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2 A rendezvous of two second messengers: The c-di-AMP receptor protein DarB controls (p)ppGpp

3 synthesis in *Bacillus subtilis*

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19

20 **Abstract**

21

22 **Many bacteria use cyclic di-AMP as a second messenger to control potassium and osmotic**
23 **homeostasis. In *Bacillus subtilis*, several c-di-AMP binding proteins and RNA molecules have been**
24 **identified. Most of these targets play a role in controlling potassium uptake and export. In addition, c-**
25 **di-AMP binds to two conserved target proteins of unknown function, DarA and DarB, that exclusively**
26 **consist of the c-di-AMP binding domain. Most likely these proteins transduce their signal by regulatory**
27 **interactions with other proteins. Here, we have investigated the function of the c-di-AMP-binding**
28 **protein DarB in *B. subtilis*, a protein consisting of two CBS (cystathionine-beta synthase) domains. We**
29 **have used an unbiased search for DarB interaction partners and identified the (p)ppGpp**
30 **synthetase/hydrolase Rel as a major interaction partner of DarB. (p)ppGpp is another second**
31 **messenger that is formed upon amino acid starvation and under other stress conditions to stop**
32 **translation and active metabolism. The interaction between DarB and Rel only takes place if the**
33 **bacteria grow at very low potassium concentrations and intracellular levels of c-di-AMP are low.**
34 **Indeed, c-di-AMP inhibits the binding of DarB to Rel. The interaction results in the Rel-dependent**
35 **accumulation of pppGpp. Our results link potassium and c-di-AMP signaling to the stringent response**
36 **and thus to the global control of cellular physiology.**

37

38

39 **Introduction**

40

41 All living cells contain high concentrations of potassium ions ^{1,2}. This ion is required for the activity of
42 many enzymes and protein complexes, among them the ribosome, for buffering the negative charge of
43 the DNA and for osmoadaptation ^{1,3}. On the other hand, potassium may become toxic if the intracellular
44 concentration becomes too high ¹. Therefore, potassium homoestasis has to be carefully controlled. In

45 many bacteria, a second messenger – cyclic di-AMP (c-di-AMP) – is involved in the control of potassium
46 homeostasis^{4,5}. The nucleotide is synthesized at high potassium concentrations whereas low c-di-AMP
47 levels indicate a potassium limitation⁶. The control of the homeostasis of potassium and other
48 osmolytes is the reason that c-di-AMP is essential for many of the bacteria that produce this signaling
49 nucleotide⁷. c-di-AMP acts by binding to a variety of targets to control their activity^{7,8}. Among the
50 targets of c-di-AMP are several proteins, like the potassium importers and exporters and a two-
51 component sensor kinase as well as a riboswitch that are involved in the control of potassium
52 homeostasis. Of all known second messenger nucleotides c-di-AMP is unique in binding and controlling
53 both a protein and the mRNA molecule that encodes it. This is the case for the *Bacillus subtilis* KtrA and
54 KimA potassium transporters that are both bound and thus inhibited by c-di-AMP. In addition, the
55 corresponding mRNAs each carry a c-di-AMP responsive riboswitch, and binding of c-di-AMP prevents
56 the expression of the transporters^{4,6,9}.

57 In *B. subtilis* and the related pathogen *Listeria monocytogenes*, the analysis of c-di-AMP-binding
58 proteins identified two potential signal transduction proteins of unknown function, DarA and DarB^{4,10,11}.
59 DarA belongs to the large family of PII-like signaling proteins that control a variety of processes mainly in
60 nitrogen metabolism¹². The DarB protein consists of a tandem of two CBS (cystathionine-beta synthase)
61 domains, an arrangement called Bateman domain¹³. CBS domains bind AMP, ATP, or other adenosine-
62 derived molecules. CBS domains are present in a variety of proteins, including osmolyte and metal ion
63 transporters, enzymes, and transcription regulators. Recently, CBS domain-containing osmolyte and
64 magnesium transporters were found to bind c-di-AMP. In the case of the osmolyte transporters, the
65 proteins are inactivated upon c-di-AMP binding^{4,14,15}. Interestingly, in contrast to most other c-di-AMP-
66 binding proteins, DarA and DarB do not contain any other domain that might be controlled by the
67 binding of the second messenger. It is therefore likely that these proteins interact with other proteins in
68 a c-di-AMP-dependent manner to control their activity.

69 In this study, we performed an unbiased search for potential interaction partners of the DarB
70 protein. This search identified the Rel protein that synthesizes and degrades the alarmone nucleotide
71 (p)ppGpp. The accumulation of this signaling nucleotide results in a global switch off of cellular activities
72 in bacteria, including DNA replication, nucleotide biosynthesis, transcription of household genes, and
73 translation ^{16,17}. Thus, the integration of c-di-AMP and (p)ppGpp signaling allows a global cellular
74 response to the availability of potassium.

75

76 **Results**

77

78 **Identification of RelA as an interaction partner of DarB**

79

80 We assumed that DarB might act by interaction with other proteins. A *L. monocytogenes* strain lacking c-
81 di-AMP is unable to grow on complex media, but suppressor mutants with the inactivated homolog of
82 DarB (CbpB) were able to grow on complex medium ¹⁸. This observation suggests that the apo-form of
83 DarB exerts some harmful interactions. In both *B. subtilis* and *L. monocytogenes*, DarB is encoded in a
84 conserved operon with the transcription factor CcpC, the regulator of the citric acid cycle ^{19,20}. We
85 hypothesized that DarB might control the activity of CcpC. However, attempts to detect an interaction
86 between the two proteins failed suggesting that DarB exerts a different function (Supplementary Fig. 1).

87 To get a first unbiased glimpse on the function of DarB, we identified potential interaction
88 partners by passing a *B. subtilis* crude extract over a DarB-saturated column. The proteins were then
89 eluted from the column, and the co-purified proteins were identified by mass spectrometry. In
90 agreement with previous results, CcpC was not identified in the fraction that co-elutes with DarB. In
91 contrast, the analysis identified the GTP pyrophosphokinase Rel as a top scoring protein (Supplementary
92 Table 1). This protein was not detected in the negative control and was therefore considered as a
93 putative interaction partner of DarB. Rel catalyzes the production of the alarmones ppGpp and pppGpp

94 by transferring pyrophosphate derived from ATP to GDP and GTP, respectively, under conditions of
95 amino acid starvation. Moreover, Rel degrades both alarmones if amino acids become available²¹.

96 In order to gain further evidence for the interaction between DarB and Rel, we used the bacterial
97 two-hybrid system in which an adenylate cyclase is reconstituted if cloned proteins interact with each
98 other resulting in β -galactosidase activity. As shown in Fig. 1a, both DarB and Rel exhibited self-
99 interaction, in agreement with structural analysis of these proteins²². In addition, co-expression of DarB
100 and Rel resulted in the reconstitution of a functional adenylate cyclase, thus confirming the interaction
101 of the two proteins. None of the two proteins showed an interaction with the Zip protein, which was
102 used as the negative control. Thus, the interaction between DarB and Rel is specific.

103 Furthermore, we performed size exclusion chromatography-multiangle light scattering (SEC-
104 MALS) experiments with DarB and the purified Rel protein (see Supplementary Fig. 2) to get *in vitro*
105 confirmation for the interaction. As shown in Supplementary Fig. 3b, the two protein co-elute *in vitro*. In
106 contrast, no co-elution was detectable when DarB was saturated with c-di-AMP (Supplementary Fig. 3c).
107 This observation suggests that only apo-DarB is capable of interacting with Rel. It is in agreement with
108 the initial pull-down experiment and the bacterial two-hybrid analysis that both revealed an interaction
109 between the two proteins in the absence of c-di-AMP.

110 To obtain additional evidence for the specificity of the interaction, we mutated the DarB protein
111 in a way to prevent the interaction with Rel. An inspection of the DarB structure (PDB code 1YAV) as well
112 as of the structure of the DarB-c-di-AMP complex (Heidemann and Ficner, unpublished results)
113 suggested that surface residues close to the c-di-AMP binding site might interfere with Rel binding. We
114 exchanged Ala-25 and Arg-132 to Gly and Met, respectively, in single mutants, and combined the two
115 mutations. The resulting DarB^{A25G,R132M} was tested for c-di-AMP and Rel binding. Isothermal titration
116 calorimetry (ITC) experiments indicated that the mutated protein binds c-di-AMP (Supplementary Fig.
117 4a) demonstrating that the protein folds correctly. However, a SEC-MALS analysis showed that the

118 mutant protein binds much weaker to Rel as compared to the wild type protein. Moreover, this residual
119 interaction is not affected by c-di-AMP (Supplementary Fig. 3d, 3e).

120 Taken together, these data indicate that DarB specifically binds to Rel, and that this interaction is
121 inhibited by c-di-AMP.

122

123 **Biochemical and physiological regulation of the interaction**

124

125 To further investigate the role of c-di-AMP in the interaction between DarB and Rel, we assayed the
126 binding of purified DarB to immobilized Strep-tagged Rel in the absence or presence of c-di-AMP. While
127 DarB was co-eluted with Rel in the absence of c-di-AMP, no DarB was retained on the column when c-di-
128 AMP was present (see Fig. 1b). LC-MS analysis of the gel segments corresponding to the size of DarB
129 confirmed this result (Fig. 1b, segments 1 and 2). No interaction between Rel and the negative control
130 CcpC was detected (Fig. 1b, see also Supplementary Fig. 5, Supplementary Table 2). Similarly, the
131 interaction was abolished if the DarB protein had mutations affecting A25 alone or in combination with
132 the R132M substitution (Supplementary Fig. 6). These results support the specific interaction between
133 Rel and DarB, and they confirm that Rel interacts with the apo-form of DarB but not with the DarB/ c-di-
134 AMP complex.

135 c-di-AMP is a second messenger that functions in potassium homeostasis, and the intracellular
136 levels of the nucleotide correlate with the potassium concentration ¹. We tested therefore, how the
137 external potassium supply would affect the interaction between DarB and Rel *in vivo*. For this purpose,
138 we used a strain that expressed His-tagged Rel from the chromosome and Strep-tagged DarB from a
139 plasmid. This strain was cultivated in minimal medium at low (0.1 mM) and high (5 mM) potassium
140 concentrations, and the protein extract was passed over a StrepTactin column to isolate Strep-DarB in
141 complex with its potential interaction partners (Fig. 1c). The presence of the Rel protein in the elution
142 fractions was analyzed by a Western blot using antibodies specific for the His-tag. While His-Rel was co-

143 eluted with DarB at the low potassium concentration, no interaction was detected when the bacteria
144 had been cultivated at the high potassium concentration. Again, the presence and absence of Rel in
145 eluates from cultures grown at 0.1 or 5 mM potassium, respectively, was verified by mass spectrometry
146 (Supplementary Table 3). No Rel was detectable in the eluate of the culture grown at the high potassium
147 concentration. Since the intracellular c-di-AMP concentration is low at an external potassium
148 concentration of 0.1 mM ⁶, we conclude that the interaction between DarB and Rel occurs at low
149 potassium concentrations when c-di-AMP is not bound to DarB. This conclusion is in excellent agreement
150 with the observed inhibition of the interaction by c-di-AMP (see Fig. 1b).

151

152 **DarB does not interact with other small alarmone synthetases**

153

154 In addition to Rel, *B. subtilis* encodes two additional (p)ppGpp synthesizing enzymes, the small alarmone
155 synthetases SasA and SasB ²³. In contrast to Rel, which is a multidomain protein (see below), the latter
156 proteins consist of a stand-alone synthetase domain. To test whether these proteins are also capable of
157 interacting with DarB, we made use of the two-hybrid system as described above for Rel. In agreement
158 with the known formation of homotetramers ²⁴, we observed self-interactions for both SasA and SasB.
159 This also indicates that the fusion proteins have folded correctly. Again, we confirmed the interaction
160 between Rel and DarB. However, no interaction of SasA and SasB with DarB could be detected (see
161 Supplementary Fig. 7). The absence of an interaction between DarB and the small alarmone synthetases
162 is supported by the fact that the proteins did not co-elute with Strep-DarB in the *in vivo* experiments
163 described above. Thus, the interaction of DarB is most likely specific for Rel.

164

165 **DarB interacts with the N-terminal portion of RelA**

166

167 The Rel protein is a multidomain protein that consists of a N-terminal hydrolase (HYD) domain, the
168 synthetase (SYN) domain, the TGS domain (for: ThrRS, GTPase and SpoT), a zinc finger domain (ZFD), and
169 the C-terminal RNA recognition motif (RRM) domain (see Fig. 2a)²⁵. While the HYD and SYN domains are
170 required for the degradation and synthesis of (p)ppGpp, respectively, the C-terminal domains are
171 involved in the interaction with the ribosome and the control of the enzymatically active domains²⁵. To
172 test the contribution of the N- and C-terminal regions of Rel to the interaction with DarB, we analysed
173 the protein-protein interactions using the bacterial two-hybrid system (see Fig. 2b). The Rel fragment
174 consisting of the SYN domain and the C-terminal regulatory domains showed a very faint interaction with
175 DarB. In contrast, a very strong interaction was observed for the N-terminal fragment consisting of the
176 HYD and the SYN domains (Rel^{NTD}). Thus, in contrast to the interaction of Rel with the ribosome which is
177 mediated by the C-terminal RRM domain, DarB seems to bind to the N-terminal part of Rel.

178 To confirm the binding of the N-terminal region of Rel to DarB, we assayed binding of DarB to the
179 immobilized truncated Rel^{NTD} protein that lacked the C-terminal part. As observed for the full-length
180 protein, this HYD-SYN fragment of Rel bound to DarB, and this interaction was prevented by the addition
181 of c-di-AMP (Fig. 2c, Supplementary Table 2).

182 In order to confirm the complex formation of Rel^{NTD} and DarB *in vitro*, a SEC-MALS experiment
183 was performed. The separated elution profiles of the two proteins correspond to a monomer for Rel^{NTD}
184 and a dimer for DarB. A dimer formation by DarB is in agreement with the results from the two-hybrid
185 analysis (Fig. 1a) and the available crystal structure of the apo-protein (PDB 1YAV). By contrast, Rel^{NTD}
186 was unable to exhibit self-interactions in the two-hybrid screen (see Fig. 2b). Co-elution of Rel^{NTD} and
187 DarB resulted in an earlier eluting peak, indicating the formation of a complex of 94.2 kDa consisting of
188 DarB and Rel^{NTD} (see Fig. 2d, Supplementary Fig. 8). The subsequent SDS page analysis of the elution
189 fractions confirmed that both proteins co-eluted from the column (see Supplementary Fig. 8). To
190 determine the kinetic parameters of the interaction, we performed isothermal titration calorimetry (ITC)
191 experiments (Fig. 2e). Titration of DarB against Rel^{NTD} revealed an equimolar stoichiometry of the two

192 proteins in the complex. Moreover, we determined the affinities of DarB for c-di-AMP and Rel. While the
193 K_D for the binding of c-di-AMP was about 45 nM (Supplementary Fig. 4a,b), we observed a K_D of 650 nM
194 for the interaction of DarB and Rel (see Fig. 2e). This about 15-fold higher affinity of DarB for c-di-AMP is
195 crucial for the c-di-AMP-mediated regulation of the DarB-Rel interaction.

196

197 **Genetic support for the DarB-RelA interaction**

198

199 So far, no function other than binding to c-di-AMP and to Rel has been identified for DarB. To get better
200 insights into the physiological role(s) of DarB, we constructed strains that either lacked DarB ($\Delta darB$,
201 GP3409) or that overexpressed the protein ($darB^+$, 168 + pGP3306) and compared growth of the three
202 strains in minimal medium with 0.1 or 5 mM potassium (see Fig. 3a). All three strains grew very similar
203 with 5 mM potassium (growth rates of 0.43, 0.39, and 0.43 h^{-1} for the wild type, the $darB^+$, and the $\Delta darB$
204 mutant, respectively). In contrast, at the low potassium concentration, we observed a delayed growth for
205 the strain overexpressing DarB as compared to the wild type and the $darB$ deletion mutant (0.09 vs. 0.21
206 and 0.18 h^{-1} , respectively). This is the condition when c-di-AMP is present in low amounts, and thus a
207 large fraction of the DarB protein is present as apo-protein with the capacity to bind to Rel. It is therefore
208 tempting to speculate that this interaction might be the reason for the growth defect. To test this idea,
209 we deleted the *rel* gene in the wild type strain and in the strain carrying the expression vector for *darB*
210 and compared the growth in minimal medium at a low potassium concentration. As shown in Fig. 3b, the
211 deletion of *rel* suppressed the growth defect that resulted from the overexpression of DarB (growth
212 rates of 0.15 and 0.18 h^{-1} for the *rel* mutant and the *rel* mutant with overexpression of DarB,
213 respectively). Overexpression of the DarB mutant proteins that are defective in the interaction with Rel
214 did not result in growth inhibition (Fig. 3c). Taken together, all these observations indicate that the

215 growth-inhibiting effect of DarB overexpression is the result of its interaction with Rel and suggests that
216 DarB might control Rel activity.

217 Transcription of rRNA promoters is decreased under conditions of the stringent response^{26,27}.
218 We observed that RNA extracted from the strain overexpressing DarB, in contrast to the wild type,
219 lacked the rRNA intermediate migrating above the 16S band (Supplementary Fig. 9). This band
220 corresponds to the size of the pre-16S rRNA²⁸. This decrease in the pre-16S rRNA pool most likely results
221 from the reduced rRNA promoter activity and drainage of the pre-RNA pool. This suggests that the
222 overexpression of DarB affects *rrn* transcription by stimulation of (p)ppGpp synthesis *in vivo*. The
223 downregulation of rRNA genes results in problems in ribosome assembly and might explain the observed
224 Rel-dependent growth defect of the DarB overexpression strain during exponential growth.

225
226 **DarB controls Rel synthetase and hydrolase activities**
227

228 The results presented above suggest that the interaction between DarB and Rel might affect synthesis of
229 (p)ppGpp by Rel. To test this idea, we used purified Rel protein to assay its synthetase and hydrolase
230 activities. The purified Rel protein had little biosynthetic activity, as indicated by the production of 1.1
231 pmol pppGpp per pmol of Rel per minute (Fig. 4a). This is in good agreement with the absence of Rel
232 synthetase activity if not triggered by uncharged tRNAs at the ribosome²⁵. In contrast, Rel activity was
233 enhanced threefold if purified DarB protein was added to the assay mixture. If DarB was saturated with
234 c-di-AMP prior to incubation with Rel, the Rel protein retained its background activity and no
235 enhancement was detected. No pppGpp synthesis was detected with DarB alone indicating that DarB is
236 unable to synthesize pppGpp and that the addition of DarB activates the synthetase activity of Rel. To
237 exclude the possibility that the activation was just a result of non-specific protein crowding, we also
238 assayed pppGpp synthesis by Rel in the presence of bovine serum albumin. In this case, Rel exhibited
239 only background activity (see Fig. 4a). These results demonstrate that the interaction between apo-DarB

240 and Rel stimulates the synthesis of pppGpp and that this stimulation is prevented in the presence of c-di-
241 AMP.

242 We also tested whether DarB affects the hydrolase activity of Rel. In this case, we determined
243 the formation of GTP resulting from the hydrolysis of pppGpp. For the Rel protein alone, we determined
244 a turnover rate of 8.7 pmol of GTP per pmol of Rel per minute (Fig. 4b). In the presence of DarB, this
245 activity was reduced six-fold. In the presence of c-di-AMP, DarB has little effect on the pppGpp hydrolase
246 activity of Rel. As observed for the synthetase activity, these effects are specific since DarB has no
247 pppGpp hydrolase activity, and the control protein (BSA) does not affect the hydrolytic activity of Rel.
248 Taken together, these data demonstrate that DarB affects Rel activity by stimulation and inhibition of
249 (p)ppGpp synthesis and degradation, respectively.

250 The data presented above suggest that the interaction of DarB with Rel results in a net increase
251 of the intracellular (p)ppGpp levels. To verify this assumption, we compared the intracellular (p)ppGpp
252 concentrations in a wild type strain, the *darB*⁺ strain overexpressing DarB, and in the *darB* mutant (Fig.
253 4c). Indeed, overexpression of DarB resulted in a significant increase of (p)ppGpp (95 pmol OD₆₀₀⁻¹ ml⁻¹
254 vs. 59 pmol OD₆₀₀⁻¹ ml⁻¹). In the *darB* mutant, the (p)ppGpp concentration was reduced as compared to
255 the wild type strain. The determination of the (p)ppGpp concentration in a *rel* mutant strain revealed
256 that Rel was the major source of (p)ppGpp production under the conditions of our experiment, and that
257 the residual (p)ppGpp synthesis was not affected by DarB overexpression. These observations are in
258 agreement with the physiological observations. They confirm that the accumulation of (p)ppGpp is the
259 cause of the growth inhibition of the strain that overexpresses DarB.

260
261 **Discussion**
262
263 In this work, we report a novel link between potassium concentration, c-di-AMP signaling and the
264 stringent response in the Gram-positive model organism *B. subtilis*. The observation that at low external

265 potassium and intracellular c-di-AMP concentrations, the apo form of DarB binds to the alarmone
266 synthetase Rel and triggers (p)ppGpp accumulation independent of the ribosome complements the
267 earlier observations that on the other hand (p)ppGpp binds to the c-di-AMP degrading
268 phosphodiesterases GdpP and PgpH to inhibit the degradation of c-di-AMP^{29,30,31}. Together, these data
269 allow to develop a model (Fig. 5a) in which at low potassium concentrations the intracellular c-di-AMP
270 levels are low and the c-di-AMP targets including DarB are present in the apo form. DarB then binds to
271 Rel and stimulates the synthesis of the alarmone (p)ppGpp in a ribosome-independent manner. The
272 accumulation of (p)ppGpp results in a re-organisation of cellular physiology including the stop of
273 translation. This direct link between the potassium concentration, the stringent response and ribosome
274 activity is very important for the cell since potassium is essential for ribosome assembly and translation
275 at the ribosome^{32,33}. On the other hand, the accumulation of (p)ppGpp interferes with the degradation
276 of c-di-AMP. This is likely to be important if potassium becomes available again. Then, c-di-AMP synthesis
277 can be initiated and as long as (p)ppGpp is present, the second messenger is protected from
278 degradation. This allows to achieve a c-di-AMP concentration that is appropriate to adjust the cellular
279 potassium homeostasis by binding to c-di-AMP responsive riboswitches that control the expression of
280 high affinity potassium transporters as well as to the potassium importers and exporters to inhibit and
281 activate these proteins, respectively.

282 There is a huge body of evidence that (p)ppGpp synthesis by Rel is triggered by uncharged tRNA
283 in the ribosomal A-site upon amino acid starvation^{17,25}. Our work supports the idea of ribosome-
284 independent stimulation of the stringent response, as shown for phosphate and fatty acid starvation in
285 *E. coli*³⁴. Our work now extends this concept also to potassium starvation in *B. subtilis*. Similar to the
286 presence of uncharged tRNAs, a lack of potassium results in a stop of translation³³, and does thus
287 require similar global responses to reprogram translation, gene expression, DNA replication and cellular
288 metabolism. It is interesting to note that c-di-AMP is a second messenger that reports on potassium
289 availability in most Gram-positive and also in many Gram-negative bacteria with the notable exception of

290 α -, β -, and γ -proteobacteria^{7,8}. In α - and β -proteobacteria, a regulatory protein, PtsN (also referred to as
291 enzyme IIA^{Ntr}) is capable of interacting with the single (p)ppGpp synthetase/hydrolase of these bacteria.
292 The interaction depends on the nitrogen supply and the resulting phosphorylation state of PtsN and
293 leads to the accumulation of (p)ppGpp^{35,36,37}. PtsN has also been implicated in the control of potassium
294 homeostasis: in *E. coli*, non-phosphorylated PtsN binds and inhibits TrkA, a subunit of low-affinity
295 potassium transporters as well as to the two-component sensor kinase KdpD, thus stimulating its activity
296 and the expression of the high-affinity Kdp potassium transport system^{38,39}. It is thus intriguing to
297 speculate that the regulatory link between potassium homeostasis and the stringent response is
298 conserved in bacteria even though the specific molecular mechanisms may be completely different.

299 An interesting aspect of this study is the mode of DarB regulation by c-di-AMP. Our biophysical
300 interaction analyses indicated an equimolar stoichiometry of the two proteins. Since DarB forms dimers,
301 and the mutations that interfere with Rel binding are located at the upper and lower side of the DarB
302 dimer, it is tempting to speculate that the proteins form a sandwich-like complex with a central DarB
303 dimer and a molecule of Rel on each face of the dimer (see Fig. 5b). This resulting 2:2 stoichiometry is
304 also best compatible with the results of the SEC-MALS analysis. Moreover, the differential affinities of
305 DarB to c-di-AMP and Rel as well as the fact that two molecules of c-di-AMP bind to each DarB dimer in
306 the region that is also important for Rel binding (Heidemann and Ficner, unpublished results) suggest
307 that c-di-AMP and Rel compete for DarB binding. Since c-di-AMP has a 15-fold higher affinity for DarB
308 than Rel, it is tempting to speculate c-di-AMP inhibits Rel binding in a competitive manner.

309 For all other processes controlled by c-di-AMP as well as other second messengers such as c-di-
310 GMP, the nucleotide directly binds to its targets to control their activity, as has been shown for
311 potassium uptake or export, osmolyte export, or pyruvate carboxylase activity^{4,11,14,15,40,41}. This raises the
312 question why Rel needs DarB as a mediator of c-di-AMP mediated control. First, Rel is already composed
313 of multiple domains, and it might have been difficult in evolution to integrate a further level of signaling
314 directly into the protein. Second, potassium starvation is completely different from, but as serious for

315 the cell as amino acid starvation. This makes it advantageous to have the two regulatory pathways for
316 Rel activity separated from each other. Moreover, it is the apo form of DarB that binds and regulates Rel
317 activity. An important function for apo-DarB has already been suggested by the observation that a *L.*
318 *monocytogenes* mutant lacking c-di-AMP readily acquires mutations affecting the DarB counterpart CbpB
319 ¹⁸. Similarly, mutations inactivating the DarA ortholog PstA were found in *L. monocytogenes* ¹⁸ suggesting
320 that this protein might also interact with its partners in the apo form under conditions of potassium
321 starvation.

322 DarB is conserved in several Gram-positive bacteria, including *L. monocytogenes* and
323 *Enterococcus faecalis*. This suggests that the novel mode of control of the stringent response may also
324 apply to these pathogens. In addition to DarB, *B. subtilis* Rel has been shown to interact with the
325 competence protein ComGA, resulting in the inhibition of the hydrolase activity of Rel ⁴². Moreover, a
326 recent study demonstrated the transient accumulation of (p)ppGpp upon heat stress ⁴³. It will be
327 interesting to study whether yet additional factors may control Rel activity to trigger the stringent
328 response under specific stress conditions.

329

330 **Methods**

331

332 **Strains, media and growth conditions.** *E. coli* DH5 α and Rosetta DE3 ⁴⁴ were used for cloning and for the
333 expression of recombinant proteins, respectively. All *B. subtilis* strains used in this study are derivatives
334 of the laboratory strain 168. *B. subtilis* and *E. coli* were grown in Luria-Bertani (LB) or in sporulation (SP)
335 medium ^{44,45}. For growth assays and the *in vivo* interaction experiments, *B. subtilis* was cultivated in
336 MSSM medium ⁶. In this medium KH₂PO₄ was replaced by NaH₂PO₄ and KCl was added as indicated. The
337 media were supplemented with ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), chloramphenicol (5
338 μ g/ml), or erythromycin and lincomycin (2 and 25 μ g/ml, respectively) if required.

339 **Phenotypic characterization.** To assay growth of *B. subtilis* mutants at different potassium
340 concentrations, the bacteria were inoculated in LB medium and precultured in MSSM medium with 0.1
341 mM KCl. The cultures were grown until exponential phase, harvested, washed three times in MSSM basal
342 salts solution before an optical density at 600 nm (OD₆₀₀) was adjusted to 1.0. For growth analysis in
343 liquid medium, the cells were used to inoculate a 96 well plate (Microtest Plate 96 Well, Sarstedt)
344 containing MSSM medium with ammonium and the required potassium concentrations. Growth was
345 tracked in an Epoch 2 Microplate Spectrophotometer (BioTek Instruments) at 37°C with linear shaking at
346 237 cpm (4 mm) for 20 h, and an OD₆₀₀ was measured in 10 min intervals.

347 **DNA manipulation.** Transformation of *E. coli* and plasmid DNA extraction were performed using
348 standard procedures⁴⁴. All commercially available plasmids, restriction enzymes, T4 DNA ligase and DNA
349 polymerases were used as recommended by the manufacturers. Chromosomal DNA of *B. subtilis* was
350 isolated as described⁴⁵. *B. subtilis* was transformed with plasmid and genomic DNA according to the two-
351 step protocol⁴⁵. Introduction of mutations in the *darB* allele was achieved by the Combined Chain
352 Reaction by using an additional 5' phosphorylated primer to introduce the mutation⁴⁶.

353 **Construction of mutant strains by allelic replacement.** Deletion of the *darB* and *rel* genes was achieved
354 by transformation of *B. subtilis* 168 with a PCR product constructed using oligonucleotides to amplify
355 DNA fragments flanking the target genes and an appropriate intervening resistance cassette as described
356 previously⁴⁷. The integrity of the regions flanking the integrated resistance cassette was verified by
357 sequencing PCR products of about 1,100 bp amplified from chromosomal DNA of the resulting mutant
358 strains, GP3409 and GP3419, respectively. Similarly, a strain allowing expression of Rel fused to C-
359 terminal His-tag was constructed by first generating an appropriate PCR product and subsequent
360 transformation of *B. subtilis* 168. The resulting strain was GP3429.

361 **Plasmid constructions.** The *ccpC*, *darB*, *rel*, *sasA* and *sasB* alleles were amplified using chromosomal DNA
362 of *B. subtilis* 168 as the template and appropriate oligonucleotides that attached specific restriction sites
363 to the fragment. Those were: KpnI and BamHI for cloning *rel* in pGP172⁴⁸, BamHI and Sall for cloning *rel*

364 in pWH844⁴⁹, XbaI and KpnI for cloning all genes in the BACTH vectors⁵⁰, BamHI and KpnI sites for
365 cloning *rel* into pGP888⁵¹ for genomic integration. The truncated *rel* variants were constructed as
366 follows: *rel*-SYN-RRM contained aa 168-734, *rel*-HYD-SYN contained aa 1-391. For the overexpression of
367 DarB, *darB* was amplified using chromosomal DNA of *B. subtilis* 168 as the template and appropriate
368 nucleotides that attached BsaI and XbaI restriction sites to the fragments and cloned between the BsaI
369 and XbaI sites of the expression vector pET-SUMO (Invitrogen, Germany). The resulting plasmid was
370 pGP2972. All plasmids are listed in Supplementary Table 4.

371 **Protein expression and purification.** *E. coli* Rosetta(DE3) was transformed with the plasmid pGP2972,
372 pGP3437, pGP3441, pGP3460 encoding wild type or mutant 6xHis-SUMO-DarB for purification of DarB or
373 with the plasmids pGP3348 or pGP3350 for expression of Strep-tagged full-length Rel and Rel^{NTD},
374 respectively, or pGP706²⁰ for expression of 6xHis-CcpC. For purification of 10xHis-SUMO-Rel, pVHP186²¹
375 was transformed into *E. coli* Rosetta(DE3). Expression of the recombinant proteins was induced by the
376 addition of isopropyl 1-thio-β-D-galactopyranoside (final concentration, 1 mM) to exponentially growing
377 cultures (OD₆₀₀ of 0.8) of *E. coli* carrying the relevant plasmid. His-tagged proteins were purified in 1 x
378 ZAP buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5), if not stated otherwise, and Strep-tagged proteins in
379 buffer W (100 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, pH 8.0). 10xHis-SUMO-RelA was purified in
380 buffer A (750 mM KCl, 5 mM MgCl₂, 40 μM MnCl₂, 40 μM Zn(OAc)₂, 20 mM imidazole, 10% glycerol, 4
381 mM β-mercaptoethanol, 25 mM HEPES:KOH pH 8) as described previously²¹. Cells were lysed by four
382 passes at 18,000 p.s.i. through an HTU DIGI-F press (G. Heinemann, Germany). After lysis, the crude
383 extract was centrifuged at 100,000 x g for 60 min and then passed over a Ni²⁺-nitrilotriacetic acid column
384 (IBA, Göttingen, Germany) for 6xHis-tagged proteins, or a StrepTactin column (IBA, Göttingen, Germany)
385 for purification of Strep-tagged proteins. The protein was eluted with an imidazole gradient or D-
386 desthiobiotin (2.5 mM), respectively. After elution, the fractions were tested for the desired protein
387 using SDS-PAGE. For the purification of Rel, the column was washed with 8 column volumes of 4M NaCl
388 to remove RNA prior to elution of the protein with 100 mM and 250 mM imidazole. To remove the

389 SUMO tag from the proteins, the relevant fractions were combined, and the SUMO tag was removed
390 with the SUMO protease (ratio 100:1) during overnight dialysis against 1 x ZAP buffer for DarB or against
391 storage buffer (720 mM KCl, 5 mM MgCl₂, 50 mM arginine, 50 mM glutamic acid, 10% glycerol, 4 mM β-
392 mercaptoethanol, 25 mM HEPES:KOH pH 8)²¹ for Rel. The cleaved SUMO moiety and the protease were
393 removed using a Ni²⁺nitrilotriacetic acid column (IBA). The purified Rel was concentrated in a Vivaspin
394 turbo 15 (Sartorius) centrifugal filter device (cut-off 50 kDa). The protein was loaded on a HiLoad 16/600
395 Superdex 200 pg column pre-equilibrated with storage buffer and the fractions containing pure Rel
396 protein were collected and concentrated in a Vivaspin turbo 15 (Sartorius). The purity of protein
397 preparations and the absence of RNA were assessed by SDS-PAGE and on a 1% agarose gel (in 1x TAE
398 buffer; 40 mM Tris-base, 1% acetic acid, 1 mM EDTA pH 8.0), respectively. The protein samples were
399 stored at -80°C until further use (but no longer than 3 days). The protein concentration was determined
400 according to the method of Bradford⁵² using the Bio-Rad dye binding assay and bovine serum albumin as
401 the standard.

402 **Initial pulldown for identification of potential binding partners.** In order to identify potential binding
403 partners of DarB, *E. coli* Rosetta (DE3) was transformed with pGP2972 (6xHis-SUMO-DarB) or the empty
404 vector control pET-SUMO and the protein was overexpressed and purified as described above until the
405 step where the protein was bound to the Ni²⁺nitrilotriacetic acid column. After extensive washing, *B.*
406 *subtilis* 168 crude extract (from LB) was added to the column to allow binding of *B. subtilis* proteins to
407 the DarB protein (apo-DarB due to overexpression in *E. coli*). Again, after extensive washing, DarB,
408 together with its potential binding partners, was eluted from the column with an imidazole gradient. The
409 elution fractions from the eluates were subjected to mass spectrometry analysis.

410 **In vivo detection of protein-protein interactions.** To detect interaction partners of the DarB *in vivo*,
411 cultures of *B. subtilis* GP3429 containing either pGP767 (DarB-Strep), or the empty vector control
412 (pGP382), were cultivated in 500 ml MSSM medium containing the indicated potassium concentrations
413 until exponential growth phase was reached (OD₆₀₀ ~ 0.4-0.6). The cells were harvested immediately and

414 stored at -20°C. The Strep-tagged protein and its potential interaction partners were then purified from
415 crude extracts using a StrepTactin column (IBA, Göttingen, Germany) and D-desthiobiotin as the eluent.
416 The eluted proteins were separated on an SDS gel and potential interacting partners were analyzed by
417 staining with Colloidal Coomassie and Western blot analysis. The eluents were further analyzed by mass
418 spectrometry analysis.

419 **Protein identification by mass spectrometry.** Excised polyacrylamide gel pieces of protein bands were
420 digested with trypsin as described previously ⁵³. Peptides were purified using C18 stop and go extraction
421 (stage) tips as described ^{54,55}. Dried peptide samples were reconstituted in 20 µl LC-MS sample buffer
422 (2% acetonitrile, 0.1% formic acid). 2 µl of each sample were subjected to reverse phase liquid
423 chromatography for peptide separation using an RSLCnano Ultimate 3000 system (Thermo Fisher
424 Scientific). Peptides were loaded on an Acclaim[®] PepMap 100 pre-column (100 µm x 2 cm, C18, 3 µm,
425 100 Å; Thermo Fisher Scientific) with 0.07% trifluoroacetic acid. Analytical separation of peptides was
426 done on an Acclaim[®] PepMap RSLC column (75 µm x 50 cm, C18, 3 µm, 100 Å; Thermo Fisher Scientific)
427 running a water-acetonitrile gradient at a flow rate of 300 nl/min. All solvents and acids had Optima
428 grade for LC-MS (Fisher Scientific). Chromatographically eluting peptides were on-line ionized by nano-
429 electrospray (nESI) using the *Nanospray Flex Ion Source* (Thermo Scientific) and continuously transferred
430 into the mass spectrometer (*Q Exactive HF*, Thermo Scientific). Full scans in a mass range of 300 to 1,650
431 m/z were recorded with the *Q Exactive HF* at a resolution of 30,000 followed by data-dependent top 10
432 HCD fragmentation at a resolution of 15,000 (dynamic exclusion enabled). LC-MS method programming
433 and data acquisition was performed with the XCalibur software 4.0 (Thermo Fisher Scientific).

434 **LC-MS data analysis.** MS/MS2 data were searched against a *B. subtilis* specific protein database (UniProt
435 Proteome ID UP000001570) using the *Proteome Discoverer Software* 2.2. The digestion mode was
436 trypsin/P, and the maximum of missed cleavage sites was set two. Carbamidomethyl at cysteines was set
437 as fixed modification, and oxidation at methionines and N-terminal acetylation of proteins as variable
438 modifications. Mass tolerances of precursors and fragment ions were 10 ppm and 20 ppm, respectively.

439 False discovery rates were calculated using the reverse-decoy mode, and the filter for peptide spectrum
440 matches was set to 0.01.

441 ***In vitro* analysis of protein-protein interactions.** To study the interaction between DarB and Rel, *E. coli*
442 Rosetta (DE3) was transformed with pGP2972 (6xHis-SUMO-DarB), pGP3444 (6xHis-SUMO-DarB^{A25G}),
443 pGP3448 (6xHis-SUMO-DarB^{R132M}), pGP3460 (6xHis-SUMO-DarB^{A25G,R132M}), pGP3348 (Strep-Rel), or
444 pGP3350 (Strep-Rel^{NTD}), respectively, and the proteins were overexpressed as described above. For
445 purification of Rel or Rel^{NTD}, the crude extract was passed over a StrepTactin column (IBA, Göttingen,
446 Germany) and washed with buffer W (pH 8.5) until the wash fractions appeared clear (confirmation with
447 Bradford assay). Purified DarB, if stated preincubated 30 min with c-di-AMP (4x excess), or the control
448 protein CcpC were added to the column, incubation happened overnight at 4°C under constant rotation.
449 Purification was continued by extensive washing of the column with buffer W before Rel, together with
450 binding partners, was eluted with D-desthiobiotin. For verification of the presence of DarB in the elution
451 fractions, fixed and stained gel bands were excised and submitted to mass spectrometry analysis.

452 **SEC-MALS.** The interaction of the full-length Rel protein or Rel^{NTD} with DarB or DarB^{A25G,R132M} was
453 analyzed by size-exclusion chromatography and multi-angle light scattering (SEC-MALS). For this
454 purpose, the purified tag-free proteins were either alone or pre-mixed in a 1:1 ratio in storage buffer (for
455 full-length Rel) or 1x ZAP buffer (for Rel^{NTD}) (~ 1mg/ml each) applied onto the column. The buffer was
456 filtered (0.1 µm filters) and degassed in line (Model 2003, Biotech AB/Sweden) prior to protein
457 separation on a S200 Superdex 10/300GL column on an Äkta Purifier (both GE Healthcare).
458 Subsequently, the eluate was analyzed in line with a miniDawn Treos multi angle light scattering system
459 followed by an Optilab T-rEX RI detector (both from Wyatt Technology, Europe) before fractionation. The
460 elution fractions were analyzed with SDS-PAGE. Data analysis was performed using the ASTRA 6.1
461 software (Wyatt Technology) and also compared to a gel filtration standard (Bio-Rad).

462 **Determination of binding affinities and of the stoichiometry of the DarB-Rel^{NTD} complex by isothermal
463 titration calorimetry.** ITC experiments were carried out with a VP-ITC microcalorimeter (MicroCal Inc.,

464 Northampton, MA) in order to determine the affinity of DarB to Rel^{NTD} and the oligomerization state of
465 the complex. In a typical setup, Rel^{NTD} (5 μ M in 50 mM Tris-HCl, pH 8.3, 200 mM NaCl) was placed in the
466 sample cell, and DarB (100 μ M in the same buffer) was placed in the titration syringe. All experiments
467 were carried out at 20°C with a stirring speed of 307 rpm. The parameters used for the titration
468 series are given in Supplementary Table 3. Data analysis was carried out using MicroCal PEQ-ITC Analysis,
469 Malvern Panalytical software.

470 **Bacterial two-hybrid assay.** Primary protein-protein interactions were identified by bacterial two-hybrid
471 (BACTH) analysis⁵⁰. The BACTH system is based on the interaction-mediated reconstruction of *Bordetella*
472 *pertussis* adenylate cyclase (CyaA) activity in *E. coli* BTH101. Functional complementation between two
473 fragments (T18 and T25) of CyaA as a consequence of the interaction between bait and prey molecules
474 results in the synthesis of cAMP, which is monitored by measuring the β -galactosidase activity of the
475 cAMP-CAP-dependent promoter of the *E. coli* *lac* operon. Plasmids pUT18C and p25N allow the
476 expression of proteins fused to the T18 and T25 fragments of CyaA, respectively. For these experiments,
477 we used the plasmids pGP2974-pGP2977, which encode N-and C-terminal fusions of T18 or T25 to *darB*.
478 Accordingly, plasmids pGP2982-pGP2985 were used for *ccpC*, pGP3344-pGP3347 for *rel*, pGP3415-
479 pGP3418 for *rel*(SYN-RRM), pGP3419-pGP3422 for *rel*(Rel^{NTD}), pGP3336-pGP3339 for *sasA*, and
480 pGP3411pGP3414 for *sasB*. These plasmids were used for co-transformation of *E. coli* BTH101 and the
481 protein-protein interactions were then analyzed by plating the cells on LB plates containing 100 μ g/ml
482 ampicillin, 50 μ g/ml kanamycin, 40 μ g/ml X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and
483 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The plates were incubated for a maximum of 36 h at
484 28°C.

485 **Total RNA preparation.** For RNA isolation, the cells were grown in MSSM minimal medium containing 0.1
486 mM KCl to an OD₆₀₀ of 0.4 to 0.6 and harvested. Preparation of total RNA was carried out as described
487 previously⁵⁶. RNA was visualized using a 1% agarose formaldehyde gel in MOPS buffer (20 mM MOPS, 5
488 mM Na-Acetate, 1 mM EDTA, pH 7). The gel was stained with ethidium bromide.

489 **Quantification of (p)ppGpp in *B. subtilis* cell extracts.** Bacteria were precultured in LB medium and in
490 MSSM medium with 0.1 mM KCl, and this preculture was used to inoculate the main culture in MSSM
491 medium with 0.1 mM KCl. The cultures were grown until the exponential phase. The nucleotides were
492 extracted and quantified by the SPE Extraction (modified from ⁵⁷). Briefly, 2 ml of the culture were mixed
493 with 75 μ l 100% formic acid and incubated on ice for 30 min. After addition of 2 ml 50 mM ammonium
494 acetate (pH 4.5), precipitates were removed by centrifugation at 3,000 \times g for 5 min. The supernatant
495 was transferred onto a prewashed SPE column (OASIS Wax cartridges 1 cc, Waters). Prewashing was
496 done with 1 ml methanol (4,500 \times g for 1 min) and 1 ml 50 mM ammonium acetate (pH 4.5) (3,800 \times g for
497 1 min). The supernatant was loaded in 1 ml steps (4 times) by 1 min centrifugation at 3,800 \times g each
498 time. The SPE column was washed with 1 ml 50 mM ammonium acetate (pH 4.5, 3,800 \times g for 1 min) and
499 1 ml methanol (3,800 \times g for 1 min). After elution with 1 ml 80% ddH₂O, 20% methanol, 3% NH₄OH into a
500 new tube and centrifugation (3,800 \times g for 1 min), the samples were frozen in liquid nitrogen and freeze-
501 dried. The nucleotides were analyzed by liquid chromatography coupled tandem mass spectrometry on a
502 QTRAP 5500 instrument (Sciex, Framingham, Massachusetts) equipped with an electrospray ionization
503 source (ESI). Data were recorded in the multiple reaction monitoring (MRM) mode. Separation was
504 performed on a Hypercarb column (30 \times 4.6 mm, 5 μ m particle size; Phenomenex, Aschaffenburg,
505 Germany) using a linear gradient of solvent A (10 mM ammonium acetate pH 10) and solvent B
506 (acetonitrile) at a flow rate of 0.6 ml/min, with solvent B with a gradient of 4 to 60% B being delivered
507 within 8 minutes. The ppGpp and pppGpp standards were purchased from Jena Bioscience.

508 **Rel synthetase activity assay.** Rel, and the wild type and A25G-R132M mutant DarB proteins were
509 purified as described above. The assay was carried out in HEPES:Polymix buffer (20 mM HEPES:KOH pH
510 7.5, 2 mM DTT, 5 mM Mg(OAc)₂, 95 mM KCl, 5 mM NH₄Cl, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM
511 spermidine)²¹. The activity of Rel was measured alone or in the presence of DarB, or the control protein
512 BSA. The assay was carried out at 37°C and the reaction was started by addition of 1 mM ATP and 1 mM
513 GTP (Jena Bioscience), and samples for the nucleotide measurement were taken after 15 min. The

514 nucleotides were extracted and quantified by SPE Extraction modified from ⁵⁷ as described above, with
515 the exception that 500 μ l of the assay mix was mixed with 1,500 μ l assay buffer and with 75 μ l 100%
516 formic acid and incubated on ice for 30 min.

517 **Rel hydrolase activity assay.** The pppGpp hydrolysis assay was carried out in HEPES:Polymix buffer (20
518 mM HEPES:KOH pH 7.5, 2 mM DTT, 5 mM Mg(OAc)₂, 95 mM KCl, 5 mM NH₄Cl, 0.5 mM CaCl₂, 8 mM
519 putrescine, 1 mM spermidine), 1 mM MnCl₂) ²¹. The activity of Rel was measured alone or in the
520 presence of DarB, or the control protein BSA. The assay was carried out at 37°C and the reaction was
521 started by addition of 1 mM pppGpp (Jena Bioscience), and samples for the nucleotide measurement
522 were taken after 15 min. The nucleotides were extracted and quantified as described above.

523

524 **Data availability**

525 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via
526 the PRIDE ⁵⁸ partner repository with the dataset identifier PXD018087.

527

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672 **Contributions**

673

674 L.K., R.F., and J.S. conceptualized the study. L.K., C.H., D.W., H.B., J.L.H., A.D., and K.S. developed the
675 methodology, performed the experiments and analyzed the data. L.K. and J.S. wrote the original draft of
676 the manuscript. J.L.H., A.D., K.S., and R.F. reviewed and edited the manuscript. R.F. and J.S. acquired
677 funding. R.F., A.D., and J.S. provided supervision.

678

679 **Ethics declaration**

680

681 **Competing interests**

682 The authors declare no competing interests.

683 **Figure Legends**

684 **Fig. 1 DarB interacts with Rel *in vitro* and *in vivo*.** **a**, Bacterial two-hybrid (BACTH) experiment testing for
685 the interaction of DarB with Rel. N- and C-terminal fusions of DarB and Rel to the T18 or T25 domains of
686 the adenylate cyclase (CyaA) were created and the proteins were tested for interaction in *E. coli* BTH101.
687 Dark colonies indicate an interaction that results in adenylate cyclase activity and subsequent expression
688 of the reporter β -galactosidase. **b**, *In vitro* Strep-Rel pulldown experiment. Strep-Rel was immobilized
689 onto a StrepTactin column and incubated with DarB, DarB preincubated with c-di-AMP, or the control
690 protein CcpC. The eluates (E) and wash (W) fractions were analyzed by SDS-PAGE and the presence of
691 DarB in the elution fractions was further verified by MS analysis (excised gel bands are numbered with 1
692 and 2). **c**, *In vivo* interaction experiment of DarB-Strep with Rel-His. *B. subtilis* expressing Rel-His₆ was
693 transformed with plasmid-borne DarB-Strep and grown in minimal medium containing low (0.1 mM) or
694 high (5 mM) potassium concentration. DarB together with its potential binding partners was purified
695 with a StrepTactin column and the elution and wash fractions were analyzed by Western blot analysis.
696 DarB and Rel were detected by using antibodies against the Strep-tag and the His-tag, respectively. HPr
697 served as a negative control. Abbreviations: EV, empty vector; CE, cell extract; cdA, c-di-AMP.

698

699 **Fig. 2. DarB binds the N-terminal domain (NTD) of Rel.** **a**, The domain organization of Rel and the
700 truncated Rel variants used in this study. Abbreviations: HYD, hydrolase domain; SYN, synthetase
701 domain; TGS, TGS domain (for: ThrRS, GTPase and SpoT); ZFD, a zinc finger domain; RRM domain (for
702 Ribosomal Recognition Motif). **b**, Bacterial two-hybrid (BACTH) assay to test for the interaction between
703 DarB and the full-length and truncated Rel-variants. N- and C-terminal fusions of DarB and the Rel
704 variants to the T18 or T25 domain of the adenylate cyclase (CyaA) were created and the proteins were
705 tested for interaction in *E. coli* BTH101. Dark colonies indicate an interaction that results in adenylate

706 cyclase activity and subsequent expression of the reporter β -galactosidase. **c**, *In vitro* pulldown
707 experiment with the NTD of Rel. Strep-Rel^{NTD} was immobilized onto a StrepTactin column and incubated
708 with DarB, DarB preincubated with c-di-AMP, or the control protein CcpC. The eluate and wash fractions
709 were analyzed by SDS-PAGE and the presence of DarB in the elution fractions was further verified by MS
710 analysis (excised gel bands are numbered with 3 and 4). **d**, The DarB-Rel^{NTD} complex was analyzed by size
711 exclusion chromatography and multi-angle light scattering (SEC-MALS). Rel^{NTD} and DarB were used in
712 equimolar concentrations. Dark blue line, DarB; black line, Rel^{NTD}; blue line, mixture of DarB and Rel. The
713 calculated molar masses determined by MALS are listed below the chromatogram. **e**, The molar ratio of
714 the DarB-Rel^{NTD}-complex was assessed by Isothermal titration calorimetry (ITC). The cell and the syringe
715 contained 10 μ M Rel^{NTD} and 100 μ M DarB (blue) or 100 μ M c-di-AMP-bound DarB (DarB^{cdA}) (pink),
716 respectively. Abbreviation: cdA, c-di-AMP.

717

718 **Fig. 3. Overexpression of DarB is toxic.** Growth experiments of **(a)** wild type *B. subtilis* (black), GP3407
719 (Δ darB; grey), wild type + pGP3306 ($darB^+$, blue), **(b)** GP3419 (Δ rel, bright green) and GP3419 (Δ rel,
720 $darB^+$, dark green), and **(c)** wild type + pGP3437/3441/3601 ($darB^{A25G}$, $darB^{R132M}$, $darB^{A25G,R132M}$, red) in
721 MSSM minimal medium with 0.1 mM KCl (upper panel) or 5 mM KCl (lower panel). Growth was
722 monitored in an Epoch 2 Microplate Spectrophotometer (BioTek Instruments) at 37°C with linear shaking
723 at 237 cpm (4 mm) for 12 h.

724

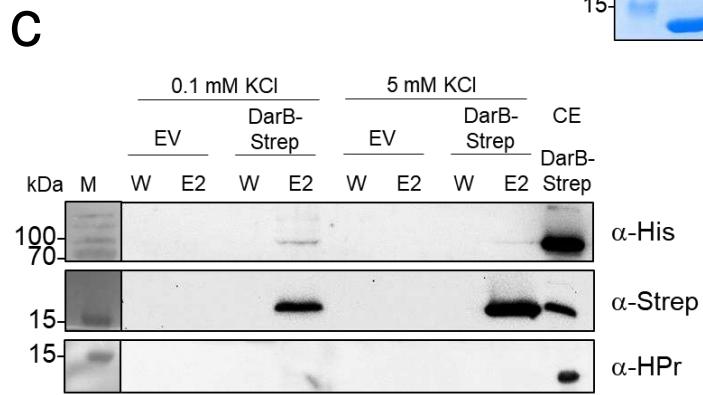
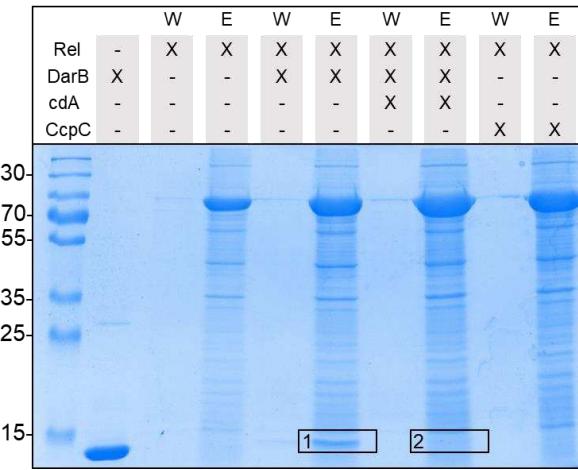
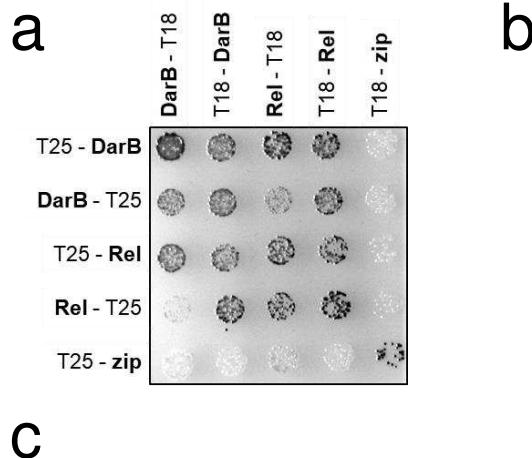
725 **Fig. 4. DarB stimulates Rel-dependent accumulation of pppGpp.** The activity of Rel was assessed in an *in*
726 *vitro* activity assay. **a**, Rel synthetase activity assay. Purified Rel was incubated with ATP and GTP, in the
727 absence or presence of DarB, c-di-AMP-saturated DarB, or bovine serum albumin (BSA) (10-fold molar
728 excess) and the production of pppGpp was determined by liquid chromatography coupled tandem mass

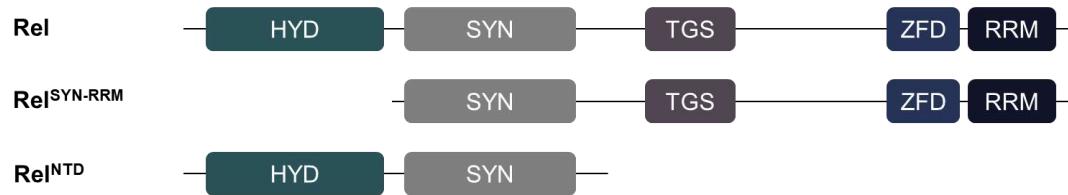
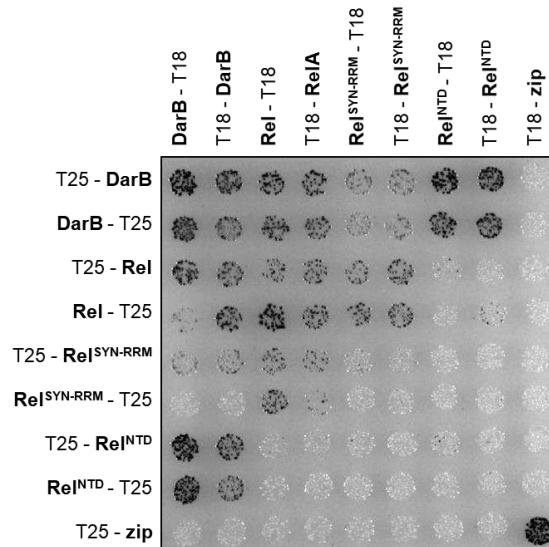
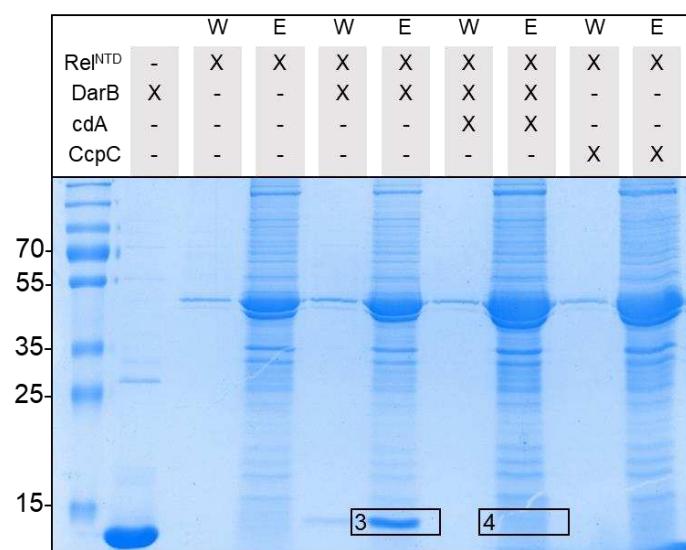
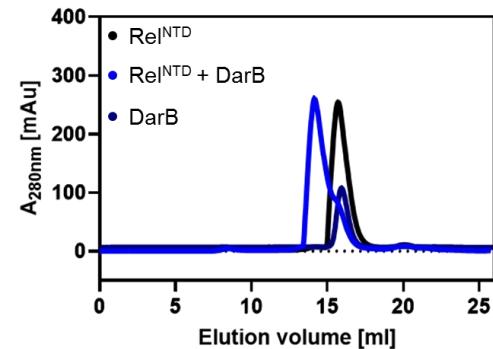
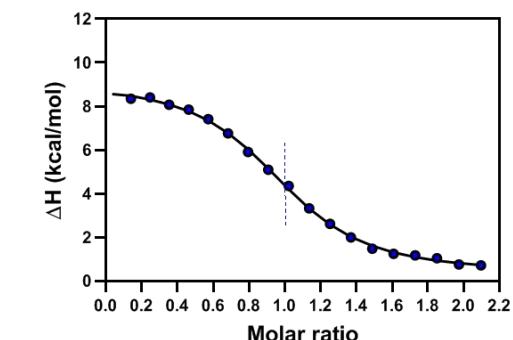
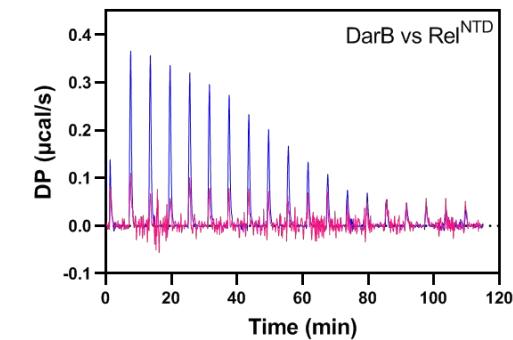
729 spectrometry on a QTRAP 5500 instrument (Sciex, Framingham, Massachusetts) equipped with an
730 electrospray ionization source (ESI). BSA served as a negative control. Statistical analysis was performed
731 using a one-way ANOVA, followed by Tukey's multiple comparisons test (**** $P < 0.0001$). **b**, Rel
732 hydrolase activity assay. Purified Rel was incubated with pppGpp, in the absence or presence of DarB, c-
733 di-AMP-saturated DarB, or bovine serum albumin (BSA) (10-fold molar excess) and the production of GTP
734 was monitored. **c**, Determination of intracellular (p)ppGpp levels in wild type *B. subtilis*, GP3407 (Δ *darB*),
735 wild type + pGP3306 (*darB*⁺), GP3419 (Δ *rel*) and GP3419 + pGP3306 (Δ *rel*, *darB*⁺). Bacteria were grown in
736 MSSM minimal medium with 0.1 mM KCl until the exponential growth phase, and the nucleotides were
737 analyzed as described above. Abbreviation: cdA, c-di-AMP.

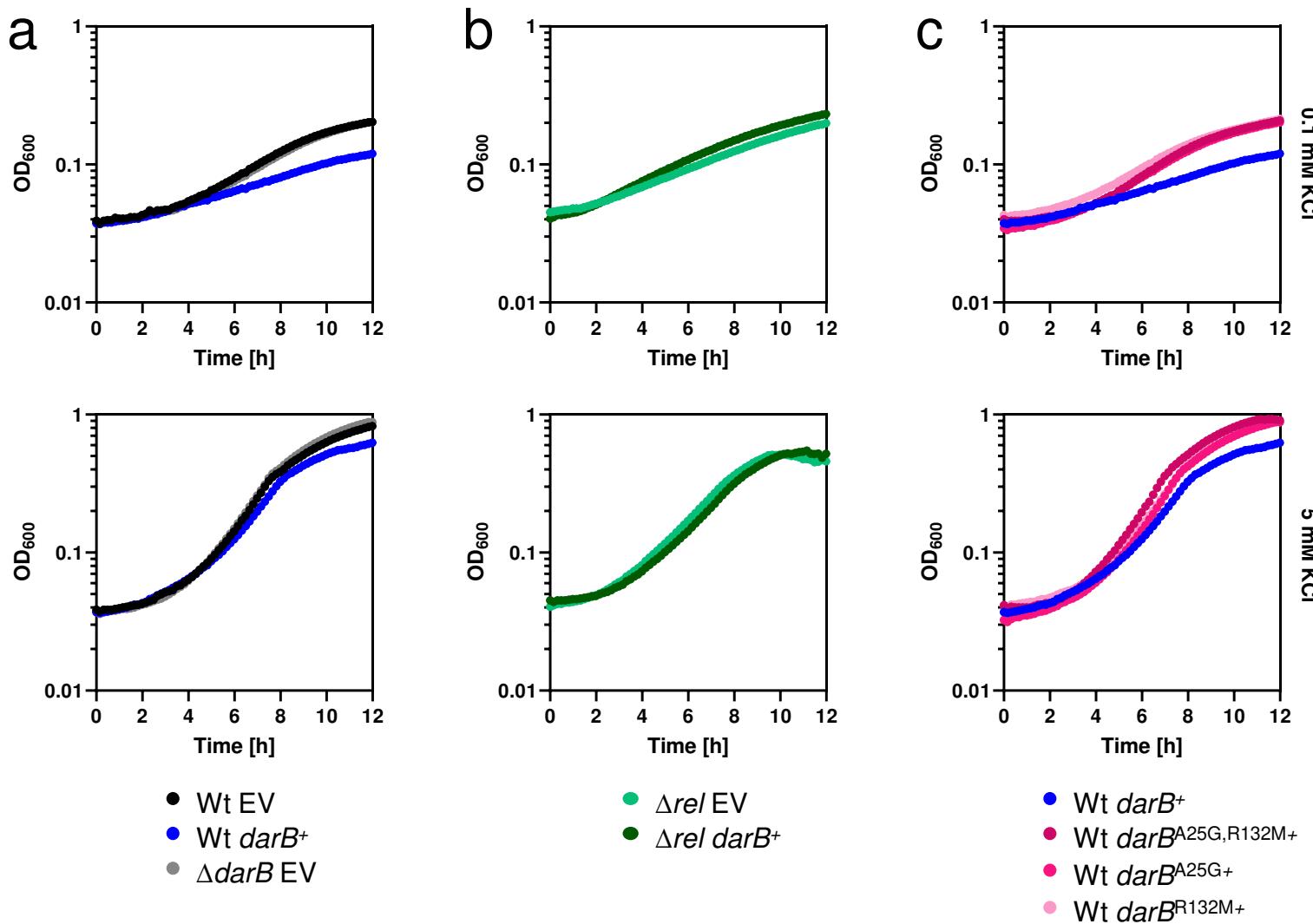
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739 **Fig. 5. The link between c-di-AMP and (p)ppGpp signaling in *B. subtilis*.** **a**, The model depicts the
740 bidirectional and dynamic process of the cellular response to potassium limitation. When potassium
741 becomes limiting, the diadenylate cyclases respond and produce less c-di-AMP and c-di-AMP receptor
742 proteins are present in the apo-form. Apo-DarB binds to Rel and stimulates (p)ppGpp synthesis.
743 (p)ppGpp accumulation induces the stringent response and inhibits the c-di-AMP-degrading
744 phosphodiesterases GdpP and PgpH. This leads to increasing intracellular c-di-AMP amounts. DarB can
745 then bind c-di-AMP and is thus no longer able to interact with Rel. **b**, The DarB-Rel complex as suggested
746 by the presented data. One DarB dimer is bound by two Rel monomers, one on each side. The
747 interaction occurs via the HYD-SYN domains of Rel. DarB, blue; Rel, grey. Abbreviation: cdA, c-di-AMP.

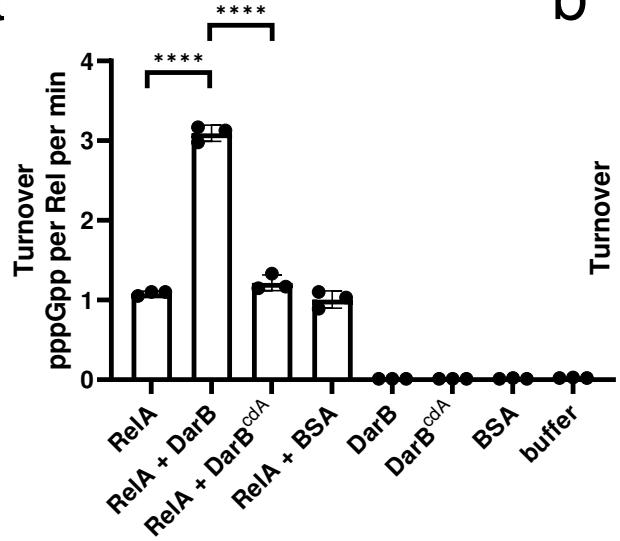
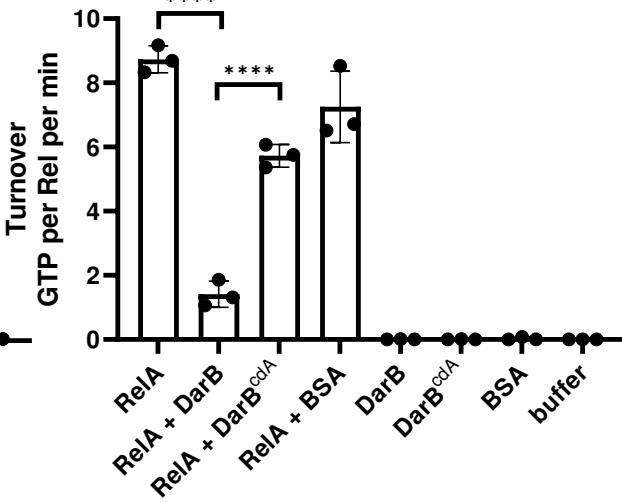
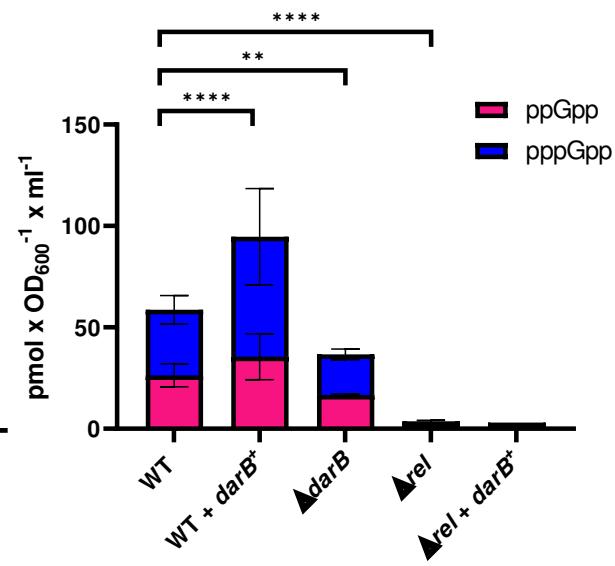
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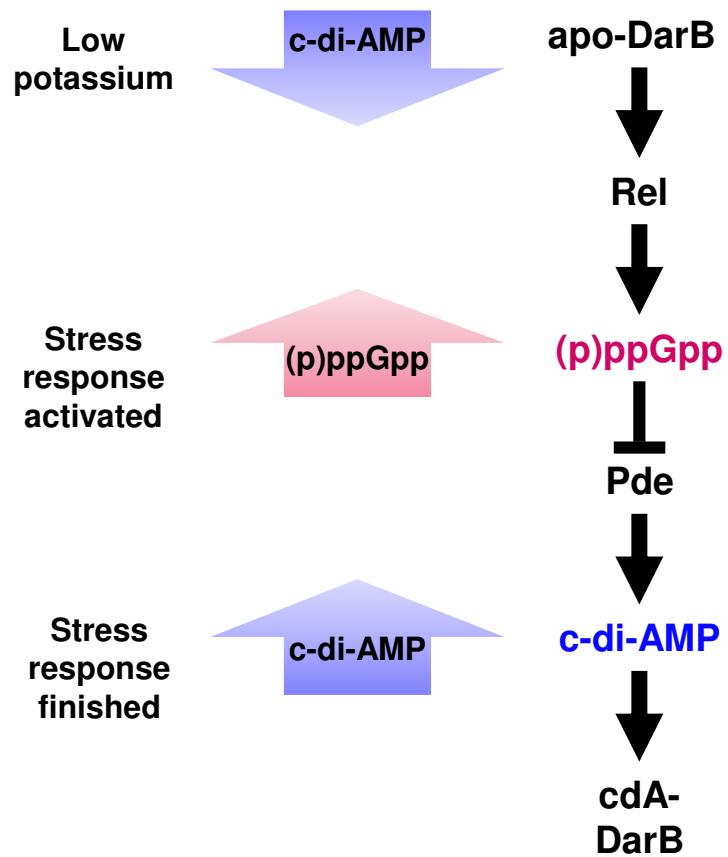


a**b****c****d****e**



Krüger *et al.*, Figure 3

a**b****c**

a**b**