TECHNISCHE UNIVERSITÄT MÜNCHEN
Lehrstuhl für Mikrobielle Ökologie

Investigation of propanediol and fucose degradation by *Salmonella* Typhimurium

Lena Staib

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Nomenclature

$\frac{v}{v}$	Volume per volume
$\frac{w}{v}$	Weight per volume
μ	Micro
°C	Degree Celsius
1,2-PD	1,2-propanediol
%	Per cent
$	US-Dollar
lux	Luciferase
pdu	Genes for 1,2-propanediol utilisation
A	Ampere
a_w	Activity of water
AA	Amino acids
ad.	Fill up to
Amp	Ampicillin
Aq	Water
Aq_{bidest}	Double distilled water
Arc	Aerobic respiration control protein
AT	Austria
BLAST	Basic local alignment search tool
bp	base pairs
c	Centi
c_{final}	Final concentration
cAMP	Cyclic adenosine monophosphate
CCD	Charged-coupled device
cDNA	Complementary DNA
cfu	Colony forming unit
cfu	Colony forming units
Cm	Chloramphenicol
CoA	Coenzyme A
CRP	cAMP receptor protein
d	Day
D-Glc	D-glucose
DAPI	4',6-diamidino-2-phenylindole
DE	Germany
DEPC	Diethylpyrocarbonate

DHAP	Dihydroxy-acetone-phosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E_0	Standard redox potential
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
Fnr	Fumatare-nitare reduction regulatory protein
FUT2	α-(1,2)-fucosyltransferase
g	Gramme
G/C-content	Guanine-cytosine content in DNA
GC	Gas chromatography
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
h	Hour
HIP/PAP	Hepatocarcinoma-intestine-pancreas/ prancreatitis associated protein
HMO	Human milk oligosaccharide
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase
IVIS	*In vivo* imaging system
k	Kilo
Kan	Kanamycin
kb	Kilo base pairs
l	Litre
L-Fuc	L-fucose
L-Rha	L-rhamnose
LB	Lysogeny broth
LPS	Lipopolysaccharides
M	Mega
m	Meter
M cell	Microfold cell
Mb	Mega base pairs
min	Minute
mol	Mole
mRNA	Messenger RNA
MS	Mass spectrometry
n	Nano
Nal	Nalidixic acid
NCBI	National center for biotechnology information
NeAA	Non-essential amino acids
NGM	Nematode growth medium
NO	Nitric oxide
OD_{600}	Optical density at 600 nm

p	Pico
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potentium hydrogenii
RegIIIβ	Regenerating islet-derived III beta
RegIIIγ	Regenerating islet-derived III gamma
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SCFA	Short chain fatty acids
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulphate
SE	Sweden
Spec	Spectinomycin
SPI	*Salmonella* pathogenicity island
Strep	Streptomycin
T	Temperature
t	Time
T$_A$	Annealing temperature
T$_m$	Melting temperature
T3SS	Type three secretion system
TCA	Tricarboxylic acid
Tet	Tetracycline
TLR	Toll-like receptor
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UK	United Kingdom
US	United States
USA	United States of America
UV	Ultra-violet
V	Volt
VB-NCE	Vogel-Bonner no carbon-E
VB-NCE-YE	Vogel-Bonner no carbon-E with yeast extract
x g	Multiple of gravity

Summary

The human organism is composed of about 10^{13} eukaryotic (animal) cells. The gastrointestinal tract (GIT) has a surface area of about 300 m^2 and is inhabited by a community of microorganisms that is equal to or even exceed the total number of human cells in the body (Sender *et al.*, 2016). The number of microorganisms increases from the proximal to the distal gut and reaches its highest density in the colon. This community of resident microorganisms in the gut is called microbiota, which is of great importance to the human body. Besides other effects, it helps in digesting otherwise indigestible carbohydrates, affects the development of the immune system, and creates a highly competitive environment exacerbating colonisation by enteric pathogens such as *Salmonella* Typhimurium. Thus, *S.* Typhimurium has developed strategies to overcome colonisation resistance, since each year millions of cases of salmonellosis are reported worldwide.

In addition to the expression of specific virulence factors, enteropathogenic bacteria have to adapt their metabolism to the nutritional and physiological environment encountered outside and inside their host. The present study focusses on the utilisation of the alternative carbon source 1,2-propanediol (1,2-PD) in *S.* Typhimurium. 1,2-PD is the fermentation end product of bacterial growth with L-fucose (L-Fuc) or L-rhamnose (L-Rha) respectively. Both sugars are available in the GIT as they are present in food of herbal origin and constituents of mucins.

Besides *S. enterica* only *Listeria monocytogenes, Clostridium perfringens, Shigella sonnei, Yersinia enterocolitica*, and some enteropathogenic and enterotoxigenic *Escherichia coli* have been found to harbour the genes for 1,2-PD utilisation. Comprehensive database analyses conducted within the scope of this thesis revealed that the gene clusters required for 1,2-PD (*pdu*) and L-Fuc (*fuc*) degradation are conserved among *S. enterica* serovars sequenced thus far. Further, the genes required for tetrathionate respiration (*ttr*) were only found in the genomes of *S. enterica* and *Y. enterocolitica*. Tetrathionate serves as electron acceptor for the anaerobic respiration of 1,2-PD in *S.* Typhimurium. It was demonstrated that the *pdu* gene cluster, consisting of 21 genes, is expressed as a single polycistronic mRNA and controlled by the promoter P_{pduA}. The gene products of *pduC* and *fucA* are essential for the degradation of the respective substrate as shown by growth assays. The formation and immediate utilisation of 1,2-PD was demonstrated during anaerobic growth of *S.* Typhimurium 14028 with L-Fuc by monitoring the expression of genes required for L-Fuc and 1,2-PD degradation during all growth phases using *lux*-promoter fusion strains. Already minor concentrations of 1,2-PD were found to be sufficient to induce *pdu* gene expression, which is not or only weakly repressed by the simultaneous availability of 1,2-PD and alternative carbon sources such as L-Fuc or yeast extract, for example. Until the late exponential phase the presence of equimolar D-glucose (D-Glc) concentrations relative to 1,2-PD was found to repress the transcription of the *pdu* genes, because D-Glc was metabolised prior to 1,2-PD as demonstrated by monitoring the light emission of P_{pduA}::*gfp*-reporter strains. The relevance of 1,2-PD degradation

was also investigated in more complex substrates such as in the gut contents of pigs, fucosylated milk, mucus-secreting cell culture and the invertebrate model. Cell culture assays using mucus-secreting cell lines confirmed the necessity of mobility for host cell invasion.

Zusammenfassung

Der menschliche Körper besteht aus circa 10^{13} eukaryotischen (tierischen) Zellen. Der Gastorintestinaltrakt hat eine Oberfläche von etwa 300 m^2, welche von Mikroorganismen besiedelt ist. Die Anzahl der Mikroorganismen gleicht oder übersteigt die der Körperzellen (Sender *et al.*, 2016). Die mikrobielle Populationsdichte nimmt im proximal-distalen Verlauf des Gastrointestinaltraks zu und erreicht im Dickdarm ihr Maximum. Die im Darm ansässige Mikroflora wird auch als Mikrobiota bezeichnet und ist von höchster Wichtigkeit für den menschlichen Körper, da sie, unter Anderem, bei der Verdauung von, anderenfalls unverdaulichen, Kohlenhydraten behilflich ist, die Entwichklung des Immunsystems unterstützt und, durch die Erschaffung einer höchst kompetitiven Umgebung, die Ansiedelung von pathogenen Mikroorganismen, wie besispielsweise *Salmonella* Typhimurium erschwert. Daher hat *S.* Typhimurium Strategien entwickelt, um diesem Wettbewerb, der sogenannten Kolonisationsresistenz, zu entgehen, denn jährlich erkranken weltweit Millionen von Menschen an Salmonellose.

Neben der Expression spezifischer Virulenzfaktoren, müssen Enteropathogene ihren Stoffwechsel an die Nährstoffverfügbarkeit und physiologischen Bedingungen, die innerhalb sowie außerhalb ihres Wirtsorganismus angetroffen werden, anpassen. Die vorliegende Arbeit befasst sich mit der Verwendung der alternativen Kolenstoffquelle 1,2-Propandiol (1,2-PD) durch *S.* Typhimurium. 1,2-PD ist das Produkt der bakteriellen Fermentation, der im Darm vorliegenden Zucker L-Fucose (L-Fuc) und beziehungsweise oder L-Rhamnose (L-Rha).

Die Gene, die für die Nutzung von 1,2-PD als Kohlenstoffquelle relevant sind, liegen lediglich in den Genomen der Enterobakterien *S. enterica*, *Listeria monocytogenes*, *Clostridium perfringens*, *Shigella sonnei*, *Yersinia enterocolitica* und einiger enteropathogener und enterotixigener *Escherichia coli* vor. Die hier präsentierten Daten zeigen, dass nahezu alle *S. enterica* Serovare, die bisher sequenziert wurden, die Gene zur Nutzung von 1,2-PD (*pdu*) und L-Fuc (*fuc*) besitzen. Lediglich in den Genomen der *S. enterica* Serovare und *Y. enterocolitica* konnten die Gene, die den Bakterien die Tetrathionatatmung (*ttr*) ermöglichen, gefunden werden. Tetrathionat dient in *S.* Typhimurium als Elektronenakzeptor, um 1,2-PD anaerob oxidieren zu können. Ausgehend vom Promotor P$_{pduA}$ werden die *pdu* Gene im *S.* Typhimurium Stamm 14028 als polycistronische mRNA exprimiert, welche die genetische Information von 21 Genen beinhaltet. Die Genprodukte der Gene *pduA* und *fucA* sind essentiell für den Abbau des jeweiligen Substrats, wie in Wachstumsversuchen gezeigt werden konnte. Darüberhinaus konnte die Bildung von 1,2-PD, als Produkt der Fermentation von L-Fuc und dessen unmittelbarer Verwertung unter anaeroben Bedingungen, während der Kultivierung von *lux*-Promotorfusionsstämmen in fucosehaltigem Medium und der Aufzeichnung der Promotoraktivitäten von Genen, die für die Verwendung von L-Fucose und 1,2-PD als Kohlenstoffquellen benötigt werden, durch die emittierte Biolumineszenz der *lux*-Reporterstämme dokumentiert werden. Der Promotor P$_{pduA}$ wird bereits durch geringste Konzentrationen an 1,2-PD

induziert und scheint nicht oder nur in geringem Maße durch die gleichzeitige Verfügbarkeit anderer Kohlenstoffquellen, wie beispielsweise L-Fuc oder Hefeextrakt, gehemmt zu werden. Bis zum Erreichen der späten exponentiellen Wachstumsphase führte ie Bereitstellung äquimolarer Konzentrationen von 1,2-PD und D-Glukose (D-Glc) hingegen zu einer Repression der *pdu* Genexpression, da D-Glc zuerst verstoffwechselt wurde, wie in Experimenten durch die Beobachtung der Lichtemission von P_{pduA}::*gfp*-Reporterstämmen gezeigt werden konnte. Des Weiteren wurde die Relevanz des 1,2-PD Abbaus in komplexeren Substraten, wie dem Darminhalt von Schweinen, fucosylierter Milch, Mukus sekretierenden Zelllinen und dem Invertebratenmodell untersucht. In Zellkulturversuchen konnte die Notwendigkeit der Bewegungsfähigkeit für die Invasion von Wirtszellen, unter der Verwendung von Mukus sekretierenden Zelllinien, bestätigt werden.

1 Introduction

1.1 Food-borne illnesses

Food-borne illnesses are caused by the consumption of contaminated beverages or foods. Symptoms are nausea, acute gastroenteritis accompanied by watery diarrhoea, abdominal pain, vomiting or cramps. The causative agents are bacteria, viruses and parasites. According to the Centers for Disease Control and Prevention (CDC), about 48 million cases of food-borne illnesses occur annually in the USA. This leads to at least 128,000 hospitalisations and 3,000 deaths (CDC, 2015). The European Food Safety Authority (EFSA) reported a total of 55,453 food-borne illnesses in Europe for the year 2012, resulting in 5,118 hospitalisations and 41 deaths (EFSA and ECDC, 2014). Non-typhoidal *Salmonella* followed by bacterial toxins, viruses and *Campylobacter*, were the most common causative agents for food-borne illnesses in Europe, while in the USA Noroviruses are reported to be the major cause (EFSA and ECDC, 2014; CDC, 2015). The aforementioned incidences resulted in annual costs of about \$77.7 billion in the USA (Scharff, 2012), which underlines the impact of food safety on the healthcare system. Food-borne bacterial pathogens carry many genes that enable them to adapt to various environmental conditions and pressures (Humphrey, 2004). Examples of such genes include *rpoS*, *fur*, *ompR/envZ*, and *phoPQ*, which are required for the survival of low pH conditions in the human pathogen *Salmonella* Typhimurium. The gene products of *ompR/envZ* are further necessary for the adaptation to osmotic pressure. In order to react to changes in oxygen availability, the pathogen harbours the genes *fnr* and *arcAB*, decreasing respiration under microaerophilic or anaerobic conditions. Further adaptations are, for example, heat and cold shock proteins, resistance against oxidative stress and cationic antimicrobial peptides (Rychlik and Barrow, 2005). Hence, *Salmonella* is regularly isolated from raw meat and meat products (Andrews-Polymenis *et al.*, 2010; RKI, 2014), eggs and egg-products (Cox *et al.*, 2000; RKI, 2014), but also from fruit and vegetables (Wells and Butterfield, 1997).

1.2 The genus *Salmonella*

Salmonella are Gram-negative, rod-shaped, motile (peritrichously flagellated) bacteria, belonging to the family of *Enterobacteriaceae*. *Salmonella* is positive for catalase, methyl red, Simmons' citrate and the production of H_2S, but negative for oxidase, indole and acetoin (Voges Proskauer) production, and the hydrolysis of urea (Farmer *et al.*, 1985). *Salmonella* and *Escherichia coli* are highly related, sharing about 71 % DNA sequence homology and may thus originate from a common ancestor (McClelland *et al.*, 2001; de Jong *et al.*, 2012).
The name *Salmonella* is derived from the American veterinary surgeon Daniel Elmer Salmon who,

together with his colleague Theobald Smith, described the bacterium for the first time in 1886 (Salmon and Smith, 1886).

The genus consists of *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further subdivided into six subspecies on the basis of the serologic identification of O (somatic)- and H (flagellar)-antigens (Brenner *et al.*, 2000). To date, some 2,500 serovars have been described, of which approximately 60 % belong to *S. enterica* subsp. *enterica*, which is responsible for infections of warm-blooded animals including humans (Farmer *et al.*, 1985).

1.2.1 *Salmonella* infections

S. enterica can cause diverse clinical manifestations in humans. The main sources of infection are the consumption of contaminated food or water, however transmission via the faecal-oral route is also possible (Santos *et al.*, 2001; WHO, 2013).

S. Typhi and *S.* Paratyphi cause systemic infections with varying clinical features (de Jong *et al.*, 2012). Worldwide annual occurrences of typhoid fever are estimated at 22 million cases, 200,000 related deaths, and an additional six million cases of paratyphoid fever. The regions most affected are East and Southeast Asia, Africa, the Caribbean, and Central and South America (Newton and Mintz, 2014). Typhoid and paratyphoid fever are treated by administration of antimicrobials, leading to the emergence of multi-drug resistant strains (Su *et al.*, 2004; Majowicz *et al.*, 2010; Newton and Mintz, 2014).

A usually less life-threatening disease is caused by non-typhoidal *S. enterica* serovars such as Typhimurium and Enteritidis. Symptoms like diarrhoea, nausea, vomiting and intestinal cramping occur six to 72 hours after ingestion of contaminated foods (Santos *et al.*, 2001; WHO, 2013). The resulting gastroenteritis, also called salmonellosis, is generally self-limiting in otherwise healthy subjects and does not require special treatment. For elderly, immunocompromised and very young patients salmonellosis can also be very serious due to dehydration or bacteraemia, and subsequent sepsis (WHO, 2013; de Jong *et al.*, 2012). With about 93.8 million estimated cases worldwide, salmonellosis is one of the main causes of acute gastroenteritis. Of these, 80.3 million cases are considered as food-borne, and 155,000 are fatal (Majowicz *et al.*, 2010). Salmonellosis not only occurs in developing countries characterised by the lack of a clean water supply and sanitation but also in industrialised counties. Indeed in the USA one million and in Europe 91,034 illnesses are estimated to be caused by non-typhoidal *Salmonella* infections (CDC, 2013; EFSA and ECDC, 2014). Although salmonellosis is a notifiable disease, about 60-90 % of the cases remain unknown (BfR, 2014; WHO, 2013).

1.2.2 *S.* Typhimurium

1.2.2.1 The genome

The genome of *S.* Typhimurium LT2 consists of the chromosome (4.857 Mb) and a virulence plasmid (pSLT). Both chromosome and virulence plasmid have a G/C-content of 53 %. The 94 kb pSLT harbours 108 protein-coding genes and is absent in *S.* Typhi and *S.* Paratyphi. The copy number of the plasmid is dependent on the conditions of growth and varies between one

to three copies (McClelland *et al.*, 2001). The *S.* Typhimurium LT2 chromosome harbours four functional prophages (Gifsy-1/ -2 and Fels-1/ -2) besides 62 genomic islands and encodes 4,489 proteins (McClelland *et al.*, 2001). Of the 62 genomic islands, ten have been said to contribute to *Salmonella*'s virulence, lending them the name *Salmonella* pathogenicity islands (SPI) (Marcus *et al.*, 2000; Hensel, 2004). Probably the best characterised SPIs are SPI-1 and SPI-2, responsible for the invasion of epithelial cells (SPI-1) and intracellular survival (SPI-2) (Hensel, 2004).

1.2.2.2 The infection cycle

Humans, cattle, pigs, horses, poultry, rodents, sheep and reptiles belong to the host range of *S.* Typhimurium (Tsolis *et al.*, 1999; RKI, 2014). The majority of data concerning the infection cycle of *S.* Typhimurium are derived from animal studies in mice, but it is uncertain whether these findings can be extrapolated to the disease caused in humans (Tsolis *et al.*, 1999). *S.* Typhimurium causes gastroenteritis in humans and calves but a systemic, typhoid-like disease in susceptible mice (Tsolis *et al.*, 1999). The latter do not develop diarrhoea, which is the most prominent symptom in humans and calves. The mice die due to organ failure or bacteraemia, a complication that finds only rare occurrences in humans and cattle (Tsolis *et al.*, 1999). The development of the streptomycin mouse model offers the possibility of studying enteric salmonellosis in mice (Barthel *et al.*, 2003).

However *Salmonella* virulence and pathogenicity are mediated and influenced by the interplay of different sets of well-orchestrated virulence factors, which are dependent on the host, the host cell type, and the state of infection (Tsolis *et al.*, 1999; Bumann, 2002).

After ingestion but prior to causing infection of the terminal ileum, *S.* Typhimurium is confronted with the acidic pH of the stomach, decreasing oxygen availability, increasing osmotic pressure and a mounting number of other microorganisms (Carter and Collins, 1974; Foster, 1991; Rychlik and Barrow, 2005). In mice, the primary sites of infection are microfold cells (M cells) of Peyer's Patches (Carter and Collins, 1974). The mechanism of uptake into non-phagocytic cells is distinct from receptor-mediated phagocytosis applied by neutrophils or macrophages, and actively induced by the pathogen (Brumell *et al.*, 1999; Galán, 1999). The capability of *Salmonella* to trigger its internalisation is encoded by SPI-1, which was most likely acquired horizontally (Collazo and Galán, 1997; Galán, 1999). In total 12 SPIs have been described so far of which ten are present in *S.* Typhimurium (Hensel, 2004). SPI-1 and SPI-4 were described to interact in terms of host cell penetration (Gerlach *et al.*, 2007, 2008). SPI-4 encodes a giant adhesin and the associated secretion system, but the genetic information for a type three secretion system (T3SS) and the effector molecules, which are delivered to the host cell, are located on SPI-1 (Gerlach *et al.*, 2007; Collazo and Galán, 1997; Marcus *et al.*, 2000). Thus, active invasion of M cells and other non-phagocytic host cells is enabled by the translocation of effector proteins by SPI-1 T3SS, leading to cytoskeletal changes of the host cell. These cytoskeletal changes are visible as membrane ruffling and subsequent internalisation of the pathogen by enhanced macropinocytosis (Jones *et al.*, 1994; Brumell *et al.*, 1999). Shortly after *S.* Typhimurium invasion the M cells die, allowing the pathogen to access the underlying lymphoid tissue, where the bacteria encounter dendritic cells, macrophages, naive B cells, CD4^{+} and CD8^{+} T-cells (Jones *et al.*, 1994; Miller *et al.*, 2007).

Secreted SPI-1 effector proteins not only contribute to cell invasion, but also induce responses of the host cell, such as the stimulation of cytokine production. These lead to the development of diarrhoea and the induction of programmed cell death in macrophages (Galán, 1999; Hersh *et al.*, 1999).

For the systemic spread, which requires intracellular survival and replication of *Salmonella* in phagocytic host cells, gene products of SPI-2 are required (Marcus *et al.*, 2000; Hensel, 2000). The pathogen resides in the host cell's cytoplasm within a membrane compartment called the *Salmonella* containing vacuole (SCV), which is formed during phagocytosis of epithelial cells, macrophages, or dendritic cells (Hensel, 2000). In a minor portion of epithelial cells, 'hyperreplicating' bacteria were observed by Knodler *et al.* (2010). The authors reported fast replication of flagellated *S.* Typhimurium in the cytoplasm of epithelial cells with consequent pyroptotic (caspase-1 dependent) cell death, and extrusion of the cells from the monolayer. It is suggested that this 'hyperreplication' mechanism is used by the pathogen to provide a sufficient number of invasion-primed bacteria for secondary infections or shedding propagation to other hosts, as well as to induce inflammation (Knodler *et al.*, 2010).

In macrophages, the bacteria are able to replicate (Richter-Dahlfors *et al.*, 1997) within the SCV, but only persist in dendritic cells (Jantsch *et al.*, 2003). Via the SPI-2 encoded T3SS and secreted effector molecules *Salmonella* is able to influence the host cell. It can inhibit the fusion of the SCV with endo- and lysosomes, and induce or delay cell death in order to gain time for replication or release of accumulated bacteria (Marcus *et al.*, 2000; Kim *et al.*, 1998; Fink and Cookson, 2007; Klumpp and Fuchs, 2007).

1.3 Gut niche occupation by *S.* Typhimurium

Upon ingestion, *S.* Typhimurium is exposed to a series of hostile environments including the attainment of the densely populated gut, and the subsequent immune responses triggered by the pathogen itself (Rychlik and Barrow, 2005; Stecher *et al.*, 2007; Knodler *et al.*, 2010).

The bacteria need to be able to respond to a change in temperature, and the low pH in the stomach. As soon as the bacteria reach the gut they encounter changes in osmotic pressure, the impact of bile salts on the outer membrane, decreasing oxygen availability, the presence of bacteriocins or antimicrobial peptides and the increasing density of other microorganisms along the gastrointestinal tract (GIT) (Rychlik and Barrow, 2005). Aside from the acquisition of nutrients, replication, and the avoidance or circumvention of the host's immune system are essential prerequisites to infection (Falkow, 1997; Lawley and Walker, 2013).

1.3.1 Host and microbiota

Epithelial surfaces of the GIT are covered with a viscoelastic mucus gel protecting the underlying tissue from mechanical damage. This serves as a nutrient source for bacteria, but also forms a barrier to prevent the direct contact of bacteria and the epithelium (Corfield *et al.*, 1992). Besides being an obstacle for microorganisms, the mucus layer also prevents free diffusion of antimicrobials and oxygen, which consequently accumulate close to the epithelium (Donaldson *et al.*, 2016).

Intestinal mucus is secreted by goblet cells and consists of mucins comprised of oligosaccharides (glycans), attached to a protein backbone. The protein backbone is made of variable counts of repetitive proline, serine and threonine units (PTS-domain) (Corazziari, 2009; Hansson, 2012; McGuckin *et al.*, 2011; Moran *et al.*, 2011). The glycosylation pattern of mucins is tissue-specific, and not conserved among species (Hansson, 2012; Moran *et al.*, 2011). This depends on the number of PTS domains as well as on the steric hindrance of adjacent glycans. Furthermore, the availability of glycosyltransferases and their substrates in the goblet cells determine the glycosylation pattern (Sheng *et al.*, 2012; McGuckin *et al.*, 2011). Here it is distinguished between secreted gel-forming, secreted non-gel-forming, and cell surface mucins. Gel-forming mucins expand after secretion, forming polymeric net-like structures. The single mucins are linked via disulphide bonds of cysteine-rich domains on N- or C-termini (Ambort *et al.*, 2012). Cell surface mucins are of a stiff and elongated structure, contributing to the viscosity of the mucus if released from the cell's surface. They are important for cell signalling from the surface to the cytoplasm via transmembrane linkages to cytoskeletal proteins (Moran *et al.*, 2011). Due to its high capacity to bind water, mucus consists of about 98 % water (Hansson, 2012; Johansson *et al.*, 2011). In rats the mucus layer is thinnest in the small intestine ($\sim$123 μm) and thickest in the colon ($\sim$830 μm). The mucus of the small intestine is one-layered and tightly attached to the epithelium, while two mucus layers cover the stomach and large intestine. The inner mucus layer of the small intestine is very thin ($\sim$20 μm) or absent, whereas it amounts to $\sim$116 μm in the colon (Atuma *et al.*, 2001). The outer mucus layer of the colon is loose, and allows bacterial colonisation. Glycans exposed on the surface of cells or mucins are important adhesion-sites for bacterial flagella, pili or fimbriae (Chessa *et al.*, 2009; Juge, 2012). In contrast to the outer mucus layer, the inner mucus layer is dense and firmly attached to the epithelium. As recently demonstrated, it is usually free from bacteria (Atuma *et al.*, 2001; Johansson *et al.*, 2011), confirming the dogma that microorganisms contact the epithelium only in disease states (Donaldson *et al.*, 2016). Round *et al.* (2011) describe an exception of the latter assumption. The authors found *Bacteroides fragilis*, a common gut symbiont of humans, to reside within the colonic crypts of mice (Round *et al.*, 2011). *B. fragilis* is able to suppress antimicrobial immune responses and to utilise host-derived mucin glycans (Round *et al.*, 2011; Lee *et al.*, 2013). Otherwise, upon infection or bacterial invasion of the mucus gel, the secretion of mucus is enhanced, and bacteria entrapped within the mucus are cleared from the GIT (Govindarajan *et al.*, 2012; Moran *et al.*, 2011).

Besides this mechanical barrier, the human GIT is inhabited by up to 10^{14} bacterial cells per gram of contents. The density of bacterial cells increases from the small (10^1-10^7 bacterial cells per gram) to the large bowel (10^{12}-10^{14} bacterial cells per gram) (Qin *et al.*, 2010; Sekirov *et al.*, 2010). These bacteria are called gut microbiota or microbiome. 1,000 bacterial species have been found to inhabit the human intestine, and each individual harbours at least 160 bacterial species (Qin *et al.*, 2010). Thus, the bacterial composition of the microbiota varies between subjects. Nonetheless, anaerobic bacteria of the two divisions *Bacteroidetes* and *Firmicutes* (Eckburg *et al.*, 2005) generally dominate the microbiota. Besides *Bacteroidetes* and *Firmicutes*, *Actinobacteria, Fusobacteria, Lentisphaerae, Proteobacteria, Verrucomicrobia* and *Deinococcus-Thermus* are present in minor proportions (Qin *et al.*, 2010; Maccaferri *et al.*, 2011).

In the small intestine proteins, carbohydrates and fat are digested, and monosaccharides and amino

acids are absorbed. The main functions of the large intestine are the absorption of water, electrolytes and microbiota-derived short-chain fatty acids (SCFA) (Martins dos Santos *et al.*, 2010). Since nutrient sources change along the GIT, the composition of the microbiota is similarly subject to change. Besides amino acids, mono- and disaccharides are available in the small intestine where they support the growth of *Proteobacteria* (especially *Enterobacteriaceae*) and *Lactobacillales*. In the caecum and large bowel complex carbohydrates are predominant, which are diet- or host-derived. These nutrients are indigestible for the host and bacteria such as *E. coli*. Thus, the microbiota changes in favour of bacteria, like *Bacteroides* and *Clostridiales* which are able to break-down more complex polysaccharides. These more complex polysaccharides also include the glycan structures of mucins (Macfarlane *et al.*, 2005; Johansson *et al.*, 2011; Kamada *et al.*, 2013).

In the proximal to distal course of the GIT, the availability of easy fermentable carbohydrates becomes scarce. Thus, in the large bowel dietary oligo- and polysaccharides, sugar alcohols such as sorbitol and xylol, proteins and peptides, mucins and extruded epithelial cells all constitute available nutrient sources for microorganisms (Cummings and Macfarlane, 1991; Macfarlane *et al.*, 1998). Microbial breakdown and fermentation of the nutrient sources referred to above lead to the production of the SCFA propionate, acetate and butyrate, H_2 gas, NH_3, amines and phenols, for example. In turn, the SCFA serve as an energy source for the host, and are almost exclusively absorbed by the host epithelium (Cummings and Macfarlane, 1991; Macfarlane *et al.*, 2005; Macfarlane and Macfarlane, 2006).

In sum, the microbiota and its enormous enzymatic capability to access and metabolise otherwise indigestible nutrient sources is beneficial to the host, as it is provided with, for instance, vitamins and SCFA (Cummings and Macfarlane, 1991; Nicholson *et al.*, 2012; Hooper *et al.*, 2002). However, microbiota-released monomers support the growth of many intestinal bacteria (Chang *et al.*, 2004), including that of pathogenic intruders.

The microbiota not only provides nutrients, but is also known to train the mucosal immune system, which in turn shapes the composition of the microbiota (Sekirov *et al.*, 2010; Nicholson *et al.*, 2012). Further, it can actively contribute to protecting against colonisation of the gut by pathogenic bacteria (Stecher and Hardt, 2011). Stecher and Hardt (2011) describe three strategies, by which the microbiota can avoid niche occupation by enteric pathogens. 1) The direct inhibition of the pathogens by consuming available oxygen and releasing inhibitory metabolites such as SCFA or bacteriocins. 2) The stimulation of the host's immune defence. In this situation the microbiota releases lipopolysaccharides (LPS) or peptidoglycan, which serve as microbial patterns. Host cells recognise these microbial patterns, which results in enhanced mucus secretion and the release of antimicrobial peptides such as defensins and immunoglobulin A (IgA). 3) The depletion of favoured nutrients (Stecher and Hardt, 2011). 'Freter's nutrient-niche hypothesis' claims that a great variety of growth-supporting (microbiota derived) substrates are present in the gut. However, it is suggested that the concentration of each substrate is very low and thus growth limiting. The ability of a certain bacterial species to colonise and persist within the GIT is determined by its ability to use one or a small number of nutrients more efficiently than its competitors (Freter *et al.*, 1983; Stecher and Hardt, 2011).

1.3.2 Metabolic adaptation of enteropathogens

Pathogenic bacteria reach specific regions or tissues within the human or animal body, where they replicate and subsequently cause infection. Thus, enteric pathogens are able to adapt to the changing environments they encounter during their life cycle in order to survive, and occupy a niche within the GIT. Extracellular pathogens are frequently exposed to changing environmental conditions, whereas intracellular pathogens inhabit more stable environments (Eisenreich *et al.*, 2010). In mammalian cells catabolic reactions such as the glycolysis and the pentose-phosphate pathway (PPP), take place in the cytosol, and the tricarboxylic-acid (TCA) cycle, the β-oxidation of lipids, and the glutaminolysis in the mitochondria. The inner membrane of the mitochondria is also home to the cell's energy metabolism, as the enzyme complexes of the respiratory chain are situated there. Biosynthesis of nucleosides, amino acids, fatty acids or lipids, as well as gluco-neogenesis primarily occurs in the cytosol. Hence, there is a frequent exchange of metabolites between the two compartments. Metabolism is further supported by vesicles such as peroxisomes, lysosomes, and secretory vesicles (Eisenreich *et al.*, 2015).

Pathogenic bacteria, like *Listeria monocytogenes*, are adapted to replicate within the host-cytosol. Thus, they gain direct access to the metabolites present there. Other pathogens such as *S.* Typhimurium proliferate within a membrane compartment, the SCV. The membrane compartment separates the pathogen from the host-cytosol. Transport systems are required to facilitate the translocation of nutrients across the membrane barrier (Abu Kwaik and Bumann, 2015; Fuchs *et al.*, 2012; Eisenreich *et al.*, 2015). Successful intracellular pathogens have adapted their metabolism to that of the host cell. Parallel utilisation of different carbon sources, like glycerol, various carbohydrates, amino acids, fatty acids and nucleosides seems to be a common strategy of intracellular pathogens, albeit each pathogen has developed individual strategies to survive and proliferate within its host cell (Steeb *et al.*, 2013; Eisenreich *et al.*, 2015). *L. monocytogenes* is capable of the uptake and metabolism of amino acids, C_3-, C_4-, and C_5-carbon substrates, and phosphorylated hexoses. In mineral media growth only occurs if arginine, the branched-chain amino acids (BCAA) leucine, valine, isoleucine, and the sulphur-containing amino acids cysteine and methionine are provided (Premaratne *et al.*, 1991), although all genes required for the synthesis of the amino acids are present in the *L. monocytogenes* genome (Glaser *et al.*, 2001). Also the vitamins riboflavin, biotin, thiamine and thiotic acid have to be added to the mineral medium, as these cannot be synthesized by the pathogen (Glaser *et al.*, 2001). During intracellular proliferation, glycerol and glucose-6-P seem to be preferred carbon sources for *L. monocytogenes* (Eylert *et al.*, 2008; Chatterjee *et al.*, 2006; Eisenreich *et al.*, 2015).

S. Typhimurium is able to use more than 80 different carbon sources, as well as multiple electron acceptors for aerobic and anaerobic respiration, allowing the pathogen to adapt to varying environmental conditions (Bumann, 2009; Gutnick *et al.*, 1969). In infection studies in mice, Becker *et al.* (2006) found that metabolism is one of the most important activities of *Salmonella* during infection. The authors state that about half of the proteins detected in proteome studies during infection were involved in metabolism (Becker *et al.*, 2006; Bumann, 2009, 2010). This result was surprising, as so far, metabolic enzymes have not been considered as virulence factors (Bumann, 2009). The development of vaccines or antimicrobial drugs, targeting metabolic enzymes of *Sal-*

monella is hampered by the robustness of the *Salmonella* metabolism (Becker *et al.*, 2006). Owing to metabolic redundancy, the pathogen can compensate the loss of a certain metabolic pathway by another, which yields the same metabolites or intermediate products (Becker *et al.*, 2006; Bumann, 2009, 2010).

S. Typhimurium harbours all genes required for the central metabolic pathways such as glycolysis, the Entner-Doudoroff pathway (ED), the pentose phosphate pathway (PPP), the TCA-cycle, the biosynthesis of all amino acids, vitamins and co-factors, nucleotides, fatty acids and lipids, and the main anaplerotic reactions such as the glyoxylate shunt, malic enzyme, phosphoenolpyruvate (PEP) carboxy kinase and PEP carboxylase (Götz *et al.*, 2010; Eisenreich *et al.*, 2015). The pathogen is able to utilise C_2, C_3-, C_4-, and C_5-substrates as carbon sources, just as glycerol, fatty acids, pyruvate, lactate and, for example, fumarate (a C_4-dicarboxylate) can be metabolised under certain growth conditions within mammalian host cells (Götz *et al.*, 2010; Steeb *et al.*, 2013).

When *S.* Typhimurium replicates in HeLa cells or murine macrophage cell lines, glycolysis and ED pathway are upregulated, suggesting glucose (D-Glc) or C_6-sugars as the main carbon source (Götz *et al.*, 2010; Bowden *et al.*, 2009; Eriksson *et al.*, 2003). While the TCA-cycle was found to be repressed in murine macrophages and HeLa cells (Götz *et al.*, 2010), intracellular proliferation in systemically infected mice depends on a functional TCA-cycle (Becker *et al.*, 2006; Tchawa Yimga *et al.*, 2006). In the SCV of Caco-2 cells, *S.* Typhimurium primarily feeds on D-Glc, but not on glucose-6-P. Mutant strains impaired in D-Glc uptake ($\Delta ptsG$ and $\Delta manXYZ$) were found to incorporate less labelled (^{13}C) C-atoms into amino acids than the wild type. Bacteria disabled in glucose-6-P uptake ($\Delta uhpT$) did not show differential ^{13}C-incorporation into amino acids compared to the wild type (Götz *et al.*, 2010). Where D-Glc cannot be taken up by the *Salmonella* mutant strain, it uses host-derived $^{13}C_3$-substrates such as glycerol, pyruvate, or lactate for *de novo* synthesis of amino acids. Additionally, the uptake of host-derived amino acids is enhanced in the mutant strains, leading to direct incorporation of the latter into proteins (Götz *et al.*, 2010). Thus, it can be concluded that amino acids are synthesised *de novo* if rich carbon sources such as D-Glc are available while amino acid uptake from the host is increased where the bacteria feed on less energy-rich substrates (Götz *et al.*, 2010).

During systemic infection in mice, *S.* Typhimurium uses at least 31 different organic and 13 inorganic host-derived carbon and energy sources in parallel (Steeb *et al.*, 2013). As the single compounds are only available in small amounts, none could support growth as a single source of carbon and energy. Among the simultaneously utilised nutrient sources are glycerol, various hexoses and pentoses, sialic acid, fatty acids, nucleosides, and aerobic and anaerobic electron acceptors (Steeb *et al.*, 2013; Eisenreich *et al.*, 2015; Becker *et al.*, 2006). Glycerol and fatty acids might be derived from lipid oxidation (Steeb *et al.*, 2013; Abu Kwaik and Bumann, 2015). Mutants compromised in aromatic amino acid, methionine or pyrimidine synthesis show virulence defects in infected animals (Leung and Finlay, 1991; McFarland and Stocker, 1987; Fields *et al.*, 1986). For *S.* Typhimurium, residing within the SCV in murine macrophages, genes encoding transport proteins for gluconate, glucuronate or galacturonate, and galactonate were found to be upregulated. Hence, the authors suggest that these carbohydrates serve as the main carbon sources for the intracellular pathogen (Eriksson *et al.*, 2003).

After the successful invasion of a pathogen, it affects host cell metabolism in three ways: 1) The

pathogen depletes metabolites from the cytoplasm or incoming vesicles after endocytosis (Steeb *et al.*, 2013; Eisenreich *et al.*, 2015). 2) The pathogen secretes effector molecules that enhance the carbon metabolism of the host cell while simultaneously preventing apoptosis of the latter (Knodler *et al.*, 2005; Yin *et al.*, 2012). 3) Other effector molecules secreted by the pathogen trigger inducible nitric oxide synthase (iNOS) synthesis by the host cell, which leads to the conversion of nitric oxide (NO) to nitrate (Spees *et al.*, 2013). Nitrate can serve as an electron acceptor for anaerobic respiration of *S.* Typhimurium. Anaerobic respiration enables the pathogen to respire non-fermentable metabolites or fermentation end products. As anaerobic bacteria of the microbiota rely on fermentation, the pathogen gains access to a nutrient niche, which is not exploited by the microbiota (Lopez *et al.*, 2012; Thiennimitr *et al.*, 2011; Winter *et al.*, 2013).

It is assumed that some of the gene clusters enabling the utilisation of alternative carbon and energy sources, like for example ethanolamine (*eut* genes), 1,2-propanediol (1,2-PD) (*pdu* genes) or *myo*-inositol (*iol* genes), have been acquired by horizontal gene transfer since the G/C-content of these 'metabolic islands' differs from that of the genome (Dobrindt *et al.*, 2004; Rohmer *et al.*, 2011). The importance of the ability to use alternative substrates not consumed by members of the microbiota remains obscure. In general, information about available and utilised substrates during proliferation of *S.* Typhimurium in the lumen of the GIT is scarce (Fuchs *et al.*, 2012; Abu Kwaik and Bumann, 2013; Staib and Fuchs, 2014). Although most metabolic enzymes have been found to be dispensable for virulence, the expression of genes involved in cofactor biosynthesis, anaerobic energy metabolism and the degradation of diverse nutrients is upregulated during infection. Thus, metabolism seems to be directly linked to virulence (Becker *et al.*, 2006; Bumann, 2009; Fuchs *et al.*, 2012).

1.3.3 Subversion of defence mechanisms by *S.* Typhimurium

As mentioned above, bacteria of the microbiota stimulate the host's immune defence, secrete inhibitory metabolites, and deplete oxygen and nutrients (Stecher and Hardt, 2011). *S.* Typhimurium would not be a successful pathogen, if it were susceptible to all of the defence mechanisms encountered in the GIT. In contrast to the microbiota, the pathogen is well equipped with genes whose products counteract some of the defence mechanisms. Additionally, the pathogen is adapted to the conditions in the inflamed intestine (Stecher *et al.*, 2007; Santos *et al.*, 2009) and even triggers inflammation (Knodler *et al.*, 2010) (**Fig. 1.1**). The chemokine-like activity of antimicrobial peptides recruits leukocytes (e.g. neutrophils). Thereby, colonisation resistance and host defence are linked (Peschel, 2002). Direct host-pathogen interaction, for example upon successful penetration of the mucus layer, and host cell invasion by *S.* Typhimurium, leads to the secretion of cytokines (Santos *et al.*, 2009). Two lines of innate immune responses are activated by the secretion of cytokines. 1) The recruitment of neutrophils, which eliminate or kill bacteria, can also cause tissue damage. This contributes to the development of diarrhoea and inflammation. These lead to clearance of the gut, a reduction of bacteria (members of the microbiota and pathogens), and a decrease in inhibitory metabolites (Santos *et al.*, 2009; Stecher *et al.*, 2007, 2008). Using a mouse enteritis model, a recent study demonstrated that the bacteria of the microbiota are severely affected by the immune responses triggered during *Salmonella* infection (Deatherage Kaiser *et al.*,

2013). Ten days after infection, *Bacteroidetes* were nearly eliminated from the gut. The alteration of the microbiota, subsequently caused a change of the metabolite composition in the gut lumen. During inflammation higher concentrations of sugar moieties, like lactose, raffinose, melibiose and galactitol accumulated, which are usually consumed by members of the microbiota. The content of high mannose and fucosylated glycans in the gut was also found to have increased upon infection. Strikingly, the genes required for L-fucose (L-Fuc) degradation were found to be expressed in *S*. Typhimurium, residing in the inflamed intestine of mice. This suggests that *S*. Typhimurium metabolises L-Fuc, alongside D-Glc, galactose, maltose and mannitol in the microbiota-depleted gut (Deatherage Kaiser *et al.*, 2013). Furthermore, the recruitment of neutrophils and the expression of iNOS results in NO-radical and reactive oxygen species (ROS) formation. The reaction between NO radicals and ROS yields peroxynitrite, which in consequence makes the generation of nitrate or S- and N-oxides possible (Winter *et al.*, 2013; Spees *et al.*, 2013). *Enterobacteriaceae* can use nitrate as well as S- and N-oxides as electron acceptors for anaerobic respiration (Winter *et al.*, 2013). Hence, commensal *E. coli* strains gain a growth advantage over other members of the microbiota during inflammation because most of the latter rely on the fermentation of complex carbohydrates and amino acids rather than anaerobic respiration (Winter *et al.*, 2013; Bliska and van der Velden, 2012). Only during intestinal inflammation does nitrate respiration become possible for *S*. Typhimurium, upon which it provides the pathogen with a growth benefit in the lumen of the gut (Rivera-Chavez *et al.*, 2013; Lopez *et al.*, 2012; Bliska and van der Velden, 2012). Nitrate reductases are also present in *Vibrio cholerae, Campylobacter jejuni, Bacillus cereus, Enterococcus faecalis, Clostridium perfringens* and *Shigella* spp. (Lopez *et al.*, 2015; Staib and Fuchs, 2014), which might confer a possible growth advantage during infection to those bacteria as well. 2) The secretion of antimicrobial proteins and peptides leads to enhanced mucus secretion, the expression of iNOS, and the secretion of lipocalin-2. Enhanced mucus secretion increases the availability of high-energy nutrients derived from mucins (Stecher *et al.*, 2008; Corazziari, 2009). Lipocalin-2 prevents bacterial iron-acquisition by binding the bacterial iron-chelator enterobactin (Nairz *et al.*, 2007; Cairo *et al.*, 2011; Santos *et al.*, 2009; Raffatellu and Bäumler, 2010). Enterobactin is commonly found in *E. coli* and *S. enterica* (Raffatellu and Bäumler, 2010). In contrast to *E. coli*, *S*. Typhimurium harbours the alternative iron-chelator salmochelin (a glycosylated form of enterobactin), which is not bound by lipocalin-2 (Raffatellu and Bäumler, 2010; Stecher and Hardt, 2011). Further, *S*. Typhimurium was found to be resistant to many antimicrobial peptides and defensins secreted by members of the microbiota and the host (Peschel, 2002; Bader *et al.*, 2005; Shi *et al.*, 2004). Upon infection of the intestinal epithelium, enhanced secretion of C-type lectins, such as RegIIIγ (regenerating islet-derived) or RegIIIβ in mice or HIP/PAP (hepatocarcinoma-intestine-pancreas/ pancreatitis associated protein) in humans , are observed (Cash *et al.*, 2006; Stelter *et al.*, 2011). Peptidoglycan carbohydrate seems to be the target of the lectins (Cash *et al.*, 2006; Stelter *et al.*, 2011). While RegIIIγ is only bactericidal for Gram$^+$ bacteria (Cash *et al.*, 2006), RegIIIβ killed both Gram$^+$ and Gram$^-$ bacteria. However, RegIIIβ does not seem to be fatal for *S*. Typhimurium, rather it interferes with the intestinal translocation of *S*. Enteritidis in mice (Stelter *et al.*, 2011; van Ampting *et al.*, 2012). In comparison to mock-infected mice, oral RegIIIβ treatment resulted in increased luminal colonisation by *S*. Typhimurium. Survival of the pathogen could be a result of the stiff lipopolysaccharide (LPS) layer expressed on the bacterial

surface. Thus, the access of RegIIIβ to the peptidoglycan is limited, which might attenuate its antimicrobial effect on *S.* Typhimurium (Stelter *et al.*, 2011).

To reach epithelial cells, chemotaxis, which enables the pathogen to move towards attractants or away from repellents, plays a crucial role in *Salmonella* infections. *S.* Typhimurium follows a D-galactose gradient towards the epithelium (Stecher *et al.*, 2008). Flagella and fimbriae expressed by the pathogen, are used to swim in the direction of the D-galactose gradient and thus the epithelium, to propel the pathogen into the mucus layers, and to attach to terminal α-(1,2)-fucose moieties of mucins and the surface of epithelial cells (Chessa *et al.*, 2009; Stecher *et al.*, 2008). Furthermore, flagellin is recognised by host epithelial cells as a pathogen associated molecular pattern (PAMP). Flagellin binds to Toll-like receptor (TLR) 5, inducing proinflammatory responses (Stecher *et al.*, 2004).
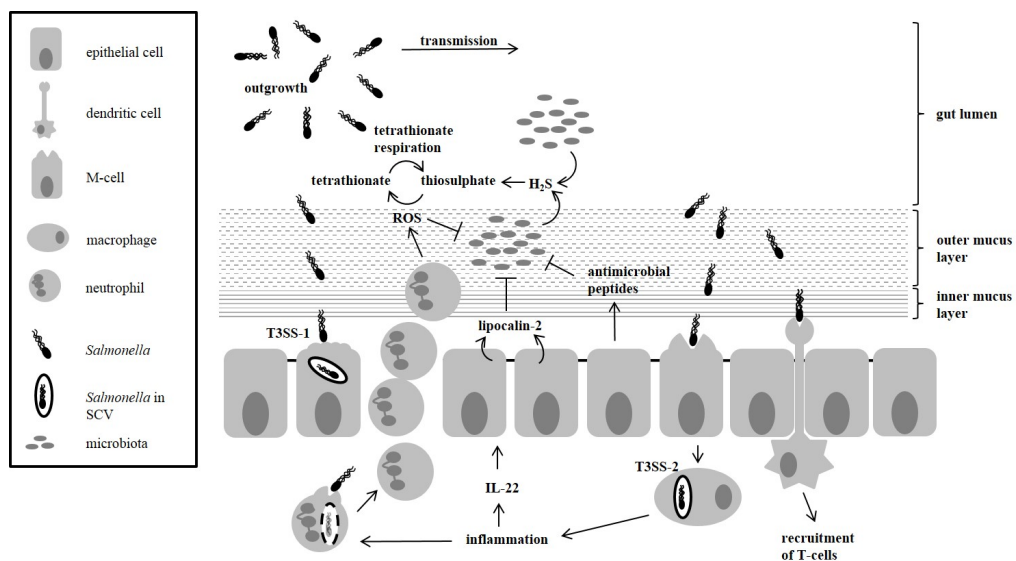
Figure 1.1: **Subversion of host defence by *S.* Typhimurium.**
The figure shows how *S.* Typhimurium manages to outgrow the microbiota. As described above, the pathogen is resistant to a number of antimicrobial peptides, ROS and the host-released lipocalin-2. By inducing inflammation, the pathogen creates environmental conditions, which are unfavourable for bacteria of the microbiota, but beneficial for itself. Transmission of the pathogen follows due to inflammation-induced diarrhoea. [Figure based on Stecher and Hardt (2011); Thiennimitr *et al.* (2012)].

1.3.4 Exploitation of exceptional nutrient sources

S. Typhimurium harbours the gene clusters responsible for ethanolamine, *myo*-inositol, sialic acid, and 1,2-PD degradation, which either differ in G/C-content to the overall genome or are absent in closely related species. Thus, it is assumed that these metabolic traits have been acquired by horizontal gene transfer (Almagro-Moreno and Boyd, 2009; Dobrindt *et al.*, 2004; Rohmer *et al.*, 2011).

Sialic acids are commonly found as part of mucin- and cellular glycoconjugates, and are involved in cell-cell recognition (Varki, 1993; Almagro-Moreno and Boyd, 2009). Proliferation of *S.* Typhi-

murium was impaired in gnotobiotic mice with a lack of free sialic acid in their gut, a result of the co-colonisation with a sialidase-deficient *Bacteroides thetaiotaomicron* strain (Ng *et al.*, 2013).

Myo-inositol, a polyol, is a component of the lipid phosphatidylinositol found in eukaryotic cell membranes. Its phosphorylated form phytate (inositol hexakisphosphate) occurs in plants and soil (Reddy, 2001; Rodrıguez and Fraga, 1999). The *iol* genes are thought to contribute to the virulence of *S.* Typhimurium in mice, pigs, chicken and calves (Carnell *et al.*, 2007; Chaudhuri *et al.*, 2009, 2013; Lawley *et al.*, 2006).

Ethanolamine is also a building block of a phospholipid, namely phosphatidylethanolamine, which is abundantly present in mammalian and bacterial membranes (Randle *et al.*, 1969).

A study by Tsoy *et al.* (2009) revealed the presence of the *eut* genes in the genomes of 84 bacteria belonging to different phyla (*Acidobacteria, Actinobacteria, Bacteroidetes, Chlorophlexi, Firmicutes, Fusobacteria*, and *Proteobacteria*). The *eut* genes are required for ethanolamine utilisation. The *eut* operon was found to exist in an extended (17 genes) version and various truncated versions. The long version, which is found in *Enterobacteriaceae*, contains the genes *eutKLMNS* (Tsoy *et al.*, 2009) that encode proteins, forming a carboxysome-like microcompartment. The microcompartment is thought to entrap enzymes and substrates optimising enzymatic reactions by spatial limitation of the reaction partners, and toxic, volatile intermediates to protect the cell from damage (Kofoid *et al.*, 1999; Penrod and Roth, 2006; Garsin, 2010).

The genes of the *eut* locus are organised consecutively, in counter-clockwise orientation on the *S.* Typhimurium genome. In *L. monocytogenes* the *eut* genes are still found in the same region of the genome, but are interrupted by genes required for 1,2-PD degradation (*pdu*) and vitamin B_{12} synthesis (*cob/cbi*) (Buchrieser *et al.*, 2003), whereas in *C. perfringens* the *eut, cob/cbi* and *pdu* genes are scattered over the chromosome (**Fig. 1.2**).
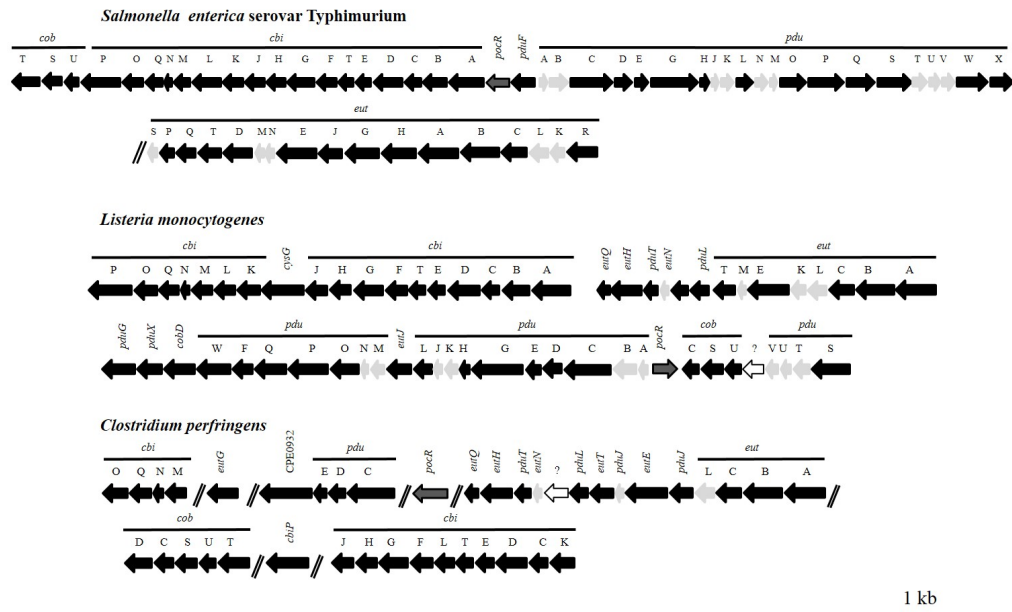
Figure 1.2: **Genetic organisation of *eut*, *pdu* and *cob/cbi* genes in enteropathogens**

Genes required for the degradation of ethanolamine, 1,2-PD and the synthesis of vitamin B_{12} in *S. enterica, L. monocytogenes* and *C. perfringens* are shown. Black arrows indicate genes encoding proteins of enzymatic function. Proteins forming the shell of the microcompartment are depicted in grey and unknown genes in white. The gene encoding the regulator PocR is indicated in grey surrounded by a solid black line. Gene functions can be obtained from **Tab. 5.11** in the Appendix. This graph has been published in Staib and Fuchs (2014).

The transcriptional regulator EutR is expressed constitutively in small amounts (Sheppard and Roth, 1994; Roof and Roth, 1992). Only in the presence of vitamin B_{12} and ethanolamine does EutR induce the expression of the *eut* operon. The main promoter of the *eut* operon is located in front of *eutS*. Induction of the gene expression from this promoter results in a polycistronic mRNA transcript, which harbours the genetic information of the genes *eutS-K*. The global regulator CRP (cAMP receptor protein), which senses a shortage in carbon and energy sources, was found to be required for *eut*-gene expression (Blackwell and Turner, 1978; Roof and Roth, 1992; Kofoid *et al.*, 1999).

Vitamin B_{12} is crucial for ethanolamine degradation, since it is necessary as a cofactor for ethanolamine ammonia lyase (EutBC), and to induce *eut*-gene expression by EutR (Scarlett and Turner, 1976; Roof and Roth, 1992). Only four enzymes that use vitamin B_{12} as a cofactor are known. These are tetrahydropteroylglutamate-homocysteine methyltransferase (*metH*), epoxyqueuosine reductase (*yjeS*), ethanolamine ammonia-lyase (*eutABC*) and the propanediol dehydratase (*pduCDE*) (Jeter, 1990).

Ethanolamine ammonia lyase is the first enzyme of the ethanolamine degradation pathway. The catalysed reaction yields acetaldehyde and ammonia (**Fig. 1.3**). Acetaldehyde can be reduced to ethanol by EutG, and ethanol is either secreted or can be metabolised to acetyl-CoA by EutE. Under aerobic growth conditions, acetyl-CoA can enter the central metabolism of the cell, whereas it

is converted to acetate via acetyl-phosphate under anaerobic conditions. Acetate is consequently secreted. Thus, under anaerobic conditions and the absence of a suitable electron acceptor allowing anaerobic respiration, ethanolamine can only serve as a nitrogen, not carbon source (Roth *et al.*, 1996).

De novo synthesis of the cofactor vitamin B_{12} only occurs under anaerobic growth conditions in *S.* Typhimurium. If the bacteria are grown aerobically with ethanolamine as the sole source of carbon and nitrogen, vitamin B_{12} or a precursor such as cyanocobalamin has to be provided in the medium (Jeter *et al.*, 1984; Escalante-Semerena and Roth, 1987). To use ethanolamine as a carbon and energy source under anaerobic conditions more efficiently, the compound needs to be oxidised by anaerobic respiration. Only tetrathionate, not fumarate or nitrate, supports anaerobic respiration of ethanolamine (Roth *et al.*, 1996).
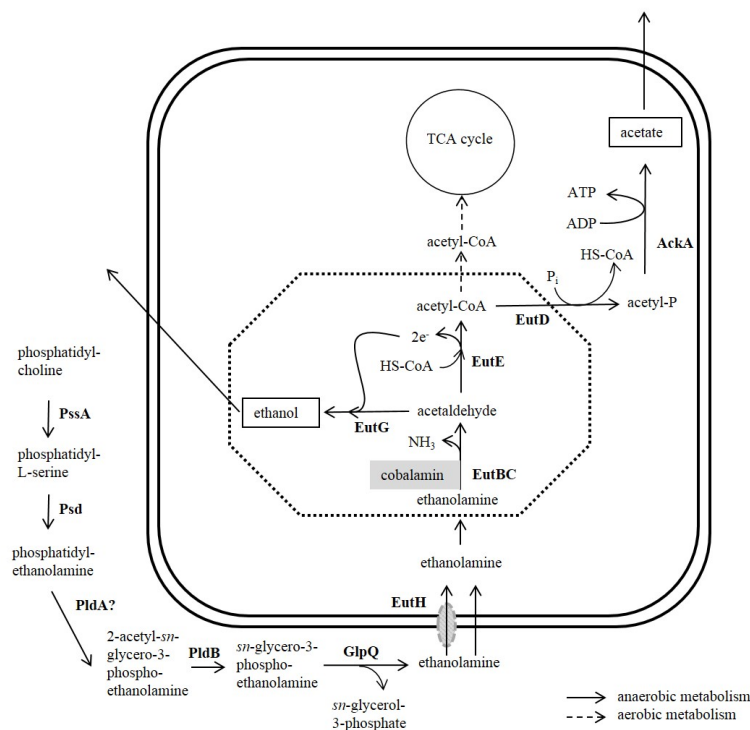
Figure 1.3: **Ethanolamine metabolism of *S.* Typhimurium.**

      The figure shows the metabolism of ethanolamine in *S.* Typhimurium. Enzymes are printed bold. Dashed arrows indicate aerobic degradation and solid lines show the anaerobic metabolism of ethanolamine in the absence of tetrathionate as a terminal electron acceptor. The microcompartment encoded by the *eut* operon is indicated as dashed octagon. PssA: phosphatidyl serine synthase; Psd: phosphatidyl serine decarboxylase; PldA: phospholipase A_1; PldB: lysophospholipase; GlpQ: glycerophosphoryl diester phosphodiesterase; EutH: ethanolamine permease; EutBC: ethanolamine ammonia lyase; EutG: alcohol dehydrogenase; EutE: aldehyde oxidoreductase; EutD: phosphotransacylase; AckA: acetate kinase; . [Figure modified Garsin (2010); Staib and Fuchs (2014)]

1.3.4.1 Tetrathionate respiration in *S.* Typhimurium

The *ttr* genes, which are located on SPI-2 (Hensel *et al.*, 1999), enable *S.* Typhimurium to perform tetrathionate respiration under anaerobic or microaerobic conditions (Hensel *et al.*, 1999). Analyses of the G/C-content of SPI-2 revealed that the genomic region shows a mosaic structure and is composed of three regions, which might be of different origin (Hensel *et al.*, 1999). The first region embraces the SPI-2 virulence genes (*ssaU-ssrB*), the second includes the genes required for tetrathionate respiration (*ttrRS* and *ttrBCA*), and the third harbours genes with a thus far uncharacterised function (Hensel *et al.*, 1999; Hensel, 2000). Studies in mice have led to the assumption that only genes of the first region are relevant for systemic pathogenesis of *S.* Typhimurium (Hensel *et al.*, 1999; Hensel, 2000). Hence, tetrathionate respiration does not contribute to *S.* Typhimurium virulence, but is thought to be important when the bacteria are exposed to tetrathionate-containing environments (Hensel *et al.*, 1999).

The genes *ttrBCA* encode the tetrathionate reductase, which is thought to be anchored in the cytoplasmic membrane via TtrC (Hensel *et al.*, 1999). Transcription of the tetrathionate reductase depends on TtrR and TtrS, forming a two component regulatory system encoded by *ttrRS* within the *ttr* gene cluster. During tetrathionate respiration, electrons are transferred from NADH + H$^+$ through the cellular quinone pool to tetrathionate as the final electron acceptor (Rivera-Chavez *et al.*, 2013), which is then reduced to two molecules of thiosulphate (Hensel *et al.*, 1999). The global regulator Fnr (fumarate-nitrate reduction regulatory protein) (Hensel *et al.*, 1999), but not ArcA or CRP, affects *ttr* expression since anaerobic conditions are required for *ttr* gene expression and tetrathionate respiration. Nitrate was found to slightly inhibit *ttr* expression, via a thus far unknown mechanism (Price-Carter *et al.*, 2001), because nitrate is an energetically more favourable electron acceptor (nitrate-nitrite E_0 = 433 mV) than tetrathionate (tetrathionate-thiosulphate E_0 = 170 mV) (Lopez *et al.*, 2012; Bliska and van der Velden, 2012).

Growth defects of *S.* Typhimurium mutants unable to utilise ethanolamine were observed in bovine raw milk and egg yolk (Srikumar and Fuchs, 2011). As *S.* Typhimurium is a food-borne pathogen, a contribution of ethanolamine degradation to the replication in food or the environment is also plausible. In the same study, significantly reduced proliferation rates were documented for the *S.* Typhimurium mutants 14028 Δ*eutR* and 14028 Δ*pocR* in the *Caenorhabditis elegans* model. The 14028 Δ*eutR* mutant cannot use ethanolamine, and the 14028 Δ*pocR* is compromised in synthesising vitamin B$_{12}$ and 1,2-PD degradation. The degradation pathways of ethanolamine and 1,2-PD in *S.* Typhimurium share the following features: 1) A polyhedral microcompartment is formed to retain volatile intermediates. 2) Vitamin B$_{12}$ is required as a cofactor for the central enzyme to convert the amine or diol to an aldehyde. 3) Tetrathionate is the only electron acceptor allowing anaerobic respiration of the respective substrate (Scarlett and Turner, 1976; Roth *et al.*, 1996; Kofoid *et al.*, 1999; Bobik *et al.*, 1999). Furthermore, the *pdu* genes were shown to be relevant for *S.* Typhimurium replication in macrophages (Klumpp and Fuchs, 2007). A further indication of the *in vivo* relevance of ethanolamine and 1,2-PD degradation in *S.* Typhimurium was published earlier (Stojiljkovic *et al.*, 1995; Conner *et al.*, 1998). Virulence defects were identified when *S.* Typhimurium strains deficient for ethanolamine (Stojiljkovic *et al.*, 1995) or 1,2-PD (Conner *et al.*, 1998) utilisation were orally administered to mice.

1.4 Research objective

Can the human gut be seen as a land of milk and honey for bacteria, or rather as a nutritional wasteland? At least for pathogens, considering the high density of bacteria in the human gut competing for food, the ability to use a variety of carbon and energy sources clearly cannot be disadvantageous. Host-derived sugars are released from complex structures such as mucins, due to the activity of enzymes secreted by members of the microbiota. These sugars not only serve as nutrients for bacteria of the microbiota, indeed they are also available for pathogens (Cummings and Macfarlane, 1991; Keeney and Finlay, 2013).

The genes for 1,2-PD degradation were previously found to be induced in murine and human cell cultures, to contribute to the replication of *S.* Typhimurium in macrophages, and to be relevant for infection in nematodes and mice (Heithoff *et al.*, 1999; Klumpp and Fuchs, 2007; Conner *et al.*, 1998; Srikumar and Fuchs, 2011). Some details about the regulation of the *pdu* gene cluster have been published previously (Rondon and Escalante-Semerena, 1992; Rondon *et al.*, 1995; Bobik *et al.*, 1992; Chen *et al.*, 1994, 1995), but the concentration of 1,2-PD required for the induction of the *pdu* gene expression remains unknown thus far. Whether the consecutive, clockwise oriented *pdu* genes are expressed as a polycistronic mRNA from a single promoter, as it is the case for the *eut* genes, has not yet been published. In the last publication concerning the promoter region of the *pdu* gene cluster, the authors suggest a single promoter for the clockwise-orientated genes of the *pdu* gene cluster, although at that time only *pduA*, *pduB* and *pduC* had been identified (Chen *et al.*, 1995). Thus, whether additional downstream promoters exist was not known at the time.

It is assumed that 1,2-PD is available to enteropathogens in the gut, because 1,2-PD is the fermentation end product of bacterial growth with L-Fuc or L-Rha (Daniel *et al.*, 1998). At least L-Fuc is constantly available in the gut, as it is a constituent of mucin glycoconjugates (Muraoka and Zhang, 2011). The ability of *S.* Typhimurium to break down L-Fuc and yield 1,2-PD was demonstrated (Badia *et al.*, 1985), and the interconnection between tetrathionate respiration, ethanolamine and 1,2-PD metabolism in the inflamed intestine has since become more concrete (Winter *et al.*, 2010; Bobik *et al.*, 1997).

At first, the thesis focused on the collection of data concerning metabolites, which are available to enteropathogens in the human gut. In the following, metabolic properties of selected enteropathogens were compared, with a special focus on metabolic pathways, required for 1,2-PD and L-Fuc degradation. Afterwards, the distribution of the *pdu* and *fuc* gene clusters among *salmonellae* was investigated in order to ascertain, whether 1,2-PD degradation is a general or an exclusive property. In *S.* Typhimurium strain 14028 genetic determinants of the *pdu* gene expression were considered, which included the search for possible promoter structures within the *pdu* gene cluster, and whether the *pdu* genes are transcribed as a single polycistronic mRNA or as multiple transcripts. Further, the minimal concentration of 1,2-PD, sufficient to induce *pdu* gene expression, was elucidated, and whether the *pdu* gene expression is repressed by the simultaneous presence of other carbon sources. The importance of central enzymes within the degradation pathways of 1,2-PD and L-Fuc was investigated, and whether *S.* Typhimurium is able to compensate for the loss of these genes. Further examinations were conducted, which focused on the relevance of 1,2-PD degradation in more complex substrates, and the interaction of *S.* Typhimurium and mucus-secreting cell lines.

2 Material and Methods

2.1 Material

Information about chemicals, enzymes, antibodies, and the suppliers are provided in **Tab. 5.1** and **Tab. 5.3** in the Appendix.

2.2 Microbiology

Bacterial strains used in this study are listed in **Tab. 5.4** in the Appendix.

2.2.1 Culture conditions

2.2.1.1 Cultivation in rich medium

Lysogeny broth (LB) (**Tab. 2.1**) was sterilised by autoclaving (121 °C, 20 min). After chilling to approximately 55 °C, further supplements such as antibiotics (see **Tab. 5.9** in the Appendix) were added aseptically. Where a solid medium was prepared, 15 $\frac{g}{l}$ agar were added to the LB-broth prior to autoclaving. Liquid cultures were inoculated using a single colony or a 1:1,000 dilution of an overnight culture, and shaken at 180 rpm.

Table 2.1: LB (Bertani, 1951).

Reagent	Amount
Tryptone from casein	10 g
Yeast extract	5 g
NaCl	5 g
Adjust pH 7.4	
Aq$_{bidest}$	ad 1 l

2.2.1.2 Cultivation in poor media

A mineral medium was prepared as described by Vogel and Bonner (1956), which is subsequently termed VB-NCE (Vogel-Bonner no carbon-E). It is composed of three stock solutions ($MgSO_4$, K_2HPO_4 and $NaNH_4HPO_4$; **Tab. 2.2**), which were separately prepared and autoclaved. Residual components of the mineral medium were filter sterilised using a 0.2 μm pore filter (**Tab. 2.2**). To support initial growth of bacteria 0.2 % ($\frac{w}{v}$), yeast extract were added (Price-Carter *et al.*, 2001)

and the resulting medium was termed VB-NCE-YE, which was used in growth assays. Unless stated otherwise, VB-NCE-YE was further enriched with trace elements (**Tab. 5.8** in the Appendix) (Price-Carter *et al.*, 2001) and carbon sources in the following concentrations: 27.8 mM D-Glc, 25 mM 1,2-PD, or 25 mM L-Fuc. Milks and gut content of pig were diluted 1:10 in VB-NCE mineral medium. In the case of anaerobic cultivation, 46 ml medium were transferred to a 100 ml culture vessel and stored overnight in the anaerobic chamber (37 °C) with an atmosphere of 80 % N_2, 10 % CO_2, 10 % H_2 (A35, Don Whitley, Shipley, YSW, UK) to enable oxygen reduction in the medium. Since tetrathionate is unstable when it is applied as a solution, it was added aseptically in a final concentration of 40 mM shortly before inoculation of the medium, while the bottles were kept in the anaerobic chamber. Bacterial overnight cultures were diluted 1:10 in VB-NCE and turbidity (optical density at 600 nm; OD_{600}) was measured in 1 ml of the dilutions using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Uppsala, SE) using VB-NCE as a reference. Where varying OD_{600} values were detected, the amount of the culture used as inoculum was adjusted. Generally, 2 ml of 1:10 diluted bacteria were used, resulting in a final dilution of the overnight culture of 1:250. The cultures were incubated in the anaerobic chamber at 37 °C without agitation. Samples were taken as triplicates of 200 μl each, and pipetted into the cavities of a 96-well microtiter plate (Greiner bio-one, Kremensmünster, AT). OD_{600} and, where applicable, bioluminescence in relative light units (RLU) luminescence at OD_{490} (RLU_{490}) were measured using a Wallac Victor[3] multilabel counter (Perkin Elmer, Waltham, MA, USA).

Where bacteria were cultivated aerobically, media were transferred to sterile Erlenmeyer-flasks and covered with aluminium foil to prevent contamination during storage, incubation, and transport outside the laminar flow cabinet. Shortly before inoculation, 200 nM cyanocobalamin (vitamin B_{12}) were added. Vitamin B_{12} is sensitive to light, therefore the flasks needed to be sheltered from direct incidence of light. Inoculation and sampling followed as described for anaerobic cultivation under the laminar flow cabinet. The cultures were incubated at 37 °C on a shaker (Certomat MO, Sartorius, Göttingen, DE) at 180 rpm.

Table 2.2: Vogel-Bonner mineral medium (Vogel and Bonner, 1956).

Reagent	c_{final}
20 x MgSO$_4$·7 H$_2$O	16.4 mM
20 x K$_2$HPO$_4$	1.148 M
20 x NaNH$_4$HPO$_4$·4 H$_2$O	334.8 mM
Prepare three separate solutions in Aq$_{bidest}$ that are autoclaved	
VB-NCE	
1 x MgSO$_4$·7 H$_2$O	0.82 mM
1 x K$_2$HPO$_4$	0.0574 mM
1 x NaNH$_4$HPO$_4$·4 H$_2$O	16.74 mM
Adjust pH to 7	
Aq$_{bidest}$	ad 1 l

Reagent	c_{final}
Autoclave solution	
VB-NCE-YE	
1000 x Trace elements (see **Tab. 5.8**)	1 x
20 % ($\frac{w}{v}$) Yeast extract	0.2 % ($\frac{v}{v}$)
Carbon source [a]	
Vitamin B$_{12}$ or Na-tetrathionate [b]	
VB-NCE	ad 1 l

[a] 1,2-PD, 25 mM, L-Fuc, 25 mM, D-Glc, 27.8 mM

[b] aerobic: Vitamin B$_{12}$ 200 nM, anaerobic: Na-tetrathionate, 40 mM

2.2.1.3 Turbidity measurements

To determine the cell density of a bacterial culture, OD$_{600}$ was measured. Two different methods were applied for OD$_{600}$ measurements. 1) One ml of a bacterial culture was transferred into a disposable cuvette (Rotilabo®, Carl Roth, Karlsruhe, DE) and measured in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech). Where the OD$_{600}$ was above 1, the measurement was repeated after diluting the sample 1:10 in an uninoculated medium. 2) 200 μl of a bacterial suspension were pipetted into the cavity of a 96-well microtiter plate (Greiner bio-one) and subsequently OD$_{600}$ was measured (0.1 s) using Wallac Victor^3 1420 multilabel counter (Perkin Elmer). For later analyses of the results, it should be kept in mind that the Wallac Victor^3 1420 is a relative and the spectrophotometer an absolute reader, meaning the obtained OD$_{600}$ values are not directly comparable (personal communication Perkin Elmer technical support).

2.2.1.4 Motility test

To investigate motility of bacteria, soft agar was prepared, consisting of 2 g tryptone, 1 g yeast extract, 1 g NaCl and 0.8 g agar in 200 ml Aq$_{bidest}$. After autoclaving, the soft agar was poured into squared petri dishes (120 mm x 120 mm x 17 mm, Greiner bio-one). Ten μl of bacterial overnight cultures were plotted concentrically on a plate, which was incubated at 37 °C with the lid facing upwards. The next day, the turbid, transmigrated area was determined by examination of the petri dish using oblique illumination (Dinkelberg, Stuttagrt, DE).

2.3 Molecular biology

2.3.1 Preparation of nucleic acids

2.3.1.1 Plasmid isolation

Plasmids were isolated from bacterial cultures grown overnight in LB-medium supplemented with the respective antibiotic(s). The plasmid isolation kits QIAprep® Spin Miniprep (Qiagen, Hilden, DE) and Invisorb® Spin Plasmid Mini Two (Stratec, Birkenfeld, DE) were used as specified by the manufacturer. For elution, Aq_{bidest} was heated to 55 °C and 30-50 μl were applied to the membrane. The column was incubated for 1 to 3 minutes prior to 1 min centrifugation at 17,000 x g.

2.3.1.2 Isolation of genomic DNA

To isolate genomic DNA, LB-medium containing the appropriate antibiotic(s) was inoculated with a single colony and incubated overnight at 37 °C. Cells of 1.5 ml overnight culture were pelleted at room temperature (RT) at 17,000 x g for 20 min. After aspiration of the supernatant, the pellet was resuspended in 400 μl lysis solution (**Tab. 2.3**) and 100 μl lysozyme (10 $\frac{mg}{ml}$), and incubated on ice for 15 min. Afterwards, 10 μl of 10 % SDS ($\frac{w}{v}$) and 5 μl of proteinase K (10 $\frac{mg}{ml}$) were added and incubated overnight at 55 °C. For precipitation of the DNA, 500 μl 2-propanol were used. After transferring the DNA to a new reaction tube using a needle, it was washed in absolute, and 70 % ($\frac{v}{v}$) ethanol. The DNA pellet was air-dried and dissolved in 150 μl Aq_{bidest} with 1 μl RNase (10 $\frac{mg}{ml}$).

Table 2.3: Lysis solution for DNA isolation.

Reagent	c_{final}
Tris-HCl pH 8.0	100 mM
EDTA	5 mM
NaCl	200 mM

2.3.1.3 Isolation of RNA

Total RNA was isolated based on the protocol of Chomczynski and Sacchi (2006). *S.* Typhimurium was grown aerobically in 50 ml VB-NCE-YE medium supplemented with trace elements, vitamin B_{12} and either D-Glc or 1,2-PD to an OD_{600} of 2.0, which was measured in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech). The bacteria were harvested in 50 ml reaction tubes and centrifuged at 3,220 x g, 4 °C for 10 min. The supernatant was aspired and the pellet dissolved in the remaining liquid. The suspension was transferred to 1.5 ml reaction tubes, which were centrifuged again at 4 °C and maximum speed (17,000 x g) for 1 min. The remaining liquid was discarded. The cells were kept on ice during all steps of the protocol, unless stated otherwise. In the following, the pellet was resuspended in 1 ml TRIzol by gentle mixing. TRIzol lyses

cells, inhibits RNases, and contains phenol in which RNA can be dissolved. The TRIzol solution was transferred to a 2 ml phase lock tube (5 PRIME, Hilden, DE) to which 400 μl chloroform were added. After inverting the tube, it was incubated 2 to 5 min at RT. Following incubation, a 15 min centrifugation step followed at maximum speed (17,000 x g) and RT, resulting in phase separation. The upper, aqueous phase, which contains dissolved RNA, was pipetted into a new 1.5 ml reaction tube, containing 450 μl 2-propanol for precipitation. After mixing by gentle inversion, the tube was incubated for 30 min at RT. The supernatant was removed after centrifugation (RT, 30 min, 17,000 x g), and the pellet was washed using 350 μl 70 % ($\frac{v}{v}$) ethanol with subsequent centrifugation at 17,000 x g, 4 °C for 5 min. The supernatant was aspired and the pellet air-dried at RT. The nucleic acid was dissolved in 25 μl of Aq$_{DEPC}$ during a 5 min incubation at 65 °C and occasional vortexing. Nucleic acid concentrations were determined using NanoDrop 1000 (Thermo Scientific, Braunschweig, DE). The preparation sample was stored at -20 °C.

Table 2.4: Preparation of Aq$_{DEPC}$.

Reagent	Volume
Diethylpyrocarbonate (DEPC)	1 ml
Aq$_{bidest}$	ad 1 l
Stir overnight at RT	
Autoclave twice	

2.3.1.4 DNase digestion of RNA

For DNase digestion approximately 40 μg nucleic acid solution were dissolved in 79 μl Aq$_{DEPC}$, and denatured at 65 °C for 5 min. Afterwards, the sample was kept on ice for a further 5 min. Remaining on ice, 10 μl of 10 x DNase buffer were added as well as 10 μl DNase (1 $\frac{U}{\mu l}$). The mixture was incubated at 37 °C for 75 min. To obtain pure RNA, 100 μl Roti®-Aqua-P/C/I (Carl Roth) and the DNase digestion sample were mixed for 15 s by gentle inversion of the tube, and centrifuged at 15 °C for 12 min at 17,000 x g. The aqueous phase was transferred to a 1.5 ml reaction tube. A 1:30 mixture of ethanol and 3 M Na-acetate (pH 6.5) was added in a 2.5 fold excess. To precipitate RNA, the tube was incubated overnight at -20 °C, and centrifuged at 17,000 x g, 4 °C for 30 min. The supernatant was aspired, and the pellet was washed using 70 % ($\frac{v}{v}$) ethanol. After a centrifugation step (10 min, 4 °C, 17,000 x g) the supernatant was removed, and the pellet air-dried at RT. RNA was dissolved in 40 μl Aq$_{DEPC}$ and incubated at 65 °C. NanoDrop 1000 (Thermo Scientific) was used for concentration and purity determination of the total RNA. To ensure that DNA-free RNA was obtained, PCR analysis was performed. In the case of visible bands on the agarose gel after PCR amplification, the DNase digestion protocol needed to be repeated.

2.3.1.5 cDNA synthesis

For complementary DNA (cDNA) synthesis, qSkript^{TM} cDNA SuperMix (Quanta biosciences, Gaithersburg, MD, USA) was used as specified by the manufacturer. Shortly, 1 μg of RNA was added to 4 μl qSkript^{TM} cDNA SuperMix (5 x concentrated) in a total volume of 20 μl. The SuperMix contains MgCl$_2$, dNTPs, buffer, qScript reverse transcriptase, random primers, and a recombinant RNase inhibitor protein. After combining RNA and the qSkript^{TM} mixture, it was incubated in a PCR cycler using the following programme: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C and holding at 8 °C. Following the amplification, 80 μl Aq$_{DEPC}$ were added to the cDNA sample to obtain a final volume of 100 μl. For the consecutive PCR reaction, the cDNA was diluted 1:10 in Aq$_{bidest}$.

2.3.1.6 Determination of nucleic acid concentrations

Concentrations of nucleic acid samples were determined spectrophotometrically using NanoDrop 1000 (Thermo Scientific). Aq$_{bidest}$ was used as reference. For the measurement 1 μl of water or nucleic acid sample was applied to the lower measurement pedestal of the NanoDrop device, and the extinction ratios of $\frac{260\ nm}{280\ nm}$ and $\frac{260\ nm}{230\ nm}$ (λ = 260 nm: Absorption of nucleic acids, λ = 280 nm: Absorption of proteins, λ = 230 nm: Absorption of salts, sugars, organic solvents) were analysed. The value of $\frac{260\ nm}{280\ nm}$-ratio should be 2.0 for pure RNA and 1.8 for pure DNA. Where lower values are obtained, contamination with proteins or phenol might be on hand. Values $\leq$ 2.0 of the $\frac{260\ nm}{230\ nm}$-ratio of pure nucleic acids, point to contaminations with sugars, salts or organic solvents.

An alternative to determine the concentration of nucleic acids is agarose gel electrophoresis, which can be performed with an appropriate molecular weight marker (size reference), that allows for quantitation of DNA. If the nucleic acid concentration of the size reference is known, the intensity of the sample band can be compared to that of the size reference, after staining the nucleic acids using a quantitative dye such as ethidium bromide or GelRed^{TM}, for example.

2.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the concentration and size determination of nucleic acids, as well as to separate plasmids and DNA fragments. Due to the net-like structure formed by the agarose gel and the slightly negative charge of nucleic acids, DNA and RNA fragments were separated according to their size in horizontal gel chambers (Peqlab, Erlangen, DE) to which an electric field of 100 mA was applied. By choosing appropriate agarose concentrations, the separation of small or large fragments was favoured. Here, agarose concentrations of 1-2 % ($\frac{w}{v}$) in 1 x TAE buffer (Tris-acetate-EDTA, 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) were used. Nucleic acid samples and 6 x Loading Dye (Thermo Scientific) were mixed in a 1:5 ratio and transferred to the pockets of the agarose gel. GeneRuler^{TM} DNA ladder mix (Thermo Scientific) served as a reference for the determination of fragment size and nucleic acid concentration. To stain the

nucleic acids in the gel, it was incubated in a staining bath containing an intercalating fluorescent dye (1 x GelRed^{TM} in 0.1 M NaCl). By subsequent transillumination at 320 nm (UVsolo TS, Biometra, Göttingen, DE), the nucleic acid bands in the gel were visualised and documented.

2.3.3 Polymerase chain reaction

2.3.3.1 Standard PCR

To specifically multiply DNA-segments, polymerase chain reaction (PCR) was conducted. The approach is based on the use of a thermostable DNA-polymerase and the application of DNA-oligonucleotides (primers), which are homologous to the 3'- and 5'-end of the desired segment. As a template, either isolated genomic DNA, plasmid DNA, cDNA or in 50 μl Aq$_{bidest}$ resuspended bacterial colonies were used. The latter is referred to as colony PCR. The composition of the reaction mixture, as well as the performance protocol of standard PCR reactions are listed below (**Tab. 2.5** and **Tab. 2.6**). Primers of 18 to 24 bp were designed using Primer3web (Whitehead Institute for Biomedical Research, MA, USA) and ordered at MWG (Eurofins Genomics, Ebersberg, DE) (see **Tab. 5.5** in the Appendix). The optimal temperature for annealing (T$_A$) was selected at 0.5 °C below the melting temperature (T$_m$) of the primers. T$_m$ is calculated according to **Equation 2.1**. Where primers with different T$_m$ were used, the annealing temperature was adapted to the primer with lower T$_m$. To generate PCR-fragments for gene deletions that require primers of more than 50 bp in length, annealing temperatures of 51 °C were applied and only modified in the case of unspecific amplification.

$$T_m = 2\,^\circ C \cdot (A + T) + 4\,^\circ C \cdot (C + G) \tag{2.1}$$

The time for elongation depends on the size of the PCR-amplicon. In general, a pace of 1 $\frac{kb}{min}$ was assumed for the *Taq* polymerase used. After amplification, purity and size of the PCR-products were determined by agarose gel electrophoresis.

Table 2.5: Reaction mixture for PCR.

Reagent	c_{final}
Taq Polymerase buffer	1 x
MgCl$_2$	1.5 mM
dNTP Mix	0.8 mM
Forward primer	20 pmol
Reverse primer	20 pmol
DNA template	50-100 ng
Taq polymerase	1.25 $\frac{U}{\mu l}$
Aq$_{bidest}$ ad 50 μl	

Table 2.6: Standard PCR programme.

	Temperature	time	
Initial denaturation	95 °C	5 min	Cycle 1
Denaturation	95 °C	10 sec	
Annealing	T_A	30 sec	Cycle 1-35
Elongation	72 °C		
Final elongation	72 °C	10 min	Cycle 35

2.3.3.2 Purification of PCR-products

PCR-products were purified using the MSB® Spin PCRapache (Stratec) as specified by the manufacturer. Elution of the PCR products was performed in 30 to 50 μl Aq$_{bidest}$.

2.3.4 Enzymatic modification of DNA/ Cloning

PCR-products or plasmids (see **Tab. 5.6** in the Appendix) were used for restriction digestion by applying the appropriate restriction endonuclease(s) (further referred to as restriction enzymes) after purification. All restriction enzymes and buffers were purchased from Thermo Scientific. Generally, 5 to 10 U of restriction enzyme were used to digest 1 μg of DNA in a total volume of 20 μl. Enzyme specific reaction buffers and conditions were used as specified by the manufacturer. Successful restriction was verified by agarose gel electrophoresis. Where necessary, target fragments were recovered from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen) according to the protocol of the manufacturer. Where gel excision was not required, plasmids and DNA-fragments were purified using the MSB® Spin PCRapache (Stratec) as described above (**Section 2.3.3.2**). To avoid religation of plasmids after restriction digestion, the plasmids were treated with shrimp alkaline phosphatase (SAP). One U SAP was used in the respective buffer for 1 μg of plasmid DNA in a total volume of 20 μl. After 20 min incubation at 37 °C, the enzymes were thermally inactivated at 65 °C for 15 min. For ligation a ratio of plasmid to insert DNA of 1:1 was chosen in a total volume of 20 μl. By applying 5 U T4-DNA ligase and the recommended buffer, ligation was performed for 1 h at RT with a following thermal inactivation of the enzyme at 65 °C for 20 min. Prior to transformation, the sample was desalinated for 20 min on an MF^{TM}-membrane filter (Merck, Darmstadt, DE) with a pore size of 0.025 μm placed on Aq$_{bidest}$ in a petri dish.

2.3.5 Genetic modification of bacteria

2.3.5.1 Preparation of electrocompetent cells

In order to enhance the efficiency of extracellular DNA uptake by electroporation, bacterial cells were treated as described below. LB-medium (100 ml), supplemented with the appropriate antibiotic(s), was inoculated with 5 ml of an overnight culture, and incubated aerobically at 37 °C and 180 rpm. The culture was grown to an OD$_{600}$ of 0.4-0.6 and subsequently kept on ice for 15 min

to stop cell division. Unless otherwise stated, all further steps were carried out on ice. The chilled culture was transferred to 50 ml reaction tubes, and centrifuged in a pre-cooled centrifuge at 9,464 x g for 7 min at 4 °C. The supernatant was aspired and the pellets resuspended in 25 ml ice-cold glycerol (5 % ($\frac{v}{v}$)). The contents of the two reaction vessels were united and centrifuged again. The pellet was dissolved in 25 ml ice-cold glycerol (5 % ($\frac{v}{v}$)) and centrifugation was repeated. Following aspiration of the supernatant, the cells were resuspended in 2.5 ml ice-cold glycerol (5 % ($\frac{v}{v}$)). After the consecutive centrifugation step, the pellet was resuspended in 500 μl 10 % ($\frac{v}{v}$) ice-cold glycerol, and distributed to reaction tubes in 40 μl aliquots. The electrocompetent cells were stored at -80 °C.

2.3.5.2 Transformation/ electroporation

The transformation technique applied was electroporation, where electrocompetent bacterial cells are exposed to an electric shock (2.5 kV, 25 μF, 200 Ω) enabling DNA fragments or plasmids to pass the cell membranes given the transient formation of pores. Electrocompetent cells were thawed on ice, and 40 μl of sterile Aq$_{bidest}$ were added to the cells as well as the recombinant DNA. Following brief incubation on ice, the mixture was transferred to electroporation cuvettes (Peqlab) and exposed to an electric shock (Gene Pulser^{TM}, Bio-Rad, München, DE). Immediately after the electric shock, 1 ml of SOC-medium (**Tab. 5.7** in the Appendix) was added to the cells and, unless otherwise stated, the transformed culture was incubated at 37 °C for 1 h. Serial dilutions were spread on LB-agar plates supplemented with the respective antibiotic(s) and incubated at 37 °C overnight.

2.3.5.3 Conjugation

Bacteria are capable of transferring DNA from one cell (donor) to another (recipient) by direct cell to cell contact. This mechanism of horizontal gene transfer is called conjugation.

Conjugation was used to pass the different derivatives of the plasmid pUTs from *E. coli* S17.1 to *S.* Typhimurium. The approach applied was conjugation on agar plates. The donor and recipient strain were spread on selective media, and both incubated at 37 °C. The next day, the strains were mixed in a donor to recipient ratio of 1:5 on a pre-warmed antibiotic-free LB-agar plate, and incubated for at least 6 h at 37 °C. The incubation temperature was chosen according to the optimal growth temperature of the recipient strain. After incubation, bacteria were completely recovered from the agar plate and resuspended in 1 ml LB-medium. Serial dilutions of 10^0 to 10^{-2} were spread on LB-agar plates containing two antibiotics in order to select for exconjugants, carrying the plasmid-mediated resistance. Another possibility included plating the serial dilutions on *Salmonella* selective agar plates, to which the respective antibiotic was added to select for the plasmid-encoded selection marker. Positive clones were twice subcultivated on selective media and confirmed by PCR.

2.3.5.4 Phage transduction

Besides transformation and conjugation, phage transduction is another mechanism by which DNA fragments can be transferred to a recipient strain. Here, the reproduction cycle of bacteriophage P22 was used to deliver the desired chromosomal region from one strain to the other. Phage transduction was used according to Maloy (1990), to transfer kanamycin insertion mutations generated by the gene deletion procedure described by Datsenko and Wanner (2000), and to circumvent the possibility of side effects caused by random recombination events of the Red recombinase system. Shortly, P22 phage lysates were prepared in order to gain phages carrying the target DNA fragment, which should be transferred to the recipient (wild type) strain. An overnight culture of the donor strain was diluted 1:100 in LB-broth, and incubated aerobically at 37 °C. After reaching an OD_{600} of 0.2, 5 ml of the culture were transferred to a glass tube to which 5 μl of a P22 suspension were added. The mixture was incubated for 6 h at 37 °C without agitation. Afterwards, the suspension was introduced to a reaction tube with a screw-cap, containing 50 μl chloroform. The sealed tube was stored at 4 °C for another 2 h to achieve complete cell lysis. The cell debris was pelleted (8,947 x g, 4 °C, 10 min), and the supernatant filter sterilised using a 0.2 μm pore filter. Until further use, the phage lysate was stored at 4 °C. To transfer the kanamycin-insertion into a wild type background, 200 μl of an overnight culture of the recipient strain was mixed with 10 μl of the phage lysate. After 1 h incubation at 37 °C (without agitation), the suspension was spread out on selective media and incubated at 37 °C. The next day, emerged colonies were spread on green indicator plates (**Tab. 2.7**), and kept at 37 °C overnight. In the case of phage-induced cell lysis, colonies appear in dark green, otherwise in white. Bacteria were subcultivated on green plates until white (phage-free) colonies emerged. These were selected, and passaged twice on selective media. Consequently, colony PCR was performed to confirm successful transduction.

Table 2.7: Green plates (Maloy, 1990).

Reagent	Amount
Tryptone from casein	8 g
Yeast extract	1 g
NaCl	5 g
Agar	15 g
Aq$_{bidest}$	ad 950 ml
autoclave (121 °C, 20 min)	
chill to approx. 55 °C	
add aseptically	
40 % ($\frac{w}{v}$) D-Glc	21 ml
2.5 % ($\frac{w}{v}$) Alizarin yellow GG	25 ml
2 % ($\frac{w}{v}$) Aniline blue	3.3 ml
50 $\frac{mg}{ml}$ Kanamycin	1 ml

2.3.5.5 Construction of *S.* Typhimurium reporter strains

Reporter strains of *S.* Typhimurium 14028 were constructed using the suicide vector pUTs-*lux* or pUTs-*gfp* (Starke *et al.*, 2013), which were integrated into the *S.* Typhimurium genome via homologous recombination. Replication of the plasmid in *trans* is dependent on the *pir* gene product, because the pUTs vector carries the origin of replication of the phage R6K.

The promoter region, located approximately 500 bp upstream of the start-codon of the respective gene, was amplified by PCR using genomic *S.* Typhimurium DNA as a template. The PCR-amplicons and the vector were digested using two restriction enzymes for site directed cloning (for restriction sites see **Tab. 5.6** in the Appendix). In the following, the promoter was ligated in front of the promoter-less reporter gene, encoded on the pUTs vector. The recombinant plasmids were delivered to *E. coli* S17.1 λ*pir* by electroporation. Afterwards, the bacteria were spread on LB-agar plates supplemented with chloramphenicol. Emerged colonies were selected and analysed for successful cloning by colony PCR. Conjugation was used to transfer the reporter gene *S.* Typhimurium. Due to the absence of the *pir* gene product (protein π) in *S.* Typhimurium, the replication of the plasmid in *trans* is not possible. *S.* Typhimurium strains that are able to grow on media containing chloramphenicol, carry a chromosomal integration of the plasmid due to the homologous recombination of the promoter regions. Exconjugants were selected on Brilliance *Salmonella* agar (Oxoid, Wesel, DE) containing chloramphenicol, and tested for genomic integration of the vector by colony PCR using chromosomal and vector-specific oligonucleotides.

2.3.5.6 Construction of *S.* Typhimurium insertion and deletion mutants

To generate non-polar deletion or insertion mutants of *S.* Typhimurium 14028, the method described by Datsenko and Wanner (2000) was applied. The approach is based on multiple homologous recombination events, enabled by the Red recombinase system of phage λ, encoded on plasmid pKD46. The recombinase system is used to exchange the respective gene by a kanamycin resistance cassette (kan^R). In a second recombination step, the kanamycin resistance cassette can be removed from the genome, resulting in gene deletion.

For kan^R-insertion or gene deletion mutagenesis in *S.* Typhimurium 14028 Nal^R, the kanamycin resistance cassette and FRT sites were amplified by PCR using the vector pKD4 as a template. To enable homologous recombination, the primers used added 50 bp extensions to the 5'- and 3'-end of the amplicon, representing the 5'- and 3'-end of the target gene. The primers were designed to retain a small residue of the gene (18 bp at the 5'- and 36 bp of the 3'-end) after deletion, in order to maintain the translational start signals of the gene and to avoid polar effects on downstream genes, resulting from the possible overlap of coding regions (Link *et al.*, 1997). Expression of the Red recombinase system was induced in a culture of *S.* Typhimurium, which carried the plasmid pKD46, by adding 1 mM arabinose to the growth medium. This culture was used for the preparation of electrocompetent cells, to which the previously mentioned PCR fragment was delivered by electroporation. After transformation, bacteria were incubated at 30 °C and 180 rpm for 1 h. Sub-

sequently, they were spread out on selective media containing kanamycin and incubated at 37 °C to eliminate pKD46 from the cells. Emerged colonies were tested for kan^R insertion by PCR, and positive clones were twice subcultivated on selective media. To remove the kanamycin cassette from the genome, the vector pCP20 was introduced to the cells by electroporation, which encodes a flippase recombinase from yeast. To obtain sufficient transformation rates, pCP20 needed to be isolated shortly before use. After transformation, the cells were incubated at 37 °C for 1 h. Serial dilutions were prepared and spread out on LB-agar plates containing chloramphenicol as selective supplement, and further incubated at 30 °C. Subcultivation of emerged colonies was performed at 37 °C, in order to remove pCP20 from the cells. Successful elimination of the kanamycin resistance cassette from the genome, was determined by parallel plating on selective media containing kanamycin, and LB-agar plates without kanamycin. Subsequently, PCR was performed to prove successful gene deletion. The PCR amplicons sequenced for validation (GATC, Konstanz, DE).

2.3.5.7 Complementation of gene deletions

To complement gene deletions, plasmids encoding the respective protein were constructed using pBR322. The gene deleted beforehand was amplified by PCR, using genomic *S*. Typhimurium DNA of the wild type strain as a template. If located in the direct vicinity of the gene, the promoter was also amplified. The primers carried restriction sites on their 5'-ends, and the amplicons were cloned into pBR322. The cloning sites were located within the vector-encoded *bla* gene, which was interrupted by successful ligation. For multiplication, the recombinant plasmids were introduced to *E. coli* S17.1 by electroporation. Positive clones were selected on solid media containing tetracycline. PCR was performed to identify bacterial colonies carrying correctly ligated plasmids, and these colonies were further used for plasmid isolation. *S*. Typhimurium kan^R-insertion or deletion strains were transformed with the respective pBR322-derivative.

2.3.6 Determination of bioluminescence and fluorescence

Insights into gene expression can be obtained by fusing reporter systems and promoters of genes or operons. The emission of light or fluorescence can be measured and the signal strength is proportional to the strength of gene expression.

2.3.6.1 Quantification of gene expression

The *lux*-reporter strains were cultivated in a VB-NCE-YE medium containing either L-Fuc, 1,2-PD or D-Glc as a carbon source. Samples were taken hourly, and were pipetted in triplicates into the cavities of white 96-well microtiter plates (Greiner bio-one) at 200 μl each. OD_{600} and bioluminescence (RLU_{490}) were measured using a Wallac Victor^3 1420 multilabel counter and its software, Wallac workstation (Perkin Elmer). To determine a threshold for significant light emission, the reporter strains described above (**Section 2.3.5.5**) were grown in a VB-NCE-YE

medium supplemented with D-Glc. Additionally, *E. coli* S17.1 λ*pir* carrying pUTs-P$_{pduA}$::*lux* in *trans* was grown in VB-NCE-YE supplemented with D-Glc. Consequently, a threshold value of 2,000 RLU$_{490}$ was determined.

2.3.6.2 Visualisation of gene expression

To illustrate the expression of genes, the In Vivo Imaging System (IVIS, Xenogen Corporation, Almeda, CA, USA) and the Olympus BX-51 (Olympus, Hamburg, DE) fluorescence microscope were used to obtain qualitative data.

2.3.6.3 Fluorescence microscopy

Fluorescence microscopy was used to investigate the induction of the *pdu* operon via the expression of green fluorescent protein (GFP). Samples were taken every 30 min and OD$_{600}$ was determined by Wallac Victor[3] (Perkin Elmer) measurement. Afterwards, the content of the cavities of the microtiter plate was transferred to 1.5 ml reaction tubes, and centrifuged at 10 °C for 5 min at maximum speed (17,000 x g). The supernatant was reduced to about 40 μl, and the bacteria resuspended in the remaining liquid. Until observation under the fluorescence microscope, the samples were kept on ice. Five μl of a sample were placed on a glass slide and covered with a cover slip. Bacteria were observed in bright field and under excitation by UV-light, using the green fluorescence channel (λ_{ex} = 487 nm, λ_{em} = 509 nm) and 1000 x magnification. Pictures were taken using the F-View Soft Imaging System (Olympus) and its software cell^F.

2.3.6.4 Xenogen In Vivo Imaging System (IVIS)

The IVIS was used to visualise *lux*-reporter activity of cell culture experiments and in *C. elegans* assays. Therefore, cell cultures or nematodes were inoculated with the respective *Salmonella* reporter strain and then measured in the detection chamber. Exposure times of 1 to 10 s were chosen. The signal of emitted light is detected and amplified by a CCD (charged-coupled device) camera (C2400-75H, Hamamatsu Photonics, Herrsching, DE), and luminescence is calculated by the number of photons emitted in 1 second per cm^2 and displayed as counts. Evaluation of the data was performed using the Living Image^{TM} software V 4.01 (Xenogen Corporation).

2.4 Cell culture

2.4.1 Cell lines

In the current research project the adherent human cell lines Caco-2 (ATCC$^®$ HTB-37^{TM}), LS174T (ATCC$^®$ CL-188^{TM}) and HT29-MTX were used. All three originate from human colorectal adenocarcinoma.

2.4.2 Culture conditions and media

All media, solutions and materials used for cell culture experiments are listed in **Tab. 5.2** in the Appendix. The medium used for the maintenance of cell cultures was RPMI 1640, supplemented with 10 % ($\frac{w}{v}$) foetal calf serum (FCS) and non-essential amino acids (NeAA). For washing steps, Dulbecco's phosphate buffered saline (PBS) supplemented with 0.1 mM EDTA (in the following referred to as PBS-EDTA) was used. The cells were kept in cell culture bottles for adhesive cells of 25 cm^2 to 175 cm^2 culture surface. The atmosphere in the incubator was set to 37 °C and 5 % CO_2. The medium was exchanged three times weekly. After reaching 80-90 % confluency, cells were detached by trypsination, and either subcultivated or prepared for storage in liquid nitrogen.

2.4.2.1 Subcultivation of human cell lines

For subcultivation, cells were washed twice using PBS-EDTA. An appropriate amount of 0.25 % trypsin (Life Technologies, Carlsbad, CA, USA) was added and incubated at 37 °C and 5 % CO_2, until the cells detached. Trypsin activity was prohibited by adding a culture medium containing FCS. Cells were harvested by centrifugation at 1,000 x g for 4 min at RT. After aspiration of the supernatant and subsequent replacement of the medium, cells were seeded in culture dishes.

2.4.2.2 Storing of human cell lines

To store human cell lines, the cells were resuspended in a freezing medium (10 % ($\frac{v}{v}$) DMSO in FCS) after trypsination. The reaction tubes containing 1 ml of cells, were stored at -80 °C for 24 h and thereafter transferred to the liquid nitrogen container.

2.4.3 Invasion assay

To perform invasion assays, $3.7 \cdot 10^4$ cells were seeded into each well of a 24 well culture dish. Cells were stained with trypan blue after trypsination, and counted in a 'Neubauer Improved' (Brand, Wertheim, DE) counting chamber. After reaching confluency the cells were maintained for another twelve days to ensure differentiation and mucus secretion in the case of LS174T cells. The invasion assay was performed according to Prouty and Gunn (2000) with some adaptations in protocol. Briefly, the confluent human cell culture was washed twice using PBS-EDTA, and inoculated with *S.* Typhimurium. The bacteria were grown overnight in LB-medium supplemented with the respective antibiotic. The bacterial culture was diluted 1:1,000 in a serum-free cell culture medium, which was consequently applied on the human cells. The inoculated cell culture was incubated for 1 h at 37 °C and 5 % CO_2. Afterwards the supernatant was aspired, and cells were washed twice with PBS-EDTA to remove surface adherent bacteria. To kill extracellular bacteria, gentamycin (100 $\frac{\mu g}{ml}$) dissolved in an FCS-containing cell culture medium was added, and incubated for another hour at 37 °C and 5 % CO_2. Cells were washed twice to remove residual gentamycin using PBS-EDTA, and subsequently lysed by applying PBS containing 1 % ($\frac{v}{v}$) triton X-100. The cell lysates were diluted in PBS and plated in triplicates on LB-agar. LB-agar was

poured in 120 x 120 x 17 mm petri dishes (Greiner bio-one). After curing, droplets (10 μl) of each diluted (10^0 to 10^{-3}) cell lysate were plotted on the upper end of the dish, which was set upright to generate separate dilution lines. After incubation at 37 °C for 24 h, emerged colonies were counted.

2.4.4 Immunofluorescence staining

For immunofluorescence staining and consecutive confocal microscopy, the cultured human cell lines HT29-MTX and LS175T were used.

The cells were seeded ($1 \cdot 10^5 \frac{cells}{well}$) in eight well ibidi-$\mu$-slides (ibiTreat or coated with collagen IV; ibidi, München, DE). Afterwards, the cells were maintained for 10 days (LS175T) or 21 days (HT29-MTX) at 37 °C and 5 % CO_2. The medium was exchanged daily by removing 200 μl of exhausted medium and adding 200 μl fresh medium (total volume per well 250 μl) in the same corner of each well to avoid detachment of cells or aspiration of the mucus layer. For infection assays with *gfp*-labelled *S.* Typhimurium, the cell culture was washed twice using PBS, and 250 μl serum-free cell culture medium were added and inoculated with 10 μl of an overnight culture of *S.* Typhimurium. After incubation times of 30, 60, 90 and 120 min at 37 °C and 5 % CO_2, the cells were fixated in 10 % ($\frac{v}{v}$) formalin-PBS for 15 min at 4 °C. Cells were washed twice with PBS, and primary antibodies (anti-MUC2 and anti-MUC5, diluted 1:200 in antibody dilution buffer, **Tab. 2.8**) were added. After 1 h incubation at 4 °C, cells were washed twice using PBS. To stain the nuclei and the mucus, DAPI (1 $\frac{\mu g}{ml}$) and the fluorescence labelled secondary antibodies (goat-anti-mouse Alexa Fluor$^®$ 488 and goat-anti-rabbit Alexa Fluor$^®$ 488) were applied. Prior to use, the secondary antibodies were diluted 1:200 in antibody dilution buffer, to which also DAPI was added. Cells were washed twice with PBS after 1 h incubation at 4 °C, and covered with 250 μl PBS. The μ-slides were placed under an inverted confocal microscope (IX81, Olympus, Hamburg, DE), and investigated using the 40 x magnification lens (water immersion). Z-stacks were recorded from the bottom of the well to the top of the mucus layer, and three-dimensional projections were reconstructed using Volocity 6.0 (Perkin Elmer) software.

Table 2.8: Antibody dilution buffer.

Reagent	Amount
10 x PBS	4 ml
BSA	0.4 g
Aq$_{bidest}$	ad 40 ml
While stirring add	
Triton X-100 (100 %)	120 μl
Store aliquots at -20 °C	

2.5 *C. elegans* assay

To avoid a heterogeneous population of larval states of nematodes, the *C. elegans* strain JK509 was used (kindly provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440), Twin Cities, MN, USA). This nematode strain is sterile at 25 °C and fertile when kept at 15 °C.

C. elegans JK509 was fed on *E. coli* OP50 (kindly provided by the CGC (*Caenorhabditis* Genetics Center)) until a sufficient number of hermaphrodites or larval state L4 nematodes were observed. To synchronise the growth stadium of *C. elegans*, eggs were isolated by washing nematodes and eggs off the agar plates, using M9-buffer (**Tab. 2.9**) supplemented with 0.1 % ($\frac{v}{v}$) triton X-100. Since the eggs were sticking to the NGM (Nematode Growth Medium, **Tab. 2.9**) agar plates, they were scraped using a cell culture spatula, and the plates were rinsed with buffer, which was collected in a 15 ml reaction tube. After centrifugation at 2,912 x g for 2 min at RT, the supernatant was reduced to about 1 ml and refilled with M9-buffer to a total volume of 10 ml. Centrifugation and reduction of the supernatant was repeated. Afterwards, 3 ml of freshly prepared bleaching solution (23.2 ml sterile Aq$_{bidest}$, 1.25 ml 5 M KOH, 0.5 ml 12 % NaOCl) were added to the pelleted nematodes, and the tube was shaken thoroughly for 3 min. Using a microscope, complete lysis of the nematodes was checked. Where any intact nematodes remained, shaking was continued for another 30 s. Following a centrifugation step, the supernatant was reduced in half without aspirating the liquid from the top, where the eggs are located, but from below the meniscus of the liquid. The reaction tube was refilled with M9-buffer to a total volume of 12 ml, and the pellet dissolved carefully. Centrifugation and washing steps were repeated until no smell of chloride was left. After a final washing step, and adjustment of the volume to 8 ml, the presence of eggs was checked microscopically. The tube was incubated on a shaker at 180 rpm (Certomat MO, Sartorius) and 15 °C. The next morning, the egg isolation was centrifuged, washed, and microscopically investigated for L1 larvae. Following a final centrifugation step the supernatant was reduced to 1 ml, and the solution transferred in droplets to *E. coli* OP50 NGM plates, which were incubated at 15 °C until L4 larvae had emerged, which were used for *Salmonella* infection assays. Adult nematodes were incubated at 25 °C to avoid further reproduction.

For the infection assays, an *S.* Typhimurium 14028 P$_{fucO}$::*lux* overnight culture was spread on NGM agar plates in portions of 100 μl per plate, and incubated at RT. The L4 larvae were either picked or washed off the *E. coli* OP50 plates, and transferred to the *Salmonella* infection plates after a washing step in an M9-buffer. Nematodes of one half of the infection plates remained feeding on *Salmonella* for 4 days. Nematodes of the other plates were relocated to NGM plates with *E. coli* OP50 after 4 h. On day 4 post infection, the nematodes were washed off the plates using an M9-buffer supplemented with 0.1 % ($\frac{v}{v}$) triton X-100, and distributed in 200 μl aliquots into the cavities of white microtiter plates (Greiner bio-one). Luminescence was determined using a Wallac Victor[3] multilabel counter (Perkin Elmer) and the IVIS (Xenogen).

Table 2.9: Nematode growth medium and M9-buffer (Stiernagle, 2006).

	Reagent	Amount
1 M KPO$_4$-buffer pH 6		
	KH$_2$PO$_4$	27.075 g
	K$_2$HPO$_4$	8.9 g
	Aq$_{bidest}$	ad 250 ml
NGM-agar		
	NaCl	1.5 g
	Peptone	1.25 g
	Aq$_{bidest}$	ad 0.5 L
	autoclave (121 °C, 20 min) and	
	chill to 55 °C	
	5 $\frac{mg}{ml}$ Cholesterol	0.5 ml
	1 M CaCl$_2$	0.25 ml
	1 M MgSO$_4$	0.5 ml
	1 M KPO$_4$-buffer	12.5 ml
	Nystatin solution	2.5 ml
M9-buffer		
	KH$_2$PO$_4$	3 g
	NA$_2$HPO$_4$	6 g
	NaCl	5 g
	1 M MgSO$_4$	1 ml
	Aq$_{bidest}$	ad 1 l
	filter sterilise (0.2 μm pore filter)	

2.6 Software and statistical analysis

In the main, evaluation and graphical display of the results were performed in Microsoft Excel. For statistical analysis, a two-sample Student's *t*-test was used, where applicable, to determine significant differences. P values of 0.05 or less were considered as significantly different from the null hypothesis. To compare protein and gene sequences, the BLAST algorithm (NCBI) and protein BLAST analysis tool (NCBI) were applied in order to identify homologous genes and proteins. For the comprehensive database query, protein sequences of *S. enterica* serovar Typhimurium LT2 (NC_003197) were used. All proteins encoded by the *fuc* and *pdu* gene clusters were used as template sequences in an NCBI protein BLAST. To specify the output, *S. enterica* (taxid: 28901) was chosen as target in the field 'organism'. The algorithm was changed from default to show 500, rather than 100 aligned sequences. Here the emphasis was placed on homology of the input and output sequences. Thus, a protein was referred to as absent in the respective serovar, in the case of less than 60 % query coverage and less than 90 % sequence homology.

Multiple sequence alignments after DNA sequencing, for example, were carried out using Clustal-W2 on the EMBL-EBI server (www.ebi.ac.uk/Tools/msa/clustalw2/). To predict possible σ^{70} promoters, the promoter prediction software BPROM (www.softberry.com) was used.

3 Results

3.1 Utilisation of alternative carbon and energy sources by enteropathogens

Due to its dense population by the microbiota, the human intestine can be considered rather as a competitive than a nutrient-rich environment for pathogens (Derrien *et al.*, 2004; Hansson, 2012). A comprehensive literature study was conducted to collect information on nutrients supporting bacterial growth in the mammalian GIT. **Figure 3.1** summarises the metabolites which are potentially available to the microbiota and pathogenic bacteria. As already mentioned, enteric pathogens have developed or gained metabolic pathways to overcome the nutrient limitation that is part of the colonisation resistance (Stecher and Hardt, 2011). These metabolic pathways enable the simultaneous utilisation of various substrates, of which some are not or only poorly metabolised by the microbiota (Fabich *et al.*, 2008).

Following the completion of the literature study regarding nutrients available to bacteria in the GIT, a survey for metabolic traits of enteropathogens was initiated. Therefore, protein and nucleotide sequences were compared using the protein-BLAST algorithm on the NCBI website to investigate whether or not certain metabolic pathways are present or absent in Gram-positive and Gram-negative food-borne enteropathogens. Nucleotide or protein sequences of Gram-negative representatives were only compared to sequences of other Gram-negative bacteria, and not to those of Gram-positive species, and vice versa. Recent literature regarding the metabolism of the selected pathogens was also taken into account. The results of this survey are presented in **Tab. 3.1**.

Molecular hydrogen, which is released as a by-product of fermentation processes in the gut can serve as an energy substrate to enteropathogens expressing membrane-bound hydrogenases linked to a hydrogen-oxidising respiratory pathway (Maier, 2005). Genes encoding proteins with ureolytic activities can be found in the genomes of some enteropathogens (**Tab. 3.1**), suggesting urea as a potential source of nitrogen besides peptides and amino acids (Matsumoto *et al.*, 2012). As for most bacteria, sugars are the most favourable carbon and energy source for enteropathogens. While dietary monosaccharides are already absorbed in the small intestine (Martins dos Santos *et al.*, 2010), sugars available in the large intestine are mostly derived from glycolytic activities of exoenzymes, for example glycosidases. The latter are secreted by members of the microbiota and help degrade otherwise indigestible complex carbohydrates of the diet, host derived mucins, or cell debris (Xu *et al.*, 2003; Kamada *et al.*, 2013). Examples of microbiota-released sugars from dietary polysaccharides include xylose, melibiose, rhamnose and fucose, which can also be metabolised by some enteropathogens (**Tab. 3.1**). At the interface of the lumen and the mucus barrier of the gut, sugars such as mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose

and glucose are available as they are components of mucin glycoconjugates (Cummings and Macfarlane, 1991; Vimal *et al.*, 2000). Another component released from mucins is sialic acid (Vimal *et al.*, 2000). Enteropathogens harbouring the *nanRATEK* operon are potentially able to use sialic acid as a carbon source (Almagro-Moreno and Boyd, 2010) (**Tab. 3.1**). As mentioned above, the vast majority of the microbiota relies on fermentable carbon sources. *B. thetaiotaomicron*, a common member of the gut flora in mice and humans, is equipped with a tremendous repertoire of glycosidases (Wexler, 2007). Hence, it is not only a supplier of carbon sources in the gut, but also a serious competitor for nutrients as it uses similar carbon and energy sources like pathogens, as for example N-acetylglucosamine, N-acetylgalactosamine, galactose and fucose (Salyers and Pajeau, 1989). Pathogens equipped with metabolic pathways, allowing the utilisation of non-fermentable substrates or fermentation end products, gain access to a nutrient niche not exploited by the microbiota (Lopez *et al.*, 2012; Thiennimitr *et al.*, 2011; Winter *et al.*, 2013). Examples of such pathways are the degradation of ethanolamine and 1,2-PD. The genes required for ethanolamine and 1,2-PD degradation, as well as those for tetrathionate respiration, have been found to be upregulated in the gut lumen of chickens (Harvey *et al.*, 2011). The fermentation of L-Fuc and L-Rha leads to the formation of 1,2-PD under anaerobic conditions. However, the degradation of L-Rha and L-Fuc to 1,2-PD is not an exclusive property of enteropathogens, in fact many microorganisms of the microbiota are also equipped with this catabolic characteristic. It is thought that the degradation of ethanolamine and 1,2-PD leads to an advantage of pathogens over the microbiota during colonisation of the gut (Sampson and Bobik, 2008; Thiennimitr *et al.*, 2011). Since vitamin B_{12} is essential as a cofactor during ethanolamine and 1,2-PD degradation (Scarlett and Turner, 1976; Jeter, 1990), and given that tetrathionate can serve as electron acceptor for anaerobic respiration (Winter *et al.*, 2010), the presence of the genes or proteins required for the synthesis of the cofactor and the electron acceptor was compared in different enteropathogens (**Tab. 3.1**). The genes of the *pdu* gene cluster are present in *L. monocytogenes, C. perfringens, S. enterica, S. sonnei, Yersinia enterocolitica* and some enteropathogenic *E. coli*. Genes required for ethanolamine degradation are present in the genomes of *E. faecalis, L. monocytogenes, C. perfringens, S. enterica*, the four chosen *Shigella* spp. and *E. coli*. Thus, only *L. monocytogenes, C. perfringens, S. enterica, S. sonnei* and some EHEC are capable of degrading ethanolamine and 1,2-PD. The genes providing the ability to synthesise vitamin B_{12} either *de novo* or from extracellular precursors (*cobUST*) (Roth *et al.*, 1996; Maggio-Hall and Escalante-Semerena, 1999) were found in all enteropathogens able to utilise ethanolamine and 1,2-PD, except in *E. faecalis*. However, anaerobic respiration of ethanolamine and 1,2-PD using tetrathionate as an electron acceptor is only possible for *S. enterica* and *Y. enterocolitica* carrying the *ttr* genes (**Tab. 3.1**). Without anaerobic respiration, ethanolamine and 1,2-PD degradation provides only a little energy but no carbon source. In the case of aerobic growth and the extracellular availability of vitamin B_{12} precursors, ethanolamine and 1,2-PD can serve as energy and carbon sources (Roth *et al.*, 1996).
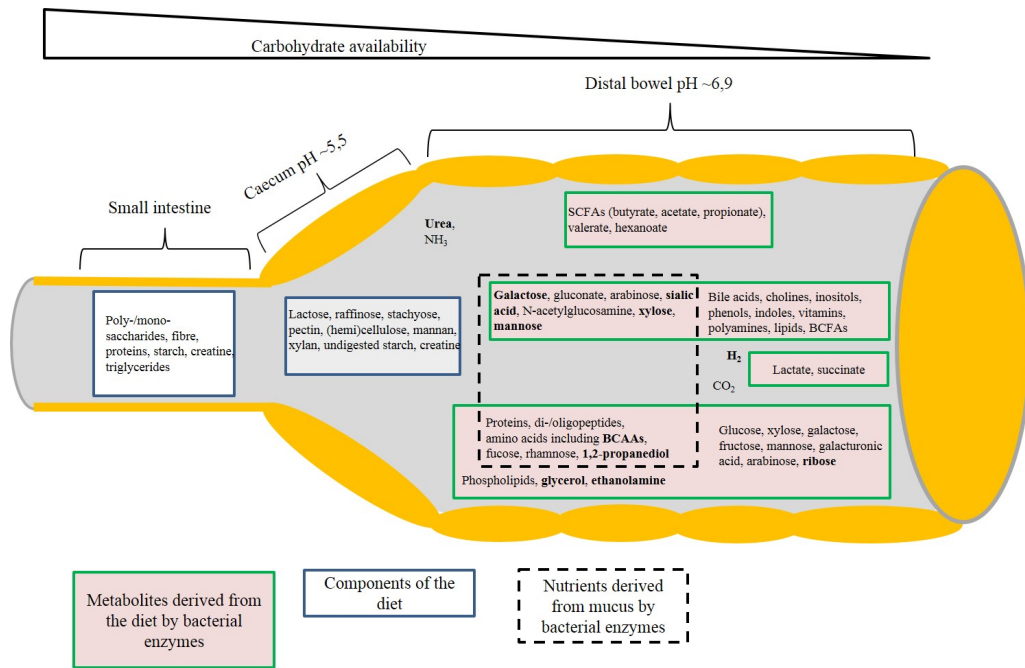
Figure 3.1: **Nutrient availability in the mammalian GIT.**
A variety of metabolites is present in the gut, which can serve as nutrients for members of the microbiota as well as for pathogenic bacteria. The metabolites originate from ingested food and its degradation products, from mucus due to the activity of bacterial enzymes and the turnover of bacterial and mammalian cells. Mucus covering the epithelial surfaces of the mammalian GIT is indicated in orange. Metabolites printed in bold letters, are specifically used by enteropathogens. [Sources: Macfarlane *et al.* (1998, 2000, 2005); Macfarlane and Macfarlane (2006); Wang *et al.* (2007); Cummings and Macfarlane (1991); Dandekar *et al.* (2012); Matsumoto *et al.* (2012)]. The figure is part of a review article published recently (Staib and Fuchs, 2014).

Table 3.1: Metabolic properties of enteropathogens [previously published in Staib and Fuchs (2014)].

Bacteria	Genes relevant for degradation of substrate or synthesis of cofactor												
	Fucose (fucI, fucA, fucK, fucO)	Rhamnose (rhaABCD)	Propanediol (pdu)	Tetrathionate reductase (ttrABC, ttrSR)	Cobalamin synthesis (cob/cbi)	Ethanolamine (eutABC)	Sialic acid (nanRATEK)§[1]	myo-Inositol (iolR, iolABCDE)	Melibiose (melA, melB)	Xylose (xylABEFGH, xylR)	Glycerol †	Hydrogenase (hya, hyf, hyc, hyp, hybC, hydB)	Urease (ureABCDEFG)
Gram-positive													
Enterococcus faecalis	-	+	-	-	-	+	-	+	-	+[4]	-[5]	-	-
Bacillus cereus	(+)[a]	-	-	-	-	-	-	+	-	+[4]	-[5]	-	+[c]
Listeria monocytogenes	+	+	+	-	+	+	-	+	-	-	+[5]	-	-
Clostridium perfringens	+[2]	+	+	-	+	+	+	+	+	-	+[5]	+	+[e]
Clostridium difficile	-	+	-	-	+	+	+	-	-	+[4]	-[5]	+	-
Gram-negative													
Salmonella enterica	+	+	+	+[3]	+	+	+	+[7]	+	+	+[5]	+	+
Shigella flexneri	+	+	-	-[3]	(+)[b]	+	+	-	+	+	-[5]	+	-
Shigella dysenteriae	+	+	-	-[3]	(+)[b]	+	+	-	+	+	-[5]	+	-
Shigella boydii	+	+	-	-[3]	(+)[b]	+	+	-	+	+	-[5]	+	-
Shigella sonnei	+	+	+	-[3]	(+)[b]	+	+	-	+	+	+[5]	+	-
Yersinia enterocolitica	-	-[c]	+	+	+	-	+	+[7]	-	+	+[5]	+	+
Campylobacter jejuni	+	-	-	-	-	-	+	-	-	-	-[5]	+	-
Vibrio cholerae	-	-	-	-[3]	(+)[b]	-	+	-	-	-	-[5]	+	-
Vibrio parahaemolyticus	-	+	-	+[3]	(+)[b]	-	+	-	-	-	-[5]	-	+
Escherichia coli [enteropathogenic (EPEC), enterotoxigenic (ETEC), uropathogenic (UPEC), enterohaemorrhagic (EHEC)]	+	+	-/+[d]	-	(+)[b]	+	+	-	+	+	-[5]	+	+[f]

+: Genes present, -: Genes absent

[a]: fucK, fucI present, [b]: cobUST present, [c]: strain dependent, [d]: present in ETEC, EPEC, [e]: neighbouring toxin genes on plasmid[6], [f]: EHEC

§References: [1] Almagro-Moreno and Boyd (2009), [2] Zuniga et al. (2005), [3] Barrett and Clark (1987), [4] Gu et al. (2010), [5] Zhang et al. (2009), [6] Dupuy et al. (1997), [7] Kröger and Fuchs (2009)

†: Cobalamin-dependent glycerol dehydratase; anaerobic degradation of glycerol Lawrence and Roth (1996)

3.2 Distribution of the genes relevant for 1,2-propanediol and L-fucose metabolism

3.2.1 The *pdu* and *fuc* genes are highly conserved among *S. enterica*

A comprehensive database query was conducted in order to determine whether the gene clusters required for L-Fuc and 1,2-PD degradation are exclusive or general properties of *S. enterica*. Using the protein BLAST tool on the NCBI website and the amino acid sequences of all proteins encoded by the *pdu* and *fuc* gene clusters of *S.* Typhimurium LT2 (NC_003197.1), the results are presented in **Tab. 3.2**. In total 82 serovars of *S. enterica* subsp. *enterica* were analysed for the presence of homologous proteins. In the case of query coverage below 60 % and an identity score below 90 % the proteins were considered to be absent from the respective serovar. The proteins encoded by the *pdu* and *fuc* genes were found to be ubiquitous in the investigated serovars of *S. enterica* subsp. *enterica*, with the exceptions of serovar Litchfield and Stanleyville (see **Tab. 5.12**, and **Tab. 5.13** in the Appendix). Many or nearly all proteins encoded by the *pdu* gene cluster were missing in serovar Litchfield and Stanleyville, while all proteins encoded by the *fuc* gene cluster were found with sufficient homologies to meet the applied requirements. For both serovars only a shotgun sequence is available, which might explain the apparent absence of proteins. For all other serovars, only single proteins were not found or were dismissed, due to insufficient homology or query coverage.

Table 3.2: The number of *S. enterica* subsp. *enterica* serovars found to express the proteins encoded by the *pdu* and *fuc* genes in sufficient homology to the *S.* Typhimurium LT2 template sequence are presented. In the case of a query coverage below 60 % and an identity score below 90 % the proteins were considered to be absent from the respective serovar. In total 82 serovars were investigated for the presence of the proteins.

Proteins encoded by the *pdu* genes	Number of *S. enterica* subsp. *enterica* serovars meeting the above set requirements
PocR	79 / 82
PduF	82 / 82
PduA	79 / 82
PduB	78 / 82
PduC	79 / 82
PduD	80 / 82
PduE	81 / 82
PduG	78 / 82
PduH	81 / 82
PduJ	81 / 82
PduK	81 / 82
PduL	81 / 82
PduM	80 / 82

Proteins encoded by the *pdu* genes	Number of *S. enterica* subsp. *enterica* serovars meeting the above set requirements
PduN	79 / 82
PduO	80 / 82
PduP	80 / 82
PduQ	77 / 82
PduS	80 / 82
PduT	78 / 82
PduU	79 / 82
PduV	80 / 82
PduW	78 / 82
PduX	79 / 82

Proteins encoded by the *fuc* genes	Number of *S. enterica* subsp. *enterica* serovars meeting the above set requirements
FucO	76 / 82
FucA	79 / 82
FucI	82 / 82
FucK	81 / 82
FucU	82 / 82
FucR	80 / 82

Homologs of both pathways were also found in the *S. enterica* subspecies *salamae, indica, diarizonae* and *arizonae*. *S. enterica* subspecies *houtenae* only contains homologs of proteins encoded by the *fuc* genes, but not of the *pdu* gene cluster. Since only single serovars of these subspecies were found, it was analysed whether all proteins encoded by the respective gene cluster could be found, or if they were only partially present. The results are shown in **Tab. 3.3**. The six proteins encoded by the *fuc* genes were present in each of the five serovars, but PduK (polyhedral body component), PduM (unknown function), PduV (unknown function) and PduT (polyhedral body component) seemed to be missing, or were present in only one of the five subspecies.

Table 3.3: Number of proteins encoded by the *pdu* and *fuc* genes in *S. enterica* subspecies *salamae, indica, diarizonae, arizonae* and *houtenae* are presented. The amino acid sequences of *S.* Typhimurium LT2 served as template for NCBI protein BLAST. In the case of less than 60 % query coverage and an identity score below 90 % sequence homology, the protein was considered to be absent in the respective serovar.

	pdu	*fuc*
Salmonella enterica subsp. *salamae* 58:l,z13,z28:z6	20 / 23	6 / 6
Salmonella enterica subsp. *indica* 6,14,25:z10:1,(2),7	23 / 23	6 / 6
Salmonella enterica subsp. *diarizonae* 60:r:e,n,x,z15	20 / 23	6 / 6
Salmonella enterica subsp. *arizonae* 62:z4,z23	19 / 23	6 / 6
Salmonella enterica subsp. *houtenae*	np	6 / 6

np: not present

3.3 Genetic determinants for the growth of *S.* Typhimurium with 1,2-PD and L-Fuc

Under optimal *in vitro* conditions (LB-medium, 37 °C, 180 rpm), *S.* Typhimurium 14028 reached stationary phase within ten hours, and a cell density of approximately $1 \cdot 10^{10}$ $\frac{cfu}{ml}$ (data not shown). To investigate the growth behaviour of *S.* Typhimurium on single carbon sources, bacterial growth assays in less nutrient-rich media were performed.

3.3.1 *fucA* is essential for growth with L-fucose

S. Typhimurium 14028 was cultivated in a VB-NCE-YE medium supplemented with 25 mM L-Fuc, tetrathionate and trace elements under anaerobic conditions. The results of three independent growth assays, using *S.* Typhimurium 14028 Nal^R/pBR322, 14028 $\Delta fucA$::kan^R and its complementation strain (14028 $\Delta fucA$::kan^R/pBR-*fucA*) are shown in **Fig. 3.2**. 14028 $\Delta fucA$::kan^R reached stationary phase after 6 h with an OD$_{600}$ of 0.108. The introduction of the plasmid pBR-*fucA* to 14028 $\Delta fucA$::kan^R partly restored the ability to grow with L-Fuc. Stationary phase was also reached after approximately 6 h but with an OD$_{600}$ of 0.166. In the case of complete complementation, a growth phenotype comparable to 14028 Nal^R/pBR322 would have been expected, but 14028 Nal^R/pBR322 reached higher OD$_{600}$ values of 0.305 at the onset of stationary phase. The low OD$_{600}$ values are the result of turbidity measurements in 96-well microtiter plates using a Wallac Victor^3 1420 multilabel counter (Perkin Elmer), and thus cannot be directly compared to measurements conducted in 1 ml cuvettes. This is because a Wallac Victor^3 1420 is a relative and a spectrophotometer an absolute reader (personal communication Perkin Elmer technical support). To exclude possible influences on the growth behaviour mediated by the presence of pBR322,

the the empty pBR322 plasmid was introduced to 14028 $\Delta fucA::kan^R$ and 14028 Nal^R. Further, 14028 $\Delta fucA::kan^R$ and 14028 $\Delta fucA::kan^R$/pBR-*fucA* were grown anaerobically in VB-NCE-YE supplemented with trace elements, tetrathionate and D-Glc. No significant differences were observed for the two strains grown with D-Glc. As D-Glc and L-Fuc are equal carbon sources comprised of C_6 bodies, no outstanding differences regarding the final cell densities of 14028 Nal^R/pBR322 grown with L-Fuc or the strains grown with D-Glc were detected. The cultures grown with D-Glc instead of L-Fuc served as controls to monitor possible effects of the genetic modifications on regular growth (**Fig. 3.2**, black charts). Due to the deletion of *fucA*, encoding the fuculose-phosphate aldolase, 14028 $\Delta fucA::kan^R$, showed an attenuated growth phenotype which was partially restored by the introduction of the plasmid pBR-*fucA*.
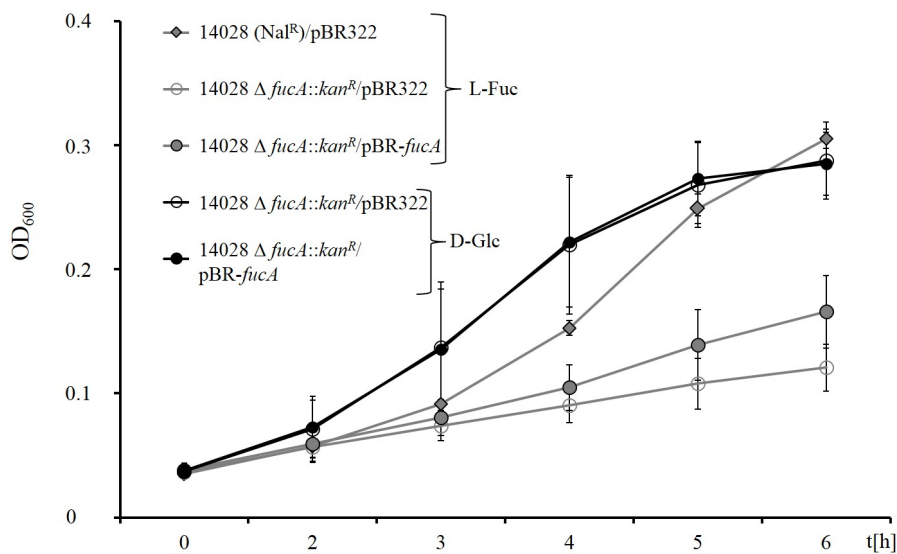
Figure 3.2: **Anaerobic growth of *S.* Typhimurium 14028 with L-Fuc.**
The growth behaviour of *S.* Typhimurium 14028 Nal^R/pBR322, 14028 $\Delta fucA::kan^R$ and 14028 $\Delta fucA::kan^R$/pBR-*fucA* was analysed. The bacteria were grown anaerobically (Whitley A35, 80 % N_2, 10 % CO_2, 10 % H_2, 37 °C) in a VB-NCE-YE medium supplemented with 25 mM L-Fuc (grey charts), 40 mM tetrathionate and trace elements without agitation. Cultivation vessels remained in the anaerobic cabinet during the whole experiment. Samples were taken anaerobically at the indicated time points and OD_{600} was measured using a Wallac Victor^3 outside the anaerobic chamber. The strains 14028 $\Delta fucA::kan^R$ and 14028 $\Delta fucA::kan^R$/pBR-*fucA* were grown in VB-NCE-YE medium supplemented with 27.8 mM D-Glc (black charts), tetrathionate and trace elements. The bacteria grown with D-Glc that served as controls and 14028 Nal^R/pBR322 grown with L-Fuc showed regular growth. The growth with L-Fuc was partially restored by the introduction of pBR-*fucA*. The curves represent the means of three independent repetitions. Vertical bars indicate the standard deviations.

3.3.2 L-fucose and 1,2-propanediol are used simultaneously

To verify that 1,2-PD is formed by *S.* Typhimurium during anaerobic degradation of L-Fuc, the gene expression of the *fuc* and *pdu* genes was monitored during all growth phases. Therefore, the reporter strains 14028 P_{fucO}::*lux* and 14028 P_{pduA}::*lux* were grown anaerobically in L-Fuc medium as described above. OD_{600} and bioluminescence (RLU_{490}) were measured in parallel (Wallac Victor[3], Perkin Elmer). The results are shown in **Fig. 3.3**. After 2 h luminescence was detected at values of $1.3 \cdot 10^5$ RLU_{490} for the 14028 P_{fucO}::*lux* reporter strain, indicating the expression of *fucO*. The signal of this reporter strain increased over the next few hours and reached its maximum of $2.9 \cdot 10^6$ RLU_{490} at 5 h past inoculation. Afterwards, luminescence decreased to values of $1.8 \cdot 10^5$ RLU_{490} when the culture reached stationary phase. FucO is a 1,2-PD oxidoreductase converting L-lactaldehyde to 1,2-PD during anaerobic L-Fuc degradation (Hacking and Lin, 1976). Expression of the *pdu* genes, from promoter P_{pduA}, was detected with RLU_{490} values of $1.2 \cdot 10^5$ after 3 h of anaerobic cultivation of the strain 14028 P_{pduA}::*lux* with L-Fuc. The transcriptional activity reached a maximum of $2.3 \cdot 10^6$ RLU_{490} at 5 h past inoculation in the middle of the logarithmic growth phase. In the following 2 h luminescence declined, and the culture reached stationary phase.
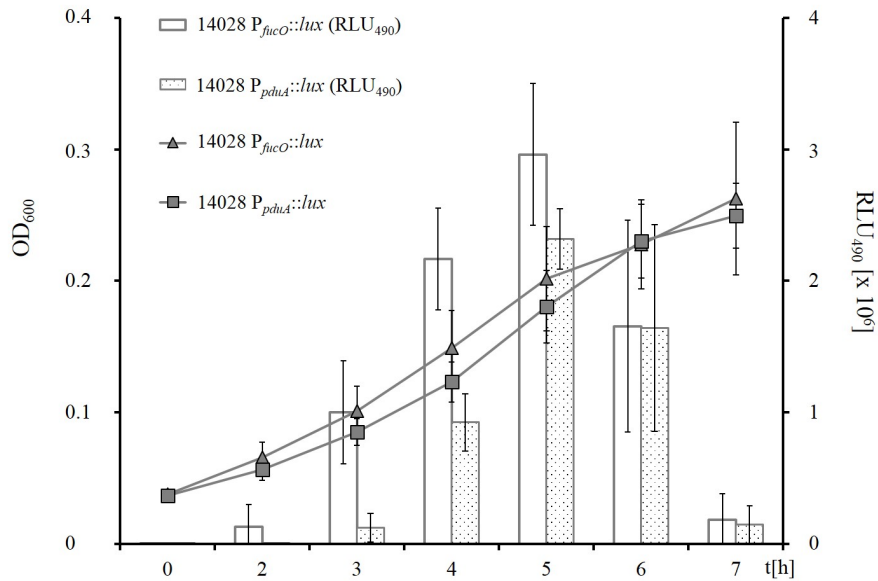
Figure 3.3: **Expression of *fucO* and the *pdu* genes during growth with L-Fuc.**

     S. Typhimurium 14028 P_{fucO}::*lux* and 14028 P_{pduA}::*lux* were grown anaerobically in VB-NCE-YE medium supplemented with 25 mM L-Fuc, 40 mM Na-tetrathionate and trace elements. Incubation and sampling procedure was maintained as described above for growth with L-Fuc. OD_{600} and luminescence (RLU_{490}) were measured in parallel using a Wallac Victor[3]. Growth of the strains is figured as line charts and bar charts show the RLU_{490} values detected during the experiment. RLU_{490} values are indicating the strength of gene expression from the promoters P_{fucO} and P_{pduA}. Gene expression was concordant with the growth of both strains whereas *pdu* expression was detected 1 h after *fucO* expression. Vertical bars represent the standard deviations of three independent experiments.

3.3.3 Deletion of *pduC* eliminates growth with 1,2-propanediol

Growth with 1,2-PD as a sole source of carbon and energy was investigated, after *pdu* gene expression was demonstrated by growing *S.* Typhimurium 14028 Nal[R] anaerobically with L-Fuc. Further, it was analysed whether the loss of the central enzyme of the 1,2-PD degradation pathway PduC can be compensated by gene products of other genes of *S.* Typhimurium 14028. To determine the growth behaviour with 1,2-PD, 14028 Nal[R] was grown aerobically (**Fig. 3.4**) and anaerobically (**Fig. 3.5**) in a VB-NCE-YE medium supplemented with 1,2-PD, trace elements and vitamin B_{12} or tetrathionate respectively. To investigate anaerobic growth, the culture vessels remained in the anaerobic cabinet (Whitley A35, 80 % N_2, 10 % CO_2, 10 % H_2, 37 °C) throughout the entire investigation. Samples were taken in the anaerobic chamber, and OD_{600} was measured outside the anaerobic cabinet using a Wallac Victor[3] and plotted over the time. To test whether the loss of the large subunit of the diol-dehydratase PduC, which is encoded by *pduC*, can be compensated by other gene products of *S.* Typhimurium 14028, *pduC* was deleted in *S.*

Typhimurium 14028 Nal^R. The resulting growth phenotype with 1,2-PD was severely attenuated under aerobic and anaerobic conditions (**Fig. 3.4** and **Fig. 3.5**). The effect of the *pduC* deletion was more pronounced when 14028 Δ*pduC* was grown aerobically with 1,2-PD (**Fig. 3.4**) and compared to 14028 Nal^R. Aerobically, 14028 Δ*pduC* reached stationary phase after 6 h (OD$_{600}$ = 0.4), whereas 14028 Nal^R reached an OD$_{600}$ of 1.04 at the onset of the stationary phase after 28 h. Anaerobically, 14028 Nal^R reached OD$_{600}$ values od 0.242 at the onset of stationary phase (t = 7 h), whereas the Δ*pduC* mutant only reached an OD$_{600}$ of 0.173 (**Fig. 3.5**). The attenuation after the deletion of *pduC* confirms the data of Walter *et al.* (1997), who eliminated growth with 1,2-PD by introducing a point mutation in *pduC* (Walter *et al.*, 1997).
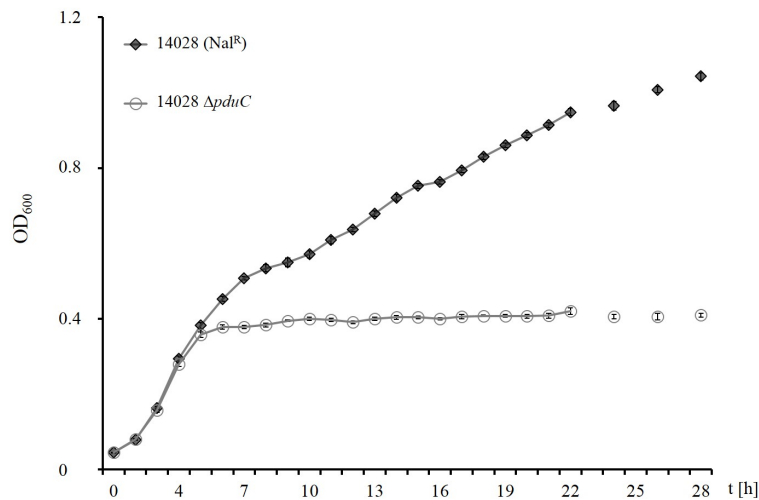
Figure 3.4: **Aerobic growth of Δ*pduC* with 1,2-PD.**
> *S.* Typhimurium 14028 Nal^R and 14028 Δ*pduC* were grown aerobically in a VB-NCE-YE supplemented with 50 mM 1,2-PD, trace elements and 200 nM vitamin B$_{12}$. The *pduC* deletion mutant reached stationary phase after 6 h (OD$_{600}$ = 0.4) whereas 14028 Nal^R reached an OD$_{600}$ of 1.04 after 28 h. The graph shows the results of three independent experiments. Vertical bars represent the standard deviations.

Further experiments were carried out in an anaerobic environment (Whitley A35, 80 % N$_2$, 10 % CO$_2$, 10 % H$_2$, 37 °C) to reflect *in vivo* conditions in the gut. After 7 h the cultures reached stationary phase, and OD$_{600}$ values of 0.242 for 14028 Nal^R/pBR322 0.242, 0.213 for 14028 Δ*pduC*/pBR-*pduC*, and 0.173 for 14028 Δ*pduC*/pBR32 were determined for this particular time point (**Fig. 3.5**). Already after 4 h, 14028 Δ*pduC*/pBR322 showed reduced growth rates in comparison with 14028 Δ*pduC*/pBR-*pduC* and 14028 Nal^R/pBR322, indicating a shortage of nutrients for 14028 Δ*pduC*/pBR322 since only yeast extract, but not 1,2-PD can be metabolised. To investigate whether the plasmid pBR322 influences the growth behaviour, the empty plasmid was introduced to 14028 Δ*pduC* and 14028 Nal^R beforehand. As a control, the Δ*pduC* mutant and the complemented strain were grown in VB-NCE-YE with D-Glc to exclude pleiotropic effects resulting from the mutation procedure (black charts in **Fig. 3.5**). The bacteria grown with

D-Glc exhibited regular growth while the deletion of *pduC* led to an attenuated growth phenotype in bacteria grown with 1,2-PD under anaerobic conditions, and was partially restored after the introduction of the plasmid pBR-*pduC* to 14028 Δ*pduC*.
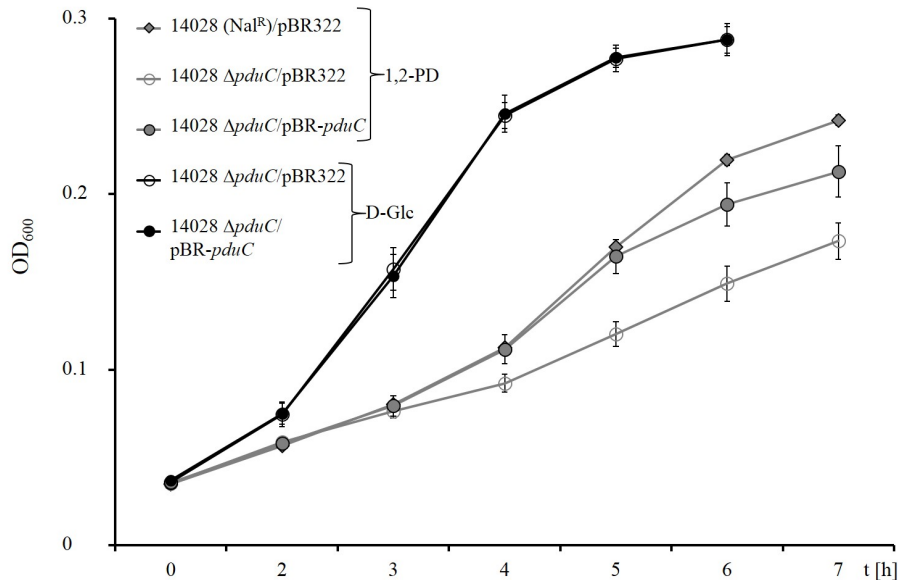
Figure 3.5: **Anaerobic growth assays of strain 14028 with 1,2-PD.**

S. Typhimurium 14028 Nal[R]/pBR322, 14028 Δ*pduC*/pBR322 and 14028 Δ*pduC*/pBR-*pduC* were cultivated in an oxygen-free environment (80 % N_2, 10 % CO_2, 10 % H_2, 37 °C) without agitation. A VB-NCE-YE medium was enriched with 25 mM 1,2-PD, tetrathionate and trace elements (grey charts). OD_{600} was measured using a Wallac Victor[3], and plotted over the time. As controls 14028 Δ*pduC*/pBR322 and 14028 Δ*pduC*/pBR-*pduC* were grown in VB-NCE-YE medium containing D-Glc (black charts) instead of 1,2-PD. The strains grown with D-Glc showed regular growth. Of the strains grown with 1,2-PD the Δ*pduC* mutant reached lower OD_{600} values ($OD_{600} = 0.173$) after 7 h than its complementation strain ($OD_{600} = 0.213$) and 14028 Nal[R]/pBR322 ($OD_{600} = 0.242$). Hence, the expression of *pduC* from the plasmid did not completely restore wild type growth with 1,2-PD. Vertical bars represent the standard deviations of three independent experiments.

3.3.4 P*pduA* promoter activity represents growth with 1,2-propanediol

The gene expression of the *pdu* genes in *S.* Typhimurium was investigated under aerobic (**Fig. 3.6**) and anaerobic (**Fig. 3.7**) growth conditions using the reporter strain 14028 P*pduA*::*lux*. Bioluminescence (RLU_{490}) and OD_{600} were recorded in parallel. Priorly, 2,000 RLU_{490} were determined as background luminescence or leaky expression of the reporter gene by growing 14028 P*pduA*::*lux* in VB-NCE-YE medium supplemented with D-Glc. Aerobically and anaerobically, the transcriptional activity reached maximum values in the middle of the exponential growth phase, and gradu-

ally decreased until the cultures reached stationary phase. Under aerobic growth conditions (**Fig. 3.6**), luminescence was detected after 5 h ($RLU_{490} = 1.5 \cdot 10^5$; $OD_{600} = 0.359$) and reached its maximum value at 12 h post inoculation with $3 \cdot 10^6$ RLU_{490} at an OD_{600} of 0.693. Anaerobically (**Fig. 3.7**), luminescence was detected 2 h past inoculation at an OD_{600} of 0.063 with RLU_{490} values of $6.7 \cdot 10^4$. The highest RLU_{490} values of $2.5 \cdot 10^6$ were detected at 5 h post inoculation at an OD_{600} of 0.154. Stationary phase was reached at an OD_{600} of 0.212, and RLU_{490} values had decreased to $4.9 \cdot 10^5$.
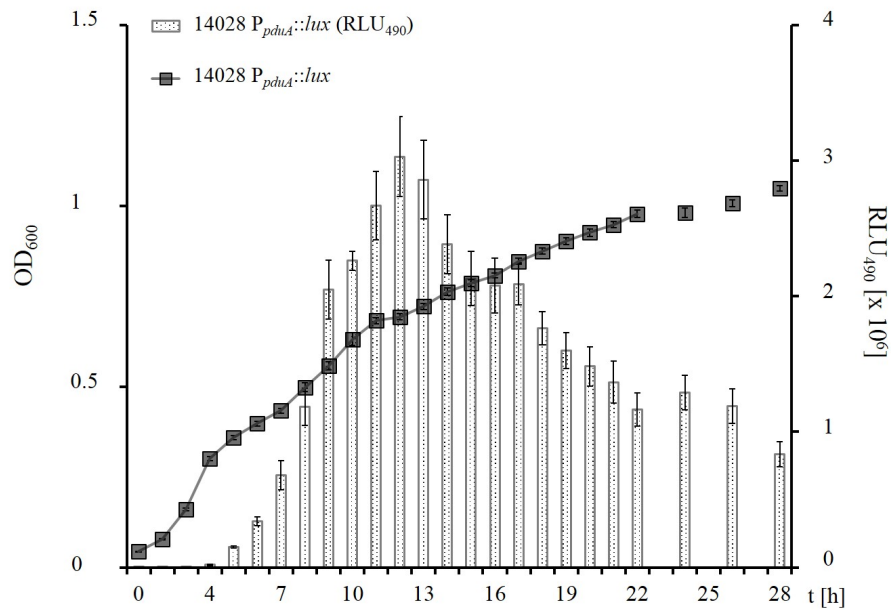
Figure 3.6: **Gene expression of *pdu* during aerobic growth with 1,2-PD.**
Aerobic growth of 14028 Nal^R P_{pduA}::*lux* in a VB-NCE-YE medium supplemented with 50 mM 1,2-PD, 200 nM vitamin B_{12} and trace elements revealed that gene expression of the *pdu* genes from the promoter P_{pduA} was reflecting the growth behaviour of the bacteria. The maximum RLU_{490} values were measured midway during the exponential growth and declined until stationary phase was reached. The graph represents a summary of three independent experiments with vertical bars as standard deviations.
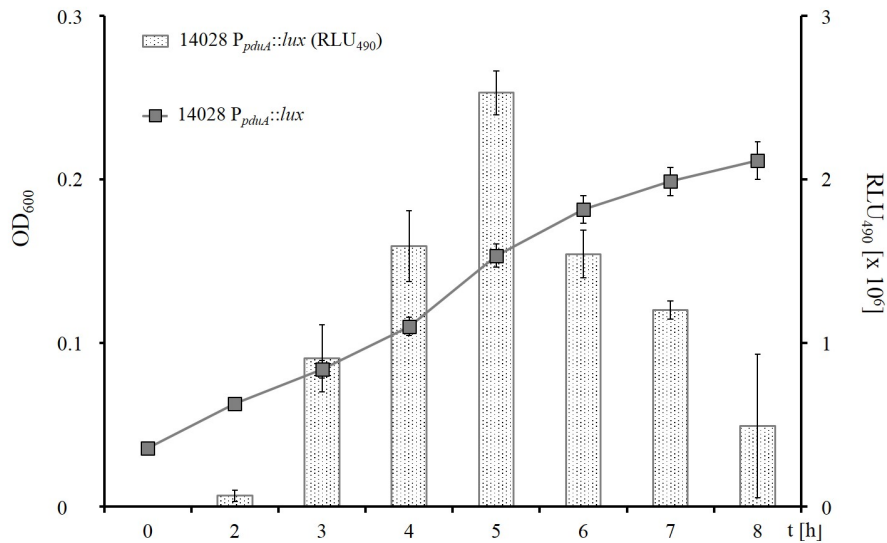
Figure 3.7: **Gene expression of *pdu* during anaerobic growth with 1,2-PD.**

S. Typhimurium 14028 P_{pduA}::*lux* was grown anaerobically in a VB-NCE-YE medium supplemented with 25 mM 1,2-PD, 40 mM Na-tetrathionate and trace elements. OD_{600} and RLU_{490} were measured using a Wallac Victor[3]. Growth of the strain is figured as line charts and the bar chart shows the RLU_{490} values that were detected during the experiment. RLU_{490} values are representing the *pdu* gene expression from the promoter P_{pduA}. The gene expression of the *pdu* genes reached the highest values in the middle of the exponential growth phase. Vertical bars represent the standard deviations of three independent experiments.

3.3.5 Role of *pdu* during cultivation in milks

Mother's milk contains highly fucosylated human milk oligosaccharides (HMOs) (Ashida *et al.*, 2009). Generally, faunal milks are rarely thought to contain fucosylated oligosaccharides compared to human mother's milk (Al Mijan *et al.*, 2011). Hence, cow's and mare's milk were also investigated. Mare's milk was chosen since it contains high albumin levels and is closer in similarity to human milk than to cow's milk (http://www.haidhof.de/stutenmilch/gutes.html).

Salmonella lacks the enzymes such as fucosidases for example, necessary to cleave the glycosidic bonds of the milk oligosaccharides to release L-Fuc monomers. Thus, mother's, mare's and cow's milk were diluted separately in a VB-NCE medium (1:10). Subsequently, 0.01 % ($\frac{w}{v}$) L-cysteine and three 10 μl inoculation loops of *Bifidobacterium infantis*, grown anaerobically at 37 °C on TOS-agar (Merck), were added to each diluted milk sample. The inoculated milk media were incubated without agitation in the anaerobic cabinet (37 °C, 80 % N_2, 10 % CO_2, 10 % H_2, Whitley A35) for 4 days. Afterwards, the cultures were centrifuged (11,648 x g, 10 min, RT), and the supernatants were filter sterilised (0.2 μm pore filter). After the adjustment of the pH to 7, the media were filter sterilised again. In the following the fermented milk media are referred to as spent-milk media.

To cultivate *S*. Typhimurium in the spent-milk media, trace elements, 40 mM Na-tetrathionate and 0.025 % ($\frac{v}{v}$) casamino acids (CAA) were added. To obtain triplicates of each approach, 12 aliquots á 3 ml were prepared of each milk spent-medium in sterile glass tubes that were placed in the anaerobic chamber. *S*. Typhimurium overnight cultures of the strains 14028 Nal^R, 14028 $\Delta pduC$, 14028 $\Delta pduC$/pBR322 and 14028 $\Delta pduC$/pBR-*pduC* were added to the spent-milk samples in final dilutions of 1:250. Serial dilutions (10^{-4} to 10^{-7}) of the inocula were plated in droplets on LB-agar plates (120 mm x 120 mm x 17 mm, Greiner bio-one). Ten μl of each sample were plotted in triplicates on the top end of the agar plates next to each other, and the latter were set in an upright position to create separate dilution lines of the droplets. The agar plates were incubated at 37 °C overnight, and emerged colonies were counted. After 48 h of cultivation in the anaerobic cabinet, the spent-milk samples inoculated with *S*. Typhimurium were diluted in a VB-NCE medium and the serial dilutions 10^{-2} to 10^{-5} were applied on squared LB-agar plates as described previously, incubated overnight at 37 °C, and the colonies counted. Because the initial cell count of the inocula had been determined, the multiplication rates of the cultures in each respective spent-milk medium were calculated. This approach was used to investigate whether 1,2-PD degradation provides a growth benefit to *S*. Typhimurium in spent-milk media. The plasmid pBR322 was also introduced to the *pduC* deletion strain to exclude the fact that possible influences on the growth behaviour resulted from the presence of pBR322. The results of three independent experiments are shown in **Fig. 3.8**. Only for mother's milk did the multiplication rates of the different genotypes vary. For 14028 Nal^R 44 times more colonies were counted compared to the inoculum after 48 h of cultivation in the milk spent-medium. 14028 $\Delta pduC$ and 14028 $\Delta pduC$/pBR322 did not show differences in multiplication (35 x the inoculum). Interestingly, 14028 $\Delta pduC$/pBR322 grew more slowly (20 x the inoculum in 48 h) than the $\Delta pduC$ strain without the plasmid pBR322, although no selection pressure using antibiotic supplementation was applied. If 1,2-PD utilisation provided a benefit to the bacteria, the $\Delta pduC$ deletion mutant would be expected to show attenuated growth in the spent-milk media in comparison with 14028 Nal^R and 14028 $\Delta pduC$/pBR322.
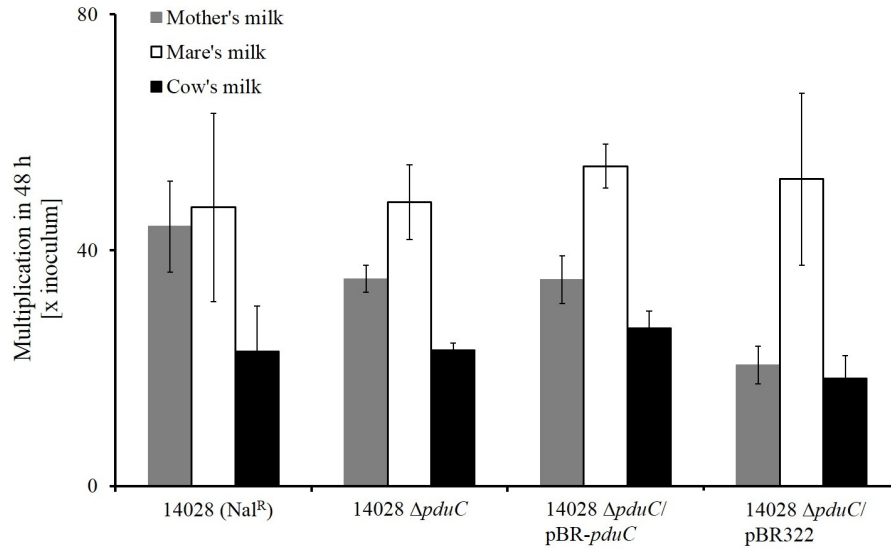
Figure 3.8: *S.* Typhimurium 14028 growth in spent-milk media.

> *S.* Typhimurium strains were anaerobically grown in milk-media that were fermented by *B. infantis* beforehand. The chart shows the means of three independent experiments with vertical bars representing the standard deviations. In cow's milk the multiplication numbers of the different strains was lower compared to mother's and mare's milk with only slight differences between the genotypes. For mare's milk the growth of all strains seemed to be equal as well. Differences between the single genotypes were visible only in the *S.* Typhimurium strains grown with mother's milk. 14028 Nal[R] showed the highest multiplication number followed by the Δ*pduC* mutant and its complementation strain (Δ*pduC*/ pBR-*pduC*). Interestingly, 14028 Δ*pduC*/pBR322 grew slower than the *pduC* deletion strain without the plasmid although no antibiotics were added to the medium.

3.3.6 Impact of *pduC* deletion on growth in gut content of pigs

Specimens of the small intestines of freshly slaughtered pigs were kindly provided by Rupert Bassitta (Chair of Animal Hygiene, WZW, TUM, DE). The contents of the specimens were removed and stored in 50 ml reaction tubes in the fridge or freezer. The residual gut tissue was ground in a VB-NCE medium using an Ultra-Turrax T50 (IKA®-Labortechnik, Staufen, DE). Gut content and ground tissue were diluted 1:10 in a VB-NCE-YE medium supplemented with trace elements. Five ml of each were inoculated with *S.* Typhimurium 14028 Δ*pduC* (final dilution of the inoculum 1:250) to deplete all available nutrients except 1,2-PD. The cultures were incubated at 30 °C under anaerobic conditions for 7 days. Since the anaerobic cabinet was not yet available the culture bottles were closed with lids containing rubber septa, and the head-space of each bottle was flooded with N_2 for 5 min. Therefore, two needles were placed in each septum, of which one was connected to the N_2 supply via a hose. After 7 days of incubation, the cultures were centrifuged at 11,648 x g, 10 min at RT. Both prior to and following the adjustment of the pH to 7, the supernatants were filter sterilised, subsequently being referred to in the following as gut media. For the growth assays, the gut media were diluted 1:100 in a VB-NCE medium, supplemented with trace elements and either 40 mM tetrathionate (anaerobic cultivation) or 200 nM vitamin B_{12} (aerobic cultivation). Overnight cultures of 14028 Nal^R and 14028 Δ*pduC* were diluted 1:10 in a VB-NCE medium and added to the prepared gut media in a final dilution of the inoculum of 1:250. The cultures were incubated for 48 h at 37 °C in glass tubes, under continuous agitation (180 rpm, Certomat MO, Sartorius) in an incubator or in anaerobic jars without agitation. In the following, the cultures were diluted 1:10 in LB-broth, and distributed in triplicates of 200 μl each into the cavities of 96-well microtiter plates (Greiner bio-one). OD$_{600}$ was measured hourly using a Wallac Victor^3 multilabel counter (Perkin Elmer). Between the measurements the microtiter plates were incubated on shakers at 37 °C and 1,000 rpm (DTS-2, ELMI, Riga, LVA). The resulting growth curves are shown in **Fig. 3.9**. The Δ*pduC* mutant did not show a differential growth behaviour compared to 14028 Nal^R in both media. A prolonged lag-phase of 14028 Δ*pduC* was expected in LB-broth, in the case of a growth disadvantage of the Δ*pduC* in the gut medium.
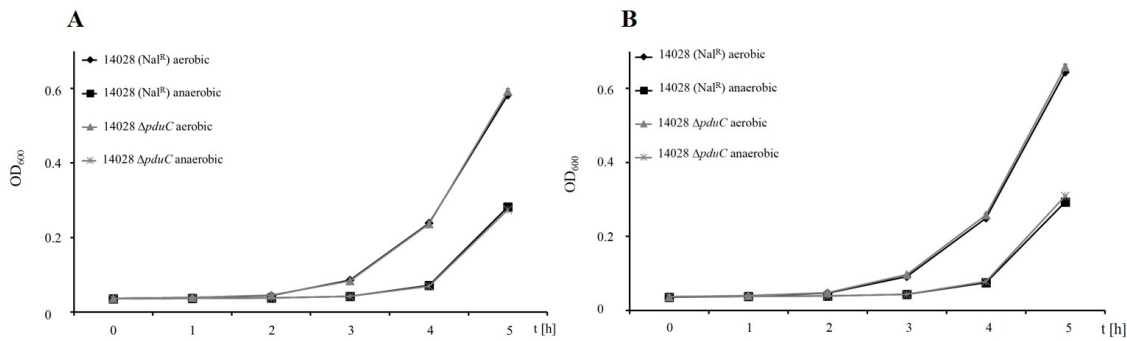
Figure 3.9: **Growth of 14028 in gut content of pig.**

Ground caecal tissue of a pig **A** or gut content **B** were diluted 1:10 in a VB-NCE-YE medium and anaerobically incubated with 14028 $\Delta pduC$ for 48 h. After filter sterilisation and adjustment of the pH the media were diluted 1:100 in a VB-NCE medium and inoculated with 14028 Nal^R and 14028 $\Delta pduC$ in final dilutions of the inocula of 1:250. Of each strain cultures were incubated aerobically and anaerobically for 48 h at 37 °C. In case of anaerobic cultivation 40 mM Na-tetrathionate and in case of aerobic cultivation 200 nM vitamin B_{12} were added. Growth was monitored by the incubation of 1:10 dilutions of each culture in LB-broth and OD_{600} measurement in a 96-well microtiter plate (Victor[3], Perkin Elmer). Chart **A** shows the results of bacteria previously grown in ground gut medium, part **B** of the same strain-set cultivated in media of gut content. No differences were detected between the growth behaviours of the two strains in either medium.

3.4 The *pdu* genes are transcribed as one polycistronic mRNA

Information concerning the exact transcription of the *pdu* gene cluster has not been published thus far. Hence, comprehensive sequence analyses to predict possible promoters within this genomic region were conducted *in silico*. Therefore, the nucleotide sequence of *S.* Typhimurium strain LT2 (NC_003197.1) was used as template for the prediction of mainly σ^{70} promoters by the software tool BPROM (www.softberry.com). Promoter sequences were expected to be located in the 500 bp sections upstream of the translational start site of each gene of the *pdu* gene cluster. Possible promoters with probability scores for the -10 and -35 region of ≥ 20 were found in front of nearly all genes except for *pduC, pduH, pduN, pduP, pduS* and *pduX*. Binding sites of transcription factors are part of the output of BPROM. With the exception of P_{pduA}, no binding sites of transcription factors were found within the predicted promoter sequences. Thus, after the survey it appeared conceivable that the whole gene cluster (*pduA-X*) could be transcribed from the promoter P_{pduA} as one mRNA, containing the genetic information of 21 genes. To test this hypothesis, RNA was isolated from *S.* Typhimurium 14028 Nal^R, grown aerobically in a VB-NCE-YE medium supplemented with 25 mM 1,2-PD, vitamin B_{12} and trace elements. The isolated RNA served as a template for cDNA synthesis and PCR. The results of the performed PCR reactions, in which

genomic 100 ng DNA (gDNA), 100 ng RNA and 100 ng cDNA were used as templates, are shown in **Fig. 3.10 B-D**. The primers used for the PCR reactions, spanned the intergenic regions between the 3'-end of the upstream and the 5'-end of the adjacent downstream *pdu* gene, and are indicated as numbered horizontal lines below the organisation of the *pdu* gene cluster in part **A** of **Fig. 3.10**. The lengths of the amplicons, obtained from PCR reactions using cDNA, match the sizes of the PCR products obtained using gDNA as a template. Hence, it can be concluded that all 21 genes of the *pdu* gene cluster are transcribed as a polycistronic mRNA. Multiple transcription start sites would have resulted in gaps on the cDNA agarose gel (**D**). This result confirms the single transcription start site of the *pdu* gene cluster, published previously (Chen *et al.*, 1995).

A PCR amplificate not matching the size of the expected PCR product is visible in **Line 17** on the agarose gel of RNA and cDNA (**Fig. 3.10 C + D**), which could be an artefact of the 40 PCR cycles. Data were reproduced in a second independent experiment.
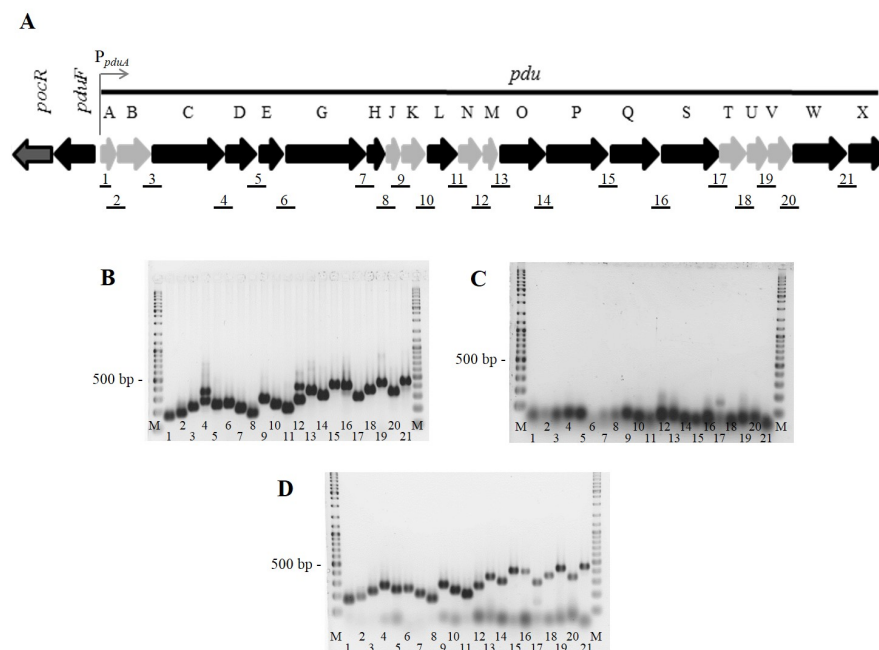
Figure 3.10: **Transcription of the *pdu* gene cluster.**

The genetic organisation of the 21 genes is shown in (**A**). PCR amplicons of the primers designed to span the intergenic regions between the 3'- and 5'-ends of adjacent genes are indicated as black horizontal lines below the genes and match the line numbers of the PCR products in **B-D**. For RNA isolation and consecutive reverse transcription *S.* Typhimurium was grown aerobically in VB-NCE-YE supplemented with 1,2-PD, vitamin B_{12} and trace elements to an OD_{600} of 2.0. After cDNA synthesis PCR was performed using gDNA (100 ng) (**B**) as a positive control, total RNA (100 ng) as a negative control (**C**) and cDNA (100 ng) (**D**) as templates. For annealing a temperature of 55 °C and extension times of 1 min were chosen. PCR products were separated in a 2 % ($\frac{w}{v}$) agarose gel. GeneRuler^{TM} DNA ladder mix (Thermo Scientific) was used as a marker (M).

3.5 Estimation of L-fucose contents in the gut

Reliable data about the L-Fuc content in the human gut are not available so far. An estimation of the L-Fuc content in the small intestine of mice is presented in the following. The calculation is based on numbers and values reported in the literature.

Brush borders from mouse ileum were prepared using 10^6 $\frac{cells}{ml}$ which resulted in 468 μg of protein (Laux *et al.*, 1986). The L-Fuc content was determined to be 1.3 $\frac{\mu g}{mg}$ of protein. The molar ratio of 1.3 μg L-Fuc in 1 ml would hence be 7.92 μM (**Equation 3.1**) and to obtain 1 mg of protein $2.1 \cdot 10^6$ cells would be needed ($\frac{1\ mg}{0.468\ mg} = 2.1$).

$$M = \frac{m}{M_w \cdot V} \tag{3.1}$$

M : Molar ratio

m : Mass in g $(1.3 \cdot 10^{-6}\ g)$

M_w : Molecular weight L-Fuc $(164.16\ \frac{g}{mol})$

V : Volume in l $(0.001\ l)$

A crypt is built of ∼250 cells and a villus of ∼3,500 cells which results in 4,200 cells per crypt and adjacent villus (Crosnier *et al.*, 2006). About 120 crypts were found per circumference of mouse ileum with a mean thickness of 4 μm (Martin *et al.*, 1998). The whole small intestine of a mouse is of about 25 cm in length. Taking this dataset as a basis it was calculated that 500 crypts and villi would be necessary to obtain 1.3 μg L-Fuc isolated from $2.1 \cdot 10^6$ cells (**Equation 3.2**). The whole small intestine of a mouse would thus consist of $75 \cdot 10^5$ crypts and villi (**Equation 3.3**) and result in a L-Fuc concentration of 118.8 mM (**Equation 3.4**).

$$\frac{2.1 \cdot 10^6\ \text{cells}}{4,200\ \text{cells}} = 500 \tag{3.2}$$

$$\frac{25\ cm}{4 \cdot 10^{-4}\ cm} = 62.5 \cdot 10^3 \tag{3.3}$$

$$(62.5 \cdot 10^3) \cdot 120\ \text{crypts \& villi} = 75 \cdot 10^5\ \text{crypts \& villi}$$

$$\frac{75 \cdot 10^5\ \text{crypts \& villi}}{500} = 15 \cdot 10^3 \tag{3.4}$$

$$(15 \cdot 10^3) \cdot 7.92\ \mu M = 118.8 \cdot 10^3\ \mu M$$

The L-Fuc content of the human intestine is assumed to be at least 10 x higher since the human gut has ∼10 x more cells than that of a mouse (Baker *et al.*, 2014). Additionally, the goblet cell number increases from the small to the large intestine (Kim and Khan, 2013) and plant fibres containing L-Fuc residues (Bobik *et al.*, 1997) are supposed to be present in the large bowel. Thus,

the L-Fuc concentration in the colon is thought to be higher than in the small intestine.

3.6 P_{pduA} is induced by low 1,2-PD levels

Using fluorescence microscopy and an adequate reporter strain, the transcriptional activity of genes can be investigated on a single cell level. In order to determine the minimal concentration of 1,2-PD, which is required to induce gene expression from P_{pduA}, the *S.* Typhimurium 14028 P_{pduA}::*gfp* reporter strain was grown anaerobically in a VB-NCE-YE medium, supplemented with tetrathionate, trace elements and decreasing 1,2-PD concentrations (1 mM to 0.01 mM). As a negative control, 14028 P_{pduA}::*gfp* was grown in a VB-NCE-YE medium supplemented with D-Glc or in a VB-NCE-YE medium, to which no carbon source was added (w/o). Overnight cultures of 14028 P_{pduA}::*gfp* were used in final dilutions of 1:250 in the respective media, and incubated in the anaerobic cabinet (Whitley A35, 37 °C, 80 % N_2, 10 % CO_2, 10 % H_2) without agitation. Samples were taken every 30 min, from time point 0 to 330 min. After turbidity measurements (OD_{600}, Wallac Victor[3]) (see **Fig. 5.13** in the Appendix), the bacteria were analysed by fluorescence microscopy. Therefore, 100 cells were sighted in bright field and the GFP-channel of the microscope. **Tab. 3.4** summarises the results of three independent repetitions of the experiment. At 1,2-PD concentrations of 0.05 mM and higher, transcriptional activity of P_{pduA} was observed, but not for samples of cultures grown with less than 0.05 mM 1,2-PD, D-Glc, or a VB-NCE-YE medium without (w/o) the addition of a carbon source.

Table 3.4: Quantitation of observed fluorescence signals of 100 *S.* Typhimurium 14028 P_{pduA}::*gfp* cells grown in a VB-NCE-YE medium supplemented with increasing concentrations of 1,2-PD. As negative controls 14028 P_{pduA}::*gfp* was grown in a VB-NCE-YE medim supplemented with D-Glc and a VB-NCE-YE medium containing no additional carbon source.

Carbon source added	c [mM]	time [min]							
		0	30	60	90	120	150	180	270
1,2 PD	1	-	-	-	-	100 %	100 %	100 %	100 %
1,2 PD	0.5	-	-	-	-	100 %	100 %	100 %	100 %
1,2 PD	0.1	-	-	-	-	100 %	100 %	100 %	100 %
1,2 PD	0.05	-	-	-	-	100 %	100 %	100 %	100 %
1,2 PD	0.01	-	-	-	-	-	-	-	-
D-Glc	25	-	-	-	-	-	-	-	-
w/o		-	-	-	-	-	-	-	-

w/o: VB-NCE-YE without the addition of a carbon source.

-: No fluorescence observed.

A representative selection of images is shown in **Fig. 3.11**. The pictures were obtained 270 min post inoculation in VB-NCE-YE medium containing 0.01-0.5 mM 1,2-PD. The minimum concentration of 1,2-PD, which is necessary to induce the gene expression of the *pdu* genes from the

promoter P_{pduA}, was narrowed down to the range between 0.05 and 0.01 mM because fluorescence was still observed at an 1,2-PD concentration of 0.05 mM, but not at a concentration of 0.01 mM. As mentioned above, the OD_{600} was measured at each time point of sampling (t = 0 to 270 min in 30 min intervals) and plotted over the time (see **Fig. 5.13** in the Appendix) to gain information as to whether strains grown in media supplemented with lower 1,2-PD concentrations showed reduced growth compared to strains grown with higher 1,2-PD concentrations. In the time range investigated, all strains grew equally well, including the strains that were cultivated in a VB-NCE-YE medium without the addition of a carbon source. Hence, it can be assumed that when the gene expression of the *pdu* genes was induced, the bacteria were still feeding on the nutrients provided by the yeast extract that was contained in the VB-NCE-YE medium.
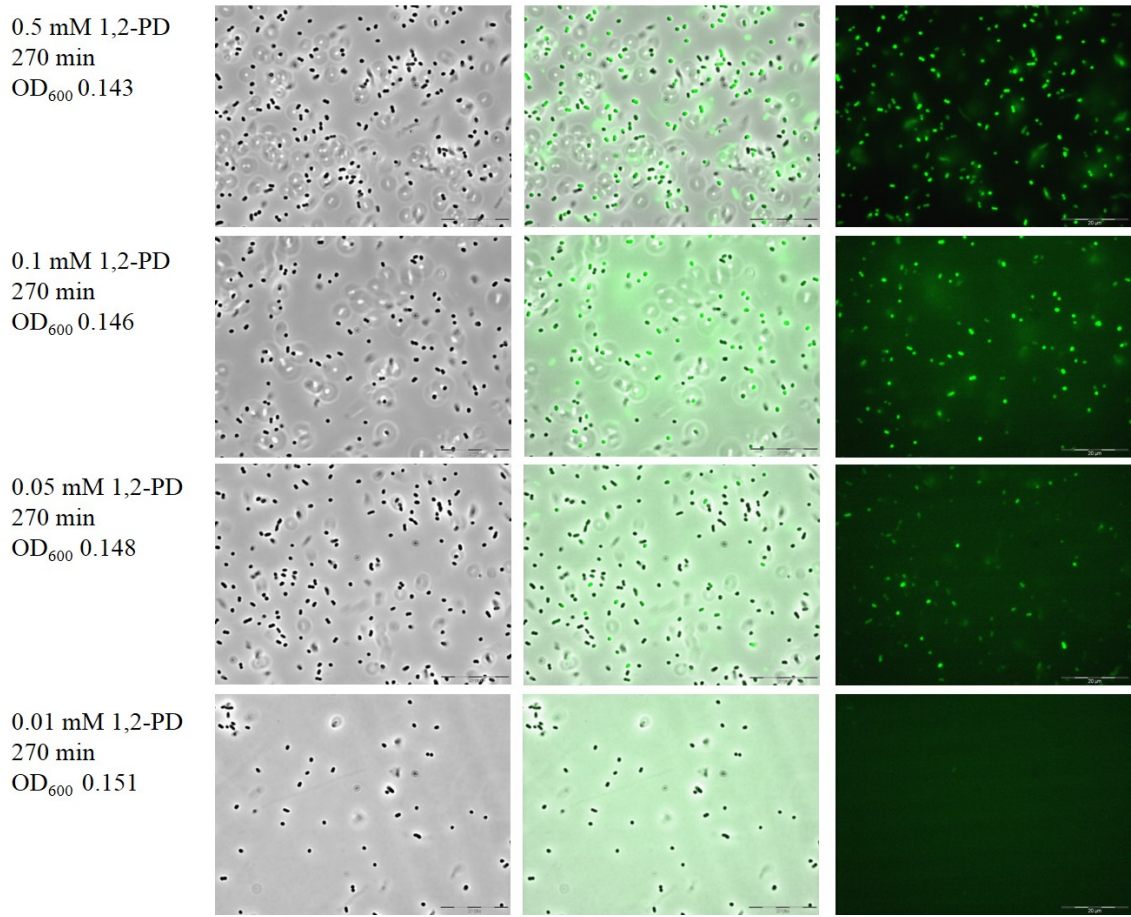
Figure 3.11: **Microscopic investigation of 14028 P_{pduA}::*gfp* at varying 1,2-PD concentrations.** *S.* Typhimurium 14028 P_{pduA}::*gfp* was grown anaerobically in VB-NCE-YE medium supplemented with trace elements, tetrathionate and 0.01 to 0.5 mM 1,2-PD, as indicated on the left. The cells were investigated under a fluorescence microscope for transcriptional activity of P_{pduA}. The photographs shown here represent the results of three independent repetitions of the experiment. The samples were taken 270 min post inoculation. Observation of the cells in bright field (left), under UV-excitation (right) and an overlay (centre) are presented. It can be concluded that the minimum concentration of 1,2-PD that is necessary to induce gene expression from promoter P_{pduA} ranges from 0.01 to 0.05 mM.

3.6.1 Expression of the *pdu* genes does not seem to be completely repressed by D-Glc

As demonstrated in the previous section, the gene expression of the *pdu* gene cluster in *S.* Typhimurium had already been induced by the presence of small amounts of 1,2-PD while bacteria continued to feed on nutrients derived from a source other than 1,2-PD. Thus, the question arose as to whether the simultaneous presence of D-Glc and 1,2-PD leads to a catabolite repression of P_{pduA}.

In a first attempt, equimolar concentrations of 1,2-PD and D-Glc (1 mM each) were added to a VB-NCE-YE medium, together with tetrathionate and trace elements. Overnight cultures of 14028 P_{pduA}::*gfp* were used in final dilutions of 1:250. As a negative control, 14028 P_{pduA}::*gfp* was grown in a VB-NCE-YE medium, without the addition of a carbon source. Bacteria were cultivated anaerobically (Whitley A35, 37 °C, 80 % N_2, 10 % CO_2, 10 % H_2) without agitation, and samples were taken every 30 min from time point 0 to 330 min post inoculation. Prior to the investigation under the fluorescent microscope, turbidity was measured using a Wallac Victor[3] (see **Fig. 5.14** in the Appendix). One-hundred cells were analysed in bright field and the GFP-channel of the microscope, and faintly fluorescent cells were already be observed after 90 min (**Fig. 3.12**) and the fluorescence intensities increased over time. The increase of the fluorescence signal at the late exponential phase might have been attributed to decreasing D-Glc concentrations, because D-Glc was metabolised prior to 1,2-PD. The possible catabolite repression of P_{pduA} by D-Glc was further investigated in another experimental setup, in which increasing D-Glc (1 to 25 mM), but steady (1 mM) 1,2-PD concentrations were tested (**Tab. 3.5**) to determine whether fluorescence is still detectable under conditions of D-Glc excess. Again, overnight cultures of 14028 P_{pduA}::*gfp* were used in final concentrations of 1:250. The cultivation, sampling procedure and monitoring of the cells was maintained as described in the preceding experiment. 14028 P_{pduA}::*gfp* grown in VB-NCE-YE medium containing D-Glc but no 1,2-PD served as a negative control. The observed fluorescence signals were much weaker in the samples of higher D-Glc concentrations compared to those with equimolar concentrations of 1,2-PD and D-Glc, and thus did not allow for a quantitative evaluation of the data. A qualitative analysis of the samples was still possible (**Tab. 3.5**), however, because no fluorescence was observed in samples of the negative controls. As already assumed above, *S.* Typhimurium was still feeding on either D-Glc or yeast extract, when GFP-fluorescence emerged, given that 1,2-PD was provided in the medium (growth curves are not shown). Thus, the *pdu* gene expression does not seem to be completely repressed by the simultaneous presence of D-Glc when 1,2-PD is present in the medium.
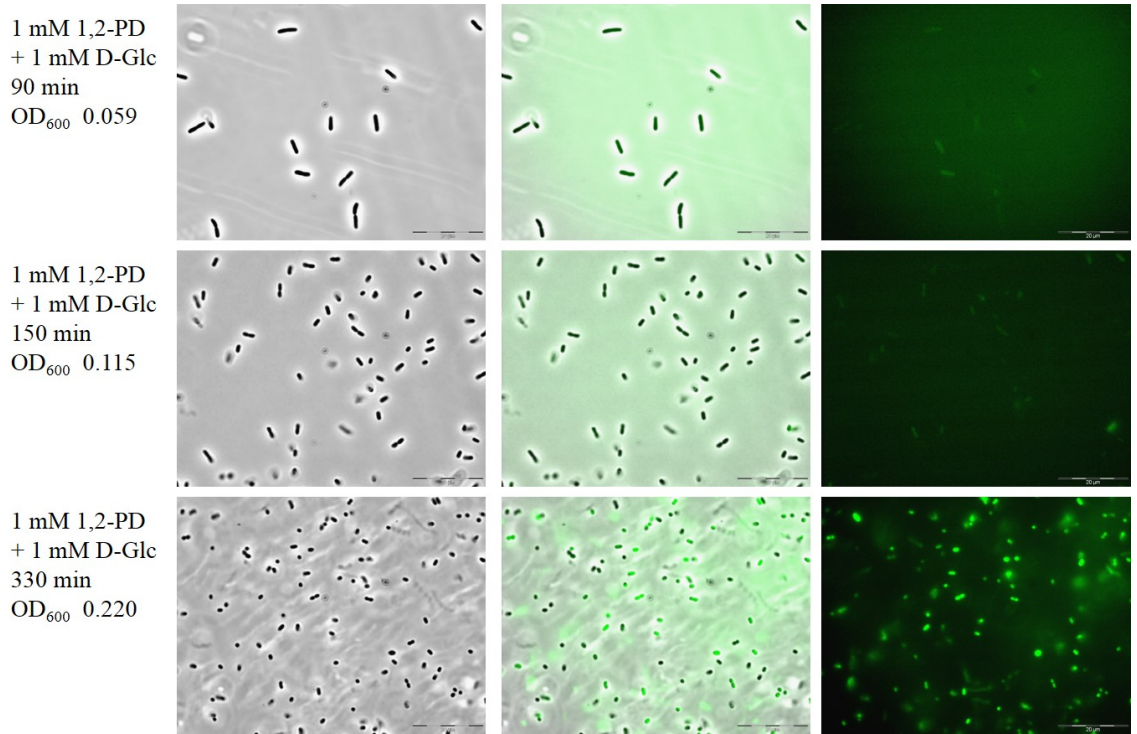
Figure 3.12: **Microscopic investigation for catabolite repression of P$_{pduA}$ by D-Glc.**
S. Typhimurium 14028 P$_{pduA}$::*gfp* was cultivated anaerobically in a VB-NCE-YE medium supplemented with trace elements, tetrathionate and equimolar concentrations of D-Glc and 1,2-PD (1 mM each). The photographs were taken of samples harvested 90, 150 and 330 min post inoculation and investigated under the fluorescence microscope for *gfp* expression. Photographs show the cells in bright field (left), under UV-excitation (right) and an overlay (centre). Fluorescence was detected after 90 min and intensities increased over the time. The pictures are representative for three independent repetitions of the experiment.

Table 3.5: Qualitative analysis of observed fluorescence signals of *S.* Typhimurium 14028 P$_{pduA}$::*gfp* grown in a VB-NCE-YE medium supplemented with steady 1,2-PD and increasing D-Glc concentrations to test for a possible catabolite repression of P$_{pduA}$ by D-Glc.

c_{D-Glc} [mM]	$c_{1,2-PD}$ [mM]	time [min]									
		0	30	60	90	120	150	180	210	240	270
1	1	-	-	-	-	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓	✓	✓
5	1	-	-	-	-	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$
10	1	-	-	-	-	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$
25	1	-	-	-	-	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$	✓$_{(f)}$
25	0	-	-	-	-	-	-	-	-	-	-
w/o		-	-	-	-	-	-	-	-	-	-

✓: Fluorescence observed; ✓$_{(f)}$: Faintly fluorescent; -: No fluorescence observed

3.7 Cell culture assays

3.7.1 *S.* Typhimurium invasiveness is influenced by *invA* and mucus

Epithelial cells of the GIT are covered by one or two mucus layers, which protect the cells from damage and direct contact to microorganisms (Atuma *et al.*, 2001). The *inv* locus is located on SPI-1 and encodes proteins required for cell invasion, so called invasins (Galán and Curtiss, 1989). The deletion of *invA* leads to a non-invasive phenotype of *S.* Typhimurium in cell culture assays (Galán *et al.*, 1992). In the following experiment, invasiveness of 14028 Nal^R and 14028 Δ*invA*, incubated with either Caco-2 or mucus-secreting LS174T cells, was investigated. It was assumed that the mucus produced by LS174T cells would prevent or at least decrease the number of bacteria recovered after cell lysis. Further, it was tested whether the deletion of *invA* leads to the previously described phenotype. The hypothesis that mucus hinders *S.* Typhimurium invasion was confirmed as shown in **Fig. 3.13**. Bacterial cell counts recovered from lysed cells were significantly lower in LS174T cells in comparison with Caco-2 cells for both genotypes, and the invasiveness of 14028 Δ*invA* was reduced compared to 14028 Nal^R in both human cell lines.
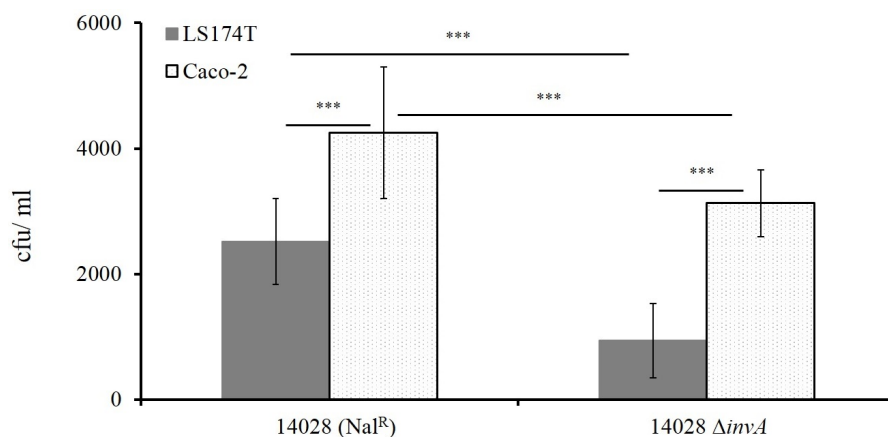
Figure 3.13: **Invasion assay in cell culture.**

    S. Typhimurium 14028 Nal^R and 14028 Δ*invA* were incubated for 60 min with either Caco-2 or LS174T cell cultures. After gentamycin treatment killing extracellular bacteria and the lysis of the human cells, the cfu of intracellular *Salmonella* were determined. Significantly less bacteria were recovered from mucus secreting LS174T cells compared to Caco-2 cells. The *invA* deletion led to reduced invasiveness in both human cell lines. Average values of three independent repetitions are shown. Black vertical bars indicate the standard deviations.

3.7.2 Invasion of LS174T cells is preferred over HT29-MTX

The cell lines HT29-MTX and LS174T have priorly been reported to produce mucus (Tom *et al.*, 1976; Lesuffleur *et al.*, 1990). The composition of the mucus produced by HT29-MTX cells is thought to be more similar to gastric mucus, since MUC5AC is the prevalent mucin (Nollevaux *et al.*, 2006), whereas LS174T cells produce mainly MUC2, the dominant mucin of the small and large intestine (van Klinken *et al.*, 1996).

Cell cultures of HT29-MTX and LS174T were maintained for 21 (HT29-MTX) or ten days (LS174T) after reaching confluency, to obtain proliferated, mucus secreting cells. After 2 h incubation with *S.* Typhimurium MvP 103, the cells were fixated using formalin, and immunofluorescence staining was conducted to visualise mucus and nuclei. Due to safety level restrictions of the laboratory containing the confocal microscope, the S1 strain, *S.* Typhimurium 14028 MvP 103 was used. The plasmid pGreen-TIR (subsequently referred to as pGreen), leading to the constitutive expression of *gfp*, was delivered to 14028 MvP 103 by electroporation. The culture-slides were investigated under a confocal microscope (IX81, Olympus) at 400 x magnification. Z-stacks were recorded and assembled in Volocity 6.0 (Perkin Elmer) software, to obtain 3-dimensional photographs. Even after 2 h *S.* Typhimurium did not invade HT29-MTX cells (**Fig. 3.14 A**), as green fluorescent bacteria were only visible on top of the mucus. In the case of mucus penetration and host-cell invasion, bacteria were expected to be located within the mucus layer or, after invasion of the host cells, in close vicinity to the nuclei of the cell culture as it was observed when 14028 MvP 103/pGreen was incubated for 2 h with LS174T cells (**Fig. 3.14 B**).
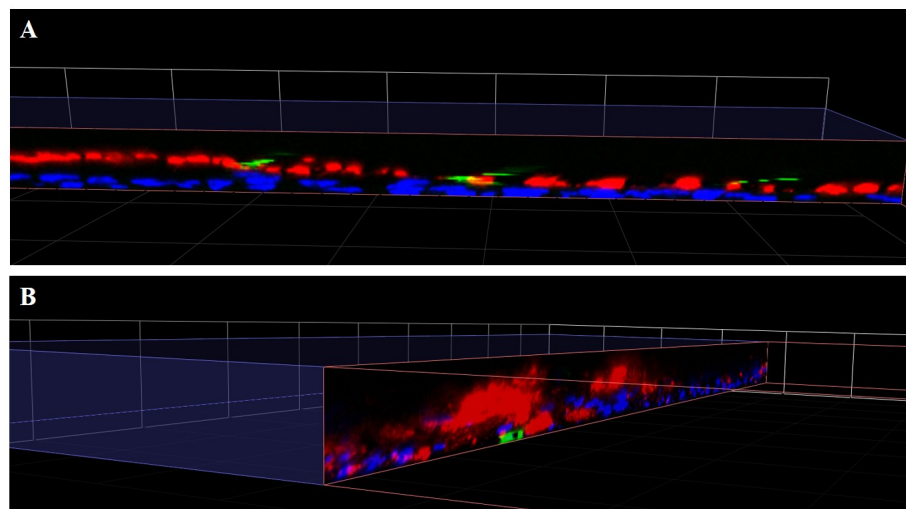
Figure 3.14: **Invasion of HT29-MTX and LS174T cells.**
　　　S. Typhimurium MvP103/pGreen was incubated for 2 h with HT29-MTX (**A**) and LS174T (**B**) cells. Bacteria constitutively expressed GFP from the plasmid pGreen and are thus visible in green. Mucus is stained in red by immunofluorescence staining and nuclei in blue (DAPI). The mucus layer of HT29-MTX cells was not penetrated by the bacteria that were only found on top of the mucus layer (**A**). Successful invasion is documented in photograph **B** where green fluorescent bacteria are located within the same z-stacks as the nuclei of LS174T cells. Photographs are representatives of three independent repetitions.

3.7.3 Motility is crucial for *S.* Typhimurium to overcome the mucus layer

To test whether *S.* Typhimurium actively invades the mucus layer of LS174T cells, a mutation of the flagellar apparatus was introduced to *S.* Typhimurium MvP 103. The genetic modification was generated by Bärbel Stecher in the *S.* Typhimurium strain SL1344 (*fliGHI*::Tn*10*) and resulted in non-motile bacteria (Stecher *et al.*, 2004). The mutation was transferred to 14028 MvP 103 by phage transduction, and the motility of the original strain as well as the 14028 MvP 103 *fliGHI*::Tn*10* mutant was tested in soft agar. Bacteria with an intact falgellar apparatus were able to transmigrate the whole agar plate, whereas the mutant strain remained at the spot of inoculation. To visualise the bacteria in the following experiments, the plasmid pGreen was introduced to 14028 MvP 103 and 14028 MvP 103 *fliGHI*::Tn*10* by electroporation. **Fig. 3.15 A** and **B** shows representative results of immunofluorescence staining experiments of LS174T cells. The mucus was stained in red using a fluorescence-labelled secondary antibody, and the nuclei were stained in blue using DAPI. Prior to fixation, motile and non-motile *S.* Typhimurium were incubated with the cell culture for 30 min, each. Invasion of the mucus was only observed for motile *S.* Typhimurium (**Fig. 3.15 B**), and not for the non-motile 14028 MvP 103 *fliGHI*::Tn*10* strain (**Fig. 3.15 A**). Thus, it can be concluded that *S.* Typhimurium actively penetrates the mucus layer by using its flagella.
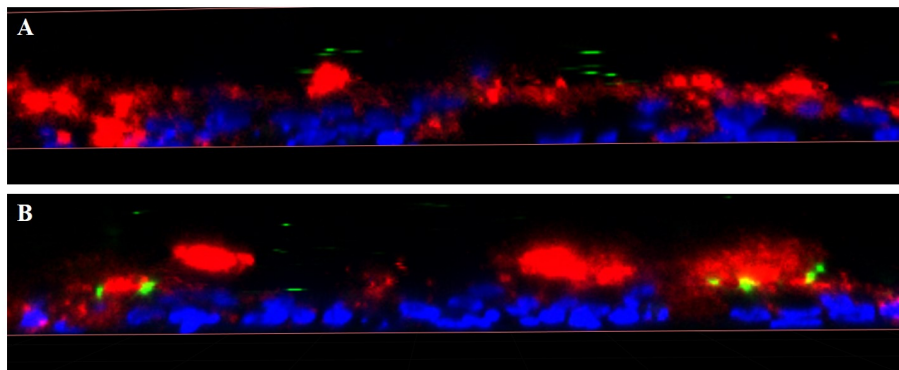
Figure 3.15: **LS174T invasion of motile and non-motile *S.* Typhimurium.**
Ten day old LS174T cells were incubated with *S.* Typhimurium MvP 103/pGreen for 30 min. Mucus is stained in red and nuclei in blue (DAPI). Invasion was monitored for the non-motile 14028 MvP 103 *fliGHI*::Tn*10* and motile 14028 MvP 103 strain. After three repetitions representative photographs were selected for each bacterial genotype. **A)** Non-motile *S.* Typhimurium 14028 MvP 103 *fliGHI*::Tn*10* were only visible above the mucus secreted by the cell culture. Invasion of the mucus was not observed. **B)** Motile *S.* Typhimurium 14028 MvP 103 were found within the mucus layer indicating successful mucus penetration.

3.8 Evidence of L-Fuc in the gut of *C. elegans*

While the presence of a glycocalyx in *C. elegans* has been described (McGhee, 2007), the sugars present in the glycoconjugates of this glycocalyx have not been further specified. To investigate whether L-Fuc is present in the glycocalyx of the nematode, feeding assays were performed. The nematodes were fed on *S.* Typhimurium 14028 P_{fucO}::*lux* for either 4 h or 4 days at 25 °C. *C. elegans* was removed from the agar plates, using an M9-buffer supplemented with triton X-100, and the nematodes were pipetted in aliquots of 200 μl into the wells of a microtiter plate, and investigated for bioluminescence using a Victor[3] multilabel counter (Perkin Elmer) and the IVIS (Xenogen). The results of the first experiment are shown in **Fig. 3.16**. Cavities B 2-9 contained nematodes fed on infection plates for 4 h, and wells D 2-9 contained nematodes fed on 14028 P_{fucO}::*lux* for 4 days. Luminescence was only detected in the wells of the microtiter plate that contained nematodes fed for 4 days on the *lux*-reporter strain. With 2,370 RLU_{490} (Wallac Victor[3]), well D 9 showed the highest value of detected luminescence, indicating the transcriptional activity of P_{fucO}. The inhomogeneous luminescence values in the cavities were probably caused by unequal numbers of nematodes in the different wells. Cavity D 9 contained the highest number of nematodes. As this first attempt was promising, it is recommended that the investigation of the sugar composition of the *C. elegans* glycocalyx be part of follow-up studies.
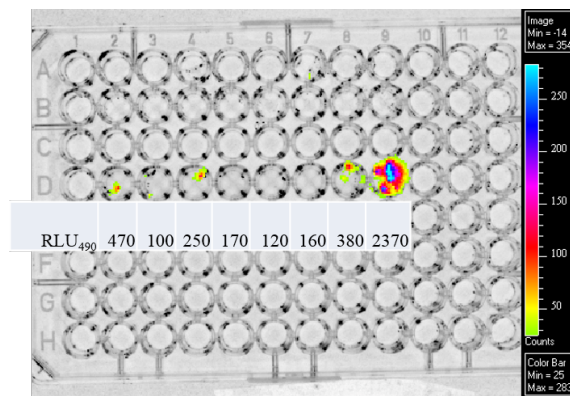
Figure 3.16: *C. elegans* **infection assay with 14028 P_{fucO}::*lux*.**
C. elegans L4 larvae were fed on the *S.* Typhimurium 14028 P_{fucO}::*lux* reporter strain to monitor L-Fuc degradation. Nematodes were fed for 4 h (wells B 2-9) or 4 days (cavities D 2-9) on infection plates. Luminescence (RLU_{490}) was measured using the IVIS and Wallac Victor[3] multilabel counter (table below the wells). The exposure time was 10 s for the IVIS and 0.1 s for Wallac Victor[3] measurements. Light emission was only detected in wells containing nematodes that were incubated on 14028 P_{fucO}::*lux* for 4 days. Due to unequal numbers of nematodes per well the highest signal was detected in well D 9 containing the most nematodes. Because this figure is showing the results a single experiment further investigations are required to provide statistical confirmation.

3.9 Summary of the results

Once ingested, enteric pathogens encounter a highly competitive environment. The shortage in nutrient supply can only be overcome by the utilisation of carbon and energy sources that are not exploited by members of the microbiota. Many enteropathogens have developed or acquired metabolic pathways to use small molecules that cannot be fermented. Ten of the 15 selected pathogens possess enzymes required for ethanolamine degradation, but only *S.* Typhimurium is able to oxidise this anaerobically, by using tetrathionate as electron acceptor. The *pdu* genes were found to be present in five of the selected species, of which only *S.* Typhimurium and *Y. enterocolitica* are capable of tetrathionate respiration as well as the synthesis of the required cofactor vitamin B_{12}.

The degradation of L-Fuc and 1,2-PD in *S.* Typhimurium was investigated in more detail. The proteins encoded by the *fuc* and *pdu* genes were found to be ubiquitous in serovars of the *S. enterica* taxid sequenced thus far. Further, the database search revealed that the *pdu* gene cluster is present in some other enteropathogenic species, in which experimental evidence about its utilisation is still lacking. By applying adequate reporter systems in *S.* Typhimurium, it was demonstrated that anaerobic L-Fuc degradation results in 1,2-PD production, and that 1,2-PD is simultaneously metabolised by the bacteria. Thus, the assumption that L-Fuc and 1,2-PD are utilised sequentially (Obradors *et al.*, 1988), in turn leading to a biphasic growth, was not confirmed. Further, it was shown that the *pdu* genes downstream of the promoter P_{pduA} are transcribed as a single polycistronic mRNA, encoding 21 proteins, and that its expression is induced by very low 1,2-PD concentrations. The induction of the *pdu* gene expression by P_{pduA} was not completely inhibited by the simultaneous presence of 1,2-PD and D-Glc, or other nutrient sources such as yeast extract or L-Fuc. The deletion of genes, encoding central enzymes in the degradation process of L-Fuc or 1,2-PD, was found to abolish growth of *S.* Typhimurium with the respective substrate. The expression of the gene deleted beforehand in *trans* partly restored the ability of the bacteria to grow with the substrate, even though wild type levels of growth were not reached after complementation. The hypothesis that the $\Delta pduC$ deletion mutant would have a disadvantage in comparison to its wild type and complementation strain, when cultivated in pre-fermented milks or gut content media of pigs, was not confirmed.

Invasion assays using human cell lines demonstrated that motility is crucial for the penetration of the mucus layer, which covers the epithelium of the gut. *S.* Typhimurium appeared to invade cells secreting intestinal MUC2 rather than those releasing gastric MUC5AC. In general, the invasiveness of *S.* Typhimurium *in vitro* can be said to depend on the functionality of the *inv* locus, motility and the secretion of mucus by the cell culture.

Whether L-Fuc is a part of the *C. elegans* glycocalyx remains speculative and requires further investigation.

4 Discussion

4.1 L-Fuc and 1,2-PD metabolism

4.1.1 Origin of free L-Fuc in the gut

L-Fuc and L-Rha are 6-deoxy-monosaccharides ($C_6H_{12}O_5$). L-Rha is part of pectin while L-Fuc is found as a common terminal sugar in human gastric mucins (Podolsky, 1985), in human milk oligosaccharides (HMOs) (Ashida *et al.*, 2009), and the carbohydrate moiety of glycoproteins and glycolipids (Yorek *et al.*, 1995). Due to the frequent incorporation of the fucosylated glycoproteins and glycolipids into the glycocalyx, L-Fuc is thought to play a role in cell to cell communication (Yorek *et al.*, 1995). L-Fuc monomers represent about 4-14 % of the oligosaccharide component of gastric mucins (Allen and Griffiths, 2001; Hugdahl *et al.*, 1988). The *FUT2* gene (encoding an α-(1,2)-fucosyltransferase) catalyses the addition of α-(1,2)-fucose residues to mucins and is expressed in human goblet cells. *Fut2* is the orthologue gene in mice and prominently expressed in the large intestine, but not in the small intestine of the animals (Hurd *et al.*, 2005). This might help explain the finding that mucins of the large intestine of mice have a higher level of fucosylation than those of the small intestine (Thomsson *et al.*, 2002). Furthermore, there is evidence for *B. thetaiotaomicron*-induced fucosylation of glycoproteins in the distal small and large intestine. *B. thetaiotaomicron* is an abundant member of the microbiota in humans and mice (Bry *et al.*, 1996; Hooper *et al.*, 1999). It is theorised that it possesses the largest repertoire of glycosidic enzymes among bacteria sequenced thus far (Wexler, 2007). Mucins serve as nutrient sources for bacteria in the gut (Cummings and Macfarlane, 1991) and enzymes needed to release L-Fuc monomers from the complex glycan structures of mucins or HMOs are secreted by bacteria of the microbiota. This means that the availability of L-Fuc monomers in the gut is microbiota dependent (Ng *et al.*, 2013). Examples of fucosidase secreting members of the microbiota include *B. bifidum* (Ashida *et al.*, 2009; Katayama *et al.*, 2004), *B. longum* (Sela *et al.*, 2008), *B. thetaiotaomicron* (Hooper *et al.*, 1999) and other *Bacteroides* species (Xu *et al.*, 2003; Turner and Roberton, 1979). Aside from members of the microbiota, pathogenic bacteria such as *B. cereus* and *C. perfringens* for example, were also found to express fucosidases (Miura *et al.*, 2005).

4.2 Distribution of the *fuc* and *pdu* genes among enteropathogens

C. jejuni is a food-borne pathogen causing gastroenteritis in humans. The gallbladder and intestinal tract are the primary sites of *C. jejuni* infection (Hugdahl *et al.*, 1988). For a long period of time it has been assumed that *C. jejuni* is unable to use carbohydrates as carbon and energy sources, since the glycolytic pathway seems to be incomplete. Hence, it has been described to primarily feed on amino acids and be capable of performing gluconeogenesis. *C. jejuni* harbours an intact TCA-cycle and the genes required for purine, thiamine and amino acid synthesis (Parkhill *et al.*, 2000). *C. jejuni* is attracted by mucins and bile (Hugdahl *et al.*, 1988). The attraction towards mucin transpired to be related to terminal L-Fuc residues, and after the removal of the mucin component from bile, the bacterium was repelled rather than attracted. Thus, mucus-derived L-Fuc serves as chemoattractant to *C. jejuni*, just as *S.* Typhimurium follows a D-galactose gradient towards the host epithelium (Hugdahl *et al.*, 1988; Stecher *et al.*, 2008). *C. jejuni* is not only attracted by L-Fuc, but it also binds the fucosylated H(O)-antigen prior to invasion of the host cell. The H(O)-antigen is a surface structure located in the intestinal mucosa, which is used by many pathogens to recognise the surface of a host cell (Ruiz-Palacios *et al.*, 2003; Bode, 2006; Kobata, 2010). α-1,2-linked fucosyloligosaccharides are contained in high amounts in mother's milk and pathogens such as *C. jejuni* attach to the HMO-derived fucosylated oligosaccharide without reaching the epithelium (Ruiz-Palacios *et al.*, 2003). It has been reported that *C. jejuni* mutants, which are unable to use L-Fuc, are outcompeted by the wild type in inoculated chickens (Muraoka and Zhang, 2011) . With the exception of *fucP*, a L-Fuc permease (*cj0486*), and *ald*, which encodes a lactaldehyde dehydrogenase (*cj0490*), no homologous genes to those of the L-Fuc degradation pathway that are known from other bacteria, such as *S.* Typhimurium, *E. coli, K. pneumoniae, Roseburia inulinivorans*, and *B. thetaiotaomicron*, have been identified in *C. jejuni* (Muraoka and Zhang, 2011; Stahl *et al.*, 2011, 2012). In general, *C. jejuni* has a rather small genome (1.641 MB) compared to *S.* Typhimurium (4.857 Mb), for example (Parkhill *et al.*, 2000; McClelland *et al.*, 2001). This might explain the lack of some metabolic pathways found in other bacteria or pathogens. In *C. jejuni*, it is suggested that L-Fuc is metabolised via L-fuconate to pyruvate, and pyruvate can either be formed from L-fuconate, requiring one enzymatic reaction, or from lactaldehyde and lactate, needing two more enzymatic reactions (Stahl *et al.*, 2012).

While the relevance of L-Rha degradation *in vivo* remains to be elucidated, defects in L-Fuc utilisation have been shown to reduce the competitiveness of *S.* Typhimurium in mice (Ng *et al.*, 2013). Furthermore, *S.* Typhimurium and *C. jejuni* have been found to attach to α-(1,2)-fucose residues *in vitro* (Chessa *et al.*, 2009; Muraoka and Zhang, 2011). Another pathogen, which benefits from L-Fuc availability is enterohaemorrhagic *E. coli* (EHEC). Its two-component-system FusKR senses free L-Fuc, and represses the expression of genes located on the pathogenicity island LEE (locus of enterocyte effacement) as well as those required for L-Fuc degradation. Instead of L-Fuc, EHEC relies on unique carbon sources such as galactose, which are not metabolised by

the microbiota, to possibly circumvent the competition for nutrients (Pacheco *et al.*, 2012; Keeney and Finlay, 2013). The repressing effect of FusKR on virulence gene expression is abolished by host signals that activate other response regulators of the pathogen in close proximity to the epithelium (Keeney and Finlay, 2013). Thus, mutants impaired of sensing free L-Fuc seem to be unable to correctly time virulence and metabolic gene expression, resulting in a colonisation defect due to unnecessary energy expenditure (Pacheco *et al.*, 2012; Keeney and Finlay, 2013).

In 1996, about 138 natural *Salmonella* isolates (partly different isolates of the same serovar) were tested experimentally, of which at least 129 were able to degrade 1,2-PD (Lawrence and Roth, 1996). The focus of the research project was the ability to synthesise vitamin B_{12}, but 1,2-PD and ethanolamine degradation were investigated as well, since vitamin B_{12} is required for the degradation of both substrates in *S.* Typhimurium (Roth *et al.*, 1996). Genome sequencing technologies and the availability of the data made it possible to screen the genomes of 82 *S. enterica* subsp. *enterica* serovars for the presence of the *pdu* and *fuc* gene clusters (**Section 3.2**). The database query performed revealed that both genomic regions are highly conserved among *S. enterica* subsp. *enterica* serovars, as well as in *S. enterica* subspecies other than *enterica*. Due to the high conservation, it is theorised that the absence of some genes in single serovars is the result of incomplete sequencing rather than a loss or deletion. As the genes have been maintained, they must provide a benefit to the pathogen. The question remains, however, whether these highly conserved gene clusters are beneficial for survival in the environment or during infection of a host. The *cob/cbi* and *pdu* region of *S.* Typhimurium is assumed to be acquired by a single horizontal gene transfer. This assumption is supported by the fact that they are missing in *E. coli*, and are of a different G/C-content (about 57-58 %) than the rest of the *S.* Typhimurium chromosome (about 53 %) (Chen *et al.*, 1994; Roth *et al.*, 1993). Further, virulence defects have been observed for *pdu* mutants of *S.* Typhimurium in cell culture experiments (Heithoff *et al.*, 1999; Klumpp and Fuchs, 2007) as well as in mice (Conner *et al.*, 1998).

An association of *S.* Typhimurium *pdu* gene expression, the poisoning of food and pathogenicity has recently been predicted by genome and literature mining (Korbel *et al.*, 2005). Thus to find out more about the relevance of the *pdu* gene cluster during infection of a host, a closer look was taken at the genomes of other enteropathogens (**Tab. 3.1**). For *L. monocytogenes* and *C. perfringens* the presence of the *pdu* and *cob/cbi* genes has already been reported (Buchrieser *et al.*, 2003; Korbel *et al.*, 2005). Besides in *S. enterica*, *L. monocytogenes*, and *C. perfringens*, the *pdu* genes are also present in *S. sonnei*, *Y. enterocolitica* and some enteropathogenic (EPEC) as well as enterotoxigenic (ETEC) *E. coli* (**Tab. 3.1**). Experimental demonstration of 1,2-PD utilisation is rather lacking, except for *S.* Typhimurium (Badia *et al.*, 1985; Obradors *et al.*, 1988). *L. monocytogenes* is assumed to grow with 1,2-PD, since its apathogenic relative *L. innocua* is able to use 1,2-PD as a (poor) substrate (Xue *et al.*, 2008). Investigations of 1,2-PD metabolism in *Y. enterocolitica* are recommended, because the gene clusters for vitamin B_{12} synthesis, 1,2-PD degradation, and tetrathionate respiration were only found to be present in the genomes of *Y. enterocolitica* and *S. enterica* (**Tab. 3.1**).

4.3 L-Fuc and 1,2-PD degradation by *S.* Typhimurium

S. Typhimurium is not able to release L-Fuc from complex oligo- or polysaccharides, but is, like its relative *E. coli*, able to grow with L-Fuc and L-Rha as sole sources of carbon and energy. The genes required for the degradation of L-Fuc are organised alike in *S.* Typhimurium and *E. coli* (**Fig. 4.1 A**). The genes *fucR* (repressor), *fucA* (L-fuculose-1-phosphate aldolase) and *fucO* (1,2-PD oxidoreductase) are transcribed separately, whereas the genes *fucPIK* (L-Fuc permease, fucose isomerase and L-fuculose kinase) are thought to form an operon, and thus to be mutually expressed from the same promoter (Chen *et al.*, 1984, 1987). The induction of the *fuc* genes is not fully understood, however. Possible effectors that remove FucR from its DNA binding site in order to enable gene expression are L-Fuc (Hooper *et al.*, 1999), L-fuculose-1-phosphate (Chen *et al.*, 1987) or L-lactaldehyde (Chen *et al.*, 1987). L-lactaldehyde could be derived from L-Rha degradation, which indicates a possible cross-induction of the *rha* and *fuc* systems in *E. coli* (**Fig. 4.1 A+C**) (Chen *et al.*, 1987). FucP is responsible for the uptake of L-Fuc, which is converted to L-fuculose by FucI (**Fig. 4.2**). The gene product of *fucK* phosphorylates L-fuculose to form L-fuculose-1-phosphate. L-lactaldehyde and dihydroxy-acetone-phosphate (DHAP) are the result of the enzymatic reaction of FucA, which uses L-fuculose-1-phosphate as substrate. Under aerobic conditions, pyruvate is formed from L-lactaldehyde and DHAP. Pyruvate enters the tricarboxylic acid cycle, and thus the central metabolism. If the bacteria are cultivated anaerobically with L-Fuc, L-lactaldehyde is not converted to pyruvate, but to 1,2-PD by FucO (Badia *et al.*, 1985). The degradation of L-Rha is similar to that of L-Fuc, and is induced by the presence of L-Rha (Power, 1967). Under anaerobic conditions the L-lactaldehyde is converted to 1,2-PD by a cross-induction of the *rha* and *fuc* gene clusters, since a 1,2-PD oxidoreductase is not encoded by the *rha* genes but by *fucO* (**Fig. 4.1 D**) (Chen *et al.*, 1987; Badia *et al.*, 1985; Obradors *et al.*, 1988). 1,2-PD is also secreted by members of the microbiota, such as *B. thetaiotaomicron*, for example, as a fermentation end product during anaerobic growth with L-Rha or L-Fuc (Daniel *et al.*, 1998; Turner and Roberton, 1979; Louis *et al.*, 2007; Badia *et al.*, 1985). A similar set of genes for L-Fuc and L-Rha degradation is used by *B. thetaiotaomicron* (**Fig. 4.1 B+E**) (Hooper *et al.*, 1999). FucP is expressed constitutively and FucR repression is counteracted by the presence of L-Fuc, allowing *fuc*-gene expression (Hooper and Gordon, 2001). In contrast to *E. coli*, the lactaldehyde reductase of the *rha* system (RhaO) must be used to reduce L-lactaldehyde to 1,2-PD, since no gene encoding such an enzyme has so far been identified among the *fuc* genes in *B. thetaiotaomicron* (Patel *et al.*, 2008).

Unlike in *E. coli* cultures, the concentration of secreted 1,2-PD in the medium did not remain stable, but the diol vanished when *S.* Typhimurium was maintained under anaerobic conditions in the same medium. Instead of 1,2-PD, propionate and n-propanol were detected as end products in the medium, although no bacterial growth was observed (Obradors *et al.*, 1988). Vitamin B_{12} serves as a cofactor for the propanediol dehydratase (PduCDE) and thus, the degradation of 1,2-PD by *S.* Typhimurium is dependent on the availability of the cofactor. The necessity of vitamin

B_{12} in 1,2-PD degradation has already been described in *Klebsiella pneumoniae* (then known as *Aerobacter aerogenes*) (Abeles and Lee, 1961). Despite the fact that *S*. Typhimurium converts 1,2-PD to propionate and n-propanol under anaerobic conditions (Obradors *et al.*, 1988), growth of *S*. Typhimurium with 1,2-PD was initially only observed under aerobic conditions, and the provision of vitamin B_{12} or its precursors (Jeter, 1990). PduCDE reduces 1,2-PD to propional-dehyde, which is further converted to propionyl-CoA. Pyruvate is generated from propionyl-CoA via the 2-methylcitric acid cycle (Bobik *et al.*, 1997), and the pyruvate can enter the tricarboxylic acid (TCA) cycle as acetyl-CoA. The resulting reducing equivalents are aerobically respired to O_2 (Price-Carter *et al.*, 2001; Horswill and Escalante-Semerena, 1999, 2001) (**Fig. 4.2**). Anaer-obically, the formed propionaldehyde is reduced to either propionate or n-propanol, which are both secreted (Obradors *et al.*, 1988) and thus, 1,2-PD cannot serve as a carbon source, since all C-atoms of 1,2-PD are secreted. Still, it continues to serve as a (very) poor energy source, by providing one mole ATP per converted molecule of 1,2-PD and an electron sink (the reduction of propionaldehyde to propanol), which could help the cell to balance redox reactions during fer-mentative growth (Walter *et al.*, 1997). To use 1,2-PD under anaerobic conditions more efficiently, the compound needs to be oxidised by anaerobic respiration. Although none of the well known electron acceptors for anaerobic respiration, such as fumarate or nitrate, support the growth of *S*. Typhimurium with 1,2-PD (Bobik *et al.*, 1997), anaerobic growth is supported by tetrathionate, a less well-studied electron acceptor (Bobik *et al.*, 1997). During anaerobic respiration, the genes required for the 2-methylcitric acid cycle (*prp*) are of major importance, since anaerobic growth with 1,2-PD is prohibited upon their deletion (Price-Carter *et al.*, 2001). As described for aer-obic degradation, the 2-methylcitric acid cycle converts propionyl-CoA to pyruvate. In this case, the reducing equivalents are oxidised by tetrathionate, which is in turn reduced to thiosulphate (Price-Carter *et al.*, 2001; Horswill and Escalante-Semerena, 1999, 2001). Thirteen years after the finding that tetrathionate serves as an electron acceptor for anaerobic respiration of 1,2-PD in *S*. Typhimurium (Bobik *et al.*, 1997), including the search for a link between tetrathionate avail-ability and 1,2-PD degradation, the inflammation of the gut was introduced as a feasible solution (Winter *et al.*, 2010). The caecal mucosa converts hydrogen sulphide (H_2S), which is produced in large quantities by the microbiota to thiosulphate ($S_2O_3^{2-}$) in order to detoxify H_2S. During inflammation and the recruitment of neutrophils to the gut lumen, ROS are formed, which reduce thiosulphate to tetrathionate ($S_4O_6^{2-}$). During infection with *S*. Typhimurium, tetrathionate does not accumulate, suggesting that it is used as an electron acceptor (Winter *et al.*, 2010). Con-sequently, tetrathionate was added to the media used for anaerobic cultivation of *S*. Typhimurium with L-Fuc or 1,2-PD, and media used for aerobic cultivation were supplemented with vitamin B_{12} (**Section 2.2.1.2**). During anaerobic growth of the *lux*-promoter fusions 14028 P_{fucO}::*lux* and 14028 P_{pduA}::*lux* with L-Fuc (**Section 3.3.2**), early *pdu* gene expression was observed. Only one hour after the induction of *fucO* gene expression, luminescence emitted by the P_{pduA}::*lux* pro-moter fusion strain was detected, indicating *pdu* gene expression from P_{pduA}. The data confirm that L-Fuc degradation by *S*. Typhimurium results in the production of 1,2-PD. A biphasic growth

phenotype, which was suggested earlier (Obradors *et al.*, 1988) was not observed (**Fig. 3.3**). It is theorised that the availability of tetrathionate in the medium allowed *S.* Typhimurium to instantly use 1,2-PD after its formation.
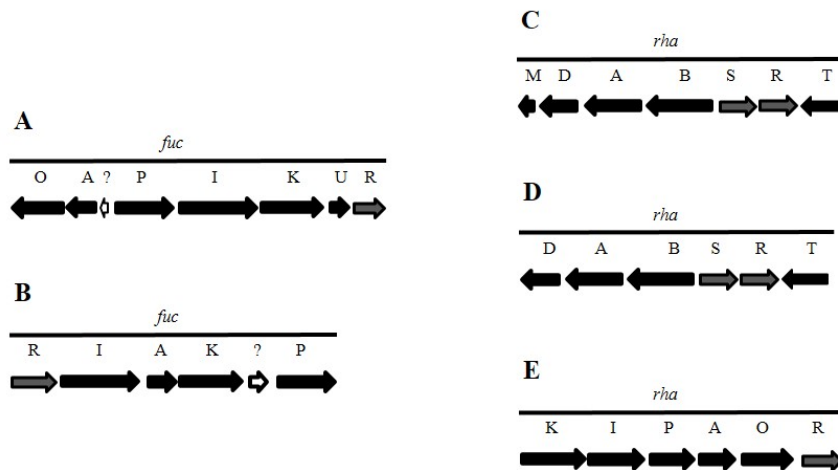
Figure 4.1: **Gene clusters for L-Fuc and L-Rha degradation.**

The gene clusters for L-Fuc degradation of *E. coli/ S.* Typhimurium (**A**) and *B. thetaiotaomicron* (**B**) are shown on the left side and the genetic organisation of the genes required for L-Rha degradation on the right side for *E. coli* (**C**) *S.* Typhimurium (**D**) and *B. thetaiotaomicron* (**E**). *fucR*: repressor, *fucP*: L-Fuc permease, *fucI*: fucose isomerase, *fucK*: L-fuculose kinase, *fucA*: L-fuculose-1-phosphate aldolase, *fucO*: 1,2-PD oxidoreductase, *fucU*: protein of unknown function; *rhaM*: rhamnose mutarotase, *rhaD*: rhamnulose-1-phosphate-aldolase, *rhaA*: rhamnose-isomerase, *rhaB*: rhamnulo-kinase, *rhaS*: transcriptional activator, *rhaR*: repressor, *rhaT*: rhamnose-transporter and *?*: hypothetical protein of unknown function. Gene annotation is different between *E. coli/ S.* Typhimurium, and *B. thetaiotaomicron*. The annotation for *B. thetaiotaomicron* is *rhaK*: rhamnulo-kinase, *rhaI*: rhamnose-isomerase, *rhaP*: rhamnose-permease, *rhaA*: rhamnulose-1-phosphate-aldolase, *rhaO*: 1,2-PD oxidoreductase, *rhaR*: repressor.

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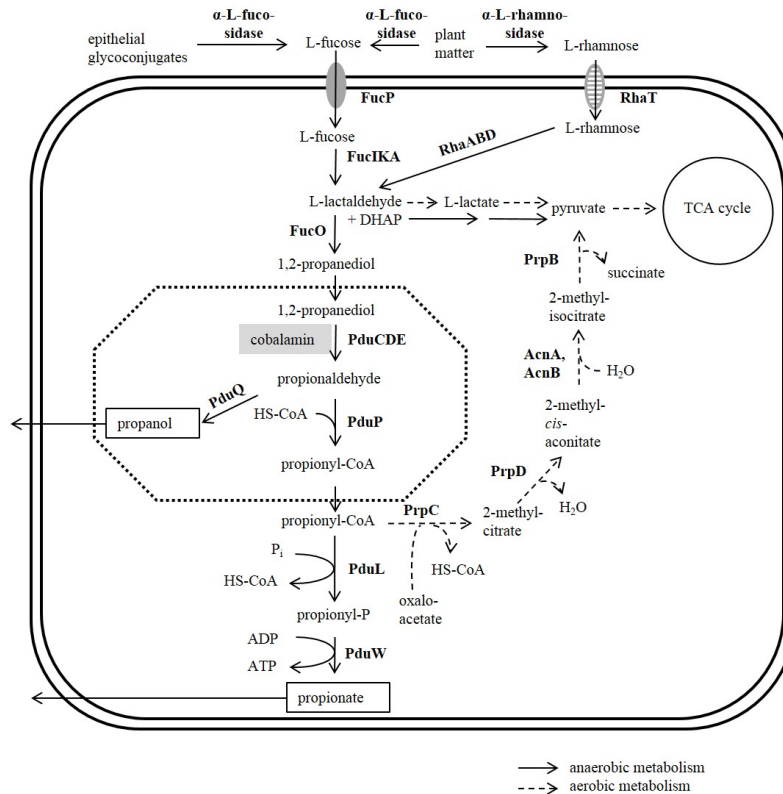
Figure 4.2: **1,2-PD metabolism of *S*. Typhimurium.**

The figure shows the metabolism of 1,2-PD in *S*. Typhimurium. Enzymes are printed bold. Dashed arrows indicate aerobic degradation of 1,2-PD and solid lines show the anaerobic metabolism of 1,2-PD in the absence of tetrathionate as a terminal electron acceptor. The microcompartment, encoded by the *pdu* gene cluster, is indicated as a dashed octagon. RhaT: rhamnose-proton symporter; RhaABD: rhamnose isomerase, rhamnulokinase, rhamnulose-1-phosphate aldolase; FucP: fucose permease; FucIKA: fucose isomerase, fucose kinase, fuculose phosphate aldolase; FucO: 1,2-PD oxidoreductase; PduCDE: 1,2-PD dehydratase; PduQ: propanol dehydrogenase; PduP: propionaldehyde dehydrogenase; PduL: phosphotransacylase; PduW: propionate kinase; PrpC: methylcitrate synthase; PrpD: 2-methylcitrate dehydratase; AcnA/AcnB: aconitases A and B; PrpB: 2-methylisocitrate lyase.

4.3.1 Induction of *pdu* gene expression

The genes for cobalamin and cobinamide biosynthesis (*cob/cbi*), as well as 1,2-PD utilisation (*pdu*) are located adjacently on the chromosome of *S*. Typhimurium. The *cob/cbi* genes are orientated counter-clockwise, and the *pdu* genes clockwise (Jeter, 1990; Rondon and Escalante-Semerena, 1992). Between the two gene clusters, the genes encoding a 1,2-PD diffusion facilitator (*pduF*), and the common regulator of the *cob/cbi* and *pdu* genes *pocR* are located. Both are orientated counter-clockwise (**Fig. 4.3**). Five promoters were suggested to be involved in the regulation

of the *cob/cbi* and *pdu* gene expression, and that their transcriptional activity depends on nutrient availability (Chen *et al.*, 1995). Low amounts of *pocR* are expressed constitutively and in case other carbon sources such as pyruvate or D-Glc are still available the promoters in front of *pduA*, *pduF* and *pocR* are repressed by the low levels of PocR under aerobic growth conditions (**Fig. 4.3 A**). An involvement of global regulators, like for instance CRP and ArcA/ArcB (aerobic respiration control protein) was assumed (Ailion *et al.*, 1993) because the gene expression of *pocR* is induced upon starvation conditions, and *de novo* synthesis of vitamin B_{12} only occurs under anaerobic growth conditions in *S.* Typhimurium (Jeter *et al.*, 1984). CRP senses a shortage in carbon and energy sources, and ArcA/ArcB anaerobic conditions (Ailion *et al.*, 1993). In the case of starvation or growth with poor carbon sources (**Fig. 4.3 B**), P_1 and P_{pocR} are activated by IHF (integrated host factor) and CRP to produce the 1,2-PD diffusion facilitator PduF, and higher amounts of PocR (Ailion *et al.*, 1993; Rondon and Escalante-Semerena, 1996; Chen *et al.*, 1995). If 1,2-PD is also available (**Fig. 4.3 C**), all five promoters are induced by the PocR-1,2-PD complex, leading to *pdu*, *cob/cbi*, *pduF* and *pocR* gene expression (Chen *et al.*, 1995). The activating effect of CRP on PocR expression is described to be weaker under anaerobic growth conditions with a rich carbon source, and the influence of the ArcA/ArcB system increases. During anaerobic growth with poor carbon sources, both ArcA/ArcB and CRP work together to induce *pocR* gene expression (Chen *et al.*, 1995; Ailion *et al.*, 1993). A reduction of *pdu* and *cob/cbi* gene expression was observed in *S.* Typhimurium strains, which are deficient for the synthesis of the antioxidant glutathione, when the bacteria were cultivated aerobically. During anaerobic cultivation, no effect of the strains impaired in glutathione synthesis had been observed. Thus far, the role of glutathione remains speculative, but it seems fair to assume it protects enzymes from damage by the formed propionaldehyde or helps to maintain reduced cysteinyl residues of proteins (like PduF and PocR) by protecting them from oxidation (Rondon *et al.*, 1995).

As low amounts of *pocR* are constitutively expressed, even minor concentrations of 1,2-PD might be adequate to activate PocR, which then induces *pduF*, *cob/cbi* and *pdu* gene expression (Chen *et al.*, 1995). In fact, only 50 μM 1,2-PD were sufficient to induce the gene expression from P_{pduA} under anaerobic growth conditions (**Section 3.6**). Thus, the induction of the *pdu* genes is very sensitive, since the amount of 1,2-PD in the gut was estimated to be approximately 119 mM in mice and 1.19 M in humans (**Section 3.5**). For the estimation of the 1,2-PD concentration in the gut, a 1:1 conversion of L-Fuc to 1,2-PD was presumed (Badia *et al.*, 1985). It was not anticipated that *pdu* gene expression was observed as long as other nutrient sources such as yeast extract, or even D-Glc, had not yet been exhausted (**Section 3.6.1**). No promoter activity was observed in media containing only D-Glc or yeast extract, but no 1,2-PD. These results were obtained during anaerobic cultivation of the bacteria, and thus confirm the findings of previous studies holding that the activating effect of CRP is reduced under anaerobic conditions and the availability of D-Glc (Chen *et al.*, 1995; Ailion *et al.*, 1993). The aforementioned experiment, growing 14028 P_{pduA}::*lux* with L-Fuc, also confirms the CRP regulation model (Chen *et al.*, 1995; Ailion *et al.*, 1993) because at the onset of luminescence emission from the P_{pduA}::*lux* promoter fusion strain that indicates *pdu*

gene expression, L-Fuc and yeast extract had not yet been depleted (**Section 3.3.2**). The ability of *S.* Typhimurium to metabolise multiple substrates in parallel has been published recently (Steeb *et al.*, 2013), and thus it would be plausible that both substrates can be utilised at the same time. Examinations of the culture medium would help to solve the question of substrate availability during all growth phases. Such analyses could, for instance, include the determination of the 1,2-PD concentration, or the residual amount of D-Glc or L-Fuc at the onset of reporter-strain signalling. Gas-chromatography (GC) or GC-mass spectrometry (MS) analysis would be suitable methods in this case.

Independently of 1,2-PD utilisation, the formation of polyhedral protein bodies in the cytoplasm can be observed when *S.* Typhimurium is cultivated in media containing 1,2-PD. The microcompartments have a cross-section of approximately 150 nm, and capture 1,2-PD dehydratase, propionaldehyde and vitamin B_{12} (Bobik *et al.*, 1999). Since propionaldehyde leads to DNA damage, inhibition of respiratory processes and growth arrest (Sampson and Bobik, 2008), the formation of such a microcompartments is reasonable. The intracellular compartments not only serve as a trap for propionaldehyde, but also as a barrier to vitamin B_{12} (Havemann *et al.*, 2002). Vitamin B_{12} is needed inside the compartment, but is synthesised in the cytoplasm, and thereby the production of the toxic aldehyde is slowed down (Havemann *et al.*, 2002). The genes encoding the constituents of the protein-shells are *pduA*, *pduBB'*, *pduJ* and *pduN*. PduU and PduT are also part of the shell, but are not essential for the formation of the microcompartment. Still, PduT and PduU are important, since they are thought to be involved in transport processes across the protein shell (Cheng *et al.*, 2011).

An attempt to explain the early induction of *pdu* gene expression could be related to the formation of the microcompartment. Perhaps the synthesis of the microcompartment's protein shell consumes a lot of energy, which can only be provided as long as the bacteria continue to feed on richer carbon and energy sources than 1,2-PD. Where tetrathionate is not available under anaerobic conditions, 1,2-PD is a rather poor substrate, only resulting in one mole ATP per mole 1,2-PD (Walter *et al.*, 1997). This hypothesis lacks experimental confirmation, but would certainly be worth investigating.
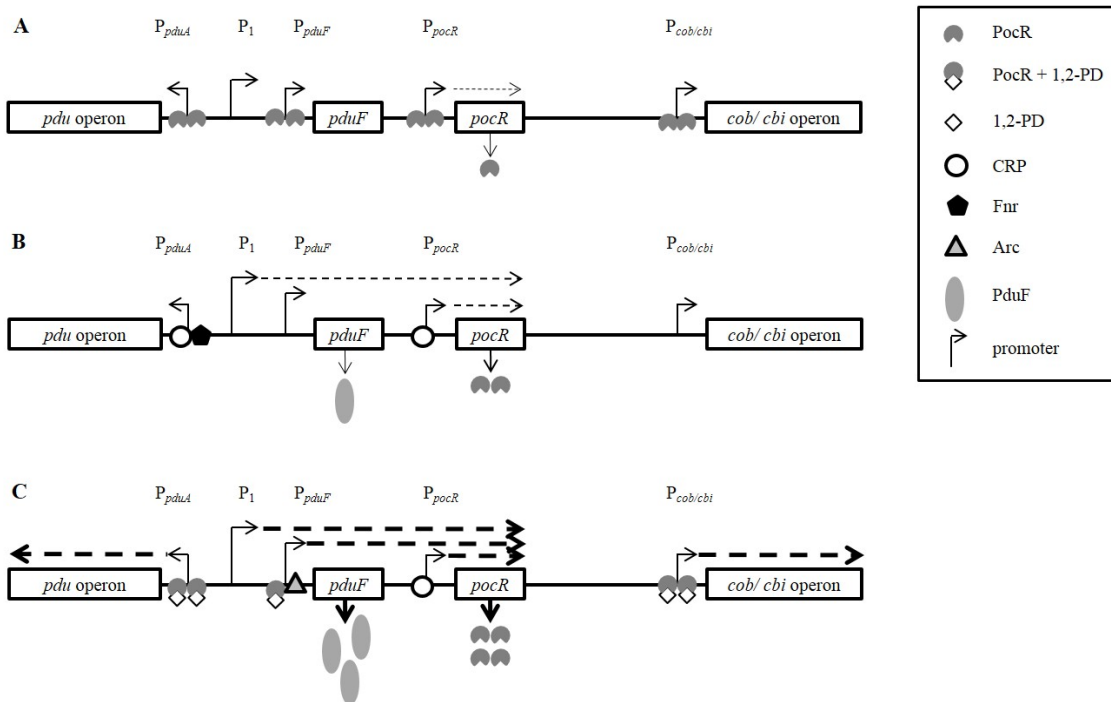
Figure 4.3: **Regulation of gene expression by PocR.**

Horizontal dashed arrows indicate the gene expression from the respective promoter in *S.* Typhimurium. Promoters are shown as solid arrows that point in the direction of transcription. **(A)** Due to the presence of alternative carbon sources and oxygen all genes are repressed by PocR, except for some basal expression from P$_{pocR}$. **(B)** When grown with poor carbon sources or under anaerobic conditions, the promoter P$_1$ is induced leading to the expression of PduF and PocR. **(C)** All promoters are induced in case of growth with poor carbon sources or the absence of oxygen and the simultaneous availability of 1,2-PD. [Figure modified Chen *et al.* (1995)]

4.3.2 Transcription of the *pdu* genes

As mentioned above, the *pdu* locus of *S.* Typhimurium is comprised of 23 genes, of which two are orientated counter-clockwise (*pduF*, *pocR*), and 21 clockwise (Bobik *et al.*, 1999). Before the discovery that the *pdu* locus encodes 23 proteins, it has been assumed that the genes orientated in a clockwise direction, starting with *pduA*, could be transcribed as an operon with a single promoter in front of *pduA* (Chen *et al.*, 1995). At that time, only *pduA*, *pduB* and *pduC* had been identified (Chen *et al.*, 1994, 1995). Thus the results obtained within the scope of this thesis confirm the assumption that all 21 genes, located between *pduF* and *yeeA* on the *S.* Typhimurium chromosome, are transcribed as a single polycistronic mRNA (**Section 3.4**). Still, the presence of additional weak promoters downstream of P$_{pduA}$ cannot be excluded, since the σ^{70} promoter prediction tool BPROM found possible promoters in front of almost every gene of the *pdu* gene cluster. Therefore further studies will be necessary to investigate the presence of additional promoters.

4.4 FucA and PduC are essential for L-Fuc and 1,2-PD degradation

Point mutations in *pduC* and *pduD* have been shown to eliminate 1,2-PD-dehydratase activity (Walter *et al.*, 1997). The deletion of *pduC* led to a severely attenuated growth phenotype in media containing 1,2-PD as a carbon- and energy source (**Section 3.3.3**). Growth with 1,2-PD was partly restored when the complementation plasmid pBR-*pduC* was introduced to the deletion strain 14028 Δ*pduC* (**Fig. 3.5**). Similar results were obtained for growth with L-Fuc by replacing *fucA* by a kanamycin-cassette (**Section 3.3.1**). The insertion mutant 14028 Δ*fucA*::*kan*^{R} also demonstrated an attenuated growth phenotype when grown with L-Fuc, and growth was improved, but not be restored to wild type levels with the introduction of pBR-*fucA* (**Fig. 3.2**). Since 1,2-PD is composed of a C_3 body, it can be assumed that it provides less energy than L-Fuc or D-Glc. Even from the fermentation of D-Glc the bacteria were found to gain more energy compared to the anaerobic respiration of 1,2-PD, as higher cell densities were reached by the controls grown anaerobically with D-Glc (black charts in **Fig. 3.5**). After the relevance of *pduC* for 1,2-PD degradation was determined, it was tested whether 1,2-PD utilisation provides a growth benefit to *S.* Typhimurium in more complex media containing pre-fermented milks or the gut contents of pigs (**Sections 3.3.5** and **3.3.6**). The milks were chosen because fucosylated milk oligosaccharides are abundant in human milk (Ashida *et al.*, 2009; Ruiz-Palacios *et al.*, 2003) and, in smaller quantities, in animal milks (Al Mijan *et al.*, 2011). The milks were pre-fermented using *B. infantis*, since *S.* Typhimurium lacks the necessary enzymes to release monomers from the milk oligosaccharides, and because the fucosidase activity of *B. infantis* is induced when it is grown with human milk oligosaccharides. Furthermore, *B. infantis* ferments L-Fuc and secretes 1,2-PD (Sela *et al.*, 2008). No influence of the *pduC* deletion was observed in comparison to the wild type and complementation strain in milks (**Fig. 3.8**), which was expected to result in a growth disadvantage of the Δ*pduC* strain. The inability of the *pduC* mutant to use 1,2-PD as a substrate was anticipated to be more pronounced in human than in animal milk, since human milk should contain the highest amount of L-Fuc. Analogous to earlier descriptions of human faeces, the gut contents of pigs was assumed to contain L-Fuc, which can be derived from mucins and ingested plant matter (Podolsky, 1985; Bry *et al.*, 1996; Bobik *et al.*, 1997). To exhaust nutrient sources other than 1,2-PD, the medium containing the gut contents was incubated with the 14028 Δ*pduC* mutant, which is still able to ferment L-Fuc to 1,2-PD. After the incubation with the deletion strain, the medium was filter sterilised, processed, and supplemented with tetrathionate or vitamin B_{12}, to enable aerobic or anaerobic 1,2-PD degradation. As previously described for the milk experiments, a disadvantage of the Δ*pduC* mutant was expected in comparison to the wild type. The growth disadvantage of the mutant strain should have been visible as a prolonged lag-phase, after the bacteria were transferred from the gut media to LB-broth. The expected phenotype was not observed (**Fig. 3.9**), which points to equal replication numbers of wild type and deletion strain in the gut media. The missing phenotype of the Δ*pduC* mutant strain, which was introduced to the milk and gut media,

could be attributed to incomplete fermentation of L-Fuc to 1,2-PD, or to insufficient depletion of other carbon and energy sources, which are available in faeces and milk. In future studies, the composition of the pre-fermented media should be analysed in more detail. This would help to elucidate whether *B. infantis* actually released L-Fuc monomers from milk oligosaccharides, and if 1,2-PD was formed from L-Fuc or L-Rha under the conditions applied. A suitable method for such an investigation would be GC-MS analysis.

4.5 Invasiveness is influenced by motility, the *inv* locus, and mucus secretion

Based on the assumption that *S.* Typhimurium consumes mucus-derived carbon sources such as L-Fuc, or 1,2-PD, which is a degradation product of L-Fuc, the interaction of the pathogen with cell lines that secrete mucins was studied. Given that the emphasis of the present study was placed on the extracellular metabolism of *S.* Typhimurium, the invasion of the cell culture by the bacteria needed to be prevented or at least delayed. The interruption of *invA* in *S.* Typhimurium leads to non-invasive bacteria, which are also less virulent in mice (Galán and Curtiss, 1989). The *inv* locus is encoded by SPI-1, and InvA is supposed to be part of the T3SS-1, which forms a channel through the inner membrane. This channel is necessary for the translocation of structural proteins, for the formation of the syringe-like T3SS, and the delivery of effector proteins to the host cytoplasm (Galán *et al.*, 1992; Hueck, 1998; Suarez and Russmann, 1998; Radtke *et al.*, 2010). Taking these findings into account, a 14028 Δ*invA* mutant was generated and tested with Caco-2 and LS174T cell cultures. Cell invasion of the Δ*invA* mutant strain was significantly attenuated in comparison to the wild type in both cell lines, but intracellular bacteria were still isolated after gentamycin treatment and lysis of the cell cultures (**Fig. 3.13**). The presence of alternative mechanisms for host cell invasion in *S.* Typhimurium have been suggested earlier, and thus the result is in accordance to the data obtained in previous studies (Galán and Curtiss, 1989), which further state that the interruption of *invA* does not influence the attachment of the pathogen to cultured epithelial cells (Galán and Curtiss, 1989).

In order to test whether 1,2-PD or L-Fuc are available and degraded by *S.* Typhimurium in human cell cultures, assays using the reporter strains 14028 Δ*invA* P$_{pduA}$::*lux* and 14028 Δ*invA* P$_{fucO}$::*lux* were performed. The reporter strains were incubated with Caco-2 and mucus secreting LS174T cells (see **Fig. 5.15** and **5.16** in the Appendix). Light emission was detected 210 min post inoculation for the strain 14028 Δ*invA* P$_{fucO}$::*lux* in LS174T cells, but the signal was very weak, and at about the same time (t= 240 min), luminescence was also detected in wells containing this strain incubated with Caco-2 cells. Due to the weak signals occurring in LS174T and Caco-2 cells after 3-4 h, and the fact that signalling of extra- or intracellular bacteria could not be distinguished, it would be rather bold to claim that the gene expression was induced by L-Fuc derived from mucins or the glycocalyx. Proliferation studies, applying 14028 Δ*invA*, Δ*invA*Δ*fucA*::*kan^R*/pBR322, Δ*invA*Δ*fucA*::*kan^R*/pBR-*fucA*, Δ*invA*Δ*pduC*::*kan^R*/pBR322 and Δ*invA*Δ*pduC*::*kan^R*/pBR-*pduC*

on LS174T cells did not result in a significantly attenuated phenotype of the *fucA* or *pduC* deletion mutants in comparison to the complementation strains, and the Δ*invA* deletion strain (see **Fig. 5.17** in the Appendix). In this cell culture assay, the multiplication of extracellular bacteria was analysed in the supernatants. No statements about the intracellular cell count can be made, because no invasion assays were performed after the removal of the supernatants. As mentioned earlier in this thesis, *S.* Typhimurium is not able to cleave the complex glycosidic bonds of mucins in order to release sugar monomers. Thus, it is tempting to speculate that L-Fuc monomers or 1,2-PD were not available to the bacteria in the cell culture assays, due to the lack of the microbiota, which would have been able to release the monosaccharides from the glycan structures (Corfield *et al.*, 1992; Chang *et al.*, 2004). Significantly less *S.* Typhimurium 14028 Nal^R were recovered from LS174T than from Caco-2 cells (**Fig. 3.13**), although the *inv* locus remained intact. In order to determine the influence of mucus on host cell invasion, cell culture assays were performed applying immunofluorescence staining of mucins. To enable the detection of the bacteria under the confocal microscope, the plasmid pGreen-TIR was introduced, which leads to constitutive GFP-expression. It was not anticipated to observe that the mucus layer of HT29-MTX cells was not invaded by *S.* Typhimurium, but rather that of LS174T cells (**Fig. 3.14**). To reinforce this finding, an invasion assay should have been performed, as described above for Caco-2 and LS174T cells, which quantitatively compares the invasiveness of *S.* Typhimurium in HT29-MTX and LS174T cells. Earlier studies have resulted in a more efficient invasion of mucus secreting HT29-MTX cells, compared to Caco-2 cells, which do not secrete mucus (Gagnon *et al.*, 2013). The authors explain this result by the enhanced adhesion to mucins via specific receptors, involving fimbrial adhesins of the pathogen, and mannose residues of the mucins (Gagnon *et al.*, 2013). Mucins secreted by HT29-MTX and LS174T cells are different, because in the main HT29-MTX cells secrete MUC5AC, which is prevalent in the stomach, whereas only low amounts of MUC2 are produced (Nollevaux *et al.*, 2006). Conversely, LS174T cells mainly produce MUC2, which has the highest abundance in the small and large intestine (van Klinken *et al.*, 1996; Moran *et al.*, 2011). It is expected that the affinity of *S.* Typhimurium to MUC2 is higher than to MUC5AC, which would explain the observed penetration of the mucus layer secreted by LS174T cells, whereas that of HT29-MTX cells was not invaded. Pathogens like *S.* Typhimurium are known to coordinate virulence gene expression by environmental cues, like for example osmolarity, and temperature (Bang *et al.*, 2005; Du *et al.*, 2011). It is theorised that mucins also serve as such signals, enabling the pathogen to determine its location within the GIT. To confirm this hypothesis, further studies on this topic are required, which should include the afore mentioned invasion assay comparing HT29-MTX and LS174T cells.

In another series of experiments, the contribution of flagella to the mucus invasion of LS174T cells was investigated. Immunofluorescence staining was used to determine the location of motile and non-motile *S.* Typhimurium 14028 MvP 103 in LS174T cell culture assays (**Section 3.7.3**). Non-motile bacteria were obtained by phage transduction of a transposon-insertion mutation within the *fliGHI* genes (Stecher *et al.*, 2004). Soft-agar swarming assays confirmed the inability of the MvP

103 *fliGHI* mutant to actively move. Consequently, bacteria were transformed with the plasmid pGreen and used in the cell culture assay with LS174T cells. The motile bacteria were located within the mucus layer after 30 min, whereas the non-motile mutant strain remained on top of it (**Fig. 3.15**). These observations are consistent with previous *in vivo* experiments, which describe enhanced fitness of motile *S.* Typhimurium in the gut, due to its possibility of actively moving towards nutrients (Stecher *et al.*, 2008).

4.6 L-Fuc availability in the *C. elegans* glycocalyx

Its short life-span, the ease of culturing, its transparency, genetic tractability, and susceptibility for a variety of mammalian pathogens, including *Salmonella*, makes the nematode *C. elegans* an attractive model organism (Clark and Hodgkin, 2014). The nematodes die after approximately 10 days feeding on *S.* Typhimurium, whereas they survive for about 20 days when they are fed on *E. coli* OP50 (Labrousse *et al.*, 2000; Aballay and Ausubel, 2001; Sem and Rhen, 2012). While the presence of a glycocalyx covering the intestinal epithelium of *C. elegans* has been described, the sugar composition of the glycocalyx has not been further elucidated (McGhee, 2007). Since L-Fuc is a common component of the mucins and glycoconjugates in the human intestine (Muraoka and Zhang, 2011), it has been assumed that it is also a constituent of the *C. elegans* glycoprotein-polysaccharides. To evaluate this hypothesis, *C. elegans* was fed on the *S.* Typhimurium 14028 P$_{fucO}$::*lux* reporter strain and on *E. coli* OP50 as a negative control. In the first experiment, luminescence was detected in the cavities of the microtiter plate, which contained the highest number of nematodes (**Fig. 3.16**), but in the following experiments, this result was not reproducible. Reasons for the failure of follow-up experiments may relate to the detection of an artefact in the first assay, or to the complexity of the host-microbe interactions in such an artificial background. Interaction studies using fluorescence-labelled lectins on fixated nematode tissues are available (Borgonie *et al.*, 1994), and the lectin, referred to as UEA-1 binds to α-L-Fuc. Yolk of *C. elegans* could be stained with UEA-1, indicating the presence of L-Fuc. Further lectins binding D-mannose and D-galactose stained the intestinal brush border of the nematode (Borgonie *et al.*, 1994). Further studies are required to resolve whether L-Fuc residues are part of the glycocalyx in the intestine of *C. elegans*, and whether *S.* Typhimurium feeds on L-Fuc in the nematode's gut. The nematodes could be fed on *S.* Typhimurium deletion strains, which are impaired to use L-Fuc, and on the wild type strain. If both strains constitutively express differential fluorescent proteins, the proliferation of both strains could be monitored during the experiment, because the nematode is transparent. In order to determine the competitive index, the different *S.* Typhimurium genotypes could also be equipped with plasmids, carrying different selection markers. However, the inability of *S.* Typhimurium to cleave complex glycosidic bonds should be kept in mind where such experiments are performed.

4.7 Conclusion and perspectives

In order to obtain a clearer understanding of the regulation of the *pdu* gene expression further experiments are required to resolve the role of the global regulators CRP and ArcA/ArcB, as well as their interaction with the promoter regions of P_{pduA} and P_{pocR} under aerobic and anaerobic conditions. The binding of the transcription factors to the DNA could be studied in more detail by performing electrophoretic mobility shift assays (EMSA) or DNaseI footprinting (Sawers *et al.*, 1997). Such studies would help to explain the regulation and early induction of *pdu* gene expression while other substrates are still available.

To determine whether *S.* Typhimurium feeds on mucus-derived L-Fuc or 1,2-PD, cell culture assays with mucus secreting cell lines and *Salmonella* reporter strains need to be performed that make it possible to determine the localisation of the bacteria as soon as fluorescence is emitted. The deletion of the *inv* locus reduces but does not prohibit host cell invasion. Thus, it is not possible to distinguish whether the fluorescence signal originates from extra- or intracellular bacteria. Because of the difficulty to separate the bacteria replicating intracellularly, extracellularly in the mucus or extracellularly in the medium, the determination of a competitive index between wild type, $\Delta pduC$::kan^R or $\Delta fucA$::kan^R insertion mutants is difficult. The culture medium can be replaced by PBS to force the bacteria to use mucus- or host cell-derived sugars, but after a while the cell culture starts to detach and dies. To elucidate, whether the bacteria are located in the mucus, are attached to the surface of the cells, or have already invaded the cell culture, a method to remove the mucus layer from the cell culture continues to be necessary. Afterwards, the cell culture can be treated with gentamycin to kill the remaining extracellular bacteria, and to determine the count of intracellular bacteria. According to the literature, it is possible to detach the mucus layer from a cell culture by incubating the latter with 10 mM N-acetylcysteine for one hour, under constant agitation (Alemka *et al.*, 2010). This approach was tested in the present study, and the cell culture, which was treated with N-acetylcysteine was used for immunofluorescence staining of mucins. Compared to untreated cells, no difference - such as the lack of the mucus layer - was visible (data not documented). Therefore the use of this technique was discontinued. Studies to find an alternative procedure to separate the mucus layer and the cell culture are needed.

For all cell culture assays it should be kept in mind that *S.* Typhimurium is not capable of cleaving L-Fuc from mucin structures. A pre-digestion of the mucus by a member of the microbiota, like for example *B. thetaiotaomicron* is required. Therefore, it is first necessary to test how *B. thetaiotaomicron* behaves in a cell culture medium and whether it does in fact cleave mucin structures, releasing L-Fuc, or rather feeds on the sugars contained in the cell culture medium. This points to the need for tests whether it is likely to maintain the cell culture and *B. thetaiotaomicron* in PBS, or if another kind of mineral medium to force the bacterium to use mucus-derived sugars is suitable. Where a mineral medium or PBS is used, it is important to make sure that the cell culture does not detach or die during the time of investigation or incubation. GC or GC-MS analysis are suitable methods to investigate whether L-Fuc or 1,2-PD are present in the supernatant of the cell

culture after incubation with *B. thetaiotaomicron*, for example.

To obtain *in vivo* data, experiments with illeal loops of mice should be performed. In light of this thesis, these investigations were planned at the chair Host-Microbe Interactomics at Wageningen UR, The Netherlands, in cooperation with the group of Prof. Jerry M. Wells. Unfortunately, due to unforeseen delays in schedule concerning the availability of mice and the training of the partner's employees to perform illeal loop assays, it was not possible to realise the investigations within the time frame of this thesis. Reporter (P_{pduA}::*gfp*, P_{pduA}::*mCherry*, P_{fucO}::*gfp*, and P_{fucO}::*mCherry*) and kan^R insertion strains ($\Delta pduC$::kan^R, $\Delta fucA$::kan^R) of *S.* Enteritidis were constructed, and it was planned to inject the reporter strains into illeal loops of mice. After different incubation times, the tissue would have been observed under a confocal microscope to examine the transcriptional activity of the respective promoter fusions, and the localisation of the bacteria. In case of promoter signalling in illeal loops, *S.* Enteritidis $\Delta pduC$::kan^R and *S.* Enteritidis $\Delta fucA$::kan^R mutant strains would have been orally administered to mice, together with a *S.* Enteritidis wild type strain. At different days post infection, mice would have been sacrificed and the gut contents would have been recovered. This would have enabled a total count of *Salmonella*, and an investigation into the proportion of mutant strains in the samples. The results might have helped in answering the question whether the respective metabolic pathway is beneficial to *S.* Typhimurium *in vivo*. Similarly, this would also have allowed for an investigation into the liver and spleen of the animals for the general presence of *Salmonella*, as well as the share of kan^R insertion mutants. To recover *Salmonella* from stool and tissue samples, selective media could have been used, such as Brilliance *Salmonella* agar (Oxoid), for example.

The ubiquitous occurrence of the *pdu* gene cluster in *S. enterica* serovars indicates its relevance for the organism, however to date the actual role of this metabolic pathway remains elusive. *In vivo* and *in vitro* assays, using deletion mutants of the *pdu* genes, suggest a contribution to *S.* Typhimurium virulence (Conner *et al.*, 1998; Heithoff *et al.*, 1999; Klumpp and Fuchs, 2007; Harvey *et al.*, 2011). A database which provides information about the fitness of *S.* Typhimurium SL1344 transposon-insertion mutants in mice, chickens, pigs and calves is available (Chaudhuri *et al.*, 2013). According to the database, within the *pdu* gene cluster only the interruption of *pocR, pduF* and *pduC* led to a reduced fitness score in chickens, pigs and calves. An interruption of *pduK*, and the region between *pduQ* and *pduS* resulted in increased fitness levels in pigs. Transposon-insertions within the *ttr* and *eut* genes did not seem to affect the fitness of the bacteria in a positive or negative manner (Chaudhuri *et al.*, 2013). For the *fuc* genes, transposon-insertion mutants of *fucP* and *fucA* resulted in a decreased fitness level (Chaudhuri *et al.*, 2013), and thus support the finding that the utilisation of L-Fuc plays a role during proliferation in the inflamed intestine (Deatherage Kaiser *et al.*, 2013). Further, a disadvantage in colonisation of *S.* Typhimurium *fucI* mutants within *B. thetaiotaomicron* mono-associated mice has been described recently (Ng *et al.*, 2013). But the question that arises here is whether the 1,2-PD metabolism could also be critical for life outside a host. That is, could the contribution to virulence be a mere side effect, with the main reason for *Salmonella* maintaining this huge gene cluster, also requiring the conservation of the

cob/cbi and *ttr* clusters, in fact being due to a benefit to survive in the environment or groceries? The upregulation of the *fuc*, *rha*, *eut*, *pdu* and *cob/cbi* gene expression had been reported for *S.* Typhimurium SL1344 growing in soft rot lesions of cilantro and lettuce, caused by a plant pathogen (Goudeau *et al.*, 2013). The comparison of the transcription of genes in murine macrophages (RAW 264.7) and in the early stationary phase, however, revealed that the expression of *pocR*, the *pdu* genes, *fucR*, *eutMDTQPS* and the majority of the *cbi* genes were downregulated in *S.* Typhimurium 4/74 (Srikumar *et al.*, 2015). Gene expression of *ttrRS* was found to be upregulated, and slightly increased numbers of transcripts were found for *eutRKLCBAHGJEN, fucO, fucA, fucPIK, fucU, ttrBCA* and *cobUST* (Srikumar *et al.*, 2015). Where vitamin B_{12} precursors are available in murine macrophages, ethanolamine utilisation could be possible. To shed light upon the question in which environment 1,2-PD serves as a source of carbon and energy for *S.* Typhimurium, further studies are required, involving experiments in animals such as mice or even pigs or cattle, as well as the investigation of *Salmonella* metabolism in food. In this context, it is suggested that transcriptome analyses in animals receiving different diets are conducted, which could include further studies on L-Fuc availability. Certainly if indications on the compounds that are available in the respective substrate are to be obtained, then these would require the use of GC-MS analyses, for example.

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5 Appendix

5.1 Supplier information

Information about suppliers of chemicals, enzymes, antibodies and other consumables are listed below.

Table 5.1: Supplier information of chemicals, buffers and solutions.

Supplier	Chemical
Affymetrix USB	Chloramphenicol
Ambion	TRIzol reagent
AppliChem	L-fucose
	Cyanocobalamin
	Spectinomycin
Baker	Ethanol
BD	Bacto^{TM}-Casamino acids
	Bacto^{TM}-Peptone
Biotium	GelRed^{TM}
Biozym	LE agarose
Fluka	Sodium selenite
	Nalidixic acid
Merck	Aniline blue
	Magnesium sulphate heptahydrate
	Calcium chloride
	Iron-II-sulphate heptahydrate
	Manganese sulphate monohydrate
	Copper sulphate pentahydrate
	Cobalt chloride hexahydrate
	D-glucose monohydrate
	TOS-propionate agar base
	MUP selective supplement
	L-cysteine
	Peptone of casein
	Trypan blue

Supplier	Chemical
	Dulbecco's phosphate buffered saline without (w/o) $CaCl_2$ and $MgCl_2$
	RPMI 1640 with (w) stable glutamine
	Trypsin
	Non-essential amino acids
	Fetal calf serum (FCS)
	Gentamycin sulphate
Oxoid	Yeast extract
	Brilliance *Salmonella* agar base
	Salmonella selective supplement
	Agar
Roth	Ampicillin sodium salt
	Diethylpyrocarbonate (DEPC)
	Di-potassium hydrogen phosphate
	Zinc sulphate
	Sodium ammonium hydrogen phosphate tetrahydrate
	Roti$^{®}$ aqua P/C/I
	2-propanol
	Chloroform
	Sodium chloride
	Dimethyl sulfoxide (DMSO)
	Kanamycin sulphate
Serva	High strength agar
Sigma-Aldrich	Alizarin yellow GG
	Nystatin solution
	Sodium molybdate
	nickel sulphate heptahydrate
	1,2-propanediol
	Sodium tetrathionate dihydrate
	Dulbecco's phosphate buffered saline w/o $CaCl_2$ and $MgCl_2$
	Goat serum
	Streptomycin
	Tetracycline hydrochloride
Thermo Scientific	GeneRuler^{TM} DNA Ladder Mix
	6 x Loading Dye
VWR	Triton X-100

Table 5.2: Supplier information for cell culture material.

Label	Order number	Supplier
RPMI 1640 w stable glutamin	FG 1215	Merck
PBS Dulbeccos w/o Ca^{2+}, Mg^{2+}	L 1825	Merck
Non-essential amino acids	K 0293	Merck
2.5 % ($\frac{w}{v}$) trypsin	15090-046	Life Technologies
RPMI 1640 w/o glucose	11879-020	Life Technologies
Foetal calf serum	S 0115	Merck
Trypan blue 0.5 % ($\frac{w}{v}$)	L 6323	Merck
Cell culture plastic		
6 well plates	90026	TPP
24 well plates	92424	TPP
Bottles 25 cm^2	92406	TPP
Bottles 75 cm^2	90076	TPP
Bottles 175 cm^2	83.3912.002	Sarstedt
μ-Slide 8 well collagen IV coated	80822	ibidi
μ-Slide 8 well collagen ibiTreat	80826	ibidi
Serological pipettes		
50 ml	94550	TPP
25 ml	760180	Greiner bio-one
10 ml	607180	Greiner bio-one
5 ml	606180	Greiner bio-one
Spatulas 24 cm	99002	TPP
Spatulas 30 cm	99003	TPP

Table 5.3: Supplier information about enzymes and antibodies used in the study.

Enzyme	Supplier
*Ahd*I	Thermo Scientific
*Ase*I	Thermo Scientific
*Kpn*I	Thermo Scientific
*Not*I	Thermo Scientific
*Pvu*I	Thermo Scientific
*Sac*I	Thermo Scientific
*Sma*I	Thermo Scientific
Taq DNA polymerase	In house isolation (Schauer, 2010)
DNaseI	Thermo Scientific
RNaseA	Sigma-Aldrich
T4 DNA ligase	Thermo Scientific
Proteinase K	Roche
Lysozym	Sigma-Aldrich
qScript^{TM} cDNA SuperMix	Quanta
Antibody	**Supplier**
Anti-MUC2 (produced in rabbit)	Sigma-Aldrich (HPA006197)
Anti-MUC5 (produced in mouse)	Sigma-Aldrich (WH0004586M7)
Goat-anti-mouse Alexa Fluor® 488	Invitrogen (A-10667)
Goat-anti-rabbit Alexa Fluor® 488	Invitrogen (A-11001)

5.2 List of bacterial strains

Table 5.4: Bacterial strains used in the study.

Strain	Relevant genotype or characteristics	Reference
S. Typhimurium		
14028	Wild type	ATCC
14028 Nal^R	Spontaneous mutant of 14028 wt on nalidixic acid	This study
14028 P_{pduA}::*lux*	Genomic integration of pUTs-*lux* (Cm^R) behind P_{pduA} via homologous recombination in Nal^R background	This study
14028 P_{fucO}::*lux*	As above behind P_{fucO}	This study
14028 P_{cspA}::*lux*	As above behind P_{cspA}	This study
14028 *invJ*::*lux* d	As above behind *invJ*	This study
14028 P_{pduA}::*gfp*	Genomic integration of pUTs-*gfp* (Cm^R) behind P_{pduA} via homologous recombination in Nal^R background	This study
14028 Δ*invA*	Deletion of *invA* in Nal^R background	This study
14028 Δ*pduC*	Deletion of *pduC* in Nal^R background	This study
14028 Δ*invA*Δ*pduC*::kan^R	As above in Δ*invA* (Nal^R) background	This study
14028 Δ*fucA*::kan^R	Kanamycin (kan^R) insertion in place of *fucA*	This study

Strain	Relevant genotype or characteristics	Reference
14028 $\Delta invA\Delta fucA$::kan^R	As above in $\Delta invA$ (Nal^R) background	This study
14028 $\Delta invA\Delta pduC$::kan^R	As above in $\Delta invA$ (Nal^R) background	This study
14028 MvP 101	$sseD$::$aphT$, Kan^R	Medina *et al.* (1999)
14024 MvP 103	$sseC$::$aphT$, Kan^R	Medina *et al.* (1999)
14024 MvP 103 $\Delta fliGHI$	$sseC$::$aphT$, Kan^R, $\Delta fliGHI$::Tn*10*, Tet^R	This study
SL1344 M913	$\Delta fliGHI$::Tn*10*, Strept^R, Tet^R	Stecher *et al.* (2008)

E. coli

Strain	Relevant genotype or characteristics	Reference
S17.1 λpir	λ-*pir* lysogen of S17.1 (Tp^{R}Strept^{R}Spec^R *thi pro hsd*R$^-$M$^+$ *recA* RP4::2-Tc::Mu-Km::Tn17)	Simon *et al.* (1983)
OP50		CGC (*Caenorhabditis* Genetics Center)

Other bacteria

Strain	Relevant genotype or characteristics	Reference
Bifidobacterium infantis		G-No. 1401, WG Dr. M. Wenning
S. Enteritidis HMI-1241		WUR, Wageningen, Prof. Dr. J. M. Wells

Strain	Relevant genotype or character-istics	Reference
S. Enteritidis $\Delta pduC$::kan^R	Kanamycin (kan^R) insertion in place of *pduC* in *S.* Enteritidis HMI-1241	This study
S. Enteritidis $\Delta fucA$::kan^R	As above in place of *fucA*	This study
S. Enteritidis P$_{pduA}$::$mCherry$	Genomic integration of pUTs-*mCherry* (Cm^R) behind P$_{pduA}$ via homologous recombination	This study
S. Enteritidis P$_{fucO}$::$mCherry$	As above behind P$_{fucO}$	This study

5.3 List of primers

Table 5.5: List of primers.

Internal label	Sequence 5'- 3'	Target
Primers for RT-PCR		
24_*pduA*_RT_F	CAGCCAATGTGATGTTAGTG	*pduA*
25_*pduA*_RT_R	TTCTACATCGGTGTGAGG	*pduA*
26_*pduAB*_RT_F	CCTCACACCGATGTAGAA	*pduAB*
27_*pduAB*_RT_R	GACAAATTCCGTTAAACTGC	*pduAB*
28_*pduBC*_RT_F	CACCAGCTTTAGTAACGAAGC	*pduBC*
29_*pduBC*_RT_R	CAAAGCCGTCCTGATTCAC	*pduBC*
32_*pduCD*_RT_F	CGCTGGGAAGAGATTAAAAAC	*pduCD*
33_*pduCD*_RT_R	CGACGGCGATAATGACTTC	*pduCD*
34_*pduDE*_RT_F	GAAGTACCAGGCAAAGTCG	*pduDE*
35_*pduDE*_RT_R	GTAGTCGCTGACCTTTGC	*pduDE*
48_*pduEG*_RT_F	GTATCGTTCAACGAAAGAAG	*pduEG*
49_*pduEG*_RT_R	CTGTGTGTAATCGTCAGTG	*pduEG*
50_*pduGH*_RT_F	CGGGCGAGGAAATATTCG	*pduGH*
51_*pduGH*_RT_R	GATGCTGAAGCAGGAAAGG	*pduGH*
52_*pduHJ*_RT_F	CGCAACACCGGTAATAACG	*pduHJ*

Internal label	Sequence 5'- 3'	Target
53_*pduHJ*_RT_R	GTACGTTGGCGGATTTAACC	*pduHJ*
54_*pduJK*_RT_F	GATGTTGAGGCCATTTTACC	*pduJK*
55_*pduJK*_RT_R	CAGGCCATTCCACTGTTC	*pduJK*
56_*pduKL*_RT_F	GAATCAGAACTGGTCAGCTG	*pduKL*
57_*pduKL*_RT_R	CAGCCGCTCATAATCCTG	*pduKL*
58_*pduLM*_RT_F	CACATTGATACCGATGAAGC	*pduLM*
59_*pduLM*_RT_R	GCTGACAGAACAGTGCTG	*pduLM*
60_*pduMN*_RT_F	GCAAATGCATCGTGACCG	*pduMN*
61_*pduMN*_RT_R	CCGCTGAGCAAAACCAGTTC	*pduMN*
62_*pduNO*_RT_F	CAAATGAGGCCATTGACCTC	*pduNO*
63_*pduNO*_RT_R	CTTCCAGGGCCGAAATCTC	*pduNO*
64_*pduOP*_RT_F	GCGACGGCATATTAATTGGG	*pduOP*
65_*pduOP*_RT_R	CATCGCGCTGATAATGGC	*pduOP*
66_*pduPQ*_RT_F	CACGCTGCAAACCTCGATATTC	*pduPQ*
67_*pduPQ*_RT_R	CTGAACCACTGTGTGGATG	*pduPQ*
68_*pduQS*_RT_F	CAATGATATCGCGGCGATC	*pduQS*
69_*pduQS*_RT_R	CTTTGAGCATCGGTTCACATTC	*pduQS*
70_*pduST*_RT_F	CAGCATATTGGTGCCAGC	*pduST*
71_*pduST*_RT_R	TTCCCCGGACAGATGGTC	*pduST*
72_*pduTU*_RT_F	CAATAACGCCGTGACGGTTG	*pduTU*
73_*pduTU*_RT_R	GGCACCGGTAAAGCGATC	*pduTU*
74_*pduUV*_RT_F	GTTTCTGCCGTCGAGTATGC	*pduUV*
75_*pduUV*_RT_R	CCGCAACCAGCGAAATACG	*pduUV*
76_*pduVW*_RT_F	GCGCACAGCAGATTTTTATTACC	*pduVW*
77_*pduVW*_RT_R	CAATGTCCCGCAGACTGTTAATG	*pduVW*
78_*pduWX*_RT_F	CATTTATCCAGACGGAGAACG	*pduWX*
79_*pduWX*_RT_R	CACATCGACGCGAATCTCG	*pduWX*

Deletion primerts

1_*invA*_del_F	GTCGTACTATTGAAAAGCTGTCTT AATTTAATATTAACAGGATACCTA TA<u>GTGTAGGCTGGAGCTGCTTC</u>	*invA*
2_*invA*_del_R	TAATTCAGCGATATCCAAATGTTG CATAGATCTTTTCCTTAATTAAGC CC<u>CATATGAATATCCTCCTTA</u>	*invA*
5_*invA*_conf_F	GCCATACCCTCTTCACTC	*invA*
6_*invA*_conf_R	GATACAGATACTTGGCAATGC	*invA*

Internal label	Sequence 5'- 3'	Target
40_*pduC*_del_F	GAAGCACTGGCGAAACGCCCTGTG AATCAGGACGGCTTTGTTAAGGAG TG<u>GTGTAGGCTGGAGCTGCTTC</u>	*pduC*
41_*pduC*_del_R	GTTTTTAATCTCTTCCCAGCGTTC TCCCTGCAGGCGATAACCTGTTGC CG<u>CATATGAATATCCTCCTTA</u>	*pduC*
16_*pduC*_conf_F	CTTCCGTCGGCGTACTGATG	*pduC*
17_*pduC*_conf_R	CGCCTTGATGCCTTCTTCTTC	*pduC*
90_*fucA*_del_F	GCGCGGCAGATTATTGATACCTGT CTGGAAATGACGCGGTTAGGGTTA AA<u>GTGTAGGCTGGAGCTGCTTC</u>	*fucA*
91_*fucA*_del_R	CTCCAGTACGATAGCGATCGCCTC GTCATCCAGTACCGGTACCGGATC GA<u>CATATGAATATCCTCCTTA</u>	*fucA*
92_*fucA*_conf_F	AATTATTGCCACGAAAACGG	*fucA*
93_*fucA*_conf_R	TGTCACTTTATCGACCACAC	*fucA*
2043_*kan*_R	CGATGCCTGCTTGCCG	*kan*

Cloning of pUTs-fusions

Internal label	Sequence 5'- 3'	Target
14_P*fucO*_*Sac*I_F	GAG<u>GAGCTC</u>TGTCTTTATTGATGCT GACGG	P*fucO*
15_P*fucO*_*Sma*I_R	GAG<u>CCCGGG</u>CGCCTGTCTCCTGAC	P*fucO*
18_*invJ*_*Sac*I_F	GAG<u>GAGCTC</u>GATAACGCCACGGG AATCTC	*invJ*
19_*invJ*_*Kpn*I_R	GAG<u>GGTACC</u>GTCTCACACGCAAT GA- CATC	*invJ*
22_P*pduA*_*Sac*I_F	GAG<u>GAGCTC</u>CACCTTCTGAGCAA AGTTCG	P*pduA*
23_P*pduA*_*Kpn*I_R	GAG<u>GGTACC</u>GTTGGGACTATAAGAA GATGC	P*pduA*
36_*invJ*_conf_F	GTTAGAAAGCGTAAGCGG	*invJ*
37_P*pduA*_conf_F	CGCTCAAACAACCGATACC	*pduF*
38_P*fucO*_conf_F	GGCGGATTGCTGATTACG	*fucA*
103_P*pduA*_*Not*I_R	ATAAGAAT<u>GCGGCCGC</u>GTTGGGAC TATAAGAAGATGC	P*pduA*
105_P*pduA*_*Not*I_F	ATAAGAAT<u>GCGGCCGC</u>CACCTTCT GAGCAAAGTTCG	P*pduA*
3545_*luxC59*_R	GATTGCACTAAATCATCACTTTCGG	*luxC*(pUTs)

Internal label	Sequence 5'- 3'	Target
3123_*luxC1*_R	CATAAGGCAATATTTGCTC	*luxC*(pUTs)
3696_pUTs-test_F	TGGAATTCTGACTCTTATAC	pUTs
3697_pUTs-test_R	CAGGTATTTATTCGGCGC	pUTs

Cloning of pBR322 complementation plasmids

Internal label	Sequence 5'- 3'	Target
96_*fucA*_compl_F _*Pst*I	AA<u>CTGCAG</u>ATCCTCATCGGTTC AGAAG	P$_{fucA}$+*fucA*
97_*fucA*_compl_R _*Ahd*I	GAG<u>GACTCCCCGTC</u>AATCATTCT GTTCGCCATC	*fucA*
98_pBR_Amp_F	ACCAGTCACAGAAAGCATC	pBR-Amp^R
99_pBR_Amp_R	CAGTTACCTTCGGAAAAAGA	pBR-Amp^R
83_Lig_*pduC*::pBR_R	GTTAAGGGATTTTGGTCATG	pBR
16_*pduC*_conf_F	CTTCCGTCGGCGTACTGATG	*pduC*
17_*pduC*_conf_R	CGCCTTGATGCCTTCTTCTTC	*pduC*

5.4 Plasmids and vectors

The following plasmids and vectors have been used or constructed in this study. The enzymes required for cloning were purchased from Thermo Scientific (Braunschweig, DE).

Table 5.6: List of plasmids and vectors.

Plasmid	Relevant genotype or characteristics	Reference
pUTs-*lux* (Cm^R)	Suicide vector, promoter-less *luxCDABE* genes, R6K *ori*, *oriT* (RP4), Cm^R	Starke *et al.* (2013)
pUTs-*gfp* (Cm^R)	Suicide vector, R6K *ori*, *oriT* (RP4), Cm^R	Starke *et al.* (2013)
pUTs-*mCherry* (Cm^R)	Suicide vector, R6K *ori*, *oriT* (RP4), Cm^R	Starke *et al.* (2013)
pKD46	Lambda Red recombinase expression plasmid, temperature sensitive replication *oriR*101/ *repA*101(ts), arabinose inducible promoter P_{araB}, lambda Red genes (P_{araB}-*gam-bet-exo*), Amp^R	Datsenko and Wanner (2000)
pKD4	*pir*-dependent, FRT-recognition sites, Kan^R	Datsenko and Wanner (2000)
pCP20	FLP-recombinase plasmid, Cm^R, Amp^R	Datsenko and Wanner (2000)
pBR322	Cloning vector, Amp^R, Tet^R	Bolivar *et al.* (1977)
pGreen TIR	GFP-cloning vector, translation initiation region (TIR), P_{lac}-TIR-*gfp*, Amp^R	Miller and Lindow (1997)
pUTs-P_{pduA}::*lux* (Cm^R)	Cloning of promoter region of *pduA* infront of *luxCDABE* using *Sac*I and *Kpn*I, Cm^R	This study
pUTs-P_{fucO}::*lux* (Cm^R)	Cloning of promoter region of *fucO* infront of *luxCDABE* using *Sac*I and *Sma*I, Cm^R	This study
pUTs-P_{pduA}::*gfp* (Cm^R)	Cloning of promoter region of *pduA* infront of *gfp* using *Not*I, Cm^R	This study
pUTs-P_{fucO}::*gfp* (Cm^R)	Cloning of promoter region of *fucO* infront of *gfp* using *Not*I, Cm^R	This study
pUTs-P_{pduA}::*mCherry* (Cm^R)	Cloning of promoter region of *pduA* infront of *mCherry* using *Sac*I and *Kpn*I, Cm^R	This study

Plasmid	Relevant genotype or characteristics	Reference
pUTs-P$_{fucO}$::*mCherry* (Cm^R)	Cloning of promoter region of *fucO* infront of *mCherry* using *Sac*I and *Sma*I, Cm^R	This study
pBR322-*pduC*	Complementation plasmid for $\Delta pduC$ deletion strain,*Pvu*I and *Ase*I used for cloning, Tet^R	This study
pBR322-*fucA*	Complementation plasmid for *fucA::kan* insertion mutant,*Pst*I and *Ahd*I used for cloning, Tet^R	This study

Maps of plasmids used for cloning are illustrated on the next pages. Positions and orientations of genes are shown as block arrows while features, like promoters or mobility regions, are indicated as boxes on the vector-backbone.

Hin dIII
Sph I
Sbf I
Pst I
Bsp MI
Sal I
Xba I
Bam HI
Ava I
Bme T110I
Sma I
Xma I
Eco ICRI
Sac I

Plac

ori

TIR

lacZ-GFP fusion

pGreenTIR

3389 bps

Amp
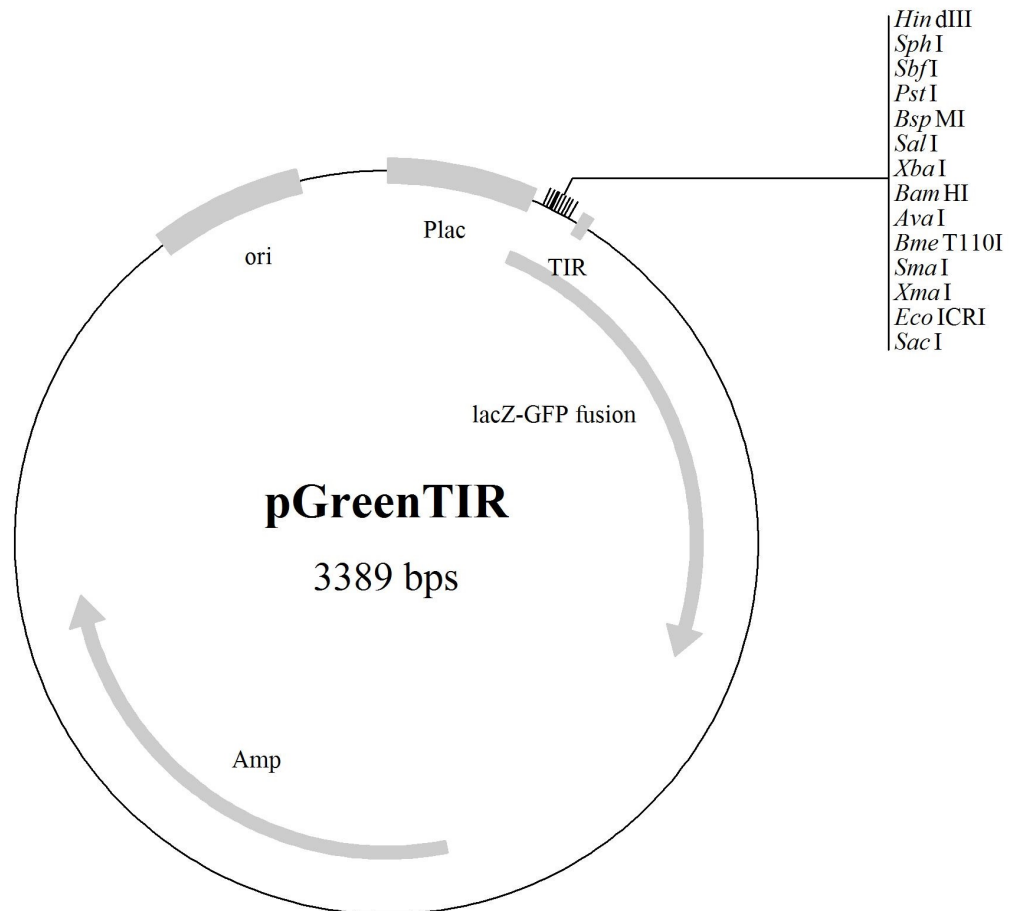
Figure 5.1: pGreen-TIR (Miller and Lindow, 1997) was used to localise *S.* Typhimurium during confocal microscopy. Bacteria carrying the plasmid are constitutively expressing GFP.

Start	End	Label	Description
2	216	P_{lac}	*lac*-promoter
217	1050	*lacZ*-GFP fusion	coding sequence of *lacZ::gfp* fusion protein
291	307	TIR	translation initiation region
1589	2449	Amp	β-lactamase
3042	3261	*ori*	origin of replication
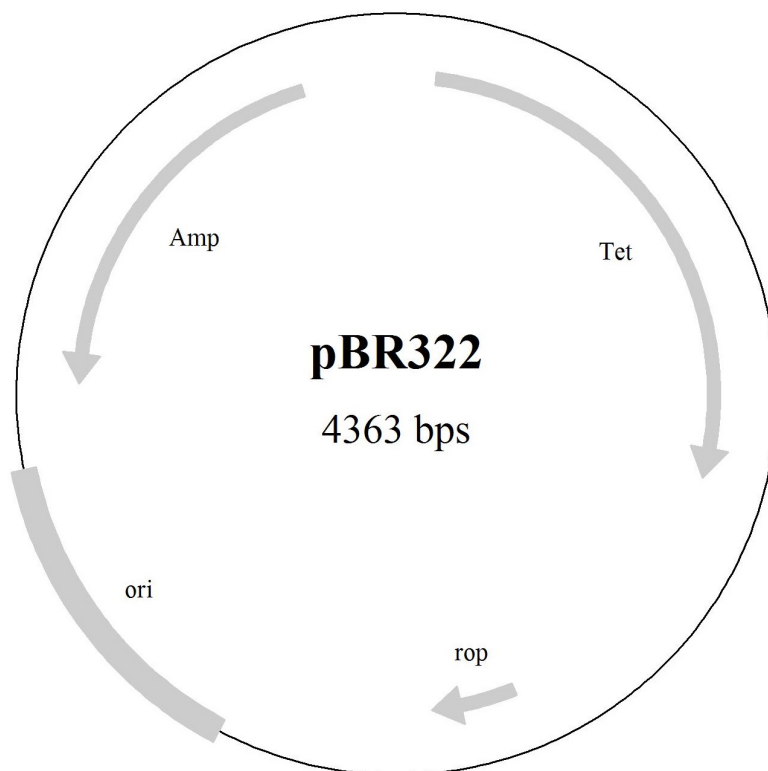
Figure 5.2: For complementation of deleted or interrupted genes the respective coding sequence was cloned into vector pBR322 (Bolivar *et al.*, 1977).

Start	End	Label	Description
86	1276	Tet	tetracycline resistance gene
1918	2105	*rop*	repressor of primer
			maintains low copy number
3133	2519	*ori*	origin of replication (pMB1)
4155	3295	Amp	β-lactamase
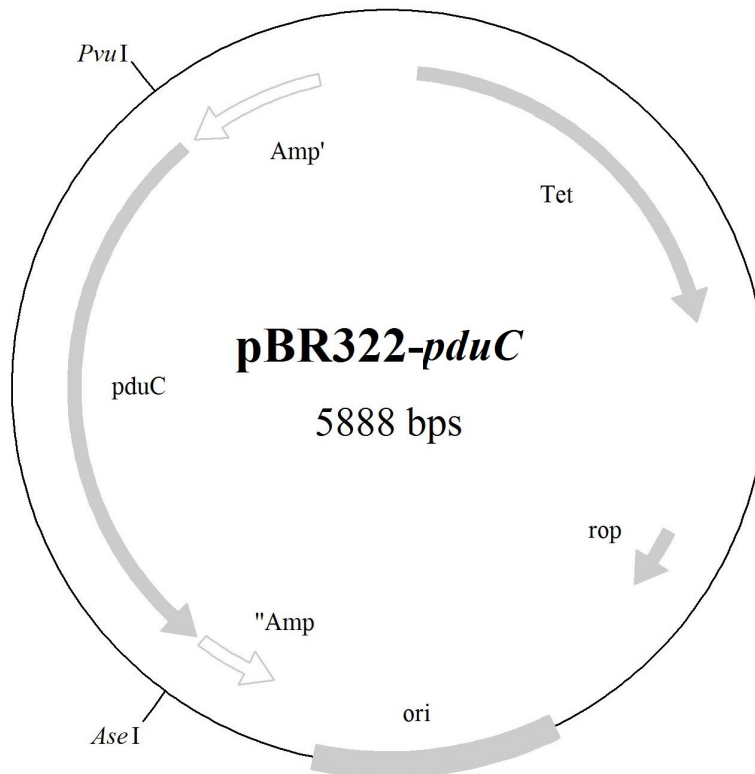
Figure 5.3: Complementation plasmid for *S.* Typhimurium Δ*pduC*.

Start	End	Label	Description
86	1276	Tet	tetracycline resistance gene
1918	2105	*rop*	repressor of primer
			maintains low copy number
3133	2519	*ori*	origin of replication (pMB1)
3540	3295	”Amp	3'-end of interrupted β-lactamase
5525	3561	*pduC*	coding sequence *pduC* of *S.* Typhimurium
5680	5264	'Amp	5'-end of interrupted β-lactamase
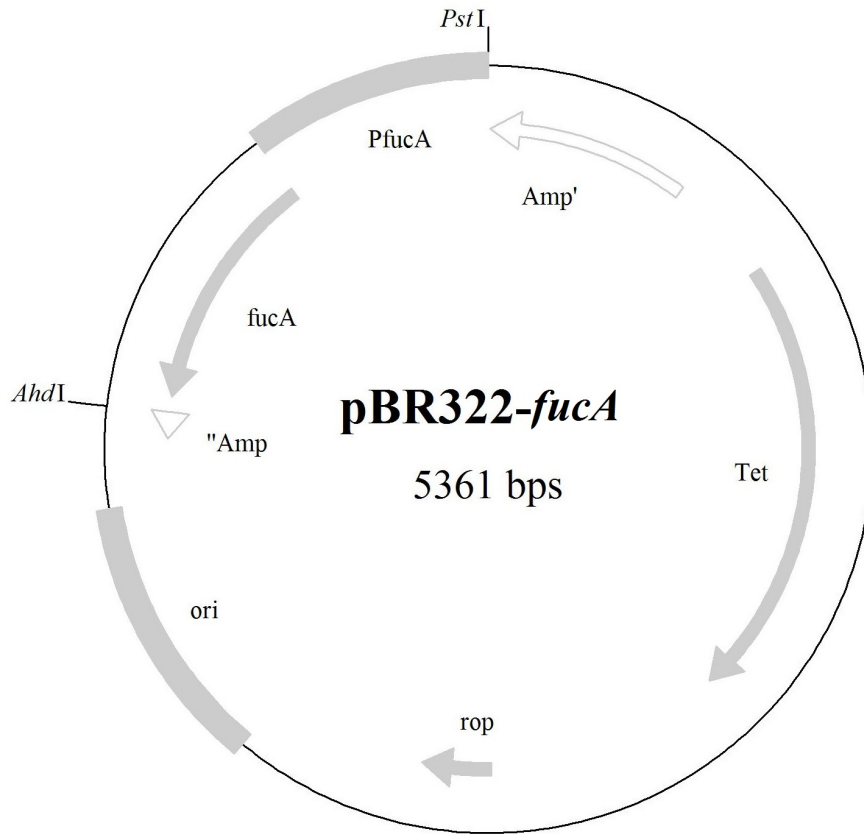
Figure 5.4: Complementation plasmid for *S.* Typhimurium Δ*fucA*::*kan^R*.

Start	End	Label	Description
840	2030	Tet	tetracycline resistance gene
2672	2859	*rop*	repressor of primer
			maintains low copy number
3887	3273	*ori*	origin of replication (pMB1)
4120	4049	”Amp	3'-end of interrupted β-lactamase
4809	4162	*fucA*	coding sequence *fucA* of *S.* Typhimurium
546	5	'Amp	5'-end of interrupted β-lactamase
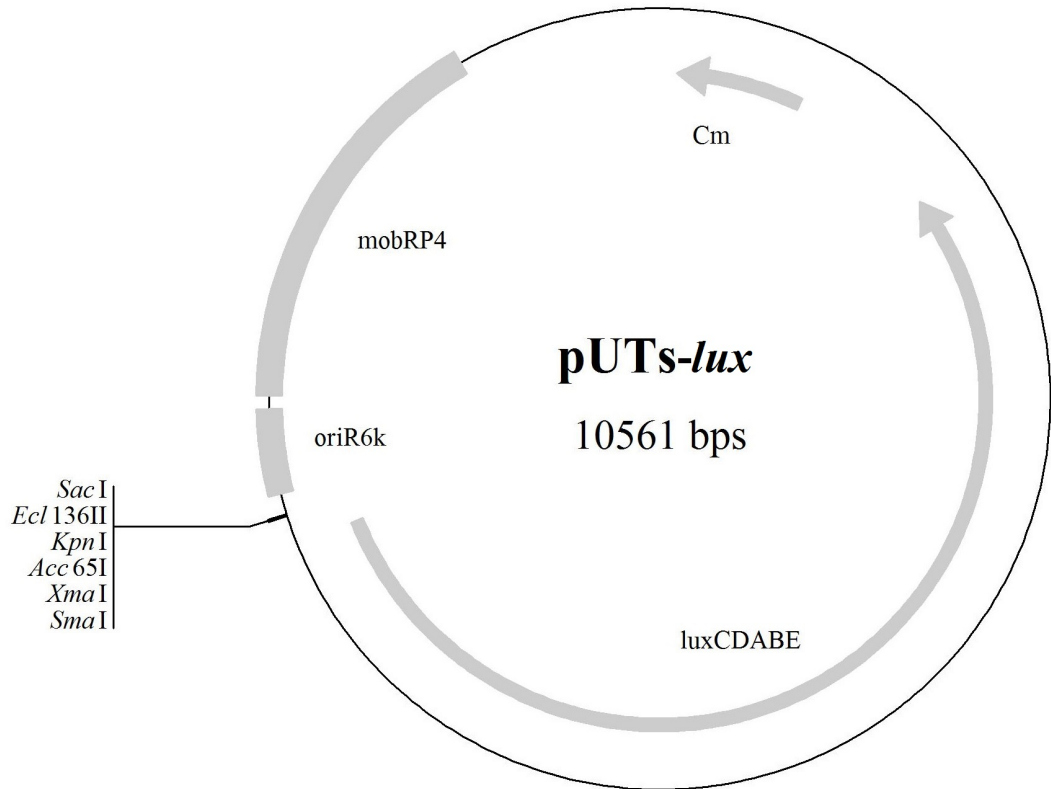
Figure 5.5: pUTs-*lux* (Starke *et al.*, 2013) was used to create *lux*-promoter fusions in *S.* Typhimurium. Via homologous recombination the plasmid is integrated into the genome. Resulting reporter strains were used to monitor gene expression.

Start	End	Label	Description
746	87	Cm	chloramphenicol resistance gene
7280	1548	*luxCDABE*	promoter-less luciferase operon
7877	7508	*ori*R6k	origin of replication
9662	7935	*mob*RP4	mobility region
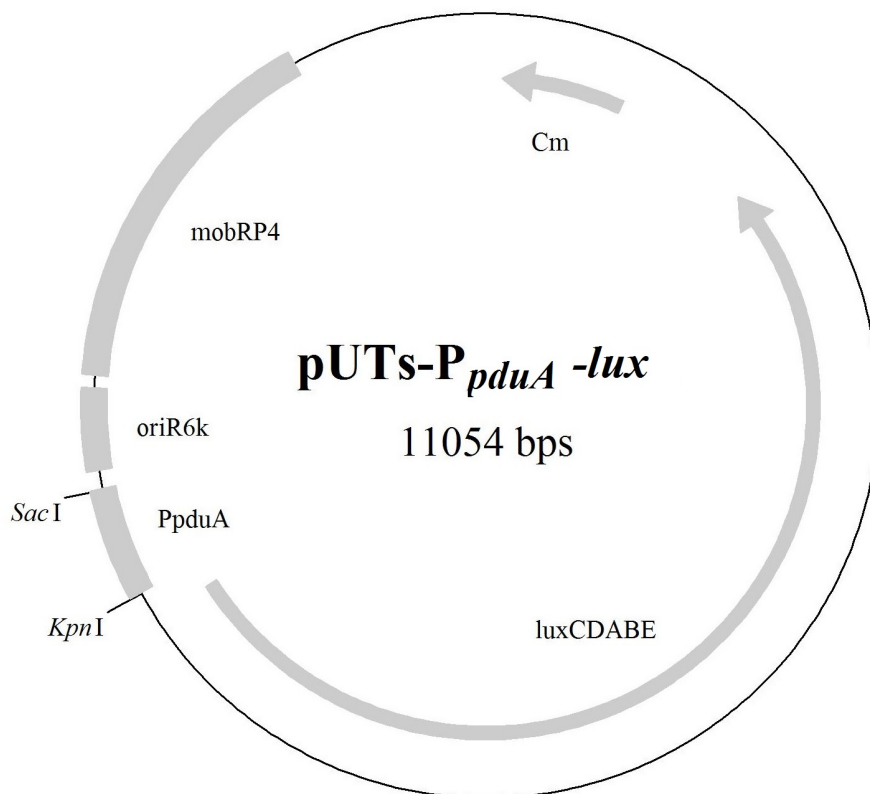
Figure 5.6: To generate the strain 14028 Nal^R P$_{pduA}$::*lux* the promoter region of *pduA* (*S.* Typhimurium LT2) was cloned (*Sac*I/ *Kpn*I) into pUTs-*lux*. The resulting plasmid was named pUTs-P$_{pduA}$-*lux*.

Start	End	Label	Description
746	87	Cm	chloramphenicol resistance gene
7280	1548	*luxCDABE*	promoter-less luciferase operon
7915	7417	P$_{pduA}$	promoter region of *pduA* (*S.* Typhimurium)
8370	8001	*ori*R6k	origin of replication
10155	8428	*mob*RP4	mobility region
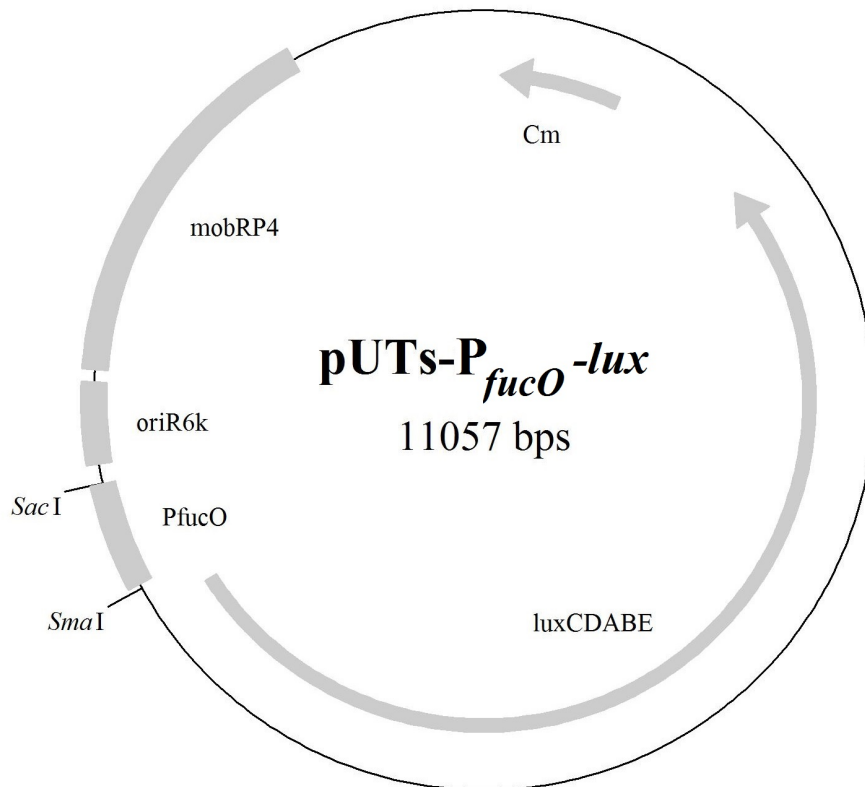
Figure 5.7: To generate the strain 14028 Nal^R P$_{fucO}$::*lux* the promoter region of *fucO* (*S.* Typhimurium LT2) was cloned (*Sac*I/ *Sma*I) into pUTs-*lux*. The resulting plasmid was named pUTs-P$_{fucO}$-*lux*.

Start	End	Label	Description
746	87	Cm	chloramphenicol resistance gene
7280	1548	*luxCDABE*	promoter-less luciferase operon
7912	7430	P$_{fucO}$	promoter region of *fucO* (*S.* Typhimurium)
8373	8004	*ori*R6k	origin of replication
10158	8431	*mob*RP4	mobility region
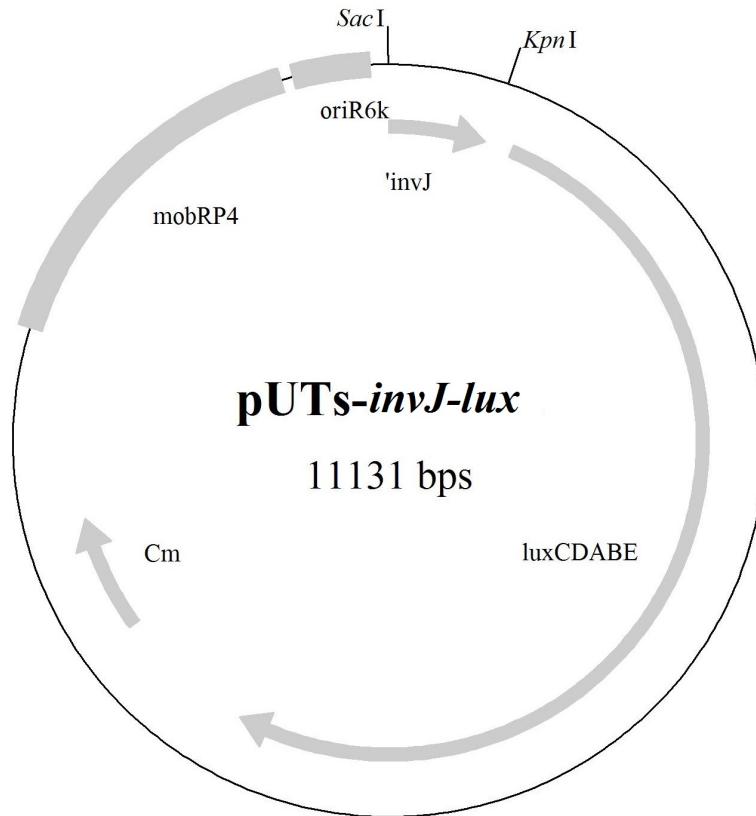
Figure 5.8: To generate the strain 14028 Nal^R *invJ*::*lux* the 3'-region of *invJ* (*S.* Typhimurium LT2) was cloned (*Sac*I/ *Kpn*I) into pUTs-*lux*. The resulting plasmid was named pUTs-*invJ-lux*.

Start	End	Label	Description
7243	7902	Cm	chloramphenicol resistance gene
709	6441	*luxCDABE*	promoter-less luciferase operon
2	553	'*invJ*	3'-region of *invJ* (*S.* Typhimurium)
10673	11042	*ori*R6k	origin of replication
8888	10615	*mob*RP4	mobility region

			Eco RI
			Ecl 136II
			Sac I
			Kpn I
			Ecl 136II
			Sac I
			Eco RI
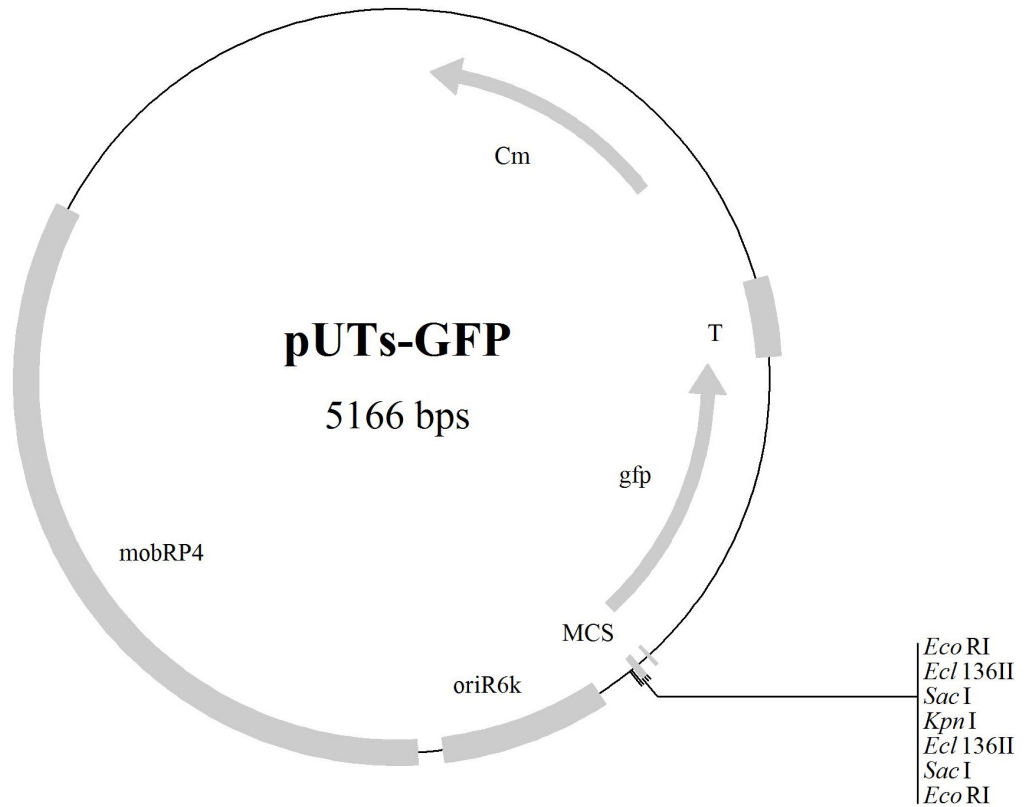
Figure 5.9: In order to monitor gene expression of bacteria under the fluorescence microscope promoter fusions were generated by introducing promoter sequences to the plasmid pUTs-*gfp* (Starke, 2015).

Start	End	Label	Description
746	87	Cm	chloramphenicol resistance gene
1237	1065	T	T1 *rrnB* terminator
1963	1247	*gfp*	promoter-less *gfp* coding sequence
2017	2005	MCS	multiple cloning site
2482	2113	*ori*R6k	origin of replication
4267	2540	*mob*RP4	mobility region
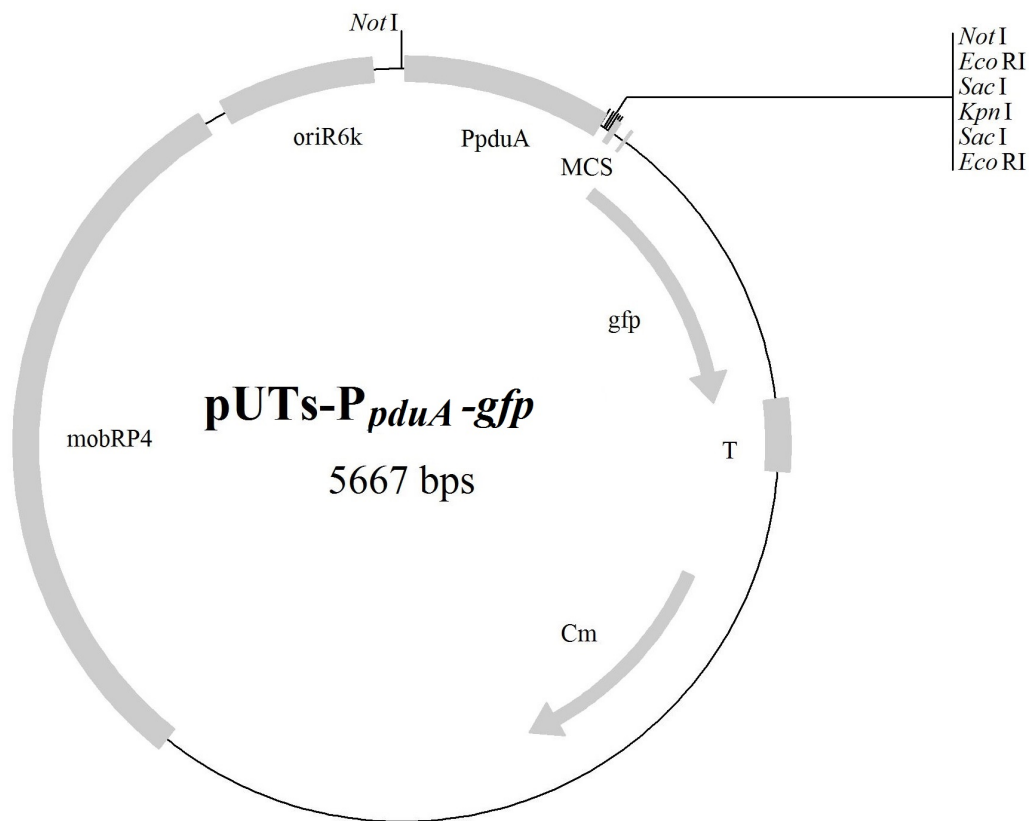
Figure 5.10: To generate the strain 14028 Nal^R P$_{pduA}$::*gfp* the promoter region of *pduA* (*S.* Typhimurium LT2) was cloned (*Not*I) into pUTs-*gfp*.

Start	End	Label	Description
1798	2457	Cm	chloramphenicol resistance gene
1307	1479	T	T1 *rrnB* terminator
7	499	P$_{pduA}$	promoter region of *pduA* (*S.* Typhimurium)
581	1297	*gfp*	promoter-less *gfp* coding sequence
527	539	MCS	multiple cloning site
5228	5597	*ori*R6k	origin of replication
3443	5170	*mob*RP4	mobility region
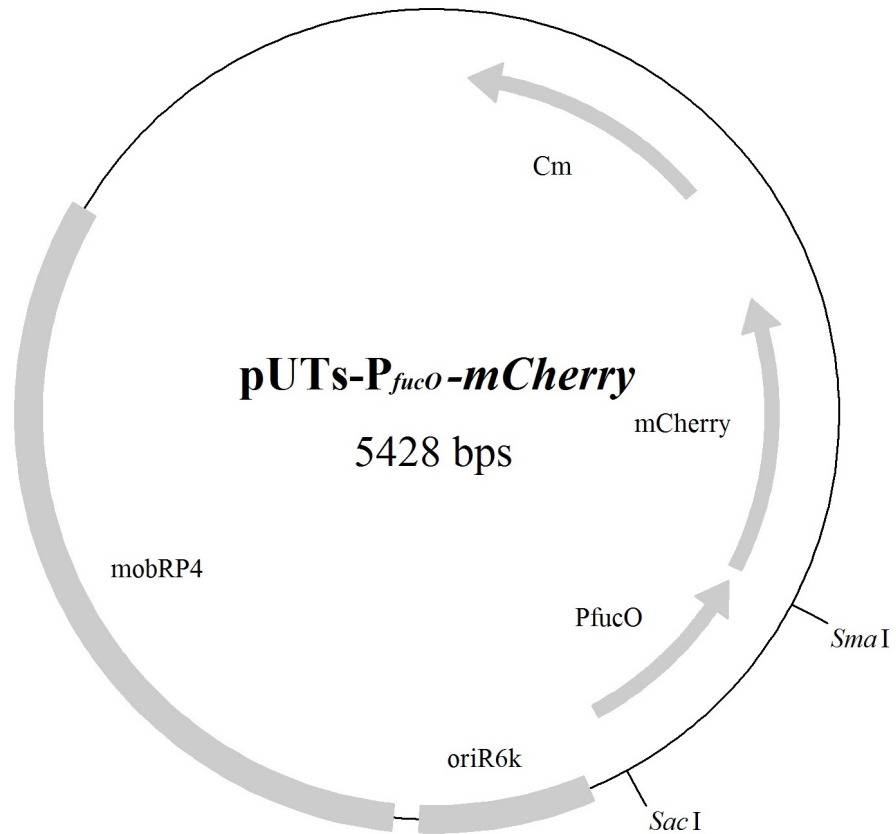
Figure 5.11: To generate the strain *S*. Enteritidis P*fucO*::*mCherry* the promoter region of *fucO* (*S*. Typhimurium LT2) was cloned (*Sac*I/ *Sma*I) into pUTs-*mCherry* (Starke and Fuchs, 2014). The resulting plasmid was named pUTs-P*fucO*-*mCherry*.

Start	End	Label	Description
746	87	Cm	chloramphenicol resistance gene
1736	1056	*mCherry*	promoter-less red fluorescent protein mCherry
2283	1801	P*fucO*	promoter region of *fucO* (*S*. Typhimurium)
2744	2375	*ori*R6k	origin of replication
4529	2802	*mob*RP4	mobility region
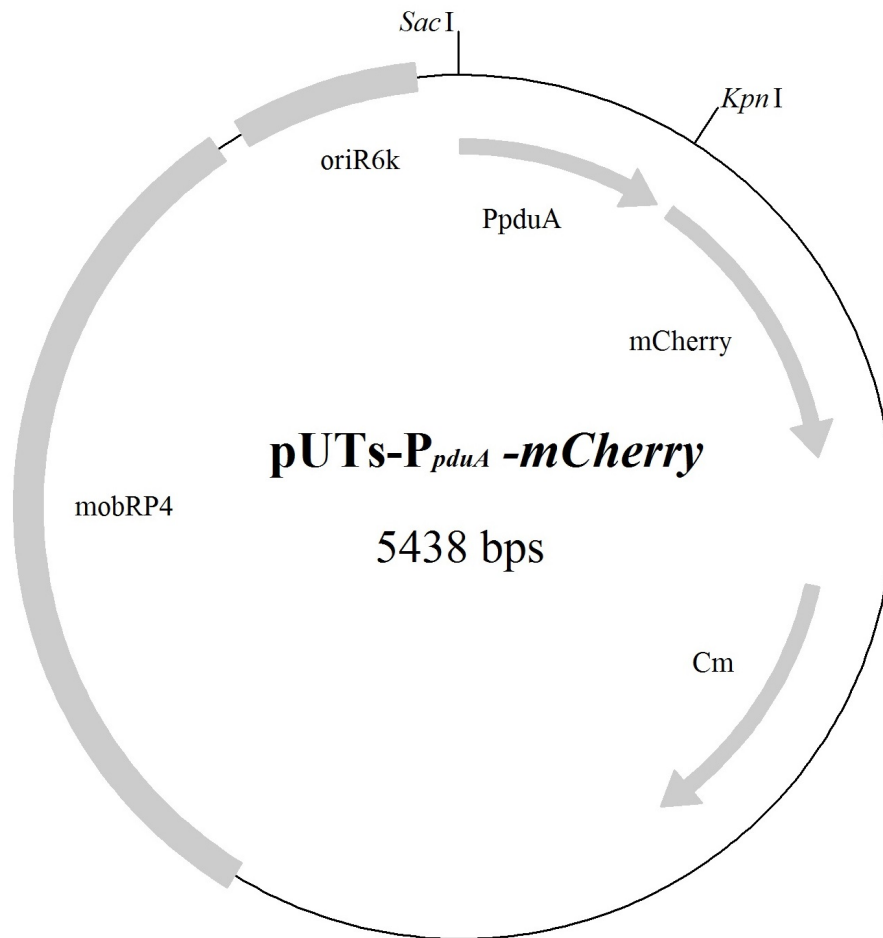
Figure 5.12: To generate the strain *S*. Enteritidis P$_{pduA}$::*mCherry* the promoter region of *pduA* (*S*. Typhimurium LT2) was cloned (*Sac*I/ *Kpn*I) into pUTs-*mCherry* (Starke and Fuchs, 2014). The resulting plasmid was named pUTs-P$_{pduA}$-*mCherry*.

Start	End	Label	Description
1150	2209	Cm	chloramphenicol resistance gene
533	1240	*mCherry*	promoter-less red fluorescent protein mCherry
5437	499	P$_{pduA}$	promoter region of *pduA* (*S*. Typhimurium)
4980	5349	*ori*R6k	origin of replication
3195	4922	*mob*RP4	mobility region

5.5 Media composition

Table 5.7: SOC-medium (Hanahan, 1983).

	Reagent	Amount
SOB-medium		
	Tryptone from casein	20 g
	Yeast extract	5 g
	NaCl	5 g
	KCl	0.2 g
	Aq$_{bidest}$	ad 1 L
	autoclave (121 °C, 20 min)	
SOC-medium		
	SOB-medium	47 ml
	1 M MgCl$_2$	1 ml
	1 M MgSO$_4$	1 ml
	1 M D-glucose	1 ml
	filter sterilise (0.2 μm pore filter)	

Table 5.8: Trace element solution (1000 x) for VB-NE medium in Aq$_{bidest}$ (Price-Carter *et al.*, 2001).

Reagent	c$_{final}$
CaCl$_2$	0.3 mM
ZnSO$_4$	0.1 mM
Fe-(II)-SO$_4 \cdot 7$ H$_2$O	0.045 mM
Na$_2$SeO$_3$	0.2 mM
NaMoO$_4$	0.2 mM
MnSO$_4 \cdot$H$_2$O	2 mM
CuSO$_4 \cdot 5$ H$_2$O	0.1 m M
CoCl$_2 \cdot 6$ H$_2$O	3 mM
NiSO$_4 \cdot 6$H$_2$O	0.1 mM

Table 5.9: Antibiotics used in growth media.

Antibiotic	Abbreviation	c_{final}	Solvent
Ampicillin	Amp	100 $\frac{\mu g}{ml}$	Aq_{bidest}
Chloramphenicol	Cm	20 $\frac{\mu g}{ml}$	100 % ethanol
Kanamycin	Kan	50 $\frac{\mu g}{ml}$	Aq_{bidest}
Nalidixic acid	Nal	20 $\frac{\mu g}{ml}$	Aq_{bidest}
Spectinomycin	Spec	100 $\frac{\mu g}{ml}$	Aq_{bidest}
Streptomycin	Strep	100 $\frac{\mu g}{ml}$	Aq_{bidest}
Tetracyclin	Tet	12 $\frac{\mu g}{ml}$	70 % ethanol

Table 5.10: M9 mineral medium (Sambrook and Russell, 2001).

Reagent	c_{final}
10 x M9-salts	
$Na_2HPO_4 \cdot 2\,H_2O$	0.493 M
KH_2PO_4	0.22 M
NaCl	0.086 M
NH_4Cl	0.19 M
Aq_{bidest}	ad 1 L
filter sterilise (0.2 μm pore filter)	
M9- mineral medium	
M9-salts	1 x
1 M $MgSO_4$	2 mM
1 M $CaCl_2$	0.1 mM
1 mM Cyanocobalamin	200 nM
or	
1 M Na-tetrathionate	40 mM

5.6 Supplementary information

5.6.1 Gene clusters

Table 5.11: *eut, pdu*, and *cob/cbi* gene functions.

Gene	Function
1,2-PD utilisation	
pduA	Polyhedral body component
pduB	Polyhedral body component
pduC	Diol dehydratase large α-subunit
pduD	Diol dehydratase medium β-subunit
pduE	Diol dehydratase small γ-subunit
pduF	Propanediol diffusion facilitator
pduG	Diol dehydratase reactivation factor large subunit
pduH	Diol dehydratase reactivation factor small subunit
pduJ	Polyhedral body component
pduK	Polyhedral body component
pduL	Phosphotransacylase
pduM	Unknown function
pduN	Polyhedral body component
pduO	Adenosyltransferase
pduP	Propionaldehyde dehydrogenase
pduQ	Propanol dehydrogenase
pduS	NADH:ubiquinone oxidoreductase
pduT	Polyhedral body component
pduU	Polyhedral body component
pduV	Unknown function
pduW	Propionate kinase
pduX	L-threonine kinase
pocR	Transcriptional activator of *pdu* genes
Ethanolamine utilisation	
eutA	Reactivating factor
eutB	Ethanolamine ammonia lyase large subunit
eutC	Ethanolamine ammonia lyase small subunit
eutD	Phosphotransacylase
eutE	Aldehyde oxidoreductase
eutG	Alcohol dehydrogenase
eutH/ eat	Ethanolamine permease
eutJ	Putative chaperonin
eutK	Polyhedral body component
eutL	Polyhedral body component
eutM	Polyhedral body component
eutN	Polyhedral body component

Gene	Function
eutP	Ethanolamine utilisation protein
eutQ	Ethanolamine utilisation protein
eutR	Transcriptional regulator
eutS	Polyhedral body component
eutT	Corrinoid cobalamin adenosyltransferase
eutV	Sensor histidine kinase
eutW	Response regulator
Vitamin B$_{12}$ synthesis	
cbiA	Cobyrinic acid a,c-diamide synthase
cbiB	Cobalamin biosynthesis protein
cbiC	Precorrin-8X methymutase
cbiD	Cobalt-precorrin-6A synthase
cbiE	Cobalt-precorrin-6Y C$_5$ methytransferase
cbiT	Cobalt-precorrin-6Y C$_{15}$ methyltransferase
cbiF	Cobalamin biosynthesis protein
cbiG	Cobalamin biosynthesis protein
cbiH	Precorrin-3B C$_{17}$ methyltransferase
cbiJ	Cobalt-precorrin-6X reductase
cbiK	Vitamin B$_{12}$ biosynthetic protein
cbiL	Cobalt-precorrin-2 C$_{20}$ methyltransferase
cbiM	Cobalt transport protein
cbiN	Cobalt transport protein
cbiQ	Cobalt transport protein
cbiO	Cobalt transport protein
cbiP	Cobyric acid synthase
cobU	Adenosylcobinamide synthase
cobS	Cobalamin synthase
cobT	Nicotinate-nucleotide dimethylbenzimidazole phosphoribosyltransferase
cobA	Adenosyl transferase
cobC	Alpha ribazole-5'-P phosphatase
cobB	Threonine-phosphate decarboxylase
butB/ tonB	Cobalamin transport over outer membrane
btuFCD	Cobalamion transport over inner membrane *btuD* has ATPase function

Table 5.12: Database query for the presence of the *pdu* encoded proteins in *S. enterica* serovars. In the case of less than 60 % query coverage and less than 90 % sequence homology to the protein sequence of strain LT2 the protein was considered as absent in the respective serovar (previously published in Staib and Fuchs (2015)).

Database query regarding 1,2-propanediol and L-fucose utilization in *salmonellae* Sept. 2014
NCBI protein BLAST
max target sequences: 500
non-redundant protein sequences=Database
Organism: *Salmonella* (taxid 28901)
60% Query coverage; 90% Identity score

1=present
0=absent

S. enterica subsp. enterica

Column headers (left to right): serovar, PocR, PduF, PduA, PduB, PduC, PduD, PduE, PduG, PduH, PduI, PduJ, PduK, PduL, PduM, PduN, PduO, PduP, PduQ, PduO, PduS, PduT, PduU, PduV, PduW, PduX

Serovars:
4,[5],12,i:-
9,12:l,v:-
Abaetetuba
Abony
Adelaide
Agona
Alachua
Albany
Amsterdam
Anatum
Aqua
Basildon
Bareilly
Berta
Bovismorbificans
Braenderup
Brockney
Cerro
Chester
Choleraesuis
Cubana
Derby
Dublin
Eastbourne
Enteritidis
Gallinarum
Gaminara
Give
Hadar
Hartford
Havana
Heidelberg
Hvittingfoss
Indiana
Infantis
Inverness
Javiana
Johannesburg
Kentucky
Litchfield
London
Manhattan
Mbandaka
Meleagridis
Miami
Minnesota
Mississippi
Montevideo
Muenchen
Muenster
Namur
Nchanga
Newport
Norwich
Ohio
Onarimon
Panama
Paratyphi A
Paratyphi B
Paratyphi C
Pomona
Poona
Pullorum
Reisen
Rubislaw
Saintpaul
Schwarzengrund
Senftenberg
Sloterdijk
Sneenga
Stanley
Stanleyville
Tallahassee
Tennessee
Thompson
Typhi
Typhimurium
Uganda
Urbana
Virchow
Wandsworth
Weltevreden
Worthington

Bottom subspecies rows:

	serovar	PocR	PduF	PduA	PduB	PduC	PduD	PduE	PduG	PduH	PduI	PduJ	PduK	PduL	PduM	PduN	PduO	PduP	PduQ	PduO	PduS	PduT	PduU	PduV	PduW	PduX
Salmonella enterica subsp. *salamae*	58:l,z13,z28:z6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Salmonella enterica subsp. *indica*	6,14,25:z10:1,(2),7	1	1											0	0	0							1	0	1	0
Salmonella enterica subsp. *diarizonae*	60:r:z35	1	1										0		0	0						0	0	0	0	0
Salmonella enterica subsp. *arizonae*	62:z4,z23:-	1	1																								

Right-side annotations:
- Genome=shotgun sequence AccNo. APAN01000034, no further annotation of *pdu* genes
- Genome=shotgun sequence AccNo. APAR01000026, no proper annotation of *pdu* genes

Table 5.13: Database query for the presence of the *fuc* encoded proteins in *S. enterica* serovars. In the case of less than 60 % query coverage and less than 90 % sequence homology to the protein sequence of strain LT2 the protein was considered as absent in the respective serovar (previously published in Staib and Fuchs (2015)).

Database qery regarding L-fucose utilization in *salmonellae* Sept. 2014
NCBI protein BLAST
max target sequences: 500 — 1=present
non-redundant protein sequences=Database — 0=absent
Organism: *Salmonella* (taxid 28901) — FucP=pseudogene!
60 % Query coverage; 90 % Identity score

	serovar	FucO	FucA	FucI	FucK	FucU	FucR
S. enterica subsp. *enterica*	4,[5],12:i	1	1	1	1	1	1
	9,12:l,v	1	1	1	1	1	1
	Abaetetuba	1	1	1	1	1	1
	Abony	1	1	1	1	1	1
	Adelaide	1	0	1	1	1	1
	Agona	1	1	1	1	1	1
	Alachua	0	0	1	1	1	0
	Albany	1	1	1	1	1	1
	Anatum	1	1	1	1	1	1
	Aqua	1	1	1	1	1	1
	Baildon	1	1	1	0	1	1
	Bareilly	1	1	1	1	1	1
	Berta	1	1	1	1	1	1
	Bovismorbificans	1	1	1	1	1	1
	Braenderup	1	1	1	1	1	1
	Bredeney	1	1	1	1	1	1
	Cerro	1	1	1	1	1	1
	Chester	1	1	1	1	1	1
	Choleraesuis	1	1	1	1	1	1
	Cubana	1	1	1	1	1	1
	Derby	1	1	1	1	1	1
	Dublin	1	1	1	1	1	1
	Eastbourne	1	1	1	1	1	1
	Enteritidis	1	1	1	1	1	1
	Gallinarum	1	1	1	1	1	1
	Gaminara	1	1	1	1	1	1
	Give	1	1	1	1	1	1
	Hadar	1	1	1	1	1	1
	Hartford	1	1	1	1	1	1
	Havana	1	1	1	1	1	1
	Heidelberg	1	1	1	1	1	1
	Hvittingfoss	0	1	1	1	1	1
	Indiana	1	1	1	1	1	1
	Infantis	1	1	1	1	1	1
	Inverness	1	1	1	1	1	1
	Javiana	1	1	1	1	1	1
	Johannesburg	0	1	1	1	1	0
	Kentucky	1	1	1	1	1	1
	Litchfield	1	1	1	1	1	1
	London	1	1	1	1	1	1
	Manhattan	1	1	1	1	1	1
	Mbandaka	1	1	1	1	1	1
	Meleagridis	1	1	1	1	1	1
	Miami	1	1	1	1	1	1
	Minnesota	1	1	1	1	1	1
	Mississippi	0	1	1	1	1	1
	Montevideo	1	1	1	1	1	1
	Muenchen	1	1	1	1	1	1
	Muenster	1	1	1	1	1	1
	Namur	1	1	1	1	1	1
	Nchanga	1	1	1	1	1	1
	Newport	1	1	1	1	1	1
	Norwich	1	1	1	1	1	1
	Ohio	1	1	1	1	1	1
	Oranienburg	1	1	1	1	1	1
	Panama	1	1	1	1	1	1
	Paratyphi A	1	1	1	1	1	1
	Paratyphi B	1	1	1	1	1	1
	Paratyphi C	1	1	1	1	1	1
	Pomona	1	1	1	1	1	1
	Poona	1	1	1	1	1	1
	Pullorum	1	1	1	1	1	1
	Rissen	1	1	1	1	1	1
	Rubislaw	1	1	1	1	1	1
	Saintpaul	1	1	1	1	1	1
	Schwarzengrund	1	1	1	1	1	1
	Senftenberg	1	1	1	1	1	1
	Sloterdijk	1	1	1	1	1	1
	Soerenga	1	1	1	1	1	1
	Stanley	1	1	1	1	1	1
	Stanleyville	1	1	1	1	1	1
	Tallahassee	1	1	1	1	1	1
	Tennessee	1	1	1	1	1	1
	Thompson	1	1	1	1	1	1
	Typhi	1	1	1	1	1	1
	Typhimurium	1	1	1	1	1	1
	Uganda	0	1	1	1	1	1
	Urbana	1	1	1	1	1	1
	Virchow	1	1	1	1	1	1
	Wandsworth	0	0	1	1	1	1
	Weltevreden	1	1	1	1	1	1
	Worthington	1	1	1	1	1	1

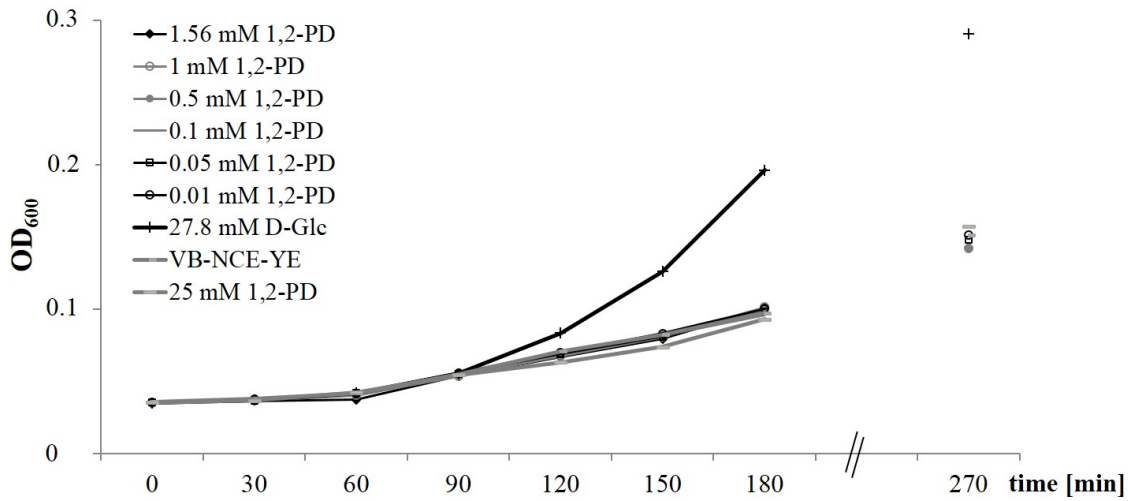|		FucO	FucA	FucI	FucK	FucU	FucR
Salmonella enterica subsp. *salamae*	58:1,z13,z28:z6	1	1	1	1	1	1
Salmonella enterica subsp. *indica*	6,14,25:z10:1,(2),7	1	1	1	1	1	1
Salmonella enterica subsp. *diarizonae*	60:r:e,n,x,z15	1	1	1	1	1	1
Salmonella enterica subsp. *arizonae*	62:z4,z23	1	1	1	1	1	1
Salmonella enterica subsp. *houtenae*		1	1	1	1	1	1

Figure 5.13: **Growth behaviour of 14028 P_{pduA}::gfp.**

In order to determine the least amount of 1,2-PD, which is necessary to induce gene expression from P_{pduA}, 14028 P_{pduA}::gfp was cultivated anaerobically (Whitley A35, 37 °C, 80 % N$_2$, 10 % CO$_2$, 10 % H$_2$) without agitation in VB-NCE-YE medium, which was supplemented with tetrathionate, trace elements and decreasing 1,2-PD concentrations (1 mM to 0.01 mM). OD$_{600}$ was determined in the time intervals indicated above (Wallac Victor[3]) by pipetting triplicates of each culture (200 μl per well) into the cavities of a 96-well microtiter plate.
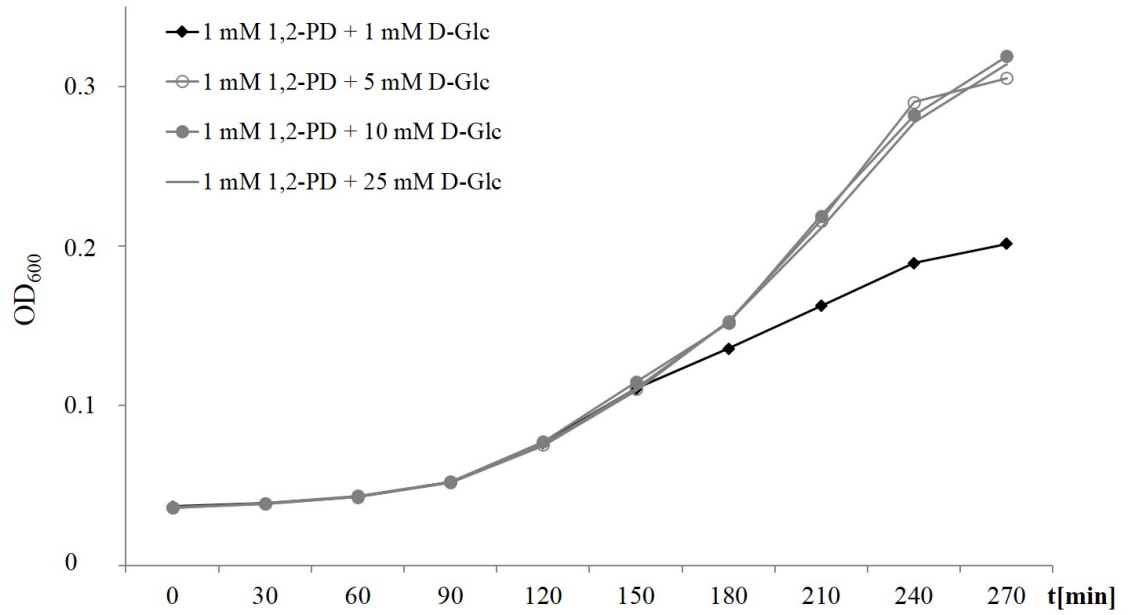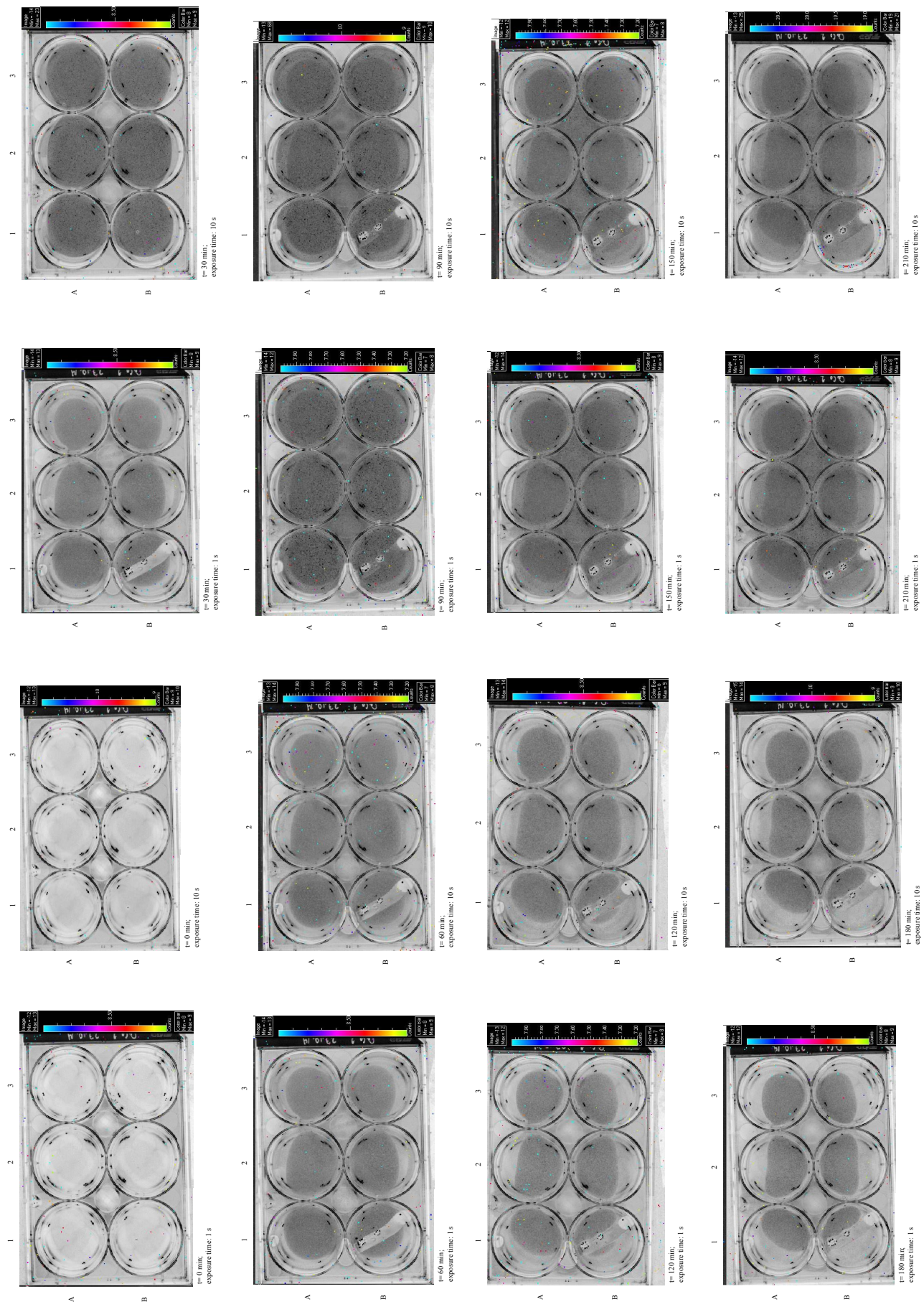
Figure 5.14: **Growth behaviour of 14028 P$_{pduA}$::*gfp* with 1,2-PD and D-Glc.**

To investigate the possible catabolite repression of P$_{pduA}$ by D-Glc, 14028 P$_{pduA}$::*gfp* was cultivated anaerobically (Whitley A35, 37 °C, 80 % N_2, 10 % CO_2, 10 % H_2) without agitation in VB-NCE-YE medium, which was supplemented with tetrathionate, trace elements and 1 mM 1,2-PD, and with increasing concentrations of D-Glc (1, 5, 10, and 25 mM). Cell densities (OD$_{600}$) were determined in the time intervals indicated above using a Wallac Victor[3]. Triplicates of each culture were pipetted (200 μl per well) into the cavities of a 96-well microtiter plate.

t= 30 min;
exposure time: 10 s

t= 90 min;
exposure time: 10 s

t= 150 min;
exposure time: 10 s

t= 210 min;
exposure time: 10 s

t= 30 min;
exposure time: 1 s

t= 90 min;
exposure time: 1 s

t= 150 min;
exposure time: 1 s

t= 210 min;
exposure time: 1 s

t= 0 min;
exposure time: 10 s

t= 60 min;
exposure time: 10 s

t= 120 min;
exposure time: 10 s

t= 180 min;
exposure time: 10 s

t= 0 min;
exposure time: 1 s

t= 60 min;
exposure time: 1 s

t= 120 min;
exposure time: 1 s
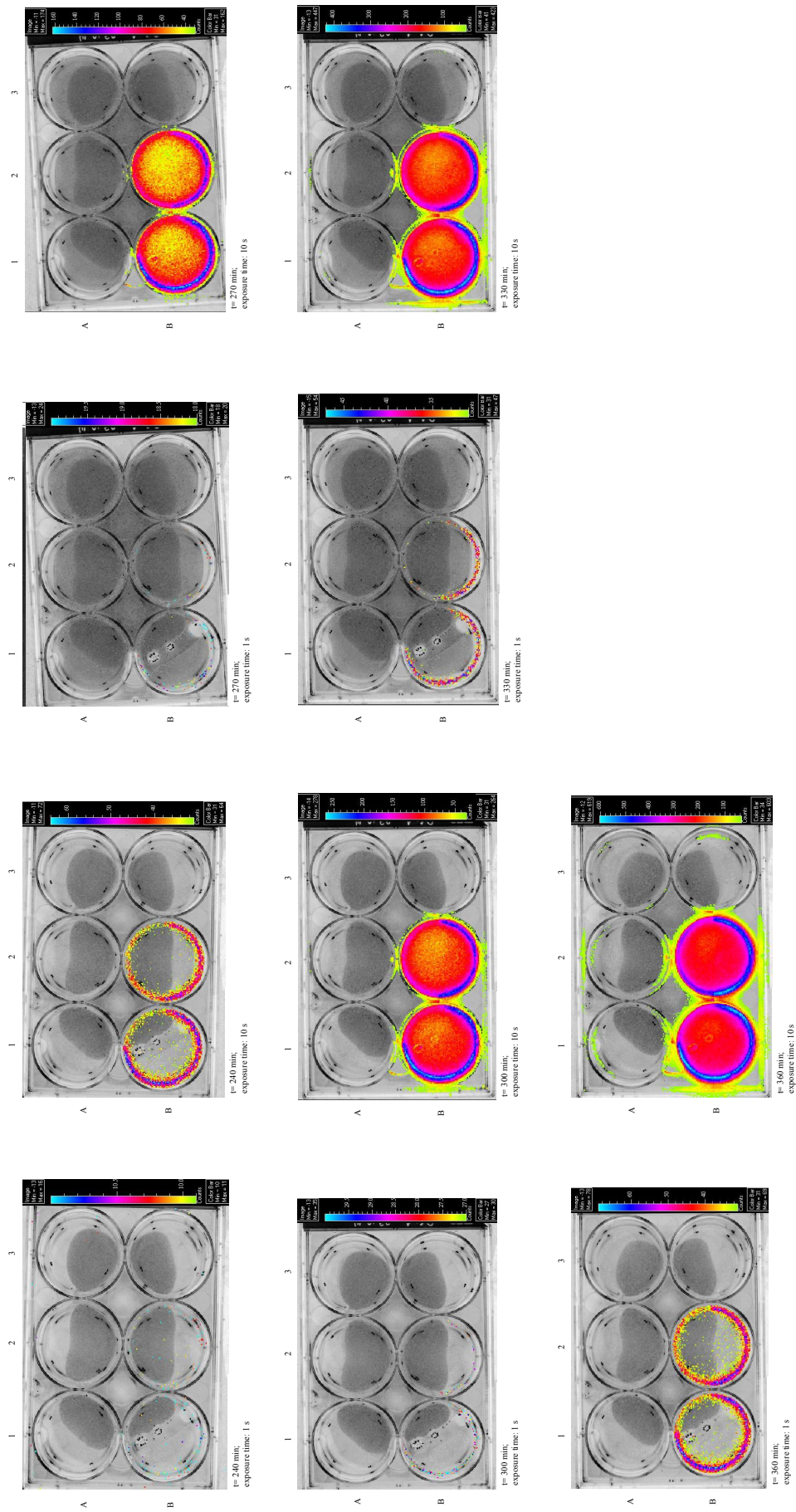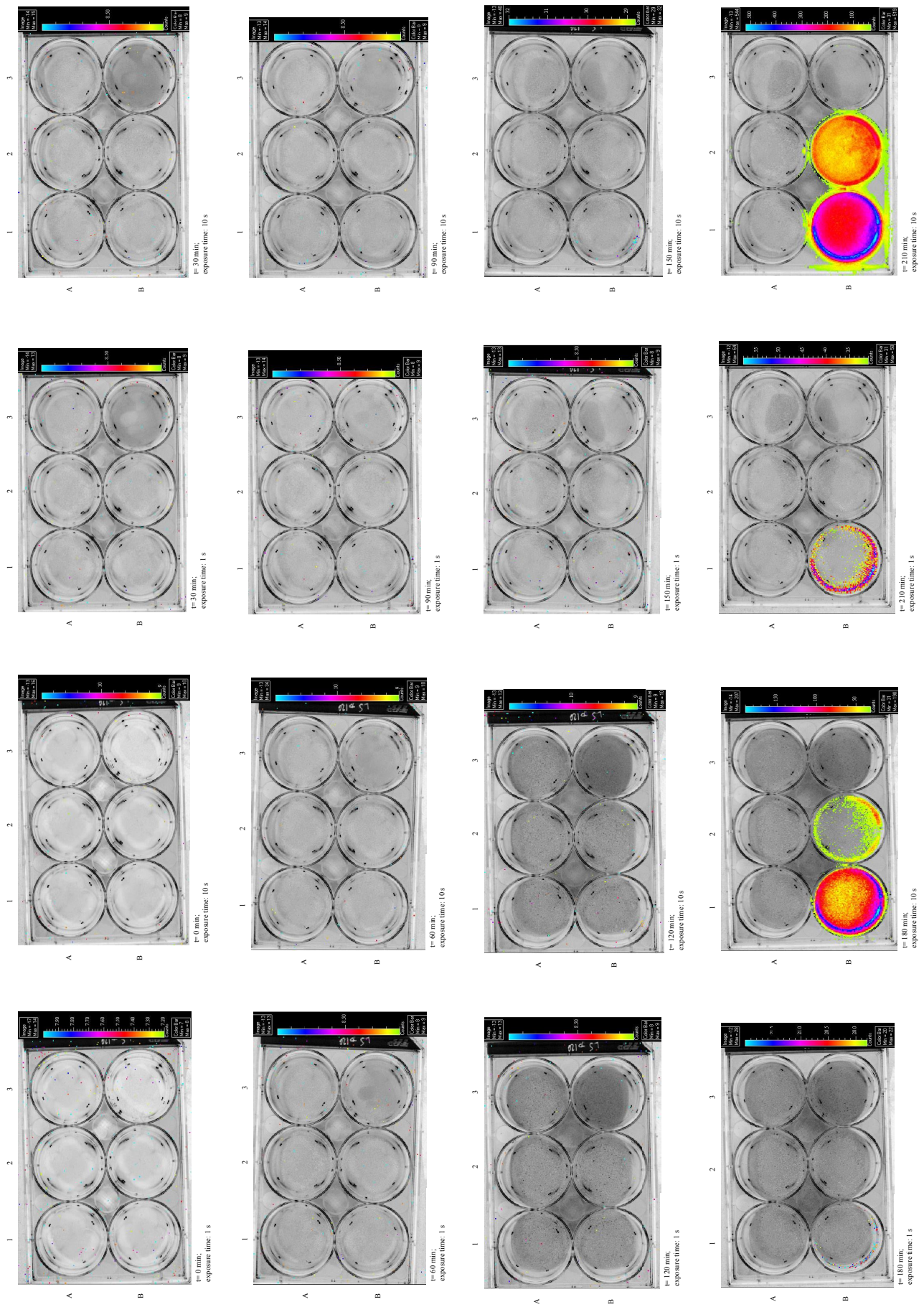
t= 180 min;
exposure time: 1 s

Figure 5.15: **Luminescence assay with Caco-2 cells.**
Caco-2 cells were incubated for 6 h with 14028 $\Delta invA$ P$_{pduA}$::lux (wells A 1+2) and 14028 $\Delta invA$ P$_{fucO}$::lux (wells B 1+2). Overnight cultures of the bacteria were diluted 1:100 in PBS containing Ca^{2+} and Mg^{2+} and 1 ml of this dilution was added to the wells containing the cell culture. Prior to inoculation the Caco-2 cells were washed twice. The culture plates were incubated at 37 °C and 5 % CO$_2$ for 6 h. Luminescence was measured every 30 min using the IVIS and exposure times of 1 s or 10 s were chosen. As negative controls, well A 3 contains Caco-2 cells, inoculated with 14028 Nal^R and well B 3 does not contain Caco-2 cells but 1 ml of 14028 $\Delta invA$ P$_{fucO}$::lux. This assay was part of the research internship of Madlaina v. Hößlin.
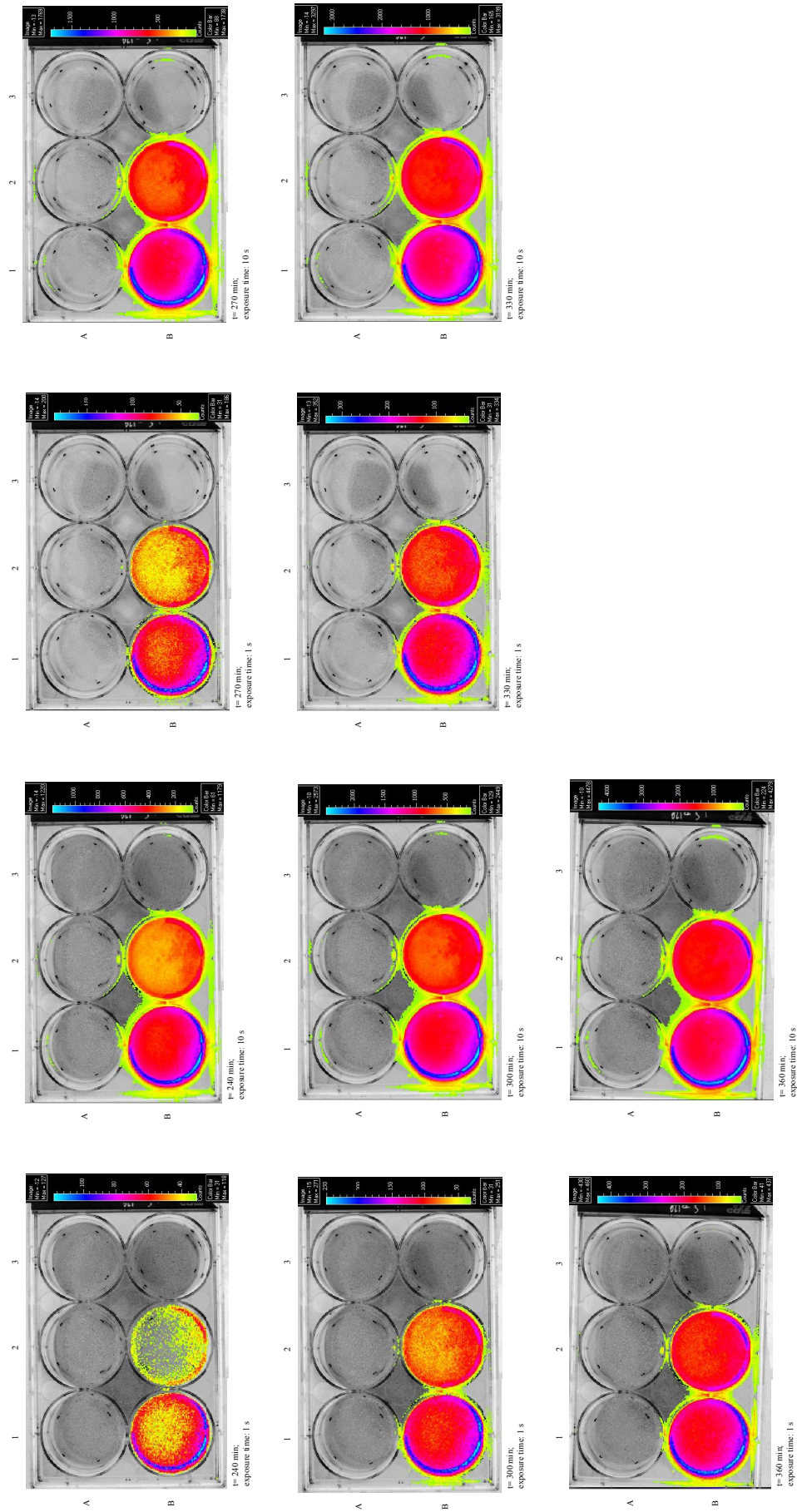
t= 30 min;
exposure time: 10 s

t= 90 min;
exposure time: 10 s

t= 150 min;
exposure time: 10 s

t= 210 min;
exposure time: 10 s

t= 30 min;
exposure time: 1 s

t= 90 min;
exposure time: 1 s

t= 150 min;
exposure time: 1 s

t= 210 min;
exposure time: 1 s

t= 0 min;
exposure time: 10 s

t= 60 min;
exposure time: 10 s

t= 120 min;
exposure time: 10 s

t= 180 min;
exposure time: 10 s

t= 0 min;
exposure time: 1 s

t= 60 min;
exposure time: 1 s

t= 120 min;
exposure time: 1 s

t= 180 min;
exposure time: 1 s

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Figure 5.16: **Luminescence assay with LS174T cells.**

LS174T cells were incubated for 6 h with 14028 $\Delta invA$ P$_{pduA}$::lux (wells A 1+2) and 14028 $\Delta invA$ P$_{fucO}$::lux (wells B 1+2). Overnight cultures of the bacteria were diluted 1:100 in PBS containing Ca^{2+} and Mg^{2+} and 1 ml of this dilution was added to the wells containing the cell culture. Prior to inoculation the LS174T cells were washed twice. The culture plates were incubated at 37 °C and 5 % CO$_2$ for 6 h. Luminescence was measured every 30 min using the IVIS and exposure times of 1 s and 10 s were chosen. As negative controls, well A 3 contains LS174T cells, inoculated with 14028 Nal^R and well B 3 only contains 1 ml of 14028 $\Delta invA$ P$_{fucO}$::lux without human cells. This assay was part of the research internship of Madlaina v. Hößlin.
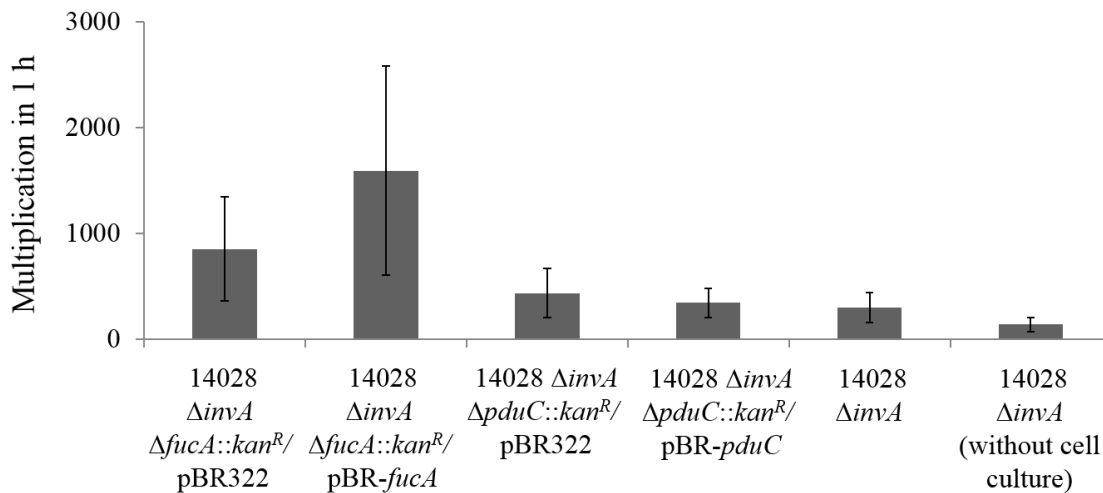
Figure 5.17: **Multiplication assay in the supernatants of LS174T cells.**

LS174T cells were incubated with 14028 $\Delta invA$, $\Delta invA\Delta fucA$::kan^R/pBR322, $\Delta invA\Delta fucA$::kan^R/pBR-$fucA$, $\Delta invA\Delta pduC$::kan^R/pBR322 and $\Delta invA\Delta pduC$::kan^R/pBR-$pduC$. Prior to inoculation, the cell culture was washed twice. The overnight cultures were diluted 1:100 in PBS containing Ca^{2+} and Mg^{2+} and 1 ml of the dilution was applied to each well of the cell culture plate. The cell counts of the inocula were determined. After 1 h incubation at 37 °C and 5 % CO_2, the supernatants were aspired and collected in order to determine the proliferation of the extracellular bacteria. As a control, 14028 $\Delta invA$ was incubated in a well, which did not contain LS174T cells. Vertical bars represent the standard deviations of three independent bacterial cultures of the same genotype, which were applied to the cell culture. This assay was part of the research internship of Madlaina v. Hößlin.

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THANK YOU

Professor Dr. Thilo M. Fuchs

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All current and previous members of ZIEL Mikrobiologie, chair Microbial Ecology

Dr. Andrea Rütschle
Lisa Holland
Anna Mühlig
Dr. Viktoria Krey

Dr. Monika Spiller

Rupert Bassitta
Prof. Dr. Jerry M. Wells
Daniel Gardner

Angela Felsl
Jessica Hellinckx
Dr. Tanja Kern
Dr. Mandy Knüpfer
Nicoletta Nolle
Johannes Rothhardt
Katharina Springer
Patrick Schiwek

Madlaina von Hößlin

E3/03-Gang:
Mario von Neubeck
Etienne Doll
Christopher Huptas

Dr. Sören Ocvirk
Julia Vörös
Dorothea Wörner
Prof. Dr. Dirk Haller

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Axel + Julia
Lina
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Helga

Marta
Stephanie
Frank

5.8 List of publications

Staib, L., and Fuchs T.M. (2014). From food to cell: nutrient exploitation strategies of entero-pathogens. *Microbiology* (2014), 160, 1020-1039.

Staib, L., and Fuchs, T.M. (2015). Regulation of fucose and 1,2-propanediol utilization by *Salmonella enterica* serovar Typhimurium. *Front. Microbiol.* 6:1116.

5.9 Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung bzw. Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

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am Lehrstuhl für Mikrobielle Ökologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung Institute for Food and Health, Technische Universität München, Freising, Germany
unter der Anleitung und Betreuung durch

Professor Dr. Thilo M. Fuchs

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß §6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

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Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von §28 (Nichtigkeit der Promotion) und §29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich
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Freising, den