



Citation for published version:

Höfler, C, Heckmann, J, Fritsch, A, Popp, P, Gebhard, S, Fritz, G & Mascher, T 2016, 'Cannibalism stress response in *Bacillus subtilis*', *Microbiology*, vol. 162, no. 1, pp. 164-176. <https://doi.org/10.1099/mic.0.000176>

DOI:

[10.1099/mic.0.000176](https://doi.org/10.1099/mic.0.000176)

Publication date:

2016

Document Version

Peer reviewed version

[Link to publication](#)

This is the author's accepted version. The version of record is available at:
<http://dx.doi.org/10.1099/mic.0.000176>

University of Bath

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Microbiology

Cannibalism Stress Response in *Bacillus subtilis*

--Manuscript Draft--

Manuscript Number:	MIC-D-15-00294R1
Full Title:	Cannibalism Stress Response in <i>Bacillus subtilis</i>
Short Title:	Cannibalism Stress Response in <i>Bacillus subtilis</i>
Article Type:	Standard
Section/Category:	Regulation
Corresponding Author:	Thorsten Mascher Technische Universität Dresden Dresden, GERMANY
First Author:	Carolin Höfler
Order of Authors:	Carolin Höfler Judith Heckmann Anne Fritsch Philipp Popp Susanne Gebhard Georg Fritz Thorsten Mascher
Abstract:	<p>When faced with carbon source limitation, the Gram-positive soil organism <i>Bacillus subtilis</i> initiates a survival strategy called sporulation, which leads to the formation of highly resistant endospores that allow <i>B. subtilis</i> to survive even long periods of starvation. In order to avoid commitment to this energy-demanding and irreversible process, <i>B. subtilis</i> 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 <i>B. subtilis</i>.</p>

Cannibalism Stress Response in *Bacillus subtilis*

Carolin Höfler^a, Judith Heckmann^a, Anne Fritsch^a, Philipp Popp^{a,3}, Susanne Gebhard^{a,1}, Georg
Fritz^{a,2}, and Thorsten Mascher^{a,3,*}

^aDepartment Biology I, Ludwig-Maximilians-Universität München, Großhaderner Str. 2-4,
82152 Planegg-Martinsried, Germany

¹Present address: Department of Biology and Biochemistry, University of Bath, Claverton
Down, Bath BA2 7AY, United Kingdom

²Present address: LOEWE-Center for Synthetic Microbiology, Philipps-Universität Marburg,
Hans-Meerwein-Str. 6, 35043 Marburg, Germany

³Present address: Technische Universität Dresden, Institute of Microbiology, Zellescher Weg
20b, 01217 Dresden, Germany

Keywords: Cell envelope stress response, antimicrobial peptides, stationary phase survival,
Bce system, ECF σ factors.

Subject category: Regulation

Running Title: Cannibalism stress response in *B. subtilis*

Word count: 5221

*Corresponding author: Prof. Dr. Thorsten Mascher, Tel.: +49 351 463-40420,
Fax: +49 351 463-37715, Email: thorsten.mascher@tu-dresden.de

24 **Abstract**

25 When faced with carbon source limitation, the Gram-positive soil organism *Bacillus subtilis*
26 initiates a survival strategy called sporulation, which leads to the formation of highly resistant
27 endospores that allow *B. subtilis* to survive even long periods of starvation. In order to avoid
28 commitment to this energy-demanding and irreversible process, *B. subtilis* employs another
29 strategy called cannibalism to delay sporulation as long as possible. Cannibalism involves the
30 production and secretion of two cannibalism toxins, the sporulation delaying protein, SDP,
31 and the sporulation killing factor, SKF, which are able to lyse sensitive siblings. The lysed
32 cells are thought to then provide nutrients for the cannibals to slow down or even prevent
33 them from entering sporulation. In this study, we uncovered the role of the cell envelope
34 stress response (CESR), especially the Bce-like antimicrobial peptide detoxification modules,
35 in cannibalism stress response during stationary phase. SDP and SKF specifically induce Bce-
36 like systems and some ECF σ factors in stationary phase cultures, but only the latter provide
37 some degree of protection. A full Bce response is only triggered by mature toxins, but not by
38 toxin precursors. Our study provides insights into the close relationship between stationary
39 phase survival and the CESR of *B. subtilis*. [199 words]

40

41 **Introduction**

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

50 While it is generally accepted that the CESR network has evolved to maintain envelope
51 integrity in the face of AMPs produced by competing species, little is known about the extent
52 to which it is also involved in responding to endogenously produced AMPs. For instance,
53 although it is known that the AMPs are co-expressed with dedicated immunity proteins that
54 prevent cells from autolysis (Dubois *et al.*, 2009; Ellermeier *et al.*, 2006; Gonzalez-Pastor *et*
55 *al.*, 2003), it is conceivable that the level of self-protection via these mechanisms can be
56 insufficient, raising the need for additional protection by the CESR network. In fact, we
57 recently reported that in early stationary phase a subpopulation of *B. subtilis* cells strongly
58 induces one of the CESR modules, the LiaRS system, even in the absence of competitors and
59 without any external addition of AMPs (Dominguez-Escobar *et al.*, 2014; Jordan *et al.*, 2007).
60 Here, we set out to test whether other systems of the CESR network of *B. subtilis* also
61 displayed such an intrinsic induction behavior during stationary phase and, if so, whether this
62 was causally related to the endogenous production of AMPs.

63 To study these questions, we focused on the expression of the core of the CESR network,
64 comprising the AMP-resistance modules, BceRS and PsdRS, as well as the ECF σ factors σ^M ,
65 σ^X and σ^W . While the BceRS and PsdRS systems regulate ABC transporters (BceAB and
66 PsdAB, respectively) that specifically confer resistance against a number of AMPs (Staroń *et*
67 *al.*, 2011), the regulons of the ECF σ factors are known to play a more promiscuous role in
68 cell envelope stress response to antimicrobial compounds (Helmann, 2002; Kingston *et al.*,
69 2013; Mascher *et al.*, 2007; Missiakas & Raina, 1998). σ^M , σ^X and σ^W each regulate a set of
70 about 30-60 target genes with partially overlapping specificity (Kingston *et al.*, 2013;
71 Mascher *et al.*, 2007), and all are activated in a growth-phase and growth medium-dependent
72 manner (Huang *et al.*, 1998): While σ^M and σ^X are induced mainly in late logarithmic growth
73 phase, σ^W only becomes active in early stationary phase (Huang *et al.*, 1998; Nicolas *et al.*,
74 2012).

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

83 In this study, we show that the BceRS and PdsRS system are, in fact, intrinsically activated
84 during stationary phase growth of *B. subtilis*, and single out the inducers amongst a number of
85 endogenously produced AMP candidates. The biological role of these AMPs has previously
86 been implicated in a process termed “cannibalism”, in which the stationary phase population
87 bifurcates into a fraction of AMP-producing cells that feed on another fraction of non-
88 producing cells (Chung *et al.*, 1994; Gonzalez-Pastor *et al.*, 2003). Our data reveals that the
89 CESR network not only serves as a defense against extrinsic attacks from competing species,
90 but also plays a novel role in the intrinsic cannibalism stress response. Interestingly, we show
91 that activation of the BceRS and PdsRS modules by cannibalism toxins critically hinges on
92 the presence of the cognate immunity proteins, providing further insight into the mode of
93 stimulus perception by these systems. [709 words]

94

95 **Methods**

96 **Media and growth conditions.** *B. subtilis* and *E. coli* were routinely grown in Luria-Bertani
97 (LB) medium or MCSE (Radeck *et al.*, 2013) including 0.2% fructose (w/v) as C-source at
98 37°C with agitation. The final composition of MCSE is as follows: 1×MOPS (from
99 10×MOPS buffer: 83.72 g l⁻¹ MOPS, 33 g l⁻¹ (NH₄)₂SO₄, 3.85 mM KH₂PO₄, 6.15 mM
100 K₂HPO₄; adjusted to pH 7 with KOH), 50 mg l⁻¹ Tryptophan, 22 mg l⁻¹ ammonium ferric

101 citrate, 1×10^{-3} M-salts (232 mg l⁻¹ MnSO₄·4H₂O, 12.3 g l⁻¹ MgSO₄·7H₂O), 0.8% (w/v) K-
102 glutamate, 0.6% (w/v) Na-succinate, 0.2% (w/v) fructose. MCSE results in well-defined
103 growth behavior and supports sporulation of *B. subtilis* under the growth conditions applied.
104 Selective media for *B. subtilis* contained chloramphenicol (5 µg ml⁻¹), kanamycin (10 µg ml⁻¹)
105 ¹), spectinomycin (100 µg ml⁻¹), or erythromycin (1 µg ml⁻¹) plus lincomycin (25 µg ml⁻¹) for
106 macrolide-lincosamide-streptogramin B (MLS) resistance. Selective media for *E. coli*
107 contained ampicillin (100 µg ml⁻¹) or chloramphenicol (35 µg ml⁻¹). Solid media additionally
108 contained 1.5% (w/v) agar.

109

110 **Bacterial strains and plasmids.** Transcriptional promoter fusions to bacterial luciferase
111 (*luxABCDE*) were constructed in pAH328 (Schmalisch *et al.*, 2010) or the pAH328 derivative
112 pBS3Clux (Radeck *et al.*, 2013) using *NotI/SalI* or *EcoRI/SpeI* restriction enzymes,
113 respectively. All strains used in this study are listed in Table 1. All *B. subtilis* strains in this
114 study are derivatives of the laboratory wild type strain W168. All plasmids and
115 oligonucleotides are listed in Table 2 and 3, respectively.

116

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

123

124 **Allelic replacement mutagenesis of *sdpAB*, *sdpC*, *sdpI*, *skfA-H*, *skfA*, *skfBC*, *skfEF*,**
125 ***skfGH*, *skfH*, *sunA* and *yydF-J* using LFH-PCR.** Long Flanking Homology PCR (LFH-

126 PCR) technique was performed as described previously (Mascher *et al.*, 2003). The
127 constructed strains are listed in Table 1 and the corresponding primers are listed in Table 3.

128

129 **Luminescence Assay.** Promoter activities were detected by following luminescence in a
130 Synergy™2 multi-mode microplate reader from BioTek® (Winooski, VT, USA) using
131 Gen5™ software. Strain cultivation was performed as follows: Freshly prepared and pre-
132 warmed (37°C) MCSE medium was inoculated 1:500 from overnight cultures and incubated
133 at 37°C with agitation until OD₆₀₀ 0.2. The culture was subsequently diluted to an OD₆₀₀ of
134 0.05 with MCSE and 100 µl were transferred to one well of a 96-well plate (black walls, clear
135 bottom; Greiner Bio-One, Frickenhausen, Germany). OD₆₀₀ and luminescence were recorded
136 every ten minutes for 18 hours. Incubation was performed at 37°C with agitation (medium
137 intensity). Raw luminescence data were normalized to cell density by dividing luminescence
138 per OD₆₀₀ at each data point (relative luminescence units (RLU) / OD₆₀₀). For each individual
139 sample, OD₆₀₀ and luminescence were background-corrected by subtracting the respective
140 mean values measured for MCSE medium only and TMB1578 (pAH328 empty) over every
141 time point. Subsequently, RLU/OD₆₀₀ values were calculated for each measurement and mean
142 values and SEM (standard error of the mean) were determined from at least three independent
143 biological replicates. [834 words]

144

145 **Results and Discussion**

146 ***Intrinsic induction of CESR target promoters during stationary phase growth***

147 Initially, we aimed at investigating if other modules within the CESR network displayed
148 induction profiles similar to the LiaRS system, which – when grown into stationary phase –
149 displayed a clear induction pattern in the absence of any external stimulus (Dominguez-
150 Escobar *et al.*, 2014). To this end, we fused the target promoters of the BceRS system (P_{bceA}),
151 of the PsdRS system (P_{psdA}) and selected target promoters of σ^M , σ^X , and σ^W (P_{ydaH}, P_{sigX}, and

152 P_{pspA} , respectively) and one promoter which is regulated by all three σ factors, P_{bcrC} , to a
153 promoter-less *luxABCDE* reporter (Radeck *et al.*, 2013; Schmalisch *et al.*, 2010). The
154 resulting promoter-*lux* fusions were integrated into the chromosome of *B. subtilis* W168 wild
155 type cells. Automated incubation of the resulting reporter strains in a microplate reader
156 revealed that all but the σ^W target promoter P_{pspA} displayed a marked increase in luminescence
157 activity between two and four hours after the onset of stationary phase (Fig. 1; t=7-8 h). The
158 amplitude of this intrinsic stationary phase induction was highest for the BceRS and PsdRS
159 target promoters (both approx. 500-fold induction; Fig. 1a, b), but also the ECF target
160 promoters displayed a 10-20-fold increase in promoter activity (Fig. 1d). From these
161 observations, we conclude that large parts of the CESR network in *B. subtilis* perceive one or
162 multiple stimuli that are endogenously produced between two to four hours after entry into
163 stationary phase.

164

165 ***AMPs and cannibalism toxins induce CESR systems***

166 Both the BceRS and PsdRS system have been shown to respond to different peptide
167 antibiotics that interfere with the cell wall biosynthetic pathway during exponential growth
168 (Breukink & de Kruijff, 2006; Staroń *et al.*, 2011). In order to elucidate the mechanism
169 behind the observed intrinsic stationary phase activation, we asked whether it could be caused
170 by endogenously produced antimicrobial peptides of *B. subtilis* W168. The first AMP we
171 considered was Sublancin 168 (SunA), which is a SP β prophage-derived bacteriocin
172 described as an S-linked glycopeptide active against Gram-positive bacteria (Oman *et al.*,
173 2011). Its production is known to be repressed during exponential growth phase by the
174 transcriptional regulators AbrB and Rok (Albano *et al.*, 2005; Strauch *et al.*, 2007). Another
175 peptide that might trigger stationary phase induction of the CESR is the YydF peptide, which
176 has been shown to be an endogenous inducer of the LiaRS system (Butcher *et al.*, 2007; Wolf
177 *et al.*, 2010). Its production is also negatively controlled by AbrB during logarithmic growth

178 (Butcher *et al.*, 2007). Subtilosin A (SboA) is another bacteriocin produced by *B. subtilis*
179 W168. Although it is known to be transcriptionally regulated by AbrB and by the two-
180 component regulatory proteins ResDE (Nakano *et al.*, 2000; Strauch *et al.*, 2007), it has been
181 reported to be produced only under anaerobic growth conditions (Nakano *et al.*, 2000).
182 Indeed, we found the *sboA* promoter to be inactive over the whole time course under our
183 cultivation conditions (data not shown). The last two potential AMPs were the two
184 cannibalism toxins sporulation delaying protein, SdpC and sporulation killing factor, SkfA
185 (referred to as SDP and SKF hereafter).

186 To study the effect of the AMPs on the induction of the CESR network, we analyzed P_{bceA} ,
187 P_{psdA} and P_{bcrC} promoter activation in mutants deleted for each gene encoding the respective
188 antimicrobial peptides (Fig. 2). Deletion of *sunA* (Sublancin 168) had no effect on any
189 promoter activity and deletion of *yydF-J* only showed a minor effect on P_{bceA} promoter
190 activity. In contrast, *sdpC* and *skfA-H* mutants revealed the most prominent reduction in
191 luciferase activity for all three promoters tested. Deletion of *sdpC* resulted in an approx. 10-
192 fold reduced P_{bceA} activity (Fig. 2b, blue curve), and deletion of *skfA-H* decreased the activity
193 about 100-fold (Fig. 2b, green curve). The effect of an *sdpC* deletion on P_{psdA} induction was
194 moderate (about 3-fold decrease), but P_{psdA} activity was almost completely lost in a *skfA-H*
195 mutant (Fig. 2d). In contrast, P_{bcrC} activity was more strongly decreased in the *sdpC* mutant
196 (about 4-fold, Fig. 2f) than in the *skfA-H* deletion strain (max. 2-fold). Moreover, in an *sdpC*
197 *skfA-H* double mutant, stationary phase activity of P_{bceA} and P_{psdA} was fully abolished, while
198 P_{bcrC} still displayed mild induction. Hence, we could identify the two cannibalism toxins SDP
199 and SKF as strong inducers of all three CESR target promoters in stationary phase. While
200 induction of ECF σ factors was expected, given the described role in mounting a secondary
201 layer of defense against SDP (Butcher & Helmann, 2006) this is the first time that an intrinsic
202 growth phase-dependent induction has been observed for Bce-like systems. Since the effect
203 was most prominent for the *bceA* promoter, subsequent investigations of the cannibalism

204 stress response were restricted to the BceRS system alone, but key findings were also verified
205 for the PsdRS system, demonstrating similar behavior (data not shown).

206

207 ***Toxin production correlates with P_{bceA} induction***

208 We next tested how stationary phase induction of P_{bceA} was correlated with the activation of
209 *sdpC* and *skfA* expression. SDP is under dual control of first its own promoter P_{sdpC} and
210 second under the promoter driving the whole *sdpABC* operon P_{sdpA} (Fig. 3). We tested both
211 promoter activities over the whole time course and found P_{sdpA} to be the stronger promoter
212 under our cultivation conditions (data not shown). Therefore, we assumed that P_{sdpA} is the
213 crucial promoter driving also expression of *sdpC*. Thus, we studied the luminescence activity
214 from P_{sdpA} - and P_{skfA} -*luxABCDE* reporter fusions throughout growth of the W168 wild type
215 strain to test correlation between SDP/SKF production and P_{bceA} induction (Fig. 4). P_{sdpA} was
216 induced about 10-fold, while P_{skfA} displayed a 100-fold induction. While both the *sdpA* and
217 *skfA* promoters were induced 5-6 h after the beginning of the experiment, the *bceA* promoter
218 became active approx. 2 h later. This indicates that the toxins first had to be produced,
219 processed and likely also accumulated to a certain threshold concentration in order to activate
220 the BceRS system.

221

222 ***The BceRS system does not mediate resistance against cannibalism toxins***

223 Based on its role in mediating resistance against the peptide antibiotic bacitracin, we reasoned
224 that the BceRS system might also confer resistance against SDP. The immunity protein of the
225 *sdpABC-sdpRI* operon is SdpI (Fig. 3). Both the toxin biosynthesis operon *sdpABC* and the
226 immunity operon *sdpRI* are under control of the transition state repressor AbrB and the master
227 regulator of sporulation Spo0A (Ellermeier *et al.*, 2006). SdpI reveals receptor/signal
228 transducing properties, and its synthesis is induced by a combined interplay between SDP,
229 SdpI and SdpR (Ellermeier *et al.*, 2006). In brief, SdpR constitutes an autorepressor blocking

230 transcription of *sdpRI* in the absence of SDP. Upon SDP synthesis and export, SDP binds to
231 SdpI at the membrane, which enables the latter to recruit SdpR into the SDP-SdpI membrane
232 complex. This titration of SdpR away from the DNA induces transcription of *sdpRI*, which
233 results in immunity against SDP (Ellermeier *et al.*, 2006). Accordingly, cannibalism-inactive
234 cells are expected to neither produce and secrete SDP nor induce enhanced SdpI expression.
235 Consequently, it is believed that these cells are highly sensitive to SDP and prone to lysis
236 while toxin-producing cells are resistant against SDP (Ellermeier *et al.*, 2006).

237 In order to study the contribution of the BceRS system towards resistance against SDP, we
238 first performed growth measurements of wild type and a mutant carrying unmarked deletions
239 of all three Bce-like systems ($\Delta bceRSAB \Delta psdRSAB \Delta yxdJKLM-yxeA$) of *B. subtilis* W168
240 (Gebhard *et al.*, 2014) (TMB1518, referred to as “3**x**bce mutant” hereafter) shown in Fig.
241 5(a). Although this mutant strain lacks all important peptide antibiotic detoxification modules
242 present in *B. subtilis*, this did not affect growth compared to wild type (Fig. 5a, blue and black
243 curve, respectively). In contrast, comparison of wild type growth to an *sdpI* mutant revealed a
244 severe growth defect upon entry into stationary phase (Fig. 5a, orange curve). Given that the
245 3**x**bce mutant seems to be unaffected in its growth behavior, we conclude that the BceRS
246 system is not involved in mediating resistance against SDP. Furthermore, we observed no
247 P_{bceA} induction in the 3**x**bce mutant, demonstrating that SDP/SKF cannot be sensed in the
248 absence of the signal transduction system and resistance is not mediated by any of the Bce-
249 like systems (data not shown). This is further supported by the finding that a mutant deficient
250 in both the 3**x**bce resistance modules and the *sdpI* immunity protein (Fig. 5a, pink curve) did
251 not show a stronger growth defect than the *sdpI* mutant alone. To further validate that the
252 BceRS system is indeed not involved in resistance against SDP, we additionally tested the
253 viability of stationary phase cultures (data not shown). We again observed no difference in
254 susceptibility between the 3**x**bce *sdpI* mutant and the single *sdpI* deletion, underpinning the
255 aforementioned result.

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

263 Taken together, we found no evidence for a role of Bce-like systems in mediating resistance
264 against SDP and SKF despite its strong induction. We therefore next focused our attention on
265 the specificity of this induction.

266

267 ***Mature SKF toxin strongly acts as inducer***

268 Of the two cannibalism toxins, SKF was the stronger inducer of the *bceA* promoter. Given
269 that the BceRS system did not confer resistance against SKF, we wondered about the
270 physiological relevance of the intrinsic induction of the CESR systems in stationary phase. In
271 order to approach this question, we first had to understand the true nature of the stimulus
272 sensed by the BceRS system. Was it the mature toxin itself or could the unprocessed
273 precursor also lead to its activation? SKF is a ribosomally synthesized AMP and requires
274 posttranslational modification to be fully active (Gonzalez-Pastor *et al.*, 2003; Liu *et al.*,
275 2010). Our knowledge of this process is still limited and direct evidence for the functions
276 described in the following sentences is still lacking. But it is assumed that the radical SAM
277 (S-adenosyl-methionine) enzyme SkfB mediates the first step in SKF maturation by forming a
278 thioether bond between the cysteine residue Cys4 and the α -carbon of the methionine residue
279 Met12 resulting in pre-SkfA (Flühe *et al.*, 2013; Liu *et al.*, 2010) (Fig. 3). SkfH, a putative
280 thioredoxin oxidoreductase-like protein and the last gene encoded in the *skfA-H* operon is
281 presumed to mediate formation of a disulfide bond leading to SkfA* (Liu *et al.*, 2010) (Fig.

282 3). Export and immunity was postulated to be mediated by SkfEF, forming an ABC
283 transporter in the membrane (Gonzalez-Pastor *et al.*, 2003). Likewise, SkfC was hypothesized
284 to be responsible for the cyclization reaction prior to or during export of the SKF peptide (Liu
285 *et al.*, 2010). SkfG is so far poorly understood and its function is unknown.

286 In order to gain deeper insight into the physiological properties of the genes encoded in the
287 *skfA-H* operon, we next studied the intrinsic P_{bceA} induction in different *skf* mutants (Fig. 6a,
288 b). In a *skfA* mutant lacking the structural gene of the SKF toxin, P_{bceA} induction is almost not
289 detectable (Fig. 6b, dark grey curve). Similar results were obtained in a mutant deleted for
290 *skfBC*, the products of which were hypothesized to be involved in maturation of the toxin
291 precursor (Flühe *et al.*, 2013). This suggests that SkfBC perform critical steps in the
292 maturation process of SKF. Likewise, P_{bceA} induction cannot be detected in a *skfEF* mutant,
293 lacking the putative immunity transporter. In contrast, deletion strains lacking either *skfGH* or
294 *skfH* alone were able to activate the BceRS system in stationary phase, albeit 10-fold reduced
295 compared to the wild type reporter strain (see Fig. 1). SkfH is hypothesized to be responsible
296 for one important disulfide bond formation in the maturation process of SKF (Liu *et al.*,
297 2010). Thus, it seems that SkfH performs a critical step in the maturation of SKF.
298 Additionally, comparison of the *skfGH* mutant and the *skfBC* or *skfEF* deletion, respectively,
299 revealed that potential modification of SKF by SkfBC and/or export via SkfEF seem to play
300 more crucial roles in the SKF maturation pathway than SkfGH alone, since P_{bceA} induction is
301 abolished in both the *skfBC* and *skfEF* mutant. In conclusion, SkfBC and SkfEF are necessary
302 for production of a fully active SKF toxin, while SkfGH seem to play a minor role, at least as
303 judged by the activation of the BceRS system in a *skfGH* mutant.

304 In order to elucidate if the mature SKF toxin or even its precursor acts as an inducer of the
305 *bceA* promoter, we combined the *sdpC* deletion with the *skfGH* deletion (Fig. 6c, d, orange
306 curve). The resulting double mutant is supposed to be deficient for SDP and lacks crucial
307 steps of SKF maturation. Fig. 6(d) shows that the *sdpC skfGH* double mutant first displayed

308 significantly decreased BceRS activation, when compared to the *sdpC* deletion mutant
309 (orange vs. grey curve) but after some time (12-13 h), P_{bceA} becomes active although to a
310 much lower extent. This observation might suggest that accumulation of immature SKF
311 precursor could already act as a weak inducer since the time point of induction is much later
312 and the dynamics considerably lower.

313

314 ***Mature SDP toxin acts as inducer***

315 The absence of any role for the BceRS system in mediating resistance against SDP provokes
316 the question why the BceRS system is triggered by this compound. In order to better
317 understand this stimulus leading to P_{bceA} induction, we investigated BceRS activation in
318 individual *sdp* mutants (Fig. 6).

319 SDP is encoded in the *sdpABC* operon and repressed by AbrB during exponential growth
320 phase and in times of nutrient availability (Chen *et al.*, 2006; Fujita *et al.*, 2005). Upon entry
321 into stationary phase, repression by AbrB is released by active Spo0A, and transcription of the
322 corresponding genes is triggered. Like SKF, SDP is a ribosomally synthesized AMP that
323 requires posttranslational modifications to mature into an active form (Gonzalez-Pastor *et al.*,
324 2003; Liu *et al.*, 2010; Perez Morales *et al.*, 2013), a process presumably mediated by SdpA
325 and SdpB (Perez Morales *et al.*, 2013). SdpA is thought to be a soluble protein attached to the
326 cytosolic face of the membrane, whereas SdpB is a transmembrane protein (Perez Morales *et*
327 *al.*, 2013). Together, they are thought to mediate the final step of processing the SDP
328 precursor peptide into active SDP by posttranslational cleavage of the N- and C-terminus
329 (Fig. 3).

330 To better understand the stimulus leading to P_{bceA} induction by SDP, we first tested if the
331 BceRS system is triggered by the mature SDP toxin or by its precursor. We initially
332 monitored P_{bceA} induction in an *sdpAB* mutant (Fig. 6c, d, blue curve): Compared to the wild
333 type reporter strain (Fig. 1) the induction was only slightly reduced. This is due to the fact that

334 SKF is still present and acting as the main inducer. Consequently, we next compared P_{bceA}
335 induction in a *skfA-H* mutant and a *skfA-H sdpAB* deletion. As a consequence, a deletion
336 strain of $\Delta skfA-H \Delta sdpAB$ would lack SKF and only produce immature, unprocessed SDP
337 precursor that could potentially trigger the BceRS system. Fig. 6(c) and (d) show that the
338 *bceA* promoter induction was completely abolished in the double mutant (green curve),
339 indicating that the SDP precursor is most likely not the inducer of the *bceA* promoter, but
340 rather the mature SDP.

341 Next, we tested *bceA* promoter induction in an *sdpI* mutant, lacking the autoimmunity against
342 SDP (Fig. 5b, c). Surprisingly, P_{bceA} induction was completely abolished in this strain. This
343 unexpected finding provoked the question if the *sdp/skf* operons are still expressed in an *sdpI*
344 mutant since a loss of auto-immunity has previously been reported to sometimes abolish toxin
345 production (Foulston & Bibb, 2010). Both P_{sdpA} and P_{skfA} showed a strong increase about 10-
346 fold and 100-fold, respectively (Fig. 5c, green and blue curve, respectively), comparable to
347 wild type results (see Fig. 4), demonstrating that the two toxin promoters are fully induced
348 and the toxins are most likely also produced. Because of the severe growth defects of the *sdpI*
349 mutant, we wondered whether the silence in the BceRS system is maybe a result of this
350 growth defect. However, addition of bacitracin ($10 \mu\text{g ml}^{-1}$) to stationary phase cultures could
351 still fully activate the BceRS system (Fig. 5c), demonstrating that the BceRS system itself is
352 still functional in the *sdpI* mutant.

353 We next addressed the question if SDP itself is still produced as a potent toxin in the *sdpI*
354 mutant. To this end, we performed a spot-on-lawn assay using a *spo0A* deletion strain as
355 sensitive lawn (Fig. 5d). Since cannibalism toxin production and immunity is regulated in a
356 Spo0A-dependent manner, a *spo0A* mutant is unable to produce both SDP and SKF and is
357 therefore sensitive against both toxins. We spotted stationary phase cultures of wild type as
358 well as *sdp* and *skf* mutants on a plate containing $\Delta spo0A$ lawn cells and compared zones of
359 inhibition after incubation overnight. Wild type spots showed a clear zone of inhibition on the

360 *spo0A* lawn indicating production of functional cannibalism toxins. We then used a *skfA*
361 deletion strain lacking SKF toxin but still expressing SDP. We found that the *skfA* mutant
362 showed a clear inhibition zone just like wild type, indicating production of functional SDP
363 toxin in the absence of SKF. Accordingly, we took an *sdpC* deletion strain lacking SDP but
364 still producing SKF. However, Δ *sdpC* was unable to kill *spo0A* deficient cells, demonstrating
365 that SDP rather than SKF is the major cannibalism toxin on solid medium, which is in
366 agreement with a previous study (Liu *et al.*, 2010). Importantly, a significant zone of
367 inhibition comparable in size to the wild type can be observed around spots of an *sdpI*
368 deletion mutant. This result unequivocally demonstrates that functional SDP toxin is still
369 produced in an *sdpI* mutant. Nevertheless, BceRS activation was abolished in this strain. This
370 observation indicates a link between toxin sensing by the BceRS system and the presence of
371 the immunity protein SdpI. While understanding the molecular mechanism behind this finding
372 is beyond the scope of this work and will require further investigations, it already points
373 towards an indirect way of sensing as will be discussed below. **[3074 words]**

374

375

376 **Conclusion**

377 Our results demonstrate that the BceRS system is intrinsically activated in late-stationary
378 phase due to the production of two cannibalism toxins, SDP and SKF, with SKF being the
379 stronger inducer. The *skfA-H* deletion resulted in a 100-fold reduced BceRS activity, whereas
380 the *sdpC* deletion caused only a 10-fold reduced P_{bceA} induction (Fig. 2b). The exact
381 physiological role of the BceRS system in the cannibalism stress response, however, remains
382 unclear. Our data suggests that it provides no role in resistance against either SDP or SKF.
383 However, it seems that the immunity determinants SdpI and SkfEF, respectively, are
384 important for triggering the BceRS response since in corresponding deletion strains BceRS
385 activation is abolished (Figs 5+6). For SkfEF, this finding is less surprising since this ABC-
386 transporter is thought to also export the SKF toxin. Hence, in its absence no mature inducer
387 reaches the extracellular environment to trigger a BceRS response. But at present, this
388 assumption is hard to investigate without a detectable SKF-dependent phenotype.
389 SDP was shown to be the weaker inducer of the *bceA* promoter, displaying only a 10-fold
390 reduced BceRS response in an *sdpC* mutant compared to the wild type (Fig. 2). Remarkably,
391 in an *sdpI* deletion, we observed a complete loss of the BceRS response despite the fact that
392 both toxin loci are fully expressed (Figs 4b+5c) and SDP is most likely functionally produced
393 (Fig. 5d).

394 Taken together, these findings indicate that SdpI is required for SDP and potentially also SKF
395 perception by the BceRS system (Fig. 7). This mode of an indirect sensing of SDP only in
396 complex with SdpI resembles the mode for bacitracin perception for the BceRS system that
397 was suggested recently (Kingston *et al.*, 2014). Here, it has been proposed that only the
398 complex of bacitracin to its membrane target, undecaprenol pyrophosphate, can act as a
399 trigger of the BceRS response. Our findings on an SdpI-dependent sensing of SDP (and
400 potentially also SKF) support this model of AMP perception by the BceRS system, in which

401 the toxin/AMP has to be bound to a membrane target before it can be perceived by the BceRS
402 system. Analyzing this novel mechanism will be the subject of further investigations.
403 Nevertheless, our results provide clear evidence for a tight link between signaling systems
404 that mediate the CESR in *B. subtilis* and intrinsic AMP production as part of the stationary
405 phase survival strategy of this organism. **[394 words]**

406

407 **Acknowledgements**

408 This project was funded by the DFG priority program SPP1617 “Phenotypic Heterogeneity
409 and Sociobiology of Bacterial Populations” (grant MA 2837/3-1 to TM). **[22 words]**

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

411

<i>E. coli</i> strain	Genotype	Reference
DH5 α	<i>recA1 endA1 gyrA96 thi hsdR17rK- mK+relA1 supE44</i>	(Sambrook & Russell, 2001)
XL1-blue	ϕ 80 Δ <i>lacZ</i> Δ <i>M15</i> Δ (<i>lacZYA-argF</i>) <i>U169</i> <i>endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[Tn10 proAB+</i> <i>lacI^q Δ(lacZ)M15] hsdR17(rK- mK+) tet^R</i>	lab stock
<i>B. subtilis</i> strain	Genotype	Reference
W168	<i>trpC2</i>	lab stock
TMB1518	W168 Δ <i>bceRSAB psdRSAB yxdJKLM yxeA</i> (clean)	(Gebhard <i>et al.</i> , 2014)
TMB1528	W168 <i>sdpI::mls</i>	this study
TMB1578	W168 <i>sacA::luxABCDE</i> (without promoter)	this study
TMB1619	W168 <i>sacA::pCHlux103 (P_{bceA}-lux)</i>	this study
TMB1620	W168 <i>sacA::pCHlux104 (P_{berC}-lux)</i>	this study
TMB1768	W168 <i>sdpC::kan</i>	this study
TMB1770	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) sdpC::kan</i>	this study
TMB1773	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) skfA-H::spec</i>	this study
TMB1775	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) yydF-J::spec</i>	this study
TMB1843	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) sunA::kan</i>	this study
TMB1985	W168 <i>sacA::pJHlux102 (P_{sdpA}-lux)</i>	this study
TMB2009	W168 <i>sacA::pJHlux104 (P_{psdA}-lux)</i>	this study
TMB2015	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) sdpC::kan skfA-H::spec</i>	this study
TMB2016	W168 <i>sacA::pJHlux105 (P_{skfA}-lux)</i>	this study
TMB2047	W168 <i>sacA::pJHlux104 (P_{psdA}-lux) sdpC::kan</i>	this study
TMB2048	W168 <i>sacA::pJHlux104 (P_{psdA}-lux) skfA-H::spec</i>	this study
TMB2118	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) sdpI::mls</i>	this study
TMB2164	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) skfA-H::spec sdpAB::mls</i>	this study
TMB2166	W168 Δ <i>bceRSAB psdRSAB yxdJKLM yxeA</i> (clean) <i>sdpI::mls</i>	this study
TMB2207	W168 <i>sacA::pCHlux104 (P_{berC}-lux) sdpC::kan</i>	this study
TMB2208	W168 <i>sacA::pCHlux104 (P_{berC}-lux) skfA-H::spec</i>	this study
TMB2209	W168 <i>sacA::pCHlux104 (P_{berC}-lux) sunA::kan</i>	this study
TMB2210	W168 <i>sacA::pCHlux104 (P_{berC}-lux) yydF-J::spec</i>	this study
TMB2211	W168 <i>sacA::pJHlux102 (P_{sdpA}-lux) sdpI::mls</i>	this study
TMB2212	W168 <i>sacA::pJHlux105 (P_{skfA}-lux) sdpI::mls</i>	this study
TMB2221	W168 <i>sacA::pCHlux104 (P_{berC}-lux) sdpC::kan skfA-H::spec</i>	this study
TMB2222	W168 <i>sacA::pJHlux104 (P_{psdA}-lux) sdpC::kan skfA-H::spec</i>	this study
TMB2223	W168 <i>sacA::pJHlux104 (P_{psdA}-lux) yydF-J::spec</i>	this study
TMB2224	W168 <i>sacA::pJHlux104 (P_{psdA}-lux) sunA::kan</i>	this study
TMB2240	W168 <i>spo0A::spec</i>	this study
TMB2257	W168 <i>sacA::pCH3Clux02 (P_{sigX}-lux)</i>	this study
TMB2259	W168 <i>sacA::pCH3Clux04 (P_{ydaH}-lux)</i>	this study
TMB2260	W168 <i>skfA::mls</i>	this study
TMB2262	W168 <i>skfEF::mls</i>	this study
TMB2265	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) skfA::mls</i>	this study
TMB2266	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) skfBC::spec</i>	this study
TMB2267	W168 <i>sacA::pCHlux103 (P_{bceA}-lux) skfEF::mls</i>	this study

TMB2268	W168 <i>sacA</i> ::pCHlux103 (<i>P_{bceA}-lux</i>) <i>skfH</i> ::kan	this study
TMB2299	W168 <i>sacA</i> ::pASp3Clux01 (<i>P_{pspA}-lux</i>)	this study
TMB2339	W168 <i>sacA</i> ::pCHlux103 (<i>P_{bceA}-lux</i>) <i>skfGH</i> ::kan	this study
TMB2806	W168 <i>sacA</i> ::pCHlux103 (<i>P_{bceA}-lux</i>) <i>sdpC</i> ::kan <i>skfGH</i> ::mls	this study
TMB2909	W168 <i>ΔbceRSAB psdRSAB yxdJKLM yxeA</i> (clean) <i>skfEF</i> ::mls	this study

412

413

414 **Table 2:** Vectors and plasmids used in this study

415

Plasmid/vector	Genotype ^a	Primers used for cloning	Reference or source
pAH328	<i>sacA</i> '...'sacA, <i>luxABCDE</i> , <i>bla</i> , <i>cat</i>		(Schmalisch <i>et al.</i> , 2010)
pBS3Clux	pAH328 derivative; <i>sacA</i> '...'sacA, <i>luxABCDE</i> , <i>bla</i> , <i>cat</i>		(Radeck <i>et al.</i> , 2013)
pCHlux103	pAH328 derivative, <i>sacA</i> :: <i>P_{bceA}-lux</i> , <i>cat</i>	TM2513/2514	This study
pCHlux104	pAH328 derivative, <i>sacA</i> :: <i>P_{bcrC}-lux</i> , <i>cat</i>	TM2515/2516	This study
pJHlux102	pAH328 derivative, <i>sacA</i> :: <i>P_{sdpA}-lux</i> , <i>cat</i>	TM2785/2786	This study
pJHlux104	pAH328 derivative, <i>sacA</i> :: <i>P_{psdA}-lux</i> , <i>cat</i>	TM2781/2782	This study
pJHlux105	pAH328 derivative, <i>sacA</i> :: <i>P_{skfA}-lux</i> , <i>cat</i>	TM2783/2784	This study
pCH3Clux02	pAH328 derivative, <i>sacA</i> :: <i>P_{sigX}-lux</i> , <i>cat</i>	TM3262/3263	This study
pCH3Clux04	pAH328 derivative, <i>sacA</i> :: <i>P_{ydaH}-lux</i> , <i>cat</i>	TM3266/3267	This study
pASp3Clux01	pAH328 derivative, <i>sacA</i> :: <i>P_{pspA}-lux</i> , <i>cat</i>	TM3268/3269	This study

416 ^aResistance cassettes: *bla* = ampicillin, *cat* = chloramphenicol

417

418 **Table 3:** Oligonucleotides used in this study.

419

Primer name	Sequence 5' – 3' ^a
<i>Construction of transcriptional promoter-lux fusions</i>	
TM2513 P _{bceA} NotI fwd	agcggccgcACGCGGTGAAATACAGCGAAG
TM2514 P _{bceA} SalI rev	taagtcgacTATATTGGATAATCTCATTATAAAAAAG
TM2515 P _{bcrC} NotI fwd	agcggccgcGGCCTTCAAAAAGCACATACG
TM2516 P _{bcrC} SalI rev	taagtcgacTTACATTTTTATATTTAGTAGACTAATC
TM2785 P _{sdpA} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> GATGACGCTTACGGAATTATCTG
TM2786 P _{sdpA} SpeI rev	ctataaactagfTTTTTTGATGTAGATTACCTCCTC
TM2781 P _{psdA} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> TGATGCTGCAAACGGCCC
TM2782 P _{psdA} SpeI rev	ctataaactagfTTTCTTTATTATAAAAAGGAAGTCAGC
TM2783 P _{skfA} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> ATGACAGATTCGTATTGCCGG
TM2784 P _{skfA} SpeI rev	ctataaactagfTCAATTTTTGCATAGAGTCTATTGAC
TM3262 P _{sigX} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> ACTCCGGGTCTGGCATAACC
TM3263 P _{sigX} SpeI rev	ctataaactagfTCACTTTTTTGTCGTATGAATAGCTTG
TM3266 P _{ydaH} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> TTTGAGAGAGAAGCTTACCGC
TM3267 P _{ydaH} SpeI rev	ctataaactagfAATTTTCATCCTAGAGATAAGACTGG
TM3268 P _{pspA} EcoRI fwd	ttataggaattc <u>cgcgccgcttagag</u> TCCGGTGACATCAATTGACTC
TM3269 P _{pspA} SpeI rev	ctataaactagfAAAGCTAATTCGGTAACCCTTG
<i>Allelic replacement mutagenesis (LFH-PCR)</i>	
TM2748 sdpC up fwd	GAAGGTTATATTGACACCTATAATCC
TM2749 sdpC up rev	CCTATCACCTCAAATGGTTTCGCTGGTTACCATGGAAACAATCAATAGCC
TM2750 sdpC do fwd	CGAGCGCCTACGAGGAATTTGTATCGGCTGCTGCAAAAACCCTAAAATTG
TM2751 sdpC do rev	CAAATATCTAAATGTCTAAATGTTTTTTTGTAAG
TM2744 skf up fwd	TGGTGCGTTAGGGTTATGATTGC
TM2745 skf up rev	CCTATCACCTCAAATGGTTTCGCTGCTCACAGATTCCCATTCTTTTTGG
TM2746 skf do fwd	CGAGCGCCTACGAGGAATTTGTATCGGGAGATGTTGGTTGGGATAAGATG

TM2747 <i>skf</i> do rev	GATTTGCTGCCGTTTTGGTAAGAC
TM2723 <i>sunA</i> up fwd	GTATCACGATGGATATTTATAGATGC
TM2724 <i>sunA</i> up rev	CCTATCACCTCAAATGGTTCGCTGGTTTTTCGAGTTCCTCTAGTTTAACTTC
TM2725 <i>sunA</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGGAGCTGTTGCTTGTCAAAACATC
TM2726 <i>sunA</i> do rev	GGGAGAATAATTGTTAAGAAAAGAATG
TM3138 <i>sdpAB</i> up fwd	CAGACAATTGAATGCTTCCC
TM3139 <i>sdpAB</i> up rev	CCTATCACCTCAAATGGTTCGCTGGCTAAAGTAATAAGAAGAAAATAATAG
TM3140 <i>sdpAB</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGGGTGAATCAGTCAAGTTTCTTAC
TM3141 <i>sdpAB</i> do rev	GTGGAAATTCTATGCAGCTAG
TM0307 <i>spo0A</i> up fwd	TATCAGAGATTCTGCTGCTGGC
TM0308 <i>spo0A</i> up rev	CCTATCACCTCAAATGGTTCGCTGAGCGACAGGCATTCCTGTCC
TM0309 <i>spo0A</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGGTTGCGGATAAGCTGAGG
TM0310 <i>spo0A</i> do rev	GGAAGAACCTGAGACACCG
TM3315 <i>skfA</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGCGTGTTTGTGCACTCCGCATC
TM3316 <i>skfA</i> do rev	GCTTCCCTAAGCTGTATTTGAACC
TM3317 <i>skfBC</i> up fwd	GTACAGTACGATTGCCTTGATCG
TM3318 <i>skfBC</i> up rev	CCTATCACCTCAAATGGTTCGCTGGAACCGCTAACTCTGGCAAATC
TM3319 <i>skfBC</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGGAAACATATGCATCATGATCAGCC
TM3320 <i>skfBC</i> do rev	CTGCCATTTGACTTGGTAATCG
TM3321 <i>skfEF</i> up fwd	CAGTACTTATTGGTACATAGCGG
TM3322 <i>skfEF</i> up rev	CCTATCACCTCAAATGGTTCGCTGCATCACCATTTTCGATAGCATTTC
TM3323 <i>skfEF</i> do fwd	CGAGCGCCTACGAGGAATTTGTATCGCATAGGGAGCCTAAGTTGGTG
TM3324 <i>skfEF</i> do rev	CATCGTTTTAGTAATGATCTGACC
TM3325 <i>skfH</i> up fwd	GAATTGTCAGACATTCTCAATCAG
TM3326 <i>skfH</i> up rev	CCTATCACCTCAAATGGTTCGCTGCTTGGCCATTTCAGTCAACATTTG
TM3393 <i>skfGH</i> up fwd	GTGCCAGAACAGTGAAGAAAATG
TM3394 <i>skfGH</i> up rev	CCTATCACCTCAAATGGTTCGCTGGAACAGATAACGACAATTTATCACC

TM0137 kan fwd CAGCGAACCATTTGAGGTGATAGG

TM0138 kan rev CGATACAAATTCCTCGTAGGCGCTCGG

TM0139 mls fwd CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC

TM0140 mls rev CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG

TM0141 spec fwd CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACT
GGCAAGAG

TM0142 spec rev CGATACAAATTCCTCGTAGGCGCTCGGGCTAGCGAGGGCAAGGGTTTATTGTT
TTCTAAAATCTG

Check primers

TM2505 *sacA* front check fwd CTGATTGGCATGGCGATTGC

TM2506 *sacA* front check rev ACAGCTCCAGATCCTCTACG

TM2507 *sacA* back check fwd GTCGCTACCATTACCAGTTG

TM2508 *sacA* back check rev TCCAAACATTCCGGTGTTATC

TM2262 pAH328 check fwd GAGCGTAGCGAAAAATCC

TM2263 pAH328 check rev GAAATGATGCTCCAGTAACC

420 ^aRestriction sites are highlighted in bold italics; BioBrick overhang sequences are underlined;
 421 overhang sequences for resistance cassettes are marked in italics.

422

423

424 **Figure legends**

425 **Figure 1: Intrinsic late-stationary phase induction of $P_{bceA-lux}$, $P_{psdA-lux}$ (a, b) and ECF**
426 **σ factor target promoters in W168 (c, d).**

427 Promoter activity was detected by following luminescence of 100 μ l cultures growing in a
428 microplate reader (Biotek[®], Synergy[™]2; 96-well plate, 37°C, shaking) over time. The upper
429 graphs (a, c) show the growth curves (OD₆₀₀) of the respective strains in MCSE medium. The
430 lower graphs (b, d) show the promoter activities as relative luminescence units (RLU) per
431 OD₆₀₀. Late-stationary phase induction is shown for both the P_{bceA} (black) and P_{psdA} (orange)
432 after 7-8 h of growth (b). Induction of P_{bcrC} controlled by σ^M , σ^X and σ^W after 7-8 h of growth
433 is shown in green (d). Intermediate induction of σ^X - and σ^M -dependent promoters (P_{sigX} and
434 P_{ydaH}) is shown in red and purple, respectively, after 7-8 h of growth. The σ^W -dependent P_{pspA}
435 (blue) stays uninduced under our cultivation conditions. Please note that the small peak at t=5
436 in this and all the following figures does not represent a regulated transition phase promoter
437 induction, since it was observed for any promoter studied in MCSE so far, including a set of
438 known constitutive promoters (Radeck *et al.*, 2013). All graphs show mean values and SEM
439 (standard error of the mean) of at least three independent replicates.

440

441 **Figure 2: Late-stationary phase induction of $P_{bceA-lux}$ (a, b), $P_{psdA-lux}$ (c, d) and $P_{bcrC-lux}$**
442 **(e, f) in deletion backgrounds.**

443 Promoter activity was detected by following luminescence in a microplate reader (for details
444 see legend Fig. 1). Panels (b), (d) and (f) show the effect of different strains deleted for
445 various antimicrobial peptide loci on each promoter: $\Delta sunA$ (Sublancin) in light brown,
446 $\Delta yydF-J$ (YydF peptide) in dark purple, $\Delta sdpC$ (SDP) in blue, $\Delta skfA-H$ (SKF) in green,
447 $\Delta sdpC\Delta skfA-H$ in red. $\Delta sunA$ had no effect on either promoter. $\Delta yydF-J$ showed only minor
448 effects on P_{bceA} , P_{psdA} and P_{bcrC} activity in stationary phase. Deletion of $sdpC$ revealed 10-fold
449 decrease on P_{bceA} activity and approx. 7-fold on P_{psdA} and P_{bcrC} activity. The $skfA-H$ deletion
450 resulted in approx. 100-fold reduced P_{bceA} and P_{psdA} activity but only 4-fold reduced P_{bcrC}
451 induction.

452

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

454 Panels (a) and (c) show main transcripts of the $sdpABC-sdpRI$ and $skfA-H$ operons, each based
455 on recent microarray studies (Nicolas *et al.*, 2012). Panels (b) and (d) show the hypothesized
456 schematic maturation pathway of SDP and SKF precursors until release of the final toxin.
457 According to Perez Morales *et al.*, 2013 pro-SdpC is translocated across the membrane by the

458 general secretory pathway (Sec) and the leader peptide thereby cleaved by the SipS/T
459 peptidase (b). SdpAB further cleave SdpC* at the N-and C-termini to release the final SDP
460 toxin to the environment. Similarly, pro-SkfA is hypothesized to be modified by SkfB to give
461 pre-SkfA which is assumed to be further processed by SkfH to prepare for export and
462 cyclization by SkfEF and SkfC, respectively (d). These assumptions are based on Liu *et al.*,
463 2010 and lack further evidence.

464

465 **Figure 4: Correlation of P_{sdpA} and P_{skfA} activities with P_{bceA} induction.**

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

472

473 **Figure 5: Effect of an $sdpI$ and a triple $bceRSAB psdRSAB yxdJKML-yxeA$ mutant on**
474 **SDP sensitivity.**

475 (a) Growth in W168 (black) and $\Delta bceRSAB \Delta psdRSAB \Delta yxdJKML-yxeA$ (referred to as
476 $\Delta 3xbce$ hereafter, blue) was similar whereas growth in $\Delta sdpI$ (orange) was impaired starting
477 after entry into stationary phase. However, growth was not further impaired in $\Delta 3xbce \Delta sdpI$
478 (pink) indicating no additional role of the BceRS system in resistance against SDP. P_{bceA} , P_{sdpA}
479 and P_{skfA} growth and induction (b, c) were detected by following luminescence in a plate
480 reader (for details see legend Fig. 1). P_{bceA} is not intrinsically induced in $\Delta sdpI$ (black filled
481 circles) whereas P_{sdpA} and P_{skfA} are activated after 5-6 h upon start of the experiment (green
482 and blue, respectively) indicating correct expression of the respective loci. Upon induction
483 with bacitracin ($10 \mu\text{g ml}^{-1}$) at $t=9$ h, P_{bceA} is fully activated (black open circles). Negative
484 data points and values smaller than 50 RLU/OD₆₀₀ are not depicted. Error bars smaller than
485 symbols are not shown. In panel (d), stationary phase cells of W168 and mutants were applied
486 to a plate containing a lawn of $\Delta spo0A$ cells. From left to right: W168, $\Delta skfA$ (SKF), $\Delta sdpC$
487 (SDP) and $\Delta sdpI$ (immunity protein against SDP). Halo indicates production of mature SDP.
488 An $sdpC$ mutant strain is unable to kill $spo0A$ deficient cells. SDP seems to be the major
489 cannibalism toxin on solid medium.

490

491 **Figure 6: P_{bceA} activity in different sdp and skf mutants.**

492 Promoter activity was detected by following luminescence in a microplate reader (for details
493 see legend Fig. 1). P_{bceA} activity in $\Delta skfA$ (dark grey), $\Delta skfBC$ (middle grey) and $\Delta skfEF$ (light
494 grey) is abolished (b). P_{bceA} response in $\Delta skfGH$ (orange) and $\Delta skfH$ (red) is about 10-fold
495 reduced (b) compared to W168 (see Fig. 1). The time delay of promoter induction in $\Delta skfGH$
496 (orange) is due to an approx. 2 h prolonged lag phase but stays the same regarding stationary
497 phase induction point. P_{bceA} induction in $\Delta sdpAB\Delta skfA-H$ (d, green curve) as well as
498 $\Delta sdpC\Delta skfGH$ (d, orange curve) is lost indicating that posttranslational modification of SDP
499 and SKF by SdpAB and SkfGH, each, is needed to activate the BceRS system.

500

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

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

511

512

513 **References**

514 **Albano, M., Smits, W. K., Ho, L. T., Kraigher, B., Mandic-Mulec, I., Kuipers, O. P. &**
515 **Dubnau, D. (2005).** The Rok protein of *Bacillus subtilis* represses genes for cell surface and
516 extracellular functions. *J Bacteriol* **187**, 2010-2019.

517
518 **Breukink, E. & de Kruijff, B. (2006).** Lipid II as a target for antibiotics. *Nat Rev Drug*
519 *Discov* **5**, 321-332.

520
521 **Butcher, B. G. & Helmann, J. D. (2006).** Identification of *Bacillus subtilis* σ^W -dependent
522 genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. *Mol*
523 *Microbiol* **60**, 765-782.

524
525 **Butcher, B. G., Lin, Y.-P. & Helmann, J. D. (2007).** The *yidFGHIJ* operon of *Bacillus*
526 *subtilis* encodes a peptide that induces the LiaRS two-component system. *J Bacteriol* **189**,
527 8616-8625.

528
529 **Chen, G., Kumar, A., Wyman, T. H. & Moran, C. P., Jr. (2006).** Spo0A-dependent
530 activation of an extended -10 region promoter in *Bacillus subtilis*. *J Bacteriol* **188**, 1411-
531 1418.

532
533 **Chung, J. D., Stephanopoulos, G., Ireton, K. & Grossman, A. D. (1994).** Gene expression
534 in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation
535 of sporulation. *J Bacteriol* **176**, 1977-1984.

536
537 **Cutting, S. M. & Van der Horn, P. B. (1990).** Genetic analysis. In *Molecular Biological*
538 *Methods for Bacillus*, pp. 27-74. Edited by C. R. Harwood & S. M. Cutting. Chichester,
539 United Kingdom: John Wiley & Sons, Ltd.

540
541 **Dominguez-Escobar, J., Wolf, D., Fritz, G., Höfler, C., Wedlich-Söldner, R. & Mascher,**
542 **T. (2014).** Subcellular localization, interactions and dynamics of the phage-shock protein-like
543 Lia response in *Bacillus subtilis*. *Mol Microbiol* **92**, 716-732.

544
545 **Dubois, J. Y., Kouwen, T. R., Schurich, A. K., Reis, C. R., Ensing, H. T., Trip, E. N.,**
546 **Zweers, J. C. & van Dijl, J. M. (2009).** Immunity to the bacteriocin sublancin 168 Is
547 determined by the SunI (YolF) protein of *Bacillus subtilis*. *Antimicrobial agents and*
548 *chemotherapy* **53**, 651-661.

549
550 **Ellermeier, C. D., Hobbs, E. C., Gonzalez-Pastor, J. E. & Losick, R. (2006).** A three-
551 protein signaling pathway governing immunity to a bacterial cannibalism toxin. *Cell* **124**,
552 549-559.

553
554 **Flühe, L., Burghaus, O., Wieckowski, B. M., Giessen, T. W., Linne, U. & Marahiel, M.**
555 **A. (2013).** Two [4Fe-4S] clusters containing radical SAM enzyme SkfB catalyze thioether

556 bond formation during the maturation of the sporulation killing factor. *Journal of the*
557 *American Chemical Society* **135**, 959-962.

558
559 **Foulston, L. C. & Bibb, M. J. (2010)**. Microbisporicin gene cluster reveals unusual features
560 of lantibiotic biosynthesis in actinomycetes. *Proc Natl Acad Sci U S A* **107**, 13461-13466.

561
562 **Fujita, M., Gonzalez-Pastor, J. E. & Losick, R. (2005)**. High- and low-threshold genes in
563 the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187**, 1357-1368.

564
565 **Gebhard, S., Fang, C., Shaaly, A., Leslie, D. J., Weimar, M. R., Kalamorz, F., Carne, A.**
566 **& Cook, G. M. (2014)**. Identification and characterization of a bacitracin resistance network
567 in *Enterococcus faecalis*. *Antimicrobial agents and chemotherapy* **58**, 1425-1433.

568
569 **Gonzalez-Pastor, J. E., Hobbs, E. C. & Losick, R. (2003)**. Cannibalism by sporulating
570 bacteria. *Science* **301**, 510-513.

571
572 **Harwood, C. R. & Cutting, S. M. (1990)**. *Molecular Biological Methods for Bacillus*.
573 Chichester: John Wiley & Sons.

574
575 **Helmann, J. D. (2002)**. The extracytoplasmic function (ECF) sigma factors. *Adv Microb*
576 *Physiol* **46**, 47-110.

577
578 **Huang, X., Fredrick, K. L. & Helmann, J. D. (1998)**. Promoter recognition by *Bacillus*
579 *subtilis* σ^W : autoregulation and partial overlap with the σ^X regulon. *J Bacteriol* **180**, 3765-
580 3770.

581
582 **Jordan, S., Rietkötter, E., Strauch, M. A., Kalamorz, F., Butcher, B. G., Helmann, J. D.**
583 **& Mascher, T. (2007)**. LiaRS-dependent gene expression is embedded in transition state
584 regulation in *Bacillus subtilis*. *Microbiology* **153**, 2530-2540.

585
586 **Kallenberg, F., Dintner, S., Schmitz, R. & Gebhard, S. (2013)**. Identification of regions
587 important for resistance and signalling within the antimicrobial peptide transporter BceAB of
588 *Bacillus subtilis*. *J Bacteriol* **195**, 3287-3297.

589
590 **Kingston, A. W., Liao, X. & Helmann, J. D. (2013)**. Contributions of the σ^W , σ^M and σ^X
591 regulons to the lantibiotic resistome of *Bacillus subtilis*. *Mol Microbiol* **90**, 502-518.

592
593 **Kingston, A. W., Zhao, H., Cook, G. M. & Helmann, J. D. (2014)**. Accumulation of
594 heptaprenyl diphosphate sensitizes *Bacillus subtilis* to bacitracin: implications for the
595 mechanism of resistance mediated by the BceAB transporter. *Mol Microbiol* **93**, 37-49.

596
597 **Liu, W. T., Yang, Y. L., Xu, Y., Lamsa, A., Haste, N. M., Yang, J. Y., Ng, J., Gonzalez,**
598 **D., Ellermeier, C. D. & other authors (2010)**. Imaging mass spectrometry of intraspecies

599 metabolic exchange revealed the cannibalistic factors of *Bacillus subtilis*. *Proc Natl Acad Sci*
600 *U S A* **107**, 16286-16290.

601

602 **Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. & Helmann, J. D. (2003)**. Cell wall
603 stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol*
604 *Microbiol* **50**, 1591-1604.

605

606 **Mascher, T., Hachmann, A. B. & Helmann, J. D. (2007)**. Regulatory overlap and
607 functional redundancy among *Bacillus subtilis* extracytoplasmic function (ECF) σ factors. *J*
608 *Bacteriol* **189**, 6919-6927.

609

610 **Missiakas, D. & Raina, S. (1998)**. The extracytoplasmic function sigma factors: role and
611 regulation. *Mol Microbiol* **28**, 1059-1066.

612

613 **Nakano, M. M., Zheng, G. & Zuber, P. (2000)**. Dual control of *sbo-alb* operon expression
614 by the Spo0 and ResDE systems of signal transduction under anaerobic conditions in *Bacillus*
615 *subtilis*. *J Bacteriol* **182**, 3274-3277.

616

617 **Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E.,**
618 **Marchadier, E., Hoebeke, M. & other authors (2012)**. Condition-dependent transcriptome
619 reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* **335**, 1103-1106.

620

621 **Oman, T. J., Boettcher, J. M., Wang, H., Okalibe, X. N. & van der Donk, W. A. (2011)**.
622 Sublancin is not a lantibiotic but an S-linked glycopeptide. *Nature chemical biology* **7**, 78-80.

623

624 **Perez Morales, T. G., Ho, T. D., Liu, W. T., Dorrestein, P. C. & Ellermeier, C. D. (2013)**.
625 Production of the cannibalism toxin SDP is a multistep process that requires SdpA and SdpB.
626 *J Bacteriol* **195**, 3244-3251.

627

628 **Radeck, J., Kraft, K., Bartels, J., Cikovic, T., Dürr, F., Emenegger, J., Kelterborn, S.,**
629 **Sauer, C., Fritz, G. & other authors (2013)**. The *Bacillus* BioBrick Box: generation and
630 evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *J*
631 *Biol Eng* **7**, 29.

632

633 **Rietkötter, E., Hoyer, D. & Mascher, T. (2008)**. Bacitracin sensing in *Bacillus subtilis*. *Mol*
634 *Microbiol* **68**, 768-785.

635

636 **Sambrook, J. & Russell, D. W. (2001)**. Molecular Cloning - a laboratory manual. Cold
637 Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

638

639 **Schmalisch, M., Maiques, E., Nikolov, L., Camp, A. H., Chevreux, B., Muffler, A.,**
640 **Rodriguez, S., Perkins, J. & Losick, R. (2010)**. Small genes under sporulation control in the
641 *Bacillus subtilis* genome. *J Bacteriol* **192**, 5402-5412.

642
643 **Schrecke, K., Staroń, A. & Mascher, T. (2012).** Two-component signaling in the Gram-
644 positive envelope stress response: intramembrane-sensing histidine kinases and accessory
645 membrane proteins. In *Two component systems in bacteria*, pp. 199-229. Edited by R. Gross
646 & D. Beier. Hethersett, Norwich, UK: Horizon Scientific Press.

647
648 **Silver, L. L. (2003).** Novel inhibitors of bacterial cell wall synthesis. *Current opinion in*
649 *microbiology* **6**, 431-438.

650
651 **Silver, L. L. (2006).** Does the cell wall of bacteria remain a viable source of targets for novel
652 antibiotics? *Biochem Pharmacol* **71**, 996-1005.

653
654 **Staroń, A., Finkeisen, D. E. & Mascher, T. (2011).** Peptide antibiotic sensing and
655 detoxification modules of *Bacillus subtilis*. *Antimicrobial agents and chemotherapy* **55**, 515-
656 525.

657
658 **Strauch, M. A., Bobay, B. G., Cavanagh, J., Yao, F., Wilson, A. & Le Breton, Y. (2007).**
659 Abh and AbrB control of *Bacillus subtilis* antimicrobial gene expression. *J Bacteriol* **189**,
660 7720-7732.

661
662 **Walsh, C. (2003).** Antibiotics - actions, origins, resistance. Washington, D.C.: ASM press.

663
664 **Wolf, D., Kalamorz, F., Wecke, T., Juszczak, A., Mäder, U., Homuth, G., Jordan, S.,**
665 **Kirstein, J., Hoppert, M. & other authors (2010).** In-depth profiling of the LiaR response
666 of *Bacillus subtilis*. *J Bacteriol* **192**, 4680-4693.

667
668

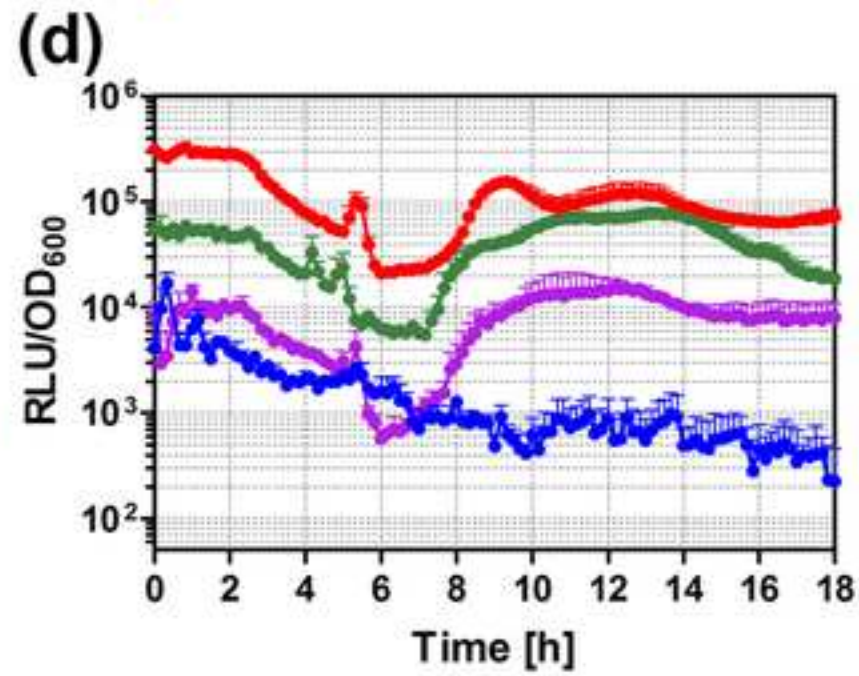
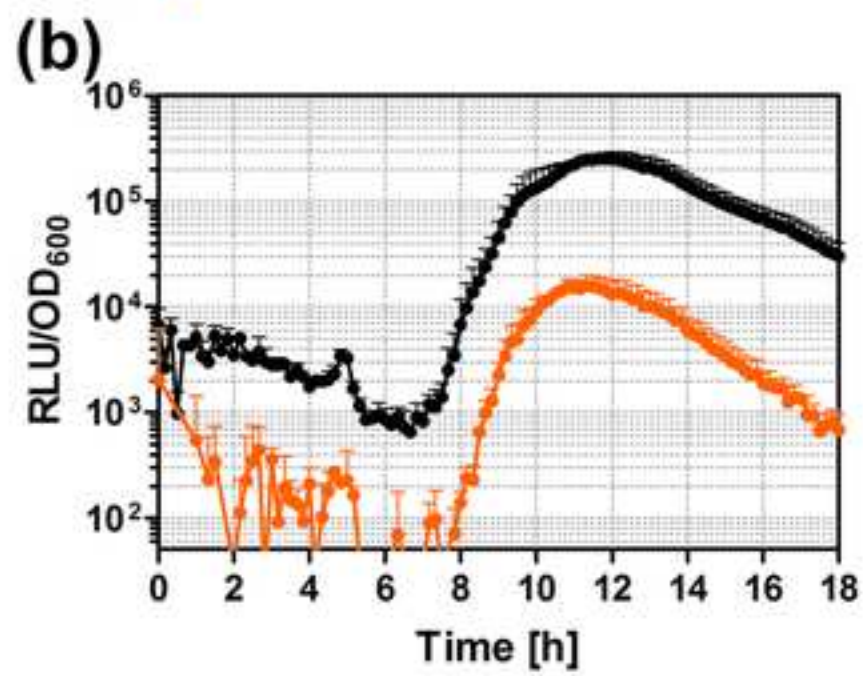
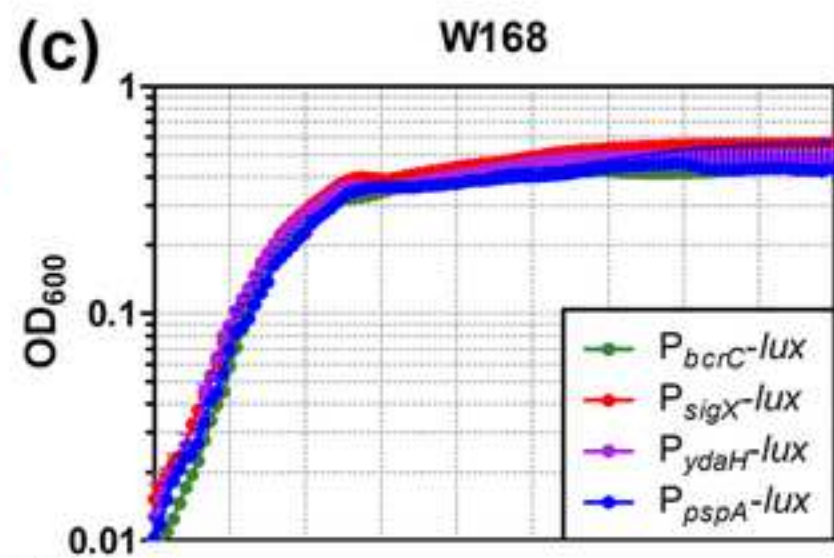
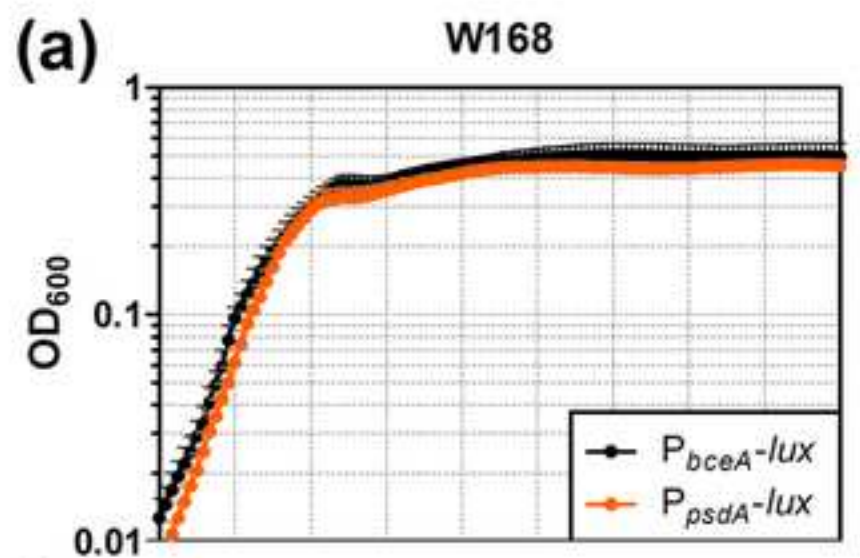
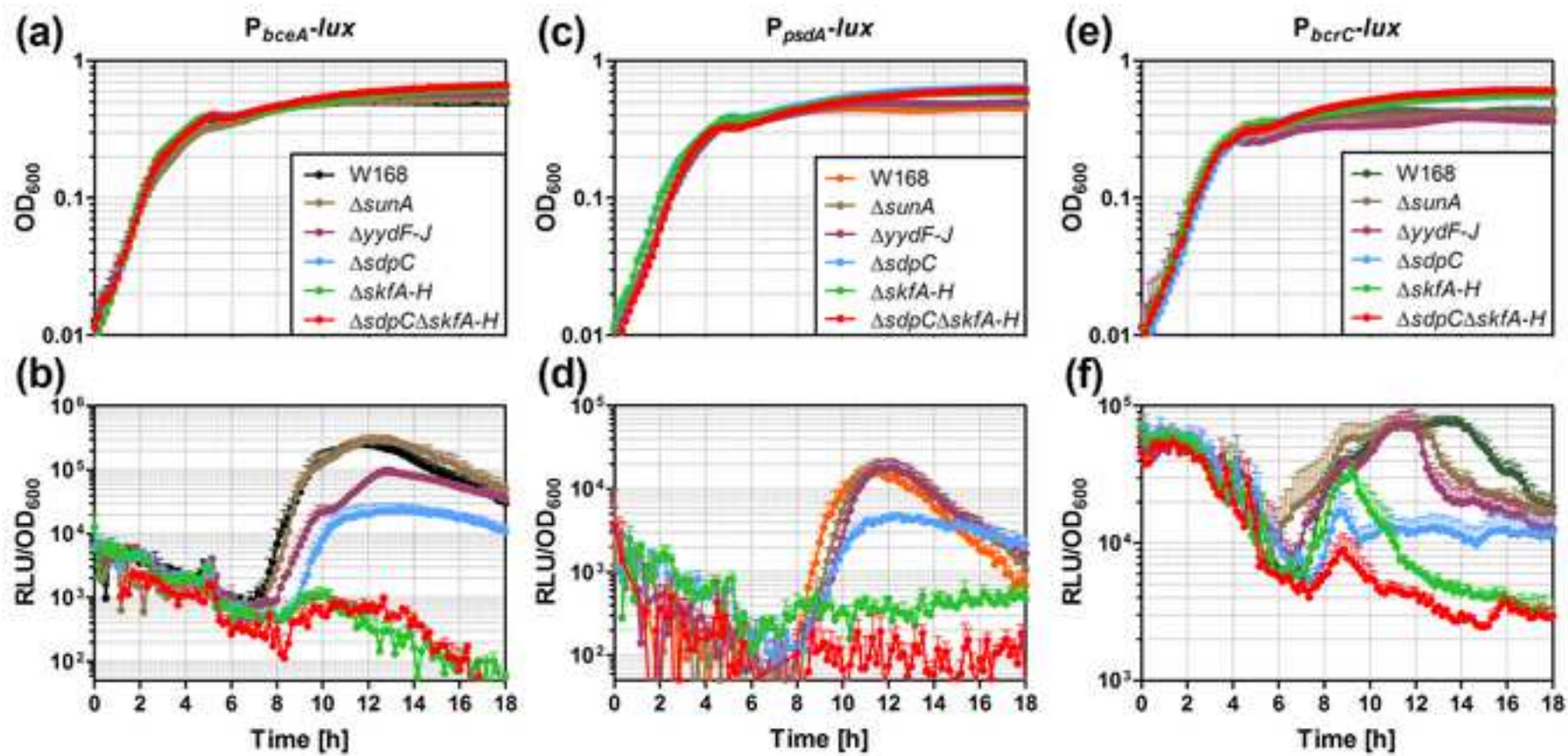
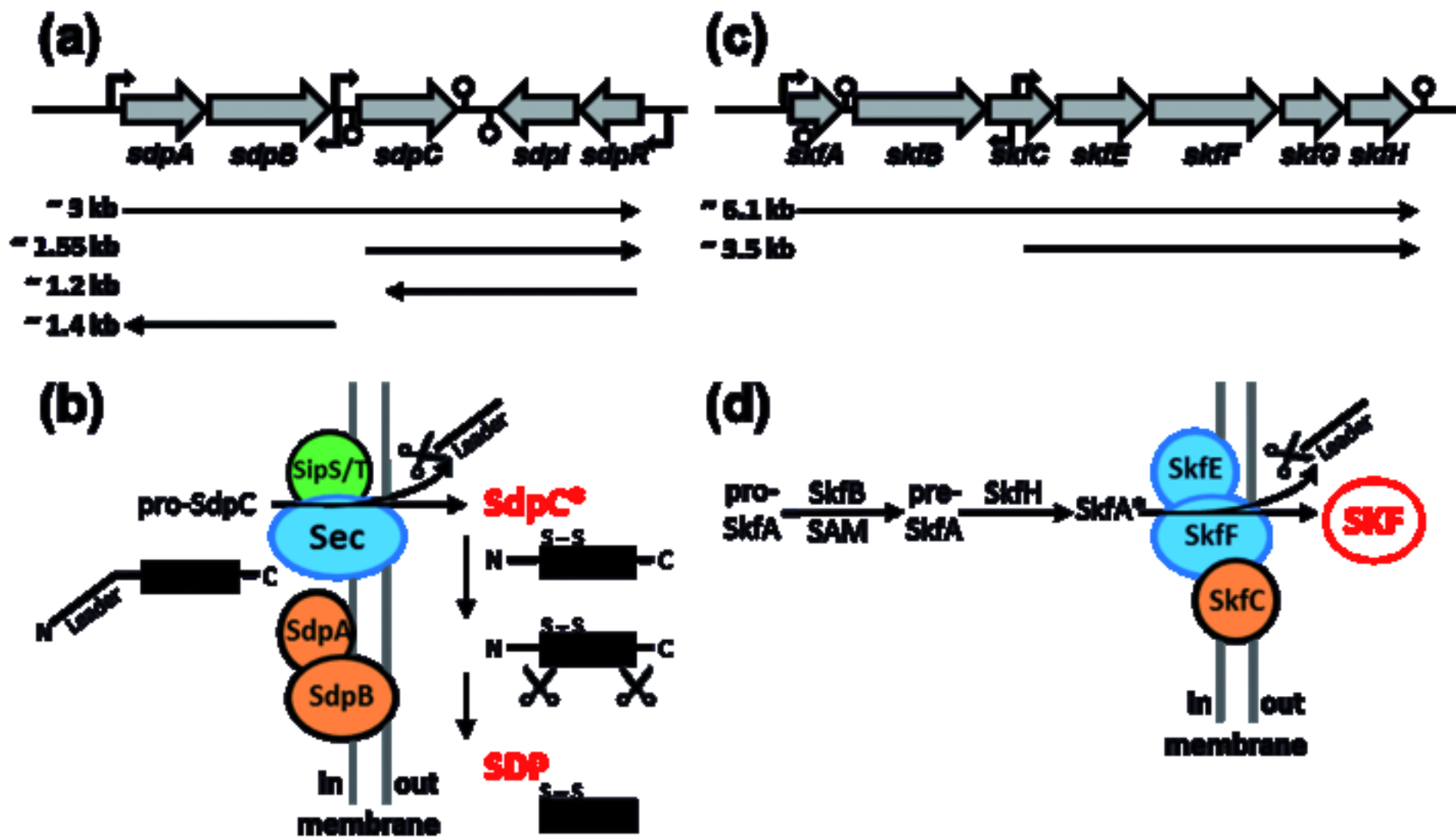


Figure 2

[Click here to download Figure: Fig2.tif](#)





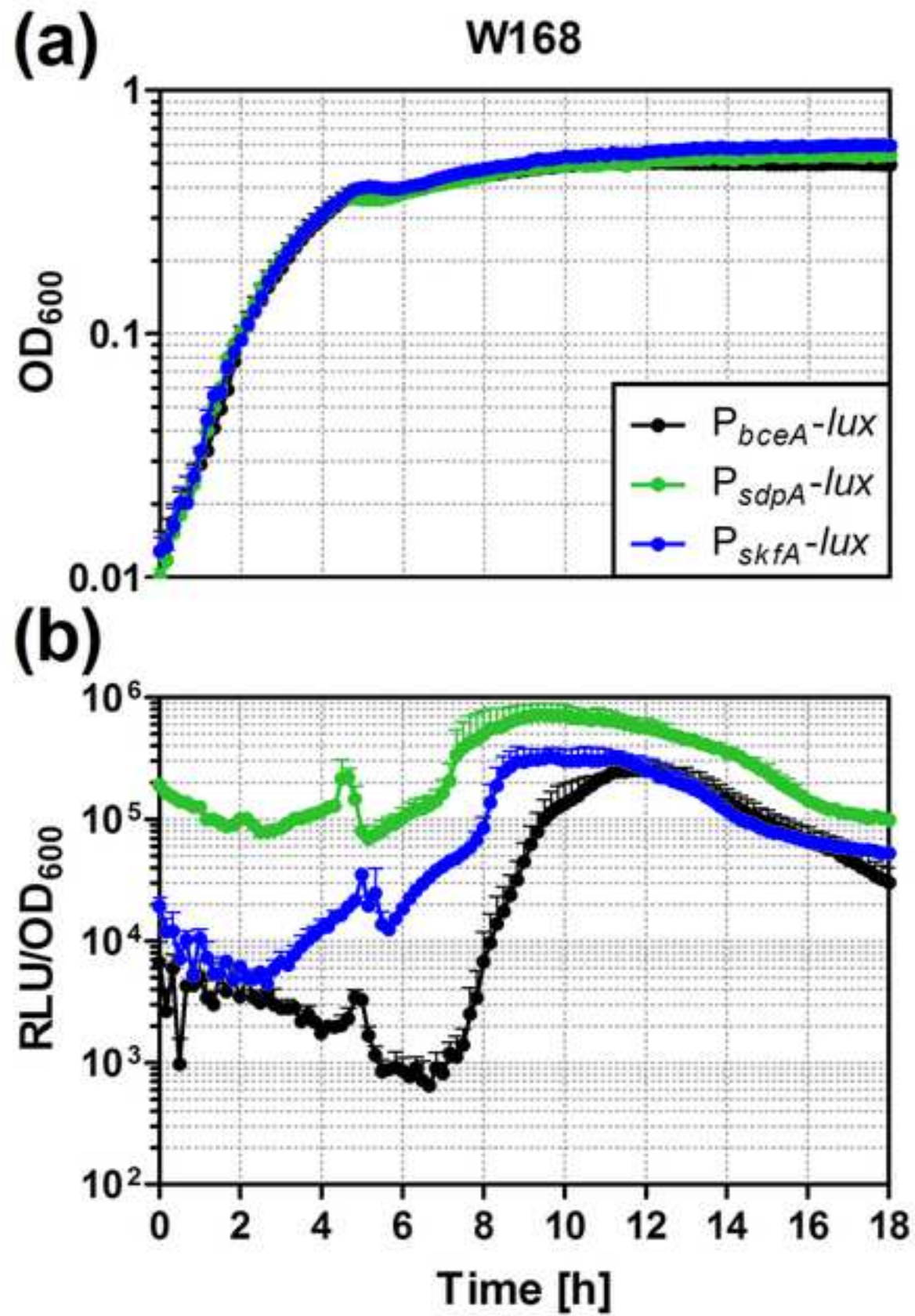
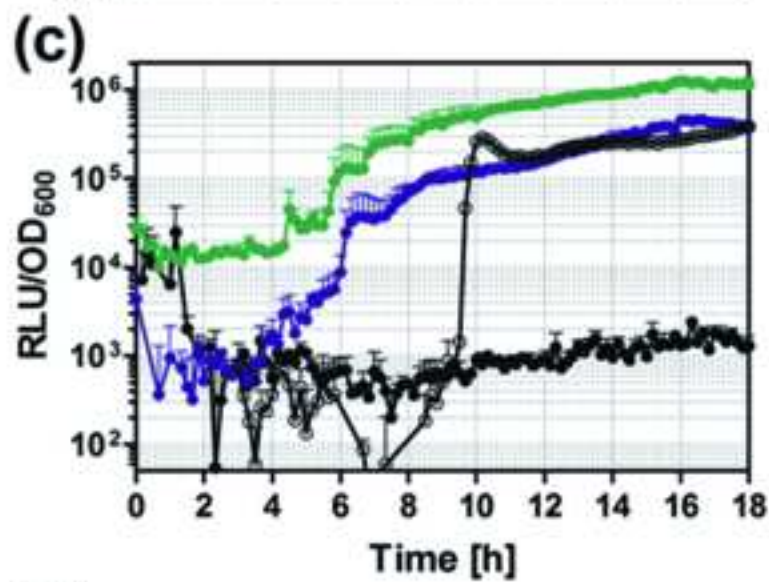
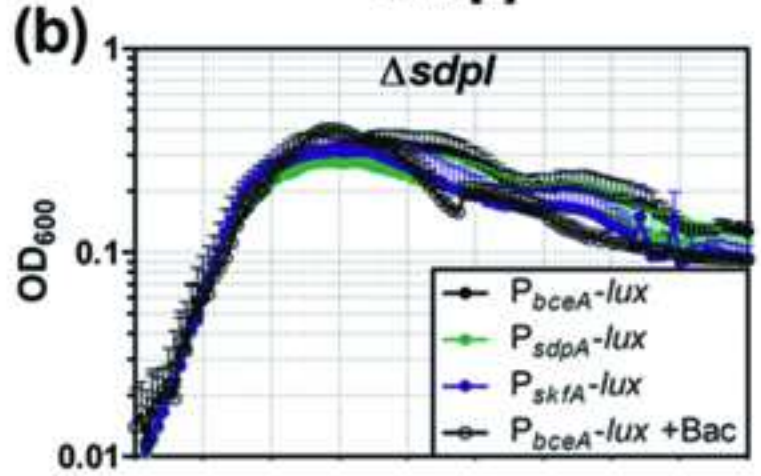
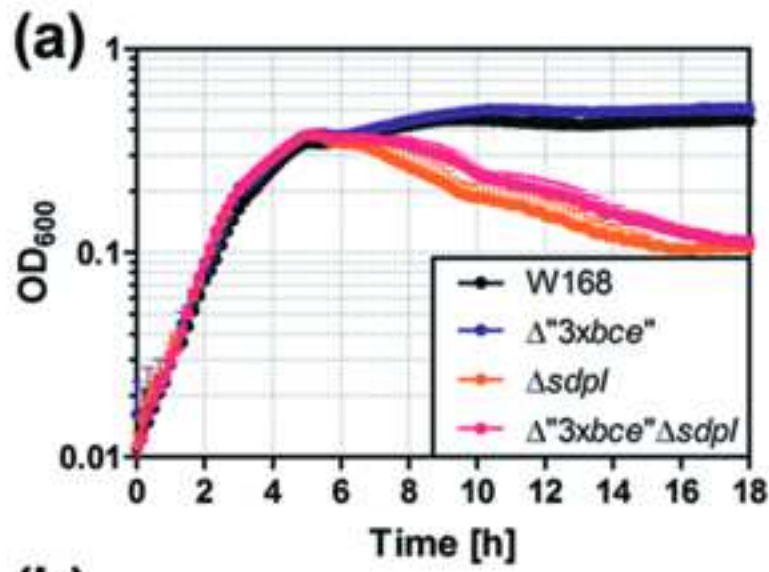


Figure 5
Click here to download Figure: Fig5.tif



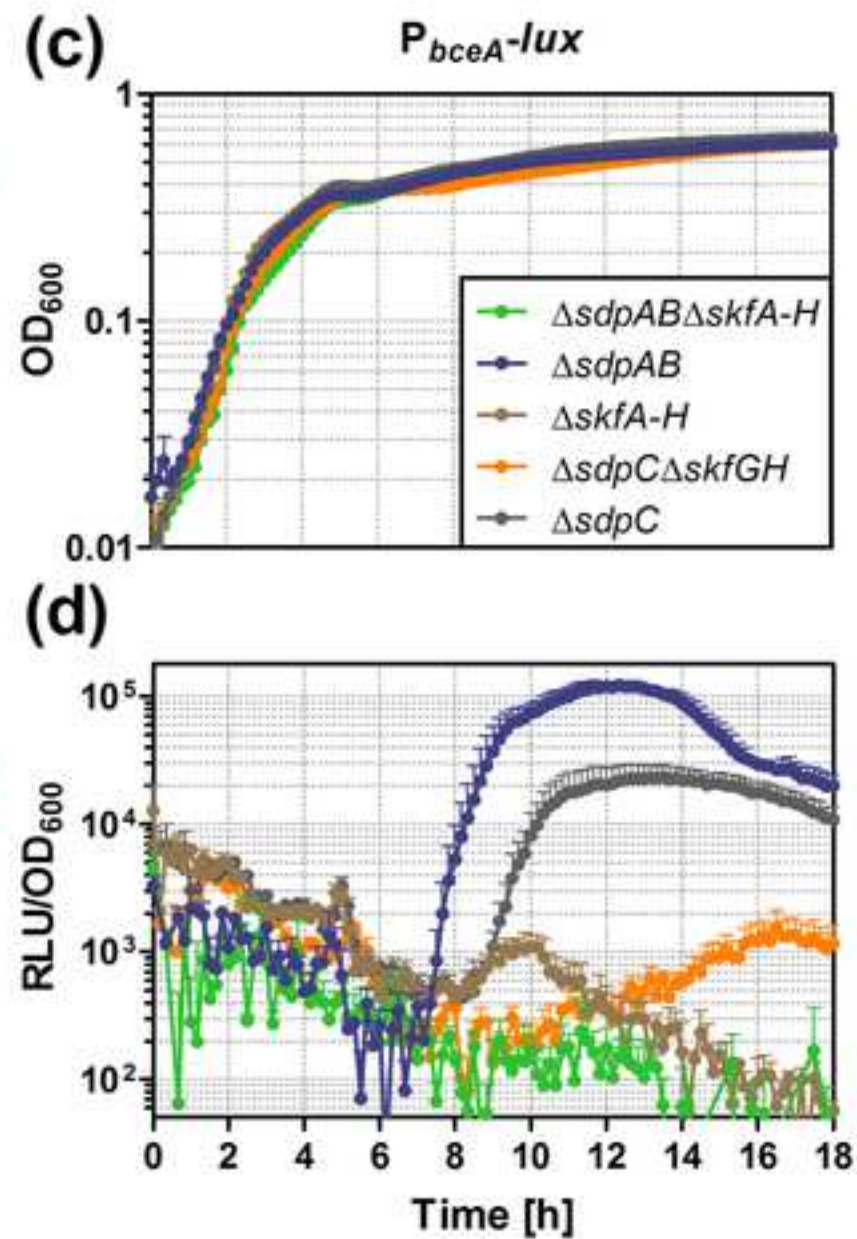
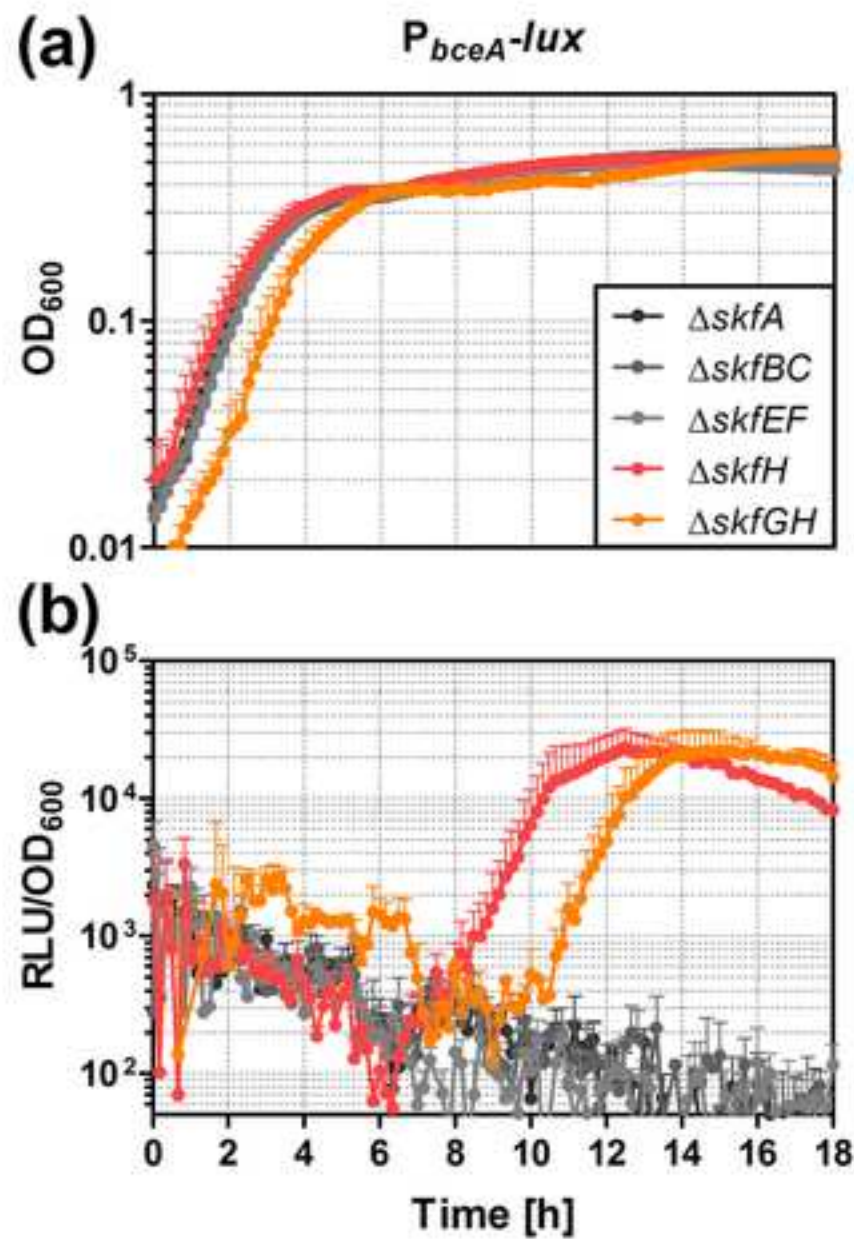


Figure 7
Click here to download Figure: Fig7.tif

