TECHNISCHE UNIVERSITÄT MÜNCHEN
LEHRSTUHL FÜR MIKROBIELLE ÖKOLOGIE

Molecular characterization of the genetic locus responsible for cereulide toxin production in emetic *Bacillus cereus*

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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

genehmigten Dissertation.

Vorsitzender:		Univ.-Prof. Dr. A. Gierl
Prüfer der Dissertation:	1.	Univ.-Prof. Dr. S. Scherer
	2.	Univ.-Prof. Dr. E. Märtlbauer
		(Ludwig-Maximilians-Universität München)

Die Dissertation wurde am 06.02.2008 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 29.04.2008 angenommen.

“There are some things so serious, you have to laugh at them.”
~Niels Bohr~

Table of Contents

List of Figures

List of Tables

Summary

The emetic toxin cereulide, a heat- and acid stable depsipeptide, is responsible for the vomiting type of gastrointestinal disease caused by *Bacillus cereus*. Cereulide is assembled by a non-ribosomal peptide synthetase (NRPS), encoded by the *ces* gene cluster. Recent increases in reported outbreaks, especially the emetic type, illustrate the need to advance our understanding of the mechanism of toxin expression.

The present study has shown the NRPS genes encoding the cereulide synthetase in emetic *Bacillus cereus* are transcribed as a 23kb polycistronic transcript from a main promoter upstream of the *cesP* CDS encoding a 4'-phosphopantetheinyl transferase (4'-PP), which is interesting as NRPS thiolation domains can frequently be activated by 4'-PPs not directly associated to the respective NRPS operon. An intercistronic promoter region identified in *cesB*, only active upon addition of amino acids, may indicate a mechanism to activate distal genes in response to the availability of NRPS substrates. Sequencing of the promoter from a dozen emetic strains and comparison to the reference strain showed that the large difference in toxicity cannot be explained by differences in the central promoter. The main promoter from the reference strain is similarly active in non-emetic, emetic-like and emetic *B. cereus*, and in an emetic *Bacillus weihenstephanensis* strain, indicating the regulatory mechanism may be more global in nature, and not unique to emetic strains. Studies of promoter activity in foods support reports implicating farinaceous rather than proteinaceous foods with emetic outbreaks, as the promoter was much more active in starchy foods.

SYBR Green I quantitative real time reverse transcription PCR (RT-qPCR) assays used to investigate *ces* gene transcriptional kinetics indicate the lower toxicity of weakly emetic strains is partially but not wholly regulated at the transcriptional level. Surprisingly, the tightly regulated growth-phase dependent expression level of cereulide synthetase was independent of that of the pleiotropic virulence factor regulator *plcR*, but rather appeared linked to sporulation. As well, *ces* transcription and toxin biosynthesis were reduced by sodium chloride addition, and transcription decreased with glucose. However toxicity cannot be predicted by growth rate or transcript levels, as shown by temperature trials: maximum cereulide accumulated at 24°C, though peak gene expression occurred at 30°C and highest growth rate at 42°C, underlining the risk of emetic disease posed by temperature-abused foods.

Zusammenfassung

Das *Bacillus cereus* Toxin Cereulid, ein hitze- und säurestabiles Depsipeptid, verursacht eine Lebensmittelvergiftung des emetischen Typs. Cereulid wird nicht ribosomal durch die Cereulidsynthetase, welche von den *ces* Genen kodiert wird, gebildet. Da Cereulid immer häufiger in Verbindung mit ernsthaften Lebensmittelvergiftungen genannt wird, ist ein besseres Verständnis der genetischen Grundlage der Cereulidsynthese erforderlich.

Diese Arbeit zeigt, dass die Cereulidsynthetasegene (*ces*) im emetischen *B. cereus* von einem zentralen Promotor als 23kb-polycistronisches Transkript transkribiert werden. Interessant ist, dass dieser zentrale Promotor am 5'-Ende der *cesP* kodierenden DNS Sequenz liegt, da NRPS-Thiolationsdomänen häufig von anderen, nicht direkt mit derselben NRPS assoziierten, 4'-Phosphopantetheinyltransferasen aktiviert werden. Eine intrinsische Promotorregion in *cesB* zeigte nur durch Zugabe von bestimmten Aminosäuren Aktivität. Dies könnte ein Hinweis auf einen Mechanismus sein, bei dem distale Gene als Reaktion auf verfügbare NRPS-Substrate stärker exprimiert werden. Der zentrale Promotor von einem Dutzend Stämme wurde sequenziert und mit dem Promotor des emetischen Referenzstamms verglichen: alle Sequenzen waren gleich. Die sehr großen Unterschiede in der Toxizität können daher nicht von unterschiedlich starken Promotoren herrühren. Zusätzlich zeigte der Zentralpromotor des Referenzstamms ähnliche Aktivität sowohl in nicht emetischen, emetisch-ähnlichen und emetischen *B. cereus* Stämmen als auch in einem emetischen *Bacillus weihenstephanensis* Stamm. Diese Ergebnisse deuten darauf hin, dass sich der Regulationsmechanismus nicht auf emetische Stämme beschränkt, sondern eher allgemeiner Natur ist. Untersuchungen auf Promotoraktivität in Lebensmitteln unterstützen bisherige Berichte, die die emetische *B. cereus* Erkrankung in Verbindung mit stärke- und nicht mit eiweißhaltigen Lebensmitteln brachten, da der Promotor eine viel höhere Aktivität in stärkehaltigen Lebensmitteln zeigt.

Quantitative Realtime RT-PCR (RT-qPCR) wurde eingesetzt, um die Kinetik der *ces*-Gentranskription zu untersuchen. Diese Analysen zeigten, dass die niedrige Toxizität schwach emetischer Stämme teilweise, aber nicht völlig auf Transkriptionsebene reguliert wird. Die Transkription der Cereulidsynthetasegene war unter allen getesteten Bedingungen in die Exponentielphase maximal. Überraschender Weise ist

die Regulation der Expressionslevel der Cereulidsynthese unabhängig von dem pleiotrophen Regulator *plcR* wohingegen ein Zusammenhang zur Sporulation zu bestehen scheint. Des Weiteren wurden im Rahmen vorliegende Arbeit die Effekte extrinsischer Faktoren, wie z.B. Salz, Aminosäuren und Kultivierungstemperatur auf die *ces* Genexpression und Toxinbildung untersucht.. Bei Zugabe von Valin, Leucin und Threonin wurde eine erhöhte Genaktivität beobachtet, wohingegen die *ces* Expression bei Zusatz von NaCl oder Glukose vermindert war.. Temperaturversuche zeigten, dass die Toxizität eine Probe weder durch die Wachstumsrate noch durch das Transkriptlevel vorhergesagt werden kann: maximale Cereulidtoxinmengen wurden bei 24°C detektiert, trotz höchster Genexpression bei 30°C und schnellster Wachstumsrate bei 42°C. Diese Ergebnisse unterstreichen das erhöhte Intoxikationsrisiko mit Cereulid durch Verzehr unausreichend gekühlter Lebensmittel.

1 Introduction

1.1 The Bacillus cereus group

Bacillus cereus is the species that gives the name to the *B. cereus* group of endospore-forming, facultative anaerobic, Gram positive, motile rods. This group of micro-organisms comprises the species *Bacillus anthracis, B. cereus, Bacillus mycoides, Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis* (Gordon *et al.*, 1973; Lechner *et al.*, 1998; Priest & Alexander, 1988; Turnbull & Kramer, 1991). Though diverse with respect to their virulence factors, they are genetically closely related (Helgason *et al.*, 2000; Kolsto *et al.*, 2002). Based on genotypic and phenotypic characteristics, *B. cereus* group members cluster into 3 major branches: 1. emetic and clinical isolates of *B. cereus*, *B. anthracis* and *B. thuringiensis*, 2. non-emetic *B. cereus* and *B. thuringiensis*, and 3. *B. weihenstephanensis* and *B. mycoides* (Ehling-Schulz *et al.*, 2005a).

B. thuringiensis produces insecticidal toxins commercially used as bio-control agents, whereas *B. anthracis* causes the fatal animal and human disease anthrax (Aronson & Shai, 2001; Turnbull, 2002); in both these species the toxin genes are located on a mega virulence plasmid (Rasko *et al.*, 2005). *B. weihenstephanensis*, a psychrotolerant dairy spoilage organism, is the most recently described species in the group (Lechner *et al.*, 1998). *B. cereus* has been increasingly associated with food borne disease (Ehling-Schulz *et al.*, 2004a).

B. cereus can cause two types of food-borne illness; enterotoxins result in diarrhoeal illness while an emetic toxin induces vomiting. The emetic type of illness is triggered by ingested toxin produced by cells growing in food (Kramer & Gilbert, 1989), whereas the enterotoxigenic type result from toxin(s) produced during vegetative growth of cells in the small intestine (Granum & Lund, 1997). Production of the chromosomally encoded, heat-labile enterotoxins is broadly distributed among *Bacillus cereus* group members (Hansen & Hendriksen, 2001; Prüß *et al.*, 1999) whereas production of the heat- and acid stable emetic toxin, encoded by genes on a mega virulence plasmid (Ehling-Schulz *et al.*, 2006), is limited to the species *B. cereus* (Agata *et al.*, 1996).

B. cereus is often detected in foods that have been heated, thereby minimizing the competitive flora while allowing spores of *B. cereus* to survive and germinate (Granum, 2001). Enterotoxic *B. cereus* is often found in dairy-based foods, while most emetic outbreaks have been linked to rice-based dishes. Although it has been suggested that emetic strains are less commonly found in the environment (Altayar & Sutherland, 2006), they have been involved in several fatalities (Dierick *et al.*, 2005; Hoffmaster *et al.*, 2006; Mahler *et al.*, 1997). Recently, a pyschrotrophic emetic toxin producing strain identified as *Bacillus weihenstephanensis* was reported (Thorsen *et al.*, 2006).

1.2 Emetic Bacillus cereus

The emetic type of illness may be underreported as it closely resembles *Staphylococcus aureus* intoxication, with the onset of symptoms (nausea, vomiting) within ½ to 5 hours and the duration of illness lasting 6 to 24 hours. The emetic toxin is heat stable for 90 minutes at 121°C and stable to pH 2-11 as well as resistant to proteolysis by trypsin and pepsin. This makes it of particular concern for the food industry, especially in mass catering facilities, as existing emetic toxin is not inactivated by processing methods [for review see (Granum, 2001), (Ehling-Schulz *et al.*, 2004a) and (Schoeni & Wong, 2005)].

The emetic *B. cereus* toxin is a cyclic dodecadepsipeptide closely related to valinomycin and has been named cereulide (Agata *et al.*, 1994). NMR studies revealed the structure as three repeats of the (D-O-Leu-D-Ala-L-O-Val-L-Val) pattern (Agata *et al.*, 1994). Cereulide is suggested to cause emesis by binding the 5-HT_3 receptor and stimulating the vagus afferent (Agata *et al.*, 1995). The ionophoretic properties of cereulide were found to be similar to valinomycin and to stimulate swelling and respiration in respiring mitochondria in the presence of potassium (Mikkola *et al.*, 1999). Cereulide has also been shown to inhibit human natural killer cell activity (Paananen *et al.*, 2002).

Detection of the emetic toxin had previously been performed by oral administration to house musk shrews (*Suncus murinus*) (Agata *et al.*, 1995) and rhesus monkeys (Shinagawa *et al.*, 1995). Filtered, heated culture supernatant of strains isolated from vomiting outbreaks caused vacuolation in HEp-2 cells (Hughes *et al.*, 1988), which

was used to develop a HEp-2 cell assay for cereulide detection (Dietrich *et al.*, 1999; Hughes *et al.*, 1988). Extracts of emetic *B. cereus* strains or contaminated food caused loss of motility to boar sperm (Andersson *et al.*, 1998b), a characteristic used to develop a semi-quantitative assay , which was calibrated against a method based on liquid chromatography and ion trap mass spectrometry (Haggblom *et al.*, 2002). A PCR method is available to distinguish emetic from non-emetic strains and other typical food-borne pathogens, by use of primers targeting a specific region of the cereulide synthetase gene cluster unique to emetic *B. cereus* strains (Ehling-Schulz *et al.*, 2004b). Recently a quantitative real time Taqman assay was reported for rapid identification of emetic *B. cereus* strains in foods from a recent food borne outbreak (Fricker *et al.*, 2007).

1.3 Non-ribosomal peptide synthesis (NRPS)

Based on its chemical structure $[D\text{-}O\text{-}Leu\text{-}D\text{-}Ala\text{-}L\text{-}O\text{-}Val\text{-}L\text{-}Val]_3$ it is not surprising that cereulide is synthesized enzymatically by a nonribosomal peptide synthetase (NRPS) (Ehling-Schulz *et al.*, 2005b). NRPS are responsible for catalyzing the nucleic acid independent synthesis of a broad range of bioactive low molecular weight peptides, with a chain length of 2 to 48 residues (Marahiel *et al.*, 1997). Other *Bacillus* sp. produce peptide antibiotics via NRPS enzymes, such as surfactin in *B. subtilis*, and tyrocidine and gramicidin S in *B. brevis*, however few transcriptional analyses have been performed.

Non-ribosomal peptide synthetases (NRPS) are large multifunctional enzymes made up of repetitive modules, each of which catalyzes one complete cycle of chain elongation and any optional modification (Stachelhaus *et al.*, 2002). The modules exhibit a conserved domain structure, and selectively catalyze the activation and thioester formation of one amino, α-hydroxy or carboxylic acid monomer (Turgay *et al.*, 1992).

Each module contains a C (condensation, absent in the initiation module), A (adenylation), T (thiolation or peptidyl carrier protein), and TE (thioesterase, present only in the termination module) domain (von Dohren *et al.*, 1997). These modules are normally arranged collinear to the peptide sequence at the gene level (von Dohren *et al.*, 1999). The A domain of a module is responsible for substrate recognition and generation of the corresponding aminoacyl adenylate. The T domain is converted to

its holo form by the covalent binding of the cysteamine thiol group of a 4'-PP (4' phosphopantetheinyl transferase) cofactor to a conserved serine residue. The T domain is responsible for binding of the aminoacyl adenylate to the sulfhydryl residue of the 4'-PP. The C domain is responsible for peptide bond formation and chain translocation, it accepts acyl groups from the preceding modules, catalyzing the nucleophilic attack of a peptidyl-S-4'-PP donor by a monomeric aminoacyl-S-4'-PP, elongating the peptide chain incrementally (Stachelhaus *et al.*, 1998). A C-terminal TE (thioesterase) catalyzes the release of the final product from the NRPS enzyme (Trauger *et al.*, 2000). Additional elements such as epimerization and methylation domains, which modify the incorporated constituents, may also be present (for review see (Finking & Marahiel, 2004; Sieber & Marahiel, 2005)). Typically, the order of modules corresponds directly to that of the components in the final peptide product.

The cereulide non-ribosomal peptide synthetase has recently been identified (Ehling-Schulz *et al.*, 2005b). Sequence analysis showed a high sequence homology to virulence plasmids of *B. cereus*, *B. thuringiensis* and *B. anthracis* (Ehling-Schulz *et al.*, 2006). The mega-plasmid on which the *ces* genes are located has recently been sequenced and renamed pCER270, a member of a group of pXO1-like plasmids (Rasko *et al.*, 2007).

The complete genetic locus for the cereulide NRPS gene *(ces)* in the emetic *B. cereus* reference strain F4810/72 has been sequenced, and consists of a 23kb gene cluster with seven coding DNA sequences (CDS), the first of which (*cesH*) codes for a putative hydrolase, the second (*cesP*) for a 4' phosphopantetheinyl transferase, the third (*cesT*) for a thioesterase, the fourth (*cesA*) for the modules incorporating D-O-Leu and D-Ala, the fifth (*cesB*) for the L-O-Val and L-Val incorporating modules, and the sixth and seventh (*cesC* and *cesD*) for a putative ATP binding cassette (Ehling-Schulz *et al.*, 2006) (Figure 1). The cereulide NRPS is unique in that the substrates for the *cesA1* (D-O-Leu) and *cesB1* (L-O-Val) modules are α-keto acids which are then chirally reduced (Magarvey *et al.*, 2006). Many studies have been performed on the biochemical characterization and genetic sequencing of non-ribosomal peptide synthetases, but few have examined regulation of these enzyme complexes on a transcriptional level.

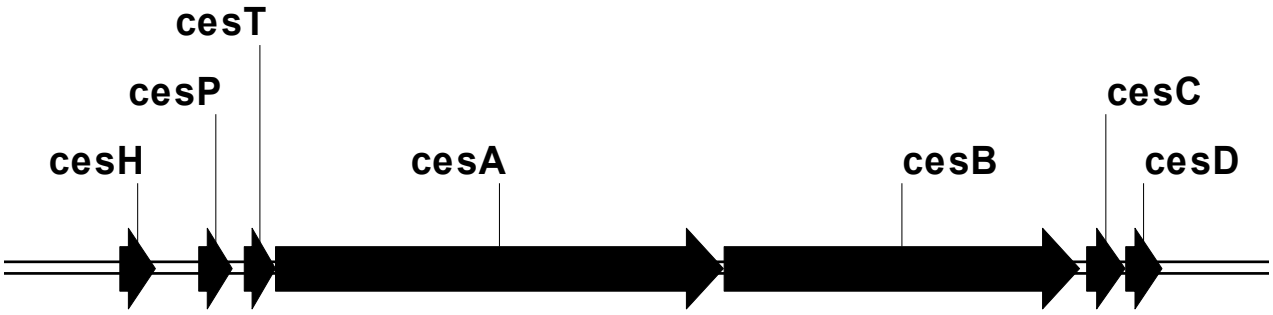


Figure 1. **Arrangement of the cereulide synthetase encoding *ces* gene cluster.**

The *ces* gene cluster in emetic Bacillus cereus located on the mega virulence plasmid pBCE270 comprises a putative hydrolase (*cesH*), a 4' phosphopantetheinyl transferase (*cesP*), the genes encoding the functional NRPS modules (*cesA* and *cesB*) and a putative ABC transporter (*cesC* and *cesD*) (according to (Ehling-Schulz *et al.*, 2006).

1.4 Research objective

The genetic locus of the cereulide synthetase *ces* gene cluster of emetic *B. cereus* encoding has been identified, sequenced and biochemically characterized (Ehling-Schulz *et al.*, 2005b; Ehling-Schulz *et al.*, 2006; Magarvey *et al.*, 2006). However, information on the regulation of cereulide production is still lacking: many NRPS have been sequenced and studied biochemically, yet few transcriptional studies exist.

The availability of the complete sequence of the cereulide synthetase *(ces)* genes permitted a study of regulation at the genetic level for the first time. A complete transcriptional analysis was undertaken to identify the promoter and characterize the transcript. The basis for low emetic toxin production by some strains was analysed on a genetic sequence and expression level. Also investigated were cereulide synthetase *ces* and pleiotropic virulence factor regulator *plcR* gene expression in both highly and weakly emetic strains, along with growth, degree of sporulation and cereulide accumulation. Effects of pre-culture conditions and media supplementation, and as well NaCl levels and culture temperature, on growth rate, *ces* gene expression and toxicity were investigated in order to provide further insights into the kinetics and regulation of cereulide toxin production. This is of utmost importance in understanding which conditions are favourable and unfavourable for emetic toxin production, in order to reduce the risk and incidence of food borne disease.

2 Materials and Methods

2.1 Bacterial strains and culture conditions

2.1.1 Bacterial strains

Bacillus cereus F4810/72 (Turnbull *et al.*, 1979), the emetic reference strain, was chosen as the model emetic organism. This strain was originally isolated from vomit by the Public Health Laboratory Service in London, UK. Strain IH41385 (Andersson *et al.*, 1998a) was used as the model strain representing weakly emetic strains. Other strains used were the non-emetic *B. cereus* type strain ATCC14579^T, the highly emetic strain F3080B/87, and the emetic-like strain NVH1519/00 (Ehling-Schulz *et al.*, 2005a). A mutant of the weakly emetic strain IH41385 without the virulence plasmid pBCE270 containing the *ces* genes (IH41385ΔpBCE270), was created by long-term storage on rich LB agar, with routine re-inoculation on same, causing it to lose the plasmid, as described elsewhere for *B. anthracis* strains (Marston *et al.*, 2005). A sporulation deficient mutant of the emetic reference strain (F4810/72 Δ*spo0A*) was used to investigate effects of sporulation ability on cereulide production (Lücking *et al.*, unpublished). As well, the recently described psychrotolerant *Bacillus weihenstephanensis* strain MC67 (Thorsen *et al.*, 2006) was used for promoter activity studies. Details of these and of additional strains used for sequencing of the *ces* promoter region are provided in the Appendix (Table 8).

Escherichia coli strains TOP10 and the non-methylating strain INV110 (Invitrogen, Germany) were used for subcloning. Vectors used were pAD123 containing a promoter-less *gfpmut3a* and pAD43-25 with *gfpmut3a* downstream of a constitutive promoter (Dunn & Handelsman, 1999) and pXen1, containing a promoter-less luciferase (*luxABCDE*) optimized for use in Gram positive hosts (Francis *et al.*, 2000).

2.1.2 Culture conditions

Strains were routinely cultured on standard plate count agar for 14h at 30°C or 37°C for *Bacillus* or *E. coli* respectively. For cultivation in liquid media, LB was chosen as the standard media after initial experiments detected equivalent cereulide production as in 10% skim milk (data not shown), a media previously commonly implemented for cereulide production studies (Finlay *et al.*, 2000). Precultures were prepared by inoculating one colony forming unit (cfu) in 3ml LB media and culturing 14h with

150rpm rotary shaking at either 30°C or 37°C for *Bacillus* or *E. coli* respectively. All liquid cultures were grown in lysogeny broth (LB) containing tryptone ($10gL^{-1}$; Oxoid Ltd., UK), yeast extract ($5gL^{-1}$; Oxoid Ltd.) and sodium chloride ($10gL^{-1}$; Carl Roth GmbH & Co KG, Germany) (Bertani, 1951). The appropriate antibiotics were added where necessary. *B. cereus* main cultures were grown from 3mL overnight pre-cultures inoculated 1:10000 in 100mL LB in 500mL baffled flasks with rotary shaking (150rpm) at 30°C. Growth was monitored by measurement of optical density at 600nm (OD_{600}) in a Perkin-Elmer 550E UV/VIS spectrophotometer (Perkin-Elmer, USA); once the OD_{600} exceeded 1, the culture was diluted 1:10 in media in order to remain within the linear measurement capability of the instrument, and OD_{600} values extrapolated. Spores were observed using phase contrast microscopy and expressed as a percentage of the total cells.

Kinetics

For transcriptional kinetic experiments the pre-culture was grown from one cfu on agar at 30°C either in 3mL LB 14h (vegetative), or in 100mL LB with 150rpm rotary shaking 16h (sporulated). Liquid cultures were prepared by inoculating 100mL LB with 100µL of 1:100 diluted (in LB) pre-culture, which gave an initial cell density of approximately 10^3 cfu mL^{-1}. Cultures were grown in 500mL baffled flasks with rotary shaking (150rpm). Growth was monitored by measurement of optical density at 600nm (OD_{600}) in a Perkin-Elmer 550E UV/VIS spectrophotometer (Perkin-Elmer, USA); once the OD_{600} exceeded 1, the culture was diluted 1:10 in media in order to remain within the linear measurement capability of the instrument, and OD_{600} values extrapolated. Samples for growth measurement, RNA extraction, and toxin testing were taken throughout exponential growth. For RNA extraction the cells were collected (10,000g, 4°C, 2min), immediately frozen in liquid nitrogen and stored at -80°C. Samples for toxicity testing were autoclaved (121°C, 15min) and stored refrigerated.

Environmental parameters

Growth experiments were performed using variations in temperature (12°C, 15°C, 24°C, 37°C, 42°C 48°C), various NaCl levels ($0-100gL^{-1}$), and added supplements previously shown to increase cereulide production (0.2% w/v D-glucose, $0.3gL^{-1}$ L-leucine, L-threonine, L-valine) (Agata *et al.*, 1999). Rice water was prepared with 20g white long grain rice in 1 litre water and autoclaved, amino acid supplements

which were shown to increase cereulide production on rice water agar (Jaaskelainen *et al.*, 2004) were added as above, with or without threonine.

Additionally, pre-cultures were used as inoculants that were either sporulated (reference condition), unsporulated vegetative cells or germinated spores. Spores were prepared as described elsewhere (de Vries *et al.*, 2004) by harvesting spores in foam collected from a 4 day old *B. cereus* flask culture (30°C, 150rpm), followed by eight washing (MilliQ water) and centrifugation (10000g, 4°C, 30min) steps, and storage at 4°C in 10mM KPO_4 pH 7.0. Prior to use as an inoculant, the spore suspension was heated (80°C 15min) to inactivate any vegetative cells.

Fermentation

The emetic reference strain was also grown in a Biostat fermenter (Sartorius BBI, Germany) in LB media, with 30% oxygen saturation, pH 6.5 and a temperature of 30°C as described in detail elsewhere (Winkler, 2006). Pre-culture conditions were standard (3ml LB, 150rpm, 16h), the preculture was diluted 1:100 and inoculation therefore 1:1000 for an initial cell density of 10^3 cfu mL^{-1}. Samples were taken hourly as described above.

2.2 Cereulide toxicity assay

Samples for toxicity testing were taken throughout growth. Cells and heat labile toxins were inactivated by heating (120°C 15min), after which the samples were stored chilled (4°C). The toxicity was determined using a modified HEp-2 cytotoxicity assay in 96-sample microwell plates (Finlay *et al.*, 1999). Samples were two fold serially diluted in Eagle minimum essential medium with Earle salts supplemented with 1% foetal calf serum, 1% sodium pyruvate and 0.4% penicillin-streptomycin (all Roche Applied Science, Germany), and 2% ethanol as a solvent for cereulide. Valinomycin (1-500ngml^{-1}; Sigma Aldrich, Germany) served as an internal standard. HEp-2 cells (6 x 10^5 cells ml^{-1}) were added and the plates incubated 48h at 37°C in 5%CO_2. Mitochondrial activity of viable cells was subsequently determined at 450nm using tetrazolium salt WST-1 (Roche Applied Science); toxicity titres were calculated as described by Dietrich et al. (Dietrich *et al.*, 1999).

2.3 DNA Isolation

Genomic DNA was prepared from 3ml overnight culture using a standard protocol (Ausubel *et al.*, 1997) with phenol/chloroform and CTAB/NaCl liquid/liquid extraction, adapted with a longer initial incubation for proteinase K digestion (3h) at 55°C. Plasmid was isolated from 3ml *E. coli* overnight cultures using the GenElute kit (Sigma Aldrich).

2.4 Polymerase chain reaction (PCR)

Completeness of DNA digestion after RNA isolation was confirmed by agarose gel electrophoresis analysis of PCR reactions using both universal 16SrDNA primers (Liesack & Stackebrandt, 1992) and primers EM1F and EM1R, shown to be both specific and very sensitive for the *ces* gene cluster (Ehling-Schulz *et al.*, 2004b). Standard PCR was performed in a 50µL reaction volume using 100ng template and 1.25U ThermoStart Taq polymerase (ABgene, Germany) with 0.5µM each primer in 1x buffer (1.5mM MgCl2, 0.4mM each dNTP). The polymerase was activated (95°C 15 min), followed by 35 amplification cycles (95°C 30s, 60°C 45s, 72°C 1min) and a final elongation step (72°C 5 min).

Putative promoter regions of the *ces* genes were amplified using PCR in order to provide a sequencing ladder for primer extension, and for cloning into fusion vectors for promoter activity studies. PCR was performed using 10ng DNA template in a 50µL reaction volume as described.

For sequencing promoter regions of various highly and weakly emetic strains, PCR was performed as described to amplify the promoter region using the forward primer (5'-CCTAAGCATGTTAGAGATTTAC-3') and reverse primer (5'-GTTCAACCGCATAAATCTTTATG-3'). Sequencing of the weakly emetic strain IH41385 *ces* gene locus was performed by amplification using PCR as described using primers designed from the emetic reference strain sequence (Appendix, Table 9). PCR amplicons were purified (Qiaquick PCR Purification kit, Qiagen, Germany) and sequence determination performed by Sequiserve (Vaterstetten, Germany). Where differences in sequencing were detected, the *ces* gene region was re-sequenced using a high fidelity polymerase (Roche Applied Science, Germany) as per the manufacturer's instructions to amplify the fragment in question.

2.5 Cloning and sequencing

2.5.1 TOPO TA cloning

For sequencing PCR amplicons, fragments generated were either sequenced directly, or subcloned into vector pCR2.1 using the TOPO TA cloning kit (Invitrogen) prior to sequencing by Sequiserve (Vaterstetten, Germany).

2.5.2 Fluorescent IRD800 dye sequencing

In order to precisely locate the promoters during primer extension experiments, approximately 1kbp flanking the putative transcriptional start were amplified by PCR and cloned into plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen). The vector was then used to transform chemically competent TOP10 *E. coli* cells (Invitrogen). Plasmid DNA was prepared from 5ml overnight cultures with the GenElute Miniprep Kit (Sigma Aldrich) and was used directly as sequencing template. The same reverse IRD800 labelled primer was used as for the respective primer extension reaction, and sequencing was performed with the termination reaction kit SequiTherm Excel II (Epicentre, USA) according to manufacturer's instructions. 1μl reaction solution was loaded onto an 8% urea polyacrylamide gel and run on the LiCor 4200 (LiCor Biosciences, USA) sequencer alongside primer extensions as a ladder. Control sequencing reactions were performed with the pSAD2 vector included in the SequiTherm kit and M13 IRD800 labelled universal forward and reverse primers.

2.6 RNA Purification.

2.6.1 RNA Isolation

Samples for RNA extraction for RT-PCR, primer extension and 5' RACE were taken at an OD_{600} of 10. For RNA extraction cells were collected (10000g, 4°C, 2min), immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using a method adapted from a plant tissue protocol (Logemann *et al.*, 1987) combined with initial beating in a Ribolyser (Hybaid, UK) with 0.1mm zirconium-silica beads (Carl Roth GmbH & Co KG, Germany) to disrupt the cells. For transcriptional

kinetics, RNA was isolated from 1mL culture samples using 1mL TRIzol reagent (Invitrogen, USA) combined with beating in a Ribolyser (Hybaid, UK) with 0.1mm zirconium-silica beads (Carl Roth GmbH & Co KG) to disrupt the cells. Further steps were as per the manufacturer's instructions.

2.6.2 DNA Digestion

Contaminating DNA was removed from 5µg total RNA by treatment with 10U RQ1 DNase (Promega, Germany) in 1x buffer in 100µL volume at 37°C for 45min. The reaction was stopped and RNA isolated by extraction with 100µL chloroform and precipitation with ethanol; the RNA pellet was subsequently twice washed with 70% ethanol and resuspended in DEPC treated ddH_2O. RNA purity and quantity were determined by absorbance at 260nm and 280nm and by agarose gel electrophoresis and stored at -80°C. Efficacy of DNA removal for RT-PCR was confirmed by using RNA as template for standard PCR with 16S oligonucleotide primers previously reported by Martineau *et al*. (Martineau *et al.*, 1996).

2.7 Reverse transcription PCR (RT-PCR)

cDNA for RT-PCR and for 5' RACE analysis was produced using SuperScript III reverse transcriptase (Invitrogen). 100ng total RNA together with 2pmol gene specific reverse primer (Table 1) and 1µL 10mM each dNTP in a 10µL volume were incubated 75°C 2min, 70°C 1min, 65°C 1min, 60°C 1min, 55°C 1min. 10µL RT mix were added (4µL 5x RT buffer, 1µL 0.1M DTT, 100U SuperScript III, and 20U RNaseOUT, all Invitrogen), and the reaction incubated at 55°C 1h, before inactivation at 70°C for 15min. For negative controls the reverse transcriptase was omitted.

Subsequent PCR reactions for RT-PCR consisted of 2µL RT reaction (template), 50pmol each forward and reverse primer (Table 1), 0.4mM each dNTP and 1.25U ThermoStart Taq polymerase (ABgene, UK) in a 50µL reaction volume. Fragments of expected length greater than 2kb were amplified using the Expand High Fidelity PCR System (Roche Applied Science) following the manufacturer's instructions.

Table 1. **Oligonucleotide primers used for RT-PCR.**

Primer	Sequence (5´-3´)	Target region
H-F	AGTGGTTGGATGTGGGATAAAC	*cesH*
H-R	CCATGACCAATTTTAGGAATGATAAC	*cesH*
HP-F	CAGTGCGTTGGTTAAACCGA	*cesHP*
HP-R	GTTCAACCGCATAAATCTTTATG	*cesHP*
PTA-F	GGTTAGCTGTTCGGAAATTG	*cesPTA*
PTA-R	GATCTCCACTATCGAACCAGC	*cesPTA*
A1-A2-F	GATGCCTGGTATTGGCAT	*cesA1-A2*
A1-A2-R	GATCAAGTACAGGTATATCTTCAG	*cesA1-A2*
A2-F	GTGAGGAATTTAGCAAGCAACTG	*cesA2*
A2-R	AACTGGCAGCGCATCCAGCT	*cesA2*
AB-F	GTGACAATTCATCAACAACTGCT	*cesAB*
AB-R	CATACTGGAATCATTGCATCTTCTGGC	*cesAB*
B-F	GCCAGAAGATGCAATGATTCCAGTATG	*cesB*
B-R	CACCTTATATGATAATGTGTCACC	*cesB*
BC-F	GGTGACACATTATCATATAAGGTG	*cesBC*
BC-R	GGCTCCATGATTTCTACGTTAATAAG	*cesBC*
CD-F	AAGGTATGAAACAAAAGTTAGCCAT	*cesCD*
CD-R	GAAAGAACCGTTACTAGGGAAATC	*cesCD*
T-R	CAGCCACAGATCAATTATGTTG	*cesCD & T*
T-F	GTGGTATTTCAGATTTAATGAAATCC	*cesCD & T*

2.8 Rapid amplification of cDNA ends (5'RACE)

RACE was performed using the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) with primers indicated (Table 2); SuperScript III (Invitrogen) was used to synthesize cDNA using the touchdown procedure described above (2.7) in order to overcome secondary structures. Initial cDNA created were used as template for the PCR reactions as per the RACE protocol from Invitrogen. Briefly, after cDNA synthesis, RNA template was digested and the single stranded cDNA purified using columns. The cDNA was poly-C tailed and used as PCR template for initial PCR reactions with a forward anchor primer binding to the poly-C 5' tail and a nested gene specific reverse primer. Subsequently nested PCR was

performed with a forward amplification primer binding to the anchor primer with a second nested gene specific reverse primer. RACE products were visualized by agarose gel electrophoresis, TOPO TA cloned (Invitrogen), and sequenced (Sequiserve) to determine the transcriptional start site. All RACE experiments were repeated with RNA samples from separate cultures.

Table 2. **Oligonucleotide primers used for primer extension and 5' RACE.**

Primer	Sequence (5´-3´)	Target region
cesP-IRD	GGACTCGTTATATTGGCTAACAG	*cesP*
cesT-IRD	GCTCTATTTCAATCCTATATTCTC	*cesT*
cesT-IRD2	CCATTAGCACTTCCACCTG	*cesT*
cesB-IRD	GATACCTTTGCTACTTGTCGTTC	*cesB*
cesH-R1	CCATGACCAATTTTAGGAATGATAAC	cDNA *cesH*
cesH-R2	GTGGTAATGCCCTTGCCATTATC	first PCR *cesH*
cesH-R3	GAATCGGTTTAACCAACGCACTG	first and nested PCR *cesH*
cesH-R4	CTGCTATGTCCATGTTCAGGTAGGTC	nested PCR *cesH*
cesP-R1	GTTCAACCGCATAAATCTTTATG	cDNA *cesP*
cesP-R2	TTTATGCTCTATGTGTTTCATTG	first PCR *cesP*
cesP-R3	CATTTTCCTTAATACATTATTAACTTTTCTTCG	nested PCR *cesP*
cesP-R4	CTTTCAGCGTCCAAATCTC	cDNA *cesP*
cesP-R5	CCATTCGTATGAGCAGTTCTCC	first PCR *cesP*
cesP-R6	CAATTTCCGAACAGCTAACCG	nested PCR *cesP*
cesT-R1	CGCTTCACCGTCTAATTGCTGA	cDNA *cesT*
cesT-R2	CTAGCGGTAGAAGCTCAATATTCGGATC	first PCR *cesT*
cesT-R3	GATCCAACAATCTTCGCCAC	nested PCR *cesT*
cesB-R1	CGCATTGTGAATCACTTCG	cDNA *cesB*
cesB-R2	ACGTGGTTCACATTCGGAAGGCGAAATAC	first PCR *cesB*
cesB-R3	GTTAAAGGGAGCGATGACATTG	nested PCR *cesB*
cesB-R4	GGCATCCAGTTTAAAGCAATATCATGAGC	cDNA *cesB*
cesB-R5	GTACTTGCTCGTAGAAATTTCTCTTG	first PCR *cesB*
cesB-R6	CCTTTCAGCATCCTCTCTGCTTCCATC	nested PCR *cesB*

2.9 Primer extension

Transcriptional start sites were confirmed using primer extension (PE). First strand cDNA synthesis was carried out by the addition of 10pmol IRD800 labelled gene specific reverse primer (Table 2) to 10µg RNA in a 7µL reaction volume, incubation 75°C 2min, 70°C 1min, 65°C 1min, 60°C 1min, 55°C 1min. 13µL RT mix were added (4µL 5x RT buffer, 1µL 0.1M DTT, 2µL 10mM dNTPs, 200U SuperScript III, and 20U RNaseOUT, all Invitrogen), and the reaction incubated at 55°C for 1h before inactivation for 15min at 70°C. For analysis 9µL SequiTherm Excel II Stop/Loading Buffer (Epicentre, USA) were added and the reaction incubated for 3min at 92°C prior to loading onto an 8% urea polyacrylamide gel. 1kb regions containing the putative promoters were amplified using PCR, cloned into the TOPO TA vector pCR2.1 (Invitrogen), sequenced using the SequiTherm Excel II DNA sequencing kit (Epicentre) and used as a sequencing ladder and size marker.

2.10 Transcript stability

Cultures were grown to mid-log phase (OD_{600} 10) and samples taken before (time zero) and in increments after addition of 200µgmL^{-1} rifampicin. RNA was isolated as described above (2.6) and used as template for real time PCR in the SmartCycler as described for quantitative real time PCR (2.15.3). Relative expression was determined using primers for *cesA* (cesA_for 5'-GATTACGTTCGATTATTTGAAG-3', cesA_rev 5'-CGTAGTGGCAATTTCGCAT-3') and normalized to 16S rDNA using primers previously reported (Martineau *et al.*, 1996). Relative expression was calculated using the REST evaluation method of Pfaffl (Pfaffl, 2001), using time point zero as the calibrator (RE=1.00).

2.11 Bio-informatics analysis

Two programs were used to analyze the *ces* sequence for putative transcriptional start sites and factors: the promoter prediction program at http://www.fruitfly.org/seq_tools/promoter.html (Reese, 2001) and the DBTBS search tool at http://dbtbs.hgc.jp/ (Ishii *et al.*, 2001). The terminator search tool (Brendel & Trifonov, 1984) available at Heidelberg Unix Sequence Analysis Resources (HUSAR;

http://genius.embnet.dkfz-heidelberg.de) and Mfold (Zuker, 2003) at Rensselaer Wadsworth (http://www.bioinfo.rpi.edu/applications/mfold/), were used for the prediction of termination structures. In silica sequence manipulation was performed using Vector NTI (Invitrogen). Frameshift slippery sites were predicted using the program FSFinder (http://wilab.inha.ac.kr/FSFinder/) (Moon *et al.*, 2004). Theoretical three dimensional protein structure models were created using SwissModel (Arnold *et al.*, 2006) at the Expert Protein Analysis System (ExPASy) server of the Swiss Institute of Bioinformatics (http://swissmodel.expasy.org/).

2.12 Construction of transcriptional fusions

2.12.1 Fusion vector construction

The putative *ces* promoter regions were fused to the reporter genes by ligating PCR amplicons of the sequentially deleted putative promoters into the multiple cloning site of the vectors. The red-shifted green fluorescent protein (GFP) *gfpmut3a* (Cormack *et al.*, 1996) reporter, without a promoter in vector pAD123 (Dunn & Handelsman, 1999) was used as a basis to perform sequential deletion analysis of the putative promoters to identify the active promoter region. The promoter-less luciferase *luxABCDE* reporter vector pXen1 (Francis *et al.*, 2000) was used as a basis to examine promoter activity in media and food of the central promoter identified using deletional analysis. For both vectors, PCR amplicon inserts were created with restriction enzyme recognition sites for EcoR1 and BamHI in the forward and reverse primer respectively. Amplicons were purified (Qiaquick PCR Purification Kit, Qiagen, Germany) and restricted (15µL eluted PCR amplicon in 25µL 2x Tango buffer with 50U each EcoR1 and BamHI (Fermentas, Germany) and re-purified as described again.

Plasmid vector isolated from six 3mL *E. coli* cultures was pooled and restricted in a total volume of 250µL 2x Tango buffer using 100U each of EcoR1 and BamHI for 45min 37°C, followed by inactivation at 65°C 20min. Vector was subsequently dephosphorylated with 1µL CIAP (calf intestinal alkaline phosphatase, Fermentas) at 37°C 30min prior to extraction with an equal volume chloroform. Plasmid DNA was precipitated with 100% ethanol and washed with 70% ethanol before drying and resuspending in 50µL water. Ligation was carried out with a 3:1 molar ratio of insert

to vector (total 300-600ng DNA) in a 10µL volume of 1x ligation buffer with 1U T4 DNA ligase (Invitrogen).

2.12.2 Bacterial transformation

E. coli strains TOP10 and INV110 were prepared as calcium chloride chemically competent cells and transformed using a standard heat shock protocol (Sambrook & Russell, 2001). TOP10 cells were used for the initial transformation of ligation reactions. However, to increase the rate of transformation in *B. cereus,* the plasmids were passaged through and prepared from the non-methylating INV 110 strain prior to electroporation of *B. cereus.*

B. cereus were transformed by electroporation; cells were prepared from 100mL early log phase culture (OD_{600} 0.4-0.7) in LB supplemented with 2% glycine grown in baffled flasks at 30°C with rotary shaking 150rpm. Cells were collected in pre-cooled 50mL centrifuge tubes (3500g, 4°C, 10min) and sequentially washed in ice cold 2.5%, 5%, and 10% glycerol before being resuspended in 10% glycerol, frozen in liquid N_2 and stored at -80°C. For transformation, up to 1µg plasmid was added to a 50µL cell aliquot in an Ecu-102 cuvette (Peqlab, Germany) and exposed to a pulse of 2.0kV, 5000Vcm^{-1}, 25µF, 200Ω for 4.6-4.8ms. Immediately 1mL LB was added and the cells permitted to recover (30°C, 2h) before plating onto pre-warmed LB_{CM7} agar.

2.12.3 Confirmation of correct ligation

Ligation was confirmed by transforming *E. coli* with 2µL ligation reaction and plating onto LB media with ampicillin (100µgml^{-1}) and culturing 14h 37°C. Transformants were picked and used to perform colony PCR with the respective forward primer used to initially create the PCR amplicon insert, and a reverse primer in the reporter gene: for the pAD123-based vectors it was situated in the *gfpmut3a* gene (GFP_R, 5'-GCCATGTGTAATCCCAGCA-3'), for the luciferase studies it was located in the *luxA* gene (LuxA_R, 5'-CCACACTCCTCAGAGATGCG-3'). Those transformants producing a PCR amplicon of the correct size were used to transform *E. coli* INV110. Plasmid DNA was prepared from overnight cultures of *E. coli* INV110 and *Bacillus* electroporated as described above. *Bacillus* were transformed with GFP fusion vectors to perform sequential deletion analyses to identify active promoters, or with luciferase fusion vectors to investigate promoter activity in various media and food.

2.13 Green fluorescent protein assay

B. cereus strains transformed with sequentially deleted promoter GFP fusion vectors as described above (2.12) were inoculated into a 3ml pre-culture grown for 14h at 30°C with rotary shaking 150rpm. The main cultures in baffled flasks were inoculated with 100µL of a 1:100 dilution of the pre-culture, and incubated at 30°C with 150rpm rotary shaking. 1.8ml samples were collected after 24h incubation, centrifuged (8000g, 4°C, 2min), washed with phosphate buffered saline (PBS) and the cells collected (8000g, 4°C, 2min). The cells were resuspended in 1mL PBS of which 200µL were distributed into four wells of black micro-well plates (NUNC, Germany) and measured in a Victor2 or Victor3 fluorometer (Perkin Elmer LAS, Germany) using 485nm as excitation and 520nm as emission wavelengths. GFP values were standardized to the culture OD_{600} at time of sampling. Background fluorescence was checked using as a negative control strains transformed with pAD123 (Dunn & Handelsman, 1999) containing the promoter-less *gfpmut3a*; the positive control was pAD43-25 (Dunn & Handelsman, 1999), where the constitutive uracil phosphoribosyltransferase (*upp*) promoter region drives expression of *gfpmut3a* (Cormack *et al.*, 1996).

2.14 Luciferase assay

B. cereus F4810/72 were transformed with the luciferase reporter gene vector pMDX[P_1/*luxABCDE*] derived from pXen1 (Francis *et al.*, 2000). Pre-cultures were inoculated and grown in 3mL LB at 30°C 14h with 150rpm rotary shaking.

Promoter activity kinetics were performed in a Victor2 fluorometer (Perkin Elmer). The overnight pre-culture (20µl) was used to inoculate 2ml media (LB_{Cm5}), of which 200µl was distributed into each of several wells in a white clear-bottomed microwell plate (NUNC, Germany). The plate was incubated at 30°C with 400rpm rotary shaking in a microwell plate incubator and shaker with a heated lid. Luminescence and OD (585nm) were measured for 0.1s on an hourly basis in the fluorometer. Relative luminescence was luminescence normalized to the OD_{585} of the culture as measured in the Victor2 (Perkin Elmer).

For investigation of activity on various media and foods, Petri dishes were inoculated with four 25µl drops, sealed to prevent moisture evaporation and incubated 30°C

24h. Luciferase activity from F4810/72 transformed with pMDX[P_1/*luxABCDE*] was visualized using a Hamamatsu 2400-32 image collector and processor with ImageMaster software (Xenogen Corporation, USA), the promoter-less vector pXen1 (Francis *et al.*, 2000) in the same strain was used as the negative control.

2.15 Quantitative real time PCR

2.15.1 Primer design

The primers developed for RT-qPCR are listed in Table 3. Primers were designed according to recommended criteria (Shipley, 2005) for *cesP*, *cesA* and *cesB* as well as *plcR* genes. Those directed against the *ces* genes were designed from the nucleotide sequence available under accession number DQ360825 (Ehling-Schulz *et al.*, 2006) at GenBank's database (http://www.ncbi.nlm.nih.gov/Genbank/). Primers targeting the *plcR* gene were designed from the *plcR* gene sequence of the emetic reference strain (Fricker *et al.*, 2008). Primers targeting the *rrn* gene were previously reported (Martineau *et al.*, 1996). Standard curves to calculate reaction efficiency were created for each target gene using decimal dilutions of a pool of sample cDNA (produced as above from sample RNA) as template and the amplification efficiency (E) calculated as per Pfaffl using $E=10^{(-1/slope)}$, which should be between 1.60 and 2.10 (Pfaffl, 2003). Prior to relative expression analysis, raw Ct values were examined to ensure stable amplification of the reference gene and melting curves analyzed to ensure fluorescence was obtained from the correct amplicon.

2.15.2 Reverse transcription

First strand synthesis of cDNA was performed in separate reactions for each gene using 100ng total RNA, 0.1µM gene specific reverse primer (Table 3), 0.5mM each dNTP, 10mM DTT, 100U SuperScript II, and 20U RNaseOUT in 1x first strand buffer (all Invitrogen). RNA and primer were incubated 75°C 2 min, 70°C 1 min, 65°C 1 min, 60°C 1 min, 55°C 1 min, 50°C 1 min, 45°C 1 min, and 42°C 1 min before adding the buffer and enzymes and incubating at 42°C 1h. Final enzyme inactivation was 70°C for 15 min, and cDNA stored at -20°C.

Table 3. **Oligonucleotide primers developed for RT-qPCR.**

Primer	Target	Sequence (5' – 3')	Nucleotide positions	Reference (Sequence)	Amplicon Length (bp)	Annealing Temp. (°C)
plcR_for	*plcR*	GTTATCCGAGAACATATGTCATC	5261106-5261084	(Fricker *et al.*, 2008)	244	57
plcR_rev	*plcR*	CTTTTTCAGCTCATTCCATACTC	5260884-5260863	(Fricker *et al.*, 2008)		57
cesP_for	*cesP*	GGTTATGCATCTTGTATACCG	5324-5345	DQ360825	260	57
cesP_rev	*cesP*	GATGAAGTGGAGATGATATAGAC	5561-5583	DQ360825		57
cesA_for	*cesA*	GATTACGTTCGATTATTTGAAG	9116-9137	DQ360825	197	53
cesA_rev	*cesA*	CGTAGTGGCAATTTCGCAT	9296-9312	DQ360825		53
MC67_cesA_for	*cesA*	GATTACGTTCGATTATTTGAAA	9116-9137	DQ360825	197	53
MC67_cesA_rev	*cesA*	CGTAACGGCAATTTCGCAT	9296-9312	DQ360825		53
cesB_for	*cesB*	TTAGATGGTATTCTTCACTTGGC	20181-20203	DQ360825	308	57
cesB_rev	*cesB*	TTGATACAAATCGCATTCTTATAACC	20463-20488	DQ360825		57
16SA1	*rrn*	GGAGGAAGGTGGGGATGACG		(Martineau *et al.*, 1996)	241	63
16SA2	*rrn*	ATGGTGTGACGGGCGGTGTG		(Martineau *et al.*, 1996)	241	63

2.15.3 Quantitative real time PCR

Quantitative real time PCR (qPCR) was performed using the SmartCycler (Cepheid, USA). Reactions were performed in 25µL volume using 1µL cDNA template from above (equivalent to 10ng total RNA) and 80 nM each primer in 1x qPCR Mastermix with SYBR Green I (ABgene). After polymerase activation (15 min 95°C), 40 amplification cycles were performed with a temperature ramp rate of 2.5C° s^{-1}, (95°C 30s, Tm 30s with optics on, 72°C 45s), followed by a melt curve from the Tm to 95°C at 0.2C° s^{-1} with optics on. All reactions were performed in triplicate. Melt curve analysis and agarose gel electrophoresis with subsequent ethidium bromide staining were used to ascertain the specificity of the reactions.

2.15.4 Relative expression calculation

Relative expression (RE) of *plcR* and *ces* to the *rrn* reference at all sampling times was calculated according to Pfaffl's REST method (Pfaffl, 2001; Pfaffl *et al.*, 2002) using the difference in Ct (cycle at which fluorescence of the reaction reaches the threshold level) values of the sample in question and a calibrator for both target and reference genes, to calculate the relative expression RE. For expression kinetics, the Ct values of the emetic reference strain F4810/72 at an OD_{600} of 1.0 (all *ces*) or 20 (*plcR*) was set as the calibrator for each respective gene for all strains. Sample to sample variation was corrected by using the 16S rDNA gene as a reference (*rrn*), which was expressed consistently during cultivation to 72h (data not shown).

$$RE = \frac{E_{target}^{(Ct_{target} - Ct_{control})}}{E_{reference}^{(Ct_{reference} - Ct_{control})}}$$

The REST method was also used to compare transcriptional regulation of samples grown under different conditions; for this the maximum transcript level observed from expression kinetics in each of the tested conditions was compared to the maximum under parallel reference conditions (strain F4810/72, sporulated pre-culture, LB media, 30°C), which was used as the calibrator.

3 Results

3.1 Transcript characterization

3.1.1 Cereulide synthetase genes a polycistronic transcript

A complete transcriptional analysis using RT-PCR was performed on the *ces* gene cluster. RT-PCR primers were designed such that the amplicons from each pair overlap; thereby the RT-PCR analysis covers the entire *ces* gene cluster (Figure 2). Analyses revealed transcripts between the genes for the modules incorporating D-O-Leu and D-Ala, and L-O-Val and L-Val, as well as transcripts in the non-coding regions between the CDS *cesP*, *cesT*, *cesA*, *cesB*, *cesC* and *cesD* (Figure 3). The amplicons from the cDNA were of the same size as those obtained using DNA as a template, whereas control reactions using RNA which had not undergone reverse transcription showed no amplicon. A primer located in the *cesD* coding region was used in combination with an antisense primer located after a predicted hairpin termination structure downstream of *cesD* (Figure 3, lane T); no transcript was observed, indicating that this inverted repeat represents the terminator of *ces* gene transcription. Thus, *cesPTABCD* are transcribed as a single 23 kb transcript. As well, the hypothetical pXO1 proteins pXO1-11, pXO1-23 and pXO1-10 (a hypothetical protein, reverse transcriptase and hypothetical methyltransferase, respectively (Ehling-Schulz *et al.*, 2006)) present downstream of the *ces* gene cluster were transcribed in the emetic reference strain (data not shown).

Rifampicin was added to mid-exponential phase cultures in order to halt transcription and investigate the stability of the *ces* gene transcript. RT-qPCR performed on RNA isolated from these samples was evaluated with the REST method (Pfaffl *et al.*, 2002) for relative transcript levels. Studies on rifampicin-treated cultures showed the polycistronic transcript to be unstable (Figure 4). Within 10 minutes 80% of *cesA1* mRNA was degraded.

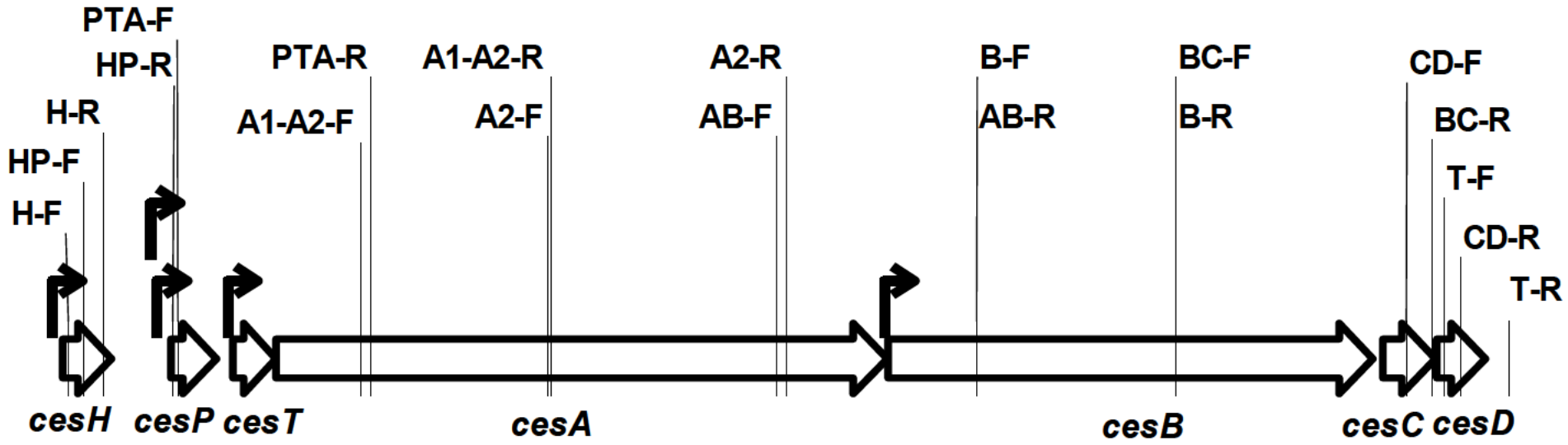


Figure 2. **The ces operon with RT-PCR primers.**

The *ces* operon showing location of the overlapping primer pairs used for transcript analysis, as well as the promoters detected by RACE and PE for *cesH* and *cesT*, the central promoters upstream of *cesP* and the intercistronic promoter upstream of *cesB* (bent arrow).

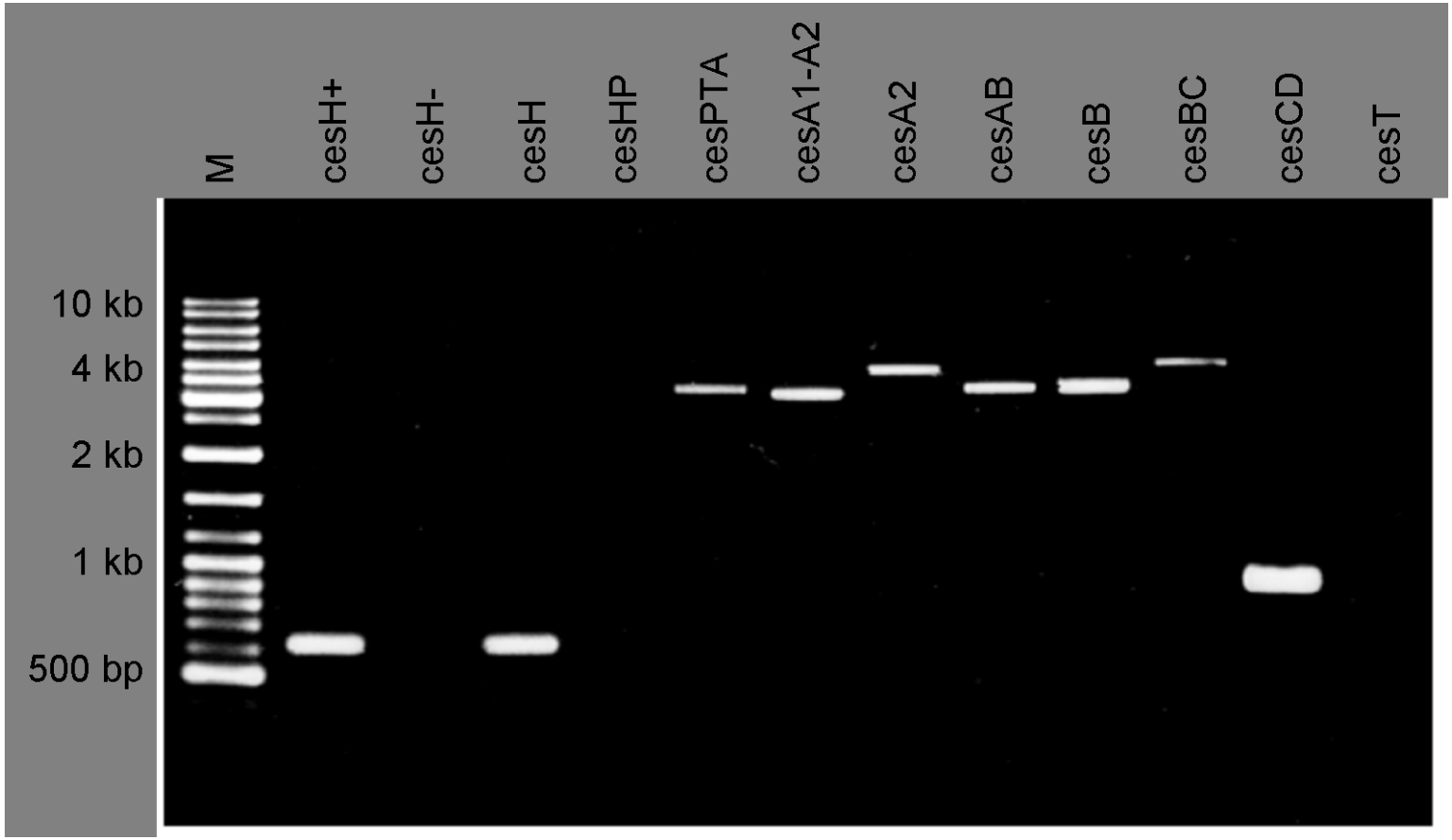


Figure 3. **Transcriptional analysis of *ces* gene cluster.**

RT-PCR shows the presence of consecutive transcripts between *cesP* and *cesD* but no transcripts between *cesH* and *cesP.* No transcripts were obtained from a forward primer located in *cesD* and a reverse primer located directly downstream of *cesD* after a predicted hairpin termination structure (see lane T). For each gene negative controls (RNA as template; see e.g. lane H-) and positive controls (DNA as template; see e.g. lane H+) were included. M: marker ladder mixture (Fermentas).

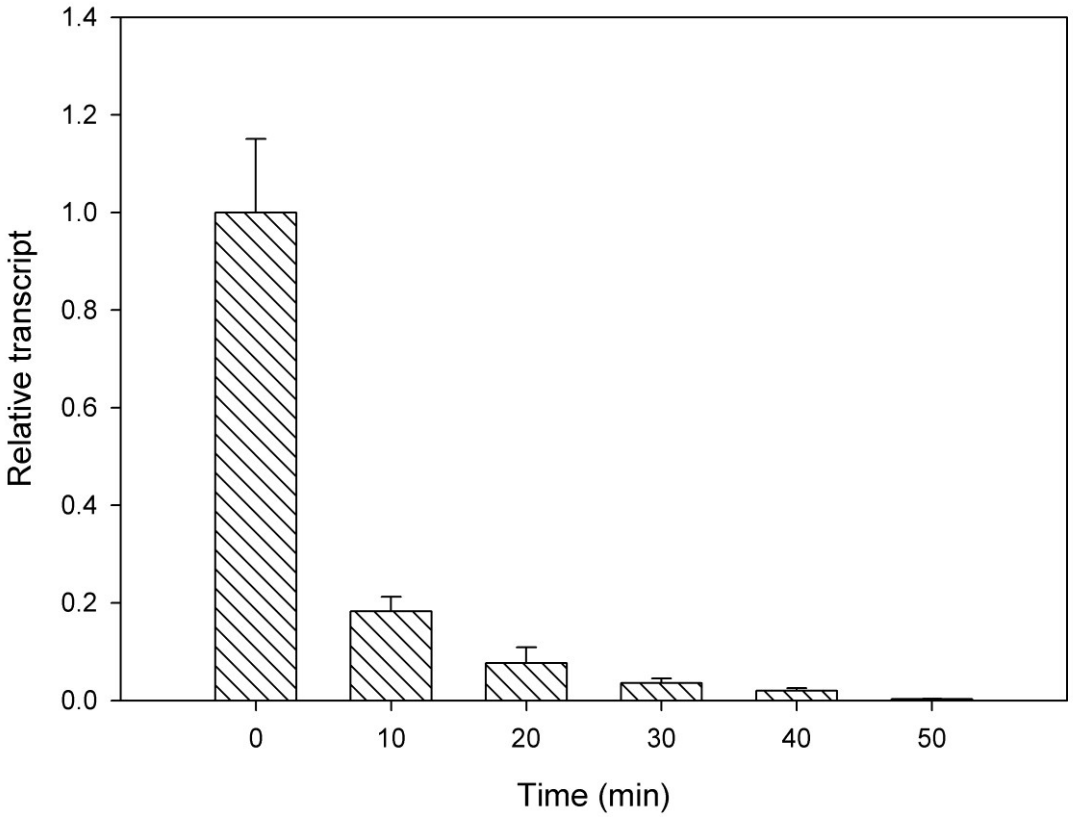


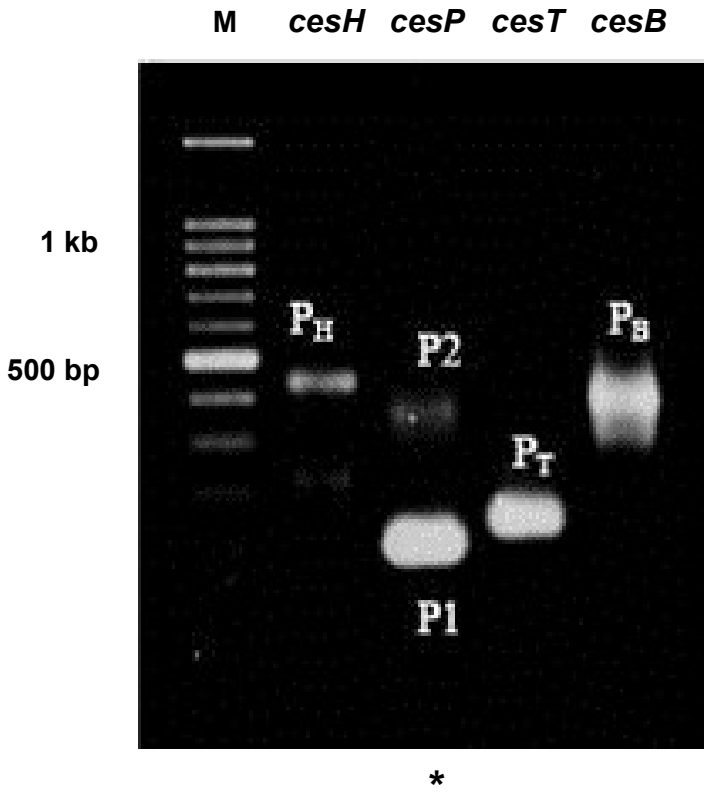
Figure 4. **Transcriptional stability of *ces* gene cluster.**

Transcriptional stability of *cesA* as determined by RT-qPCR analyzed using the REST method, shows a half life less than 10 min.

3.2 Promoter characterization

3.2.1 Central and intercistronic promoters detected

Transcription initiation sites were mapped using 5' RACE and primer extension (PE). From these studies, *cesH* was found to have a putative promoter 76bp upstream from the translational start, and *cesP* was found to have two putative promoters, one located 100bp upstream (P_1) from the start codon and a second promoter 256bp (P_2) from the translational start (Figure 5). Additional intercistronic promoters that might enhance transcription or be active under specific conditions were found upstream of *cesT* (56bp, Figure 6) and *cesB* (80bp upstream) but not upstream of *cesA* or *cesC*.



*cesP

```
                -35                          -10   (P2)+1
AATGATTTATCTATATTCTGTAAACAAATGTTTTCAAAATCAATATAAA

AAGTTAACGGAATACCAATTCTGTTAGCCAATATAACGAGTCCCCAAAA

AAGTAATCGTTGCCGTACCCGTATGAAATTGAAATAAATAAAATTATTA
                      -35                      -10
ATATTCTAATAATTCGTATTGCGTGAATATTAATAATTGTTTATAATGA
     +1(P1)
AAATTGTATAATTTTGGTTAATTAACTTGTGCGAAGAAAAGTTAATAAT
                                                RBS
GTATTAAGGAAAATGAATGTTTTTGTATTTTACTTTTTTTAAAAGAGGG
   CesP M  K  H  I  E  H  A  N  S  I  K  I  Y  A
ATTTTTCAATGAAACACATAGAGCATAAAAATTCCATAAAGATTTATGC
```

Figure 5. **5' RACE mapping of *ces* promoter sites.**

RACE products detected for *cesH* and *cesPTABCD* indicate that intercistronic promoters are present (P_T, P_B). RACE products were subcloned and sequenced; promoters are marked (P). The central *cesP* promoters P_1 and P_2 are indicated by an asterisk and the *cesP* promoter region sequence is depicted to the right. Promoters (P_1, P_2) and transcription start sites (+1) are indicated. The translation start (ATG) is printed in bold face. A putative ribosome binding site (RBS) and putative -10 and -35 σ^A recognition sites (deduced from the consensus sites for *B. subtilis*) are underlined.

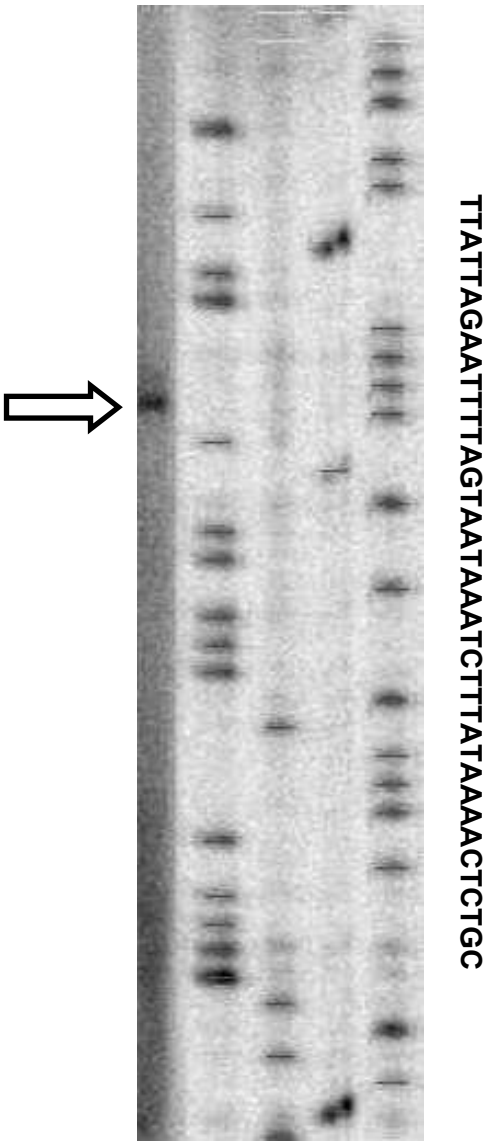


Figure 6. **Primer extension (PE) mapping of *ces* promoter sites.**

Primer extension gel of *cesT* promoter 56bp upstream shown alongside *ces* region, sequenced with a primer (6041-6079bp) different than that used for PE.

The central *ces* promoter region, including transcriptional start sites P_1 and P_2, of 12 weakly and highly emetic strains were sequenced and compared to the emetic reference strain F4810/72. No differences were found in the 420bp sequenced upstream of the translational start codon (Figure 7).

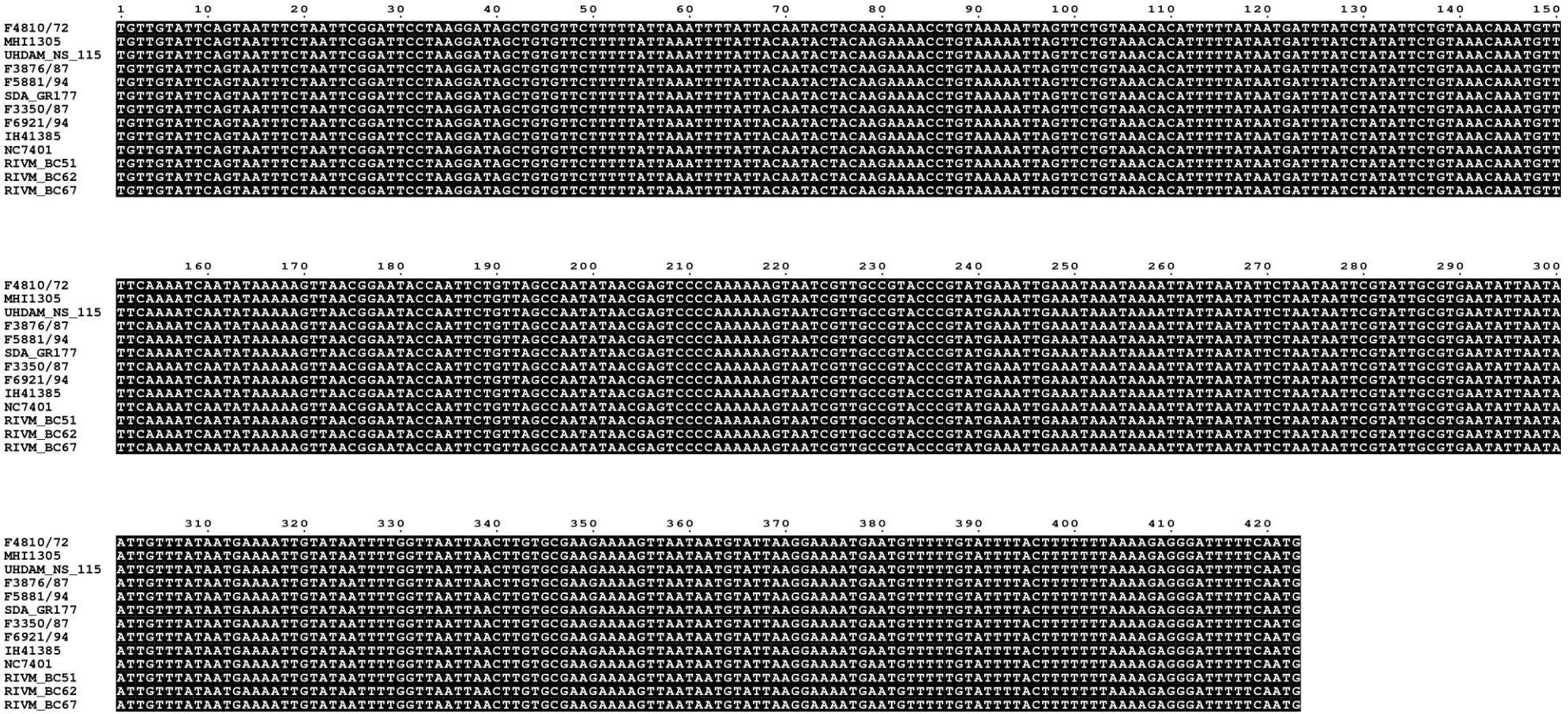


Figure 7. **Sequencing of *ces* promoter region.**

Promoter region sequenced 420bp upstream of the *cesP* start codon (ATG, position 421-423) in 12 highly and weakly emetic *B. cereus* strains: MHI1305, UHDAM NS 115, F3876/87, F5881/94, SDA GR177, F3350/87, F6921/94, IH41385, NC7401, RIVM BC51, RIVM BC62, and RIVM BC67 in addition to the reference strain F4810/72.

3.2.2 Sequence analysis

RT-PCR shows the strong presence of transcripts in the non-coding region, indicating co-transcription of *cesPTABCD* via frameshifting and stop codon read-through. Promoter studies indicated an intercistronic *cesB* promoter. In the *cesA* region there are six potential ATG start codons after the *cesT* stop codon and +1 frameshift prior to the conserved A1 core motif in *cesA*. In *cesB* there are eight potential ATG start codons downstream of the *cesA* stop codon and upstream of the conserved A1 core motif in *cesB*, however it is not yet known which of these eight is the actual translational start codon. The putative intercistronic promoter located in *cesB* is at position 17248 in the *ces* sequence DQ360825 (Ehling-Schulz *et al.*, 2006) at GenBank's database (http://www.ncbi.nlm.nih.gov/Genbank/); this putative *cesB* transcriptional start site is 80bp upstream of the third of the eight possible start codons, and 51bp upstream of a predicted RBS (AGGAGG), however no canonical -10 or -35 sites were found. The RACE and primer extension studies also showed a transcriptional start site located at base pair 16762, which is 272bp upstream of the first ATG after the *cesA* stop codon, though this is still within the CDS of *cesA*.

3.2.3 Deletion analysis shows strong main promoter

In order to investigate the activity of putative promoter regions and perform deletion analyses to narrow down the active promoter, these were amplified by PCR and cloned into promoter-trap vectors based on pAD123 (Dunn & Handelsman, 1999), containing a promoter-less red-shifted green fluorescent protein (GFP) gene, *gfpmut3a* (Cormack *et al.*, 1996). Fusion vectors created are shown in Table 4. Strains of *B. cereus* transformed with the resultant fusion vectors (pMKD series) showed no difference in growth characteristics, or in cereulide toxin production of emetic strains, from that of their respective WT (Figure 8). Of the fusion vectors created, the short *cesH* promoter construct showed moderate activity, equivalent to that of the positive control, pAD43-25 (Dunn & Handelsman, 1999) containing the constitutive promoter *B. cereus* promoter *upp* (data not shown).

Table 4. **Promoter fusion vectors.**

Vector	Promoter	Promoter Region	Reference
pAD123	none		(Dunn & Handelsman, 1999)
pAD43-25	*upp*		(Dunn & Handelsman, 1999)
pMKD [P_H/*gfpmut3a*]	P_H	3173-3346	this study
pMKD [P_T/*gfpmut3a*]	P_T	6081-6170	this study
pMKD [P_1-P_{2+}/*gfpmut3a*]	P_1 & P_2	4684-5162	this study
pMKD [P_1-P_2/*gfpmut3a*]	P_1 & P_2	4798-5146	this study
pMKD [P_2/*gfpmut3a*]	P_2	4798-4930	this study
pMKD [P_1/*gfpmut3a*]	P_1	4909-5146	this study
pMKD [P_0/*gfpmut3a*]	P_0 (3' of P_1)	5060-5146	this study
pMKD [P_{B2}/*gfpmut3a*]	P_B	16733-17612	this study
pXen1	none		(Francis *et al*., 2000)
pMDX [P_{P1}/*luxABCDE*]	P_1	4909-5146	this study

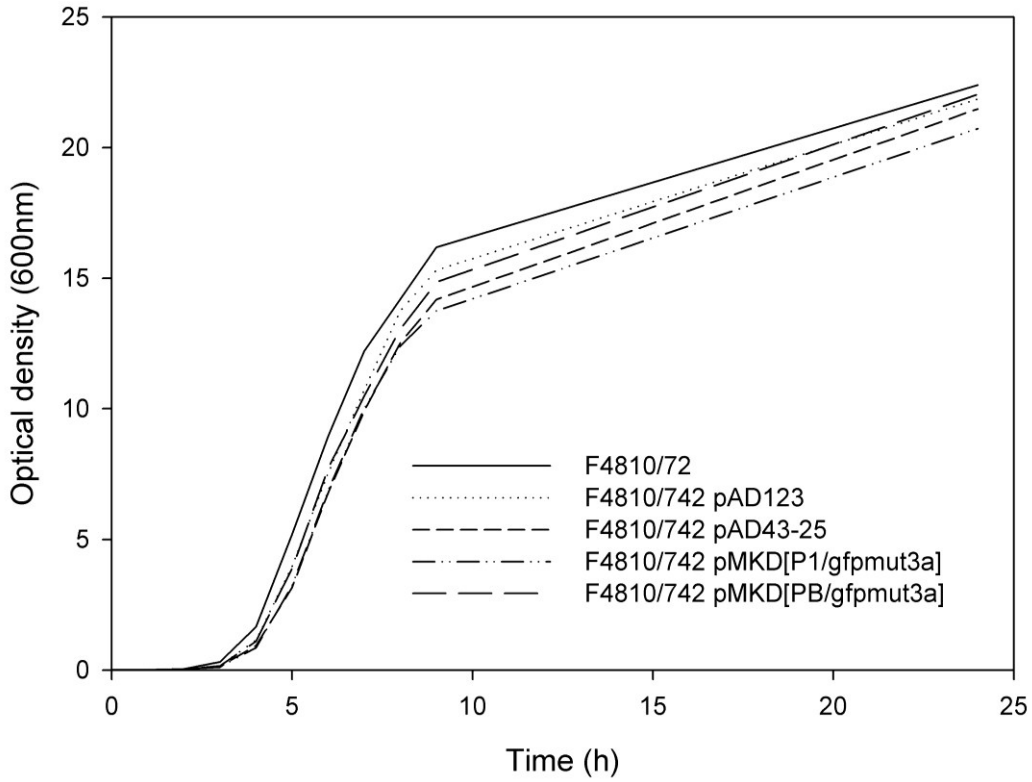


Figure 8. **Growth curves of *B. cereus* F481072 with GFP reporter gene plasmids.**

Emetic reference strain F4810/72, WT, and transformed with GFP fusion vectors, without (pAD123), and with various promoters grown in LB at 30°C 150 rpm. Graphed curve represents mean values of two independent experiments.

The promoter region of *cesP* was highly active whereas no significant activity of the intercistronic *cesT* promoter was detected under the tested conditions (it was similar to the promoter-less vector pAD123) these conditions. The translational start of *cesB* is not yet known, and there are eight possible methionine (Met) start codons upstream of the first conserved NRPS A core motif. Therefore a dozen *cesB* putative promoter regions were amplified and cloned into pMKD fusion vectors. Of these *cesB* intercistronic promoter fusions, the only one active in the tested conditions was a construct which essentially covered the entire region downstream of *cesA* and upstream of the last ATG prior to the first conserved *cesB* A domain, including both putative promoters determined by 5' RACE and PE.

From sequential deletion analysis of the central promoters P_1 and P_2, it can be seen that P_1 is the main promoter active in the tested conditions (Figure 9, Figure 10). The longest construct (P_1-P_2+) was less active than when a similar but shorter construct in which both promoters were still present (P_1-P_2). Promoter P_2 alone was not active in these conditions, and as well, downstream of the P_1 transcriptional start site, P_0 was not active either. Therefore P_1 appears to be the central promoter active under these culture conditions.

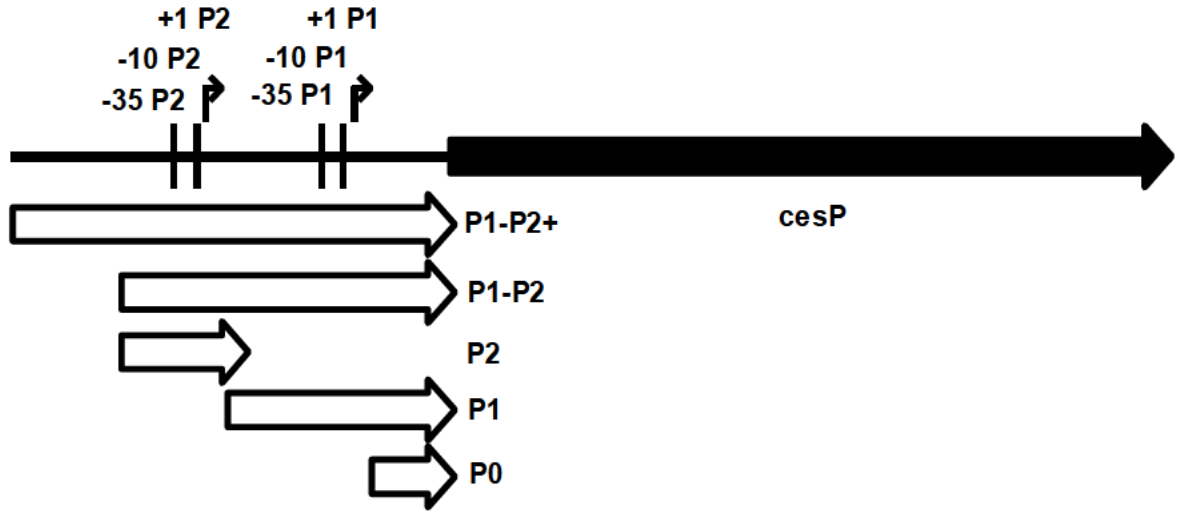


Figure 9. **Deletional analysis of central *ces* promoter.**

Putative promoter regions upstream of *cesP* transcriptional starts P_1 and P_2 were sequentially 5' and 3' shortened and cloned upstream of the reporter gene *gfpmut3a* in vector pMKD (for construct description see Table 4).

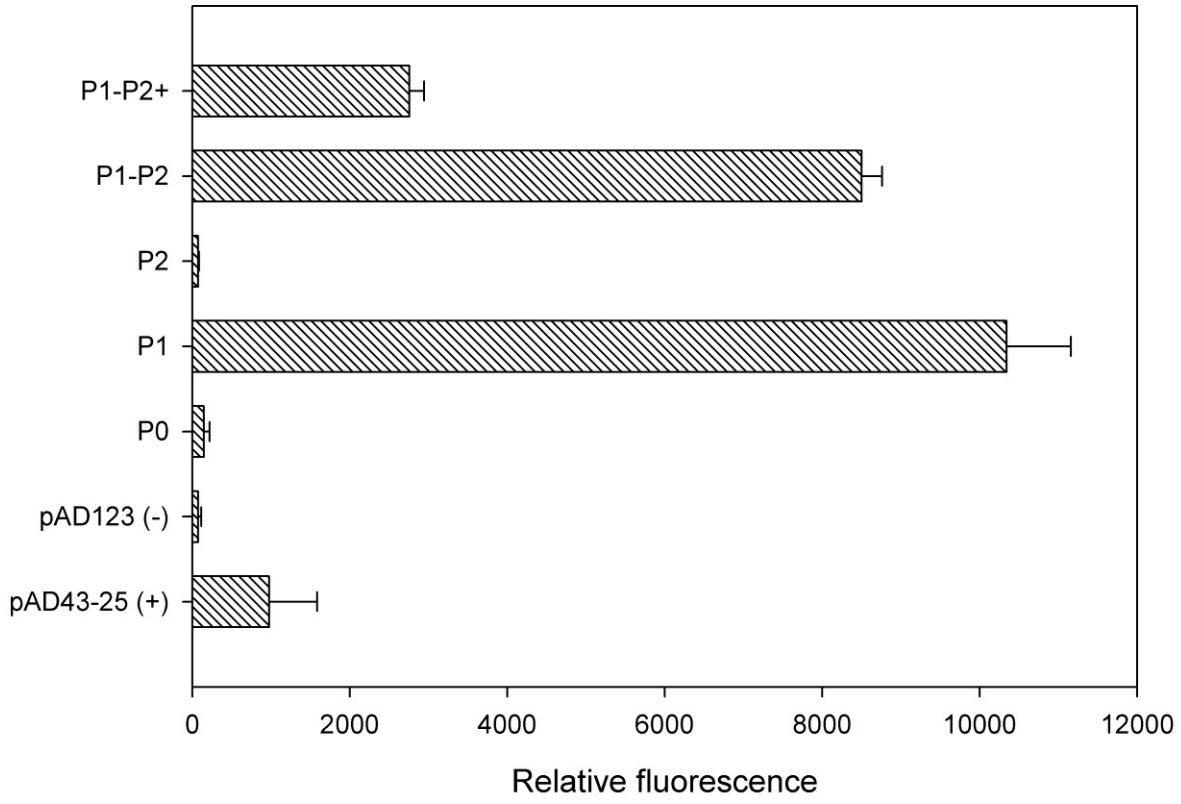


Figure 10. **GFP reporter activity of deletional constructs of central promoter.**

GFP activity from 24h cultures of *B. cereus* F4810/72 transformed with promoter-*gfpmut3a* fusion vectors. Fluorescence was normalized to OD_{600} of culture. All values are means and error bars standard deviations of four independent experiments.

The P_1 promoter region from the recently described emetic *B. weihenstephanensis* strain MC67 (Thorsen *et al.*, 2006) was also used to create promoter fusion vector pMKD[P_1_MC67/*gfpmut3a*] and transform the reference strain F4810/72. The MC67 P_1 promoter (P_1_MC67) had much lower activity than the same promoter region amplified from the reference strain. However, the P_1 promoter region amplified from the reference strain F4810/72 was similarly active in both the reference and MC67 strains (Figure 11).

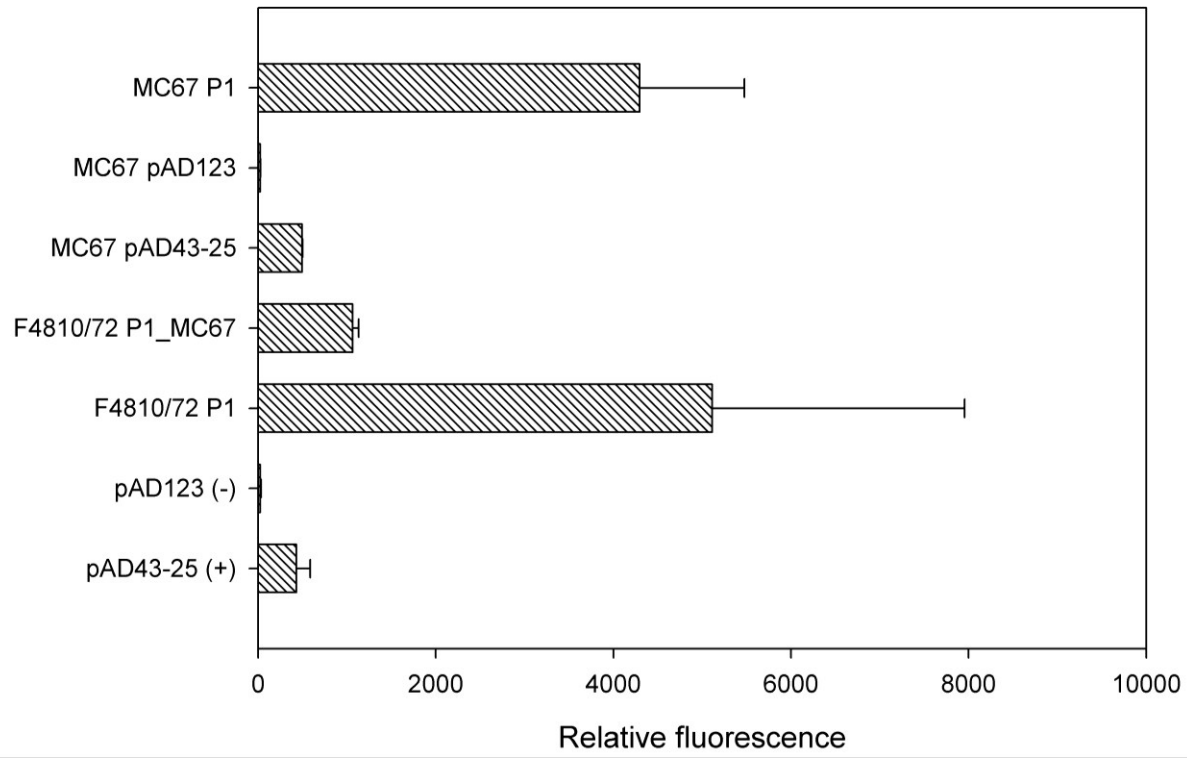


Figure 11. **Activity of central promoter from *B. weihenstephanensis* MC67.**

Promoter constructs in the reference strain F4810/72 and the emetic *B. weihenstephanensis* strain MC67. P_1 amplified from the reference strain F4810/72. P_1_MC67 amplified from *B. weihenstephanensis* strain MC67. All values are means and error bars standard deviations of two to three independent experiments.

3.2.4 Comparison of highly and weakly emetic strains

In order to determine if differences in toxicity had a genetic basis the entire cereulide synthetase gene cluster of the weakly emetic strain IH41385 was sequenced. Several differences in the coding sequence result in changes to the amino acid in strain IH41385 compared to F4810/72, most noticeably, a stop codon introduced in *cesB* (Table 5). The effect of this stop codon did not prove significant, however, when investigated with RT-qPCR for transcriptional kinetics. The transcriptional kinetic analyses indicated that the level of *cesB* expression in IH41385 was proportional to that of *cesA* and *cesP*, when comparing all three to the expression in F4810/72: *cesB*

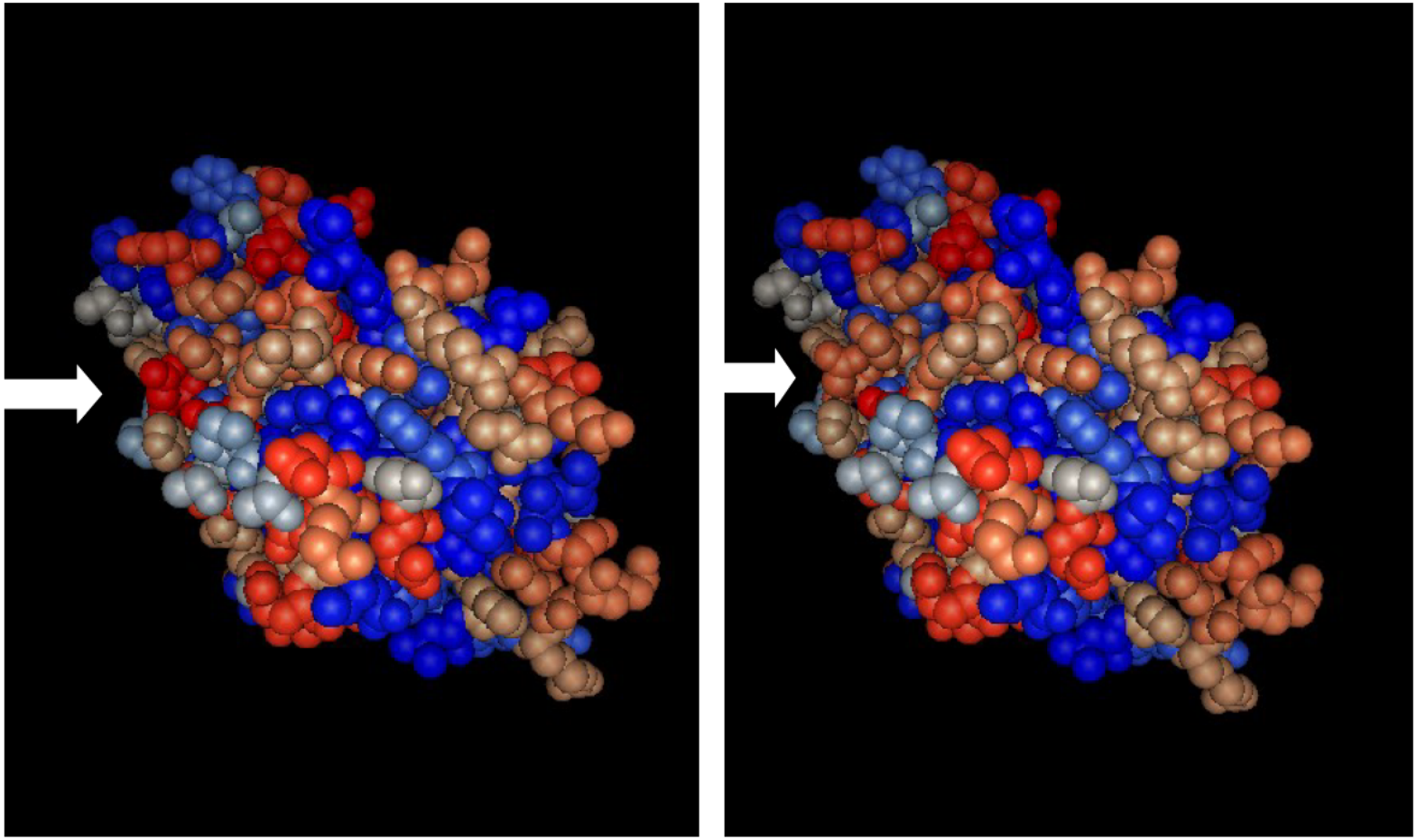
was lower in IH41385, but in the same proportion as the lower expression of *cesP* and *cesA* as well.

Table 5. **Differences in *ces* sequence of F4810/72 versus IH41385.**

Position in *ces* (GenBank DQ360825)	*ces* gene	Base in F4810/72	Amino acid F4810/72	Base in IH41385	Amino acid IH41385	Effect in IH41385
5748	*cesP*	G	aspartate	A	asparagine	
5910	after *cesP*	C	non coding	A	non coding	non coding
6198	*cesT*	T	glycine	C	glycine	silent
6293	*cesT*	T	valine	A	glutamate	
8975	*cesA*	G	valine	A	isoleucine	
14661	*cesA*	C	serine	T	phenylalanine	
15343	*cesA*	T	histidine	A	glutamine	
19982	*cesB*	T	cysteine	A	opal STOP	STOP
22643	*cesB*	G		A		silent
24731	*cesB*	G		A		silent
25468	*cesC*	G	arginine	A	histidine	

The IH41385 *ces* sequence was used to create theoretical *in silica* three dimensional models of the NRPS modules. This was only possible to perform with the 4'-PP module, since no suitable template structure (required to create a new theoretical model) were available in the SwissModel protein structure repository for any of the other modules in the cereulide synthetase. Structure comparison of the 4'-PP, with one amino acid change, from aspartate in F4810/72 to asparagine in IH41385, did not lead to readily observable differences (Figure 12). However, the amino acid changes in the other cereulide synthetase modules could affect the folding, binding efficiency and overall effectiveness of the resultant NRPS enzyme in its ability to catalyze the assembly of cereulide.

To investigate whether elements regulating the P_1 promoter from the reference strain were unique to emetic strains, the P_1 promoter fusion (pMKD[P_1/*gfpmut3a*]) was used to transform non-emetic, emetic-like and emetic *B. cereus* strains. GFP activity measured as fluorescence indicated that the promoter was similarly active in all the strains tested (Figure 13).



F4810/72 **IH41385**

Figure 12. **Theoretical 3D protein models of 4'-PP module from F4810/72 and IH41385.**

Comparison of 4'-PP module of emetic reference strain F4810/72 and weakly emetic IH41385 showing the single change: polar asparagine (F4810/72) versus acidic aspartate (IH41385).

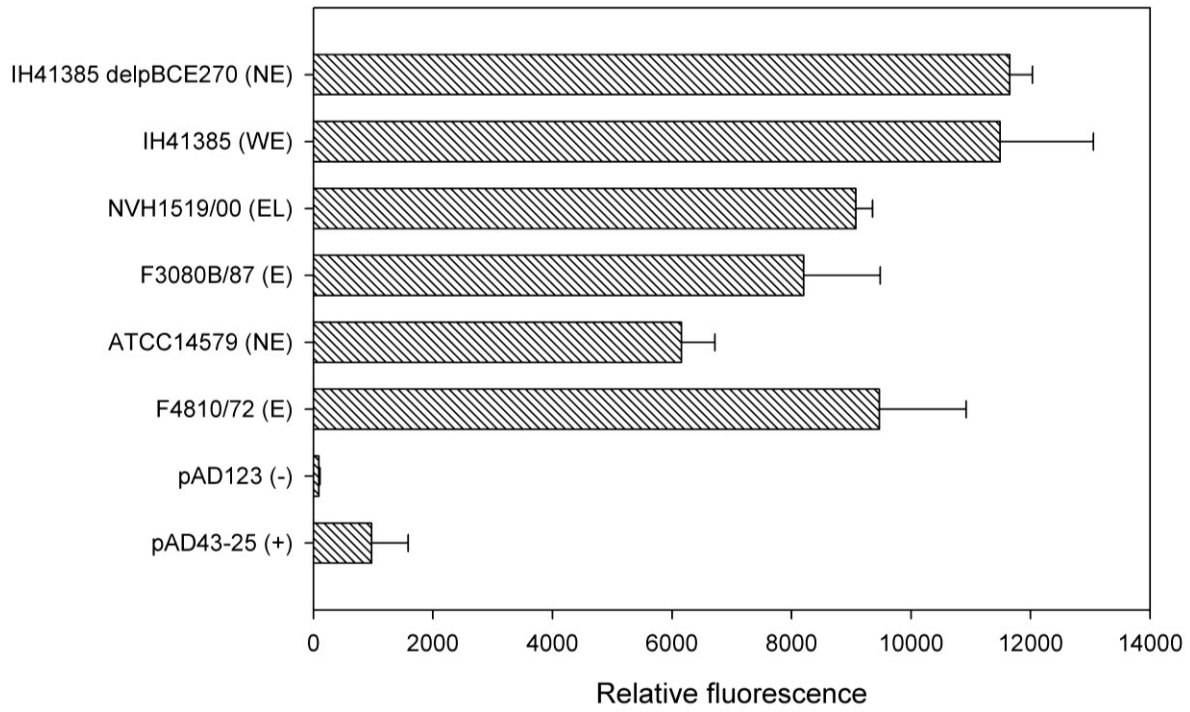


Figure 13. **Central promoter activity in various *B. cereus* strains.**

Promoter fusion vector pMKD[P_1/*gfpmut3a*] with the central *cesP* promoter, P_1, transformed into various *B. cereus* strains. IH41385delpBCE270, weakly emetic strain without plasmid containing *ces* gene cluster; IH41385, weakly emetic strain; NVH1519/00, emetic-like strain; F3080B/87, highly emetic strain: ATCC14579 non-emetic type strain; F4810/72 highly emetic reference strain; pAD123, promoter-less vector as negative control; pAD43-25, constitutive promoter *upp* driving *gfpmut3a* as positive control. All values are means and error bars standard deviations of two to three independent experiments.

3.2.5 Promoter activity in supplemented media

Promoter construct activity was measured in LB supplemented with 0.2% D-glucose with or without an amino acid cocktail of 0.3gL^{-1} of L-valine, -leucine and –threonine (Figure 14, Figure 15, Figure 16). The hydrolase promoter P_H activity increased in response to glucose supplementation, though the effect was somewhat milder when amino acids were supplemented as well. Overall the activity of the hydrolase promoter was similar to the constitutive *upp* promoter in pAD43-25 (Figure 14).

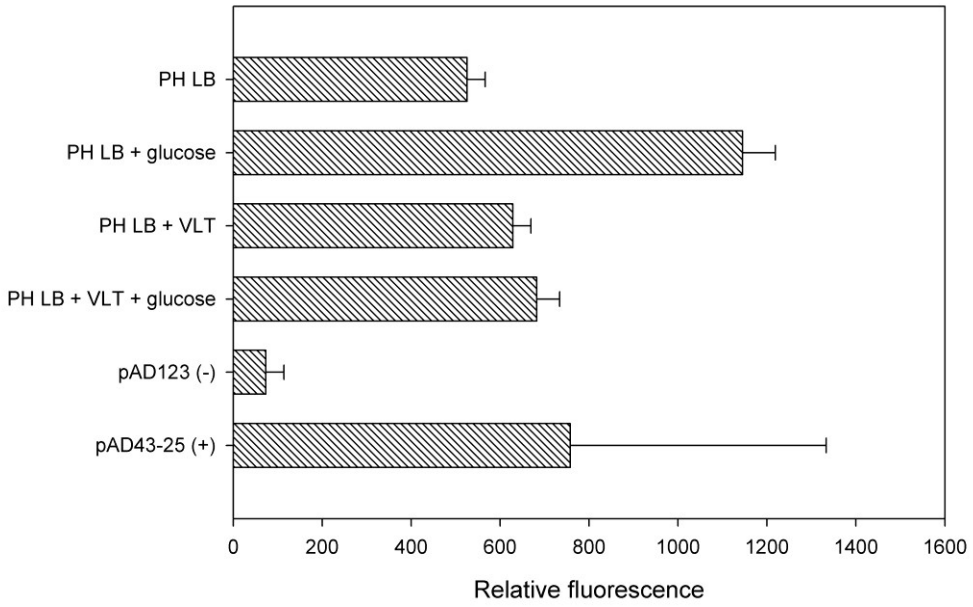


Figure 14. ***cesH* promoter activity affected by media supplements.**

Promoter fusion vector pMKD[P_H/*gfpmut3a*] with the putative hydrolase *cesH* promoter, P_H activity measured as GFP fluorescence after 24h 30°C culture growth in LB media supplemented with 0.2% D–glucose, with or without VLT amino acids (0.3gL^{-1} L-valine, -leucine and –threonine). The promoter-less vector pAD123 was the negative control; pAD43-25 with constitutive *upp* promoter driving *gfpmut3a* was the positive control. All values are means and error bars standard deviations of two to three independent experiments.

The central *ces* P_1 promoter also increased its activity in the presence of glucose supplementation, with or without additional amino acid supplementation (Figure 15). Although not active in normal LB media, the *cesB* promoter construct showed increased activity in response to amino acid supplementation in combination with glucose (Figure 16).

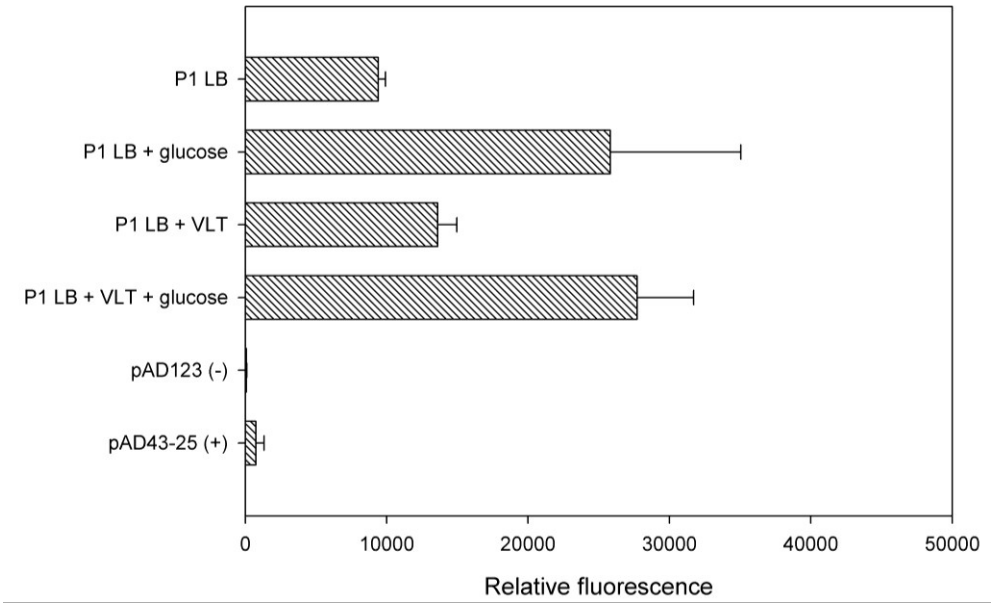


Figure 15. **Central *ces* promoter P_1 activity affected by media supplements.**

Promoter fusion vector pMKD[P_1/*gfpmut3a*] with the central *cesP* promoter, P_1 activity measured as GFP fluorescence in LB supplemented with 0.2% D–glucose, with or without VLT amino acids (0.3gL^{-1} L-valine, -leucine and –threonine). All values means and error bars standard deviations of two to three independent experiments.

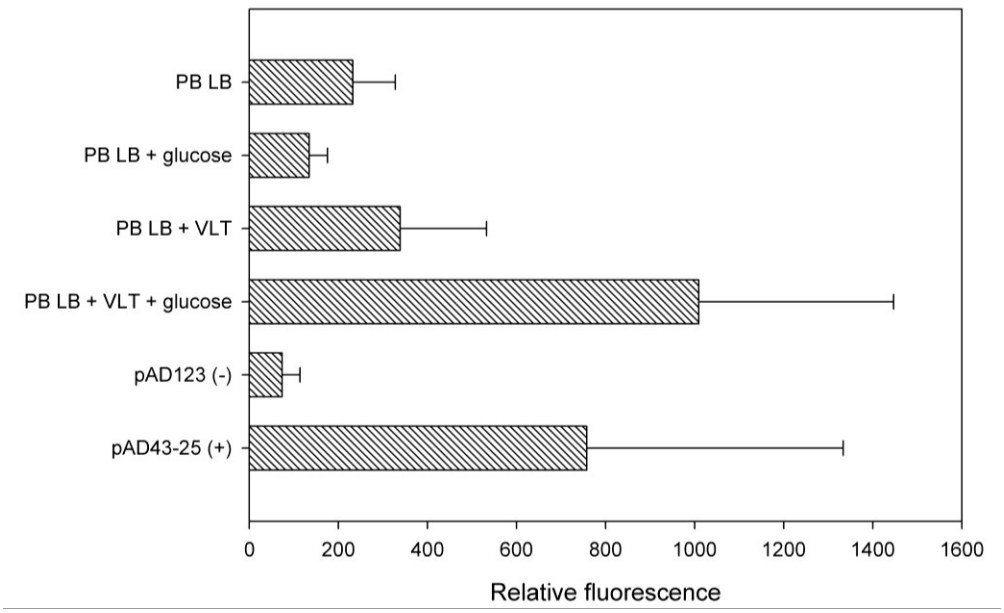


Figure 16. **Intercistronic *cesB* promoter activity affected by media supplements.**

Promoter fusion vector pMKD[P_B/*gfpmut3a*] with intercistronic *cesB* promoter region, P_B activity measured as GFP fluorescence in LB supplemented with 0.2% D–glucose,

with or without VLT amino acids (0.3gL^{-1} L-valine, -leucine and –threonine). All values means and error bars standard deviations of two to three independent experiments.

3.2.6 Promoter activity in media and foods

Since emetic outbreaks have frequently been associated with farinaceous rather than proteinaceous foods, the promoter activity on several selected foods, including cream cheese, ground beef, rice and semolina noodles was tested. As well, the activity on several types of media commonly used to culture *B. cereus* was observed: blood, brain heart infusion (BHI), mannitol-egg yolk-polymyxin B (MYP), polymyxin B-egg yolk-mannitol-bromthymol blue (PEMB), plate count (PC) and lysogeny broth (LB). The central promoter P_1 was cloned upstream of the luciferase gene (*luxABCDE*) optimized for use in Gram positive bacteria in pXen1 (Francis *et al.*, 2000) to create pMDX[P_1/*luxABCDE*] and transform the emetic reference strain F4810/72. Promoter activity as measured by luminescence was very strongly influenced by the growth medium (Figure 17, Figure 18).

On media based on or including rich proteinaceous ingredients (blood, brain heart infusion) promoter activity was less than on media that did not include or contained only minimal levels of such ingredients, but had higher sugar levels (glucose or mannitol) (Figure 17).

Correspondingly, little *ces* promoter activity was observed on proteinaceous fatty foods such as ground beef and cream cheese, whereas farinaceous, mainly carbohydrate, foods such as corn starch, instant mashed potato flakes, rice and noodles, high levels of *ces* promoter activity were observed (Figure 18). Within the group of farinaceous foods, those that induced the highest levels of promoter activity were also those most commonly associated with outbreaks, white rice and noodles.

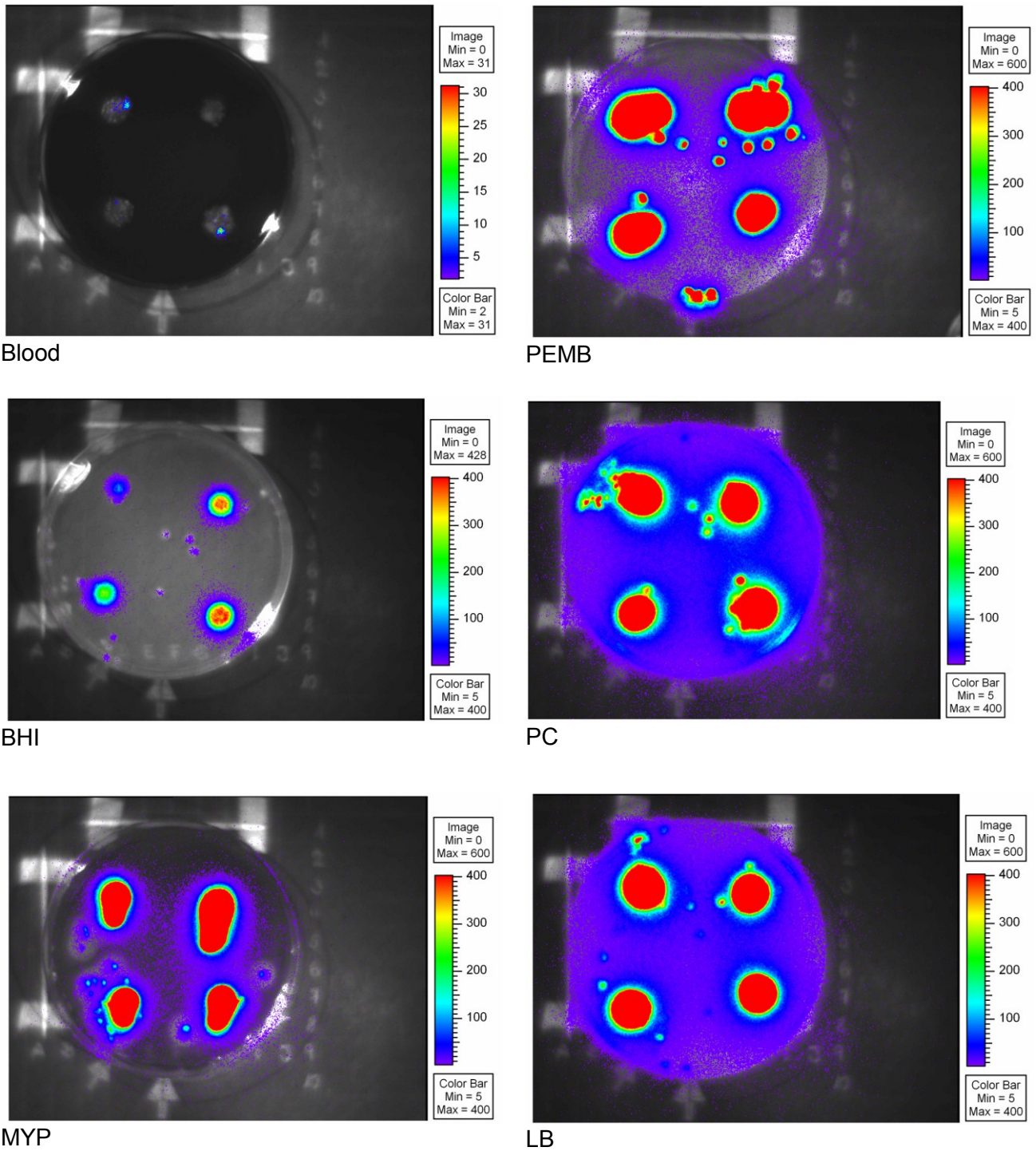
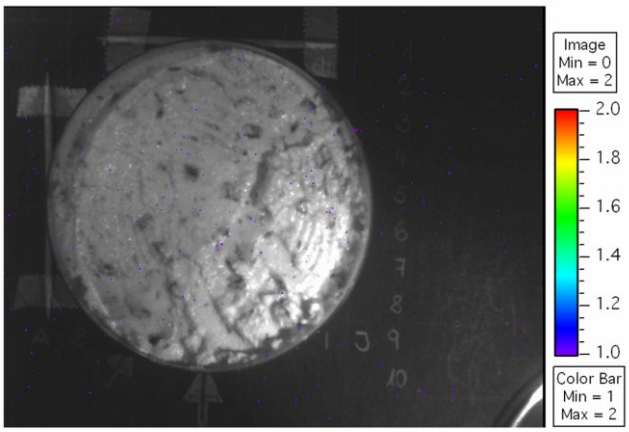
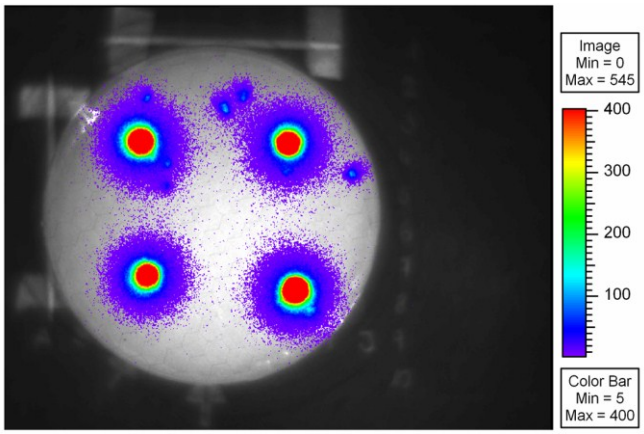


Figure 17. **Central *ces* promoter activity in various culture media.**

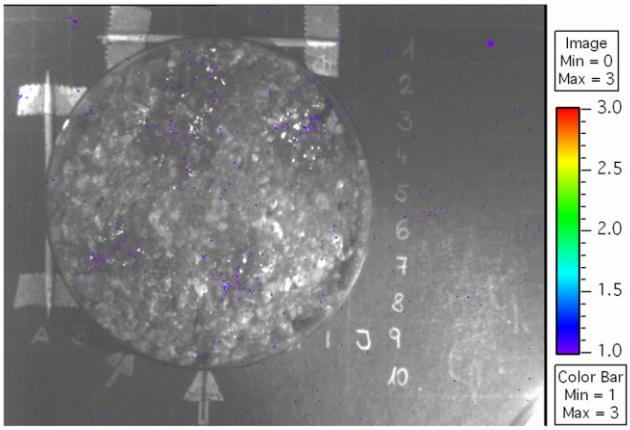
Promoter activity measured as luminescence after 24h 30°C culture growth on various solid media. *B. cereus* F4810/72 transformed with pMDX[P_1/*luxABCDE*] with the central promoter, P_1 upstream of the luciferase cassette. Media tested were blood, lysogeny broth (LB), brain heart infusion (BHI), mannitol yolk polymyxin B (MYP), plate count (PC) and polymyxin B egg yolk mannitol bromthymol blue (PEMB).



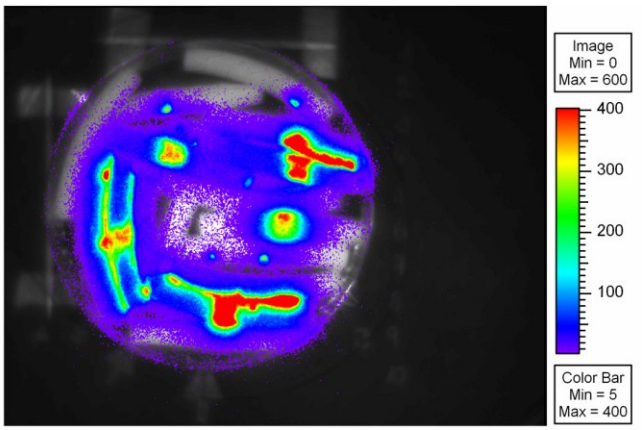
Cream Cheese



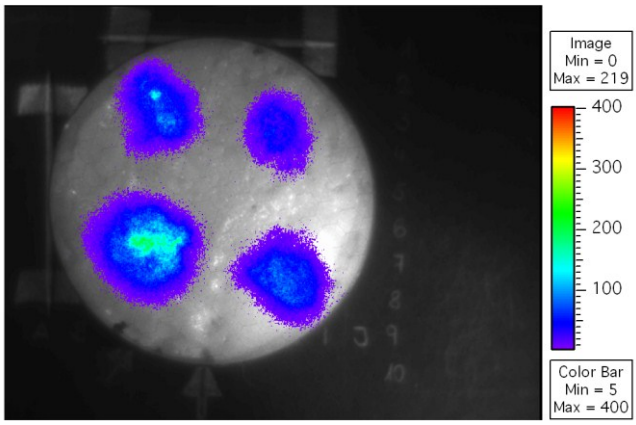
Corn Starch



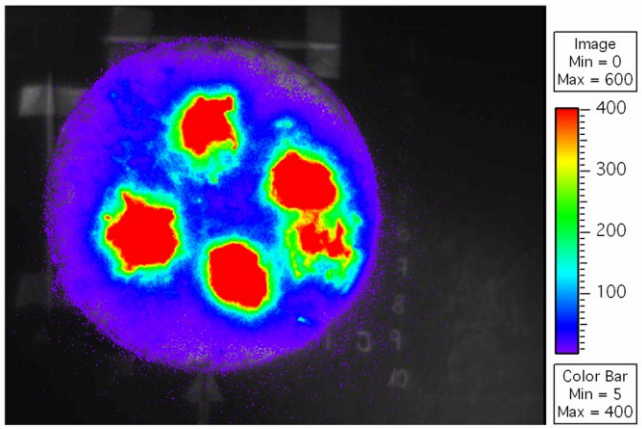
Ground Beef



Noodles



Mashed Potato Flakes



Sushi Rice

Figure 18. **Central *ces* promoter activity in various foods.**

Promoter activity measured as luminescence after 24h 30°C culture growth on various foods. Strain *B. cereus* F4810/72 was transformed with pMDX[P_1/*luxABCDE*] with the central *cesP* promoter, P_1 upstream of the luciferase cassette. Foods tested were cream cheese, fresh ground beef, instant mashed potato from flakes, corn starch, semolina noodles and white sushi rice.

3.3 Transcriptional kinetics

The highly emetic reference strain F4810/72 demonstrated a typical growth pattern in LB media at 30°C as measured by optical density at 600nm (Figure 20, Figure 21); the weakly emetic strain IH41385 grew in a similar fashion (Figure 22). The cultures' initial cell density at inoculation was 10^3 cfu ml^{-1}, and exponential growth progressed after a lag phase of 5-6h. Logarithmic growth continued until 20h, at which stationary phase was reached, characterized by a stable culture density; at this point the cultures were sporulated. Sporulation occurred synchronously in late logarithmic phase and progressed rapidly. The cultures remained in stationary phase until approximately 24h, after which progressive lysis occurred, indicated by a decrease in culture density and the formation of foam. Cereulide toxin could first be detected in strain F4810/72 using the cell assay after 12 hours in mid-logarithmic phase, and levels increased throughout exponential phase until stationary phase at which it was maximal and remained stable thereafter.

3.3.1 SYBR Green I assay

A SYBR Green I assay was developed to monitor the relative expression of the cereulide synthetase *(ces)* and pleiotropic regulator (*plcR*) genes. Primers were designed for *cesP*, *cesA* and *cesB* as well as *plcR* genes (Table 3). Primers directed against *cesP*, *cesA* and *cesB* were designed from the nucleotide sequence available under accession number DQ360825 in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/). Those designed for *cesA* target a novel α-ketoreductase in the *cesA1* domain which is unique to the cereulide synthetase gene cluster (Ehling-Schulz *et al.*, 2006; Magarvey *et al.*, 2006). The *cesA* primers for the *B. weihenstephanensis* strain MC67 were optimized by sequencing the PCR fragment obtained with the *B. cereus ces* primers and optimizing the oligonucleotide primers to that of the sequenced MC67 amplicon.

The assays were tested for their range and efficiency of amplification by creating standard curves of serial dilutions from an initial pool of cDNA synthesized using the respective gene specific reverse primers. The linear detection range for the respective primers using cDNA as a template for standard curves was equivalent to 100ng to 10pg total RNA for *ces* & *plcR* primers, and 10ng to 10fg for the *rrn* primers. In this assay the amplification efficiencies using cDNA were 1.96 for *cesP*, 1.77 for both *cesA* and *cesB*, 1.95 for the *plcR*, and 2.04 for the *rrn* gene. To confirm the efficacy of DNase digestion and confirm absence of contaminating DNA, each RNA sample was used as PCR and real time qPCR template without reverse transcriptase. No SYBR fluorescent signals were detected, nor were any PCR bands visible on gels (data not shown).

In order to study the *ces* gene expression SYBR Green I based reverse transcription quantitative real time PCR (RT-qPCR) was performed. Samples were taken for RT-qPCR to detect mRNA levels of the cereulide synthetase *(ces)* and *plcR* (pleiotropic regulator) normalized to *rrn* (16S rDNA). The same amount (100ng) of DNA-free total RNA was used for all reverse transcription reactions with gene specific reverse primers to maximize detection of low level transcripts.

In order to normalize and compare the relative expression using the Pfaffl method, a calibrator (with a relative expression value of 1.00) is required. The calibrator chosen was the emetic reference strain expression at an OD_{600} of 1.0 for the *ces* genes, and at an OD_{600} of 20 for *plcR*, as these were consistent throughout experiments. All relative expression kinetics of the reference and weakly emetic strains were calibrated to these. Transcript of *plcR* and all targeted *ces* (*cesP, cesA* and *cesB*) were detected in both *B. cereus* strains F4810/72 and IH41385 throughout exponential growth, the pattern of *plcR* and *ces* gene expression was similar in both when grown in baffled flasks (Figure 20, Figure 22). For *plcR,* expression was highest during early exponential phase, dropped off sharply after mid-logarithmic phase and remained at this low level. Transcript of *ces* could first be detected at an OD_{600} of approximately 0.5, increasing to a maximum during mid-exponential growth, after which it decreased to levels similar to that in very early exponential phase. Cereulide synthetase expression kinetics observed using RT-qPCR were confirmed by *ces* P_1 promoter-luciferase reporter gene assays in F4810/72 (Figure 19).

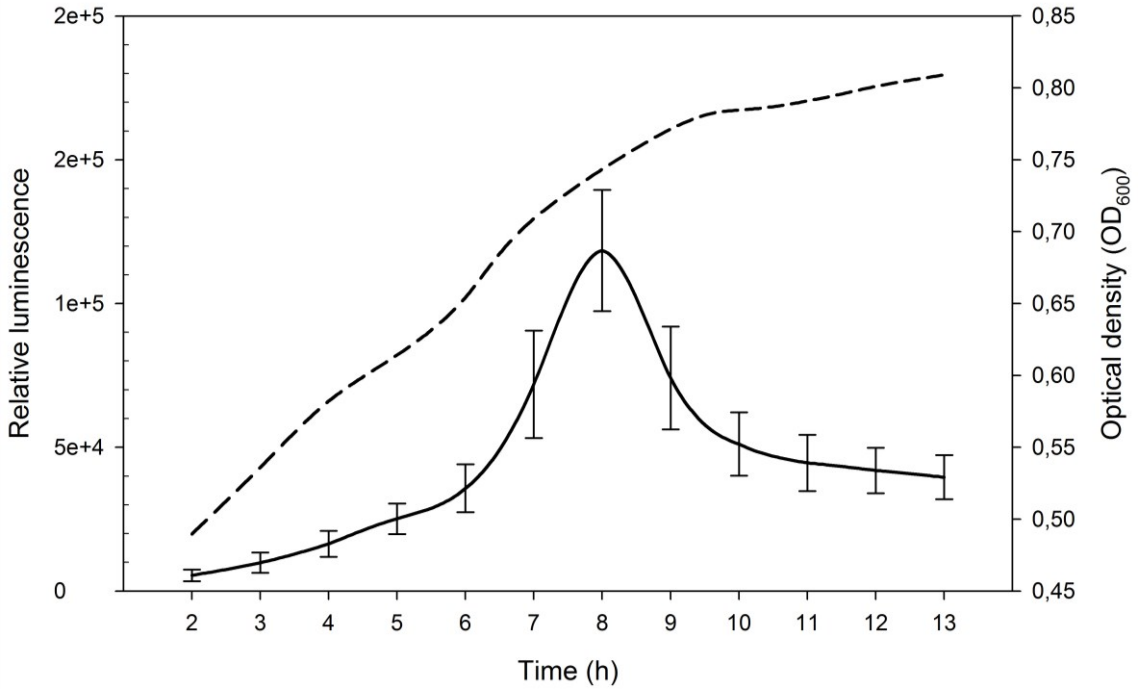


Figure 19. **Kinetics of *ces* P_1 promoter activity measured by luminescence.**

Activity measured by relative reporter gene luciferase expression during growth at 30°C in LB media with rotary shaking 150rpm in baffled flask, of *B. cereus* F4810/72 transformed with pMDX[P_1/*luxABCDE*] Dashed line optical density (OD_{600}) and solid line luminescence. All values are means and error bars standard deviations of two independent experiments.

3.3.2 Growth-phase dependent expression independent of plcR levels

The emetic reference strain F4810/72 had an exponential growth rate of 1.8 and sporulated synchronously late in exponential growth; *ces* transcript and cereulide expression were analyzed throughout (Table 6). Relative expression (RE) of *cesP* was 192, *cesA* 139 and *cesB* 97 times higher during mid- compared to early exponential phase (Figure 20). During fermentation *cesA* relative expression peaked during mid-exponential phase at 363, reflecting a 2.6 fold up-regulation during fermentation compared to flask cultivation (Figure 21). Similar cereulide toxin levels were detected in baffled flask cultures (662µgml^{-1}) and the fermenters (701µgml^{-1}) despite the higher *cesA* transcript levels in the fermenter, and its faster growth rate (4.1). Interestingly, *plcR* transcript levels (RE 140) were the same in flasks as well as fermenters.

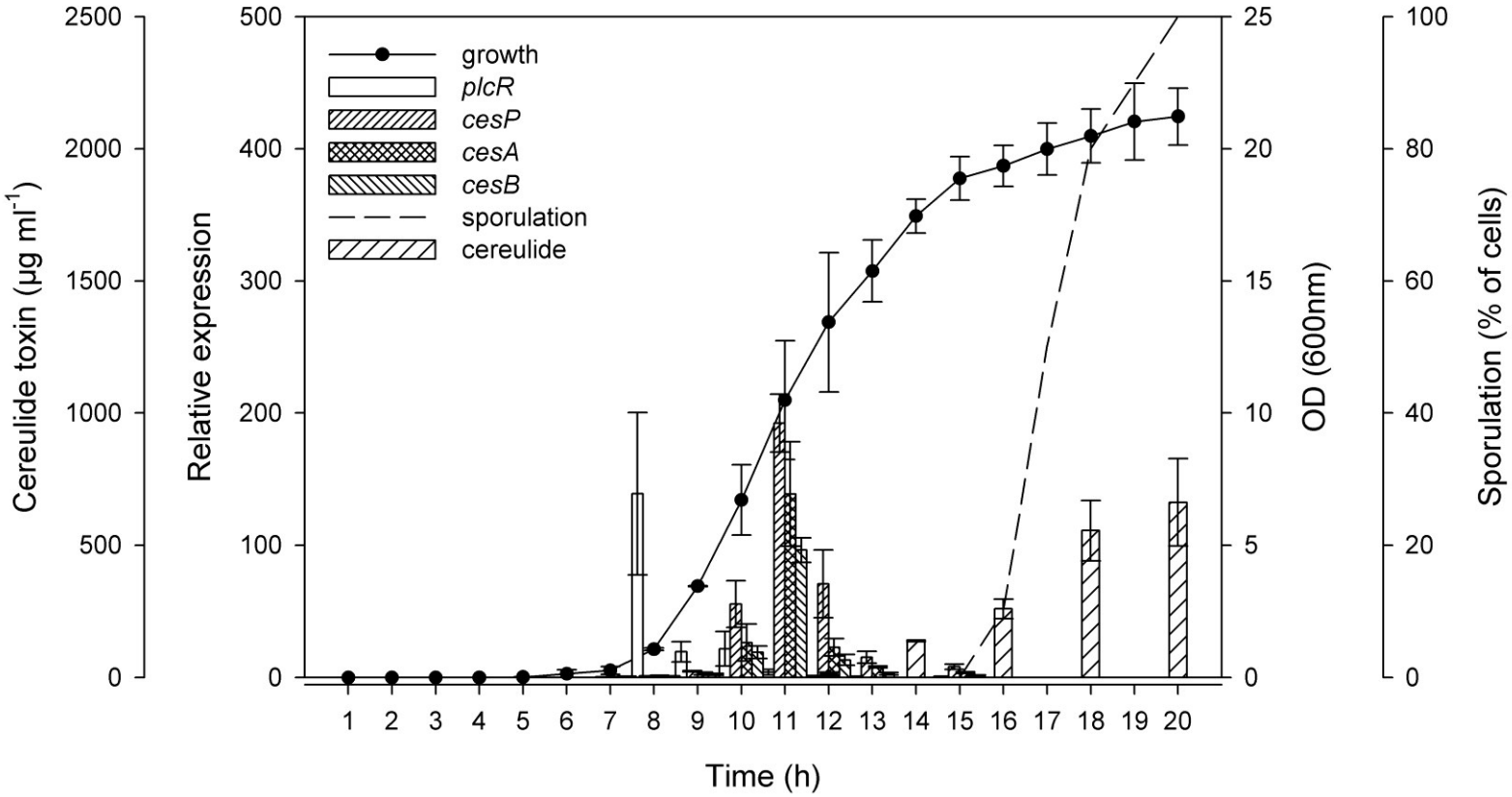


Figure 20. ***B. cereus* F4810/72 expression kinetics using RT-qPCR.**

Kinetics of relative *ces* and *plcR* gene expression, sporulation and cereulide toxin synthesis during growth at 30°C in LB media with rotary shaking 150rpm in baffled flask of *B. cereus* emetic reference strain F4810/72. All values are means and error bars standard deviations of three independent experiments.

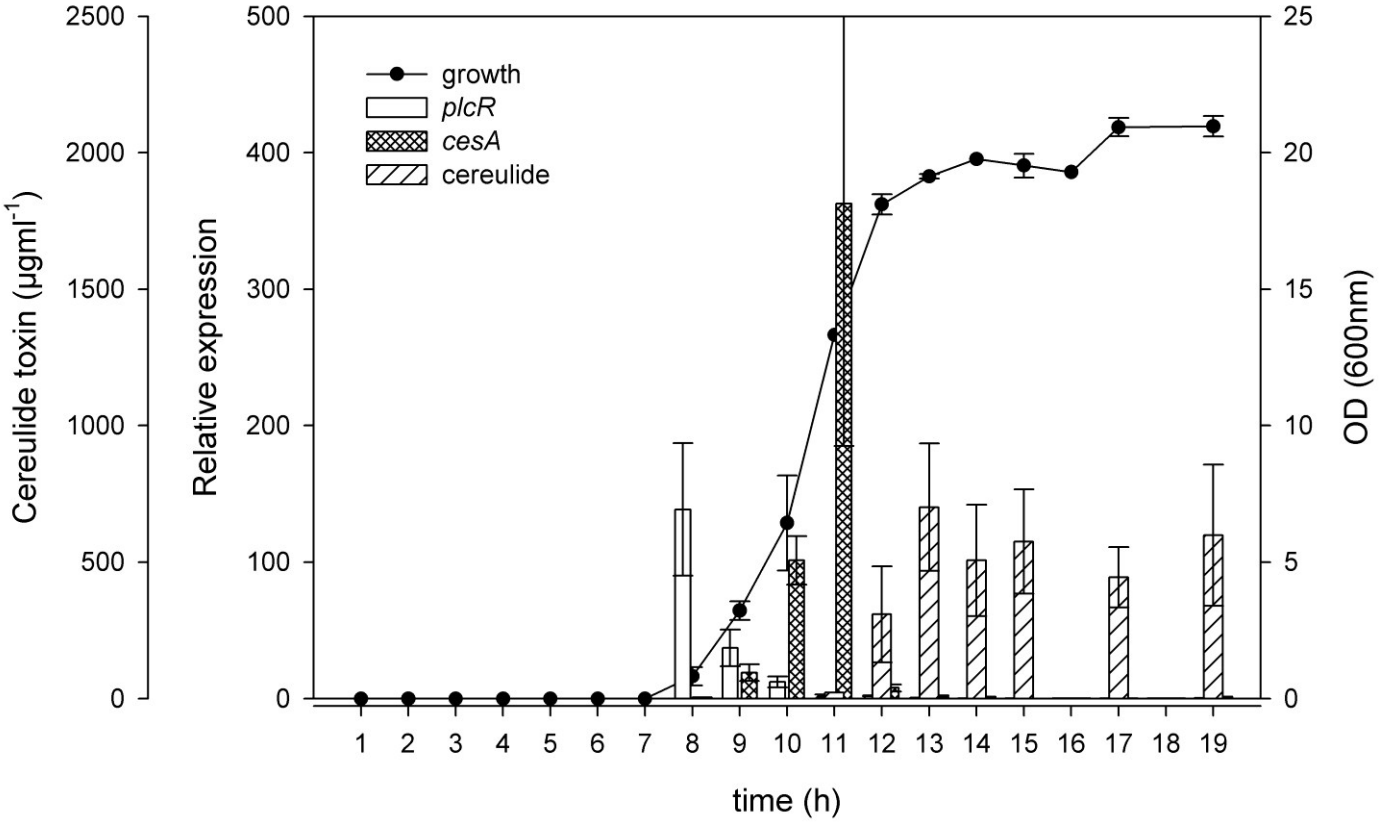


Figure 21. ***B. cereus* F4810/72 fermenter expression kinetics using RT-qPCR.**

Kinetics of relative *ces* and *plcR* gene expression, sporulation and cereulide toxin synthesis during F4810/72 growth at 30°C in LB media in a Biostat fermenter. All values are means, and error bars standard deviations, of two independent experiments.

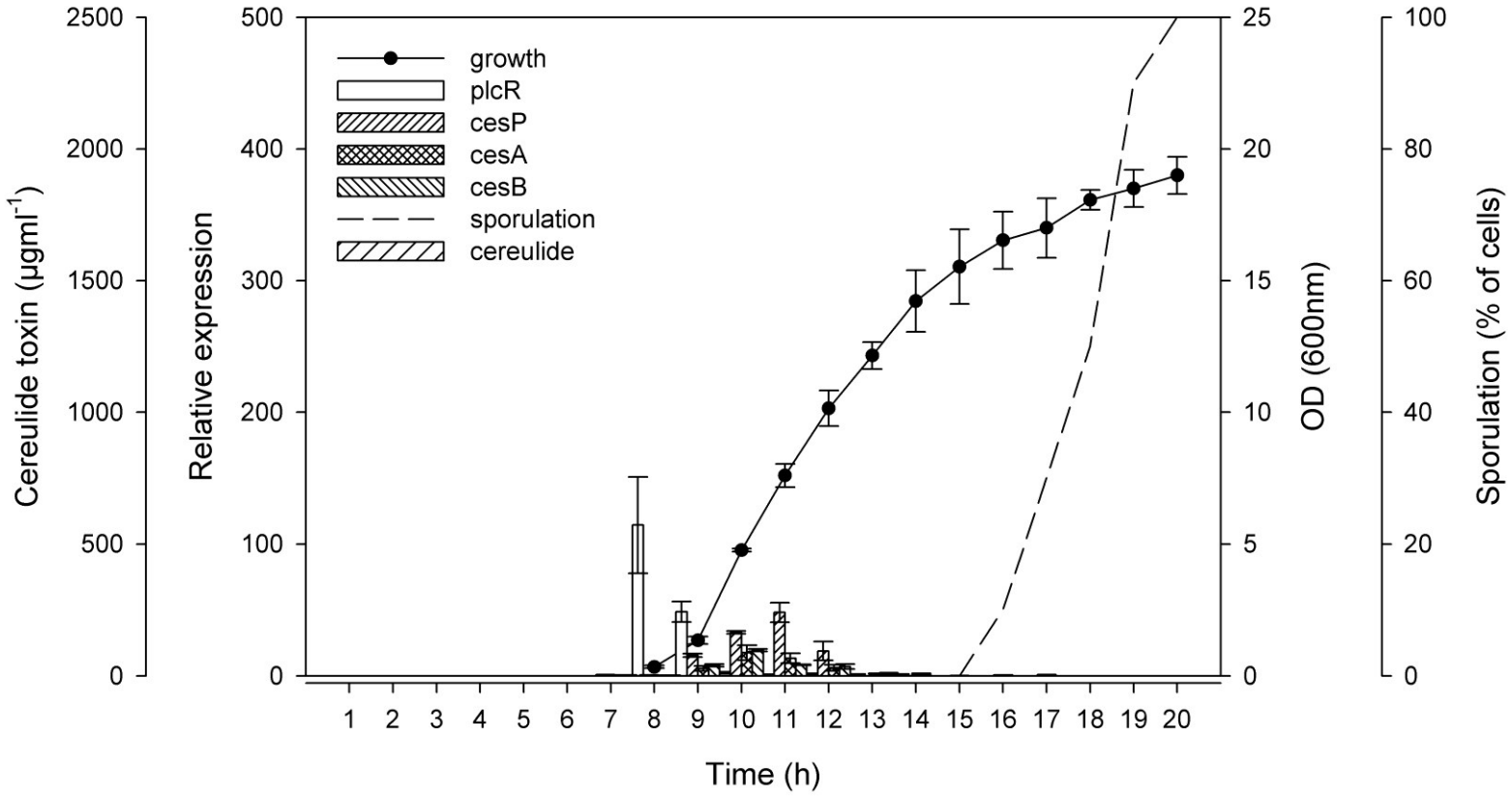


Figure 22. ***B. cereus* IH41385 expression kinetics using RT-qPCR.**

Kinetics of relative *ces* and *plcR* gene expression, sporulation and cereulide toxin synthesis during growth at 30°C in LB media in shaken baffled flask of weakly emetic strain IH41385. All values means and error bars standard deviations of two independent experiments.

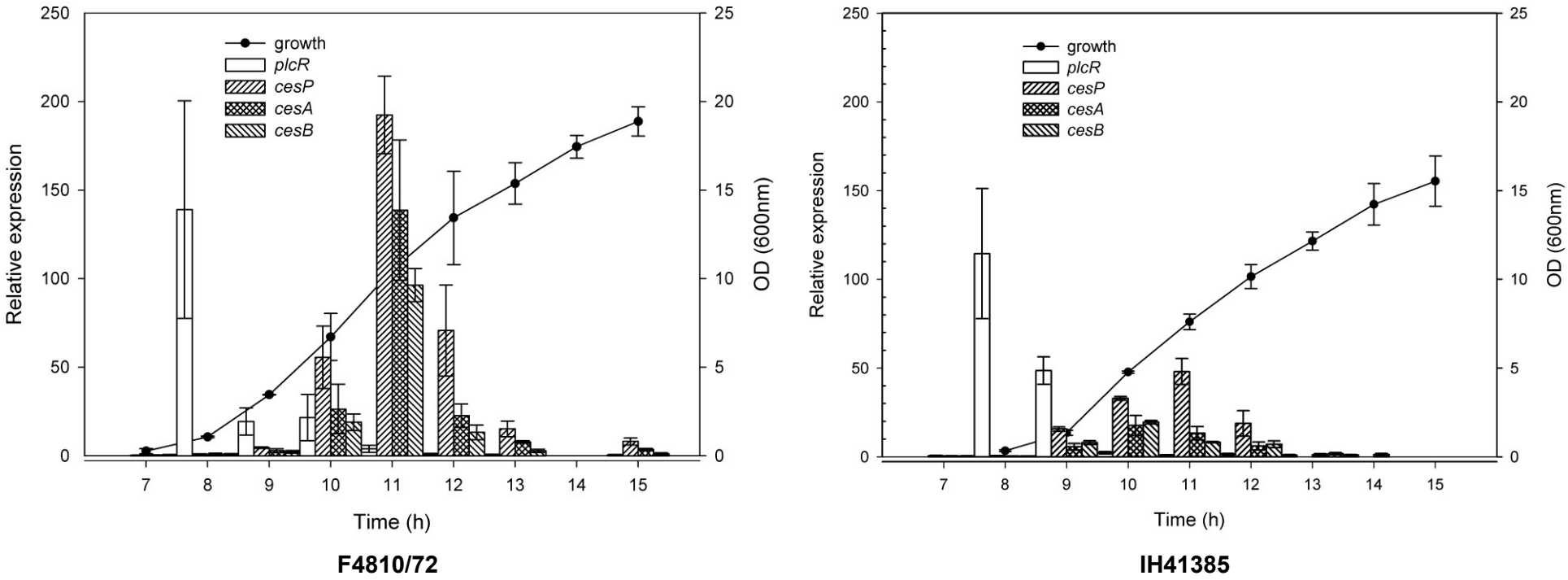


Figure 23. **Expanded view of kinetics from F4810/72 and IH41385.**

Relative *ces* and *plcR* gene expression during growth at 30°C in LB media, rotary shaking 150rpm in baffled flask of *B. cereus* emetic reference strain F4810/72 versus IH41385. All values are means, and error bars standard deviations, of two to three independent experiments.

3.3.3 Lower expression of weakly emetic strain

Both F4810/72 and IH41385 cultures had similar growth rates (1.8 and 1.6 respectively) and sporulated synchronously in late exponential phase, with the cultures' cells fully sporulated by the time stationary phase was reached (OD_{600} 19.5 to 22). Most significantly, *plcR* transcript levels in the weakly emetic strain IH41385 were similar to that of the highly emetic strain F4810/72 (Figure 22, Figure 23). However, *ces* expression in the weakly emetic strain was much lower, with a maximum RE of 48, 18 and 19 for *cesP*, *cesA* and *cesB* respectively, showing a four, eight and five fold down-regulation compared to the emetic reference strain (Table 6). The maximum cereulide titre detected in IH41385 was 3.2µgml^{-1}. This was at the lower detection limit of this HEp2 cell assay and 345 times less than the reference strain's toxin titre.

An opal stop codon confirmed in *cesB* of IH41385 did not result in a cessation of transcription. The expression of *cesB* using RT-qPCR was studied using primers downstream of the stop codon. The relative level of cesB transcript in IH41385 was not proportionately lower than that of cesP or cesA in relation to the reference strain F4810/72 (Figure 23).

3.3.4 Sporulation deficient mutant shows depressed *ces* expression

A *spo0A* deletion mutant of F4810/72, F4810/72Δ*spo0A* (Lücking *et al.*, unpublished), showed raised levels of *plcR* expression, not just in early exponential phase, but throughout growth (Figure 24), Transcript levels peaked during early logarithmic phase (RE 295) a 2.2 fold up-regulation compared to the WT F4810/72 strain, and maintained a RE of 36 to 79 throughout. However, although the rate was similar to the wild type (WT), the F4810/72Δ*spo0A* cultures grew to a lower maximum OD_{600} (14.3 to 16.5) compared to the OD_{600} levels the parent WT strain F4810/72 or the weakly emetic IH41385 cultures reached. As well, *cesP and cesA* expression were severely reduced in F4810/72Δ*spo0A*, 16 and 17 fold down-regulated, respectively, compared to the WT F4810/72 (Table 6). No cereulide toxin was detected in any *spo0A* deletion mutants, nor did sporulation occur.

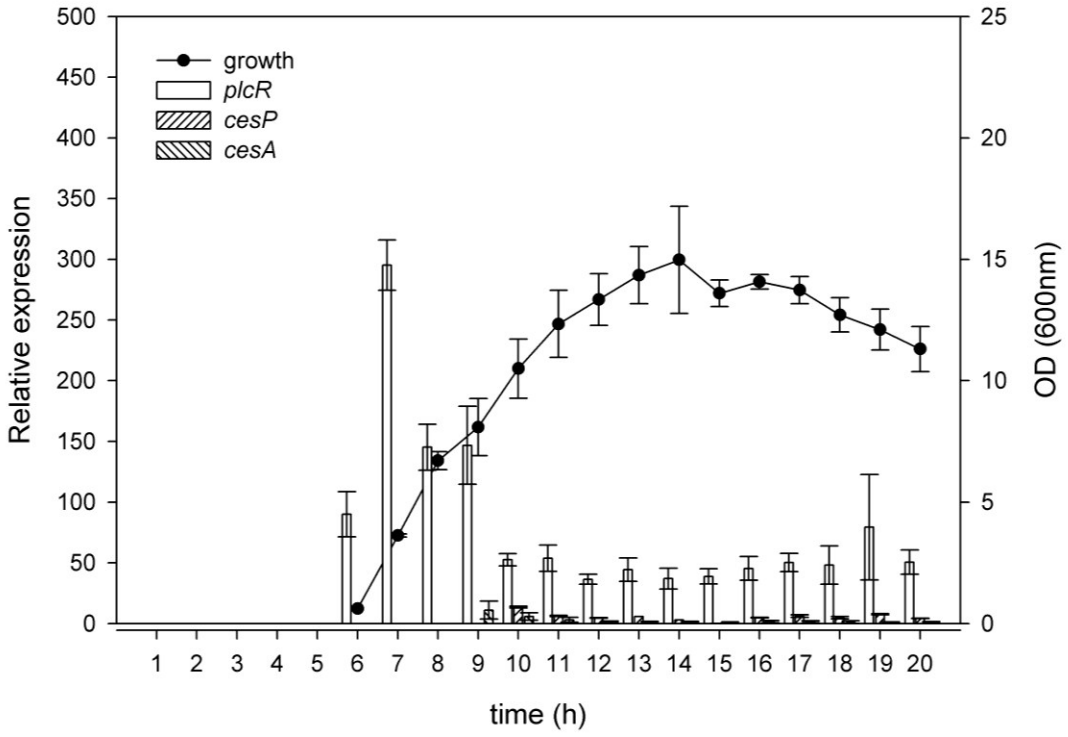


Figure 24. ***B. cereus* F4810/72 Δ*spo0A* expression kinetics using RT-qPCR.**

Kinetics of relative *ces* and *plcR* gene expression during growth at 30°C in LB media, rotary shaking 150rpm in baffled flask of *B. cereus* F481072Δ*spo0A*. All values are means, and error bars standard deviations, of two independent experiments.

3.3.5 Emetic *B. weihenstephanensis* shows drastically reduced expression

The emetic *Bacillus weihenstephanensis* strain MC67 did not grow very densely in LB media in baffled flasks, only reaching maximum OD_{600} values of 16.5-16.8 (Figure 25). During growth in LB media this strain formed long chains of 8 or more still-attached cells and did not sporulate. Maximum transcription levels were also lower for *cesA* (RE 1.5) which reflected a 162 fold down-regulation compared to *B. cereus* F4810/72 (Table 6); though with such low expression levels one might not refer to an expression peak. Interestingly, relatively high levels of toxin were detected (140-196 $\mu g ml^{-1}$) from cultures of MC67.

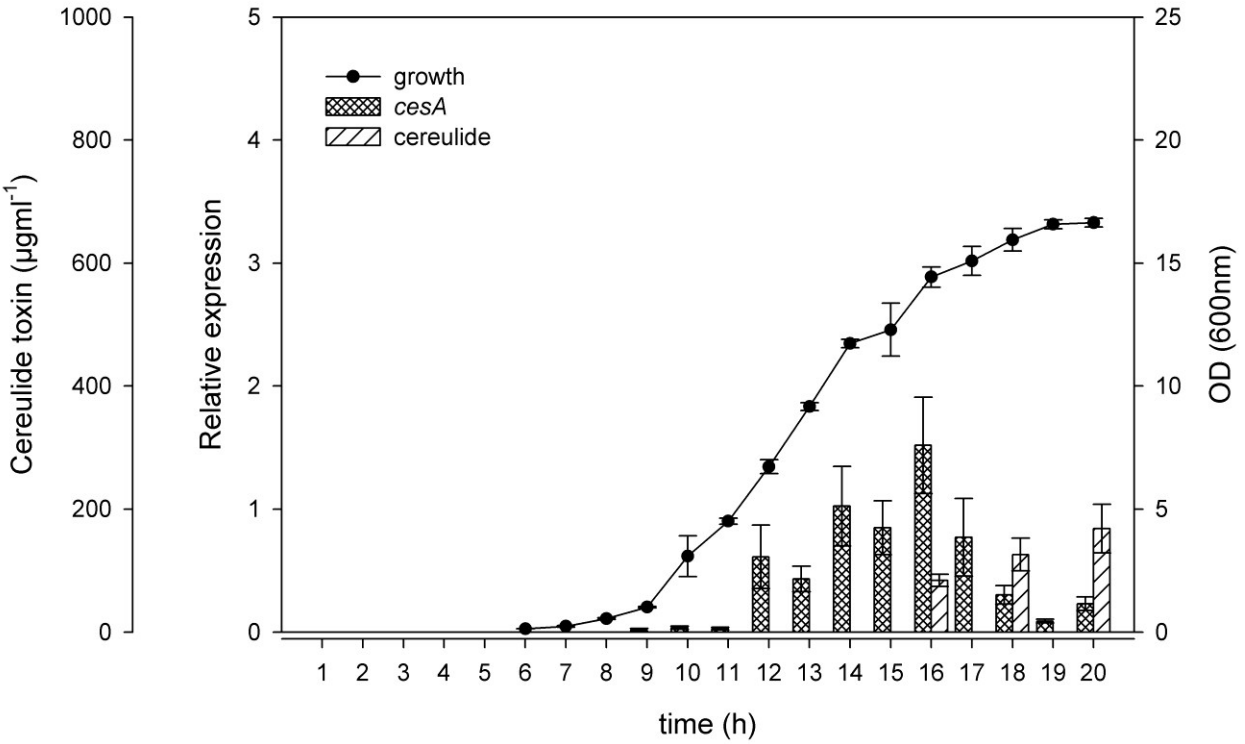


Figure 25. ***B. weihenstephanensis*** **MC67 expression kinetics using RT-qPCR.**

Kinetics of relative *ces* gene expression and cereulide toxin synthesis during growth at 30°C in LB media, rotary shaking 150rpm in baffled flask of *B. weihenstephanensis* MC67. All values are means, and error bars standard deviations, of two independent experiments.

Table 6. **Growth rate, *ces* regulation, and cereulide production of various strains.**

Strain	Growth Rate	*plcR*	*cesP*	*cesA*	*cesB*	Cereulide (µgml^{-1})
B. cereus F4810/72	1.8	1.0	1.0	1.0	1.0	662
B. cereus F4810/72 fermentation	4.1	1.0	NT	2.6	NT	151
B. cereus IH41385	1.6	1.3	- 5.1	- 6.8	- 4.3	2.0
B. cereus F4810/72 Δspo0A	1.8	2.2	- 15.6	- 17.2	NT	ND
B. weihenstephanensis MC67	1.5	NT	NT	- 162	NT	168

* ND, not detected; NT, not tested

3.3.6 Environmental effects

Cultures of emetic reference strain F4810/72 were grown in baffled flasks as described, with samples taken hourly for OD_{600} measurement, microscopy, RT-qPCR, and toxicity tests. Evaluated were the effects of pre-culture conditions, media components and temperature, on the exponential growth rate, maximum *cesA* transcript levels and maximum toxin titre (Table 7). In order to compare effects on the maximum *cesA* gene expression, complete transcriptional kinetics were performed and analyzed for each growth experiment, and the maximum *cesA* expression level for each condition was compared to a calibrator. In this case the calibrator (RE = 1.00) was designated as the maximum expression of the emetic reference strain F4810/72 cultivated under reference conditions (30°C, sporulated pre-culture, LB media) and in parallel to the conditions under evaluation. In all conditions tested, the pattern of expression was the same, with maximum expression during mid-exponential growth, dropping off thereafter. Cereulide, if detectable levels were present, was found at maximum levels in late exponential, approaching stationary, phase. As well, sporulation occurred under all conditions permissible for growth, with the cultures sporulated by the time stationary phase was reached.

During growth of the emetic reference strain F4810/72 under various conditions at 30°C there was no significant difference in growth rates (1.71-1.76) (Table 7); effects were observed at different temperatures or when NaCl concentration increased to $50gL^{-1}$ and above. Rice water cultures with amino acid supplementation (as above) became dark brown, whereas the non-supplemented cultures remained pale yellow. Neither transcript nor toxin were found in any of the rice water media based cultures grown at 24°C or 30°C up to 30h, though toxin is frequently isolated from rice-based dishes. However strain F4810/72 did grow well, as observed by cell counting in a haemocytometer; all rice water cultures reached a cell density of $5x10^7$ $cfuml^{-1}$.

Experiments showed that when a non-sporulated vegetative cell pre-culture was used to inoculate the main culture *cesA* expression was down regulated (3.1 fold), as were those cultures inoculated with a spore suspension (down regulated 2.1 fold) (Figure 26, Table 7). Growth rate of the standard (sporulated pre-culture) culture was 1.71, compared to 1.99 for the vegetative cell inoculant culture and 1.57 for cultures inoculated with only spores. Toxin titres reflected transcript levels at $372\mu gml^{-1}$, $662\mu gml^{-1}$ and $419\mu gml^{-1}$ cereulide for cultures with vegetative, sporulated and germinated spores as pre-cultures respectively.

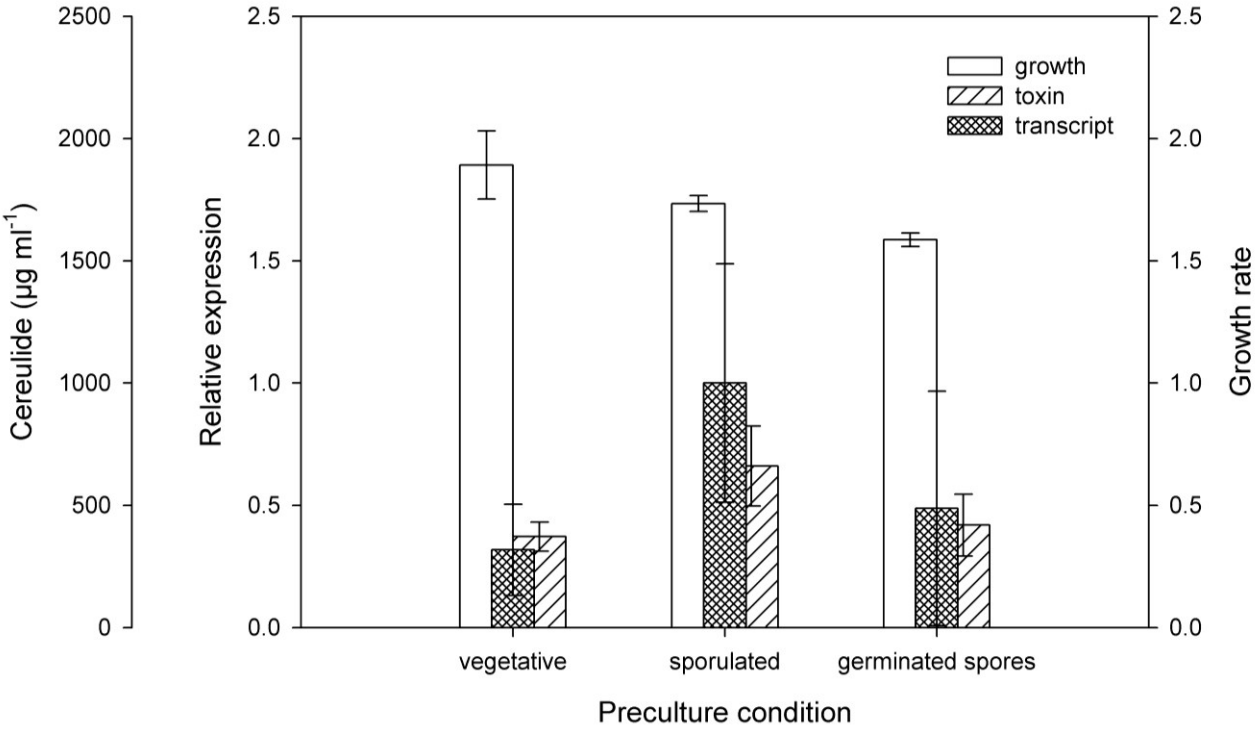


Figure 26. **Effects of preculture growth conditions on expression kinetics.**

Exponential growth rate, relative *cesA* gene expression, and toxin accumulation in *B. cereus* F4810/72 grown in baffled flasks with rotary shaking 150rpm. All values are means, and error bars standard deviations, of four independent experiments.

The effect of supplementation with amino acids and glucose on *ces* gene expression was investigated. To compare with the reference LB experiments, LB media was supplemented with glucose, amino acids, or both. The growth rates of cultures in all four media types were very similar, 1.7 to 1.9 (Figure 27, Table 7). In the glucose supplemented media *cesA* expression was down-regulated 2.5 fold, whereas cultures with amino acid supplementation, either alone or in combination with glucose, showed *cesA* expression essentially unchanged (-1.1 and -1.3 respectively). Although the growth rates were similar, toxin titres increased with supplementation of glucose (1193µgml^{-1}), amino acids (1260µgml^{-1}) and both (1443µgml^{-1}), compared to cereulide produced in non-supplemented LB (673µgml^{-1}).

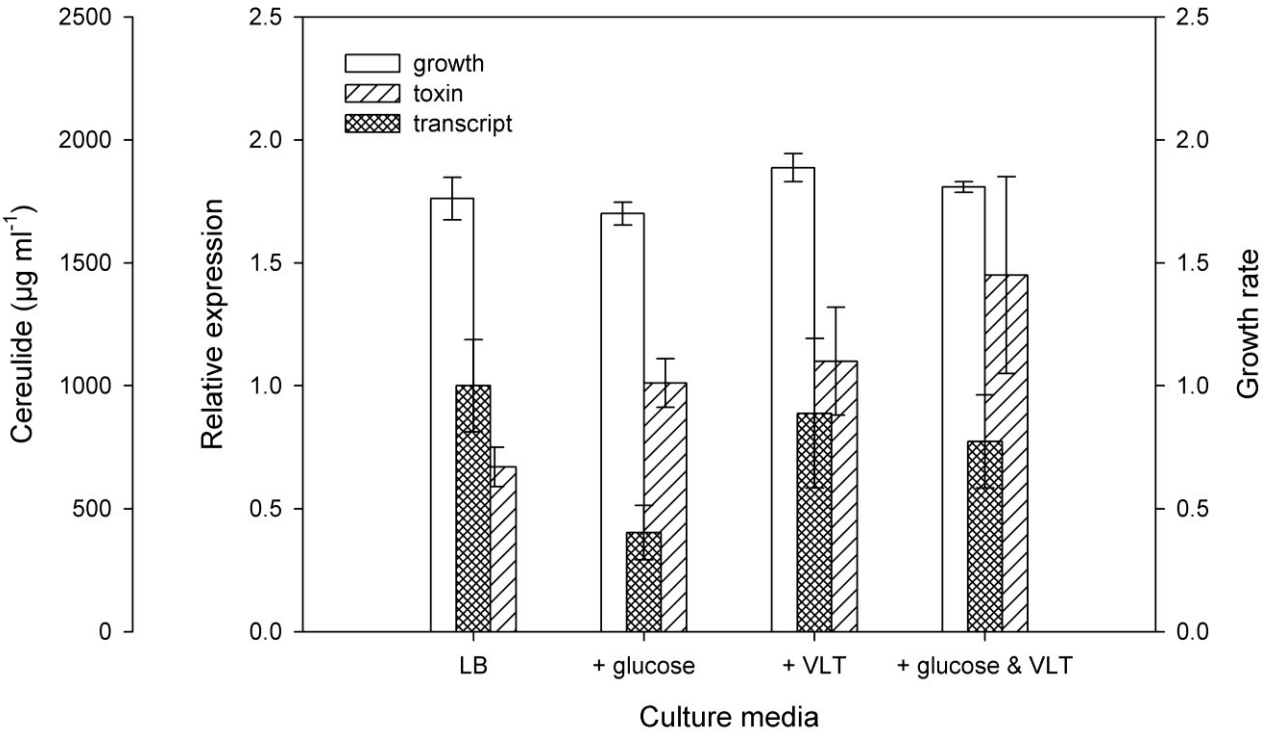


Figure 27. **Effects of media supplementation on expression kinetics.**

Exponential growth rate, relative *cesA* gene expression, and toxin accumulation in *B. cereus* F4810/72 grown in baffled flasks with rotary shaking 150rpm. All values are means, and error bars standard deviations, of four independent experiments.

The effect of NaCl concentration was investigated using LB media with $10gL^{-1}$ NaCl as reference, and compared to LB with $0gL^{-1}$ to $100gL^{-1}$ NaCl (Figure 28, Table 7). At $0gL^{-1}$ and $25gL^{-1}$ growth rates did not change much (1.83 and 1.73 respectively). Although *cesA* was up-regulated slightly, 1.3 fold, in the culture without NaCl, the cereulide levels remained similar, $706\mu gml^{-1}$ compared with $662\mu gml^{-1}$ for $10gL^{-1}$. In media with $25gL^{-1}$ NaCl *cesA* was down-regulated (4 fold) and the toxin titre dropped from $662\mu gml^{-1}$ (LB $10gL^{-1}$) to $282\mu gml^{-1}$. In LB with $50gL^{-1}$ NaCl growth rates dropped to 0.98, the final OD_{600} attained was also less (ca. 13.4-14.5) and *ces* transcription was down-regulated 12 fold. Very little toxin was produced in $50gL^{-1}$ NaCl LB, with a titre of $11\mu gml^{-1}$. At NaCl levels of $75gL^{-1}$ the bacteria did not grow well, with a lag phase of over 20h (compared to 5h for all others), forming long chains and failing to sporulate; *cesA* was down-regulated 15 fold and only minimal, $0.5\mu gml^{-1}$, toxin was detected. At concentrations of $100gL^{-1}$ NaCl the bacterial cultures no longer grew at all.

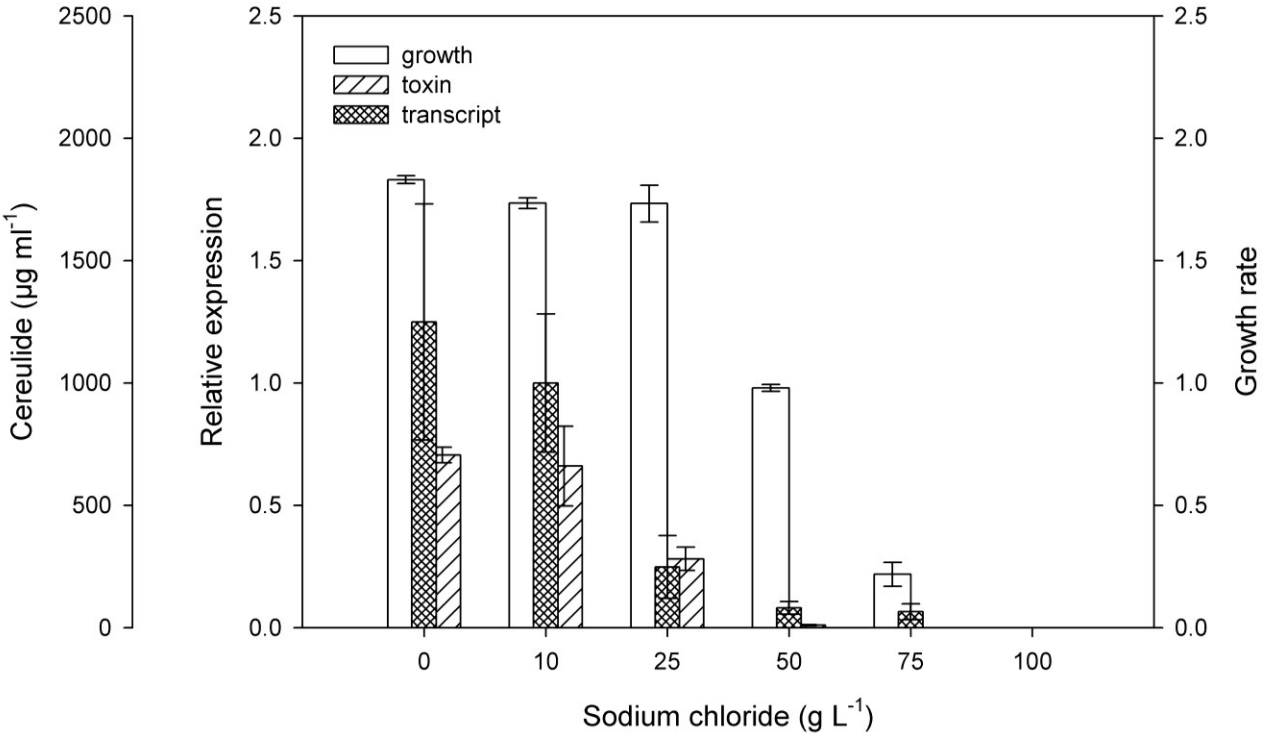


Figure 28. **Effects of sodium chloride levels on expression kinetics.**

Exponential growth rate, relative *cesA* gene expression, and toxin accumulation in *B. cereus* F4810/72 grown in baffled flasks with rotary shaking 150rpm. All values are means, and error bars standard deviations, of four independent experiments.

To investigate temperature effects, cultures were grown at 12°C, 15°C, 24°C, 30°C, 37°C, 42°C and 48°C. Although the growth rates increased with increasing temperatures up to 42°C, transcription was maximal at moderate temperature (30°C) and cereulide toxin accumulation was maximal at a slightly cooler temperature (24°C) (Figure 29, Table 7). At all temperatures the culture grew, though at 12°C and 15°C it was drastically slowed. Neither *cesA* transcript nor toxin were detected at all during 10 days of growth at 12°C, and the cultures grew to only half the density as at 30°C. At 15°C growth reached a similar culture density in stationary phase (200h) as at 30°C, at this time the cultures were sporulated. Cereulide was detected at a relatively high level (309µgml^{-1}) at 15°C despite *ces* expression being four fold down-regulated compared to 30°C. The highest cereulide toxin accumulated at 24°C (2079µgml^{-1}) though growth rates were lower and *ces* expression somewhat down-regulated (1.6

fold). Growth rates increased with temperatures above 30°C. Although at 37°C growth rate was similar to that at 30°C both the *ces* relative expression and cereulide toxin decreased significantly, with *ces* ten fold down-regulated and the toxin titre at 15h only 34µgml^{-1}. At 42°C the growth rate increased, with stationary phase reached and the culture fully sporulated within 15h, yet *ces* expression was 55 fold down-regulated and no toxin was detected. Growth rate decreased at 48°C, the upper limit of growth for F4810/72, with *ces* transcript almost non-existent and no cereulide detected. At 48°C the cultures did not grow to an OD_{600} above 4.3.

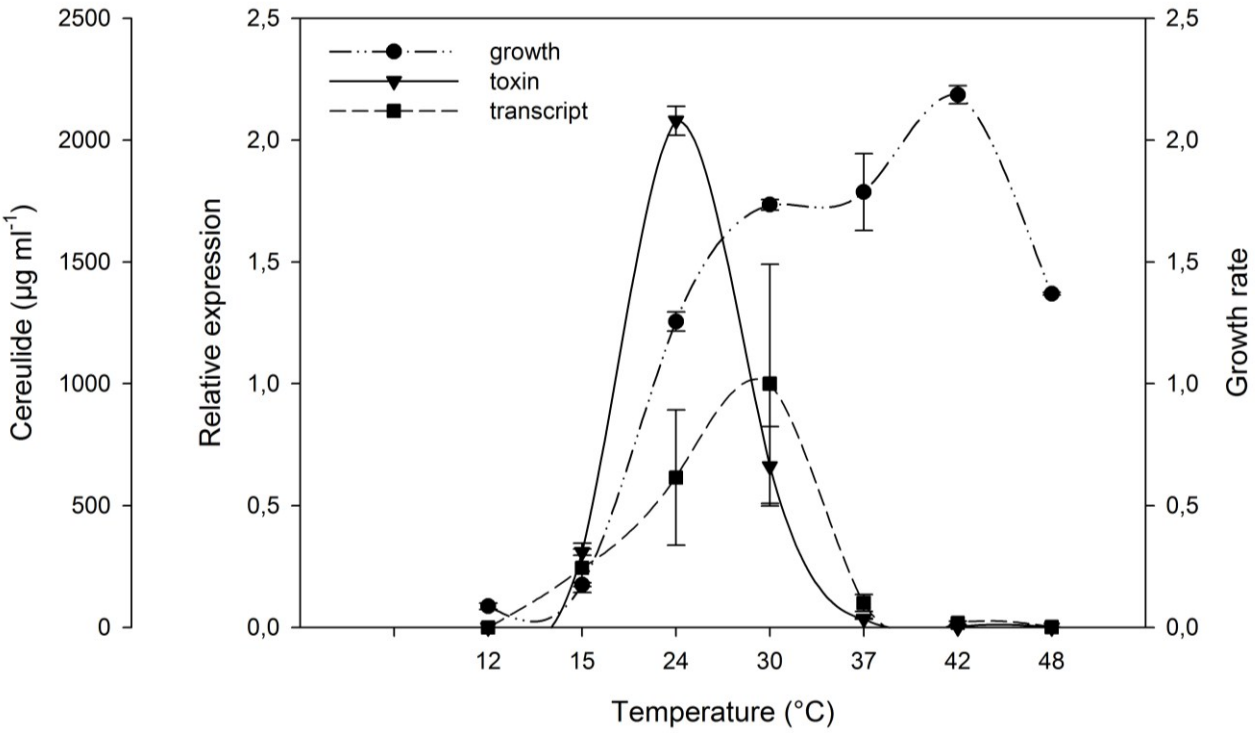


Figure 29. **Effects of temperature on expression kinetics.**

Exponential growth rate, relative *cesA* gene expression, and toxin accumulation in *B. cereus* F4810/72 grown in baffled flasks with rotary shaking 150rpm. All values are means, and error bars standard deviations, of two independent experiments.

Table 7. **Effects of conditions on F4810/72 growth, transcription and toxicity.**

Experimental Condition	Exponential Growth Rate	*ces* Regulation	CereulideToxin Titre (µg ml^{-1})
Standard conditions Sporulate pre-culture, 10 g L^{-1} NaCl, 30°C	1.7-1.8	1.0	662
Vegetative pre-culture	2.0	- 3.1	382
Spore suspension pre-culture	1.6	- 2.1	419
0 g L^{-1} NaCl	1.8	1.2	706
25 g L^{-1} NaCl	1.7	- 4.0	282
50 g L^{-1} NaCl	1.0	- 12	11
75 g L^{-1} NaCl	0.2	- 15	0.5
100 g L^{-1} NaCl	ND	ND	ND
0.2% w/v D-glucose	1.7	- 2.5	1193
0.3 g L^{-1} L-Val, -Leu, -Thr	1.9	- 1.1	1260
0.2% D-glucose + 0.3 g L^{-1} L- Val, -Leu, -Thr	1.8	- 1.3	1443
12°C	0.09	ND	ND
15°C	0.18	- 4.1	309
24°C	1.3	- 1.6	2079
37°C	1.8	- 10	35
42°C	2.2	- 55	ND
48°C	1.4	ND	ND

* ND, none detected

4 Discussion

4.1 4'-Phosphopantetheinyl transferase integral to cereulide NRPS

The cereulide synthetase (*ces*) gene cluster comprises, besides the typical genes such as a putative 4´-phosphopantetheinyl transferase (4'-PP, *cesP*) essential for priming the NRPS and the structural genes responsible for the assembly of the peptide product (*cesA, cesB*), a putative type II thioesterase (*cesT*) (Ehling-Schulz *et al.*, 2006). An additional CDS (*cesH*) that shows high homology to putative hydrolases and acyltransferases is located at the N terminus; at the C terminus the cereulide synthetase genes are flanked by a thioesterase at the 3' end of *cesB*, and putative ABC transporter (*cesC/D*) (Ehling-Schulz *et al.*, 2006).

Transcriptional analysis of the *ces* gene cluster presented here shows that *cesH* is transcribed from its own promoter while *cesPTABCD* are co-transcribed as a single large 23kbp polycistronic operon (Figure 3). A predicted hairpin structure after the *cesC/D* genes appears to be the termination structure: no transcript was detected using RT-PCR. Co-transcription of NRPS genes has recently been reported in other species, for the anguibactin synthetase in *Vibrio anguillarum* (Di Lorenzo *et al.*, 2004), acinetobactin in *Acinetobacter baumannii* (Mihara *et al.*, 2004) and syringopeptin in *Pseudomonas syringae* (Wang *et al.*, 2006). As well, a similar organization has been reported for other *Bacillus* sp. NRPS genes. In *B. brevis* gramicidin S synthesis is encoded by three CDS (*grsT*, *grsA* and *grsB*) which are co-transcribed from a central promoter upstream of *grsT* (Kratzschmar *et al.*, 1989). Tyrocidine is synthesized by three enzymes encoded in one operon by *tycA*, *tycB* and *tycC*; these are also co-transcribed from a single promoter upstream of *tycA* (Marahiel *et al.*, 1987). In *B. subtilis*, surfactin is synthesized by the products of a 27kbp operon containing *srfA-A*, *srfA-B*, *srfA-C* and *srfA-TE*, transcribed from a promoter upstream of *srfA-A* (Cosmina *et al.*, 1993).

Two transcriptional start sites of *cesPTABCD* were identified upstream of the translational start site of *cesP* (P_2 256bp and P_1 100bp) and additional intercistronic promoters were identified for *cesT* and *cesB* (Figure 5, Figure 6). It was interesting that the main *ces* promoter is located upstream of *cesP* and not *cesA*, as frequently 4'-phosphopantetheinyl (4'-PP) transferase domains, which activate the thiolation domains to bind the cognate substrate, are highly conserved and not necessarily

specific to their respective NRPS. In gramicidin S, the 4'-PP transferase gene (*gsp*) is located upstream of the structural *grsTAB* genes, and transcribed from its own promoter P_{gsp}, yet can be used to complement in trans the deleted 4'-PP gene (*sfp*) required for surfactin biosynthesis (Borchert *et al.*, 1994). In the case of cereulide, it appears that the 4'-PP transferase encoded by *cesP* is an integral part of the polycistronic *ces* gene cluster; an intercistronic promoter found for *cesT* showed no activity above the background of the respective promoter fusion plasmid under the tested conditions.

RACE and primer extension studies showed two putative transcriptional start sites for *cesB*. Interestingly, only a large construct (Table 4, pMKD[P_B/*gfpmut3a*]) that included both of these putative *cesB* intercistronic promoters showed GFP activity in these conditions. Even then, this construct displayed promoter activity 50-fold less that of the P_1 promoter (Figure 15, Figure 16). The 13 *Staphylococcus aureus* type I capsule genes encoded in a 14.6kbp region are co-transcribed from a central promoter; five intercistronic promoters were also detected, however their expression level was 45-198 fold less than that from the main promoter (Ouyang & Lee, 1997).

Both main and intercistronic promoters were reported for the expression of *dtxR*, an iron-dependent transcriptional regulator in *Corynebacterium diphtheriae*; the main promoter was dependent on environmental factors, whereas the intercistronic promoter in a 224bp region between structural genes (142bp upstream from the translational start) was unaffected by iron levels (Oram *et al.*, 2006). The *priA-cpgA* region of the *B. subtilis* genome also appears to show complex regulation: most of the genes in the *def-cpgA* region are transcribed as a single transcript under tested conditions, yet three transcriptional start sites were found (Hinc *et al.*, 2006). Early *Streptomyces clavuligerus* cephamycin biosynthetic genes (*lat*, *pcbAB* and *pcbC*) are coexpressed as a polycistronic transcript from a promoter upstream of *lat*, as well *pcbC* is expressed, but at much lower levels, from an intercistronic promoter (Alexander *et al.*, 2000). These intercistronic promoters, such as found for *cesB*, might ensure adequate expression of distal genes.

The occurrence of unusually long NRPS gene transcripts with multiple promoters and long leader sequences has been previously described for the *mcy* gene cluster of the genetic locus responsible for microcystin biosynthesis in the cyanobacterium *Microcystis aeruginosa* (Kaebernick *et al.*, 2002); both microcystin and the *Nodularia spumigena* cyanobacterial hepatoxin nodularin (Moffitt & Neilan, 2004) are

bidirectionally transcribed from a central promoter. The two different *mcy* main promoters appeared to be differentially regulated by light, (Kaebernick *et al.*, 2002) while regulation of the *ces* promoters is still cryptic. In addition to the primary *cesP* promoter, the intercistronic *cesT* and *cesB* promoters identified in the *ces* operon might be active under particular conditions, as has been reported for the *his* operon in *E. coli* (Grisolia *et al.*, 1983).

There are several +1 frameshifts in the *ces* gene (from *cesP* to *cesT* to *cesA* to *cesB*). Frameshifting has mainly been investigated in viruses, but occurs in prokaryotes as well, most frequently associated with IS elements (Baranov *et al.*, 2005). Although +1 frameshifting is less common than -1 frameshifting, it has been described in both prokaryotes and eukaryotes (Farabaugh, 1996). Using the program FSFinder (http://wilab.inha.ac.kr/FSFinder/) (Moon *et al.*, 2004) no +1 frameshift slippery sites were found in the *ces* genetic locus using either prokaryotic or eukaryotic patterns, but three -1 frameshift patterns (slippery sites) were found within 12bp of each other in the overlapping 32bp region of *cesT* and *cesA*, as well several between *cesA* and *cesB*. This could account for *cesA* being co-transcribed with *cesT*, perhaps by two -1 frameshifts, and perhaps explain why no intercistronic promoters were predicted or detected for *cesA* although a putative Shine Dalgarno ribosome binding site (RBS) (AGGAGG) was found 48bp upstream of the *cesA* start codon, although within the previous CDS (*cesT*). Frameshifts were also observed in other NRPS genes analysed *in silica*, such as the -1 frameshift in microcystin *mcyABC* (GenBank accession number AF183408) and between *grsA* and *grsB* in gramicidin S (GenBank X15577) (data not shown). In summary, -1 frameshifts might represent a mechanism in NRPS genes to ensure cotranscription of the complete NRPS gene cluster.

4.2 Promoter strength cannot account for toxicity differences

Sequencing of the central promoter region of a dozen highly and weakly emetic strains show the differences in toxicity cannot be accounted for by promoter strength: the sequences were identical (Figure 7). Transcriptional kinetic studies found that a weakly emetic strain had lower levels of *cesP*, *cesA* and *cesB* mRNA than the emetic reference strain, however the difference was not in linear correlation to the difference in toxicity (Figure 20, Figure 22, Table 6). The stability of the *cesA* mRNA as detected

by RT-qPCR indicated a half-life of less than ten minutes; it has previously been noted from a genome-wide survey of mRNA decay in *B. subtilis* that no general 5' features nor function were found useful to predict stability, though 80% of the mRNAs studied were shown to have a half-life less than seven minutes (Hambraeus *et al.*, 2003). However, it has also been reported that specific domain-domain interactions, rather than coordinated transcription and translation, seem to be play a major role in determining correct assembly and activity of NRPS (Guenzi *et al.*, 1998), therefore the correct folding and stability of the cereulide synthetase may be a key factor in determining final toxin levels. As the main promoter sequences were shown to be the same (except strain MC67 (Fuerst, 2006)), it would prove interesting to sequence the entire *ces* gene cluster of these dozen strains in order to evaluate potential differences in NRPS folding and subsequent biosynthetic efficiency of assembly of the cereulide. Kinetics of growth, transcription, and toxin production presented in this study indicate 3-4 fold higher cereulide production, yet 1.6 fold less transcript, at 24°C compared to 30°C (Figure 29, Table 7), which may indicate a more stable folding of the NRPS enzyme at 24°C compared to 30°C; this may illustrate and influence of correct NRPS folding on final toxin levels.

4.3 Regulation appears to be chromosomal but independent of the central virulence regulator plcR

The central promoter showed similar activity in non-emetic, emetic-like and emetic *B. cereus* strains as well as in an emetic *B. weihenstephanensis* strain MC67 (Figure 11, Figure 13). Emetic-like *B. cereus* are those that show similar phenotypic characteristics to the emetic strains (Ehling-Schulz *et al.*, 2005a) and harbour a pXO1-like mega-plasmid, but lack the *ces* gene cluster and therefore do not produce cereulide (Ehling-Schulz *et al.*, 2006). That the P_1 promoter was active in strain IH41385ΔpBCE270 and the non-emetic type strain ATCC14579 indicate that the mechanism of regulation is not encoded on the pXO1-like plasmid pBCE270, encoding the cereulide synthetase genes. IH41385ΔpBCE270 lacks the mega-plasmid, whereas ATCC14579 has been reported not to harbour a pXO1-like mega-plasmid at all, but rather a 15kbp plasmid pBClin15 (Ivanova *et al.*, 2003), therefore it could be speculated that the *ces* regulatory components are chromosomally located.

Analysis of the *ces* sequence for putative binding sites for known *Bacillus cereus* group virulence regulation factors (Fouet & Mock, 2006) such as PlcR (Agaisse *et al.*, 1999), Spo0A (Strauch *et al.*, 1990), AbrB (Strauch *et al.*, 2005) or CodY (Sonenshein, 2005) did not detect any conserved sites. However, a putative binding site for DegU was found 153bp upstream of the P_1 promoter, this may provide a hint that cereulide synthesis is linked to competence. DegU acts to prime transcription of *comK* (Hamoen *et al.*, 2000) which is a transcriptional activator of late competence genes, and is also both a positive and negative regulator of multi-cellular behaviour in *B. subtilis* (Verhamme *et al.*, 2007). Regulation of competence also includes the two component signalling system of ComP/ComA, which also serve to activate transcription of the NRPS product surfactin (*srfA*) (Nakano & Zuber, 1991); however, *comP* independent expression of *srfA* was reported in media supplemented with glucose, which however required *spo0A*, *spo0B*, *spo0F* and the *spo0K* operons, though not via their effects on *abrB* expression (Marahiel *et al.*, 1993). Control of the *B. subtilis* gene *aprE* encoding subtilisin is regulated in a complex fashion involving AbrB, DegU, Hpr, SinI, and SinR and Spo0A (Sanchez & Olmos, 2004). The results presented here, glucose activation of the P_1 promoter with a *degU* binding site upstream, could lead one to hypothesize that the cereulide synthetase genes are linked to natural competence; this would correlate with findings that indicate the *ces* genes located on an potentially ancient pXO1-like plasmid may have been acquired over time (Ehling-Schulz *et al.*, 2006).

In general, little is known about emetic *Bacillus cereus* cereulide synthetase expression, and why some strains are highly- whereas others are only weakly toxic. In order to further knowledge, studies were undertaken to elucidate the kinetics of cereulide synthetase *(ces)* gene expression in relation to growth, sporulation and toxin accumulation in both highly and weakly emetic strains. RT-qPCR assays were developed for the *ces* and *plcR* genes, with primers whose efficiency was within the acceptable range of 1.60 and 2.10 (Pfaffl, 2001). As the pleiotropic regulator *plcR* is a known virulence regulator of *B. cereus*, its expression was investigated as well, to compare its expression levels in strains of highly differing toxicity (IH41385 3.2μgml^{-1}, F4810/72 662 μgml^{-1}). The patterns of expression for *plcR* and the *ces* genes were the same for both the highly and weakly emetic strains (Figure 23). Both *plcR* and the cereulide NRPS *ces* were regulated at the transcriptional level. That *plcR* transcription peaks in early logarithmic phase agrees with a report showing the

highest level in the first sample taken (Zigha *et al.*, 2006). During fermentation the same pattern of expression was observed as in baffled flasks; the level of *plcR* transcript was the same, yet *cesA* transcript was 2.6 fold higher (Figure 21, Table 6). This agrees with earlier results that indicate that oxygenation is important to cereulide production (Finlay *et al.*, 2002a; Jaaskelainen *et al.*, 2004), as oxygen was better controlled in the fermenter.

The weakly emetic strain IH41385 showed a relative expression of *plcR* similar to F4810/72, however maximal *ces* expression was four to eight fold lower (Figure 23, Table 6). Interestingly, the similar *plcR* transcription level, yet reduced *ces* expression, of the weakly emetic strain would indicate that, although *plcR* is a well-characterized extra-cellular virulence factor regulator of *B. cereus* (Agaisse *et al.*, 1999; Gohar *et al.*, 2002), it may not necessarily be directly involved in cereulide NRPS regulation. Recent studies have shown that although *plcR* does regulate *cytK* (Brillard & Lereclus, 2004) in *B. cereus*, differing CytK toxin levels in rare strains cannot be solely accounted for by the *plcR* box in the *cytK* promoter region (Fagerlund *et al.*, 2007). A cholesterol dependent cytolysin (CDC), anthrolysin, in the close relative *B. anthracis* has been shown not to be *plcR* regulated (Ross & Koehler, 2006), although the analogous CDCs in *B. cereus* and *B. thuringiensis* are part of the *papR/plcR* regulon. It is possible that regulation of cereulide expression is linked to another regulator or other factors such as sporulation and glucose and amino acid availability, as hinted to by these results. Additionally, the several hundred-fold lower cereulide production of the weakly emetic strain IH41385 (3.2μgml^{-1} compared to 662μgml^{-1}) may not be solely accounted for by the lower *ces* expression. Other factors such as substrates like α-keto acids (Magarvey *et al.*, 2006) produced by other metabolic pathways, and their lesser availability to the cereulide NRPS enzyme for assembly of the cereulide peptide, may contribute to the lower toxicity of IH41385. As well, the amino acid changes in strain IH41385 versus F4810/72 discovered by sequencing the *ces* gene locus in IH41385 (Table 5) could have an effect on the secondary and tertiary structure of the cereulide synthetase enzyme, affecting its efficiency of binding and synthesis of cereulide. The weakly emetic strain IH41385 has been reported to produce toxin levels of 0.5-1.0ngmg^{-1} biomass fresh weight (Carlin *et al.*, 2006) compared to the reference strain, reported to produce 240-600ngmg^{-1}. These results correlate with the results reported here, with the strain IH41385 producing only 0.2% as much toxin as the reference strain under the same cultivation

conditions. From the results presented here it would appear that *ces* is not directly PlcR regulated.

4.4 Impact of sporulation

The emetic *B. weihenstephanensis* strain MC67, despite showing *ces* expression levels less than 1% of the level of the emetic reference strain F4810/72, produced toxin at about 25% of the level of strain F4810/72 (Figure 25, Table 6). Most interestingly, MC67 did not sporulate in LB media at 30°C, which was expected as it did not on LB agar either, even after 10 days, though it did on PEMB agar within 24h (Fuerst, 2006). This made it interesting to investigate this strain in LB media, as it has previously been shown to produce six times the toxin of the emetic strain when grown on BHI agar at 25°C for 10 days (Thorsen *et al.*, 2006). However, since cereulide biosynthesis commences upon entry into stationary phase, a 10 day old culture on plates may reflect many cycles of growth, sporulation, germination and outgrowth. Under the tested conditions MC67 did not sporulate and produced less cereulide, perhaps indicating a link between sporulation and cereulide toxin production.

The relation of sporulation to cereulide gene expression was investigated by comparing the effects of preculture conditions on expression, and by investigating expression in a strain of F4810/72 in which a key sporulation gene, *spo0A*, had been deleted (F4810/72Δ*spo0A*). The results of the expression studies using vegetative and sporulated precultures indicate that sporulation ability has a link to *ces* expression and cereulide toxin production (Figure 26, Table 7). The importance of sporulation genes to *ces* expression and cereulide production was clearly illustrated by the relative expression kinetics of the *spo0A* deletion mutant of the emetic reference strain F4810/72. In this strain the expression of *plcR* was 2.2 fold higher than in the WT strain, whereas *ces* expression was 17 fold less; no cereulide was detected nor did sporulation occur (Figure 24, Table 6). Although it has previously been speculated that cereulide expression is independent of sporulation, this conclusion was made from strain NC7401 cultures grown at room temperature in trypticase soy (TS) broth for 120h, based on the spore count not increasing; however cereulide toxin did not substantially increase either (Haggblom *et al.*, 2002).

From the transcriptional and toxin kinetic results, it could be postulated that *ces* regulation is not directly *plcR* regulated but somehow connected to an early sporulation-linked gene. Spo0A is involved in the regulation of 121 genes in *B. subtilis* (Molle *et al.*, 2003), both positively and negatively, directly and indirectly, at both low and high threshold levels (Fujita *et al.*, 2005). In the complex sporulation mechanism Spo0A is a master regulator, both positive as is the case with *spoIIA, spoIIE* and *spoIIG*, and negative as is the case with the transition state regulator *abrB* (Sonensheim *et al.*, 2002). AbrB was recently reported to negatively control expression of *B. anthracis* toxin genes *pagA, lef* and *cya*, which peak in late exponential phase (Saile & Koehler, 2002); however, growth phase was determined without dilution of the culture. What is frequently declared as late exponential phase is equivalent to mid-exponential when the culture density is determined as in the study presented here (data not shown); this infers that the *abrB* regulated *B. anthracis* toxin genes display similar transcription kinetics as *ces*, peaking in mid-exponential phase. The link between cereulide toxin production and sporulation, and the involvement of *ces* in pathogenicity and its location on a *B. anthracis* pXO1-like mega-plasmid, invite speculation about the possibility of a similar regulatory mechanism. One could hypothesize about a theoretical *ces* operon repressor, also negatively regulated by Spo0A, analogous to *abrB*, which inhibits expression of *ces* after mid-exponential phase. If Spo0A were not present even in low threshold levels, as in the deletion mutant F4810/72Δ*spo0A*, exponential phase *ces* promoter activity would not occur as the hypothetical repressor would continue to block *ces* gene transcription. In summary, the connection between sporulation ability and cereulide toxin production, as well as the location of the *ces* gene cluster on a pXO1-like mega-plasmid, invite the hypothesis about a repressor mechanism analogous to *abrB* in *B. anthracis*, one which is also regulated by Spo0A, and in turn regulates toxin genes located on a virulence mega-plasmid.

4.5 Effect of culture conditions on cereulide synthetase gene expression and toxin levels

The relation of sporulation to cereulide gene expression was investigated by comparing the effects of pre-culture conditions on expression. The results presented here indicate positive effects of a sporulated pre-culture on *ces* transcription and cereulide production in the subsequent main culture. Interestingly, upon supplementation of LB media with glucose, *ces* transcription is down-regulated. This could be an indication of the involvement of *spo0A*, whose strong sporulation-specific promoter P_{ps} is repressed by glucose (Yamashita *et al.*, 1989). However, the levels of glucose used were such that the sporulation-specific expression of *spo0A* would not be repressed altogether (which occurs at 2%) (Chibazakura *et al.*, 1991) so sporulation itself would not be affected.

Toxicity results of the present study using amino acid and glucose supplementation support previous reports which reported increased cereulide production when 0.2% (w/v) glucose 0.3gL^{-1} and L-valine, -threonine and -leucine were added to media (Agata *et al.*, 1999). Interestingly, liquid rice water cultures with this amino acid supplementation, previously shown to increase toxin production in rice water agar to the equivalent of that detected on rich media (Jaaskelainen *et al.*, 2004), did not produce detectable toxin in these experiments at either 24°C or 30°C after up to 30h. The assumption was that liquid rice media was not nutritionally sufficient for cereulide production or that it would require more than 30h at either temperature. The addition of amino acids to LB, while not significantly affecting transcription or growth rate, did increase cereulide accumulation (Figure 27, Table 7). This would indicate that increased toxin resulted not from *ces* gene regulation or increased growth, but rather from higher rates of assembly of the emetic toxin by the cereulide NRPS, perhaps due to higher levels of available substrate. The promoter-GFP fusion studies indicated that the *cesB* region with putative promoter function was activated in media with amino acid plus glucose supplementation (Figure 16); this could ensure expression of the distal genes in the presence of sufficient energy for metabolism and substrate for the cereulide synthetase product. The central promoter P_1, and less dramatically the *cesH* promoter, responded with increased activity to supplementation with D-glucose (Figure 14, Figure 15), hinting that cereulide synthesis is not triggered by starvation or stress.

Cereulide synthetase promoter P_1 activity in response to growth media composition was also measured using the emetic strain F4810/72 transformed with a reporter gene vector placing the luciferase reporter gene under the control of the P_1 promoter, pMDX[P_1/*luxABCDE*]. The results from investigations indicate increased *ces* promoter activity in glucose-supplemented liquid LB media, and on solid growth media containing glucose. As shown from the plates with media and food inoculated with F4810/72 transformed with pMDX[P_1/*luxABCDE*], carbohydrate based media (PEMB, MYP, PC, LB) show much higher activity than protein-rich media such as blood and BHI agar (Figure 17). However, BHI and blood agar are still frequently used to study emetic *B. cereus* and cereulide production, though others have also reported less toxin detected on this media (Finlay *et al.*, 1999; Szabo *et al.*, 1991). The P_1 promoter is barely active (although growth was observed) in ground beef and cream cheese, whereas in starchy foods such as corn starch, instant mashed potato from flakes, cooked semolina noodles and sushi rice, which provide ready carbohydrates, promoter activity is very strong (Figure 18). These findings correlate with reports of emetic poisoning cases that implicate temperature-abused farinaceous, rather than proteinaceous foods (Agata *et al.*, 2002; Dierick *et al.*, 2005; Ehling-Schulz *et al.*, 2004a; Mahler *et al.*, 1997; Rajkovic *et al.*, 2006) and underscore the risk of emetic *B. cereus* poisoning, for instance, in mass catering quantities of rice- or pasta-based dishes that have been improperly cooled.

Highly relevant to food borne disease, are those results involving salt and temperature trials, as implicated foods are often rice-based dishes that have been temperature abused, and with sauces that may be salty; standard pasta with tomato sauce contains up to 3.7g NaCl per 210g portion, or almost $18gL^{-1}$. There was little difference in growth rate, *ces* expression and cereulide levels between LB without salt and reference conditions, indicating that $10gL^{-1}$ NaCl does not exert much effect (Figure 28, Table 7). Although the growth rate did not drop significantly at $25gL^{-1}$ NaCl, *ces* expression was much lower and toxin titres dropped to 35%. At a salt concentration of $50gL^{-1}$ the growth rate was significantly affected (half), as was transcription of *ces*, and the toxin titre was only 1.5% of that detected in reference media. Although cultures did grow in $75gL^{-1}$ NaCl it was severely retarded (formed long chains and failed to sporulate) as reflected by the down-regulated *ces* expression and lack of cereulide detected. This is the first time it has been shown that addition of simple table salt (NaCl) has a direct influence on cereulide toxin

production, not simply by lack of growth, but through down-regulation of the *ces* genes. This would indicate that salted food may offer some protection from cereulide production if emetic strains are present.

Temperature had a significant influence on *ces* NRPS transcription and on toxin production, though not concurrent with the growth rate (Figure 29, Table 7). Cultures were grown at temperatures between 12°C and 48°C; 12°C was previously shown to be the lower limit for toxin production (Finlay *et al.*, 2000) and 48°C the upper limit for growth of emetic strains (Carlin *et al.*, 2006). At 12°C growth was severely restricted and neither transcript nor toxin were detected, however one research group has reported toxin production as low as 12°C, however in 10% skim milk rather than liquid media, after 4d using the same initial inoculant level (10^3 cfu mL^{-1} at time of inoculation) (Finlay *et al.*, 2000). At 15°C only minimal transcript and toxin levels were detected, correlating with others who also report minimal cereulide at 15°C compared to temperatures in the range of 25°C to 30°C (Szabo *et al.*, 1991). That significantly less cereulide was detected at lower temperatures contradicts only Finlay *et al.*, who calculated increased toxin production at 15°C compared to 20°C or 30°C, however not in liquid media but in boiled rice and in skim milk (Finlay *et al.*, 2000; Finlay *et al.*, 2002b). Others studies generally agree that most cereulide is produced at close to room temperature, rather than refrigeration or high (ca 40°C) temperatures (Agata *et al.*, 2002; Haggblom *et al.*, 2002; Jaaskelainen *et al.*, 2004)

Using the results of the present study to examine cereulide synthetase expression and toxin production as a factor of the growth rate (nominal *ces* and cereulide), at 15°C nominal *ces* expression would be highest (ratio=1.4) as well as nominal toxin production (ratio=1766). It could be argued that in relation to growth rate, transcription and toxin accumulation are highest at 15°C, however due to the drastically reduced growth at this low temperature, there may simply not be sufficient cells per mL to present a risk, for instance, in refrigerated foods. Therefore what is most important in relation to food safety awareness is under what conditions high amounts of toxin accumulates. In this study, maximum toxin production occurred at 24°C, though neither growth nor transcription was at the highest level. Transcriptional levels at 24°C and 30°C correlated fairly linearly with their exponential growth rates, however toxin levels were four times higher at 24°C than at 30°C. Examined in relation to the growth rate, nominal toxin production at 24°C (ratio=1663) was more

similar to 15°C than 30°C (ratio=383), whereas nominal *ces* expression (ratio=0.49) was similar to 30°C (ratio=0.58).

The results presented here illustrate that *ces* transcription and cereulide biosynthesis cannot be predicted by growth rates. This is an important point for the food industry and mass catering facilities with respect to implementation of HACCP (hazard analysis at critical control points) plans. At 24°C neither nominal- expression nor toxin accumulation were highest, yet at this temperature significantly more total toxin per mL culture was produced. This underlines the role of temperature abuse with respect to reports of emetic disease caused by temperature abuse (frequently room temperature, close to 24°C) of prepared foods (Ehling-Schulz *et al.*, 2004a) where heating inactivates vegetative cells, but allows *B. cereus* spores to germinate and grow. At temperatures above 30°C up to 42°C growth rates increased but both transcription and emetic toxin production decreased significantly, with none detectable at 42°C. At the upper limit for growth, 48°C, neither transcript nor cereulide were detected. These results also correlate well with reports that cereulide production on trypticase soy agar after 11d incubation was highest at 21°C, much lower at 10°C and negligible at 40°C and 42°C (Haggblom *et al.*, 2002). The results of the present study are consistent with the range of temperatures at which emetic *B. cereus* grow well, and with reported outbreaks that tend to implicate prepared foods which have been temperature abused (not refrigerated appropriately) rather than foods which have been stored at chilled temperatures.

5 Conclusion and Perspectives

This is the first time that an analysis of transcriptional regulation of the emetic toxin genes has been performed. It shows that the polycistronic transcript of the cereulide synthetase genes is driven by a central promoter upstream of the *ces* gene cluster's 4'PP transferase, and that this P_1 promoter is sensitive to environmental influences such as nutrient availability. The regulation of the cereulide synthetase genes appears to be chromosomal rather than located on the mega-plasmid carrying the *ces* gene cluster. The results presented show the dramatic difference in toxicity of highly and weakly emetic strains cannot be solely accounted for by regulation of gene expression or promoter strength, but may be affected be sequence differences causing a difference in protein folding. The independence of cereulide synthetase relative expression levels from transcript amounts of the pleiotropic regulator *plcR* and the positive effect of sporulation capability, open up the possibility of *ces* gene regulation being connected to other regulatory factors, perhaps linked to sporulation or competence.

Also shown is the critical importance that environmental conditions have on cereulide production, and that high sodium chloride levels can decrease both cereulide gene expression and toxin formation. The key role of carbohydrate based growth media composition on cereulide synthetase transcription and of temperature abuse on toxin production is of utmost importance when considering processing and handling procedures and HACCP plans in the food industry and mass catering. The reverse transcription quantitative real time PCR assay developed in this study enables the sensitive detection of mRNA of several cereulide synthetase genes, crucial in determining the effect of disrupting putative regulators on *ces* gene expression. The luciferase reporter fusion vectors developed in this work permit the study of cereulide gene expression kinetics under real life conditions, such as in food or on food handling surfaces or packaging materials, in order to develop safer handling, processing and storage procedures. In summary, this study provides a solid starting point and the tools for further study, to gain deeper insight into and unravel the mechanisms of cereulide synthesis regulation.

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7 Appendix

7.1 Bacillus strains

Table 8. **Bacillus strains used in this study.**

Strain	Species	Phenotype	Cereulide	Origin	Reference
ATCC 14579^T	*B. cereus*	non-emetic		Environment (soil)	*Bacillus cereus* type strain
F4810/72 Δ*spo0A*	*B. cereus*	non-emetic	0 [a]	*spo0A* deletion mutant of F4810/72	Lücking *et al*., unpublished
IH41385 ΔpBCE270	*B. cereus*	non-emetic	0 [a]	strain lacking ΔpBCE270 plasmid	this study
NVH1519/00	*B. cereus*	emetic-like		Food borne outbreak (deer meat stew)	(Ehling-Schulz *et al.*, 2005a)
F3080B/87	*B. cereus*	emetic	500 [b]	Food borne outbreak (chicken korma with rice)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
F3350/87	*B. cereus*	emetic		Food borne outbreak	(Ehling-Schulz *et al.*, 2005a)
F3876/87	*B. cereus*	emetic		Clinical (pyrexia)	(Ehling-Schulz *et al.*, 2005a)
F4810/72	*B. cereus*	emetic	662 [a] 240-600 [b]	Food borne outbreak (vomit)	(Turnbull *et al.*, 1979)
F5581/94	*B. cereus*	emetic		Food borne outbreak (fried rice)	(Ehling-Schulz *et al.*, 2005a)
F6921/94	*B. cereus*	emetic		Food borne outbreak (rice)	(Ehling-Schulz *et al.*, 2005a)
IH41385	*B. cereus*	emetic	ND-3 [a] 0.5-1 [b]	Clinical (dialysis fluid)	(Andersson *et al.*, 1998a; Carlin *et al.*, 2006)
MHI1305	*B. cereus*	emetic	170-200 [b]	Food borne outbreak (Indian rice dish)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
NC7401	*B. cereus*	emetic	180-600 [b]	Food borne outbreak (vomit)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
RIVM BC51	*B. cereus*	emetic		Food borne outbreak (rice)	(Ehling-Schulz *et al.*, 2005a)
RIVM BC62	*B. cereus*	emetic		Clinical (vomit)	(Ehling-Schulz *et al.*, 2005a)
RIVM BC67	*B. cereus*	emetic	20-30 [b]	Clinical (human faeces)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
SDA GR177	*B. cereus*	emetic	70 [b]	Food environment (dairy)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
UHDAM NS 115	*B. cereus*	emetic	1200-1600 [b]	Environment (spruce tree)	(Carlin *et al.*, 2006; Ehling-Schulz *et al.*, 2005a)
MC67	*B. weihenste-phanensis*	emetic	196 [a]	Environment (soil)	(Thorsen *et al.*, 2006)

[a] Cereulide ($\mu g^{-1}ml$) from liquid LB cultures quantified using HEp-2 cell assay in this study
[b] Cereulide (ng^{-1} mg biomass) from TSA plates quantified using LC-MS assay (Carlin *et al.*, 2006)

7.2 Oligonucleotide primers

Table 9. **Oligonucleotide primers used to sequence *ces* in strain IH41385.**

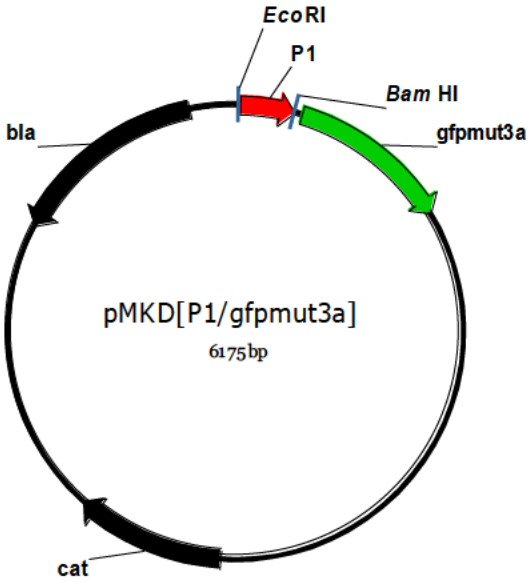
Primer	Sequence (5' – 3')	Nucleotide positions in DQ360825
Mark_for	CTGGTTAAAGAAGTAGCTTAGACTAAAC	3163-3190
Hydr_for2	GATAATGGCAAGGGCATTACC	3740-3760
Hydr_for3	CCTAAGCATGTTAGAGATTTAC	4433-4454
PPP1_for	GTTAATAATGTATTAAGGAAAATGAATG	5076-5103
IKA_rev	GTAATAGGAGTAAGTTCAACCGC	5181-5203
IKA_for	GTGCATGAAGAATACACGTGTC	5706-5727
Dra_L_for	GAAGTGGCGAAGATTGTTGG	6216-6235
KABcl_for	GAGATGCAAGAACATTCACGAG	7052-7073
IEA_rev	GCTTGCTTAGCGGCACGC	7207-7224
KIDB_for	GTCAAGAGATTGTTATACATAGTATTG	7578-7604
LeuAc_for1	GATGCCTGGTATTGGCAT	8302-8319
Sfu_L_for2	TACAGTTCCAACTTTTAGATCGGTAG	9202-9227
Sfu_L_rev	GACGTAGTGGCAATTTCGCAT	9294-9314
KCA2_for	GTAATGGATTTAGATGCGATGCG	9809-9831
LeuAT_CesA1rev5	TACTAAACTAGCAAGCTCTTC	10754-10774
KDMJ_for	GTGCATCACATTGTTTCGGATGG	11234-11256
FAA_rev	GTATAATCTGCGTATTGAATCGGT	11341-11364
FAB_for	GCTACTAGTCGATGAATTACAGAT	11824-11847
H_04081_for	ATAGTGAGGCAACTATTCTCTTAACA	12501-12526
H_2372_for	ACATGCAATGTTACAGGCCTTGGA	12946-12969
H_271_for	GTCAAGATAAGAGGCTTCCGAATTG	13421-13445
H_272_rev	CTACATCTTCTAATGCAGCAATTGC	13466-13490
Bob_for	GGTAACTGGGGAAATCGG	13987-14004
Bob_rev	CCGATTTCCCCAGTTACC	13987-14004
For_Ava2	CAGCATGTTACACAACCAACTGC	14576-14598
SspI_for	GTGACAATTCATCAACAACTGCT	15188-15210
Mar_seq_rev	TGCTCTGATTCATCGTACTGACT	15629-15651
Fred_for	GCAGGGAAGTATAAATCGTTC	15881-15901
PCesB_for	GAGCATGAAGTGATAACACTAGGT	16451-16474
PCesB_for3	GAACGACAAGTAGCAAAGGTATC	17109-17131
PCesB_rev2	GTTAAAGGGAGCGATGACATTG	17275-17296
PCesB_rev1	CGCATTGTGAATCACTTCG	17594-17612
Rev1_VspI	GATAAGATCAAGCCATCTTGTCGG	18300-18323

Table 9 continued. **Oligonucleotide primers used for sequencing IH41385.**

Primer	Sequence (5' – 3')	Nucleotide positions in DQ360825
F-PSF_forseq	GATAATGAGACTGTGGCAGAAGGAG	18729-18753
Tony_rev	GCTTCTTCGTTTTGATAATAACC	18795-18817
F-PSF_revseq	GATACACAACTTTATCATCACGATG	19824-19848
Marg_for	GGTGGTGCATCTTTCACTG	20388-20406
Pvu_rev	CGATGTCATACCACATACTCCAGTT	20487-20511
Forward_Cvalin	GTGTTTGCCGAGTGAGAATGC	21010-21030
Cvalin_test1_for	GAGGGAGAGTTACCGACGTTAGAG	21750-21773
C3_Val_rev4	GCCGTTAATAGCGTCATAAACATCG	21892-21916
Reverse_Val_C1	GCTGCGCAACACCTCGACGT	22655-22674
ValBclIR	GTTCCAATCGGAATGCTGTCTTG	23430-23452
Alf_for	CGCTCTCAATAAATGGGAAAG	23932-23952
Tvalin_test1_rev	GTTTTCTGGTAACAGCGTTTCTAC	24000-24023
ValXba_for1	AGTTGTAAAGGGGGCCAAG	24626-24644
For_ABC4	GGTAAGTGGAGTAGAAGCATATT	25196-25218
PABC_rev1	CAAATGTCGAATTGTTGTGG	25366-25385
Rev3_ABC	GCCTTTACTATGTGCTTTTAGAGATG	26159-26184
For6_ABC	GTGGTATTTCAGATTTAATGAAATCC	26261-26286
Kg12919_for	GTCTAATGAAATCATTAGGAATC	26297-26319
Hpin1_rev	GAAAGAACCGTTACTAGGGAAATC	26535-26558
OE_20081_for	TGCGAATACAGAAAGCTTAGCCA	26688-26710
Macy_for	TTTTCGCTCTCCTTATTTGATG	27470-27491
Mh_SnaF	GAGCAAGAGTGAATCAAATGAG	28027-28048
Edna_for	GTGAGAGGAATGAAGGAGTAACG	28705-28727
Edna_rev	GGTTATCCATATAGACACTACG	28815-28836
Snap_for	GATTCTGGTAAGACTCAAAAGGTCAC	29315-29340
Mh_Ans2F	GAGCCTATCTGTGAAGCACAC	29775-29795
Bart_for	CGGTAACACACGATACATTGC	30455-30475
Mh_Ans2R	CGGTCCACCTATATTGTAG	31378-31396
Mh_AhaR	GAAGCTCTGTATTATACGG	31788-31806
Scott_rev	CCGATTTCCTCGTAATTCTTAGC	31944-31966

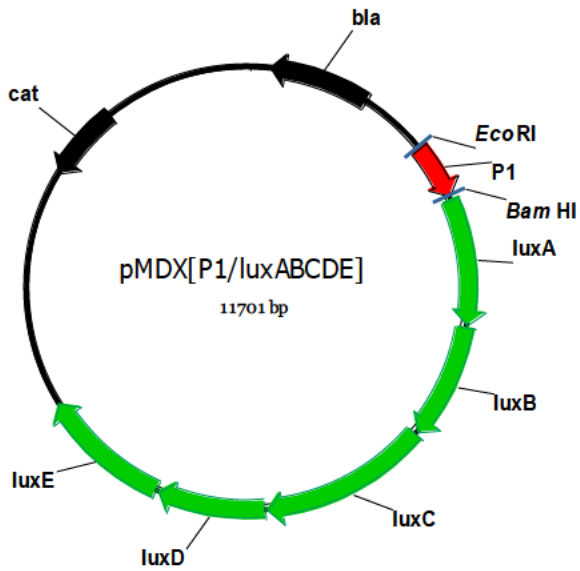
7.3 Plasmids

Two representative examples of promoter trap reporter gene vectors created during this work are shown. Both were created by directional cloning (EcoR1/BamH1) of the promoter under investigation into the multiple cloning site of vectors pAD123 and pXen1, for the pMKD and pMDX series, respectively.



pMKD[P_1/*gfpmut3a*]

E. coli / B. cereus promoter trap shuttle vector with ampicillin resistence in *E. coli* and chloramphenicol in *Bacilli*. Cereulide synthetase central P_1 promoter cloned upstream of reporter gene *gfpmut3a* (red-shifted green fluorescent protein).



pMDX[P_1/*luxABCDE*]

E. coli / B. cereus promoter trap shuttle vector with ampicillin resistence in *E. coli* and chloramphenicol in *Bacilli*. Cereulide synthetase central P_1 promoter cloned upstream of reporter gene *luxABCDE* (luciferase).

7.4 Genetically modified organisms

Table 10 **Genetically modified organisms created in this study.**

WSGMO	Strain description
7245	*Escherichia coli* DH5α pAD123
7250	*Escherichia coli* DH5α pAD43-25
7252	*Bacillus cereus* F4810/72 pMKD *[cesP1/gfp]*
7255	*Bacillus cereus* F4810/72 pAD43-25
7260	*Bacillus cereus* ATCC 14579 pMKD *[cesP1/gfp]*
7264	*Bacillus cereus* NVH 1519/00 pMKD *[cesP1/gfp]*
7267	*Bacillus cereus* IH41385 ΔpBCE
7271	*Escherichia coli* DH5α pXen-1
7276	*Bacillus cereus* F4810/72 pAD123
7279	*Bacillus weihenstephanensis* M67 pAD43-25
7280	*Bacillus weihenstephanensis* MC67 pAD123
7281	*Bacillus weihenstephanensis* M67 pMKD [*cesP1/gfp*]
7308	*Bacillus cereus* F4810/72 pXen-1
7309	*Bacillus cereus* F4810/72 pMDX [*cesP1/luxABCDE*]
7310	*Bacillus cereus* F4810/72 pMDX [*cspA/luxABCDE*]
7316	*Bacillus cereus* F4810/72 pMKD *[cesP1 MC67/gfp]*
7317	*Bacillus cereus* F4810/72 Δspo0A pMKD *[cesP1/gfp]*
7318	*Bacillus cereus* F4810/72 pMKD *[cesPB/gfp]*
7319	*Bacillus cereus* F4810/72 pMKD *[cesPH/gfp]*
7320	*Bacillus cereus* F4810/72 pMKD *[cesPT/gfp]*
7321	*Bacillus cereus* F4810/72 pMKD *[cesP1-P2/gfp]*
7322	*Bacillus cereus* F4810/72 pMKD *[cesP1-P2+/gfp]*
7323	*Bacillus cereus* F4810/72 pMKD *[cesP2/gfp]*
7324	*Bacillus cereus* F4810/72 pMKD *[cesP0/gfp]*
7325	*Bacillus cereus* ATCC14579 pMKD *[cesP1-P2/gfp]*
7326	*Bacillus cereus* F3080B/87 pMKD *[cesP1-P2/gfp]*
7327	*Bacillus cereus* NVH1519/00 pMKD *[cesP1-P2/gfp]*
7328	*Bacillus cereus* IH41385 pMKD *[cesP1-P2/gfp]*
7329	*Bacillus cereus* IH41385 ΔpBCE270 pMKD *[cesP1-P2/gfp]*

8 Acknowledgements

I would like to thank Prof. Dr. Siegfried Scherer for the opportunity to complete my doctoral studies at the Microbiology Unit of the ZIEL (Zentral Institut für Ernährungs- und Lebensmittelforschung) at the Technical University of Munich, campus Weihenstephan.

I would like to extend sincere thanks and appreciation to my doctoral supervisor, PD Dr. Monika Ehling-Schulz, for all the advice and support she gave in so many areas over the years I was in her research group.

I would also like to mention the others who made coming to work every day a pleasure: doctoral candidates Martina Fricker, Genia Lücking, and lab technologist Romy Renner. As well I thank M.Sc. student Bernd Straßer for his contribution to part this work. It was a pleasure to work with all the staff and students at the ZIEL Microbiology over the years.

I would also like to extend my deepest appreciation to my parents Irmgard and Hermann, my brother Norbert, and especially to my partner Heiko, for their constant encouragement and support from afar and near.

I thank Dr. Daniel Zeigler of the Bacillus Genetic Stock Center at the Ohio State University and Dr. Jo Handelsman of the Department of Plant Pathology at the University of Wisconsin-Madison for the kind gifts of the vectors pAD123 and pAD43-25. I would also like to thank Dr. Kevin Francis and the Xenogen Corporation for the gift of vector pXen1. Thank you to Line Thorsen of the Department of Food Science at The Royal Veterinary and Agricultural University in Denmark for the gift of the emetic *Bacillus weihenstephanensis* strain MC67. Thank you to my colleague Genia Lücking for the gift of the *spo0A* deletion mutant of *Bacillus cereus* strain F4810/72.

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