Response of *Bacillus subtilis* to Antimicrobial Peptide Stress

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München, 24.09.2015

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Carolin Höfler
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(* shared first authorship)

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CHAPTER II


Sara Kesel and Andreas Mader performed the experiments. Carolin Höfler was involved in strain constructions. Sara Kesel and Madeleine Leisner analyzed the data obtained from the microscopy experiments. Thorsten Mascher and Madeleine Leisner conceived and designed the experiments and wrote the manuscript.

CHAPTER III


Julia Dominguez-Escobar, Diana Wolf and Carolin Höfler carried out the experiments, drew the figures and constructed the tables. Julia Dominguez-Escobar, Diana Wolf and Georg Fritz analyzed the data. Roland Wedlich-Söldner and Thorsten Mascher designed the experiments. Julia Dominguez-Escobar, Diana Wolf, Roland Wedlich-Söldner and Thorsten Mascher wrote the manuscript.

CHAPTER IV


Carolin Höfler performed the majority of the experiments, drew the figures and tables and supervised the students Judith Heckmann, Anne Fritsch and Philipp Popp, who constructed some strains and performed some experiments. Susanne Gebhard, Georg Fritz and Thorsten Mascher designed the experiments. Carolin Höfler, Susanne Gebhard, Georg Fritz and Thorsten Mascher wrote the manuscript.

We hereby confirm the above mentioned declaration.

Carolin Höfler

Prof. Dr. Thorsten Mascher
Summary

Bacteria share their often complex habitats with many different microorganisms with whom they must constantly compete for nutrients. Thus, they have evolved various mechanisms to attack rivaling species and defend themselves accordingly. The Gram-positive spore-forming soil organism *Bacillus subtilis* is a member of the Firmicutes phylum and is able to produce and secrete many antimicrobial peptides (AMPs) to kill its competitors. These peptides are usually produced in stationary growth phase when nutrient availability is limited. Their main target is the bacterial cell envelope, which is the first structural barrier for defense. Therefore, close monitoring of cell envelope integrity is pivotal for survival. In addition to extracytoplasmic function sigma factors (ECF σ factors), *B. subtilis* employs four different two-component systems (2CSs) to counteract cell envelope stress (LiaSR, BceRS, PsdRS and YxdJK).

The first part of this thesis deals with the LiaSR 2CS of *B. subtilis*. It consists of a histidine kinase LiaS and its cytosolic response regulator LiaR. Additionally, there is an accessory inhibitor protein LiaF located in the membrane, which keeps LiaS in its OFF-state under non-inducing conditions. The LiaSR system is encoded within two adjacent operons regulated by two promoters and harboring six genes (*liaIH-liaGFSR*). The first operon, *liaIH*, is under control of the LiaR-dependent target promoter *PliaI*. It is tightly regulated and has very low basal activity under non-inducing conditions. In contrast, the second promoter *PliaG* controlling expression of the adjacent operon *liaGFSR* is relatively strong and constitutive.

The LiaSR system responds to a great variety of envelope-targeting AMPs, e.g. the cyclic AMP bacitracin. Upon its addition, *PliaI* and hence, expression of *liaIH* is strongly induced. The LiaSR system responds to AMP damage, rather than the compound itself and mounts a secondary layer of defense against envelope perturbations. We could demonstrate that the system responds not only fast and strongly to different external bacitracin concentrations but also shows a heterogeneous response: At low bacitracin concentrations the majority of the population remains in its OFF-state while only few cells activate the LiaSR system.

The second part of this thesis deals with the subcellular localization, interactions and dynamics of the phage-shock protein-like Lia response in *B. subtilis*. Despite extensive studies on the LiaSR system over the last decade, its physiological role remains unclear. In this study, we used fluorescence and time-lapse microscopy to study the subcellular localization and interaction of the small membrane protein LiaI and phage-shock protein A homolog LiaH under inducing and non-inducing conditions. LiaI localizes into few distinct foci at the membrane which are highly dynamic under non-inducing conditions, while LiaH exhibited disperse cytosolic localization. Upon bacitracin induction, the number of LiaI foci increase, they become static at the membrane and recruit LiaH into these protein complexes. Our data indicated that LiaI scans the membrane for envelope damage and stops at sites of AMP-generated damage. Once recruited to these spots, LiaH is hypothesized to serve as a “patch” from the inside to shield against AMP-induced damage.
The LiaSR system is not only triggered by the external addition of cell wall antibiotics. It is also intrinsically activated during transition from exponential to stationary growth phase. The induction was previously shown to be at least partially due to Spo0A-mediated de-repression of \( P_{\text{lia}} \). The LiaSR system is kept inactive during logarithmic growth phase by the transcriptional regulator AbrB, which itself is negatively regulated by Spo0A, the master regulator of sporulation at the onset of stationary phase. Since Spo0A-mediated de-repression alone is not sufficient for the transition phase induction of the LiaSR system, we sought to identify other potential inducers, i.e. AMPs produced by \( B. \ subtilis \) itself during this growth stage. In the course of studying this intrinsic transition phase induction of the LiaSR system, we observed that other 2CSs, the BceRS and PsdRS, as well as the ECF \( \sigma \) factors \( \sigma^M \), \( \sigma^X \) and \( \sigma^W \) are also induced in stationary phase by an unknown stimulus.

The third part of this thesis deals with the identification of the stimuli leading to this intrinsic activation of the different systems. Using a lux-reporter system to monitor target promoter activity of each system, we searched for AMPs produced by \( B. \ subtilis \) W168 itself. As previously reported, the YydF peptide was shown to be an inducer of the LiaSR system responsible for its heterogeneous activation. In this study, we found that the BceRS and PsdRS 2CSs, as well as the ECF \( \sigma \) factors \( \sigma^M \), \( \sigma^X \) and \( \sigma^W \), are activated by the two cannibalism toxins, SDP and SKF, in stationary phase. The most prominent effect was observed for the BceRS system. Therefore, we focused on this system in the last part of this thesis to gain deeper insight into the physiological relevance of this process. While the BceRS response is stronger for SKF compared to SDP, we found no evidence that the BceRS system is involved in mediating resistance against the two toxins. Surprisingly, their own membrane immunity determinants, SkfEF and SdpI, respectively, seem to be important for BceRS activation since induction is lost in the corresponding deletion strains. This observation suggests that the AMPs have to be bound to a membrane target in order to be perceived by the BceRS system.
Zusammenfassung


Im zweiten Teil dieser Arbeit geht es um die subzelluläre Lokalisation, Interaktionen und Dynamiken der Phagenschockprotein-ähnlichen Lia Antwort in B. subtilis. Trotz umfassender Studien zum LiaSR System in den vergangenen 10 Jahren ist seine physiologische Rolle bisher unklar. In dieser Arbeit wandten wir Fluoreszenz- und Time-lapse Mikroskopietechniken an um die subzelluläre Lokalisation und Interaktion vom kleinen Membranprotein LiaI und dem Phagenschockprotein A Homolog LiaH unter induzierenden und nicht-induzierenden Bedingungen
zu untersuchen. LiaI bildet wenige aber dennoch distinkte Foci an der Membran, die unter nicht-induzierenden Bedingungen hochdynamisch sind, während LiaH dispers im Cytosol lokalisiert. Nach Bacitracin-Induktion erhöht sich die Anzahl an LiaI Foci, welche an der Membran statisch werden und welche LiaH in diese Proteinkomplexe rekrutieren. Unsere Daten deuten darauf hin, dass LiaI die Membran „abscannet“ um Membranschäden zu detektieren und an den Stellen stoppt, die durch ein antimikrobielles Peptid geschädigt wurden. Sobald LiaH an diese Stellen rekrutiert wurde, könnte LiaH als eine Art „Flicken“ von innen heraus dienen, um die beschädigte Stelle abzudecken.

Das LiaSR System wird nicht nur durch externe Zugabe von Zellwandantibiotika induziert. Es zeigt auch eine intrinsische Aktivierung beim Übergang von der exponentiellen in die stationäre Wachstumsphase. Vor einiger Zeit wurde gezeigt, dass der Induktion zumindest teilweise eine Spo0A-vermittelte De-Repression von $P_{lia}$ zugrunde liegt: Das LiaSR System wird während der logarithmischen Wachstumsphase durch den transkriptionellen Regulator AbrB inaktiv gehalten, welcher selbst negativ reguliert wird vom Masterregulator der Sporulation, Spo0A, zu Beginn der stationären Phase. Da die Spo0A-vermittelte De-Repression allein für die Übergangsphase-Induktion des LiaSR Systems nicht ausreichend ist, strebten wir danach weitere potenzielle Induktoren zu identifizieren, nämlich antimikrobielle Peptide, die von $B. subtilis$ selbst in dieser Wachstumsphase produziert werden. Während wir die intrinsische Übergangsphase-Induktion im LiaSR System untersuchten, stellten wir fest, dass sowohl andere Zweikomponentensysteme, wie das BceRS und PsdRS System, als auch die ECF Sigma Faktoren $\sigma^M$, $\sigma^X$ und $\sigma^W$ ebenso in der stationären Phase von einem bislang unbekannten Stimulus aktiviert werden.

CHAPTER I

Introduction

Aims
CHAPTER I Introduction

The soil is a complex habitat for many different microorganisms such as the Gram-positive organism \textit{Bacillus subtilis}, a member of the Firmicutes phylum, and others such as Actinobacteria. Bacteria have to adapt quickly to changing environmental conditions such as heat, moisture or oxygen. Additionally, they compete for limited nutrients in order to survive. Therefore, they have evolved a variety of antimicrobial peptides (AMPs) which often target the bacterial cell envelope. In response to suppress the growth of competitors, bacteria have developed signal transducing systems to monitor such extrinsic substances and to counteract these severe stress conditions accordingly (Msadek, 1999).

1.1 The bacterial cell envelope and cell wall biosynthesis

The envelope is an essential structure for the cell. It determines the shape, protects the cell from environmental stresses and counteracts the internal osmotic pressure (Höltje, 1998, Delcour \textit{et al.}, 1999). The cell envelope of Gram-positive and Gram-negative bacteria differs significantly (Fig. 1.1). While Gram-positive bacteria have, in addition to their cytoplasmic membrane, a rather thick peptidoglycan layer with teichoic acids (Foster & Popham, 2002) resulting in an overall negative charge of the cell wall, Gram-negative bacteria harbor only a thin peptidoglycan layer lacking teichoic acids. But in contrast to Gram-positives, Gram-negatives possess a periplasmic space and an additional outer membrane (Silhavy \textit{et al.}, 2010).

Here, I will focus on Gram-positive bacteria since all the work presented in this thesis was performed in \textit{B. subtilis}.

Although the composition of the peptidoglycan layer differs between species, the overall structure is identical. The cell wall forms a polymer consisting of sugars and amino acids building up a netlike structure outside the cytoplasmic membrane. The sugars are composed of alternating residues of $\beta$-(1,4) linked \textit{N}-acetylglucosamine (GlcNAC) and \textit{N}-acetylmuramic acid (MurNAC). The MurNAC molecules are connected pentapeptide bridges leading to the characteristic 3D mesh-like strong and rigid layer (Vollmer \textit{et al.}, 2008).
The peptidoglycan biosynthesis starts in the cytoplasm where the peptidoglycan building blocks are synthesized and then covalently attached to a carrier molecule, bactoprenol (undecaprenol-monophosphate). Bactoprenol transports the peptidoglycan monomers across the membrane to the extracellular space where they are inserted into the growing cell wall (Fig. 1.2). The first step of peptidoglycan biosynthesis is the conversion of fructose-6-phosphate to GlcNAc in the cytoplasm. Next, GlcNAc is activated by the addition of uridine diphosphate (UDP) resulting in UDP-GlcNAc, which is further converted to UDP-MurNAc. Then, the pentapeptide chain including a D-alanyl-D-alanine dipeptide (D-Ala-D-Ala) is attached to UDP-MurNAc followed by its connection to the lipid carrier molecule, bactoprenol, at the inner surface of the membrane (Bouhss et al., 2008). This complex is called lipid I. Another GlcNAc molecule is coupled to the MurNAc residue of lipid I resulting in lipid II. The following steps all involving bactoprenol are called the lipid II cycle (Delcour et al., 1999, Foster & Popham, 2002). The complete peptidoglycan subunit linked by a pyrophosphate to the lipid carrier bactoprenol is then flipped to the outer surface of the membrane where it is incorporated into the nascent peptidoglycan net through transglycosylation and transpeptidation to produce new glycan strands (Barrett et al., 2007, Sauvage et al., 2008). The remaining undecaprenol-pyrophosphate (UPP) is dephosphorylated and transferred back to the inner surface of the membrane. Thus, the lipid carrier is recycled and ready for the next round of coupling and transfer of a new peptidoglycan subunit (Chang et al., 2014).

Because of its essential nature, cell wall biosynthesis is the target for many AMPs (Fig. 1.2), as described in the next section.

![Fig. 1.2: Cell wall biosynthesis of Gram-positive bacteria and inhibition by selected antibiotics. Important steps of cell wall biosynthesis at their specific cellular level are schematically indicated. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; UDP, uridine diphosphate; UMP, uridine monophosphate; UPP (undecaprenol pyrophosphate); P, phosphoryl group; P_i, inorganic phosphate. Amino acids are depicted as small grey circles; bactoprenol (undecaprenol monophosphate) is indicated by the waved line. Antibiotics targeting essential steps are highlighted in red and their site of action is shown. Lantibiotics comprise a group of several antibiotics (nisin, subtilin, gallidermin, actagardine and mersacidin). Amino acids are depicted as three-letter code. This figure is taken from (Jordan et al., 2008) with modifications.](image-url)
1.2 Cell wall active antimicrobial peptides

Nearly all stages of cell wall biosynthesis are prone to AMP attacks (Fig. 1.2) (Schneider & Sahl, 2010). Production of AMPs, though, is not restricted to bacteria but has been reported in basically all groups of organisms including fungi, plants and animals (Peschel & Sahl, 2006). They are active against other bacteria of the same species or across genera (Cotter et al., 2005). Noteworthy, producers simultaneously express dedicated immunity proteins in order to avoid self-killing (Gonzalez-Pastor et al., 2003, Ellermeier et al., 2006, Dubois et al., 2009). AMPs often have a cationic and amphipathic nature but vary in size, secondary structure and sequence (Peschel & Sahl, 2006). AMPs produced by bacteria can be ribosomally or non-ribosomally synthesized. In the following paragraphs, I would like to describe selected AMPs that are relevant for this thesis.

**Non-ribosomally synthesized AMPs** are assembled within protein complexes which modify and release the active peptide (Stein, 2005). One relevant example is bacitracin, a cyclic lipopeptide antibiotic produced by *B. subtilis* and *B. licheniformis*. It is primarily active against Gram-positive bacteria and requires a divalent metal ion, usually zinc, to exhibit its full activity (Ming & Epperson, 2002). It inhibits cell wall biosynthesis by tightly binding to UPP (Fig. 1.2) (Stone & Strominger, 1971). It encloses the pyrophosphate group entirely and prevents accessibility for phosphatases and thus, recycling of the lipid carrier is restricted, ultimately leading to cell death (Stone & Strominger, 1971, Storm & Strominger, 1973, Economou et al., 2013). To counteract its damage, resistance mechanisms have been developed to remove bacitracin from its site of action. The major resistance determinant in *B. subtilis* is composed of the BceRS two-component system (2CS) regulating the adenosine triphosphate-binding cassette (ABC) transporter BceAB (Mascher et al., 2003, Ohki et al., 2003) (see sections 1.3 and 1.4.2 for details). A secondary mechanism for bacitracin resistance is the upregulation of BcrC, an alternative phosphatase that dephosphorylates UPP on the extracellular side of the membrane (Cao & Helmann, 2002, Bernard et al., 2005). Subsequent lipid carrier recycling ensures successful completion of cell wall biosynthesis and survival of the cell.

**Ribosomally synthesized AMPs** are gene-encoded peptides that usually require posttranslational modifications to become fully active (Papagianni, 2003). They vary broadly in structure and can form linear or cyclic peptides. They often have a cationic and amphiphilic nature, which facilitates contact with the negatively charged bacterial envelope and enables membrane permeabilization (Papagianni, 2003). Examples of this class are the heavily modified lantibiotics. Their characteristic feature is the presence of lanthionine or methyllanthionine residues. They are structurally diverse, forming elongated, flexible molecules such as nisin, or more globular shapes like mersacidin (Sahl et al., 1995, Bierbaum & Sahl, 2009). Another set of ribosomally synthesized AMPs are the two cannibalism toxins SDP (sporulation delaying protein) and SKF (sporulation killing factor) which will be described in detail later (see below).

One previously reported lantibiotic peptide produced by *B. subtilis* 168 is sublancin, encoded within the SPβ-prophage. Its structure contains a characteristic dehydroalanine residue and a methyllanthionine bridge (Paik et al., 1998). Usually, lantibiotics and other ribosomally
synthesized AMPs harbor an N-terminal leader peptide sequence for export and a C-terminal core which finally constitutes the mature peptide (Willey & van der Donk, 2007). This initial precursor peptide is then posttranslationally modified by enzymes to release the mature and active peptide. However, the B. subtilis 168 genome does not harbor such enzyme loci for lantibiotics (Oman et al., 2011). Oman and coworkers studied sublancin biosynthesis and postulated that it is not a lantibiotic but rather a very uncommon S-linked glycopeptide (Oman et al., 2011). The biosynthetic loci for sublancin contain the gene sunA and two genes, bdbA and bdbB, encoding two thiol-disulfide oxidoreductases. Additionally, the immunity protein SunI (formerly YolF) is encoded within the same locus. Furthermore, it has been proposed that SunS (formerly YolJ) is a glycosyltransferase mediating addition of glucose to the cysteine residue at position 22 (Oman et al., 2011). The active and mature peptide is then transported across the membrane by the ABC transporter SunT. Expression of sunA is known to be repressed during exponential growth phase by the transcriptional regulators AbrB and Rok (Albano et al., 2005, Strauch et al., 2007). Transcription of sunA is initiated due to AbrB inhibition at the onset of stationary phase by the master regulator of sporulation, Spo0A (see also section 1.5, Fig. 1.9).

Subtilosin A is another bacteriocin produced by B. subtilis 168. It is also known to be repressed by AbrB and Rok during logarithmic growth but transcription is additionally dependent on the two-component regulatory proteins ResDE (Nakano et al., 2000, Albano et al., 2005). Thus, it is induced under stress conditions like nutrient or oxygen depletion in stationary phase (Nakano et al., 2000). Subtilosin A (SboA) is encoded within the sbo-alb operon overlapping with sboX, which constitutes another bacteriocin-like product and the albABCDEFG genes required for processing, export and immunity of the circular peptide. AlbA and AlbF have been shown to be essential for SboA biosynthesis and probably processing and maturation of the linear SboA precursor into a cyclic thioether-bridged peptide (Zheng et al., 2000). AlbB, AlbC and AlbD have been reported to be involved in mediating immunity, while the functions of SboX and AlbG still remain elusive (Zheng et al., 2000). Due to their homology to zinc-dependent proteases, AlbE and AlbF represent putative peptidases and a role in leader-peptide cleavage has been proposed (Flühe et al., 2012).

The YydF peptide encoded in the yydFGHIJ operon represents another peptide which is produced by B. subtilis 168 at the onset of stationary phase. Its production is again repressed by AbrB during exponential growth. At the transition from logarithmic to stationary phase, AbrB repression is released and transcription of the yydFGHIJ operon is initiated (Butcher et al., 2007). It has been debated whether Rok is a regulator of yydFGHIJ expression or not (Albano et al., 2005, Butcher et al., 2007). Based on sequence homology studies, the YydF precursor is proposed to be modified by YydG, a predicted Fe-S oxidoreductase and to be proteolytically cleaved by the peptidase YydH. Export and immunity are hypothesized to be mediated by the predicted ABC transporter YydIJ (Butcher et al., 2007). It could be demonstrated that in the absence of YydIJ, the LiaSR system is highly induced probably due to accumulation of the YydF peptide causing cell envelope damage (Butcher et al., 2007).

One very remarkable AMP causing envelope damage by collapsing the proton motive force is the cannibalism toxin SdpC (for simplicity reasons the mature form will be called SDP hereafter) (Lamsa et al., 2012). SDP is a 42-amino acid linear AMP that is ribosomally synthesized and first
transcribed as an inactive precursor, pro-SdpC (Liu et al., 2010, Perez Morales et al., 2013). Pro-SdpC contains an N-terminal extension including its signal peptide sequence required for export which is proteolytically cleaved during export via the general secretory pathway (Linde et al., 2003, Perez Morales et al., 2013). Subsequent disulfide bond formation is needed for the full activity of the peptide but is not essential. Further N- and C-terminal processing of the precursor peptide requires two extra proteins, SdpA and SdpB, encoded within the same operon (Fig. 1.3A).

SdpA is a cytosolic protein which also localizes to the membrane in a SDP-dependent manner. SdpB is predicted to constitute a membrane protein harboring six transmembrane helices (Perez Morales et al., 2013). Together, they are hypothesized to mediate the final processing of the SDP precursor peptide, thereby releasing the active and mature SDP toxin to the environment (Perez Morales et al., 2013). The gene sdpC is encoded in the sdpABC operon and is regulated by two promoters, P_{sdpA} and P_{sdpC} (Fig. 1.3A). Its dedicated immunity protein, SdpI, is expressed from an opposing operon, sdpRI, under control of P_{sdpR} (Fig. 1.3A). SdpI is a membrane protein proposed to be involved both in immunity and signal transduction. Although the mechanism of how SdpI provides immunity remains elusive, it was shown to be an important resistance determinant (Ellermeier et al., 2006). Its signaling properties come into play, when SDP is produced. SDP is proposed to bind to SdpI at the membrane and this complex then sequesters SdpR away from the DNA thereby inducing transcription of sdpRI (Ellermeier et al., 2006). SdpR is a negative regulator of its own promoter in the absence of SDP (Ellermeier et al., 2006). This repression is then relieved in the presence of SDP. Expression of the sdpABC and sdpRI operons is induced at the onset of stationary phase by indirect activation through the master regulator of sporulation, Spo0A (see also section 1.5, Fig. 1.9) (Gonzalez-Pastor et al., 2003). During exponential growth phase, transcription

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**Fig. 1.3:** Schematic representation of the genomic context of the sdpABC-sdpRI (A) and skfABCefgh (C) operon and processing of the SdpC (B) and SkfA (D) precursor into mature SDP and SKF. Genomic context of the sdpABC-sdpRI and skfABCefgh operon including all mapped promoters and terminators as well as the main transcripts are shown (A, C). Steps of SDP/SKF processing are indicated in (B, D). See text for details. This figure is based on (Liu et al., 2010, Nicolas et al., 2012, Perez Morales et al., 2013) and taken from (Höfler et al., 2015).
is blocked by the transition state regulator AbrB. This inhibition is then released by increasing concentrations of active Spo0A (Spo0A~P), thereby repressing AbrB activity (see also section 1.5, Fig. 1.9) (Fujita et al., 2005, Chen et al., 2006). Importantly, a broadly heterogeneous activation of Spo0A is the trigger for this transcription initiation (Chung et al., 1994). SDP is a cannibalism toxin able to lyse sensitive siblings. Cannibalism and its contribution to survival under stress conditions will be explained further in section 1.6.

In contrast to the sdpABC-sdpRI operon, information about the second cannibalism toxin, SkfA, is still limited (for simplicity reasons called SKF hereafter). SKF is encoded within the skfABCDEFGH operon (Fig. 1.3C) and is a ribosomally assembled AMP which needs posttranslational modifications in order to become fully active (Fig. 1.3D) (Gonzalez-Pastor et al., 2003). SKF was first described to be able to kill the Gram-positive plant pathogen Xanthomonas oryzae (Lin et al., 2001). SKF is a 26-amino acid cyclic sactipeptide harboring disulfide and thioether bonds (Liu et al., 2010, Arnison et al., 2013). The gene products of the skfABCDEFGH operon have been postulated to be involved in SKF maturation. SkfB is a radical SAM enzyme containing a 4Fe-4S cluster, which is needed for an unusual thioether bond formation in SKF between the cysteine residue Cys4 and the α-carbon of the methionine residue Met12 (Liu et al., 2010). SkfC is a member of the CAAX protease family and is assumed to be responsible for leader peptide cleavage and the cyclization reaction of the peptide. SkfE and SkfF are hypothesized to constitute an ABC transporter for export of and immunity against SKF. SkfE is a predicted ATPase while SkfF constitutes a permease. While the role of SkfG remains elusive so far, SkfH is a thioredoxin-oxidoreductase-like protein which is assumed to be involved in disulfide bond formation (Liu et al., 2010). The skfABCDEFGH operon is expressed in a growth phase-dependent manner and induced at the beginning of stationary phase and/or under nutrient limiting conditions. Like SDP, it is negatively regulated by AbrB and activated by increasing levels of phosphorylated Spo0A (see also Fig. 1.9) (Burbuly et al., 1991, Gonzalez-Pastor et al., 2003, Molle et al., 2003). Expression of SKF is regulated by one main promoter, PskfA, and a second one within skfC, as determined by a recent genome-wide transcriptome study (Nicolas et al., 2012).

All the above mentioned peptides share some characteristic features: They are encoded in operons with genes associated with their posttranslational modification and processing, export and immunity. Their promoters are regulated by transcriptional regulators (Rok, AbrB and Spo0A) usually in a growth phase- and nutrient-dependent manner. Dedicated immunity proteins ensure that toxin producers are resistant against their own peptides and only sensitive siblings are lysed. However, the spectrum of resistance mechanisms, which bacteria have evolved against AMPs, is large and is explained in more detail in the following section.
1.3 Resistance mechanisms against antimicrobial peptides

AMPs often lead to the induction of stress response countermeasures, which are specifically induced upon stress signal occurrence. There are several mechanisms bacteria employ to counteract such stresses.

One mechanism describes the destruction or modification of the antibiotic, thereby rendering it inactive (Breukink & de Kruijff, 2006). For instance, the β-lactam ring of β-lactam antibiotics structurally resembles the D-Ala-D-Ala moiety of the UDP-MurNAc pentapeptide of the peptidoglycan units. As a consequence, they are recognized by transpeptidases and block crosslinking of the glycan chains (Fig. 1.2) (Strominger & Tipper, 1965). Resistance is achieved by β-lactamases, which hydrolyze the β-lactam rings (Ghuysen, 1991).

Another mechanism many bacteria use is to shield the target of the antibiotic such that the antimicrobial substances cannot access the target. Bacteria are able to reduce the accessibility of lipid II by changing the cell wall composition (Davies et al., 1996, Maisnier-Patin & Richard, 1996, Verheul et al., 1997, Crandall & Montville, 1998, Mantovani & Russell, 2001, Kramer et al., 2004). Usually, the cell wall of Gram-positive bacteria is negatively charged due to the phosphate groups of the teichoic acids. AMPs are cationic and amphipathic molecules with positive charges that facilitate contact with their targets at the cell surface. Therefore, one possible resistance mechanism for the cell is to incorporate positive charges into the growing cell wall. This leads to an electrostatic repulsion between cationic AMPs (CAMPs) and the bacterial cell envelope (Peschel & Sahl, 2006). Incorporation of positive charges is achieved by coupling D-alanine residues to teichoic acids resulting in an overall positive charge (Neuhaus & Baddiley, 2003). For example, B. subtilis regulates its cell wall charge via upregulation of the dltABCDE operon resulting in D-alanylation of teichoic acids (Perego et al., 1995, Neuhaus & Baddiley, 2003, Reichmann et al., 2013). These positive charges of the cell wall repel the positively charged antimicrobial substances and as a consequence, access to the antibiotic target structures is denied.

A second mode of action involving the dlt system is based on steric hindrance of CAMPs. Through D-alanylation of the teichoic acids, the cell wall becomes more compact and dense, leading to impermeability of the CAMPs to the membrane (Revilla-Guarinos et al., 2014).

The next possible resistance mechanism bacteria have evolved to cope with antibiotic stress is the presence of resistance pumps. Usually, these pumps constitute ABC transporters which can be coupled to a 2CS for signal transmission. Such ABC transporters can also be found within the cell envelope stress response network of B. subtilis, which will be described in more detail in the following sections. ABC transporters can also function as exporters for the synthesized AMP, in addition to being immunity determinants. One example is YydJ, which is postulated to be an ABC transporter/immunity determinant of the YydF peptide (Butcher et al., 2007). Another example is SkfEF constituting a possible exporter/immunity protein of the cannibalism toxin SKF.
As mentioned above, the cell envelope of a microbial cell constitutes a key target for many AMPs produced and secreted by other species. In order to survive in competitive, AMP-rich environments, bacteria need to sense cell wall/membrane damage and the respective cell wall active compounds to protect themselves from irreversible damage. For this, they have evolved a set of cell envelope stress response (CESR) systems to cope with stress signals from the environment. These signals provoke a stimulus-specific response within the cell typically through differential gene expression to mediate resistance by mounting protective countermeasures.

Bacteria employ three major routes of transmembrane signaling: one-component systems (1CSs), two-component systems (2CSs) and alternative extracytoplasmic function sigma factors (ECF σ factors) (Staroń & Mascher, 2010). In 1CSs, the input and output domains are located on a single polypeptide chain. Signal perception by the input domain modulates the activity of the output domain which then acts as a transcriptional regulator binding to its target genes to repress or activate transcription (Ulrich et al., 2005). 1CSs usually play a minor role in transmembrane signaling due to their restricted protein architecture. In contrast, input and output domains of 2CSs and ECF σ factors are not encoded by a single polypeptide chain. Instead, they are separated on two proteins which facilitate signal transduction derived from the extracellular environment (Mascher et al., 2006, Staroń et al., 2009).

Typical 2CSs consist of a membrane-spanning histidine kinase (HK) and a cognate cytoplasmic response regulator (RR). The HK acts as a sensor protein which is able to detect specific stimuli from the environment via its extracellular N-terminal input domain. Subsequently, it undergoes a conformational change leading to autophosphorylation of a conserved histidine residue in its C-terminal transmitter domain. The phosphoryl group is then transferred to the RR resulting in phosphorylation of a conserved aspartate residue in the receiver domain. In its phosphorylated (activated) state, the RR binds to specific target promoters and can modulate the transcription of target genes. In order to set the system back to the pre-stimulus state, dephosphorylation of the RR can be accomplished by the phosphatase activity of the HK, by the RR itself or by external phosphatases (Stock et al., 2000, Mascher et al., 2006).

ECF σ factors are controlled by their cognate anti-σ factor consisting of a cytoplasmic and an extracellular domain linked by one transmembrane helix. In the absence of stress signals, the anti-σ factor solidly binds to its ECF σ factor and keeps it inactive. However, in the presence of a specific stimulus, the anti-σ factor gets inhibited and releases the ECF σ factor which can interact with the RNA polymerase (RNAP) and activate transcription of downstream target genes (Helmann, 2002).

The CESR network of B. subtilis consists of seven ECF σ factors and four 2CSs. They have been well studied and characterized over the last decade both at the level of differential gene expression and protein production (Wecke & Mascher, 2011) and have been described to respond to various cell wall antibiotics (Fig. 1.4) (Jordan et al., 2008). Out of the seven ECF σ factors, three appear to play important roles in the CESR, σM, σX and σW, each regulating a set of ~30-60 target genes with
partially overlapping specificity (Mascher et al., 2007, Kingston et al., 2013). Furthermore, three out of the four 2CSs, the BceRS, PsdRS and YxdJK systems, specifically respond to a variety of AMPs and mediate resistance against them (Staroń et al., 2011). These three 2CSs are referred to as Bce-like 2CSs because they share the same protein architecture and mechanistic features. They are all linked to and regulate the expression of genes encoding ABC transporters which are strongly induced by specific AMPs and represent the resistance determinant of each system (Jordan et al., 2008, Staroń et al., 2011). Moreover, the ABC transporters are required for sensing the AMPs and for transmission of the information to the cognate 2CS (Rietkötter et al., 2008, Staroń et al., 2011). All 2CSs, together with their cognate ABC transporters, comprise the so-called detoxification modules of B. subtilis (Staroń et al., 2011). The fourth 2CS, the LiaSR system, responds to a broader range of AMPs and is presumably involved in envelope damage sensing (Wolf et al., 2012).

This thesis focuses mainly on the BceRS-like and LiaSR 2CSs as well as in parts on the ECF σ factors σ^μ, σ^X and σ^W of B. subtilis. Therefore, these systems will be described in detail in the following sections.

![Cell envelope stress response network of B. subtilis](image)
1.4.1 The LiaSR two-component system

LiaSR-like systems are highly conserved within the Firmicutes phylum of Gram-positive bacteria. The best characterized system until now is the LiaSR system in *B. subtilis* (Fig. 1.5). It has been originally discovered and described in the course of studying the bacitracin stimulon (Mascher *et al.*, 2003). Its name, LiaSR, derives from “lipid II cycle Interfering antibiotic sensor and response regulator” and is one of the 2CSs within the CESR network (Mascher *et al.*, 2004, Jordan *et al.*, 2006). As its name implies, it is primarily induced by AMPs interfering with the lipid II cycle of cell wall biosynthesis, e.g. bacitracin, vancomycin or cationic AMPs (CAMPs) as shown in Fig. 1.2 and 1.4 (Mascher *et al.*, 2004, Pietiäinen *et al.*, 2005). Additionally, it responds to more unspecific stimuli such as alkaline shock, detergents or organic solvents (e.g. ethanol, phenol) although to a much weaker extent (Petersohn *et al.*, 2001, Wiegert *et al.*, 2001, Mascher *et al.*, 2004, Pietiäinen *et al.*, 2005, Tam le *et al.*, 2006). Given the lack of substrate specificity of the LiaSR system, a direct role in mediating resistance against these AMPs could not be shown. However, current data indicate some level of interdependence between the different CESR systems regarding resistance to bacitracin. If the BceRS system is deleted, cells are up to 85-fold more sensitive to bacitracin compared to wild type. If liaIH is deleted additionally, sensitivity increases up to 512-fold although a liaIH single mutant is as sensitive as wild type (personal communication, Georg Fritz). Hence, double mutants reveal hidden layers of resistance of the different CESR systems. Therefore, the LiaSR system is proposed to be rather a damage sensing system and to represent a secondary resistance layer when the primary layer (BceAB) is missing (Rietkött er *et al.*, 2008, Wolf *et al.*, 2012) (personal communication, Georg Fritz).

The HK, LiaS, constitutes an intramembrane-sensing HK with two transmembrane helices linked by a short extracellular loop (Mascher, 2006, Mascher, 2014). The second helix is connected to a cytoplasmic HAMP (short for: present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases) domain which is presumably involved in intramolecular signal conversion (Hulko *et al.*, 2006, Mascher, 2014). Upon activation, LiaS undergoes a conformational change leading to autophosphorylation at a conserved histidine residue. The phosphoryl group is...
then transferred to an aspartate residue of its cognate RR, LiaR, within its conserved N-terminal receiver domain. This activation of LiaR leads to binding of target promoters on the DNA via its C-terminal DNA binding domain containing a characteristic helix-turn-helix motif (Jordan et al., 2006).

The LiaSR 2CS is genetically and functionally associated with an accessory protein, LiaF. This membrane protein has been shown to keep LiaS inactive under non-inducing conditions. Current research implies that such accessory proteins are the actual sensor component and the transmembrane helices within the kinase serve as signal transfer regions that connect the signal perceived by the accessory protein with the phosphorylation status of the RR (Mascher, 2014).

The lia operon consists of six genes in total, liaH-GFSR. The genes encoding the 2CS are under control of a constitutive promoter upstream of liaG (P\textsubscript{liaG}, Fig. 1.5) (Jordan et al., 2006). This ensures appropriate amounts of the respective signaling proteins, Lia(F)SR, in case of cell envelope stress stimuli. Inducing conditions then lead to a strong LiaR-dependent activation of the promoter upstream of liaI (P\textsubscript{liaI}). Activation of P\textsubscript{liaI} results in the expression of two transcripts: the first transcript covers liaIH, being the major transcript of 1.1 kb in size. The second one comprises the whole lia operon, liaIH-GFSR of about 4 kb. This is due to a weak terminator downstream of liaH leading to substantial read-through from P\textsubscript{liaI} into the downstream genes (Mascher et al., 2004). P\textsubscript{liaI} is the only relevant target promoter of LiaR; however, the exact physiological role of LiaIH has not been characterized in detail yet. Recent studies demonstrate that the small membrane protein Lial serves as a membrane anchor for the phage-shock protein A homolog, LiaH, upon cell envelope stress conditions (Dominguez-Escobar et al., 2014). While LiaH has been shown to localize in the cytosol under non-inducing conditions and Lial localizes in highly motile foci within the cytoplasmic membrane, this pattern changes upon inducing conditions: Lial and LiaH co-localize into distinct static foci at the membrane presumably to protect the envelope from the AMP-induced membrane damage (Dominguez-Escobar et al., 2014).

The LiaSR system is not only induced upon external addition of cell wall antibiotics and other less specific stimuli. It has been shown to be also intrinsically induced in a growth phase-dependent manner (Fig. 1.6) (Jordan et al., 2007). During transition from exponential to stationary growth phase, B. subtilis cells undergo a complex differentiation cascade in order to adapt to changing environmental conditions, e.g. nutrient limitation. This cascade is regulated by the master regulator of sporulation, Spo0A. It orchestrates the conversion from vegetative cells to highly resistant dormant endospores (see also section 1.5) (Msadek, 1999, Phillips & Strauch, 2002, Errington, 2003, McKenney et al., 2013). During exponential growth phase, the transition state regulator AbrB represses P\textsubscript{liaI} activity by direct binding to the liaI promoter region (Fig. 1.6, right). Additionally, under non-inducing conditions, LiaF inhibits LiaS and keeps it in its OFF-state. During transition from exponential to stationary phase, Spo0A becomes active (Spo0A-P) and inhibits AbrB (Fig. 1.6, left), thereby releasing AbrB on P\textsubscript{liaI}. But this indirect activation of P\textsubscript{liaI} is not sufficient to induce transcription of liaH, since the system needs to be activated simultaneously by an intrinsic stimulus to release the LiaS inhibition by LiaF. This results in the activation of LiaR and subsequent initiation of transcription of liaIH (Jordan et al., 2007). One previous report suggested that the YydF peptide
encoded in the yydFGHIJ operon is able to induce the LiaSR system in the absence of its own immunity ABC transporter YydIJ (Butcher et al., 2007). Expression of the YydF peptide was shown to be repressed by AbrB during vegetative growth. At the onset of stationary phase, AbrB repression is released by Spo0A and the peptide is most probably produced (Butcher et al., 2007). Indeed, our own unpublished data suggest that YydF is an inducer of the LiaSR system during transition phase. However, transition phase induction is significantly weaker compared to the strong induction by cell wall antibiotics (approx. 10-15-fold vs. 100-fold) (Jordan et al., 2007).

In conclusion, the LiaSR system is induced by a wide range of AMPs and some rather unspecific stimuli perturbing the cell membrane as well as during transition from exponential to stationary growth phase. This intrinsic activation can be attributed to the YydF peptide. Upon intrinsic activation of the LiaSR system, subsequent gene expression involves at least five regulatory proteins: LiaF, LiaS and LiaR as well as AbrB and Spo0A (Jordan et al., 2007). The biological significance of liaIH induction has been investigated recently (Dominguez-Escobar et al., 2014). LiaI has been suggested to scan the membrane and recruit LiaH in the presence of membrane damage to co-localize into static foci to shield against AMP-generated membrane damage.

1.4.2 Bce-like two-component systems of B. subtilis

The remaining three 2CSs of the cell envelope stress response in B. subtilis, the BceRS, PsdRS and YxdJK systems, are all genetically and functionally linked to genes encoding ABC transporters (Dintner et al., 2011). They represent so-called AMP detoxification modules since they have been shown to respond to and mediate resistance against a wide range of AMPs. The best characterized module is BceRSAB, which specifically responds to bacitracin and to a lesser extent to the lantibiotics actagardine and mersacidin (Staroń et al., 2011). The 2CS consists of an intramembrane-sensing HK, BceS, and its cognate cytosolic RR, BceR. Like LiaS, BceS is not the sensor component of the system. It is again an accessory protein, the ABC transporter BceAB, which comprises the sensing part of the system (Fig. 1.7). It consists of an ATPase domain (BceA) and a permease domain (BceB) harboring ten transmembrane helices and a large unique
exocellular loop structure between transmembrane helix seven and eight (Rietkötter et al., 2008). Under inducing conditions, bceAB expression is upregulated to confer resistance to the peptide antibiotic. However, this upregulation of bceAB requires the BceAB ABC transporter itself for signal perception. It has been shown that BceS alone is unable to sense stimuli and therefore insufficient for signal transduction (Bernard et al., 2007, Rietkötter et al., 2008, Dintner et al., 2014). Hence, only in presence of BceAB, BceS is able to be activated by autophosphorylation upon peptide antibiotic stress. Subsequent phosphoryl group transfer to BceR results in DNA binding to the bceA promoter region and initiation of bceAB transcription. This autoregulation of BceAB ensures that resistance to an AMP can be maintained any time. Notably, it has been shown recently that BceAB senses the transport flux of bacitracin, thereby directly monitoring its current detoxification capacity (Fritz et al., 2015). This enables a cost-efficient and precise regulation of antibiotic resistance depending on the current capacity (Fritz et al., 2015).

The other two Bce-like 2CSs described in B. subtilis, PsdRS and YxdJK, are paralogous in sequence and genomic context organization to the BceRS 2CS, but differ in their substrate specificity. The PsdRS 2CS responds primarily to lantibiotics such as nisin, subtilin, actagardine and gallidermin and to the lipopeptide enduracidin, which all interfere with the lipid II cycle (Staroń et al., 2011). The PsdRS 2CS also responds to bacitracin, but to a much weaker extent. This is due to cross-activation of the PsdR RR by the paralogous BceS HK (Mascher et al., 2003, Rietkötter et al., 2008).

Little is known about the YxdJK 2CS and its cognate ABC transporter YxdLM. The expression of yxdLM is dependent on the YxdJ RR and it has been shown to respond to the human cationic antimicrobial peptide LL-37 (Joseph et al., 2004, Pietiäinen et al., 2005, Staroń et al., 2011). The question remains why a soil-living bacterium has evolved a system that responds to a human peptide. Since LL-37 is, so far, the only inducer of the YxdJK 2CS, the biological significance remains elusive.

1.4.3 ECF σ factors σ^M, σ^X and σ^W – a short overview


In general, σ factors are part of the RNA polymerase (RNAP). They are able to bind to the core enzyme and direct the RNAP holoenzyme to appropriate promoters to initiate transcription. All
bacteria contain primary (or housekeeping) σ factors that are responsible for the expression of most of the genes. Additionally, many bacteria living in complex habitats, such as the soil, also contain alternative σ factors, which are only activated under specific conditions. ECF σ factors constitute a subgroup of alternative σ factors. They are able to replace primary σ factors to redirect the RNAP and activate transcription of a different set of genes from specific alternative promoters (Helmann, 2002). ECF σ factors are kept inactive under non-inducing conditions by their cognate anti-σ factor through direct protein-protein interactions (Brown & Hughes, 1995). They are usually co-expressed with their anti-σ factors (see Fig. 1.4). In the presence of a stimulus, the ECF σ factor is released from the anti-σ factor, resulting in binding to the RNAP core enzyme and activating transcription from alternative promoters (Helmann, 2002). Besides this extrinsic induction by specific compounds, intrinsic induction of the ECF σ factors, i.e. without any external stimulus, occurs in a growth phase- and growth medium-dependent manner (Huang et al., 1998). While σW and σX are induced generally in late logarithmic growth phase, σW only becomes active in early stationary phase (Huang et al., 1998, Nicolas et al., 2012).

One of the best characterized ECF σ factors is σW regulating the activity of about 30 promoters which control expression of approx. 60 genes (see Fig. 1.4). These genes often encode proteins or peptides involved in detoxification. Thus, σW has been postulated to mediate intrinsic immunity against a wide range of antibiotic compounds (Cao et al., 2001, Butcher & Helmann, 2006). σW is particularly activated by cell wall antibiotics such as vancomycin or cephalosporin C (Cao et al., 2002) as well as alkaline shock (Wiegert et al., 2001). However, σW does not seem to be required for resistance against these compounds despite its strong induction (Cao et al., 2002). Nevertheless, a sigW mutant has been shown to be more susceptible to a broad range of AMPs produced by other Bacillus species (Butcher & Helmann, 2006).

The second ECF σ factor involved in the CESR is σM (see Fig. 1.4). It is predominantly induced by acid, heat, salt and superoxide stress conditions as well as by specific cell wall antibiotics such as bacitracin or vancomycin (Cao et al., 2002, Mascher et al., 2003, Thackray & Moir, 2003). Similar to σW, σM regulates the expression of about 60 genes within 30 operons upon stress conditions (Eiamphungporn & Helmann, 2008). These genes of the σM regulon have distinct functions varying from cell wall synthesis, shape determination, cell division, DNA damage monitoring and detoxification (Eiamphungporn & Helmann, 2008). One example is the expression of bcrC, which is upregulated upon bacitracin exposure. Elevated levels of BcrC contribute to resistance against bacitracin (Cao & Helmann, 2002). Its expression is dependent on σM, however, bcrC has been identified to constitute an in vitro target of σX-directed transcription and was shown to be part of the σW-regulon as well (Cao & Helmann, 2002, Pietiäinen et al., 2005).

The third ECF σ factor is σX (see Fig. 1.4). It controls the expression of about ten operons encoding proteins primarily involved in cell envelope composition and cell surface modification (Cao & Helmann, 2004). One of the target operons of σX is the dltABCDE operon. It is responsible for introducing positively charged amino acids (D-alanylation) into the teichoic acids, thereby reducing the overall negative charge of the cell wall (Neuhaus & Baddiley, 2003). This significantly decreases susceptibility to cationic AMPs and constitutes an important resistance mechanism. In
fact, a $\text{sig}X$ mutant is prone to autolysis and more sensitive to nisin which has been shown to form pores in the membrane (van Heusden et al., 2002, Cao & Helmann, 2004).

In conclusion, ECF $\sigma$ factors are involved in the complex CESR of $B. \text{subtilis}$ controlling an “antibiosis regulon” which counteracts cell wall stress. The ECF $\sigma$ factor response is complex, since it has been shown that $\sigma^W$, $\sigma^M$ and $\sigma^X$ control an overlapping set of genes (see Fig. 1.4) (Mascher et al., 2007, Kingston et al., 2013). With this overlapping specificity it is possible to induce appropriate countermeasures against stresses although one $\sigma$ factor might be missing.

1.5 Sporulation – taking the last exit

$B. \text{subtilis}$ is a widely used Gram-positive model organism to study basic and complex cellular mechanisms. It is able to form endospores, which involves a complex sporulation cascade. Many genes and gene products are associated with sporulation and a combined interplay is necessary to coordinate the steps in the spore development. Fig. 1.8 gives an overview of the sporulation cycle. The sporulation process starts when vegetatively growing cells suffer from nutrient exhaustion (Trach et al., 1991). Formation of highly resistant and dormant endospores is the only way bacteria can overcome starvation conditions in a long-term perspective. The whole cycle is regulated by the master regulator of sporulation, Spo0A. As depicted in Fig. 1.8, the cycle starts with an asymmetric polar division into two compartments, the mother cell and the forespore (or prespore), which is orchestrated by genes regulated by Spo0A and $\sigma^H$ (Levin & Losick, 1994). Within both compartments, spore development is tightly organized by an elaborate set of genes activated by a series of compartment-specific $\sigma$ factors (Errington, 2003, Tan & Ramamurthi, 2014).

Once the spore has matured and the spore coat has formed, the mother cell lyses and releases the spore to the environment (Errington, 2003, McKenney et al., 2013, Tan & Ramamurthi, 2014). Outgrowth of the spore occurs under nutrient-rich conditions, upon which it can re-enter its vegetative cycle. As already mentioned, starvation and high cell densities promote sporulation by activation of the transcriptional regulator, Spo0A.

**Fig. 1.8:** The sporulation cycle of $B. \text{subtilis}$. Sporulation includes a complex differentiation cascade and involves different stages of forespore (prespore) formation and maturation until the final release of the spore. See text for details. This figure is taken and modified from (Errington, 2003).
Indeed, Spo0A as being the master regulator of sporulation is part of a well-studied phosphorelay system (Burbulys et al., 1991). Several kinases (KinA, KinB, KinC, KinD and KinE) are involved in that system and activated by different stimuli. KinA and KinB primarily respond to changes in ATP levels whereas KinC and KinD are activated by unknown signals but appear to be relevant for controlling the expression of cannibalism genes and are important for biofilm formation (LeDeaux et al., 2010, Eswaramoorthy et al., 2010, Devi et al., 2015). KinE does not play a role in sporulation (Fujita et al., 2005). Upon activation of KinA and KinB, they undergo subsequent autophosphorylation and indirectly phosphorylate Spo0A. This is achieved by two intermediates Spo0F and Spo0B (Burbulys et al., 1991, Higgins & Dworkin, 2012, Boguslawski et al., 2015). KinA and KinB transfer the phosphoryl group to Spo0F which then, in turn, phosphorylates Spo0B. Thereupon, Spo0B→P transfers the phosphoryl group to Spo0A and sporulation is subsequently initiated.

This tight regulation of sporulation is crucial since sporulation is a very energy-demanding process and irreversible once the asymmetric septum is formed (Parker et al., 1996). Additionally, different levels of Spo0A→P lead to different gene expression. High levels of Spo0A→P initiate sporulation, while low concentrations of Spo0A→P regulate a different set of genes in the cell. This includes genes for production, export and immunity to toxins or AMPs mostly indirectly via repression of the transition state regulator AbrB (Fig. 1.9). The two cannibalism toxins, SDP and SKF, are induced by this pathway. This difference in activation of different sets of genes is due to the binding affinity of Spo0A→P to different promoter regions (Fujita et al., 2005). Genes which require a high level of Spo0A→P to be induced were shown to have low binding constants, while genes which need lower levels of Spo0A→P revealed high binding affinities or were indirectly activated by Spo0A-mediated relief of repression by AbrB. Cell-to-cell variations in (in)active Spo0A lead to a highly heterogeneous population at the onset of stationary growth phase where nutrients become scarce (Figs 1.9 and 1.10).

One important effect of Spo0A→P at the beginning of transition phase adaptation is the repression of the negative regulator AbrB (Fig. 1.9) (Perego et al., 1988). AbrB is active during logarithmic growth phase and known to repress many genes involved in sporulation or AMP production (Strauch et al., 1989, Albano et al., 2005, Stein, 2005). When Spo0A→P levels begin to increase, abrB is directly repressed (Strauch et al., 1990). Both cannibalism operons (sdpABC-sdpRI and sfbABCDEFGH) are under direct negative control of AbrB and indirectly
activated by Spo0A. Cannibalism describes a strategy that cells usually employ before they undergo sporulation. This will be described in the following section.

1.6 Cannibalism – a strategy to delay sporulation

Cannibalism is a social behavior and occurs during the early stages of sporulation of *B. subtilis*. It has been proposed to be a sporulation delay strategy to overcome temporary nutrient limitation dependent on the master regulator of sporulation, Spo0A (Gonzalez-Pastor *et al.*, 2003). Cannibalism can be particularly beneficial for the cells since the sporulation cascade is very energy-consuming and irreversible once the asymmetric septum is formed (Parker *et al.*, 1996). The basis for cannibalism is the heterogeneity of a sporulating population. It can be divided into two subpopulations: (1) sporulating cells with active Spo0A and (2) nonsporulating cells with inactive Spo0A (Fig. 1.10).

Induction of the cannibalism operons *sdpABC-sdpRI* and *skfABCEFGH* is regulated in an AbrB- and Spo0A-dependent manner (see also section 1.2) (Fujita *et al.*, 2005, Chen *et al.*, 2006). Transcription of both operons is repressed by AbrB during logarithmic growth and repression is released by rising levels of active Spo0A during early stationary phase. Hence, low levels of Spo0A~P indirectly activate expression of the *sdpABC-sdpRI* and *skfABCEFGH* operons (Strauch *et al.*, 1990, Fujita *et al.*, 2005, Chen *et al.*, 2006). Additionally, the *skf* regulatory region harbors a high-affinity binding site for Spo0A resulting in activation at low doses of Spo0A~P. This means that *skfABCEFGH* expression is not only indirectly activated by Spo0A via AbrB relief but also directly induced by Spo0A (Fujita *et al.*, 2005, Gonzalez-Pastor, 2011). On the other hand, it has been demonstrated that high levels of Spo0A~P can repress the expression of *sdpABC-sdpRI* by binding of Spo0A~P to a low affinity binding site within the *sdp* regulatory region (Fujita *et al.*, 2005). These cell-to-cell

![Fig. 1.10: Simplified schematic overview of the underlying mechanisms driving cannibalism.](Image) Cannibalism is based on a heterogeneous activation of Spo0A at the onset of stationary growth phase. Spo0A-ON cells activate transcription of the *skfABCEFGH* and *sdpABC-sdpRI* operons whereas in Spo0A-OFF siblings expression of these operons is repressed. Lysed cells supply the cannibal subpopulation with nutrients which delays sporulation significantly. This figure is adapted from (Engelberg-Kulka *et al.*, 2006).
variations of different levels of active Spo0A lead to a stochastic activation of the \textit{sdpABC-sdpRI} and \textit{skiABCEFGH} operons within one bacterial population giving rise to two subpopulations (Spo0A-ON and Spo0A-OFF) (Chung \textit{et al.}, 1994, Chastanet \textit{et al.}, 2010). Spo0A-ON cells do not only produce and secrete the respective cannibalism toxins but also simultaneously express immunity proteins to ensure that the producers are not killed by their own toxins (see also section 1.3). Accordingly, in Spo0A-OFF cells the cannibalism operons are still repressed by AbrB. As a consequence, neither toxins nor immunity proteins are made, rendering Spo0A-OFF cells sensitive to the toxins produced by their siblings (Gonzalez-Pastor \textit{et al.}, 2003, Ellermeier \textit{et al.}, 2006). As a result, they are prone to lyse and release nutrients to the starved environment. The cannibal cells are then able to feed on these nutrients and sporulation is arrested until the new nutrients are exhausted again. This cycle of starvation, initiation of cannibalism and resumption of growth continues until the majority of cells has transformed into spores.

The biological significance of cannibalism becomes evident when considering the fact that spore formation is highly energy-demanding and reversible only up to a certain point (Parker \textit{et al.}, 1996). After this checkpoint, the cells are not able to resume vegetative growth although nutrients might be available again. Additionally, completion of sporulation takes several hours. This might result in having a disadvantage over other microorganisms in their natural environment or just over nonsporulating sister cells. Cannibalism helps to maintain a mixed population during stationary phase with few spores, some cells already committed to sporulation and many cells vegetatively growing.

1.7 Aims of this thesis

The LiaSR system of \textit{B. subtilis} responds to a great variety of peptide antibiotics including the cyclic AMP bacitracin. It has been shown previously that the LiaSR system is highly induced by bacitracin. Whole population studies revealed a heterogeneous induction pattern of the LiaSR system depending on the extracellular bacitracin concentration. In CHAPTER II, the aim was to investigate this heterogeneity of the LiaSR response to different external concentrations of bacitracin on single cell level. We used quantitative fluorescence microscopy including time-lapse microscopy to elucidate the heterogeneous induction of the LiaSR system in a bacitracin-dependent manner.

Although the LiaSR system has been extensively studied over the last decade, its physiological role remained unclear. Despite its strong and fast induction by various cell wall antibiotics, the LiaSR system only seems to provide some secondary resistance layer when the primary resistance determinant is missing. While LiaF, LiaS and LiaR have been characterized well over the last years, the function of LiaI and LiaH remained elusive. The aim of CHAPTER III was to gain deeper insight into the physiological role of the LiaSR system by using time-lapse microscopy to study the subcellular localization and interaction of LiaI and LiaH in the presence and absence of bacitracin.
In addition to its strong and fast activation by external cell wall antibiotics, the LiaSR system was shown to be induced intrinsically without any external stimulus at the transition from exponential to stationary growth phase. This transition phase induction is partly due to Spo0A-mediated derepression of the LiaSR system via inhibition of its negative regulator AbrB during logarithmic growth phase but is not sufficient for induction. In the course of studying this intrinsic activation, we observed that the BceRS and PsdRS 2CSs as well as the ECF σ factors σM, σX and σW are also induced in stationary growth phase by an unknown stimulus. In CHAPTER IV, the aim was to determine the stimuli leading to the intrinsic activation of these systems. We used a lux-reporter system to monitor target promoter activity of each system in a microplate reader and screened for a variety of peptide antibiotics and toxins produced by *B. subtilis* 168 in stationary growth phase.
CHAPTER II

Immediate and Heterogeneous Response of the LiaFSR Two-Component System of *Bacillus subtilis* to the Peptide Antibiotic Bacitracin

Sara Kesel, Andreas Mader, Carolin Höfler, Thorsten Mascher, Madeleine Leisner

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Immediate and Heterogeneous Response of the LiaFSR Two-Component System of Bacillus subtilis to the Peptide Antibiotic Bacitracin

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Abstract

Background: Two-component signal transduction systems are one means of bacteria to respond to external stimuli. The LiaFSR two-component system of Bacillus subtilis consists of a regular two-component system LiaRS comprising the core Histidine Kinase (HK) LiaS and the Response Regulator (RR) LiaR and additionally the accessory protein LiaF, which acts as a negative regulator of LiaRS-dependent signal transduction. The complete LiaFSR system was shown to respond to various peptide antibiotics interfering with cell wall biosynthesis, including bacitracin.

Methodology and Principal Findings: Here we study the response of the LiaFSR system to various concentrations of the peptide antibiotic bacitracin. Using quantitative fluorescence microscopy, we performed a whole population study analyzed on the single cell level. We investigated switching from the non-induced ‘OFF’ state into the bacitracin-induced ‘ON’ state by monitoring gene expression of a fluorescent reporter from the RR-regulated liaI promoter. We found that switching into the ‘ON’ state occurred within less than 20 min in a well-defined switching window, independent of the bacitracin concentration. The switching rate and the basal expression rate decreased at low bacitracin concentrations, establishing clear heterogeneity 60 min after bacitracin induction. Finally, we performed time-lapse microscopy of single cells confirming the quantitative response as obtained in the whole population analysis for high bacitracin concentrations.

Conclusion: The LiaFSR system exhibits an immediate, heterogeneous and graded response to the inducer bacitracin in the exponential growth phase.

Introduction

Two-component systems (TCS) are a fundamental principle of bacterial signal transduction that enables cells to respond to environmental stimuli [1–3]. These phosphotransfer systems involve two conserved components, a histidine protein kinase (HK) and a response regulator protein (RR). Extracellular stimuli are sensed by the HK, leading to its autophosphorylation [4]. The phosphoryl group is then transferred from the HK to the RR. The RR, now in its ‘active’ form, elicits the specific response. Bacteria such as Escherichia coli or Bacillus subtilis posses about 30 HKs and RR (summarized in [24] and [25]). The Lia system, is comprised of the LiaRS TCS, with the HK LiaS and the RR LiaR, and additionally the accessory protein LiaF (Figure 1). The latter is only triggered after a particular stimulus concentration has been overcome. The response itself can thereby be homogeneous (the whole population behaves in the same way) or heterogeneous with parts of the population behaving differently than the others. Regardless of the observed output, regulation of both types of systems can involve a number of auxiliary protein components. Systems involving accessory proteins [15–17], often referred to as three-component systems, also include peptide antibiotic-sensing systems of Gram-positive bacteria [18,19,20].

One such system is the LiaFSR cell envelope stress response module of Bacillus subtilis [21,22], which strongly responds to various peptide antibiotics such as bacitracin, nisin, vancomycin or daptomycin [23], but also to other less specific envelope perturbing conditions, such detergents or alkaline shock (summarized in [24] and [25]). The Lia system is comprised of the LiaRS TGS, with the HK LiaS and the RR LiaR, and additionally the accessory protein LiaF (Figure 1). The latter is associated with all LiaRS-like TCS and acts as a negative regulator of LiaR-mediated gene regulation [21]. The mechanism by which response [7], others result in an all-or-nothing response [14]. The latter is only triggered after a particular stimulus concentration has been overcome. The response itself can thereby be homogeneous (the whole population behaves in the same way) or heterogeneous with parts of the population behaving differently than the others. Regardless of the observed output, regulation of both types of systems can involve a number of auxiliary protein components. Systems involving accessory proteins [15–17], often referred to as three-component systems, also include peptide antibiotic-sensing systems of Gram-positive bacteria [18,19,20].

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LiaF interferes with LiaRS-dependent signal transduction is not yet understood. The genes of the LiaFSR system, together with a forth protein of unknown function, LiaG, are encoded in the liaGFSR operon, which is expressed from the constitutive liaG promoter \((P_{\text{liaG}})\) in the absence of inducing conditions [21]. Activation of LiaR results in induction of the \(p_{\text{lia}}\) promoter \((P_{\text{lia}})\) resulting in a strong upregulation of the LiaH operon, but also the complete lia locus (Figure 1) [21,22]. The exact physiological role of LiaI and LiaH is not well understood, but the proteins seem to be involved in sensing and counteracting membrane damage [22].

In contrast to other cell wall antibiotic sensors of \(B.\ subtilis\), such as the BceRS and PsdRS systems that directly sense peptide antibiotics and specifically mediate resistance against them [26], the Lia system seems to respond only indirectly to some quality of the damage caused by the diverse set of inducing conditions [27].

Here we focus on the activation of the \(P_{\text{lia}}\) by LiaR in response to the external stimulac bacitracin, which is the strongest and most robust inducer of LiaRS activity [23,26]. As seen recently in other studies [28,29], signal transduction of TCS can result in heterogeneous expression of genes regulated by these TCS. Heterogeneous gene expression in genetically identical cells can result in phenotypically different outcomes, a phenomenon also known as phenotypic heterogeneity [30]. Gene expression in itself is a stochastic or ‘noisy’ process [31]. Two different kinds of noise can be distinguished: intrinsic noise, due to noise in transcription or translation of the particular gene studied; or extrinsic noise as caused by fluctuations in the amount of other cellular components affecting gene expression [31]. Independent of the source of the noise, the arising heterogeneity can be manifested in broad gene expression distributions or by bifurcation into distinct subpopulations [32], as has been observed in \(B.\ subtilis\) in case of the transition state and stationary phase differentiation [32,33].

For the LiaFSR system, averaged data obtained by whole population studies revealed that the response of the \(P_{\text{lia}}\) is dependent on the external antibiotic concentration [23]. However, a quantitative single cell analysis of the Lia response addressing heterogeneity in gene expression has not yet been performed. Using quantitative fluorescence microscopy [33,34], we focused on a whole population study analyzed at the single cell level. We monitored gene expression from \(P_{\text{lia}}\) over time and found heterogeneity at low bacitracin concentrations. While expression levels from \(P_{\text{lia}}\) increased with the externally provided bacitracin amount, we found the immediate response of the LiaFSR system independent of the antibiotic concentration. We defined a switching threshold from the non-induced ‘OFF’ state to the bacitracin-induced ‘ON’ state. The number of cells in the ‘ON’ state, as well as the basal expression rate of the \(P_{\text{lia}}\) increased with bacitracin concentration. In addition, a well defined time window for switching into the ‘ON’ state was observed at all bacitracin concentrations.

Results

Gene expression increases at high bacitracin concentrations

In this study, we aimed at a deeper understanding of the response of the LiaFSR system to various concentrations of the peptide antibiotic bacitracin. We used the \(B.\ subtilis\) strain \(TMB\) 1172 [35], which carries a translational fusion of \(P_{\text{lia}}\) with the green fluorescent protein GFPmut1. This GFP reporter has been integrated chromosomally in addition to the naturally occurring genes under the control of \(P_{\text{lia}}\) and regulated by the RR LiaR (Figure 1). Therefore, we were able to study the response of the LiaFSR system by analyzing the expression of the GFP reporter, as it represents the expression of the LiaR regulated target genes. In particular, we studied the fluorescence development of the GFP reporter in dependence of bacitracin, a model component used to study cell envelope stress response modules of \(Bacillus\ subtilis\) [19,36]. We chose the stable GFP variant, GFPMut1, shown to have a half-life of more than 24 h [37,38], as we were only interested in the onset of gene expression. Thereby, we excluded possible variations in gene expression due to GFP decay.

Our cells were grown until mid-exponential phase before being induced with bacitracin to ensure that the recorded \(P_{\text{lia}}\) response was only due to external induction via bacitracin rather than intrinsic induction via the transition state regulator AbrB or the master regulator of sporulation Spo0A as present in the stationary phase [39]. Prior to bacitracin induction, we quantified the fluorescence intensity (FI) of non-induced cells representing the autofluorescence level (\(F_{\text{auto}}\)) and found it to be narrowly distributed with \(F_{\text{auto}}\) 8±1 FU (Figure 2A). After bacitracin induction, we monitored the fluorescence development for two hours with five to seven minute intervals. At high bacitracin concentrations all cells shifted from the autofluorescence level to intermediate and finally high GFP expression levels. The maximal fluorescence intensities were reached at 60 min after bacitracin induction as shown in Figure 2B–F. While at 30 μg/ml bacitracin maximal fluorescence intensities of 272 FU on average were reached, \(F_{\text{max}}\) decreased with lower bacitracin concentrations (Table 1). \(F_{\text{max}}\) thereby represents the average FI of all cells at time point 60 min (see Materials and Methods). As seen in earlier publications [23,36], we verified that even the highest bacitracin concentrations used had no negative effects on cell growth, thereby ruling out the risk of affecting gene expression (Figure S1). In addition, we performed control experiments using a promoter-less GFP mutant to ensure that the observed increase in fluorescence is

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**Figure 1. Core of the LiaFSR system.** Arrows denote upregulation and T-shaped lines indicate inhibition. The LiaFSR system of \(Bacillus\ subtilis\) consists of the two-component signal transducing system LiaRS and the accessory membrane protein LiaF, a LiaRS-specific inhibitor. Stress represented e.g. by cell wall antibiotics such as bacitracin is sensed by LiaS/F and leads to expression of the liaIH- liaGFSR (‘lia locus’ in the Figure) locus mediated by LiaR. To study the response of the Lia system to external stressors, we report activity of \(P_{\text{lia}}\) using the fluorescent marker GFP expressed under the control of the \(lia\) promoter, chromosomally inserted ectopically in addition to the native Lia system. CM indicates the cytoplasmic membrane. doi:10.1371/journal.pone.0053457.g001
due to bacitracin induction. As expected, no GFP expression could be detected in the promoter-less mutant (data not shown).

The general response of $P_{liaI}$ was similar for all bacitracin concentrations (Figure 2C–F). First, the whole cell population responded within less than 10 min as at T10 a clear shift to higher fluorescence values was observable. Only at very low bacitracin concentrations (0.1 mg/ml) hardly any fluorescence could be detected within the 120 min observation period, as cells stayed at $F_{I_{auto}} = 8 \pm 1$ FU (Figure S2). Second, $F_{I_{max}}$ was reached within 60 min. Third, after 60 min fluorescence levels decreased again probably due to ongoing cell division. Taken together, our data demonstrate that the LiaFSR system exhibits a graded and fast response to the external stimulus bacitracin: the $F_{I_{max}}$ as obtained after 60 min of induction increased with the stimulus concentration. In addition, cells started expression of the fluorescent protein even at low inducer concentrations within less than 10 min, in contrast to other systems such as e.g. the arabinose utilization system.

**Figure 2. Expression profiles of the $P_{liaI}$ response in dependence of the bacitracin concentration.** Addition of bacitracin induced GFP expression. At $T_{eq}$, all cells reached their maximum fluorescence intensities. While at high bacitracin concentrations all cells shifted to high fluorescence values, at low bacitracin concentrations (1 and 0.3 µg/ml) a fraction of cells did not express GFP. The observed decrease of fluorescence intensities after $T_{eq}$ is attributed to ongoing cell division. A) Autofluorescence (~8 FU) of *Bacillus subtilis* cells recorded shortly before bacitracin addition. B) Representative images of *B. subtilis* cells 60 min after bacitracin induction. Bacitracin concentration is given in the right upper corner of each image in µg/ml. C–F) Histograms of GFP expression from the liaI promoter for different time points, at C) 30 µg/ml bacitracin ($T_{7} = 7$ min after bacitracin induction), D) 3 µg/ml bacitracin, E) 1 µg/ml bacitracin, and F) 0.3 µg/ml bacitracin.

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system where for low inducer concentrations cells responded only 20 min after induction [40].

Heterogeneity in gene expression is established at low bacitracin concentrations

As we had observed that \(F_{\text{max}}\) decreased with lower bacitracin concentrations, the question arose whether this was due to general lower fluorescence intensities in all cells at \(T_{\text{off}}\) or due to a heterogeneous GFP expression in the population at low inducer concentrations, with only a fraction of cells expressing GFP at high levels. While for high bacitracin concentrations (30 and 3 \(\mu\)g/ml) all switched from \(F_{\text{low}}\) to \(F_{\text{max}}\) by 60 min post-induction, this could not be observed at low bacitracin levels in the range of the autofluorescence. Therefore, a clear heterogeneity in gene expression levels was present at 60 min after bacitracin induction at low antibiotic concentrations (Figure 2B). Interestingly, no bimodality was observed at any time point at low bacitracin concentrations, as FI levels of cells expressing GFP ranged continuously from \(F_{\text{low}}\) to \(F_{\text{max}}\), making it difficult to separate the non-induced cells from cells with induced GFP expression corresponding to higher GFP levels. Therefore, we defined the switching threshold from the non-induced ‘OFF’ state to the induced ‘ON’ state in the following way: At high bacitracin induction all cells switched into the induced ‘ON’ state. Although \(F_{\text{max}}\) was not reached until \(T_{\text{f60}}\), all cells had clearly shifted away from the autofluorescence level \(F_{\text{low}}\) at \(T_{\text{f10}}\) (3 \(\mu\)g/ml bacitracin) and \(T_{\text{10}}\) (3 \(\mu\)g/ml bacitracin). We used these intermediate states as seen in experiments with high inducer concentrations (30 and 3 \(\mu\)g/ml bacitracin) to determine the switching threshold by applying a Gaussian fit to the histograms shown in Figure 3 (see Material and Methods, Table S1). This resulted in a switching threshold of 30 FU: cells showing expression levels above 30 FU (= three-fold above background) were considered as being in the ‘ON’ state. This threshold definition best reflected the observed fluorescence expression distributions (Figure 2C–F). Subsequently, we determined the fraction of cells in the ‘ON’ state as a function of time \(f_{\text{ON}}(T)\) (see Materials and Methods), which was well described by a sigmoid function (Figure 4 left, Table 1, Table S3). Maximal switching into the ‘ON’ state was observed at about 11 min for 3 and 30 \(\mu\)g/ml bacitracin and about 14 min for 1 and 0.3 \(\mu\)g/ml bacitracin. One possible explanation for this observation is heterogeneous timing [36]. Here, the time point of switching for individual cells is distributed over a longer time period. As the fraction of cells in the ‘ON’ state saturated 20 min after bacitracin induction, even for low bacitracin concentrations, and no further increase of the fraction of cells in the ‘ON’ state could be observed thereafter, we find this explanation unlikely. Instead, we assume that cells still responding at low antibiotic concentrations need more time to do so (Figure 4 left, Table S4). The maximal switching rate \(p_{\text{ONmax}}\) was about 10 to 20%/min for high bacitracin concentrations (Table 1, Table S5), and was significantly reduced at 0.3 \(\mu\)g/ml bacitracin with about 4%/min. Therefore, the small number of cells entering the ‘ON’ state at this bacitracin concentration can be ascribed to the reduced switching rate.

Independent on the bacitracin concentration added, switching into the ‘ON’ state started approximately five minutes after bacitracin induction, ending 20 min later. This indicates the presence of a well-defined switching window of about 20 min in which cells can enter the ‘ON’ state. As soon as bacitracin, or any damage caused by it, is sensed by the LiaFSR system, cells start to switch into the ‘ON’ state. The shut-down of the LiaFSR response can be understood in the context of the complete bacitracin stress response network that the Lia system is embedded in: several TCS are present in \textit{B. subtilis} [19] that sense the antibiotic bacitracin leading to the activation of bacitracin detoxification systems that remove the antibiotic from its site of action [19,32]. This in turn lowers the inducing stress that is sensed by the LiaFSR system, resulting in the observed ‘switch-off’ at about 20 min. Although, the fraction of cells in the ‘ON’ state does not increase any further 20 min after bacitracin induction, an increase in fluorescence intensities can be observed until \(T_{\text{f60}}\). We attribute this to the stability of the GFP-mRNA: as long as GFP-mRNA is present, cells switching into the ‘ON’ state is dependent on the external antibiotic concentration and reaches a saturating level at 3 \(\mu\)g/ml bacitracin. Above this concentration all cells enter the ‘ON’ state.

### Table 1. Quantitative Analysis of the LiaFSR response.

<table>
<thead>
<tr>
<th>Bacitracin [(\mu)g/ml]</th>
<th>(F_{\text{max}}) [FU]</th>
<th>(f_{\text{ONmax}}) [%]</th>
<th>(p_{\text{ONmax}}) [%/min]</th>
<th>(t_{p_{\text{ONmax}}}) [min]</th>
<th>(F_{\text{basalmax}}) [FU]</th>
<th>(P_{\text{max}}) [FU/min]</th>
<th>(t_{P_{\text{max}}}) [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>272±2</td>
<td>99±0.2</td>
<td>10.6±1.1</td>
<td>11.3±1.3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>139±4</td>
<td>100±0</td>
<td>19.5±13.8</td>
<td>10.6±1.9</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>44±4</td>
<td>78±2.4</td>
<td>9.7±2.9</td>
<td>14.2±1.1</td>
<td>21.6±0.7</td>
<td>2.3±0.4</td>
<td>8.0±0.35</td>
</tr>
<tr>
<td>0.3</td>
<td>26±2</td>
<td>26±2</td>
<td>3.9±2.6</td>
<td>14.3±1.5</td>
<td>11.9±0.4</td>
<td>0.3±0.3</td>
<td>6.2±1.3</td>
</tr>
<tr>
<td>0.1</td>
<td>8±1</td>
<td>2.3±0.1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(F_{\text{max}}\) = average maximal fluorescence intensity at \(T_{\text{f60}}\), \(f_{\text{ONmax}}\) = maximal fraction of cells in the ‘ON’ state, \(p_{\text{ONmax}}\) = maximal switching rate, \(t_{p_{\text{ONmax}}}\) = time point of maximal switching, \(F_{\text{basalmax}}\) = average maximal basal fluorescence intensity, \(P_{\text{max}}\) = maximal expression rate, \(t_{P_{\text{max}}}\) = time point of maximal expression rate.

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Response of \textit{B. subtilis} LiaFSR System to Bacitracin
Basal expression rate of \( P_{\text{liaI}} \) is dependent on bacitracin concentration

We observed that the maximal switching rate \( P_{\text{fONmax}} \) was reduced at 0.3 \( \mu \text{g/ml} \) bacitracin as compared to higher bacitracin concentrations and was reached at later time points. This raised the question whether the smaller switching rate at low bacitracin concentrations was due to a reduced \( P_{\text{liaI}} \) promoter activity. We addressed this question by analyzing the basal expression rate \( (P_a) \). As GFPmut1 and LiaI represent different proteins, it is possible that GFPmut1 and LiaI have different proteolysis rates. Therefore, the concentration of GFPmut1 controlled by \( P_{\text{liaI}} \) is not necessarily a direct measure for the concentration of LiaI. However, the expression rates, i.e. the production rate of LiaI and GFPmut1, are expected to be similar, as the complete native \( P_{\text{liaI}} \) including all native signals for LiaI expression is present.

As a first step, we selected the cells that had not switched into the ‘ON’ state, as present in experiments with 1 and 0.3 \( \mu \text{g/ml} \) bacitracin. The average basal fluorescence value of cells that had not switched \( (F_{\text{basal}}) \) shifted to higher values with time, saturating at the maximal basal fluorescence value \( F_{\text{basalmax}} \). This increase of fluorescence values of not-induced cells could be well described by a sigmoid fit function \( f(T) \) (Table S6), similar to the fraction of cells in the ‘ON’ state. However, \( f(T) \) was shifted towards earlier times as compared with \( f_{\text{ON}}(T) \), indicating that the basal expression rate \( P_a \) had a maximum and that the maximum expression rate was shifted to earlier times as compared with the maximum switching rate \( P_{\text{fON}} \). The maximal fluorescence values of not-induced cells as obtained at 20 min after bacitracin induction showed significantly higher values as compared to the autofluorescence (Figure 5 A,C), with about 22 and 12 FU for 1 and 0.3 \( \mu \text{g/ml} \) bacitracin, respectively (Table 1).

We determined the basal expression rate \( P_a \) as the first derivative with respect to time of the mean grey value of those cells that had not entered the ‘ON’ state (Figure 5 B and D), which was well described by a Gaussian function (Table S7). The maximum basal expression rate, \( P_{\text{a(max)}} \) (Material and Methods), at 1 \( \mu \text{g/ml} \) was 2.3\( \pm 0.4 \) FU/min exceeding the value of 0.3\( \pm 0.3 \) FU/min at 0.3 \( \mu \text{g/ml} \) bacitracin by a factor of eight (Table S8). This indicated that the graded response of the LiaFSR system was merely due to a decreased basal expression rate at low bacitracin concentrations. As the maximal basal expression rate was reached at about 7 min at 1 and 0.3 \( \mu \text{g/ml} \) bacitracin as compared to the maximal switching rate at about 14 min (Table 1, Table S9), switching into the ‘ON’ state can be attributed to the increase of the basal expression rate at these bacitracin concentrations. As the basal expression rate is reduced again to zero times as compared with \( f_{\text{ON}}(T) \), indicating that the basal expression rate \( P_a \) had a maximum and that the maximum expression rate was shifted to earlier times as compared with the maximum switching rate \( P_{\text{fON}} \). The maximal fluorescence values of not-induced cells as obtained at 20 min after bacitracin induction showed significantly higher values as compared to the autofluorescence (Figure 5 A,C), with about 22 and 12 FU for 1 and 0.3 \( \mu \text{g/ml} \) bacitracin, respectively (Table 1).

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approximately 15–20 min after bacitracin induction, the duration of the switching window is well defined. The time delay between Panax and PfONmax of about 6 to 8 min (Figure 5 E, F) is in the range of the maturation time of the used fluorescent protein GFPmut1 with 8 min (Figure S3, Table S10), demonstrating the immediate response of the LiaFSR system to the antibiotic bacitracin.

Switching initiation is similar for individual cells

So far, we have quantitatively analyzed the Pf switches response of the whole bacterial population grown in stirred liquid cultures as given by the averaged values of the single cells. In order to study the switching behavior of individual cells we developed a new protocol for fluorescent time-lapse microscopy of exponentially growing B. subtilis cells. Bacteria were fixed via attachment to microfluidic chambers coated with a specific silane (Materials and Methods) and flushed with fresh medium including the antibiotic bacitracin.

Figure 4. Fraction of cells in the ‘ON’ state as a function of time (fON(T)) and switching rate (PfON). For definition of the switching threshold see description in the Materials and Method section. The fraction of cells in the ‘ON’ state (fON) increased with time, finally saturating at its maximal level. The maximal fraction of cells in the ‘ON’ state (fONmax) decreased with the bacitracin concentration. Similarly, the maximal switching rate (PfONmax) decreased at low bacitracin concentrations (e.g. 0.3 μg/ml). A, C, E, G) Fraction of cells in the ‘ON’ state as a function of time (fON). Solid line: best fit to a sigmoid function as previously described in [33] (Table S2). B, D, F, H) Switching rate (PfON). The switching rate was determined as the first derivative with respect to time of the fraction of cells in the ‘ON’ state. Solid line: best fit to a Gaussian function (Table S3). A and B: 30 μg/ml bacitracin; C and D: 3 μg/ml bacitracin; E and F: 1 μg/ml bacitracin, G and H: 0.3 μg/ml bacitracin.

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As bleaching of the GFPmut1 molecules in single cells was significant, we corrected the obtained fluorescent values as described in the Materials and Methods section. Since the GFP expression levels for low bacitracin concentrations were in the range of the bleaching, we were only able to monitor the switching behavior of individual cells over time at 30 mg/ml bacitracin.

Analyzing bleach-corrected fluorescence values (Material and Methods), we observed that cells started switching at about five minutes after bacitracin induction and all cells had switched into the ‘ON’ state within 15 min, as seen in experiments performed in liquid cultures. As expected, individual cells reached fluorescence values at 60 min post-induction between 200 and 600 FU (Figure 6). But in contrast to the experiments of whole populations described above, FI values increased until 80 min (200–800 FU) indicating that cell division was reduced for cells grown directly on the microscopic slide rather than in flask cultures. Nevertheless, the same overall switching behavior could be observed for individual cells growing in the microfluidic chamber as compared to cells grown in liquid culture, demonstrating the suitability of this approach. In a next step we compared the individual switching curves by applying a sigmoid function to the fluorescence development of single cells over time. This study revealed that cells initiated switching into the ‘ON’ state within the same time frame, but the individual switching curves showed a high variation with individual switching rates ranging from 6–15 FU/min (Figure 6). In accordance with our findings of whole population studies, our single cell data obtained by time-lapse microscopy demonstrate the fast response of the LiaFSR system to bacitracin.

**Discussion**

In this report, we quantitatively investigated the response of the LiaFSR system to an external signal, the peptide-antibiotic bacitracin, by performing a population study analyzed on the single cell level. Quantitative fluorescence microscopy (QFM) as described in this study, has been used previously to analyze switching of *Bacillus subtilis* into the competent state [33]. In this


Figure 6. Switching characteristics of single cells at 30 μg/ml bacitracin. Fluorescence development of single cells over time at 30 μg/ml bacitracin was comparable to the data obtained by single cell analysis of the above described population study: All cells switched into the induced ‘ON’ state, exceeding the threshold fluorescence intensity within 15 min. In contrast to the whole population study the maximal fluorescence intensity was reached only after 80 min. A) Fluorescence development of one individual cell is shown. Top: bright field images at different time points. Bottom: fluorescence images at different time points. B) Fluorescence development of 13 individual cells is shown. C) Sigmoidal fits have been applied to eight fluorescence intensity traces in Figure 6B. The fluorescence intensity was normalized to the maximum fluorescence intensity and the time axis was shifted to T45, where cells had half-maximum fluorescence intensity. Blue and red line: two individual fluorescence traces representing cells with the slowest and highest individual switching rates in this cell batch. doi:10.1371/journal.pone.0053457.g006

In the particular case it was shown that the sensitivity of this approach is high enough to detect an increase of promoter activity by a factor of two. This result was confirmed independently, using fluorescence in situ hybridization (FISH), demonstrating the usability of quantitative fluorescence microscopy [41]. Another quantitative method to analyze single cells is flow cytometry. We performed flow cytometry experiments in order to study the LiaFSR response to various bacitracin concentrations (Figure S4), confirming our results obtained by QFM. Fluorescence values of single cells obtained by flow cytometry for low bacitracin concentrations were difficult to separate from the buffer background even after applying gating procedures. Therefore, we chose to focus on quantitative fluorescence microscopy to analyze our data in order to obtain the complete information of the LiaFSR response for high and low bacitracin concentrations.

We observed an immediate response of the system with cells switching in the bacitracin-induced ‘ON’ state within 20 min, irrespective of the externally provided bacitracin concentration. The switching rate shows its maximum approximately 7 min after the maximum of the basal expression rate. Importantly, this response time is in the range of the maturation time of the green fluorescent reporter with 8 min [42], indicating an almost instant burst of LiaR-dependent transcription initiation at Pnad. This is in contrast to other studies, in which maximum RR-regulated mRNA concentrations [1] or the concentration of promoter-bound RR [10] could be detected only within 20-30 min after exposure to the externally provided signal. Functional characterization of all two-component signal transduction systems in E. coli revealed a wide span in auto-phosphorylation rates of the HK ranging from about 2 min to 10 min. Phospho-transfer to RRs by phosphorylated cognate HKs took place within less than ½ min [9]. As maximal switching into the ‘ON’ state of the LiaFSR system can be observed within 15 min after bacitracin addition, even at the lowest bacitracin concentration, this demonstrates that no further regulatory elements are involved in the bacitracin-dependent LiaFSR response. This is in line with our finding that the basal expression rate of the lad promoter is dependent on the bacitracin concentration, indicating that the LiaR concentration is directly affecting gene expression from Pnad. Recently, it was found that even at very high bacitracin concentrations (50 μg/ml) only about 20 molecules of LiaR are present within a single cell [43], while in the absence of bacitracin LiaR was not detectable. The amount of available LiaR controlling expression from Pnad is therefore dependent on the bacitracin concentration. The low number of LiaR molecules can explain the observed variations in gene expression, in particular the heterogeneity present at low bacitracin concentrations, as cell-to-cell differences (noise [31]) in the exact number of LiaR directly affect gene expression from Pnad.

Performing a population study analyzed at the single cell level, in combination with time-lapse microscopy, we quantitatively analyzed the response of the LiaFSR two-component system to bacitracin. As described above, the LiaFSR system responds within less than 15 min to the external stimulus. Cell-to-cell differences are present at all bacitracin concentrations and decrease at low bacitracin levels. The maximum switching rate as well as basal expression rate depends on the bacitracin concentration, reflecting the graded response of the LiaFSR system. For a stress sensor system, this kind of response is reasonable. Changing environmental conditions, including the presence of stressors, require fast stress sensing systems such as the LiaFSR system, that are shut-off as soon as the stressor is no longer present. Taken together, our data demonstrate that the LiaFSR system exhibits an immediate, heterogeneous and graded response to the peptide antibiotic bacitracin in the exponential growth phase.

Materials and Methods

Growth conditions

_Bacillus subtilis_ strain TMB 1172 [35] carries a translational fusion of Pnad with the green fluorescent reporter protein GFPmut1. TMB 1172 was grown in LB medium at 37°C, shaken at 300 rpm. Overnight cultures were diluted to OD₆₀₀ of 0.1. Cells were grown to mid-logarithmic phase, then were again diluted to OD₆₀₀ of 0.1 into fresh medium and grown for additional 30 min to ensure optimal growth conditions before induction with the peptide-antibiotic bacitracin (Sigma) at Tₐ₀ = 30 min and applying...
the cells to the microscopic slides. This way any cross-over from intrinsic stationary phase induction [35] could be avoided. Experiments for each bacitracin concentration were performed in triplicates on three different days. For each time point a minimum of 100 cells was analyzed. The bacitracin concentrations used in this study are far below the minimal inhibitory concentration (MIC) [23,36] and have been shown to have no effect on growth (Figure S1).

Construction of promoter-less-gfp mutant strain

The promoter less vector pGFPamy [44] was transformed into B. subtilis as a negative control. The vector carries a chloramphenicol resistance cassette for selection in B. subtilis, and integrates into the amyE locus by double crossing-over, resulting in a stable integration of the promoter-less-gfp fusion. The plasmid was linearized with PstI and used to transform B. subtilis 168 with chloramphenicol selection (5 μg/ml). Successful integration into the amyE locus was confirmed by starch test.

Flow cytometry

For flow cytometry experiments, the cultures were grown as described above. Samples were taken every 10 min for 120 min and diluted 1:100 in PBS (phosphate buffered saline). The experiments were performed using a Partec CyFlow Space instrument and the software FlowMax. GFP was excited with a laser at 488 nm and its emission measured at 518 nm. The analysis of the cells was done at a flow-rate of 2 μl/s. In between measurements, the instrument was rinsed with PBS to eliminate cross-contamination. In addition to the different concentrations of bacitracin, not induced samples and PBS alone were analyzed for cross-contamination. In addition to the different concentrations of bacitracin, not induced samples and PBS alone were analyzed for control purposes. To discriminate dead from healthy cells, appropriate gating procedures have been applied. 50000 cells lying in the appropriate gate have been analyzed for each time point.

Fluorescence Microscopy

Cells were sampled throughout growth as indicated in the main text. For image acquisition of the whole cell population, cells were permitted to attach to microscopic slides (eight-well IBIDI chamber, uncoated) and covered with 1% Agarose-patches.

For time-series of single cells, cells were allowed to attach to microfluidic chambers coated with 100% 1-[3-(Trimethoxysilyl)-propyl]indole (Sigma). Cells were induced already attached to the microfluidic channels and washed with fresh medium in the presence of bacitracin at a flow-rate of 0.3 ml/h.

Image acquisition was done using a Zeiss Axiovert 200 M microscope equipped with an Andor Digital Camera and a Zeiss EC Plan-Neofluar 100×/1.3 Oil immersion objective. Andor software was used for image acquisition. The stability of the absolute fluorescence values was verified using a microscope intensity calibration kit (Invitrogen, FokalCheck fluorescence microscope test slide #3). Microspheres showed a deviation of mean grey value of less than 1% under the experimental conditions used for detection of GFP fluorescence. Homogeneity of illumination was tested using fluorescent slides and the maximum deviation was less than 5%.

Image Analysis

Images were processed using ImageJ software. Image background was corrected using a rolling ball algorithm with a radius of 50. An intensity threshold tool was used to delimit the boundaries of the cells in the bright field image. The boundaries of the cells were obtained with the wand tool of the ImageJ software and transferred to the fluorescence image using the ROI manager of ImageJ. Only cells that were fully lying within the bright field image and were not in the process of cell division were considered. Furthermore, dead cells as observable by different contrast in the bright field image as compared to healthy cells were excluded from the single cell analysis. The remaining single cells were then analyzed with respect to their mean grey value. Data preparation was performed using the Software IGOR PRO 4.06 and Adobe Illustrator CS4.

Definitions and calculation methods

\[ F_{\text{max}}: \text{average autolfuorescence/fluorescence intensity of cells not induced by bacitracin, given as fluorescence units (FU) as obtained by the mean grey value. The average autolfuorescence level of cells prior bacitracin induction was } F_{\text{auto}} = 0 \pm 1 \text{ FU (Figure 2A).} \]

\[ F_{\text{max}}: \text{average maximal GFP expression/fluorescence intensity as observed at } T_{\text{GFP}}. \text{ Upon induction with bacitracin cells expressed the GFP reporter. The resulting fluorescence intensities were obtained as the mean grey value of each single cell. The error of } F_{\text{max}} \text{ is given as the standard error.} \]

Switching threshold: The switching threshold separates cells being in the ‘OFF’ state (no/basal expression) from cells being in the ‘ON’ state (induced GFP-expression). We used the intermediate states seen in experiments with high inducer concentrations (30 and 3 μg/ml bacitracin) to determine the switching threshold. A Gaussian fit was applied to the histograms shown in Figure 3 (Table S1). The values of the center of these distributions, in addition to the average fluorescence values of all cells at this time point, were averaged. The resulting value of 30 FU was then defined to be the switching threshold: any cell with fluorescent value above 30 FU (mean grey value) was considered as being in the ‘ON’ state.

\[ f_{\text{ON}}: \text{fraction of cells in the ‘ON’ state. We determined the fraction of cells in the ‘ON’ state as a function of time using the switching threshold. The fraction of cells in the ‘ON’ state was well defined by a sigmoid function with } f_{\text{ON}}(T) = f_{\text{max}} + \frac{1}{1 + \exp(-k(T_{\text{half}}-T))}. \text{ The fit parameter of this function can be found in Table S2. The maximal switching threshold in the ‘ON’ state is determined using this fit function (Table S2). The error of the fraction of cells in the ‘ON’ state has been calculated according to: square root of } (p(1-p)/n-1). \]

\[ f_{\text{ON}}: \text{average switching rate of cells switching into the ‘ON’ state. The switching rate was determined as the first derivative of the fraction of cells in the ‘ON’ state with respect to time. To reduce the error, the maximal switching rate } (P_{\text{max}}) \text{ was determined using two different calculation methods: a) } P_{\text{max}} = \text{maximum of the 1st derivative of the exact data points of } f_{\text{ON}}; \text{ b) by obtaining } \Lambda \text{ of the Gaussian fit applied to the data Figure 4 right according to } P_{\text{ON}} = \gamma_0 + \Lambda \exp(-\langle x-x_0 \rangle/width)^2) \text{ (Table S5). The high error for data determined at 3 μg/ml bacitracin is attributed to the steep increase of the fraction of cells in the ‘ON’ state leading to a high fitting error. Additional data points in order to reduce the error could not be attained, as cells stored on ice for later image acquisition tended to lyse at bacitracin concentrations } >1 \mu \text{g/ml. Therefore image acquisition was performed immediately after sampling of the cells. The exact results of both calculation methods as well as the average values are given in Table S5. The error of the switching rate was calculated according to: Error } f_{\text{ON}} \text{ at time point } t_2 = \text{square root of } [(error at } (t_2)/(error at (t_1))^2], \text{ with } t_1 \text{ and } t_2 \text{ the time points of the derivated time interval. The individual errors here are the errors of } f_{\text{ON}} \text{ as described above. Please note that error propagation has to be} \]
taken into account when deriving data points, leading to the high errors in Figure 4, E and H.

\( t(\text{PfONmax}) \): Time point of maximal switching rate. To reduce the error the time point of the maximal switching rate has been determined in three different ways: a) \( T_{\text{half}} \) of the sigmoidal fit applied to Figure 4 left according to \( f_{\text{ON}}(T) = f_{\text{base}} + f_{\text{max}}/1 + \exp(-k(T_{\text{half}}-T)) \); b) Time point of \( \text{PfONmax} \), \( \text{maximum of the 1}\text{st derivative of the exact data points of fON} \), c) by obtaining \( x_0 \) of the Gaussian fit applied to the data in Figure 4 right according to \( \text{PfON}(T) = y_0 + A \exp \left(-\left(x-x_0\right)/\text{width}\right)^2 \) (Table S4).

\( \text{FIbasalmax} \): average maximal basal fluorescence intensity of cells in the ‘OFF’ state. The error is given as the standard error.

\( \text{FIbasal} \): average basal fluorescence intensity of cells in the non-induced ‘OFF’ state. We determined the \( \text{Pa} \) as the first derivative with respect to time of the mean grey value of those cells that had not entered the ‘ON’ state. To reduce the error the maximal basal expression rate \( \text{Pamax} \) has been determined in two different ways:

\( a) \text{Pa} = \text{maximum of the 1}\text{st derivative of the exact data points of } \text{FIbasal} \), b) by obtaining \( x_0 \) of the Gaussian fit applied to Figure 5 B, D according to \( f_{\text{ON}}(T) = y_0 + A \exp \left(-\left(x-x_0\right)/\text{width}\right)^2 \) (Table S8). The error of the basal expression rate was calculated according to: Error \( \text{Pa} \) at time point \( t_2 = \text{square root of } \left\{ \text{error at } t_2 \right\}^2 + \text{error at } t_2 \), with \( t_1 \) and \( t_2 \) the time points of the derived time interval. The individual errors here are the errors of \( \text{FIbasal} \) as described above. Please note that error propagation has to be taken into account when deriving data points, leading to the high errors in Figure 5 B and D.

\( t(\text{Pamax}) \): The time point of the maximal basal expression rate has been determined in three different ways:

\( a) T_{\text{half}} \) of the sigmoidal fit applied to Figure 5 A, C according to \( f(T) = f_{\text{base}} + f_{\text{max}}/1 + \exp(k(T_{\text{half}}-T)) \) (Table S9), b) \( \text{Pamax} = \text{maximum of the 1}\text{st derivative of the exact data points of } \text{FIbasal} \), c) by obtaining \( x_0 \) of the Gaussian fit applied to Figure 5 right according to \( f_{\text{ON}}(T) = y_0 + A \exp \left(-\left(x-x_0\right)/\text{width}\right)^2 \) (Table S9).

A summary of all data described here, as well as the average data obtained from the different calculation methods for \( t(\text{PfONmax}), t(\text{Pamax}), \text{PfONmax} \) and \( \text{Pamax} \) can be found in Table 1. The obtained data for each calculation method for \( t(\text{PfONmax}), t(\text{Pamax}), \text{PfONmax} \) and \( \text{Pamax} \) are given in Figure S5.

Bleach correction of single cell time-series

In time-series of individual cells, bleaching of GFP in these cells occurred. Hence, we applied a bleach correction to our time-series data. After each time-series a new spot was chosen at an appropriate distance to ensure that no bleaching had occurred yet on this spot. Twenty successive images were taken. One image was immediately taken after the previous one. For each cell of this spot the obtained 'bleach curve' was fitted exponentially. The resulting rates were averaged. The data obtained in the actual time-series were then divided by \( e^{-tk} \), with \( n \) being the number of pictures already taken of this spot and \( k \) the average of the rates determined by the exponential fit of the 'bleach curves'.

GFPmut1 maturation

To determine the time delay between expression of the GFP reporter and the onset of fluorescence, strain TMB 1172 was grown in LB medium as described above. For induction of GFPmut1 expression bacitracin was added after 60 min at a final concentration of 30 \( \mu \text{g/mL} \) and erythromycin was added at 80 min, inhibiting protein biosynthesis. Increase of fluorescence after 80 min must therefore be due to folding of already synthesized GFP (Figure S3). Assuming a first-order kinetic we fitted the data with a single exponential function and obtained a characteristic maturation time of 7.9±0.69 min.

Supporting Information

Table S1 Fit parameter for the fluorescence distributions given in Figure 3.

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Table S2 Fit parameters for the fraction of cells in the ‘ON’ state \( f_{\text{ON}} \).

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Table S3 Fit parameter for the switching rate \( \text{PfON} \).

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Table S4 Time point of maximal switching rate \( t(\text{PfONmax}) \).

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Table S5 Maximal switching rate \( \text{PfONmax} \).

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Table S6 Fit parameters for the basal fluorescence level \( \text{FIbasal} \).

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Table S7 Fit parameter for the basal expression rate \( \text{Pa} \).

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Table S8 Maximal basal expression rate \( \text{Pamax} \).

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Table S9 Time point of maximal basal expression rate \( t(\text{Pamax}) \).

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Table S10 Maturation of GFPmut1.

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Figure S1 Influence of bacitracin on cell growth. Cells were grown as described in the Material and Methods section in the presence of bacitracin at different final concentrations (Black: 0 \( \mu \text{g/mL} \), grey: 0.1 \( \mu \text{g/mL} \), blue: 0.3 \( \mu \text{g/mL} \), yellow: 1 \( \mu \text{g/mL} \), green: 3 \( \mu \text{g/mL} \), red: 30 \( \mu \text{g/mL} \)). At these concentrations bacitracin has no influence on cell growth.

Figure S2 Expression profiles of the Lia response at 0.1 \( \mu \text{g/mL} \) bacitracin. At these very low inducing concentration of bacitracin nearly all cells stay in the non-induced ‘OFF’ state. A) Representative image of \( B. subtilis \) cells 60 min after bacitracin induction. B) Histograms of GFP expression from the lia promoter for different time points \( (T_m = 10 \text{ min after bacitracin induction}) \). Red arrows indicate the few cells in the ‘ON’ state at this bacitracin concentration.

Figure S3 Maturation of GFPmut1. Arrow indicates the addition of 400 \( \mu \text{g/mL} \) erythromycin at 80 min leading to immediate translation inhibition. Therefore any fluorescence development arising after erythromycin addition can be attributed to the maturation of the GFP fluorophore. Grey: cells grown in the absence of erythromycin. Black: Cells grown in the presence of erythromycin. Solid lines: best fit to an exponential function (Table S10).
Figure S4  Flow cytometry analysis of the $P_{\text{liaI}}$ response of LiaFSR to bacitracin. Flow cytometry analysis verified the results obtained by quantitative fluorescence microscopy as shown in main Figure 2. Addition of bacitracin induced GFP expression. A) At T=0 all cells reached their maximum fluorescence intensities. While at high bacitracin concentrations all cells shifted to high fluorescence values, at low bacitracin concentrations (1 and 0.3 μg/ml) a fraction of cells did not express GFP and stayed at the autofluorescence value. As low fluorescence intensities of induced cells were hard to distinguish from the background fluorescence of not induced cells using flow cytometry, we chose quantitative fluorescence microscopy for detailed analysis of the LiaFSR response. Data shown here represent the mean grey value of each single cell: mean FI [FU]. A) Background signal of the buffer PBS in the gated area. B) Autofluorescence of not induced Bacillus subtilis cells (C–F) Histograms of GFP expression from the liaI promoter for different time points, at C) 30 μg/ml bacitracin ($T_{30}=30$ min after bacitracin induction), D) 3 μg/ml bacitracin, E) 1 μg/ml bacitracin, and F) 0.3 μg/ml bacitracin. (EPS)

Figure S5  Maximal switching rate $P_{\text{fONmax}}$ and maximal basal expression rate $P_{\text{mmax}}$ for various bacitracin concentrations. As switching into the ‘ON’ state took place in a very short time period of less than 10–15 min, only few data points between the ‘OFF’ and the ‘ON’ state could be obtained. As cells treated with high bacitracin concentrations, although not showing any fitness defects, tended to lyse when stored on ice, a shorter experimental time resolution was not possible. Therefore, to reduce the error by simply fitting to the data, the maximal switching rate as well as the maximal basal expression rate was determined using several calculation methods as described in the Material and Methods section. This Figure gives an overview of the data obtained by the various methods used. A) Time point of maximum switching rate $t(P_{\text{fONmax}})$; Black, grey and light grey bars represent data obtained as described in Table S4 a–c. Blue: averaged data of the time point of maximal switching. B) Maximal switching rate $P_{\text{fONmax}}$; Black, and light grey bars represent data obtained as described in Table S9 a–c. Blue: averaged data of $t(P_{\text{mmax}})$. C) Time point of maximum basal expression rate $(t(P_{\text{mmax}}))$; Black, grey and light grey bars represent data obtained as described in Table S6 a–c. Blue: averaged data of $P_{\text{mmax}}$. D) Maximum basal expression rate $P_{\text{mmax}}$; Black and light grey bars represent data as described in Table S8 a and b. Blue: average data of maximal $P_{\text{mmax}}$. (EPS)

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Author Contributions

Conceived and designed the experiments: TM ML. Performed the experiments: CH AM SK. Analyzed the data: SK ML. Wrote the paper: TM ML.

References

CHAPTER III

Subcellular localization, interactions and dynamics of the phage-shock protein-like Lia response in *Bacillus subtilis*

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Subcellular localization, interactions and dynamics of the phage-shock protein-like Lia response in *Bacillus subtilis*

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Summary

The *liaIH* operon of *Bacillus subtilis* is the main target of the envelope stress-inducible two-component system LiaRS. Here, we studied the localization, interaction and cellular dynamics of Lia proteins to gain insights into the physiological role of the Lia response. We demonstrate that LiaI serves as the membrane anchor for the phage-shock protein A homologue LiaH. Under non-inducing conditions, LiaI localizes in highly motile membrane-associated foci, while LiaH is dispersed throughout the cytoplasm. Under stress conditions, both proteins are strongly induced and colocalize in numerous distinct static spots at the cytoplasmic membrane. This behaviour is independent of MreB and does also not correlate with the stalling of the cell wall biosynthesis machinery upon antibiotic inhibition. It can be induced by antibiotics that interfere with the membrane-anchored steps of cell wall biosynthesis, while compounds that inhibit the cytoplasmic or extracytoplasmic steps do not trigger this response. Taken together, our data are consistent with a model in which the Lia system scans the cytoplasmic membrane for envelope perturbations. Upon their detection, LiaS activates the cognate response regulator LiaR, which in turn strongly induces the *liaIH* operon. Simultaneously, LiaI recruits LiaH to the membrane, presumably to protect the envelope and counteract the antibiotic-induced damage.

Introduction

The bacterial cell envelope, consisting of the cytoplasmic membrane, the cell wall, and – in Gram-negative bacteria – the outer membrane, is the first and major barrier of defence against threats from the environment. Hence, closely monitoring envelope integrity is crucial for survival of a bacterial cell in its natural habitat. Accordingly, complex regulatory networks have evolved in both Gram-negative and Gram-positive bacteria to respond to envelope stress by mounting protective countermeasures. The underlying signal transduction is predominantly mediated by two-component systems and alternative sigma factors of the extracytoplasmic function (ECF) protein family (Raivio, 2005; Jordan et al., 2008; MacRitchie et al., 2008). An additional protective layer is provided by the so-called phage-shock protein (PSP) response, which is centred around homologues of the ubiquitously distributed phage-shock protein A (PspA). Proteins belonging to the PspA/IM30 protein family are found in Gram-negative and Gram-positive bacteria, as well as in archaea and plant chloroplasts (reviewed in Model et al., 1997; Darwin, 2005; Darwin, 2007; Bultera et al., 2010; Joly et al., 2010; Vohtknecht et al., 2012; Yamaguchi and Darwin, 2012).

The PSP response has been extensively studied in *Escherichia coli* (Model et al., 1997; Darwin, 2005; Joly et al., 2010). It is induced by filamentous phage infection, high osmolarity, heat shock, secretion stress or changes in lipid biosynthesis as well as in presence of protonophores, like CCCP (Brissette et al., 1990; Bergler et al., 1994; Model et al., 1997; Jones et al., 2003; DeLisa et al., 2004). In *E. coli*, the PSP regulon consists of the *pspAB-CDE* operon and the separate genes *pspF* and *pspG* (Joly et al., 2010). Transcription of the *psp* genes is initiated in a σ54-dependent manner and controlled by the enhancer PspF. Under non-stress conditions, PspF is inhibited by cytosolic PspA (Elderkin et al., 2002; 2005; Joly et al., 2009). Upon induction, PspA is recruited to the plasma membrane via two transmembrane anchors, PspB and...
PspC. This allows PspA to counteract membrane damage and at the same time releases the PspF enhancer from its inhibitory complex, thereby allowing expression of all psp genes (Huvet et al., 2011). A similar regulatory mechanism has been demonstrated for the PSP response in the closely related pathogenic bacterium Yersinia enterocolitica (Maxson and Darwin, 2006; Gueguen et al., 2009; 2011; Yamaguchi et al., 2010; 2013; Horstman and Darwin, 2012). In summary, data obtained so far indicates that the PSP response is regulated by an intricate set of transient protein interactions centred around PspA, which switches its interaction partners from PspF (non-inducing conditions) to PspB/C (stress conditions).

Despite our detailed molecular understanding of the PSP response, its physiological role is still not well defined. Mutants in psp genes often show only mild phenotypes but available data so far points to a function of PspABC in protecting and stabilizing the membrane against leakage and loss of membrane potential (Kobayashi et al., 2007; Vranken et al., 2008; Horstman and Darwin, 2012). Moreover, the PSP response also seems to be important for virulence (Karlinsey et al., 2010; Yamaguchi and Darwin, 2012) and protein secretion (Jones et al., 2003; DeLisa et al., 2004; Seo et al., 2007; Wang et al., 2011; Mehner et al., 2012).

The genome of the Gram-positive model organism Bacillus subtilis encodes two PspA homologues, termed PspA and LiaH. Despite the clear sequence homology, the underlying regulation is significantly different from the E. coli/Y. enterocolitica blueprint (see below). Expression of pspA is controlled by the ECF sigma factor σW in response to envelope stress, SPP1 phage infection and alkaline shock (Wiegert et al., 2001; Wenzel et al., 2012), but its physiological role has not been determined. In contrast, the Lia response of B. subtilis has been intensively studied in the last decade (reviewed in Jordan et al., 2008; Schrecke et al., 2012). LiaH is encoded in the liaIH operon, which is controlled by the LiaFSR three-component system (Wolf et al., 2010). LiaFSR is part of the regulatory network orchestrating cell envelope stress response in B. subtilis (Mascher et al., 2003). It consists of a classical bacterial two-component system, LiaSR, and the LiaSR-specific inhibitor protein, LiaI (Jordan et al., 2006; Wolf et al., 2010; Schrecke et al., 2013). In B. subtilis, this three-component system is encoded by the last genes of the liaH-liaGFSR locus, which is expressed as two transcriptional units. While expression of the last four genes is ensured from a weak constitutive promoter, P_{lia}, the liaIH operon is expressed from the strictly LiaR-dependent promoter P_{liaH}, which represents the only target of LiaFSR-dependent signalling (Mascher et al., 2004; Wolf et al., 2010). When challenged with cell wall antibiotics, such as bacitracin, nisin or vancomycin, this promoter is strongly induced in a concentration-dependent manner (Mascher et al., 2003; 2004; Jordan et al., 2006). Moreover, the lia operon is also more weakly induced by detergents, ethanol, alkaline shock, and secretion stress (Wiegert et al., 2001; Mascher et al., 2004; Hyryläinen et al., 2005; Pietiäinen et al., 2005). Hence, the inducer spectrum of the Lia response is very reminiscent of the range of stimuli triggering the PSP response.

Additionally, P_{lia} is embedded in the complex differentiation cascade ultimately leading to endospore formation. Here, the transition state repressor AbrB binds P_{lia} during logarithmic growth, thereby maintaining its low basal activity. During transition state, AbrB repression is relieved by the action of Spo0A, the master regulator of sporulation, and LiaR is activated by an unknown intrinsic stimulus (Jordan et al., 2007). P_{lia} seems to be the only relevant target promoter controlled by LiaR, which induces expression of the liaIH operon and thereby mounts a PSP-like response (Wolf et al., 2010). This operon encodes a small-membrane protein, LiaI, and the PspA homologue LiaH respectively. The latter forms large oligomeric ring structures (Wolf et al., 2010), reminiscent of those observed for other PspA-like proteins (Aseeva et al., 2004; Hankamer et al., 2004; Standar et al., 2008; Otters et al., 2013).

So far, the physiological role of LiaIH remains unclear. Despite its strong induction by antibiotics interfering with the lipid II cycle of cell wall biosynthesis, the Lia system does not mediate any resistance against them (Wolf et al., 2010). The only exception is the membrane-damaging antibiotic daptomycin, which triggers the Lia response that in turn provides some degree of protection against this compound (Hachmann et al., 2009; Wolf et al., 2010). On the other hand, mild sensitivity phenotypes have been observed in liaIH mutant against cell wall antibiotics interfering with cytoplasmic (fosfomycin) or extracellular steps (some β-lactams) of cell wall biosynthesis, as well as some generators of oxidative stress, none of which act as inducers of the Lia response (Wolf et al., 2010). Taken together, these and other preliminary data suggest a protective role of the Lia response by maintaining the integrity of the cytoplasmic membrane, rather than of the cell wall (D. Wolf and T. Mascher, unpublished).

Here, we aimed at increasing our knowledge of the physiological role of the Lia response by studying the interactions, cellular localization and protein dynamics of its main effectors, LiaI and LiaH.

**Results**

LiaI is a small-membrane protein with two transmembrane helices

Previously, we have demonstrated that the liaIH operon represents the only relevant target of LiaR-dependent
LiaI is a membrane protein that interacts with LiaH. Based on hydrophobicity plots and secondary structure predictions, LiaI seems to be a membrane protein of 126 amino acids with two putative transmembrane regions (TMRs) and a C-terminal cytoplasmic domain of about 60 amino acids (Fig. 1A). To verify these predictions of LiaI topology, we constructed translational fusions of two different liaI gene fragments, encoding the full-length protein (base pairs 1–338) and an N-terminal fragment (base pairs 1–126) that terminates between the two postulated TMRs, to the reporter genes phoA and lacZ (Fig 1A; fusion points are indicated by stars). The alkaline phosphatase PhoA is only active in extracytoplasmic space while activity of the β-galactosidase LacZ indicates a cytoplasmic localization (Manoil, 1991; Daley et al., 2005). On selective 5-bromo-4-chloro-3-indolylphosphate (BCIP 50 μg ml−1) agar, we observed activity of phosphatase A (resulting in blue colonies) only for E. coli strains containing the short liaI fragment fused to phoA, but not the full-length fragment. The opposite behaviour was observed for the lacZ-fusions in B. subtilis: No colony coloration was found on X-Gal (100 μg ml−1) agar plates with cells containing the short liaI fragment fused to lacZ but with cells containing the full-length liaI-lacZ fusion (Fig 1A and data not shown). These results indicate that LiaI is a membrane protein with both the N- and C-terminus in the cytoplasm and two TMRs, which are connected by a small extracellular loop.

LiaI functions as a membrane anchor for LiaH

The genes liaI and liaH form an operon that is conserved in Firmicutes bacteria harbouring LiaFSR homologues (Jordan et al., 2006) and is strongly induced under cell envelope stress conditions (Mascher et al., 2003; 2004), indicative of a functional link between the two encoded proteins. Moreover, the genes for homologous PspA proteins in proteobacteria are also genetically associated with genes encoding small-membrane proteins that function as membrane anchors for PspA proteins (Joly et al., 2009). Hence, we propose a similar cellular role for LiaI. Especially the cytoplasmic C-terminus with its 60 amino acids length represents a suitable docking interface for LiaH.

Initially, we investigated the interaction between LiaI and LiaH by bacterial two-hybrid assay (BACTH) (Karimova et al., 1998; 2000; see Experimental procedures for details). In addition to the full-length liaI, two versions of liaI were cloned in the BACTH vectors, encoding a full-length LiaI and a truncated version that terminates after the second TMR. We observed a strong interaction between LiaH and full-length LiaI (Fig. 1B) and also between both LiaI versions, indicative of the functional expression of all alleles. Since no interaction was observed between LiaH and the truncated version of LiaI containing both TMRs but lacking the cytoplasmic...
Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTH101</td>
<td>F− cya−99 araD139 galE15 galK16 rpsL1 (strS) hsdR2 mcrA1 mcrB1</td>
<td>Lab stock</td>
</tr>
<tr>
<td>CC118</td>
<td>F−Δ(ara-leu-7897 araD139::lacX74 phoA520 galE glnK thi rpsE rpoB argE recA1</td>
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</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
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<td>tpC2</td>
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<tr>
<td>3417</td>
<td>W168 mrecC::pSG5276 (P_ara gfp-mrecE) Ω cm(^h)</td>
<td>Leaver and Errington (2005)</td>
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<tr>
<td>RWSB432</td>
<td>W168 liaI::pDW5101 (liaI-GFP) Ω amyE::RWB4 (mRFPRuby-mreB) Ω cm(^h) spec(^n)</td>
<td>This study</td>
</tr>
<tr>
<td>TMB321</td>
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<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>TMB688</td>
<td>W168 pdW3802 (cytoplasmic C-terminal part of liaI) mle(^h)</td>
<td>This study</td>
</tr>
<tr>
<td>TMB841</td>
<td>W168 pGP380 (empty vector control) mls(^h)</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
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<tr>
<td>TMB2206</td>
<td>W168 ΔliaI ΔliaH amyE::pCH5402 (P_ara-liaI-gfp)</td>
<td>This study</td>
</tr>
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a. Resistance cassettes: cm, chloramphenicol; str, streptomycin; mls, macrolide-lincosamide-streptogramin B; spec, spectinomycin.

C-terminus, we conclude that the latter is necessary for the interaction with LiaH. The specificity of the BACTH results on LiaI-LiaH interaction was validated by a lack of interaction between LiaI and other membrane proteins, including LiaG or LiaF (data not shown).

To verify these observations, we next studied the interaction between LiaI and LiaH in more detail. By using the SPINE (Strep-Protein INteraction Experiment) approach (Herzberg et al., 2007), we were able to demonstrate an in vivo interaction between LiaI and LiaH by in vivo cross-linking followed by co-purification and detection of both proteins via immunoblotting (Fig. 1C). Strain TMB688, which constitutively expresses a soluble Streptacin-tagged derivative of LiaI (Table 1), was grown in the presence of bacitracin (20 μg mL\(^{-1}\)) to induce the LiaRS system and hence LiaH production. At an OD\(_{600}\) of 1.0 formaldehyde was added to cross-link interacting proteins in vivo (Herzberg et al., 2007). After incubation of the cytoplasmic cell extract with Strep-Tactin sepharose and subsequent thorough washing, we detected both proteins in the elution fractions by using LiaH- and Strep-tagII-specific antibodies respectively (Fig. 1C). In contrast, no signals for LiaH or the tag-specific antibodies were detected in the elution fractions of the control strain TMB841 (data not shown). We therefore conclude that LiaI and LiaH interact when the Lia-system is induced by cell envelope stress.

Given that both proteins interact in vivo and LiaI is a membrane protein, one would expect that LiaH should be detectable in membrane fractions under Lia-inducing conditions. To corroborate this hypothesis we incubated the wild-type strain in the presence and absence of bacitracin to compare the induced and uninduced state of expression of the liaI-H operon respectively. Equal amounts of the cytoplasmic and membrane protein fraction harvested from both uninduced and induced cultures were separated by SDS-PAGE, followed by LiaH-specific Western analysis (see Experimental procedures for details). While no LiaH-specific signal could be observed in any fraction from uninduced cultures, a band of the appropriate size was detected in the cytoplasmic, and especially the membrane fraction under inducing conditions, thereby verifying not only the induction of LiaH production but also its specific association with the cytoplasmic membrane (Fig. 1D, lanes 1–4).

Based on the results described so far, it seems reasonable to assume that this membrane localization of the cytoplasmic protein LiaH depends on its membrane anchor LiaI. We therefore repeated the experiment described above with strain TMB1394, harbouring an in-frame markerless deletion of liaI (Table 1). Compared to the wild-type strain, the overall amount of LiaH was slightly reduced in the liaI mutant (lanes 2 + 4 compared to lanes 6 + 8 and data not shown), indicating translational coupling between the two overlapping genes. Moreover, the ratio of the LiaH-specific signal was clearly shifted from the membrane fraction towards the cytoplasmic fraction and simultaneously decreased in the membrane fraction of the ΔliaI strain (Fig. 1D, lanes 5–8), indicative of an important role of LiaI for the membrane tethering of LiaH. However, the results also indicate that upon induction LiaH was still able to associate with the cytoplasmic membrane, even in the absence of its identified membrane anchor LiaI. This observation might indicate that LiaH, just as its proteobacterial counterpart PspA, has more than...
one interaction partner in the cytoplasmic membrane (Darwin, 2005). Alternatively, elevated levels of LiaH alone could be sufficient for its association with the membrane, as has been observed in a mutant of Y. enterocolitica that lacks the two known membrane anchors of PspA, PspB and PspC (Yamaguchi et al., 2010). Both scenarios could be envisioned for LiaH and further experiments will be required to clarify this observation.

Taken together, our data demonstrate that LiaI is a membrane protein with two TMRs that functions as a membrane anchor for LiaH through its cytoplasmic C-terminal domain. Moreover, our data can be viewed as a first indication that B. subtilis LiaH is embedded in a larger protein interaction network that involves additional, so far unknown, proteins to ensure its proper positioning at the cytoplasmic membrane under conditions of cell envelope stress.

**LiaI and LiaH colocalize in discrete foci under envelope stress conditions**

The data described above indicate that – under envelope stress conditions – the cytoplasmic protein LiaH is recruited to the cytoplasmic membrane with the help of LiaI and presumably additional proteins. This would suggest that LiaI and LiaH should at least to some extent also colocalize within the cell. We therefore aimed at directly visualizing this localization by generating strains in which functional C-terminal LiaI-/LiaH-GFP fusions were integrated directly into the native lia locus and therefore also expressed under the native stress-inducible liaI promoter. The cellular localization of LiaH and LiaI in strains TMB1328 and TMB1421, respectively, was then analysed by total internal reflection fluorescence microscopy (TIRFM). With this technique, only an about 200 nm wide section of the cell closest to the glass slide, containing the cell wall, cytoplasmic membrane, but only a small part of the cytoplasm, will be excited by the laser. Compared to epifluorescence, TIRFM therefore greatly reduces background fluorescence and bleaching of fluorophores for membrane-associated proteins (Spira et al., 2012).

In uninduced cells, only very faint signals could be detected for both proteins (Fig. 2A, left panels). While LiaH-GFP gave a diffuse signal, in line with a cytoplasmic localization, LiaI-GFP was always detected in a few distinct foci at the cytoplasmic membrane. While the number of LiaI-foci varied between cells, we never observed a dispersed signal at the membrane. Under inducing condition (addition of bacitracin, 20 μg ml⁻¹ final concentration), the number of LiaI-foci greatly increased. Importantly, LiaH-GFP now showed a similar cellular distribution, indicative of a colocalization and possibly complex formation of LiaI and LiaH in the presence of envelope stress (Fig. 2A, right panels).

**Fig. 2.** LiaI is the membrane anchor of LiaH and both localize in discrete foci.

A. Localization of LiaI-GFP (TMB1421) and LiaH-GFP (TMB1328) under control of their native promoter in uninduced (left) and bacitracin-induced cells (right). Bright-field images (left) and TIRF images (right).

B. Colocalization of LiaI and LiaH (TMB1441). The linescan plotted on the right was taken along the dotted line. Colocalization (orange), LiaI-GFP foci (green) and LiaH-mRFPRuby foci (red) (number of foci counted = 200).

C. Localization of LiaH-GFP in wild-type (TMB1328) and ΔliaI (TMB1407) cells. Bright-field images (top row) and corresponding TIRF images (bottom row). Quantification of LiaH-GFP foci per μm in wild-type and ΔliaI cells (means and standard deviations derived from 40 cells). The two conditions are statistically different in a t-test (P < 0.001). Cells were grown until OD₆₀₀ 0.2–0.4 in LB (supplemented with 0.05% xylose in B) at 30°C and induced with bacitracin (20 μg ml⁻¹) for 30 min.

Scale bars for all microscopic images: 2 μm.
To determine whether the observed localization pattern (Fig. 2A) as well as the interaction (Fig. 1C and D) of LiaI and LiaH under stress conditions indeed reflects the formation of complexes, we generated strain TMB1441, which contains a LiaI-GFP fusion gene integrated in the amyE locus under the control of the xylose-inducible promoter P\textsubscript{xyI}, together with a LiaH-mRFP\textsubscript{ruby} fusion protein placed under the control of the native liaI promoter. While the amount of ectopically expressed LiaI-GFP was now xylose-dependent and uncoupled from envelope stress, its behaviour under stress and non-stress conditions was indistinguishable from LiaI-GFP in strain TMB1421, in which the expression of the corresponding gene was under control of the native P\textsubscript{liaI} promoter (data not shown). As observed with LiaH-GFP in strain TMB1328, LiaH-mRFP\textsubscript{ruby} exhibited a weak and diffuse signal in non-stressed cells, while LiaI-GFP formed distinct foci at the cytoplasmic membrane (data not shown). Upon bacitracin stress, LiaH was recruited to the membrane patches, with 80% of foci containing both LiaI-GFP and LiaH-mRFP\textsubscript{ruby} (Fig. 2B). These numbers are in line with recent observations for the cell wall biosynthesis machinery of \textit{B. subtilis} (Dominguez-Escobar et al., 2011), thereby supporting the notion of a physical interaction between LiaI and LiaH at the membrane. This assumption was further supported by a reduction of the number of LiaI-GFP foci upon liaI deletion (Fig. 2C). In summary, our results support the notion that LiaI recruits LiaH to the membrane under envelope stress conditions. 

**LiaI foci are highly motile in non-stressed cells and become static under envelope stress conditions**

Having observed formation of LiaI/LiaH patches at the cytoplasmic membrane we next studied the dynamics of these foci in real time. For this purpose, we analysed membrane recruitment of LiaI-GFP at different time points post induction through cell wall stress. Upon addition of bacitracin (20 \(\mu\)g ml\(^{-1}\)) the first visible foci already appeared within 3 min and foci number constantly increased over 15–20 min (Fig. 3A and B). The observed timing was in line with a recent report on kinetics of P\textsubscript{liaI} induction, which demonstrated a rapid response of the LiaRS system to bacitracin shock (Kesel et al., 2013). Interestingly, at early induction time points (3–7 min) LiaH foci were often motile (kymographs in Fig. 3A), while they became static after prolonged induction (Fig. 3A).

Since our results indicate that LiaI recruits LiaH under stress conditions, we reasoned that LiaI foci should also become static in the presence of envelope stress. Indeed, while LiaI foci in unstressed cells showed fast and randomly oriented movement (Fig. 3C and supplemental Movie S1), most LiaI foci became immobile in the presence of bacitracin (Fig. 3D and supplemental Movie S2).

Taken together, our data suggest that a small number of LiaI foci constantly scan the cytoplasmic membrane during normal growth. Under envelope stress conditions, LiaI and LiaH expression is strongly increased. LiaI, maybe together with additional unknown proteins, recruits LiaH to the membrane into large immobile protein complexes. Both stalling and complex formation might either occur at sites of membrane damage, or be the result of the strong upregulation of LiaH expression itself in the presence of envelope stress conditions. The first idea would require that LiaI performs some sensory function in perceiving envelope damage, while the second would be independent of damage. To discriminate the two possibilities, we introduced a copy of the liaH operon, including its native LiaR-dependent promoter P\textsubscript{liaI} and encoding a C-terminal translational LiaH-GFP fusion protein, ectopically into the amyE locus, thereby placing it under the additional control of the xyle-dependent promoter P\textsubscript{xyI} (Fig. 4A). This set-up allows for the separation of a mere dose-dependent versus a stress-dependent localization of LiaH-GFP.

In the absence of xylose and envelope stress, weak and polar LiaH-GFP foci can be seen (Fig. 4A). Induction with xylose leads to a significant increase in strength of these mostly polarly localized foci. Moreover, some cells also contain individual and very weak lateral foci. In contrast, induction with bacitracin again leads to occurrence of numerous discrete LiaH-GFP foci (Fig. 4A), a distribution identical to the one observed before (Figs 2 and 3). To rule out any interference from the presence of the second native copy of the liaH operon, we also performed similar experiments in an isogenic liaH deletion strain. While the intensity of the foci was slightly weaker throughout, we basically achieved the same results (Fig. 4A). This result provides a first clear hint that foci formation is indeed coupled to envelope stress and not merely a result of an increase in protein amounts as a result of induction.

To substantiate our findings, we next investigated the dynamics of an ectopically expressed copy of LiaI-GFP, the expression of which was uncoupled form envelope stress, by time-lapse TIRF microscopy (Fig. 4B). Induction with xylose results in a strong increase in the number LiaI foci, which were highly motile. Hence, the behaviour of LiaI foci was comparable to the situation described for the uninduced wild-type (Fig. 3C), despite the significant increase in protein amounts and hence foci numbers. If bacitracin was simultaneously added, the number of LiaI-GFP foci did not increase further, but now all foci remained static. This again supports the hypothesis that foci stalling is primarily a consequence of envelope stress rather than a result of the increased protein amounts in the cell.
Recruitment of LiaH to static membrane foci is specifically induced by antibiotics that interfere with the lipid II cycle of cell wall biosynthesis

We have previously shown that the Lia system can be specifically triggered by cell wall antibiotics that interfere with the lipid II cycle of cell wall biosynthesis, such as bacitracin, nisin, or vancomycin, resulting in an over 200-fold increased liaIH expression for the first three compounds, and an about 50-fold induction in case of vancomycin (Mascher et al., 2004; Staroń et al., 2011 and references therein). But despite its strong induction, the Lia system does not mediate resistance against these inducers (Wolf et al., 2010). Of the three cell wall antibiotics for which B. subtilis shows a Lia-dependent change in susceptibility – fosfomycin, some β-lactams, and daptomycin – only the latter acts as an inducer. We therefore wanted to determine how cellular localization and dynamics of LiaI and LiaH were influenced by treatment with different antibiotics that affect cell wall biosynthesis and envelope integrity (Fig. 5A).

Both the strong induction and the recruitment of LiaH-GFP to membrane foci were readily observed for the known inducers of the Lia response, bacitracin, daptomycin, vancomycin and nisin. But despite the fact that a liaIH mutant shows increased fosfomycin sensitivity (Wolf et al., 2010), LiaH remained dispersed in the cytoplasm after a challenge with fosfomycin or ampicillin (Fig. 5A). Hence, cell wall antibiotics that affect cytoplasmic or extracellular steps of cell wall biosynthesis neither act as inducers of the Lia response, nor do they affect localization of LiaH (Fig. 5A) and LiaI (data not shown). Taken together, these observations demonstrate that the localization of LiaH into static membrane foci correlates with the inducer profile of the Lia response and hence the strong upregulation of LiaI and LiaH production, but not with the antibiotic sensitivity phenotypes associated with LiaIH.
Movement and localization of LiaI foci are independent of the cell wall biosynthesis machinery

In rod-shaped bacteria, the cell wall biosynthesis machinery is co-ordinated by the actin-like MreB, which spatially organizes the enzymatic activities required for proper bacterial growth (Chastanet and Carballido-Lopez, 2012; White and Gober, 2012). Recently, it was reported that contrary to previous models cell wall biosynthesis is not driven by treadmilling of MreB filaments, but instead that intracellular MreB patches are actively moved by peptidoglycan biosynthesis (Dominguez-Escobar et al., 2011; Garner et al., 2011). Accordingly, addition of cell wall antibiotics resulted in slowing or arrest of MreB motility (Dominguez-Escobar et al., 2011). It had previously been suggested that in E. coli MreB is crucial for PspA-mediated stress response, but not stress-induction of the...
pspA operon (Engl et al., 2009). Because of the known link between cell wall biosynthesis and MreB, and in light of the induction of the Lia response by cell wall antibiotics, especially since the motility of LiaI and LiaH foci was also negatively affected by the action of cell wall antibiotics, we wondered if the cellular localization of LiaIH and the dynamics of the MreB-associated cell wall biosynthesis machinery might overlap in the presence of severe envelope stress. To address this question, we studied the colocalization of mRFPRuby-MreB and LiaH-GFP (Fig. 5B and supplementary Movie S3). While both LiaI and MreB form motile patches at the membrane, their modes of movement are very different. While MreB patches are moved perpendicular to the long cell axis in a highly directed manner (Domínguez-Escobar et al., 2011; Garner et al., 2011), LiaI diffuses randomly within the membrane on a much faster timescale. Despite the fact that the mobility of both MreB and LiaI patches can be stopped in the presence of cell wall antibiotics, this response occurs at very different antibiotic concentration. In fact, LiaI foci seem to be significantly more susceptible to antibiotic-induced stalling (Fig. 5B): In the presence of bacitracin (20 μg ml⁻¹) movement of LiaI patches was already stalled, while MreB remained motile, clearly demonstrating that MreB, and hence the cell wall biosynthesis machinery was not associated with LiaIH, even under conditions of severe envelope stress (Fig. 5B and supplementary Movie S3).

These data, together with the antibiotic profile triggering the Lia response described above (Fig. 5A), indicate that the positions of LiaIH foci are independent of the cell wall biosynthesis machinery. Instead, it is reasonable to assume that LiaIH motility is most likely affected by changes in membrane properties as a result of antibiotic action. But the exact molecular nature of this stimulus remains to be identified.

The intrinsic stationary-phase induction of the Lia response is heterogeneously distributed but also results in the colocalization of LiaI and LiaH in foci

In addition to the very strong (about 200-fold) and immediate induction of the Lia response by the extrinsic addition of some cell wall antibiotics, the expression of liaIH operon also increases a moderate 10-fold at the onset of stationary phase, as quantified by β-galactosidase assays as an average over the whole population (Jordan et al., 2007). This intrinsic transition state induction of the Lia response is still poorly understood. We could previously demonstrate that under these conditions the activity of both the LiaRS system and Spo0A, the master regulator of differentiation and sporulation in B. subtilis are required (Jordan et al., 2007). But the nature of the intrinsic trigger still remains elusive, as does the reason for the much weaker promoter activities under these conditions.

In the course of the present studies, we noticed that LiaH-GFP and LiaI-GFP also localized in discrete foci in the stationary growth phase, similarly to the bacitracin-induced cells during mid-exponential phase (Fig. 6A). Remarkably, both induction and foci formation could only be observed in a small subset of cells, especially in late stationary-phase cultures (about 6% at an OD₆₀₀ of 7.2; Fig. 6B). To verify this observation, we analysed the behaviour of a transcriptional P₄₃₃₄₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃₉₄₃¢© 2014 John Wiley & Sons Ltd, Molecular Microbiology, 92, 716–732
constant $D$ by linear fits, as one expects a linear dependence of the MSD on the time lag for two-dimensional Brownian motion (MSD = $4D\tau$). Accordingly, the apparent diffusion constant of LiaI was reduced from $D = (2.7 \pm 0.4)$.

**Fig. 7.** Quantitative analysis of LiaI protein dynamics. A and B. Representative trajectories obtained by single particle tracking for LiaI-GFP under uninducing (A) and inducing (B) conditions. Each plot shows 10 trajectories (colours) followed over 10 frames recorded at a frame rate of 71 ms.

C. Mean squared displacement (MSD) analysis of LiaI protein dynamics in uninduced and bacitracin-induced cells as a function of the lag time $\tau$ between two observations. Symbols represent mean experimental MSDs and error bars the corresponding standard errors to the mean, based on $n = 57$ (LiaI-GFP –bac) and $n = 85$ (LiaI-GFP +bac) single particle trajectories, as described in Experimental procedures. Solid and dotted lines are fits of a simple two-dimensional diffusion model (MSD = $4D\tau$) and an anomalous diffusion model (MSD = $4\alpha\tau^\alpha$) respectively. The apparent diffusion constants $D$ obtained from the simple model are indicated next to the fits. The anomalous diffusion model describes the data significantly better than the simple one, as indicated by an $F$-test with $P$-values of 0.008 (LiaI unind.) and $3 \times 10^{-9}$ (LiaI ind.).

D and E. Best-fit parameters of the anomalous diffusion model and their corresponding confidence intervals [66.8% (light green), 95% (dark green) and 99% (black)]. The colour codes for log($\chi^2$), where a low $\chi^2$ indicates high quality of the fit for a given combination of model parameters.
\[ \times 10^{-2} \, \mu m^2 \, s^{-1} \] in the uninduced samples to \[ D = (0.4 \pm 0.1) \times 10^{-2} \, \mu m^2 \, s^{-1} \] in induced samples.

However, in both cases it is clearly visible that the MSD is not fully linear, but has a rather concave shape. In fact, it is known that most experimental diffusion measurements within cell membranes do not display simple linear relationships, but that an anomalous diffusion model (MSD = 4Γ τα) is often more suitable to explain the data (Saxton and Jacobson, 1997). In general terms, anomalous diffusion can be the result of both obstacles to diffusion and traps with a distribution of binding energies or escape times. Fits to the MSD curves in Fig. 7C showed that the anomalous diffusion model in fact explains our data significantly better than simple diffusion (P-values < 0.008, see caption to Fig. 7). While the anomalous diffusion constants Γ obtained for LiaI were similar to the apparent diffusion constants D under both conditions, the scaling exponent α was reduced from \[ α = 0.9 \pm 0.1 \] under uninducing to \[ α = 0.5 \pm 0.1 \] under inducing conditions (Figs. 7C–E).

While for eukaryotic membrane proteins anomalous diffusion seems to be the rule rather than the exception (Saxton and Jacobson, 1997), it has also been reported that in bacteria the three-dimensional cell geometry can lead to a sublinear increase of the MSD curves even when the particles undergo simple Brownian diffusion (Deich et al., 2004; Niu and Yu, 2008). Accordingly, it seems likely that the deviation of the anomalous scaling exponent obtained for LiaI under non-inducing conditions (\[ α = 0.9 \pm 0.1 \]) from the Brownian diffusion case (\[ α = 1 \]) can be attributed to such geometrical factors. However, the strong reduction of \[ α \] for LiaI foci under cell envelope stress conditions (\[ α = 0.5 \pm 0.1 \]) clearly indicates subdiffusive motion, because the cell geometry does not change upon antibiotic treatment.

These findings show that treatment with bacitracin does not only reduce the mobility of LiaI by a factor of eight, but also suggest that either damaged areas within the cell envelope or oligomerization with LiaH serve as obstacles and/or diffusional traps for LiaI foci under cell envelope stress conditions. The apparent diffusion coefficient for LiaI obtained under non-inducing conditions (\[ D = (2.7 \pm 0.4) \times 10^{-2} \, \mu m^2 \, s^{-1} \]) is near the lower end of the range of diffusion constants measured for other bacterial membrane proteins (Deich et al., 2004; Mullineaux et al., 2006; Lenn et al., 2008). This might suggest that LiaI forms larger complexes or locates within less mobile fractions of the bacterial membrane, but the limited amount of reference data does not permit a final conclusion.

**Discussion**

In this study, we demonstrated that LiaI is a small-membrane protein with two transmembrane helices that acts as a membrane anchor for the phage-shock protein homologue LiaH through its C-terminal cytoplasmic domain (Fig. 1). Our analyses further showed that under non-inducing conditions, LiaI is organized in a few membrane-anchored foci that appear to rapidly scan the cytoplasmic membrane in a diffusive pattern (Figs 2 and 3). Upon envelope stress, the liaH- operon is strongly induced (Mascher et al., 2004; Jordan et al., 2006). Accordingly, the number of LiaI foci rapidly increases (Fig. 3). Moreover, these foci seem to recruit LiaH to the membrane and become mostly static under envelope stress conditions (Figs 2–4). These observations indicate that LiaI foci could be sensors of some aspect of membrane-linked envelope stress, reminiscent of PspC (Darwin, 2005; 2007). Complex formation could then be the result of strongly increased LiaH amounts under inducing conditions, e.g. in the presence of envelope stress, potentially at the sites of envelope damage (Fig. 4).

**Functionality of GFP-fusion proteins of LiaI and LiaH**

Translational GFP-fusions are a powerful way to study the subcellular localization of proteins in vivo, and such approaches have revealed numerous important insights into their biological function in the past (Margolin, 2000; Phillips, 2001). While the self-contained domain structure of GFP often does not interfere with the functionality of its fusion partner, introduction of GFP-fusion proteins can nevertheless generate artefacts, leading to misinterpretations. Basically, such artefacts can be the result of any of the following three issues: (i) alterations of the protein amount due to copy-number effects, e.g. if expressed from a replicative plasmid, (ii) generation of artificial situations due to uncoupling the production of a fusion-protein from its native regulation, e.g. if expressed ectopically from an inducible or constitutive promoter without the corresponding partner genes and/or outside its normal regulatory context, and (iii) disruption of the proteins’ functionality by the fused GFP, in case that the fusion end is functionally important.

By introducing the gfp-fusion genes at their native locus, we avoided copy-number effects and ensured that expression of the fusion proteins, together with their coexpressed partner proteins, remained under their native regulation. While functionality of LiaI is indicated by its ability to interact with LiaH, we currently have no reliable read-out for LiaH-GFP functionality. While we cannot rule out the possibility of interfering with LiaH functionality due to the GFP-fusion, a vast body of evidence argues against such issues. PspA/IM30-like proteins are highly conserved, such that even distantly related family members can complement for each other, as recently demonstrated by the functional complementation of an E. coli pspA
mutant with VIPP1 from *Arabidopsis* (Zhang et al., 2012).

Over the years, a number of functional GFP-fusions of PspA or VIPP1 proteins have been generated and rigorously evaluated in different organisms, such as *E. coli*, *Y. enterocolitica*, *Chlamydomonas reinhardtii*, and *Arabidopsis thaliana* (Zhang et al., 2012). Moreover, we observe a relocalization of LiaH from a diffuse cytoplasmic distribution to membrane-associated patches both upon bacitracin stress and at the onset of stationary phase without any external inducer, but not with other cell wall antibiotics. It is hard to imagine that such a specific response based on demonstrated protein–protein interactions could be the result of a translational GFP-fusion, especially in light of similar redistributions of functional PspA-GFP fusion proteins in the other organisms. Nevertheless, in the absence of a clear LiaH-dependent phenotype that could be used to demonstrate the full functionality of translational GFP-fusions to LiaH, this potential caveat needs to be taken into account when interpreting the data presented in this work.

**Foci formation and motility is a universal feature of PspA/IM30 proteins**

Our observations are reminiscent of a recent report on the cellular dynamics of the proteins that mount the PSP response in *Y. enterocolitica*, the phage-shock protein PspA, the enhancer protein PspF, and the membrane proteins PspB and PspC (Yamaguchi et al., 2013). Under non-inducing conditions, all four proteins appear as highly motile foci either in the cytoplasm (PspA, PspF) or at the cytoplasmic membrane (PspB and PspC), with PspA and PspF directly interacting with each other. Under stress-inducing conditions, PspA switches its partner and re-associates with PspC that together with PspB can then be found in large static complexes at the cytoplasmic membrane (Yamaguchi et al., 2013). Moreover, membrane association of PspA requires induction of the PSP response (Yamaguchi et al., 2010), just as described here for the Lia response. A complex formation of PspA in lateral and highly motile patches has previously also been observed for *E. coli* (Engl et al., 2009; Lenn et al., 2011).

Overall, the behaviour of PSP proteins from *Y. enterocolitica* is very similar to the dynamics of Lia proteins in *B. subtilis*. One difference is that LiaH is present in very low amounts dispersed in the cytoplasm under non-inducing conditions, where it exerts no known function and does not seem to have a protein-interaction partner. Taken together, the available data on the cellular dynamics of PSP-proteins emphasizes the striking similarities between the Lia responses of *B. subtilis*, a low G+C Gram-positive bacterium, and the PSP response in Gram-negative γ-proteobacteria, despite the mechanistically different molecular regulation mechanisms (Fig. 8). This similarity seems to even reach beyond bacterial PspA-/IM30 family members. Two recent reports on the eukaryotic PspA-homologue VIPP1 from the alga *C. reinhardtii* and the plant *A. thaliana* demonstrated the formation of VIPP1-spots and even filaments within chloroplasts (Nordhues et al., 2012; Zhang et al., 2012). In the latter case, VIPP1 foci also showed dynamic movement, but in contrast to PspA and LiaH only under stress conditions (Zhang et al., 2012). Collectively, a unifying picture emerges that PspA/IM30 proteins exhibit their function in larger mobile or static complexes through protein–protein interactions at membrane interfaces. The close similarity of PspA-like proteins is also supported by the recent demonstration that *Arabidopsis* VIPP1 can functionally complement an *E. coli* pspA mutant (Zhang et al., 2012).

**Coupling of stress response and gene regulation**

A bacterial stress response usually is the combination of two cellular processes: (i) recruiting stress response pro-
teins to their site of action, and – often coupled to this – (ii) upregulation of the corresponding genes through a signal transducing mechanism. Along those lines, the model in Fig. 8 highlights an important difference between the proteobacterial PSP response and the Lia response in *B. subtilis*. In proteobacteria, the ultimate stress response and underlying gene regulation are tightly interlinked since they are mediated through the same mediator protein, PspC. In response to envelope stress PspC recruits PspA to the membrane and thereby elicits its protective role against cell envelope stress. At the same time, the latter releases its inhibitory grip on PspF, which is then able to induce expression of the pspA operon from a *σ*^54^-dependent promoter (Darwin, 2005; Joly *et al*., 2010). Hence PspC, as the sensory input of the PSP response is also the mediator of the output – recruitment of PspA to the membrane. Accordingly, the PspC dynamics follows that of PspA: the foci become static under inducing conditions (Yamaguchi *et al*., 2013).

In contrast, the regulation is very different in *B. subtilis* and presumably other Firmicutes bacteria that harbour LiaH homologues under control of the LiaFSR three-component system (Jordan *et al*., 2008; Schrecke *et al*., 2012). It seems that the membrane protein LiaI scans the envelope for damage and turns static once the cells encounter envelope stress and LiaH is recruited to the membrane. Since the LiaFSR-dependent stress regulation is fully functional in a liaIH deletion strain (Jordan *et al*., 2006), it seems that in *B. subtilis* – in contrast to the proteobacterial PSP response – there is just a regulatory but not a physical interaction between LiaFSR and LiaH. Consequently, the ultimate stress response (recruitment of LiaH to the membrane) is uncoupled from stress-induced gene regulation, as also indicated by the data shown in Fig. 4. But it will require additional work on the cellular dynamics of LiaFSR before such hypotheses can be explored further.

**Experimental procedures**

**Bacterial strains and growth conditions**

All bacterial strains and plasmids are listed in Table 1 and Table S1 respectively. In general, *B. subtilis* and *E. coli* were grown in LB medium or on plates at 37°C with aeration. *B. subtilis* strains used in this study are derivatives of the laboratory wild-type strain W168.

**DNA manipulations and cloning**

Molecular cloning techniques were performed as described (Sambrook and Russell, 2001). For topology investigation vectors pHA-4 and pAC7 were used. Fragments of *liaI* were amplified (primers see Table S2) and cloned into pHA-4 to generate plasmids pDW401 and pDW402. LacZ fusions (pDW1001 and pDW1002, see Table S1) were constructed by cloning *liaI* fragments, fused with the promoter *PphoA* (primer TM856, see Table S2), into the promoter-less vector pAC7. The replicative vector pGP380 was used for protein overexpression to study protein interactions via SPINE. The cytoplasmic C-terminus of *liaI* was amplified (Table S2), restricted and cloned into pGP380 (Herzberg *et al*., 2007). *B. subtilis* W168 was transformed with the resulting plasmid pDW3802, generating the strain TMB688 (Table 1). As a control, *B. subtilis* W168 transformed with the empty vector pDG380 was used (TMB841). For BACTH analysis, genes were amplified from chromosomal DNA of *B. subtilis* (Table S2) and cloned into pUT18, pUT18C, pKT25 and pKT25N (Karimova *et al*., 1998) (Table S1).

GFp- and mRFPruby-fusion constructs were designed for fluorescence microscopy (Table S1). Fragments of *liaH* and *liaI* were amplified and cloned into pSG1151 (Lewis and Marston, 1999; Feucht and Lewis, 2001) to construct C-terminal GFP fusions via endogenous recombination into the native lia-locus (pDW5101 and pDW5102). Full-length versions of *liaI* and *liaH* were amplified and cloned into pSG1154 (Lewis and Marston, 1999; Feucht and Lewis, 2001) to generate C-terminal GFP fusions under the control of *PphoA*, which were integrated into the amyE locus of *B. subtilis* W168 (pSJ5401 and pSJ5402). The vector pRWB2 (Dominguez-Escobar *et al*., 2011) was used to fuse N-terminal mRFPruby to *liaH* under the control of *PphoA* and later to integrate the plasmid (pDW6401) into the endogenous lia-locus by transformation of *B. subtilis* W168, generating strain TMB1714.

**Construction of markerless liaI deletion**

Markerless deletion of *liaI* was constructed using the vector pMAD (Arnaud *et al*., 2004). Genomic regions approximately 1 kb up- and downstream of the gene were amplified using primers listed in Table S2, and restricted. The two fragments were cloned into pMAD, generating pDW106. Mutants generation was performed as previously described (Arnaud *et al*., 2004). In brief: *B. subtilis* W168 was transformed with pDW106 and incubated at 30°C with MLS selection on LB agar plates supplemented with X-Gal (100 μg ml^-1^). Blue colonies were selected and incubated for 6–8 h at 42°C in LB medium with MLS selection, resulting in the integration of pDW106 into the chromosome. Again, blue colonies were picked from LB (X-Gal) plates and incubated at 30°C for 6 h in LB medium without selection. Subsequently, the liquid culture was shifted to 42°C for 3 h, and the cells were then plated on LB (X-Gal) plates, this time without selective pressure. White colonies that had lost the plasmid were picked and checked for MLS sensitivity. The resulting strain TMB1394 was subsequently analysed by PCR and sequenced for the integrity of the desired genetic modifications.

**PhoA/LacZ activity assay**

The topology of LiaI was tested by using the PhoA/LacZ activity assay. *E. coli* strain CC118 was transformed with plasmids containing *phoA*-fusions (pDW401and pDW402 Table S1) and streaked on agar plates with 5-brom-4-chlor-3-indolylphosphate (BCIP 50 μg ml^-1^), ampicillin (100 mg ml^-1^) and arabinose (0.2% w/v). Plasmids containing *lacZ*-fusions
(pDW1001 and pDW1002; Table S1) were transformed into *B. subtilis* W168 and streaked on agar plates with kanamycin (10 μg ml⁻¹) and X-Gal (100 μg ml⁻¹). Plates were incubated at 37°C overnight, followed by blue-white screening (Manoil, 1991).

In vivo cross-linking (SPINE)

SPINE (Strep-protein interaction experiment) was performed as described (Herzberg *et al*., 2007) by using formaldehyde, a cross-linking agent (final concentration 0.6%). In brief, cells were grown in LB at 37°C to an OD₆₀₀ of 1.0. Formaldehyde was added and after 20 min cells were harvested and washed in cell disruption buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). Again, cell pellets were resuspended in cell disruption buffer, then PMSF (1 mM) was added and cells were disrupted by sonication. After centrifugation, strep-tag purification was performed by using Strep-tactin columns (1 ml Strep-Tactin® sepharose, IBA, Goettingen, Germany). Proteins were separated on 12.5% SDS-PAGE and cross-linked proteins were detected by Western blot.

**Cell fractionation**

*Bacillus subtilis* cells were grown in LB at 37°C to an OD₆₀₀ of 1.0 and then fractionated into cytoplasmic and membrane components as described (Schöbel *et al*., 2004): the cell pellet was resuspended in cell disruption buffer and disrupted by sonication. After centrifugation, the supernatant was ultracentrifuged at 4°C, 30 000 g for 1 h and the supernatant was again collected (= cytoplasmic fraction). The membrane pellet was washed and ultracentrifuged again (4°C, 30 000 g, 30 min). Finally, the membrane pellet was resuspended in cell disruption buffer. Protein concentrations of all collected fractions were determined by using BCA reagent (PIERCE) and 30 μg total protein was loaded per lane.

**Immunoblotting**

Immunoblotting by using α-Strep-Tactin horseradish peroxidase conjugate was performed according to the manufacturer’s protocol (IBA, Goettingen). Western analysis with α-LiaH (1:5000) and α-rabbit horseradish peroxidase conjugate secondary antibody (1:7500) was done as described (Jordan *et al*., 2007).

**Bacterial two-hybrid analysis**

BACTH analysis was carried out as described (Karimova *et al*., 1998). *E. coli* BTH101 was co-transformed with plasmids containing genes of interest and either T18 or T25 fragments. After transformation, cells were spotted on agar plates with kanamycin (25 μg ml⁻¹), ampicillin (50 μg ml⁻¹), X-Gal (40 μg ml⁻¹) and IPTG (0.5 mM) and incubated at 30°C for 40 h. Blue colonies indicate potential protein–protein interactions. Empty vectors or vectors carrying fusions of the leucine zipper of GCN4 to the T18/T25 fragments were used as negative and positive controls respectively.

**Sample preparation for microscopy**

Overnight pre-cultures of *B. subtilis* were grown in LB medium supplemented with xylose (0.05%) and appropriate antibiotic selection from freshly isolated colonies on plate. Day cultures were performed by diluting pre-culture to an OD₆₀₀ of 0.01–0.05 in LB and grown at 30°C. Expression of fluorescent xylose-inducible fusions was induced by addition of xylose to 0.05%. Samples for microscopic observation were taken at exponential phase (OD₆₀₀ of 0.3–0.6) or stationary phase (OD₆₀₀ of 1.7–2.0) and immobilized on 1.2% agarose-coated microscope slides as described (Glaser *et al*., 1997). To stain the bacterial membranes, the vital membrane dye FM4–64 was added to obtain the final concentration of 2 μg ml⁻¹ in uninduced cells. To induce with bacitracin, cells were diluted to an OD₆₀₀ in uninduced cells of 0.2, then 1 ml of culture was harvested and resuspended in LB medium with 20 μg ml⁻¹ bacitracin, cells were incubated again to an OD₆₀₀ of 0.4 and samples were taken for microscopic observations.

**Microscopy**

All images were acquired on a custom TIRFM set-up from Till Photonics based on a fully automated iMIC Stand with climate control chamber and an Olympus 1.45 NA 100× objective. DPSS lasers with output powers of 75 mW at 488 nm (Coherent Sapphire) and 75 mW at 561 nm (Cobalt Jive) were used as light sources. Lasers were selected through an AOTF and directed through a broad-band fibre to the iMIC. A galvanometer-driven 2-axis scanner head was used to adjust TIRFM incidence angles. Images were collected with an AndorXON DU-897 EM CCD camera at maximum gain setting (300) attached to a 2× magnification lens. Acquisition was controlled by the Live Acquisition (Till Photonics) software package. For two-colour TIRFM experiments a double colour filter set was used. Incidence angles and z-position were adjusted individually for both channels to obtain comparable evanescent wave penetration depth and focus position. Time-lapse movies were taken on at least three different days for each strain. To follow LiaH dynamics exposure times of 100 ms and frame rates of 200 ms, for LiaI 50 ms and frame rates of 71 ms and for MreB exposure times of 100 ms and frame rates of 2 s were used. All images were processed in Metamorph v7.1.2 (Molecular Devices) using local background subtraction (flatten background function) and Gaussian filtering (kernel 1-3-1; 3-7-3; 1-3-1). Kymographs, linescans, colour overlay and image montages were performed with the respective functions in Metamorph. Images were rotated and zoomed for visualization purposes only. Number of foci per length quantification was made in ImageJ 1.43 (Wayne Rasband, National Institute of Health, USA).

**Single particle tracking and MSD analysis**

For the automated detection and quantitative analysis of particle trajectories a two-dimensional (2D) particle tracker Mosaic (Sbalzarini and Koumoutsakos, 2005) was used. For LiaI-GFP movies of 71 ms between frames (200 frames with exposure times of 50 ms) were analysed. For particle detection a radius of 3, a cut-off of 0, a percentile of 0.1–0.2% and for particle linking a link range of 2 and a displacement of 2
pixels were used. Trajectories of less than 10 frames were filtered. The tracking software returns the x and y positions of the traces in the respective frame of the movie, from which the mean squared displacement (MSD) was calculated. To that end, the squared displacement for each trajectory $\Delta^2(x(t), t) = [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2$ was averaged over all available t, resulting in the MSD of each trajectory $<\Delta^2>$. Finally, the MSD was averaged over about 100 trajectories for each protein species and condition. The resulting MSD curves were fitted with the normal and anomalous diffusion models described in the main text by using a trust-region reflective Newton method (MATLAB, The MathWorks). Standard deviations on estimated parameters were determined from 66.8% confidence intervals as described (Press et al., 1992). Since the fitted models are nested, an F-test was performed to test whether the anomalous diffusion describes the data significantly better than the normal diffusion model.

Comparative genomic analysis

Domain-based analysis of protein sequences was performed using the SMART database (Schultz et al., 1998) and the microbes-online website. Secondary predictions of the protein LiaI was carried out using database JPred3 (Cole et al., 2008) (http://www.compbio.dundee.ac.uk/www-jpred/).

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References


CHAPTER IV

Cannibalism Stress Response in *Bacillus subtilis*

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Cannibalism Stress Response in Bacillus subtilis

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**Keywords:** Cell envelope stress response, antimicrobial peptides, stationary phase survival, Bce system, ECF $\sigma$ factors.

**Subject category:** Regulation

**Running Title:** Cannibalism stress response in \textit{B. subtilis}

**Word count:** 5221

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4.1 Abstract

When faced with carbon source limitation, the Gram-positive soil organism *Bacillus subtilis* initiates a survival strategy called sporulation, which leads to the formation of highly resistant endospores that allow *B. subtilis* to survive even long periods of starvation. In order to avoid commitment to this energy-demanding and irreversible process, *B. subtilis* employs another strategy called cannibalism to delay sporulation as long as possible. Cannibalism involves the production and secretion of two cannibalism toxins, the sporulation delaying protein, SDP, and the sporulation killing factor, SKF, which are able to lyse sensitive siblings. The lysed cells are thought to provide nutrients for the cannibals to slow down or even prevent them from entering sporulation. In this study, we uncovered the role of the cell envelope stress response (CESR), especially the Bce-like antimicrobial peptide detoxification modules, in cannibalism stress response during stationary phase. SDP and SKF specifically induce Bce-like systems and some ECF σ factors in stationary phase cultures, but only the latter provide some degree of protection. A full Bce response is only triggered by mature toxins, but not by toxin precursors. Our study provides insights into the close relationship between stationary phase survival and the CESR of *B. subtilis*.

4.2 Introduction

In their natural environment, microorganisms constantly compete for nutrients. In order to defend their habitat against invading species, many bacteria produce and secrete antimicrobial peptides (AMPs) that interfere with the integrity or biosynthesis of the cell envelope. AMP action leads to an arrest in cell growth and often to cell lysis (Silver, 2003, Walsh, 2003, Silver, 2006). To defend against such antimicrobial attacks, many bacteria induce a complex cell envelope stress response (CESR). In *Bacillus subtilis*, the underlying regulatory network is orchestrated by four two-component systems (TCS) and seven extracytoplasmic function (ECF) σ factors (Helmann, 2002, Jordan et al., 2007, Schrecke et al., 2012).

While it is generally accepted that the CESR network has evolved to maintain envelope integrity in the face of AMPs produced by competing species, little is known about the extent to which it is also involved in responding to endogenously produced AMPs. For instance, although it is known that the AMPs are co-expressed with dedicated immunity proteins that prevent cells from autolysis (Gonzalez-Pastor et al., 2003, Ellermeier et al., 2006, Dubois et al., 2009), it is conceivable that the level of self-protection via these mechanisms can be insufficient, raising the need for additional protection by the CESR network. In fact, we recently reported that in early stationary phase a subpopulation of *B. subtilis* cells strongly induces one of the CESR modules, the LiaRS system, even in the absence of competitors and without any external addition of AMPs (Jordan et al., 2007, Dominguez-Escobar et al., 2014). Here, we set out to test whether other systems of the CESR network of *B. subtilis* also displayed such an intrinsic induction behavior during stationary phase and, if so, whether this was causally related to the endogenous production of AMPs.
To study these questions, we focused on the expression of the core of the CESR network, comprising the AMP-resistance modules, BceRS and PsdRS, as well as the ECF σ factors σ\textsuperscript{M}, σ\textsuperscript{X} and σ\textsuperscript{W}. While the BceRS and PsdRS systems regulate ABC transporters (BceAB and PsdAB, respectively) that specifically confer resistance against a number of AMPs (Staroń et al., 2011), the regulons of the ECF σ factors are known to play a more promiscuous role in cell envelope stress response to antimicrobial compounds (Missiakas & Raina, 1998, Helmann, 2002, Mascher et al., 2007, Kingston et al., 2013). σ\textsuperscript{M}, σ\textsuperscript{X} and σ\textsuperscript{W} each regulate a set of about 30-60 target genes with partially overlapping specificity (Mascher et al., 2007, Kingston et al., 2013), and all are activated in a growth phase- and growth medium-dependent manner (Huang et al., 1998): While σ\textsuperscript{M} and σ\textsuperscript{X} are induced mainly in late logarithmic growth phase, σ\textsuperscript{W} only becomes active in early stationary phase (Huang et al., 1998, Nicolas et al., 2012).

So far, no growth phase dependency has been observed for the BceRS and PsdRS modules. Both systems respond to and mediate resistance against a variety of peptide antibiotics: The BceRS system responds to the cyclic peptide antibiotic bacitracin and to a lesser extent also to the lantibiotics actagardine and mersacidin (Mascher et al., 2003, Rietkötter et al., 2008), while the PsdRS system responds primarily to lantibiotics, such as nisin or gallidermin (Staroń et al., 2011). Since the B. subtilis strain W168 is known to produce and secrete a variety of similar AMPs, it was conceivable that they might also act as inducers of the BceRS and PsdRS modules.

In this study, we show that the BceRS and PsdRS system are, in fact, intrinsically activated during stationary phase growth of B. subtilis, and single out the inducers amongst a number of endogenously produced AMP candidates. The biological role of these AMPs has previously been implicated in a process termed “cannibalism”, in which the stationary phase population bifurcates into a fraction of AMP-producing cells that feed on another fraction of non-producing cells (Chung et al., 1994, Gonzalez-Pastor et al., 2003). Our data reveals that the CESR network not only serves as a defense against extrinsic attacks from competing species, but also plays a novel role in the intrinsic cannibalism stress response. Interestingly, we show that induction of the BceRS and PsdRS modules by cannibalism toxins critically hinges on the presence of the cognate immunity proteins, providing further insight into the mode of stimulus perception by these systems.

### 4.3 Methods

#### 4.3.1 Media and growth conditions

B. subtilis and E. coli were routinely grown in Luria Bertani (LB) medium or MCSE (Radeck et al., 2013) including 0.2% fructose (w/v) as C-source at 37°C with agitation. The final composition of MCSE is as follows: 1× MOPS (from 10× MOPS buffer: 83.72 g l\textsuperscript{-1} MOPS, 33 g l\textsuperscript{-1} (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 3.85 mM KH\textsubscript{2}PO\textsubscript{4}, 6.15 mM K\textsubscript{2}HPO\textsubscript{4}; adjusted to pH 7 with KOH), 50 mg l\textsuperscript{-1} Tryptophan, 22 mg l\textsuperscript{-1} ammonium ferric citrate, 1× III’-salts (232 mg l\textsuperscript{-1} MnSO\textsubscript{4}.xH\textsubscript{2}O, 12.3 g l\textsuperscript{-1} MgSO\textsubscript{4}.xH\textsubscript{2}O), 0.8% (w/v) K-glutamate, 0.6% (w/v) Na-succinate, 0.2% (w/v) fructose. MCSE results in well-defined growth behavior and supports sporulation of B. subtilis under the growth conditions applied. Selective
media for *B. subtilis* contained chloramphenicol (5 µg ml\(^{-1}\)), kanamycin (10 µg ml\(^{-1}\)), spectinomycin (100 µg ml\(^{-1}\)), or erythromycin (1 µg ml\(^{-1}\)) plus lincomycin (25 µg ml\(^{-1}\)) for macrolide-lincosamide-streptogramin B (MLS) resistance. Selective media for *E. coli* contained ampicillin (100 µg ml\(^{-1}\)) or chloramphenicol (35 µg ml\(^{-1}\)). Solid media additionally contained 1.5% (w/v) agar.

**4.3.2 Bacterial strains and plasmids**

Transcriptional promoter fusions to bacterial luciferase (*luxABCDE*) were constructed in pAH328 (Schmalisch *et al.*, 2010) or the pAH328 derivative pBS3Clux (Radeck *et al.*, 2013) using *Not*I/*Sal*I or *EcoR*I/*Spe*I restriction enzymes, respectively. All strains used in this study are listed in Table 1 (at the end of section 4.3). All *B. subtilis* strains in this study are derivatives of the laboratory wild type strain W168. All plasmids and oligonucleotides are listed in Table 2 and 3, respectively (at the end of section 4.3).

**4.3.3 DNA manipulations**

All plasmids were constructed by standard cloning techniques and ligation mixtures were transformed into *E. coli* competent cells (DH5α, XL1-blue). The plasmids were verified by sequencing and transformed into *B. subtilis* as described previously (Harwood & Cutting, 1990). Plasmid integration into the *B. subtilis* chromosome was checked by colony-PCR. Preparation of chromosomal DNA from *B. subtilis* for transformation was prepared according to standard procedure (Cutting & Van der Horn, 1990).

**4.3.4 Allelic replacement mutagenesis of *sdpAB, sdpC, sdl, skfA-H, skfA, skfBC, skfEF, skfGH, skfH, sunA* and *yydF-J* using LFH-PCR**

Long Flanking Homology PCR (LFH-PCR) technique was performed as described previously (Mascher *et al.*, 2003). The constructed strains are listed in Table 1 and the corresponding primers are listed in Table 3 (at the end of section 4.3).

**4.3.5 Luminescence Assay**

Promoter activities were detected by following luminescence in a Synergy™2 multi-mode microplate reader from BioTek® (Winooski, VT, USA) using Gen5™ software. Strain cultivation was performed as follows: Freshly prepared and pre-warmed (37°C) MCSE medium was inoculated 1:500 from overnight cultures and incubated at 37°C with agitation until OD\(_{600}\) 0.2. The culture was subsequently diluted to an OD\(_{600}\) of 0.05 with MCSE and 100 µl were transferred to one well of a 96-well plate (black walls, clear bottom; Greiner Bio-One, Frickenhausen, Germany). OD\(_{600}\) and luminescence were recorded every ten minutes for 18 hours. Incubation was performed at 37°C with agitation (medium intensity). Raw luminescence data were normalized to cell density by dividing luminescence per OD\(_{600}\) at each data point (relative luminescence units (RLU) / OD\(_{600}\)). For each individual sample, OD\(_{600}\) and luminescence were background-corrected by subtracting the respective mean values measured for MCSE medium only and TMB1578 (pAH328 empty) over every time point. Subsequently, RLU/OD\(_{600}\) values were calculated for each measurement and
mean values and SEM (standard error of the mean) were determined from at least three independent biological replicates.

**Table 1:** Strains used in this study.

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<thead>
<tr>
<th><strong>E. coli</strong></th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>recA1 endA1 gyrA96 thi hsdR17K- mK+ relA1 supE44 φ80ΔlacZΔM15 Δ(lacZYA-argF)U169</td>
<td>(Sambrook &amp; Russell, 2001)</td>
</tr>
<tr>
<td>XL1-blue</td>
<td>endA1 gyrA96(alk) thi-1 recA1 relA1 lac gln44 F[λr10 proAB lacIΔ757 Δ(lacZ)M15] hsdR17(rK mK') tetR</td>
<td>lab stock</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. subtilis</strong></th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>W168</td>
<td>trpC2</td>
<td>lab stock</td>
</tr>
<tr>
<td>TMB1518</td>
<td>W168 ΔbceRSAB psdRSAB yxdJKLM yxeA (clean)</td>
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</tr>
<tr>
<td>TMB1528</td>
<td>W168 sdpt::mls</td>
<td>this study</td>
</tr>
<tr>
<td>TMB1578</td>
<td>W168 sacA::luxABCDE (without promoter)</td>
<td>this study</td>
</tr>
<tr>
<td>TMB1619</td>
<td>W168 sacA::pChlux103 (P_bceA::lux)</td>
<td>this study</td>
</tr>
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<td>TMB1620</td>
<td>W168 sacA::pChlux104 (P_bceC::lux)</td>
<td>this study</td>
</tr>
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<td>TMB1768</td>
<td>W168 sdPC::kan</td>
<td>this study</td>
</tr>
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<td>TMB1985</td>
<td>W168 sacA::pHlux1102 (P_sdpC::lux)</td>
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<td>TMB2009</td>
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<td>TMB2015</td>
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<td>TMB2164</td>
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<td>TMB2166</td>
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<td>TMB2212</td>
<td>W168 sacA::pHlux105 (P_sdA::lux) sdpt::mls</td>
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<td>TMB2240</td>
<td>W168 spo0A::spec</td>
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<td>TMB2257</td>
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<td>W168 sacA::pCH3Clux04 (P_sdA::lux)</td>
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<td>TMB2260</td>
<td>W168 sktA::mls</td>
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<tr>
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Table 2: Vectors and plasmids used in this study.

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<tr>
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<td>sacA::…sacA, luxABCDE, bla, cat</td>
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<td>PBS3Clux</td>
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<td>pCHlux103</td>
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<td>pCHlux104</td>
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<td>pJHlux102</td>
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aResistance cassettes: bla = ampicillin, cat = chloramphenicol

Table 3: Oligonucleotides used in this study.

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<td>Construction of transcriptional promoter-lux fusions</td>
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<td>TM2513 P_{bceA} NotI fwd</td>
<td>agcgccgctgacaagcgcgttataaatagc</td>
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<td>TM2514 P_{bceA} SalI rev</td>
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<td>TM2515 P_{bcrC} NotI fwd</td>
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<tr>
<td>TM2516 P_{bcrC} SalI rev</td>
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<td>TM2785 P_{sdpA} EcoRI fwd</td>
<td>ttataggaacctagccctctctag</td>
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<tr>
<td>TM2786 P_{sdpA} SpeI rev</td>
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58
Table 3: continued.

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<th>Template</th>
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<td>Allelic replacement mutagenesis (LFH-PCR)</td>
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Restriction sites are highlighted in bold italics; BioBrick overhang sequences are underlined; overhang sequences for resistance cassettes are marked in italics.

4.4 Results and Discussion

4.4.1 Intrinsic induction of CESR target promoters during stationary phase growth

Initially, we aimed at investigating if other modules within the CESR network displayed induction profiles similar to the LiaRS system, which – when grown into stationary phase – displayed a clear induction pattern in the absence of any external stimulus (Dominguez-Escobar et al., 2014). To this end, we fused the target promoters of the BceRS system (P_{bceA}), of the PsdRS system (P_{psdA}) and selected target promoters of σ^M, σ^K, and σ^W (P_{ydlAH}, P_{sigX}, and P_{pspA}, respectively) and one promoter which is regulated by all three σ factors, P_{bcrC}, to a promoter-less luxABCDE reporter (Schmalisch et al., 2010, Radeck et al., 2013). The resulting promoter-lux fusions were integrated into the chromosome of B. subtilis W168 wild type cells. Automated incubation of the resulting reporter strains in a microplate reader revealed that all but the σ^W target promoter P_{pspA} displayed a marked increase in luminescence activity between two and four hours after the onset of stationary phase (Fig. 4.1; t=7-8 h). The amplitude of this intrinsic stationary phase induction was highest for the BceRS and PsdRS target promoters (both approx. 500-fold induction; Fig. 4.1b), but also the ECF target promoters displayed a 10-20-fold increase in promoter activity (Fig. 4.1d). From these observations, we conclude that large parts of the CESR network in B. subtilis perceive one or multiple stimuli that are endogenously produced between two to four hours after entry into stationary phase.
Fig. 4.1: Intrinsic late stationary phase induction of $P_{\text{bceA-lux}}$, $P_{\text{psdA-lux}}$ (a, b) and ECF $\sigma$ factor target promoters in W168 (c, d). Promoter activity was detected by following luminescence of 100 µl cultures growing in a microplate reader (Biotek®, Synergy™2; 96-well plate, 37°C, shaking) over time. The upper graphs (a, c) show the growth curves (OD$_{600}$) of the respective strains in MCSE medium. The lower graphs (b, d) show the promoter activities as relative luminescence units (RLU) per OD$_{600}$. Late stationary phase induction is shown for both the $P_{\text{bceA}}$ (black) and $P_{\text{psdA}}$ (orange) after 7-8 h of growth (b). Induction of $P_{\text{bcrC}}$ controlled by $\sigma^M$, $\sigma^X$ and $\sigma^W$ after 7-8 h of growth is shown in green (d). Intermediate induction of $\sigma^X$- and $\sigma^M$- dependent promoters ($P_{\text{sigX}}$ and $P_{\text{ydaH}}$) is shown in red and purple, respectively, after 7-8 h of growth. The $\sigma^W$- dependent $P_{\text{pspA}}$ (blue) stays uninduced under our cultivation conditions. Please note that the small peak at t=5 h in this and all the following figures does not represent a regulated transition phase promoter induction, since it was observed for any promoter studied in MCSE so far, including a set of known constitutive promoters (Radeck et al., 2013). All graphs show mean values and SEM (standard error of the mean) of at least three independent replicates.

4.4.2 AMPs and cannibalism toxins induce CESR systems

Both the BceRS and PsdRS system have been shown to respond to different peptide antibiotics that interfere with the cell wall biosynthetic pathway during exponential growth (Breukink & de Kruijff, 2006, Staron et al., 2011). In order to elucidate the mechanism behind the observed intrinsic stationary phase activation, we asked whether it could be caused by endogenously produced AMPs of *B. subtilis* W168. The first AMP we considered was Sublancin 168 (SunA), which is a SPβ prophage-derived bacteriocin described as an S-linked glycopeptide active against Gram-positive bacteria (Oman et al., 2011). Its production is known to be repressed during exponential growth phase by the transcriptional regulators AbrB and Rok (Albano et al., 2005, Strauch et al., 2007). Another peptide that might trigger stationary phase induction of the CESR is the YydF peptide, which has been shown to be an endogenous inducer of the LiaRS system (Butcher et al., 2007). Its production is also negatively controlled by AbrB during logarithmic growth (Butcher et al., 2007). Subtilosin A (SboA) is another bacteriocin produced by *B. subtilis* W168. Although it is known to be transcriptionally regulated by AbrB and by the two-component regulatory proteins ResDE (Nakano et al., 2000, Strauch et al., 2007), it has been reported to be produced only under anaerobic growth conditions (Nakano et al., 2000). Indeed, we found the sboA promoter to be inactive over the whole
time course under our cultivation conditions (data not shown). The last two potential AMPs were the two cannibalism toxins sporulation delaying protein, SdpC and sporulation killing factor, SkfA (referred to as SDP and SKF hereafter).

To study the effect of the AMPs on the induction of the CESR network, we analyzed P_{bceA}, P_{psdA} and P_{bcrC} promoter activation in mutants deleted for each gene encoding the respective antimicrobial peptides (Fig. 4.2).

Deletion of sunA (Sublancin 168) had no effect on any promoter activity and deletion of yydF-J only showed a minor effect on P_{bceA} promoter activity. In contrast, sdpC and skfA-H mutants revealed the most prominent reduction in luciferase activity for all three promoters tested. Deletion of sdpC resulted in an approx. 10-fold reduced P_{bceA} activity (Fig. 4.2b, blue curve), and deletion of skfA-H decreased the activity about 100-fold (Fig. 4.2b, green curve). The effect of an sdpC deletion on P_{pada} induction was moderate (about 3-fold decrease), but P_{pada} activity was almost completely lost in a skfA-H mutant (Fig. 4.2d). In contrast, P_{bcrC} activity was more strongly decreased in the sdpC mutant (about 4-fold, Fig. 4.2f) than in the skfA-H deletion strain (max. 2-fold). Moreover, in an sdpC skfA-H double mutant, stationary phase activity of P_{bceA} and P_{pada} was fully abolished, while P_{bcrC} still displayed mild induction. Hence, we could identify the two cannibalism toxins SDP and SKF as strong inducers of all three CESR target promoters in stationary phase.

While induction of ECF σ factors was expected, given the described role in mounting a secondary layer of defense against SDP (Butcher & Helmann, 2006), this is the first time that an intrinsic growth phase-dependent induction has been observed for Bce-like systems. Since the
effect was most prominent for the $bceA$ promoter, subsequent investigations of the cannibalism stress response were restricted to the BceRS system alone, but key findings were also verified for the PsdRS system, demonstrating similar behavior (data not shown).

### 4.4.3 Toxin production correlates with $P_{bceA}$ induction

We next tested how stationary phase induction of $P_{bceA}$ was correlated with the activation of $sdpC$ and $skfA$ expression. SDP is under dual control of first its own promoter $P_{sdpC}$ and second under the promoter driving the whole $sdpABC$ operon $P_{sdpA}$ (Fig. 4.3).

![Fig. 4.3: Schematic overview of SDP and SKF maturation and genomic context.](image)

Panels (a) and (c) show main transcripts of the $sdpABC$-$sdpRI$ and $skfA$-$H$ operons, each based on recent microarray studies (Nicolas et al., 2012). Panels (b) and (d) show the hypothesized schematic maturation pathway of SDP and SKF precursors until release of the final toxin. According to Perez Morales et al., 2013, pro-$SdpC$ is translocated across the membrane by the general secretory pathway (Sec) and the leader peptide thereby cleaved by the SipS/T peptidase (b). $SdpAB$ further cleave $SdpC^*$ at the N- and C-termini to release the final SDP toxin to the environment. Similarly, pro-$SkfA$ is hypothesized to be modified by $SkfB$ to give pre-$SkfA$ which is assumed to be further processed by $SkfH$ to prepare for export and cyclization by $SkfEF$ and $SkfC$, respectively (d). These assumptions are based on Liu et al., 2010 and lack further evidence.

We tested both promoter activities over the whole time course and found $P_{sdpA}$ to be the stronger promoter under our cultivation conditions (data not shown). Therefore, we assumed that $P_{sdpA}$ is the crucial promoter driving also expression of $sdpC$. Thus, we studied the luminescence activity from $P_{sdpA^*}$ and $P_{skfA^*}$-$luxABCDE$ reporter fusions throughout growth of the W168 wild type strain to test correlation between SDP/SKF production and $P_{bceA}$ induction (Fig. 4.4). $P_{sdpA}$ was induced about 10-fold, while $P_{skfA}$ displayed a 100-fold induction. While both the $sdpA$ and $skfA$ promoters were induced 5-6 h after the beginning of the experiment, the $bceA$ promoter became active approx. 2 h later. This indicates that the toxins first had to be produced, processed and likely also accumulated to a certain threshold concentration in order to activate the BceRS system.
4.4.4 The BceRS system does not mediate resistance against cannibalism toxins

Based on its role in mediating resistance against the peptide antibiotic bacitracin, we reasoned that the BceRS system might also confer resistance against SDP. The immunity protein of the sdpABC-sdpRI operon is SdpI (Fig. 4.3). Both the toxin biosynthesis operon sdpABC and the immunity operon sdpRI are under control of the transition state repressor AbrB and the master regulator of sporulation Spo0A (Ellermeier et al., 2006). SdpI reveals receptor/signal transducing properties, and its synthesis is induced by a combined interplay between SDP, SdpI and SdpR (Ellermeier et al., 2006). In brief, SdpR constitutes an autorepressor blocking transcription of sdpRI in the absence of SDP. Upon SDP synthesis and export, SDP binds to SdpI at the membrane, which enables the latter to recruit SdpR into the SDP-SdpI membrane complex. This titration of SdpR away from the DNA induces transcription of sdpRI, which results in immunity against SDP (Ellermeier et al., 2006). Accordingly, cannibalism-inactive cells are expected to neither produce and secrete SDP nor induce enhanced SdpI expression. Consequently, it is believed that these cells are highly sensitive to SDP and prone to lysis while toxin-producing cells are resistant against SDP (Ellermeier et al., 2006).

In order to study the contribution of the BceRS system towards resistance against SDP, we first performed growth measurements of wild type and a mutant carrying unmarked deletions of all three Bce-like systems (ΔbceRSAB ΔpsdRSAB ΔyxdJKLM-yxeA) of B. subtilis W168 (Gebhard et al., 2014) (TMB1518, referred to as “3xbce mutant” hereafter) shown in Fig. 4.5(a).

Although this mutant strain lacks all important peptide antibiotic detoxification modules present in B. subtilis, this did not affect growth compared to wild type (Fig. 4.5a, blue and black curve, respectively). In contrast, comparison of wild type growth to an sdpI mutant revealed a severe growth defect upon entry into stationary phase (Fig. 4.5a, orange curve). Given that the 3xbce mutant seems to be unaffected in its growth behavior, we conclude that the BceRS system is not involved in mediating resistance against SDP. Furthermore, we observed no P_bceA induction in the 3xbce mutant, demonstrating that SDP/SKF cannot be sensed in the absence of the signal transduction system and resistance is not mediated by any of the Bce-like systems (data not shown).
This is further supported by the finding that a mutant deficient in both the 3xbce resistance modules and the SdpI immunity protein (Fig. 4.5, pink curve) did not show a stronger growth defect than the sdpI mutant alone. To further validate that the BceRS system is indeed not involved in resistance against SDP, we additionally tested the viability of stationary phase cultures (data not shown). We again observed no difference in susceptibility between the 3xbce sdpI mutant and the single sdpI deletion, underpinning the aforementioned result.

Next, we tested if the BceRS system instead might be involved in mediating resistance against SKF. Towards that end, we deleted skfEF, which encode the putative ABC transporter that is thought to be responsible for export and immunity of SKF and followed growth of a skfEF mutant over time (data not shown). In contrast to the sdpI deletion, there was no growth defect observable for the skfEF mutant. Next, we combined the 3xbce mutant with the skfEF deletion to see whether the additional 3xbce deletion affects growth. But again, the 3xbce skfEF mutant did not show any growth defect.

Taken together, we found no evidence for a role of Bce-like systems in mediating resistance against SDP and SKF despite its strong induction. We therefore next focused our attention on the specificity of this induction.
4.4.5 Mature SKF toxin strongly acts as inducer

Of the two cannibalism toxins, SKF was the stronger inducer of the bceA promoter. Given that the BcERs system did not confer resistance against SKF, we wondered about the physiological relevance of the intrinsic induction of the CESR systems in stationary phase. In order to approach this question, we first had to understand the true nature of the stimulus sensed by the BcERs system. Was it the mature toxin itself or could the unprocessed precursor also lead to its activation? SKF is a ribosomally synthesized AMP and requires posttranslational modification to be fully active (Gonzalez-Pastor et al., 2003; Liu et al., 2010). Our knowledge of this process is still limited and direct evidence for the functions described in the following sentences is still lacking. But it is assumed that the radical SAM (S-adenosyl-methionine) enzyme SkfB mediates the first step in SKF maturation by forming a thioether bond between the cysteine residue Cys4 and the α-carbon of the methionine residue Met12 resulting in pre-SkfA (Flühe et al., 2013; Liu et al., 2010) (Fig. 4.3). SkfH, a putative thioredoxin oxidoreductase-like protein and the last gene encoded in the skfA-H operon is presumed to mediate formation of a disulfide bond leading to SkfA* (Liu et al., 2010) (Fig. 4.3). Export and immunity was postulated to be mediated by SkfEF, forming an ABC transporter in the membrane (Gonzalez-Pastor et al., 2003). Likewise, SkfC was hypothesized to be responsible for the cyclization reaction prior to or during export of the SKF peptide (Liu et al., 2010). SkfG is so far poorly understood and its function is unknown.

In order to gain deeper insight into the physiological properties of the genes encoded in the skfA-H operon, we next studied the intrinsic \( P_{bceA} \) induction in different skf mutants (Fig. 4.6a+b). In a skfA mutant lacking the structural gene of the SKF toxin, \( P_{bceA} \) induction is almost not detectable (Fig. 4.6b, dark grey curve). Similar results were obtained in a mutant deleted for skfBC, the products of which were hypothesized to be involved in maturation of the toxin precursor (Flühe et al., 2013). This suggests that SkfBC perform critical steps in the maturation process of SKF. Likewise, \( P_{bceA} \) induction cannot be detected in a skfEF mutant, lacking the putative immunity transporter. In contrast, deletion strains lacking either skfGH or skfH alone were able to activate the BcERs system in stationary phase, albeit 10-fold reduced compared to the wild type reporter strain (see Fig. 4.1). SkfH is hypothesized to be responsible for one important disulfide bond formation in the maturation process of SKF (Liu et al., 2010). Thus, it seems that SkfH performs a critical step in the maturation of SKF. Additionally, comparison of the skfGH mutant and the skfBC or skfEF deletion, respectively, revealed that potential modification of SKF by SkfBC and/or export via SkfEF seem to play more crucial roles in the SKF maturation pathway than SkfG alone, since \( P_{bceA} \) induction is abolished in both the skfBC and skfEF mutant. In conclusion, SkfBC and SkfEF are necessary for production of a fully active SKF toxin, while SkfGH seem to play a minor role, at least as judged by the activation of the BcERs system in a skfGH mutant.
In order to elucidate if the mature SKF toxin or even its precursor acts as an inducer of the $bceA$ promoter, we combined the $sdpC$ deletion with the $skfGH$ deletion (Fig. 4.6d, orange curve). The resulting double mutant is supposed to be deficient for SDP and lacks crucial steps of SKF maturation. Fig. 4.6d shows that the $sdpC$ $skfGH$ double mutant first displayed significantly decreased BceRS activation, when compared to the $sdpC$ deletion mutant (orange vs. grey curve) but after some time (12-13 h), $P_{bceA}$ becomes active although to a much lower extent. This observation might suggest that accumulation of immature SKF precursor could already act as a weak inducer since the time point of induction is much later and the dynamics considerably lower.

### 4.4.6 Mature SDP toxin acts as inducer

The absence of any role for the BceRS system in mediating resistance against SDP provokes the question why the BceRS system is triggered by this compound. In order to better understand this stimulus leading to $P_{bceA}$ induction, we investigated BceRS activation in individual $sdp$ mutants (Fig. 4.6).

SDP is encoded in the $sdpABC$ operon and repressed by AbrB during exponential growth phase and in times of nutrient availability (Chen et al., 2006; Fujita et al., 2005). Upon entry into stationary phase, repression by AbrB is released by active Spo0A, and transcription of the corresponding genes is triggered. Like SKF, SDP is a ribosomally synthesized AMP that requires posttranslational modifications to mature into an active form (Gonzalez-Pastor et al., 2003; Liu et al., 2010; Perez Morales et al., 2013), a process presumably mediated by SdpA and SdpB (Perez Morales et al., 2013). SdpA is thought to be a soluble protein attached to the cytosolic face of the membrane, whereas SdpB is a transmembrane protein (Perez Morales et al., 2013). Together, they are thought to mediate the final step of processing the SDP precursor peptide into active SDP by posttranslational cleavage of the N- and C-terminus (Fig. 4.3).
To better understand the stimulus leading to $P_{bceA}$ induction by SDP, we first tested if the BceRS system is triggered by the mature SDP toxin or by its precursor. We initially monitored $P_{bceA}$ induction in an $sdpAB$ mutant (Fig. 4.6d, blue curve): Compared to the wild type reporter strain (Fig. 4.1) the induction was only slightly reduced. This is due to the fact that SKF is still present and acting as the main inducer. Consequently, we next compared $P_{bceA}$ induction in a $skfA-H$ mutant and a $skfA-H sdpAB$ deletion. As a consequence, a deletion strain of $\Delta skfA-H \Delta sdpAB$ would lack SKF and only produce immature, unprocessed SDP precursor that could potentially trigger the BceRS system. Fig. 4.6(d) shows that the $bceA$ promoter induction was completely abolished in the double mutant (green curve), indicating that the SDP precursor is most likely not the inducer of the $bceA$ promoter, but rather the mature SDP.

Next, we tested $bceA$ promoter induction in an $sdpI$ mutant, lacking the autoimmunity against SDP (Fig. 4.5b, c). Surprisingly, $P_{bceA}$ induction was completely abolished in this strain. This unexpected finding provoked the question if the $sdpABC-\text{sdpRI}/skfA-H$ operons are still expressed in an $sdpI$ mutant since a loss of auto-immunity has previously been reported to sometimes abolish toxin production (Foulston & Bibb, 2010). Both $P_{sdpA}$ and $P_{skfA}$ showed a strong increase about 10-fold and 100-fold, respectively (Fig. 4.5c, green and blue curve, respectively), comparable to wild type results (see Fig. 4.4), demonstrating that the two toxin promoters are fully induced and the toxins are most likely also produced. Because of the severe growth defects of the $sdpI$ mutant, we wondered whether the silence in the BceRS system is maybe a result of this growth defect. However, addition of bacitracin (10 $\mu$g ml$^{-1}$) to stationary phase cultures could still fully activate the BceRS system (Fig. 4.5c), demonstrating that the BceRS system itself is still functional in the $sdpI$ mutant.

We next addressed the question if SDP itself is still produced as a potent toxin in the $sdpI$ mutant. To this end, we performed a spot-on-lawn assay using a $spo0A$ deletion strain as sensitive lawn (Fig. 4.5d). Since cannibalism toxin production and immunity is regulated in a Spo0A-dependent manner, a $spo0A$ mutant is unable to produce both SDP and SKF and is therefore sensitive against both toxins. We spotted stationary phase cultures of wild type as well as $sdp$ and $skf$ mutants on a plate containing $\Delta spo0A$ lawn cells and compared zones of inhibition after incubation overnight. Wild type spots showed a clear zone of inhibition on the $\Delta spo0A$ lawn indicating production of functional cannibalism toxins. We then used a $skfA$ deletion strain lacking SKF toxin but still expressing SDP. We found that the $skfA$ mutant showed a clear inhibition zone just like wild type, indicating production of functional SDP toxin in the absence of SKF. Accordingly, we took an $sdpC$ deletion strain lacking SDP but still producing SKF. However, $\Delta sdpC$ was unable to kill $spo0A$ deficient cells, demonstrating that SDP rather than SKF is the major cannibalism toxin on solid medium, which is in agreement with a previous study (Liu et al., 2010). Importantly, a significant zone of inhibition comparable in size to the wild type can be observed around spots of an $sdpI$ deletion mutant. This result unequivocally demonstrates that functional SDP toxin is still produced in an $sdpI$ mutant. Nevertheless, BceRS activation was abolished in this strain. This observation indicates a link between toxin sensing by the BceRS system and the presence of the immunity protein SdpI. While understanding the molecular mechanism behind this finding is beyond
the scope of this work and will require further investigations, it already points towards an indirect way of sensing as will be discussed below.

4.5 Conclusion

Our results demonstrate that the BceRS system is intrinsically activated in late stationary phase due to the production of two cannibalism toxins, SDP and SKF, with SKF being the stronger inducer. The $skfA-H$ deletion resulted in a 100-fold reduced BceRS activity, whereas the $sdpC$ deletion caused only a 10-fold reduced $P_{bceA}$ induction (Fig. 4.2b). The exact physiological role of the BceRS system in the cannibalism stress response, however, remains unclear. Our data suggest that it provides no role in resistance against either SDP or SKF. However, it seems that the immunity determinants SdpI and SkfEF, respectively, are important for triggering the BceRS response since in corresponding deletion strains BceRS activation is abolished (Figs 4.5+4.6). For SkfEF, this finding is less surprising since this ABC transporter is thought to also export the SKF toxin. Hence, in its absence no mature inducer reaches the extracellular environment to trigger a BceRS response. But at present, this assumption is hard to investigate without a detectable SKF-dependent phenotype.

SDP was shown to be the weaker inducer of the $bceA$ promoter, displaying only a 10-fold reduced BceRS response in an $sdpC$ mutant compared to the wild type (Fig. 4.2b). Remarkably, in an $sdpI$ deletion, we observed a complete loss of the BceRS response despite the fact that both toxin loci are fully expressed (Figs 4.4+4.5c) and SDP is most likely functionally produced (Fig. 4.5d).

Taken together, these findings indicate that SdpI is required for SDP and potentially also SKF perception by the BceRS system (Fig. 4.7). This mode of an indirect sensing of SDP only in complex with SdpI resembles...
the mode for bacitracin perception by the BceRS system that was suggested recently (Kingston et al., 2014). Here, it has been proposed that only the complex of bacitracin to its membrane target, undecaprenol pyrophosphate, can act as a trigger of the BceRS response. Our findings on an Sdpl-dependent sensing of SDP (and potentially also SKF) support this model of AMP perception by the BceRS system, in which the toxin/AMP has to be bound to a membrane target before it can be perceived by the BceRS system. Analyzing this novel mechanism will be the subject of further investigations.

Nevertheless, our results provide clear evidence for a tight link between signaling systems that mediate the CESR in B. subtilis and intrinsic AMP production as part of the stationary phase survival strategy of this organism.

4.6 Acknowledgements

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4.7 References of CHAPTER IV


CHAPTER V

Discussion

Future perspectives
5 Discussion

The cell envelope stress response network of *B. subtilis* involves a variety of 2CSs and ECF σ factors. The LiaSR system is a 2CS which responds to cell envelope targeting peptide antibiotics. Bacitracin has been shown to be one of the strongest inducers of the LiaSR system (Mascher *et al.*, 2003). Recently, it has been demonstrated that the LiaSR system provides a secondary resistance layer of resistance against bacitracin if the primary layer is missing (Georg Fritz, personal communication). Besides its strong induction by bacitracin, the LiaSR system is also intrinsically induced during transition from exponential to stationary growth phase. This induction was shown to be broadly heterogeneous occurring in only 5-10% of the cells and is considerably weaker (Jordan *et al.*, 2007, Dominguez-Escobar *et al.*, 2014).

In the first part of this thesis, we wanted to gain deeper insight into the underlying mechanisms responsible for the heterogeneity within the LiaSR system. First, we found that the LiaSR system is not only heterogeneously activated during transition phase but also upon addition of low bacitracin concentrations (Kesel *et al.*, 2013). Second, we could show that LiaI is a highly dynamic membrane protein under non-inducing conditions while it becomes static upon addition of bacitracin (Dominguez-Escobar *et al.*, 2014). Thereby, it recruits LiaH from the cytoplasm to the membrane forming a complex presumably to close the cell envelope damaged site and maintain cell envelope integrity (Dominguez-Escobar *et al.*, 2014). In the following section 5.1 and 5.2, I would like to discuss where heterogeneity in the LiaSR system might come from and the role of the LiaIH dynamics under bacitracin stress conditions.

In the course of investigating the transition phase induction of the LiaSR system, we observed that the BceRS, the PsdRS as well as the ECF σ factors σ* M*, σ* X* and σ* W* also showed an intrinsic activation in stationary growth phase. Since these systems are known to respond to various cell envelope perturbing agents, we reasoned that they must be activated by one or several endogenously produced peptides.

In the second part of this thesis, we wanted to determine the stimuli responsible for this intrinsic induction of the different cell envelope stress response systems with our main focus on the BceRS system. First, we found that the cannibalism toxins SDP and SKF were the main inducers of this stationary phase activation (Höfler *et al.*, 2015). However, we found no evidence that the BceRS system is involved in mediating resistance although we could demonstrate that it only responds to the mature toxins. Second, we found a link between the presence of the immunity determinants of the cannibalism toxins (Sdpi and SkfEF) and BceRS activation. They seemed to be needed for stimulus perception by the BceRS system. In the following section 5.2, I would like to discuss the physiological role and relevance of the BceRS activation by SDP and SKF and give some future perspectives.
5.1 Heterogeneous activation of the LiaSR system and its origins

Bacteria employ 2CSs to respond to different environmental stimuli via transfer of a phosphoryl group from the HK to its cognate RR. Subsequently, differential gene expression is induced to mount protective countermeasures. *B. subtilis* employs a number of 2CSs to respond to cell envelope stress, one of which is the LiaSR system. Besides the HK LiaS and the RR LiaR, it includes a third protein, LiaF, blocking LiaS activity under non-inducing conditions.

Signal transduction of 2CSs can lead to heterogeneous expression of genes regulated by these 2CSs although the cells of a population are genetically identical (Smits *et al.*, 2006, Botella *et al.*, 2011, Ghosh *et al.*, 2011). As a result, heterogeneous gene expression can lead to phenotypic heterogeneity. One example is the transition state and stationary phase differentiation cascade of *B. subtilis* with a fraction of cells already Spo0A-active and others still being Spo0A-inactive. Heterogeneous induction of the LiaSR system in response to low bacitracin concentrations during exponential growth phase on single cell level could be demonstrated in this thesis (Kesel *et al.*, 2013) (CHAPTER II). From this study, some open questions aroused: Where does heterogeneity in the LiaSR system come from? Does it derive from the LiaSR system itself? And if so, which proteins would be involved?

5.1.1 Heterogeneity might originate from the LiaSR system itself

Heterogeneous gene expression in bacteria is a common feature to adapt to fluctuating environmental conditions or in terms of cell differentiation. *B. subtilis* is a paradigm for studies on gene expression heterogeneity. Individual cells within a certain population are able to differentiate into different cell types, e.g. spores, matrix producers, cannibals, competent cells or peptide antibiotic producers (Lopez & Kolter, 2010). It is assumed that the regulation of gene expression by so-called 2CS connectors is critical for heterogeneity. Such connectors are thought to modulate the phosphorylation state and thereby the activity of HKs and RRs, thus affecting the output, i.e. gene expression. Connectors are present both in Gram-positive and Gram-negative bacteria and are critical for a variety of physiological functions, including sporulation and transition from exponential to stationary growth phase (Mitrophanov & Groisman, 2008). A well investigated example is the phosphorelay cascade of Spo0A in *B. subtilis*. The cascade leads to sporulation by starting with autophosphorylation of the sensor kinases KinA and KinB and a subsequent transfer of the phosphoryl group to Spo0F. Then, Spo0F~P transfers the phosphoryl group to Spo0B, which in turn, phosphorylates Spo0A. In cells that reach a certain threshold concentration of Spo0A~P, sporulation is triggered. Within that phosphorelay cascade, several connectors are present which directly inhibit the system (Fig. 5.1).

The first two connectors, Sda and Kipl, inhibit the autophosphorylation step of KinA (Wang *et al.*, 1997, Burkholder *et al.*, 2001). A second step of regulation can take place at the level of Spo0F phosphorylation. Here, the proteins RapA, B, E and H promote dephosphorylation of Spo0F (Perego & Brannigan, 2001, Smits *et al.*, 2007). The last possible regulatory step can occur via
dephosphorylation of Spo0A by the connector protein Spo0E. Both, Rap and Spo0E proteins are hypothesized to function by inducing the autodephosphorylation activity of Spo0F-P and Spo0A-P, respectively (Perego & Brannigan, 2001). All these connectors have in common that they are themselves induced by conditions that inhibit sporulation, either by nutrient availability (KipI) or induction of competence (e.g. RapA and RapE) (Wang et al., 1997). In fact, this is important for the fine-tuning of Spo0A (de)phosphorylation and thereby tight regulation. There are two subpopulations of sporulating (high Spo0A-P levels) and non-sporulating cells (low Spo0A-P levels). If environmental conditions change and nutrients become available again, the non-sporulating cells are able to resume growth, thus having an advantage over the sporulating cells. Indeed, heterogeneity is assumed to be an advantageous characteristic giving the population the opportunity to avoid commitment to the timely and costly developmental sporulation pathway (Dubnau & Losick, 2006). Strikingly, the two connector proteins RapA and Spo0E have been shown to directly affect Spo0A-associated heterogeneity. Deletions of rapA and spo0E abolish heterogeneity by inducing the activity of Spo0A in almost all cells of the population (Veening et al., 2005).

Such connector proteins show many similarities with LiaF, modulating the phosphorylation state of LiaS and thereby regulating the kinase activity. It is known that LiaF is an inhibitor protein of LiaS under non-inducing conditions and maybe even promoting dephosphorylation of LiaS (Jordan et al., 2006, Schrecke et al., 2013). Therefore, LiaF would constitute and function as such a connector protein modulating the phosphorylation state of LiaS and thus affecting gene expression of the liaIH-GFSR operon. Recently, it has been demonstrated that the ratio of LiaF:LiaS:LiaR under native conditions is 18:4:1 (Schrecke et al., 2013). This shows that LiaF needs to be in excess over LiaS in order to maintain the phosphatase activity of LiaS and to keep the system silent in the
absence of a stimulus (Schrecke et al., 2013). Upon addition of bacitracin, LiaF releases its inhibitory effect on LiaS which undergoes autophosphorylation resulting in activation of the LiaSR system. Heterogeneous activation of the LiaSR system was shown to occur only upon the addition of low antibiotic concentrations (Kesel et al., 2013). This could be explained as follows: If there are only few bacitracin molecules in the environment, not all cells perceive the bacitracin stimulus. Also the ratio of LiaF:LiaS:LiaR is not a fixed ratio and it changes upon bacitracin addition. There are possibly cell-to-cell variations (or noise) in the number of proteins relative to each other. Therefore, low concentrations of bacitracin could presumably only induce the LiaSR system in cells with a slightly lower amount of LiaF molecules.

During the intrinsic transition phase induction of the LiaSR system, again LiaF releases LiaS inhibition only in a certain subpopulation of cells. Here, the stimulus for activation of the LiaSR system was shown to be the endogenously produced YydF peptide (Butcher et al., 2007). Since the expression of yydF was shown to be dependent on AbrB and Spo0A (Butcher et al., 2007), again cell-to-cell variations in the number of peptide producing cells might occur due to the heterogeneous activation of Spo0A. This means that not all cells produce and secrete YydF and therefore, not all cells do respond. Furthermore, it has been shown that under native conditions the detection of LiaR molecules was challenging despite the presence of the constitutive promoter $P_{liaG}$ (Schrecke et al., 2013). One the one hand, this could be explained by the presence of two stem-loop structures at the end of liaS (Schrecke et al., 2013). These could lead to premature termination of transcription. On the other hand, the reason for the little amount of LiaR molecules under non-inducing conditions might be the instability of the 3’end of the liaFSR transcript which could be subject to RNase degradation (Schrecke et al., 2013). Thus, the number of LiaR molecules per cell controlling expression from $P_{liaI}$ depends on the external bacitracin concentration. At low antibiotic concentrations, the low number of LiaR proteins might explain the variations in gene expression leading to heterogeneity (Kesel et al., 2013, Schrecke et al., 2013). Cell-to-cell differences in the exact number of LiaR molecules therefore directly affect gene expression from $P_{liaI}$.

Taken together, heterogeneous gene expression is frequently used by bacteria to adapt to changing environmental conditions. This can be achieved by precisely controlled signaling pathways which modulate the transcription of individual genes. Heterogeneity in the expression levels of individual genes or the occurrence of bistability where some genes are only expressed in a subpopulation of cells have been observed. Heterogeneous activation of the LiaSR system at low antibiotic concentrations or during transition phase might derive from LiaF and its relative amount to LiaS modulating its activity or the little amount of LiaR proteins regulating expression from $P_{liaI}$. 
5.2 LiaI dynamics vary under stress and non-stress conditions

Bacteria respond to cell envelope stress by mounting protective countermeasures. The most common signal transduction pathways are 2CSs and ECF σ factors (Jordan et al., 2008). Additionally, another protective layer is provided by the phage-shock protein (PSP) response. The PSP response has been thoroughly studied in *E. coli* and more recently in *Yersinia enterocolitica* (Model et al., 1997, Darwin, 2005, Joly et al., 2010, Yamaguchi et al., 2013, Flores-Kim & Darwin, 2015). It is mainly induced by phage infection, high osmolarity, heat shock and changes in lipid biosynthesis (Brissette et al., 1990, Bergler et al., 1994, Model et al., 1997). In this thesis, the dynamics of LiaI and the PspA homolog LiaH was investigated in the presence and absence of bacitracin (CHAPTER III). We found that LiaI is highly dynamic under non-inducing conditions possibly scanning the membrane for damaged sites. Upon stress conditions, LiaI recruits LiaH from the cytoplasm to the membrane forming a complex. However, LiaH was still detected at the membrane in the absence of LiaI. Therefore, we assume that LiaH has more than one interaction partner. Since the PSP response is well-understood in *E. coli*, but only little is known for *B. subtilis*, first I would like to compare both systems and highlight some similarities in the following section. Furthermore, I would like to provide some future perspectives for other potential interaction partners of LiaH and draw some conclusions.

5.2.1 The PSP response of *E. coli* – similarities in *B. subtilis*

The PSP regulon in *E. coli* consists of the *pspABCDE* operon and the two distinct genes *pspF* and *pspG* (Joly et al., 2010). Under non-inducing conditions, the transcriptional activator protein PspF is kept inactive by the cytosolic PspA protein. In the presence of stress signals, PspA is recruited to the membrane proteins PspB and PspC in order to counteract membrane damage. PspF is released and acts as an activator for *pspABCDE* transcription (Fig. 5.2).

Despite this detailed knowledge about the molecular mechanisms, the physiological role of the PSP response remains unclear. However, studies on the PSP response suggest a role in stabilizing and

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**Fig. 5.2: Comparison of the PSP response in γ-proteobacteria (A) and Firmicutes bacilli (B).** Proteins are depicted as circles at their known or predicted cellular location, with the letters corresponding to the respective genes, indicated below. The phage shock proteins PspA and LiaH are depicted in their oligomeric ring form structure. LiaG is depicted as a dotted circle because homologs are only present in *B. subtilis* and its closest relatives. Double-ended arrows indicate protein-protein interactions, regular arrows activation and T-shaped lines inhibition. CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane. This figure is taken and modified from (Wolf et al., 2010).
protecting the membrane against proton leakage and loss of membrane potential (Kobayashi et al., 2007, Vrancken et al., 2008, Horstman & Darwin, 2012). It is hypothesized that PspA binds to phosphatidyl-glycerol (PG) and phosphatidyl-serine (PS) within the lipid bilayer (Kobayashi et al., 2007). This is thought to cause a change in rigidity of the inner membrane which is suggested to facilitate proton motive force (PMF) maintenance (Kobayashi et al., 2007). Another role for PspA, potentially including PspG, was suggested by Engl et al., 2009. It was found that PspA and PspG formed static complexes at the cell poles and rather mobile foci along the cell. They hypothesized that the polar complexes modulate PMF-consuming processes such as chemotaxis (Engl et al., 2009). Additionally, they proposed a role for PspA in cell wall biogenesis since lateral movement of PspA seems to involve cytoskeletal proteins such as MreB. Therefore, the PSP response is hypothesized to play a role in the repair of stress-induced membrane damage and PMF maintenance (Engl et al., 2009, Joly et al., 2010).

In *B. subtilis*, there are two PspA homologs, PspA and LiaH. Here, expression of *pspA* is mediated by the ECF σ factor σ\(^W\) under envelope stress conditions. However, its physiological role remains elusive. In contrast, the LiaSR system has been studied extensively over the last decade and the function of many proteins of the *liaIH-GFSR* operon has been elucidated. The 2CS consisting of LiaSR included an accessory inhibitory protein LiaF. Transcription and expression of the last four genes of the operon is ensured by the constitutive promoter P\(_{liaG}\). Expression of *liaIH* is strictly dependent on the LiaR-regulated promoter P\(_{liaI}\). LiaH is a PspA homolog and was shown to form large oligomeric ring structures similar to those observed for other PspA-like proteins (Aseeva et al., 2004, Hankamer et al., 2004, Standar et al., 2008, Wolf et al., 2010, Otters et al., 2013).

In this thesis, we were able to show that LiaI and LiaH were shown to interact upon cell envelope stress, i.e. addition of bacitracin, thereby LiaI being a membrane anchor for LiaH (see CHAPTER III, Fig. 2). LiaH was predominantly found to localize in the cytoplasm under non-inducing conditions and seemed to be recruited to the membrane upon cell envelope stress (see CHAPTER III, Fig. 3), where LiaI and LiaH co-localize in distinct foci. This is a first link to the PSP response in *E. coli*. PspA\(_{E.c.}\) and LiaH both localize in the cytoplasm under native conditions which changes upon envelope stress: Both proteins get recruited into (a) complex(es) at the membrane. In *E. coli*, PspA gets recruited into the PspB-PspC complex while, in *B. subtilis*, LiaH forms a complex with LiaI. Both events finally lead to the initiation of gene transcription in a direct or indirect manner. In *E. coli*, the release of PspF by PspA leads to the transcription of the *pspABCDE* operon whose gene products are thought to counteract membrane damage. Within the LiaSR system of *B. subtilis*, other phage-shock protein homologs are missing. Here, the release of LiaF inhibition of LiaS ultimately results in transcription initiation by LiaR binding to P\(_{liaI}\). From our knowledge gained during this study, we hypothesize that LiaIH serve as a "patch" from the inside at the sites of membrane damage to shield against AMP-induced membrane damage.

Interestingly, we found that LiaH was still able to associate with the cytoplasmic membrane in a *liaI* mutant, pointing towards the fact that LiaH might have more than one interaction partner (see CHAPTER III, Fig. 2). So far unpublished data gained over the last years also indicated a link of
LiaH and the $\sigma^W$-regulated proteins encoded in the yvlABCD operon (Fig. 5.3), which will be discussed below.

### 5.2.2 LiaH and its connection to the YvlABCD interaction network

In order to search for other LiaH interaction partners besides LiaI, a genome-wide analysis was performed to determine other phage-shock protein homologs in \textit{B. subtilis}. YvlC, a member of the yvlABCD operon was discovered to be a homolog to PspC from \textit{E. coli} (PhD thesis, Diana Wolf). Remarkably, YvlB was identified to be a paralog to LiaG of \textit{B. subtilis}. Little is known about the function and localization of the Yvl proteins but preliminary data obtained from bacterial two-hybrid analyses revealed interactions between some Lia proteins, PspA and Yvl proteins giving rise to the following scheme (PhD thesis, Diana Wolf; Master thesis, Marion Kirchner) (Fig. 5.3).

Fig. 5.3: Interconnection of proteins involved in cell envelope protection. Proteins encoded in ECF $\sigma^W$-dependent operons are shown in green. Proteins encoded in the lia locus are marked in blue. Thin arrows indicate weak interactions; thick arrows illustrate strong interactions as judged from bacterial two-hybrid analyses. Based on sequence analyses, the location of each protein is displayed. Final evidence for the above indicated interactions and potential protein localization is lacking. This figure is based on Diana Wolf, PhD thesis, 2012 and adapted from Marion Kirchner, Master thesis, 2013.

As indicated in Fig. 5.3, YvlB is a key player in this interaction network. It seems to comprise the central hub connecting the LiaSR response and PspA as part of the PSP response. Indeed, preliminary data from bacterial two-hybrid analyses already showed that YvlB interacts with LiaH and LiaI. It is conceivable that YvlB guides LiaH to the membrane where it interacts with LiaI under stress conditions. Together, they might form a complex at the membrane under inducing conditions. This would be in agreement with the observation that YvlB localizes both to the membrane and to the cytoplasm in a growth phase-dependent manner (Master thesis, Korinna Kraft). YvlB was predominantly found in the cytoplasm during middle sporulation stages and at the membrane during exponential and early sporulation stages. As mentioned, YvlB is a LiaG paralog harboring a COG3595 domain consisting of $\beta$-sheets forming a $\beta$-propeller motif. This domain is assumed to be responsible for protein-protein interactions or signal transduction (Fülöp & Jones,
1999, Menke et al., 2010). In this respect, the signal that leads to the halt of LiaI scanning in the membrane, might also serve as a signal for YvlB which could help LiaI to recruit LiaH.

The bacterial two-hybrid analyses already pointed towards some interaction between YvlB and PspA. Since PspA in E. coli is recruited to the membrane under stress conditions it would be interesting to test this finding in B. subtilis as well and additionally look for co-localization with LiaH. A preliminary study from a Master student (Annika Sprenger) already revealed that PspA in B. subtilis localizes to the membrane in few distinct foci under non-inducing conditions. However, PspA localization upon induction has not been tested. Although the results of the bacterial two-hybrid analyses depicted in Fig. 5.3 let us to assume that there is no direct interaction between PspA and LiaH, it would be conceivable that these two proteins may interact directly upon inducing conditions or that interaction is mediated by their common interaction partner YvlB. Therefore, it would be interesting to test, if LiaH and PspA co-localize at the membrane under envelope stress conditions.

Other possible LiaH interaction partners at the membrane could be YvlA, YvlC or YvlD assuming their predicted localization is correct. In a preliminary study about the localization of YvlA, YvlB and YvlC from the Master student Korinna Kraft, it was shown that all three proteins exhibit distinct localization patterns. YvlA and YvlC were predominantly found at the membrane throughout the different stages of growth (from exponential until late stationary phase including spore formation). Here, YvlC localization is in agreement with the localization pattern of its homolog PspC in E. coli. Remarkably, YvlB was found both in the cytoplasm (during middle sporulation stages) and at the membrane (during exponential and early sporulation stages). This, indeed, suggests a central role for YvlB potentially mediating interaction of two or more proteins in a growth phase-dependent manner. Therefore, one could assume some level of interaction between LiaH and YvlA or YvlC via YvlB, which potentially helps to recruit LiaH to the membrane. Hence, all these very preliminary and highly speculative data suggest that there are, in fact, a number of possible candidates which might be involved in LiaH recruitment to the membrane upon inducing conditions.

In this study, we were also able to show that LiaI foci display fast and random movement in the membrane in unstressed cells (see CHAPTER III, Fig. 3). Under inducing conditions, most of these foci become static (see CHAPTER III, Fig. 3). LiaH reveals disperse cytoplasmic localization under non-inducing conditions and only gets recruited to the membrane upon addition of stressors. In the following section, I would like to compare this finding to the PSP response in Yersinia enterocolitica and highlight some similarities and differences between the two systems.

5.2.3 The PSP response of Y. enterocolitica – similarities and differences in B. subtilis

The Y. enterocolitica PSP response is reminiscent to the Lia response in B. subtilis (Fig. 5.4). In Y. enterocolitica, the cytosolic phage-shock protein PspA, the enhancer protein PspF and the two membrane proteins PspB and PspC exhibit high dynamics under non-inducing conditions (Yamaguchi et al., 2013). This is reminiscent of the membrane protein LiaI in B. subtilis scanning the membrane in the absence of a stimulus. Also LiaH appears to be located diffusely in the
cytoplasm as judged from our fluorescence microscopy data. In *Y. enterocolitica*, upon stress, PspA is recruited to PspC at the membrane which then forms large stationary foci together with PspB (Yamaguchi *et al.*, 2013). These findings indeed match (at least to a certain point) with the dynamics of the LiaSR system where LiaH is recruited to LiaI at the membrane when cells encounter stress. They appear to co-localize into static foci potentially with other, so far unknown proteins to counteract the specific stress signal. One difference between the PSP response in *Y. enterocolitica* and the Lia response in *B. subtilis* is that PspA*Y.e.* has an interaction partner in the cytoplasm while for LiaH in *B. subtilis* no interaction partners have been found so far. As already discussed in the previous section (see Fig. 5.3), LiaH after all might have interaction partners. The bacterial two-hybrid analyses already pointed towards an interaction between LiaH and YvlB and potentially other Yvl proteins within that network. These preliminary observations need further investigation in the future.

### 5.3 A novel mode of BceRS activation by the cannibalism toxins SDP and SKF

In addition to the LiaSR system, this thesis also addressed the question about the intrinsic stimuli responsible for the stationary phase induction of the BceRS and PsdRS 2CSs as well as the ECF σ factors σM, σX and σW. We found that all systems responded to the two cannibalism toxins SDP and SKF. Since the effect was most prominent for the BceRS 2CS, we focused on that system to gain deeper insight into the physiological relevance of this process.

We found that only the mature toxins are able to fully trigger the BceRS response and that the toxin precursors only play minor roles in activation of the BceRS system (see CHAPTER IV, Fig. 4.6). Ppbce induction was further shown to correlate with toxin production (see CHAPTER IV, Fig. 4.4). The toxin promoters, PsdpA and PskfA, revealed increased activity about two hours before the BceRS system started to respond (see CHAPTER IV, Fig. 4.4). We assumed that this time delay is needed in order to produce a fully functional toxin with all the posttranslational modifications needed. Given the fact that the BceRS system mediates resistance against the AMP

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**Fig. 5.4: Graphical illustration of the Lia response in *B. subtilis* (A) and the PSP response in *Y. enterocolitica* (B).** This scheme illustrates the protein dynamics of LiaI and the recruitment of LiaH during the Lia stress response of *B. subtilis* (A) and the protein dynamics of PspBC and relocalization of PspA in the PSP response of *Y. enterocolitica* (B). The arrows indicate the mobility of the proteins within the cytoplasmic membrane or within the cytoplasm. This figure is taken from (Dominguez-Escobar *et al.*, 2014).
bacitracin, it stood to reason that it would also confer resistance against SDP and SKF. But our comprehensive study let us to conclude that it does not mediate resistance against the toxins.

Remarkably, the BceRS system was not activated in an sdpl mutant although functional SDP and SKF are produced (see CHAPTER IV, Fig. 4.5c+d). In conclusion, the BceRS system seems to require SdpI in order to be activated by the two toxins.

The outstanding questions arising from this study are: (i) Why is $P_{bceA}$ induction completely abolished in an sdpl or skfEF mutant? (ii) How do the BceRS system and the resistance determinants interact or rely on each other so that induction is lost in the absence of the latter?

In the case of SkfEF, loss of BceRS activation can be explained as follows: Since SkfEF is hypothesized to constitute an exporter of the toxin (Gonzalez-Pastor et al., 2003, Liu et al., 2010), translocation of the toxin to the outer surface of the membrane will presumably not occur if SkfEF are missing. Hence, the BceRS system will not be activated. Additionally, we did not observe any growth defect in a skfEF mutant, indicating that SKF is not active in the intracellular environment.

In contrast, an sdpl mutant exhibited a severe growth defect starting from entry into stationary phase where SDP and SKF are supposed to be produced. SdpI is proposed to be a membrane and signal transduction protein (Ellermeier et al., 2006). Ellermeier and colleagues suggested a model in which SdpI binds to SDP at the membrane and that this complex then sequesters SdpR away from the DNA to the membrane thereby inducing transcription of the two-gene operon sdpl (Ellermeier et al., 2006). This leads to increasing amounts of Sdp protein in the membrane needed for resistance and survival. If this protein is missing, cells lose their ability to induce appropriate countermeasures in response to the lethal toxin damage. Since we were not able to observe any BceRS response in the absence of SdpI, we conclude that the BceRS system somehow requires SdpI in order to sense both SDP and SKF (Fig. 5.5).

![Fig. 5.5: Model of BceRS activation via SDP/SKF-SdpI complex formation.](image)

Notably, this model of BceRS activation is reminiscent of the $P_{bceA}$ induction by bacitracin when bound to its target UPP. Recently, bacitracin was hypothesized to be sensed either directly or indirectly (Dintner et al., 2014, Kingston et al., 2014). Dintner and colleagues were able to demonstrate a direct binding of bacitracin to the ABC transporter BceAB in vitro. This finding is in good agreement with a recent study by Fritz and colleagues who demonstrated that BceAB is a bacitracin flux sensor which monitors its detoxification capacity in order to precisely adjust the
protein levels needed for protection (Fritz et al., 2015). These two studies demonstrated a direct bacitracin binding to BceAB. In contrast, Kingston and colleagues rather postulated that bacitracin first has to be bound to its membrane target UPP in order to be recognized by the BceRS system (Kingston et al., 2014). Such an indirect sensing mechanism including a membrane target protein could be also conceivable for SDP/SKF recognition. Only the presence of Sdpl, the membrane target of SDP, enables the BceRS system to respond to the two cannibalism toxins. Binding of Sdpl to SDP/SKF could alter membrane rigidity or integrity which could be the trigger for activation of the BceRS system. This would describe a novel mode of AMP perception in which the toxin has to be bound to a membrane target which somehow “presents” the AMP to the BceRS system prior to detection.

5.4 Future perspectives and concluding remarks

In this study, we identified two novel inducers of the BceRS system. SDP and SKF are two cannibalism toxins produced at the onset of stationary phase and under nutrient-limiting conditions in order to kill sensitive siblings. The lysed cells then provide nutrients for the cannibalistic subpopulation and enable them to maintain vegetative growth and delay entry into the sporulation cycle. Besides, we were also able to demonstrate that SDP and SKF induction is not solely Bce-specific but rather a common phenomenon of the different 2CSs and ECF σ factors tested, which are involved in the cell envelope stress response. However, the mechanism behind the BceRS activation by the toxins remains unclear. At this point, I would like to mention a few open questions and provide some future perspectives. The main outstanding question is, as already mentioned, the mechanism of BceRS activation by SDP and SKF. In order to address this question, it would be interesting to isolate and purify the two toxins and induce growing wild type and sdpI mutant cultures with certain concentrations of SDP and SKF. It would be possible to test whether the BceRS system only responds to the toxins in the presence of Sdpl as expected from our results. Furthermore, it would be interesting to perform in vitro binding studies in order to determine if SDP/SKF molecules are able to bind to BceAB or whether Sdpl is, in fact, needed for a successful interaction.

All data gained and presented in CHAPTER IV were performed on population level and so far, detailed single cell studies have been neglected. To further investigate and correlate P_bceA induction with toxin production, it would be tempting to perform time-lapse microscopy with double labeled promoters. In growing microcolonies, it would be exciting to observe whether the BceRS system responds in cells lying next to a toxin producer or not. Another question would be, if the BceRS system is also activated in a toxin producer or whether only sensitive cells trigger the BceRS response. Labeling proteins (BceAB, BceS, Sdpl, SklEF) and peptides (SDP, SKF) instead of their promoters would provide further insights into the localization pattern of these proteins and peptides. Co-localization studies would give a hint on potential interaction partners in or at the membrane. By using TIRFM (total internal reflection microscopy) it would be also possible to reveal protein dynamics, if any, upon addition of SDP or SKF. Since Sdpl is proposed to be an integral
membrane protein and signal transduction protein, a scanning mechanism reminiscent of LiaI scanning the membrane in the absence of an inducer would be possible. The presence of SDP (and also potentially SKF) might lead to a complex formation with Sdpi possibly resulting in stalling of Sdpi and a change in membrane rigidity or integrity which could, in turn, trigger the BceRS response.

In conclusion, during this study, many novel and interesting insights have been gained about the activation of the BceRS system in stationary growth phase. Novel AMPs, such as SDP and SKF, have been identified to be inducers of the BceRS system. So far, we have no evidence that the BceRS system is involved in resistance against these peptides and induction seems to rely on the presence of the immunity protein Sdpi. The whole mechanism behind this is still unclear and needs further investigation in the future.
References of CHAPTER I and CHAPTER V


References


References


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An dieser Stelle möchte ich ein paar Menschen nennen, ohne deren Unterstützung die letzten Jahre nicht möglich gewesen wären.


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10/2005 – 06/2008 | Bachelor studies „Biology“ at the Ruprecht-Karls-University, Heidelberg


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Publications


Conferences – Posters


Conferences - Talks


Prizes