resistance of spores. Although the effects seem to be less pronounce than that of temperature and medium, some other factors have been identified that exert an (at least slight) effect on the spore resistance to pressure-mediated germination of *B. subtilis*, i.e., aeration, the pH, and the water activity (different effect of glycerol and NaCl) of the sporulation medium (Nguyen Thi Minh et al., 2011). The latter effect might be related to osmotic stress conditions provoking alterations the inner membrane (levels of individual

fatty acids and phospholipids, (Lopez et al., 2002)). Both the effect of pH (*B. cereus*, (Oh and Moon, 2003)) and NaCl concentration (*B. subtilis*, (Black et al., 2007b)) appear to be dependent on the HPT process intensity. However, not much is known about the precise mechanism and such effects in other spore-formers.
Notably, sporulation conditions such as variable nutrient availability and sub-optimal temperatures, which are commonly prevalent in soil or sediment, the natural habitat of many spore-formers, can affect (typically increase) the resistance properties of spores from various species. This can have direct implications on the evaluation of food safety as it may lead to the underestimation of risk originating from pathogenic spore-formers.

1.5.7.2 Germination conditions

Spores commonly have to grow in order to be counted as survivor of an inactivation process such as an HPT treatment. Since conditions at which spores are incubated after a treatment can significantly influence their recovery and, thus, the determined inactivation efficiency of the treatment, such conditions need to be considered.
Generally, all factors that can affect the germination, outgrowth, and/or growth of spores can, potentially, influence spore recovery. These factors are numerous and include the availability of nutrients, the presence of compounds aiding in or inhibiting growth, the gas atmosphere, as well as incubation temperature and time. Since the effects of recovery conditions on HPT inactivation studies examining *C. botulinum* type E spores were investigated in this study, they are described in detail in the introduction and discussion sections of this manuscript.

1.5.7.3 Matrix

Environmental characteristics can strongly affect the survival of spores during HPT treatments. One of the most important influence factors is the pH of the matrix surrounding the spores, which plays an important role in spore inactivation, not only in food. Due to the shift of the dissociation equilibrium (for details see section 1.4.2.3) caused by alterations in both pressure and temperature, it is important to consider this factor even when conducting spore inactivation studies in simple aqueous model systems. The use of buffer solutions that are not pressure and temperature stable, i.e. not suitable to largely maintain their pH during HPT treatments, can result in highly accelerated spore inactivation rates (Mathys et al., 2008c). Generally, a low pH is thought to increase HPT spore inactivation (*B. coagulans*, (Roberts and Hoover, 1996); *B. cereus* (Gao and Ju, 2010)). However, this effect may be species-dependent and putatively occurs in a process intensity-dependent manner, since variations in the pH (3 – 8) did not result in significant alterations in *B. subtilis* spore inactivation by HPT treatments at 100 and 600 MPa, 40 °C (Wuytack and Michiels, 2001). Additionally, it was shown that the pH value and the inactivation of proteolytic *C. botulinum* type B spores do only correlate within certain limits, i.e., no significant difference between inactivation at pH 5.15 and 6, but markedly increased inactivation rate at pH 4 (Margosch et al., 2004a). Another important role of the pH can be found in its synergistic action with some (antimicrobial) substances such as citric and acetic acids (*B. amyloliquefaciens*, (Ratphitagsanti et al., 2010)) or nisin (*B. coagulans*, (Gao and Ju, 2011)). A temporally decrease of the pH of food products under pressure likely contributes to spore inactivation. However, pressure-induced changes in the pH of complex food matrices cannot be easily

determined and food matrices generally exert protective rather than synergistic effects on the HPT inactivation of bacterial endospores.

Another factor of major importance for microbial inactivation in food is the total water content and the water activity (a_w). Low water activities typically exert a baroprotective effect, which is related to the general fact that the presence of water is necessary for an effective inactivation of microorganisms. Although some mechanisms how the a_w could affect inactivation are known, e.g., that a reduced a_w can protect proteins from denaturation (Cioni and Strambini, 1994; Di Primo et al., 1995; Priev et al., 1996; Ruan et al., 2003; Zancan and Sola-Penna, 2005), the exact mechanism underlying baroprotective effects of low a_w values are unknown. Notably, the a_w value alone is insufficient as parameter to predicted baroprotective effects, since substances reducing the a_w to an identical level can exert different effects on microbial inactivation (e.g., NaCl and sucrose, (Molina-Hoppner et al., 2004)). Consistently, parameters such as degree of saturation of solutions might be more suitable as relevant factor for describing baroprotective effects (Koseki and Yamamoto, 2007).

Additional food properties, which can influence the HPT resistance of spores include protein content and fat content. For example, soybean protein and sucrose significantly protect *B. cereus* spores against HPT inactivation (540 MPa, 71 °C, 16.8 min, (Gao and Ju, 2010)). In contrast, soybean oil showed only a slight and less significant protective effect (Gao and Ju, 2010). The protective effect of the fat content is still a matter of discussion. Although spore protection can be often observed in fat-rich environments, it has been frequently attributed to the low water activity of the product due to the concentration of solutes in the aqueous phase rather than to the fat itself (e.g., *G.* (*B.) stearothermophilus* in cocoa mass, (Ananta et al., 2001)).

Similar to the HPT resistance of spores, which does generally not correlate with their heat resistance assessed in model buffer suspensions, protection conferred by the food matrix against HPT and heat does not necessarily correlate. For example, this was shown for proteolytic *C. botulinum* types A and B and *C. sporogenes* spores in Bolognese sauce, cream sauce, and rice water agar (Bull et al., 2009).

For practical considerations on food safety the variety of different effects exerted by various food components, their interconnection in complex food matrices, which may additionally be species- and HPT treatment intensity-dependent indicates that it is extremely difficult to precisely predict the efficacy of HPT processes to inactivate spores in food. Thus, modeling approaches can help to characterize matrix effects and roughly estimate possible inactivation levels of a certain spore type in a narrow range of products, a final, reliable evaluation of the reachable degree of food safety has to be made conducting challenge tests in a specific food, under specific process conditions, and with spores of relevant spore-forming species.

1.5.7.4 Experimental design

There is a multitude of studies that have dealt with the inactivation of bacterial endospores by HPT treatments, and numerous spore inactivation kinetics have been recorded using model spore suspensions and various food products as matrix. As discussed above, the use of a suitable p/T-stable buffer (see 1.5.7.3) and a precise control of the HPT process parameters (especially the temperature, see 1.4.4.1) are crucial to obtain comparable results.

However, there are some additional factors related to the experimental design and the control of process parameters, which can influence inactivation results and impede their comparability.
One of the factors of major importance is represented by the use of heat activation of spore samples prior to HPT processing, which can have a significant influence on spore germinability and, consequently, resistance to HPT treatments. Since effects of a sublethal heat shock applied prior to HPT treatments on the HPT inactivation of *C. botulinum* type E spores were investigated in this study, they are described in detail in the introduction and discussion sections of this manuscript.
Furthermore, it is commonly necessary to conduct inactivation experiments with high inoculation levels to demonstrate an effective inactivation by a certain number of log cycles, e.g., 6 log inactivation for *C. botulinum* type E (see section 1.3.6.2). However, higher initial inoculum can result in lower spore inactivation levels detected after HPT treatments (10^4 -10^9 *B. subtilis* spores, 100 MPa, 45 – 75 °C, 10 – 120 min, (Furukawa et al., 2002)), which was suggested to occur due to increased spore clump formation in highly concentrated spore suspension. On the other hand, very high inoculum levels could also provoke an opposite effect (detection of higher inactivation levels), since clustered spores might germinate more rapidly due to nGR-independent germination pathways allowing spores to sense germination in their environment (see section 1.2.2.4.2, Fig. 1-4). Although this was shown to be not the case for non-proteolytic *C. botulinum* type B (Webb et al., 2012), it cannot be excluded that this occurs in highly concentrated suspensions containing spores produced by other species. This could be the case, e.g., for *Bacillus* spores, which readily release Ca-DPA under pressure and possess the Ca-DPA-responsive CLE, CwlJ. Notably, the latter mechanism is highly speculative, but would result in the same conclusion as that for clump formation at high spore densities, i.e., that inoculation levels for inactivation kinetics should be chosen as low as possible (but high enough to demonstrate the stipulated log reduction or enable the proper calculation of D-values). However, these dependencies illustrate that the conduction of challenge tests using low spore inocula is indispensable.
Another factor, which was shown to influence spore inactivation by HPT processes could be the type of sample packaging (Patazca et al., 2013). This has been attributed to differences in temperatures samples are subjected to at different places in flexible plastic pouches (Patazca et al., 2013), which could be related to differences in the heat transfer between the sample and the pressure transmitting fluid (PTF). In the simplest case, packaging systems can affect the apparent spore resistance due to adhesion effects of spores to the packaging material. Exact reasons for the reported effect of sample packaging were not specified in the respective study and remain (at least) unclear. However, it illustrates that even such apparently simple factors should not be neglected when designing experimental setups.

1.5.8 Strategies to enhance HHP-mediated spore inactivation

Strategies to inactivate bacterial endospores by HHP without the need for its combination with high temperatures include the introduction of two-step processes or the addition of synergistically acting compounds.
Two-step processes can consist of a mild pressure treatment to induce spore germination followed by a treatment at higher pressure and moderate temperature or by a mild heat

treatment to inactivate germinated spores. Alternatively, a heat activation step at a moderate temperature followed by a high pressure treatment (Mills et al., 1998) and a combination with irradiation treatments before or after pressurization (Crawford et al., 1996) has been explored in some studies. Although optimization of the process parameters of the two steps can result in the successful inactivation of *Bacillus* spores by several log cycles (Raso et al., 1998b), reliable sterilization processes completely inactivating such spores were not established. Due to the generally lower effectiveness of pressure to induce germination of *Clostridium* spores (see section 1.5.3.2), an application of such processes to effectively inactivate spores from this species appears to be even more difficult.

Reasons for difficulties in achieving complete inactivation can most likely be found in the great heterogeneity in pressure sensitivity and the effectiveness of HHP-induced germination among spores of different species and within a population (Heinz and Knorr, 1996; Wuytack et al., 1998), i.e., complete germination can commonly not be achieved (Aouadhi et al., 2012). Furthermore, germination does not follow log-liner kinetics and dwell times (*B. subtilis* optimum for germination around 300 MPa, 30 °C, 60 min) that may be required for triggering the germination of a large portion of the spore population can be very long (Reineke et al., 2012). This altogether, makes two-step approaches, especially for the inactivation for *Clostridium* spores unlikely to be suitable for an industrial application.

A possibility to enhance the induction of germination and inactivation of *Clostridium* (and *Bacillus*) spores is to add suitable nutrient germinants, e.g., to a food, combined with a heat activation step and a subsequent HPT treatment, e.g., L-asparagine, KCl, 80 °C for 10 min and 586 MPa, 73 °C for 10 min to inactivate *C. perfringens* (Akhtar et al., 2009). Although this approach enhanced inactivation levels at relatively mild treatment conditions, it also failed to provoke complete (> 6 log in this case) reduction in viable cell counts. Additionally, it might not be desired to alter food product formulations, requirements for nutrient-triggered germination vary considerably between species and within populations, and it is questionable whether the presence of nutrients for an optimal germination, e.g., of a pathogenic spore-former, presents a feature a food product should have. All this makes it difficult to use this approach for a broad spectrum of foods and against spores from various species. While significant spore reduction can be achieved, additional strategies are required to ensure food safety upon storage.

An effective way to decrease the process temperature required for effective spore inactivation during HPT processes is the addition of antimicrobial compounds such as essential oils (Gayan et al., 2012) or sucrose laurate (only effective at pH ≤ 6.0; (Stewart et al., 2000)). Especially nisin has been demonstrated to be very effective in combination with HHP treatments, e.g., inactivating *C. perfringens* (Gao et al., 2011), *A. acidoterrestris* in apple juice (Sokolowska et al., 2012), or *B. subtilis* and *B. cereus* in milk, although it can also fail to provoke complete inactivation (Black et al., 2008). Such a combination of antimicrobial substances and HHP processing might be reasonable in some cases. However, economical, legal, and food product specific limiting factors have to be considered, e.g., that preservatives are often not desired or permitted in food products, especially not in high amounts, and that the use of certain substances that can affect sensory food properties might be limited to only a few food products. Additionally, an important marketing argument for HHP-treated food, i.e., the possibility of labeling food as ‘additive-free’, could not be used anymore.

2 INTRODUCTION

2.1 Motivation and Aim

Consumers tend to demand for minimally processed, healthy food with low amounts of additives (Kinsey and Senauer, 1996; Schmidt, 2006; Sloan, 1998). Additionally, food products should have a long shelf-life, which is desired by many customers and, maybe more importantly, by the food industry (logistics/costs). At the same time, levels of legally permitted chemical preservatives tend to decrease and authorities aim at a reduction of food ingredients that act as traditional preservatives and as such present important factors within the hurdle concept for food safety (e.g., salt (EuropeanCommision, 2012) and sugar (Norton et al., 2006) reducing the water activity). This makes the production of safe high-quality foods more and more challenging (Markland et al., 2013).

High hydrostatic pressure (HHP) treatments at or below ambient temperatures are frequently applied nowadays to compensated for the reduction of other hurdles, additionally increase food safety, enable the production of shelf-stable heat sensitive food products, and/or for processing reasons (e.g., increase meat recovery, shell removal from Crustaceans). However, and effective inactivation of bacterial endospores, i.e., sterilization process, requires the addition of heat. Such processes can be carried out at the sterilization temperature of 121.1 °C, i.e., pressure assisted thermal sterilization (PATS), or below this temperature, i.e., high pressure thermal (HPT) processing (for further information see section 1.1). However, although HPT processing has several advantages in comparison with conventional retorting, particularly less detrimental effects on organoleptic and nutritional food quality, (section 1.4.3), it is not yet applied at the industrial level. Among others (section 1.1), one major reason for this can be found in the lack of knowledge on basic mechanisms of spore inactivation by HPT treatments (section 1.5.3), including spores from the highly food safety-relevant organism *Clostridium botulinum*.

Depending on the food product composition, its intended storage temperate, and its origin, different types of *C. botulinum* may present the primary threat to food safety. For the safety of refrigerated processed food of extended durability (REPFED) (section 1.3.6.1) primarily from aquatic environments of temperate regions of the northern hemisphere (sections 1.3.5.1 and 1.3.5.2, Fig. 1-16), that are packed under anaerobic conditions and have a composition allowing the growth (section 1.3.6.2), *C. botulinum* type E presents a major threat. This organism belongs to the genetically and physiologically distinct, non-proteolytic *C. botulinum* group II (section 1.3.2), and produces the botulinum neurotoxin E (BoNT/E) (section 1.3.3). Preformed toxin food can cause foodborne botulism, a rare (section 1.3.4.3), but severe (section 1.3.4.2) illness. Under unfavorable conditions *C. botulinum* type E forms endospores (section 1.2.2), which are less resistant than those formed by other *C. botulinum* types (section 1.3.2), but can, under certain circumstances, survive traditional food processing such as hot-smoking processes (section 1.3.5.3, Fig. 1-16) and, in contrast to proteolytic *C. botulinum* types, grow at refrigerated temperatures around 3 °C (section 1.2.2).

Despite of these industry-related reasons that made it high time to extend the knowledge on effects of high pressure on *C. botulinum* type E spores, the primary motivation for this study was the tremendous gap of knowledge on effects exerted by high hydrostatic pressure on *Bacillus* versus *Clostridium* spores. While the overwhelming majority of data is available for the non-pathogenic, rarely food spoilage-associated model organism, *B. subtilis*, knowledge

on the effect of high pressure on *Clostridium* spores including those from highly food safety-relevant organism *C. botulinum* type E is scarce, i.e. comprising only a few HPT inactivation curves that were not always obtained under defined conditions (Reddy et al., 1999; Skinner et al., 2014).

Thus, the general aim of this study was to contribute to closing the gap of knowledge regarding the HPT-mediated inactivation of spores from *C. botulinum* type E. The generated data should be valuable for the design of future spore inactivation studies, for the interpretation of results for other *Clostridium* spores, for future considerations about food safety, and, finally, contribute to the establishment of HPT food processing at the industrial level.

2.2 Working Hypotheses

Work done in this study can be roughly divided in three sections, i.e., the determination of (i) effects of the experimental design on the outcome of *C. botulinum* type E HPT inactivation studies, (ii) detailed inactivation kinetics and mechanistic insights, and (iii) reasons for most significant effects observed in the first part of the study. Main working hypotheses (►) of the three different parts and reasons for such hypotheses are stated below.

2.2.1 Influence Factors on HPT Inactivation

- It is likely that laboratory sporulation conditions (particularly media and temperatures) differ from the growth and sporulation conditions of naturally occurring spores and spores derived thereof behave differently, which limits the extrapolation of results towards food safety.
- Modification of sporulation conditions can lead to perturbations in the ordered course of the sporulation phosphorelay (Veening et al., 2006) (section 1.2.2.3.2, Fig. 1-2), which can cause problems in proper spore assembly and alterations in structural features, which, in turn, might affect spore germination and resistance properties.
- Sporulation media resembling the natural habitat of *C. botulinum* were shown to yield highly HPT-resistant spores (*C. botulinum* type A, (Margosch et al., 2006; Margosch et al., 2004a).
- Sporulation characteristics and resistance properties of spores from several *Clostridium* species were shown to be drastically altered depending on the type of sporulation medium (Dixit et al., 2005; Roberts, 1965) and the sporulation temperature (section 1.2.4.1).
- It is impossible to draw direct conclusions from existing data for processes other than HHP, or other organisms, on the effect of sporulation temperature on the pressure resistance of *C. botulinum* type E endospores (section 1.2.4.1).

► Medium composition and the temperature during sporulation can influence the HPT resistance of *C. botulinum* type E spores, which cannot be predicted from existing

data, might lead to an increased resistance of naturally grown spores, and could impede proper risk assessment.

- Spore suspension purification can be required, since the production of spore crops containing 100 % spores is often impossible, including *C. botulinum* type E (time-shifted sporulation, (section 1.2.2.3.2)).
- The use of mixtures of vegetative cells and spores can complicate the correct determination of spore-specific D-values building the basis for food safety evaluation (section 1.3.6.2).
- Spore suspensions are frequently stored before they are treated, e.g., by HPT treatments.
- Spore maturation processes might continue during storage, which could influence their resistance properties.

► Resistance properties of *C. botulinum* type E spores can be changed by spore suspension purification treatments and might change over time during storage, which can impede proper risk assessment.

- The formation of spore clumps can result in the detection of lower numbers of survivors of an inactivation process (section 1.5.4).
- Recovery conditions can largely influence the number of detected survivors of an inactivation process (section 1.5.7.2).
- Detection of wrong numbers of survivors affects the “measured” or “apparent” spore resistance and impedes proper safety assessment.

► Sample dilution and recovery conditions can change resistance properties of *C. botulinum* type E spores and impede proper risk assessment.

2.2.2 HPT Inactivation Kinetics and Mechanism

- Inactivation kinetics commonly build the basis for food safety evaluation (e.g., 6-D concept for *C. botulinum* type E in REPFED, section 1.3.6)
- Spore inactivation does not necessarily follow 1st order kinetics and was frequently shown to exhibit significant tailing effects (section 1.5.4).
- *C. botulinum* type E spores are known to be relatively heat susceptible (section 1.3.2)
- Pressure and temperature frequently act synergistically to inactivate spores.

► Despite of possible tailing effects in inactivation kinetics, *C. botulinum* type E spores can be inactivated by HPT treatments at relatively mild temperatures.

- p/T-dependent areas were described where spores are stabilized by pressure resulting in a pronounced tailing effect and even in lower inactivation levels than at ambient pressure at the same temperature (section 1.5.3.1)

► Specific combinations at very high pressures and very high temperatures could exist, where *C. botulinum* type E spores are stabilized resulting in a lower inactivation.

- Two-step high pressure processes present a strategy to enhance inactivation of *Bacillus* spores at very mild process conditions (section 1.5.8).
- Germinant receptors of *Bacillus* and *Clostridium* spores largely differ in their requirements for activation (section 1.2.2.4.1).
- The responsiveness *Clostridium* spores to germinate at low pressures combined with ambient temperatures is very low or absent (section 1.5.3.2).

► Low pressure levels do not induce germination in *C. botulinum* type E spores, which, consequently, cannot be exploited in two-step processes.

- The success of a preservation process is reflected by its effectiveness in decreasing the probability that a single spore grows out.
- Common *C. botulinum* contamination levels in food are presumably low, i.e., growth is likely to initiate from only a few spores (section 1.3.5).
- Even a small inoculum of *C. botulinum* may develop into a hazard (section 1.3.4).
- Spore populations are frequently very heterogeneous, i.e., individual lag or detection times cannot be predicted from population measurements (section 1.5.5).

► Times to detect growth form individual *C. botulinum* type E spores could be heterogeneous, but low pressure levels might not affect such times and, thus, have no impact on food safety considerations.

- The germination machinery in *Clostridium* spores significantly differs from that in *Bacillus* spores (section 1.2.2.4).
- *Bacillus* and *Clostridium* spores are likely to follow different inactivation pathways at high pressure levels combined with high temperatures (section 1.5.3).

► HPT inactivation kinetics of *C. botulinum* type E spores and underlying mechanisms are likely to completely differ from that described for the model organism *B. subtilis*.

- Available data for proteolytic *C. botulinum* types A and B and non-proteolytic type B indicate that there can be large strains-specific differences in the HPT resistance.

► Spores from different *C. botulinum* type E strains are likely to possess largely different HPT resistance properties.

2.2.3 Characterization of Medium/Temperature Effects

Working hypotheses for this last part were partially derived from results obtained in previous experiments of this study.

- The sporulation media used in this study provoking the development of *C. botulinum* type E spores with different HPT resistance properties significantly differ in their cation contents (Fig. 4-17).
- Divalent cations are typically accumulated in high amounts during sporulation (Loshon and Setlow, 1993) in a mineral type- and concentration-dependent manner (Slepecky and Foster, 1959).
- Cations can influence sporulation, germination and spore resistance of spores from various species (Tab. 5-2).
- Calcium, magnesium, and manganese are thought to be the most important cation types in the spore core (section 1.2.3.1).

► Calcium, magnesium, and/or manganese concentrations in the sporulation medium could influence the HPT resistance of *C. botulinum* type E spores.

- Environmental cation concentrations and temperatures during sporulation are likely to be highly variable in the natural habitat of *C. botulinum* type E.
- The efficiency of cation uptake during sporulation was previously suggested to be temperature-dependent in some *Bacillus* spores.

► Effects of sporulation temperature and medium cation contents on the HPT resistance of *C. botulinum* type E spores could be interconnected.

- Coat maturation proceeding after sporulation might be one effect explaining altered HPT resistance of *C. botulinum* type E spores during long-term storage.
- Cations affect the HPT resistance of *C. botulinum* type E spores and the coat contains several cation-dependent enzymes including some that are possibly involved in cross-linking.
- Rapid DPA release occurs concomitant or precedes inactivation of *C. botulinum* type E spores. Although it is not sure (even not very likely) that cortex lytic enzymes play a role in this effect in are *C. botulinum* type E spores, it cannot be completely excluded on the basis of the current state of knowledge. However, the coat is thought to harbor this part of the germination machinery.

- Calcium can increase the expression of spore coat genes, which was speculated to confer protection of *B. subtilis* spores against heat (Oomes et al., 2009).
- Temperature was shown to affect the level of at least one coat protein in *B. subtilis* (Melly et al., 2002).

► The coat of *C. botulinum* type E spores could play a role in their HPT resistance.

2.3 Approach

Against the background of the general aim, it was important to extent the knowledge on factors that can influence the outcome of HPT inactivation studies, which was completely absent for *C. botulinum* type E spores and extremely scarce for any other *Clostridium* spores. Thus, the first step of this study comprised the investigation of possible influence factors that can either directly affect inherent spore resistance properties, i.e., the sporulation medium and temperature, alter their resistance properties during spore suspension handling, or influence their recovery, i.e., the detectability of survivors of HPT treatments. As described in section 1.5.7 and summarized in Fig. 1-23, these tested influence factors are the putative main factors related to sporulation conditions, experimental design, and recovery conditions. During these experiments, the other possible main influence factors related to the matrix and process control (Fig. 1-23) were considered, trying to minimize their possible effects, i.e., by using largely p/T-independent buffer solutions (section 1.4.2.3, also see section 1.5.7.3) and close-to adiabatic isothermal holding times (section 1.4.4). Such precautions should largely facilitate the comparability of results obtained in this study with those from other (future) studies.

After the identification of influence factors on the (apparent) HPT resistance of *C. botulinum* type E spores, detailed inactivation kinetics in broad range of p/T-combinations were recorded. This presents the first comprehensive data set on the inactivation of C. botulinum type E in a broad range of p/T/t conditions. Complementation of this data with DPA release kinetics and the determination of heat and lysozyme susceptible spore fractions after HPT treatments provided some insight that makes it possible to speculate on underlying mechanisms of the HPT inactivation of *C. botulinum* type E and compare possible inactivation pathways with that described for other organisms.

Finally, experiments were conducted to further characterize effects of sporulation medium and temperature on the HPT resistance of *C. botulinum* type E spores observed in the beginning of this study. This included the determination of the role of sporulation medium cation contents in medium-dependent effects on the HPT resistance. One step further, the interconnection of both sporulation temperature and cations present during sporulation was assessed. Although the role of the coat in HPT resistance is not clear, some dependencies described in literature and found here might point towards an involvement of spore coat layers in the HPT resistance of spores. Thus, the effect of decoating treatments on the HPT resistance of *C. botulinum* type E spores was determined. Based on the results of these experiments and having determined that sporulation temperature and medium cation contents might somehow act on overlapping subset of resistance factors in *C. botulinum* type E spores, it seemed greatly interesting to investigate effects of both intrinsic influence factors during sporulation on the expression profile of coat-associated genes.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

Strains use in this study, their alternative names, and (if available) their origin of isolation and gene bank accession number are listed in Tab. 3-1. All strains used throughout this study were *C. botulinum* type E strains, i.e., belonging to the non-proteolytic physiological *C. botulinum* group II (for characteristics see 1.3.2). Notably, although some experiments were also conducted with other strains, data shown in the results section exclusively contains information on the three fish and aquatic environment-associated strains TMW 2.990, TMW 2.992, and TMW 2.994.

Tab. 3-1: Strains

TMW	Alternative name	Origin of isolation	NCBI Accession No. / reference
2.990	Beluga	Fermented whale flippers, botulism outbreak, Canada	NZ_ACSC00000000.1
2.991	S5	n/a	
2.992	1576	From environmental samples from the Scandinavian region	(Johannsen, 1963)
2.993	EG; Strain Gordon	n/a	
2.994	Baumgart	Isolated from salt water fish	(Baumgart, 1972)
2.995	1537/62 Johannsen	n/a	
2.996	1103 Terry Roberts	n/a	
2.997	REB 1718; LGL	n/a	
2.998	E 2622; LGL	n/a	

3.1.2 Instruments, Materials, and Chemicals

Instruments used in this study and their manufacturers are listed in Tab. 3-2. Characteristics of the pressure units used are explicitly summarized in Tab. 3-3. For convenience and to facilitate reproducibility of the results presented in this study, material for pressure treatments and the majority of chemicals and kits purchased during this study together with their supplier are listed in the Appendix (Tab. 7-1 and Tab. 7-2).

Tab. 3-2: Instruments

Instrument	Supplier	Business contact
Anaerobic chamber, Type WA6600	Hereus Instruments GmbH	Hanau, Germany (now ThermoScientific)
Dual vessel HP unit (TMW-RB)	Knam Schneidetechnik GmbH	Langenargen, Germany
ICP-OES Optima 5200 Dual View (DV); cation concentration measurement	Perkin Elmer	Waltham, MA, USA
LightCycler®	Roche Diagnostics Deutschland GmbH	Mannheim, Germany
Micro-scale HP unit Mini Foodlab FBG 5620	Stansted Fluid Power Ltd	London, UK
Phase contras microscope Axiostar plus	Carl Zeiss Microimaging GmbH	Munich, Germany
Refrigerated/heating circulator type FC 600 for high pressure system TMW-RB	JULABO Labortechnik GmbH	Seelbach, Germany
Single vessel HP unit U111	Unipress	Warsaw, Poland
Spectral photometer NovaspecII	Amersham Biosciences	Freiburg, Germany
Sunrise™ 96-well plate reader	Tecan Group Ltd	Männedorf, Switzerland
Themostatted Column Compartment (TCC-100)	Dionex Corporation	Sunnyvale, California, USA
Ultimate 3000 HPLC system (autosampler, pump)	Dionex Corporation	Sunnyvale, California, USA
Ultimate 3000 variable wavelength detector ()	Dionex Corporation	Sunnyvale, California, USA

Characteristics of the pressure units used are also described in detail in literature (TMW-RB: (Lenz and Vogel, 2014); U111: (Reineke et al., 2011); FBG 5620: (Mathys et al., 2009; Reineke et al., 2008)) and summarized in Tab. 3-3:

Tab. 3-3: Characteristics of HP units used in this study

	TMW-RB	U111	FBG 5620
V [mL]	2 × 7	3.7	0.3
$(\Delta p/\Delta t)_{max}$ [MPa/s]	10	25	350
p_{max} [MPa]	800	900	1200
T_{max} [°C]	80	> 120	> 120
PTL	60:40 polyethylene glycol:water	sebacate	sebacate
Temperature control	thermostating vessel jackets	Silicon oil bath	Heating/cooling block with four heating elements

3.1.3 Sporulation and Recovery Media

The pH values of growth media all refer to standard conditions of 25°C at atmospheric pressure and were adjusted using 1 – 5 M NaOH or HCl, respectively. All media were autoclaved at 121°C for 15 min prior to use. After sterilization, growth media were quickly cooled down avoiding agitation and incubated for minimum 24 to 36 h in an anaerobic workstation to allow for temperature and gas atmosphere equilibration. Where not specified explicitly, 15 g/L agar-agar were added for the preparation of solid agar plates. Sugars or sugar mixes were always filter sterilized and aseptically added after autoclaving a medium. The compositions of the most important media for growth, sporulation, and recovery used in this study are listed below. Additional media tested for their use in sporulation steps (data not shown) are listed in the Appendix section of this manuscript (7.1).

A1 Medium [1 L]		(Roberts, 1965)
Trypticase (Casein digest)	50.0 g	
Peptones (Bacto-Peptone)	5.0 g	
Glucose*	4.0 g	
Sodium Thioglycolate	2.0 g	
add distilled water	900 mL	
add sugar solution* (autoclaved separately)	100 mL	
		pH: 7.0 ± 0.2

Anaerobic Egg Yolk Agar (AEY) [1 L]		M12 BAM Media Index**
Proteose Peptone	20 g	
Tryptone	5 g	
Yeast Extract	5 g	
NaCl	5 g	
Agar-agar	20 g	
Glucose**	4 g	
add distilled water	1000 mL	
		pH: 7.0 ± 0.2
at 48 – 50 °C: add 80 mL yolk-saline*, mix, dry 2 – 3 d at room temperature, store at 4 °C		

*Wash 2 fresh eggs with stiff brush and soak in 70% EtOH for 1h. Crack eggs aseptically and retain yolks. Drain yolk sac content into sterile 50 mL screw cap tube. Add yolk to equal volume of sterile 0.85% saline.

**In contrast to the original formulation, 4 g/L glucose were added as energy source.

Biphasic Cooked Meat Medium (BCM) [1L]		(Peck et al., 1992)
Lower solid phase:		
Robertson's cooked meat medium (double)	300 mL	(SGL, London, UK)
Glucose	0.3 g	
Agar-agar	4.5 g	
Upper liquid phase:		
Distilled water, deoxygenated	40 mL	
Macerate Cooked Meat medium prior to use		
		pH: 7.2 ± 0.2

M140 Sporulation Broth [1 L]		(BAM Media Index)*
Polypeptone	15.0 g	
Yeast extract	3.0 g	
Starch, soluble	3.0 g	
$MgSO_4$ (anhydrous)	0.1 g	
Sodium thioglycollate	1.0 g	
Na_2HPO_4	11.0 g	
Glucose*	4.0 g	
		pH: 7.8 ± 0.1

**In contrast to the original formulation, 4 g/L glucose were added as energy source.

RCM (Reinforced Clostridial Medium) broth [1 L]		(Hirsch and Grinsted, 1954)
Meat extract	10.0 g	
Peptones (from casein)	10.0 g	
Yeast extract	3.0 g	
Sodium chloride	5.0 g	
Sodium acetate	3.0 g	
L-Cysteinium chloride	0.5 g	
Agar-agar	0.5 g	
add distilled water	900 mL	
add sugar solution (filter sterilized)	100 mL	
		pH: 6.8 ± 0.2 at 25°C
Sugar solution for RCM		Final concentration [1 L]
Glucose (Glucose x H_2O)	5 g (5.5 g)	0.5% (w/v)
Soluble starch	1 g	0.1% (w/v)
add distilled water	100 mL	

TPY (Trypticase-Peptone-Yeast Extract) broth [1 L]		(Artin et al., 2008)
Trypticase (pancreatic digest of casein)	50 g	
Peptone (soya peptone)	5 g	
Yeast extract	20 g	
Sodium thioglycolate	1 g	
add distilled water	1 L	
		pH: 7.0 ± 0.2

TPYG/TPYC [1 L]		(Artin et al., 2008) /FDA
Trypticase (pancreatic digest of casein)	50 g	
Peptone (soya peptone)	5 g	
Yeast extract	20 g	
Sodium thioglycolate	1 g	
add distilled water	900 mL	
add sugar solution **G** or **C** (filter sterilized)	100 mL	
		pH: 7.0 ± 0.2

TPY**G** sugar solution = glucose solution

Glucose solution		Final concentration [1 L]
Glucose (Glucose x H_2O)	4 g (4.4 g)	0.4% (w/v)
add distilled water	100 mL	

TPY**C** sugar solution = Glucose-Maltose-Cellobiose-Starch (CMCS) sugar mix

Sugar mix		Final concentration [1 L]
Glucose (Glucose x H_2O)	4 g (4.4 g)	0.4% (w/v)
Maltose (Maltose x H_2O)	1 g (1.05 g)	0.1% (w/v)
Cellobiose	1 g	0.1% (w/v)
Soluble starch	1 g	0.1% (w/v)
add distilled water	100 mL	

<u>Further TPYC/TPYG modifications:</u>

TPYG**T** or TPYC**T** = TPYC or TPYG + trypsin

Stir to suspend 1.5 g trypsin (1:250) in 100 mL distilled water. Let particles settle and filter-sterilize supernatant through 0.45 µm membrane. Add 67 mL trypsin solution just before use.

mTPYC = TPYC reducing the tryptone, proteose peptone, and yeast extract amounts to 12.5, 1.25 and 5 g/L (one fourth of the original formulation) containing final concentrations of 18, 7, and 0.2 mg/L Ca^{2+}, Mg^{2+}, and Mn^{2+}.

mTPYC**-** = **m**TPYC with Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations adjusted to 30, 2000, and 0.2 mg/L using $CaCl_2 \times 2\ H_2O$, $MgCl_2$, and $MnSO_4 \times H_2O$.

mTPYC**+** = **m**TPYC with Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations adjusted to 1000, 10, and 1 mg/L using $CaCl_2 \times 2\ H_2O$, $MgCl_2$, and $MnSO_4 \times H_2O$.

SFE (Sediment Fish Extract) medium [1 L]

Sediment extract*	950 mL
Peptone from casein	2.5 g
Soya tryptone	20.0 g
Meat extract	20.0 g
Yeast extract	3.0 g
$CaCO_3$	2.0 g
Sodium thioglycolate	1.0 g
Cysteine x HCl (L-Cysteine x HCl x H_2O)	0.5 g (0.56 g)
Add 50 mL sterile filtered sugar mix (as described for TPYC)	
	pH: 7.0 ± 0.2

*Mix approx. 400 g fresh water sediment with 100 g fish (in this study: whole rainbow trout) and 1 L distilled water. Shake vigorously to mix. Steam mixture for 1 h. Shake again, allow the soil to sediment and filter the supernatant through filter paper (in this study: 589/2 white ribbon filter paper, pore size 4-12 µm; Schleicher & Schuell, Dassel, Germany). Use 950 mL filtrate as base for the medium. Dissolve sugars for sugar solution in 50 mL distilled water, sterile filter and add to the other 950 mL to give a total volume of 1 L SFE medium.

DMM (Defined minimal medium) [1 L] (Whitmer and Johnson, 1988)

$NaH_2PO_4 \times H_2O$	4.7 g
K_2HPO_4	11.14 g
$(NH_4)_2SO_4$	2.64 g
$FeSO4 \times 7\ H_2O$	0.28 mg
$ZnC1_2$	0.135 mg
$CaCl_2 \times 2\ H_2O$	1.47 mg
$MgSO_4 \times 7\ H_2O$	74.0 mg
$MnCl_2$	0.2 mg
Resazurin	2.0 mg
$NaHCO_3$	1.0 g
Methionine, Histidine, Isoleucine, Leucine, Glycine, Tryptophan, Valine, Serine	0.1 g of each aa
Glutamate	0.5 g

Tyrosine	0.05 g
Cysteine hydrochloride	1.0 g
Biotin	0.2 mg
Thiamine	0.4 mg
Pyridoxamine	1.0 mg
Folic acid	0.25 mg
Choline	50 mg
Calcium pantothenate	50 mg
Nicotinamide	1.0 mg
Glucose	10 g
Adenine	0.01 g
Sodium acetate	1.0 g
	pH: 7.2 ± 0.1

MDM (Modified defined minimal medium)
= DMM omitting calcium pentothenate addition and adjusting the medium to final Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations of 10, 10, and 0.1 mg/L using $CaCl_2 \times 2\ H_2O$, $MgCl_2$, and $MnSO_4 \times H_2O$.

3.1.4 Dilution Media

Identical to the growth and sporulation media, the pH values of dilution media all refer to standard conditions of 25 °C at atmospheric pressure and were adjusted using 1 – 5 M NaOH or HCl, respectively. All media were autoclaved at 121°C for 15 min prior to use. After sterilization, growth media were quickly cooled down avoiding agitation and incubated for minimum 24 to 36 h in an anaerobic workstation to allow for temperature and gas atmosphere equilibration.

Peptone Water (PW) [1 L]	
Peptone from Casein	10 g
NaCl	5 g
add distilled water	1000 mL
	pH: 7.0 ± 0.1

TS Plus (TS+) [1 L]	
Tryptone	15.0 g
Sodium chloride	8.5 g
Antifoam B Emulsion	0.1 mL
add distilled water	1000 mL
	pH: 7.0 ± 0.1

S Plus (S+) [1 L]	
Sodium chloride	8.5 g
Antifoam B Emulsion	0.1 mL
add distilled water	1000 mL
	pH: 7.0 ± 0.1

3.1.5 Buffer Solutions

Identical to the growth and sporulation media, the pH values of buffer solutions all refer to standard conditions of 25 °C at atmospheric pressure and were adjusted using 1 – 5 M NaOH or HCl, respectively. All buffer solutions were autoclaved at 121°C for 15 min prior to use. After sterilization, growth media were quickly cooled down avoiding agitation and incubated for minimum 24 to 36 h in an anaerobic workstation to allow for temperature and gas atmosphere equilibration. Where the actual temperature of a buffer differed from the standard temperature, the pH value that had to be adjusted was corrected by a factor calculated from the $\Delta pK_a/°C$ value of the buffering substance.

3.1.5.1 Buffers for High Pressure Treatments

Tris-His Buffer (THB)	(Margosch, 2004)
Tris-HCl	10 mM
Histidine-HCl	20 mM
add distilled water	1000 mL
	pH: 4.0 – 6.0

Phosphate Buffered Saline (PBS)	
NaCl	8 g
KCl	0.2 g
$Na_2HPO_4 \times 2\ H_2O$	1.44 g
KH_2PO_4	0.2 g
add distilled water	1000 mL
	pH: 7.4

Sodium Phosphate Buffer (SPB)	
$Na_2HPO_4 \times 2\ H_2O$	50 mM
NaH_2PO_4	50 mM
Titrate solutions of Na_2HPO_4 with NaH_2PO_4	
	pH: 5.8 – 8.0

Target pH	Na_2HPO_4 / NaH_2PO_4 ratio
5.8	7.9 / 92.1
6.0	12.0 / 88.0
6.2	17.8 / 82.2
6.4	25.5 / 74.5
6.6	35.2 / 64.8
6.8	46.3 / 53.7
7.0	57.7 / 42.3
7.2	68.4 / 31.6
7.4	77.4 / 22.6
7.6	84.5 / 15.5
7.8	89.6 / 10.4
8.0	93.2 / 6.8

Imidazole Buffer(IB)

Imidazole	50 mM
	pH: 6.2 – 7.8

Imidazole Phosphate Buffer (IPB)

SPB	50 mM
IB	50 mM
Adjust pH values of SPB and IB and mix 1:1	
	pH: 6.2 – 7.8

3.1.6 Oligonucleotides and PCR Mixes

3.1.6.1 Standard PCR

Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). For the purpose of storage for a longer period, primers (obtained from Eurofins MWG Operon, Ebersberg, Germany) were resuspended with TE buffer to a final concentration of 100 pmol/µl.

Primers for BoNT genes were used for an initial check of the selected strains. Primer sequences were obtained from the official FDA website.

Tab. 3-4: BoNT primer sequences.

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon length (bp)	T_m (°C)
BoNT_A	GTG ATA CAA CCA GAT GGT AGT TAT AG	AAA AAA CAA GTC CCA ATT ATT AAC TTT	983	60.1 54.3
BoNT_B	GAG ATG TTT GTG AAT ATT ATG ATC CAG	GTT CAT GCA TTA ATA TCA AGG CTG G	492	58.9 59.7
BoNT_E	CCA GGC GGT TGT CAA GAA TTT TAT	TCA AAT AAA TCA GGC TCT GCT CCC	410	59.3 61.0
BoNT_F	GCT TCA TTA AAG AAC GGA AGC AGT GCT	GTG GCG CCT TTG TAC CTT TTC TAG G	1137	63.4 64.6

Tab. 3-5: Master Mix for standard PCR.

Master Mix (x 1):		
H_2O_{DEPC}	21.0 µl	42.6
Buffer + $MgCl_2$ (10xC) (final conc. 1xC)	**2.5** µl	**5.0**
dNTP mix 10 mM each (final conc. 160/200 µM)	**0.4** µl	**1.0**
Primer forward (100 pmol/µl) (final conc. 0.8/0.6 µM)	**0.2** µl	**0.3**
Primer reverse (100 pmol/µl) (final conc. 0.8/0.6 µM)	**0.2** µl	**0.3**
Taq Polymerase (5 U/µl) (final conc. 0.04/0.03 U/µl)	**0.2** µl	**0.3**
Template DNA	**0.5** µl	**0.5**
	= 25 µl	= 50 µl

(BoNT primers as described in the Materials section; Template: approx. 0.05-0.5 µg genomic DNA for a 25 µl reaction mixture)

3.1.6.2 Real-Time qPCR

The tables below contain information on the primers for target and reference genes as well as the compositions of Primer Mixes and Master Mixes used for qPCR experiments. Primer sequences and their conservation in genes from closely related non-proteolytic *C. botulinum* strains are also indicated in the appendix section 7.6.

Tab. 3-6: Primer Mix for qPCR analysis.

Primer Mix for qPCR:	
H_2O_{DEPC}	40 µl
Primer forward	5 µl
Primer reverse	5 µl

Tab. 3-7: Primer sequences qPCR analysis.
Note that the (old) locus tags provided are those found in *C. botulinum* type E strain Alaska.

Gene locus	F R	**Primer sequence** (5' → 3')	Start	bp	T_m [°C]	G+C	**Amp [bp]**	**E**
spoIIIAH	F	TCAATGTAGCCTTATCATCTTCAGCT	351	26	62.41	38.46	130	1.06
CLH_2173	R	GCAGCAAAGTTGAATAAGGGAGG	480	23	62.27	47.83		
cotS	F	GAGGGAATTTGAAATTGAAAGGCAGT	6	26	62.95	38.46	106	1.01
CLH_0457	R	AGCATCGTTCGCCTTTGTTAGT	106	22	63.02	45.45		
coat protein S	F	TTTCACACCATCTATCCAGGG	721	21	59.24	47.62	128	1.05
CLH_0878	R	CTGCTAATGAATGGGTTTACAGAT	848	24	59.23	37.50		
spsE	F	CATTGGGATTTGAAAGTGCAGT	608	22	59.89	40.91	107	1.01
CLH_1466	R	AGAAGCTTTATGATCAGGCCC	714	21	59.95	47.62		
spsC	F	GCAGAAGGTGGAGCAGTTACTT	577	22	62.72	50.00	100	1.05
CLH_1508	R	CCTTTGATTGGCCATGCATAGC	676	22	62.69	50.00		
spoIVA	F	TGTAGGAGTAGTTGGACCGGT	57	21	62.12	52.38	145	1.14
CLH_1156	R	ACTCTTACCTGACCCACTTTGTG	201	23	62.37	47.83		
cotH	F	GGCTCAATTGAATGGAGAACAACC	1620	24	62.78	45.83	100	1.04
CLH_0075	R	CATTCCTTTGCCACCACCCA	1719	20	63.01	55.00		
sleB	F	ACATGCTTGATTTGGAGTAGCTTT	160	24	61.43	37.50	111	0.98
CLH_0291	R	CGTGTTATAAATCCCAGCTTTCCA	270	24	61.53	41.67		
yAbG	F	GCAGTTGGAAGGTGTATTCC	391	20	59.81	50.00	102	1.12
CLH_0458	R	CCCATCATGACCTGTTAGAACT	492	22	59.68	45.45		
sodA	F	TTCGACCTTTGCCCAATCAA	28	20	60.16	45.00	133	1.07
CLH_1802	R	TCCTCTTAGCAACAATCTTATTCCT	160	25	60.02	36.00		
spo0A	F	GCTTGCCCTTATCGTTGTGA	59	20	60.66	50.00	105	1.04
CLH_2160	R	TGGAAAGAGCAATAAGACATGCT	163	23	60.59	39.13		
16S rRNA	F	AATCCGCTATGAGATGGGCC	213	20	61.83	55.00	118	1.00
CLH_3310	R	CCGTGTCTCAGTCCCAATGT	330	20	61.82	55.00		

Tab. 3-8: Master Mix for qPCR analysis.

Master Mix (x 1) for qPCR:	
H_2O_{DEPC}	5.25 µl
Primer mix	0.75 µl
SYBR© Green	7.5 µl

3.2 Methods

3.2.1 Standard Methods

Standard procedures and conditions that were constant in all of the conducted experiments and provided below. Since other procedure and conditions varied depending on the different aims of the specific experiments, they are provided for every main experiment from section 3.2.2 on.

3.2.1.1 Strains Used

If not stated otherwise, experiments were conducted with the three fish and aquatic environment-associated *C. botulinum* type E strains TMW 2.990, TMW 2.992, and TMW 2.994.

3.2.1.2 General Growth, Sporulation, and Recovery Conditions

Generally, all experimental steps involving growth, sporulation, and recovery of *C. botulinum* type E were performed in an anaerobic cabinet with a constant gas atmosphere or 85% N_2, 10% CO_2, and 5% H_2. Additionally, such steps were commonly performed avoiding the exposure of cells/spores to light. This was done due to the fact that *C. botulinum* types A, B, E, F (Eklund et al., 1969) , C (Eklund et al., 1971), and D (Eklund et al., 1972), including non-proteolytic type E strains 8E, beluga, and 066B (Eklund et al., 1969)) can carry prophages, i.e., are lysogenic. In addition to substances interacting with DNA (mitomycin C, acridine orange) (Eklund et al., 1969), ionic detergents (deoxycholate, sodium dodecyl sulfate, (Takumi et al., 1980)), and non-ionic detergents (Tween 80, Brij-58, a low effect, (Takumi et al., 1980)), UV light (Inoue and Iida, 1968) can induced lysis. Notably, detergents also can enhance sporulation, but their presence should be avoided as they lead to deficiency in the outer cell wall layer (Takumi et al., 1980) with other possible (unknown) effects on spore properties.

3.2.1.3 Long Term Storage

For the purpose of long term storage, a potion of 15 mL of a 48 h culture (grown in RCM broth) was centrifuged (7000 x g; 10 min; 4°C), resuspended in 1 mL fresh medium, mixed with 500 µl sterile 80% glycerol and stored in cryotubes at -80°C. In parallel, this procedure was done with spore suspensions to have both cells and spore available in the form of frozen -80 °C stocks. (20% glycerol for spore stocks). In the majority of cases, overnight cultures were inoculated from spores stocks.

3.2.1.4 Preparation of Overnight Cultures

For the preparation of overnight cultures, pure, cryoconserved cultures were slightly defrosted. Routinely, 50 mL of TPYG medium were inoculated with 500 µl of -80 °C cell stock suspension. When necessary, a heat shock at 60 °C for 15 min was applied to heat activate spores. A tube containing 15 mL growth medium only was included as a negative (medium contamination) control and used as reference during measurements of the optical density at a wavelength of 600 nm (OD_{600}). Cultures were incubated overnight (24 ± 4 h) at 28 °C. When cryoconserved spore stocks were used, the incubation period can be prolonged to up to 48 h, and preceded by heat shock of the culture at 60 °C for 20 min, if necessary.

3.2.1.5 Test for Toxin Genes

The preparation of overnight cultures was performed is specified in the standard protocol above. Routinely, DNA was isolated from cells situated in their mid-exponential to early stationary phase as monitored measuring the OD_{600} of the cell suspension. DNA isolation and purification was conducted using the commercially available E.Z.N.A. bacterial DNA isolation kit according to the instructions provided along with it. DNA concentration and purity was roughly estimated optically (260/280 ratio > 1.8) putting 1.5 µl of a sample on a NanoDrop® device (ThermoScientific).

Tab. 3-9: Standard PCR program.

PCR Steps		*Number of cycles*	*Temperature*	*Time*
initial denaturation		1	94°C	180 s[1]
amplification	denaturation	30 - 35	94°C	30 s[2]
	annealing		55°C (*BoNT/E*)	45 s[3]
	elongation		72°C	90 s[4]
final elongation		1	72°C	7 min[5]

(Lid temperature: 94 °C)

Amplification products were visualized in 0.5xTBE or 1xTAE gels with 0.5% agarose that were loaded with 14 µl sample + 3 µl (5x)loading buffer (reference: 10 µl 100 bp ladder), ran at approx. 5 V/cm, and stained with ethidium bromide or alternative substances.

3.2.1.6 Standard Procedure for Spore Suspension Preparation

In this paragraph, the standard procedure used for the preparation of spore suspensions is described. Notably, sporulation media, temperatures, spore suspension handling, and recovery conditions varied depending on the different aims of the specific experiments. These specific conditions are specified from section 3.2.2 on. In the case, nothing is specified for a specific experiment, the protocol can be presumed to comprise sporulation in SFE medium at 20 °C, EtOH purification (the addition of an equal volume of pure ethanol and incubation at room temperature for 2 h (vortexing every 20 min)), spore suspension storage for 7 d at 4°C, and recovery in TPYC.

Overnight cultures were centrifuged (5,000 × g, 10 min, 4 °C). Cell pellets were resuspended in 50 mL of sporulation medium and added to another 450 mL of the same sporulation medium. Spores were grown for minimum 14 d at a constant temperature under anaerobic conditions avoiding the exposure to light. Sporulation efficiency was monitored regularly using phase contrast microscopy. Spore crops were harvested by centrifugation (10,000 × g, 15 min, 4 °C). The supernatant was discarded and spores were resuspended in in 1/10 volume (50 mL) of ice-cold distilled water.

The purification protocol applied in this study basically consisted of a total of six wash cycles after harvesting, i.e., of repeated centrifugation at 10,000 × g for 10 min at 4 °C followed by resuspension in distilled water (or buffer solution). Generally, careful handling ensured that the six wash cycles applied in this study only marginally affected the spore yield. Three of the

wash cycles were employed directly after harvesting, where an equal volume (50 mL) of ice-cold distilled water was used for resuspension after the first and the third centrifugation step. Saline with a surfactant (S+) was used for resuspension after the second centrifugation step. Thereafter, spore suspensions were centrifuged again (10,000 × g, 10 min, 4 °C).
This was followed by a purification step. Thereafter, three additional wash cycles (10,000 × g, 4 °C, 10 min) in distilled water were followed by a last centrifugation step (5,000 × g, 4 °C, 15 min). Spores were then resuspended in IPB (imidazole phosphate buffer, pH 7) to give an initial viable spore count of approx. $10^6 – 10^7$ spores/mL. Thereafter, spore suspensions were stored for a specific time at a specific temperature before HPT treated.

3.2.1.7 Standard Recovery Conditions

Spore inactivation was evaluated via the determination of CFU (colony forming units) in pour plates containing appropriate dilutions (routinely, three dilutions plated in duplicate). If not stated explicitly, the dilution medium used was tryptone saline plus surfactant (TS+), the medium used for pour plating was TPYC agar (15 g/L agar-agar) supplemented with 10 µg/mL hen egg white lysozyme (min. 100,000 u/mg) and plates were incubated anaerobically at 28 °C for 7 d.

3.2.1.8 HPT Treatments

Different HP units (Tab. 3-3) and p/T/t treatment parameters were used for some of the experiments conducted. Which unit was used is specified in the respective section describing the setup of the single experiments in detail. Generally, the sample packaging was always the same in preparing samples for HP treatments in a specific HP unit.
For unit TMW-RB, 200 µl of a spore suspension were filled in a heat shrink tubing (DERAY®, shrink temperature 200°C, d_i = 3 mm). The tubes were heat sealed at both edges avoiding contact of the sealer with the suspensions as well as large air bubbles. Shrink tubes with spore suspensions were placed in 1.8 mL Nunc cryovials containing the same buffer as used for suspending the spores. Again, air bubbles inside the vials were avoided. Samples were stored on ice until treated. Temperature inside the pressure vessels was controlled via thermostating vessel jackets using an external refrigerated/heating circulator (FC 600; JULABO) with the initial temperature set to 2.5 × 10^{-3}°C × target pressure [MPa] below the target temperature. Standard compression and decompression rates for the unit TMW-RB were set to 200 and 800 MPa/min, respectively.

3.2.1.9 Independent Replicates and Statistic Significance

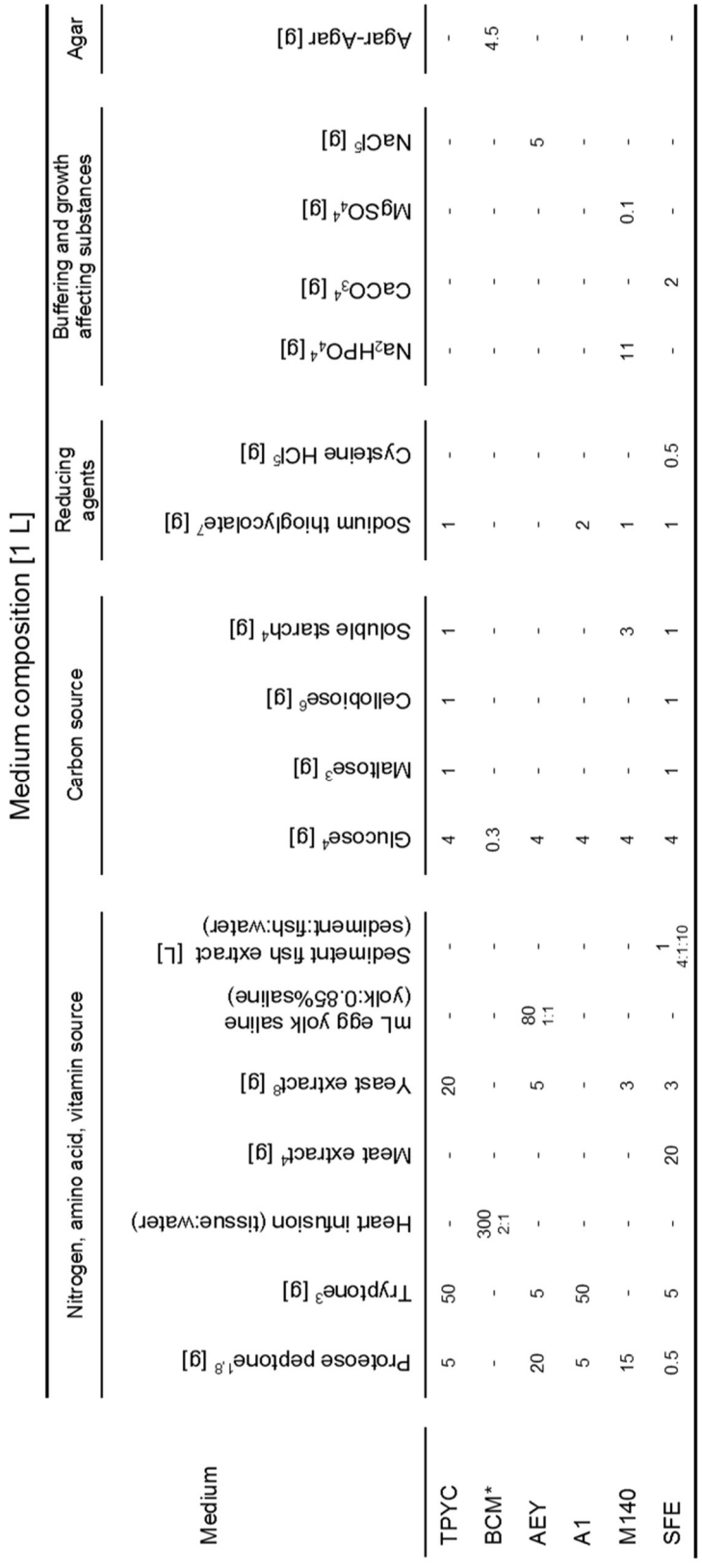
If not stated explicitly to be different, all experiments were conducted independently in triplicate. If not stated otherwise, differences are described as statistically significant at $p \leq 0.05$ determined using a two-sample two-tailed Student’s t-test assuming normal distributed unpaired data with unequal variance.

3.2.2 Influence Factors on HPT Resistance

3.2.2.1 Effect of Sporulation Medium on Spore Resistance

Strains and sporulation conditions

Strains TMW 2.990, TMW 2.992, and TMW 2.994 were used to evaluate the effect of the sporulation medium on the HPT resistance. The sporulation temperature was constantly 28 °C. Spore suspension handling and recovery conditions were consistent with the standard conditions stated above. In contrast to the standard protocol describe above (section 3.2.1), growing cultures were centrifuged (10,000 × g, 4°C, 15 min), the supernatant was discarded and cell pellets were resuspended with an equal volume of different sporulation media (50 mL), which was used as inoculum for another 450 mL of the same sporulation medium. Tested sporulation media included those previously used for the growth/sporulation of *C. botulinum* type E, i.e., TPYC (Artin et al., 2008), BCM (Peck et al., 1992), AEY (Hobbs et al., 1967), A1 (Roberts, 1965). Additionally, M140 (*C. perfringens* sporulation broth, BAM Media Index, + 4 g/L glucose) and SFE (modified from the proteolytic *C. botulinum* sporulation medium WSH, (Margosch et al., 2006)) were used. Sporulation characteristics were examined microscopically. To facilitate the comparison of the different sporulation media and to make it easier to spot differences in their major components, their composition is summarized in Tab. 3-10.



Medium	Nitrogen, amino acid, vitamin source							Carbon source				Reducing agents	Buffering and growth affecting substances					Agar
	Protease peptone[1,8] [g]	Tryptone[3] [g]	Heart infusion (tissue:water)	Meat extract[4] [g]	Yeast extract[8] [g]	mL egg yolk saline (yolk:0.85%saline)	Sediment fish extract [L] (sediment:fish:water)	Glucose[4] [g]	Maltose[3] [g]	Cellobiose[6] [g]	Soluble starch[4] [g]	Sodium thioglycolate[7] [g]	Cysteine HCl[5] [g]	Na_2HPO_4[4] [g]	$CaCO_3$[4] [g]	$MgSO_4$[4] [g]	NaCl[5] [g]	Agar-Agar [g]
TPYC	5	50	-	-	20	-	-	4	1	1	1	1	-	-	-	-	-	-
BCM*	-	-	300 2:1	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	4.5
AEY	20	5	-	-	5	80 1:1	-	4	-	-	-	-	-	-	-	-	5	-
A1	5	50	-	-	-	-	-	4	-	-	-	2	-	-	-	-	-	-
M140	15	-	-	-	3	-	-	4	-	-	3	1	-	11	-	0.1	-	-
SFE	0.5	5	-	20	3	-	1 4:1:10	4	1	1	1	1	0.5	-	2	-	-	-

Tab. 3-10: Compositional comparison of different sporulation media.
Composition of complex sporulation media used. *Lower solid phase; ready to use media or medium components obtained from [1]Oxoid Ltd., Basingstoke, Hampshire, UK, [2]Fluka (Steinheim, Germany), [3]GERBU (Wieblingen, Germany), [4]Merck (Darmstadt, Germany), [5]Roth (Karlsruhe, Germany), [6]Serva (Heidelberg, Germany), [7]AppliChem (Darmstadt, Germany); for M140: polypeptone peptone BBL from [8]BD (Heidelberg, Germany).

Heat and high pressure treatments

Pressure treatments were conducted using the HP unit TMW-RB. For heat treatments, samples were packed in heat shrink tubes identical to those used for HPT treatments. Sealed tubes were put into thin walled glass tubes containing buffer and a thin wire thermocouple to monitor the temperature. Glass tubes were then immersed in a water bath for heat treatments. First, the heat and pressure resistance on the three test strains was examined at 20, 40, 60, and 80 °C combined with ambient pressure (0.1 MPa = heat alone), 200, 400, 600, and 800 MPa for a constant holding time of 10 min. To test the effect of the sporulation medium on the spore resistance to high pressure, heat, and combined HPT treatments, a target pressure of 800 MPa was combined with an elevated temperature (80 °C) for a dwell time of 10 min.

3.2.2.2 Effect of Sporulation Temperature on Spore Resistance

Strains and sporulation conditions

Strains TMW 2.990, TMW 2.992, and TMW 2.994 were used to evaluate the effect of the sporulation temperature on the HPT resistance. The experimental setup was essential identical to the standard protocol described above (section 3.2.1), except following differences: purified spore suspensions were stored for maximum 3 d at 4°C and sporulation temperatures were varied, i.e., 13, 18, 23, 28, 33 and 38°C were used, with 28 °C being designated as standard reference temperature for these experiments. Depending on the temperature spores were formed (> 95 %) within 7 – 14 d.

Heat and high pressure treatments

The HPT unit TMW-RB was used. Target pressure-temperature combinations used were 200 MPa at 40 and 80°C and 800 MPa at 40 and 80°C held for a constant dwell of 10 min. Additionally, experiments with varying holding times (1, 60, 150, 300 and 600 s) were conducted for the maximum pressure / temperature used (800 MPa / 80°C) to investigate if sporulation temperature-mediated effects observed for 10 min occur consistently within shorter pressure holding times.

3.2.2.3 Effect of Spore Suspension Purification and Storage

Strains and sporulation conditions

Strains TMW 2.990, TMW 2.992, and TMW 2.994 were used to evaluate the effect of spore suspension purification and storage on the HPT resistance.

Effects of purification steps

For better clarity, a flowchart illustrating the standard conditions for spore suspension production in this study contains information on the altered steps during these experiments. Consistent with Fig. 3-1, steps of spore suspension preparation, treatment, and handling consisted of (i) growth and sporulation, (ii) harvesting, (iii) purification, (iv) storage, (v) HPT treatment, (vi) dilution, and (vii) recovery of surviving spores. Parallel arrows indicate steps that were modified to investigate their impact on the outcome of HPT *C. botulinum* type E spore inactivation studies. Effects of such variations were evaluated systematically, i.e., only on parameter was changed at a time, while all other handling steps remained unaltered. Lighter (green) colors of parallel arrows in Fig. 3-1 indicate standard procedures, which were held constant when the effect of a specific parameters was investigated.

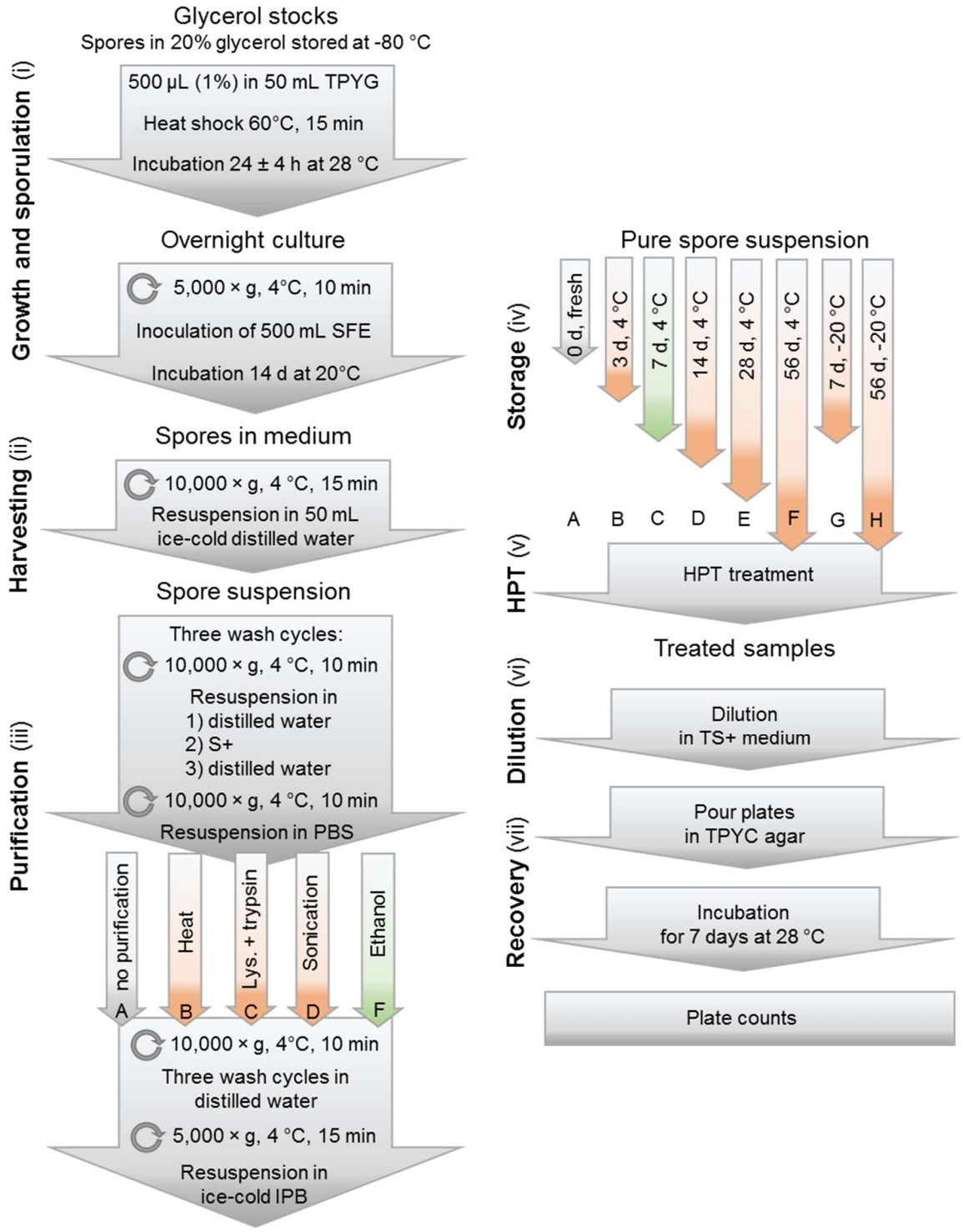


Fig. 3-1: Experimental setup for testing purification and storage effects.

Spore suspension purification alternatively consisted of (A) no purification step, (B) heating in a thin walled glass tube in a water bath at 60 °C for 30 min, (C) the resuspension in PBS containing 200 µg/ml lysozyme[4] (hen egg white (c-type) lysozyme, min. 100,000 u/mg) and 100 µg/ml trypsin[1] (trypsin from bovine pancreas, ≥ 9,000 BAEE units/mg protein) and incubation for 2.5 h at 45 °C, (E) sonication in a thin walled glass tube in an ice-water bath for 5 min, or (F) the addition of an equal volume of pure ethanol and incubation at room temperature for 2 h (vortexing every 20 min) (standard purification step for later experiments).

Purified spore suspensions were (A) immediately HPT treated, stored at 4 °C for (B) 3 d, (C) 7 d (standard storage time in this study), (C) 14 d, (D) 28, and (E) 56 d, or stored at -20 °C for (F) 7 d and (G) 56 d before they were HPT treated.
HPT treatments were conducted using the high pressure unit TMW-RB described before applying a target pressure of 800 MPa at target temperatures of 40 and 70 °C for 10 min.

3.2.3 Pressure/Temperature-Dependent Inactivation

Sporulation conditions

Consistent with the standard protocol, spores were produced at 20 °C in SFE medium. All other steps were also performed according to the standard protocol.

Strains and HPT treatments

Strains TMW 2.990, TMW 2.992, and TMW 2.994 were used. All three HP units with their characteristics specified in Tab. 3-3 were used, i.e., TMW-RB, U111, and FBG 5620. However, the used strain and HP unit depended on the p/T parameter combination and the aim of a specific experiment. An overview of the dependencies is provided in Tab. 3-11.

Tab. 3-11: Experimental setup for inactivation kinetics and mechanistic studies.

	High pressure unit					
	U111		FBG 5620		TMW-RB	
p_{target} | T_{target} combinations applied [MPa | °C]	300 |	30,45,60,75			300 |	30,45,60,75
	450 |	30,45,60,75			450 |	30,45,60,75
	600 |	30,45,60,75			600 |	30,45,60*,75*
	750 |	30,45,60,75			750 |	30,45,60*,75*
	900 |	60,75	900 |	30,45,60,75		
			1200|	45,60,75		
t_{dwell} applied [s]	1, 60, 150, 300, 600					
C. botulinum strain	TMW 2.990 TMW 2.992 TMW 2.994		TMW 2.990		TMW 2.990	
determination of	heat susceptible lysozyme germinable inactivated spores		heat susceptible lysozyme germinable inactivated spores		DPA release inactivated spores*	

3.2.3.1.1 Determination of inactivation kinetics

HPT treatments

Unit U111 (Reineke et al., 2011) was used for target pressure levels of 300 – 750 MPa combined with target temperatures of 30 – 75 °C and treatments at 900 MPa/60 and 75 °C (Tab. 3-11). Cryovails contained four shrink tubes with spore suspensions (2 × strain TMW 2.990, 1 × 2.992, 1 × 2.994). The temperature was monitored using a thermocouple located in the center of a cryovail containing the same buffer solution in which spores were suspended (IPB). Isothermal dwell times were realized immersing the pressure vessel into a temperature controlled silicon oil bath (immersion thermostat CC2-E with SilOil M40.165.10; Huber GmbH, Germany) and adjusting the pressure build-up start temperature individually for every target pressure and temperature used (Reineke et al., 2011). Process parameters

were recorded in 0.5 s intervals. Experiments were conducted in duplicate using independently grown spore crops from all three strains.

Unit FBG 5620 was used for experiments at 900 MPa/30 – 75 °C and at 1200 MPa/45 – 75 °C (Tab. 3-11). Temperature control via a heating/cooling block allowed for realizing almost ideal adiabatic pressure build-up phases and isothermal dwell times at such high pressure levels combined with low target temperatures (Mathys et al., 2009; Reineke et al., 2008). Process parameters were recorded every 0.39 s. Experiments were conducted in duplicate with two independently grown spore crops from strain TMW 2.990.

Dwell time in both units started when a pressure level 50 MPa below the target pressure was exceeded. Due to the fast compression and related to the accuracy of the ramp control this measure increased the precision of pressure-/temperature-profiles achieved and eliminated the possibility of an unwanted prolongation of the dwell time in case the actual target pressure was not exceeded at the end of pressure ramp. Due to its suitable phase behavior within the p/T parameter range tested (Reineke et al., 2008), Bis(2-ethylhexyl) sebacate (Nr. 84822; Sigma-Aldrich, USA) was used as pressure transferring liquid (PTL).

Average pressure build-up and release rates were 25 and 132 MPa/s for unit U111 and 212 and 69 MPa/s for unit FBG 5620. In unit U111, the average pressure levels held during the dwell times were 8 MPa lower than the target pressure. Average sample temperatures reached during the dwell times of treatments with a target temperature of 30, 45, 60, and 75 °C were 30.3 °C, 44.7 °C, 59.3 °C, and 74.0 °C, respectively. In unit FBG 5620, the average pressure levels held during the dwell times were 23 MPa higher than the target pressure. Average temperatures reached during the dwell times of treatments with a target temperature of 30, 45, 60, and 75 °C were 29.1 °C, 44.2 °C, 59.7 °C, and 75.1 °C, respectively. Pressure and temperature profiles recorded during 900 MPa/60 °C treatments in both units are depicted in Fig. 3-2A (U111) and Fig. 3-2B (FBG 5620).

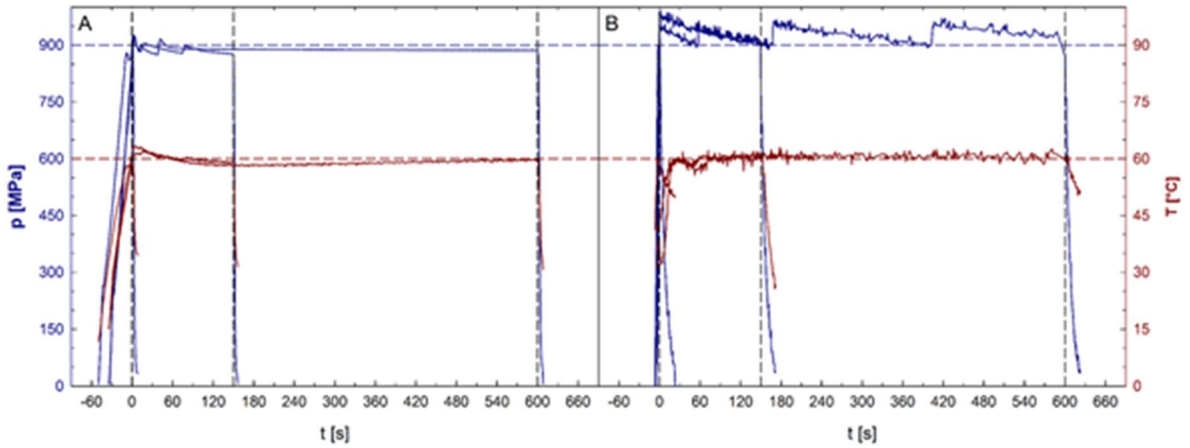


Fig. 3-2: Typical p/T profiles in HP unit U111 and FBG 5620
Pressure (upper plots, left y-axis) and sample temperature (lower plots, right y-axis) profiles in the high pressure units U111 (A) and FBG 5620 (B). Horizontal dashed lines indicate the target pressure of 900 MPa (upper line) and the target temperature of 60 °C (lower line). Time point 0 represents the start of the dwell time. Vertical dashed lines indicate ends of dwell times after 1, 150, and 600 s.

Thermal post-treatments

One of the duplicate TMW 2.990 samples per spore crop was subjected to a second, thermal treatment after the HPT treatments. Shrink tubes were incubated 1 h at room temperature followed by 20 min at 60 °C in a waterbath. Samples were kept on ice after treatments.

Determination of colony forming units

The determination of survivors was done as described for the standard procedure, except of the use of TPYC plates with and without 10 µg/mL hen egg white lysozyme.

3.2.3.1.2 Determination of released dipicolinic acid (DPA) after pressure treatments

HPT treatments

Unit TMW-RB was used for experiments to determine the HPT-mediated DPA release from *C. botulinum* TMW 2.990 spores after pressure treatments at 300 – 750 MPa combined with target temperatures of 30 – 75 °C (Tab. 3-11). Temperature inside the pressure vessels was controlled via thermostating vessel jackets connected to an external refrigerated/heating circulator. The design of this unit did not allow for monitoring the actual sample temperature during HPT treatments. Thus, individual target pressure-/target temperature-dependent starting temperatures were determined empirically recording temperature profiles in the center of a dummy cryovial containing IPB. For the actual experiments, an external thermocouple was used to monitor the temperature in the center of cryovials containing IPB and shrink tubes. Ice-cold cryovials were transferred to the pre-heated pressure vessel filled with PTL. Short before (time point extrapolated from dummy test runs) the determined starting temperature was reached, the external thermocouple was removed, the lid closed and the pressure ramp started. To be able to compare spore inactivation levels after treatments conducted using different high pressure units, spore counts after 750 MPa treatments at 60 and 75 °C were also determined after treatments in unit TMW-RB.

Determination of dipicolinic acid (DPA) release

Similar to previous studies (Margosch et al., 2004a; Reineke et al., 2013b), high-performance liquid chromatography (HPLC) was used to determine the relative amount of DPA released from pressure treated spores. In accordance with the method used by Fichtel et al. (2007b) (except for a post-column complexation), a Dionex Ultimate 3000 HPLC system with a TCC-100 equipped with a Gemini C18 column (column temperature: 20 °C) was used. The column was protected with a Gemini C18 guard column (4 × 3 mm). Sodium bisulphate buffer solution adjusted to pH 1.2 with sulphuric acid filtered through PVDF membrane disc filters and degassed by ultrasonication was used as mobile phase. Isocratic elution was used for standard runs (20% methanol degassed with helium). A gradient program (20 – 40 % methanol) was used for sample runs. Flow rate was set to 2 mL min^{-1}. An Ultimate 3000 variable wavelength detector was used to measure DPA absorbance at 275 nm.

DPA concentrations in the surrounding medium of untreated, pressure treated, and autoclaved *C. botulinum* TMW 2.990 spores were determined. Additionally, the total amount of DPA detectable in autoclaved *C. botulinum* TMW 2.992 and TMW 2.994 spore samples was analyzed. Untreated and high pressure treated *C. botulinum* TMW 2.990 samples were kept at room temperature for 2 h after a treatment and stored at -80 °C prior to HPLC analysis. For analysis, samples were filtered through cellulose acetate syringe filters (pore size 0.2 µm), diluted 1:1 with $NaSO_4/H_2SO_4$ (pH 1.2), adjusted to pH 1.2 with H_2SO_4, and filtered again. To extract all available DPA, spore suspension samples of each batch were placed in 15 mL polypropylene screw cap tubes and autoclaved (208 kPa, 121.1 °C) for 30 min (Pellegrino et al., 2002) followed by 1:1 dilution, H_2SO_4 addition, and filtration. Samples (200 µL) were placed in polypropylene HPLC vials (250 µL). 20 µL sample were injected per run.

The use of glassware was avoided to minimize possible adsorption effects of DPA to surfaces (Fichtel et al., 2007b). 2,6-pyridinedicarboxylic acid (dipicolinic acid) was used as standard. Pressure-induced DPA release was calculated relative [%] to the total DPA content (autoclaved samples) (Margosch et al., 2004a; Reineke et al., 2013b). Experiments were conducted independently in triplicate.

3.2.3.1.3 Calculation of isoeffect curves

For the calculation of isoeffect curves of heat susceptible, lysozyme activated, and inactivated spores after HPT treatments under isothermal-isobaric conditions, mean spore inactivation levels at four kinetic points (60, 150, 300, and 600 s) were considered in comparison with inactivation levels occurring during compression/decompression (1 s treatments), i.e., excluding effects under non-isobaric and non-isothermal conditions. Kinetic modeling was basically done as described before (Reineke et al., 2012; Reineke et al., 2013b). Since no shoulder formation was observed for the inactivation kinetics of *C. botulinum* type E spores, an n^{th} order reaction model (Eqn. 1, (Kessler, 2002)) was used where the number of surviving spores (N) depends on the decrease in survivors with time (dN/dt), the corresponding rate constant (k), and the reaction order (n).

$$\frac{dN}{dt} = -k \cdot N^n$$

(Eq. 3-1)

To derive isoeffect lines for pressure-temperature diagrams, kinetic analysis of the experimental inactivation data was done. The rate constants were regressively obtained (TableCurve 3D, SPSS Inc., USA) by fitting the inactivation results with n^{th}-order kinetics (TableCurve 2D, SPSS Inc., USA). For n^{th}-order decay reactions, Eq 3-1 was integrated:

$$\frac{N}{N_0} = \left[1 + (n-1) \cdot k \cdot t \cdot N_0^{(n-1)}\right]^{\frac{1}{1-n}}$$

(Eq. 3-2)

and logarithmized:

$$\log_{10}\left(\frac{N}{N_0}\right) = \log_{10}\left[1 + (n-1) \cdot k \cdot t \cdot N_0^{(n-1)}\right]^{\frac{1}{1-n}}$$

(Eq. 3-3)

To identify the reaction order, all individual kinetics were fit over a range of reaction orders (n = 1.0 – 1.7). The minimal cumulative standard error (∑ SD) identified the optimal reaction order (TableCurve 2D, SPSS Inc., USA). After the identification of the reaction order (n), the rate constants (k) were obtained regressively (TableCurve 2D, SPSS Inc., USA). To get a functional relationship of the rate constant with pressure and temperature dependence k(p,T), empirical equations have often been suggested (Ardia, 2004; Ardia et al., 2004a; Margosch et al., 2006). Using a Taylor series expansion up to 3rd order terms,

$$\ln(\mathrm{k}) = \mathrm{a} + \mathrm{b} \cdot p + \mathrm{c} \cdot T + \mathrm{d} \cdot p^2 + \mathrm{e} \cdot T^2 + \mathrm{f} \cdot p \cdot T + \mathrm{g} \cdot p^3 + \mathrm{h} \cdot T^3$$

(Eq. 3-4)

the rate constant k(p,T) could be calculated. To solve the functional relationship between pressure and temperature, k was replaced with k(p,T) and the reduction rate (N/N_0) and time (t) were set as constants. The isoeffect lines were calculated with MathCAD 15; Mathsoft Engineering & Education, USA). The calculated parameters for the Taylor series expansion and its confidence intervals are given in Table 1 of the appendix material.

Since the DPA release data were differently structured, an alternative modeling approach was used for the calculation of isoeffect lines for the release of DPA from spores after HPT treatments. In contrast to the inactivation isoeffect lines, a significant DPA amount was already released during pressure build-up, which made it necessary to consider both effects occurring under static conditions (isothermal, isobaric dwell times) as well as non-isobaric and non-isothermal conditions (during pressure build-up) in the model. Hence a Weibullian power law:

$$\log_{10}\left(\frac{N_t}{N_0}\right) = -b \cdot t^n \qquad \textbf{(Eq. 3-5)}$$

was used to calculate the functional relationship of the scale (b) and shape parameter (n) in dependence of pressure and temperature.

By applying the Weibullian power law, a non-linear regression fit was done for each individual DPA release kinetic and the scale and shape parameter were determined. To get a functional relationship of the scale parameter b with pressure and temperature, it was assumed that the shape parameter n is constant with varying pressure and temperature (Van Boekel, 2009). Therefore, the average of all values for the shape parameter was calculated (0.23) and the non-linear regression fit of each inactivation kinetic was repeated with this fixed value for the shape parameter.

To get a functional relationship of the scale parameter b with pressure and temperature the same method as described above for the n^{th} order reaction model was used.

3.2.3.2 Detection Time Variability of HPT treated spores

Strains, spores suspension preparation and high pressure treatments

Strain TMW 2.990 was used exclusively. Other procedures were identical to the standard procedures described above. The HP unit TMW-RB was used. Treatment conditions were 200, 300, 400, and 600 MPa at 20 °C, 600 MPa at 80 °C, and 800 MPa at 60 °C for a constant dwell time of 10 min.

Detection time determination

Determination of the detection time was essentially done as described by Stinger et. al. (Stringer et al., 2011). Treated and untreated samples were serially diluted using S+ and, in the final dilution step, TPYC medium to give approx. 20 viable spores/mL. 50 µL of a diluted spore suspension were placed in one well of a 96-well plate containing 200 µL TPYC. 94 wells per plate were used for treated or untreated samples. Two control wells contained 250 µL TPYC without spores. Growth from spores was monitored over an incubation period of up to 336 h at 25°C measuring the optical density (OD_{600nm}) in 15 min intervals using a 96-well plate reader (Sunrise™) placed in an anaerobic cabinet. Detection time was defined as time span required to reach an OD_{600} of 0.2. Data from two independent experiments were pooled (188 wells per treatment condition in total).

3.2.4 Determination of Medium/Temperature Effects

3.2.4.1 Determination of the Role of Cations in Sporulation Media

Strains and high pressure treatments

Strains and the experimental setup for heat and high pressure treatments were identical to those described above for the determination of the effect of sporulation media on spore resistance (see 3.2.2.1). Treatment conditions tested were 0.1 MPa/80 °C (heat), 800 MPa/20 °C (HHP), and 800 MPa/80 °C (HPT or HHPT in some graphs) for constant holding time of 10 min.

Effect of medium supplementation with calcium, magnesium and manganese

The cation contents of two complex sporulation media previously determined to confer different spore resistance properties (SFE and M140) and TPYC (the standard sporulation medium in many studies) were determined using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) (Perkin Elmer ICP-OES Optima 5200 Dual View (DV); method according to DIN EN ISO 11885 E22). Based on the results from this analysis the effect of supplementation of M140 with cation concentrations typical for SFE on spore resistance was determined. For that purpose, $MgSO_4$ addition to M140 was omitted and $CaCl_2 \times 2\ H_2O$, $MgCl_2$ and $MnSO_4 \times H_2O$ was used to adjust Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations to the respective levels found in SFE. Spores from the three strains used were grown in SFE, M140, and supplemented M140 medium at 28 °C and compared with respect to their heat and HPT resistance.

Sporulation in defined medium and role of cations

In analogy to the experiments with complex media, defined minimal medium (DMM) was used to examine the effect of single cation source deficiency and excessive cation addition (10-, 100- and 200-fold Ca^{2+}, Mg^{2+}, or Mn^{2+} concentrations) on the HPT resistance. In contrast to the procedure described above, growing cells were centrifuged (10,000 × g, 4°C, 15 min), supernatant was discarded and cell pellets were resuspended with an equal volume of defined medium. Defined minimal medium developed previously (Whitmer and Johnson, 1988) for non-proteolytic *C. botulinum* group II was slightly modified (MDM), i.e., omitting calcium pentothenate addition and adjusting the medium to final Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations of 10, 10, and, 0.1 mg/L, respectively (close to original formulation) using the respective salts mentioned above. Spores were grown at 28 °C. HPT process parameters tested for MDM spores were 800 MPa, 80°C, 10 min.

3.2.4.2 Determination of the Sporulation Medium/Temperature Interconnection

Strains and high pressure treatments

The experimental setup was identical to that described for the experiments to examine the effect of sporulation temperature on spore resistance (section 3.2.2.2). However, for these experiments, only strain TMW 2.990 was used. Pressure treatments were conducted at 200 MPa at 40 and 80°C (10 min), at 800 MPa at 40°C (10 min), and at 800 MPa at 80 °C (5 min).

Interconnection between sporulation medium and temperature effects

The standard sporulation medium used for these experiments was TPYC, which typically contained around 70, 30, and 1 mg/L Ca^{2+}, Mg^{2+} and Mn^{2+}. This standard medium was modified (mTPYC) reducing the tryptone, proteose, and yeast extract amounts to 12.5, 1.25

and 5 g/L (one fourth of the original formulation), without changing the amounts of sugars and sodium thioglycolate. The final mTPYC contained 18, 7 and 0.2 mg/L Ca^{2+}, Mg^{2+}, and Mn^{2+}, respectively, as determined by ICP-OES analysis (described above). Furthermore, $CaCl_2 \times 2\ H_2O$, $MgCl_2$, and $MnSO_4 \times H_2O$ were used to adjust Ca^{2+}, Mg^{2+}, and Mn^{2+} concentrations to 30, 2000, and 0.2 mg/L (referred to as mTPYC-) and to 1000, 10, and 1 mg/L (referred to as mTPYC+), respectively. Cation concentrations were selected upon their putative negative and positive effect on the pressure resistance of *C. botulinum* type E spores determined in previous experiments. Spores were produced in mTPYC, mTPYC-, and mTPYC+ at 18, 28, and 38°C and tested for their HPT resistance.

3.2.5 Methods to Assess the Role of the Spore Coat in Resistance

3.2.5.1 Decoated Spores

A protocol that was slightly modified from earlier studies, where spore were decoated (Fitz-James, 1971; Hofstetter et al., 2012; Riesenman and Nicholson, 2000) was used for removing outer layers of *C. botulinum* type E spores. Spores grown and purified according to the standard protocol described above were centrifuged, resuspended in a solution containing 0.5% SDS, 0.1 M dithiothreitol, and 0.1 M NaCl and incubated at 37 °C for 1.5 h vortexing the suspension every 30 min. Survivors of HPT treatments at 800 MPa/40 °C and 800 MPa/80 °C were enumerated. Strain TMW 2.990 was used and treatments were conducted in the HP unit TMW-RB.

3.2.5.2 Expression of Coat-Associated Genes via Real-time qPCR

3.2.5.2.1 Conditions tested

Real-time qPCR experiments were conducted to estimate to which extent the expression of coat-associated genes are affected by alterations in the sporulation temperature and the sporulation medium cation content. The conditions tested were identical to those, where maximum effects on the HPT resistance of *C. botulinum* type E spores was found earlier in this study, i.e., low temperatures (20 °C, mTPYC), beneficial cation supplementation of the sporulation medium (28 °C, mTPYC+), and a combination thereof (20 °C, mTPYC+). Relative expression levels were then compared with those at standard growth conditions (28 °C, TPYC).

3.2.5.2.2 Genetic Information

Experimental design basically relied on the data available at NCBI for sequences of the non-proteolytic *C. botulinum* group II strains *C. botulinum* E1 strain Beluga (six shotgun sequences, NZ_ACSC01000000; strain TMW 2.990 in this study) and that available for *C. botulinum* Alaska E43 (whole genome sequence, NC_010723, CP001078.1). Since the available information on strain Beluga is separated in several (six) contigs and annotation appears to be far more reliable for strain Alaska, genes of interest were commonly first searched in the genome of strain Alaska and then compared to sequences present in strain Beluga. The two mentioned sequences were the first sequences published and for long the only information available.

Additional genetic information on group II *C. botulinum* strains is still scarce, but, fortunately, was started to be extended in the past few years. Genetic information for the non-proteolytic *C. botulinum* type B strain Eklund 17B was published some years ago (two almost identical

sequences, except for 45 single nucleotide polymorphisms (SNPs) and some potential assembly differences), i.e., the complete chromosome sequence FR745875.1 (Stringer et al., 2013) and the complete genome sequence CP001056.1 (Brinkac et al., 2008). Additionally, the whole genome sequence of the non-proteolytic *C. botulinum* type F strain Eklund 202 F was published in 2014 (CP006903.1, (Smith et al., 2015)). For *C. botulinum* type E, two additional complete genome sequences for strains NCTC 8266 (CP010520) and NCTC 8550 (CP010521) and one draft genome sequence (whole-genome shotgun project, three contigs, JXMR00000000.1) for strain NCTC 11219 are available since 2015 (Clauwers et al., 2015), which are very helpful for interpreting the results obtained in this study.

3.2.5.2.3 Selection of Target Genes

Factors considered for the selection of genes of interest were their previous characterization as coat protein in the model organism *B. subtilis* and their respective annotation in *C. botulinum* type E strain Alaska and/or Beluga or high homologies of their encoded protein sequences to proteins previously suggested to be important for coat assembly and/or structure in other *Clostridium* species. Other criteria were the degree of conservation among non-proteolytic *C. botulinum* strains (some strain-specific differences appear to exist between strain Beluga and Alaska).

3.2.5.2.4 Selection of Reference Genes

The two reference genes chosen for the normalization of qPCR results were that coding for 16S rRNA, which has been previously shown to be stable expressed in proteolytic *C. botulinum* even during different growth phases (Kirk et al., 2014) and the sporulation master regulator, Spo0A (section Fig. 1-2), which, naturally shows different expression profiles depending on the growth/sporulation phase, but is likely to show similar expression levels at an identical sporulation phase.

3.2.5.2.5 Primer Design

Sequences of target and reference genes for qPCR were obtained from the NCBI database. The free online tool Primer3web (version 4.0.0, (Untergasser et al., 2012)) was used to find primers with suitable characteristics (length, amplicon size, T_m, repeats, runs, G+C content, etc.) following the generally acknowledged rules for qPCR primer design (e.g., https://www.sigmaaldrich.com/.../qpcr_technical_guide.pdf; (Thornton and Basu, 2011)). Primer pairs suggested by the web tool were manually analyzed for their characteristic. Primer pairs with suitable characteristics and best matching the characteristics of the whole primer set were selected. The two best matching primer pairs (if available three) per gene were then tested using the free web version of Beacon Designer™ (Premier Biosoft, California, USA; http://www.premierbiosoft.com/qpcr/) for their tendency to form self-dimers, and cross dimers (between forward and reverse primers), or hairpin structures (especially critical/avoidable when involving a 3′ end). Primer pairs with the highest −ΔG (Gibbs free energy; minimum requirement: −3 kcal/mol or higher) were selected for further analysis. The tendency of the amplicon to form secondary structures and the amplicon T_m (needs to be lower than desired qPCR annealing temperature) were checked using the online tool UNAFold (Integrated DNA Technologies, Inc., Iowa, USA; http://eu.idtdna.com/UNAFold). Since the unspecific fluorescent dye SYBR® green was used, primer specificity was checked using nucleotide BLAST (BLASTN 2.5.0+, NCBI, (Zhang et al., 2000)), i.e., to compare primer

sequences with the genomes of the sequenced *C. botulinum* type E strains (section 3.2.5.2.2).

3.2.5.2.6 Growth and Harvesting Conditions

An overnight culture of *C. botulinum* strain TMW 2.990 was spread plated on a TPYG plate. One colony was picked and inoculated into 25 mL of TPYG medium and again grown overnight. Two separate flasks containing 100 mL mTPYC+ medium were inoculated each with 5 mL of the overnight culture. Additionally, each of another two flasks with 100 mL mTPYC medium were inoculated with 5 mL of the overnight culture. One TPYC and one mTPYC+ flask were incubated at 28 °C. The remaining two flasks were incubated at 20 °C. The time point for harvesting was dependent on the environmental condition examined and normalized against the growth phase. For this purpose, detailed growth curves (OD_{600}) under the different conditions were recorded to estimate their approximate courses. In parallel, sample were examined microscopically. The time required from the transition point from exponential to stationary growth until the first phase-bright spore was detected was determined for every growth condition. The sampling time point for every conditions was defined as 10% of this time span (e.g., 1.5 h after the transition point from exponential to stationary growth for 28 °C, mTPYC and 2.5 h after this transition point for 20 °C, mTPYC). This was done to facilitate the comparison of expression levels and to take samples at a time point, where expression levels of the sporulation master regulator Spo0A and the alternative sigma factor σ^E, which putatively controls the expression of most of the selected target genes, were still high (both are thought to peak around the transition point in proteolytic *C. botulinum*, (Kirk et al., 2014)). On the other hand, it can be assumed that there was enough time for σ^E to initiate the transcription of genes belonging to its regulon. The only exception was made for *sps* genes (putatively σ^K-controlled), where the sampling time point was set to 80% of the time span described above. Once the sampling time point was reached, 1 mL of the respective culture was transferred into an 1.5 mL reagent tube containing an 0.2 mL of an ice-cold mix of 90% ethanol and 10% phenol. After incubation on ice for 20 min, samples were harvested by centrifugation (10,000 × g, 10 min, 4 °C). The supernatant was discarded and the pellets were frozen with liquid nitrogen and stored at -80 °C until they were used for RNA isolation.

3.2.5.2.7 RNA isolation, Purification, and Transcription into cDNA

Frozen cell pellets were resuspended in 0.1 mL of a mixture of RNase free TE Buffer containing 500 µg/mL lysozyme (hen egg white (c-type) lysozyme, min. 100,000 u/mg; Serva) and 1000 U/mL mutanolysin (Sigma Aldrich) and incubated at 37 °C for 30 min. Thereafter, RNA Pure© solution was added (1.4 mL, Qiagen, Hilden, Germany), samples were vortexed, and centrifuged (12000 x g; 10 min; 4°C). The supernatant was divided into two separate tubes (treated in parallel). To extract the RNA, chloroform was added (140 µl per tube), samples vortexed, placed on ice (5 min), and centrifuged (12000 x g; 15 min; 4°C). The upper, clear phase was transferred to a new tube recombining contents of the two parallel treated tubes. An equal volume (approx. 750 µl) ice cold isopropanol was added, sample shaken, placed on ice (15 min), and centrifuged (12000 x g; 15 min; 4°C). Supernatant was discarded, pellets washed (750 µl ethanol (70%)), and centrifuged (12000 x g; 10 min; 4°C). This ethanol wash step was repeated one time. Pellets were air dried (5 to 10 min), resuspended in DEPC water (60 µl), and incubated in a water bath (55 °C, 10 min). Samples were divided into four

aliquots (20 µl for further purification and qPCR, 2 µl for Nanodrop measurement (A_{260}/A_{280} ratio), 4 µl to run on a gel, 34 µl stock), frozen with liquid nitrogen, and stored at -80 °C.
As rough estimate for the RNA integrity, RNA samples were checked on an agarose gel (1.2% in 0.5 x TBE Buffer), i.e., checking the detectability of 16S and 23S bands. All materials needed for gel electrophoresis (chamber, inlet, etc.) were cleaned before the integrity check with SDS (0.5 - 1.0%) for minimum 30 min, SDS rinsed off and rinsed twice with H_2O_{DEPC}. 4 µl RNA sample were mixed with 2 µl loading buffer and run against a III standard (Fermentas, St. Leon-Rot, Germany).
The sample (20 µl) in thermocycler caps was treated with DNase (5 µl) in RNase-free DNase Buffer (4 µl). After 1 h at 37°C, DNase stop solution (4 µl) was added followed by DNase inactivation at 65°C for 10 min. RNA samples were divided into two 10 µ aliquots (one for analysis, one control without reverse transcriptase). Both aliquots were mixed with 2 µl random primers, incubated at 70°C (5 min) and placed on ice (5 min). 2 µl dNTP, 4 µl RT Buffer, 0.5 µl RT (not for control), and 1.5 µl DEPC water were added, followed by three incubation steps (25 °C/5 min, 42 °C/1 h, 70 °C/15 min). 1 µl of every rewritten cDNA sample was used for standard PCR with a housekeeping gene, to check the absence of DNA in the negative control.

3.2.5.2.8 Real Time qPCR

Relatively quantification of gene expression was performed using a Lightcycler© (Roche, Mannheim, Germany) and SYBR© Green. 13.5 µl of the master mix and 1.5 µl cDNA sample (16S rRNA samples were routinely diluted 1,000-fold) were placed in Lightcycler© capillaries, mixed by pipetting, spun down (10s centrifugation), and place into the qPCR machine. One negative (H_2O_{DEPC} instead of cDNA template) and two positive controls (DNA (1:100)) were measured together with the different cDNA samples (one positive and one negative without RT per gene and sporulation condition).

qPCR Steps		*Number of cycles*	*Temperature*	*Time*
initial denaturation		1	95 °C	600 s
amplification	denaturation	30 - 45	95 °C	15 s
	annealing		57 °C	10 s
	elongation		72°C	60 s
melting curve			gradient 60 – 95 °C	0.1 °C/s
cooling			40 °C	

All melting curves were checked manually for their correct appearance.

3.2.5.2.9 Determination of Efficiencies and Calculation of Relative Gene Expression

The efficiency (E) of the amplification reaction for each gene was determined analyzing dilution of standard DNA (1:10 – 1:100,000) in duplicate. The slope of the line was calculated automatically from plots of crossing points against DNA concentrations and checked manually.

$$E = 10^{-1/slope}$$

(Eq. 3-6)

The genes were quantified relatively by comparing the crossing points of a target gene with those of the reference genes using following equation for the relative gene expression ratio (Pfaffl, 2001).

$$\text{Relative Gene Expression Ratio} = \frac{(E_{target})^{\Delta CP_{target\ gene}(control-\ sample)}}{(E_{ref})^{\Delta CP_{reference\ gene}(control-\ sample)}}$$

(Eq. 3-7)

4 RESULTS

4.1 Influence Factors on HPT Inactivation

4.1.1 Sporulation Medium, Sporulation Characteristics, and Resistance

4.1.1.1 Strain-Dependent Differences in Sporulation, Heat, and Pressure Resistance

Sporulation onset was similar in all media tested and the first spores were visible after approx. two days of incubation. However, the time required to reach a high percentage of sporulated cells, generally over 98% free, phase bright spores, varied considerably with the sporulation medium used. Fastest sporulation was observed in BCM (168 ± 11 h), whereas sporulation lasted longest in SFE (261 ± 16 h). Sporulation time span in M140 ranged in between (204 ± 10 h).

Spores of the three *C. botulinum* type E strains tested in our study showed similar sporulation characteristics but different HPT resistance. Whereas the resistance to heat stress (80 °C, 10 min at ambient pressure) differed slightly, differences in the HPT resistance tended to increase as treatment pressure and temperature increased and was generally in the order TMW 2.990 (Beluga) > 2.992 > 2.994. The application of 200 MPa combined with 80 °C did not result in significantly higher inactivation levels as compared to 80 °C heat treatment at ambient pressure. Generally, temperatures over 60 °C and pressure levels over 600 MPa result in inactivation of a large portion of the spore population. The harshest conditions applied, i.e. 80 °C combined with 800 MPa led to a dramatic reduction in viable spore counts (over 6 log in all three strains) but no complete inactivation (Fig. 4-1).

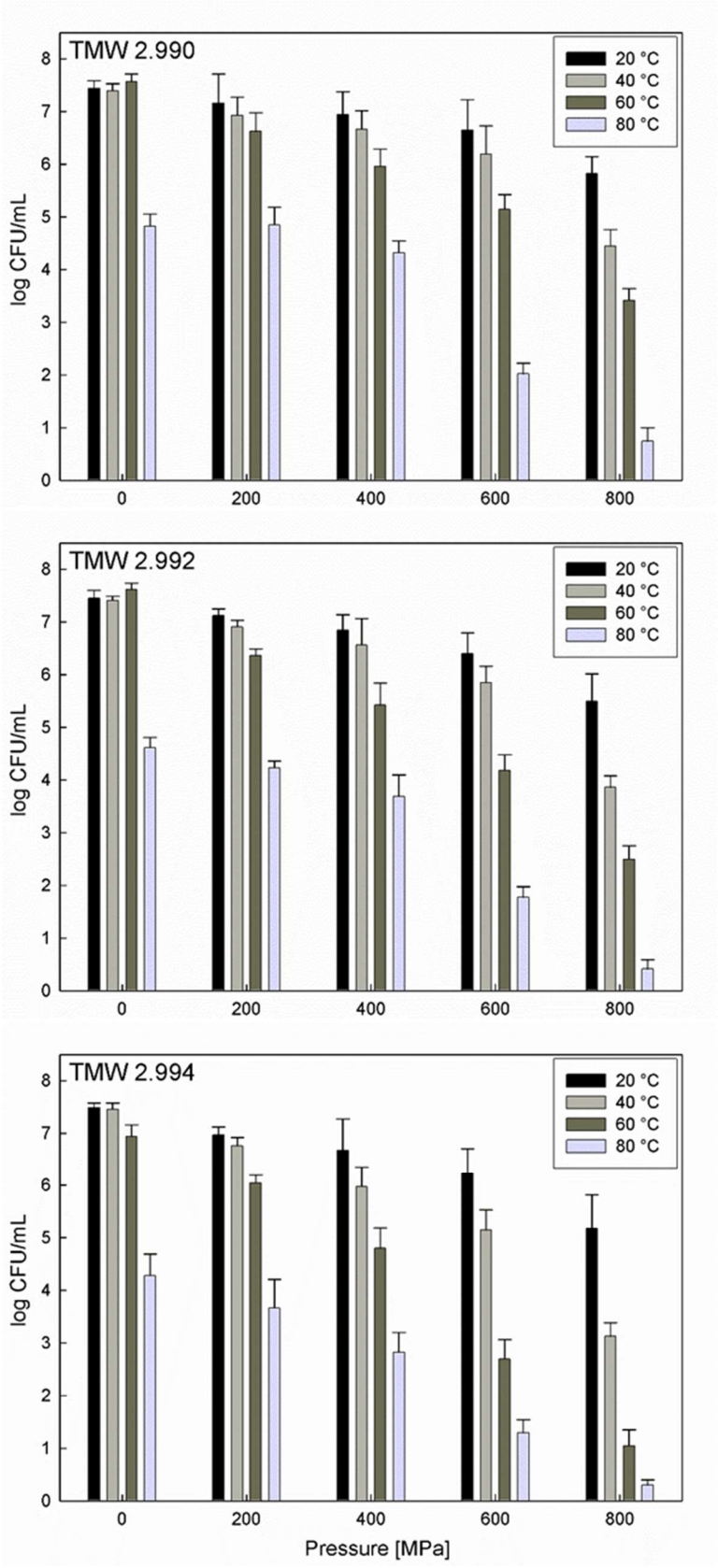


Fig. 4-1: Comparison between heat and HPT inactivation.
Counts of spores surviving heat treatments and HPT treatments with a constant dwell time of 10 min. Initial spore counts were adjusted to 7.4 log CFU/mL. Different bars indicate different treatment temperatures (20 – 80 °C). Different bar groups indicate different pressure levels (200 – 800 MPa). Data set for 0 MPa refers to heat treatments at ambient pressure (0.1 MPa). Error bars indicate standard deviation of three independent experiments.

4.1.1.2 Effect of Sporulation Media on Spore Resistance

Sporulation characteristics as observed by phase contrast microscopy were greatly dependent on the sporulation medium. The differences were only minor strain-dependent indicating that the three strains have similar requirements for an effective sporulation. Sporulation occurred most readily in TPYC, A1 and SFE. However, the other sporulation media also yielded 96 – 99% phase bright spores within 12 days of incubation.

HPT treatment conditions of 800 MPa at 80 °C for 10 min resulted in the inactivation of about 5.5 log10 (strain TMW 2.990 grown in SFE) to complete inactivation (strain TMW 2.994 grown in AEY or M140) of initially 7.4 log10 viable spores per mL depending on strain and sporulation medium used. According to the comparable sporulation characteristics of the three strains in different media the pattern of resistance conferred by the different sporulation media was similar for the strains tested (Fig. 4-2; Tab. 4-1).

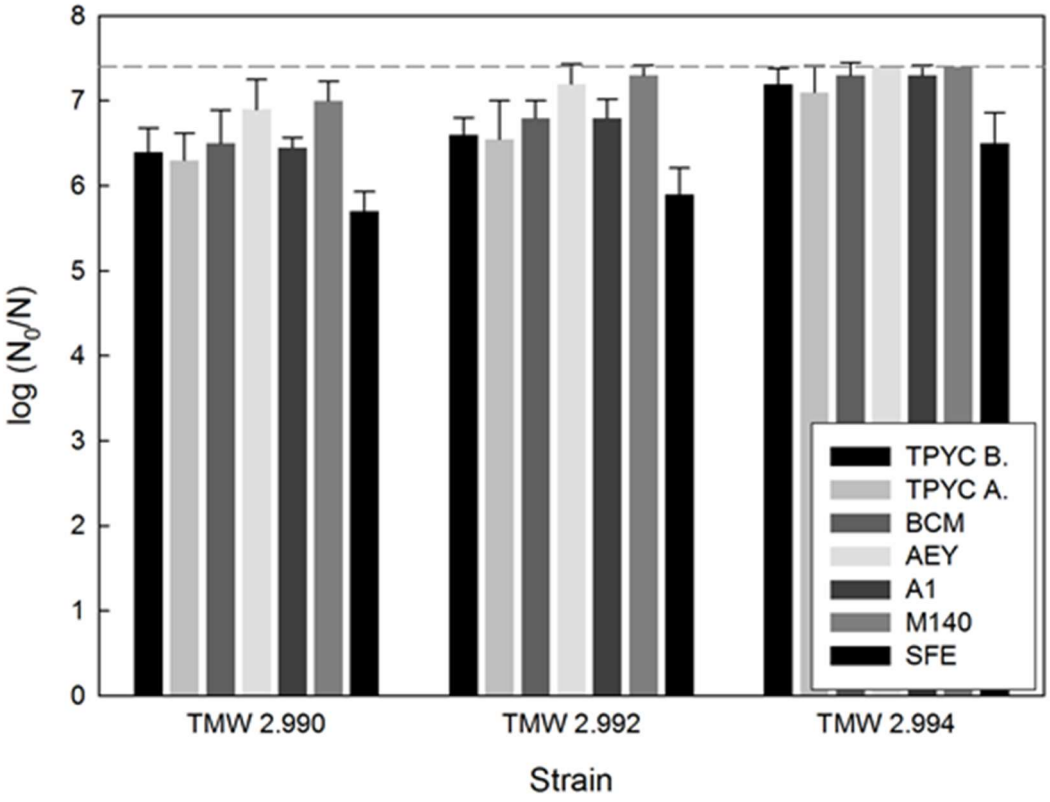
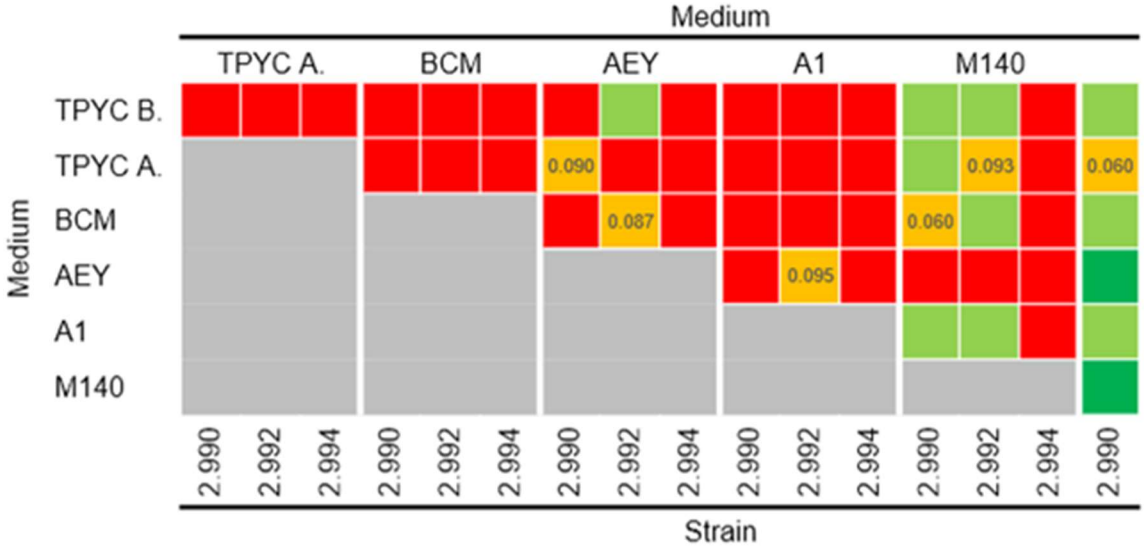


Fig. 4-2: Effect of sporulation medium on HPT resistance.
Log inactivation of viable spores/mL grown in different sporulation media and subjected to a HPT treatment of 800 MPa at 80°C for 10 min. In the case of TPYC, spores were grown in liquid broth (B.) and on agar plates (A.). Initial spore count (7.4 log) is indicated by the gray dashed line.

Tab. 4-1: Significance of medium-dependent differences.
Significance of sporulation medium related differences in the HPT resistance of the strains tested. Red: p > 0.10, no significance, orange: p < 0.10 marginal, light green: p < 0.05 fair, dark green: p < 0.01 good significance of medium related difference in resistance.

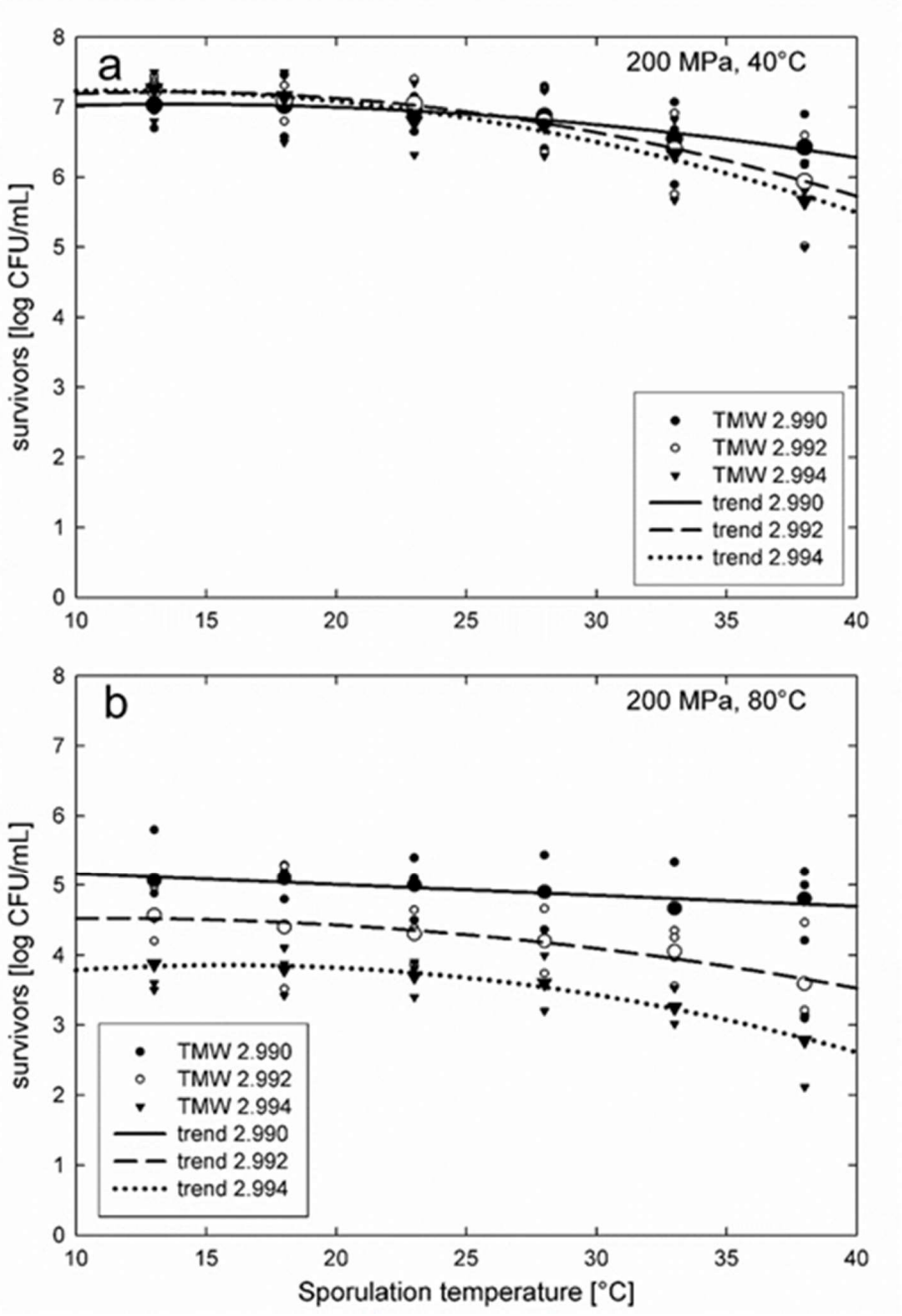


4.1.2 Sporulation Temperature and Spore Resistance

Spores from the three strains TMW 2.990, 2.992 and 2.994 were produced at 13, 18, 23, 28, 33 and 38°C in TPYC and tested for their resistance to HHP/HPT treatments (200 and 800 MPa at 40 and 80 °C, constant dwell of 10 min).

Resistance to low pressure levels at moderate temperatures

Treatments at 200 MPa and 40 °C resulted in low spore inactivation, regardless of the strain used, i.e., means of 0.6, 0.7, and 0.8 log reduction of viable spore counts for strains TMW 2.990, 2.992, and 2.994 sporulated at 28°C (Fig. 4-3a). A decrease in the sporulation temperature from 28 to 13°C did not lead to significant differences in pressure resistance with only a slight trend towards increased resistance for the strains TMW 2.992 and 2.994 (approx. 0.2, 0.5, and 0.5 log lower inactivation for TMW 2.990, 2.992, and 2.994 spores, respectively). When the sporulation temperature was increased from 28 to 38°C there was a clear trend towards decreased HHP resistance (approx. 0.4, 0.9, and 1.1 log higher inactivation for TMW 2.990, 2.992, and 2.994 spores, respectively). Although no significant differences in the HHP resistance to 200 MPa / 40°C treatments were observed between the different strains used, the resistance of TMW 2.992 and 2.994 spores tended to be more affected by shifts in the sporulation temperature compared to TMW 2.990 spores (Fig. 4-3a).


a
200 MPa, 40°C
survivors [log CFU/mL]
TMW 2.990
TMW 2.992
TMW 2.994
trend 2.990
trend 2.992
trend 2.994
b
200 MPa, 80°C
survivors [log CFU/mL]
Sporulation temperature [°C]

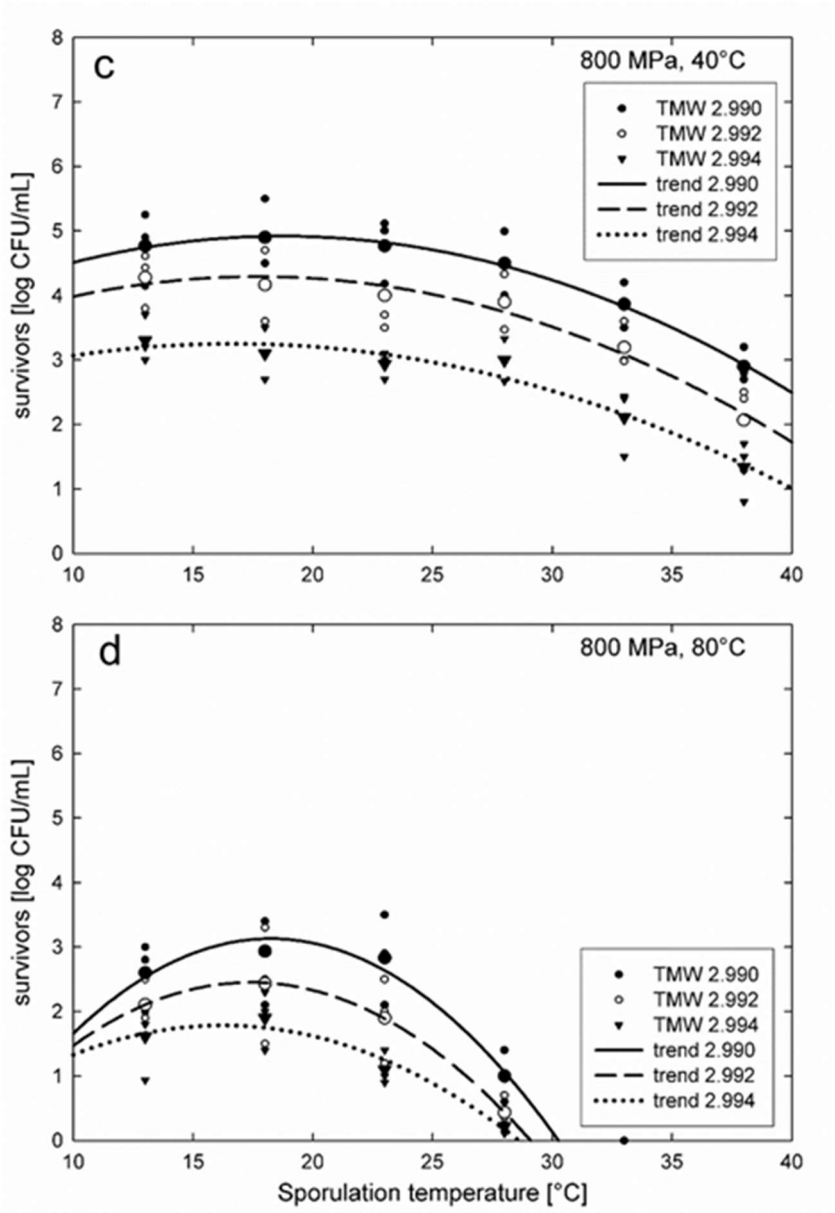


Fig. 4-3: Effects of sporulation temperature on spore resistance.
Viable spores (CFU/mL) of the *C. botulinum* type E strains TMW 2.990 (solid circles), TMW 2.992 (open circles), and TMW 2.994 (solid triangles) surviving 10 min pressure treatments at (a) 200 MPa and 40°C, (b) 200 MPa and 80°C, (c) 800 MPa and 40°C, (d) 800 MPa and 80°C for a constant dwell time of 10 min. Large symbols indicate mean values, small symbols indicate single data from three independent experiments. Sporulation temperature-dependent trends in the pressure resistance are indicated as lines (2nd order polynomial fits of mean values) for the strains TMW 2.990 (solid line), TMW 2.992 (dashed line), and TMW 2.994 (dotted line). Initial spore count: 7.5 log CFU/mL.

Resistance to low pressure levels at elevated temperatures

In contrast to treatments at a moderate temperature of 40°C, treatments at 200 MPa / 80°C led to significant spore inactivation, i.e., means of 2.6, 3.3, and 3.9 log reduction of viable spore counts for strains TMW 2.990, 2.992, and 2.994 sporulated at 28°C, which also differed considerably among the strains used (Fig. 4-3b). Inactivation levels found for TMW 2.990 spores were consistently significantly lower than that found for TMW 2.994 spores. Mean inactivation levels for spores from strain TMW 2.992 were always between those found for the other two strains, but differences between these strains were not significant. Similar to

the results at 40°C, the HHP resistance of the two more sensitive strains tended to be more dependent on the sporulation temperature. Whereas sporulation at lower temperatures (13 vs. 28°C) led to almost identical inactivation levels of spores from the three strains (approx. 0.2, 0.4, and 0.3 log lower inactivation for TMW 2.990, 2.992, and 2.994 spores, respectively), an increase in the sporulation temperature (38 vs. 28°C) resulted in the production of more pressure susceptible spores by the strains TMW 2.992 and 2.994 (approx. 0.1, 0.6, and 0.8 log higher inactivation for TMW 2.990, 2.992, and 2.994 spores, respectively) (Fig. 4-3b).

Resistance to high pressure levels at moderate temperatures

Inactivation levels of spores produced at 28°C caused by 800 MPa / 40°C treatments were slightly higher than that found after treatments at 200 MPa / 80°C, i.e. 3.0, 3.6, and 4.5 log reduction of viable spore counts for strains TMW 2.990, 2.992, and 2.994 (Fig. 4-3c). Similar to 200 MPa / 80°C, 800 MPa / 40°C treatments caused significantly different inactivation of TMW 2.990 compared to TMW 2.994 spores, no matter which sporulation temperature was used. Similar to the results for a lower pressure, the resistance to 800 MPa / 40°C treatments was not significantly altered by sporulation temperatures below 28°C. However, resistance to such treatment conditions tended to peak at a sporulation temperature of 18°C for TMW 2.990 spores (0.4 log less inactivation vs. 28°C spores) and 13°C for TMW 2.992 and 2.994 (0.4 and 0.3 log less inactivation vs. 28°C spores). In contrast to the treatments at 200 MPa / the HHP resistance to 800 MPa was highly dependent on the sporulation temperature when temperatures above 28°C were used, which was found consistently for spores from all three strains (approx. 1.6, 1.8, and 1.7 log higher inactivation for TMW 2.990, 2.992 and 2.994 spores, respectively) (Fig. 4-3c).

Resistance to high pressure levels at elevated temperatures

A combination of 800 MPa and 80°C resulted in the highest spore inactivation levels, i.e. 6.5, 7.0, and 7.3 log reduction of viable spore counts for strains TMW 2.990, 2.992, and 2.994 grown at 28°C, which were highly dependent on the temperatures the spores were produced at (Fig. 4-3d). Strain-dependent differences in the inactivation levels provoked by 800 MPa / 80°C treatments showed the same trend (strain order) compared to those in result of treatments at other intensities, but were not significant. The resistance to 800 MPa / 80°C treatments markedly increased, when spores were grown at lower temperatures, while sporulation temperatures above 28°C resulted in the production of highly susceptible spores, which were completely inactivated by such treatments. HHP resistance tended to peak at 18 °C for all strains (approx. 1.9, 2, and 1.7 log lower inactivation for TMW 2.990, 2.992, and 2.994 spores, respectively), i.e., a further decrease of the sporulation temperature to 13°C led to the production of spores, which tended to possess slightly lower HHP resistance (Fig. 4-3d).

Sporulation temperature dependent inactivation kinetics

Furthermore, the dependence of sporulation temperature-mediated alterations in the pressure resistance on the holding time was investigated using the treatments parameters, where the largest differences were observed in the previous experiment, i.e., at 800 MPa and 80°C. Inactivation kinetics of spores from the different strains TMW 2.990 (Fig. 4-4a), 2.992 (Fig. 4-4b) and 2.994 (Fig. 4-4c) indicate that the trends in sporulation temperature-mediated effects on the HPT resistance observed for 10 min treatments can be found consistently within holding times between 1 and 10 min. However, differences in the resistance between

spores grown at the standard temperature for these experiments, i.e., 28°C, and temperatures below and above were not always significant at shorter holding times. Generally, differences increased when pressure dwells were extended, especially to or above 5 min. Additionally, the observed inactivation kinetics for all three strains suggest that a decrease in the sporulation temperature tended to (slightly) increase tailing of survivor curves, whereas inactivation of spores produced at elevated temperatures tended to follow log linear inactivation curves.

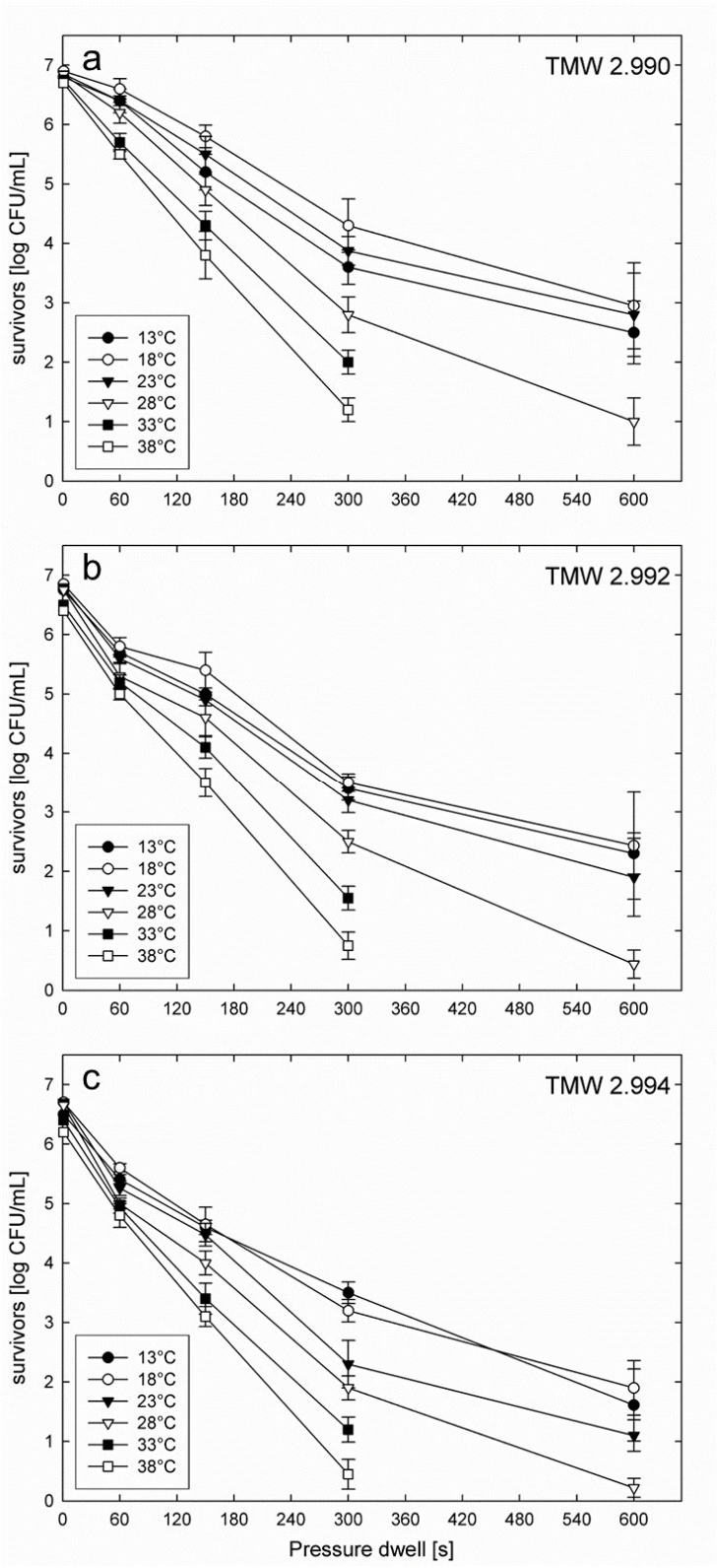


Fig. 4-4: Sporulation temperature-dependent HPT inactivation kinetics.
Viable spores (CFU/mL) of the *C. botulinum* type E strains (a) TMW 2.990, (b) TMW 2.992, and (c) TMW 2.994 surviving HPT treatments at 800 MPa and 80°C for 1 – 600 s. The HPT resistance of spores grown at 13 (solid circles), 18 (open circles), 23 (solid triangles), 28 (open triangles), 33 (solid squares), and 38°C (open squares) is compared. Initial spore count: 7 log CFU/mL. Values at pressure dwell 0 indicate the effect of a holding time of 1 s, i.e., pressure build-up and release only. 10 min values for 33 and 38°C spores leading to complete inactivation are not depicted. Error bars indicate the standard deviation from three independent experiments.

4.1.3 Spore Suspension Purification/Storage and HPT Resistance

4.1.3.1 Purification

The careful adjustment of the initial spore concentration resulted in similar plate count results without an additional purification step for all three strains tested, i.e., mean values of 20.0 ± 0.6 × 10^6, 19.7 ± 1.0 × 10^6, and 20.3 ± 1.8 × 10^6 CFU/mL for the strains TMW 2.990, TMW 2.992, and TMW 2.994, respectively.

To be able to describe the effects of the different purification treatments on pour plate counts independent from variations in counts without an additional purification step among the three independent replicate experiments, the differences between CFU/mL without purification and CFU/mL after a specific purification step were calculated individually for every spore suspension. Results for the three *C. botulinum* type E strains TMW 2.990 (left), TMW 2.992 (middle), and TMW 2.994 (right graph) strains are indicated using gray boxes in Fig. 4-5. In addition to the mean (white dashed lines in boxes) and median (black lines in boxes) values, this figure also includes data (mean values) derived from microscopic analyses (gray lines). The upper gray line in each of the three adjacent graphs indicates the (mean) difference between the number of vegetative cells and spores in a spore suspension visible microscopically and the number actually forming colonies in a pour plate, i.e., ⟨∑ [(spores and cells/mL determined microscopically) - (CFU/mL without purification determined via plate counts)]⟩ / (number of spore suspension preparations). Thus, this line indicates the theoretical plate count result if all vegetative cells and spores observed under the microscope would have grown and formed a single colony. The middle gray line indicates the (mean) difference between spores present in a spore suspension and colonies formed in a pour plate, i.e., ⟨(∑ [(spores/mL determined microscopically) - (CFU/mL without purification determined via plate counts)]⟩ / (number of spore suspension preparations). Thus, this line indicated the theoretical plate count result if all microscopically visible spores but no vegetative cells would have survived a purification treatment, and all spores present would have formed a single colony. The lower gray line indicates the microscopically determined negative number of vegetative cells in a spore suspension, i.e., ∑ [(cells/mL determined microscopically) × (-1)]) / (number of spore suspension preparations). Thus this line indicates the theoretical plate count result if all microscopically visible vegetative cells would have been inactivated by a specific purification treatment and all viable spores forming colonies before the purification step still form colonies in plates after a treatment.

Maximum differences in cell counts provoked by a specific purification treatment were strain-dependent, which is likely to reflect strain-specific differences in the sporulation efficiency, i.e., the spore/vegetative cell ratio present in unpurified spore suspensions as indicated by the difference between the upper and the middle gray lines. However, very similar patterns observable in Fig. 4-5A-C indicate that the purification treatments have similar effects regardless of the strain used.

Values obtained using plate counts were constantly lower than those obtained counting vegetative cells and spores under the microscope. Such differences varied slightly depending on the spore preparation and strain, but were generally very similar, i.e., between 2.4 and 4.3 % for strain TMW 2.990, between 2.4 and 4.4 % for strain TMW 2.992, and between 2.3 and 6.1 % for strain TMW 2.994. Sonication treatments tended to slightly increase cell counts in comparison to unpurified suspensions, but detected differences were not significant. In

contrast, lysozyme + trypsin and ethanol treatments significantly reduced the number of CFU near to the theoretical level of an inactivation of microscopically visible vegetative cells without affecting the viability of spores forming colonies before the purification steps. This is indicated by similar levels of the lower gray line and the mean values (white dashed lines inside of the boxes) reached after lysozyme + trypsin and ethanol treatments in each graph of Fig. 4-5. The reduction CFU provoked by heat treatments was also significant and approaching the lower gray line but constantly slightly less efficient, i.e., slightly above the lower gray line for all three strains tested.

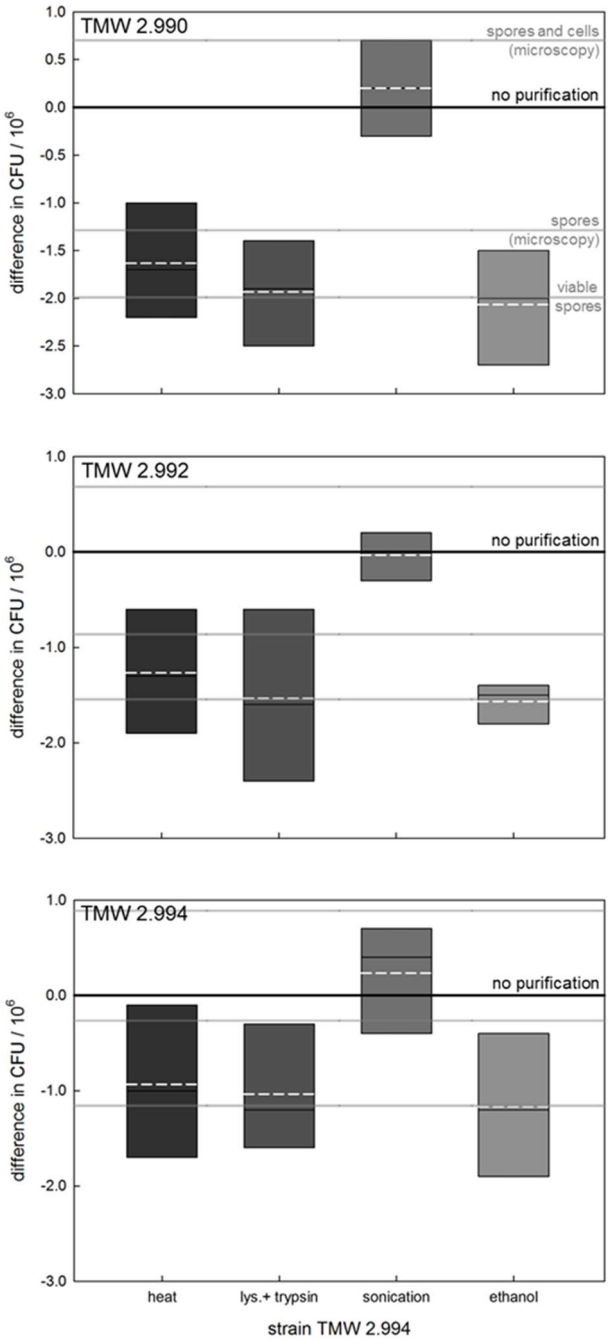


Fig. 4-5: Effect of purification steps on viable cell/spore counts.
Effect of purification treatment on viable spore/cell counts. Each graph contains the results for one of the three *C. botulinum* type E strains used, i.e., TMW 2.990 (left), TMW 2.992 (middle), and TMW 2.994 (right). Difference between viable CFU/mL with (box plots) and without (continuous black reference lines) a purification treatment (dark gray trough light gray: heat, lysozyme+trypsin,

sonication, ethanol). Black lines in boxes indicate media values. White dashed lines in boxes indicate mean values. Light gray continues lines indicate expected values, if all vegetative cells and spores survived a purification treatment (upper lines), if all vegetative cells are inactivated and all spores form colonies in plates (middle lines), and, if all vegetative cells are inactivated and viable spores form colonies in plates (lower lines).

Initial spore concentration was between 7.2 and 7.3 log CFU/mL depending on the strain and purification methods used. Inactivation levels ($\log_{10}(N_0/N)$) for "unpurified" spore suspensions (i.e., only purified by wash cycles) reached during isobaric, (close-to) isothermal holding times of 10 min at 800 MPa/40 °C were approx. 2.8, 3.2, and 4.1 for strains TMW 2.990, 2.992, and 2.994, respectively. After HPT treatments at 800 MPa/70 °C, such levels were approx. 5.4, 5.5, and 6.0 for the three tested strains. Purification treatments-dependent alterations in the determined inactivation levels are indicated as differences in log reduction determined for purified minus log reduction values determined for unpurified samples [i.e., $\log(N_0/N)_{purified}$-$\log(N_0/N)_{unpurified}$] in Fig. 4-6.

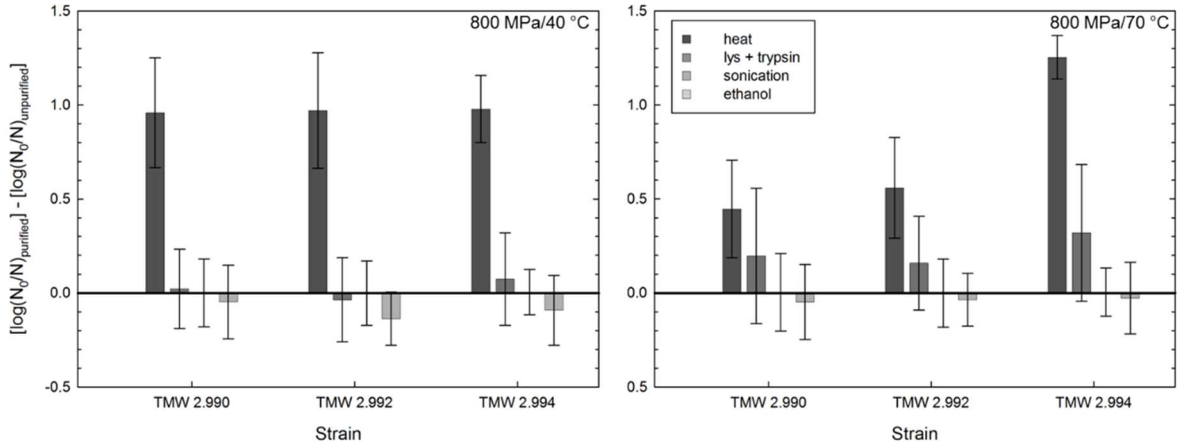


Fig. 4-6: Purification method-dependent HPT inactivation.
Effect of different purification treatments on the log reduction of viable counts of cells/spores [i.e., $\log_{10}(N_0/N)_{purified}$]in comparison with the log reduction occurring in samples with no additional purification step [i.e., $\log_{10}(N_0/N)_{unpurified}$]. Differences in the log reduction are indicated for the three *C. botulinum* type E strains TMW 2.990, 2.992, and 2.994 treated at 800 MPa at 40 °C (left graph) or 70 °C (right graph) for a constant holding time (10 min). Bar colors (from dark to light gray) indicate the purification treatment which is put into relation with unpurified samples, i.e., heat, lysozyme+trypsin, sonication, and ethanol treatments. Error bars indicate the square root of the sum of error squares.

Mean inactivation levels using ethanol purification were constantly the lowest regardless of the strain and the HPT treatment intensity. However, differences between spores treated with ethanol, sonication, or no additional purification step, i.e., wash cycles only, were never significant. Although this is not appropriate in practice, D-values could be calculated form only the mean values at the two points during isothermal, isobaric holding times determined here, i.e., 1 s and 10 min. The maximum difference in such D-values between ethanol treated and unpurified spore suspensions would be only 8 s (strain TMW 2.992, 800 MPa/40°C; corresponding to a 4 % increase in the D-value). Very similar cell counts (CFU/mL) for unpurified, sonicated, and ethanol treated spore suspensions after HPT treatments indicate that spore resistance is not reduce by ethanol or sonication. However, the presence of only a small fraction of HPT susceptible vegetative cells in unpurified and sonicated spore suspensions is not high enough to influence log reduction values to an extent that makes

mean differences occurring under the conditions tested significant. This is related to the relatively high sporulation efficiency achieved for all three strains under the given sporulation conditions in this study, and, thus, can likely be different when high sporulation efficiencies cannot be reached.

Log reduction values determined for enzymatically treated spores suspensions did also not significantly differ from that for unpurified spore suspension. However, there was a slight difference observable between inactivation levels for enzymatically treated spores HPT treated either at 800 MPa/40 °C or 800 MPa/70 °C. Although not significant, there was a trend that purification protocols employing incubation in the presence of trypsin and lysozyme tended to increase HPT inactivation levels at 800 MPa/70 °C. Again, the theoretical calculation of D-values from only two data points (mean values) would result in only a small maximum difference in D-values between ethanol and enzymatically treated spores, i.e., only 5 s (strain TMW 2.994, 800 MPa/70°C; corresponding to a 6 % increase in the D-value).

Most strikingly, the application of a heat treatment during spore suspension purification significantly increased the detected log reduction values by around 1 log cycle for all three strains treated at 800 MPa at 40 °C (0.96, 0.97, and 0.98, for TMW 2.990, 2.992, and 2.994). After HPT inactivation treatments at 800 MPa at 70 °C, this trend was also observable, although the increase in log reduction values was less pronounced, at least for strains TMW 2.990 and 2.992 (0.45, 0.56, and 1.25, for TMW 2.990, 2.992, and 2.994). The theoretical calculation of D-values in this case would lead to a maximum difference in D-values between ethanol and heat-treated spores of 1 min (strain TMW 2.990, 800 MPa/40°C; corresponding to a 37 % increase in the D-value).

4.1.3.2 Storage Conditions

Under the storage conditions tested in this study, no significant differences for viable spore counts were observed for storage times up to two weeks at 4 °C. Since this is long enough to conduct several experiments with spores from the same batch, this factor was not of major importance for subsequent experiments.

4.2 Pressure/Temperature-Dependent Inactivation

4.2.1 Spore Inactivation in Different High Pressure Units

To be able to compare the results from experiments under isothermal-isobaric conditions conducted in the high pressure units U111 and FBG 5620 and the results obtained for the HPT-mediated release of DPA from spores conducted in unit TMW-RB, it was essential that treatments with identical target parameters (p/T/t) in the different units exert comparable effects on spores. Spore inactivation levels obtained after treatments in the different high pressure units excluding effects under non-isobaric and non-isothermal conditions (log (N_t/N_{1s})) are shown in Fig. 4-7.

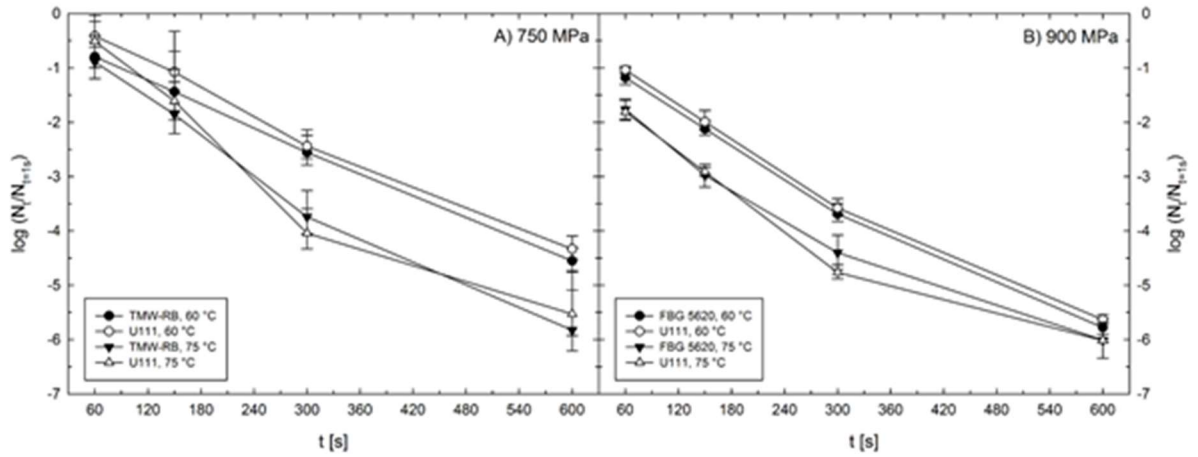


Fig. 4-7: Comparison of inactivation kinetics in different HP units.
Mean inactivation levels of *C. botulinum* TMW 2.990 spores after 60 – 600 s HPT treatments (log (N_t/N_{1s})) in different high pressure units. A) Inactivation after treatments in units TMW-RB (solid symbols) and U111 (open symbols) at a target pressure of 750 MPa and target temperatures of 60 (circles) and 75 °C (triangles). B) Inactivation after treatments in units FBG 5620 (solid symbols) and U111 (open symbols) at a target pressure of 900 MPa and target temperatures of 60 (circles) and 75 °C (triangles). Error bars indicate standard deviation from two (unit U111 and FBG 5620) or three (unit TMW-RB) independent experiments.

The absence of significant differences between inactivation results obtained using the three different high pressure units demonstrates that stress intensities at identical target parameters are comparable independently of the unit used. Most importantly this indicates that the empirical adjustment of starting temperatures and the experimental setting of experiments in unit TMW-RB (used to examine the HPT-mediated DPA release) were suitable to obtain similar spore inactivation levels compared with the other two units, where the actual sample temperatures were precisely controlled.

4.2.2 Inactivation Kinetics and DPA Release

Spore counts of *C. botulinum* TMW 2.990 before and after HPT treatments at 300 – 1200 MPa combined with temperatures of 30 – 75 °C for dwell times of 1 – 600 s (high pressure units U111 and FBP 5620; Table 1) are shown in Fig. 4-8. Kinetics for 1200 MPa at 30 °C are missing, because it was not possible to realize isothermal treatment conditions applying such high pressure levels combined with low target temperatures. This was related to the fact that the adiabatic heat of compression, i.e. the temperature raise in the sample during pressure build-up, exceeds the target temperature (30 °C) by far. Additionally, water in the sample would have been in the frozen state (ice VI) at 1200 MPa/30 °C, which would have largely influenced inactivation results. Fig. 4-8 also contains data for the relative amount of DPA released from spores in response to 300 – 750 MPa treatments at 30 – 75 °C for dwell times of 1 – 600 s (high pressure unit TMW-RB).

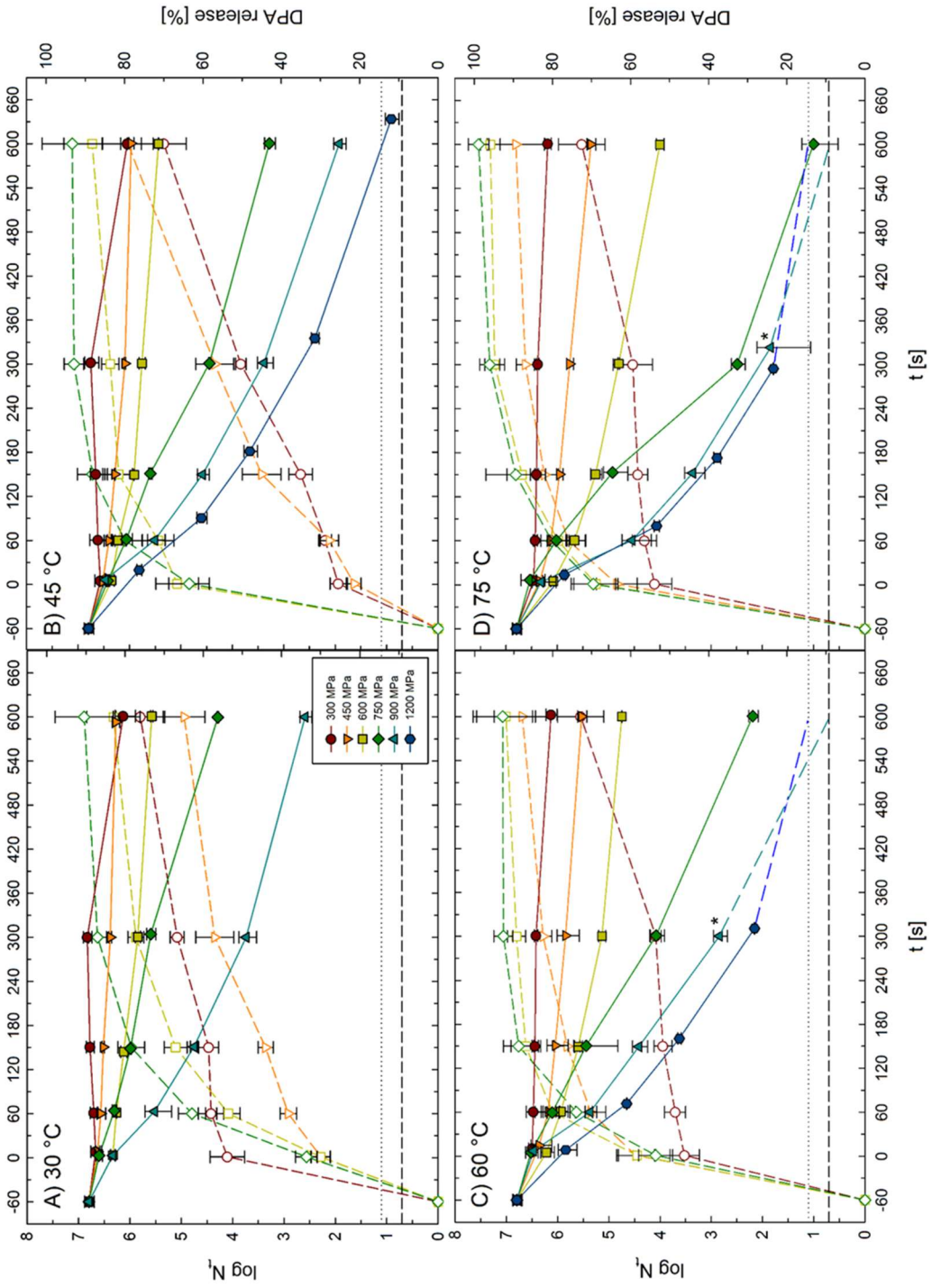


Fig. 4-8: Inactivation and DPA release kinetics.
Kinetics of surviving *C. botulinum* TMW 2.990 spores and accompanying release of DPA after HPT treatments at 300 – 1200 MPa combined with A) 30 °C, B) 45 °C, C) 60 °C, and D) 75 °C for 1 – 600 s. Solid lines and symbols indicate viable spore counts (left y-axis) after HPT treatments. Short dashed lines and open symbols indicate DPA release (right y-axis). Colors and symbol shapes indicate the pressure intensity of a treatment, i.e. 300 (red circles), 450 (orange downward triangles), 600 (yellow squares), 750 (green diamonds), 900 (cyan upward triangles), and 1200 MPa (blue hexagons). Inactivation by 1200 MPa/30 °C and DPA release after 900 and 1200 MPa treatments was not determined and is therefore not depicted. Horizontal lines at the bottom of each graph represent detection limits for viable spore counts after treatments in unit FBG 5620 (dotted line, 1.125 log) and unit U111 (short dashed line, 0.7 log). For spore counts below the detection limit plots are extended towards this limit with medium dashed lines. Asterisks mark mean values for spore counts from the units FBG 5620 and U111. Error bars indicate standard deviation from two (viable spore counts, units U111 and FBG 5620) or three (DPA release, unit TMW-RB) independent experiments.

Spore inactivation generally increased with increasing treatment intensity and duration. Thus, data presented here do not point towards the existence of any p/T zones where inactivation is significantly retarded. Pressure levels of 300 and 450 MPa had a weak effect on spore counts regardless of the temperature at which the treatments were conducted (30 – 75 °C). When only isothermal-isobaric dwell times are considered (log (N_t/N_{1s})), spore inactivation by such treatments did not exceed 1 log cycle, and maximum inactivation levels reached after treatments at 300 and 450 MPa were 0.6 log (at 30 °C, 600 s) and 1 log (at 45 °C, 600 s), respectively. Spore inactivation was markedly increased when pressures above 600 MPa were used. Maximum log inactivation values at 600 and 750 MPa target pressure were 2.1 and 5.6, respectively (reached at 75 °C, 600 s). Maximum log inactivation at 900 or 1200 MPa were also found in combination with 75 °C (600 s), which in both cases resulted in a complete inactivation of the spore population (>6 log). The absolute maximum of inactivation reached at 30 °C was 3.8 log (900 MPa, 600 s; note: 1200 MPa/30 °C experiments not conducted). The highest inactivation levels reached at target temperatures of 45, 60, and 75 °C were 4.9, 5.8, and >6 log, respectively (all in combination with 1200 MPa, 600 s).
Similar to the inactivation levels, DPA release increased with treatment intensity and dwell time. As an exception, the maximum relative amount of DPA released from spores after treatments at 300 MPa was 76 % after 600 s at 30 °C, which was slightly higher than the amounts released after 300 MPa treatments at 45, 60, or 75 °C. Maximum relative DPA amounts released after 450, 600, and 750 MPa were 92, 96, and 99 % each after 600 s treatments at 75 °C. Comparing DPA release after treatments at identical target temperatures, maximum DPA release reached after 30, 45, 60, and 75 °C treatments were 90, 93, 93, and 99 %, respectively (all after 600 s at 750 MPa). Fastest DPA release occurred at 75 °C where 52, 64, 67, and 70 % of the total DPA content of spores was released in response to a short (1 s) pressure pulse at 300, 450, 600, and 750 MPa, respectively. However, short dwell times at 300 MPa/30 °C were also relatively effective in triggering the release of DPA (54 % after pressure build-up), which exceeded the release after 1s treatments at 300 MPa/45 °C (25 %), 300 MPa/60 °C (46 %), 450 MPa/30 °C (30 %), 600 MPa/30 °C (30 %), and 750 MPa/30 °C (34 %) by far.

4.2.3 Heat Susceptible Fraction after HPT Treatments

Heat treatments (60 °C) subsequent to the HPT treatments were employed to give an estimate on the number of spores that lost their high heat resistance in response to HPT but were not inactivated during such treatments. This fraction can contain (i) spores that germinated during or within 2 h after a HPT treatment following the physiological pathway as well as (ii) spores that somehow lost their heat resistance (commonly associated with DPA release and core rehydration) or (iii) spores that are sublethally damaged (e.g., in molecules essential for outgrowth) and for which a heat treatment at 60 °C becomes lethal.
The difference in the number of viable spores (CFU/mL) detected after HPT treatments and that after a subsequent additional heat treatment at 60 °C ($\log(N_t/N_{t+heat})$) was generally low and did not exceed 0.59 log observed after 300 MPa/30 °C/600 s treatments, which corresponds to 74 % of the spore population surviving this HPT process (Fig. 4-9). Very small but significant differences were also found after treatments at 300 MPa/45 °C, 450 MPa/30 °C, and 450 MPa/45 °C, i.e., up to 0.41, 0.47, and, 0.25 log for a dwell time of 600 s,

respectively. This corresponds to 61, 66, and 52 % of the surviving population without applying a second heat treatment. After treatments at the remaining pressure/temperature combinations (>450 MPa/>45 °C), plate count results were virtually identical, no matter if HPT treatments were followed by a second thermal treatment or not.

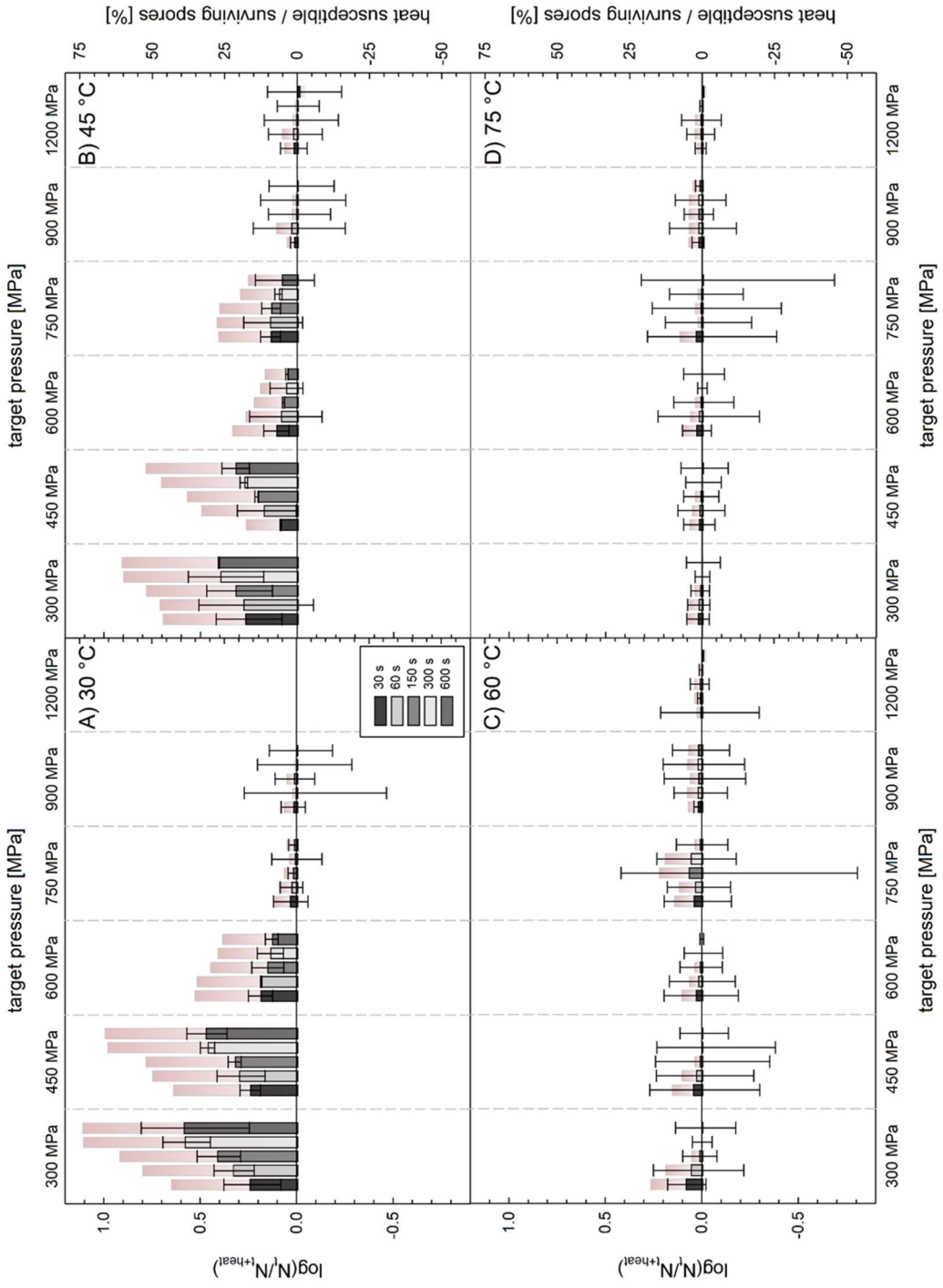


Fig. 4-9: Heat susceptible spore fraction after HPT treatments.
Difference in survivors of *C. botulinum* TMW 2.990 spores after HPT treatments with and without a subsequent heating step at 60 °C (log (Nt/Nt+heat); left y-axis). HPT treatments at A) 30 °C, B) 45 °C, C) 60 °C, and D) 75 °C. Columns (x-axis) indicate different target pressures (30 °C/1200 MPa experiments no conducted). Different color intensities of bars indicate different dwell times. Positive and negative values correspond to lower and higher numbers of CFU, respectively, after the additional heat treatment. Error bars indicate the square root of the sum of square of errors for HPT treated and HPT and heat treated samples derived from two independent experiments. Color gradient bars indicate the number of heat susceptible spores (or cells) relative to the number of spores (or cells) surviving a HPT treatment ((Nt-Nt+heat)/Nt × 100 [%]; right y-axis).

4.2.4 The Effect of Lysozyme in the Recovery Medium

Lysozyme catalyzes the hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine peptidoglycan residues in bacterial cell walls. However, bacterial spores are commonly resistant to this enzyme, which is related to the presence of coat layers acting as outer shell that impedes the access of this enzyme to the peptidoglycan layer underneath it, i.e., the cortex. During germination, the cortex is lysed by spore inherent cortex lytic enzymes, which presents an important step towards full core rehydration and outgrowth. Thus, plate counts with and without lysozyme in the recovery medium were carried out to detect spores with both defects in their cortex lytic machinery and coat layers permitting access of lysozyme to the peptidoglycan layer underneath it. Besides of subtle but significant differences that appeared to exist when spores were treated at 600 and 750 MPa, which tended to increase with increasing treatment temperature and dwell time, CFU/mL found in pour plates with and without lysozyme were generally not significantly different from each other (Fig. 4-10). The maximum difference observed was 0.23 log (log(Nt+lysozyme/Nt) after treatments at 600 MPa/75 °C for 600 s corresponding to 69 % of the surviving spore fraction detected in plates without lysozyme ((Nt+lysozyme/Nt)/Nt × 100). Slightly lower lysozyme-dependent log differences in spore counts were found for samples treated at 750 MPa/75 °C/600 s (0.21 log, 61 %), 600 MPa/60 °C/600 s (0.19 log, 56 %), and 600 MPa/75 °C/600 s (0.16 log, 45 %).

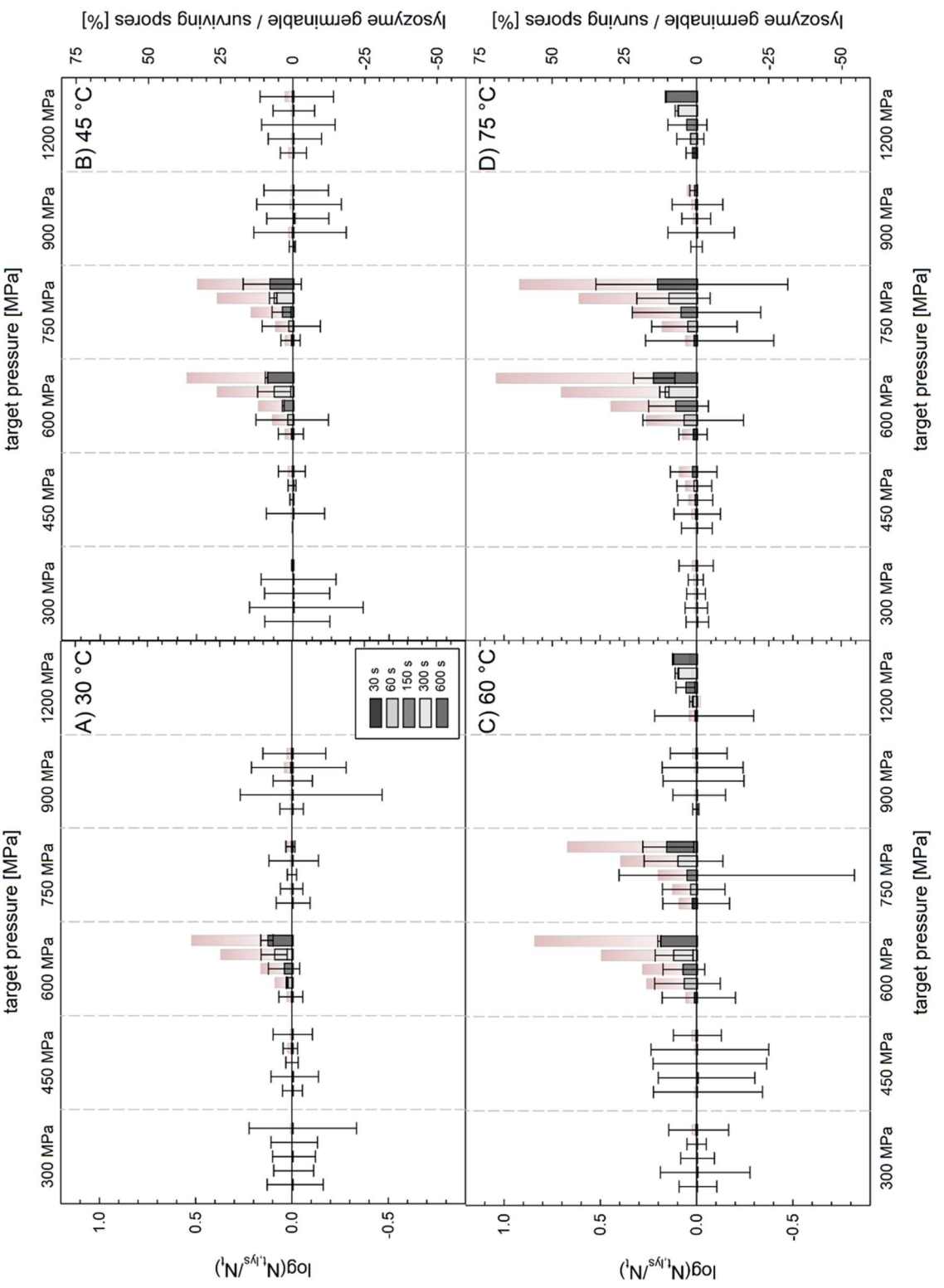


Fig. 4-10: Lysozyme susceptible spore fraction after HPT treatments.
Difference in survivors of *C. botulinum* TMW 2.990 spores after HPT treatments enumerated on plate with and without lysozyme ($\log(N_{t+lysozyme}/N_t)$; left y-axis). HPT treatments at A) 30 °C, B) 45 °C, C) 60 °C, and D) 75 °C. Columns (x-axis) indicate different target pressures (30 °C/1200 MPa experiments no conducted). Different color intensities of bars indicate different dwell times. Positive and negative values correspond to higher and lower numbers of CFU on plates containing lysozyme. Error bars indicate the square root of the sum of square of errors for HPT treated samples plated on plates with and without lysozyme. Color gradient bars indicate the number of lysozyme-dependently germinable spores relative to the number of spores surviving a HPT treatment ($(N_{t+lysozyme}/N_t)/N_t \times 100$ [%]; right y-axis).

4.2.5 Isoeffect Curves

To facilitate the comparison of data obtained for *C. botulinum* TMW 2.990 spore inactivation, the heat susceptible fraction after HPT treatments, and the effect of the presence of lysozyme in the recovery medium, 1 log and 3 log isoeffect lines were calculated as it has been described previously (Reineke et al., 2012). Fig. 4-11 shows the empirical determination of the reaction order (n = 1.1) (Fig. 4-11A) and the comparison between calculated and experimental data (Fig. 4-11B), which resulted in a good fit (adjusted R^2 = 0.97 for spore inactivation). Random residual distribution indicates the absence of a large heteroscedastic error.

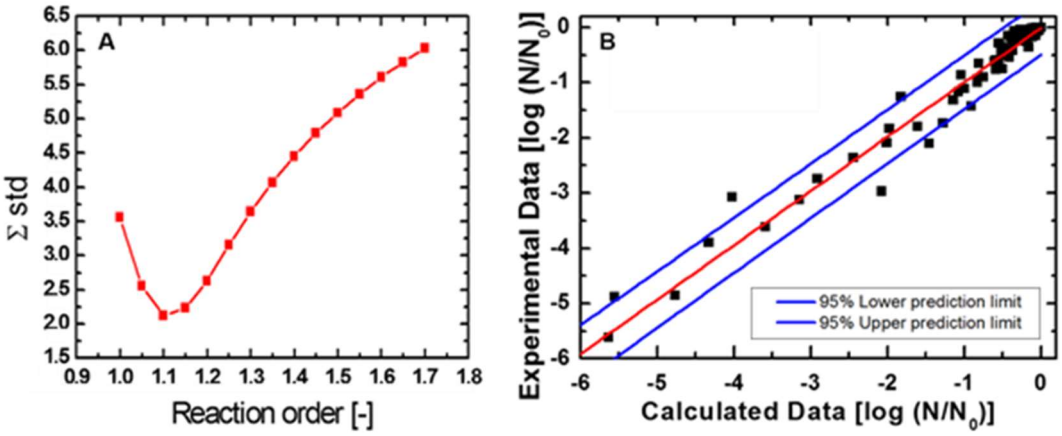


Fig. 4-11: Statistical values for isoeffect curve modeling.
Sum square error plotted against reaction order (A) and comparison between calculated and experimental data (B) for the inactivation of *C. botulinum* TMW 2.990 spores.

Fig. 4-12 shows the calculated isoeffect lines for a 1 log and 3 log inactivation after 3, 5, 8, and 10 min isothermal-isobaric dwell time determined directly after HPT treatments without lysozyme in the recovery medium (A and D), with an additional heating step after the HPT process (B and E), and with lysozyme in the recovery medium (C and F).

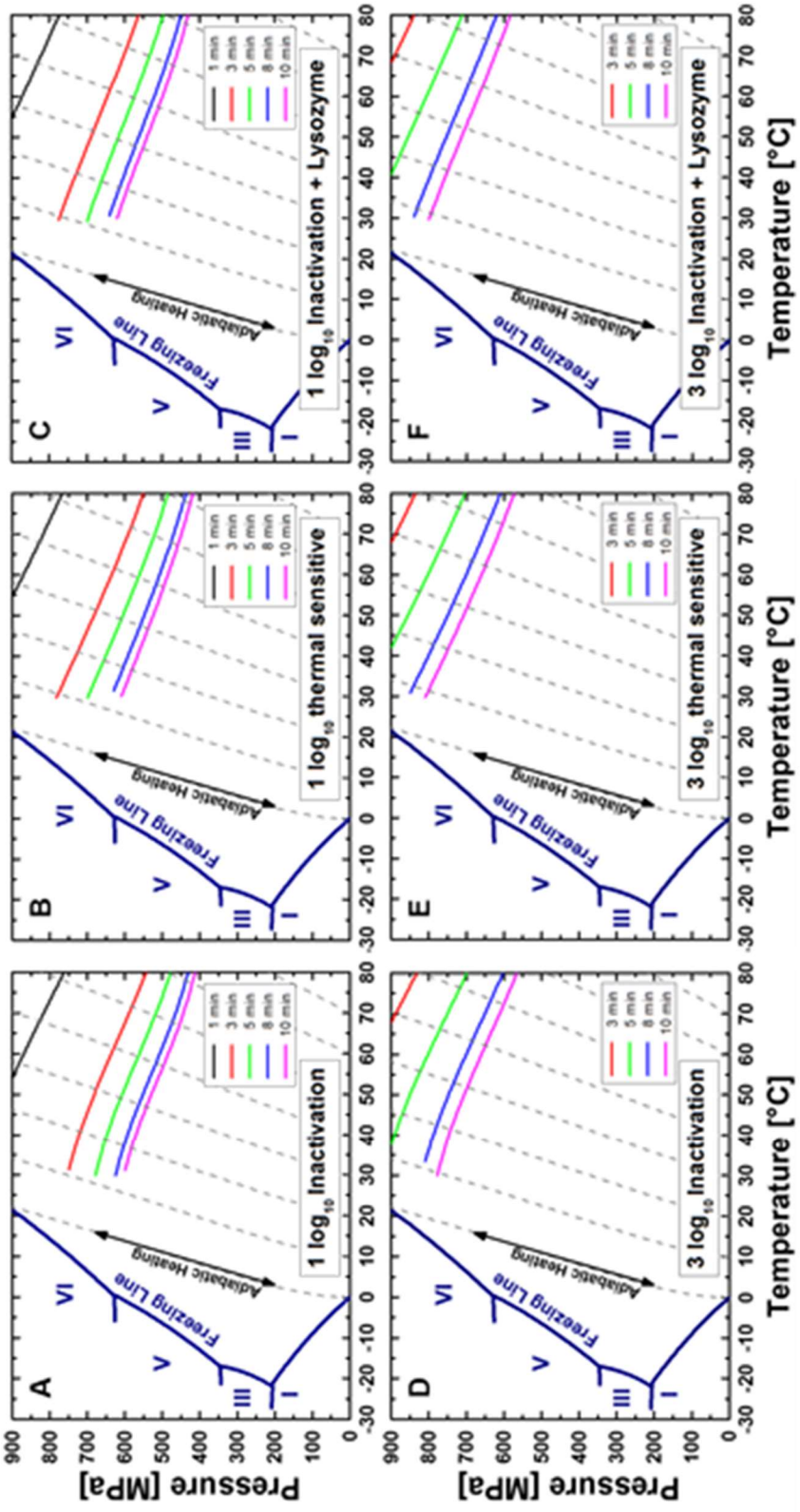


Fig. 4-12: Isorate curves for inactivation, heat- and lysozyme-susceptibility.
Isoeffect lines for a 1 log (A,B,C) and 3 log (D,E,F) inactivation of *C. botulinum* TMW 2.990 determined directly after HPT treatments in standard TPYC plates (A,D), including a second heat treatment at 60 °C (B,E), and plated in standard TPYC plates containing lysozyme. Different colors indicate different dwell times under isothermal-isobaric conditions, i.e. 1 min (black), 3 min (red), 5 min (green), 8 min (blue), and 10 min (pink).

In accordance with the small differences between the spore counts of samples analyzed directly after HPT treatment and determined after a second heat treatment (Fig. 4-9) or when lysozyme was added to the recovery medium (Fig. 4-10), calculated isoeffect lines shown in Fig. 4-12 display almost identical shapes regardless of an additional heat treatment or the recovery conditions. This illustrates the absence of large portions of heat susceptible or lysozyme-dependently germinable spores after HPT treatments described above.

Fig. 4-13 shows the comparison between calculated and experimental data (Fig. 4-13A) for the release of DPA from *C. botulinum* TMW 2.990 spores after HPT treatments, which resulted in an adequate fit (adj. $R^2 = 0.93$). However, the fact that the residuals are not entirely stochastically distributed indicates the possible presence of a heteroscedastic error. This is related to the fact that the model assumes isothermal-isobaric conditions to enable the calculation of isoeffect lines. However, due to the considerable DPA amount already released during pressure build-up, non-isobaric and non-isothermal conditions had to be included in the model. Fig. 4-13B depicts calculated isoeffect lines for a 90 % release of DPA for different dwell times dependent on the target pressure und process temperature. To facilitate the comparison with inactivation isoeffect lines (Fig. 4-12), lines for 6 min corresponding to 5 min dwell + 1 min come-up (red) and 11 min corresponding to 10 min dwell + 1 min come-up (black) are included. Similar to the results for inactivation, high pressures levels at low temperatures or low pressure levels at high temperatures are required to provoke the released of the same amount of DPA from spores. This dependency appears to be almost linear for longer dwell times.

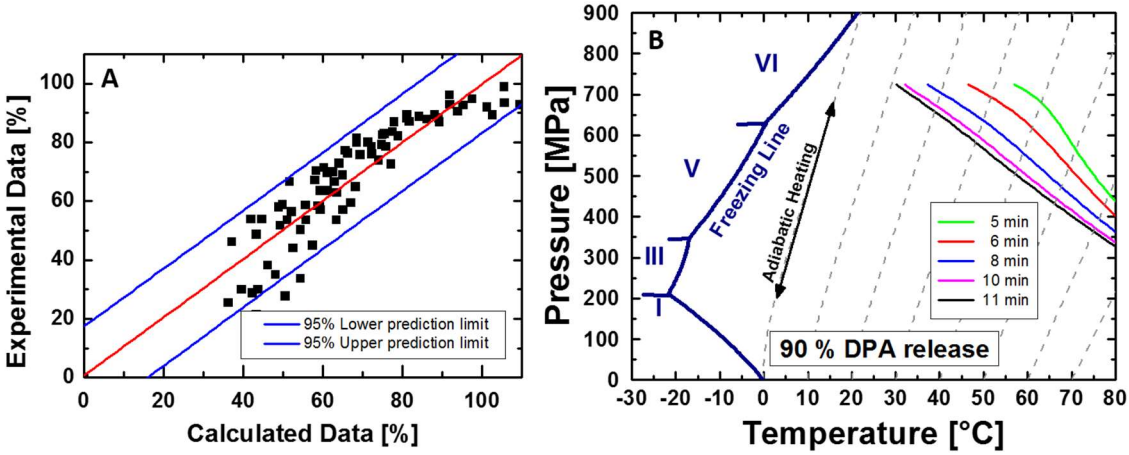


Fig. 4-13: Goodness of model and isorate curves for DPA release.
Comparison between calculated and experimental data (A) and isoeffect lines (B) for a 90 % DPA release from HPT treated *C. botulinum* TMW 2.990 spores determined 2 h after the respective treatment. Data for the calculation are derived from experiments in unit TMW-RB at 300 – 750 MPa and 30 – 75 °C including DPA release data occurring during pressure-come up. Different colors of isoeffect lines indicate different total process time (including 1 min come-up), i.e. after 5 min total process time (green), 6 min (= 5 min dwell + 1 min come-up) (red), 8 min (blue), 10 min (pink), and 11 min (= 10 min dwell + 1 min come-up) (black).

4.2.6 Strain-Specific HPT Resistance and DPA Content

In addition to *C. botulinum* type E strain TMW 2.990, inactivation levels of strains TMW 2.992 and TMW 2.994 after isothermal-isobaric HPT treatments at 300 – 750 MPa and 30 – 75 °C for 1 – 600 s were determined. Both strains were generally less resistant to HPT treatments than strain TMW 2.990. Considering all investigated p/T/t conditions, average differences in

log inactivation occurring during isothermal-isobaric dwell times ($\Delta \log N_{1s}/N_t$) between strain TMW 2.990 and TMW 2.992 and between TMW 2.990 and TMW 2.994 were 0.3 and 0.4 log cycles, respectively. Despite of the usual similar shapes of inactivation curves, the extent of differences appeared to be process intensity-dependent. With a few exceptions, strain-specific differences in inactivation levels tended to increase with increasing pressure, temperature, and dwell time.

Largest strain-specific differences in inactivation levels were observed after HPT treatments at 600 – 750 MPa at 60 °C (Fig. 9A) or 75 °C (Fig. 9B). Absolute maxima of differences between strain TMW 2.990 and the other strains were 1.1 log for TMW 2.992 (750 MPa/60 °C/300 s) and 2 log for TMW 2.994 (600 MPa/60 °C/600 s) (Fig. 9). Second largest differences between strain TMW 2.990 and TMW 2.992 were 0.8 log (600 MPa/60 and 75 °C/600 s). Second largest differences between strain TMW 2.990 and TMW 2.994 were 1.8 (600 MPa/75 °C/600 s) and 1.3 log (750 MPa/60 °C/300 s). Thus, with the exception of treatments at 750 MPa/75 °C, strain-dependent differences appeared to progressively increase with increasing pressure and/or temperature reaching maximum levels when pressure levels above 600 MPa and process temperatures above 60 °C were used (Fig. 4-14).

This trend can also be observed for average differences occurring after all isothermal-isobaric dwell times (60 and 600 s) in a specific pressure/temperature range. Average differences in inactivation levels between the strains TMW 2.990 and TMW 2.992 ranged between 0.1 and 0.3 log when spores from both strains were subjected to HPT treatments at 300 MPa/45 – 75 °C, 450 MPa/45 °C, 600 MPa/30 – 45 °C, and 750 MPa/45 °C. Larger differences between the inactivation levels, i.e. in a range between 0.3 and 0.7 log, were found after HPT treatments at 450 MPa/60 – 75 °C, 600 MPa/60 – 75 °C, and 750 MPa/30, 60 and 75 °C.

Average differences in inactivation levels between the strains TMW 2.990 and TMW 2.994 occurring during isothermal-isobaric dwell times of 60 – 600 s ranged between 0.1 and 0.3 log cycles after HPT treatments at 300 MPa/45 – 75 °C, 600 MPa/30 °C, and 750 MPa/45 °C. A range between 0.3 and 0.7 log was found after HPT treatments at 450 MPa/60 – 75 °C, 600 MPa/45 °C, and 750 MPa/30, 60 and 75 °C. Between 0.8 – 1.2 log were detected after 600 MPa/60 and 75 °C treatments. In contrast, no significant differences were observed between any of the strains after HPT treatments at 300 or 450 MPa at 30 °C (data not shown).

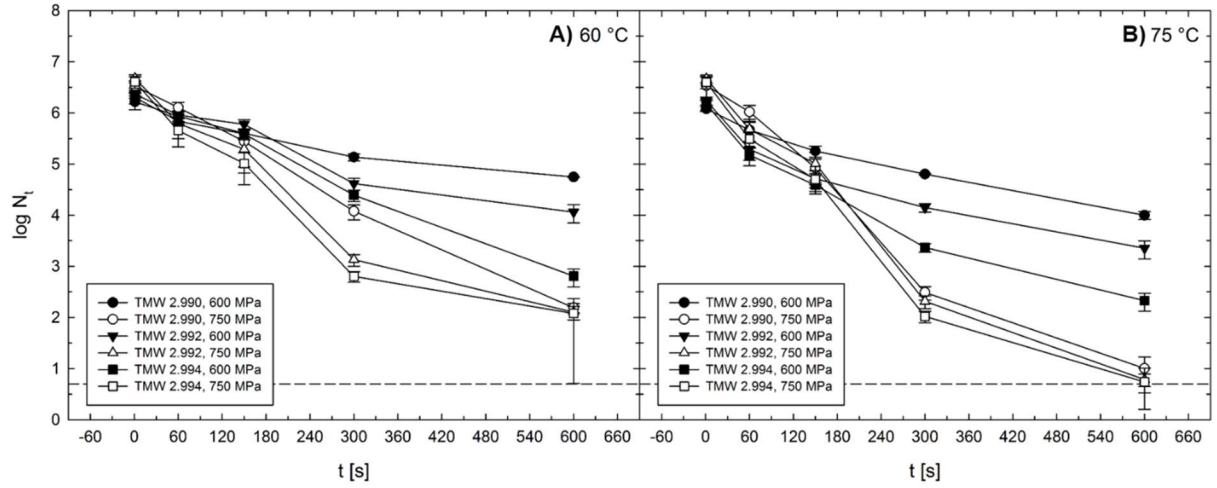


Fig. 4-14: Strain-specificity of inactivation kinetics.
Mean levels of *C. botulinum* type E strains TMW 2.990 (circles), TMW 2.992 (triangles), and TMW 2.994 (squares) spores surviving 1 – 600 s HPT treatments (log N_t) in unit U111 at target pressure of 600 MPa (solid symbols) and 750 MPa (open symbols). (A) In combination with 60 °C and (B) in combination with 75 °C. The dashed line indicates the detection limit. Error bars indicate standard deviation from two independent experiments.

In addition to comparing inactivation levels, data obtained here allows for a comparison of strains reading their total amount of DPA detectable in autoclaved spore samples (HPLC analysis). The determination of total spore counts using a counting chamber (0.01 mm depth) and phase contrast microscopy enabled the calculation of DPA concentration per spore. Three different spore crops were analyzed and average DPA levels per spore were 155 ± 23, 132 ± 9, and 72 ± 14 amol (= 10^{-18} mol) for *C. botulinum* strains TMW 2.990, TMW 2.992, and TMW 2.994, respectively. This corresponds to an average of 9.3 ± 1.4 × 10^7, 8.0 ± 0.5 × 10^7, and 4.3 ± 0.8 × 10^7 DPA molecules per spore.

4.2.7 Detection Time Variability of HPT treated spores

The effect of pressure treatments with different intensities on the time to detect growth from individual *C. botulinum* type E spores was evaluated after treatments at 200, 300, 400, and 600 MPa at 20 °C, which were conditions that were shown in this study to provoke no significant inactivation of *C. botulinum* type E (≤ 1 log). Additionally, 600 MPa at 80 °C and 800 MPa at 60 °C were used resulting in approx. 5 and 4 log reduction of viable spore counts, respectively, based on the data also obtained previously in this study.
No growth was detected in minimum 27, maximum 34, and a mean number of 32 wells per plate (94 inoculated wells). Assuming that the number of spores per well follows a Poisson distribution as a result of the dilution process, this would correspond to 1.25, 1.02, and 1.08 viable spores per well on average (Baranyi et al., 2009).
The earliest time point where growth appeared from untreated *C. botulinum* type E spores was 17.5 h, while the last spore completed outgrowth approx. 14 h later reaching the same OD around 31.5 h after nutrient addition. The maximum growth rate was relatively constant, no matter at which time point spores started to grow. Approx. 20% of the spores grew to OD 0.2 between the 22nd and 23rd hour of incubation, which coincides with the mean (22.9 h) and median (22.5 h) detection times of untreated spores. The frequency distribution of detection

times of such spores was slightly positively skewed and resembled a Weibull rather than a normal distribution (Fig. 4-15A).

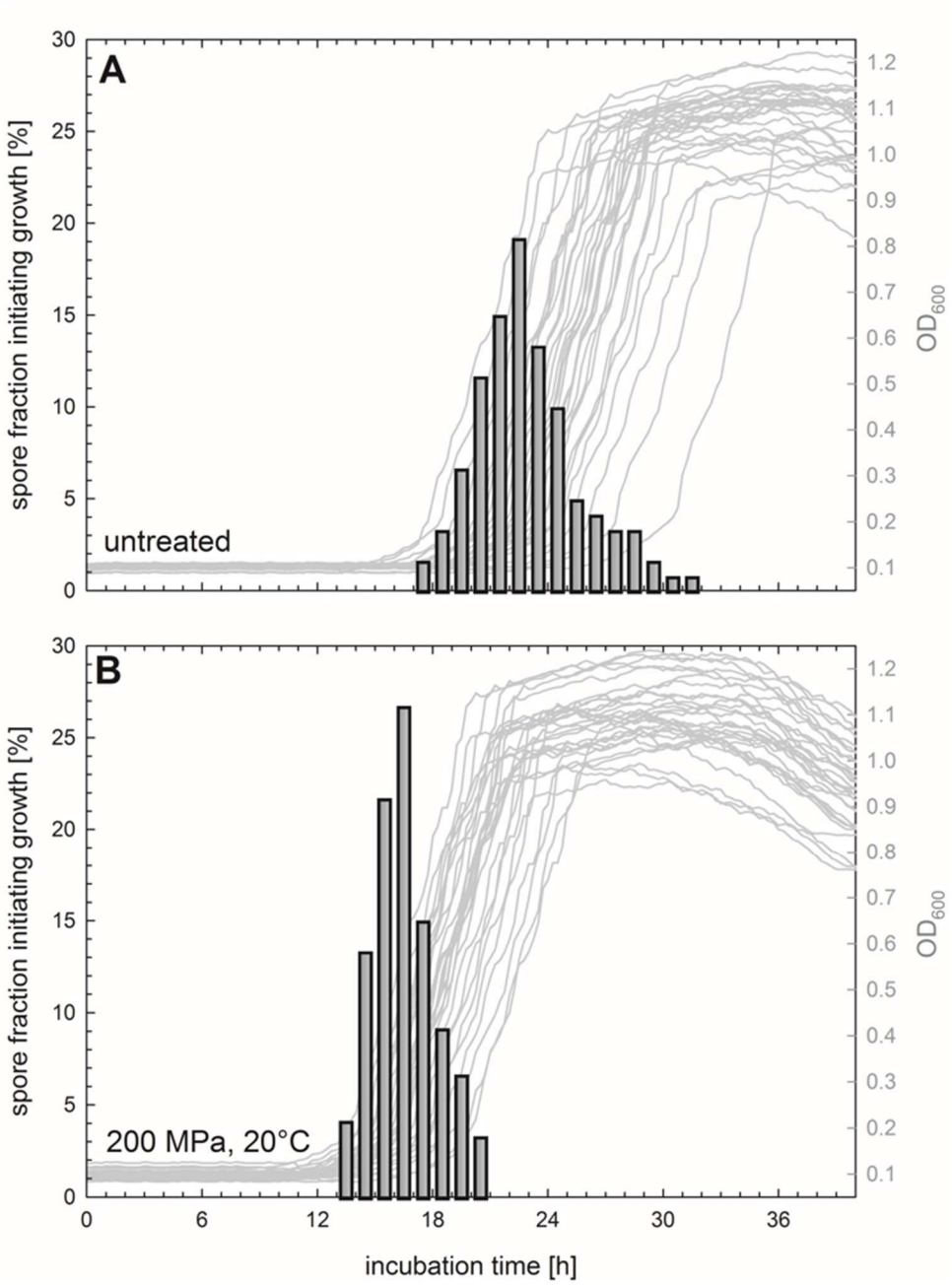


Fig. 4-15: Growth detection times of untreated and 200 MPa-treated spores.
Frequency distributions of detection times of individual *C. botulinum* type E spores untreated (A) and treated at 200 MPa, 20 °C for 10 min (B). Gray solid lines describe representative growth curves obtained by measuring OD600nm. Bars indicate the percentage of spores initiating growth (OD600nm = 0.2) within a respective incubation time interval of 1 h. Data for bars is derived from an average of 124 growth curves.

When spores were treated for 10 min at 200 MPa and 20 °C, the frequency distribution clearly became narrower and the degree of skewness was decreased (mean: 16.6 h, median: 16.5 h) (Fig. 4-15B). In addition to the mean time required to generate growth, the time span between the first (13.5 h) and the last (20.5 h) spore growing was markedly reduced.

In contrast to the treatment at 200 MPa, high pressure treatments at 300 MPa and 20 °C resulted in very similar growth behavior as compared to untreated spores. Mean, median and incubation time required for the appearance of first and last spore growing (21.5, 20.1, 17.4, 28.9 h) was a smidgen lower, but the frequency distribution of detection times showed a very similar shape as compared to untreated spores (Fig. 4-16).

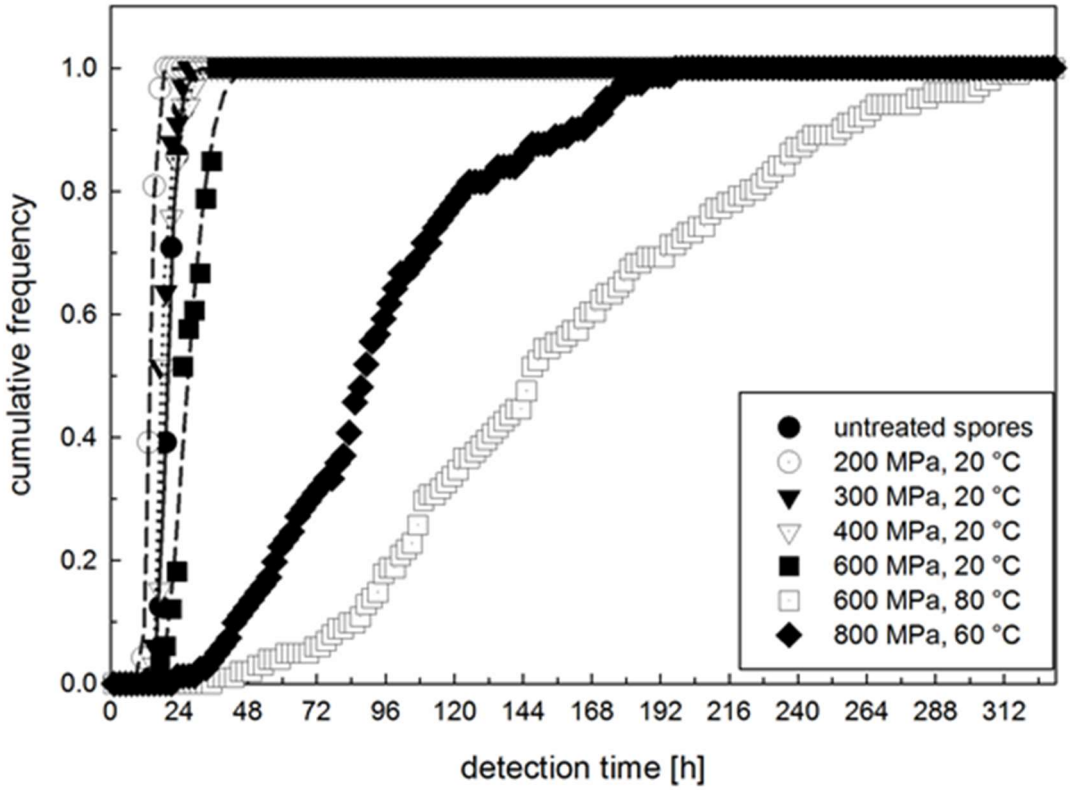


Fig. 4-16: Cumulative frequency distribution of detection times.
Cumulative frequency distributions of untreated (black circles, solid line) and spores treated at 20 °C and 200 MPa (open circles, first dashed line), 300 MPa (solid triangles, first dotted line), 400 MPa (open triangles, second dotted line) and 600 MPa (solid squares, second dashed line), at 80 °C, 600 MPa (open squares) and at 60 °C, 800 MPa (solid diamonds).

Increasing the pressure level to 400 MPa and 600 MPa, respectively, caused a slight and moderate increase in characteristic detection times (mean, median, first, last spore after 400 and 600 MPa: 23.1 and 30.4, 21.9 and 27.9, 17.8 and 19.9, 33.7 and 39.4) (Fig. 4-16).
When the treatment temperature was increased to 60 and 80 °C combined with pressure levels of 800 and 600 MPa, we observed a drastic prolongation of the detection time. Mean and median times required to detect growth were approx. four-fold (74 h) and seven-fold (139 h) longer as compared to untreated spores. The frequency distribution of detection times broadened tremendously and first growth was detected after 26.0 h and 40.1 h, while an OD of 0.2 was reached after 199.4 and 321.4 h in the last well (Fig. 4-16).

4.3 Cation/Temperature Effects on Spore Resistance

4.3.1 Role of Cations in Sporulation Medium-Dependent Resistance

To be able to assess the role that sporulation medium cation contents play in the different effects of sporulation media on the heat and pressure resistance of *C. botulinum* type E spores, cation contents of the media used had to be known. Therefore, the standard sporulation medium used in many studies examining non-proteolytic *C. botulinum* strains, TPYC, the sporulation media conferring highest and lowest resistance, i.e., M140 and SFE, as well as the pure sediment fish extract used as base for the SFE medium were analyzed for their mineral content. The media differed significantly with respect to their contents of divalent cations as it is depicted in Fig. 4-17. Comparing SFE and M140, the Ca^{2+} content was approx. 180-fold higher, the Mg^{2+} content approx. 2-fold lower and the Mn^{2+} content 8-fold higher in SFE.

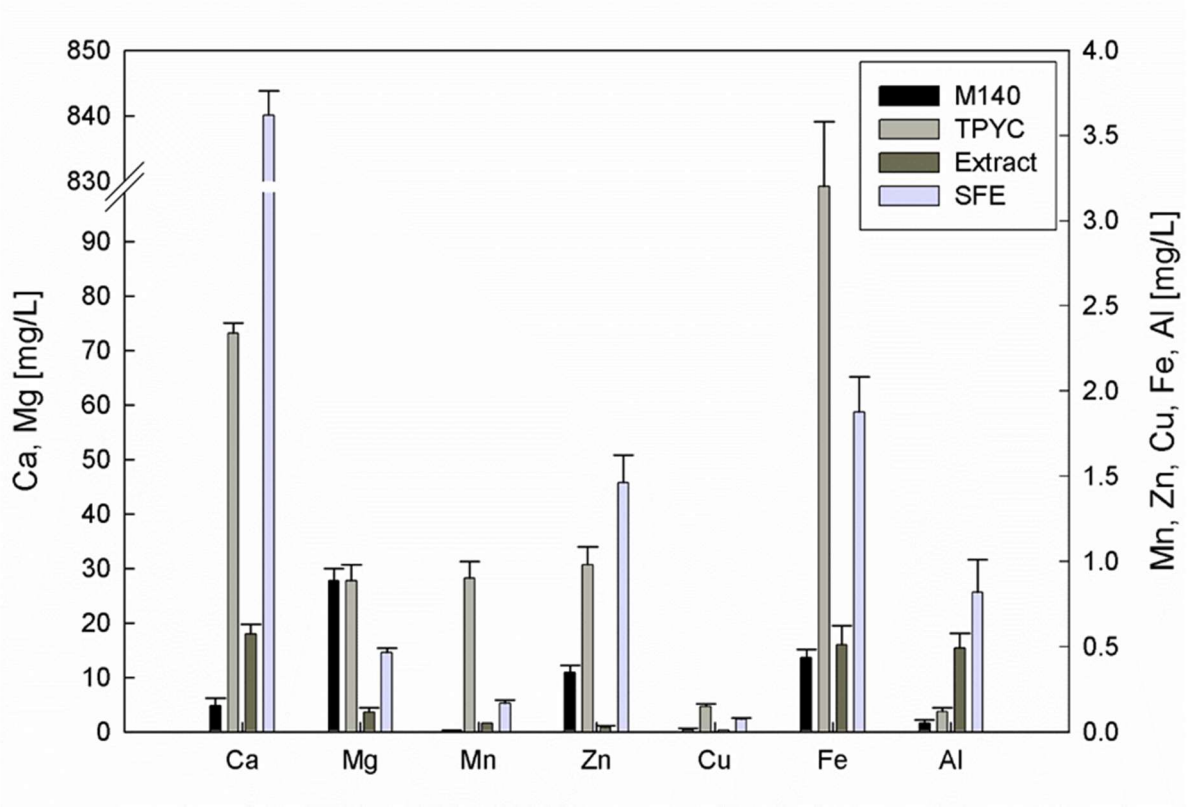


Fig. 4-17: Mineral contents of sporulation media.
Concentrations of different minerals in the sporulation media M140, TPYC, SFE and the extract base for SFE [mg/L] as determined by ICP-OES.

4.3.1.1 Supplementation of M140 medium with typical SFE cation amounts

As Ca^{2+}, Mg^{2+}, and Mn^{2+} are presumably important for spore resistance the effect of supplementation of a medium conferring low HPT resistance, i.e., M140, with cation contents that can be found in a medium conferring high resistance, i.e., SFE, was determined. M140 without $MgSO_4$ contained 4.8, 7.5, and 0.03 mg/L Ca^{2+}, Mg^{2+}, and Mn^{2+}, respectively. These values were adjusted to 840, 14.6, and 0.17 mg/L using $CaCl_2 \times 2\ H_2O$, $MgCl_2$, and $MnSO_4 \times H_2O$. Spores from strain TMW 2.990 grown in cation supplemented M140 tended to be

more heat, HHP, and HPT resistant than spores grown in M140 without supplementation (Fig. 4-18, Tab. 4-2) with fair statistical significance for HPT and only slight differences with marginal significance for heat and HHP. The inactivation levels of supplemented M140 spores generally become more similar to that of SFE spores, although there is still a marginally significant difference with respect to heat and HPT resistance. For HHP, no significant differences were observed anymore. Thus, spores grown in supplemented M140 show enhanced, but no full resistance as it is conferred by SFE. The positive effect in dependence of the type of stress applied was in the order HPT > heat > HHP with mean differences of 0.8, 0.55, and 0.50 log viable spores per mL, respectively. The identical order and similar differences in resistance were observed for the other two strains used (data not shown).

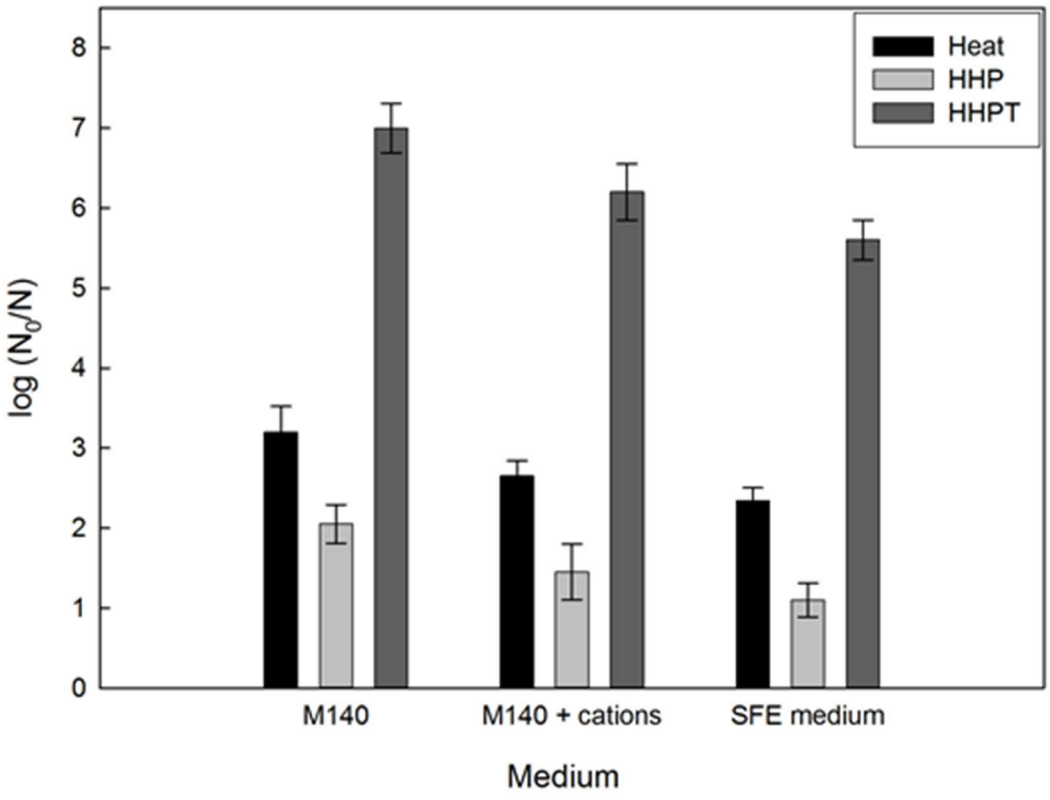
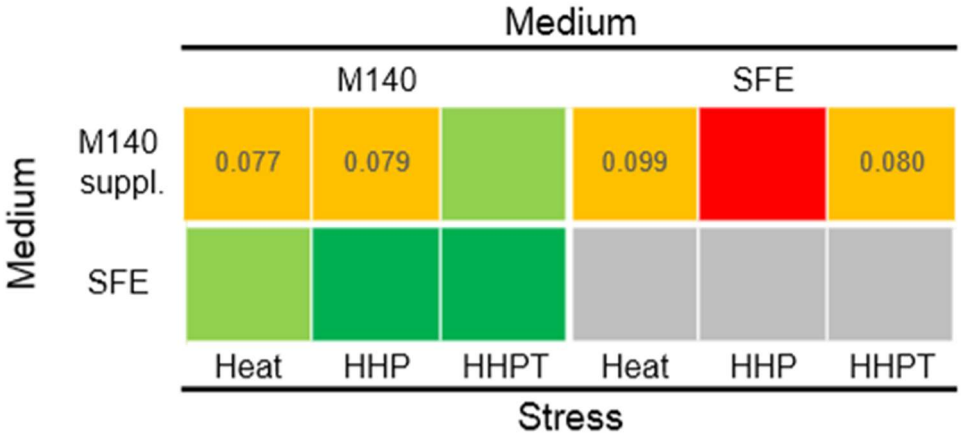


Fig. 4-18: Cation supplementation and HPT resistance.
Comparison of *C. botulinum* TMW 2.990 spore inactivation in M140, M140 supplemented with SFE-like cation contents and SFE. Bars indicate log reduction of initially 7.5 log viable spores/mL, heat: 0.1 MPa, 80°C, 10 min, HHP: 800 MPa, 20°C, 10 min. (Note: to indicate that the same pressure conditions are prevalent as during HHP treatments and only the temperature was increased (from 30 to 80 °C), HPT treatments are designated as HHPT in the figure legends).

Tab. 4-2: Significance of cation-dependent differences in spore resistance.
Significance of sporulation medium-related differences in heat, HHP, and HPT resistance between *C. botulinum* TMW 2.990 spores produced in M140, M140 supplemented with cation amounts typical for SFE, and SFE. Red: p > 0.10, no significance, orange: p < 0.10 marginal, light green: p < 0.05 fair, dark green: p < 0.01 good significance of medium related difference in resistance. (Note: to indicate that the same pressure conditions are prevalent as during HHP treatments and only the temperature was increased (from 30 to 80 °C), HPT treatments are designated as HHPT in the figure legends).



4.3.1.2 Sporulation in defined medium and role of cations

4.3.1.2.1 Single cation source deficiency

Since the used media vary greatly with respect to their cation contents, and supplementation of M140 with SFE-like Ca^{2+}, Mg^{2+}, and Mn^{2+} contents influences spore resistance properties, the role of these divalent cations was further examined. The medium MDM (modified defined minimal medium) was used to determine the effect of single cation source deficiency on spore resistance. Generally, sporulation was less effective in MDM as compared to complex media. Bars in Fig. 4-19 indicate the difference in heat and HHP resistance between *C. botulinum* TMW 2.990 spores grown in standard MDM versus spores from MDM without Ca^{2+}, Mg^{2+}, or Mn^{2+}. Negative numbers indicate lower resistance and positive numbers indicate higher resistance than that of standard MDM spores. Treatments at 800 MPa at 80°C resulted in complete inactivation of initially 7.5 log spores/mL whenever any cation source was omitted (data not shown). Generally, the absence of either cation source led to decreased spore resistance. The absence of Ca^{2+} resulted in a dramatic decrease in heat resistance, i.e., over 2 log higher inactivation levels, whereas HHP resistance was lowered by approx. 1 log. In contrast, a lack of Mg^{2+} resulted in a slight decrease in heat and HHP resistance. Omitting the addition of Mn^{2+} affected HHP resistance more than heat resistance as observed by an approx. 1.9 and 0.8 log difference in spore inactivation, respectively.

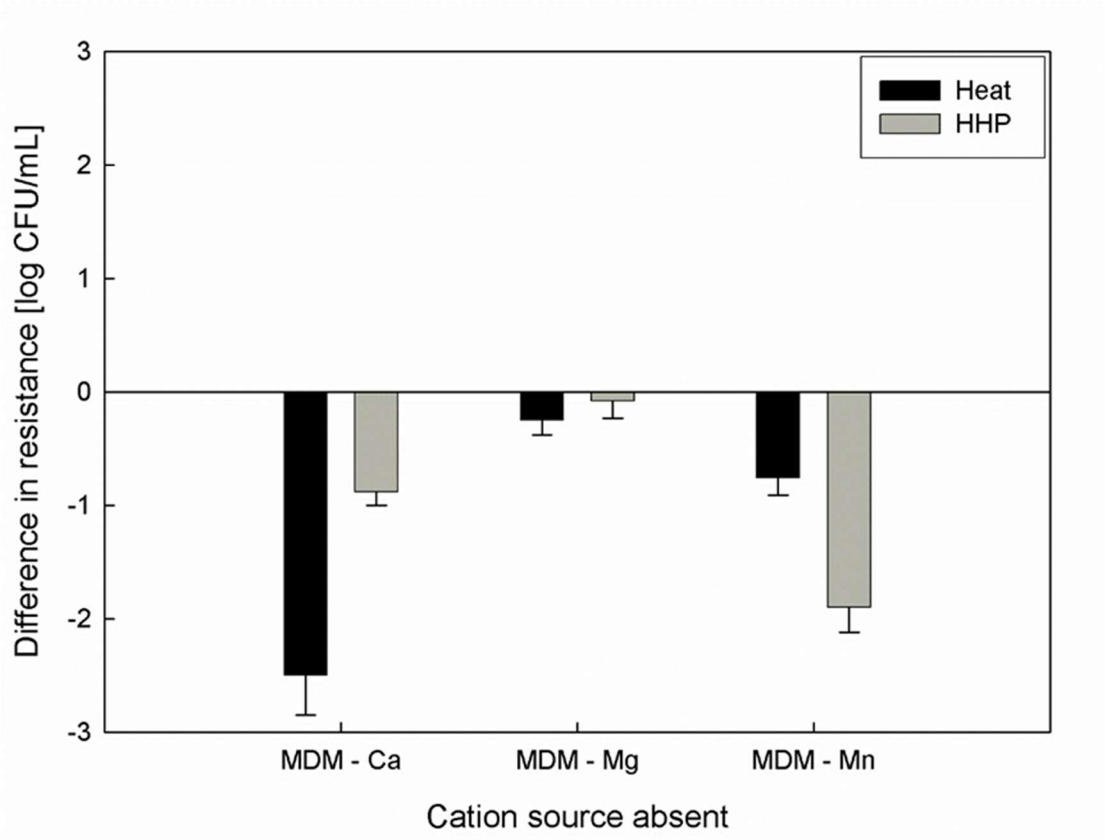


Fig. 4-19: Absence of specific cations and heat/HHP resistance.
Comparison of *C. botulinum* TMW 2.990 spore inactivation in standard MDM und MDM deficient in a single type of cations. Bars indicate [(log reduction of standard MDM spores) – (log reduction of cation deficient MDM)]. Thus, negative values indicate lower spore resistance. 7.5 log initial CFU, heat: 0.1 MPa, 80°C, 10 min, HHP: 800 MPa, 20°C, 10 min.

4.3.1.2.2 Excessive cation addition

A stepwise 10-, 100-, and 200-fold increase of cation amounts in MDM resulted in the production of spores with markedly different resistance properties in a cation type, cation concentration, and stress type-dependent manner. Additionally, high Ca^{2+} contents speeded up sporulation slightly, while high Mg^{2+} and Mn^{2+} concentrations did not lead to large alterations in sporulation rates.

Spore heat resistance steadily increased with increasing Ca^{2+} concentration in the sporulation medium from 10 to 100 to 1000 mg/L. A further increase to 2000 mg/L did not lead to the production of significantly more heat resistant spores compared to spores grown in MDM with 1000 mg/L Ca^{2+}. In contrast, an increase of Ca^{2+} in the sporulation medium had a slightly negative effect on HHP resistance with spores grown in the presence of 2000 mg/L being significantly less HHP resistant than spores obtained from medium supplemented with 10 or 100 mg/L Ca^{2+}. Furthermore, an increased Ca^{2+} content led to largely unaltered HPT resistance with concentrations of 1000 and 2000 mg/L being slightly but not significantly more resistant than *C. botulinum* TMW 2.990 spores from medium with 10 mg/L (Fig. 4-20A).

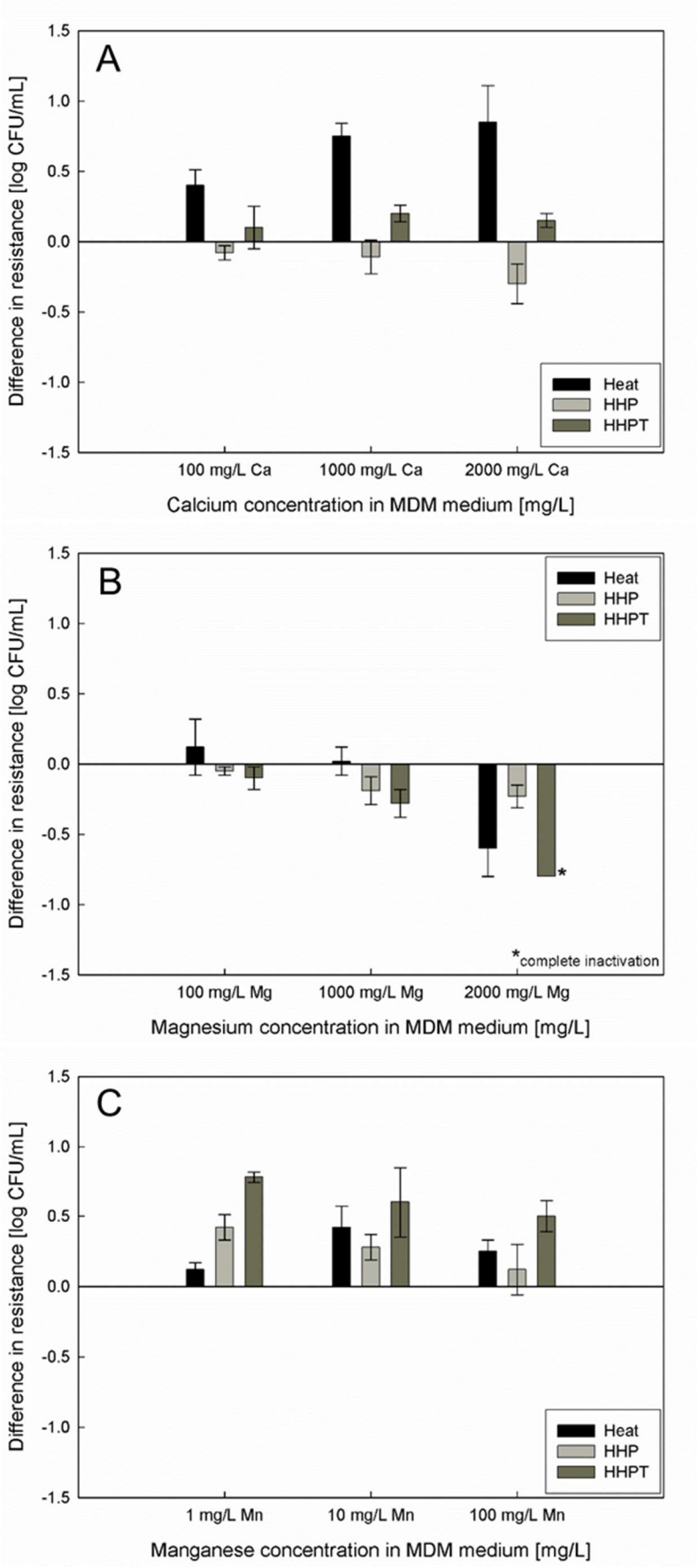


Fig. 4-20: High cation amounts and spore resistance.
Comparison of *C. botulinum* TMW 2.990 spore inactivation in standard MDM and MDM supplemented with additional calcium (A), magnesium (B), and manganese (C). 7.5 log initial CFU, heat: 0.1 MPa, 80°C, 10 min, HHP: 800 MPa, 20°C, 10 min, HPT: 800 MPa, 80°C, 10 min. (Note: to indicate that the

same pressure conditions are prevalent as during HHP treatments and only the temperature was increased (from 30 to 80 °C), HPT treatments are designated as HHPT in the figure legends).

In contrast to Ca^{2+}, high Mg^{2+} levels (2000 mg/L) generally led to decreased spore resistance to all three treatments applied, with the largest effect on HPT resistance (complete inactivation) followed by heat and HHP resistance. At lower concentrations (100 and 1000 mg/L), heat resistance remained largely unaffected, whereas HHP and HPT resistance decreased slightly (Fig. 4-20B).
Increased Mn^{2+} levels generally affected spore resistance positively. Mn^{2+} amounts of 1 mg/mL resulted in the highest increase in HHP and HPT resistance, which tended to decrease when higher amounts were added to the sporulation medium. Heat resistance conferred by the presence of Mn^{2+} during sporulation seemed to peak at 10 mg/L (Fig. 4-20C).
Comparable experiments using the other two strains resulted in slightly lower and slightly higher differences in resistance for spores from strain TMW 2.992 and TMW 2.994, respectively. However, trends observed for these strains were identical to those shown for TMW 2.990 and are, therefore, not depicted.

4.3.2 Interconnection between Sporulation Medium and Temperature

In addition to the effects of sporulation medium, its cation content, and the sporulation temperature as separate influence factors, it was examined, whether the sporulation medium mineral content influences the observed effects of the sporulation temperature on the HPT resistance of spores from the most resistant strain (TMW 2.990). For this purpose, sporulation temperatures (18, 28, and 38°C) and sporulation media (mTPYC, mTPYC-, and mTPYC+) were selected upon existing data and their putatively significant effect on the resistance of spores treated at p/T/t combinations of 800 MPa/40°C/10 min (Fig. 4-21a) and 800 MPa/80°C/5 min (Fig. 4-21b).

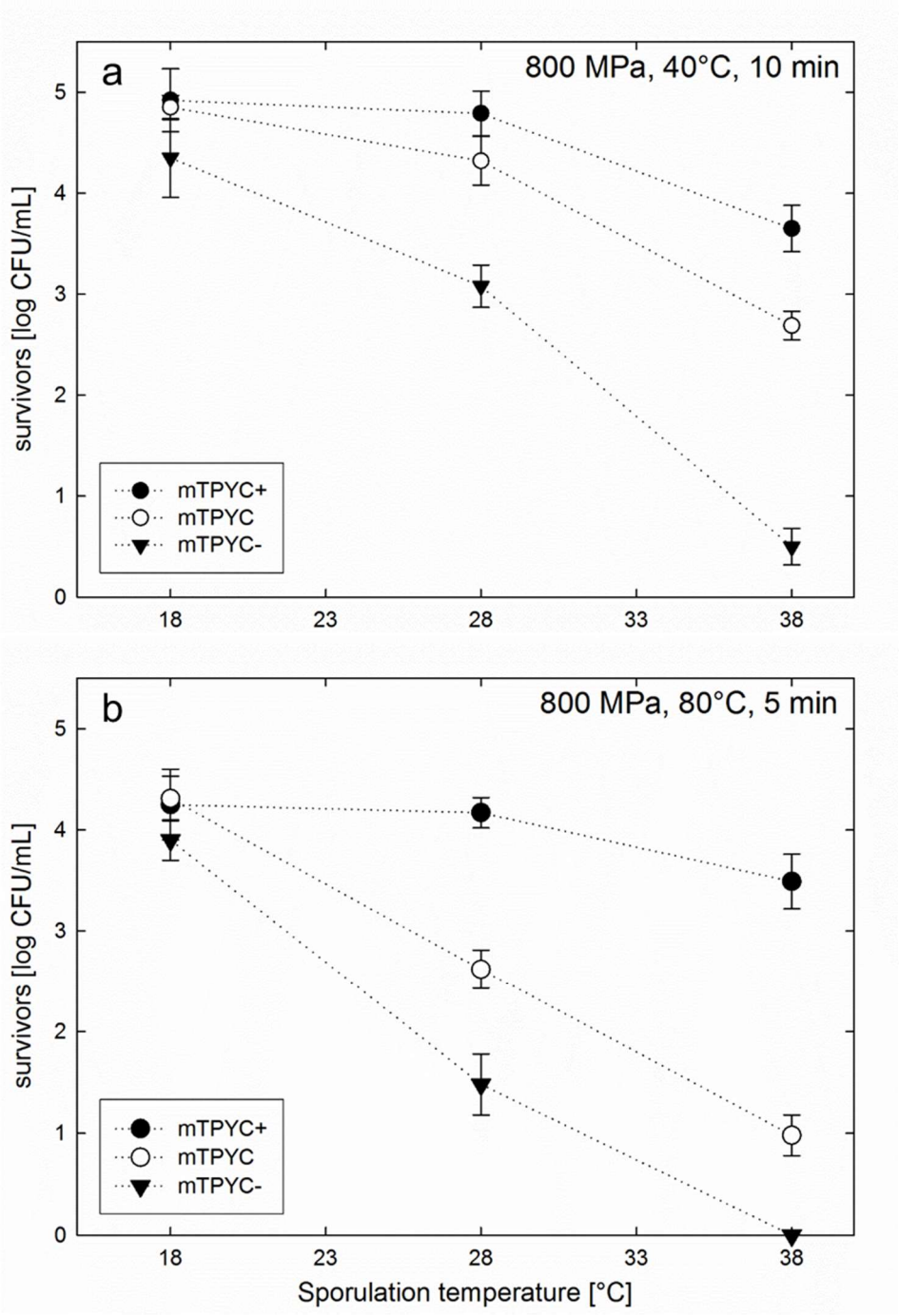


Fig. 4-21: Interconnection of medium cations, temperature, and spore resistance.
Viable spores (CFU/mL) of the *C. botulinum* type E strain TMW 2.990 after pressure treatments at (a) 800 MPa at 40°C for 10 min and (b) 800 MPa at 80°C for 5 min depending on the sporulation temperature and medium, i.e., mTPYC- (solid circles), mTPYC (open circles), and mTPYC+ (solid triangles). Dotted lines indicate tendencies between different sporulation temperatures. Initial spore count: 7 log CFU/mL. Error bars indicate the standard deviation from three independent experiments.

Results indicate that the minerals present during sporulation can significantly affect the extent of sporulation temperature-mediated alterations in sporal HPT resistance. At 28°C, the standard sporulation temperature used in these experiments, medium supplementation with cation concentrations previously shown in this study (Fig. 4-20) to exert a positive effect on sporal HPT resistance indeed protected spores from being inactivated, whereas an adverse supplementation significantly decreased the HPT resistance. When spores were grown at

38°C the positive effect of a putatively optimal and a the negative effect of a putatively unfavorable sporulation medium mineral content tended to be larger compared to differences observed for 28°C spores (Fig. 4-21). When the sporulation temperature was decreased to 18°C, differences in the resistance of spores grown in the different media diminished and HPT resistance was only slightly negatively affected by an unfavorable mineralization. In contrast, the putatively optimal mineralization did not lead to any significant changes in the resistance. Thus, highest resistance to the two treatments applied was provoked by either advantageous mineralization in combination with sporulation at 28°C or sporulation in non-supplemented (or optimally supplemented) medium at 18°C.

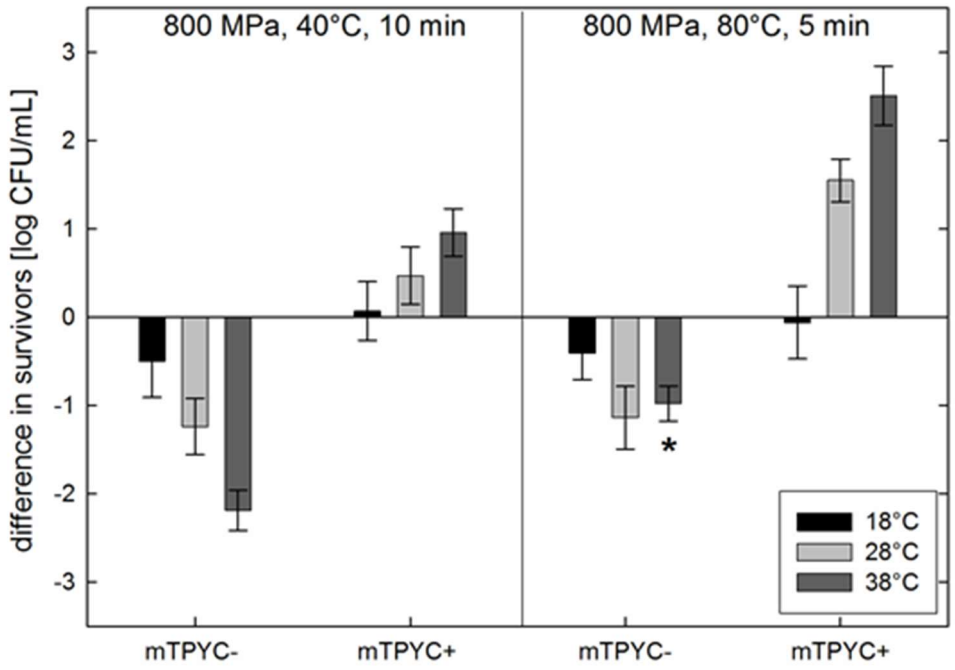


Fig. 4-22: Differential values for cation/temperature/spore resistance interconnection.
This figure is derived from data presented in Fig. 4-21, but expresses difference to facilitate the comparison of effects. Effect of medium supplementation with cations on the HHP resistance of *C. botulinum* type E strain TMW 2.990 spores grown at different temperatures. Indicated as difference between the [number of mTPYC- or mTPYC+ spores] - [number of mTPYC spores] surviving a HHP treatment of 800 MPa / 40°C, 10 min (left) and 800 MPa / 80°C, 5 min (right). Negative values indicate decreased resistance. Different bar colors indicated different sporulation temperatures. Error bars indicate the square root of the sum of error squares. *Note: Complete inactivation of 38°C, mTPYC- spores after 800 MPa / 80°C, 5 min treatments.

4.3.3 Role of Spore Coat in Resistance

4.3.3.1 Decoated Spores

Decoating treatments in a solution containing 0.5% SDS, 0.1 M dithiothreitol, and 0.1 M NaCl and incubated at 37 °C for 1.5 h, did not result in a significantly reduced viability of *C. botulinum* type E (strain TMW 2.990). However, the HPT resistance was reduced significantly resulting an approx. 3 log cycles lower level of survivors after HPT treatments at 800 MPa/40°C and the inactivation of the entire spore population at 800 MPa/80 °C (Fig. 4-23).

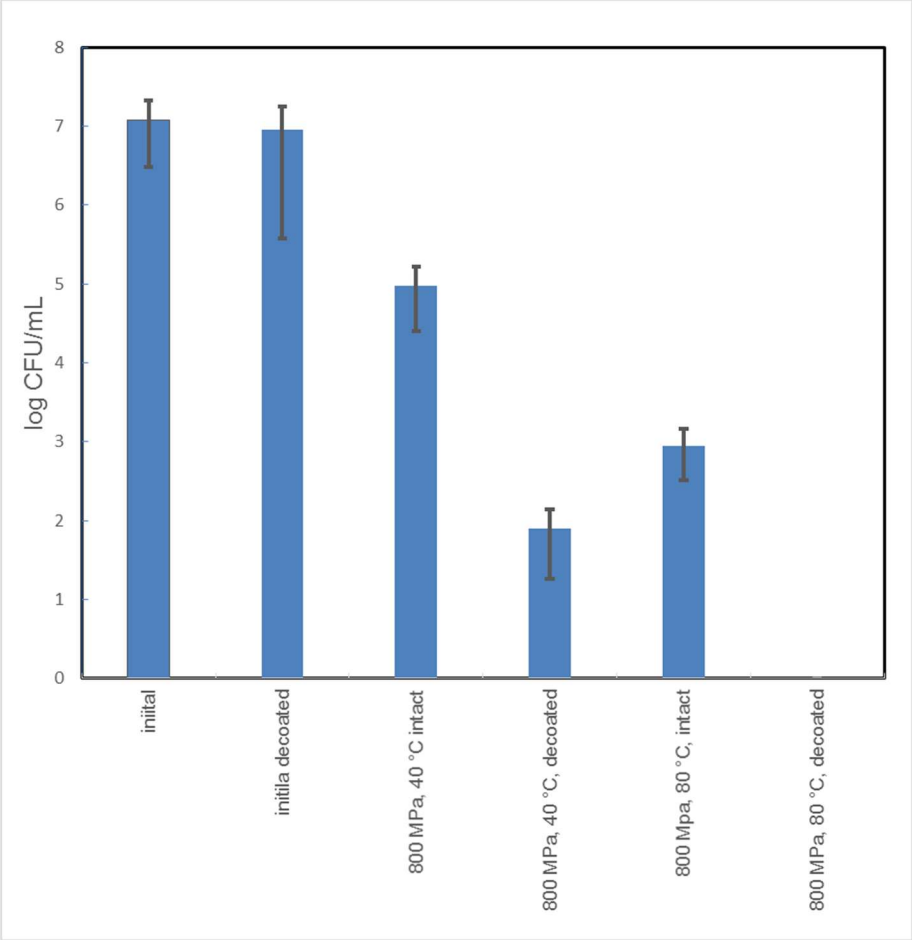


Fig. 4-23: HPT inactivation of decoated spores.
Effect of decoating treatment on the viability (CFU/mL) and the HPT resistance of *C. botulinum* type E strain TMW 2.990 spores.

4.3.3.2 Expression of Coat-Related Genes

From the ten investigated coat-related genes, only three genes (*spoIIIAH*, *yabG*, and *sodA*) were significantly affected in their expression levels. Only one gene under one condition was reduced in its expression, i.e., *sodA* upon “optimal” mineralization conditions (-5.6-fold). In contrast, a decrease in the sporulation temperature resulted in an almost 7.7-fold overexpression, whereas a combination of increased cation amounts in the medium and a lower temperature resulted in a slight (not significant) overexpression of the identical gene. Genes coding for SpoIIIAH and YabG, were overexpressed under any conditions tested, where the highest values were obtained for *spoIIIAH*, i.e., 12.3-fold at 20 °C/in mTPYC medium, 16.7-fold at 28 °C/in mTPYC+ medium, and 15.6-fold at 20 °C/in mTPYC+ medium. The respective overexpression of *yabG*, was 5.3-fold, 6.0-fold, and 5.7-fold under the three conditions tested relative to the reference conditions 28 °C/mTPYC. The relative expression values for all genes and all conditions tested are summarized in Tab. 4-3.

Tab. 4-3: Effect of sporulation conditions on coat-associated gene expression.
Effect of a sporulation temperature decrease (20 °C, mTPYC), "optimal" sporulation medium mineralization (28 °C, mTPYC+), or both (20 °C, mTPYC+) on the expression of coat-associated genes in *C. botulinum* strain TMW 2.990. Relative expression values were calculated using (Eq. 3-7. *16rRNA* and *spo0A* (for *sps* genes only 16rRNA) were used as reference. Relative expression values for the different conditions were then divided by relative expression values found at standard sporulation conditions (28 °C, mTPYC). Values in the table are provided as x-fold over-/or underexpression (e.g., a value of 0 means equal expression levels as in standard samples, and a relative expression level of 0.5 would be designated as -2-fold in the table). Dark green significant differences with > 2-fold overexpression upon temperature decrease or cation availability. Light green: slight overexpression > 1-fold overexpression. Yellow: no significant effect on expression level. Light red: slight underexpression, > 1-fold. Dark red: Significant underexpression: > 2-fold. ± Values indicate the standard deviation of differences found in three independent experiments.

Gene	20°C mTPYC	28 °C mTPYC+	20 °C mTPYC+
spoIIIAH	12.3 ± 0.5	16.7 ± 1.1	15.6 ± 0.7
spoIVA	1.7 ± 1.3	0.5 ± 0.7	1.6 ± 0.5
cotH	1.2 ± 0.8	0.7 ± 0.6	1.5 ± 0.9
cotS	-1.7 ± 1.3	0.5 ± 0.7	-1.0 ± 0.7
coat protein S	0.2 ± 0.2	0.5 ± 0.9	-0.2 ± 0.5
yabG	5.3 ± 0.8	6.0 ± 0.6	5.7 ± 0.5
sodA	7.7 ± 1.4	-5.6 ± 0.8	1.8 ± 1.2
spsE	-0.4 ± 0.2	1.2 ± 0.4	0.9 ± 0.6
spsC	-0.2 ± 0.5	1.1 ± 0.7	0.8 ± 0.7
sleB	0.4 ± 0.3	-0.5 ± 0.2	-0.2 ± 0.5

5 DISCUSSION

Results of this study allow the refinement of initial working hypotheses (►) described in section 2.2 (►), based on which further theses in a wider sense (►) as well as more radical and highly speculative theses (►) can be put. Refined working hypotheses and theses (including some rather provocative theses (►)) are listed below, where differences between initial and refined working hypotheses are indicated by underlined key words.

Influence factors on HPT inactivation:

- ► Sporulation medium composition and temperature influence the HPT resistance of *C. botulinum* type E spores. Conditions that are putatively closer to those found in the natural habitat of this organism (SFE medium (4.1.1) and low temperatures (4.1.2)) lead to an increased resistance, which complicates proper risk assessment. Such effects are can be found in other spore formers, but their extent is species-dependent.
- ► Resistance properties of *C. botulinum* type E spores are significantly reduced only by heat treatments applied during spore suspension purification and slightly change over time during long-term storage.
- ► Sample dilution and recovery conditions do not significantly change the outcome of inactivation studies using *C. botulinum* type E spores and do not need special attention in risk assessment.
  - ► General predictions from existing data on influence factors during sporulation in one species for the behavior of spores from another species cannot be easily drawn, which putatively reflects the adaptation to ecological niches and different habitats.
  - ► The application of heat purification or activation treatments before HPT treatments leads to an overestimation of spore lethality.
    - ► The majority of inactivation levels of spores from pathogenic species reported today to be achievable by a specific HPT treatment are too high leading to an overestimation of food safety.
      - ► The worldwide average incidence of foodborne illness associated with pathogenic spore-forming species would increase by 50% shortly after the industrial implementation of HPT technology (ignoring that the safety margin of a 12D-concept for *C. botulinum* might be sky high anyway).

Pressure/temperature-dependent inactivation:

- ► The absence of pronounced tailing effects in inactivation kinetics enables a rapid inactivation of *C. botulinum* type E spores by HPT treatments at relatively mild temperatures.
- ► Specific combinations where *C. botulinum* type E spores are stabilized do apparently not exist at high pressure levels up to 1.2 GPa.
- ► Low pressure levels induce germination in *C. botulinum* type E spores, though to a very low extent.
- ► Times to detect growth form individual *C. botulinum* type E spores are heterogeneous, and treatments at low pressure levels affect such times and, thus, have an impact on food safety considerations.
  - ► The sequence of physical treatments consisting of the sequential application of a heat treatment before (4.1.3.1) or after (4.2.3) a HPT treatment is decisive

for the success of two-step processes involving mild heat and a HPT step to inactivate spores.

- Low efficiency of germination induction at low pressure levels makes two-step approaches involving a mild HHP treatment before a heat treatment likely unsuitable for achieving stipulated log-inactivation levels of *Clostridium* spores.
- Two-step approaches involving a mild HHP treatment after a heat treatment have the potential to induce germination of dormant survivors and increase the risk originating from pathogenic *Clostridium* spores.
  - Two-step processes involving a mild HHP treatment should be generally avoided when *Clostridium* spores are among the target organisms.
- Rapid inactivation without significant tailing of survivor curves or areas of spore stabilization makes HPT treatments excellent as alternative process to replace conventional heat sterilization processes.
  - The exploitation of adiabatic heating and synergistic effects of heat and pressure on spore inactivation, enables the production of a variety of safe food with superior nutritional and sensory characteristics.
    - Increased shelf-life will decrease the amount of food waste and eliminate food shortage worldwide. Together with the preservation of nutritionally valuable substances this leads to the worldwide availability a healthy food products.
      - The worldwide average life expectancy will increase by 50% after the industrial implementation of HPT technology.

- HPT inactivation kinetics of *C. botulinum* type E are completely different. Despite of some commonalities in the behavior of *B. subtilis* and *C. botulinum* type E under pressure there are obviously large differences in the underlying mechanisms.

  Commonalties include that
  - the ability to retain DPA is of major importance for HPT resistance
  - CLEs are not required for a rapid, non-physiological DPA release
  - p/T zones with differences in the inactivation mechanism overlap

  Major differences include that *C. botulinum* type E shows a
  - low effectiveness of low pressures to induce germination
  - faster inactivation and DPA release at >500 MPa/>60 – 70 °C
  - no complete retardation of inactivation at >500 MPa/<60 – 70 °C
    - Numerous influence factors on the experimental setup level, species-, genera-, and p/T/t-dependent inactivation pathways, and, last but not least, complex effects of the food matrix on spore inactivation impede a reliable estimation of HPT processes to provide food safety.
    - A generic approach for the evaluation of HPT inactivation of bacterial endospores is not possible.
    - Inactivation data from non-pathogenic model organisms (surrogates) can never serve as basis for the evaluation of food safety provided by a specific process.

- Spores from different *C. botulinum* type E strains appear to possess no largely different HPT resistance properties.
  - *C. botulinum* type E constitutes a homogenous group forming spores with very similar resistance properties.

- True surrogate organisms for *C. botulinum* type E do not exist.

Characterization of medium/temperature effects:

- Calcium, magnesium, and/or manganese concentrations in the sporulation medium significantly influence the HPT resistance of *C. botulinum* type E spores.
  - The effect of cations on spore resistance are dependent on the stress type (heat, HHP, HPT), cation type, and concentration.
  - The presence of calcium is most important for heat resistance, whereas manganese is most important for HHP resistance.

- Effects of sporulation temperature and medium cation contents on the HPT resistance of *C. botulinum* type E spores are interconnected.
  - Decreased temperature and disadvantageous cation contents and, the other way round, increased temperature and advantageous cation concentrations compensate for each other's negative effects on spore resistance.
  - A simultaneous decrease in the temperature and the presence of advantageous cation concentrations does not further enhance spore resistance.
    - The interconnection effects of medium cation contents and sporulation temperature on the HPT resistance of *C. botulinum* type E spores is related to the temperature-dependent uptake of cations in the core.
    - Compensation of adverse effects and the absence of synergistic effects between medium cation contents and sporulation temperature is due to their action on an overlapping subset of targets in *C. botulinum* type E spores.

.

- The coat of *C. botulinum* type E spores is likely to play a role in their HPT resistance.
  - Decoated spores show decreased resistance to HPT treatments.
  - The gene sodA is upregulated at lower temperatures but downregulated in the presence of advantageous cation contents.
  - Genes coding for the coat-associated proteins SpoIIIAH and YabG are significantly overexpressed in the presence of advantageous cation concentrations, low sporulation temperature, and a combination of both.
    - Increased activities of the Mn-dependent cross-linking-associated enzyme, SodA, is unlikely to present a HPT resistance factor in *C. botulinum* type E spores, but it can play a role in manganese being the most important cation for HHP resistance.
    - SpoIIIAH and YabG are part of a molecular subset affected by both cation contents and temperature and resistance factors for the HPT resistance of *C. botulinum* type E spores.
    - A predetermined breaking point in the coat marked by SpoIIIAH, the structure of the innermost coat layer influenced by SpoIIIAH, and/or the cross-linking degree of the coat enhanced by the action of YabG play a role in HPT resistance.

Results obtained in this study in the context of findings from previous studies as well as pros and cons speaking for or against a particular thesis stated above are discussed below.

5.1 Influence Factors on HPT Inactivation

To be able to accurately assess the inactivation *C. botulinum* Type E spores by HPT treatments, several factors have to be considered which might influence determined inactivation rates. Essentially, such factors can be divided in five groups (section 1.5.7, Fig. 1-23), i.e.,(i) factors that affect the stress resistance of the whole or a part of the spore population during their development, i.e., conditions prevalent during sporulation such as sporulation temperature and medium, (ii) factors that can modify the HPT resistance of spores, e.g., during purification or storage of spore suspensions, (iii) factors that do not affect spore resistance properties but influence the apparently "measured" resistance, e.g., recovery conditions that may influence the quantification of survivors, (iv) factors that have an unintended influence on the HPT process intensity, e.g., the occurrence of large temperature peaks due to adiabatic heating, (v) protective or synergistic effects of compounds in the matrix surrounding spore during a HPT treatment. The experimental setup in this study was selected to minimize the effect of influence factor related to categories (iv) and (v). The putatively major factor related to groups (i), (ii), and (iii) were assessed in this study for their potential effects on the outcome of HPT spore inactivation studies using *C. botulinum* Type E spores.

5.1.1 Sporulation Medium

5.1.1.1 Strain-Dependent Differences in Heat and Pressure Resistance

Since the effects of the sporulation medium was planned to be tested not only on pressure, but also on heat resistance, the first experiments were conducted to assess the effects of temperatures between 20 and 80 °C combined with pressure of 0.1 up to 800 MPa for constant dwell time of 10 min. Data shown in Fig. 4-1 indicates that HPT treatments are capable of inactivating spores from all three *C. botulinum* type E strains tested. Synergistic effects between pressure and temperature appear to be treatment intensity-dependent, but can already begin to occur at 200 MPa (in the case of 60 °C treatments).

Available data on high pressure resistance of spores from different *C. botulinum* types indicate that the (heat and HHP) resistance is generally in the order proteolytic types A, B > non-proteolytic type B > (sensitive type B strains) ≥ non-proteolytic type E (Margosch et al., 2004a; Reddy et al., 2003; Reddy et al., 2006). In this initial experiment, levels found for the inactivation of *C. botulinum* type E spores clearly exceed those previously found for other *C. botulinum* types (Margosch et al., 2004a; Reddy et al., 2003; Reddy et al., 2006), which is in accordance with the general order mentioned above.

Comparable data on the HPT inactivation of non-proteolytic *C. botulinum* type E are scarce. In the beginning of this study, there was only one older study published where the HPT inactivation of *C. botulinum* type E spores was tested in a limited range of p/T combinations under not precisely specified process conditions (Reddy et al., 1999). Inactivation levels found here were slightly lower than to those reported earlier for type E strains Beluga and Alaska (Reddy et al., 1999), where results from high pressure treatments up to 827 MPa (5

min) of two type E strains (Alaska and Beluga) in phosphate buffer (67 mM, pH 7) indicated that (i) spores from both strains can hardly be inactivated at temperatures below 35 °C, or pressure levels of 689 MPa (35 – 50 °C, 5 min) and below (not shown), (ii) slight inactivation (below 1.5 log CFU) can start to occur at temperatures between 35 and 40 °C (strain-dependent), and (iii) Beluga spores are constantly more resistant than Alaska spores (Reddy et al., 1999). Similarly, we found that higher inactivation levels are achieved with increasing pressure, temperature, and holding time, that high pressure levels combined with elevated temperatures are required to effectively inactivate type E spores, and that Beluga spores are relatively resistant, i.e., more resistant than spores from the other two strains tested.
The maximum difference in the amount of type E spores surviving a 10 min treatment found here was around 2.5 log CFU (strain TMW 2.990 (Beluga) vs. TMW 2.994), depending on the pressure level and temperature applied in our study, which is in the above mentioned range for strains Beluga and Alaska, i.e., approx. 2 log CFU difference for many treatment conditions (Reddy et al., 1999). This is in contrast to the vast strain-dependent differences reported earlier for non-proteolytic (Reddy et al., 2006) and proteolytic (Margosch et al., 2004a) *C. botulinum* type B spores. Interestingly, there seem to exist sensitive non-proteolytic type B spores that possess similar resistance properties type E spore by means of their inactivation levels and their protection by food matrix (Reddy et al., 2006). On the other hand, there are highly resistant non-proteolytic type B spores (Reddy et al., 2006) that are still generally less resistant than spores from proteolytic type A (Reddy et al., 2003), but share some traits such as the low protective effect of food matrix on their inactivation. In summary, data from this and previous studies indicate that *C. botulinum* type E might present a rather homogeneous group with respect to their high pressure resistance properties when compared to other *C. botulinum* types. Naturally, the number of strains tested so far is too low to draw definitive conclusion regarding this issue.

5.1.1.2 Correlation between Sporulation Velocity and Pressure Resistance

The time span required for similar percentages of sporulated *C. botulinum* type E cells in the low resistance medium M140 was significantly shorter than that in the high resistance medium SFE. However, sporulation velocity does not correlate with the observed HPT resistance over the full range of media used, as resistance tended to be in the order M140 < AEY < BCM = A1 < TPYC < SFE (not all differences significant, Tab. 4-1), whereas sporulation speed tended to be in the order BCM > AEY > A1 > M140 > TPYC > SFE. Nonetheless, there is a clear trend observable, i.e., that shorter sporulation seems somehow to be connected to a reduced high pressure resistance. As perturbations of the sporulation phosphorelay were shown to lead to shorter sporulation and reduced spore heat resistance of *B. subtilis* mutant strains (Veening et al., 2006), the effect observed here might be related to sporulation medium-dependent effects on the ordered course of the developmental program during sporulation (section 1.2.2.3.2).

5.1.1.3 Sporulation Medium State of Matter and Spore Resistance

Results further indicate that there is virtually no difference in the HPT resistance of *C. botulinum* type E spores grown in liquid TPYC or on TPYC agar plates. Similarly, it has been shown for other spore formers that the use of solid media has no significant influence on the sporulation of *C. perfringens* (De Jong et al., 2002) and does not affect the low (10 °C) sporulation temperature-mediated decrease in heat resistance of *B. weihenstephanensis*

spores (Baril et al., 2011)). In contrast, the medium state of matter has previously been reported to significantly affect sporulation characteristics of *Clostridium* sp. RKD (biphasic media stimulated spore production; (Dixit et al., 2005)) and *C. botulinum* type E (inclusion of particulate material in the agar phase, e.g., soil, vegetable matter, meat/fish chunks suggested to be advantageous (Bruch et al., 1968)). Furthermore, the use of solid agar plates as compared to liquid media with the same organic nutrient composition has been described to significantly influence sporulation (Ryu et al., 2005), germination properties, spore structure (Faille et al., 2007), and resistance properties (Rose et al., 2007) of *Bacillus* spores. However, results from this study indicated that differences in nutrient or water availability occurring on agar plates vs. liquid medium do not significantly affect the HPT resistance of *C. botulinum* type E spores.

5.1.1.4 Effect of Sporulation Medium Composition on Spore Resistance

As shown in Fig. 4-2, the sporulation medium composition can significantly influence the HPT resistance of *C. botulinum* type E spores. Reasons for such effects are unknown. However, explanations for altered spore properties and resistance in response to sporulation in different media might be found in variations of the composition or structure of the spore components modulating the ability of spores to retain DPA and the dehydrated state of the core.
Consistent with the results found here, early studies showed that the sporulation of non-proteolytic *C. botulinum* type E (Roberts, 1965), and, of mesophilic *Clostridium* species in general, strongly depends on the strain examined and the type of sporulation medium used. It has also been found that a chemically defined medium compared to complex TPGY differentially affects growth and sporulation of *C. botulinum* type E (unaltered efficiency but faster sporulation, i.e., 3 h instead of 9 h), which seems not to be the case for other *C. botulinum* serotypes or *C. sporogenes* (Hawirko et al., 1979; Peck et al., 1992). Additionally, the type of medium has been described as decisive for the heat resistance of *C. botulinum* type A (Tsuji and Perkins, 1962) and *Clostridium* sp. RKD spores (Dixit et al., 2005). However, the correlation between sporulation medium and high pressure resistance of *C. botulinum* type E spores has, until now, not been investigated. In this study, TPYC was used, which only differs from TPYG (Hawirko et al., 1979; Peck et al., 1992) in the amount of sugar sources added, and led to the production of HPT resistant type E spores. Only spores grown in SFE tended to be more resistant.

5.1.1.5 Production of Resistant Spores in SFE

Remarkably, the sporulation medium SFE conferred the highest resistance regardless of the strain tested. As sediment fish extract was used as a base for SFE, this medium partially resembles the natural environment of *C. botulinum* type E. Consistent with the results presented here, it has previously been found that the soil extract medium, WSH, promotes proper sporulation and leads to the formation of HPT resistant *C. botulinum* type A spores (Margosch et al., 2006; Margosch et al., 2004a). Since it is considerably difficult to collect sufficiently high numbers of spores from their natural habitat, the production of laboratory spore crops is apparently indispensable for the conduction of resistance studies (Carlin, 2011). Thus, media such as SFE could be appropriate for the production of *C. botulinum* type E spores to be used in HPT resistance studies, although they still represent laboratory spores and the relationship between spore formers and their natural environment is likely to be more complex than that under laboratory conditions due to factors such as varying temperature,

humidity, redox conditions, spore density (Siala et al., 1974), and interacting micro- and macroflora (Carlin, 2011).

It can only be speculated on mechanisms how SFE contributes to the production of highly resistant spores. Generally, the modification of sporulation conditions can lead to perturbations in spore assembly and alterations in structural features of spores, such as membrane fluidity, coat structure, or protoplast composition, which can affect spore germination and resistance properties. However, as the sporulation media used in this study were complex in composition (Tab. 3-10) and considerably different in the amounts of the single ingredients added, it conclusions on compounds exerting positive or negative effects on the HPT resistance of *C. botulinum* type E endospores cannot easily be drawn. Factors that could be excluded from such considerations are the effects of inoculum, culture volume, external atmosphere (constant in the anaerobic cabinet), and medium pH (adjusted to 7.2 ± 0.1 at 20 °C in all media tested). Additionally, all ingredients were obtained from a specific manufacturer (except polypetone for M140) and taken from the same batch. Thus, it can be assumed that they were similar in composition. In earlier studies, it was shown that sporulation characteristics, spore dormancy, and resistance properties can be influenced by various compounds present during sporulation, including single amino acids (Perkins and Tsuji, 1962), peptone source (even for peptones from different manufacturers, (Roberts and Ingram, 1965)), sugar type (De Jong et al., 2002) and concentration (Decaudin and Tholozan, 1996), as well as organic and fatty acids (Sugiyama, 1951). Such factors cannot be excluded to contribute to the observed effect on HPT resistance.

5.1.2 Sporulation Temperature

Since effects of the sporulation temperature detected in this study were strongly dependent on the HPT treatment conditions (Fig. 4-3), interrelations are discussed separately for the parameter combinations used.

Low pressure / moderate temperature treatments (200 MPa / 40°C)

Sporulation temperature did not significantly influence spore inactivation levels achieved by low pressure / moderate temperature treatments (200 MPa / 40°C) (Fig. 4-3a). This was observed for all three strains tested, and is in accordance with earlier results for *B. cereus* spores (Raso et al., 1998a). In contrast, it has been reported that low and high sporulation temperatures, respectively, impair and enhance *B. subtilis* germination induced by a pressure/temperature combination of 150 MPa and 37°C (Black et al., 2005). Although, sensitivity to pressure-induced germination does not necessarily correlate with pressure inactivation, especially at low pressures and ambient temperatures, this indicates that the sporulation temperature can influence the resistance of *B. subtilis* spores to pressure treatments with mild processing conditions. At a higher pressure level (350 MPa / 40°C), low sporulation temperatures were reported to lead to a reduced HPT resistance of *B. subtilis* spores (Nguyen Thi Minh et al., 2011), while increased sporulation temperatures exerted no significant effect on the HPT resistance of *B. subtilis* spores (Nguyen Thi Minh et al., 2011). However, available data indicates that there are significant species-specific differences in the effect of sporulation temperature on the resistance of bacterial endospores to low pressure / moderate temperature treatments. Germination is thought to presents the first step in the inactivation of spores by pressure treatments (at least well characterized in Bacillus spores,

(section 1.5.3)). Although it was reported that physical DPA channel opening could start to occur at pressure levels as low as 200 MPa and temperatures below 50°C in *B. subtilis* spores (Reineke et al., 2012), it is generally assumed that lower pressure levels at moderate temperatures primarily induce a germination pathway similar to the physiologic, nutrient-triggered germination. Provided that this similar in *C. botulinum* type E spores (which is in fact unknown), results presented here point towards unaltered germination properties (e.g., germinant receptor activity) of spores grown at different temperatures or changed germination, but no significant differences in the inactivation of germinated spores by a treatment at 200 MPa / 40°C for 10 min.

Low pressure / elevated temperature treatments (200 MPa / 80°C)

At 200 MPa / 80°C, similar to treatments at 200 MPa / 40°C, we did not observe any significant influence of the sporulation temperature on the HPT resistance of *C. botulinum* type E spores (Fig. 4-3b). This has also been observed for the HPT resistance of *B. cereus* spores grown at low temperatures to low pressure / elevated temperature treatments (55 – 150 MPa / 60°C, (Raso et al., 1998a)). However, this has been reported to be not the case when pressure is increased to 200 – 300 MPa / 60°C, where low and high sporulation temperature, respectively, increase and decrease the HPT resistance of *B. cereus* spores (Raso et al., 1998a). Such a negative effect of elevated sporulation temperatures on the HPT resistance has also been reported for *B. subtilis* spores (100 – 300 MPa / 55°C, (Igura et al., 2003)), but was observed only as a slight trend for the two less resistant *C. botulinum* strains TMW 2.992 and 2.994 in this study. At 200 MPa / 80°C, process temperature presumably guides spore inactivation. In contrast to many *Bacillus* spores, where a positive correlation between sporulation temperature and heat resistance is widely acknowledged (at least within an organism-specific sporulation temperature range, e.g., (Condon et al., 1992; Khoury et al., 1987)), heat resistance of non-proteolytic *C. botulinum* type B spores has been reported to remain largely unaffected by changes in the sporulation temperature (Peck et al., 1995a). This points towards species-dependent differences responsible for the divergent results between *Bacillus* and *C. botulinum* type E spores. At moderate pressure and elevated temperature (over 50°C) nutrient-like germination is retarded in *Bacillus* spores presumably due to decreasing germinant receptor activity with increasing treatment temperature (Paidhungat et al., 2002), and rapid DPA release, which does not continue after pressurization, indicates that a direct DPA channel opening occurs (*B. subtilis*, (Reineke et al., 2013b)). Provided that the inactivation mechanism is similar for *Bacillus* and *C. botulinum* type E spores (which is again in fact unknown), unaltered HPT resistance to 200 MPa / 80°C treatments would indicate that the pressure induced DPA release via DPA channels and the ability to retain DPA under such conditions are not significantly affected by the sporulation temperature.

High pressure / moderate temperature treatments (800 MPa / 40°C)

When a higher pressure level at moderate temperature (800 MPa / 40°C) was applied, HPT resistance of *C. botulinum* type E spores grown at low temperatures was not significantly different from that of spores grown at the standard temperature (28°C), whereas elevated sporulation temperatures resulted in significantly decreased resistance (Fig. 4-3c). Low and high sporulation temperatures have been reported to exert similar effects on *B. weihenstephanensis* spores treated at 500 MPa / 34°C (Garcia et al., 2010), and *B. cereus* spores treated at 690 MPa / 25°C and 40°C (Raso et al., 1998a), with the exception of a slight

increase in resistance of *B. cereus* spores grown in the cold to 690 MPa / 40°C (Raso et al., 1998a). Most studies comparing heat and pressure resistance show that pressure and heat act synergistically to inactivate spores (Ahn et al., 2007; Bull et al., 2009). At pressure levels over 600 MPa this synergism tends to diminish, and DPA release from *B. subtilis* spores and subsequent inactivation is governed by treatment temperature (Reineke et al., 2013b). Accordingly, high pressure levels (over 400 – 500 MPa) combined with ambient temperatures (up to 40°C) were frequently shown to stabilize spores resulting in low inactivation levels due to a retarded germination (e.g., *B. subtilis*, (Reineke et al., 2012; Wuytack et al., 1998); *G. stearothermophilus*, (Ardia, 2004; Mathys et al., 2009); *B. amyloliquefaciens*, (Rajan et al., 2006a)). Notably, results indicate similar inactivation levels of *C. botulinum* type E spores produced at 28°C by treatments at 800 MPa / 40°C and 200 MPa / 80°C. In contrast, spores produced at higher temperatures showed significantly lower HPT resistance to 800 MPa / 40°C, but not to 200 MPa / 80°C. This indicates that an increased sporulation temperature induces changes in the structure of spores, which are specifically detrimental to their HPT resistance to treatments at elevated pressure levels.

High pressure / elevated temperature treatments (800 MPa / 80°C)

Compared to 28°C, C*. botulinum* type E spores produced at low and high temperatures showed significantly higher and lower HPT resistance, respectively, when subjected to treatments at 800 MPa / 80°C ((Fig. 4-3d). This is in accordance with earlier reports for spores from other organisms treated at similar pressure levels, i.e., *B. cereus* (690 MPa / 60°C, (Raso et al., 1998a)), *B. amyloliquefaciens, B. sporothermodurans*, *B. coagulans* (600 MPa / 110°C, (Olivier et al., 2012)) and *B. subtilis* spores (800 MPa / 70°C, (Margosch, 2004; Margosch et al., 2004b)). Interestingly, the HPT resistance of *C. botulinum* type E spores tended to peak around 18 – 23°C, indicating that there is no linear correlation between sporulation temperature and HPT resistance. Although it was shown that a stabilizing effect on spores can occur at extremely high pressure levels combined with high temperatures (e.g., *B. amyloliquefaciens* spores at 800 – 1200 MPa and 120°C) resulting in a lower inactivation compared to heat treatments alone (Margosch et al., 2006), germination and inactivation rates were frequently reported to strongly and steadily increase with increasing pressure levels combined with elevated temperatures. In *Bacillus* spores, this has been attributed to a direct spore inactivation with physiologic germination being bypassed or at least playing a diminished role (de Heij et al., 2003). Accordingly, it was found here that the process temperature presents an important factor for the effective inactivation of spores at high pressure levels ((Fig. 4-3c,d). This, however, does not mean that the sporulation temperature necessarily exerts a similar effect on the resistance to heat alone and heat combined with pressure. In fact, sporulation temperature and thermal resistance positively correlate with each other in numerous spore-forming species (at least within a species-specific sporulation temperature range). There are only a few exceptions (partially contradictory to results from other studies) where high sporulation temperatures reduced the heat resistance of *B. licheniformis*, *B. weihenstephanensis*, (Baril et al., 2012b), and proteolytic *C. botulinum* spores (Sugiyama, 1951), and high or low sporulation temperatures did not significantly affect the heat resistance of *C. perfringens*, (Rey et al., 1975) and non-proteolytic *C. botulinum* type B spores (Peck et al., 1995a). A positive effect of decreased sporulation temperatures on heat resistance has not yet been reported for any species. Thus, the negative effect of elevated sporulation temperatures on the HPT resistance of *C. botulinum* type E spores found

here, suggests that different resistance factors are important for heat and HHP resistance, which seems to represent a conserved feature among spores from different species, even with largely different inherent resistance properties.

Species-dependent differences in sporulation temperature effects

Species-dependent differences in the effects of specific sporulation temperatures might be related to the different heat stability of the total macromolecular content, e.g., thermophiles are likely to be equipped with more heat stable molecules (e.g., enzymes) than mesophiles. However, since spore macromolecules are generally identical to that in vegetative cells, i.e., not inherently heat resistant (Beaman and Gerhardt, 1986), differences in the spore composition, e.g., the type or amount of protective molecules and the composition or structure of protective spore components, are also likely to play a role in sporulation temperature-mediated effects on spore resistance. Spore resistance properties and, therefore, the amount or structure of molecules or compartments involved in resistance, i.e., resistance factors, have frequently been demonstrated to be highly influenced by various environmental factors during sporulation with temperature playing a prominent role. Notably, the sporulation temperature rather than the growth temperature prior to sporulation is decisive for the development of sporal resistance properties indicating that altered resistance properties are not a result of a process of selecting for fitter or more resistant vegetative cells (heat resistance of *B. subtilis*, (Condon et al., 1992), and *B. weihenstephanensis*, (Baril et al., 2011)). A summary of available data on the effect of sporulation temperatures on the HPT resistance of spores and a comparison with the data found here is provided in Tab. 5-1.

Tab. 5-1: Sporulation temperature effects on HPT resistance

Summary of available data on sporulation temperature effects on the HHP resistance. Unless mentioned explicitly sporulation temperature is indicated on top of each column. (ST) = standard temperature used in the respective study; ↑ = HHP resistance increased; ↓ = HHP resistance decreased; (≈) = HHP resistance unaltered; *B. w.* = *B. weihenstephanensis*, *B. a.* = *B. amyloliquefaciens*, *B. s.* = *B. sporothermodurans*, *B. c.* = *B. coagulans*; *initial temperature prior to pressurization; ** Pressure-induced germination measured. Especially at low pressures at ambient temperatures, sensitivity to pressure-induced germination does not necessarily correlate with HHP inactivation as germination is generally higher, but not all germinated spores are inactivated.

Organism	p [MPa]	T [°C]	Dwell [min]	Matrix	Initial [spores/mL]	Sporulation temperature [°C] 12 (or 13)	20 (or 19 or 18)	23	30 (or 28)	37 (or 33)	44 (or 38)	48 - 50	Reference
B. subtilis	150	37*	1 – 7	Tris-HCl pH 7.5	OD_{600} = 1.0			↑ >20% ger. @ 7 min	↑ >40% ger. @ 7 min	(ST) >80% germinated after 7 min**	↓ 90% ger. @ 7 min		(Black et al., 2005)**
								consistently lower germ. for 1.5 - 7 min			consis. 1.5 - 7 min		
	100	55	30	Dest. water	10^6				(ST) ≈ 1 log reduction	↓ >2 log reduction = lowest resistance	↓ ≈ 1.3 log reduction		
	200	55	30	Dest. water	10^6				(ST) ≈ 1.7 log reduct.	↓ ≈ 3.5 log reduct.	↓ ≈ 3.5 log reduct.		(Igura et al., 2003)
										similar resist. of 37 and 44°C spores			
	300	55	30	Dest. water	10^6				(ST) ≈ 2 log reduction	↓ < 4.5 log reduct.	↓ > 4.5 log reduct.		
										slight difference of 37 vs. 44°C spores			
	350	40	60	Dest. water	10^9 (spray-dried)		↓ (19°C) 3.37 log red.		(ST) ≈ 2.3 log red. (not mentioned)			(≈) (49°C) 2.31 log red.	(Nguyen Thi Minh et al., 2011)
	500	50*	0.5 - 5	Tris–HCl pH 7.5	OD_{600} = 1.0			↓ ≈ 95% germinated after 2.5 min	↓ >90% germinated after 2.5 min	(ST) 75% germinated after 2.5 min**	↑ 50% germinated after 2.5 min		(Black et al., 2007b)**
								consistently between 0.5 and 2.5 min, ≈ identical germination after 5 min					
	800	70	1s -16 min	mashed carrot pH 5.15	10^7				(ST) 2.5 log reduction after 1s		↓ 5 log red. after 1s	↓ (48°C) 6 log red. after 1s (= detection limit)	(Margosch, 2004)

B. cereus	55 - 400	25, 40	15	Mac-Ilvaine buffer pH7	10^8			(≈) log inactivation 0 – 0.5 at 25°C 0 – 2.5 at 40°C	(ST)	(≈) log inactivation 0 – 0.5 at 25°C 0 – 2.5 at 40°C		(Raso et al., 1998)
								inact. level HHP but not spo. temp.-dependent, consistent for 250 MPa, 25°C, 0.5 – 30 min				
	550, 690	25	15	Mac-Ilvaine buffer pH7	10^8			(≈) slightly less than 1 and 1.5 log reduct.	(ST) Slightly over 1 and 1.3 log reduction	↓ Approx. 1.4 and 2.3 log inactivation		
								resistance of 20°C spores marginally higher vs. 30°C spores				
	550, 690	40	15	Mac-Ilvaine buffer pH7	10^8			↑ ≈ 3.1 - 3.4 log red.	(ST) ≈ 4.4 - 5.7 log red.	↓ ≈ 5.3 - 6.5 log red.		
	55 - 150	60	15	Mac-Ilvaine buffer pH7	10^8			(≈) < 0.5 – 2.8 log red.	(ST) < 0.5 – 2.8 log reduction	↓ 1.8 – 3.8 log red.		
	200, 250, 300	60	15	Mac-Ilvaine buffer pH7	10^8			↑ ≈ 3 - 3.2 log red.	(ST) 3.5, 4 and 5.2 log reduction	↓ 3.9, 4.6, 5.4 log red.		
								general trends confirmed for 690 MPa, 40°C, 0.5 – 30 min				
	400 - 690	60	15	Mac-Ilvaine buffer pH7	10^8			↑ 3.7 – 6 log red.	(ST) 6 – over 7 log reduction (detect limit)	↓ 6 – over 7 log red. (below detect. limit)		
								general trends confirmed for 690 MPa, 60°C, 0.5 – 30 min	400 MPa: resistance 30 ≈ 37°C spores			
B. w.	150	27	0.5	Phosp. buffer pH 7.4	OD_{600} = 0.6-1.0	↑ 50% germination	↑ 15% germination		(ST) >99% germination**			(Garcia et al., 2010)**
	500	34	2	Phosp. buffer pH 7.4	OD_{600} = 0.6-1.0	(≈)	(≈)		(ST) 35% germinated**			
B. a.	600	110	2 - 3	Nutrient agar	10^5 to 10^6 /g				↑ D = 4.0 (3 min)	(ST) D value 0.58 (2 min dwell)		(Olivier et al., 2012)
B. s.	600	110	0.75	Nutrient agar	10^5 to 10^6 /g				↑ D value 0.24	(ST) D value 0.14		
B. c.	600	110	4 - 6	Nutrient agar	10^5 to 10^6 /g					(ST) D value 0.5 6 min dwell	↓ (50°C) D = 0.24 (4 min)	
C. botulinum type E	200	40	10	IPB buffer pH 7	5×10^7	(≈) (13°C) (0.5 log inactivation)	(≈) (18°C) (0.5 log inactivation)	(≈) (23°C) (0.6 log inactivation)	(ST) 28°C (0.6 log inactivation)	(≈) (33°C) (0.9 log inactivation)	(≈) (38°C) (1 log inactivation)	(Lenz and Vogel, 2015)
	200	80	10	IPB buffer pH 7	5×10^7	(≈) (13°C) (2.4 log inactivation)	(≈) (18°C) (2.4 log inactivation)	(≈) (23°C) (2.5 log inactivation)	(ST) 28°C (2.6 log inactivation)	(≈) (33°C) (2.8 log inactivation)	(≈) (38°C) (2.7 log inactivation)	
	800	40	10	IPB buffer pH 7	5×10^7	(≈) (13°C) (2.7 log inactivation)	(≈) (18°C) (2.6 log inactivation)	(≈) (23°C) (2.7 log inactivation)	(ST) 28°C (3 log inactivation)	↓ (33°C) (3.6 log inactivation)	↓ (38°C) (4.6 log inactivation)	
	800	80	1 - 10	IPB buffer pH 7	5×10^7	↑ (13°C) (4.9 log inactivation)	↑ (18°C) (4.6 log inactivation)	↑ (23°C) (4.7 log inactivation)	(ST) 28°C (6.5 log inactivation)	↓ (33°C) (7.5 log = complete)	↓ (38°C) (7.5 log = complete)	

The listed results indicate that this effect is frequently treatment intensity-, i.e., p/T-dependent, but largely independent from the treatment duration. Although there can be exceptions (Black et al., 2007b) and the sporulation temperature does not significantly influence pressure-mediated inactivation at many process conditions, spore resistance generally tends to decrease with increasing sporulation temperatures (Black et al., 2005; Garcia et al., 2010; Igura et al., 2003; Margosch, 2004; Nguyen Thi Minh et al., 2011; Olivier et al., 2012; Raso et al., 1998a).

Possible explanations for sporulation temperature effects on resistance

In addition to the medium, the sporulation temperature is putatively one of the most important and certainly one of the best characterized influence factors during sporulation and was shown to affect a large variety of spore properties of both *Bacillus* and *Clostridium* spores. In spores of different *Bacillus* species, sporulation temperature has been reported to negatively correlate with (i.e., low temperatures increase) surface roughness (Lindsay et al., 1990), spore size (Baweja et al., 2008; Garcia et al., 2010), wet density (Lindsay et al., 1990), the percentage of L-alanine substitution in the cortex peptidoglycan (at least slightly, (Atrih and Foster, 2001a; Melly et al., 2002)), the level of at least one coat protein (Melly et al., 2002), and the overall size and integrity of the exosporium (Faille et al., 2007). For both *Bacillus* and *Clostridium* species, it was shown that spore properties negatively correlating with the sporulation temperature include the core water content (Beaman and Gerhardt, 1986) (Melly et al., 2002; Paredes-Sabja et al., 2008a; Popham et al., 1995b) (not significantly, (Atrih and Foster, 2001a)), and the degree of unsaturation of the inner membrane (Aguilar et al., 1998; Evans et al., 1998; Gaughran, 1947; Peck et al., 1995a) (not significant (Planchon et al., 2011)).

Furthermore, sporulation temperature has been reported to positively correlate (i.e., low temperatures decrease) with the core/(core+cortex) volume ratio (Lindsay et al., 1990), the DPA content (no linear relationship, (Lindsay et al., 1990)) (Planchon et al., 2011) (not significant, (Melly et al., 2002)) (only at elevated temperatures, (Baweja et al., 2008)), the mineral contents (not linear, (Igura et al., 2003)) (Lindsay et al., 1990) (not significant, (Atrih and Foster, 2001a)), and cross-linked muramic acid, tripeptide side chains and tetrapeptides in the cortex (slight and sometimes not significant, (Atrih and Foster, 2001a; Melly et al., 2002)). Additionally, a decrease in the sporulation temperature was shown to decrease the average acyl chain length in the inner membrane of *Clostridium* spores (Evans et al., 1998; Peck et al., 1995a).

In contrast, α/β-type SASP levels putatively remain unaffected by the sporulation temperature (Melly et al., 2002; Movahedi and Waites, 2000) and the degree of δ-lactam residues in the cortex tend to peak at the optimum growth temperature (Atrih and Foster, 2001a; Melly et al., 2002).

Since the homeoviscous adaptation of membrane properties is a conserved mechanism among various bacteria, it seems likely that alterations in the inner membrane are involved sporulation temperature-mediated effects. However, at least in *Bacillus*, *des* mutants have been reported to have no significantly altered HPT resistance (Black et al., 2007a; Black et al., 2007b). Alternatively or additionally, the effect of the sporulation temperature on spore components involved in germination could play a role. For example, cold stress was shown to induce σ^B (Mendez et al., 2004) that activates germinant receptor operons (*B.*

weihenstephanensis, (Garcia et al., 2010) and contributes to proper germination (*B. cereus*, (van Schaik and Abee, 2005)). Additionally, effects of the sporulation temperature and divalent cations appear to be interconnected, at least in some *Bacillus* (Igura et al., 2003) and *C. botulinum* type E spores as shown in this study. In addition to the sporulation temperature-dependent uptake of minerals during sporulation, both influence factors may act on an overlapping subset of spore resistance factors. However, underlying mechanisms are far from being completely understood and many other sporulation temperature-mediated alterations in spore properties mentioned above could play a role.
A detailed summary on various effects of the sporulation temperature on spore characteristics is provided in section 1.2.4.1.

5.1.3 Effect of Spore Suspension Purification and Storage

The results obtained in this study are discussed along the main steps of inactivation studies evaluated for their impact on spore HPT resistance in this study, i.e., purification (iii) and storage (iv) of spore suspensions according to Fig. 3-1.

5.1.3.1 Purification

As delineated in the introduction section, the use of spore suspensions that are virtually free of vegetative cells is important for an accurate assessment of the effectiveness of sterilization processes. Although sporulation conditions were carefully selected and generally resulted in a high sporulation efficiency of the *C. botulinum* type E strains used, variations in this efficiency were unavoidable (> 90 up to 99 %). Without applying an additional purification step (Fig. 3-1(iii)A), aeration during harvesting, centrifugation, and repeated wash cycles in distilled water are likely to stress and remove vegetative *Clostridium* cells. However, this did not reliably lead to the complete removal of vegetative cells, which was observed microscopically and is reflected by a decrease in cell counts after heat, enzymatic, and ethanol purification steps (Fig. 4-5).

Sonication

The fact that the detected CFU in sonicated samples are not significantly lower than that in unpurified samples indicates that that this treatment, at least under the conditions used here, does not significantly affect the viability of vegetative cells or spores. In some cases, mean CFU levels even tended to increase. However, this was never significant and would be difficult to explain, since the formation of spore clumps, which could dissociate and cause such an effect, was microscopically not observable for the *C. botulinum* type E spores investigated here.
Sonication treatments are frequently applied in spore inactivation studies before, between, and/or after wash cycles without any further purification steps (e.g., *C. perfringens*, (Goodenough and Solberg, 1972)), or prior to, during, and/or after an enzymatic treatment (*C. sporogenes*, (Yang et al., 2009); psychrotrophic *Clostridium* spp., (Broda et al., 1998); *C. difficile*, (Fraise et al., 2015; Perez et al., 2011); (*G. stearothermophilus*, (Ahn et al., 2015)). Major reasons stated for the application of a sonication step are to accelerate enzymatic digestion of unwanted cell material (30 min, proteolytic *C. botulinum*, (Grecz et al., 1962)), to release of spores from mother cells (Broda et al., 1998) (Yang et al., 2009), and to separate spore agglomerates (Yang et al., 2009), which can hardly be avoided for some organisms

and might cause shoulder formation/lag-phase (e.g., *G. stearothermophilus* (Mathys et al., 2008a) (Mathys et al., 2008b)) and/or tailing (Cerf, 1977)) (Juneja et al., 1995) of survivor curves. Studies, where sonication has been used include some studies on the HPT inactivation of *C. botulinum* including type E (50 % amplitude in an ice waterbath, 20 min, prior to six wash cycles, non-proteolytic *C. botulinum*, including E (Skinner et al., 2014) and proteolytic *C. botulinum* type A (Patazca et al., 2013); before inactivation studies, 20 min, non-proteolytic *C. botulinum* (Juneja et al., 1995)).

Comparable data to that reported here on the effect of sonication treatments is scarce and heterogeneous to some extent, which might be related to species- and/or treatment intensity-specific differences (e.g., application of energy, final temperatures). For example, it has been suggested that, due to a viability loss over time, *C. difficile* spores should be sonicated only small aliquots needed for the actual experiment in a thin walled glass vessel for a maximum of 5 min (Fraise et al., 2015). However, the viability of *B. cereus*, *B. licheniformis* (Burgos et al., 1972), *C. perfringens* (Goodenough and Solberg, 1972), and proteolytic *C. botulinum* spores (for treatments ≤ 3.5 h (Grecz et al., 1962)) has been reported to be not affected by sonication, which seems to be also the case for *C. botulinum* type E spores as determined here (at least under the conditions applied here).

In addition to spore viability, data presented here indicates that sonication treatments do also not significantly affect spore resistance to HPT treatments (Fig. 4-6). However, due to the relatively high sporulation efficiency and, thus, the presence of only a relatively small fraction of remaining vegetative cells after the first three wash cycles, no significant differences between the log reduction values calculated for unpurified, enzymatically treated, sonicated, or ethanol treated spore suspensions that were subjected to HPT treatments were detected. Comparable data is scarce, focused on effects of sonication on heat resistance, and, similar to spore viability, does do allow to draw general conclusion from it. For example, no effect on heat resistance of *C. perfringens* spores (12 min, 75 w, (Goodenough and Solberg, 1972)), but a decreased heat resistance of *B. cereus* and *B. licheniformis* spores (Burgos et al., 1972) were reported. Additionally, a heat shock (80 °C, 10 min) in combination with sonication (but not heat shock alone) has been reported to alter heat resistance properties of *C. perfringens* spores in food (beef slurry, (Evelyn and Silva, 2015)). Reasons for the latter finding are unknown. However, although this is purely speculative, the fact that sonication is capable of partially removing the exosporium (at least from *C. difficile* spores) leaving a thin exosporium layer attached to an otherwise intact spore coat structure and, this way, influencing spore hydrophobicity (Escobar-Cortes et al., 2013) might play a role, since spore might then possibly localize in differentially protective areas within a food matrix. If this would be the case, it would make sonication treatments unsuitable or at least questionable for their use during spore inactivation studies in heterogeneous food products. This would potentially also concern *C. botulinum* type E spore, which possess a large exosporium layer (Hodgkiss and Ordal, 1966). Moreover, data shown here indicates that sonication alone (at least under the conditions applied here) is not suitable for purifying *C. botulinum* type E spore suspensions, since it left vegetative cells still viable as described above (microscopy and Fig. 4-5).

In contrast to sonication, purification steps involving heat, enzymes, and ethanol appear to be technically suitable for the production of pure spore suspensions, since they inactivated the majority of vegetative cells present in cell/spore mixtures (microscopy and Fig. 4-5).

Heat

Heat treatments at pasteurization temperatures (60 – 80 °C; organism-dependent) exploiting differences in the heat susceptibility of spores and vegetative cells has been for long and is still commonly carried out to inactivate vegetative cells (Alcock, 1984). For example, 10 min at 70 °C for *C. difficile* (Perez et al., 2011) (Fraise et al., 2015), 20 min at 75 °C for *C. perfringens* (Juneja et al., 2003) (Evelyn and Silva, 2015), or 10 min at 80 °C for *C. sporogenes* (Membre et al., 2015) and various *Bacillus* species (Tola and Ramaswamy, 2014) (Zhang et al., 2014) (Ahn et al., 2015). Differences in the heat resistance of the relatively susceptible spores from *C. botulinum* type E and their vegetative forms are smaller than those found for other spore-forming species, the temperature for a heat purification step had to be relatively low. Since spore inactivation can begin not directly at, but somewhat above temperatures of 60 °C and the lethality of vegetative cells begins to decrease below that temperature, 60 °C (30 min) were used to inactivate cells in spore suspensions in this study. The heat treatment applied here effectively inactivated vegetative cells, which was observed microscopically and is reflected by the significant decrease in CFU as shown in Fig. 4-5. The fact that the mean values consistently tended to be slightly higher than the theoretically calculated level (lower gray line in Fig. 4-5) does not necessarily have a meaning, since differences were never significant. However, this could also reflect the possibility that superdormant spores, which would normally not germinate in plates, are activated by heat and increase the number of detected CFU/mL. However, data from an earlier study suggest that effects of sublethal heat shocks on germination of *C. botulinum* type E are strain-dependent and were not observed for various strains including Beluga (= TMW 2.990) (60°C for 60 min, (Roberts and Ingram, 1965)).

Nonetheless, heat activation of spores, which could reduce shoulder formation in survivor curves (Palop et al., 1997), has been occasionally stated as additional major reason for applying heat shocks during inactivation studies of spores (Byun et al., 2011) including *C .botulinum* type E spores (60°C, 10 min, (Juneja et al., 1995)). Heat shock requirements that lead to an effective activation of spores can greatly differ among *Clostridium* species, i.e., from around 40°C for psychrophilic (*C. frigoris*, (Yang et al., 2009) (Spring et al., 2003)) to around 80 °C (proteolytic *C. botulinum*, *C. sporogenes*, (Yang et al., 2009)) up to 90 °C (*C. sporogenes*, (Byun et al., 2011)) for mesophilic species. For non-proteolytic *C. botulinum* strains, an effective temperature was reported to lie around 60°C (Juneja et al., 1995), which is the temperature used in this study.

However, a heat step during purification led to a significant decrease in the HPT resistance of *C. botulinum* type E spores regardless of the HPT treatment intensity (800 MPa, 40 or 70 °C, 10 min) and strain used (Fig. 4-6). Except of reports where a heat shock was shown to increase germination induced by very low pressure levels (e.g., 25 – 100 MPa, *Bacillus* species, (Gould and Sale, 1970)), comparable data is scarce and focused on the effect of heat shocks on the heat resistance of spores. For example, sublethal heat shocks have been reported to increase the heat resistance of *C. perfringens*, (Heredia et al., 1997; Juneja et al., 2003) and *B. megaterium* spores (Sedlak et al., 1993), which is the contrary to the drastically decreased HPT resistance observed here. Furthermore, heat shocks were shown to (reversibly) increase the sporulation capacity and counteract strain degeneration in various *Clostridium* species (Ponce et al., 2009) (Kashket and Zhi-Yi, 1995)). Additionally, an accelerated/enhanced germination has been reported for various *Bacillus* species (Evans

and Curran, 1943) (Sogin et al., 1972) (Leuschner and Lillford, 1999) (Wang et al., 2008). For non-proteolytic *C. botulinum* including type E it was shown that the germination rate and final extent of nutrient-triggered germination can be increased, while requirements for non-essential co-germinants and that for optimal germination temperatures are reduced (Plowman and Peck, 2002; Stringer et al., 2011). However such effects appear to be species and even strain-dependent, since no effect on germinability (non-toxigenic, non-proteolytic *C. botulinum*) (Evans et al., 1997) and effects on *C. botulinum* Type E strain VH, but not other strains including strain Beluga (= TMW 2.990) were observed after a heat shock at 60 °C for 60 min (Roberts and Ingram, 1965).

Exact reasons for effects provoked by sublethal heat shocks are no clear. The frequently reported effects on germination, which appear to occur not identical in all spores of a population (Russell, 1971) and different for dormant and superdormant spores (Ghosh et al., 2009), are very likely to involve changes in the configuration of macromolecules (Keynan and Evenchik, 1969). This could include the activation, i.e., increased responsiveness of germination receptor, e.g., due to a change in their conformation (Johnstone, 1994; Plowman and Peck, 2002) (Zhang et al., 2010) or increased accessibility to germination receptor (Zhang et al., 2010), since no effects have been reported on nGR-independent germination processes (*Bacillus* species, (Zhang et al., 2010)). This, however, is unlikely to be the reason for the observed effects of a heat shock on the HPT resistance of *C. botulinum* type E spores, since nGR-dependent Pressure-mediated germination is generally not very efficient in *C. botulinum* type E spores and primarily associated with low pressure levels at around ambient temperatures (Lenz et al., 2015).

Endothermic peaks in differential scanning calorimetry (DSC) thermographs in the range of typical spore activation temperatures have been previously assigned to changes in different regions of a spore. Suggested assignment of endothermic peaks include an initial activation stage for germination at around 60 °C (*B. subtilis*, (Ablett et al., 1999), *B. megaterium* (Belliveau et al., 1992), *B. subtilis*, (Nguyen Thi Minh et al., 2010)), which can be assigned to a transition of outer spore regions (coat) (*B. subtilis*, (Leuschner and Lillford, 2003)). Furthermore, material relaxation corresponding to a structure change from a less to a more mobile/less dense state of the cortex at 70 °C (Nguyen Thi Minh et al., 2010) and a thermal evaporation process at 89–90 °C (Stecchini et al., 2006) have assigned to the respective endothermic peaks. Although it has to be noted that these data have been obtained exclusively for *Bacillus* spores, which differ in their heat activation requirements form *C. botulinum* type E spores, this assignment points towards the possibility that changes in the coat structure of *C. botulinum* type E spores play a role in the decrease of the HPT resistance. Additionally, any changes affecting the ability of spores to retain DPA in their core could be involved in the observed effect on resistance. For example, this might include changes such as phase transition of the densely packed inner membrane region during a sublethal heat shock.

Although a sublethal heat shock might be technically suitable to eliminate vegetative cells and activate spores, which could reduce shoulder formation in survivor curves, such as treatment should be avoided, especially, but probably not exclusively, for testing the effectiveness of HPT processes to inactivate *C. botulinum* type E spores. The two major reasons for this are that (i) it is rather unlikely, that spore populations sitting in their natural environment such as soil or sea sediment are situated in a uniformly activated state, and (ii)

results presented here indicate that such a treatment can largely affect the HPT resistance of spores. An exception might be the application of heat treatments of the inoculum to avoid select of non-degenerated cells and activate stock cultures (Kashket and Zhi-Yi, 1995) (Ponce et al., 2009).

Enzymes and Ethanol

In contrast to the difficulties arising from the use of sonication or heat treatments, the application of enzymatic or ethanol treatments appears suitable for the production of pure *C. botulinum* type E spore suspensions without significant effects on spore viability or HPT treatments. However, the slight (though not significant) trend for a decreased resistance of enzymatically treated spores to treatments at high pressure combined with elevated temperature (800 MPa/70 °C) Fig. 4-6, was the reason to choose ethanol treatments as standard procedure during this study.

5.2 Pressure/Temperature-Dependent Inactivation

5.2.1 Inactivation Kinetics

5.2.1.1 Process Control and Comparability of Results

Spore inactivation data derived under isothermal-isobaric conditions excluding effects during pressure build-up and release can be compared between different high pressure units (Fig. 4-7). This is in accordance with earlier findings, where the exclusion of non-isothermal and non-isobaric conditions resulted in a perfect fit for models derived from kinetic data using different high pressure equipment (*B. subtilis* spores, (Reineke et al., 2012)). Provided that inactivation data are comparable between different high pressure units, it can be assumed that kinetic data for DPA release are also comparable between these units (Reineke et al., 2013b). The comparability of kinetic data is not only important for the interpretation of the results showed here but also presents a prerequisite of the direct comparability of results from different spore inactivation studies, which are usually conducted using various high pressure equipment. This becomes especially important when high process temperatures are used, which is the common case when the inactivation of bacterial endospores is investigated.

Notably, although the process temperature was controlled within a narrow range during treatments in unit FBG 5620, it cannot be excluded that pressure peaks (temporary pressure levels exceeding the target pressure) during treatments in this unit (Fig. 3-2) resulted in a slight overestimation of the effectiveness of treatments, i.e., a detection of higher inactivation levels than those that might have been found if ideal isobaric conditions would have been prevalent during a treatment. This might especially be the case where the temperature can be assumed to play a minor role for inactivation (30 – 45 °C), very high pressure levels were used (750 – 1200 MPa), and experiments were conducted solely in unit FBG 5620 (i.e. 750 and 900 MPa/30 °C, 900 and 1200 MPa/45 °C).

5.2.1.2 Inactivation Kinetics

Inactivation of *C. botulinum* type E strain TMW 2.990 (Beluga) spores is retarded at <600 MPa/<60 °C (max. 0.7 log reduction; Fig. 4-8), which is in accordance with results from

previous studies for the same strain (Reddy et al., 1999) and data obtained in this study. Although spores from non-proteolytic *C. botulinum* strains are markedly less resistant to harsh environmental conditions (physical treatments) than spores from several other species, inactivation levels achieved after treatments at low pressure/moderate temperatures are similar or lower than those reported for various other spores (e.g., *B. subtilis*, (Reineke et al., 2012)). Under such processing conditions, inactivation is likely to occur as a result of pressure-induced germination and loss of physiological fitness, whereas there are only reversible or minor changes in the overall spore structure (e.g., 200 MPa, 30 – 55 °C, *G. stearothermophilus*, (Georget et al., 2014)). Thus, it seems reasonable that either germination is not efficiently triggered or the physiological fitness of *C. botulinum* type E spores is not impaired during low pressure/moderate temperature treatments. The former could be related to a low responsiveness of *C. botulinum* nutrient germinant receptors (nGRs) to pressure. Additionally, inner membrane properties (composition/rigidity/phase behavior) might play a role, since the pressure-induced formation of a gel phase with different packing properties and lateral organization has been proposed to contribute to the activation of nGRs (Georget et al., 2014).

An increase in target pressure and/or temperature accelerated inactivation, which is also consistent with previous findings (Reddy et al., 1999) and data obtained in this study. When effects occurring during pressure build-up are included ($\log(N_0/N_t)$), HPT inactivation levels at moderate temperatures were similar compared with those reported earlier (Reddy et al., 1999). For example, after 5 min at 827 MPa/45 °C were reported to result in an approximately 2.4 log inactivation of *C. botulinum* TMW 2.990 (Beluga) spores (Reddy et al., 1999), which is similar to the 2.3 – 3.4 log inactivation after 750 – 900 MPa/45 °C treatments reported here. The significant inactivation by high pressure/moderate temperature treatments is in contrast to a low or no inactivation frequently reported for *Bacillus* spores (Ardia, 2004; Mathys et al., 2009; Rajan et al., 2006b; Reineke et al., 2012; Wuytack et al., 1998).

At higher process temperatures (≥60 °C), differences between inactivation levels determined here and those reported earlier for non-proteolytic *C. botulinum* spores (Reddy et al., 1999) (Skinner et al., 2014) become apparent. For example, a 5 log inactivation after 5 min at 827 MPa/55 °C (Reddy et al., 1999) clearly exceeds the 2.7 – 3.9 log inactivation after 750 – 900 MPa/60 °C treatments found here. Such differences are putatively related to process temperatures exceeding the target temperature in the early phase of less precisely controlled HPT processes (adiabatic peak after pressure build-up). An increase in the process intensity accelerated inactivation resulting in an >6 log reduction ($\log(N_0/N_t)$) after 1200 MPa/45 °C or 750 MPa/75 °C treatments and a complete inactivation of approx. 6×10^6 spores per mL after 10 min at 900 and 1200 MPa at 60 or 75 °C (Fig. 4-8).

Spore inactivation at high pressure/elevated temperature is thought to occur due to a multi-stage mechanism beginning with sublethal injury of spores by heat and pressure (Margosch et al., 2004a; Margosch et al., 2004b) and/or DPA release due to the inner membrane losing its barrier function (Mathys et al., 2007a). In the latter case, core rehydration and the accompanying loss of heat resistance facilitates spore inactivation by moderate heat, where the pressure level plays a less important role (Margosch et al., 2004a; Margosch et al., 2004b). Inactivated spores were reported to be characterized by a physically compromised inner membrane (Mathys et al., 2007a). Germinant receptors are thought to play no role in the HPT-mediated DPA-release triggered under such conditions (Mathys et al., 2007a)

(Reineke et al., 2013a). Thus, this process is commonly referred to as non-physiological germination (Reineke et al., 2013a). In comparison with proteolytic *C. botulinum* strains (Margosch et al., 2004a; Margosch et al., 2004b) and *G. stearothermophilus* (Mathys et al., 2007a) for which these steps have been described, *C. botulinum* type E spores appear to be significantly more susceptible to both heat and pressure. In contrast to earlier studies where particular p/T combinations result in lower spore inactivation than heat alone and a pronounced pressure-dependent tailing effect (*B. amyloliquefaciens*, 800 – 1200 MPa/120 °C, (Margosch et al. 2006); proteolytic *C. botulinum*, 700 MPa/121 °C, packaging-dependent, possibly related to inhomogeneous processing, (Patazca et al., 2013)), zones of spore stabilization were not observable for *C. botulinum* type E spores.

There were virtually no vegetative cells remaining in the purified spore suspensions. Thus, neglecting the possibility that dormant spores, which are not detected by initial plate counts but activated by a specific treatment (e.g., a sublethal heat or pressure), the reduction in viable counts after HPT treatments equals the inactivation of initially viable spores. Generally, this inactivation can occur due to damages in spore components essential for spores to develop into a vegetative, growing and doubling cell. Although such damages do not necessarily lead to the release of DPA from the spore core, DPA release (and concomitant core rehydration and loss of resistance properties) was reported to present the rate-limiting step in the inactivation of *Bacillus* spores (*B. subtilis*, (Reineke et al., 2013b)). However, comparable data on the HPT-mediated release of DPA from *Clostridium* spores is limited and some species-dependent differences seem to exist (Hofstetter et al., 2013a; Hölters et al., 1999; Margosch et al., 2004a), which is discussed in the following paragraphs.

5.2.2 Strain-specific Inactivation Kinetics and DPA Contents

Completing data obtained in the beginning of this study (Fig. 4-1), exact inactivation kinetics of the three *C. botulinum* type E strains TMW 2.990, TMW 2.992, and TMW 2.994 were recorded (Fig. 4-14). The general order of the HPT resistance of spores from *C. botulinum* type E strains TMW 2.990 > TMW 2.992 > TMW 2.994 is consistent with that found earlier in this study (Fig. 4-1). Only slight variations in the maxima of strain-dependent differences can be found between these results (approx. 2 log cycles) and the results obtained earlier in this study (approx. 2.5 log cycles, 800 MPa/80 °C/600 s, Fig. 4-1) where the same strains but higher stress intensities (750 MPa/75 °C here vs. 800 MPa/80 °C before), less precise process control, and different sporulation conditions were used. A similar range of strain-dependent differences (around 2 log cycles) was reported earlier for *C. botulinum* type E strains Beluga (TMW 2.990) and Alaska (827 MPa/40 and 45 °C/5 min, (Reddy et al., 1999)). Although comparable data is scarce and the HPT resistance of only a small number of *C. botulinum* type E strains has been investigated up to now, strain-dependent differences observed so far are significantly smaller compared with those reported for other *C. botulinum* serotypes. For example, inactivation of 5.5 log (detection limit) of proteolytic *C. botulinum* type A spores by HPT treatments at 600 MPa/80 °C was not reached within 60 min for a resistant strain (TMW 2.365) but within 12 min for a sensitive strain (ATCC 25765) (in mashed carrots, (Margosch, 2004; Margosch et al., 2004a)). Furthermore, log inactivation levels of spores of non-proteolytic *C. botulinum* type B strains, which appear to be generally somewhat less resistant than spores of proteolytic type A, were reported to vary between 0.4 (resistant

strain KAP9-B) and >5.5 log cycles (sensitive strain KAP8-B) after 827 MPa/75 °C treatments (phosphate buffer, pH 7, (Reddy et al., 2006)).

The observable trend that strain-dependent differences in HPT inactivation levels differ depending on the p/T range applied might just reflect that fact that strain-specific differences become more easily detectable when the process intensity and therefore inactivation levels are increased. However, since maximum differences were observed in a p/T range where rapid non-physiological DPA-release might become the governing factor (>500 MPa/60 °C), an increase in such strain-dependent differences might be related to strain-specific differences in compartments modulating the non-physiological release of DPA. On the other hand, small differences at low pressure levels combined with low temperatures might indicate that the relatively low susceptibility to undergo pressure-induced physiologic-like germination is conserved among the three strains tested. This might point towards a high similarity in spore compartments that are thought to influence the effectiveness of pressure triggering physiologic-like germination such as the spores' nGRs.

Strain-specific DPA contents of spores

DPA levels of *C. botulinum* strains TMW 2.990, 2.992, and 2.994 were 155 ± 23, 132 ± 9, and 72 ± 14 amol DPA/spore, respectively. These values are substantially lower than those previously reported for *B. subtilis* spores (365, (Hindle and Hall, 1999); 174 – 384, (Huang et al., 2007); 193, (Fichtel et al., 2007a); 216 amol/spore, (Sanchez-Salas et al., 2011)), various *Bacillus* spores (193 – 505 (Fichtel et al., 2007a); 365 – 820 amol/spore (Kort et al., 2005)), and *Clostridium* spores (598 *C. hungatei*, 614 *C. sporogenes*, 747 *Clostridium* G5A-1, (Yang et al., 2009)). Earlier studies reporting on the DPA content of *C. botulinum* spores specified a range between 8.7 % – 13.4 % DPA/spore dry weight for type A, 7.4 – 8.1 % for type B, and 9.5 % for type E (Grecz and Tang, 1970). This is completely in the range assumed to be present in *B. subtilis* spores but contrasts the range of 3 – 4 % DPA reported somewhat earlier for *C. botulinum* type A (Day and Costilow, 1964). Differences in the determined DPA level might occur due various influence factors including the accuracy of the method used, species- and strain-specific differences, and sporulation conditions. However, a direct comparison with the spores' DPA content found here is not possible, since the spore's dry mass was not determined here. Generally, a comparison between the DPA content and the HPT resistance of the different strains used here corroborates results from various previous studies where it was shown that the DPA content does not necessarily correlate with their HPT resistance (e.g., (Reineke et al., 2011)).

5.2.3 Mechanistic Insights

5.2.3.1 DPA Release

Similar to the inactivation levels, DPA release from *C. botulinum* type E (strain TMW 2.990) spores generally increased with increasing treatment intensity and longer dwell times. Only one exception was observed, i.e., the DPA release after 300 MPa/30 °C treatments. After such treatments, DPA release occurred considerably slower compared with treatments at high pressure/high temperature but significantly faster and more effective than after moderate heat/moderate temperature treatments (Fig. 4-8). Generally, HPT treatment conditions sufficient to provoke more than 2 log cycles (>99 %) inactivation (min. 600 MPa/60 °C/600 s) were required to trigger the release of 90 % of the spores' total DPA content. After treatments

at the highest intensity tested (750 MPa/75 °C/600 s), the inactivation level reached 5.8 log cycles and 99 % of the total DPA content were released. A fast DPA release from *C.* botulinum type E spores has also been reported to occur during heat processing at 75 °C, where almost 60 and 90 % of DPA were released after 5 and 10 min dwell time, respectively, corresponding to an approx. 0.5 and 1 log inactivation (strain VH, Vancouver Herring, (Grecz and Tang, 1970)).

Comparison with other *Clostridium* spores

In comparison with *C. botulinum* type A, type E spores are significantly less resistant to heat and HPT treatments and appear to lose their DPA considerably faster. Type A spores were reported to release only approx. 25 % of their DPA after 800 MPa/80 °C/600 s treatments at pH 6 (Margosch et al., 2004a). A complete release was observed only under conditions leading to an >5 log reduction (>99.999 %), i.e. at 800 MPa/116 °C for >1 h (approx. 70 % release after 600 s (Margosch et al., 2004a)). In contrast, a substantial DPA release from *C. beijerneckii* and *C. sporogenes* spores was shown to occur within <5 min at 600 MPa/90 °C (Hofstetter et al., 2013a) and a considerable DPA release from *C. pasteurianum* spores was described after relatively mild HPT processes (Hölters et al., 1999). Albeit DPA release from *C. pasteurianum* spores appears to occur consistently slower after 300 MPa treatments and faster after 450 MPa treatments (Hölters et al., 1999), reported levels are similar to those found here for *C. botulinum* type E. For example, 300 MPa/60 °C/60 – 600 s treatments resulted in approx. 40 – 60 % DPA release from *C. pasteurianum* (Hölters et al., 1999) and 49 – 73 % from *C. botulinum* type E spores.

DPA release at low pressure levels and moderate temperatures

Low pressure levels can trigger a physiologic-like (similar to nutrient-induced) germination pathway, where the activation of nGRs provokes the release of over 90% of the large depot of DPA present in the core as a 1:1 chelate with divalent cations (predominantly Ca^{2+}) (Paidhungat and Setlow, 2000, 2001; Rode and Foster, 1966; Wang et al., 2011). Consequentially, nGR levels are the major factor determining low pressure-mediated germination rates whereas other germination related proteins play a minor role (*B. subtilis*, (Doona et al., 2014)). Typically, pressure levels between 80 – 100 MPa (Margosch et al., 2006; Torres and Velazquez, 2005) (Paidhungat et al., 2002) and 150 MPa (Gould and Sale, 1970; Reineke et al., 2012) can efficiently trigger germination of different *Bacillus* spores in the absence of nutrients (temperature and pH-dependent, (Gould and Sale, 1970; Reineke et al., 2012)). Possibly due to (reversible) conformation modifications of nGR domains at the spores' inner membrane periphery (Georget et al., 2014). Though less effective, physiologic-like germination was reported to be also triggered at pressure levels of 300 up to 600 MPa (threshold pressure) at temperatures up to 50 °C (*B. subtilis*, (Reineke et al., 2013b; Wuytack et al., 1998)).

Bacillus and *Clostridium* spores have considerable differences in nutrient germination requirements, which is putatively related to the different genetic architecture (Paredes-Sabja et al., 2011) and different conformation of their nGRs. Hence, it is not surprising that *Clostridium* nGRs also harbor a different responsiveness to HPT, and that HPT levels triggering nGR-mediated germination of *B. subtilis* spores do not provoke identical effects in *Clostridium* spores. Although the pressure levels at which physiologic-like germination is triggered and the effectiveness of this process may differ between spores from different species, the observed DPA release from *C. botulinum* type E even after very short dwell

times is in accordance with findings for *B. subtilis* where a short pressure pulse (150 MPa, 30 s) can be sufficient to commit spores to germination (Kong et al., 2014). Notably, since only the total amount of released DPA was measured, it is not possible to define whether the DPA originates from a certain germinated spore fraction or presents a partial release from the majority of spores. However, the accelerated DPA release from *C. botulinum* type E spores after 300 MPa/30 °C treatments compared with that after moderate heat/moderate temperature treatments (Fig. 4-8) might be attributed to pressure-induced germination of a distinct spore fraction.

DPA release at high pressure levels and high temperatures

At higher pressure levels and increased process temperatures, DPA release from *C. botulinum* type E spores was significantly accelerated. Although temperatures required for a substantial DPA release after HPT treatments at high pressure/elevated temperatures are somewhat lower than those reported for other spore formers, the rapid DPA release under such conditions presents a common response among *Bacillus* (Reineke et al., 2013b) and *Clostridium* (Margosch, 2004) spores. In *Bacillus* spores, high pressure/elevated temperature treatments are thought to trigger a non-physiological DPA release, which leads to a partial core rehydration but incomplete germination (Reineke et al., 2013a) and occurs independently from the presence of functional nGRs (Black et al., 2007b). Although the exact mechanism underlying this rapid DPA release remains to be elucidated, there exists some knowledge, which allows for discussing factors that might determine organism-specific differences in the non-physiological DPA release rates.

Whereas effects on the solubility of DPA seem unlikely (core DPA concentration exceeds solubility limit by far), (i) inner membrane properties and (ii) cortex lytic enzymes (CLEs) present potential factors that might play a role.

(i) Biological membranes can undergo phase transition under pressure and are generally recognized as one of the most pressure-sensitive cellular components (Winter and Jeworrek, 2009). Consequently, pore formation due to inner membrane damage or changes in the inner membrane organization opening DPA-channels (Setlow, 2003) might be involved in the HPT-mediated non-physiological DPA release (Paidhungat et al., 2002) (Wilson et al., 2008). The membrane structure and T,p-dependent phase behavior is influenced by membrane proteins and, vice versa, protein conformation (and function) can be influenced by the lipid environment (Ulmer et al., 2002) (Winter and Jeworrek, 2009), which makes it difficult to clearly spot reasons for differences in the release of DPA from spores of different species. Thus, inner membrane properties (e.g. fatty acids, density, rigidity, and phase behavior) as well as the type and abundance of specific membrane proteins could potentially influence the DPA release rate and account for species-specific differences. However, high pressure can counteract fluidizing effects on the inner spore membrane (phase transition from gel state to the liquid-crystalline phase) occurring during heat treatments at ambient pressure (Hofstetter et al., 2013b). Additionally, it was reported that inactivation and DPA release do not require disturbance of the highly ordered membrane state (*C. beijerinckii*, *C. sporogenes*, 200 or 600 MPa, 90 °C, up to 60 min) (Hofstetter et al., 2013b). This makes it likely that alterations in the structure of membrane proteins such as channel proteins (Black et al., 2005) or associated DPA binding proteins (SpoVA proteins, (Li et al., 2012) (Doona et al., 2014)) are involved in the inner membrane losing its barrier function in response to HPT treatments at high pressures/elevated temperatures. Thus, differences in such proteins (or their lipid

environment) could account for organism-specific differences in non-physiological DPA release rates.

(ii) Although the non-physiological DPA release was reported to be not limited by the activity of the two CLEs conserved among *Bacillus* species (Paredes-Sabja et al., 2011), i.e. CwlJ and SleB, such enzymes might play a role in the non-physiological germination pathway. This appears possible since CwlJ (GerQ-dependent) can be activated by DPA release (or exogenous Ca–DPA) and SleB (YpeB-dependent) can be activated by cortex deformation (due to core rehydration). Activation and cortex degradation by such enzymes can facilitate further DPA release and core rehydration (Wuytack et al., 1998) (Paidhungat et al., 2002) (Setlow, 2003) (Black et al., 2007b) (Reineke et al., 2011) (Reineke et al., 2013b). *C. botulinum* strain TMW 2.990 (Beluga) possesses SleB (NCBI accession EES50540.1) but lacks YpeB, CwlJ, and GerQ. However, YpeB appears to be required for functional SleB in *Bacillus* and *Clostridium* spores (*C. difficile*, (Burns et al., 2010) (Cartman and Minton, 2010) (Paredes-Sabja et al., 2011)). Similar to many *Clostridium* spores, *C. botulinum* TMW 2.990 presumably relies on the exo-acting lytic transglycosylase (Gutelius et al., 2014), SleC (EES49934.1), which is activated by a Csp protease (Adams et al., 2013) for cortex hydrolysis during germination (Paredes-Sabja et al., 2009b). Unlike CwlJ and/or SleB, SleC appears not to be activated in response to DPA release or core rehydration (Paredes-Sabja et al., 2008b) (Wang et al., 2012), which makes it questionable whether it plays a role in non-physiological DPA release from *Clostridium* spores. Thus, similar to *B. subtilis* spores, non-physiological DPA release from *C. botulinum* type E at high pressure/elevated temperatures to be triggered in a CLE-independent manner (Reineke et al., 2013a). However, this would also indicate that the sequential steps of a rapid DPA release, partial core rehydration, CLE activation, cortex lysis, further core rehydration, and inactivation, proposed for *Bacillus* spores treated at high pressure/elevated temperatures, might not occur identically in *C. botulinum* type E spores. Consequently, other molecules/steps might be involved. Provided that SleB is actually not functional and SleC not activated by HPT, there is still the possibility that other, unknown cortex lytic mechanisms (e.g. involving other putative CLEs: EES48735.1 or EES47840.1) are triggered by the release of DPA, directly by HPT, or in consequence of morphological changes in the spore induced by HPT. Finally, it might also be possible that spores are inactivated without the need for cortex degradation.

DPA release at high pressure levels and moderate temperatures

In contrast to the rapid DPA release at high pressure/elevated temperatures, the effective release of DPA from *C. botulinum* type E spores at high pressure levels (750 MPa) combined with moderate temperatures appears to present no common response in *Bacillus* spores. Again the barrier function of the inner spore membrane and/or the activation of CLEs could potentially play a role in such species-specific differences. However, exact reasons for this striking difference are completely unclear.

Generally, a comparison of DPA release and inactivation data indicates that a rapid and substantial release of DPA after HPT treatments presents a crucial step for an effective spore inactivation, which supports earlier findings for *B. subtilis* spores (Reineke et al., 2013b). However, comparing DPA release and inactivation data between different species (e.g., *B. subtilis* and *C. botulinum* type E spores) also shows that DPA release profiles are helpful to characterize the response of spores to pressure but are not suitable as a measure for HPT resistance properties of spores from different species (Hofstetter et al., 2013a).

5.2.3.2 Heat susceptible spore fraction after HPT treatments

A second heat treatment was employed to estimate the number of spores, which lost their spore-specific heat resistance in response to a HPT treatment but were not inactivated during this treatment. This is likely to apply primarily to physiologic-like germinated spores, which germinated during a HPT treatment and survived it or germinated after a treatment. There were virtually no vegetative cells remaining in the purified spore suspensions prepared for HPT treatments, and it is unlikely that many vegetative cells survive HPT treatments. Therefore, the inactivation of initially present vegetative cells is unlikely to play a role in the observed differences in cell counts between HPT treated and HPT/heat treated samples. However, *C. botulinum* type E spores normally tolerate 60 °C but their resistance is much lower than other spore formers and closer to that of their vegetative forms. Thus, it cannot be excluded that non-germinated, HPT-damaged but germinable spores are inactivated by a second thermal treatment.

The absence of significant differences between cell counts of HPT and HPT/heat treated samples after any HPT treatment at 60 and 75 °C (Fig. 4-9) indicates that, regardless of the pressure applied (300 – 1200 MPa), there are no germinated spores surviving or germinating after such HPT treatments. Additionally, this means that such process conditions do not create sublethally damaged but germinable and heat susceptible spores. This also applies to HPT treatments at 750 – 1200 MPa/30 °C and 900 – 1200 MPa/45 °C. This indicates that, despite of the rapid inactivation of a significant number of spores within the population, surviving spores remain largely intact, i.e., retain their heat resistance and viability. This is in accordance with the proposed inactivation mechanism via non-physiological germination, where DPA release, core rehydration and inactivation were proposed to occur in immediate succession at p >600 MPa and T >60 °C (Reineke et al., 2013b).

Small but significant differences, which tended to slightly increase with longer dwell times, were detected after 300 – 450 MPa/30 – 45 °C treatments. The largest heat susceptible spore fraction (max. around 0.6 log (N_t/N_{t+heat}), Fig. 4-9) was detected after 300 MPa/30 °C treatments, which matches the accelerated release of DPA under such treatment conditions compared with that after 450 MPa treatments at moderate temperatures (Fig. 4-8). This suggests that either pressure-induced physiologic-like germination occurs during such treatments and germination leads to the loss of resistance after the HPT treatment and/or processing conditions are not harsh enough to inactivate spores germinating during a treatment. Very small differences in cell counts of HPT versus HPT/heat treated samples were observed after treatments up to a pressure of 600 MPa at 30 °C. The fact that such differences do not tend to increase with prolonged dwell times, might indicate that geminating spores are increasingly physiologically damaged during treatments resulting in their inactivation.

This points towards physiologic-like germination (though not very effective) of *C. botulinum* type E spores triggered by moderate pressure levels of 300 – 450 MPa (up to 600 MPa) combined with moderated temperatures up to 45 °C. This pressure/temperature range overlaps with that previously reported for *B. subtilis*, where the optimum for physiologic-like germination can be found around 80 – 150 MPa but occurs up to a threshold pressure of 600 MPa (Reineke et al., 2013b; Wuytack et al., 1998). However, the effectiveness of pressure to induce physiologic-like germination of *C. botulinum* type E spores appears to be drastically lower than spores from other species (e.g., *B. subtilis*, (Reineke et al., 2013b); *G.*

stearothermophilus, (Georget et al., 2014)). This low effectiveness and its maximum at low pressures/temperatures is in accordance with other findings in this study where 300 MPa treatments at ambient temperature tended to slightly (but not significantly) shorten the time to detect growth from HPT treated *C. botulinum* type E spores (population heterogeneity discussed later). Results from other studies suggest that pressure treatments might be generally not very effective in triggering physiologic-like germination of *Clostridium* spores. For example, 100 – 200 MPa, 7 min treatments did not induce germination of *C. perfringens* spores within 60 min after pressure treatment (Akhtar et al., 2009) and pressure cycling was relatively ineffective in reducing *C. sporogenes* spore counts (60 MPa followed by 400 MPa at 60 °C, <3 log inactivation (Mills et al., 1998)).

5.2.3.3 Lysozyme

Small (max. 0.23 log after 600 MPa/75 °C) and in most cases not significant differences between *C. botulinum* type E spore counts in plates with and without lysozyme were exclusively detected at pressure levels above 600 MPa (Fig. 4-10). This indicates that HPT treatments below 600 MPa do not provoke the formation of spores with both defects in their cortex lytic machinery and coat layers but without severe damages in other spore components or molecules essential for germination and outgrowth. At 600 – 750 MPa, a small spore fraction with exactly such injuries can be observed, which tends to be larger when process temperatures are increased and dwell times prolonged. At 900 – 1200 MPa, the number of spores with such injuries decreases again, which might be due to severe damages in spore components essential for germination and/or outgrowth accompanying injuries in the cortex lytic machinery and coats. However, in the latter pressure range, inactivation levels are high, i.e., nearly complete inactivation, and difficulties in detecting differences in cell counts might play a role.

The occurrence of lysozyme-dependently germinable spore fractions after intense HPT treatments is generally in accordance with the previously described prolongation of the times to detect growth from high pressure/high temperature treated *C. botulinum* type E spores (Lenz et al., 2014), which is accompanied by an increase in the heterogeneity of detection times among individual spores and indicative of sublethal damages in the germination machinery. The result that inactivation levels (Fig. 4-8) do not correlate with lysozyme susceptible spore fractions (Fig. 4-10) corroborates earlier findings for *B. subtilis*, i.e., that the inactivation of cortex lytic enzymes does not present a prerequisite for spore inactivation (Reineke et al., 2011).

In contrast to the very weak effect of lysozyme on the recovery of HPT treated spores observed here, lysozyme was reported to significantly aid in the recovery of heat treated *C. botulinum* type E spores (Alderton et al., 1974; Lindstrom et al., 2003). For example, 10 µg/mL lysozyme in the plating medium resulted in the detection of 4 log spores/mL instead of a complete inactivation of initially 6 – 8 log spores/mL determined on plates without lysozyme after heat treatments at 85 °C for 10 min (*C. botulinum* TWM 2.990 (Beluga), (Peck et al., 1992)). This suggests that damages in the cortex lytic machinery and the coat provoked by HPT and heat alone are different and/or that such damages are more likely to occur concomitant with inactivation (due to severe injury of other spore components) during HPT compared with heat treatments. This is in accordance with the significantly different

inactivation mechanisms proposed and spore resistance factors important for heat and HPT inactivation (Setlow and Johnson, 2013).
Notably, it was reported later that lysozyme addition to liquid recovery medium for MPN counts improves only the initial (4 days) recovery of heat and high pressure injured *C. botulinum* type A and B spores and has no significant effect when added to plating agar (Reddy et al., 2010). This appears to point towards distinct *C. botulinum* type- and strain-dependent and/or treatment intensity-related differences. Another explanation for the comparably low effect of lysozyme addition found here for HPT treated and earlier for heat treated spores (Alderton et al., 1974; Lindstrom et al., 2003; Peck et al., 1992) might be found in the recovery duration, which has not been specified in some earlier studies.

5.2.3.4 Isoeffect curves

Inactivation isorate curves

Isoeffect curves for the inactivation of *C. botulinum* TMW 2.990 spores by 1 and 3 log cycles (Fig. 4-12) demonstrate that either high pressures levels at low temperatures (e.g., 1 log inactivation after 600 MPa/30 °C/10 min) or lower pressure levels at higher temperatures (e.g., 1 log inactivation after 400 MPa/80 °C/10min) can be used to achieve an inactivation of 90 and 99.9 %, respectively (initially approx. 10^7 viable spores/mL). Such inactivation levels are far below the 6 log inactivation stipulated for the inactivation of non-proteolytic *C. botulinum* spores for the production of safe food (Garcia-Graells et al., 2002; Peck et al., 1995b). However, the shape of isoeffect curves as graphical representation of the estimated parameters can facilitate the description of the pressure- and temperature-dependent behavior of *C. botulinum* type E during HPT processing.
The pressure/temperature-dependency of spore inactivation during a given dwell time appeared to be almost linear and was not drastically altered when HPT treated spores were subjected to a second heat treatment or when lysozyme was added to the recovery medium. This reflects the fact that no large heat susceptible or lysozyme-dependently germinable spore fractions were detected after HPT treatments. Accordingly, the p/T/t parameter range displayed in Fig. 4-12 is not suitable for efficiently triggering pressure-induced physiologic-like germination or provoking damages in the cortex lytic machinery and coat layers leaving other spore components essential for germination and outgrowth intact (both discussed above).

DPA release isorate curves

Similar to inactivation, the p/T dependency of a 90 % DPA release appears to be almost linear, at least for longer dwell times. This means that either high pressures levels at low temperatures or low pressure levels at high temperatures applied for a specific dwell time trigger the release of the same amount of DPA (Fig. 4-13). Isoeffect lines for 1 log, i.e. 90 %, inactivation and those for a 90 % DPA release after 5 – 10 min would intersect at points corresponding to a process temperature of around 60 – 70 °C at around 500 MPa. Above this temperature range, DPA release occurs significantly faster than inactivation. At temperatures below 60 °C, inactivation occurs faster than the release of DPA, i.e., DPA release appears to be less important for inactivation. Notably, this matches the process conditions where inactivation of *C. botulinum* type E is significantly accelerated. This indicates that a rapid release of DPA is a crucial step for the effective inactivation of *C. botulinum* type E. However, a direct comparison of concrete calculated data points of

isoeffect lines for a 1 log inactivation and those for a 90 % DPA release is impeded by two facts. Firstly, the inactivation data refer to a 90 % loss of viability of the initially viable spore fraction, whereas DPA release data refer to a 90 % release of the total amount of available DPA, i.e. from the core of all spores present including viable and dormant spores. Secondly, inactivation data used for modeling excludes, whereas DPA release data includes effects occurring under non-isobaric and non-isothermal conditions, i.e., pressure build-up and release. Nonetheless, the different slopes of isoeffect curves for inactivation and DPA release indicate that an increase in the process temperature accelerates DPA release markedly more than inactivation. This is indicative of a rapid DPA release and subsequent inactivation at high process temperatures versus a slower release of DPA in relation to the inactivation rate at lower process temperatures. This putatively reflects the presence of different, p/T-dependent mechanisms underlying the inactivation of *C. botulinum* type E spores.

Comparison with mechanisms characterized in *Bacillus* spores

In comparison with the well-characterized spore forming model organism *B. subtilis* (Reineke et al., 2012) markedly different shapes of isoeffect inactivation curves can be observed, which reflect significant differences in the resistance of the two organisms against HPT treatments. Whereas *C. botulinum* type E requires higher pressure levels at high temperatures to be inactivated, *B. subtilis* but not *C. botulinum* type E inactivation is retarded at high pressure/moderate temperatures (below approx. 60 °C). Similarly, isoeffect curves for DPA release are different between these organisms (Reineke et al., 2013b). In contrast to *B. subtilis*, low pressure levels appear to be less effective in triggering the release of DPA from *C. botulinum* type E spores and the role of the pressure level in triggering DPA release is not diminished above a certain threshold pressure (600 MPa for *B. subtilis*). Additionally, no distinct p/T zones with markedly different shapes of DPA release isoeffect curves were detected for *C. botulinum* type E. However, the latter could be related to the fact that the effect of pressure levels below 300 MPa and prolonged dwell times exceeding 10 min was not investigated here.

However, data obtained here together with the inactivation mechanism proposed for the model organism *B. subtilis* (Reineke et al., 2013a) suggest that p/T combinations provoking physiologic-like and non-physiological germination are very similar for *C. botulinum* type E and *B. subtilis*. Although HPT is markedly less effective in triggering physiologic-like germination, HPT treatments at 300 – 450 MPa/30 – 45 °C (up to 600 MPa/30 – 45 °C, possibly up to 750 MPa/45 °C) are likely to induced physiologic-like germination with the effectiveness tending to increase with decreasing pressure and temperature as well as prolonging dwell times. The generally low effectiveness of pressure to induce physiologic-like germination after treatments for up to 10 min dwell time in combination with the mild processing conditions can be assumed to account for the very low inactivation of *C. botulinum* type E spores after treatments in this parameter range. At >500 MPa/>60 – 70 °C, non-physiological germination, i.e., rapid DPA release putatively due to the inner membrane losing their barrier function, might be responsible for the effective inactivation of *C. botulinum* type E spores observed at such high pressure/elevated temperatures. In contrast to *B. subtilis*, a considerable portion of a *C. botulinum* type E spore population can be inactivated at pressure levels over 500 MPa and temperatures below 60 °C. At such parameter combinations, DPA release appears to play a less important role for inactivation compared with high pressure/high temperature treatments. This suggests that damages to the inner

membrane barrier function are less severe and injury of additional spore components essential for germination and outgrowth might be involved.

5.2.3.5 Isorate Curve Shapes for *Bacillus* and *C. botulinum* Type E

Differences in the proposed inactivation pathways for *Bacillus* and *C. botulinum* type E spores discussed above are likely to account for large differences observable in their HPT resistance of *Bacillus* and *Clostridium* spores. As an example, 3 log inactivation isorate curves for the *Bacillus* model organism, *B. subtilis*, are compared with the curves determined in this study for *C. botulinum* type E.

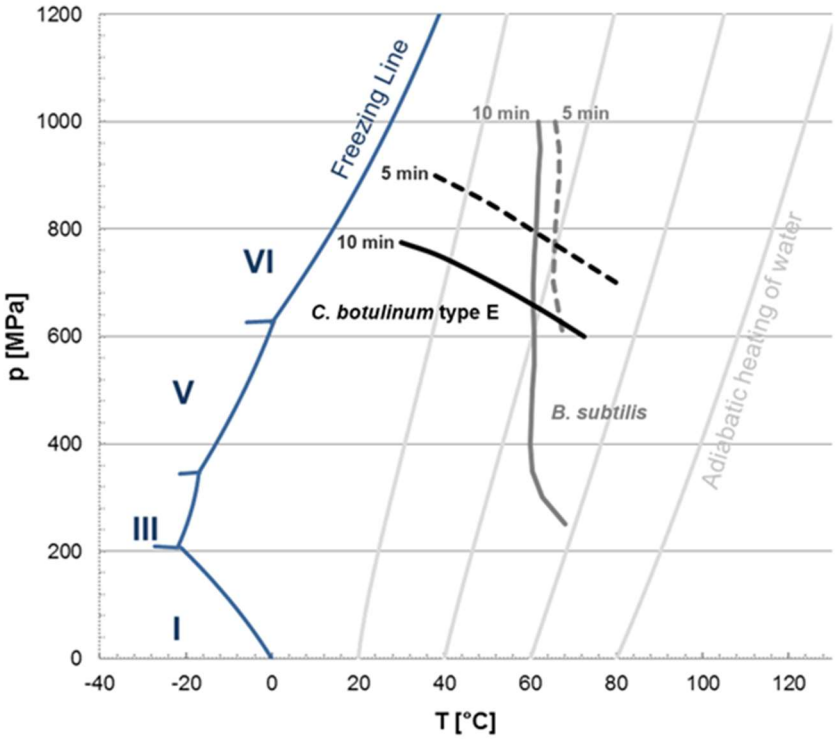


Fig. 5-1: Inactivation isorate curves for *B. subtilis* and *C. botulinum* type E.
Isorate lines for a 3 log inactivation of *B. subtilis* strain PS832 (gray, (Reineke et al., 2012)) and non-proteolytic *C. botulinum* type E strain TMW 2.990 (black, unpublished data). p/T combinations required to achieve a 3 log inactivation after 5 min HPT treatments are depicted as dashed lines. A 3 log inactivation after a holding time of 10 min requires p/T combinations marked by the solid lines.

The markedly different shape of the curves reflects large differences in resistance of these two organisms against HPT treatments, i.e., *C. botulinum* type E, which tends to be less resistant compared to other *C. botulinum* types, requires much higher pressure levels at high temperatures to be inactivated by 3 log cycles. At lower temperatures (below approx. 60 °C) in combination with high pressure levels, *B. subtilis* but not *C. botulinum* type E inactivation is retarded. This demonstrates impressively that the inactivation of spores is highly dependent on the treatment intensity and that this dependency can largely vary between different spore formers. Accordingly, isorate curves for the inactivation of other *Bacillus* or *Clostridium* species can be assumed be different from those depicted here.
Different inactivation levels typically reflect species-specific differences in resistance factors (e.g., inner membrane composition). Additionally, both species- and treatment-dependency

of inactivation levels is certainly also related to differences in inactivation pathways, i.e., the inactivation pathway a specific organism follows depends on the p/T combination, and the inactivation pathways followed by two different organism at a specific pressure/temperature combination are not necessarily identical. This has important consequences on the possibility to predict the behavior of a certain organism in repose to HPT treatments from existing data for other organisms, especially when species form different genera are compared. In contrast to species-specific differences, there can also exist huge differences in the HPT resistance between different strains within a species, but such strains might usually follow a similar inactivation pathway at a certain p/T combination, i.e. isorate curves are likely to be shifted but might have a similar shape. These general points might be considered when discussing about surrogate organisms and their use in the evaluation of food safety provided by HPT processing (section 1.5.6).

5.2.4 Detection Time Variability of HPT-treated spores

Our results indicate that HHP/HPT treatments can largely alter the detection time and the associated variability of individual *C. botulinum* type E spores, being capable of decreasing detection times and heterogeneity (200 MPa, 20 °C) (Fig. 4-15 and Fig. 4-16) and markedly prolonging very heterogeneously distributed detection times (600 MPa, 80 °C; 800 MPa, 60 °C) (Fig. 4-16). Similarly, it has been demonstrated previously that 200 MPa, 70 °C treatments (16 min, *B. subtilis*) slightly and 800 MPa, 70 °C treatments (4 min, *B. licheniformis*) strongly prolong detection times of single *Bacillus* spores and widen associated distributions (Margosch et al., 2004b). However, 70 °C treatments at ambient pressure but not HHP treatments at 100 MPa, 20°C (30 min, virtually identical detection times as compared to untreated spores) were shown to decrease *B. licheniformis* detection times and heterogeneity (Margosch et al., 2004b).

Notably, the detection time comprises a number of sequential processes spores have to undergo before growth can be detected. Dormant spores possess a set of pre-formed enzymes, which allow for breaking dormancy without the need for initiation of an active metabolism. At suitable conditions, nutrient germinants activate germinant receptors (GRs) (commitment to germination (Yi and Setlow, 2010)), which causes calcium dipicolinic acid (Ca-DPA) release and core rehydration (germination stage I) followed by hydrolyzation of the peptidoglycan cortex by activated cortex lytic enzymes (CLE) and full rehydration (germination stage II). This is followed by outgrowth, where non-fractile, heat-susceptible, fully germinated spores initiate macromolecular synthesis and degrade/emerge from remaining spore structures as immature cells. This is followed by the synthesis of the complete molecular set found in mature cells, increase in size and first cell division (Moir, 2006; Setlow, 2003). Interestingly, extensive studies on the variability of the different lag phase stages of individual *C. botulinum* type B spores revealed that there is no correlation between germination and later lag phase stages (Stringer et al., 2005; Webb et al., 2007), i.e. it is impossible to predict the time when growth occurs from germination times.

Since we focused on the determination of detection times, it is unclear which lag stages (germination, outgrowth, first cells doubling) are affected or how pressure treatments affect *C. botulinum* type E spores. Since growth rates are almost identical for any spore that initiated growth suggests that growth after the first cell division is not affected by HHP treatments (Fig. 4-15). Additionally, we did not detect considerable amounts of heat susceptible (fully

germinated) *C. botulinum* type E spores after 200 MPa HHP treatments (data not shown), which indicates that differences in detection time distributions occur due to changes in spore properties (e.g., GR activation) and rather than different growth from HHP germinated spores. Available data for *Bacillus* spores, which possess unique proteins and signal transduction pathways but have many components of the germination machinery in common with Clostridia (Paredes-Sabja et al., 2011) suggests that the conformation of GRs (involved in early germination stage I) into an activated state could play a critical role in the observed decrease in detection time after 200 MPa treatments: (i) In *Bacillus* spores, the initial lag between nutrient availability and the beginning of Ca-DPA release (including GR activation) is highly variable within a population and decisive for the germination velocity of individual spores, whereas subsequent steps (rapid and constant Ca-DPA release and rehydration) trigger a generally irreversible cascade of degradative steps (Zhang et al., 2010). (ii) Low pressure levels (100 – 300 MPa (Black et al., 2005) or even a short HHP pulse at 150 MPa, 30 s (Kong et al., 2014)) can activate GRs (*B. subtilis*) putatively by changing their conformation (Mozhaev et al., 1996), where the commitment to germination is, in contrast to nutrient germination, more variable among individual spores and frequently reversible at ambient pressure (Kong et al., 2014). Accordingly, our finding that treatments at 300 and 400 MPa resulted in only slightly lower detection times and almost identical variability compared to untreated spores (Fig. 4-16) might be due to such pressure levels being not suitable for activating GRs of *C. botulinum* type E. In contrast to 200 – 400 MPa, treatments at 600 MPa, where different pathways are thought to govern HHP triggered germination (*B. subtilis*, (Reineke et al., 2013a)), led to a slight reduction in spore counts as determined previously in this study and slightly increased detection times (Fig. 4-16), which points towards a reversible damage or damage to only a part of molecules involved in germination or outgrowth.

Similar to the effect reported for *Bacillus* spores (Margosch et al., 2004b), lethal HPT treatments (600 MPa, 80 °C and 800 MPa 60 °C resulting in an approx. 5 and 4 log reduction) markedly prolong detection times for growth from *C. botulinum* type E spores and increased their variability (Fig. 4-16). Such effects were previously shown to also occur after lethal heat treatments of *B. subtilis* (Smelt et al., 2008) and non-proteolytic *C. botulinum* type B spores (Stringer et al., 2009). Notably, the germination system was identified as being the major target for heat damage of non-proteolytic *C. botulinum* type B and times required per lag stage changed from outgrowth > cell doubling > germination for untreated spores to germination > outgrowth > cell doubling for heat treated spores (Stringer et al., 2011). Accordingly, severe damages to spore components involved in germination, e.g., GRs, could be the main reason for the increased detection times after HPT treatments observed here. The great heterogeneity in detection time frequency distributions could reflect the different initial number of germination related molecules, e.g., spores equipped with high initial numbers of GRs still have a sufficient amount of functional GRs after the treatment. However, others molecules directly involved in germination or outgrowth or spore layers, the germination machinery is situated in, might be involved in the observed effect.

Although environmental conditions (temperature, nutrient availability etc.) in food considerably influence germination and growth characteristics of *C. botulinum* type E, an earlier onset of growth is likely to lead to earlier toxin formation. Since pressure levels ≤ 300 MPa at ambient temperatures can reduce the mean detection time and narrow the associated distribution, the chance that *C. botulinum* type E spores grow out and produce toxin within a

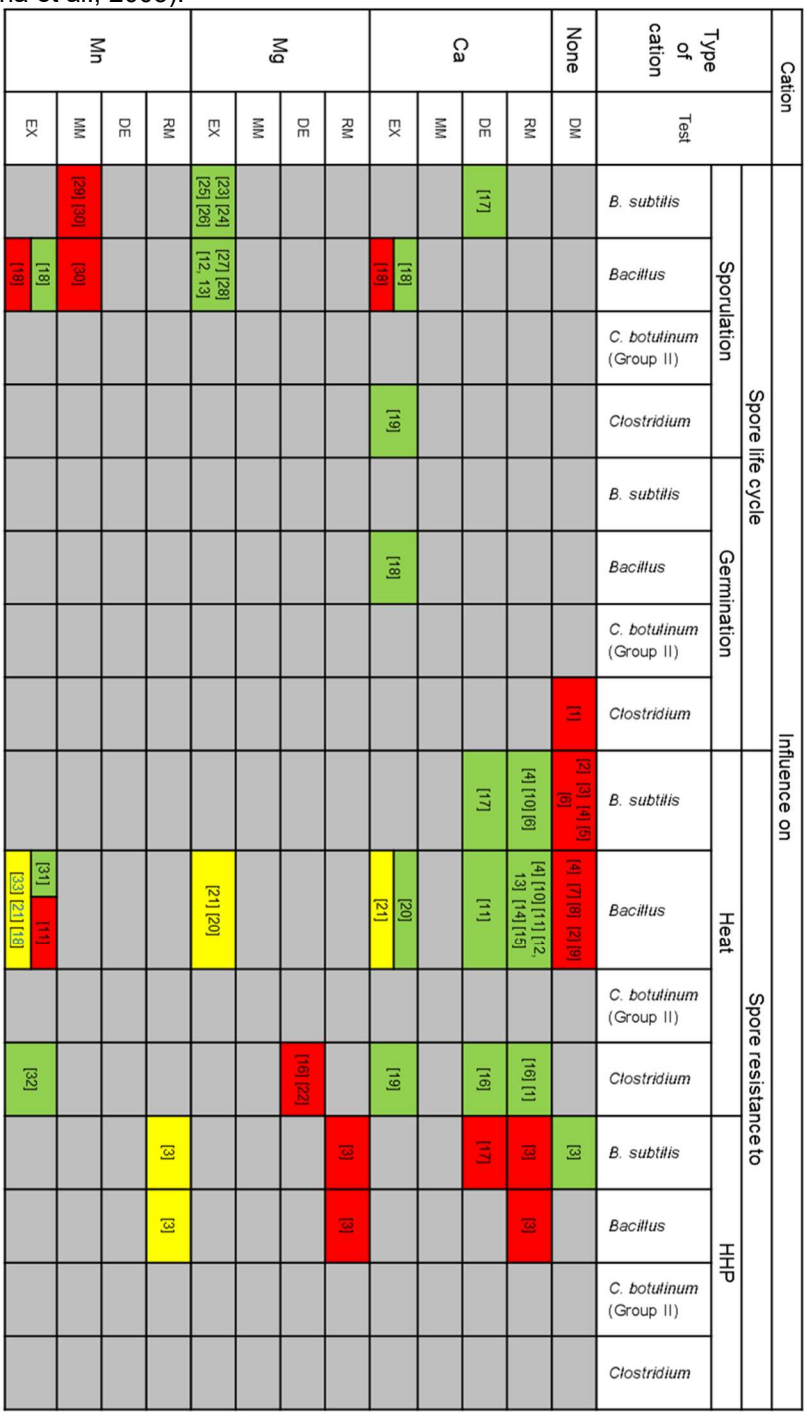
district period (e.g., product shelf-life) can be enhanced. Although such treatment conditions are far from the appropriate range to inactivate spores effectively, this should be kept in mind when a product could be contaminated with *C. botulinum* type E and low pressures are used, e.g., to inactivate vegetative pathogenic or spoilage organisms or for seafood shucking.
Moreover, it should be considered that the markedly extended detection times after lethal pressure treatments can lead to an over-estimation of *C. botulinum* spore inactivation when incubation/recovery times are too short. Therefore, MPN methodology instead of plate counts might be advantageous for the use in challenge studies to confirm safety of pressure treated foods.
Further research is necessary to clarify the effect of HHP treatments on specific stages during detection time of individual spores and to spot spore structures that modulate this response. The time a single spore requires to grow and produce toxin in a food product certainly depends on the microenvironment in the food matrix and was shown to be influenced by the history of spores (Webb et al., 2007). A deeper understanding of how factors during sporulation, food preservation and recovery affect detection times from individual spores represents a fundamental part of estimating the hazard associated with spore-forming food intoxicators such as *C. botulinum*.

5.3 Characterization of Medium/Temperature Effects

5.3.1 Role of Cations in Sporulation Medium-Dependent Resistance

Exact reasons for different sporulation media provoking different HPT resistance are unclear and difficult to explain when complex media are used. However, the mineral content of sporulation media has previously been described to present one of the most important factors influencing spore resistance properties. As Ca^{2+}, Mg^{2+}, and Mn^{2+} presumably play prominent roles (Setlow and Johnson, 2007), and amounts of such cations significantly differ in the media conferring low and high HPT resistance, i.e., M140 and SFE (Fig. 4-17), the effect of supplementation of M140 with Ca, Mg, and Mn contents typically found in SFE on the heat, HHP, and HPT resistance was tested in this study.
To provide an overview of existing data and facilitate the interpretation of results obtained in this study, effects of Ca, Mg, and Mn on spore properties that can be found in literature are summarized in Tab. 5-2.

Tab. 5-2: Existing data on the effect of cations on spores.
Green = positive correlation with cation presence/amount, Yellow = no significant effect, Red = negative correlation. Abbreviations: (DM), = spore demineralization by acid titration (RM), = spores demineralized by acid titration a remineralized with a certain type of cation, (DE) = deficiency of one type of cation in the sporulation medium, (MM) = Minimal medium with one type of cation as only cation source, (EX) = Excessive addition of a certain type of cation to the sporulation medium. References: [1] (Ando and Tsuzuki, 1983), [2] (Bender and Marquis, 1985), [3] (Igura et al., 2003), [4] (Beaman and Gerhardt, 1986), [5] (Palop et al., 1999), [6] (Setlow and Johnson, 2007), [7] (Marquis et al., 1981), [8] (Alderton and Snell, 1963), [9] (Elmabsout and Stevenson, 1979), [10] (Curran et al., 1943), [11] (Slepecky and Foster, 1959), [12] and [13] (Grelet, 1952a, b), [14] (Penna et al., 1996), [15] (Amaha et al., 1956), [16] (Sugiyama, 1951), [17] (Nguyen Thi Minh et al., 2011), [18] (Levinson and Hyatt, 1964), [19] (Mah et al., 2008), [20] (Roberts and Hitchins, 1969), [21] (Gonzalez et al., 2008), [22] (Dixit et al., 2005), [23] (Weinberg, 1955), [24] (Amaha and Ordal, 1957), [25] (Charney et al., 1951), [26] (Vasantha and Freese, 1979), [27] (Kolodziej and Slepecky, 1964), [28] (Curran and Evans, 1954), [29] (Oomes and Brul, 2004), [30] (Cazemier et al., 2001), [31] (Aoki and A., 1974), [32] (Kihm et al., 1990), [33] (Penna et al., 2003).



5.3.1.1 Supplementation of M140 with Typical SFE Cation Contents

Supplementation of the sporulation medium yielding the most heat susceptible spores, M140, with Ca, Mg, and Mn amounts typical for SFE led to a slight but significant increase in HPT resistance of *C. botulinum* type E spores (Fig. 4-18). Additionally, spores grown in supplemented M140 tended to be more resistant against heat and HHP treatments. However, such differences were not significant, which might be related to a different inactivation mechanism or simply to the different stress intensities of heat, HHP, and HPT treatments, i.e., drastically lower inactivation levels provoked by heat or HHP treatments alone could make it harder to detect significant differences. Comparable data is scarce. Cation supplementation of a sporulation medium did not enhance the heat resistance of *C. botulinum* type A spores (Tsuji and Perkins, 1962). This difference might arise from other cation concentrations used, varying, species-specific mineral requirements among different *C. botulinum* types, and other medium compounds acting synergistically or counteracting effects of cation supplementation. For *B. subtilis* spores, it was shown that the presence of a mix of multiple metal ions at high concentrations in the sporulation medium promotes the production of highly heat resistant spores (116-fold increase; (Atrih and Foster, 2001a; Oomes and Brul, 2004) and is more effective than the excessive addition of only one cation source (Oomes and Brul, 2004). Comparably lower differences between M140 and supplemented M140 observed in this study might be related to species- and stress intensity-specific reasons, or indicate that mineral contents typically present in M140 already contribute to spore resistance.

Differences in heat resistance observed in our study may arise from (i) different contents of minerals in the spore protoplast (Atrih and Foster, 2001a), where they interact with protective molecules (e.g., Ca-DPA) or lead to alterations in the core water content, representing an important resistance factor, (ii) changes in the expression levels of SASP encoding genes (Oomes and Brul, 2004) affecting resistance either directly (increased amount in the core, increased protective effect (Oomes and Brul, 2004)) or indirectly (via their influence on the developmental program of spores due to their high DNA affinity (Cabrera-Hernandez et al., 1999)), or (iii) changes in other spore compartments related to resistance, e.g., the spore cortex structure (Atrih and Foster, 2001a). As germination is reported to be a crucial step in the HHP mediated inactivation of spores (at least in *Bacillus*, (Reineke et al., 2013a), section 1.5.3), and disturbances in the metal mix during sporulation can affect the expression of genes related to germination (Hornstra et al., 2006a; Oomes and Brul, 2004), differences in the HHP and HPT resistance observed for *C. botulinum* type E spores might also be influenced by mineral mediated alterations in the amount or functionality of molecules involved in germination (e.g., germinant receptor, cortex lytic enzymes), or spore layers where the germination machinery is situated in. However, due to the lack of knowledge regarding the pressure-mediated inactivation mechanisms in *Clostridium* spores (section 1.5.3), this is purely speculative. However, results indicate that the cation content can, at least partially, explain differences in the resistance of *C. botulinum* type E spores grown in different media. To further evaluate the distinct effects of Ca^{2+}, Mg^{2+}, and Mn^{2+}, the resistance of spores grown in both the complete absence, and the presence of increased amounts of single cations, was determined.

5.3.1.2 The effect of calcium on spore resistance properties

Heat resistance

Results indicate that, for the Ca^{2+} requirements of non-proteolytic *C. botulinum* type E during sporulation, the organism specific threshold level influencing spore heat resistance exceeds that of trace amounts (in contrast to, e.g., *B. cereus* (Gonzalez et al., 2008)). When Ca^{2+} addition was omitted in MDM, heat resistance was drastically decreased (Fig. 4-19). Ca^{2+} concentrations of 1000 mg/L and above conferred the highest heat resistance (Fig. 4-20A), which is in accordance with some earlier reports, where Ca^{2+} has been associated for long and probably most frequently with heat resistance of bacterial endospores (e.g., (Sugiyama, 1951)). As Ca^{2+} and DPA are thought to be co-imported into the forespore (Hintze and Nicholson, 2010), drastically decreased DPA and accordingly Ca-DPA amounts in spores grown in Ca^{2+} deficient or low Ca^{2+} medium might contribute to a decrease in heat resistance. However, the reported presence of different calcium pools (Stewart et al., 1980), the finding that Ca^{2+} supplementation in complex media does not necessarily alter its amount in spores (proteolytic *C. botulinum* type B, (Kihm et al., 1990)), and evidence that Ca^{2+} in the sporulation medium also influences the gene expression profile of spore formers (Oomes et al., 2009) suggests that Ca^{2+}-mediated alterations in spore heat resistance might not rely solely on the physical presence of calcium in the spore protoplast or Ca-DPA complex formation.

HHP resistance

The HHP resistance of *C. botulinum* type E spores grown in the absence of Ca^{2+} was significantly decreased (approx. 0.8 log lower as compared to standard MDM spores) (Fig. 4-19), which possibly occurs due to defects in the spore structure. This effect was drastically less severe than that on heat resistance, which might be related to the different inactivation mechanisms of heat and HHP, i.e., that the ability to retain DPA rather than its protective action in the spore core represents a rate limiting step for spore inactivation by HHP (Reineke et al., 2013b). In contrast to the results obtained here, calcium deficiency has been reported to decrease the efficiency of pressure-induced germination, i.e., increase pressure resistance (Igura et al., 2003) of *B. subtilis* spores, possibly via its negative effect on the activity of cortex lytic enzymes (Paidhungat et al., 2002), germination protein suppression, and/or a decrease in protoplast DPA content (Nguyen Thi Minh et al., 2011). This apparent discrepancy might arise from species-dependent differences in inactivation mechanisms (section 1.5.3), or different treatment conditions, since HHP inactivation pathways of *Bacillus* spores are largely p/T-dependent (Reineke et al., 2012) (section 1.5.3). However, the presence of low amounts of Ca^{2+} during sporulation is apparently required for the development of fully HHP resistant *C. botulinum* type E spores. Additionally, HHP resistance generally tends to decrease with increasing amounts of Ca^{2+} (10 to 2000 mg/L) in the sporulation medium (Fig. 4-20A). Besides structural changes in spore layers, this might be related to the overexpression of germination related genes in the presence of high calcium concentrations (Oomes et al., 2009).

HPT resistance

Sporulation in Ca^{2+}-deficient medium led to the inactivation of the complete spore population by HPT treatments. However, HPT resistance remained largely unaffected when the Ca^{2+} concentration in MDM was increased, and was only slightly positively affected by increasing the concentration from 10 to 1000 or 2000 mg/L, which is in contrast to earlier findings for *B.*

subtilis spores (Margosch et al., 2004b). The reasons for the observed effect are not clear. However, at increased temperatures, the inactivation mechanism under pressure is thought to become different (e.g., due to the temperature-dependent activity of enzymes involved in germination (Reineke et al., 2011)) (section 1.5.3). Since heat resistance is positively affected by the excessive addition of Ca^{2+}, HHP resistance tends to be negatively affected, and HPT resistance remains largely unaffected, it is tempting to speculate that the effects described above contributing to heat and HHP resistance, respectively, somehow compensate for each other.

Notably, for the comparison of inactivation levels of spores grown in supplemented media, it has to be considered that the type of Ca^{2+} source supplied, i.e. the type of associated anions, might function in the regulation of sporulation influencing sporulation characteristics and resistance (Mah et al., 2008).

5.3.1.3 The effect of magnesium on spore resistance properties

Heat resistance

In contrast to Ca^{2+} deficiency, the absence of Mg^{2+} in MDM had no significant influence on the heat resistance of *C. botulinum* type E spores. The role of Mg^{2+} in heat resistance is not fully clear. It has been reported that Mg^{2+} is not essential for heat resistance (Roberts and Hitchins, 1969) but remineralization of demineralized spores can partly restore (Bender and Marquis, 1985) or even enhance (Igura et al., 2003) heat resistance of various *Bacillus* spores. The small effect of Mg^{2+} absence on heat resistance (Fig. 4-19) might indicate that the presence of other cations effectively compensates for the lack of Mg^{2+} during sporulation.

HHP resistance

Similar to heat resistance, the HHP resistance of *C. botulinum* type E spores was not significantly affected by the absence of Mg^{2+} in MDM, which indicates that Mg^{2+} in the sporulation medium is, in contrast to Ca^{2+}, not required for full HHP resistance of *C. botulinum* type E spores (Fig. 4-19). This is in contrast to the negative effect on HHP resistance described for the addition of Mg^{2+} to demineralized *B. subtilis* spores (Igura et al., 2003). However, when Mg^{2+} concentrations in the sporulation medium are increased from 10 or 100 mg/L to 1000 or 2000 mg/L, HHP resistance was influenced slightly negatively, although at the highest concentration used (2000 mg/L), this effect is significantly lower than that on heat or HPT resistance (Fig. 4-20B).

HPT resistance

Although there was only little effect on the resistance to heat or HHP alone, HPT treatments led to the inactivation of the complete spore population when the addition of Mg^{2+} was omitted in the sporulation medium (Fig. 4-19). This might be due to a different inactivation mechanism at elevated temperatures or simply due to the higher stress intensity of the combined treatment, i.e., higher inactivation levels of MDM spores (around 7 log CFU/mL), which makes it easier to detect significant differences in resistance. As there is little known about the effect of Mg^{2+} on spore resistance factors, it is difficult to speculate on reasons, e.g., structural changes, which might account for Mg^{2+} concentration-dependent differences in the HHP or HPT resistance.

5.3.1.4 The effect of manganese on spore resistance properties

Heat resistance

The absence of any single cation source in the sporulation medium led to a decrease in heat resistance of *C. botulinum* type E spores in the order $Ca^{2+} > Mn^{2+} > Mg^{2+}$ (Fig. 4-19). A similar order has been reported previously for de-/remineralized *Bacillus* spores (Bender and Marquis, 1985), which indicates that the physical presence of Mn^{2+} in mature spores can contribute to the heat resistance of spores from both species.

Generally, Mn^{2+} requirements of *C. botulinum* type E are comparatively low, which is reflected by the amounts in the standard and supplemented MDM media being 100-fold lower than the amounts of Ca^{2+} and Mg^{2+}. Nonetheless, an increase in the Mn^{2+} concentration resulted in an increase in heat resistance of *C. botulinum* type E spores (Fig. 4-20C). This could be partially related to a cation type-dependent uptake, which has previously been demonstrated to occur in proteolytic *C. botulinum* type B (Mn^{2+} but not Ca^{2+} or Mg^{2+} addition to a complex medium readily increases amounts of the respective cation in spores (Kihm et al., 1990)) and *B. subtilis* (spores readily accumulate Mn^{2+} in the presence of a salt mix (Atrih and Foster, 2001a)). The positive effect of Mn^{2+} cations on the heat resistance found here is consistent with earlier findings for non-proteolytic *C. botulinum* type B (Kihm et al., 1990), but seems to be not existent in *Bacillus* spore formers such as *B. megaterium* (no significant effect (Levinson and Hyatt, 1964); negative effect of high Mn^{2+} levels (Slepecky and Foster, 1959)). Previous studies suggest various ways how Mn^{2+} could affect the development of heat resistance of bacterial endospores, e.g., via enhancing repair of thermal injuries (Kihm et al., 1990) (at least *B. subtilis* wet heat resistance is not dependent on RecA-mediated DNA repair (Nicholson et al., 2000)), macromolecule protection by Mn^{2+} complexed with DPA, and effects on spore's developmental program influencing spore structures such as the cortex peptidoglycan (Atrih and Foster, 2001a)).

HHP resistance

The absence of Mn^{2+} during sporulation led to a considerable decrease of HHP resistance of almost 2 log cycles when compared with spores grown in a standard medium containing as little as 0.1 mg/L Mn^{2+} (Fig. 4-19). This represents the second highest effect on heat or HHP resistance of *C. botulinum* type E spores mediated by the absences of a cation source found and demonstrates the importance of Mn^{2+} for HHP resistance. Comparable data is generally scarce and absent for *Clostridium* spores. The presence of Mn^{2+} did not significantly influence the HHP resistance of mature *B. subtilis* spores (remineralization of acid titrated spores; (Igura et al., 2003)), but its presence during sporulation affected germination properties of *B. megaterium* (Levinson and Hyatt, 1964). Thus, the effect on HHP resistance observed here might be related to structural changes provoked by the presence of Mn^{2+} during sporulation, affecting pressure induced germination. Altered activities of various Mn^{2+}-dependent enzymes involved in the maturation process of spores, such as superoxide dismutase (SodA) possibly involved in coat maturation in *B. subtilis* and conserved in *C. botulinum* type E (Accession numbers Beluga (TMW 2.990): EES48688; Alaska: YP_001921184), might play a role. Interestingly, increased Mn^{2+} concentrations from 0.1 to 1 mg/L resulted in a clear positive effect on HHP resistance, which tends to diminish when the Mn^{2+} amount is further increased to 10 or 100 mg/L (Fig. 4-20C). This might be related to Mn^{2+} at higher concentrations competing with other minerals for binding sites in the spore or Mn^{2+} concentration-dependent effects on enzyme activities or gene expression. Although heat and

HHP inactivation mechanisms are different, the existence of an optimum for the Mn^{2+}-mediated HHP resistance is consistent with previous observations made for the heat resistance of *B. subtilis* spores, i.e., that a balanced metal mix is required for the highest resistance (Oomes and Brul, 2004).

HPT resistance

The absence of Mn^{2+} resulted in the inactivation of the entire spore population by HPT treatments. Interestingly, the Mn^{2+} concentration-dependent effect on HPT resistance showed the same trend as it was observed for the resistance to HHP treatments at ambient temperatures, i.e., resistance "peaks" at 1 mg/L Mn^{2+}. The fact that this is in contrast to earlier findings for a *B. subtilis* food isolate sporulated in an $MnSO_4$-rich medium (Margosch et al., 2004b), might be related to the higher Mn^{2+} concentration (275 mg/L) used (Margosch et al., 2004b), i.e., the concentration-dependent effect of Mn^{2+} on HPT resistance diminishes or even turns into a negative effect at higher Mn^{2+} concentrations, or simply species-dependent differences in mineral requirements.

5.3.2 Interconnection between Sporulation Medium and Temperature

As discussed in the previous section, in addition to the sporulation temperature, the sporulation medium and, in particular, its mineral content can individually influence the HPT resistance of *C. botulinum* type E spores. Interestingly, both the sporulation medium composition and the sporulation temperature can influence the mineral contents in mature spores (Igura et al., 2003; Lindsay et al., 1990), and, possibly, an overlapping subset of other spore structures, which might include resistance factors to HPT treatments. Furthermore, effects of sporulation temperature and medium have been already demonstrated to be interconnected in conferring resistance to some *Bacillus* spores (heat resistance of *B. cereus*, (Planchon et al., 2011) and *B. weihenstephanensis*, (Garcia et al., 2010) (Baril et al., 2011)). However, until now, this was not demonstrated for the HPT inactivation and not in *Clostridium* spores.

Results indicate that the sporulation medium mineral content can influence sporulation temperature-mediated effects on the HPT resistance of *C. botulinum* type E spores (Fig. 4-21 and Fig. 4-22). A 'disadvantageous' calcium/magnesium/manganese supplementation of the sporulation medium acted synergistically with the detrimental effect of increased sporulation temperatures on the HHP resistance, and the negative effect of such a medium mineralization was counteracted by sporulation at low temperatures. On the other hand, 'beneficial' medium mineralization counteracted the negative effect provoked by sporulation at elevated temperatures. However, beneficial medium supplementation did not further increase the positive effect of sporulation at low temperatures, i.e., the HPT resistance of spores grown in non-supplemented medium at 18°C was virtually identical to that of spores produced in optimally supplemented medium at 18 or 28°C. The finding that mineralization can compensate for negative effects provoked by the sporulation temperature and vice versa indicates (i) that both environmental factors affected identical molecular targets, i.e., distinct defects in the spore structure or weakened resistance factors were counterbalanced, or (ii) that mineralization and sporulation temperature acted on different molecular targets, but negative effects on specific resistance factors were compensated by positive effects on other resistance factors. However, the result that the highest HPT resistance was achieved by

either adding optimal cation amounts to the sporulation medium or lowering the sporulation temperature, and that, at this point, there was no positive synergism between mineralization and sporulation temperature suggests that both environmental factors influenced a similar subset of *C. botulinum* type E spore HHP resistance factors.

Since the sporulation temperature-dependent resistance to HPT (100 – 300 MPa / 55°C, 30 min) negatively correlates with the mineral content of *B. subtilis* spores, spore mineralization has been suggested to be a crucial factor involved in sporulation temperature-mediated effects on HPT resistance (Igura et al., 2003). This has been suggested to be related to a sporulation temperature-mediated decrease in mineral contents resulting in the inhibition of pressure-induced germination, potentially due to a decreased activity of cortex-lytic enzymes in *Bacillus* spores (Igura et al., 2003). The negative effect of elevated sporulation temperatures is generally consistent the findings for *C. botulinum* type E spores, and spore mineralization might also be involved in effects on HPT resistance observed in this study. However, if an increase in the sporulation temperature also increased mineral contents in *C. botulinum* type E spores, the only effect that could be easily explained is the observed negative synergistic effect of high sporulation temperatures and disadvantageous medium mineral contents, possibly due to an increased uptake of cations negatively influencing HPT resistance. This suggests that, in addition to spore mineral contents, other resistance factors modulating HPT-triggered germination or, more generally, the ability to retain DPA of *C. botulinum* type E spores serve as common targets altered by sporulation temperature and cations present during sporulation.

5.3.3 Role of Spore Coat in Sporulation Medium/Temperature Effect

5.3.3.1 Decoated Spores

In contrast to heat resistance. where the coat has been reported to play no significant role as resistance factor (Setlow and Johnson, 2007), the role in HPT resistance is not clear. However, it was speculated in previous studies, that a potential role of the coat in HPT resistance might arise from the fact that important components of the sporulation machinery are situated in or near this layer (CLE) (Bagyan and Setlow, 2002; Oomes et al., 2009) (Brul et al., 2011).

From the results of experiments with decoated spores in this study, it could be concluded that the coat, or at least the presence of outer spore layers is important for HPT resistance in *C. botulinum* type E spores. However, although the used decoating treatment has been frequently stated as specifically removing outer spore layers, it cannot be excluded that the harsh conditions present during decoating affect spore structures that are important for HPT resistance without affecting spore viability (germination, outgrowth, growth; number of CFU not reduced significantly (Fig. 4-23)).

5.3.3.2 Expression of Coat-Related Genes

The relative expression of the majority of the ten examined coat-associated genes was not significantly affected by alterations in the sporulation temperature, in sporulation medium cation contents, or both. However, results (Tab. 4-3) suggest that at least two of the examined genes coding for coat-associated proteins, i.e., SpoIIIAH and YabG, present potential parts of overlapping subsets of HPT resistance factors, which might, based on the results

presented in section 4.3.2, exist in *C. botulinum* type E spores. Results obtained for the different coat-associated genes are discussed below.

SpoIIIAH

Expression levels of *spoIIIAH* increased significantly and strongly (> 10-fold overexpression) in response to all three changes made, i.e., when either medium mineralization, the temperature, or both factors were different (optimized for the production of highly HPT resistant spores). The mother cell protein SpoIIIAH or "stage III sporulation ratchet engulfment protein" is well characterized in *B. subtilis*, where *spoIIIAH* expression is controlled by the mother cell-specific sigma factor σ^E (Eichenberger et al., 2004) (Fig. 1-2). Furthermore, it was reported that SpoIIIAH is targeted specifically to the membrane surrounding the forespore through an interaction of its C-terminal extracellular domain with the C-terminal extracellular domain of the forespore membrane protein SpoIIQ (Meisner et al., 2008) (domain may also be localized to the mother cell-forespore inter-membrane space, (Blaylock et al., 2004; Doan et al., 2005; Jiang et al., 2005)). This way, SpoIIIAH forms part (maybe by forming a ring structure) of a transmembrane channel linking the mother cell and the forespore (Meisner et al., 2008). This channel was shown to be important for the cross-compartmental talk between the two compartments during sporulation (McKenney and Eichenberger, 2012) (Setlow, 2012), the activation of the late forespore-specific σ^G (Fig. 1-2) (Eichenberger et al., 2004), the assembly of coat proteins from kinetic classes II to VI (section 1.2.3.6.6), and the mother cell-distal pole serving as nucleation site for spore coat assembly (McKenney and Eichenberger, 2012). Since some spores (*B. anthracis*) were shown to grow exclusively out of sporulation mother cell distal pole (Steichen et al., 2007), the SpoIIQ-SpoIIIAH channel was speculated to be involved in the co-ordination of the assembly of preferential germination/outgrowth site (McKenney and Eichenberger, 2012). In *C. botulinum* spores, SpoIIQ orthologues or proteins that could take a similar role as partner of SpoIIIAH are not present or not yet identified. Furthermore, it is unknown whether SpoIIIAH is involved in sporulation, coat assembly, and (possibly) germination, in a similar manner as it has been described in *Bacillus* spores. However, SpoIIIAH was reported to be conserved in *Clostridium* (Paredes et al., 2005) and the whole *spoIIIAA-AH* operon appears to be similarly organized in *Bacillus* and *Clostridium* species (Jones et al., 2008). If also the role of SpoIIIAH is conserved in *Clostridium* species, the significantly overexpression of *spoIIIAH* under conditions favorable for the production of HPT resistant spores would indicate that better compartmental cross-talk during sporulation, more effective spore coat protein assembly, and/or an altered architecture of a "predetermined breaking point" of the outer spore layers during germination and outgrowth might contribute to the increased HPT resistance of *C. botulinum* type E spores grown in cation supplemented medium or at sub-optimal growth temperatures. Additionally, similar overexpression levels in response to both separate changes in sporulation conditions and in response to a combination of both (cation presence and a lower temperature) indicates that SpoIIIAH might represent one of the overlapping subset of targets affecting HPT resistance, the existence of which was proposed earlier in this study (section 3.2.4.2).

SpoIVA

Expression levels of *spoIVA* (stage IV sporulation protein A) also tended to increase in response to lower temperatures and a combination of lower temperatures and medium supplementation, but the detected increase was not significant and close to zero when only

sporulation medium cation contents were adjusted. Similar to SpoIIIAH, SpoIVA is very well characterized in *B. subtilis*, where it is produced in the mother cell under the control of σ^E (Fig. 1-2) and already localizes at the forespore mother cell pole of the cell division septum shortly after asymmetric division of the sporangium (Jones et al., 2008; Pogliano et al., 1995). SpoIVA is described as one of four major morphogenetic proteins in *B. subtilis* (Ramamurthi et al., 2009; Ramamurthi and Losick, 2008; Setlow, 2012), which marks the outer membrane of the engulfed forespore as site for both cortex synthesis and coat assembly (Roels et al., 1992; Stevens et al., 1992) (both linked inseparably, (Catalano et al., 2001)). SpoIVA was shown to be responsible for the initial recruitment/deployment of proteins to the spore surface and anchoring the innermost coat layer, i.e. the basement, to the spore surface (de Francesco et al., 2012). This protein was reported to self-assemble into large oligomers (Mullerova et al., 2009) forming a cable-like structure in an ATP-dependent manner (Ramamurthi and Losick, 2008). This structure is thought to serve as scaffold for other coat proteins during the sequential encasement of the forespore (McKenney et al., 2010; McKenney and Eichenberger, 2012; Setlow, 2012). As curvature proceeds, the basement focus, a set of at least six kinetic class I proteins including SpoIVA, tracks with the engulfing mother cell membrane and encase the entire forespore with a basement layer (Driks et al., 1994; Price and Losick, 1999; Setlow, 2012).

In *B. subtilis*, SpoIVA directly interacts with SpoVM (Ramamurthi et al., 2006), putatively with SpoVID (Beall et al., 1993; Driks et al., 1994; Mullerova et al., 2009; Ozin et al., 2000; Wang et al., 2009), and maybe (weakly) with SafA (Mullerova et al., 2009; Qiao et al., 2012). In addition to the coat basement, correct SpoIVA localization is essential for the assembly of CotE (major outer coat morphogenetic protein, (Driks et al., 1994; Webb et al., 1995; Zheng et al., 1988) and the recruitment of additional, σ^K-controlled structural proteins (e.g., CotT, (Bourne et al., 1991), YhjR, YmaG, YodI, YppG, YsnD, YsxE, YxeE, and CotN, (Henriques and Moran, 2007)) and enzymes (e.g., YisY, (Henriques and Moran, 2007; Kim et al., 2006)). Orthologues of SpoVM, SpoVID, SafA, and many outer coat proteins are absent in *Clostridium* species (Henriques and Moran, 2007). However, orthologues of SpoIVA can be found throughout *Bacillus* and *Clostridium* species (including *C. botulinum* type E) and the fact that its C-terminal region is highly conserved suggests that the SpoIVA-dependent mechanism for coat attachment to the outer forespore membrane and possibly the first part of the *B. subtilis* localization cascade at the outer forespore membrane might be conserved in various spore-forming bacteria (Henriques and Moran, 2007; Jones et al., 2008). Thus, results obtained in this study point towards the conclusion that sporulation temperature, medium cation contents, and HPT resistance are not significantly linked to SpoIVA levels guiding the ordered initial recruitment of coat proteins to the spore coat basement.

cotH

Very similar to the SpoIVA expression levels, expression of *cotH* tended to increase in response to lower temperatures and a combination of lower temperatures and medium supplementation, but the detected increase was not significant and close to zero when only sporulation medium cation contents were adjusted.

In *B. subtilis*, CotH (and CotO) were previously described as additional morphogenetic proteins, which regulate the assembly of a partially overlapping protein subset but do not have a specific role in coat attachment (McPherson et al., 2005; Naclerio et al., 1996; Zilhao et al., 1999). However, due to its localization dependency on the major morphogenetic protein

CotE, CotH is generally classified as one of many outer coat proteins (Fig. 1-6) (Kim et al., 2006). Although CotH has been not yet unambiguously assigned to a kinetic wave, dependence on CotE suggests an assembly primarily during kinetic wave II, which is thought to be mainly controlled by σ^E. Additionally, the germination-associated protein SscA might be involved in the assembly of CotH (Kodama et al., 2011), although its exact role remains to be elucidated. CotH itself was reported to function as protease inhibitor in the mother cell cytoplasm stabilizing other coat proteins (outer coat CotC and the crust component CotG) (Isticato et al., 2004; Isticato et al., 2008; Kim et al., 2005; Sacco et al., 1995; Zilhao et al., 2004). Furthermore, it was suggested to play a role in germination and lysozyme resistance and, thus, potentially also influencing inner coat assembly.

CotH orthologues can be found in some, but not all *Bacillus* species and some, but not all *Clostridium* species including *C. perfringens*, *C. cellulolyticum*, *C. difficile*, and *C. thermocellum* (Henriques and Moran, 2007). However, CotH might have different main functions in different (even *Bacillus*) species (e.g., directing coat protein assembly and functioning as germination repressor in *B. anthracis*, (Giorno et al., 2007)). Such functions described in other organisms together with the mentioned suggestion that CotH also possibly influences inner coat layers might be more valuable than the roles described in *B. subtilis* to explain the role of CotH orthologues in *C. botulinum* type E, since this organism lacks the major morphogenetic outer coat protein CotE and most other outer coat proteins characterized in *B. subtilis*. Although it is difficult to speculate on the actual role of CotH, *cotH* expression levels seem to be not lined to HPT resistance of *C. botulinum* type E spores.

CotS

Expression levels of *cotS* tended to decrease at low growth temperatures and a combination of low growth temperatures with the presence of “optimal” medium cation amounts, whereas alterations in the cation amounts alone did not affect expression levels at all. However, none of the observed differences compared to standard sporulation conditions was significant, which was also the case for the second putative coat protein from the CotS family examined. Similar to CotH, CotS is characterized as CotE-dependent outer coat protein in *B. subtilis* (Kim et al., 2006) (McKenney et al., 2010), which again indicates the limited transferability of insights into the localization of some coat proteins in *B. subtilis* to actual dependencies in *C. botulinum*. The cotS family of coat proteins is relatively large and not always designated as CotS (e.g., members are also YtaA and YutH). Although it cannot be excluded that other structural coat proteins play a role in the response of *C. botulinum* type E to altered temperatures and medium cation contents, results obtained here suggest that at least the two tested CotS family proteins readily identified in strain Beluga, are not responsible for the HPT resistance of spores produced under such altered conditions.

YabG

The coat-associated protease YabG (sporulation peptidase, peptidase_V57 superfamily) together with the putative transmembrane channel protein SpoIIIAH were the only proteins, the genes of which were significantly overexpressed in response to temperature and medium alterations as well as a combination thereof. In *B. subtilis*, YabG was suggested to play a role in cross-linking of spore surface layers via its cooperation with cross-linking enzymes (e.g., Tgl) either generating substrates for cross-linking enzymes from protein precursors or facilitating access of such enzymes to their target sites (Kuwana et al., 2006). Additionally, YabG was reported to be involved in the proteolysis and maturation of the spore coat proteins

including the major morphogenetic coat basement protein SpoIVA (described above). Functional YabG was shown to be crucial for normal coat protein composition.
Interestingly, it was shown that increased temperature compensates for the lack of YabG, which has been suggested to be due to increased Tgl activity (Suzuki et al., 2000b) or the altered conformation of Tgl substrates at higher temperatures (Kuwana et al., 2006). Although Tgl was reported to be not conserved in *Clostridium* species (Henriques and Moran, 2007), this general observation is consistent with the increased *yabG* expression levels detected in *C. botulinum* type E grown at lower temperatures. The fact that cation supplementation of the growth medium similarly increased *yabG* expression levels and that a combination of both factors during growth led to almost identical expression levels compared with the effects of each factor alone, indicates that YabG might belong to an overlapping subset of targets affecting HPT resistance, the existence of which was proposed earlier in this study (section 3.2.4.2). Moreover, provided that the mode of action of YabG is similar in *B. subtilis* and *C. botulinum*, new light would be shed on the interconnection between SpoIVA and HPT resistance discussed above, i.e., that increased levels of mature SpoIVA could be present in *C. botulinum* type E spores grown at lower temperatures and/or in the presence of specific cations although *spoIVA* expression levels are not altered. This could lead to a different (better?) organization of the innermost coat layer, which, in turn, could confer increased HPT resistance, independent from the presence of Tgl or other cross-linking enzymes.

SodA

In addition to YabG, other important coat-associated enzymes are involved in cross-linking or maturation of other coat proteins. One of these enzymes is the superoxide dismutase, SodA, which can be also found in *C. botulinum* type E. Notably, *sodA* expression levels in response to “optimal” cation supplementation in the sporulation medium at the references temperature of 28 °C were the only ones in this study where a significant decrease was detected. In *B. subtilis*, SodA is putatively responsible for the covalent cross-link of tyrosine-rich outer coat proteins (e.g., CotG) into the insoluble matrix provoking a striated appearance of the outer coat layer (Henriques et al., 1998). Similarly, it was suggested that the *C. difficile* CotE (with 1-Cys-peroxiredoxin activity) might be, similar to SodA, involved in cross-linking of tyrosine-rich spore coat proteins and, thus, speculated to play a role in coat assembly (Permpoonpattana et al., 2011). Furthermore, it was speculated that H_2O_2 may play a key role in spore coat synthesis and could serve as a substrate for the oxidative cross-linking of spore coat monomers (Henriques et al., 1998). This process, in turn, could be modulated by enzymes such as SodA. However, the localization of SodA and its exact role in coat maturation is not completely clear. For example, the structural coat integrity of *C. difficile sodA* (also *cotD, cotE*) mutants led to the suggestion that these enzymes are located in the exosporium involved in coat polymerization and detoxification of H_2O_2 or that they are structurally redundant (Permpoonpattana et al., 2013). This makes it considerably difficult, to speculate on the possible locations and mode of actions of SodA in *C. botulinum* type E. However, the fact that significantly decreased *sodA* expression levels were detected after growth in mTPYC+ medium might reflect a response to higher manganese levels in the medium, which makes lower amounts of this Mn-dependent enzyme necessary to achieve similar activity. On the other hand, enhanced *sodA* expression at lower temperatures might be a response to increased coat rigidity and, thus, reduced accessibility of cross-linking targets of SodA. Notably, *sodA* expression levels are the only ones, where effects of low

temperature and cation supplementation appear to compensate for each other. However, contrary effects on *sodA* expression, make it unlikely that this response is connected to HPT resistance of *C. botulinum* type E spores, which was enhanced under all three environmental conditions.

spsE and spsC

The expression levels of *spsE* and *spsC* tended to be slightly (not significantly) increased in response to growth in supplemented medium, but largely unaffected at lower temperatures. As a result a combination of both environmental factors led to almost no effect of gene expression levels relative to the reference conditions (mTPYC, 28 °C). The two spore coat polysaccharide biosynthesis proteins SpsC and SpsE are described as glutamine-dependent sugar transaminase and phosphoenolpyruvate-sugar pyruvyltransferase in *B. subtilis*, respectively. Spore coat polysaccharide biosynthesis proteins are thought to be controlled by σ^K, are putatively mainly involved in glycosylation of the outermost coat layers, and, thus, can modulate spore surface properties such as hydrophobicity (Driks, 1999). However, they appear to be not essential for an intact coat structure (Driks, 1999). A role of such sugars in the protection of structures essential to spore germination from damage by wet heat, i.e., similar like protective effects of sugars added to a heating medium, has been proposed (Oomes et al., 2009), but not yet proven. Although their function in *C. botulinum* type E is not known and *C .botulinum* type E possess a completely different exosporium structure, SpsC and SpsE might have similar activities to that described in *B. subtilis*. However, a significant increase in expression levels of *sps* genes in response to increased calcium contents in the medium (mTPYC+ vs. mTPYC) as it was observed earlier for *B. subtilis* (Oomes et al., 2009), was not detected in this study. This might reflect, species- and calcium concentration-dependent differences or be a result of adverse effects exerted by altered amounts in other cations (Mg and Mn). However, the absence of significant differences in the gene expression levels of both *spsE* and *spsC* suggests that they unlikely account for detected differences in the HPT resistance of *C. botulinum* type E spores.

sleB

The expression levels of *sleB*, which codes for a cortex lytic enzyme that is well characterized in *B. subtilis* and is conserved in some *Clostridium* species (Henriques and Moran, 2007) including *C. botulinum* type E, were not significantly affected under the conditions tested. SleB is thought to play a role in the non-physiological germination pathway in *Bacillus* spores (described in detail in section 1.5.3), but might not be functional in many *Clostridium* spores (discussed in detail in section 4.2.2). Despite of its putatively absent functionality, gene expression data indicates that altered levels of this enzyme in *C. botulinum* type E spores are unlikely to account for the observed increase in HPT resistance under the conditions tested. However, it cannot be excluded, that one of the putative, yet uncharacterized cortex lytic enzymes (e.g., CLH_2909, CLH_1514, and CLH_0063 in strain Alaska) or other cell wall hydrolases (e.g., CLH_2422, CLH_2946, CLH_2947) are functional in *C. botulinum* type E spores, affected by alterations in the sporulation temperature or medium cation contents, and mediating the observed effects on HPT resistance.

Notably, there are several points that have to be considered drawing final conclusions out of the results presented here: (i) Translation efficiencies, post-translational modifications, and factors affecting correct assembly of proteins in the coat are reasons, why increased gene

expression levels have not necessarily to correlate with increased levels of a specific protein correctly assembled in the coat. (ii) Differences in relative expression levels might be dependent on the sampling time point. (iii) An observed upregulation and even a resulting increase in correctly assembled coat protein levels are not necessarily linked to increased HPT resistance. However, due to the lack of knowledge regarding the time course of gene expression as well as coat protein abundance, localization, and modifications in *C. botulinum* type E, it is difficult to estimate the impact of the mentioned influence factors. This, altogether, makes additional experiments such as the determination of gene expression kinetics, studies on the protein level, and the creation and analysis of knock-out mutants necessary to gain further insights.

6 REFERENCES

Abe, F., 2013. Dynamic structural changes in microbial membranes in response to high hydrostatic pressure analyzed using time-resolved fluorescence anisotropy measurement. Biophys Chem 183, 3-8.

Abel-Santos, E., Dodatko, T., 2007. Differential nucleoside recognition during *Bacillus cereus* 569 (ATCC 10876) spore germination. New J Chem 31, 748-755.

Ablett, S., Darke, A.H., Lillford, P.J., Martin, D.R., 1999. Glass formation and dormancy in bacterial spores. Int J Food Sci Tech 34, 59-69.

Abrahamsson, K., 1967. Occurrence of type E *Cl. botulinum* in smoked eel. *In*: Ingram M, Roberts TA, eds. Botulism 1966. Proceedings of the Fifth International Symposium on Food Microbiology. Moscow: Meat Research Institute, Agricultural Research Council, Cambridge, UK, 1967:73-5.

Adam, K.H., Flint, S.H., Brightwell, G., 2010. Psychrophilic and psychrotrophic *clostridia*: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. Int J Food Sci Tech 45, 1539-1544.

Adams, C.M., Eckenroth, B.E., Putnam, E.E., Doublié, S., Shen, A., 2013. Structural and functional analysis of the CspB protease required for *Clostridium* spore germination. Plos Pathog 9, e1003165.

Aguilar, P.S., Cronan, J.E., Jr., de Mendoza, D., 1998. A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. Journal of bacteriology 180, 2194-2200.

Aguilar, P.S., Hernandez-Arriaga, A.M., Cybulski, L.E., Erazo, A.C., de Mendoza, D., 2001. Molecular basis of thermosensing: a two-component signal transduction thermometer in Bacillus subtilis. The EMBO journal 20, 1681-1691.

Ahn, J., Balasubramaniam, V.M., Yousef, A.E., 2007. Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. International Journal of Food Microbiology 113, 321-329.

Ahn, J., Lee, H.Y., Balasubramaniam, V.M., 2015. Inactivation of Geobacillus stearothermophilus spores in low-acid foods by pressure-assisted thermal processing. J Sci Food Agr 95, 174-178.

Aizawa, S.-I., Zhulin, I.B., Márquez-Magaña, L., Ordal, G.W., 2002. Chemotaxis and Motility. In: *Bacillus subtilis* and its closest relatives. eds Sonenshein AL, Hoch JA & Losick R; (UK), FSA - Food Standards Agency; ASM Press, Washington DC, 2002, 437-452.

Akhtar, S., Paredes-Sabja, D., Torres, J.A., Sarker, M.R., 2009. Strategy to inactivate *Clostridium perfringens* spores in meat products. Food Microbiol 26, 272-277.

Ala-Huikku, K., Nurmi, E., Pajulahti, H., Raevuori, M., 1977. The occurrence of Clostridium botulinum type E in Finnish trout farms and the prevention of toxin formation in fresh-salted vacuum-packed trout fillets. Nord Vet Med 29, 386-391.

Alberto, F.O., Botella, L., Carlin, F., Nguyen-The, C., Broussolle, W., 2005. The Clostridium botulinum GerAB germination protein is located in the inner membrane of spores. Fems Microbiology Letters 253, 231-235.

Alcock, S., 1984. Elevation of heat resistance of *Clostridium sporogenes* following heat shock. Food Microbiology, 1984, 1, 39-47.

Alderton, G., Chen, J.K., Ito, K.A., 1974. Effect of lysozyme on the recovery of heated *Clostridium botulinum* spores. Appl Microbiol 27, 613-615.

Alderton, G., Snell, N., 1963. Base Exchange and Heat Resistance in Bacterial Spores. Biochem Bioph Res Co 10, 139-&.

Alimova, A., Katz, A., Gottlieb, P., Alfano, R.R., 2006. Proteins and dipicolinic acid released during heat shock activation of Bacillus subtilis spores probed by optical spectroscopy. Appl Opt 45, 445-450.

Alsaker, K.V., Papoutsakis, E.T., 2005. Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. J Bacteriol 187, 7103-7118.

Amaha, M., Ordal, Z.J., 1957. Effect of Divalent Cations in the Sporulation Medium on the Thermal Death Rate of Bacillus-Coagulans Var Thermoacidurans. Journal of bacteriology 74, 596-604.

Amaha, M., Ordal, Z.J., Touba, A., 1956. Sporulation Requirements of Bacillus-Coagulans Var Thermoacidurans in Complex Media. Journal of bacteriology 72, 34-41.

Ananta, E., Heinz, V., Schlüter, O., Knorr, D., 2001. Kinetic studies on high-pressure inactivation of *Bacillus stearothermophilus* spores suspended in food matrices. Innov Food Sci Emerg 2, 261-272.

Ando, Y., Tsuzuki, T., 1983. Mechanism of chemical manipulation of the heat-resistance of *Clostridium perfringens* spores. J Appl Bacteriol 54, 197-202.

Aoki, H., A., S.R., 1974. The formation of B. megaterium spores having increased heat and radiation resistance and variable heat shock requirements due to manganous ions, . p. 95-102. In A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research 1973. Academic Press., Inc., London.

Aouadhi, C., Simonin, H., Prevost, H., de Lamballerie, M., Maaroufi, A., Mejri, S., 2012. Optimization of pressure-induced germination of *Bacillus sporothermodurans* spores in water and milk. Food Microbiol 30, 1-7.

Appert, N., 1810. L'art de conserver pendant plusiers annes toutes les substances animales et vegetables, Patris & Co., Quay Napoleon, Paris.
Ardia, A., 2004. Process considerations on the application of high pressure treatment at elevated temperature levels for food preservation. Dissertation, TU Berlin.
Ardia, A., Knorr, D., Ferrari, G., Heinz, V., 2004a. Kinetic studies on combined high-pressure and temperature inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. Appl Biotechnol Food Sci Policy 1, 169-173.
Ardia, A., Knorr, D., Heinz, V., 2004b. Adiabatic heat modelling for pressure build-up during high-pressure treatment in liquid-food processing. Food Bioprod Process 82, 89-95.
Aronson, A.I., Ekanayake, L., Fitz-James, P.C., 1992. Protein filaments may initiate the assembly of the Bacillus subtilis spore coat. Biochimie 74, 661-667.
Aronson, A.I., Fitz-James, P., 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol Rev 40, 360-402.
Aronson, A.I., Fitzjame.Pc, 1971. Reconstitution of Bacterial Spore Coat Layers in-Vitro. Journal of bacteriology 108, 571-&.
Aronson, A.I., Hu, H., 2008. The response to a specific germinant by Bacillus anthracis spores in primary mouse macrophages is modulated by a protein encoded on the pXO1 plasmid. Arch Microbiol 190, 539-546.
Arora, G., Sajid, A., Arulanandh, M.D., Misra, R., Singhal, A., Kumar, S., Singh, L.K., Mattoo, A.R., Raj, R., Maiti, S., Basu-Modak, S., Singh, Y., 2013. Zinc regulates the activity of kinase-phosphatase pair (BasPrkC/BasPrpC) in *Bacillus anthracis*. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 26, 715-730.
Arritt, F.M., Eifert, J.D., Jahncke, M.L., Pierson, M.D., Williams, R.C., 2007. Effects of modified atmosphere packaging on toxin production by Clostridium botulinum in raw aquacultured summer flounder fillets (Paralichthys dentatus). J Food Protect 70, 1159-1164.
Artin, I., Bjorkman, P., Cronqvist, J., Radstrom, P., Holst, E., 2007. First case of type E wound botulism diagnosed using real-time PCR. J Clin Microbiol 45, 3589-3594.
Artin, I., Carter, A.T., Holst, E., Lovenklev, M., Mason, D.R., Peck, M.W., Radstrom, P., 2008. Effects of carbon dioxide on neurotoxin gene expression in nonproteolytic *Clostridium botulinum* type E. Applied and Environmental Microbiology 74, 2391-2397.
Atrih, A., Bacher, G., Korner, R., Allmaier, G., Foster, S.J., 1999. Structural analysis of Bacillus megaterium KM spore peptidoglycan and its dynamics during germination. Microbiology 145 (Pt 5), 1033-1041.
Atrih, A., Foster, S.J., 2001a. Analysis of the role of bacterial endospore cortex structure in resistance properties and demonstration of its conservation amongst species. J Appl Microbiol 91, 364-372.
Atrih, A., Foster, S.J., 2001b. In vivo roles of the germination-specific lytic enzymes of Bacillus subtilis 168. Microbiology 147, 2925-2932.
Atrih, A., Zollner, P., Allmaier, G., Foster, S.J., 1996. Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. J Bacteriol 178, 6173-6183.
Atrih, A., Zollner, P., Allmaier, G., Williamson, M.P., Foster, S.J., 1998. Peptidoglycan structural dynamics during germination of *Bacillus subtilis* 168 endospores. J Bacteriol 180, 4603-4612.
Augustin, J.C., 2011. Challenges in risk assessment and predictive microbiology of foodborne spore-forming bacteria. Food Microbiol 28, 209-213.
Aung, S., Shum, J., Abanes-De Mello, A., Broder, D.H., Fredlund-Gutierrez, J., Chiba, S., Pogliano, K., 2007. Dual localization pathways for the engulfment proteins during Bacillus subtilis sporulation. Mol Microbiol 65, 1534-1546.
Autio, T.J., Lindstrom, M.K., Korkeala, H.J., 2004. Research update on major pathogens associated with fish products and processing of fish. Food Saf Assur Vet 2, 115-134.
Bach, R., Wenzel, S., Müller-Prasuhn, G., Gläsker, H., 1971. Teichforellen als Träger von *Clostridium botulinum* und Ursache von Botulismus. 3. Mitt.: Nachweis von Clostridium botulinum Typ E in einer Teichwirtschaft mit Bearbeitungsbetrieb sowie in frischen und geräucherten Forellen verschiedener Herkunft. Arch. Lebensmittelhyg. 22, 107-112.
Bagyan, I., Setlow, P., 2002. Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. J Bacteriol 184, 1219-1224.
Bahl, H., Müller, H., Behrens, S., Joseph, H., Narberhaus, F., 1995. Expression of heat-shock genes in *Clostridium acetobutylicum*. Fems Microbiol Rev 17, 341-348.
Baker, D.A., Genigeorgis, C., Glover, J., Razavilar, V., 1990. Growth and toxigenesis of C. botulinum type E in fishes packaged under modified atmospheres. International Journal of Food Microbiology 10, 269-289.
Balasubramaniam, V.M., Balasubramanian, S., 2003. Compression heating influence of pressure transmitting fluids on bacteria inactivation during high pressure processing. Food Res Int 36, 661-668.
Ball, D.A., Taylor, R., Todd, S.J., Redmond, C., Couture-Tosi, E., Sylvestre, P., Moir, A., Bullough, P.A., 2008. Structure of the exosporium and sublayers of spores of the Bacillus cereus family revealed by electron crystallography. Mol Microbiol 68, 947-958.

Banawas, S., Paredes-Sabja, D., Korza, G., Li, Y., Hao, B., Setlow, P., Sarker, M.R., 2013. The *Clostridium perfringens* germinant receptor protein GerKC is located in the spore inner membrane and is crucial for spore germination. J Bacteriol 195, 5084-5091.

Bandow, J.E., Brotz, H., Hecker, M., 2002. Bacillus subtilis tolerance of moderate concentrations of rifampin involves the sigma(B)-dependent general and multiple stress response. Journal of bacteriology 184, 459-467.

Baranyi, J., George, S.M., Kutalik, Z., 2009. Parameter estimation for the distribution of single cell lag times. Journal of Theoretical Biology 259, 24-30.

Baril, E., Coroller, L., Couvert, O., El Jabri, M., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., Mafart, P., 2012a. Sporulation boundaries and spore formation kinetics of Bacillus spp. as a function of temperature, pH and a(w). Food Microbiol 32, 79-86.

Baril, E., Coroller, L., Couvert, O., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., Mafart, P., 2012b. Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores as function of sporulation temperature and pH. Food Microbiol 30, 29-36.

Baril, E., Coroller, L., Postollec, F., Leguerinel, I., Boulais, C., Carlin, F., Mafart, P., 2011. The wet-heat resistance of *Bacillus weihenstephanensis* KBAB4 spores produced in a two-step sporulation process depends on sporulation temperature but not on previous cell history. International Journal of Food Microbiology 146, 57-62.

Bauer, T., Little, S., Stover, A.G., Driks, A., 1999. Functional regions of the Bacillus subtilis spore coat morphogenetic protein CotE. Journal of bacteriology 181, 7043-7051.

Baumgart, J., 1972. Occurence and demonstration of *Clostridium botulinum* in salt water fish. Archiev für Lebensmittelhygiene 23, 34-39.

Baweja, R.B., Zaman, M.S., Mattoo, A.R., Sharma, K., Tripathi, V., Aggarwal, A., Dubey, G.P., Kurupati, R.K., Ganguli, M., Chaudhury, N.K., Sen, S., Das, T.K., Gade, W.N., Singh, Y., 2008. Properties of *Bacillus anthracis* spores prepared under various environmental conditions. Arch Microbiol 189, 71-79.

Beall, B., Driks, A., Losick, R., Moran, C.P., 1993. Cloning and characterization of a gene required for the assembly of the *Bacillus subtilis* spore coat. J Bacteriol 175, 1705-1716.

Beaman, T.C., Gerhardt, P., 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl Environ Microb 52, 1242-1246.

Beaman, T.C., Koshikawa, T., Pankratz, H.S., Gerhardt, P., 1984. Dehydration Partitioned within Core Protoplast Accounts for Heat-Resistance of Bacterial-Spores. Fems Microbiology Letters 24, 47-51.

Beaman, T.C., Pankratz, H.S., Gerhardt, P., 1989. Low heat resistance of Bacillus sphaericus spores correlated with high protoplast water content. FEMS Microbiol Lett 49, 1-4.

Becker, E.C., Pogliano, K., 2007. Cell-specific SpoIIIE assembly and DNA translocation polarity are dictated by chromosome orientation. Mol Microbiol 66, 1066-1079.

Beckering, C.L., Steil, L., Weber, M.H., Volker, U., Marahiel, M.A., 2002. Genomewide transcriptional analysis of the cold shock response in Bacillus subtilis. Journal of bacteriology 184, 6395-6402.

Behravan, J., Chirakkal, H., Masson, A., Moir, A., 2000. Mutations in the gerP locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores. J Bacteriol 182, 1987-1994.

Belliveau, B.H., Beaman, T.C., Pankratz, H.S., Gerhardt, P., 1992. Heat killing of bacterial spores analyzed by differential scanning calorimetry. Journal of Bacteriology 174, 4463-4474.

Bender, G.R., Marquis, R.E., 1985. Spore heat resistance and specific mineralization. Appl Environ Microb 50, 1414-1421.

Bi, C.H., Jones, S.W., Hess, D.R., Tracy, B.P., Papoutsakis, E.T., 2011. SpoIIE is necessary for asymmetric division, sporulation, and expression of sigma(F), sigma(E), and sigma(G) but does not control solvent production in *Clostridium acetobutylicum* ATCC 824. J Bacteriol 193, 5130-5137.

Bischofs, I.B., Hug, J.A., Liu, A.W., Wolf, D.M., Arkin, A.P., 2009. Complexity in bacterial cell-cell communication: Quorum signal integration and subpopulation signaling in the *Bacillus subtilis* phosphorelay. P Natl Acad Sci USA 106, 6459-6464.

Black, E.P., Koziol-Dube, K., Guan, D., Wei, J., Setlow, B., Cortezzo, D.E., Hoover, D.G., Setlow, P., 2005. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. Appl Environ Microb 71, 5879-5887.

Black, E.P., Linton, M., McCall, R.D., Curran, W., Fitzgerald, G.F., Kelly, A.L., Patterson, M.F., 2008. The combined effects of high pressure and nisin on germination and inactivation of *Bacillus* spores in milk. J Appl Microbiol 105, 78-87.

Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., Hoover, D.G., 2007a. Response of spores to high-pressure processing. Compr Rev Food Sci F 6, 103-119.

Black, E.P., Wei, J., Atluri, S., Cortezzo, D.E., Koziol-Dube, K., Hoover, D.G., Setlow, P., 2007b. Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure. J Appl Microbiol 102, 65-76.

Blaylock, B., Jiang, X., Rubio, A., Moran, C.P., Jr., Pogliano, K., 2004. Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. Genes Dev 18, 2916-2928.

Bott, T.L., Deffner, J.S., McCoy, E., Foster, E.M., 1966. Clostridium botulinum type E in fish from the Great Lakes. Journal of Bacteriology 91, 919-924.
Bourne, N., FitzJames, P.C., Aronson, A.I., 1991. Structural and germination defects of Bacillus subtilis spores with altered contents of a spore coat protein. Journal of Bacteriology 173, 6618-6625.
Boyer, A., Girault, C., Bauer, F., Korach, J.M., Salomon, J., Moirot, E., Leroy, J., Bonmarchand, G., 2001. Two cases of foodborne botulism type E and review of epidemiology in France. Eur J Clin Microbiol Infect Dis 20, 192-195.
Brandi, A., Pon, C.L., Gualerzi, C.O., 1994. Interaction of the main cold shock protein CS7.4 (CspA) of Escherichia coli with the promoter region of hns. Biochimie 76, 1090-1098.
Bridgman, P.W., 1912. Thermodynamic properties of liquid water to 80 degrees and 12000 kgm. P Am Acad Arts Sci 48, 307-362.
Brigulla, M., Hoffmann, T., Krisp, A., Volker, A., Bremer, E., Volker, U., 2003. Chill induction of the SigB-dependent general stress response in Bacillus subtilis and its contribution to low-temperature adaptation. J Bacteriol 185, 4305-4314.
Brinkac, L.M., Brown, J.L., Bruce, D., Detter, C., Munk, C., Smith, L.A., Smith, T.J., Sutton, G., Brettin, T.S., 2008. Direct submission. . Consortium: US DOE Joint Genome Institute (JGI-PGF). Submission date: 29-APR-2008 Los Alamos National Laboratory, Los Alamos, NM, USA
Broda, D.M., De Lacy, K.M., Bell, R.G., 1998. Efficacy of heat and ethanol spore treatments for the isolation of psychrotrophic Clostridium spp. associated with the spoilage of chilled vacuum-packed meats. International Journal of Food Microbiology 39, 61-68.
Bruch, M.K., Bohrer, C.W., Denny, C.B., 1968. Adaptation of biphasic bulture technique to sporulation of *Clostridium botulinum* type E. J Food Sci 33, 108-109.
Brul, S., van Beilen, J., Caspers, M., O'Brien, A., de Koster, C., Oomes, S., Smelt, J., Kort, R., Ter Beek, A., 2011. Challenges and advances in systems biology analysis of *Bacillus* spore physiology; molecular differences between an extreme heat resistant spore forming *Bacillus subtilis* food isolate and a laboratory strain. Food Microbiol 28, 221-227.
Brunsing, R.L., La Clair, C., Tang, S., Chiang, C., Hancock, L.E., Perego, M., Hoch, J.A., 2005. Characterization of sporulation histidine kinases of *Bacillus anthracis*. J Bacteriol 187, 6972-6981.
Buchanan, C.E., Gustafson, A., 1992. Mutagenesis and mapping of the gene for a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J Bacteriol 174, 5430-5435.
Buchanan, C.E., Ling, M.L., 1992. Isolation and sequence-analysis of Dacb, which encodes a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J Bacteriol 174, 1717-1725.
Bull, M.K., Olivier, S.A., van Diepenbeek, R.J., Kormelink, F., Chapman, B., 2009. Synergistic inactivation of spores of proteolytic *Clostridium botulinum* strains by high pressure and heat is strain and product dependent. Appl Environ Microb 75, 434-445.
Burgos, J., Ordonez, J.A., Sala, F., 1972. Effect of Ultrasonic-Waves on Heat Resistance of Bacillus-Cereus and Bacillus-Licheniformis Spores. Applied Microbiology 24, 497-&.
Burns, D.A., Heap, J.T., Minton, N.P., 2010. SleC is essential for germination of *Clostridium difficile* spores in nutrient-rich medium supplemented with the bile salt taurocholate. J Bacteriol 192, 657-664.
Burns, G.F., Williams, H., 1975. *Clostridium botulinum* in Scottish fish farms and farmed trout. J Hyg (Lond) 74, 1-6.
Butzin, X.Y., Troiano, A.J., Coleman, W.H., Griffiths, K.K., Doona, C.J., Feeherry, F.E., Wang, G., Li, Y.Q., Setlow, P., 2012. Analysis of the Effects of a gerP Mutation on the Germination of Spores of Bacillus subtilis. Journal of Bacteriology.
Byun, B.Y., Liu, Y.H., Tang, J., Kang, D.H., Cho, H.Y., Hwang, H.J., Mah, J.H., 2011. Optimization and evaluation of heat-shock condition for spore enumeration being used in thermal-process verification: Differential responses of spores and vegetative cells of Clostridium sporogenes to heat shock. Food Sci Biotechnol 20, 751-757.
Cabrera-Hernandez, A., Sanchez-Salas, J.L., Paidhungat, M., Setlow, P., 1999. Regulation of four genes encoding small, acid-soluble spore proteins in *Bacillus subtilis*. Gene 232, 1-10.
Camp, A.H., Losick, R., 2009. A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. Gene Dev 23, 1014-1024.
Campo, N., Rudner, D.Z., 2006. A branched pathway governing the activation of a developmental transcription factor by regulated intramembrane proteolysis. Mol Cell 23, 25-35.
Cangiano, G., Mazzone, A., Baccigalupi, L., Isticato, R., Eichenberger, P., De Felice, M., Ricca, E., 2010. Direct and indirect control of late sporulation genes by GerR of *Bacillus subtilis*. J Bacteriol 192, 3406-3413.
Cann, D.C., Wilson, B.B., Shewan, J.M., Hobbs, G., 1966. Incidence of Clostridium botulinum type E in fish products in the United Kingdom. Nature 211, 205-206.
Carlin, F., 2011. Origin of bacterial spores contaminating foods. Food Microbiol 28, 177-182.
Carr, K.A., Janes, B.K., Hanna, P.C., 2010. Role of the gerP operon in germination and outgrowth of *Bacillus anthracis* spores. Plos One 5, e9128.

Cartman, S.T., Minton, N.P., 2010. A mariner-based transposon system for *in vivo* random mutagenesis of *Clostridium difficile*. Appl Environ Microbiol 76, 1103-1109.
Catalano, F.A., Meador-Parton, J., Popham, D.L., Driks, A., 2001. Amino acids in the Bacillus subtilis morphogenetic protein SpoIVA with roles in spore coat and cortex formation. Journal of Bacteriology 183, 1645-1654.
Cato, E.P., George, W.L., Finegold, S.M., 1986. Genus Clostridium. *In:* P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2, The Williams and Wilkins Co, Baltimore (1986), pp. 1141–1200.
Cazemier, A.E., Wagenaars, S.F.M., ter Steeg, P.F., 2001. Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage *bacilli*. J Appl Microbiol 90, 761-770.
CDC, 2013. Botulism: Epidemiological Overview for Clinicians. Centers for Disease Control and Prevention, USA, 2013, http://www.cdc.gov/.
Cerf, O., 1977. Tailing of Survival Curves of Bacterial-Spores. J Appl Bacteriol 42, 1-19.
CFSPH, 2010. Botulism. The Center for Food Security and Public Health, Iowa State University, 2160 College of Vet Med, Ames, IA 50011, USA. http://www.cfsph.iastate.edu/Factsheets/pdfs/botulism.pdf.
Chada, V.G., Sanstad, E.A., Wang, R., Driks, A., 2003. Morphogenesis of bacillus spore surfaces. Journal of Bacteriology 185, 6255-6261.
Chaibi, A., Ababouch, L.H., Busta, F.F., 1996. Inhibition by monoglycerides of L-alanine-triggered Bacillus cereus and Clostridium botulinum spore germination and outgrowth. J Food Protect 59, 832-837.
Charney, J., Fisher, W.P., Hegarty, C.P., 1951. Managanese as an essential element for sporulation in the genus Bacillus. J Bacteriol 62, 145-148.
Chastanet, A., Losick, R., 2011. Just-in-time control of Spo0A synthesis in *Bacillus subtilis* by multiple regulatory mechanisms. J Bacteriol 193, 6366-6374.
Cheftel, J.C., 1995. Review: High-pressure, microbial inactivation and food preservation. Food Sci Technol Int 1, 75-90.
Chen, G., Driks, A., Tawfiq, K., Mallozzi, M., Patil, S., 2010a. Bacillus anthracis and Bacillus subtilis spore surface properties and transport. Colloid Surface B 76, 512-518.
Chen, Y., Fukuoka, S., Makino, S., 2000. A novel spore peptidoglycan hydrolase of Bacillus cereus: biochemical characterization and nucleotide sequence of the corresponding gene, sleL. Journal of Bacteriology 182, 1499-1506.
Chen, Y., Miyata, S., Makino, S., Moriyama, R., 1997. Molecular characterization of a germination-specific muramidase from Clostridium perfringens S40 spores and nucleotide sequence of the corresponding gene. Journal of Bacteriology 179, 3181-3187.
Chen, Y., Ray, W.K., Helm, R.F., Melville, S.B., Popham, D.L., 2014. Levels of germination proteins in *Bacillus subtilis* dormant, superdormant, and germinating spores. Plos One 9, e95781.
Chen, Z., Durao, P., Silva, C.S., Pereira, M.M., Todorovic, S., Hildebrandt, P., Bento, I., Lindley, P.F., Martins, L.O., 2010b. The role of Glu498 in the dioxygen reactivity of CotA-laccase from Bacillus subtilis. Dalton Trans 39, 2875-2882.
Chesnokova, O.N., McPherson, S.A., Steichen, C.T., Turnbough, C.L., Jr., 2009. The spore-specific alanine racemase of *Bacillus anthracis* and its role in suppressing germination during spore development. J Bacteriol 191, 1303-1310.
Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S.J., Moir, A., 2002. Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. Microbiology 148, 2383-2392.
Chlopin, G.W., Tammann, G., 1903. Über den Einfluß hoher Drücke auf Mikroorganismen. Z Hyg Infektionskr 45, 171-204.
Chowdhury, S., Maris, C., Allain, F.H., Narberhaus, F., 2006. Molecular basis for temperature sensing by an RNA thermometer. The EMBO journal 25, 2487-2497.
Christie, G., Lowe, C.R., 2007. Role of chromosomal and plasmid-borne receptor homologues in the response of *Bacillus megaterium* QM B1551 spores to germinants. J Bacteriol 189, 4375-4383.
Chubukov, V., Sauer, U., 2014. Environmental dependence of stationary-phase metabolism in *Bacillus subtilis* and *Escherichia coli*. Appl Environ Microbiol 80, 2901-2909.
Chulkova, I.F., Bulatova, T.I., Anisimova, L.I., 1976. [Contamination of fish by agents of botulism type F and differentiating it from type E]. Zh Mikrobiol Epidemiol Immunobiol, 74-76.
Cioni, P., Strambini, G.B., 1994. Pressure effects on protein flexibility monomeric proteins. Journal of Molecular Biology 242, 291-301.
Clarkson, J., Campbell, I.D., Yudkin, M.D., 2004. Efficient regulation of sigmaF, the first sporulation-specific sigma factor in *B. subtilis*. J Mol Biol 342, 1187-1195.
Clauwers, C., Briers, Y., Lavigne, R., Michiels, C.W., 2015. Two Complete and One Draft Genome Sequence of Nonproteolytic Clostridium botulinum Type E Strains NCTC 8266, NCTC 8550, and NCTC 11219. Genome Announc 3.
Clery-Barraud, C., Gaubert, A., Masson, P., Vidal, D., 2004. Combined effects of high hydrostatic pressure and temperature for inactivation of *Bacillus anthracis* spores. Appl Environ Microb 70, 635-637.

Clouston, J.G., Wills, P.A., 1969. Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. J Bacteriol 97, 684-690.
Cohn, F.J., 1872. Untersuchungen über Bakterien. In: Cohn F. J. (Hrsg.), Beiträge zur Biologie der Pflanzen. Band I, Heft 2, p. 145. J. U. Kers's Verlag. Breslau.
Cohn, F.J., 1876. Untersuchungen über Bakterien. IV. In: Cohn F. J. (Hrsg.), Beiträge zur Biologie der Pflanzen. Band II, Heft 2, pp. 249-276. J. U. Kers's Verlag. Breslau.
Condon, S., Bayarte, M., Sala, F.J., 1992. Influence of the sporulation temperature upon the heat resistance of *Bacillus subtilis*. J Appl Bacteriol 73, 251-256.
Condón, S., Palop, A., Raso, J., Sala, F.J., 1996. Influence of the incubation temperature after heat treatment upon the estimated heat resistance values of spores of *Bacillus subtilis*. Lett Appl Microbiol 22, 149-152.
Connan, C., Deneve, C., Mazuet, C., Popoff, M.R., 2013. Regulation of toxin synthesis in *Clostridium botulinum* and *Clostridium tetani*. Toxicon 75, 90-100.
Cooksley, C.M., Davis, I.J., Winzer, K., Chan, W.C., Peck, M.W., Minton, N.P., 2010. Regulation of neurotoxin production and sporulation by a putative agrBD signaling system in proteolytic *Clostridium botulinum*. Appl Environ Microb 76, 4448-4460.
Cortezzo, D.E., Setlow, P., 2005. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J Appl Microbiol 98, 606-617.
Costa, T., Isidro, A.L., Moran, C.P., Henriques, A.O., 2006. Interaction between coat morphogenetic proteins SafA and SpoVID. Journal of Bacteriology 188, 7731-7741.
Costa, T., Serrano, M., Steil, L., Volker, U., Moran, C.P., Jr., Henriques, A.O., 2007. The timing of cotE expression affects Bacillus subtilis spore coat morphology but not lysozyme resistance. Journal of Bacteriology 189, 2401-2410.
Costa, T., Steil, L., Martins, L.O., Volker, U., Henriques, A.O., 2004. Assembly of an oxalate decarboxylase produced under sigmaK control into the Bacillus subtilis spore coat. Journal of Bacteriology 186, 1462-1474.
Couesnon, A., Pereira, Y., Popoff, M.R., 2008. Receptor-mediated transcytosis of botulinum neurotoxin A through intestinal cell monolayers. Cell Microbiol 10, 375-387.
Couesnon, A., Raffestin, S., Popoff, M.R., 2006. Expression of botulinum neurotoxins A and E, and associated non-toxin genes, during the transition phase and stability at high temperature: analysis by quantitative reverse transcription-PCR. Microbiology 152, 759-770.
Cowan, A.E., Koppel, D.E., Setlow, B., Setlow, P., 2003. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. Proc Natl Acad Sci USA 100, 4209-4214.
Cowan, A.E., Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., Setlow, P., 2004. Lipids in the inner membrane of dormant spores of Bacillus species are largely immobile. Proc Natl Acad Sci USA 101, 7733-7738.
Crawford, Y.J., Murano, E.A., Olson, D.G., Shenoy, K., 1996. Use of high hydrostatic pressure and irradiation to eliminate *Clostridium sporogenes* spores in chicken breast. J Food Protect 59, 711-715.
Curran, H.R., Brunstetter, B.C., Myers, A.T., 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. Journal of bacteriology 45, 485-494.
Curran, H.R., Evans, F.R., 1954. The influence of iron or manganese upon the formation of spores by mesophilic aerobes in fluid organic media. J Bacteriol 67, 489-497.
Cutting, S., Oke, V., Driks, A., Losick, R., Lu, S.J., Kroos, L., 1990. A forespore checkpoint for mother cell gene-expression during development in *Bacillus subtilis*. Cell 62, 239-250.
Dahlenborg, M., Borch, E., Radstrom, P., 2003. Prevalence of Clostridium botulinum types B, E and F in faecal samples from Swedish cattle. International Journal of Food Microbiology 82, 105-110.
Daniel, R.A., Errington, J., 1993. Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in Bacillus subtilis. J Mol Biol 232, 468-483.
Daryaei, H., Balasubramaniam, V.M., 2012. Microbial decontamination of food by high pressure processing. Woodhead Publishing Series in Food Science, Technology and Nutrition., in: Demirci, A., Ngadi, M.O. (Eds.), Microbial Decontamination in the Food Industry: Novel Methods and Applications. Elsevier.
Day, L.E., Costilow, R.N., 1964. Physiology of the sporulation process in *Clostridium botulinum*. I. Correlation of morphological changes with catabolic activities, synthesis of dipicolinic acid, and development of heat resistance. J Bacteriol 88, 690-694.
de Francesco, M., Jacobs, J.Z., Nunes, F., Serrano, M., McKenney, P.T., Chua, M.H., Henriques, A.O., Eichenberger, P., 2012. Physical Interaction between Coat Morphogenetic Proteins SpoVID and CotE Is Necessary for Spore Encasement in Bacillus subtilis. Journal of Bacteriology 194, 4941-4950.
de Heij, W.B.C., van Schepdael, L.J.M.M., Moezelaar, R., Hoogland, H., Matser, A.M., van den Berg, R.W., 2003. High-pressure sterilization: Maximizing the benefits of adiabatic heating. Food Technol-Chicago 57, 37-41.
de Hoon, M.J., Eichenberger, P., Vitkup, D., 2010. Hierarchical evolution of the bacterial sporulation network. Curr Biol 20, 735-745.

De Jong, A.E.I., Beumer, R.R., Rombouts, F.M., 2002. Optimizing sporulation of *Clostridium perfringens*. J Food Protect 65, 1457-1462.
Decaudin, M., Tholozan, J.L., 1996. A comparative study on the conditions of growth and sporulation of three strains of *Clostridium perfringens* type A. Canadian Journal of Microbiology 42, 298-304.
Delgado, F.J., Contador, R., Álvarez-Barrientos, A., Cava, R., Delgado-Adámez, J., Ramírez, R., 2013. Effect of high pressure thermal processing on some essential nutrients and immunological components present in breast milk. Innovative Food Science & Emerging Technologies 19, 50-56.
Derman, Y., Isokallio, M., Lindstrom, M., Korkeala, H., 2013. The two-component system CBO2306/CBO2307 is important for cold adaptation of Clostridium botulinum ATCC 3502. Int J Food Microbiol.
Devi, A.F., Buckow, R., Singh, T., Hemar, Y., Kasapis, S., 2015. Colour change and proteolysis of skim milk during high pressure thermal–processing. Journal of Food Engineering 147, 102-110.
Di Primo, C., Deprez, E., Hoa, G.H., Douzou, P., 1995. Antagonistic effects of hydrostatic pressure and osmotic pressure on cytochrome P-450cam spin transition. Biophysical journal 68, 2056-2061.
Diaz, A.R., Stephenson, S., Green, J.M., Levdikov, V.M., Wilkinson, A.J., Perego, M., 2008. Functional role for a conserved aspartate in the Spo0E signature motif involved in the dephosphorylation of the Bacillus subtilis sporulation regulator Spo0A. Journal of Biological Chemistry 283, 2962-2972.
Disteche, A., 1959. Ph Measurements with a Glass Electrode Withstanding 1500 Kg-Cm2 Hydrostatic Pressure. Rev Sci Instrum 30, 474-478.
Dixit, A., Alam, S.I., Dhaked, R.K., Singh, L., 2005. Sporulation and heat resistance of spores from a *Clostridium* sp RKD. J Food Sci Technol 70, 367-373.
Doan, T., Marquis, K.A., Rudner, D.Z., 2005. Subcellular localization of a sporulation membrane protein is achieved through a network of interactions along and across the septum. Mol Microbiol 55, 1767-1781.
Doan, T., Morlot, C., Meisner, J., Serrano, M., Henriques, A.O., Moran, C.P., Jr., Rudner, D.Z., 2009. Novel secretion apparatus maintains spore integrity and developmental gene expression in Bacillus subtilis. Plos Genet 5, e1000566.
Dodds, K.L., 1993. Clostridium botulinum in the environment. *In:* Clostridium botulinum ecology and control in foods.A. H. W. Hauschild and K. L. Dodds (ed.), p. 21-25, Marcel Dekker, Inc., New York, N.Y.
Dodds, K.L., Brodsky, M.H., Warburton, D.W., 1992. A Retail Survey of Smoked Ready-to-Eat Fish to Determine Their Microbiological Quality. J Food Protect 55, 208-210.
Donovan, W., Zheng, L.B., Sandman, K., Losick, R., 1987. Genes encoding spore coat polypeptides from Bacillus subtilis. J Mol Biol 196, 1-10.
Doona, C.J., Ghosh, S., Feeherry, F.F., Ramirez-Peralta, A., Huang, Y., Chen, H., Setlow, P., 2014. High pressure germination of *Bacillus subtilis* spores with alterations in levels and types of germination proteins. J Appl Microbiol 117, 711-720.
Driks, A., 1999. *Bacillus subtilis* spore coat. Microbiol Mol Biol R 63, 1-20.
Driks, A., 2003. The dynamic spore. Proc Natl Acad Sci U S A 100, 3007-3009.
Driks, A., 2004. From rings to layers: surprising patterns of protein deposition during bacterial spore assembly. Journal of Bacteriology 186, 4423-4426.
Driks, A., 2007. Surface appendages of bacterial spores. Molecular Microbiology 63, 623-625.
Driks, A., Roels, S., Beall, B., Moran, C.P., Losick, R., 1994. Subcellular-localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Gene Dev 8, 234-244.
Dring, G.J., Gould, G.W., 1971. Sequence of events during rapid germination of spores of Bacillus cereus. J Gen Microbiol 65, 101-104.
Dufresne, I., Smith, J.P., Liu, J.N., Tarte, I., Blanchfield, B., Austin, J.W., 2000. Effect of headspace oxygen and films of different oxygen transmission rate on toxin production by Clostridium botulinum type E in rainbow trout fillets stored under modified atmospheres. J Food Safety 20, 157-175.
Durre, P., Hollergschwandner, C., 2004. Initiation of endospore formation in *Clostridium acetobutylicum*. Anaerobe 10, 69-74.
Ebmeier, S.E., Tan, I.S., Clapham, K.R., Ramamurthi, K.S., 2012. Small proteins link coat and cortex assembly during sporulation in Bacillus subtilis. Mol Microbiol 84, 682-696.
ECDC, 2014. European Center for Disease Prevention and Control, Annual epidemiological reports from 2007 trough 2014. http://www.ecdc.europa.eu.
Ehrenberg, C.G., 1838. Die Infusionstierchen als vollkommene Organismen. Verlag Leopold Voss, Leipzig.
Eichenberger, P., 2010. The red-ox status of a penicillin-binding protein is an on/off switch for spore peptidoglycan synthesis in Bacillus subtilis. Mol Microbiol 75, 10-12.
Eichenberger, P., Fujita, M., Jensen, S.T., Conlon, E.M., Rudner, D.Z., Wang, S.T., Ferguson, C., Haga, K., Sato, T., Liu, J.S., Losick, R., 2004. The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. Plos Biol 2, 1664-1683.
Eklund, M.W., Poysky, F.T., Boatman, E.S., 1969. Bacteriophages of *Clostridium botulinum* types a B E and F and nontoxigenic strains resembling type E. J Virol 3, 270-&.

Eklund, M.W., Poysky, F.T., Reed, S.M., 1972. Bacteriophage and the toxigenicity of Clostridium botulinum type D. Nat New Biol 235, 16-17.
Eklund, M.W., Poysky, F.T., Reed, S.M., Smith, C.A., 1971. Bacteriophage and the toxigenicity of Clostridium botulinum type C. Science 172, 480-482.
Elbisi, H.M., Ordal, Z.J., 1956. The Effect of Sporulation Temperature on the Thermal Resistance of Bacillus-Coagulans Var Thermoacidurans. Journal of bacteriology 71, 10-16.
Elmabsout, Y.E., Stevenson, K.E., 1979. Activation of Bacillus-Stearothermophilus Spores at Low Ph. J Food Sci 44, 705-709.
EMBL-EBI, 2016 MUSCLE, Multiple allignment online tool; http://www.ebi.ac.uk/Tools/msa/muscle/; EMBL (European Molecular Biology Laboratory) -EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK
Copyright © EMBL-EBI 2016.
Erlendsson, L.S., Hederstedt, L., 2002. Mutations in the thiol-disulfide oxidoreductases BdbC and BdbD can suppress cytochrome c deficiency of CcdA-defective Bacillus subtilis cells. Journal of Bacteriology 184, 1423-1429.
Ermolenko, D.N., Makhatadze, G.I., 2002. Bacterial cold-shock proteins. Cell Mol Life Sci 59, 1902-1913.
Escobar-Cortes, K., Barra-Carrasco, J., Paredes-Sabja, D., 2013. Proteases and sonication specifically remove the exosporium layer of spores of Clostridium difficile strain 630. Journal of Microbiological Methods 93, 25-31.
EuropeanCommision, 2012. Implementation of the EU Salt Reduction Framework Results of Member States survey. Luxembourg: Publications Office of the European Union, 2012. ISBN: 978-92-79-23821-5. Doi: 10.2772/2754.
Evans, F.R., Curran, H.R., 1943. The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J Bacteriol 46, 513-523.
Evans, R.I., McClure, P.J., Gould, G.W., Russell, N.J., 1998. The effect of growth temperature on the phospholipid and fatty acyl compositions of non-proteolytic *Clostridium botulinum*. Int J Food Microb 40, 159-167.
Evans, R.I., Russell, N.J., Gould, G.W., McClure, P.J., 1997. The germinability of spores of a psychrotolerant, nonproteolytic strain of *Clostridium botulinum* is influenced by their formation and storage temperature. J Appl Microbiol 83, 273-280.
Evelyn, Silva, F.V., 2015. Use of power ultrasound to enhance the thermal inactivation of Clostridium perfringens spores in beef slurry. Int J Food Microbiol 206, 17-23.
Eyring, H., 1935. The Activated Complex in Chemical Reactions. The Journal of Chemical Physics 3, 107-115.
Fach, P., Perelle, S., Dilasser, F., Grout, J., Dargaignaratz, C., Botella, L., Gourreau, J.M., Carlin, F., Popoff, M.R., Broussolle, V., 2002. Detection by PCR-enzyme-linked immunosorbent assay of Clostridium botulinum in fish and environmental samples from a coastal area in northern France. Applied and Environmental Microbiology 68, 5870-5876.
Faille, C., Tauveron, G., Gentil-Lelievre, C.L., Slomianny, C., 2007. Occurrence of *Bacillus cereus* spores with a damaged exosporium: consequences on the spore adhesion on surfaces of food processing lines. J Food Protect 70, 2346-2353.
Feig, S., Stersky, A.K., 1981. Characterization of a Heat-Resistant Strain of Bacillus-Coagulans Isolated from Cream Style Canned Corn. J Food Sci 46, 135-137.
Fenicia, L., Anniballi, F., Aureli, P., 2007. Intestinal toxemia botulism in Italy, 1984-2005. Eur J Clin Microbiol Infect Dis 26, 385-394.
Fenicia, L., Franciosa, G., Pourshaban, M., Aureli, P., 1999. Intestinal toxemia botulism in two young people, caused by Clostridium butyricum type E. Clin Infect Dis 29, 1381-1387.
Ferstl, P., 2010. Druckinduzierte Phasenübergänge in Nahrungsfetten. Dissertation, Lehrstuhl fuer Systemverfahrenstechnik,Technische Universitaet Muenchen, 2010.
Fichtel, J., Koster, J., Rullkotter, J., Sass, H., 2007a. Spore dipicolinic acid contents used for estimating the number of endospores in sediments. Fems Microbiology Ecology 61, 522-532.
Fichtel, J., Koster, J., Scholz-Bottcher, B., Sass, H., Rullkotter, J., 2007b. A highly sensitive HPLC method for determination of nanomolar concentrations of dipicolinic acid, a characteristic constituent of bacterial endospores. J Microbiol Methods 70, 319-327.
Fisher, N., Hanna, P., 2005. Characterization of Bacillus anthracis germinant receptors in vitro. J Bacteriol 187, 8055-8062.
Fitz-James, P.C., 1971. Formation of protoplasts from resting spores. Journal of Bacteriology 105, 1119-1136.
Fleming, T.C., Shin, J.Y., Lee, S.H., Becker, E., Huang, K.C., Bustamante, C., Pogliano, K., 2010. Dynamic SpoIIIE assembly mediates septal membrane fission during *Bacillus subtilis* sporulation. Gene Dev 24, 1160-1172.
Foegeding, P.M., Busta, F.F., 1983. Proposed role of lactate in germination of hypochlorite-treated Clostridium botulinum spores. Appl Environ Microbiol 45, 1369-1373.

Forrest, T.M., Wilson, G.E., Pan, Y., Schaefer, J., 1991. Characterization of Cross-Linking of Cell-Walls of Bacillus-Subtilis by a Combination of Magic-Angle Spinning Nmr and Gas-Chromatography Mass-Spectrometry of Both Intact and Hydrolyzed C-13-Labeled and N-15-Labeled Cell-Wall Peptidoglycan. Journal of Biological Chemistry 266, 24485-24491.

Fraeye, I., Knockaert, G., Van Buggenhout, S., Duvetter, T., Hendrickx, M., Van Loey, A., 2010. Enzyme infusion prior to thermal/high pressure processing of strawberries: Mechanistic insight into firmness evolution. Innovative Food Science & Emerging Technologies 11, 23-31.

Fraise, A.P., Wilkinson, M.A.C., Bradley, C.R., Paton, S., Walker, J., Maillard, J.Y., Wesgate, R.L., Hoffman, P., Coia, J., Woodall, C., Fry, C., Wilcox, M., 2015. Development of a sporicidal test method for Clostridium difficile. J Hosp Infect 89, 2-15.

Francis, M.B., Allen, C.A., Shrestha, R., Sorg, J.A., 2013. Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. Plos Pathog 9, e1003356.

Francis, M.B., Allen, C.A., Sorg, J.A., 2015. Spore Cortex Hydrolysis Precedes Dipicolinic Acid Release during Clostridium difficile Spore Germination. J Bacteriol 197, 2276-2283.

Fujita, M., Gonzalez-Pastor, J.E., Losick, R., 2005. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. J Bacteriol 187, 1357-1368.

Fujita, M., Losick, R., 2005. Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. Gene Dev 19, 2236-2244.

Fukushima, T., Yamamoto, H., Atrih, A., Foster, S.J., Sekiguchi, J., 2002. A polysaccharide deacetylase gene (pdaA) is required for germination and for production of muramic delta-lactam residues in the spore cortex of *Bacillus subtilis*. J Bacteriol 184, 6007-6015.

Furukawa, S., Noma, S., Shimoda, M., Hayakawa, I., 2002. Effect of initial concentration of bacterial suspensions on their inactivation by high hydrostatic pressure. Int J Food Sci Tech 37, 573-577.

Galperin, M.Y., Mekhedov, S.L., Puigbo, P., Smirnov, S., Wolf, Y.I., Rigden, D.J., 2012. Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. Environ Microbiol.

Gänzle, M.G., Vogel, R.F., 2001. On-line fluorescence determination of pressure mediated outer membrane damage in *Escherichia coli*. Syst Appl Microbiol 24, 477-485.

Gao, Q.Y., Huang, Y.F., Wu, J.G., Liu, H.D., Xia, H.Q., 1990. A review of botulism in China. Biomed Environ Sci 3, 326-336.

Gao, Y.L., Ju, X.R., 2010. Modelling the effects of food ingredients and pH on high-pressure processing inactivation of Bacillus cereus spores: a laboratorial study. Int J Food Sci Tech 45, 1862-1869.

Gao, Y.L., Ju, X.R., 2011. Inactivation of *Bacillus coagulans* spores subjected to combinations of high-pressure processing and nisin. T Asabe 54, 385-392.

Gao, Y.L., Qiu, W.F., Wu, D., Fu, Q., 2011. Assessment of *Clostridium perfringens* spore response to high hydrostatic pressure and heat with nisin. Appl Biochem Biotech 164, 1083-1095.

Garcia-Alvarado, J.S., Labbe, R.G., Rodriguez, M.A., 1992a. Sporulation and enterotoxin production by Clostridium perfringens type A at 37 and 43 degrees C. Applied and Environmental Microbiology 58, 1411-1414.

Garcia-Alvarado, J.S., Rodriguez, M.A., Labbe, R.G., 1992b. Influence of elevated temperature on starch hydrolysis by enterotoxin-positive and enterotoxin-negative strains of Clostridium perfringens type A. Applied and Environmental Microbiology 58, 326-330.

Garcia-Graells, C., Masschalch, B., van Opstal, I., Wuytack, E.Y., Michiels, C.W., 2002. Mild non-thermal food preservation, Presentation at the conference: Frontiers in microbial fermentation and preservation, Wageningen, Netherlands.

García-Parra, J., González-Cebrino, F., Cava, R., Ramírez, R., 2014. Effect of a different high pressure thermal processing compared to a traditional thermal treatment on a red flesh and peel plum purée. Innovative Food Science & Emerging Technologies.

Garcia, D., van der Voort, M., Abee, T., 2010. Comparative analysis of *Bacillus weihenstephanensis* KBAB4 spores obtained at different temperatures. Int J Food Microbiol 140, 146-153.

Gaughran, E.R.L., 1947. The saturation of bacterial lipids as a function of temperature. J Bacteriol 53, 506-506.

Gayan, E., Torres, J.A., Paredes-Sabja, D., 2012. Hurdle approach to increase the microbial inactivation by high pressure processing: Effect of essential oils. Food Eng Rev 4, 141-148.

Georget, E., Kapoor, S., Winter, R., Reineke, K., Song, Y., Callanan, M., Ananta, E., Heinz, V., Mathys, A., 2014. In situ investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure. Food Microbiol 41, 8-18.

Getchell, R.G., Culligan, W.J., Kirchgessner, M., Sutton, C.A., Casey, R.N., Bowser, P.R., 2006. Quantitative polymerase chain reaction assay used to measure the prevalence of Clostridium botulinum type E in fish in the lower Great Lakes. J Aquat Anim Health 18, 39-50.

Ghosh, S., Setlow, P., 2009. Isolation and characterization of superdormant spores of *Bacillus s*pecies. J Bacteriol 191, 1787-1797.

Ghosh, S., Zhang, P.F., Li, Y.Q., Setlow, P., 2009. Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. J Bacteriol 191, 5584-5591.
Ghuysen, J.M., 1991. Serine Beta-Lactamases and Penicillin-Binding Proteins. Annu Rev Microbiol 45, 37-67.
Gibbs, P.A., Davies, A.R., Fletcher, R.S., 1994. Incidence and Growth of Psychrotrophic Clostridium-Botulinum in Foods. Food Control 5, 5-7.
Giebel, J.D., Carr, K.A., Anderson, E.C., Hanna, P.C., 2009. The germination-specific lytic enzymes SleB, CwlJ1, and CwlJ2 each contribute to Bacillus anthracis spore germination and virulence. J Bacteriol 191, 5569-5576.
Giebel, J.D., Carr, K.A., Hanna, P.C., 2012. Germination of *Bacillus anthracis* spores., in: Abel-Santos, E. (Ed.), Bacterial Spores: Current Research and Applications., Caister Academic Press, Norfolk, UK., pp. Chapter 8: p. 107-119.
Giglio, R., Fani, R., Isticato, R., De Felice, M., Ricca, E., Baccigalupi, L., 2011. Organization and evolution of the cotG and cotH genes of Bacillus subtilis. Journal of Bacteriology 193, 6664-6673.
Giorno, R., Bozue, J., Cote, C., Wenzel, T., Moody, K.S., Mallozzi, M., Ryan, M., Wang, R., Zielke, R., Maddock, J.R., Friedlander, A., Welkos, S., Driks, A., 2007. Morphogenesis of the Bacillus anthracis spore. Journal of Bacteriology 189, 691-705.
Girbal, L., Soucaille, P., 1998. Regulation of solvent production in *Clostridium acetobutylicum*. Trends Biotechnol 16, 11-16.
Gola, S., Foman, C., Carpi, G., Maggi, A., Cassara, A., Rovere, P., 1996. Inactivation of bacterial spores in phosphate buffer and in vegetable cream treated with high pressures. Progr Biotechnol 13, 253-259.
Goldman, R.C., Tipper, D.J., 1978. Bacillus subtilis spore coats: complexity and purification of a unique polypeptide component. Journal of Bacteriology 135, 1091-1106.
Gonzalez-Pastor, J.E., Hobbs, E.C., Losick, R., 2003. Cannibalism by sporulating bacteria. Science 301, 510-513.
Gonzalez, I., Lopez, M., Fernandez, A., Bernardo, A., 2008. Thermal inactivation of *Bacillus cereus* spores formed in media with different mineral contents. Arch Lebensmittelhyg 59, 34-38.
Gonzalez, I., Lopez, M., Martinez, S., Bernardo, A., Gonzalez, J., 1999. Thermal inactivation of Bacillus cereus spores formed at different temperatures. International Journal of Food Microbiology 51, 81-84.
Goodenough, E.R., Solberg, M., 1972. A technique for producing large yields of vegetative cell-free refractile Clostridium perfringens spores of unaltered heat resistance. Appl Microbiol 23, 429-430.
Gould, G.W., 1999. Water and the bacterial spore: Resistance, dormancy and germination. Water Management in the Design and Distribution of Quality Foods, 301-323.
Gould, G.W., 2006. History of science-spores. J Appl Microbiol 101, 507-513.
Gould, G.W., Sale, A.J., 1970. Initiation of germination of bacterial spores by hydrostatic pressure. J Gen Microbiol 60, 335-346.
Gounina-Allouane, R., Carlin, F., Broussolle, V., 2008. Influence of the sporulation temperature on the impact of the nutrients inosine and L-alanine on Bacillus cereus spore germination. Food Microbiol 25, 202-206.
Graumann, P., Schroder, K., Schmid, R., Marahiel, M.A., 1996. Cold shock stress-induced proteins in Bacillus subtilis. J Bacteriol 178, 4611-4619.
Graumann, P., Wendrich, T.M., Weber, M.H., Schroder, K., Marahiel, M.A., 1997. A family of cold shock proteins in Bacillus subtilis is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Mol Microbiol 25, 741-756.
Graumann, P.L., Marahiel, M.A., 1998. A superfamily of proteins that contain the cold-shock domain. Trends Biochem Sci 23, 286-290.
Graumann, P.L., Marahiel, M.A., 1999a. Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in Bacillus subtilis. Archives of Microbiology 171, 135-138.
Graumann, P.L., Marahiel, M.A., 1999b. Cold shock response in Bacillus subtilis. J Mol Microb Biotech 1, 203-209.
Grecz, N., Anellis, A., Schneider, M.D., 1962. Procedure for cleaning of Clostridium botulinum spores. Journal of Bacteriology 84, 552-558.
Grecz, N., Tang, T., 1970. Relation of dipicolinic acid to heat resistance of bacterial spores. J Gen Microbiol 63, 303-310.
Grelet, N., 1952a. [The determinism of sporulation in Bacillus megatherium. IV. Mineral constituents of the synthetic medium necessary for sporulation]. Ann Inst Pasteur (Paris) 83, 71-79.
Grelet, N., 1952b. [The determinism of sporulation of the Bacillus megatherium. II. Effect of deficiency of mineral constituent in synthetic medium]. Ann Inst Pasteur (Paris) 82, 66-77.
Grenha, R., Rzechorzek, N.J., Brannigan, J.A., de Jong, R.N., Ab, E., Diercks, T., Truffault, V., Ladds, J.C., Fogg, M.J., Bongiorni, C., Perego, M., Kaptein, R., Wilson, K.S., Folkers, G.E., Wilkinson, A.J., 2006. Structural characterization of Spo0E-like protein-aspartic acid phosphatases that regulate sporulation in bacilli. Journal of Biological Chemistry 281, 37993-38003.
Griffiths, K.K., Zhang, J., Cowan, A.E., Yu, J., Setlow, P., 2011. Germination proteins in the inner membrane of dormant *Bacillus subtilis* spores colocalize in a discrete cluster. Mol Microbiol 81, 1061-1077.

Gu, S.Y., Rumpel, S., Zhou, J., Strotmeier, J., Bigalke, H., Perry, K., Shoemaker, C.B., Rummel, A., Jin, R.S., 2012. Botulinum Neurotoxin Is Shielded by NTNHA in an Interlocked Complex. Science 335, 977-981.
Guillot, C., Moran, C.P., Jr., 2007. Essential internal promoter in the spoIIIA locus of Bacillus subtilis. J Bacteriol 189, 7181-7189.
Gupta, R., Balasubramaniam, V.M., 2012. High-Pressure Processing of Fluid Foods, in: Cullen, P.J., Tiwari, B.K., Valdramidis, V. (Eds.), Novel thermal and non-thermal technologies for fluid foods, 1st ed. Elsevier Science.
Gutelius, D., Hokeness, K., Logan, S.M., Reid, C.W., 2014. Functional analysis of SleC from *Clostridium difficile*: an essential lytic transglycosylase involved in spore germination. Microbiology 160, 209-216.
Halberg, R., Kroos, L., 1994. Sporulation regulatory protein SpoIIID from Bacillus subtilis activates and represses transcription by both mother-cell-specific forms of RNA polymerase. J Mol Biol 243, 425-436.
Haraldsen, J.D., Sonenshein, A.L., 2003. Efficient sporulation in *Clostridium difficile* requires disruption of the sigma(K) gene. Mol Microbiol 48, 811-821.
Harris, L.M., Welker, N.E., Papoutsakis, E.T., 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. J Bacteriol 184, 3586-3597.
Hawirko, R.Z., Naccarato, C.A., Lee, R.P., Maeba, P.Y., 1979. Outgrowth and sporulation studies on *Clostridium botulinum* type E: influence of isoleucine. Can J Microbiol 25, 522-527.
Hayakawa, I., Kanno, T., Tomita, M., Fujio, Y., 1994. Application of high-pressure for spore inactivation and protein denaturation. J Food Sci 59, 159-163.
Hayert, M., Perrier-Cornet, J.M., Gervais, P., 1999. A simple method for measuring the pH of acid solutions under high pressure. J Phys Chem A 103, 1785-1789.
Hayes, C.S., Alarcon-Hernandez, E., Setlow, P., 2001. N-terminal amino acid residues mediate protein-protein interactions between DNA-bound alpha/ss-type small, acid-soluble spore proteins from Bacillus species. Journal of Biological Chemistry 276, 2267-2275.
Hayes, C.S., Setlow, P., 2001. An alpha/beta-type, small, acid-soluble spore protein which has very high affinity for DNA prevents outgrowth of Bacillus subtilis spores. Journal of bacteriology 183, 2662-2666.
Hayes, S., Craig, J.M., Pilcher, K.S., 1970. Detection of Clostridium-Botulinum Type-E in Smoked Fish Products in Pacific Northwest. Canadian Journal of Microbiology 16, 207-&.
Hecker, M., Volker, U., 1998. Non-specific, general and multiple stress resistance of growth-restricted Bacillus subtilis cells by the expression of the sigmaB regulon. Molecular Microbiology 29, 1129-1136.
Heinitz, M.L., Johnson, J.M., 1998. The incidence of Listeria spp., Salmonella spp., and Clostridium botulinum in smoked fish and shellfish. J Food Protect 61, 318-323.
Heinz, V., Buckow, R., 2010. Food preservation by high pressure. J Verbrauch Lebensm 5, 73-81.
Heinz, V., Knorr, D., 1996. High pressure inactivation kinetics of *Bacillus subtilis* cells by a three-state-model considering distributed resistance mechanisms. Food Biotechnol 10, 149-161.
Henriques, A.O., Beall, B.W., Roland, K., Moran, C.P., Jr., 1995. Characterization of cotJ, a sigma E-controlled operon affecting the polypeptide composition of the coat of Bacillus subtilis spores. Journal of Bacteriology 177, 3394-3406.
Henriques, A.O., Melsen, L.R., Moran, C.P., Jr., 1998. Involvement of superoxide dismutase in spore coat assembly in Bacillus subtilis. Journal of Bacteriology 180, 2285-2291.
Henriques, A.O., Moran, C.P., 2000. Structure and assembly of the bacterial endospore coat. Methods 20, 95-110.
Henriques, A.O., Moran, C.P., 2007. Structure, assembly, and function of the spore surface layers. Annu Rev Microbiol 61, 555-588.
Heredia, N., Ybarra, P., Hernandez, C., Garcia, S., 2009. Extracellular protectants produced by Clostridium perfringens cells at elevated temperatures. Lett Appl Microbiol 48, 133-139.
Heredia, N.L., Garcia, G.A., Luevanos, R., Labbe, R.G., GarciaAlvarado, J.S., 1997. Elevation of the heat resistance of vegetative cells and spores of Clostridium perfringens type A by sublethal heat shock. J Food Protect 60, 998-1000.
Heredia, N.L., Labbe, R.G., Garcia-Alvarado, J.S., 1998. Alteration in sporulation, enterotoxin production, and protein synthesis by Clostridium perfringens type A following heat shock. J Food Protect 61, 1143-1147.
Hielm, S., Bjorkroth, J., Hyytia, E., Korkeala, H., 1998a. Prevalence of *Clostridium botulinum* in Finnish trout farms: Pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Applied and Environmental Microbiology 64, 4161-4167.
Hielm, S., Hyytia, E., Andersin, A.B., Korkeala, H., 1998b. A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. Journal of Applied Microbiology 84, 133-137.
Hilbert, D.W., Piggot, P.J., 2004. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. Microbiol Mol Biol R 68, 234-262.
Hill, K.K., Smith, T.J., Helma, C.H., Ticknor, L.O., Foley, B.T., Svensson, R.T., Brown, J.L., Johnson, E.A., Smith, L.A., Okinaka, R.T., Jackson, P.J., Marks, J.D., 2007. Genetic diversity among Botulinum Neurotoxin-producing clostridial strains. Journal of Bacteriology 189, 818-832.

Hill, K.K., Xie, G., Foley, B.T., Smith, T.J., Munk, A.C., Bruce, D., Smith, L.A., Brettin, T.S., Detter, J.C., 2009. Recombination and insertion events involving the botulinum neurotoxin complex genes in Clostridium botulinum types A, B, E and F and Clostridium butyricum type E strains. BMC Biol 7, 66.
Hindle, A.A., Hall, E.A., 1999. Dipicolinic acid (DPA) assay revisited and appraised for spore detection. Analyst 124, 1599-1604.
Hines, H.B., Lebeda, F., Hale, M., Brueggemann, E.E., 2005. Characterization of botulinum progenitor toxins by mass spectrometry. Applied and Environmental Microbiology 71, 4478-4486.
Hintze, P.E., Nicholson, W.L., 2010. Single-spore elemental analyses indicate that dipicolinic acid-deficient *Bacillus subtilis* spores fail to accumulate calcium. Archives of Microbiology 192, 493-497.
Hippe, H., Andreesen, J.R., Gottschalk, G., 1999. The genus Clostridium—nonmedical. *In:* The Prokaryotes, M. Dworkin (Ed.), Springer-Verlag, Berlin (1999) Chapter 81, pp. 1–59.
Hirsch, A., Grinsted, E., 1954. Methods for the Growth and Enumeration of Anaerobic Spore-Formers from Cheese, with Observations on the Effect of Nisin. J Dairy Res 21, 101-110.
Hite, B.H., 1899. The effect of pressure in the preservation of milk. A preliminary report. Bull. West Virginia Univ. Agric. Exp. Station 58, 15–35.
Hobbs, G., Cann, D.C., Wilson, B.B., Shewan, J.M., 1965. The incidence of the organisms of the genus Clostridium in vacuum-packed fish. J. Appl. Bacteriol. 28, 265.
Hobbs, G., Stiebrs, A., Eklund, M.W., 1967. Egg yolk reaction of *Clostridium botulinum* type E in different basal media. Journal of Bacteriology 93, 1192.
Hoch, J.A., 1993. Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Annu Rev Microbiol 47, 441-465.
Hoch, J.A., 2000. Two-component and phosphorelay signal transduction. Curr Opin Microbiol 3, 165-170.
Hodgkiss, W., Ordal, Z.J., 1966. Morphology of the spore of some strains of Clostridium botulinum type E. Journal of Bacteriology 91, 2031-2036.
Hofstetter, S., Denter, C., Winter, R., McMullen, L.M., Gänzle, M.G., 2012. Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of Clostridium spp. Journal of Microbiological Methods 91, 93-100.
Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., McMullen, L.M., 2013a. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol 34, 46-51.
Hofstetter, S., Winter, R., McMullen, L.M., Gänzle, M.G., 2013b. In situ determination of *Clostridium* endospore membrane fluidity during pressure-assisted thermal processing in combination with nisin or reutericyclin. Appl Environ Microbiol 79, 2103-2106.
Hofweber, R., Horn, G., Langmann, T., Balbach, J., Kremer, W., Schmitz, G., Kalbitzer, H.R., 2005. The influence of cold shock proteins on transcription and translation studied in cell-free model systems. FEBS J 272, 4691-4702.
Hölters, C., van Almsick, G., Ludwig, H., 1999. High Pressure Inactivation of Anaerobic Spores from *Clostridium pasteurianum*, in: Ludwig, H. (Ed.), Proceedings of the International Conference on High Pressure Bioscience and Biotechnology, Heidelberg, August 30 - September 3, 1998. Springer Berlin Heidelberg, pp. 65-68.
Holtmann, G., Brigulla, M., Steil, L., Schutz, A., Barnekow, K., Volker, U., Bremer, E., 2004. RsbV-independent induction of the SigB-dependent general stress regulon of Bacillus subtilis during growth at high temperature. Journal of bacteriology 186, 6150-6158.
Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D., Jentsch, S., 2000. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell 102, 577-586.
Horn, G., Hofweber, R., Kremer, W., Kalbitzer, H.R., 2007. Structure and function of bacterial cold shock proteins. Cell Mol Life Sci 64, 1457-1470.
Hornstra, L.M., de Vries, Y.P., de Vos, W.M., Abee, T., 2006a. Influence of sporulation medium composition on transcription of ger operons and the germination response of spores of *Bacillus cereus* ATCC 14579. Appl Environ Microb 72, 3746-3749.
Hornstra, L.M., de Vries, Y.P., Wells-Bennik, M.H.J., de Vos, W.M., Abee, T., 2006b. Characterization of germination receptors of *Bacillus cereus* ATCC 14579. Appl Environ Microb 72, 44-53.
Horowitz, B.Z., 2010. Type E botulism. Clin Toxicol (Phila) 48, 880-895.
Horsburgh, G.J., Atrih, A., Foster, S.J., 2003. Characterization of LytH, a differentiation-associated peptidoglycan hydrolase of *Bacillus subtilis* involved in endospore cortex maturation. J Bacteriol 185, 3813-3820.
Houghtby, G.A., Kaysner, C.A., 1969. Incidence of Clostridium botulinum type E in Alaskan salmon. Appl Microbiol 18, 950-951.
Huang, I.H., Waters, M., Grau, R.R., Sarker, M.R., 2004. Disruption of the gene (spoOA) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. Fems Microbiol Lett 233, 233-240.

Huang, S.-s., Chen, D., Pelczar, P.L., Vepachedu, V.R., Setlow, P., Li, Y.-q., 2007. Levels of Ca(2+)-dipicolinic acid in individual *Bacillus s*pores determined using microfluidic Raman Tweezers. Journal of Bacteriology 189, 4681-4687.
Hullo, M.F., Moszer, I., Danchin, A., Martin-Verstraete, I., 2001. CotA of Bacillus subtilis is a copper-dependent laccase. Journal of Bacteriology 183, 5426-5430.
Husemann, M.H.W., Papoutsakis, E.T., 1988. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic-acid and proton concentrations. Biotechnol Bioeng 32, 843-852.
Huss, H.H., 1980. Distribution of Clostridium botulinum. Applied and Environmental Microbiology 39, 764-769.
Huss, H.H., Eskildsen, U., 1974. Botulism in farmed trout caused by Clostridium botulinum type E; a preliminary report. Nord Vet Med 26, 733-738.
Huss, H.H., Pedersen, A., 1979. Clostridium botulinum in fish. Nord Vet Med 31, 214-221.
Huss, H.H., Pedersen, A., Cann, D.C., 1974. The incidence of Clostridium botulinum in Danish trout farms. II. Measures to reduce contamination of the fish. J. Food Technol. 9, 451-458.
Hutson, R.A., Thompson, D.E., Lawson, P.A., Schocken-Itturino, R.P., Böttger, E.C., Collins, M.D., 1993. Genetic interrelationships of proteolytic Clostridium botulinum types A, B, and F and other members of the Clostridium botulinum complex as revealed by small-subunit rRNA gene sequences. Antonie Van Leeuwenhoek 64, 273-283.
Hutson, R.A., Zhou, Y., Collins, M.D., Johnson, E.A., Hatheway, C.L., Sugiyama, H., 1996. Genetic characterization of Clostridium botulinum type A containing silent type B neurotoxin gene sequences. J Biol Chem 271, 10786-10792.
Hyytiä-Trees, E., 1999. Prevalance, molecular epidemiology and growth of *Clostridium botulinum* type E in fish and fishery products. Academic Dissertation, Faculty of Vetinary Medicin, Departement of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland.
Hyytia-Trees, E., Lindstrom, M., Schalch, B., Stolle, A., Korkeala, H., 1999. Clostridium botulinum type E in Bavarian fish. Arch Lebensmittelhyg 50, 79-82.
Hyytia, E., Hielm, S., Korkeala, H., 1998. Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiology and Infection 120, 245-250.
Igura, N., Kamimura, Y., Islam, M.S., Shimoda, M., Hayakawa, I., 2003. Effects of minerals on resistance of *Bacillus subtilis* spores to heat and hydrostatic pressure. Appl Environ Microb 69, 6307-6310.
Imamura, D., 2012. Identification and Characterization of the Outermost Layer of Bacillus subtilis Spores. Yakugaku Zasshi 132, 919-924.
Imamura, D., Kuwana, R., Takamatsu, H., Watabe, K., 2010. Localization of proteins to different layers and regions of Bacillus subtilis spore coats. Journal of Bacteriology 192, 518-524.
Imamura, D., Kuwana, R., Takamatsu, H., Watabe, K., 2011. Proteins involved in formation of the outermost layer of Bacillus subtilis spores. Journal of Bacteriology 193, 4075-4080.
Inoue, K., Iida, H., 1968. Bacteriophages of Clostridium Botulinum. J Virol 2, 537-&.
Irigul-Sonmez, O., Koroglu, T.E., Ozturk, B., Kovacs, A.T., Kuipers, O.P., Yazgan-Karatas, A., 2014. In *Bacillus subtilis* LutR is part of the global complex regulatory network governing the adaptation to the transition from exponential growth to stationary phase. Microbiology 160, 243-260.
Ishida, A., Futamura, N., Matsusaka, T., 1987. Detection of Peroxidase-Activity and Its Localization in the Forespore Envelopes of Bacillus-Cereus. J Gen Appl Microbiol 33, 27-32.
Ishimori, T., Takahashi, K., Goto, M., Nakagawa, S., Kasai, Y., Konagaya, Y., Batori, H., Kobayashi, A., Urakami, H., 2012. Synergistic effects of high hydrostatic pressure, mild heating, and amino acids on germination and inactivation of *Clostridium sporogenes* spores. Appl Environ Microb 78, 8202-8207.
Isticato, R., Esposito, G., Zilhao, R., Nolasco, S., Cangiano, G., De Felice, M., Henriques, A.O., Ricca, E., 2004. Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J Bacteriol 186, 1129-1135.
Isticato, R., Pelosi, A., De Felice, M., Ricca, E., 2010. CotE binds to CotC and CotU and mediates their interaction during spore coat formation in Bacillus subtilis. Journal of Bacteriology 192, 949-954.
Isticato, R., Pelosi, A., Zilhao, R., Baccigalupi, L., Henriques, A.O., De Felice, M., Ricca, E., 2008. CotC-CotU heterodimerization during assembly of the Bacillus subtilis spore coat. Journal of Bacteriology 190, 1267-1275.
Iyer, A.V., Blinkova, A.L., Yang, S.Y., Harrison, M., Tepp, W.H., Jacobson, M.J., Johnson, E.A., Bennett, G.N., Walker, J.R., 2008. Clostridium taeniosporum is a close relative of the Clostridium botulinum Group II. Anaerobe 14, 318-324.
Jaeger, H., Janositz, A., Knorr, D., 2010. The Maillard reaction and its control during food processing. The potential of emerging technologies. Pathologie-biologie 58, 207-213.
Jenkinson, H.F., Sawyer, W.D., Mandelstam, J., 1981. Synthesis and Order of Assembly of Spore Coat Proteins in Bacillus-Subtilis. Journal of General Microbiology 123, 1-16.
Jiang, M., Shao, W., Perego, M., Hoch, J.A., 2000. Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. Mol Microbiol 38, 535-542.

Jiang, W., Hou, Y., Inouye, M., 1997. CspA, the major cold-shock protein of Escherichia coli, is an RNA chaperone. J Biol Chem 272, 196-202.
Jiang, X., Rubio, A., Chiba, S., Pogliano, K., 2005. Engulfment-regulated proteolysis of SpoIIQ: evidence that dual checkpoints control sigma activity. Mol Microbiol 58, 102-115.
Job, G., Herrmann, F., 2006. Chemical potential - a quantity in search of recognition. Eur J Phys 27, 353-371.
Johannsen, A., 1963. *Clostridium botulinum* in Sweden and the adjacent waters. Journal of Applied Microbiology 26, 43-47.
Johannsen, A., 1965. Clostridium botulinum type E i rökta fiskvaror. Svensk Vet Tidn, 17:150-1 and 162-4, 1965.
Johnson, E.A., 2007. *Clostridium botulinum*. In Food Microbiology, Fundamentals and Frontiers, 3rd edition, Doyle, M. P. and Beuchat L. R. (eds.), ASM Press, Washington, D.C., 2007, Chapter 18, p. 401 ff.
Johnson, F.H., Zobell, C.E., 1949. The retardation of thermal disinfection of *Bacillus subtilis* spores by hydrostatic pressure. J Bacteriol 57, 353-358.
Johnson, J.L., Francis, B.S., 1975. Taxonomy of the Clostridia: ribosomal ribonucleic acid homologies among the species. J Gen Microbiol 88, 229-244.
Johnstone, K., 1994. The trigger mechanism of spore germination: current concepts. Soc Appl Bacteriol Symp Ser 23, 17S-24S.
Jones, C.A., Padula, N.L., Setlow, P., 2005. Effect of mechanical abrasion on the viability, disruption and germination of spores of *Bacillus subtilis*. J Appl Microbiol 99, 1484-1494.
Jones, D.T., Woods, D.R., 1986. Acetone-butanol fermentation revisited. Microbiol Rev 50, 484-524.
Jones, S.W., Paredes, C.J., Tracy, B., Cheng, N., Sillers, R., Senger, R.S., Papoutsakis, E.T., 2008. The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol 9, R114.
Jones, S.W., Tracy, B.P., Gaida, S.M., Papoutsakis, E.T., 2011. Inactivation of sigmaF in *Clostridium acetobutylicum* ATCC 824 blocks sporulation prior to asymmetric division and abolishes sigmaE and sigmaG protein expression but does not block solvent formation. J Bacteriol 193, 2429-2440.
Juliano, P., Knoerzer, K., Fryer, P.J., Versteeg, C., 2009. *C. botulinum* inactivation kinetics implemented in a computational model of a high-pressure sterilization process. Biotechnol Prog 25, 163-175.
Juneja, V.K., Eblen, B.S., Marmer, B.S., Williams, A.C., Palumbo, S.A., Miller, A.J., 1995. Thermal-resistance of nonproteolytic type B and type E *Clostridium botulinum* spores in phosphate buffer and turkey slurry. J Food Protect 58, 758-763.
Juneja, V.K., Novak, J.S., Huang, L.H., Eblen, B.S., 2003. Increased thermotolerance of Clostridium perfringens spores following sublethal heat shock. Food Control 14, 163-168.
Kalchayanand, N., Dunne, C.P., Sikes, A., Ray, B., 2004. Germination induction and inactivation of *Clostridium* spores at medium-range hydrostatic pressure treatment. Innov Food Sci Emerg 5, 277-283.
Kanda-Nambu, K., Yasuda, Y., Tochikubo, K., 2000. Isozymic nature of spore coat-associated alanine racemase of Bacillus subtilis. Amino Acids 18, 375-387.
Kashket, E.R., Zhi-Yi, C., 1995. Clostridial strain degeneration. Fems Microbiol Rev 17, 307-315.
Kebede, B.T., Grauwet, T., Mutsokoti, L., Palmers, S., Vervoort, L., Hendrickx, M., Van Loey, A., 2014. Comparing the impact of high pressure high temperature and thermal sterilization on the volatile fingerprint of onion, potato, pumpkin and red beet. Food Res Int 56, 218-225.
Keller, J.E., Cai, F., Neale, E.A., 2004. Uptake of botulinum neurotoxin into cultured neurons. Biochemistry 43, 526-532.
Kessler, H.G., 2002. Food and Bio Process Engineering, 5th revised and extended edition. Verlag A. Kessler (Publishing House A. Kessler), München. 2002.
Keynan, A., Evenchik, Z., 1969. Activation. In *The bacterial spore*. (Eds Gould, G. W. and Hurst, A.) pp. 301-358. London, Academic Press.
Khoury, P.H., Lombardi, S.J., Slepecky, R.A., 1987. Perturbation of the heat resistance of bacterial spores by sporulation temperature and ethanol. Current Microbiology 15, 15-19.
Khoury, P.H., Qoronfleh, M.W., Streips, U.N., Slepecky, R.A., 1990. Altered Heat-Resistance in Spores and Vegetative Cells of a Mutant from Bacillus-Subtilis. Current Microbiology 21, 249-253.
Kihm, D.J., Hutton, M.T., Hanlin, J.H., Johnson, E.A., 1990. Influence of transition metals added during sporulation on heat resistance of *Clostridium botulinum* 113B spores. Applied and Environmental Microbiology 56, 681-685.
Kim, H., Hahn, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P., Driks, A., 2006. The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol 59, 487-502.
Kim, J.H., Lee, C.S., Kim, B.G., 2005. Spore-displayed streptavidin: a live diagnostic tool in biotechnology. Biochem Biophys Res Commun 331, 210-214.
King, L.A., Niskanen, T., Junnikkala, M., Moilanen, E., Lindstrom, M., Korkeala, H., Korhonen, T., Popoff, M., Mazuet, C., Callon, H., Pihier, N., Peloux, F., Ichai, C., Quintard, H., Dellamonica, P., Cua, E., Lasfargue, M., Pierre, F., de Valk, H., 2009. Botulism and hot-smoked whitefish: a family cluster of type E botulism in France, September 2009. Euro Surveill 14.

Kinsey, J., Senauer, B., 1996. Consumer trends and changing food retailing formats. Am J Agr Econ 78, 1187-1191.
Kirk, D.G., Palonen, E., Korkeala, H., Lindstrom, M., 2014. Evaluation of normalization reference genes for RT-qPCR analysis of spo0A and four sporulation sigma factor genes in Clostridium botulinum Group I strain ATCC 3502. Anaerobe 26, 14-19.
Kitamura, M., 2002. Identification of RNA species in the RNA-toxin complex and structure of the complex in Clostridium botulinum type E. Biochem Biophys Res Commun 291, 154-157.
Knoerzer, K., Buckow, R., Sanguansri, P., Versteeg, C., 2010. Adiabatic compression heating coefficients for high-pressure processing of water, propylene-glycol and mixtures - A combined experimental and numerical approach. Journal of Food Engineering 96, 229-238.
Knoerzer, K., Chapman, B., 2011. Effect of material properties and processing conditions on the prediction accuracy of a CFD model for simulating high pressure thermal (H PT) processing. Journal of Food Engineering 104, 404-413.
Knoerzer, K., Juliano, P., Gladman, S., Versteeg, C., Fryer, P.J., 2007. A computational model or temperature and sterility distributions on a pilot-scale high-pressure high-temperature process. Aiche J 53, 2996-3010.
Knorr, D., Reineke, K., Mathys, A., Heinz, V., Buckow, R., 2011. High-pressure-induced effects on bacterial spores, vegetative microorganisms, and enzymes. Food Eng Ser, 325-340.
Kobayashi, K., Hashiguchi, K., Yokozeki, K., Yamanaka, S., 1998. Molecular cloning of the transglutaminase gene from Bacillus subtilis and its expression in Escherichia coli. Biosci Biotechnol Biochem 62, 1109-1114.
Kobayashi, K., Kumazawa, Y., Miwa, K., Yamanaka, S., 1996. epsilon-(gamma Glutamyl)lysine cross-links of spore coat proteins and transglutaminase activity in Bacillus subtilis. Fems Microbiology Letters 144, 157-160.
Koch, R., 1876. Untersuchungen über Bakterien. V. Die Aetiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des *Bacillus Anthracis*. In: Cohn F. (Hrsg.), Beiträge zur Biologie der Pflanzen. Band 2, Heft 2, 277-310.
Kodama, T., Matsubayashi, T., Yanagihara, T., Komoto, H., Ara, K., Ozaki, K., Kuwana, R., Imamura, D., Takamatsu, H., Watabe, K., Sekiguchi, J., 2011. A novel small protein of Bacillus subtilis involved in spore germination and spore coat assembly. Biosci Biotechnol Biochem 75, 1119-1128.
Kodama, T., Takamatsu, H., Asai, K., Kobayashi, K., Ogasawara, N., Watabe, K., 1999. The Bacillus subtilis yaaH gene is transcribed by SigE RNA polymerase during sporulation, and its product is involved in germination of spores. Journal of bacteriology 181, 4584-4591.
Kodama, T., Takamatsu, H., Asai, K., Ogasawara, N., Sadaie, Y., Watabe, K., 2000. Synthesis and characterization of the spore proteins of Bacillus subtilis YdhD, YkuD, and YkvP, which carry a motif conserved among cell wall binding proteins. Journal of Biochemistry 128, 655-663.
Kolodziej, B.J., Slepecky, R.A., 1964. Trace Metal Requirements for Sporulation of Bacillus Megaterium. Journal of Bacteriology 88, 821-830.
Kong, L.B., Doona, C.J., Setlow, P., Li, Y.Q., 2014. Monitoring rates and heterogeneity of high-pressure germination of *Bacillus* spores by phase-contrast microscopy of individual spores. Appl Environ Microb 80, 345-353.
Korkeala, H., Stengel, G., Hyytia, E., Vogelsang, B., Bohl, A., Wihlman, H., Pakkala, P., Hielm, S., 1998. Type E botulism associated with vacuum-packaged hot-smoked whitefish. International Journal of Food Microbiology 43, 1-5.
Kort, R., O'Brien, A.C., van Stokkum, I.H., Oomes, S.J., Crielaard, W., Hellingwerf, K.J., Brul, S., 2005. Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. Appl Environ Microb 71, 3556-3564.
Koseki, S., Yamamoto, K., 2007. Water activity of bacterial suspension media unable to account for the baroprotective effect of solute concentration on the inactivation of Listeria monocytogenes by high hydrostatic pressure. International Journal of Food Microbiology 115, 43-47.
Koutchma, T., Song, Y., Setikaite, I., Juliano, P., Barbosa-Canovas, G.V., Dunne, C.P., Patazca, E., 2010. Packaging evaluation for high-pressure high-temperature sterilization of shelf-stable foods. J Food Process Eng 33, 1097-1114.
Krajcikova, D., Lukacova, M., Mullerova, D., Cutting, S.M., Barak, I., 2009. Searching for protein-protein interactions within the Bacillus subtilis spore coat. Journal of Bacteriology 191, 3212-3219.
Krebbers, B., Matser, A.M., Hoogerwerf, S.W., Moezelaar, R., Tomassen, M.M.M., van den Berg, R.W., 2003. Combined high-pressure and thermal treatments for processing of tomato puree: evaluation of microbial inactivation and quality parameters. Innovative Food Science & Emerging Technologies 4, 377-385.
Kukreja, R.V., Singh, B.R., 2007. Comparative role of neurotoxin-associated proteins in the structural stability and endopeptidase activity of botulinum neurotoxin complex types A and E. Biochemistry 46, 14316-14324.
Kumaran, D., Eswaramoorthy, S., Furey, W., Navaza, J., Sax, M., Swaminathan, S., 2009. Domain organization in Clostridium botulinum neurotoxin type E is unique: its implication in faster translocation. J Mol Biol 386, 233-245.

Kumazawa, T., Masayama, A., Fukuoka, S., Makino, S., Yoshimura, T., Moriyama, R., 2007. Mode of action of a germination-specific cortex-lytic enzyme, SleC, of Clostridium perfringens S40. Biosci Biotechnol Biochem 71, 884-892.

Kunclova, D., Liska, V., Svoboda, P., Svobodova, J., 1995. Cold-shock response of protein, RNA, DNA and phospholipid synthesis in Bacillus subtilis. Folia Microbiol 40, 627-632.

Kuwana, R., Ikejiri, H., Yamamura, S., Takamatsu, H., Watabe, K., 2004. Functional relationship between SpoVIF and GerE in gene regulation during sporulation of Bacillus subtilis. Microbiology 150, 163-170.

Kuwana, R., Okuda, N., Takamatsu, H., Watabe, K., 2006. Modification of GerQ reveals a functional relationship between Tgl and YabG in the coat of Bacillus subtilis spores. J Biochem 139, 887-901.

La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C.L., Gualerzi, C.O., 1991. Identification of a cold shock transcriptional enhancer of the Escherichia coli gene encoding nucleoid protein H-NS. Proc Natl Acad Sci U S A 88, 10907-10911.

Labell, T.L., Trempy, J.E., Haldenwang, W.G., 1987. Sporulation-specific sigma-factor sigma-29 of *Bacillus subtilis* is synthesized from a precursor protein, P-31. P Natl Acad Sci USA 84, 1784-1788.

Lafrancois, B.M., Riley, S.C., Blehert, D.S., Ballmann, A.E., 2011. Links between type E botulism outbreaks, lake levels, and surface water temperatures in Lake Michigan, 1963-2008. J Great Lakes Res 37, 86-91.

Lai, E.M., Phadke, N.D., Kachman, M.T., Giorno, R., Vazquez, S., Vazquez, J.A., Maddock, J.R., Driks, A., 2003. Proteomic analysis of the spore coats of Bacillus subtilis and Bacillus anthracis. Journal of Bacteriology 185, 1443-1454.

Lalitha, K.V., Gopakumar, K., 2000. Distribution and ecology of Clostridium botulinum in fish and aquatic environments of a tropical region. Food Microbiol 17, 535-541.

Lambert, E.A., Popham, D.L., 2008. The Bacillus anthracis SleL (YaaH) Protein Is an N-Acetylglucosaminidase Involved in Spore Cortex Depolymerization. Journal of bacteriology 190, 7601-7607.

Lambert, E.A., Sherry, N., Popham, D.L., 2012. In vitro and in vivo analyses of the Bacillus anthracis spore cortex lytic protein SleL. Microbiology 158, 1359-1368.

Larson, W.P., Hartzell, T.B., Diehl, H.S., 1918. The effect of high pressures on bacteria. Journal of Infectous Disease 22, 271-279.

Lawley, T.D., Croucher, N.J., Yu, L., Clare, S., Sebaihia, M., Goulding, D., Pickard, D.J., Parkhill, J., Choudhary, J., Dougan, G., 2009. Proteomic and genomic characterization of highly infectious Clostridium difficile 630 spores. Journal of Bacteriology 191, 5377-5386.

Lawrence, P.I., Stroming.Jl, 1970. Biosynthesis of peptadoglycan of bacterial cell walls: XVI. Reversible fixation of radioactive penicillin-G to D-alanine carboxy-peptidase of *Bacillus subtilis*. J Biol Chem 245, 3660-3666.

Lazazzera, B.A., Kurtser, I.G., McQuade, R.S., Grossman, A.D., 1999. An autoregulatory circuit affecting peptide signaling in *Bacillus subtilis*. J Bacteriol 181, 5193-5200.

Lechowich, R.V., Ordal, Z.J., 1962. The influence of the sporulation temperature on the heat resistance and chemical composition of bacterial spores. Can J Microbiol 8, 287-295.

Leguerinel, I., Couvert, O., Mafart, P., 2007. Modelling the influence of the sporulation temperature upon the bacterial spore heat resistance, application to heating process calculation. International Journal of Food Microbiology 114, 100-104.

Lenz, C.A., Reineke, K., Knorr, D., Vogel, R.F., 2015. High pressure thermal inactivation of *Clostridium botulinum* type E endospores – kinetic modeling and mechanistic insights. Frontiers in Microbiology 6.

Lenz, C.A., Schnabel, J., Vogel, R.F., 2014. The variability of times to detect growth from individual *Clostridium botulinum* type E endospores is differentially affected by high pressure treatments. High Pressure Res, 1-7.

Lenz, C.A., Vogel, R.F., 2014. Effect of sporulation medium and its divalent cation content on the heat and high pressure resistance of *Clostridium botulinum* type E spores. Food Microbiol 44, 156-167.

Lenz, C.A., Vogel, R.F., 2015. Pressure-Based Strategy for the Inactivation of Spores. Subcell Biochem 72, 469-537.

Leuschner, R.G., Lillford, P.J., 1999. Effects of temperature and heat activation on germination of individual spores of *Bacillus subtilis*. Lett Appl Microbiol 29, 228-232.

Leuschner, R.G., Lillford, P.J., 2003. Thermal properties of bacterial spores and biopolymers. Int J Food Microbiol 80, 131-143.

Levin, P.A., Fan, N., Ricca, E., Driks, A., Losick, R., Cutting, S., 1993. An unusually small gene required for sporulation by *Bacillus subtilis*. Mol Microbiol 9, 761-771.

Levinson, H.S., Hyatt, M.T., 1964. Effect of sporulation medium on heat resistance, chemical composition, and germination of *Bacillus megaterium* spores. Journal of bacteriology 87, 876-886.

Lewis, J.C., Snell, N.S., Burr, H.K., 1960. Water Permeability of Bacterial Spores and the Concept of a Contractile Cortex. Science 132, 544-545.

Li, J.H., Chen, J., Vidal, J.E., McClane, B.A., 2011. The Agr-like quorum-sensing system regulates sporulation and production of enterotoxin and Beta2 toxin by *Clostridium perfringens* type A non-food-borne human gastrointestinal disease strain F5603. Infect Immun 79, 2451-2459.

Li, J.H., McClane, B.A., 2010. Evaluating the involvement of alternative sigma factors SigF and SigG in *Clostridium perfringens* sporulation and enterotoxin synthesis. Infect Immun 78, 4286-4293.
Li, Y.F., Davis, A., Korza, G., Zhang, P., Li, Y.Q., Setlow, B., Setlow, P., Hao, B., 2012. Role of a SpoVA protein in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. J Bacteriol 194, 1875-1884.
Liang, L., He, X., Liu, G., Tan, H., 2008. The role of a purine-specific nucleoside hydrolase in spore germination of Bacillus thuringiensis. Microbiology 154, 1333-1340.
Licciardello, J.J., Nickerson, J.T., Ribich, C.A., Goldblith, S.A., 1967. Thermal inactivation of type E botulinum toxin. Appl Microbiol 15, 249-256.
Lindsay, J.A., Barton, L.E., Leinart, A.S., Pankratz, H.S., 1990. The effect of sporulation temperature on sporal characteristics of *Bacillus subtilis* A. Curr Microbiol 21, 75-79.
Lindström, M., Fredriksson-Ahomaa, M., Korkeala, H., 2009. Molecular Epidemiology of Group I and II Clostridium botulinum. *In:* Clostridia: Molecular Biology in the Post-genomic Era, Brüggemann, H. and Gottschalk, G. (eds.), Caister Academic Press (2009), ISBN: 978-1-904455-38-7.
Lindström, M., Kiviniemi, K., Korkeala, H., 2006. Hazard and control of group II (non-proteolytic) Clostridium botulinum in modern food processing. International Journal of Food Microbiology 108, 92-104.
Lindstrom, M., Nevas, M., Hielm, S., Lahteenmaki, L., Peck, M.W., Korkeala, H., 2003. Thermal inactivation of nonproteolytic *Clostridium botulinum* type E spores in model fish media and in vacuum-packaged hot-smoked fish products. Appl Environ Microbiol 69, 4029-4036.
Little, S., Driks, A., 2001. Functional analysis of the Bacillus subtilis morphogenetic spore coat protein CotE. Mol Microbiol 42, 1107-1120.
Liu, H., Krajcikova, D., Wang, N., Zhang, Z., Wang, H., Barak, I., Tang, J., 2016a. Forces and Kinetics of the Bacillus subtilis Spore Coat Proteins CotY and CotX Binding to CotE Inspected by Single Molecule Force Spectroscopy. J Phys Chem B 120, 1041-1047.
Liu, H., Qiao, H., Krajcikova, D., Zhang, Z., Wang, H., Barak, I., Tang, J., 2016b. Physical interaction and assembly of Bacillus subtilis spore coat proteins CotE and CotZ studied by atomic force microscopy. J Struct Biol 195, 245-251.
Liu, Y., Carlsson Moller, M., Petersen, L., Soderberg, C.A., Hederstedt, L., 2010. Penicillin-binding protein SpoVD disulphide is a target for StoA in Bacillus subtilis forespores. Mol Microbiol 75, 46-60.
Loepfe, C., Raimann, E., Stephan, R., Tasara, T., 2010. Reduced host cell invasiveness and oxidative stress tolerance in double and triple csp gene family deletion mutants of Listeria monocytogenes. Foodborne Pathog Dis 7, 775-783.
Long, S., Jones, D.T., Woods, D.R., 1984. Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. Appl Microbiol Biot 20, 256-261.
Lopez, C.S., Garda, H.A., Rivas, E.A., 2002. The effect of osmotic stress on the biophysical behavior of the *Bacillus subtilis* membrane studied by dynamic and steady-state fluorescence anisotropy. Arch Biochem Biophys 408, 220-228.
Lopez, D., Kolter, R., 2010. Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. Fems Microbiol Rev 34, 134-149.
Lorand, L., Graham, R.M., 2003. Transglutaminases: crosslinking enzymes with pleiotropic functions. Nature reviews. Molecular cell biology 4, 140-156.
Loshon, C.A., Setlow, P., 1993. Levels of small molecules in dormant spores of *Sporosarcina* species and comparison with levels in spores of *Bacillus* and *Clostridium* species. Canadian Journal of Microbiology 39, 259-262.
Lottering, E.A., Streips, U.N., 1995. Induction of cold shock proteins in Bacillus subtilis. Curr Microbiol 30, 193-199.
Lund, B.M., Peck, M.W., 2000. *Clostridium botulinum.* In: The microbiological safety and quality of food. B. M. Lund, T. C. Baird-Parker, & G. W. Gould (Eds.), Gaithersburg: Aspen.
Luu, H., Akoachere, M., Patra, M., Abel-Santos, E., 2011. Cooperativity and interference of germination pathways in Bacillus anthracis spores. J Bacteriol 193, 4192-4198.
Mackie, A.R., Johnson, P.E., Van der Plancken, I., Balasa, A., Husband, F.A., Grauwet, T., Hendrickx, M., Knorr, D., Mills, E.N.C., 2010. High pressure, thermal and pulsed electric-field-induced structural changes in selected food allergens. Molecular Nutrition & Food Research 54, 1701-1710.
Magge, A., Granger, A.C., Wahome, P.G., Setlow, B., Vepachedu, V.R., Loshon, C.A., Peng, L., Chen, D., Li, Y.Q., Setlow, P., 2008. Role of dipicolinic acid in the germination, stability, and viability of spores of Bacillus subtilis. Journal of Bacteriology 190, 4798-4807.
Magill, N.G., Cowan, A.E., Koppel, D.E., Setlow, P., 1994. The internal pH of the forespore compartment of Bacillus megaterium decreases by about 1 pH unit during sporulation. Journal of Bacteriology 176, 2252-2258.
Mah, J.H., Kang, D.H., Tang, J., 2008. Effects of minerals on sporulation and heat resistance of Clostridium sporogenes. International Journal of Food Microbiology 128, 385-389.
Makino, S., Moriyama, R., 2002. Hydrolysis of cortex peptidoglycan during bacterial spore germination. Med Sci Monit 8, 119-127.

Mandicmulec, I., Doukhan, L., Smith, I., 1995. The *Bacillus subtilis* Sinr protein is a repressor of the key sporulation gene-Spo0a. J Bacteriol 177, 4619-4627.
Margosch, D., 2004. Behaviour of bacterial endospores and toxins as safety determinants in low acid pressurized food. Dissertation, Technische Universitat München.
Margosch, D., Ehrmann, M.A., Buckow, R., Heinz, V., Vogel, R.F., Gänzle, M.G., 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl Environ Microb 72, 3476-3481.
Margosch, D., Ehrmann, M.A., Gänzle, M.G., Vogel, R.F., 2004a. Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J Food Protect 67, 2530-2537.
Margosch, D., Gänzle, M.G., Ehrmann, M.A., Vogel, R.F., 2004b. Pressure inactivation of *Bacillus* endospores. Appl Environ Microb 70, 7321-7328.
Markland, S.M., Farkas, D.F., Kniel, K.E., Hoover, D.G., 2013. Pathogenic psychrotolerant sporeformers: an emerging challenge for low-temperature storage of minimally processed foods. Foodborne Pathog Dis 10, 413-419.
Marquis, R.E., Carstersen, E.L., Z., C.S., Bender, G.R., 1981. Preparation and characterization of various salt forms of Bacillus megaterium spores, p. 266-268. In H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
Marshall, K.M., Bradshaw, M., Pellett, S., Johnson, E.A., 2007. Plasmid encoded neurotoxin genes in Clostridium botulinum serotype A subtypes. Biochem Biophys Res Commun 361, 49-54.
Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H., Henriques, A.O., 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the Bacillus subtilis endospore coat. J Biol Chem 277, 18849-18859.
Masayama, A., Fukuoka, H., Kato, S., Yoshimura, T., Moriyama, M., Moriyama, R., 2006. Subcellular localization of a germiantion-specific cortex-lytic enzyme, SleB, of *Bacilli* during sporulation. Genes Genet Syst 81, 163-169.
Masayama, A., Kato, S., Terashima, T., Molgaard, A., Hemmi, H., Yoshimura, T., Moriyama, R., 2010. Bacillus subtilis spore coat protein LipC is a phospholipase B. Biosci Biotechnol Biochem 74, 24-30.
Masayama, A., Kuwana, R., Takamatsu, H., Hemmi, H., Yoshimura, T., Watabe, K., Moriyama, R., 2007. A novel lipolytic enzyme, YcsK (lipC), located in the spore coat of Bacillus subtilis, is involved in spore germination. Journal of bacteriology 189, 2369-2375.
Mathys, A., 2008. Inactivation mechanisms of *Geobacillus* and *Bacillus* spores during high pressure thermal sterilization. Dissertation, Technische Universitaet Berlin.
Mathys, A., Chapman, B., Bull, M., Heinz, V., Knorr, D., 2007a. Flow cytometric assessment of *Bacillus* spore response to high pressure and heat. Innov Food Sci Emerg 8, 519-527.
Mathys, A., Heinz, V., Knorr, D., 2008a. High Pressure Sterilization-State of the Art. Proceedings of the 2008 Joint Central European Congress, Vol 1, 89-95.
Mathys, A., Heinz, V., Knorr, D., 2008b. New pressure and temperature effects on bacterial spores. Joint 21st Airapt and 45th Ehprg International Conference on High Pressure Science and Technology 121.
Mathys, A., Heinz, V., Schwartz, F.H., Knorr, D., 2007b. Impact of agglomeration on the quantitative assessment of *Bacillus stearothermophilus* heat inactivation. J Food Eng 81, 380-387.
Mathys, A., Kallmeyer, R., Heinz, V., Knorr, D., 2008c. Impact of dissociation equilibrium shift on bacterial spore inactivation by heat and pressure. Food Control 19, 1165-1173.
Mathys, A., Reineke, K., Heinz, V., Knorr, D., 2009. High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. High Pressure Res 29, 3-7.
Matsumura, T., Jin, Y., Kabumoto, Y., Takegahara, Y., Oguma, K., Lencer, W.I., Fujinaga, Y., 2008. The HA proteins of botulinum toxin disrupt intestinal epithelial intercellular junctions to increase toxin absorption. Cell Microbiol 10, 355-364.
McKenney, P.T., Driks, A., Eskandarian, H.A., Grabowski, P., Guberman, J., Wang, K.H., Gitai, Z., Eichenberger, P., 2010. A distance-weighted interaction map reveals a previously uncharacterized layer of the Bacillus subtilis spore coat. Curr Biol 20, 934-938.
McKenney, P.T., Eichenberger, P., 2012. Dynamics of spore coat morphogenesis in Bacillus subtilis. Mol Microbiol 83, 245-260.
McPherson, D.C., Kim, H., Hahn, M., Wang, R., Grabowski, P., Eichenberger, P., Driks, A., 2005. Characterization of the Bacillus subtilis spore morphogenetic coat protein CotO. Journal of Bacteriology 187, 8278-8290.
Meersman, F., Smeller, L., Heremans, K., 2002. Comparative Fourier transform infrared spectroscopy study of cold-, pressure-, and heat-induced unfolding and aggregation of myoglobin. Biophys J 82, 2635-2644.
Meisner, J., Wang, X., Serrano, M., Henriques, A.O., Moran, C.P., 2008. A channel connecting the mother cell and forespore during bacterial endospore formation. P Natl Acad Sci USA 105, 15100-15105.
Melly, E., Genest, P.C., Gilmore, M.E., Little, S., Popham, D.L., Driks, A., Setlow, P., 2002. Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. J Appl Microbiol 92, 1105-1115.

Melly, E., Setlow, P., 2001. Heat shock proteins do not influence wet heat resistance of Bacillus subtilis spores. Journal of bacteriology 183, 779-784.
Mendez, M.B., Orsaria, L.M., Philippe, V., Pedrido, M.E., Grau, R.R., 2004. Novel roles of the master transcription factors Spo0A and sigma(B) for survival and sporulation of *Bacillus subtilis* at low growth temperature. J Bacteriol 186, 989-1000.
Merivirta, L.O., Lindstrom, M., Bjorkroth, K.J., Korkeala, H.J., 2006. The prevalence of Clostridium botulinum in European river lamprey (Lampetra fluviatilis) in Finland. International Journal of Food Microbiology 109, 234-237.
Meunier, F.A., Schiavo, G., Molgo, J., 2002. Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. Journal of physiology, Paris 96, 105-113.
Meyer, C.L., Papoutsakis, E.T., 1989. Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of *Clostridium acetobutylicum*. Appl Microbiol Biot 30, 450-459.
Mihailovich, M., Militti, C., Gabaldon, T., Gebauer, F., 2010. Eukaryotic cold shock domain proteins: highly versatile regulators of gene expression. Bioessays 32, 109-118.
Miller, B.S., Kennedy, T.E., Streips, U.N., 1991. Molecular Characterization of Specific-Heat Shock Proteins in Bacillus-Subtilis. Current Microbiology 22, 231-236.
Mills, G., Earnshaw, R., Patterson, M.F., 1998. Effects of high hydrostatic pressure on *Clostridium sporogenes* spores. Lett Appl Microbiol 26, 227-230.
Minerich, P.L., Labuza, T.P., 2003. Development of a pressure indicator for high hydrostatic pressure processing of foods. Innov Food Sci Emerg 4, 235-243.
Moir, A., 2003. Bacterial spore germination and protein mobility. Trends Microbiol 11, 452-454.
Moir, A., 2006. How do spores germinate? J Appl Microbiol 101, 526-530.
Moir, A., Smith, D.A., 1990. The genetics of bacterial spore germination. Annu Rev Microbiol 44, 531-553.
Molina-Gutierrez, A., Stippl, V., Delgado, A., Gänzle, M.G., Vogel, R.F., 2002. In situ determination of the intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment. Applied and Environmental Microbiology 68, 4399-4406.
Molina-Hoppner, A., Doster, W., Vogel, R.F., Gänzle, M.G., 2004. Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal high-pressure treatments. Applied and Environmental Microbiology 70, 2013-2020.
Molle, V., Nakaura, Y., Shivers, R.P., Yamaguchi, H., Losick, R., Fujita, Y., Sonenshein, A.L., 2003. Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. J Bacteriol 185, 1911-1922.
Moller, M., Hederstedt, L., 2006. Role of membrane-bound thiol-disulfide oxidoreductases in endospore-forming bacteria. Antioxid Redox Signal 8, 823-833.
Mongkolthanaruk, W., Robinson, C., Moir, A., 2009. Localization of the GerD spore germination protein in the Bacillus subtilis spore. Microbiology 155, 1146-1151.
Monroe, A., Setlow, P., 2006. Localization of the transglutaminase cross-linking sites in the *Bacillus subtilis* spore coat protein GerQ. J Bacteriol 188, 7609-7616.
Mortojudo, J.W., Siagian, E.G., Suhadi, F., Ward, B.Q., Ward, W.M., 1973. The presence of Clostridium botulinum in Indonesian waters. J Appl Bacteriol 36, 437-440.
Moschonas, G., Bolton, D.J., Sheridan, J.J., McDowell, D.A., 2010. The effect of storage temperature and inoculum level on the time of onset of 'blown pack' spoilage. Journal of Applied Microbiology 108, 532-539.
Movahedi, S., Waites, W., 2000. A two-dimensional protein gel electrophoresis study of the heat stress response of *Bacillus subtilis* cells during sporulation. J Bacteriol 182, 4758-4763.
Movahedi, S., Waites, W., 2002. Cold shock response in sporulating Bacillus subtilis and its effect on spore heat resistance. Journal of bacteriology 184, 5275-5281.
Mozhaev, V.V., Lange, R., Kudryashova, E.V., Balny, C., 1996. Application of high hydrostatic pressure for increasing activity and stability of enzymes. Biotechnol Bioeng 52, 320-331.
Mullerova, D., Krajcikova, D., Barak, I., 2009. Interactions between Bacillus subtilis early spore coat morphogenetic proteins. FEMS Microbiol Lett 299, 74-85.
Myers, G.S.A., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q.H., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., Paulsen, I.T., 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Res 16, 1031-1040.
Naclerio, G., Baccigalupi, L., Zilhao, R., De Felice, M., Ricca, E., 1996. Bacillus subtilis spore coat assembly requires cotH gene expression. Journal of Bacteriology 178, 4375-4380.
Nakashio, S., Gerhardt, P., 1985. Protoplast Dehydration Correlated with Heat-Resistance of Bacterial-Spores. Journal of bacteriology 162, 571-578.

Nevas, M., Lindstrom, M., Hautamaki, K., Puoskari, S., Korkeala, H., 2005. Prevalence and diversity of Clostridium botulinum types A, B, E and F in honey produced in the Nordic countries. International Journal of Food Microbiology 105, 145-151.

Nguyen Thi Minh, H., Durand, A., Loison, P., Perrier-Cornet, J.M., Gervais, P., 2011. Effect of sporulation conditions on the resistance of *Bacillus subtilis* spores to heat and high pressure. Appl Microbiol Biot 90, 1409-1417.

Nguyen Thi Minh, H., Gervais, P., Perrier-Cornet, J.M., Champion, D., 2010. New insight into the thermal properties and the biological behaviour of the bacterial spores. Food Biophys 5, 238-246.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol R 64, 548-572.

Nishihara, T., Kondo, M., Nonaka, T., Higashi, Y., 1982. LOCATION OF CALCIUM AND PHOSPHORUS IN ASHED SPORES OF BACILLUS-MEGATERIUM, DETERMINED BY ELECTRON-PROBE X-RAY-MICROANALYSIS. Microbiol Immunol 26, 167-172.

Nishiura, H., 2007. Incubation period as a clinical predictor of botulism: analysis of previous izushi-borne outbreaks in Hokkaido, Japan, from 1951 to 1965. Epidemiol Infect 135, 126-130.

NIST, 2002. Standard Reference Database 23 Version 7.0. National Institute of Standards and Technology (NIST) / American Society of Mechanical Engineers (ASME) - International Association for the Properties of Water and Steam (IAPWS); Lemmon, E. W., McLinden, M. O., Huber, M. L., 2002, Standard Reference Database 23 Version 7.0, Physical and Chemical Properties Devision, © by the U.S. Secretary of Commerce on behalf of The United States of America. .

Nolling, J., Breton, G., Omelchenko, M.V., Makarova, K.S., Zeng, Q.D., Gibson, R., Lee, H.M., Dubois, J., Qiu, D.Y., Hitti, J., Wolf, Y.I., Tatusov, R.L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M.J., Bennett, G.N., Koonin, E.V., Smith, D.R., Finishing, G.S.C.P., 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J Bacteriol 183, 4823-4838.

Norton, I., Fryer, P., Moore, S., 2006. Product/process integration in food manufacture: Engineering sustained health. Aiche J 52, 1632-1640.

Oey, I., Lille, M., Van Loey, A., Hendrickx, M., 2008a. Effect of high-pressure processing on colour, texture and flavour of fruit- and vegetable-based food products: a review. Trends Food Sci Tech 19, 320-328.

Oey, I., Van der Plancken, I., Van Loey, A., Hendrickx, M., 2008b. Does high pressure processing influence nutritional aspects of plant based food systems? Trends Food Sci Tech 19, 300-308.

Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K., Inoue, K., 1999. Structure and function of Clostridium botulinum progenitor toxin. J Toxicol-Toxin Rev 18, 17-34.

Oh, S., Moon, M.J., 2003. Inactivation of *Bacillus cereus* spores by high hydrostatic pressure at different temperatures. J Food Protect 66, 599-603.

Ohyama, A., Tanimura, E., Ito, T., Yamamoto, N., Yabuuchi, E., 1979. Electron-Microscopic Studies of Clostridium-Botulinum Type-E. Japanese Journal of Medical Science & Biology 32, 126-128.

Ohye, D., Scott, W., 1957. Studies in the Physiology of Clostridium Botulinum Type E. Aust J Biol Sci 10, 85-94.

Olivier, S.A., Bull, M.K., Chapman, B., 2012. *Bacillus* spp. spores produced at lower temperatures are more resistant to high pressure thermal processes but mineralization does not predict relative resistance. Innov Food Sci Emerg 16, 96-101.

Olivier, S.A., Bull, M.K., Stone, G., van Diepenbeek, R.J., Kormelink, F., Jacops, L., Chapman, B., 2011. Strong and Consistently Synergistic Inactivation of Spores of Spoilage-Associated Bacillus and Geobacillus spp. by High Pressure and Heat Compared with Inactivation by Heat Alone. Applied and Environmental Microbiology 77, 2317-2324.

Oomes, S.J., Jonker, M.J., Wittink, F.R., Hehenkamp, J.O., Breit, T.M., Brul, S., 2009. The effect of calcium on the transcriptome of sporulating *B. subtilis* cells. Int J Food Microbiol 133, 234-242.

Oomes, S.J., van Zuijlen, A.C., Hehenkamp, J.O., Witsenboer, H., van der Vossen, J.M., Brul, S., 2007. The characterisation of *Bacillus* spores occurring in the manufacturing of (low acid) canned products. Int J Food Microbiol 120, 85-94.

Oomes, S.J.C.M., Brul, S., 2004. The effect of metal ions commonly present in food on gene expression of sporulating *Bacillus subtilis* cells in relation to spore wet heat resistance. Innov Food Sci Emerg 5, 307-316.

Orsburn, B., Melville, S.B., Popham, D.L., 2008. Factors contributing to heat resistance of Clostridium perfringens endospores. Appl Environ Microbiol 74, 3328-3335.

Ozin, A.J., Henriques, A.O., Yi, H., Moran, C.P., 2000. Morphogenetic proteins SpoVID and SafA form a complex during assembly of the *Bacillus subtilis* spore coat. J Bacteriol 182, 1828-1833.

Ozin, A.J., Samford, C.S., Henriques, A.O., Moran, C.P., Jr., 2001. SpoVID guides SafA to the spore coat in Bacillus subtilis. J Bacteriol 183, 3041-3049.

Pace, P.J., Krumbiegel, E.R., Angelotti, R., Wisniewski, H.J., 1967. Demonstration and isolation of Clostridium botulinum types from whitefish chubs collected at fish smoking plants of the Milwaukee area. Appl Microbiol 15, 877-884.

Paidhungat, M., Setlow, B., Daniels, W.B., Hoover, D., Papafragkou, E., Setlow, P., 2002. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl Environ Microb 68, 3172-3175.
Paidhungat, M., Setlow, B., Driks, A., Setlow, P., 2000. Characterization of spores of Bacillus subtilis which lack dipicolinic acid. Journal of Bacteriology 182, 5505-5512.
Paidhungat, M., Setlow, P., 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J Bacteriol 181, 3341-3350.
Paidhungat, M., Setlow, P., 2000. Role of Ger proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*. J Bacteriol 182, 2513-2519.
Paidhungat, M., Setlow, P., 2001. Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. J Bacteriol 183, 3982-3990.
Palop, A., Raso, J., Condon, S., Sala, F.J., 1996. Heat resistance of Bacillus subtilis and Bacillus coagulans: Effect of sporulation temperature in foods with various acidulants. J Food Protect 59, 487-492.
Palop, A., Sala, F.J., Condon, S., 1997. Occurrence of a highly heat-sensitive spore subpopulation of Bacillus coagulans STCC 4522 and its conversion to a more heat-stable form. Applied and Environmental Microbiology 63, 2246-2251.
Palop, A., Sala, F.J., Condon, S., 1999. Heat resistance of native and demineralized spores of Bacillus subtilis sporulated at different temperatures. Applied and Environmental Microbiology 65, 1316-1319.
Pandey, N.K., Aronson, A.I., 1979. Properties of the Bacillus subtilis spore coat. Journal of Bacteriology 137, 1208-1218.
Paredes-Sabja, D., Sarker, N., Setlow, B., Setlow, P., Sarker, M.R., 2008a. Roles of DacB and spm proteins in clostridium perfringens spore resistance to moist heat, chemicals, and UV radiation. Appl Environ Microbiol 74, 3730-3738.
Paredes-Sabja, D., Setlow, B., Setlow, P., Sarker, M.R., 2008b. Characterization of *Clostridium perfringens* spores that lack spoVA proteins and dipicolinic acid. J Bacteriol 190, 4648-4659.
Paredes-Sabja, D., Setlow, P., Sarker, M.R., 2009a. GerO, a putative Na^+/H^+-K^+ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*. J Bacteriol 191, 3822-3831.
Paredes-Sabja, D., Setlow, P., Sarker, M.R., 2009b. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. J Bacteriol 191, 2711-2720.
Paredes-Sabja, D., Setlow, P., Sarker, M.R., 2011. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. Trends Microbiol 19, 85-94.
Paredes-Sabja, D., Torres, J.A., Setlow, P., Sarker, M.R., 2008c. *Clostridium perfringens* spore germination: characterization of germinants and their receptors. J Bacteriol 190, 1190-1201.
Paredes-Sabja, D., Udompijitkul, P., Sarker, M.R., 2009c. Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning-related isolates. Appl Environ Microbiol 75, 6299-6305.
Paredes, C.J., Alsaker, K.V., Papoutsakis, E.T., 2005. A comparative genomic view of clostridial sporulation and physiology. Nat Rev Microbiol 3, 969-978.
Paredes, C.J., Rigoutsos, I., Papoutsakis, E.T., 2004. Transcriptional organization of the *Clostridium acetobutylicum* genome. Nucleic Acids Res 32, 1973-1981.
Pasteur, L., 1866. Etudes sur le vin. Des Seances del L'Academie Des Sciences 63, 509-510.
Patazca, E., Morrissey, T.R., Loeza, V., Reddy, N.R., Skinner, G.E., Larkin, J.W., 2013. Effect of packaging systems and pressure fluids on inactivation of Clostridium botulinum spores by combined high pressure and thermal processing. J Food Prot 76, 448-455.
Peck, M.W., 2006. Clostridium botulinum and the safety of minimally heated, chilled foods: an emerging issue? Journal of Applied Microbiology 101, 556-570.
Peck, M.W., 2009. Biology and genomic analysis of Clostridium botulinum. Adv Microb Physiol 55, 183-265, 320.
Peck, M.W., Evans, R.I., Fairbairn, D.A., Hartley, M.G., Russell, N.J., 1995a. Effect of sporulation temperature on some properties of spores of non-proteolytic *Clostridium botulinum*. Int J Food Microbiol 28, 289-297.
Peck, M.W., Fairbairn, D.A., Lund, B.M., 1992. The effect of recovery medium on the estimated heat inactivation of spores of nonproteolytic *Clostridium botulinum*. Lett Appl Microbiol 15, 146-151.
Peck, M.W., Fernandez, P.S., 1995. Effect of lysozyme concentration, heating at 90 degrees C, and then incubation at chilled temperatures on growth from spores of non-proteolytic Clostridium botulinum. Lett Appl Microbiol 21, 50-54.
Peck, M.W., Goodburn, K.E., Betts, R.P., Stringer, S.C., 2008. Assessment of the potential for growth and neurotoxin formation by non-proteolytic *Clostridium botulinum* in short shelf-life commercial foods designed to be stored chilled. Trends Food Sci Tech 19, 207-216.
Peck, M.W., Lund, B.M., Fairbairn, D.A., Kaspersson, A.S., Undeland, P.C., 1995b. Effect of heat treatment on survival of, and growth from, spores of nonproteolytic *Clostridium botulinum* at refrigeration temperatures. Applied and Environmental Microbiology 61, 1780-1785.

Pedersen, L.B., Murray, T., Popham, D.L., Setlow, P., 1998. Characterization of dacC, which encodes a new low-molecular weight penicillin-binding protein in *Bacillus subtilis*. J Bacteriol 180, 4967-4973.
Pelczar, P.L., Igarashi, T., Setlow, B., Setlow, P., 2007. Role of GerD in germination of *Bacillus subtilis* spores. J Bacteriol 189, 1090-1098.
Pelczar, P.L., Setlow, P., 2008. Localization of the germination protein GerD to the inner membrane in *Bacillus subtilis* spores. J Bacteriol 190, 5635-5641.
Pellegrino, P.M., Fell, N.F., Gillespie, J.B., 2002. Enhanced spore detection using dipicolinate extraction techniques. Anal Chim Acta 455, 167-177.
Penna, T.C., Machoshvili, I.A., Taqueda, M.E., 1996. Thermal resistance of Bacillus stearothermophilus spores on strips previously treated with calcium. PDA J Pharm Sci Technol 50, 227-237.
Penna, T.C.V., Machoshvili, I.A., Ishii, M., 2003. Effect of media on spore yield and thermal resistance of Bacillus stearothermophilus. Appl Biochem Biotech 106, 287-294.
Perego, M., 2001. A new family of aspartyl phosphate phosphatases targeting the sporulation transcription factor Spo0A of *Bacillus subtilis*. Mol Microbiol 42, 133-143.
Perez-Fuentetaja, A., Clapsadl, M.D., Getchell, R.G., Bowser, P.R., Lee, W.T., 2011. Clostridium botulinum type E in Lake Erie: Inter-annual differences and role of benthic invertebrates. J Great Lakes Res 37, 238-244.
Perez, J., Springthorpe, V.S., Sattar, S.A., 2011. Clospore: A liquid medium for producing high titers of semi-purified spores of *Clostridium difficile*. Journal of AOAC International 94, 618-626.
Perkins, W.E., Tsuji, K., 1962. Sporulation of Clostridium botulinum. II. Effect of arginine and its degradation products on sporulation in a synthetic medium. Journal of Bacteriology 84, 86-94.
Permpoonpattana, P., Phetcharaburanin, J., Mikelsone, A., Dembek, M., Tan, S., Brisson, M.C., La Ragione, R., Brisson, A.R., Fairweather, N., Hong, H.A., Cutting, S.M., 2013. Functional Characterization of Clostridium difficile Spore Coat Proteins. Journal of Bacteriology.
Permpoonpattana, P., Tolls, E.H., Nadem, R., Tan, S., Brisson, A., Cutting, S.M., 2011. Surface layers of Clostridium difficile endospores. Journal of Bacteriology 193, 6461-6470.
Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J.D., Volker, U., Hecker, M., 2001. Global analysis of the general stress response of Bacillus subtilis. Journal of bacteriology 183, 5617-5631.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.
Phadtare, S., Inouye, M., 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in Escherichia coli. Journal of Bacteriology 183, 1205-1214.
Phadtare, S., Inouye, M., 2004. Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of Escherichia coli. J Bacteriol 186, 7007-7014.
Phadtare, S., Yamanaka, K., Inouye, M., 2000. The cold shock response. In Stortz, G. and Hengge-Aronis, R. (eds.), Bacterial Stress Response. American Society for Microbiology, Washington, DC, pp. 33-47.
Phillips, Z.E., Strauch, M.A., 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. Cell Mol Life Sci 59, 392-402.
Pierce, K.J., Salifu, S.P., Tangney, M., 2008. Gene cloning and characterization of a second alanine racemase from Bacillus subtilis encoded by yncD. FEMS Microbiol Lett 283, 69-74.
Piggot, P.J., Hilbert, D.W., 2004. Sporulation of *Bacillus subtilis*. Curr Opin Microbiol 7, 579-586.
Planchon, S., Dargaignaratz, C., Levy, C., Ginies, C., Broussolle, V., Carlin, F., 2011. Spores of *Bacillus cereus* strain KBAB4 produced at 10 degrees C and 30 degrees C display variations in their properties. Food Microbiol 28, 291-297.
Planck, M., 1887. Ann. Phys. Chem., 32, 426.
Plomp, M., Leighton, T.J., Wheeler, K.E., Malkin, A.J., 2005a. Architecture and high-resolution structure of Bacillus thuringiensis and Bacillus cereus spore coat surfaces. Langmuir 21, 7892-7898.
Plomp, M., Leighton, T.J., Wheeler, K.E., Malkin, A.J., 2005b. The high-resolution architecture and structural dynamics of Bacillus spores. Biophys J 88, 603-608.
Plomp, M., Leighton, T.J., Wheeler, K.E., Pitesky, M.E., Malkin, A.J., 2005c. Bacillus atrophaeus outer spore coat assembly and ultrastructure. Langmuir 21, 10710-10716.
Plowman, J., Peck, M.W., 2002. Use of a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. J Appl Microbiol 92, 681-694.
Pogliano, K., Harry, E., Losick, R., 1995. Visualization of the subcellular location of sporulation proteins in Bacillus subtilis using immunofluorescence microscopy. Mol Microbiol 18, 459-470.
Ponce, A., Yang, W.W., Crow-Willard, E.N., 2009. Production and characterization of pure *Clostridium* spore suspensions. J Appl Microbiol 106, 27-33.
Popham, D.L., 2002. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell Mol Life Sci 59, 426-433.
Popham, D.L., Gilmore, M.E., Setlow, P., 1999a. Roles of low-molecular-weight penicillin-binding proteins in *Bacillus subtilis* spore peptidoglycan synthesis and spore properties. J Bacteriol 181, 126-132.

Popham, D.L., Helin, J., Costello, C.E., Setlow, P., 1996a. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. J Bacteriol 178, 6451-6458.
Popham, D.L., Helin, J., Costello, C.E., Setlow, P., 1996b. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. P Natl Acad Sci USA 93, 15405-15410.
Popham, D.L., Illadesaguiar, B., Setlow, P., 1995a. The *Bacillus subtilis* Dacb gene, encoding penicillin-binding protein-5-asterisk, is part of a 3-gene operon required for proper spore cortex synthesis and spore core dehydration. J Bacteriol 177, 4721-4729.
Popham, D.L., Meador-Parton, J., Costello, C.E., Setlow, P., 1999b. Spore peptidoglycan structure in a cwlD dacB double mutant of Bacillus subtilis. Journal of bacteriology 181, 6205-6209.
Popham, D.L., Orsburn, B.C., Melville, S.B., 2010. EtfA catalyses the formation of dipicolinic acid in *Clostridium perfringens*. Mol Microbiol 75, 178-186.
Popham, D.L., Sengupta, S., Setlow, P., 1995b. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water content and with or without major DNA-binding proteins. Appl Environ Microb 61, 3633-3638.
Price, K.D., Losick, R., 1999. A four-dimensional view of assembly of a morphogenetic protein during sporulation in Bacillus subtilis. Journal of Bacteriology 181, 781-790.
Priev, A., Almagor, A., Yedgar, S., Gavish, B., 1996. Glycerol Decreases the Volume and Compressibility of Protein Interior. Biochemistry 35, 2061-2066.
Qiao, H., Krajcikova, D., Liu, C., Li, Y., Wang, H., Barak, I., Tang, J., 2012. The interactions of spore-coat morphogenetic proteins studied by single-molecule recognition force spectroscopy. Chemistry, an Asian journal 7, 725-731.
Quinlan, R.J., Reinhart, G.D., 2005. Baroresistant buffer mixtures for biochemical analyses. Anal Biochem 341, 69-76.
Raffestin, S., Couesnon, A., Pereira, Y., Mazuet, C., Popoff, M.R., 2009. Botulinum and Tetanus Neurotoxins, Molecular Biology and Toxin Gene Regulation, Mode of Action *In:* Clostridia: Molecular Biology in the Post-genomic Era, Brüggemann, H. and Gottschalk, G. (eds.), Caister Academic Press (2009), ISBN: 978-1-904455-38-7.
Ragkousi, K., Eichenberger, P., van Ooij, C., Setlow, P., 2003. Identification of a new gene essential for germination of Bacillus subtilis spores with Ca2+-dipicolinate. Journal of Bacteriology 185, 2315-2329.
Ragkousi, K., Setlow, P., 2004. Transglutaminase-mediated cross-linking of GerQ in the coats of Bacillus subtilis spores. Journal of Bacteriology 186, 5567-5575.
Rajan, S., Ahn, J., Balasubramaniam, V.M., Yousef, A.E., 2006a. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in egg patty mince. J Food Protect 69, 853-860.
Rajan, S., Pandrangi, S., Balasubramaniam, V.M., Yousef, A.E., 2006b. Inactivation of *Bacillus stearothermophilus* spores in egg patties by pressure-assisted thermal processing. Lwt-Food Sci Technol 39, 844-851.
Ramamurthi, K.S., Clapham, K.R., Losick, R., 2006. Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. Mol Microbiol 62, 1547-1557.
Ramamurthi, K.S., Lecuyer, S., Stone, H.A., Losick, R., 2009. Geometric cue for protein localization in a bacterium. Science 323, 1354-1357.
Ramamurthi, K.S., Losick, R., 2008. ATP-driven self-assembly of a morphogenetic protein in *Bacillus subtilis*. Mol Cell 31, 406-414.
Ramirez-Peralta, A., Gupta, S., Butzin, X.Y., Setlow, B., Korza, G., Leyva-Vazquez, M.A., Christie, G., Setlow, P., 2013. Identification of new proteins that modulate the germination of spores of *bacillus* species. J Bacteriol 195, 3009-3021.
Ramirez-Peralta, A., Stewart, K.A.V., Thomas, S.K., Setlow, B., Chen, Z., Li, Y.Q., Setlow, P., 2012a. Effects of the SpoVT Regulatory Protein on the Germination and Germination Protein Levels of Spores of Bacillus subtilis. Journal of Bacteriology 194, 3417-3425.
Ramirez-Peralta, A., Zhang, P.F., Li, Y.Q., Setlow, P., 2012b. Effects of Sporulation Conditions on the Germination and Germination Protein Levels of Bacillus subtilis Spores. Applied and Environmental Microbiology 78, 2689-2697.
Ramirez, N., Liggins, M., Abel-Santos, E., 2010. Kinetic evidence for the presence of putative germination receptors in *Clostridium difficile* spores. J Bacteriol 192, 4215-4222.
Rasanayagam, V., Balasubramaniam, V.M., Ting, E., Sizer, C.E., Bush, C., Anderson, C., 2003. Compression Heating of Selected Fatty Food Materials during High-pressure Processing. J Food Sci 68, 254-259.
Raso, J., Barbosa-Canovas, G., Swanson, B.G., 1998a. Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of *Bacillus cereus*. J Appl Microbiol 85, 17-24.
Raso, J., Gongora-Nieto, M.M., Barbosa-Canovas, G.V., Swanson, B.G., 1998b. Influence of several environmental factors on the initiation of germination and inactivation of *Bacillus cereus* by high hydrostatic pressure. Int J Food Microbiol 44, 125-132.

Raso, J., Palop, A., Bayarte, M., Condon, S., Sala, F.J., 1995. Influence of Sporulation Temperature on the Heat-Resistance of a Strain of Bacillus-Licheniformis (Spanish Type Culture Collection-4523). Food Microbiol 12, 357-361.

Rasooly, R., Do, P.M., 2010. Clostridium botulinum Neurotoxin Type B Is Heat-Stable in Milk and Not Inactivated by Pasteurization. J Agric Food Chem.

Ratnayake-Lecamwasam, M., Serror, P., Wong, K.W., Sonenshein, A.L., 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. Genes Dev 15, 1093-1103.

Ratphitagsanti, W., Ahn, J., Balasubramaniam, V.M., Yousef, A.E., 2009. Influence of Pressurization Rate and Pressure Pulsing on the Inactivation of Bacillus amyloliquefaciens Spores during Pressure-Assisted Thermal Processing. J Food Protect 72, 775-782.

Ratphitagsanti, W., De Lamo-Castellvi, S., Balasubramaniam, V.M., Yousef, A.E., 2010. Efficacy of pressure-assisted thermal processing, in combination with organic acids, against *Bacillus amyloliquefaciens* spores suspended in deionized water and carrot puree. J Food Sci 75, M46-M52.

Ravagnani, A., Jennert, K.C.B., Steiner, E., Grunberg, R., Jefferies, J.R., Wilkinson, S.R., Young, D.I., Tidswell, E.C., Brown, D.P., Youngman, P., Morris, J.G., Young, M., 2000. Spo0A directly controls the switch from acid to solvent production in solvent-forming *clostridia*. Mol Microbiol 37, 1172-1185.

Reddy, N.R., Solomon, H.M., Fingerhut, G.A., Rhodehamel, E.J., Balasubramaniam, V.M., Palaniappan, S., 1999. Inactivation of *Clostridium botulinum* type E spores by high pressure processing. J Food Safety 19, 277-288.

Reddy, N.R., Solomon, H.M., Tetzloff, R.C., Rhodehamel, E.J., 2003. Inactivation of *Clostridium botulinum* type A spores by high-pressure processing at elevated temperatures. J Food Protect 66, 1402-1407.

Reddy, N.R., Tetzloff, R.C., Skinner, G.E., 2010. Effect of media, additives, and incubation conditions on the recovery of high pressure and heat-injured *Clostridium botulinum* spores. Food Microbiol 27, 613-617.

Reddy, N.R., Tetzloff, R.C., Solomon, H.M., Larkin, J.W., 2006. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov Food Sci Emerg 7, 169-175.

Redmond, C., Baillie, L.W., Hibbs, S., Moir, A.J., Moir, A., 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology 150, 355-363.

Reineke, K., 2013. Mechanisms of *Bacillus* spore germination and inactivation during high pressure processing. Dissertation. Bornimer Agrartechnische Berichte Heft 80. Leibniz-Institut für Agrartechnik Postdam-Bornim e.V., 2013. ISSN 0947-7314.

Reineke, K., Doehner, I., Mathys, A., Knorr, D., 2010. Pressure induced germination of *Bacillus subtilis* spores - A mechanistic study, 6th International Conference on High Pressure Bioscience and Biotechnology (HPBB2010), Freising-Weihenstephan, Germany, p. 43; Lecture L410.

Reineke, K., Doehner, I., Schlumbach, K., Baier, D., Mathys, A., Knorr, D., 2012. The different pathways of spore germination and inactivation in dependence of pressure and temperature. Innov Food Sci Emerg 13, 31-41.

Reineke, K., Mathys, A., Heinz, V., Knorr, D., 2008. Temperature control for high pressure processes up to 1400 MPa. Joint 21st Airapt and 45th Ehprg International Conference on High Pressure Science and Technology 121.

Reineke, K., Mathys, A., Heinz, V., Knorr, D., 2013a. Mechanisms of endospore inactivation under high pressure. Trends Microbiol 21, 296-304.

Reineke, K., Mathys, A., Knorr, D., 2011. The impact of high pressure and temperature on bacterial spores: Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. J Food Sci 76, 189-197.

Reineke, K., Schlumbach, K., Baier, D., Mathys, A., Knorr, D., 2013b. The release of dipicolinic acid — The rate-limiting step of *Bacillus* endospore inactivation during the high pressure thermal sterilization process. Int J Food Microbiol 162, 55-63.

Rey, C.R., Walker, H.W., Rohrbaugh, P.L., 1975. The influence of temperature on growth, sporulation, and heat resistance of spores of six strains of *Clostridium perfringens*. J Milk Food Technol 38, 461–465.

Rhodehamel, E.J., Solomon, H.M., Lilly, T., Kautter, D.A., Peeler, J.T., 1991. Incidence and Heat-Resistance of Clostridium-Botulinum Type-E Spores in Menhaden Surimi. J Food Sci 56, 1562-&.

Riesenman, P.J., Nicholson, W.L., 2000. Role of the spore coat layers in Bacillus subtilis spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Applied and Environmental Microbiology 66, 620-626.

RKI, 2016. Infektionsepidemiologisches Jahrbuch (annual infection epidemiologic report) for the years 2001 to 2015; Robert Koch-Institut, Berlin, Germany, 2002 - 2016.

Roberts, C.M., Hoover, D.G., 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J Appl Bacteriol 81, 363-368.

Roberts, T.A., 1965. Sporulation of *Clostridium botulinum* type E in different culture media. J Appl Bacteriol 28, 142-146.

Roberts, T.A., Hitchins, A.D., 1969. Resistance of spores, in: Gould, G.W., Hurst, A. (Eds.), The Bacterial Spore. Academic Press, Inc., New York, pp. 611–670.

Roberts, T.A., Ingram, M., 1965. Resistance of spores of *Clostridium botulinum* type E to heat and radiation. J Appl Bacteriol 28, 125-141.
Robertson, M., 1915. Notes upon certain anaerobes isolated from wounds. The Journal of Pathology and Bacteriology 20, 327-349.
Rocourt, J., Moy, G., Vierk, K., Schlundt, J., 2003. The present state of foodborne disease in OECD countries. Food Safety Department WHO, Geneva.
Rode, L.J., Foster, J.W., 1960. The action of surfactants on bacterial spores. Arch Mikrobiol 36, 67-94.
Rode, L.J., Foster, J.W., 1966. Quantitative aspects of exchangeable calcium in spores of *Bacillus megaterium*. J Bacteriol 91, 1589-1593.
Roels, S., Driks, A., Losick, R., 1992. Characterization of *spoIVA*, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. J Bacteriol 174, 575-585.
Rose, R., Setlow, B., Monroe, A., Mallozzi, M., Driks, A., Setlow, P., 2007. Comparison of the properties of *Bacillus subtilis* spores made in liquid or on agar plates. J Appl Microbiol 103, 691-699.
Ross, M.A., Setlow, P., 2000. The Bacillus subtilis HBsu protein modifies the effects of alpha/beta-type, small acid-soluble spore proteins on DNA. Journal of Bacteriology 182, 1942-1948.
Rowland, S.L., Burkholder, W.F., Cunningham, K.A., Maciejewski, M.W., Grossman, A.D., King, G.F., 2004. Structure and mechanism of action of Sda, an inhibitor of the histidine kinases that regulate initiation of sporulation in *Bacillus subtilis*. Mol Cell 13, 689-701.
Ruan, K., Xu, C., Li, T., Li, J., Lange, R., Balny, C., 2003. The thermodynamic analysis of protein stabilization by sucrose and glycerol against pressure-induced unfolding. European Journal of Biochemistry 270, 1654-1661.
Rummel, A., Eichner, T., Weil, T., Karnath, T., Gutcaits, A., Mahrhold, S., Sandhoff, K., Proia, R.L., Acharya, K.R., Bigalke, H., Binz, T., 2007. Identification of the protein receptor binding site of botulinum neurotoxins B and G proves the double-receptor concept. Proc Natl Acad Sci U S A 104, 359-364.
Russell, A.D., 1971. The destruction of bacterial spores. In *Inhibition and destruction of the microbial cell* (Ed Hugo, W. B.) pp. 451-612. London, Academic Press.
Ryu, J.H., Kim, H., Beuchat, L.R., 2005. Spore formation by *Bacillus cereus* in broth as affected by temperature, nutrient availability, and manganese. J Food Protect 68, 1734-1738.
Sabrowski, A., 2000. Quality changes in smoked vacuum-packed trout fillets during storage (Lagerungsabhängige Qualitätsveränderungen von geräucherten, vakuumverpackten Forellenfilets). Inaugural Dissertation, Institut für Lebensmittelhygiene, Fachbereich Veterinärmedizin, Hygiene, FU Berlin.
Sacco, M., Ricca, E., Losick, R., Cutting, S., 1995. An additional GerE-controlled gene encoding an abundant spore coat protein from Bacillus subtilis. Journal of Bacteriology 177, 372-377.
Sahin, O., Yong, E.H., Driks, A., Mahadevan, L., 2012. Physical basis for the adaptive flexibility of Bacillus spore coats. J R Soc Interface 9, 3156-3160.
Sala, F.J., Ibarz, P., Palop, A., Raso, J., Condon, S., 1995. Sporulation Temperature and Heat-Resistance of Bacillus-Subtilis at Different Ph Values. J Food Protect 58, 239-243.
Sames, T., 1900. Zur Kentniss der bei hoeherer Temperatur wachsenden Bakterien- und Streptothrixarten. Z. Hyg. Infektionskrankheiten, 33, 313-362.
Sanchez-Salas, J.L., Setlow, B., Zhang, P., Li, Y.Q., Setlow, P., 2011. Maturation of released spores is necessary for acquisition of full spore heat resistance during *Bacillus subtilis* sporulation. Applied and Environmental Microbiology 77, 6746-6754.
Santangelo, J.D., Kuhn, A., Treuner-Lange, A., Durre, P., 1998. Sporulation and time course expression of sigma-factor homologous genes in *Clostridium acetobutylicum*. Fems Microbiol Lett 161, 157-164.
Sarker, M.R., Akhtar, S., Torres, J.A., Paredes-Sabja, D., 2013. High hydrostatic pressure-induced inactivation of bacterial spores. Crit Rev Microbiol.
Sarker, M.R., Huang, I.H., 2006. Complementation of a *Clostridium perfringens* spo0A mutant with wild-type spo0A from other *Clostridium* species. Appl Environ Microb 72, 6388-6393.
Sauer, U., Treuner, A., Buchholz, M., Santangelo, J.D., Durre, P., 1994. Sporulation and primary sigma-factor homologous genes in *Clostridium acetobutylicum*. J Bacteriol 176, 6572-6582.
Saujet, L., Monot, M., Dupuy, B., Soutourina, O., Martin-Verstraete, I., 2011. The key sigma factor of transition phase, SigH, controls sporulation, metabolism, and virulence factor expression in *Clostridium difficile*. J Bacteriol 193, 3186-3196.
Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-major pathogens. Emerg Infect Dis 17, 7-15.
Scheeff, E.D., Axelrod, H.L., Miller, M.D., Chiu, H.J., Deacon, A.M., Wilson, I.A., Manning, G., 2010. Genomics, evolution, and crystal structure of a new family of bacterial spore kinases. Proteins 78, 1470-1482.
Scherrer, R., Gerhardt, P., 1972. Location of Calcium within Bacillus Spores by Electron-Probe X-Ray Microanalysis. Journal of bacteriology 112, 559-&.
Schiavo, G., Rossetto, O., Montecucco, C., 1994. Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. Seminars in cell biology 5, 221-229.

Schindelin, H., Herrler, M., Willimsky, G., Marahiel, M.A., Heinemann, U., 1992. Overproduction, crystallization, and preliminary X-ray diffraction studies of the major cold shock protein from Bacillus subtilis, CspB. Proteins 14, 120-124.
Schiott, T., Hederstedt, L., 2000. Efficient spore synthesis in Bacillus subtilis depends on the CcdA protein. Journal of Bacteriology 182, 2845-2854.
Schmidt, D.B., 2006. Trends in consumer attitudes toward food and health: From food biotechnology to personalized nutrition. Potential Health Benefits of Citrus 936, 254-259.
Schmidt, R., Margolis, P., Duncan, L., Coppolecchia, R., Moran, C.P., Losick, R., 1990. Control of developmental transcription factor-rho-F by sporulation regulatory proteins *spoIIAA* and *spoIIAB* in *Bacillus subtilis*. P Natl Acad Sci USA 87, 9221-9225.
Schultz, D., Wolynes, P.G., Ben Jacob, E., Onuchic, J.N., 2009. Deciding fate in adverse times: Sporulation and competence in *Bacillus subtilis*. P Natl Acad Sci USA 106, 21027-21034.
Scotcher, M.C., Bennett, G.N., 2005. SpoIIE regulates sporulation but does not directly affect solventogenesis in *Clostridium acetobutylicum* ATCC 824. J Bacteriol 187, 1930-1936.
Scotcher, M.C., Rudolph, F.B., Bennett, G.N., 2005. Expression of abrB310 and sinR, and effects of decreased abrB310 expression on the transition from acidogenesis to solventogenesis, in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microb 71, 1987-1995.
Scurrah, K.J., Robertson, R.E., Craven, H.M., Pearce, L.E., Szabo, E.A., 2006. Inactivation of *Bacillus* spores in reconstituted skim milk by combined high pressure and heat treatment. J Appl Microbiol 101, 172-180.
Sebaihia, M., Peck, M.W., Minton, N.P., Thomson, N.R., Holden, M.T.G., Mitchell, W.J., Carter, A.T., Bentley, S.D., Mason, D.R., Crossman, L., Paul, C.J., Ivens, A., Wells-Bennik, M.H.J., Davis, I.J., Cerdeno-Tarraga, A.M., Churcher, C., Quail, M.A., Chillingworth, T., Feltwell, T., Fraser, A., Goodhead, I., Hance, Z., Jagels, K., Larke, N., Maddison, M., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Sanders, M., Simmonds, M., White, B., Whithead, S., Parkhill, J., 2007. Genome sequence of a proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. Genome Res 17, 1082-1092.
Sedlak, M., Vinter, V., Adamec, J., Vohradsky, J., Voburka, Z., Chaloupka, J., 1993. Heat-Shock Applied Early in Sporulation Affects Heat-Resistance of Bacillus-Megaterium Spores. Journal of bacteriology 175, 8049-8052.
Senior, A., Moir, A., 2008. The *Bacillus cereus* GerN and GerT protein homologs have distinct roles in spore germination and outgrowth, respectively. J Bacteriol 190, 6148-6152.
Serrano, M., Zilhao, R., Ricca, E., Ozin, A.J., Moran, C.P., Jr., Henriques, A.O., 1999. A Bacillus subtilis secreted protein with a role in endospore coat assembly and function. J Bacteriol 181, 3632-3643.
Setlow, B., Melly, E., Setlow, P., 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. J Bacteriol 183, 4894-4899.
Setlow, B., Setlow, P., 1977. Levels of acetyl coenzyme A, reduced and oxidized coenzyme A, and coenzyme A in disulfide linkage to protein in dormant and germinated spores and growing and sporulating cells of *Bacillus megaterium*. J Bacteriol 132, 444-452.
Setlow, B., Setlow, P., 1980. Measurements of the pH within dormant and germinated bacterial spores. Proc Natl Acad Sci U S A 77, 2474-2476.
Setlow, B., Setlow, P., 1995. Binding to DNA protects alpha/beta-type, small, acid-soluble spore proteins of Bacillus and Clostridium species against digestion by their specific protease as well as by other proteases. Journal of Bacteriology 177, 4149-4151.
Setlow, B., Sun, D.X., Setlow, P., 1992. Interaction between DNA and Alpha/Beta-Type Small, Acid-Soluble Spore Proteins - a New Class of DNA-Binding Protein. Journal of bacteriology 174, 2312-2322.
Setlow, P., 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. Soc Appl Bacteriol Symp Ser 23, 49-60.
Setlow, P., 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annu Rev Microbiol 49, 29-54.
Setlow, P., 2003. Spore germination. Curr Opin Microbiol 6, 550-556.
Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 101, 514-525.
Setlow, P., 2012. Dynamics of the assembly of a complex macromolecular structure - the coat of spores of the bacterium Bacillus subtilis. Molecular Microbiology 83, 241-244.
Setlow, P., Johnson, E.A., 2007. Spores and their significance, in: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food microbiology fundamentals and frontiers, 3rd ed. American Society of Microbiology Press., Washington, DC, pp. 35-67.
Setlow, P., Johnson, E.A., 2013. Spores and their significance, in: Doyle, M.P., Buchanan, R.L. (Eds.), Food Microbiology: Fundamentals and Frontiers, 4th Ed. American Society of Microbiology Press, Washington, D.C., 2013, Washington, DC, pp. 45-79.
Seyler, R.W., Jr., Henriques, A.O., Ozin, A.J., Moran, C.P., Jr., 1997. Assembly and interactions of cotJ-encoded proteins, constituents of the inner layers of the Bacillus subtilis spore coat. Mol Microbiol 25, 955-966.

Shah, I.M., Dworkin, J., 2010. Induction and regulation of a secreted peptidoglycan hydrolase by a membrane Ser/Thr kinase that detects muropeptides. Mol Microbiol 75, 1232-1243.
Shah, I.M., Laaberki, M.H., Popham, D.L., Dworkin, J., 2008. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell 135, 486-496.
Sharma, S.K., Ramzan, M.A., Singh, B.R., 2003. Separation of the components of type A botulinum neurotoxin complex by electrophoresis. Toxicon 41, 321-331.
Shibata, H., Adachi, S., Hirose, Y., Ike, M., Tani, I., Hashimoto, T., 1993. Role of calcium in biphasic germination of *Bacillus cereus* T-spores sensitized to lysozyme. Microbiol Immunol 37, 187-194.
Shpigelman, A., Kyomugasho, C., Christiaens, S., Van Loey, A.M., Hendrickx, M.E., 2014. Thermal and high pressure high temperature processes result in distinctly different pectin non-enzymatic conversions. Food Hydrocolloid 39, 251-263.
Siala, A., Hill, I.R., Gray, T.R.G., 1974. Populations of spore forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis*. Journal of General Microbiology 81, 183-190.
Silvaggi, J.M., Popham, D.L., Driks, A., Eichenberger, P., Losick, R., 2004. Unmasking novel sporulation genes in Bacillus subtilis. Journal of bacteriology 186, 8089-8095.
Simpson, E.B., Hancock, T.W., Buchanan, C.E., 1994. Transcriptional control of Dacb, which encodes a major sporulation-specific penicillin-binding protein. J Bacteriol 176, 7767-7769.
Skinner, G.E., Marshall, K.M., Morrissey, T.R., Loeza, V., Patazca, E., Reddy, N.R., Larkin, J.W., 2014. Combined high pressure and thermal processing on inactivation of type E and nonproteolytic type B and F spores of *Clostridium botulinum*. J Food Prot 77, 2054-2061.
Skovgaard, N., 1979. Bacterial Association of and Metabolic-Activity in Fish in North-Western Europe. Arch Lebensmittelhyg 30, 106-109.
Slepecky, R., Foster, J.W., 1959. Alterations in metal content of spores of *Bacillus megaterium* and the effect on some spore properties. Journal of bacteriology 78, 117-123.
Sloan, A.E., 1998. Food industry forecast: Consumer trends to 2020 and beyond. Food Technol-Chicago 52, 37-+.
Smeller, L., Meersman, F., Heremans, K., 2008. Stable misfolded states of human serum albumin revealed by high-pressure infrared spectroscopic studies. European biophysics journal : EBJ 37, 1127-1132.
Smelt, J.P.P.M., Bos, A.P., Kort, R., Brul, S., 2008. Modelling the effect of sub(lethal) heat treatment of *Bacillus subtilis* spores on germination rate and outgrowth to exponentially growing vegetative cells. Int J Food Microbiol 128, 34-40.
Smith, G.R., Moryson, C.J., 1977. The low prevalence of Clostridium botulinum in the lakes, marshes and waterways of the Camargue. J Hyg (Lond) 78, 33-37.
Smith, L.D.S., 1975. *In:* The Pathogenic Anaerobic Bacteria. American lecture series ; publication no. 980 : A monograph in the Bannerstone division of American lectures in clinical microbiology, Springfield III, Publisher: Charles C, Thomas, ISBN: 0398033935.
Smith, N.A.S., Knoerzer, K., Ramos, A.M., 2014. Evaluation of the differences of process variables in vertical and horizontal configurations of High Pressure Thermal (HPT) processing systems through numerical modelling. Innovative Food Science & Emerging Technologies 22, 51-62.
Smith, T.J., Hill, K.K., Xie, G., Foley, B.T., Williamson, C.H., Foster, J.T., Johnson, S.L., Chertkov, O., Teshima, H., Gibbons, H.S., Johnsky, L.A., Karavis, M.A., Smith, L.A., 2015. Genomic sequences of six botulinum neurotoxin-producing strains representing three clostridial species illustrate the mobility and diversity of botulinum neurotoxin genes. Infect Genet Evol 30, 102-113.
Smith, T.J., Lou, J., Geren, I.N., Forsyth, C.M., Tsai, R., Laporte, S.L., Tepp, W.H., Bradshaw, M., Johnson, E.A., Smith, L.A., Marks, J.D., 2005. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun 73, 5450-5457.
Sobel, J., 2005. Botulism. Clin Infect Dis 41, 1167-1173.
Soderholm, H., Lindstrom, M., Somervuo, P., Heap, J., Minton, N., Linden, J., Korkeala, H., 2011. cspB encodes a major cold shock protein in Clostridium botulinum ATCC 3502. International Journal of Food Microbiology 146, 23-30.
Sogin, M.L., McCall, W.A., Ordal, J.Z., 1972. Effect of heat activation conditions on the germinal response of *Bacillus cereus* T spores. pp. 363-367. In Halvorson HO, Hanson R, and Campbell LL (ed.), Spores, vol. 5. American Society for Microbiology, Washington, D.C.
Sokolowska, B., Skapska, S., Fonberg-Broczek, M., Niezgoda, J., Chotkiewicz, M., Dekowska, A., Rzoska, S., 2012. The combined effect of high pressure and nisin or lysozyme on the inactivation of *Alicyclobacillus acidoterrestris* spores in apple juice. High Pressure Res 32, 119-127.
Sonenshein, A.L., 2000. Control of sporulation initiation in *Bacillus subtilis*. Curr Opin Microbiol 3, 561-566.
Sowell, M.O., Buchanan, C.E., 1983. Changes in penicillin-binding proteins during sporulation of *Bacillus subtilis*. J Bacteriol 153, 1331-1337.
Spring, S., Merkhoffer, B., Weiss, N., Kroppenstedt, R.M., Hippe, H., Stackebrandt, E., 2003. Characterization of novel psychrophilic clostridia from an Antarctic microbial mat: description of Clostridium frigoris sp. nov., Clostridium lacusfryxellense sp. nov., Clostridium bowmanii sp. nov. and Clostridium

psychrophilum sp. nov. and reclassification of Clostridium laramiense as Clostridium estertheticum subsp. laramiense subsp. nov. Int J Syst Evol Microbiol 53, 1019-1029.
Squeglia, F., Marchetti, R., Ruggiero, A., Lanzetta, R., Marasco, D., Dworkin, J., Petoukhov, M., Molinaro, A., Berisio, R., Silipo, A., 2011. Chemical basis of peptidoglycan discrimination by PrkC, a key kinase involved in bacterial resuscitation from dormancy. J Am Chem Soc 133, 20676-20679.
Stecchini, M.L., Del Torre, M., Venir, E., Morettin, A., Furlan, P., Maltini, E., 2006. Glassy state in Bacillus subtilis spores analyzed by differential scanning calorimetry. International Journal of Food Microbiology 106, 286-290.
Steil, L., Serrano, M., Henriques, A.O., Volker, U., 2005. Genome-wide analysis of temporally regulated and compartment-specific gene expression in sporulating cells of *Bacillus subtilis*. Microbiol-Sgm 151, 399-420.
Steiner, E., Dago, A.E., Young, D.I., Heap, J.T., Minton, N.P., Hoch, J.A., Young, M., 2011. Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. Mol Microbiol 80, 641-654.
Stephenson, K., Hoch, J.A., 2002. Evolution of signalling in the sporulation phosphorelay. Mol Microbiol 46, 297-304.
Stephenson, K., Lewis, R.J., 2005. Molecular insights into the initiation of sporulation in Gram-positive bacteria: new technologies for an old phenomenon. Fems Microbiol Rev 29, 281-301.
Stevens, C.M., Daniel, R., Illing, N., Errington, J., 1992. Characterization of a sporulation gene, spoIVA, involved in spore coat morphogenesis in Bacillus subtilis. Journal of Bacteriology 174, 586-594.
Stewart, C.M., Dunne, C.P., Sikes, A., Hoover, D.G., 2000. Sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA 3679 to combinations of high hydrostatic pressure and other processing parameters. Innov Food Sci Emerg 1, 49-56.
Stewart, M., Somlyo, A.P., Somlyo, A.V., Shuman, H., Lindsay, J.A., Murrell, W.G., 1980. Distribution of calcium and other elements in cryosectioned *Bacillus cereus* T spores, determined by high-resolution scanning electron probe x-ray microanalysis. Journal of Bacteriology 143, 481-491.
Steyn, C.E., Cameron, M., Witthuhn, R.C., 2011. Occurrence of *Alicyclobacillus* in the fruit processing environment - A review. Int J Food Microbiol 147, 1-11.
Stiegelmeyer, S.M., Giddings, M.C., 2013. Agent-based modeling of competence phenotype switching in *Bacillus subtilis*. Theoretical biology & medical modelling 10, 23.
Stippl, V.M., 2005. Optical In-Situ Measurement of the pH-Value During High Pressure Treatment of Fluid Food. Dissertation, Technische Universitaet Muenchen.
Stippl, V.M., Delgado, A., Becker, T.M., 2002. Optical method for the in-situ measurement of the pH-value during high pressure treatment of foods. High Pressure Res 22, 757-761.
Stragier, P., Kunkel, B., Kroos, L., Losick, R., 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science 243, 507-512.
Stragier, P., Losick, R., 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu Rev Genet 30, 297-341.
Stringer, S.C., Carter, A.T., Webb, M.D., Wachnicka, E., Crossman, L.C., Sebaihia, M., Peck, M.W., 2013. Genomic and physiological variability within Group II (non-proteolytic) *Clostridium botulinum*. Bmc Genomics 14.
Stringer, S.C., Webb, M.D., George, S.M., Pin, C., Peck, M.W., 2005. Heterogeneity of times required for germination and outgrowth from single spores of nonproteolytic *Clostridium botulinum*. Applied and Environmental Microbiology 71, 4998-5003.
Stringer, S.C., Webb, M.D., Peck, M.W., 2009. Contrasting Effects of Heat Treatment and Incubation Temperature on Germination and Outgrowth of Individual Spores of Nonproteolytic *Clostridium botulinum* Bacteria. Applied and Environmental Microbiology 75, 2712-2719.
Stringer, S.C., Webb, M.D., Peck, M.W., 2011. Lag time variability in individual spores of *Clostridium botulinum*. Food Microbiol 28, 228-235.
Stute, R., Klingler, R.W., Boguslawski, S., Eshtiaghi, M.N., Knorr, D., 1996. Effects of high pressures treatment on starches. Starch-Starke 48, 399-408.
Suen, J.C., Hatheway, C.L., Steigerwalt, A.G., Brenner, D.J., 1988. Genetic confirmation of identities of neurotoxigenic Clostridium baratii and Clostridium butyricum implicated as agents of infant botulism. J Clin Microbiol 26, 2191-2192.
Sugawara, Y., Fujinaga, Y., 2011. The botulinum toxin complex meets E-cadherin on the way to its destination. Cell Adh Migr 5, 34-36.
Sugiyama, H., 1951. Studies on factors affecting the heat resistance of spores of *Clostridium botulinum*. J Bacteriol 62, 81-96.
Sun, Y.L., Sharp, M.D., Pogliano, K., 2000. A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of Bacillus subtilis. Journal of Bacteriology 182, 2919-2927.
Sunde, E.P., Setlow, P., Hederstedt, L., Halle, B., 2009. The physical state of water in bacterial spores. P Natl Acad Sci USA 106, 19334-19339.

Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K., Murata, N., 2000a. The pathway for perception and transduction of low-temperature signals in Synechocystis. Embo J 19, 1327-1334.
Suzuki, S., Izawa, Y., Kobayashi, K., Eto, Y., Yamanaka, S., Kubota, K., Yokozeki, K., 2000b. Purification and characterization of novel transglutaminase from Bacillus subtilis spores. Biosci Biotechnol Biochem 64, 2344-2351.
Syed, Q.A., Reineke, K., Saldo, J., Buffa, M., Guamis, B., Knorr, D., 2012. Effect of compression and decompression rates during high hydrostatic pressure processing on inactivation kinetics of bacterial spores at different temperatures. Food Control 25, 361-367.
Takagi, A., Kawata, T., Yamamoto, S., 1960. Electron microscope studies on ultrathin sections of spores of the Clostridium group, with special reference to the sporulation and germination process. Journal of Bacteriology 80, 37-46.
Takamatsu, H., Chikahiro, Y., Kodama, T., Koide, H., Kozuka, S., Tochikubo, K., Watabe, K., 1998. A spore coat protein, CotS, of Bacillus subtilis is synthesized under the regulation of sigmaK and GerE during development and is located in the inner coat layer of spores. Journal of Bacteriology 180, 2968-2974.
Takamatsu, H., Imamura, A., Kodama, T., Asai, K., Ogasawara, N., Watabe, K., 2000a. The yabG gene of Bacillus subtilis encodes a sporulation specific protease which is involved in the processing of several spore coat proteins. Fems Microbiology Letters 192, 33-38.
Takamatsu, H., Imamura, D., Kuwana, R., Watabe, K., 2009. Expression of yeeK during Bacillus subtilis sporulation and localization of YeeK to the inner spore coat using fluorescence microscopy. Journal of Bacteriology 191, 1220-1229.
Takamatsu, H., Kodama, T., Imamura, A., Asai, K., Kobayashi, K., Nakayama, T., Ogasawara, N., Watabe, K., 2000b. The Bacillus subtilis yabG gene is transcribed by SigK RNA polymerase during sporulation, and yabG mutant spores have altered coat protein composition. Journal of bacteriology 182, 1883-1888.
Takamatsu, H., Kodama, T., Nakayama, T., Watabe, K., 1999a. Characterization of the yrbA gene of Bacillus subtilis, involved in resistance and germination of spores. Journal of Bacteriology 181, 4986-4994.
Takamatsu, H., Kodama, T., Watabe, K., 1999b. Assembly of the CotSA coat protein into spores requires CotS in *Bacillus subtilis*. Fems Microbiol Lett 174, 201-206.
Takumi, K., Kinouchi, T., Kawata, T., 1980. Isolation of nontoxigenic variants associated with enhanced sporulation and alteration in the cell wall from Clostridium botulinum type a 190L by treatment with detergents. Microbiol Immunol 24, 469-477.
Tani, K., Watanabe, T., Matsuda, H., Nasu, M., Kondo, M., 1996. Cloning and sequencing of the spore germination gene of *Bacillus megaterium* ATCC 12872: similarities to the NaH-antiporter gene of *Enterococcus hirae*. Microbiol Immunol 40, 99-105.
Thornton, B., Basu, C., 2011. Real-Time PCR (qPCR) Primer Design Using Free Online Software. Biochem Mol Biol Edu 39, 145-154.
Tipper, D.J., Linnett, P.E., 1976. Distribution of peptidoglycan synthetase activities between sporangia and forespores in sporulating cells of Bacillus sphaericus. J Bacteriol 126, 213-221.
Todd, J.A., Bone, E.J., Ellar, D.J., 1985. The sporulation-specific penicillin-binding protein-5a from *Bacillus subtilis* ss a Dd-carboxypeptidase *invitro*. Biochem J 230, 825-828.
Todd, J.A., Roberts, A.N., Johnstone, K., Piggot, P.J., Winter, G., Ellar, D.J., 1986. Reduced heat-resistance of mutant spores after cloning and mutagenesis of the *Bacillus subtilis* gene encoding penicillin-binding protein-5. J Bacteriol 167, 257-264.
Toepfl, S., Mathys, A., Heinz, V., Knorr, D., 2006. Review: Potential of High Hydrostatic Pressure and Pulsed Electric Fields for Energy Efficient and Environmentally Friendly Food Processing. Food Reviews International 22, 405-423.
Tola, Y.B., Ramaswamy, H.S., 2014. Combined effects of high pressure, moderate heat and pH on the inactivation kinetics of Bacillus licheniformis spores in carrot juice. Food Res Int 62, 50-58.
Tomas, C.A., Beamish, J., Papoutsakis, E.T., 2004. Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol 186, 2006-2018.
Tonello-Samson, C., 2014. Industrial application of high pressure processing in food industry. Book of Abstracts, p. 48; 8th International Conference on High Pressure Bioscience and Biotechnology, July 15th – 18th 2014, Nantes, France; AND personal communication.
Torres, J.A., Velazquez, G., 2005. Commercial opportunities and research challenges in the high pressure processing of foods. J Food Eng 67, 95-112.
Tovar-Rojo, F., Chander, M., Setlow, B., Setlow, P., 2002. The products of the spoVA operon are involved in dipicolinic acid uptake into developing spores of Bacillus subtilis. Journal of Bacteriology 184, 584-587.
Trach, K.A., Hoch, J.A., 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis* - Identification and sequence of the protein-kinase of the alternate pathway. Mol Microbiol 8, 69-79.
Tsuji, K., Perkins, W.E., 1962. Sporulation of Clostridium botulinum. I. Selection of an aparticulate sporulation medium. Journal of Bacteriology 84, 81-85.

Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R., Vogel, R.F., 2002. Effects of pressure-induced membrane phase transitions on inactivation of HorA, an ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*. Appl Environ Microb 68, 1088-1095.
Underwood, S., Guan, S., Vijayasubhash, V., Baines, S.D., Graham, L., Lewis, R.J., Wilcox, M.H., Stephenson, K., 2009. Characterisation of the sporulation initiation pathway of *Clostridium difficile* and the role in toxin production. J Bacteriol 191, 7296-7305.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., C., F.B., M., R., Rozen, S.G., 2012. Primer3 - new capabilities and interfaces. Primer3web version 4.0.0 http://bioinfo.ut.ee/primer3/. Nucleic Acids Research 40(15):e115.
Van Boekel, M.A.J.S., 2009. Kinetic modeling of reactions in foods. . CRC Press, London.
van der Voort, M., Garcia, D., Moezelaar, R., Abee, T., 2010. Germinant receptor diversity and germination responses of four strains of the *Bacillus cereus* group. Int J Food Microbiol 139, 108-115.
van Ooij, C., Eichenberger, P., Losick, R., 2004. Dynamic patterns of subcellular protein localization during spore coat morphogenesis in Bacillus subtilis. Journal of Bacteriology 186, 4441-4448.
van Ooij, C., Losick, R., 2003. Subcellular localization of a small sporulation protein in Bacillus subtilis. Journal of Bacteriology 185, 1391-1398.
van Schaik, W., Abee, T., 2005. The role of sigma(B) in the stress response of Gram-positive bacteria - targets for food preservation and safety. Curr Opin Biotech 16, 218-224.
van Schaik, W., Tempelaars, M.H., Wouters, J.A., de Vos, W.M., Abee, T., 2004. The alternative sigma factor sigmaB of Bacillus cereus: response to stress and role in heat adaptation. Journal of Bacteriology 186, 316-325.
Vasantha, N., Freese, E., 1979. Role of Manganese in Growth and Sporulation of Bacillus-Subtilis. Journal of General Microbiology 112, 329-336.
Veening, J.W., Kuipers, O.P., Brul, S., Hellingwerf, K.J., Kort, R., 2006. Effects of phosphorelay perturbations on architecture, sporulation, and spore resistance in biofilms of *Bacillus subtilis*. J Bacteriol 188, 3099-3109.
Vepachedu, V.R., Setlow, P., 2007. Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. J Bacteriol 189, 1565-1572.
Vigh, L., Maresca, B., Harwood, J.L., 1998. Does the membrane's physical state control the expression of heat shock and other genes? Trends Biochem Sci 23, 369-374.
Villarreal, L., Heredia, N., Garcia, S., 2002. Cold tolerance and changes in rates of synthesis of individual proteins of Clostridium perfringens as a result of cold pre-treatment. Acta Aliment Hung 31, 179-184.
Walker, J.R., Gnanam, A.J., Blinkova, A.L., Hermandson, M.J., Karymov, M.A., Lyubchenko, Y.L., Graves, P.R., Haystead, T.A., Linse, K.D., 2007. Clostridium taeniosporum spore ribbon-like appendage structure, composition and genes. Molecular Microbiology 63, 629-643.
Waller, L.N., Fox, N., Fox, K.F., Fox, A., Price, R.L., 2004. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of Bacillus anthracis and Bacillus subtilis. J Microbiol Methods 58, 23-30.
Wang, G., Paredes-Sabja, D., Sarker, M.R., Green, C., Setlow, P., Li, Y.Q., 2012. Effects of wet heat treatment on the germination of individual spores of *Clostridium perfringens*. J Appl Microbiol 113, 824-836.
Wang, G., Zhang, P., Paredes-Sabja, D., Green, C., Setlow, P., Sarker, M.R., Li, Y.Q., 2011. Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. J Appl Microbiol 111, 1212-1223.
Wang, J., Meng, J., Lawrence, G.W., Zurawski, T.H., Sasse, A., Bodeker, M.O., Gilmore, M.A., Fernandez-Salas, E., Francis, J., Steward, L.E., Aoki, K.R., Dolly, J.O., 2008. Novel chimeras of botulinum neurotoxins A and E unveil contributions from the binding, translocation, and protease domains to their functional characteristics. J Biol Chem 283, 16993-17002.
Wang, K.H., Isidro, A.L., Domingues, L., Eskandarian, H.A., McKenney, P.T., Drew, K., Grabowski, P., Chua, M.H., Barry, S.N., Guan, M., Bonneau, R., Henriques, A.O., Eichenberger, P., 2009. The coat morphogenetic protein SpoVID is necessary for spore encasement in *Bacillus subtilis*. Mol Microbiol 74, 634-649.
Wang, S.T., Setlow, B., Conlon, E.M., Lyon, J.L., Imamura, D., Sato, T., Setlow, P., Losick, R., Eichenberger, P., 2006. The forespore line of gene expression in *Bacillus subtilis*. J Mol Biol 358, 16-37.
Warburg, R.J., Buchanan, C.E., Parent, K., Halvorson, H.O., 1986. A detailed study of gerj-mutants of *Bacillus subtilis*. J Gen Microbiol 132, 2309-2319.
Ward, B.Q., Carroll, B.J., Garrett, E.S., Reese, G.B., 1967a. Survey of Us Atlantic Coast and Estuaries from Key Largo to Staten Island for Presence of Clostridium Botulinum. Applied Microbiology 15, 964-&.
Ward, B.Q., Garrett, E.S., Reese, G.B., 1967b. Further Indications of Clostridium Botulinum in Latin American Waters. Applied Microbiology 15, 1509-&.
Warth, A.D., 1978. Molecular structure of the bacterial spore. Adv Microb Physiol 17, 1-45.
Warth, A.D., Ohye, D.F., Murrell, W.G., 1963. The composition and structure of bacterial spores. J Cell Biol 16, 579-592.

Warth, A.D., Stroming.Jl, 1972. Structure of Peptidoglycan from Spores of Bacillus-Subtilis. Biochemistry 11, 1389-&.
Webb, C.D., Decatur, A., Teleman, A., Losick, R., 1995. Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in Bacillus subtilis. Journal of Bacteriology 177, 5906-5911.
Webb, M.D., Pin, C., Peck, M.W., Stringer, S.C., 2007. Historical and contemporary NaCl concentrations affect the duration and distribution of lag times from individual spores of nonproteolytic *Clostridium botulinum*. Appl Environ Microb 73, 2118-2127.
Webb, M.D., Stringer, S.C., Le Marc, Y., Baranyi, J., Peck, M.W., 2012. Does proximity to neighbours affect germination of spores of non-proteolytic *Clostridium botulinum*? Food Microbiol 32, 104-109.
Weber, M.H., Marahiel, M.A., 2002. Coping with the cold: the cold shock response in the Gram-positive soil bacterium Bacillus subtilis. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 357, 895-907.
Wei, J., Setlow, P., Hoover, D.G., 2009. Effects of moderately high pressure plus heat on the germination and inactivation of *Bacillus cereus* spores lacking proteins involved in germination. Lett Appl Microbiol 49, 646-651.
Wei, J., Shah, I.M., Ghosh, S., Dworkin, J., Hoover, D.G., Setlow, P., 2010. Superdormant spores of *Bacillus s*pecies germinate normally with high pressure, peptidoglycan fragments, and bryostatin. J Bacteriol 192, 1455-1458.
Weil, R., 1899. Zur Biologie des Milzbrandbacillen. Arch. Hyg., 35, 355-408.
Weinberg, E.D., 1955. The Effect of Mn++ and Antimicrobial Drugs on Sporulation of Bacillus-Subtilis in Nutrient Broth. Journal of bacteriology 70, 289-296.
Weir, J., Predich, M., Dubnau, E., Nair, G., Smith, I., 1991. Regulation of Spo0h, a gene coding for the *Bacillus subtilis* sigma-H factor. J Bacteriol 173, 521-529.
Westphal, A.J., Price, P.B., Leighton, T.J., Wheeler, K.E., 2003. Kinetics of size changes of individual *Bacillus thuringiensis* spores in response to changes in relative humidity. Proc Natl Acad Sci USA 100, 3461-3466.
Whitmer, M.E., Johnson, E.A., 1988. Development of improved defined media for Clostridium botulinum serotypes A, B, and E. Applied and Environmental Microbiology 54, 753-759.
WHO, 2002. http://www.who.int/mediacentre/factsheets/fs270/en/; World Health Organization; Access Date: 01-15-2011.
Wilkinson, S.R., Young, D.I., Morris, J.G., Young, M., 1995. Molecular-genetics and the initiation of solventogenesis in *Clostridium berijerinckii* (formerly *Clostridium acetobutylicum*) Ncimb-8052. Fems Microbiol Rev 17, 275-285.
Williams, O.B., 1929. The heat resistance of bacterial spores. J. Infectious Diseases 29:362-368.
Williams, O.B., Robertson, W.J., 1954. Studies on heat resistance. VI. Effect of temperature of incubation at which formed on heat resistance of aerobic thermophilic spores. Journal of Bacteriology 67, 377-378.
Williamson, C.H., Sahl, J.W., Smith, T.J., Xie, G., Foley, B.T., Smith, L.A., Fernandez, R.A., Lindstrom, M., Korkeala, H., Keim, P., Foster, J., Hill, K., 2016. Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing Clostridia. Bmc Genomics 17, 180.
Wilson, D.R., Dabrowski, L., Stringer, S., Moezelaar, R., Brocklehurst, T.F., 2008. High pressure in combination with elevated temperature as a method for the sterilisation of food. Trends Food Sci Tech 19, 289-299.
Winter, R., Dzwolak, W., 2005. Exploring the temperature-pressure configurational landscape of biomolecules: from lipid membranes to proteins. Philos Trans A Math Phys Eng Sci 363, 537-562; discussion 562-533.
Winter, R., Jeworrek, C., 2009. Effect of pressure on membranes. Soft Matter 5, 3157-3173.
Wistow, G., 1990. Cold shock and DNA binding. Nature 344, 823-824.
Woods, D.R., 1995. The genetic-engineering of microbial solvent production. Trends Biotechnol 13, 259-264.
Wörner, K., Szurmant, H., Chiang, C., Hoch, J.A., 2006. Phosphorylation and functional analysis of the sporulation initiation factor Spo0A from *Clostridium botulinum*. Mol Microbiol 59, 1000-1012.
Wu, J.J., Schuch, R., Piggot, P.J., 1992. Characterization of a Bacillus subtilis sporulation operon that includes genes for an Rna-polymerase sigma-factor and for a putative Dd-carboxypeptidase. J Bacteriol 174, 4885-4892.
Wuytack, E.Y., Boven, S., Michiels, C.W., 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl Environ Microb 64, 3220-3224.
Wuytack, E.Y., Michiels, C.W., 2001. A study on the effects of high pressure and heat on Bacillus subtilis spores at low pH. International Journal of Food Microbiology 64, 333-341.
Yamakawa, K., Nakamura, S., 1992. Prevalence of Clostridium botulinum type E and coexistence of C. botulinum nonproteolytic type B in the river soil of Japan. Microbiol Immunol 36, 583-591.
Yamanaka, K., Fang, L., Inouye, M., 1998. The CspA family in Escherichia coli: multiple gene duplication for stress adaptation. Mol Microbiol 27, 247-255.
Yang, W.W., Crow-Willard, E.N., Ponce, A., 2009. Production and characterization of pure Clostridium spore suspensions. Journal of Applied Microbiology 106, 27-33.

Yasuda, Y., Kanda, K., Nishioka, S., Tanimoto, Y., Kato, C., Saito, A., Fukuchi, S., Nakanishi, Y., Tochikubo, K., 1993. Regulation of L-Alanine-Initiated Germination of Bacillus-Subtilis Spores by Alanine Racemase. Amino Acids 4, 89-99.

Yi, X., Setlow, P., 2010. Studies of the Commitment Step in the Germination of Spores of *Bacillus* Species. J Bacteriol 192, 3424-3433.

Young, S.B., Setlow, P., 2003. Mechanisms of killing of Bacillus subtilis spores by hypochlorite and chlorine dioxide. Journal of Applied Microbiology 95, 54-67.

Young, S.B., Setlow, P., 2004. Mechanisms of killing of Bacillus subtilis spores by Decon and Oxone (TM), two general decontaminants for biological agents. Journal of Applied Microbiology 96, 289-301.

Yudkin, M.D., Clarkson, J., 2005. Differential gene expression in genetically identical sister cells: the initiation of sporulation in *Bacillus subtilis*. Mol Microbiol 56, 578-589.

Yule, A.M., Barker, I.K., Austin, J.W., Moccia, R.D., 2006. Toxicity of Clostridium botulinum type E neurotoxin to Great Lakes fish: implications for avian botulism. J Wildl Dis 42, 479-493.

Yura, T., Kanemori, M., Morita, M.T., 2000. The heat shock response: regulation and function. In Stortz, G. and Hengge-Aronis, R. (eds.), Bacterial Stress Response. American Society for Microbiology, Washington, DC, pp. 3-18.

Zancan, P., Sola-Penna, M., 2005. Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures. Archives of Biochemistry and Biophysics 444, 52-60.

Zhang, J., Fitz-James, P.C., Aronson, A.I., 1993. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of Bacillus subtilis. Journal of Bacteriology 175, 3757-3766.

Zhang, J.Q., Griffiths, K.K., Cowan, A., Setlow, P., Yu, J., 2013. Expression level of *Bacillus subtilis* germinant receptors determines the average rate but not the heterogeneity of spore germination. J Bacteriol 195, 1735-1740.

Zhang, P., Kong, L., Wang, G., Scotland, M., Ghosh, S., Setlow, B., Setlow, P., Li, Y.Q., 2012. Analysis of the slow germination of multiple individual superdormant *Bacillus subtilis* spores using multifocus Raman microspectroscopy and differential interference contrast microscopy. J Appl Microbiol 112, 526-536.

Zhang, P., Setlow, P., Li, Y., 2009. Characterization of single heat-activated *Bacillus* spores using laser tweezers Raman spectroscopy. Opt Express 17, 16480-16491.

Zhang, P.F., Garner, W., Yi, X.A., Yu, J., Li, Y.Q., Setlow, P., 2010. Factors Affecting Variability in Time between Addition of Nutrient Germinants and Rapid Dipicolinic Acid Release during Germination of Spores of *Bacillus* Species. Journal of Bacteriology 192, 3608-3619.

Zhang, Y.Q., Zhou, L.L., Zhang, Y.J., Tan, C.Q., 2014. Inactivation of Bacillus subtilis Spores Using Various Combinations of Ultraviolet Treatment with Addition of Hydrogen Peroxide. Photochemistry and Photobiology 90, 609-614.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000; 7(1-2):203-14.

Zhao, Y.S., Tomas, C.A., Rudolph, F.B., Papoutsakis, E.T., Bennett, G.N., 2005. Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. Appl Environ Microb 71, 530-537.

Zheligovskaya, E.A., Malenkov, G.G., 2005. Molecular dynamics simulation of metastable ices IV and XII. J Struct Chem+ 46, 276-284.

Zheligovskaya, E.A., Malenkov, G.G., 2006. Crystalline water ices. Usp Khim+ 75, 64-85.

Zheng, L., Halberg, R., Roels, S., Ichikawa, H., Kroos, L., Losick, R., 1992. Sporulation regulatory protein GerE from Bacillus subtilis binds to and can activate or repress transcription from promoters for mother-cell-specific genes. J Mol Biol 226, 1037-1050.

Zheng, L.B., Donovan, W.P., Fitz-James, P.C., Losick, R., 1988. Gene encoding a morphogenic protein required in the assembly of the outer coat of the Bacillus subtilis endospore. Genes Dev 2, 1047-1054.

Zilhao, R., Isticato, R., Martins, L.O., Steil, L., Volker, U., Ricca, E., Moran, C.P., Jr., Henriques, A.O., 2005. Assembly and function of a spore coat-associated transglutaminase of *Bacillus subtilis*. J Bacteriol 187, 7753-7764.

Zilhao, R., Naclerio, G., Henriques, A.O., Baccigalupi, L., Moran, C.P., Jr., Ricca, E., 1999. Assembly requirements and role of CotH during spore coat formation in Bacillus subtilis. Journal of Bacteriology 181, 2631-2633.

Zilhao, R., Serrano, M., Isticato, R., Ricca, E., Moran, C.P., Henriques, A.O., 2004. Interactions among CotB, CotG, and CotH during assembly of the *Bacillus subtilis* spore coat. J Bacteriol 186, 1110-1119.

Zverlov, V.V., Velikodvorskaya, G.A., Schwarz, W.H., 2003. Two new cellulosome components encoded downstream of celI in the genome of Clostridium thermocellum: the non-processive endoglucanase CelN and the possibly structural protein CseP. Microbiology 149, 515-524.

7 APPENDIX

7.1 Additional Growth and Sporulation Media

Schaedler anaerobe broth [1 L]		Oxoid (Hampshire, UK) (26.5 g/L)
Tryptone soya broth	10.0 g	
Proteose peptone	5.0 g	
Yeast extract	5.0 g	
Dextrose	5.0 g	
Tris buffer	0.75 g	
L-Cystein	0.4 g	
Hemin	0.01 g	
		pH: 7.6 ± 0.2 at 25°C

BHI (Brain Heart (Infusion)) broth [1 L]		Fluka (Steinheim, Germany) (37 g/L)
Calf brain (infusion from 200 g)	12.5 g	
Beef heart (infusion from 250 g)	5 g	
Peptone	10 g	
Sodium Chloride	5 g	
D(+)Glucose (autoclaved sepearately)	2 g	
Disodium hydrogen phospate	2.5 g	
		(pH: no specifications / ± 7.4)

Robertson's Cooked Meat Medium [1L]		(Robertson, 1915)
Bullock's Heart	248.8 g	
Tap water	248.8 g	
Mince heart finely, ground in a mortar, cook		
		pH: 7.2 ± 0.2

Robertson's Cooked Meat Medium (modified) [1 L]		BD, Franklin Lakes, NJ USA
Beef Heart Tissue Granules	98.0 g	
Peptic Digest of Animal Tissue	20.0 g	
Dextrose	2.0 g	
Sodium Chloride	5.0 g	
Add distilled water	1000 mL	
		pH: 7.2 ± 0.2

WSH (Weihenstephaner Südhang) agar [1 L] (Margosch et al., 2006)

Soil extract*	1 L
Meat extract	20.0 g
Yeast extract	3.0 g
Cysteine x HCl (L-Cystein x HCl x H_2O)	0.5 g (0.56 g)
$CaCO_3$	5.0 g
Egg white (from fresh eggs)**	1.5 eggs
Agar-agar (no agar for medium)	15.0 g

pH: 7.0 ± 0.2

*Mix approx. equal quantities per weight of soil and water. Shake to mix. Autoclave for 30 min at 1 atm. Allow the soil to sediment and filter the supernatant through filter paper. (Margosch 2004: according to Gams et al. 1998. CBS course of mycology. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands). Add distilled water to compensate weight loss. Final volume 1 L (Werres et al. 2001)

**Wash fresh eggs with a stiff brush and drain. Soak eggs 1 h in 70% ethanol. Drain ethanol. Crack eggs aseptically and remove whites.

7.2 Suppliers of Materials and Chemicals

Tab. 7-1: Material

Material	Supplier, Country	Business Contact
Sterile 15 mL polypropylene screw cap tubes; 62.554.502	SARSTEDT AG & Co.	Nuembrecht, Germany
Sterile 50 mL screw cap tubes; 62.547.254	SARSTEDT AG & Co.	Nuembrecht, Germany
SafeSeal 1.5 mL caps; 72.706	SARSTEDT AG & Co.	Nuembrecht, Germany
Sterile 92×16 mm Petri dishes with cams; 82.1473	SARSTEDT AG & Co.	Nuembrecht, Germany
1.8 mL Nunc™ Cryotube vials; 177280	Thermo Fisher Scientific	Bonn, Germany
Shrink tubes DERAY® - I 3000 for U111 experiments	DSGCanusa	Rheinbach, Germany
DERAY® - KYF190 for FBG 5620 experiments	DSGCanusa	Rheinbach, Germany
Gemini C18 column (150 mm length, 4.6 mm d_i)	Phenomenex	Aschaffenburg, Germany
Gemini C18 guard column (4 × 3 mm).	Phenomenex	Aschaffenburg, Germany
PVDF membrane disc filters (pore size 0.22 µm; 1201107) for HPLC	Berrytec GmbH	Grünwald, Germany
cellulose acetate syringe filters (pore size 0.2 µm; F2500-16)	Thermo Fisher Scientific	Bonn, Germany
polypropylene HPLC vials (250 µL; 6820.0029)	Thermo Fisher Scientific	Bonn, Germany

Tab. 7-2: List of chemicals/kits

Chemical	Company	Business contact
2-propanol	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
ABsolute™ QPCR SYBR® Green Capillary mix	Thermo Fisher Scientific	Bonn, Germany
Acetic acid, Rotipuran 100%	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Agar-agar, BD European agar	BD	Heidelberg, Germany
BBL™ Polypeptone™ Peptone	BD	Heidelberg, Germany
BHI broth	Fluka Chemie GmbH	Buchs, Switzerland
$CaCl_2 \times 2\ H_2O$	Merck KGaA	Darmstadt, Germany
Chloroform (trichloromethane)	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
CSPD	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Cystein HCl	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
D(+)Glucose monohydrate	Merck KGaA	Darmstadt, Germany
D-Cellobiose	Serva Electrophoresis GmbH	Heidelberg, Germany
DIG-dUTP	Roche	Mannheim, Germany
Dimidiumbromid	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
D-Maltose monohydrate	GERBU Biotechnik GmbH	Wieblingen, Germany
DNase, DNase buffer, DNase stop solution	Promega	Mannheim, Germany
dNTP	Promega	Mannheim, Germany
DPA (2,6-pyridinedicarboxylic acid) (99% as medium additive; ≥ 99.5% for HPLC and Tb^{3+} assays) (2321-10G-F)	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
E.Z.N.A.® Bacterial DNA Kit	Omega Bio-Tek	Norcross, GA, USA
EtOH absolute	BDH Prolabo/VWR Int.	Fontenay sous Bois, France
GBX developer and replenisher and fixer and replenisher	Kodak	Stuttgart, Germany
Lysozyme, from chicken egg white, min. 100.000 u/mg	Serva Electrophoresis GmbH	Heidelberg, Germany
Meat extract	Merck KGaA	Darmstadt, Germany
$MgCl_2$	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
$MgSO_4$	Merck KGaA	Darmstadt, Germany
$MnSO_4 \times H_2O$	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Na_2HPO_4	Merck KGaA	Darmstadt, Germany
NaCl	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
PCR Buffer+ $MgCl_2$	Promega	Mannheim, Germany
Peptone from casein	Merck KGaA	Darmstadt, Germany
Proteose peptone	Oxoid Ltd.	Hampshire, UK
Random primers	Promega	Mannheim, Germany
RCM (reinforced Clostridial medium)	Merck KGaA	Darmstadt, Germany
RNase AWAY®	Thermo Fisher Scientific	Bonn, Germany
RT, RT Buffer	Promega	Mannheim, Germany
Schaedler anaerobe broth	Oxoid Ltd.	Hampshire, UK
SDS, Dodecylsulfat-Na-salt	Serva Electrophoresis GmbH	Heidelberg, Germany

Sodium thioglycolate	AppliChem GmbH	Darmstadt, Germany
Starch, soluble	Merck KGaA	Darmstadt, Germany
Taq polymerase	Promega	Mannheim, Germany
Tb^{3+} (Terbium(III)chloride hexahydrate)	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
Tryptose	GERBU Biotechnik GmbH	Wieblingen, Germany
Yeast extract	Carl Roth GmbH + Co. KG BD	Karlsruhe, Germany Heidelberg, Germany
ACES buffer grade	AppliChem GmbH	Darmstadt, Germany
Imidazole	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
Antifoam B Emulsion	Dow Corning GmbH	Wiesbaden, Germany
Polyethylene glycol (PEG 400)	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
L-alanine (≥99%)	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
DL-Lactic acid (90%)	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
Glucose (monohydrate)	Merck KGaA	Darmstadt, Germany
Antifoam B Emulsion	Dow Corning GmbH	Wiesbaden, Germany
KCl	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
$Na_2HPO_4 \times 2\ H_2O$	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
KH_2PO_4	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Lysozyme, hen egg white (c-type), min. 100,000 u/mg	Serva Electrophoresis GmbH	Heidelberg, Germany
Trypsin from bovine pancreas, ≥ 9,000 BAEE units/mg protein	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
Maltose ($_D$-maltose)	GERBU Biotechnik GmbH	Wieblingen, Germany
Cellobiose ($_D$-cellobiose)	Serva Electrophoresis GmbH	Heidelberg, Germany
SYTO9® fluorescent stain	Thermo Fisher Scientific Inc.	Waltham, Massachusetts, USA
Sodium bisulphate buffer solution (50 mM $NaSO_4$; 233714, ReagentPlus®, 99%) for HPLC	Sigma-Aldrich Chemie GmbH	Steinheim, Germany
Sulphuric acid (H_2SO_4, X994.1, Rotipuran® 95-98%) for HPLC	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Methanol; 7342.1, Rotisolv® HPLC gradient grade	Carl Roth GmbH + Co. KG	Karlsruhe, Germany

7.3 Parameters for Modeling of Isorate Curves

Tab. 7-3: Modeling Parameters.
Summary of parameters for the Weibull model and the Taylor series as well as the confidence intervals.

Inactivation				
Parameter	Value	Std	95% Confidence	
a	0.02397187	0.04112893	-0.06906822	0.11701197
b	-0.00010637	0.00013122	-0.00040322	0.00019047
c	0.00019127	0.00156994	-0.00336017	0.00374272
d	1.23E-07	1.65E-07	-2.50E-07	4.97E-07
e	-5.04E-06	1.62E-05	-4.18E-05	3.17E-05
f	-7.81E-07	3.07E-06	-7.73E-06	6.16E-06
g	3.89E-10	2.57E-09	-5.42E-09	6.20E-09
h	1.54E-08	3.37E-08	-6.08E-08	9.16E-08
Adj R^2				0.95

Heat treatment			
Value	Std	95% Confidence	
0.03220063	0.04170704	-0.06214725	0.12654852
-0.00010579	0.00013307	-0.00040681	0.00019523
-0.00021685	0.001592	-0.0038182	0.0033845
9.31E-08	1.67E-07	-2.86E-07	4.72E-07
-8.61E-07	1.65E-05	-3.81E-05	3.64E-05
-2.10E-07	3.11E-06	-7.25E-06	6.83E-06
8.42E-10	2.61E-09	-5.05E-09	6.74E-09
6.78E-09	3.42E-08	-7.05E-08	8.41E-08
			0.94

Plated on lysozyme			
Value	Std	95% Confidence	
0.03178821	0.03954437	-0.05766737	0.1212438
-9.54E-05	0.00012617	-0.00038077	0.00019005
-0.00027502	0.00150945	-0.00368963	0.00313959
6.80E-08	1.59E-07	-2.91E-07	4.27E-07
9.24E-07	1.56E-05	-3.44E-05	3.62E-05
-2.57E-07	2.95E-06	-6.93E-06	6.42E-06
1.39E-09	2.47E-09	-4.20E-09	6.98E-09
1.77E-09	3.24E-08	-7.15E-08	7.51E-08
			0.95

7.4 Effects of Sporulation Temperature

Tab. 7-4: Effects of sporulation temperature on spore components.

Spore structure affected	Spo. Temp. ↓	Spo. Temp. ↑	Organism	Remark	Reference	HHP
Size	↑	↓	*B. anthracis*		(Baweja et al., 2008)	?
Size	↑	↓	*B. weihenstephanensis*	Not linear (far decrease of temp.= similar to standard spores)	(Garcia et al., 2010)	?
Wet density	↑	↓	*B. subtilis* A		(Lindsay et al., 1990)	?
Surface	Rough	Smooth	*B. subtilis* A		(Lindsay et al., 1990)	X
Core/(core+ cortex) volume ratio	↓	↑	*B. subtilis* A		(Lindsay et al., 1990)	?
DPA content	↓		*B. cereus*		(Planchon et al., 2011)	X
DPA content	≈	↑	*B. anthracis*		(Baweja et al., 2008)	X
DPA content	≈	≈	*B. subtilis*		(Melly et al., 2002)	X
DPA content	↓	↑	*B. subtilis* A	Not linear (far decrease of temp.= similar to standard spores)	(Lindsay et al., 1990)	X
Mineral contents	↓	↑	*B. subtilis*	Not linear (high increase of temp.= mineral content falls again)	(Igura et al., 2003)	V
Mineral contents	↓	↑	*B. subtilis* A	Not linear (high increase of temp.= mineral content falls again), only trend, mineral type dependent	(Lindsay et al., 1990)	V
Mineral contents	≈	≈	*B. subtilis*		(Atrih and Foster, 2001a)	V
Core water content	↑	↓	*B. stearothermophilus*, *B. subtilis*, *B. cereus*		(BEAMAN and GERHARDT, 1986)	X
Core water content	↑	↓	*C. perfringens*		(Paredes-Sabja et al., 2008a)	X
Core water content	↑	↓	*B. subtilis*		(Popham et al., 1995b)	X
Core water content	↑	↓	*B. subtilis*		(Melly et al., 2002)	X
Core water content	≈	≈	*B. subtilis*		(Atrih and Foster, 2001a)	X

α/β-type SASP	≈	≈	*B. subtilis*		(Movahedi and Waites, 2000)	X
α/β-type SASP	≈	≈	*B. subtilis*		(Melly et al., 2002)	X
Inner membrane unsaturation	↑		*B. subtilis*		(Aguilar et al., 1998)	X
Inner membrane unsaturation	↑		*C. botulinum*	type B	(Peck et al., 1995a)	X
Inner membrane unsaturation	↑	↓	*C. botulinum*	non-toxigenic, non-proteolytic, psychrotolerant	(Evans et al., 1998)	X
Inner membrane unsaturation	≈		*B. cereus*		(Planchon et al., 2011)	X
Inner membrane unsaturation		↓	*B. subtilis*		(Gaughran, 1947)	X
Inner membrane average acyl chain length	↓		*C. botulinum*	type B	(Peck et al., 1995a)	?
Inner membrane average acyl chain length	↓	↑	*C. botulinum*	non-toxigenic, non-proteolytic, psychrotolerant	(Evans et al., 1998)	?
Inner membrane cyclopropane fatty acids	↓	↑	*C. botulinum*	non-toxigenic, non-proteolytic, psychrotolerant	(Evans et al., 1998)	?
Cortex cross-linked muramic acid	↓(slight but significant)	↑ (slight but significant)	*B. subtilis*		(Melly et al., 2002)	X
Cortex cross-linked muramic acid	≈	≈	*B. subtilis*		(Atrih and Foster, 2001a)	X
L-alanine substitution	↑ (slight)	↓ (slight)	*B. subtilis*		(Melly et al., 2002)	X
L-alanine substitution	↑ (slight)	↓ (slight)	*B. subtilis*		(Atrih and Foster, 2001a)	X

tripeptide side chains	≈	≈	*B. subtilis*		(Melly et al., 2002)	X
tripeptide side chains	↓ (slight)	↑ (slight)	*B. subtilis*		(Atrih and Foster, 2001a)	X
tetrapeptides	↓	↑	*B. subtilis*		(Melly et al., 2002)	X
tetrapeptides	↓	↑	*B. subtilis*		(Atrih and Foster, 2001a)	X
δ-lactam		Tends to peak at optimum temp. 30°C (not significant)	*B. subtilis*		(Melly et al., 2002)	X
δ-lactam		Tends to peak at optimum temp. 30°C (not significant)	*B. subtilis*		(Atrih and Foster, 2001a)	X
Coat CotA	↑	↓	*B. subtilis*		(Melly et al., 2002)	?
Exosporium size	↑	↓	*B. cereus*		(Faille et al., 2007)	X
Exosporium damage	↓	↑	*B. cereus*		(Faille et al., 2007)	X

7.5 Conservation of *B. subtilis* Coat Proteins

Tab. 7-5: Major *B. subtilis* coat genes in *C. botulinum* type E

Protein	*B. subtilis* ORF ID	*C. botulinum* type E strain Alaska	NCBI Accesion Number (old/new)	Max score/ Query coverage	*C. botulinum* type E strain Beluga	NCBI Accesion Number (old/new)	Max score/ Query coverage	Discription
CotI Subtilist YtaA UniProt	BG13821 BSORF Bacillus subtilis Genome Database	spore coat protein CotS	YP_001919865.1 WP_012450479.1	116/75%	spore coat protein CotS	ZP_04822255.1 Record removed	114/75%	PKc-like superfamily, Multidomains: spore CotS
cotJC	BG11801	protein cotJC	YP_001922166.1 WP_012450300.1	234/93%	CotJC protein	ZP_04822876.1 Record removed	147/59%	Specific hit: Mn catalase, ferritin-like superfamily
CotS	BG11380	spore coat protein CotS	YP_001919865.1 WP_012450479.1	106/90%	spore coat protein CotS	ZP_04822255.1 Record removed	105/90%	PKc-like superfamily, Multidomains: spore CotS
CotSA	BG11381	hypothetical protein CLH_3208 and mannosyltransferase B	YP_001922573.1 WP_012450868.1 and YP_001919864.1 WP_012450868.1	49.3/69% and 45.8/48%	mannosyltransferase B	ZP_04821460.1 Record removed	45.4/48%	Specific hit GT_YqgM_like superfamily: glycosyltransferase_GTB type superfaily, multidomains: RfaG
SpoIVA	BG10275	stage IV sporulation protein A	YP_001920550.1 WP_003374033.1	541/100%	methyl-accepting chemotaxis protein	ZP_04820904.1 Record removed	30,8/14%	Specific hit Spore IV A. Spre IVA suberfamily, multidomains spore IV A

Tab. 7-6: Conservation of different coat genes among genera

Gene group	Gene of interest	Activity	*B.subtilis*	Bacilli	Clostridia	Alaska	Beluga
Maturation	Tgl	Transglutaminase	+	+	-	-	-
	YabG sporulation peptidase	Peptidase_V57 superfamily	+	+	+	+ YP_001919866.1	+ ZP_04823352.1
	CotG		+	-	-	-	-
	SodA	superoxide dismutase Sod_Fe_N and Sod_Fe_C superfamilies	+	+	+	+ YP_001921054.1 Mn/Fe superoxide dismutase (henriques: associate with exosporium)	+ ZP_04822388.1 superoxide dismutase
	CotA	Outer spore coat protein A, copper-dependent laccase	+	+	-	-	-
Germination	spore cortex-lytic protein– high similarity to SleC (C. perfringens, C. botulinum B)	conserved PG binding domain PG_binding_1 superfamily	-	-	+	YP_001919517.1	ZP_04822649.1
	putative spore-cortex-lytic enzyme (high similarity to CspA in C botulinum B)	Peptidase_S8_S53 superfamily	-	-	+	YP_001920907.1	ZP_04821450.1
	putative spore-cortex-lytic enzyme (high similarity to CspA in C botulinum B)	Peptidase_S8_S53 superfamily	-	-	+	YP_001920906.1	ZP_04820555.1
	spore-cortex-lytic enzyme sleB	Hydrolase_2 superfamily	+	+	+	YP_001919726.1	ZP_04823255.1
	sleL (yaaH)	GH18_chitinase-like superfamily Conserved PG binding LysM domain	+	+	+/-	-	-
	sleM	GH25_muramidase superfamily	-	-	+	+ muramidase Endo-N-acetylmuramidases YP_001922197.1	+ Lysozyme Endo-N-acetylmuramidases ZP_04823616.1
	CwlJ	Cell wall hydrolase	+	+	+	-	-
	gerPA - F (yisH – C)	Spore germination proteins	+	+	- (C. ultunense)	-	-
	cotD	Structural inner coat protein	+	+	-	-	-
	LipC (YcsK)	Phospholipase B(SGNH_hydrolase superfamily)	+	+/-	+/-	+ Low homology putative esterase YP_001919852.1	+ Low homology putative esterase ZP_04822315.1
Spore coat polysacharide synthesis	spsA - L	spore coat polysaccharide biosynthesis protein spsA = nucleotide-diphospho-sugar transferase = spore coat dTDP-glycosyltransferase				family 2 glycosyl transferase YP_001922370.1	glycosyl transferase, family 2 ZP_04821759.1
	spsB	dTDP glycosyl/glycerophosphate transferase				-	-
	spsC	spore coat polysaccharide biosynthesis protein glutamine-dependent sugar transaminase				YP_001920900.1 spsC	ZP_04823132.1
	spsD	TDP-glycosamine N-acetyltransferase				-	-
	SpsE	spore coat polysaccharide biosynthesis protein SpsE phosphoenolpyruvate-sugar pyruvyltransferase				YP_001920858.1 spsE	ZP_04823884.1 spsE

	SpsF	spore coat polysaccharide biosynthesis protein SpsF glycosyltransferase				YP_001920187.1 spsF cytidylyltransferase	Cytidylyltransferase ZP_04821222.1
	SpsG	glycosyltransferase				YP_001920192.1 glycosyltransferase	ZP_04821028.1 glycosyltransferase
	spsI	glucose-1-phosphate thymidylyltransferase				YP_001922563.1 glucose-1-phosphate thymidylyltransferase	ZP_04823483.1 glucose-1-phosphate thymidylyltransferase
	spsJ	dTDP-glucose 4,6-dehydratase				dTDP-glucose 4,6-dehydratase YP_001922379.1	dTDP-glucose 4,6-dehydratase ZP_04822462.1
	spsK	dTDP-4-dehydrorhamnose reductase				dTDP-4-dehydrorhamnose reductase YP_001922377.1	dTDP-4-dehydrorhamnose reductase ZP_04823252.1
	spsL;	nucleotide sugar epimerase				dTDP-4-dehydrorhamnose 3,5-epimerase YP_001922562.1	dTDP-4-dehydrorhamnose 3,5-epimerase ZP_04824157.1
DPA channel	spoVAA					-	-
	spoVAB					-	-
	spoVAC					stage V sporulation protein AC YP_001920239.1	ZP_04821430.1
	spoVAD					stage V sporulation protein AD YP_001920240.1	ZP_04823336.1
	spoVAE					YP_001920241.1	ZP_04822808.1
	spoVAF	stage V sporulation protein AF				YP_001921692.1	ZP_04823055.1
Coat genes	CotE From c difficile	Cdifficile cotE bifunctional protein: peroxiredoxin/chitinase				Only putative peroxiredoxin bcp, thioredoxin_like superfamily YP_001922441.1	ZP_04821965.1 Only putative peroxiredoxin bcp, thioredoxin_like superfamily
	C difficile CotA,	CD630_16130				-	-
	C difficile CotB, DUF2935 superfamily	CD630_15110				Only C terminal end conserved (the last 60 aa) YP_001920411.1	ZP_04823312.1 conserved hypothetical protein
	C difficile CotCB,	CD630_05980 CotJC1 Mn Catalase, ferritin_like superfamily				YP_001922166.1 protein cotJC	Interrupted but conserved CotJC protein ZP_04822876.1
	C difficile CotD	CD630_24010 CotJC2 Mn Catalase ferritin_like superfamily				YP_001922166.1 protein cotJC	Interrupted but conserved CotJC protein ZP_04822876.1
	yisY; hydrolase					alpha/beta hydrolase family protein YP_001920031.1	alpha/beta hydrolase family protein ZP_04820785.1
	Yhax putative hydrolase					Possibly related to phosphatase YidA YP_001922326.1	Possibly related to phosphatase YidA ZP_04823168.1
	sbcC;	DNA ATP-dependent repair enzyme; exonuclease YirY				exonuclease SbcC YP_001922117.1	exonuclease SbcC ZP_04820654.1
	SpoIIQ					Slight similarity to conserved membrane-associated protein YP_001919903.1	conserved membrane-associated protein ZP_04821742.1
	spoIIIAH;	stage III sporulation ratchet engulfment protein;				stage III sporulation protein AH YP_001921554.1	stage III sporulation protein AH ZP_04821915.1
	yutH					YP_001919861.1 spore coat protein, CotS family	ZP_04820627.1 spore coat protein, CotS family
	ysxE					Some similarity to spore coat protein YP_001920273.1	Some similarity to ZP_04823714.1 spore coat protein S
	yjqC;	PBSX phage manganese-containing catalase;				YP_001921891.1 CotJC protein	

		K07217 Mn-containing catalase					CotJC protein only N terminus conserved in Beluga ZP_04822876.1
	OxdD	= yoaN yoaN; oxalate decarboxylase				-	-
	yncD	= alrB; alanine racemase				alanine racemase YP_001920878.1	alanine racemase ZP_04823761.1
	cotQ	yvdP; spore coat protein; oxidoreductase				oxidase, FAD-binding YP_001920704.1 conserved fad binding sequence	oxidase, FAD-binding ZP_04823997.1 conserved fad binding sequence and C terminal end
	cotH	protease inhibitor protecting coat protein in b sub				Cot H YP_001919529.1	cotH ZP_04822683.1
Germinant receptors in B subtilis:	gerAA gerA superfamily	germination receptor germination response to L-alanine				spore germination protein YP_001922292.1	spore germination protein GerA ZP_04820962.1
	gerAB	spore germination protein AB, spore permease superfamily				-	-
	gerAC	spore germination protein AC, Spore gerAC superfamily- spore_ger_x_C multi-domain				-	-
	gerBA, gerA superfamily	g ermination response to the combination of glucose, fructose, L-asparagine, and KCl				spore germination protein YP_001922292.1 higher homology than to gerAA or gerKA	spore germination protein GerA ZP_04820962.1 higher homology than to gerAA or gerKA
	gerBB	spore permease superfamily				Low homology to amino acid permease YP_001921439.1	-
	gerBC	Spore gerAC superfamily- spore_ger_x_C multi-domain				Low homology to spore germination protein YP_001922291.1	Low homology to germination protein, Ger(x)C family ZP_04824101.1
	gerKA	germination response to the combination of glucose, fructose, L-asparagine, and KCl, gerA superfamily				YP_001922292.1	ZP_04820962.1
	gerKB	Spore permease superfamily				-	-
	gerKC	Spore gerAC superfamily- spore_ger_x_C multi-domain				-	-

7.6 Conservation of Primer Sequences Used

Sequences were obtained from the NCBI database via nucleotide BLAST. Strain reference numbers are provided in section 3.2.5.2.2. Multiples allignments were performed using the MUSCLE online tool (EMBL-EBI, 2016).

Forward Primer
Reverse Primer

spoIIIAH

```
NCTC8266 CTCTTGTTGTTCTCTTTCACTTCTAGATTGATAAAAATATTGATTTTGGCTCAATGTAGC
NCTC8550 CTCTTGTTGTTCTCTTTCACTTCTAGATTGATAAAAATATTGATTTTGGCTCAATGTAGC
AlaskaE43 CTCTTGTTGTTCTCTTTCACTTCTAGATTGATAAAAATATTGATTTTGGCTCAATGTAGC
Beluga  CTCTTGTTGTTCTCTTTCACTTCTAGATTGATAAAAATATTGATTTTGGCTCAATGTAGC
202F  TTCTTGTTGTTCTCTTTCACTTCTAGATTGATAGAAGTATTCATTTTGACTCAATGTAGC
Eklund17B TTCTTGTTGTTCTCTTTCACTTCTAGATTGATAGAAGTATTCATTTTGACTCAATGTAGC
   ******************************** ** **** ****** ***********
NCTC8266 CTTGTCATCTTCAGCTTTAGCTATTTCTCCATTTTCCTCAGTCACTCCTATATTTTCTGG
NCTC8550 CTTGTCATCTTCAGCTTTAGCTATTTCTCCATTTTCCTCAGTCACTCCTATATTTTCTGG
AlaskaE43 CTTGTCATCTTCAGCTTTAGCTATTTCTCCATTTTCCTCAGTCACTCCTATATTTTCTGG
Beluga  CTTATCATCTTCAGCTTTAGCTATTTCTCCATTTTCCTCAGTCACTCCTATATTTTCTGG
202F  TTTATCATCCTCAGCTTTAGCTATTTCTCCATTTTCTTCAGTCACCCCTATATTTTCTGG
Eklund17B TTTATCATCCTCAGCTTTAGCTATTTCTCCATTTTCTTCAGTCACCCCTATATTTTCTGG
   ** ***** ************************* ******** **************
NCTC8266 TGATAATACTGCACTTAGATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
NCTC8550 TGATAATACTGCACTTAGATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
AlaskaE43 TGATAATACTGCACTTAGATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
Beluga  TGATAATACTGCACTTAGATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
202F  TGATAATACTGCACTTAAATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
Eklund17B TGATAATACTGCACTTAAATCAGTTGGATCATTTAATCCTCCCTTATTCAACTTTGCTGC
  ***************** ****************************************
```

cot S

```
Eklund17B ATGATGAGGGAATTTGAAATTGAAAGGCAGTTCGATATTAAGATAGAAACAATTAAGGCT
202F  ATGATGAGGGAATTTGAAATTGAAAGGCAGTTTGATATTAAGATAGAAATAATTAAGGCC
NCTC8266 ATGATGAGGGAATTTGAAATTGAAAGGCAGTTCGATATTAAGATAGAAACAATTAAGGCT
NCTC8550 ATGATGAGGGAATTTGAAATTGAAAGGCAGTTCGATATTAAGATAGAAACAATTAAGGCT
AlaskaE43 ATGATGAGGGAATTTGAAATTGAAAGGCAGTTCGATATTAAGATAGAAACAATTAAGGCT
Beluga  ATGATGAGGGAATTTGAAATTGAAAGGCAGTTCGATATTAAGATAGAAACAATTAAGGCT
  ******************************** ***************** *********
Eklund17B AATAAGGGTGTTTACTATCTTAAGACTAACAAAGGAGAACGATGCTTAAAAAGGATAAAT
202F  AATAAGGGTGTTTACTATCTTAAGACTAACAAGGGAGAACGATGCTTAAAGAGGATAAAT
NCTC8266 AATAAGGGTGTTTACTATCTTAAGACTAACAAAGGCGAACGATGCTTAAAAAGGATAAAT
NCTC8550 AATAAGGGTGTTTACTATCTTAAGACTAACAAAGGCGAACGATGCTTAAAAAGGATAAAT
AlaskaE43 AATAAGGGTGTTTACTATCTTAAGACTAACAAAGGCGAACGATGCTTAAAAAGGATAAAT
Beluga  AATAAGGGTGTTTACTATCTTAAGACTAACAAAGGCGAACGATGCTTAAAAAGGATAAAT
  ******************************** ** ************** *********
```

coat protein S

```
NCTC8266 TTTCACACCATCTATCCAGGGGGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
NCTC8550 TTTCACACCATCTATCCAGGGGGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
AlaskaE43 TTTCACACCATCTATCCAGGGGGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
Beluga  TTTCACACCATCTATCCAGGGGGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
Eklund17B TTTCACACCATCTATCCAAGGTGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
202F  TTTCACACCATCTATCCAAGGTGTTAGTATAAATAGCATGTCTGAATAATATACAAATCT
  ****************** ** ***************************************
NCTC8266 ATTATTATCAATGGTAGGAATAAATTTTGGAACTTTTATACCATATCTGTAAACCCATTC
NCTC8550 ATTATTATCAATGGTAGGAATAAATTTTGGAACTTTTATACCATATCTGTAAACCCATTC
AlaskaE43 ATTATTATCAATGGTAGGAATAAATTTTGGAACTTTTATACCATATCTGTAAACCCATTC
Beluga  ATTATTATCAATGGTGGGAATAAATTTTGGAACTTTTATACCATATCTGTAAACCCATTC
Eklund17B ATTATTATTAACGGTAGGAATAAATTTTGGAACTTGTATACCATATCTGTAAACCCATTC
202F  ATTATTATTAATGGTAGGAATAAATTTTGGAACTTGTATACCATATCTGTAAACCCATTC
  ******** ** *** ******************* ************************
NCTC8266 ATTAGCAGAATATACGTATAGTAAATCTGGTAAATCATAATAAACTTTTTTTAAACAATA
```

```
NCTC8550 ATTAGCAGAATATACGTATAGTAAATCTGGTAAATCATAATAAACTTTTTTTAAACAATA
AlaskaE43 ATTAGCAGAATATACGTATAGTAAATCTGGTAAATCATAATAAACTTTTTTTAAACAATA
Beluga  ATTAGCAGAATATACGTATAGTAAATCTGGTAAATCATAATAAACTTTTTTTAAACAATA
Eklund17B ATTAGCAGAATATACATATAGTAAATCTGGCAAATCATAATAAACTTTTTTTAAGCAATA
202F  ATTAGCAGAATATACATATAGTAAATCTGGCAAATCATAATAAACTTTTTTTAAGCAATA
  *************** ************** ********************** *****
```

spsE

```
202F  GATCATACATTAGGGTTTGAAAGTGCGGTAGCTGCTACTACTTTGGGAGCAGAATTTATA
Eklund17B GATCATACATTAGGGTTTGAAAGTGCGGTAGCTGCTACTACTTTGGGAGCAGAATTTATA
NCTC8266 GATCATACATTGGGATTTGAAAGTGCAGTATCAGCTACTACTTTAGGTGCAGAATTTATA
NCTC8550 GATCATACATTGGGATTTGAAAGTGCAGTATCAGCTACTACTTTAGGTGCAGAATTTATA
AlaskaE43 GATCATACATTGGGATTTGAAAGTGCAGTATCAGCTACTACTTTAGGTGCAGAATTTATA
Beluga  GATCATACATTGGGATTTGAAAGTGCAGTATCAGCTACTACTTTAGGTGCAGAATTTATA
  *********** ** *********** *** * *********** ** ************
202F  GAGAAACACATAACATTAGATAAAAAAATGCAAGGACCAGATCATAAAGCATCTATGACA
Eklund17B GAGAAACACATAACATTAGACAAAAAAATGCAAGGACCAGATCATAAAGCATCTATGACA
NCTC8266 GAAAAACACATAACATTAGATAAAAAAATGAAGGGGCCTGACCATAAAGCTTCTATGATG
NCTC8550 GAAAAACACATAACATTAGATAAAAAAATGAAGGGGCCTGACCATAAAGCTTCTATGATG
AlaskaE43 GAAAAACACATAACATTAGATAAAAAAATGAAGGGGCCTGACCATAAAGCTTCTATGATG
Beluga  GAAAAGCACATAACATTAGATAAAAAAATGAAGGGGCCTGATCATAAAGCTTCTATGATG
  ** ** ************** ********* * ** ** ** ******** *******
```

spsC

```
NCTC8266 TCCTTCTCATTCCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
NCTC8550 TCCTTCTCATTCCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
AlaskaE43 TCCTTCTCATTCCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
Beluga  TCTTTCTCATTCCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
Eklund17B TCTTTCTCATTTCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
202F  TCTTTCTCATTCCATGCAGTTAAGAACTTAACTACAGCAGAAGGTGGAGCAGTTACTTTT
  ** ******** **********************************************
NCTC8266 AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
NCTC8550 AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
AlaskaE43 AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
Beluga  AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
Eklund17B AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
202F  AATGATAATACTTTTGGAAATCATGAAGATTTACAAAAATATATGAGATTTACAGCTATG
  ************************************************************

NCTC8266 CATGGCCAATCAAAGGATGCATTAACAAAACTTAAAGCTGGAGCATGGGAATATGATATC
NCTC8550 CATGGCCAATCAAAGGATGCATTAACAAAACTTAAAGCTGGAGCATGGGAATATGATATC
AlaskaE43 CATGGCCAATCAAAGGATGCATTAACAAAACTTAAAGCTGGAGCATGGGAATATGATATC
Beluga  CATGGCCAATCAAAGGATGCATTAACAAAGCTTAAAGCTGGAGCATGGGAATATGATATC
Eklund17B CACGGTCAATCAAAGGATGCATTAACAAAACTTAAAGCTGGAGCATGGGAATATGATATC
202F  CATGGTCAATCAAAGGATGCATTAACAAAGCTTAAAGCTGGAGCATGGGAATATGATATC
  ** ** ********************** ******************************
```

spoIVA

```
NCTC8266 GTGGACAGCTTTAACATATACAAGGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
NCTC8550 GTGGACAGCTTTAACATATACAAGGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
AlaskaE43 GTGGACAGCTTTAACATATACAAGGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
Beluga  GTGGACAGCTTTAACATATACAAGGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
Eklund17B GTGGACAGTTTTAACATATACAAAGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
202F  GTGGACAGTTTTAACATATACAAAGATATAGCTGAAAGAACCCAAGGGGACATATATGTA
  ******** ************** ************************************
NCTC8266 GGAGTAGTTGGACCGGTAAGAACTGGTAAATCAACTTTTATAAAAAAATTTATGGACCTT
NCTC8550 GGAGTAGTTGGACCGGTAAGAACTGGTAAATCAACTTTTATAAAAAAATTTATGGACCTT
AlaskaE43 GGAGTAGTTGGACCGGTAAGAACTGGTAAATCAACTTTTATAAAAAAATTTATGGACCTT
Beluga  GGAGTAGTTGGACCGGTAAGAACTGGTAAATCAACTTTTATAAAAAAATTTATGGACCTT
Eklund17B GGAGTAGTTGGACCGGTAAGAACCGGTAAATCAACTTTTATAAAAAAGTTTATGGACCTT
202F  GGAGTAGTTGGACCGGTAAGAACTGGTAAATCAACTTTTATAAAAAAGTTTATGGACCTT
  ********************** ********************** ************
NCTC8266 ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTACCA
NCTC8550 ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTACCA
AlaskaE43 ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTACCA
```

```
Beluga  ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTACCA
Eklund17B ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTACCA
202F  ATGGTAATACCAAAGATTGATAATACTTATAAGAAAGAAAGAGCGAAAGATGAGTTGCCA
  ********************************************************** ***
NCTC8266 CAAAGTGGGTCAGGTAAGAGTATTCATACAACAGAACCTAAATTCGTGCCAAATGAGGCG
NCTC8550 CAAAGTGGGTCAGGTAAGAGTATTCATACAACAGAACCTAAATTCGTGCCAAATGAGGCG
AlaskaE43 CAAAGTGGGTCAGGTAAGAGTATTCATACAACAGAACCTAAATTCGTGCCAAATGAGGCG
Beluga  CAAAGTGGGTCAGGTAAGAGTATTCATACAACAGAACCTAAATTCGTGCCAAATGAGGCG
Eklund17B CAAAGTGGTTCAGGTAAAAGTATTCACACAACAGAACCTAAATTTGTACCAAATGAGGCG
202F  CAAAGTGGTTCAGGTAAAAGTATTCACACAACAGAACCTAAATTTGTACCAAATGAGGCA
  ******** ******** ******** ***************** ** ***********
```

cotH

```
NCTC8266 AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTATGGAACTATAGAA
NCTC8550 AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTATGGAACTATAGAA
AlaskaE43 AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTATGGAACTATAGAA
Beluga  AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTATGGAACTATAGAA
Eklund17B AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTACGGAACTATAGAA
202F  AAAAGTGTATTGGCTCAATTGAATGGAGAACAACCATCAACAACTTACGGAACTATAGAA
  ********************************************** ************
NCTC8266 TCAACGGTAAATATGACTGCATTAGGTGATATAGGTAGTGGCAAAGGAGCGTCAGGAAAT
NCTC8550 TCAACGGTAAATATGACTGCATTAGGTGATATAGGTAGTGGCAAAGGAGCGTCAGGAAAT
AlaskaE43 TCAACGGTAAATATGACTGCATTAGGTGATATAGGTAGTGGCAAAGGAGCGTCAGGAAAT
Beluga  TCAACAGTAAATATGACTGACTTAGGTGATATGGGTGGTGGCAAAGGAATGCCAGGAAAT
Eklund17B TCAACAGTAAATATGACTGCCTTAGGTGATATGGGTGGTGGCAAAGGAATGCAAGGAAAT
202F  TCAACAGTAAATATGACTGCCTTAGGTGATATGGGTGGTGGCAAAGGAATGCAAGGAAAT
  ***** ************* *********** *** *********** * *******
```

sleB

```
202F  AGGGTCATATCCTCTTATAGCATCATAAACTGCATTGTAACATGCTTGATTTGGAGTAGC
Eklund17B AGGGTCATATCCTCTTATAGCATCATAAACTGCATTGTAACATGTTTGATTTGGAGTAGC
NCTC8266 AGGGTCATATCCCCTTATAGCATCATAAACAGCATTATAACATGCTTGATTTGGAGTAGC
NCTC8550 AGGGTCATATCCCCTTATAGCATCATAAACAGCATTATAACATGCTTGATTTGGAGTAGC
AlaskaE43 AGGGTCATATCCCCTTATAGCATCATAAACAGCATTATAACATGCTTGATTTGGAGTAGC
Beluga  AGGGTCATATCCCCTTATAGCATCATAAACAGCATTATAACATGCTTGATTTGGAGTAGC
  ************ ***************** ***** ******* ***************

202F  TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTCTGAAAAATTACTTCTTTTAT
Eklund17B TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTCTGAAAAATTACTTCTTTTAT
NCTC8266 TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTTTTGAAATATTACTTCTTTTAT
NCTC8550 TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTTTTGAAATATTACTTCTTTTAT
AlaskaE43 TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTTTTGAAATATTACTTCTTTTAT
Beluga  TTTAATATCTCCATTTTTAACACATGAAAATGCATTTTTTTTGAAATATTACTTCTTTTAT
  ************************************* ***** **************
202F  TGAATTTGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGATGCTACAGCTACTTT
Eklund17B TGAATTCGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGACGCTACAGCTACTTT
NCTC8266 TGAATTTGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGATGCTACAGCTACTTT
NCTC8550 TGAATTTGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGATGCTACAGCTACTTT
AlaskaE43 TGAATTTGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGATGCTACAGCTACTTT
Beluga  TGAATTTGGAAAGCTGGGATTTATAACACGATTTAAGACAACTGATGCTACAGCTACTTT
  ****** ************************************* **************
```

yabG

```
Eklund17B TTGAAAGTATATAAGCAGTTATCACTAGATGCAGTTGGAAGGTGTGTTCCTGAGAGTAAA
202F  TTGAAAGTATATAAGCAGTTATCACTAGATGCAGTTGGAAGATGTGTTCCTGAGAGTAAA
NCTC8266 TTAAAAGTATACAAGCAATTATCGCTAGATGCAGTTGGAAGGTGTATTCCTGAGAGTAAA
NCTC8550 TTAAAAGTATACAAGCAATTATCGCTAGATGCAGTTGGAAGGTGTATTCCTGAGAGTAAA
AlaskaE43 TTAAAAGTATACAAGCAATTATCGCTAGATGCAGTTGGAAGGTGTATTCCTGAGAGTAAA
Beluga  TTAAAAGTATACAAACAATTATCGCTAGAGGCAGTTGGAAGGTGTATTCCTGAGAGTAAA
  ** ******** ** ** ***** ***** *********** *** ***************
Eklund17B CAACCTGGTATTATAGTTGATTTGGTAAGAGAAATAAAACCAGATATAGTAGTTCTAACA
202F  CAACCTGGTATTATAGTTGATTTGGTTAGGGAAATAAAACCAGATATAGTAGTTCTAACA
NCTC8266 CAACCGGGAGTTATAGTTGATTTAGTAAGGGAGATAAAACCAGATATAGTAGTTCTAACA
NCTC8550 CAACCGGGAGTTATAGTTGATTTAGTAAGGGAGATAAAACCAGATATAGTAGTTCTAACA
AlaskaE43 CAACCGGGAGTTATAGTTGATTTAGTAAGGGAGATAAAACCAGATATAGTAGTTCTAACA
```

```
Beluga  CAACCGGGAGTTATAGTGGATCTAGTAAGGGAGATAAAACCAGATATAGTAGTTCTAACA
  ***** ** ******* *** * ** ** ** **************************
Eklund17B GGTCATGATGGAGTATTAAAAGACTCTAGAAATTATTTAGATTTAAATAATTATAGAAAT
202F  GGTCATGATGGAGTCTTAAAAGACTCTAGAAATTATTTGGATTTAAATAATTATAGAAAT
NCTC8266 GGTCATGATGGGGTTTTAAAGGATTCTAGAAATTATTTAGACTTAAATAACTATAGAAAT
NCTC8550 GGTCATGATGGGGTTTTAAAGGATTCTAGAAATTATTTAGACTTAAATAACTATAGAAAT
AlaskaE43 GGTCATGATGGGGTTTTAAAGGATTCTAGAAATTATTTAGACTTAAATAACTATAGAAAT
Beluga  GGTCATGATGGGGTTTTAAAGGATTCTAGAAATTATTTAGACTTAAATAACTATAGAAAT
  *********** ** ***** ** ************** ** ******** *********
```

sodA

```
Beluga  CTAAATTGAATAATTCATATATAAATCTTCGACCTTTGCCCAATCAATTATATTCCAAAT
202F  CTAAATTGAATAATTCATATATAAATCTTCTACTTTTGCCCAATCAATTATATTCCAAAT
NCTC8266 CTAAATTGAATAATTCATATATAAATCTTCTACTTTTGCCCAATCAATTATATTCCAAAT
NCTC8550 CTAAATTGAATAATTCATATATAAATCTTCTACTTTTGCCCAATCAATTATATTCCAAAT
AlaskaE43 CTAAATTGAATAATTCATATATAAATCTTCTACTTTTGCCCAATCAATTATATTCCAAAT
Eklund17B CTAAATTGAATAATTCATATATAAATCTTCGACCTTTGCCCAATCAATTATATTCCAAAT
  ***************************** ** *************************
Beluga  ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCATGTTCCCAAAC
202F  ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCGTGTTCCCAAAC
NCTC8266 ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCGTGTTCCCAAAC
NCTC8550 ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCGTGTTCCCAAAC
AlaskaE43 ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCGTGTTCCCAAAC
Eklund17B ATTTTTAACATAATCAGCTCTTAGATTTTTATATTTTAAATAATATGCGTGTTCCCAAAC
  ********************************************** ***********
Beluga  ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
202F  ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
NCTC8266 ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
NCTC8550 ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
AlaskaE43 ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
Eklund17B ATCTATACATAATATAGGAATAAGATTGTTGCTAAGAGGATTATTTTGATTTAATACACT
  ************************************************************
```

spo0A

```
Beluga  TGTAGGCTTGCCCTTATCGTTGTGAATAGTATATCCGAATAGCTTATTAATAGCCTCTAT
AlaskaE43 AGTAGGCTTGCCCTTATCGTTGTGAATAGTATATCCGAATAGCTTATTAATAGCTTCTAT
NCTC8266 AGTAGGCTTGCCCTTATCGTTGTGAATAGTATATCCGAATAGCTTATTAATAGCTTCTAT
NCTC8550 AGTAGGCTTGCCCTTATCGTTGTGAATAGTATATCCGAATAGCTTATTAATAGCTTCTAT
202F  AGTAGGCTTACCCTTATCATTGTGTATAGTATATCCAAATAGCTTGTTTATAGCTTCTAT
Eklund17B AGTAGGCTTGCCCTTATCATTGTGTATAGTATATCCAAATAGCTTGTTTATAGCTTCTAT
   ******** ******** ***** ************ ******** ** ***** *****

Beluga  TTGGCCTCTGCCCCACGCTACTTCTATAGCATGTCTTATTGCTCTTTCCACTCTTGAGGC
AlaskaE43 TTGGCCTCTGCCCCACGCTACTTCTATAGCATGTCTTATTGCTCTTTCTACTCTTGAAGC
NCTC8266 TTGGCCTCTGCCCCACGCTACTTCTATAGCATGTCTTATTGCTCTTTCTACTCTTGAAGC
NCTC8550 TTGGCCTCTGCCCCACGCTACTTCTATAGCATGTCTTATTGCTCTTTCTACTCTTGAAGC
202F  TTGACCTCTGCCCCAAGCAACTTCTATAGCATGTCTTATTGCTCTTTCCACTCTTGAAGC
Eklund17B TTGGCCTCTGCCCCAAGCAACTTCTATAGCATGTCTTATTGCTCTTTCCACTCTTGAAGC
  *** ************ ** ****************************** ******** **
```

16S rRNA

```
7656-6139Beluga  GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
2605264-2606781_202F GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
9562-11079NCTC  GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
9562-11079NCTC8550  GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
9562-11079AlaskaE43  GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
200563-202080Eklund17B GTAGTTTCGCATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGC
    ************************************************************

7656-6139Beluga  ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
2605264-2606781_202F ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
9562-11079NCTC  ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
9562-11079NCTC8550  ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
9562-11079AlaskaE43  ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
200563-202080Eklund17B ATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
    ************************************************************

7656-6139Beluga  GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
2605264-2606781_202F GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
9562-11079NCTC  GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
```

```
9562-11079NCTC8550  GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
9562-11079AlaskaE43  GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
200563-202080Eklund17B GTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
    ************************************************************
```

8 LIST OF PUBLICATIONS

Peer-reviewed original papers:

Lenz, C.A., Reineke, K., Knorr, D., Vogel, R.F., 2015. High pressure thermal inactivation of *Clostridium botulinum* type E endospores – kinetic modeling and mechanistic insights. Frontiers in Microbiology 6.*

Lenz, C.A., Vogel, R.F., 2015. Differential effects of sporulation temperature on the high pressure resistance of *Clostridium botulinum* type E spores and the interconnection with sporulation medium cation contents. Food Microbiology 46, 434-442.*

Schnabel, J., Lenz, C.A., Vogel, R.F., 2015. High pressure inactivation of *Clostridium botulinum* type E endospores in model emulsion systems. High Pressure Research, 1-11.

Lenz, C.A., Vogel, R.F., 2014. Effect of sporulation medium and its divalent cation content on the heat and high pressure resistance of *Clostridium botulinum* type E spores. Food Microbiology 44, 156-167.*

Lenz, C.A., Schnabel, J., Vogel, R.F., 2014. The variability of times to detect growth from individual *Clostridium botulinum* type E endospores is differentially affected by high pressure treatments. High Pressure Research, 1-7.*

Lenz, C.A., Hew Ferstl, C.M., Vogel, R.F., 2010. Sub-lethal stress effects on virulence gene expression in *Enterococcus faecalis*. Food Microbiology 27, 317-326.

Chapter in Edited Book

Lenz, C.A., Vogel, R.F., 2015. Pressure-Based Strategy for the Inactivation of Spores. Subcell Biochem 72, 469-537.*

Oral presentations:

Lenz, C.A., Vogel, R.F., 2016. Themal load versus food safety - How much heat is required in high pressure processing to inactivate *Clostridium botulinum* type E endospores?, 9th International Conference on High Pressure Bioscience and Biotechnology, HPBB2016. 07/2016., Toronto, Canada.

Vogel, R.F., van Nassau, T.J., Scherzinger, A.S., Lenz, C.A., 2016. Insights into the role of cell wall intergrity on the high pressure sensitivity of bacteria, 9th International Conference on High Pressure Bioscience and Biotechnology, HPBB2016. 07/2016., Toronto, Canada.

van Nassau, T.J., Lenz, C.A., Scherzinger, A.S., Vogel, R.F., 2015. Synergy between bacteriophage-encoded peptidoglycan hydrolases and high pressure on the inactivation of *Listeria monocytogenes* and *Staphylococcus aureus*, Bacteriophages 2015. 01/2015. London, UK.

Vogel, R.F., van Nassau, T.J., Scherzinger, A.S., Lenz, C.A., 2015. Synergism of high pressure and bacteriophage endolysins for the preservation of sensitive foods, 9. Internationaler Kongress „Forum Life Science", 03/2015. Garching, Germany.

van Nassau, T.J., Lenz, C.A., Scherzinger, A.S., Vogel, R.F., 2015. Synergy between endolysin and high pressure on the inactivation of *Listeria monocytogenes*, 15. Fachsymposium Lebensmittelmikrobiologie, Fachgruppe Lebensmittelmikrobiologie der VAAM, Fachgruppe Lebensmittelmikrobiologie der DGHM, 04/2015. Freising, Germany.

Lenz, C.A., Schnabel, J., Vogel, R.F., 2014. High pressure thermal inactivation of *Clostridium botulinum* type E endospores: Influence Factors, process Windows, resistance Factors, 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB2014. 07/2014. Keynote lecture, Nantes, France.

Ramos, S.J., Lenz, C.A., Vogel, R.F., 2014. High pressure thermal sterilization to inactivate *Bacillus amyloliquefaciens* spores in model systems and real foods, 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB2014. 07/2014. Nantes, France.

Lenz, C.A., Schnabel, J., Reineke, K., Knorr, D., Vogel, R.F., 2012. Where pasteurization meets sterilization: high pressure inactivation of Clostridium botulinum type E and implications for food safety, 7th International Conference on High Pressure Bioscience and Biotechnology, HPBB2012. 08/2012. Keynote lecture, Otsu, Japan.

Schnabel, J., Lenz, C.A., Usbeck, J.C., Kern, C.C., Behr, J., Vogel, R.F., 2012. Correlation of MALDI-TOF MS patterns and high pressure resistance of *Clostridium botulinum* type E endospores, 7th International Conference on High Pressure Bioscience and Biotechnology, HPBB2012. 08/2012. Otsu, Japan.

Lenz, C.A., Paralta, A.R., Vogel, R.F., 2010. Behaviour of *Clostridium botulinum* type E endospores in high pressure treatments in model systems versus real foods, 6th International Conference on High Pressure Bioscience and Biotechnology, HPBB2010. 08/2010. Freising, Germany.

Poster presentations:

Maier, M.B., Lenz, C.A., Vogel, R.F., 2016. Towards a generic approach for the evaluation of high pressure temperature inactivation of *Clostridium botulinum*, 9th International Conference on High Pressure Bioscience and Biotechnology, HPBB2016. 07/2016., Toronto, Canada.

Kafka, T.A., Reitermayer, D., Lenz, C.A., Vogel, R.F., 2014. Influence of surface hydrophobicity on high-pressure inactivation of *Lactobacillus plantarum*, 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB2014. 07/2014. Nantes, France.

Schnabel, J., Lenz, C.A., Vogel, R.F., 2014. Food matrix parameters, in particular the fat content, influence the high pressure inactivation of *Clostridium botulinum* type E endospores., 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB2014. 07/2014. Nantes, France.

Van Nassau, T.J., Lenz, C.A., Scherzinger, A.S., Vogel, R.F., 2014. Synergy between endolysins and high pressure on the inactivation of *Listeria monocytogenes* and *Staphylococcus aureus,* 8th International Conference on High Pressure Bioscience and Biotechnology, HPBB2014. 07/2014. Nantes, France.

Lenz, C.A., Schnabel, J., Vogel, R.F., 2012. The role of divalent cations in the high pressure tolerance of *Clostridium botulinum* type E endospores, 7th International Conference on High Pressure Bioscience and Biotechnology, HPBB2012. 08/2012. Otsu, Japan.

Paralta, A.R., Lenz, C.A., Vogel, R.F., 2010. High pressure inactivation of *Clostridium botulinum* type E spores in trout, 6th International Conference on High Pressure Bioscience and Biotechnology, HPBB2010. 08/2010. Freising, Germany.

Lenz, C.A., Hew, C.M., Vogel, R.F., 2009. Sublethal stress effects on virulence gene expression in *Enterococcus faecalis*, European Society of Clinical Microbiology and Infectious Diseases, ESCMID Conference, Enterococci – from Animals to Man. 11/2009. Barcelona, Spain.

Hew, C.M., Lenz, C.A., Ehrmann, M., Vogel, R.F., 2006. Virulence gene expression of two *Enterococcus faecalis* strains after high pressure treatment, European High Pressure Research Group Meeting (COST). 09/2006. Prague, Czech Republic.

*Asterisks mark written publications that contain data obtained in this study.

9 LEBENSLAUF

UNIVERSITÄT

08/13 – heute	**Wissenschaftlicher Mitarbeiter / Projektleiter** für Forschungsprojekte mit dem Schwerpunkt „Hochdruckinaktivierung von Mikroorganismen" am Lehrstuhl für Technische Mikrobiologie, TU München, Freising – Weihenstephan
04/09 – heute	**Wissenschaftlicher Mitarbeiter / Promotion:** „Effekte von hydrostatischem Hochdruck auf *Clostridium botulinum* Typ E Endosporen" am Lehrstuhl für Technische Mikrobiologie der TUM
09/08 – 03/09	**Wissenschaftliche Hilfskraft** am Lehrstuhl für Technische Mikrobiologie, der TUM
05/08 – 10/08	**Forschungsprojekt:** „Hitzetoleranz von *Streptococcus thermophilus* in trockener Umgebung" am Lehrstuhl für Technische Mikrobiologie, TU München, Freising – Weihenstephan
05/07 – 04/08	**Diplomarbeit:** „Auswirkungen von subletalem Stress auf die Expression von Virulenzgenen in *Enterococcus faecalis*" (Note: 1,0) am Lehrstuhl für Technische Mikrobiologie, TU München, Freising – Weihenstephan
10/03 – 06/04	**Auslandsstudium** *Food Science/Food Technology* am *University College Cork*, Irland
10/01 – 04/08	**Studium** der Technologie und Biotechnologie der Lebensmittel Abschluss: Diplom Ingenieur (Dipl.-Ing. (Univ.)) (Note: 2,1) an der TU München, Freising – Weihenstephan

SCHULE UND ZIVILDIENST

09/00 – 07/01	**Zivildienst**, Münchner Jugendförderung e.V., München – Feldmoching
09/91 – 06/00	**Gymnasium Olching**, Abschluss: Abitur (Note: 2,6)

10 EIDESSTATTLICHE ERKLÄRUNG

Anlage 5

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung bzw. Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung, Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel: Effect of high hydrostatic pressure on Clostridium botulinum ~~spores~~ type E endospores in am Lehrstuhl für Technische Mikrobiologie

(Lehrstuhl bzw. Fachgebiet oder Klinik)

unter der Anleitung und Betreuung durch Prof. Dr. Rudi F. Vogel

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

(X) Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

(X) Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

( ) Die vollständige Dissertation wurde in .....

veröffentlicht. Die promotionsführende Einrichtung.....
hat der Vorveröffentlichung zugestimmt.

(X) Ich habe den angestrebten Doktorgrad **noch nicht** erworben und bin **nicht** in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

( ) Ich habe bereits am .....

bei der Fakultät für .....

der Hochschule .....

unter Vorlage einer Dissertation mit dem Thema .....

.....
die Zulassung zur Promotion beantragt mit dem Ergebnis:

.....

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich

(X) einverstanden
( ) nicht einverstanden

München, den 25.9.2016

Unterschrift