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 then 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.
Cannibalism Stress Response in *Bacillus subtilis*

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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 then 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*. [199 words]

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; Silver, 2006; Walsh, 2003). 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 (Dubois et al., 2009; Ellermeier et al., 2006; Gonzalez-Pastor et al., 2003), 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 (Dominguez-Escobar et al., 2014; Jordan et al., 2007). 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^M, σ^X and σ^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 (Helmann, 2002; Kingston et al., 2013; Mascher et al., 2007; Missiakas & Raina, 1998). σ^M, σ^X and σ^W each regulate a set of about 30-60 target genes with partially overlapping specificity (Kingston et al., 2013; Mascher et al., 2007), and all are activated in a growth-phase and growth medium-dependent manner (Huang et al., 1998): While σ^M and σ^X are induced mainly in late logarithmic growth phase, σ^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 activation 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. [709 words]

Methods

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⁻¹ MOPS, 33 g l⁻¹ (NH₄)₂SO₄, 3.85 mM KH₂PO₄, 6.15 mM K₂HPO₄; adjusted to pH 7 with KOH), 50 mg l⁻¹ Tryptophan, 22 mg l⁻¹ ammonium ferric
citrate, 1× III’-salts (232 mg l⁻¹ MnSO₄·4H₂O, 12.3 g l⁻¹ MgSO₄·7H₂O), 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.

**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 NotI/SalI or EcoRI/SpeI restriction enzymes, respectively. All strains used in this study are listed in Table 1. 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.

**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).

**Allelic replacement mutagenesis of sdpAB, sdpC, sdpI, 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.

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 OD₆₀₀ 0.2. The culture was subsequently diluted to an OD₆₀₀ 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). OD₆₀₀ 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 OD₆₀₀ at each data point (relative luminescence units (RLU) / OD₆₀₀). For each individual sample, OD₆₀₀ 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/OD₆₀₀ 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. [834 words]

Results and Discussion

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^{X}, and σ^{W} (P_{ydaH}, P_{sigX}, and
and one promoter which is regulated by all three $\sigma$ factors, $P_{bcrC}$, to a promoter-less $luxABCDE$ reporter (Radeck et al., 2013; Schmalisch et al., 2010). 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 $\sigma^W$ target promoter $P_{pspA}$ displayed a marked increase in luminescence activity between two and four hours after the onset of stationary phase (Fig. 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. 1a, b), but also the ECF target promoters displayed a 10-20-fold increase in promoter activity (Fig. 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.

**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; Staroń 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 antimicrobial peptides 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; Wolf et al., 2010). 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 PbceA, PpsdA and PbcrC promoter activation in mutants deleted for each gene encoding the respective antimicrobial peptides (Fig. 2). Deletion of sunA (Sublancin 168) had no effect on any promoter activity and deletion of yydF-J only showed a minor effect on PbceA 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 PbceA activity (Fig. 2b, blue curve), and deletion of skfA-H decreased the activity about 100-fold (Fig. 2b, green curve). The effect of an sdpc deletion on PpsdA induction was moderate (about 3-fold decrease), but PpsdA activity was almost completely lost in a skfA-H mutant (Fig. 2d). In contrast, PbcrC activity was more strongly decreased in the sdpc mutant (about 4-fold, Fig. 2f) than in the skfA-H deletion strain (max. 2-fold). Moreover, in an sdpc skfA-H double mutant, stationary phase activity of PbceA and PpsdA was fully abolished, while PbcrC 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).

**Toxin production correlates with \( P_{bca} \) induction**

We next tested how stationary phase induction of \( P_{bca} \) 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. 3). 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 \( P_{bca} \) induction (Fig. 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.

**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. 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 ($\Delta bceRSAB \Delta psdRSAB \Delta yxdJKLM-yxeA$) of $B. subtilis$ W168
(Gebhard et al., 2014) (TMB1518, referred to as “3x$bce$ mutant” hereafter) shown in Fig.
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. 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. 5a, orange curve). Given that the
3x$bce$ 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 3x$bce$ 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 3x$bce$ resistance modules and the $sdpI$ immunity protein (Fig. 5a, 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 3x$bce$ $sdpI$ 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.

_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. 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.
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. 6a, b). In a skfA mutant lacking the structural gene of the SKF toxin, P_bceA induction is almost not detectable (Fig. 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. 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 SkfGH 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. 6c, d, orange curve). The resulting double mutant is supposed to be deficient for SDP and lacks crucial steps of SKF maturation. Fig. 6(d) 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.

**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. 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. 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. 6c, d, blue curve): Compared to the wild type reporter strain (Fig. 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 ΔskfA-H ΔsdpAB would lack SKF and only produce immature, unprocessed SDP precursor that could potentially trigger the BceRS system. Fig. 6(c) and (d) show 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. 5b, c). Surprisingly, P_{bceA} induction was completely abolished in this strain. This unexpected finding provoked the question if the sdp/skf 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. 5c, green and blue curve, respectively), comparable to wild type results (see Fig. 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 μg ml^{-1}) to stationary phase cultures could still fully activate the BceRS system (Fig. 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. 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 Δspo0A lawn cells and compared zones of inhibition after incubation overnight. Wild type spots showed a clear zone of inhibition on the
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, ΔsdpC 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. [3074 words]
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. 2b). The exact physiological role of the BceRS system in the cannibalism stress response, however, remains unclear. Our data suggests 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 5+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 promorter, displaying only a 10-fold reduced BceRS response in an sdpC mutant compared to the wild type (Fig. 2). 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 4b+5c) and SDP is most likely functionally produced (Fig. 5d).

Taken together, these findings indicate that SdpI is required for SDP and potentially also SKF perception by the BceRS system (Fig. 7). This mode of an indirect sensing of SDP only in complex with SdpI resembles the mode for bacitracin perception for 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 SdpI-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. [394 words]

Acknowledgements

This project was funded by the DFG priority program SPP1617 “Phenotypic Heterogeneity and Sociobiology of Bacterial Populations” (grant MA 2837/3-1 to TM). [22 words]
### Table 1: Strains used in this study.

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Table 2: Vectors and plasmids used in this study

*Resistance cassettes: bla = ampicillin, cat = chloramphenicol
Table 3: Oligonucleotides used in this study.

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 Allelic replacement mutagenesis (LFH-PCR)
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TM2725 sunA do fwd  CGAGCCGCTACGGAAATTGTATCGGAGCTGTGCTTTGCTAAAACATATC
TM2726 sunA do rev  GGGAGAATAATTGGAAGAAAAGAATG
TM3138 sdpAB up fwd  CAGACAATTGAGTTCCTCCC
TM3139 sdpAB up rev  CCTATACCTCAAAATGGTTTCGCTTGCTAAAGTAAGAAAATAATAG
TM3140 sdpAB do fwd  CGAGCGCCCTACGGAAATTGTATCGGAGCTGTGCTTTGCAAAGCACT
TM3141 sdpAB do rev  GTGGAAATTCTATGCAAGCTAG
TM3142 spo0A do fwd  CAGACAATTGCAGTTGCTGCTGGC
TM3143 spo0A do rev  CCTATACCTCAAAATGGTTTCGCTGCTAAAGTAAGAAAATAATAG
TM3144 spo0A do rev  GGAAGAACCTGAGACACCG
TM3315 skfA do fwd  CGAGCGCCCTACGGAAATTGTATCGGAGCTGTGCTTTGCAAAGCACT
TM3316 skfA do rev  GCTTCCCTAAGCTATTTGAACC
TM3317 skfBC up fwd  GTACAGTACGATTGCTTGATCG
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TM3319 skfBC do fwd  CGAGCGCCCTACGGAAATTGTATCGGAGCTGTGCTTTGCAAAGCACT
TM3320 skfBC do rev  CTGCCATTTTGACTTTGAAATCG
TM3321 skfEF up fwd  CAGTACTTATTGGTACATAGCGG
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TM3323 skfEF do fwd  CGAGCGCCCTACGGAAATTGTATCGGAGCTGTGCTTTGCAAAGCACT
TM3324 skfEF do rev  CATCGTTTTAGTAATGATCTGACC
TM3325 skfH up fwd  GAATTGTCAGACATTCTCAATCAG
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TM3393 skfGH up fwd  GTGCCAAGACAGTGAAGAAAATG
TM3394 skfGH up rev  CCTATACCTCAAAATGGTTTCGCTGGAACAGATAACGACAATTTATCAC
TM0137 kan fwd  CAGCGAACCATTTGAGGTGATAGG
TM0138 kan rev  CGATACAAATTCTCTGATAGCGCTCGG
TM0139 mls fwd  CAGCGAACCATTTGAGGTGATAGG
TM0140 mls rev  CGATACAAATTCTCTGATAGCGCTCGGGCCGACTGCGCAAAAGACATAATCG
TM0141 spec fwd  CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC
TM0142 spec rev  CGATACAAATTCTCTGATAGCGCTCGGGCCGACTGCGCAAAAGACATAATCG

Check primers

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TM2506 sacA front check rev  ACAGCTCCAGATCCTCTACG
TM2507 sacA back check fwd  GTCGCTACCATTACCAGTTG
TM2508 sacA back check rev  TCCAAACATTCGGTGTTATC
TM2262 pAH328 check fwd  GAGCGTAGCGAAAAATCC
TM2263 pAH328 check rev  GAAATGATGCTCCAGTAACC

*Restriction sites are highlighted in bold italics; BioBrick overhang sequences are underlined; overhang sequences for resistance cassettes are marked in italics.
Figure legends

Figure 1: Intrinsic late-stationary phase induction of PbceA-lux, PpsdA-lux (a, b) and ECF σ 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 (OD600) of the respective strains in MCSE medium. The lower graphs (b, d) show the promoter activities as relative luminescence units (RLU) per OD600. Late-stationary phase induction is shown for both the PbceA (black) and PpsdA (orange) after 7-8 h of growth (b). Induction of PbcrC controlled by σM, σX and σW after 7-8 h of growth is shown in green (d). Intermediate induction of σX- and σM-dependent promoters (PsigX and PpsdA) is shown in red and purple, respectively, after 7-8 h of growth. The σW-dependent PpspA (blue) stays uninduced under our cultivation conditions. Please note that the small peak at t=5 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.

Figure 2: Late-stationary phase induction of PbceA-lux (a, b), PpsdA-lux (c, d) and PbcrC-lux (e, f) in deletion backgrounds.

Promoter activity was detected by following luminescence in a microplate reader (for details see legend Fig. 1). Panels (b), (d) and (f) show the effect of different strains deleted for various antimicrobial peptide loci on each promoter: ΔsunA (Sublancin) in light brown, ΔyydF-J (YydF peptide) in dark purple, ΔsdpC (SDP) in blue, Δskf-A-H (SKF) in green, ΔsdpCΔskfA-H in red. ΔsunA had no effect on either promoter. ΔyydF-J showed only minor effects on PbceA, PpsdA and PbcrC activity in stationary phase. Deletion of sdpC revealed 10-fold decrease on PbceA activity and approx. 7-fold on PpsdA and PbcrC activity. The skf-A-H deletion resulted in approx. 100-fold reduced PbceA and PpsdA activity but only 4-fold reduced PbcrC induction.

Figure 3: Schematic overview of SDP and SKF maturation and genomic context.

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.

Figure 4: Correlation of $P_{sdpA}$ and $P_{skfA}$ activities with $P_{bceA}$ induction.
Promoter activity was detected by following luminescence in a microplate reader (for details see legend Fig. 1). $P_{sdpA}$ and $P_{skfA}$ activity is shown over time (in green and blue, respectively). $P_{bceA}$ induction is shown for comparison (black). $P_{sdpA}$ revealed a higher basal activity compared to $P_{skfA}$ and showed approx. 10-fold induction in stationary phase starting around 5 h after beginning of the experiment. $P_{skfA}$ exhibited a similar induction pattern starting slightly later (5-6 h) showing approx. 100-fold induction.

Figure 5: Effect of an $sdpI$ and a triple $bceRSAB psdRSAB yxdJKML$-yx$eA$ mutant on SDP sensitivity.
(a) Growth in W168 (black) and Δ$bceRSAB$ Δ$psdRSAB$ Δ$yxdJKLM$-yx$eA$ (referred to as Δ3xbce hereafter, blue) was similar whereas growth in Δ$sdpI$ (orange) was impaired starting after entry into stationary phase. However, growth was not further impaired in Δ3xbce Δ$sdpI$ (pink) indicating no additional role of the BceRS system in resistance against SDP. $P_{bceA}$, $P_{sdpA}$ and $P_{skfA}$ growth and induction (b, c) were detected by following luminescence in a plate reader (for details see legend Fig. 1). $P_{bceA}$ is not intrinsically induced in Δ$sdpI$ (black filled circles) whereas $P_{sdpA}$ and $P_{skfA}$ are activated after 5-6 h upon start of the experiment (green and blue, respectively) indicating correct expression of the respective loci. Upon induction with bacitracin (10 µg ml$^{-1}$) at t=9 h, $P_{bceA}$ is fully activated (black open circles). Negative data points and values smaller than 50 RLU/OD$_{600}$ are not depicted. Error bars smaller than symbols are not shown. In panel (d), stationary phase cells of W168 and mutants were applied to a plate containing a lawn of Δ$s$po0A cells. From left to right: W168, Δ$skfA$ (SKF), Δ$sdpC$ (SDP) and Δ$sdpI$ (immunity protein against SDP). Halo indicates production of mature SDP. An $sdpC$ mutant strain is unable to kill spo0A deficient cells. SDP seems to be the major cannibalism toxin on solid medium.

Figure 6: $P_{bceA}$ activity in different $sdp$ and $skf$ mutants.
Promoter activity was detected by following luminescence in a microplate reader (for details see legend Fig. 1). \( P_{bceA} \) activity in \( \Delta skfA \) (dark grey), \( \Delta skfBC \) (middle grey) and \( \Delta skfEF \) (light grey) is abolished (b). \( P_{bceA} \) response in \( \Delta skfGH \) (orange) and \( \Delta skfH \) (red) is about 10-fold reduced (b) compared to W168 (see Fig. 1). The time delay of promoter induction in \( \Delta skfGH \) (orange) is due to an approx. 2 h prolonged lag phase but stays the same regarding stationary phase induction point. \( P_{bceA} \) induction in \( \Delta sdpAB\Delta skfA-H \) (d, green curve) as well as \( \Delta sdpC\Delta skfGH \) (d, orange curve) is lost indicating that posttranslational modification of SDP and SKF by SdpAB and SkfGH, each, is needed to activate the BceRS system.

**Figure 7: Model of SDP/SKF sensing by the BceRS system.**

SdpI binding to SDP (and maybe SKF) is a prerequisite for sensing by the BceRS system. The BceRS system consists of an ABC-transporter, BceAB (short A, B) responsible for the detection of bacitracin (Bac) and is coupled to a TCS consisting of a histidine kinase BceS (short: S) and its cognate response regulator, BceR (short: R). Detection of Bac leads to an induction of \( P_{bceA} \) and subsequent transcription of AB to mediate resistance. Current research argues about Bac recognition by AB. One hypothesis is that it has to bind its target UPP (undecaprenol pyrophosphate) in the bacterial membrane in order to be sensed by AB. Taken this hypothesis for granted it could be that only the SdpI-SDP complex can be recognized by AB. ECF \( \sigma^W \) is induced by SDP (and SKF?) and provides a second layer of resistance.
References


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