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Insulation and wiring specificity of BceR-like response regulators and their target promoters in *Bacillus subtilis*

Model of signal transduction pathways of two Bce-like systems after induction with corresponding AMPs in Bacillus subtilis. The TCSs Bce and Psd and their inducing antibiotics as signal inputs are highlighted black and grey, respectively. For reasons of simplicity, the ABC transporters of both systems are not shown. Solid arrows indicate the signal transduction pathway within one system, while cross-regulation between BceS and PsdR is highlighted by the dotted arrow. On each promoter, MBS representing for the main binding site and SBS representing for the secondary binding of Bce-like RRs are filled with white on bceA promoter and slashes on psdA promoter. CM, cell membrane.

84x94mm (300 x 300 DPI)

Insulation and wiring specificity of BceR-like response regulators and their target promoters in *Bacillus subtilis*

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Abbreviated summary

An efficient insulation is essential to ensure wiring specificity of highly similar signal transducing systems. Here, we describe the regulatory features necessary to allow discrimination of two target promoters by their corresponding paralogous response regulators, involved in mediating resistance against antimicrobial peptides in *Bacillus subtilis*. We demonstrate that regulator competition in combination with hierarchical cooperative binding ensures specificity despite only slight differences in binding affinities.

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4   # target promoters in *Bacillus subtilis*

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19 Running title: Wiring specificity of Bce-like systems

20 Key words: two-component system, promoter recognition, antimicrobial peptide resistance, bacitracin,

21 DNA-binding site

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27 **Summary**

28 BceRS and PsdRS are paralogous two-component systems in *Bacillus subtilis* controlling the

29 response to antimicrobial peptides. In the presence of extracellular bacitracin and nisin,

30 respectively, the two response regulators (RRs) bind their target promoters, P_{bceA} or P_{psdA},

31 resulting in a strong up-regulation of target gene expression and ultimately antibiotic

32 resistance. Despite high sequence similarity between the RRs BceR and PsdR and their

33 known binding sites, no cross-regulation has been observed between them. We therefore

34 investigated the specificity determinants of P_{bceA} and P_{psdA} that ensure the insulation of these

35 two paralogous pathways at the RR-promoter interface. *In vivo* and *in vitro* analyses

36 demonstrate that the regulatory regions within these two promoters contain three important

37 elements: in addition to the known (main) binding site, we identified a linker region and a

38 secondary binding site that are crucial for functionality. Initial binding to the high affinity,

39 low specificity main binding site is a prerequisite for the subsequent highly specific binding

40 of a second RR dimer to the low affinity secondary binding site. In addition to this

41 hierarchical cooperative binding, discrimination requires a competition of the two RRs for

42 their respective binding site mediated by only slight differences in binding affinities.

Introduction

Antimicrobial peptides (AMPs) are predominantly produced by Gram-positive microbes to suppress the growth of competitors in their natural habitats (Berdy, 2005). The main target of AMPs is the bacterial cell envelope, especially different intermediates of the lipid II cycle. By binding to their target molecules, AMPs inhibit cell wall biosynthesis and cause cell death (Silver, 2003, Breukink & de Kruijff, 2006, Jordan *et al.*, 2008).

In Firmicutes bacteria, sensing of and resistance against AMPs is usually mediated by highly conserved Bce-like detoxification modules containing an ATP-binding-cassette (ABC) transporter and a two-component system (TCS) (Dintner *et al.*, 2011). The genome of *Bacillus subtilis* encodes three such systems: BceRS-BceAB, PsdRS-PsdAB and the poorly understood YxdJK-YxdLM-YxeA system (Joseph *et al.*, 2002, Gebhard & Mascher, 2011). The BceRS-BceAB paradigm responds to AMPs such as bacitracin, actagardine and mersacidin (Staroń *et al.*, 2011, Dintner *et al.*, 2014, Fritz *et al.*, 2015). It consists of two separate operons: the *bceRS* operon encodes the TCS comprised of a membrane anchored histidine kinase (HK), BceS, and a cytoplasmic response regulator (RR), BceR, under the control of a constitutive promoter. The *bceAB* operon encodes the ABC transporter under the control of an inducible BceR-dependent promoter, P$_{bceA}$. In the absence of AMPs, both operons are expressed at a very low level. In the presence of AMPs such as bacitracin, the ABC transporter BceAB senses this stimulus and passes the signal on to the HK BceS (Dintner *et al.*, 2014). Upon autophosphorylation, BceS then activates its cognate RR BceR by phosphoryl-group transfer. Phosphorylated BceR will then bind to P$_{bceA}$ and strongly induce *bceAB* transcription, ultimately resulting in increased BceAB production, thereby conferring AMP resistance (Mascher *et al.*, 2003, Ohki *et al.*, 2003, Bernard *et al.*, 2007, Rietkötter *et al.*, 2008, Fritz *et al.*, 2015) (Fig. 1 black system, BceAB not shown).

The main inducers of the Psd system are lipid II-binding lantibiotics such as nisin, actagardine, gallidermin and subtilin. In turn, the Psd system confers resistance against nisin, actagardine

3

69   and subtilin (Staroń *et al.*, 2011). The signal transduction pathway within Psd system (Fig. 1

70   grey system, PsdAB not shown) is similar to that described for the Bce system (Gebhard &

71   Mascher, 2011). Despite significant sequence similarity between BceRS-BceAB and PsdRS-

72   PsdAB, signaling in each system is generally well insulated from the other, although a

73   previous study has demonstrated some degree of cross-phosphorylation between BceS and

74   PsdR at high bacitracin concentrations (Rietkötter *et al.*, 2008) (Fig. 1, dotted arrow).

75   In bacteria, transcription initiation starts with promoter recognition by the σ subunit of the

76   RNA polymerase holo-enzyme at the -35 promoter element, followed by binding and

77   unwinding of the DNA double helix at the -10 promoter element (Lee *et al.*, 2012). A -10

78   promoter element with a perfect match to the σ^A consensus sequence (TATAAT) could be

79   identified in P$_{bceA}$. It is located 6 bp upstream of the transcription initiation site, which is 32 bp

80   upstream of the *bceA* start codon. However, a conserved -35 element was not found (Ohki *et*

81   *al.*, 2003). An identical σ^A-dependent -10 element was also found in P$_{psdA}$, again lacking a

82   clear -35 element at the appropriate position (Staroń *et al.*, 2011) (Fig. 2A). For such

83   promoters deviating significantly from the consensus sequence at the -35 position, the σ

84   subunit of RNA polymerase can still be recruited to these promoters by interaction with

85   activators like RRs binding to the upstream region (Jarmer *et al.*, 2001, Paget & Helmann,

86   2003). RRs usually contain an N-terminal receiver domain and a C-terminal output domain.

87   Both BceR and PsdR belong to the OmpR/PhoB subfamily of RRs with a C-terminal winged

88   helix-turn-helix DNA-binding output domain that regulates the transcription of target genes

89   by binding to their corresponding promoter regions via a specific recognition motif (Martínez-

90   Hackert & Stock, 1997, Fabret *et al.*, 1999, Galperin, 2010). Inverted repeats on P$_{bceA}$ as well

91   as on P$_{psdA}$ were mapped as BceR- and PsdR-binding sites, respectively, upstream of the

92   corresponding -10 promoter elements (Fig. 2A), which implies an interaction between BceR-

93   like RRs and the RNA polymerase holo-enzyme (Ohki *et al.*, 2003, de Been *et al.*, 2008,

94   Staroń *et al.*, 2011).

4

95   The DNA binding domains of BceR and PsdR share 51% sequence identity (66% similarity)

96   and the corresponding binding sites on P_{bceA} and P_{psdA} contain eleven out of fourteen identical

97   nucleotides (Fig. 2A) (Joseph *et al.*, 2002). Nevertheless, no cross-regulation was detected at

98   the transcriptional level between BceR-P_{psdA} and PsdR-P_{bceA} (Rietkötter *et al.*, 2008). Such a

99   regulatory insulation, that is, prevention of nonspecific regulatory cross-talk, is of course

100   desired and can arise at different molecular levels *in vivo* (Huynh & Stewart, 2011). The most

101   prominent mechanism for conferring such signaling specificity depends on the molecular

102   recognition between the two interaction partners (Podgornaia & Laub, 2013). However, in the

103   case of the Psd and Bce systems, the high degree of identity between the two regulator

104   binding sites raised the question how specificity can be ensured between two such closely

105   related systems.

106   Here we provide detailed insights into the molecular mechanisms that ensure insulation and

107   transcriptional regulation specificity between two Bce-like systems in *B. subtilis*, Bce and Psd

108   at the level of RR-promoter interaction. Using both *in vivo* and *in vitro* approaches, we

109   identified a secondary RR-binding site in both P_{bceA} and P_{psdA}, in addition to the previously

110   identified (main) binding site. Importantly, we demonstrate that the main binding site, while

111   being essential for promoter activation, does not significantly contribute to specificity of RR-

112   promoter interactions. Instead, the secondary binding site and the variable linker region

113   between the two sites are the primary specificity determinants. Moreover, our data show that

114   *in vivo* promoter discrimination is based on competition between the two RRs for their

115   respective binding sites.

116

117

118 **Results**

119

120 *Identification of the minimal* **bceA** *and* **psdA** *promoter motif*

121 P$_{bceA}$ and P$_{psdA}$ are the target promoters for the RRs BceR and PsdR, respectively (Staroń *et*

122 *al.*, 2011). When *B. subtilis* is challenged with bacitracin, BceR is activated by the

123 corresponding HK BceS and binds to a specific region of P$_{bceA}$, resulting in a strong

124 upregulation of the operon encoding the ABC transporter for resistance (Mascher *et al.*, 2003)

125 (Fig. 1). Previous work has already mapped an inverted repeat sequence for BceR binding in

126 the P$_{bceA}$ region (AAGCgTGTGACgaaaatGTCACAtGCTT) from -111 to -84 upstream of the

127 *bceA* start codon (Ohki *et al.*, 2003). For P$_{psdA}$, a highly similar PsdR binding site

128 (ATgTgACAgcatTGTaAgAT) could be identified from -99 to -80 upstream of the *psdA* start

129 codon (Staroń *et al.*, 2011). In agreement with these studies, a comparative genomics study

130 identified a putative binding site among most *bceA*-like promoters in Firmicutes bacteria, with

131 an overall consensus sequence of TnACA-N$_4$-TGTAA as a recognition site for BceR-like RRs

132 (Dintner *et al.*, 2011).

133 We first wanted to verify that these two known conserved binding motifs are indeed

134 indispensable for the RR-dependent activation of the *bceA* and *psdA* promoters and identify

135 the minimal regulatory elements for both promoter regions. Towards that goal, progressively

136 truncated *bceA* promoters starting with the 5'-position ranging from -111 to -103 and ending

137 at +82 relative to the ATG start codon of *bceA* were used to construct transcriptional *lacZ*

138 reporter fusions (Table S2), which were integrated at the *amyE* locus in *B. subtilis* 168 wild

139 type (WT) (Table S1). Progressively truncated *psdA* promoter fragments starting with 5'-

140 positions ranging from -110 to -95, all ending at position +30 relative to the ATG start codon

141 of *psdA*, were generated in a similar fashion (Fig. 2A). The promoter activity of the resulting

142 reporter strains was determined by quantitative β-galactosidase assay in the presence of

143 bacitracin (P$_{bceA}$) or nisin (P$_{psdA}$) (Staroń *et al.*, 2011).

144   Truncated *bceA* promoters from -111 until -106 showed activities comparable to the non-

145   truncated promoter fragment starting at position -122 after bacitracin induction (black bars)

146   (Fig. 2B). The truncations starting at position -105 and position -104 displayed a decreased

147   promoter activity, while a further truncation of one additional nucleotide (starting at position -

148   103) led to a complete loss of promoter activity after bacitracin induction.

149   Similar results were obtained for truncated *psdA* promoter fragments after nisin induction

150   (grey bars) (Fig. 2C). No decrease of promoter activity was observed for truncations with 5'-

151   positions starting from -110 to -100 relative to the positive control fragment, starting at

152   position -126. The promoter activities were significantly reduced for fragments truncated at

153   positions -99 to -96, while a truncation at position -95 led to a complete loss of activity after

154   nisin induction.

155   These data confirmed that the 7-4-7 nt binding motif TGTGACGaaaaTGTCACA of P$_{bceA}$ and

156   the TGTGACAgcatTGTAAGA binding motif of P$_{psdA}$ are indeed necessary for promoter

157   induction and constitute likely binding sites for BceR and PsdR, in good agreement with

158   previous reports (Ohki *et al.*, 2003, de Been *et al.*, 2008, Staroń *et al.*, 2011). These will be

159   referred to as "<u>m</u>ain <u>b</u>inding <u>s</u>ites" (MBSs) from now on. Position -104 relative to *bceA* start

160   codon and position -96 relative to *psdA* start codon determine the minimal 5'-end of active

161   RR-dependent promoter fragments.

162

163   *A secondary binding site on* **bceA** *and* **psdA** *promoters*

164   Sequence analysis of P$_{bceA}$ and P$_{psdA}$ did not identify a typical -35 region (TTGACA) upstream

165   of the -10 region as normally recognized by σ^A (Jarmer *et al.*, 2001). However, a 7 nt

166   conserved half binding site for a BceR-like RR, located 13/14 nt downstream of the MBS and

167   38 nt upstream of the -10 region, was predicted for both the *bceA* and the *psdA* promoter

168   regions (Dintner *et al.*, 2011). This observation implies the existence of a <u>s</u>econdary <u>b</u>inding

169   <u>s</u>ite (SBS) instead of a typical -35 element on *bceA*-like promoters. Based on this prediction,

7

170   we annotated a putative SBS also showing the 7-4-7 pattern, as well as a linker region (L)

171   between the MBS and the SBSs on both *bceA* and *psdA* promoters (Fig. 2A). We

172   experimentally investigated the function of the predicted promoter motifs by randomizing

173   their sequence, while maintaining the GC/AT content of the linker region. The fragments

174   were used to generate *lacZ* reporter gene fusions (Tables 1+2) and were assayed as before.

175   Both the WT *bceA* promoter (Fig. 2D) and the *psdA* promoter (Fig. 2E) showed strong

176   induction with the corresponding inducers bacitracin (black bars) or nisin (grey bars)

177   compared to the non-induced samples (white bars), but no such response to the non-cognate

178   inducer. The weak induction of P$_{psdA}$ by bacitracin (Fig. 2E) was due to the known cross-

179   phosphorylation of PsdR by BceS (Rietkötter *et al.*, 2008) (Fig. 1 dotted arrow). Randomizing

180   the MBS led to a complete loss of activity for both promoters. The same effect was obtained

181   when randomizing the sequence of the predicted SBS. However, activities of both *bceA* and

182   *psdA* promoters only showed a slight decrease by randomly mutating the corresponding linker

183   region (L) between the two binding sites (Fig. 2D and 2E).

184   The data demonstrate that on both P$_{bceA}$ and P$_{psdA}$, a SBS exists that is located downstream of

185   the MBS with a 13/14 nt linker region in between them. This SBS seemingly replaces the -35

186   region and is as indispensable as the MBS for RR-dependent promoter activity. Additional

187   assays done by randomizing either the first or the second half of each SBS were in agreement

188   with the results obtained for the completely randomized SBSs (data not shown), further

189   demonstrating that each half binding site has the same importance for P$_{bceA}$ and P$_{psdA}$ activity.

190

191   ***Major specificity determinants are located in the region containing linker and SBS***

192   So far, we have identified an extended regulatory region in P$_{bceA}$ and P$_{psA}$, consisting of two

193   binding sites, MBS and SBS, and a linker region between them. Since there is no cross-

194   regulation at the RR-promoter interface, neither between BceR-P$_{psdA}$ nor PsdR-P$_{bceA}$

195   (Rietkötter *et al.*, 2008), we therefore wanted to analyse the specificity determinants within

196   the *bceA*/*psdA* promoters. Towards that goal, a series of chimeric promoters derived from

197   P$_{bceA}$ and P$_{psdA}$ was constructed (Table S2) and fused with *lacZ*. Chimeric promoters BP1-4

198   are derived from P$_{\boldsymbol{b}ceA}$ (black) with gradually substituting P$_{\boldsymbol{p}sdA}$ (grey) at the 3'-terminal end

199   (Fig. 3A). Chimeric promoters PB1-4 are derived from P$_{\boldsymbol{p}sdA}$ (grey) with increasing of 3'-

200   fragments from P$_{\boldsymbol{b}ceA}$ (black) (Fig. 3B). To specifically eliminate any cross-talk between the

201   Bce and Psd systems, the chimeric promoters as well as WT P$_{bceA}$ and WT P$_{psdA}$ fragments as

202   references, were introduced into the WT strain and additionally the otherwise isogenic

203   Δ*bceRS* (TMB1460) and the Δ*psdRS* (TMB1462) strains (Table S1). Compared to the WT

204   strain, the Δ*bceRS* and Δ*psdRS* backgrounds remove the effect of cross-phosphorylation and

205   hence provide a clearer view of individual RR-promoter interactions.

206   P$_{bceA}$ showed the same high activity in the Δ*psdRS* mutant (Fig. 3D) as in the WT strain (Fig.

207   3C) after bacitracin induction, but no activity after nisin induction in either the WT (Fig. 3C)

208   or the Δ*bceRS* background (Fig. 3E). Correspondingly, P$_{psdA}$ was highly induced by nisin in

209   both the WT strain (Fig. 3C) and the Δ*bceRS* mutant (Fig. 3E). Importantly, the moderate

210   induction of P$_{psdA}$ by bacitracin seen in the WT (Fig. 3C) was not detected in the Δ*bceRS*

211   mutant (Fig. 3D) due to the elimination of cross-phosphorylation between BceS and PsdR.

212   These results are in agreement with previous studies that there is no cross-regulation at the

213   RR-promoter level.

214   Chimeric promoters BP1 and BP2 showed high activity after induction with bacitracin in both

215   the WT strain (Fig. 3C) and the Δ*psdRS* strain (Fig. 3D), but no activity upon nisin induction

216   in either the WT strain (Fig. 3C) or the Δ*bceRS* strain (Fig. 3E). Hence, BP2 could be

217   recognized by BceR, but not by PsdR. These results indicate that the specificity determinants

218   are located within the region upstream of and including the SBS. Interestingly, the chimeric

219   promoter BP3 could neither be induced by bacitracin in the Δ*psdRS* background (Fig. 3D) nor

220   by nisin in the Δ*bceRS* background (Fig. 3E), but showed moderate induction by bacitracin in

221   only the WT background (Fig. 3C). The observation that PB3 requires both TCSs for

222   responding to bacitracin might point towards the formation of RR heterodimers. But this

223   interpretation is purely speculative at the moment and will require follow-on studies.

224   Moreover, BP4 – possessing the whole region downstream of the MBS originating from P$_{psdA}$

225   – was not only moderately induced by bacitracin in the Δ$psdRS$ background (Fig. 3D) but also

226   by nisin in the Δ$bceRS$ background (Fig. 3E), indicating a relaxation of specificity from BceR

227   to PsdR. The results of BP2 and BP4 demonstrate that the major specificity determinants of

228   P$_{psdA}$ are located in the region containing the linker and the SBS.

229   Chimeric promoters PB1 and PB2 showed a decreased activity after induction with nisin in

230   both the WT background (Fig. 3F) and the Δ$bceRS$ mutant (Fig. 3G) relative to P$_{psdA}$, and no

231   bacitracin induction in the Δ$psdRS$ mutant (Fig. 3H), indicating no change of specificity.

232   These results corroborate that the region downstream of the SBS is not relevant for the RR-

233   promoter specificity. Interestingly, PB3 showed a significantly decreased activity in the

234   Δ$bceRS$ mutant with nisin induction (Fig. 3G) and a strongly increased activity in the Δ$psdRS$

235   mutant with bacitracin induction (Fig. 3H). Chimera PB4 was not inducible by nisin in the

236   Δ$bceRS$ strain (Fig. 3G), but instead showed high induction by bacitracin in the Δ$psdRS$ strain

237   (Fig. 3H), strongly reminiscent of the intact P$_{bceA}$. The promoter activities of PB3 and PB4 in

238   the WT strain (Fig. 3F) were in accordance with those observed in both mutant backgrounds.

239   These data indicate that the change of specificity from P$_{psdA}$ to P$_{bceA}$ can be achieved by

240   exchanging the SBS (PB3), and is further strengthened by an additional substitution of the

241   linker region (PB4).

242   The analysis of chimeric promoter constructs described above demonstrates that (i) all three

243   regulatory parts (MBS-linker-SBS) together determine the RR-specificity, with (ii) the region

244   downstream of the MBS of P$_{bceA}$/P$_{psdA}$, containing the linker and the SBS, functioning as the

245   main discriminator for BceR/PsdR recognition.

246

10

247   *Rewiring the specificity between P$_{bceA}$ and P$_{psdA}$ enables dissecting the roles of individual*

248   *specificity determinants*

249   To further investigate the functions of MBS, linker region and SBS on the *psdA* promoter for

250   PsdR recognition, additional chimeric promoters were generated with different combinations

251   of these three motifs on P$_{bceA}$ replaced by the corresponding region of P$_{psdA}$ (Fig. 4A) to rewire

252   specificity from BceR to PsdR. Promoter activities were measured as described above in the

253   WT strain (Fig. 4C), the Δ*psdRS* strain (Fig. 4D) and the Δ*bceRS* strain (Fig. 4E).

254   Compared to P$_{bceA}$, replacing only the MBS (M), the linker (L) or both (M+L) of P$_{bceA}$ with

255   the corresponding region of P$_{psdA}$ showed decreased promoter activity in the WT strain after

256   induction with bacitracin (Fig. 4C) as well as in the Δ*psdRS* mutant (Fig. 4D). No increase of

257   the promoter activity after induction by nisin was observed in either the WT strain (Fig. 4C)

258   or the Δ*bceRS* mutant (Fig. 4E). This indicates that the MBS, the linker or both of P$_{psdA}$ are

259   not enough to cause activation via PsdR. Changing the SBS (S) on P$_{bceA}$ into P$_{psdA}$ led to a

260   decrease of promoter activity after induction with bacitracin in the WT strain (Fig. 4C) as well

261   as in the Δ*psdRS* mutant (Fig. 4D) and a slight but detectable increase of promoter activity

262   after induction with nisin in the Δ*bceRS* mutant (Fig. 4E). This indicates that exchanging only

263   the SBS alone already conferred a relaxation of promoter specificity from BceR to PsdR.

264   Substitution of the linker together with the SBS (L+S) resulted in a higher promoter activity

265   compared to only exchanging the SBS (S) both after bacitracin induction (Fig. 4D) and nisin

266   induction (Fig. 4E). This indicates that the linker region (L) can enhance promoter activity

267   with both cognate PsdR and noncognate BceR. Compared to only the SBS switch (S),

268   exchanging both the MBS and the SBS simultaneously (M+S) resulted in a severe decrease of

269   the promoter activity after induction with bacitracin (Fig. 4D), while causing an increase of

270   the promoter activity after induction with nisin (Fig. 4E).

271   Taken together, these results suggest that the SBS on P$_{psdA}$ is the main discriminator for PsdR-

272   binding to P$_{psdA}$, even though the intensity of induction with the SBS substitution alone is not

273   very strong. The linker cannot determine specificity by itself but can increase promoter

274   activity with both BceR and PsdR, which explains the change of specificity that was detected

275   for construct BP4 including the linker and the SBS but not for construct BP3 with only the

276   SBS (Fig. 3C). Despite the fact that the MBS is absolutely crucial for RR-promoter

277   interaction, the MBS of P$_{psdA}$ alone cannot determine specificity. Instead, it supports the SBS

278   in strengthening the promoter activity. Not surprisingly, switching all three elements together

279   (M+L+S) resulted in the highest change of specificity after induction with nisin (Fig. 4E),

280   demonstrating that all three parts together contribute to the specificity.

281   In order to support the results obtained above, a similar approach was performed towards

282   rewiring the specificity from P$_{psdA}$ to P$_{bceA}$. A comparable series of chimeric promoters with

283   different combinations of the MBS, the linker region and the SBS of P$_{psdA}$ being replaced by

284   the corresponding regions from P$_{bceA}$ was constructed (Fig. 4B) (Table S2) and the promoter

285   activities of the corresponding *B. subtilis* reporter strains (Table S1) were determined. The

286   results are shown in Fig. 4F-4H. Overall, the combined data is in very good agreement with

287   the results obtained for rewiring the specificity from P$_{psdA}$ to P$_{bceA}$ with only minor differences

288   between the two sets.

289   Taken together, exchanging the MBS alone had no effect on the specificity of induction of

290   P$_{bceA}$ and only caused a very minor change in P$_{psdA}$ behaviour. Instead, the SBS provides the

291   major discriminator for RR binding. The data is particularly clear for the BceR-P$_{bceA}$

292   interaction, where exchange of the SBS alone was able to cause a clear change in specificity,

293   while the role of the SBS of P$_{psdA}$ for the PsdR-P$_{psdA}$ pair is less prominent. Both promoters

294   have in common that the MBSs strengthen the specificity by increasing the interactions with

295   the cognate RR, while simultaneously reducing the interactions with the non-cognate RR. In

296   addition, the linker regions fine tune promoter activity. While specific roles can therefore be

297   attributed to these three regulatory elements, it should be pointed out that the specificity of

298   BceR-like RRs for their target promoters is ultimately determined by the specific combination

299   of MBS, linker and SBS working together.

300

301   **In vitro *analysis of BceR binding to* P_{bceA} *and* P_{psdA}**

302   Next, we wanted to investigate if BceR could also discriminate between its native promoter

303   P_{bceA} and the non-cognate P_{psdA} *in vitro*. BceR carrying an N-terminal His_{10}-tag with the

304   expected molecular mass of about 27 kDa was produced in and purified from the cytoplasmic

305   fraction of *E. coli* C43(DE3) cells containing plasmid pCF120 (Table S2). Electrophoretic

306   mobility shift assays (EMSAs) were performed with purified BceR and the two promoters

307   P_{bceA} and P_{psdA}. 300 bp promoter DNA fragments (300 bp) of P_{bceA} or P_{psdA} containing the

308   MBS, the linker region and the SBS were amplified and labeled at the 5'-end with 6FAM by

309   PCR. 6FAM labeled P_{sigW} (the target promoter of an ECF sigma factor in *B. subtilis*) was used

310   as a negative control.

311   The results of EMSAs with BceR and P_{bceA} are shown in Figure 5A. Increasing concentrations

312   of BceR phosphorylated by the addition of phosphoramidate (BceR-P; see Experimental

313   procedure) were incubated with 30 fmol of 6FAM-P_{bceA} (lane 2 to lane 5), demonstrating a

314   concentration-dependent binding of BceR-P to P_{bceA}. The first shift was observed at 1.0 μM

315   BceR-P representing the initial binding event of BceR-P to P_{bceA}. An additional shift occurred

316   at BceR-P concentrations of 1.5 μM or above and presumably represents a second binding

317   event, consistent with the presence of two BceR binding sites on the DNA fragment. In

318   contrast, unphosphorylated BceR showed a much weaker binding (data not shown), which

319   demonstrated that RR-phosphorylation promotes DNA binding by increasing BceR affinity to

320   P_{bceA}.

321   EMSAs were also performed between BceR-P and P_{psdA} (Fig. 5B). Two successive shifts of

322   P_{psdA} band in lane 3 and lane 4 compared to free P_{psdA} DNA fragment (lane 1) demonstrated

323   that BceR-P can also bind to two sites in the noncognate but highly related P_{psdA} *in vitro*. In

13

324 contrast, no shift was observed for the P_{sigW} DNA fragment (Fig. 5E), confirming the overall

325 specificity of the assay: BceR-P cannot bind to promoter fragments that do not harbor the

326 binding motifs of a P_{bceA}-like promoter.

327 To further illustrate the specificity and affinities of BceR-P binding to P_{bceA} and P_{psdA}, 900

328 fmol of unlabeled promoter fragments were used as competitor DNA (Fig. 5A/5B lane 6-8).

329 Co-incubation of BceR-P with 30 fmol 6FAM-P_{bceA} and 900 fmol unlabeled P_{bceA} fragment

330 (Fig. 5A lane 6) completely abolished the retardation of the labeled P_{bceA} fragment due to the

331 competitive binding of BceR-P to an excess of unlabeled P_{bceA}. However, the shift of 6FAM-

332 P_{bceA} band was not influenced by adding a 30-fold molar excess of unlabeled P_{psdA} (Fig. 5A

333 lane 7) or P_{sigW} (Fig. 5A lane 8). This shows that despite its ability to bind to both P_{bceA} and

334 P_{psdA} in isolation, BceR is clearly able to distinguish between the two promoters and

335 preferentially binds to its cognate target. In contrast, the retardation of the 6FAM-P_{psdA} DNA

336 fragment was abolished by either addition of 30-fold excess unlabeled P_{bceA} (Fig. 5B lane 6)

337 or unlabeled P_{psdA} (Fig. 5B lane 7) fragments but not by P_{sigW} (Fig. 5B lane 8). These results

338 clearly demonstrate that, while BceR-P can interact with seemingly identical activities with

339 both target promoters in isolation (the shift occurs at comparable BceR-P concentrations), it

340 preferentially binds to its native promoter, P_{bceA}, compared to P_{psdA} *in vitro* when incubated in

341 competition. Hence, the binding affinity for its cognate target promoter P_{bceA} seems to be

342 higher than for P_{psdA}, which determines the *in vivo* specific transcription initiation.

343 Unfortunately, any efforts to purify PsdR failed, thereby preventing the performance of

344 similar *in vitro* studies on PsdR-P_{psdA}/P_{bceA} interactions.

345

346 ***Cooperative binding of BceR to two binding sites on* P_{bceA}**

347 The *in vivo* promoter activity assays demonstrated that both binding sites on P_{bceA} are

348 indispensable for BceR-P_{bceA} interaction (Fig. 2D). Moreover, the EMSA studies on complete

349 promoter fragments strongly suggest two binding events at P_{bceA} *in vitro* (Fig. 5A). To

350   discriminate between the individual binding reactions, we next performed EMSAs with BceR-

351   P on 6FAM labeled *bceA* promoter DNA-fragments carrying randomized versions of either

352   the MBS or the SBS (Fig. 5D).

353   Incubation of BceR-P with labeled P$_{bceA}$ SBS^R (P$_{bceA}$ containing a native MBS and a

354   randomized and hence inactive SBS) caused only a single shift at a BceR-P concentration of

355   1.0 µM (Fig. 5C), a concentration comparable to the threshold concentration for binding to

356   the intact P$_{bceA}$ fragment (Fig. 5A lane 3). Increasing the BceR-P concentration did not lead to

357   any additional shift. Hence, P$_{bceA}$ containing only the MBS merely allows one binding event,

358   which is the binding of BceR-P to the MBS. The identical BceR-P concentrations required for

359   shifting either the WT or the SBS^R fragments indicates that binding of BceR-P to the MBS is

360   independent of the SBS.

361   Incubation of BceR-P with labeled P$_{bceA}$ MBS^R (P$_{bceA}$ containing a randomized and hence

362   inactive MBS but an intact SBS) failed to retard the DNA-fragment within the same

363   concentration range (Fig. 5D). This suggests that either BceR-P has a very low affinity for

364   binding to the SBS alone or that binding to the SBS depends on and occurs after BceR-P

365   binding to the MBS.

366

367   ***Determination of binding kinetics of BceR-promoter interaction unravels the mechanism***

368   ***that determines BceR promoter specificity***

369   For quantitatively describing the binding kinetics of BceR-promoter interactions, we next

370   performed SPR spectroscopy in combination with Interaction Map® (IM) analysis. We

371   captured a biotin-labeled DNA-fragment comprising the P$_{bceA}$ region (see Table S3 for exact

372   sequence) to a sensor chip coated with immobilized streptavidin. Next, increasing

373   concentrations of His$_{10}$-BceR and His$_{10}$-BceR-P were injected over the chip surface. While

374   non-phosphorylated BceR did not interact with the P$_{bceA}$ promoter (Fig. 6A), BceR-P showed

375   clear binding (Fig. 6B). Since BceR has two binding sites on the DNA-fragment used for SPR,

15

376   we performed IM analyses. In order to determine and quantify the individual binding events

377   represented by the SPR curves. Briefly, the IM algorithm splits the experimental SPR data set

378   into several theoretical monovalent binding events and selects the binding curves that best fit

379   the experimental data when summed up. By plotting the association rate k_a and the

380   dissociation rate k_d within a two-dimensional distribution, heterogeneous binding data can be

381   displayed as a map, in which each peak corresponds to one component that contributes to the

382   cumulative binding curve (Altschuh *et al.* 2012). The sensorgram could be split into two

383   binding events, one characterized by a fast ON/fast OFF (k_a=1.8 × 10^6/M*s; k_d=1.0x10^{-1}/s)

384   and one characterized by a slow ON/slow OFF binding kinetics (k_a=3.2 × 10^5/M*s;

385   k_d=5.5x10^{-4}/s) that differ in their overall affinity (K_D=58 nM and K_D=1.7 nM, respectively)

386   (Fig. 6E). Each binding peak makes up an approximate peak weight of 50% revealing that

387   both DNA-binding sites are bound by equal amounts of BceR-P molecules.

388   As a next step, we determined the binding kinetics between BceR-P and P$_{bceA}$ when the MBS

389   or the SBS was randomized (MBS^R or SBS^R, respectively). Inactivation of the MBS

390   completely prevented DNA-binding of BceR-P (Fig. 6C), while a clear DNA-binding of BceR

391   could still be observed when only the SBS was randomized (Fig. 6D). In contrast to the

392   sensorgram including both intact binding sites (Fig. 6B), the IM of the corresponding

393   sensorgram suggested in principle only the slow ON/slow OFF binding event (k_a=1.5 × 10^6

394   M*s; k_d=4.9 × 10^{-4}/s, resulting in an overall binding affinity of K_D=0.4 nM (Fig. 6F).

395   However, the *in silico* sensorgram is comparable to that one of the slow ON/slow OFF

396   interaction of BceR-P to intact P$_{bceA}$ site revealing that this reflects binding of BceR-P to the

397   MBS although the overall affinity is approximately six-fold higher, mainly caused by the five-

398   fold higher ON rate. The peak weight is calculated as 80%, meaning that this interaction

399   mainly contributes to the measured sensorgram. However, the fast ON/fast OFF peak did not

400   completely disappear, but compared to the intact promoter site the peak weight is lower than

401   20% and can therefore be neglected. These data clearly show that the MBS of the P$_{bceA}$ region

16

402   is essential for binding of the RR to the DNA. Moreover, the affinity of the RR is not

403   sufficient to allow any binding of BceR to the SBS if the MBS was not previously occupied,

404   at least under the experimental regime applicable for SPR spectroscopy. Comparing the

405   binding kinetics of BceR-P to the intact and the SBS^R promoter, it can be assumed that the

406   SBS increases the overall affinity of the RR to the promoter region, and therefore is important

407   for triggering gene expression.

408   Finally, we wondered if the binding mechanism of BceR-P is also similar to the related P$_{psdA}$.

409   We captured DNA comprising the P$_{psdA}$ promoter as well as the P$_{psdA}$ promoter in which the

410   MBS or SBS were inactivated (MBS^R or SBS^R, respectively) onto the chip. First, we injected

411   increasing concentrations of non-phosphorylated BceR over the chip and observed, as

412   expected, no binding to the P$_{psdA}$ promoter (Fig. 6G). Then, increasing concentrations of

413   BceR-P were injected. The interaction of Bce-R to P$_{psdA}$ was almost comparable to the one

414   observed for the P$_{bceA}$ promoter (Fig. 6H). The IM analysis underlying this sensorgram also

415   revealed two binding events, one with fast ON/fast OFF (k$_a$=6.2 $\times$ 10^5/M*s; k$_d$=1.2x10^{-1}/s)

416   and one with slow ON/slow OFF binding kinetics (k$_a$=1.1 $\times$ 10^5/M*s; k$_d$=6.2x10^{-4}/s) likewise

417   resulting in two binding events that differ in their overall affinity (K$_D$=188 nM and K$_D$=6.2

418   nM, respectively). Compared to the affinities of BceR-P to P$_{bceA}$, the binding affinities for

419   P$_{psdA}$ are indeed in a similar range, but both P$_{psdA}$ binding sites differ in their affinity in the

420   factor of three to BceR-P (Fig. 6L). In agreement with the data obtained for the P$_{bceA}$ promoter

421   region, inactivation of the MBS completely prevented BceR-P binding to the P$_{psdA}$ promoter

422   region (Fig. 6I). Inactivation of the SBS showed a 1:1 interaction described by one peak in the

423   IM analysis that corresponds to the slow ON/slow OFF MBS site with an association rate of

424   k$_a$=1.1 $\times$ 10^5 M*s and a dissociation rate k$_d$=7.3 $\times$ 10^{-4}/s making an overall binding affinity of

425   K$_D$=6.7 nM (Fig. 6M), also fitting well to k$_a$, k$_d$, and K$_D$ of the BceR-P/P$_{psdA}$ interaction (Fig.

426   6L). These data clearly demonstrate that the binding mechanism of BceR-P to the P$_{psdA}$

427   promoter is comparable to that of BceR-P to the P$_{bceA}$ promoter, however, with slightly altered

428   binding kinetics and binding affinities differing by a factor of three.

429   Taken together, the *in vitro* data obtained for the binding of BceR-P to isolated promoter

430   fragments by EMSA (Fig. 5A/B) and SPR spectroscopy (Fig. 6B/E and Fig. 6 H/L) are in

431   good agreement with each other. They indicate a hierarchical cooperative binding of

432   phosphorylated BceR-like RRs first to the MBS and then to the SBS. While promoter

433   discrimination could not be explained by EMSAs alone, we could determine slight differences

434   in the binding affinities by SPR combined with IM analyses that could explain promoter

435   preference and discrimination of isolated RRs on single promoter fragments and therefore

436   selected activation of transcription. Moreover, DNA curvature as well as interaction of BceR-

437   P with the RNA polymerase could be further factors that finally lead to total promoter

438   activation *in vivo*.

439

440   **Discussion**

441

442   On P$_{bceA}$ and P$_{psdA}$, no typical -35 element was found in the appropriate location upstream of

443   the -10 element, indicating that the σ unit of the RNA polymerase cannot bind properly to the

444   promoter by itself to initiate transcription initiation. However, binding can nevertheless be

445   established under such conditions when the σ unit interacts with an RR that binds to upstream

446   elements of the promoter, thereby compensating weak σ unit binding (Lee *et al.*, 2012). DNA

447   binding domain structures of both PhoB and OmpR from the OmpR subfamily showed that

448   these RRs can directly interact with the σ subunit of the RNA polymerase (Martínez-Hackert

449   & Stock, 1997, Blanco *et al.*, 2002). BceR and PsdR, which belong to the same subfamily, are

450   assumed to assist the transcription initiation of RNA polymerase in a similar way.

451   Specific transcription initiation by RRs is important for maintaining the insulation of the

452   corresponding signaling systems. The similarity of Bce-like RRs DNA-binding domain and

453   their binding sites on target promoters increases the potential of unwanted cross-talk at the

454   transcription initiation level. However, we could show that Bce-type RRs in *B. subtilis* are

455   extremely specific in inducing the transcription of only their cognate ABC transporter operons.

456   While we observed binding of BceR-P to both the cognate P$_{bceA}$ and the non-cognate P$_{psdA}$

457   with very similar affinities *in vitro* (Fig. 5A and 5B), BceR can only induce the transcription

458   of *bceAB* but not of *psdAB in vivo* (Fig. 3). Moreover, our EMSA experiments showed that

459   when incubated with a mixture of both promoter fragments, BceR is able to specifically bind

460   its cognate target, even if that is present at 30-fold lower concentrations (Fig. 5). Promoter

461   discrimination between cognate and non-cognate binding sites can therefore be based on even

462   minor differences in binding affinities of isolated RRs to the otherwise highly similar binding

463   sites, as demonstrated by the SPR measurements (Fig. 6). This discriminatory ability becomes

464   especially apparent under conditions of competition between binding partners (as shown by

465   the EMSA competition experiments, Fig. 5), which is most reminiscent of the intracellular

466   environment, where both RRs and DNA target sequences are present at the same time.

467   The slight affinity preference is the ability of the RR to distinguish the cognate from non-

468   cognate promoter in the natural cellular setting. Our data strongly suggest that *B. subtilis*

469   evolved a sophisticated mechanism to maintain this ability by combining this existing target

470   site competition of homologous RRs to their respective binding sites with hierarchical and

471   cooperative DNA binding (Fig. 7). Instead of the single binding sites reported previously

472   (Ohki *et al.*, 2003, de Been *et al.*, 2008), we experimentally demonstrated the presence of two

473   binding sites in the regulatory region of the Bce-type RR target promoters (Fig. 2D and 2E),

474   as was already suggested by a comparative genomics study on Bce-like TCSs (Dintner *et al.*,

475   2011). By performing EMSAs and SPR assays of BceR with P$_{bceA}$ mutants carrying random

476   mutation in either the MBS or the SBS, we demonstrated that BceR has a high affinity and

19

477   shows independent binding to the upstream MBS (Fig. 5C and 6D). BceR has a low affinity

478   for the downstream SBS and cannot bind to it alone under our experimental conditions (Fig.

479   5D and 6C). While it is not possible to unequivocally determine the order of BceR binding to

480   its two target sites from the data presented herein, our results nevertheless strongly suggest

481   that a BceR dimer first binds to the high-affinity MBS. This first binding event might then

482   assist the subsequent binding of another dimer to the downstream low-affinity SBS. In this,

483   binding to the MBS appears to be of low specificity, while the second binding event to the

484   SBS occurs with high specificity. This is supported by our *in vivo* promoter activity assays

485   where we showed that exchanging the SBS between the P$_{bceA}$ and P$_{psdA}$ fragments resulted in a

486   much stronger influence on promoter specificity than exchanging the MBS by *in vivo*

487   promoter activity assays (Fig. 4). This hierarchical and cooperative binding to the two sites

488   enables BceR to discriminate between its cognate promoter P$_{bceA}$ and the non-cognate P$_{psdA}$,

489   which is based on: (i) The MBSs of these two promoters differ only in three bases and provide

490   a high-affinity, low-specificity docking site; (ii) The SBSs of these two promoters harbor five

491   different bases and represent low-affinity, yet high specificity interaction sites; (iii) Only this

492   combination of MBS and SBS together with the binding competition described above

493   ultimately allows BceR-P to discriminate between the cognate promoter P$_{bceA}$ and the non-

494   cognate P$_{psdA}$, thereby ultimately ensuring the wiring specificity of highly similar RRs.

495   It should be pointed out that the specificity of interaction between BceR and the MBS/SBS of

496   either the cognate or non-cognate site – as expressed by the different binding affinities

497   determined by SPR measurement *in vitro* (Fig. 6) – will *in vivo* of course be influenced by the

498   relative cellular concentrations of phosphorylated BceR-like RRs. For both P$_{bceA}$ and P$_{psdA}$, the

499   K$_D$ values differ by a factor of 30 between the MBS and the SBS, with the first in the range of

500   2-7 nM while the latter was determined in the 50-150 nM range (Fig. 6).

501   The strong discriminatory power of the SBS relative to the MBS suggests cellular BceR-P

502   concentrations in the medium (approx. 10 to 100) nanomolar range. Under such conditions,

503   the MBS of both P$_{bceA}$ and P$_{psdA}$ would be fully bound, while the small differences in binding

504   affinities to the respective SBS should be sufficient for promoter discrimination at RR

505   concentrations near the K$_D$ values. Unfortunately, no data on the cellular concentrations of

506   BceR-like RRs is available, and even a comprehensive quantitative analysis aimed at

507   determining the cellular amounts of all mRNA and protein species of the *B. subtilis* cell failed

508   to detect BceR in any of over 200 conditions tested (Buescher et al. 2012) indicative of a very

509   low basal abundance. The true physiological conditions for promoter-RR interaction therefore

510   have to remain speculative.

511   The linker regions of these two promoters showed characteristically distinct GC/AT contents:

512   P$_{bceA}$ has a high AT content, while P$_{psdA}$ has a high GC content (Fig. 2A). We showed that

513   mutating the linker region into a random sequence while maintaining the GC/AT content of

514   each promoter only slightly affected the promoter activity (Fig. 2D and 2E). However,

515   exchanging the linker region between these two promoters, which means changing the GC/AT

516   content, resulted in a more pronounced effect on the promoter activity (Fig. 4). AT-rich

517   sequences are known to mediate DNA bending (Koo *et al.*, 1986). One possibility is that the

518   AT-rich linker region on P$_{bceA}$ confers a structural difference compared to P$_{psdA}$ by bending the

519   promoter between two binding sites, which might accommodate the binding of two BceR

520   dimers.

521   The high specificity of the SBS is presumably determined mainly by its first half-site, for

522   which P$_{bceA}$ and P$_{psdA}$ differ in four out of seven bases. In contrast, the second half-sites of the

523   SBSs only differ in one base. The sequence identity of the second half-site and its location at

524   the -35 position suggests that it can probably be bound by both BceR and the σ^A subunit of the

525   RNA polymerase. Along those lines, we saw that a P$_{bceA}$ mutant with the SBS replaced by a

526   second MBS (MBS-linker-MBS) completely lost its promoter activity (data not shown),

527   further supporting the importance of the second half for transcription initiation, presumably

528   by σ^A subunit binding. Alternatively, the binding of the σ^{70} subunit to the -35 element could

529   be replaced by protein-protein interactions between the RNA polymerase and BceR/PsdR.

530   Such a mechanism was shown *in vitro* for PhoB and CRP dependent promoter activation.

531   (Kumar *et al.*, 1994). A recent study demonstrated that in PhoB regulated promoters, σ^{70}

532   forms a number contacts with DNA-bound PhoB, replacing contacts with the -35 element

533   (Blanco *et al.*, 2011).

534   Hierarchical and cooperative DNA binding is widespread among the OmpR RR subfamily.

535   For example, PhoB can bind cooperatively to two binding sites in the *pstS* promoter with

536   different individual binding affinities (Blanco *et al.*, 2012). P_{ompF} has three OmpR binding

537   sites with gradually reduced affinity from upstream to downstream, and binding of OmpR to

538   the first site is important for subsequent binding to the lower-affinity downstream sites

539   (Harlocker *et al.*, 1995). Likewise, the RR YpdB from *E. coli* also shows a two-step

540   cooperative binding mechanism to its target promoter P_{yhjX} (Behr *et al.*, 2016): binding of

541   YpdB to the upstream site A initiates subsequent binding to the downstream site B followed

542   by a rapid and successive promoter clearance. Similar to P_{bceA}-binding of BceR, binding of

543   YpdB to P_{yhjX} was completely abolished if site A was inactivated (Behr *et al.*, 2016).

544   Interestingly, highly cooperative binding of BceR-P to its target promoter was already

545   strongly suggested by a recent quantitative study on the regulatory dynamics of the Bce

546   system (Fritz *et al.*, 2015). This study indicated a high degree of cooperativity within the

547   signaling pathway, presumably caused by cooperative binding of BceR-P to multiple sites in

548   the target promoter. This cooperativity was shown to be crucial for the highly dynamic dose-

549   response behavior of *bceAB* expression in the presence of increasing amounts of bacitracin,

550   resulting in an accurate produce-to-demand strategy that adjusts cellular BceAB levels to just

551   the right amount to cope with the current presence of bacitracin (Fritz *et al.*, 2015). These

552   specific results on BceR cooperativity are in good agreement with a recent theoretical study,

553   which identified cooperativity as an important mechanism to significantly reduce crosstalk in

554   gene regulation (Friedlander *et al.*, 2016).

555   The evolution of such complex regulatory mechanisms often correlates with the regulatory

556   function of the RRs: e.g. PhoB and OmpR regulate dozens of operons in *E. coli* in the

557   presence of certain stimuli. Some of these operons need to be highly upregulated while others

558   require only moderate or subtle modulations in response to a given trigger. Controlling such

559   differential expression levels of multiple target operons by a single RR can be achieved

560   through assembly of different numbers of binding sites with sequence variations. We have

561   demonstrated for *B. subtilis* that a similar mechanism can also be used to maintain signaling

562   specificity and regulatory insulation between paralogous Bce-like systems that presumably

563   evolved by gene duplications followed by sequence diversification of both the DNA binding

564   domain and their target promoter sequence. Combining a high-affinity but low-specificity

565   MBS and a high-specificity but low-affinity SBS provides *B. subtilis* with enough sequence

566   space to ensure that Bce-like RRs can evolve the ability to discriminate cognate from non-

567   cognate promoters, thereby ensuring the signaling fidelity of highly paralogous Bce-like

568   systems on the transcription level. It will be interesting to see if such a combination of

569   competitive and hierarchical cooperative binding can also explain the target site

570   discrimination for other paralogous pairs of two-component systems.

571

572   **Experimental procedures**

573
574   ***Bacterial strains and growth conditions.*** All strains used in this study are listed in Table S1.

575   *E. coli* DH5α□and XL1-blue were used for cloning. All *B. subtilis* strains used in this study

576   are derivatives of the laboratory WT strain 168. *E. coli* and *B. subtilis* were grown routinely in

577   Luria-Bertani (LB) medium at 37°C with aeration. *B. subtilis* was transformed by natural

578   competence as previously described (Harwood & Cutting, 1990). Ampicillin (100 μg ml^{-1})

579   was used for selection of all plasmids in *E. coli*. Chloramphenicol (5 μg ml^{-1}), spectinomycin

580   (100 μg ml^{-1}) or erythromycin (1 μg ml^{-1}) plus lincomycin (25 μg ml^{-1}) for macrolide-

581 lincosamide-streptogramin B (mls) resistance were used for the selection of *B. subtilis*

582 mutants. Bacitracin was supplied as the Zn^{2+}-salt. Growth was measured as optical density at

583 600 nm wavelength (OD_{600}). Solid media contained 1.5 % (w/v) agar.

584 ***Plasmid construction and genetic techniques.*** All plasmids constructed in this study are

585 listed in Table S2. The corresponding primer sequences are provided in the supplemental

586 material (Table S3). Different promoter fragments derived from P_{bceA} and P_{psdA} were fused to

587 *lacZ* and cloned into the vector pAC6 (Stülke *et al.*, 1997) via the EcoRI/BamHI sites. The

588 details of all promoter constructs are given in Table S2. For construction of the BceR-

589 production plasmid in *E. coli*, *bceR* was amplified with primers TM2007/2008 and cloned into

590 vector pET16b with XhoI and BamHI obtaining pCF120, resulting in an N-terminal His_{10}-tag

591 fusion. Constructs for unmarked gene deletion in *B. subtilis* were cloned into the vector

592 pMAD (Arnaud *et al.*, 2004). For each operon to be deleted, 800-1000 bp fragments located

593 immediately before the start codon of the first gene ("up" fragment) and after the stop codon

594 of the last gene ("down" fragment) were amplified. The primers were designed to create a 17-

595 20 bp overlap between the PCR-products (Table S2), facilitating fusion of the fragments by

596 PCR overlap extension and subsequent cloning into pMAD. Gene deletions were performed

597 as previously described (Arnaud *et al.*, 2004). All constructs were checked for by sequencing,

598 and all *B. subtilis* strains created were verified by colony PCR using appropriate primers.

599
600 ***β-Galactosidase assays.*** Promoter activity assays were performed as described previously

601 (Mascher *et al.*, 2004). In brief, cells were inoculated from fresh overnight cultures and grown

602 in LB medium at 37°C with aeration until they reached an OD_{600} between 0.4 and 0.5. The

603 cultures were split into 2 mL aliquots and challenged with 30 µg ml^{-1} bacitracin or 2 µg ml^{-1}

604 nisin with one aliquot left untreated (non-induced control). After incubation for an additional

605 30 min at 37°C with aeration, the cultures were harvested and β-galactosidase activities were

606 determined as described previously, with normalization to cell density (Miller, 1972).

607

608   ***Overproduction and purification of His-tagged BceR.*** To produce BceR carrying an N-

609   terminal His$_{10}$-tag, *E. coli* C43 (DE3) cells harboring plasmid pCF120 were grown at 25 °C

610   with agitation until they reached an OD$_{600}$ of about 0.4. IPTG (0.5 mM) was added to the

611   culture and incubation was continued at 18 °C with agitation overnight. Cells were harvested

612   by centrifugation at 4,400 × g for 10 min. The cell pellet was washed with buffer A (20 mM

613   potassium phosphate buffer [pH7.5], 100 mM NaCl) and stored at -20 °C until use.

614   To purify His$_{10}$-tagged BceR, cells were resuspended in buffer B (50 mM potassium

615   phosphate buffer [pH 7.5], 500 mM NaCl, 5 mM β-mercapto-ethanol, 10 mM imidazole and

616   10 % (w/v) glycerol) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) plus

617   2 mg DNaseI and disrupted by three passages through a French pressure cell (Thermo Fisher)

618   at 20,000 PSI. Unbroken cells were removed by centrifugation at 17,000 × g for 20 min and

619   the cell-free supernatant was filtered through a 0.45 μm syringe filter before loading onto a 1

620   ml Ni^{2+}-NTA resin column (Qiagen) pre-equilibrated with 5 column volumes (CVs) of buffer

621   B. Loading was followed by washing with 5 CVs of buffer B and then with 5 CVs of buffer B

622   containing 100 mM imidazole. BceR was eluted with buffer B supplemented with 250 mM

623   imidazole. Fractions containing BceR were pooled and dialyzed in buffer C (50 mM Tris-HCl

624   [pH 7.5], 150 mM NaCl, 10 mM MgSO$_4$, 5 mM β-mercapto-ethanol, 5 mM imidazole and 10 %

625   (w/v) glycerol) at room temperature for 1 h. Protein concentration was determined with Roti$^{®}$-

626   Nanoquant (Carl Roth), and the proteins stored at 4 °C until use.

627

628   ***<u>E</u>lectrophoretic <u>M</u>obility <u>S</u>hift <u>A</u>ssays (EMSA).*** For electrophoretic mobility shift assays,

629   different DNA fragments (around 300bp) generated by PCR using primers TM3146 (5'

630   terminal 6FAM labeled) and TM3137 were purified by gel extraction. Unlabeled DNA

631   fragments were generated by PCR using primers TM3136/3137 and purified by gel extraction.

632   N-terminal His$_{10}$-BceR samples in the non-phosphorylated state and after phosphorylation

633   with 50 mM phosphoramidate at room temperature for 2 h were centrifuged at 16,060 × g and

634   4 °C for 10 min to remove the aggregated protein. Protein concentrations of the supernatants

635   were determined as above and the proteins were stored on ice. Binding reactions were set by

636   incubating 6FAM-labelled DNA-fragments with different concentrations of His$_{10}$-BceR at

637   room temperature for 20 min. The reaction mixture included 30 fmol labeled target DNA and

638   0, 0.5, 1.0, 1.5, 2.0 μM protein with binding buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl,

639   10 mM MgSO$_4$, 1 mM DTT, 5 μg ml^{-1} salmon sperm DNA and 4 % (w/v) glycerol) in a total

640   volume of 5.5 μl. Unlabeled competitor DNA was added to the system to a final concentration

641   of 900 fmol. Samples were loaded on a 6% native polyacrylamide gel and electrophoresis was

642   performed by 300 V for 15 min in 1× TBE buffer. 6FAM fluorescence of labeled DNA bands

643   was detected by PhosphorImager (Typhoon Trio™, GE Healthcare).

644

645   ***Surface Plasmon Resonance (SPR) spectroscopy.*** SPR assays were performed in a Biacore

646   T200 using carboxymethyl dextran sensor chips pre-coated with streptavidin (Xantec

647   SAD500-L, XanTec Bioanalytics GmbH, Düsseldorf, Germany). All experiments were

648   carried out at a constant temperature of 25°C and using HBS-EP+ buffer [10 mM HEPES pH

649   7.4; 150 mM NaCl; 3 mM EDTA; 0.05 % (v/v) detergent P20] as running buffer. Before

650   immobilizing the DNA fragments, the chips were equilibrated by three injections using 1 M

651   NaCl/50 mM NaOH at a flow rate of 10 μl min^{-1}. Then, 10 nM of the respective double-

652   stranded biotinylated DNA fragment was injected using a contact time of 420 sec and a flow

653   rate of 10 μl min^{-1}. As a final wash step, 1 M NaCl/50 mM NaOH/50% (v/v) isopropanol was

654   injected. Approximately 100-200 RU of each respective DNA fragment were captured onto

655   the respective flow cell. All interaction kinetics of BceR or BceR-P with the respective DNA

656   fragment were performed in HBS-EP+ buffer at 25°C at a flow rate of 30 μl min^{-1}. The

657   proteins were diluted in HBS-EP+ buffer and passed over all flow cells in different

658   concentrations (0.1 nM-10 nM) using a contact time of 180 sec followed by a 300 sec

659   dissociation time before the next cycle started. After each cycle the surface was regenerated

660   by injection of 2.5 M NaCl for 60 sec at 30 µl min^{-1} flow rate followed by a second

661   regeneration step by injection of 0.5% (w/v) SDS for 60 sec at 30 µl min^{-1}. All experiments

662   were performed at 25°C. Sensorgrams were recorded using the Biacore T200 Control

663   software 2.0 and analyzed with the Biacore T200 Evaluation software 2.0. The surface of flow

664   cell 1 was not immobilized with DNA and used to obtain blank sensorgrams for subtraction of

665   bulk refractive index background. The referenced sensorgrams were normalized to a baseline

666   of 0. Peaks in the sensorgrams at the beginning and the end of the injection emerged from the

667   runtime difference between the flow cells of each chip.

668   Calibration-free concentration analysis (CFCA) was performed using a 5 µM solution of

669   purified BceR-P (calculated from Lowry-based protein determination), which was stepwise

670   diluted 1:2, 1:5, 1:10, and 1:20. Each protein dilution was two-time injected, one at 5 µl min^{-1}

671   as well as 100 µl min^{-1} flow rate. On the active flow cell P$_{psdA}$-DNA was used for BceR-P-

672   binding. CFCA basically relies on mass transport, which is a diffusion phenomenon that

673   describes the movement of molecules between the solution and the surface. The CFCA

674   therefore relies on the measurement of the observed binding rate during sample injection

675   under partially or complete mass transport limited conditions. Overall, the initial binding rate

676   (dR/dt) is measured at two different flow rates dependent on the diffusion constant of the

677   protein. The diffusion coefficient of BceR-P was calculated using the Biacore diffusion

678   constant   calculator   and   converter   webtool   (https://www.biacore.com/lifesciences/

679   Application_Support/online_support/Diffusion_Coefficient_Calculator/index.html),   whereby

680   a globular shape of the protein was assumed. The diffusion coefficient of BceR-P was

681   determined as D=1.031x10^{-10} m^2/s. The initial rates of those dilutions that differed in a factor

682   of at least 1.5 were considered for the calculation of the „active" concentration, which was

683   determined as 5x10^{-8}M (1% of the total protein concentration determined by Lowry assay) for

684   BceR-P. The quite low percentage of "active" protein compared to total protein does not

685   necessarily mean that most of the protein is inactive due to misfolding and/or aggregation. It

27

686   is rather possible that not the complete amount is phosphorylated and therefore not "active"

687   and/or that, although thoroughly washed with high salts, a portion of the protein has still DNA

688   bound after the purification process. However, the „active" protein concentration was

689   ultimately used for calculation of the binding kinetic constants.

690
691   *Interaction map® (IM) analyses*

692   IM calculations were performed on the Ridgeview Diagnostic Server (Ridgeview Diagnostics,

693   Uppsala, Sweden). For this purpose, the SPR sensorgrams were exported from the Biacore

694   T200 Evaluation Software 2.0 as *.txt files and imported into TraceDrawer Software 1.5

695   (Ridgeview Instruments, Uppsala, Sweden). IM files were generated using the IM tool within

696   the software, which produces files that were then sent via e-mail to the server

697   (im@ridgeviewdiagnostics.com), where the IM calculations were performed (Altschuk *et al.*

698   2012). The resulting files were then evaluated for spots in the TraceDrawer 1.5 Software, and

699   the IM spots were quantified.

700
701   **Acknowledgements**

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Tables

Table S1. Bacterial strains used in this study.

Strain	Genotype or characteristic(s)[a]	Reference or source
E. coli strains		
DH5α	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (r_K^- m_K^+) *rel*A1 *gln*V44 Φ80' Δ*lacZ* Δ*M15* Δ*(lacZYA-argF)U169*	(Grant *et al.*, 1990)
XL1-Blue	*end*A1 *gyr*A96 *(nal^R)* *thi*-1 *rec*A1 *rel*A1 *lac* *sup*E44 [F' *pro*AB⁺ *lacI*q Δ*(lacZ)M15] hsd*R17*(r_K^- m_K^+)	Stratagene
C43 (DE3)	F⁻ *omp*T *gal* *dcm* *hsdS_B* (r_B^- m_B^-)(DE3)	(Miroux & Walker, 1996)
B. subtilis strains		
W168	Wild type, *trpC2*	Laboratory stock
TMB279	W168 *amyE*::pER603; cm^r	(Rietkötter *et al.*, 2008)
TMB299	W168 *amyE*::pER605; cm^r	(Rietkötter *et al.*, 2008)
TMB412	W168 *amyE*::pCF601; cm^r	This study
TMB607	W168 *amyE*::pJS605; cm^r	This study
TMB805	W168 *amyE*::pAS601; cm^r	This study
TMB806	W168 *amyE*::pAS602; cm^r	This study
TMB960	W168 *amyE*::pAS603; cm^r	This study
TMB961	W168 *amyE*::pAS604; cm^r	This study
TMB962	W168 *amyE*::pAS605; cm^r	This study
TMB963	W168 *amyE*::pAS606; cm^r	This study
TMB964	W168 *amyE*::pAS607; cm^r	This study
TMB965	W168 *amyE*::pAS608; cm^r	This study
TMB966	W168 *amyE*::pAS609; cm^r	This study
TMB967	W168 *amyE*::pAS610; cm^r	This study
TMB1047	W168 *amyE*::pAS613; cm^r	This study
TMB1048	W168 *amyE*::pAS614; cm^r	This study
TMB1049	W168 *amyE*::pAS615; cm^r	This study
TMB1050	W168 *amyE*::pAS616; cm^r	This study
TMB1051	W168 *amyE*::pAS617; cm^r	This study
TMB1052	W168 *amyE*::pAS618; cm^r	This study
TMB1053	W168 *amyE*::pAS619; cm^r	This study
TMB1054	W168 *amyE*::pAS620; cm^r	This study
TMB1460	W168 with unmarked deletions of the *bceRS* loci	This study
TMB1462	W168 with unmarked deletions of the *psdRS* loci	This study
TMB2244	W168 *amyE*::pMG600; cm^r	This study
TMB2245	W168 *amyE*::pMG601; cm^r	This study
TMB2247	W168 *amyE*::pMG603; cm^r	This study
TMB2248	W168 *amyE*::pMG604; cm^r	This study
TMB2249	W168 *amyE*::pMG605; cm^r	This study
TMB2250	W168 *amyE*::pMG606; cm^r	This study
TMB2252	W168 *amyE*::pMG608; cm^r	This study
TMB2253	W168 *amyE*::pMG609; cm^r	This study
TMB2303	TMB1462 *amyE*::pER603; cm^r	This study
TMB2304	TMB1462 *amyE*::pCF601; cm^r	This study
TMB2307	TMB1460 *amyE*::pER603; cm^r	This study
TMB2308	TMB1460 *amyE*::pCF601; cm^r	This study
TMB2382	TMB1460 *amyE*::pMG600; cm^r	This study
TMB2383	TMB1460 *amyE*::pMG601; cm^r	This study
TMB2385	TMB1460 *amyE*::pMG603; cm^r	This study
TMB2386	TMB1460 *amyE*::pMG604; cm^r	This study
TMB2387	TMB1462 *amyE*::pMG600; cm^r	This study

TMB2388	TMB1462 *amyE*::pMG601; cm^r	This study
TMB2390	TMB1462 *amyE*::pMG603; cm^r	This study
TMB2391	TMB1462 *amyE*::pMG604; cm^r	This study
TMB2392	TMB1460 *amyE*::pMG605; cm^r	This study
TMB2393	TMB1460 *amyE*::pMG606; cm^r	This study
TMB2395	TMB1460 *amyE*::pMG608; cm^r	This study
TMB2396	TMB1460 *amyE*::pMG609; cm^r	This study
TMB2397	TMB1462 *amyE*::pMG606; cm^r	This study
TMB2399	TMB1462 *amyE*::pMG608; cm^r	This study
TMB2400	TMB1462 *amyE*::pMG609; cm^r	This study
TMB2455	W168 *amyE*::pMG612; cm^r	This study
TMB2456	W168 *amyE*::pMG613; cm^r	This study
TMB2457	W168 *amyE*::pMG614; cm^r	This study
TMB2460	W168 *amyE*::pMG617; cm^r	This study
TMB2461	W168 *amyE*::pMG618; cm^r	This study
TMB2462	W168 *amyE*::pMG619; cm^r	This study
TMB2463	TMB1462 *amyE*::pMG614; cm^r	This study
TMB2464	TMB1460 *amyE*::pMG614; cm^r	This study
TMB2465	TMB1462 *amyE*::pMG613; cm^r	This study
TMB2466	TMB1460 *amyE*::pMG613; cm^r	This study
TMB2467	TMB1462 *amyE*::pMG619; cm^r	This study
TMB2468	TMB1460 *amyE*::pMG619; cm^r	This study
TMB2469	TMB1462 *amyE*::pMG618; cm^r	This study
TMB2470	TMB1460 *amyE*::pMG618; cm^r	This study
TMB2475	TMB1462 *amyE*::pMG605; cm^r	This study
TMB2505	W168 *amyE*::pCF608; cm^r	This study
TMB2506	W168 *amyE*::pCF609; cm^r	This study
TMB2507	W168 *amyE*::pCF610; cm^r	This study
TMB2508	W168 *amyE*::pCF611; cm^r	This study
TMB2509	W168 *amyE*::pMG621; cm^r	This study
TMB2510	TMB1460 *amyE*::pMG621; cm^r	This study
TMB2511	TMB1462 *amyE*::pMG621; cm^r	This study
TMB2512	W168 *amyE*::pMG622; cm^r	This study
TMB2513	TMB1460 *amyE*::pMG622; cm^r	This study
TMB2514	TMB1462 *amyE*::pMG622; cm^r	This study
TMB2515	W168 *amyE*::pCF612; cm^r	This study
TMB2516	TMB1460 *amyE*::pCF612; cm^r	This study
TMB2517	TMB1462 *amyE*::pCF612; cm^r	This study
TMB2518	W168 *amyE*::pCF613; cm^r	This study
TMB2519	TMB1460 *amyE*::pCF613; cm^r	This study
TMB2520	TMB1462 *amyE*::pCF613; cm^r	This study
TMB2536	W168 *amyE*::pCF614; cm^r	This study
TMB2537	TMB1460 *amyE*::pCF614; cm^r	This study
TMB2538	TMB1462 *amyE*::pCF614; cm^r	This study
TMB2539	W168 *amyE*::pCF615; cm^r	This study
TMB2540	TMB1460 *amyE*::pCF615; cm^r	This study
TMB2541	TMB1462 *amyE*::pCF615; cm^r	This study
TMB2631	W168 *amyE*::pCF616	This study
TMB2632	TMB1460 *amyE*::pCF616	This study
TMB2633	TMB1462 *amyE*::pCF616	This study
TMB2637	W168 *amyE*::pCF618	This study
TMB2638	TMB1460 *amyE*::pCF618	This study
TMB2639	TMB1462 *amyE*::pCF618	This study
TMB2640	W168 *amyE*::pCF619; cm^r	This study
TMB2641	TMB1460 *amyE*::pCF619; cm^r	This study
TMB2642	TMB1462 *amyE*::pCF619; cm^r	This study
TMB2643	W168 *amyE*::pCF620; cm^r	This study
TMB2644	TMB1460 *amyE*::pCF620; cm^r	This study
TMB2645	TMB1462 *amyE*::pCF620; cm^r	This study

818   ^{a} Resistant cassettes: cm, chloramphenicol; r, resistant.

819 **Table S2.** Vectors and plasmids used in this study.

Plasimd	Genotype or characteristic(s)	Primers used for cloning	Reference or source
Vectors			
pAC6	Vector for transcriptional promoter fusions to *lacZ* in *B. subtilis*, integrates in *amyE*; cm^r		(Stülke *et al.*, 1997)
pET16b	Vector for IPTG-inducible gene expression; carries a N-terminal His$_{10}$-tag sequence; amp^r		Novagen
pMAD	Vector for construction of unmarked deletions in *B. subtilis*, temperature sensitive replicon; mls^r		(Arnaud *et al.*, 2004)
Plasmids			
pAS601	pAC6 P$_{psdA}$ (-99 to +30) -*lacZ*	1591/0600	This study
pAS602	pAC6 P$_{psdA}$ (-97 to +30) -*lacZ*	1592/0600	This study
pAS603	pAC6 P$_{psdA}$ (-104 to +30) -*lacZ*	1688/0600	This study
pAS604	pAC6 P$_{psdA}$ (-103 to +30) -*lacZ*	1687/0600	This study
pAS605	pAC6 P$_{psdA}$ (-102 to +30) -*lacZ*	1686/0600	This study
pAS606	pAC6 P$_{psdA}$ (-101 to +30) -*lacZ*	1685/0600	This study
pAS607	pAC6 P$_{psdA}$ (-100 to +30) -*lacZ*	1684/0600	This study
pAS608	pAC6 P$_{psdA}$ (-98 to +30) -*lacZ*	1683/0600	This study
pAS609	pAC6 P$_{psdA}$ (-96 to +30) -*lacZ*	1682/0600	This study
pAS610	pAC6 P$_{psdA}$ (-95 to +30) -*lacZ*	1681/0600	This study
pAS613	pAC6 P$_{bceA}$ (-110 to +82) -*lacZ*	1869/0555	This study
pAS614	pAC6 P$_{bceA}$ (-109 to +82) -*lacZ*	1870/0555	This study
pAS615	pAC6 P$_{bceA}$ (-108 to +82) -*lacZ*	1871/0555	This study
pAS616	pAC6 P$_{bceA}$ (-107 to +82) -*lacZ*	1872/0555	This study
pAS617	pAC6 P$_{bceA}$ (-106 to +82) -*lacZ*	1873/0555	This study
pAS618	pAC6 P$_{bceA}$ (-105 to +82) -*lacZ*	1874/0555	This study
pAS619	pAC6 P$_{bceA}$ (-104 to +82) -*lacZ*	1875/0555	This study
pAS620	pAC6 P$_{bceA}$ (-103 to +82) -*lacZ*	1876/0555	This study
pCF101	pMAD Δ*bceRS*	2351/2352 2353/2354	This study
pCF103	pMAD Δ*psdRS*	2357/2358 2359/2360	This study
pCF120	pET16b *bceR*	2007/2008	This study
pCF601	pAC6 P$_{psdA}$ (-126 to +30)-*lacZ*	0674/0600	This study
pCF608	pAC6 P$_{bceA}$ (-122 to +82) main binding site mutation-*lacZ*	2262/3563 3564/0555	This study
pCF609	pAC6 P$_{bceA}$ (-122 to +82) second binding site mutation-*lacZ*	0554/3565 3566/0555	This study
pCF610	pAC6 P$_{psdA}$ (-126 to +30) main binding site mutation-*lacZ*	2262/3567 3568/0600	This study
pCF611	pAC6 P$_{psdA}$ (-126 to +30) second binding site mutation-*lacZ*	0674/3569 3570/0600	This study
pCF612	pAC6 P$_{psdA}$ (-126 to +30) second binding site switched into the corresponding region of P$_{bceA}$ -*lacZ*	0674/3553 3554/0600	This study
pCF613	pAC6 P$_{psdA}$ (-126 to +30) linker and second binding site switched into the corresponding region of P$_{bceA}$ -*lacZ*	0674/3557 3558/0600	This study
pCF614	pAC6 P$_{bceA}$ (-122 to +82) main binding site, linker and second binding site switched into the corresponding region of P$_{psdA}$ -*lacZ*	3692/0555	This study
pCF615	pAC6 P$_{psdA}$ (-126 to +30) main binding site, linker and second binding site switched into the corresponding region of P$_{bceA}$ -*lacZ*	3693/0600	This study
pCF616	pAC6 P$_{bceA}$ (-122 to +82) main binding site and second binding site switched into the corresponding region of P$_{psdA}$ -*lacZ*	3719/0555	This study

pCF618	pAC6 P$_{bceA}$ (-122 to +82) main binding site and linker switched into the corresponding region of P$_{psdA}$ -*lacZ*	3721/0555	This study
pCF619	pAC6 P$_{psdA}$ (-126 to +30) main binding site and second binding site switched into the corresponding region of P$_{bceA}$ -*lacZ*	3720/0600	This study
pCF620	pAC6 P$_{psdA}$ (-126 to +30) main binding site and linker switched into the corresponding region of P$_{bceA}$ -*lacZ*	3722/0600	This study
pER603	pAC6 P$_{bceA}$ (-122 to +82) -*lacZ*	0554/0555	(Rietkötter *et al.*, 2008)
pER605	pAC6 P$_{psdA}$ (-110 to +30)-*lacZ*	0599/0600	(Rietkötter *et al.*, 2008)
pMG600	pAC6 P$_{bceA}$ (-122 to -46) - P$_{psdA}$ (-36 to +30) (BP1) -*lacZ*	1689/3240 3241/0600	This study
pMG601	pAC6 P$_{bceA}$ (-122 to -56) - P$_{psdA}$ (-46 to +30) (BP2) -*lacZ*	1689/3242 3243/0600	This study
pMG603	pAC6 P$_{bceA}$ (-122 to -76) - P$_{psdA}$ (-66 to +30) (BP3) -*lacZ*	1689/3246 3247/0600	This study
pMG604	pAC6 P$_{bceA}$ (-122 to -88) - P$_{psdA}$ (-79 to +30) (BP4) -*lacZ*	1689/3248 3249/0600	This study
pMG605	pAC6 P$_{psdA}$ (-126 to -37) - P$_{bceA}$ (-45 to +82) (PB1) -*lacZ*	0674/3230 3231/0555	This study
pMG606	pAC6 P$_{psdA}$ (-126 to -47) - P$_{bceA}$ (-55 to +82) (PB2) -*lacZ*	0674/3232 3233/0555	This study
pMG608	pAC6 P$_{psdA}$ (-126 to -67) - P$_{bceA}$ (-75 to +82) (PB3) -*lacZ*	0674/3236 3237/0555	This study
pMG609	pAC6 P$_{psdA}$ (-126 to -80) - P$_{bceA}$ (-87 to +82) (PB4) -*lacZ*	0674/3238 3239/0555	This study
pMG612	pAC6 P$_{bceA}$ (-122 to + 82) linker mutation -*lacZ*	0146/3351 3395/0010	This study
pMG613	pAC6 P$_{bceA}$ (-122 to + 82) linker switched into the corresponding part of P$_{psdA}$ -*lacZ*	0146/3401 3400/0010	This study
pMG614	pAC6 P$_{bceA}$ (-122 to + 82) main binding site switched into the corresponding region of P$_{psdA}$ -*lacZ*	0146/3419 3354/0010	This study
pMG617	pAC6 P$_{psdA}$ (-126 to + 30) linker mutation -*lacZ*	0146/3353 3352/0600	This study
pMG618	pAC6 P$_{psdA}$ (-126 to + 30) linker switched into the corresponding region of P$_{bceA}$ -*lacZ*	0146/3403 3402/0600	This study
pMG619	pAC6 P$_{psdA}$ (-126 to + 30) main binding site switched into the corresponding region of P$_{bceA}$ -*lacZ*	0146/3357 3356/0600	This study
pMG621	pAC6 P$_{bceA}$ (-122 to + 82) second binding site switched into the corresponding region of P$_{psdA}$ -*lacZ*	2262/3551 3552/0555	This study
pMG622	pAC6 P$_{bceA}$ (-122 to + 82) linker and the second binding site switched into the corresponding region of P$_{psdA}$ -*lacZ*	2262/3555 3556/0555	This study
pJS605	pAC6 P$_{bceA}$ (-111 to +82) -*lacZ*	1307/0555	This study

820 Amp, ampicillin; cm, chloramphenicol; mls, macrolide-lincosamide-streptogramin B group antibiotics; r,
821 resistant.

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823   **Supplemental Table S3.** Primers used in this study.

Primer name	Sequence (5'-3')[a]	Use
TM0010	CTTCGCTATTACGCCAGCTGG	*lacZ* check rev
TM0146	GTCTGCTTTCTTCATTAGAATCAATCC	*cat* check rev
TM0554	GATC<u>GAATTC</u>GAACATGTCATAAGCGTGTGACG	P*bceA* (-122) fwd
TM0555	GATC<u>GGATCC</u>TATCGATGCCCTTCAGCACTTCC	P*bceA* rev
TM0599	AGTC<u>GAATTC</u>CACCCTCGTGAATGTGACAGC	P*psdA* (-110) *fwd*
TM0600	AGTC<u>GGATCC</u>CGATAGGTTCGTTGTTTGCAACACG	P*psdA* rev
TM0674	AGTC<u>GAATTC</u>TCGTGTTTTCAAGTGACACC	P*psdA* (-126) fwd
TM1307	GATC<u>GAATTC</u>CAAGCGTGTGACGAAAATGTCACAT	P*bceA* (-111) fwd
TM1591	AGTC<u>GAATTC</u>ATGTGACAGCATTGTAAGATTGG	P*psdA* (-99) fwd
TM1592	AGTC<u>GAATTC</u>GTGACAGCATTGTAAGATTGG	P*psdA* (-97) fwd
TM1681	AGTC<u>GAATTC</u>ACGACAGCATTGTAAGATTGG	P*psdA* (-95) fwd
TM1682	AGTC<u>GAATTC</u>ATGACAGCATTGTAAGATTGG	P*psdA* (-96) fwd
TM1683	AGTC<u>GAATTC</u>TGTGACAGCATTGTAAGATTGG	P*psdA* (-98) fwd
TM1684	AGTC<u>GAATTC</u>TAATGTGACAGCATTGTAAG	P*psdA* (-100) fwd
TM1685	AGTC<u>GAATTC</u>GAATGTGACAGCATTGTAAG	P*psdA* (-101) fwd
TM1686	AGTC<u>GAATTC</u>TGAATGTGACAGCATTGTAAG	P*psdA* (-102) fwd
TM1687	AGTC<u>GAATTC</u>AGTGAATGTGACAGCATTGTAAG	P*psdA* (-103) fwd
TM1688	AGTC<u>GAATTC</u>CGTGAATGTGACAGCATTGTAAG	P*psdA* (-104) fwd
TM1689	CCGATGATAAGCTGTCAAAC	pAC6 bandshifts
TM1869	ATGC<u>GAATTC</u>AGCGTGTGACGAAAATG	P*bceA* (-110) fwd
TM1870	ATGC<u>GAATTC</u>CGCGTGTGACGAAAATGTC	P*bceA* (-109) fwd
TM1871	ATGC<u>GAATTC</u>ACGCGTGTGACGAAAATGTC	P*bceA* (-108) fwd
TM1872	ATGC<u>GAATTC</u>AAGTGTGACGAAAATGTC	P*bceA* (-107) fwd
TM1873	ATGC<u>GAATTC</u>AAATGTGACGAAAATGTC	P*bceA* (-106) fwd
TM1874	ATGC<u>GAATTC</u>GTGACGAAAATGTCAC	P*bceA* (-105) fwd
TM1875	ATGC<u>GAATTC</u>ATGACGAAAATGTCAC	P*bceA* (-104) fwd
TM1876	ATGC<u>GAATTC</u>AAGACGAAAATGTCAC	P*bceA* (-103) fwd
TM2007	ATCG<u>CTCGAG</u>TTGTTTAAACTTTTGCTGATTG	*bceR* fwd
TM2008	ATCG<u>GGATCC</u>TTAATCATAGAACTTGTCCTC	*bceR* rev
TM2262	GAGCGTAGCGAAAAATCC	pAH328 checkfwd
TM2351	AATTT<u>GGATCC</u>GAGGAAGCAAAAGGAAATC	*bceRS* deletion up fwd
TM2352	CTTGATTTCATGAAACAGCG	*bceRS* deletion up rev
TM2353	ctgtttcatgaaatcaag ATATTGATGTTGAGTCGGAG	*bceRS* deletion down fwd
TM2354	AATTCCATGGTTCAAATTTCGCAGGATGAG	*bceRS* deletion down rev
TM2357	AATTT<u>GGATCC</u>CTACGATCTAAATGGTTTCC	*psdRS* deletion up fwd
TM2358	ATTTTTGAAGATGACCGCCC	*psdRS* deletion up rev
TM2359	cggtcatcttcaaaaat CACTGTGATGACCATCGTG	*psdRS* deletion down fwd
TM2360	AATTCCATGGACCGAAACGGCAAACACAC	*psdRS* deletion down rev
TM3230	GTCAGCATCCTCCCATCGAAC	PB1 up rev
TM3231	cgatgggaggatgctgac TTCCTTTTTATAATGAGATTATCC	PB1 down fwd
TM3232	TCCCATCGAACTTTCTTGCAATTC	PB2 up rev
TM3233	caagaaagttcgatggga AAGCCCGGCATTCCTTTTTATAATG	PB2 down fwd
TM3236	TTCCGCTCCCCAATCTTACAATG	PB3 up rev
TM3237	taagattggggagcggaa TTGTTCGCCGTATCGAAGG	PB3 down fwd
TM3238	ATCTTACAATGCTGTCACATTC	PB4 up rev
TM3239	gtgacagcattgtaagat GCTTTTCTTTTTTGTTCGCCG	PB4 down fwd
TM3240	TGCCGGGCTTTTCCTTCGATAC	BP1 up rev
TM3241	cgaaggaaaagcccggca TTCCTTTTTATAATAAAGAAAAAGG	BP1 down fwd
TM3242	TTCCTTCGATACGGCGAAC	BP2 up rev
TM3243	ttcgccgtatcgaaggaa GGATGCTGACTTCCTTTTTATAATAAAG	BP2 down fwd
TM3246	AAAAGAAAAGCATGTGACATTTTC	BP3 up rev
TM3247	gtcacatgcttttcttttTTGCAAGAAAGTTCGATGGGAGG	BP3 down fwd
TM3248	ATGTGACATTTTCGTCACACGC	BP4 up rev
TM3249	gtgacgaaaatgtcacatTGGGGAGCGGAATTGCAAGAAAG	BP4 down fwd
TM3351	cgaacaaatttgtataGCATGTGACATTTTCGTC	P*bceA* L-M up rev
TM3352	cgcacggcaattgcaAGAAAGTTCGATGGGAGG	P*psdA* L-M down fwd
TM3353	tgcaattgccgtgcgCAATCTTACAATGCTGTCAC	P*psdA* L-M up rev
TM3354	gacagcattgtaagaTGCTTTTCTTTTTTGTTCGCC	P*bceA* M-S down fwd
TM3356	gacgaaaatgtcacaTTGGGGAGCGGAATTGCAAG	P*psdA* M-S down fwd
TM3357	tgtgacattttcgtcACATTCACGAGGGTGTCACTTG	P*psdA* M-S up rev
TM3395	tatacaaatttgttcgCCGTATCGAAGGAAAAGC	P*bceA* L-M down fwd
TM3400	ggcgaacaatccgctcccGCATGTGACATTTTCGTCAC	P*bceA* L-S down fwd

35

TM3401	gggagcggattgttcgccGTATCGAAGG	P*bceA* L-S up rev
TM3402	cttgcaataaaagaaaaCAATCTTACAATGCTGTCAC	P*psdA* L-S down fwd
TM3403	ttttctttattgcaagAAAGTTCGATGGG	P*psdA* L-S up rev
TM3419	tcttacaatgctgtcACACGCTTATGACATGTTCG	P*bceA* M-S up rev
TM3551	ccatcgaactttcttgCAAAAAAGAAAAGCATGTGACATTTTC	P*bceA* S-S up rev
TM3552	caagaaagttcgatGGAAAAGCCCGGCATTCC	P*bceA* S-S down fwd
TM3553	ccttcgatacggcgaaCAATTCCGCTCCCCAATC	P*psdA* S-S up rev
TM3554	ttcgccgtatcgaaGGGAGGATGCTGACTTCC	P*psdA* S-S down fwd
TM3555	actttcttgcaattccgctccccaATGTGACATTTTCGTCACACG	P*bceA* S+L-S up rev
TM3556	ggaattgcaagaaagttcgatGGAAAAGCCCGGCATTCC	P*bceA* S+L-S down fwd
TM3557	tacggcgaacaaaaaagaaaagcATCTTACAATGCTGTCACATTC	P*psdA* S+L-S up rev
TM3558	ttttttgttcgccgtatcgaaGGGAGGATGCTGACTTCC	P*psdA* S+L-S down fwd
TM3563	gcgttaagtcaccgctaaCGCTTATGACATGTTCGAATTCG	P*bceA* M-M up rev
TM3564	ttagcggtgacttaacgcTGCTTTTCTTTTTTGTTCGCCG	P*bceA* M-M down fwd
TM3565	cagctagcagtcagtcagAAAAAGAAAAGCATGTGACATTTTC	P*bceA* S-M up rev
TM3566	ctgactgactgctagctgAAAAGCCCGGCATTCCTTTT	P*bceA* S-M down fwd
TM3567	tacttcggtcaccgctaaTTCACGAGGGTGTCACTTG	P*psdA* M-M up rev
TM3568	ttagcggtgaccgaagtaTTGGGGAGCGGAATTGCAAG	P*psdA* M-M down fwd
TM3569	gtcagtcgtcagtcagtcATTCCGCTCCCCAATCTTAC	P*psdA* S-M up rev
TM3570	gactgactgacgactgacGAGGATGCTGACTTCCTTTT	P*psdA* S-M down fwd
TM3665	GTCATAAGCGTGTGACGAAAATGTCACATGCTTTTCTTTTTTGTTC GCCGTATCGAAGGAAAAGCCCGGCATTCCT	P*bceA* WT fwd (for SPR)
TM3666	AGGAATGCCGGGCTTTTCCTTCGATACGGCGAACAAAAAGAAA AGCATGTGACATTTTCGTCACACGCTTATGAC	Biotin-P*bceA* WT rev (for SPR)
TM3667	CCCTCGTGAATGTGACAGCATTGTAAGATTGGGGAGCGGAATTG CAAGAAAGTTCGATGGGAGGATGCTGACTTCCT	P*psdA* WT fwd (for SPR)
TM3668	AGGAAGTCAGCATCCTCCCATCGAACTTTCTTGCAATTCCGCTCC CCAATCTTACAATGCTGTCACATTCACGAGGG	Biotin-P*psdA* WT rev (for SPR)
TM3669	GTCATAAGCGTTAGCGGTGACTTAACGCTGCTTTTCTTTTTTGTTC GCCGTATCGAAGGAAAAGCCCGGCATTCCT	P*bceA* M-M fwd (for SPR)
TM3670	AGGAATGCCGGGCTTTTCCTTCGATACGGCGAACAAAAAGAAA AGCAGCGTTAAGTCACCGCTAACGCTTATGAC	Biotin-P*bceA* M-M rev (for SPR)
TM3671	CCCTCGTGAATTAGCGGTGACCGAAGTATTGGGGAGCGGAATTG CAAGAAAGTTCGATGGGAGGATGCTGACTTCCT	P*psdA* M-M fwd (for SPR)
TM3672	AGGAAGTCAGCATCCTCCCATCGAACTTTCTTGCAATTCCGCTCC CCAATACTTCGGTCACCGCTAATTCACGAGGG	Biotin-P*psdA* M-M rev (for SPR)
TM3673	GTCATAAGCGTGTGACGAAAATGTCACATGCTTTTCTTTTTCTGA CTGACTGCTAGCTGAAAAGCCCGGCATTCCT	P*bceA* S-M fwd (for SPR)
TM3674	AGGAATGCCGGGCTTTTCAGCTAGCAGTCAGTCAGAAAAAGAAA AGCATGTGACATTTTCGTCACACGCTTATGAC	Biotin-P*bceA* S-M rev (for SPR)
TM3675	CCCTCGTGAATGTGACAGCATTGTAAGATTGGGGAGCGGAATGA CTGACTGACGACTGACGAGGATGCTGACTTCCT	P*psdA* S-M fwd (for SPR)
TM3676	AGGAAGTCAGCATCCTCGTCAGTCGTCAGTCAGTCATTCCGCTCC CCAATCTTACAATGCTGTCACATTCACGAGGG	Biotin-P*psdA* S-M rev (for SPR)
TM3677	TCACGAATTACCATCTACACCCTGCCAAAAATTTGATAAACTTAT TTTATAAAAAAATTGAAACCTTTTGAAACGAA	P*sigW* WT fwd (for SPR)
TM3678	TTCGTTTCAAAAGGTTTCAATTTTTTTATAAAATAAGTTTATCAAA TTTTTGGCAGGGTGTAGATGGTAATTCGTGA	Biotin-P*sigW* WT rev (for SPR)
TM3692	GATC<u>GAATTC</u>GAACATGTCATAAGCGTGTGACAGCATTGTAAGA TTGGGGAGCGGAATTGC	P*bceA* M+L+S-S fwd
TM3693	AGTC<u>GAATTC</u>TCGTGTTTTCAAGTGACACCCTCGTGAATGTGACG AAAATGTCACATGCTTTTCTTTTTTGTTCGC	P*psdA* M+L+S-S fwd
TM3719	GATC<u>GAATTC</u>GAACATGTCATAAGCGTGTGACAGCATTGTAAGA TGCTTTTCTTTTTTGCAAG	P*bceA* M+S-S fwd
TM3720	AGTC<u>GAATTC</u>TCGTGTTTTCAAGTGACACCCTCGTGAATGTGACG AAAATGTCACATTGGGGAGCGGAATTG	P*psdA* M+S-S fwd
TM3721	GATC<u>GAATTC</u>GAACATGTCATAAGCGTGTGACAGCATTGTAAGA TTG	P*bceA* M+L-S fwd
TM3722	AGTC<u>GAATTC</u>TCGTGTTTTCAAGTGACACCCTCGTGAATGTGACG AAAATGTCACATG	P*psdA* M+L-S fwd

824 *a* Restriction sites are underlined; overlaps to other primers for PCR fusions are shown by lower case letters.

36

825   **Figure legends**

826

827   **Figure 1. Model of signal transduction pathways of two Bce-like systems after induction**

828   **with corresponding AMPs in *Bacillus subtilis*.** The TCSs Bce and Psd and their inducing

829   antibiotics as signal inputs are highlighted black and grey, respectively. For reasons of

830   simplicity, the ABC transporters of both systems are not shown. Solid arrows indicate the

831   signal transduction pathway within one system, while cross-regulation between BceS and

832   PsdR is highlighted by the dotted arrow. On each promoter, MBS representing for the main

833   binding site and SBS representing for the secondary binding of Bce-like RRs are filled with

834   white on *bceA* promoter and slashes on *psdA* promoter. CM, cell membrane.

835

836   **Figure 2. Functional analysis of *bceA* and *psdA* promoters of *B. subtilis*.** (A) DNA

837   sequence alignment of the *bceA* promoter and the *psdA* promoter. Different motifs are framed

838   and annotated underneath the DNA sequence. Important positions on each promoter are

839   marked with arrows according to the start codon of the corresponding regulated gene. Half

840   binding sites of Bce-like RRs on each promoter are emphasized in bold face. Activities of (B)

841   truncated constructions of the *bceA* promoter (from -122: +82 to -103: +82) and (C) truncated

842   constructions of the *psdA* promoter (from -126: +30 to -95: +30) according to the start codon

843   of regulated genes. Activities of (D) P$_{bceA}$ mutants and (E) P$_{psdA}$ mutants with MBS^R (main

844   binding site random mutation), L^R (linker random mutation) and SBS^R (secondary binding site

845   random mutation) are compared with the corresponding WT promoters. All promoter

846   constructions were fused to *lacZ* and introduced into *amyE* locus of *B. subtilis* 168. Cultures

847   growing exponentially in LB were challenged with Zn^{2+}-bacitracin 30 µg ml^{-1} (black bars) or

848   nisin 2 µg ml^{-1} (grey bars) for 30 min, comparing with the non-induced condition (white bars).

849   β-galactosidase activities are expressed in Miller Units (MU) (Miller, 1972) and results are

850 shown as the mean plus standard deviation of three biological replicates. A log scale is

851 applied for reasons of clarity.

852

853 **Figure 3. Functional studies of chimeric promoters derived from P$_{bceA}$ ("B") and P$_{psdA}$**

854 **("P").** Schematic of series of chimeric promoters (A) BP1-4, *bceA* promoter fragments (black)

855 with gradual substitutions of 3' region by increased corresponding parts of *psdA* promoter

856 (grey) and (B) PB1-4 vice versa are compared with WT P$_{bceA}$ and P$_{psdA}$. The MBS and SBS of

857 P$_{bceA}$ and P$_{psdA}$ are represented as in Fig.1. Grey dashed lines indicate the fusion points of each

858 chimera. (C to H) Activities of chimeric promoters compared with WT promoters in different

859 genetic backgrounds of *B. subtilis*. Transcriptional *lacZ* fusions of WT promoters (P$_{bceA}$ and

860 P$_{psdA}$) as well as different sets of chimeras (BP1-4 and PB1-4) were integrated at the *amyE*

861 locus of *B. subtilis* wildtype (WT), Δ*psdRS* strain (TMB1462) and Δ*bceRS* strain (TMB1460).

862 Promoter activities were measured by β-galactosidase assay as described in Fig. 2. (C) BP1-4

863 in WT, (D) BP1-4 in Δ*psdRS* strain, (E) BP1-4 Δ*bceRS* strain, (F) PB1-4 in WT, (G) PB1-4 in

864 Δ*bceRS* strain and (H) PB1-4 in Δ*psdRS* strain. For reasons of clarity, the values of promoter

865 activities induced by bacitracin are represented as % relative to the native P$_{bceA}$, while the

866 values of promoter activities induced by nisin are represented as % relative to the native P$_{psdA}$

867 promoters. Both wild type promoters are set to 100% after subtraction of the uninduced

868 promoter activities. The original data sets corresponding to Fig. 3 are provided in Fig. S1.

869 Black and grey bars, induction with bacitracin and nisin, respectively.

870

871 **Figure 4. Unravelling the roles of different promoter elements in RR-promoter**

872 **specificity.** (A and B) Schematic of chimeric promoters derived from P$_{bceA}$ and P$_{psdA}$,

873 respectively. Composition of each chimeric promoter is indicated as follows: M, main binding

874 site; L, linker; S, secondary binding site. MBS and SBS from P$_{bceA}$ and P$_{psdA}$ are indicated as

875 in Fig.1. (C to H) Activities of chimeric promoters compared with WT promoters in different

38

876   genetic backgrounds of *B. subtilis*. Transcriptional *lacZ* fusions of WT promoters (P*bceA* and

877   P*psdA*) as well as different sets of chimeras from (A) and (B) were integrated at *amyE* locus in

878   *B. subtilis* WT strain, Δ*psdRS* strain (TMB1462) and Δ*bceRS* strain (TMB1460). Promoter

879   activities were measured by β-galactosidase assay as described for Fig. 2. (C) P*bceA*-derived

880   chimeras in WT, (D) P*bceA*-derived chimeras in Δ*psdRS* strain, (E) P*bceA*-derived chimeras in

881   Δ*bceRS* strain, (F) P*psdA*-derived chimeras in WT, (G) P*psdA*-derived chimeras in Δ*bceRS* strain

882   and (H) P*psdA*-derived chimeras in Δ*psdRS* strain. Black bars and grey bars represent samples

883   induced with bacitracin and nisin, respectively. Data representation as described for Fig. 3;

884   original data sets are provided in Fig. S2.

885

886   **Figure 5. *In vitro* binding of BceR-P to P*bceA* and P*psdA*.** Increasing concentrations of

887   phosphorylated 10×His-BceR were incubated with 30 fmol of different 6FAM-labeled

888   promoter DNA fragments as follows: (A) P*bceA* from -122 to +82, (B) P*psdA* from -126 to +30,

889   (C) P*bceA* SBS^R (SBS inactivated), (D) P*bceA* MBS^R (MBS inactivated), and (E) P*sigW* as a

890   negative control. Schematics of *bceA*-like promoters and corresponding mutants are shown in

891   the lower left corner of each gel. The concentrations of phosphorylated BceR are indicated

892   above the gel by [BceR-P] in μM. 900 fmol of unlabelled competitor (comp.) DNA fragments

893   containing P*bceA*, P*psdA* and P*sigW* were added for lanes 6, 7 and 8, respectively, in (A) and (B).

894

895   **Figure 6. Surface plasmon resonance spectroscopy of and IM analysis BceR-P binding**

896   **within the P*bceA* and P*psdA* promoter region.** (A) BceR binding to P*bceA*, (B) BceR-P binding

897   to P*bceA*, (C) BceR-P binding to P*bceA* MBS^R (MBS inactivated), and (D) BceR-P binding to

898   P*bceA* SBS^R (SBS inactivated), (E) IM and *in silico* sensorgrams of BceR binding to P*bceA*,; (F)

899   IM and *in silico* sensorgrams of BceR binding to P*bceA* SBS^R (SBS inactivated), (G) BceR

900   binding to P*psdA*, (H) BceR-P binding to P*psdA*, (I) BceR-P binding to P*psdA* MBS^R, (K) BceR-P

901   binding to P*psdA* SBS^R), (L) IM and *in silico* sensorgrams of BceR binding to P*psdA*, and (M)

902   IM and *in silico* sensorgrams of BceR binding to P$_{psdA}$ SBS^R (SBS inactivated). SPR

903   sensorgrams: 0.2 nM (red line), 0.5 nM (brown line), 1 nM (dark blue line), 2.5 nM (magenta

904   line), 5 nM (green line), 7.5 nM (lime green line), and 10 nM (blue line), respectively, of each

905   of purified BceR or BceR-P was passed over the chip. The sensorgrams show each one

906   representative example of three independently performed experiments. IM analyses: the blue

907   spots in the IMs represent the fast ON/fast OFF interaction, which corresponds to the SBS, the

908   green spots the slow ON/slow OFF interaction corresponding to the higher affine MBS. The

909   respective calculated sensorgrams are shown in the same colors. The calculated overall

910   affinities (K$_D$), as well as the ON (k$_a$) and OFF (k$_d$) rates, are indicated below the respective *in*

911   *silico* sensorgram. The grey shapes of the IM peaks represent the weighing factors meaning

912   the darker the grey scale, the stronger the contribution.

913

914   **Figure 7. Model of the specific transcriptional activation of P$_{bceA}$ by BceR and RNA**

915   **polymerase.** Initially, a BceR dimer (black), but not a PsdR dimer (grey) preferentially binds

916   to the MBS of P$_{bceA}$. This interaction then facilitates the binding of a second BceR dimer to

917   the SBS directly upstream of the -10 element of P$_{bceA}$. This second binding event then

918   mediates the binding of the σ^A subunit of the RNA polymerase holo-enzyme to the promoter

919   region to ultimately initiate transcription. Presumably, the structure of the DNA is altered by

920   the linker region between two binding sites (DNA bending). See discussion for details.

921

922   **Figure S1. Functional studies of chimeric promoters derived from P$_{bceA}$ ("B") and P$_{psdA}$**

923   **("P").** Schematic of series of chimeric promoters (A) BP1-4, *bceA* promoter fragments (black)

924   with gradual substitutions of 3' region by increased corresponding parts of *psdA* promoter

925   (grey) and (B) PB1-4 vice versa are compared with WT P$_{bceA}$ and P$_{psdA}$. The MBS and SBS of

926   P$_{bceA}$ and P$_{psdA}$ are represented as in Fig.1. Grey dashed lines indicate the fusion points of each

927   chimera. (C to H) Activities of chimeric promoters compared with WT promoters in different

928 genetic backgrounds of *B. subtilis*. Transcriptional *lacZ* fusions of WT promoters (P$_{bceA}$ and

929 P$_{psdA}$) as well as different sets of chimeras (BP1-4 and PB1-4) were integrated at the *amyE*

930 locus of *B. subtilis* wildtype (WT), Δ*psdRS* strain (TMB1462) and Δ*bceRS* strain (TMB1460).

931 Promoter activities were measured by β-galactosidase assay as described in Fig. 2. (C) BP1-4

932 in WT, (D) BP1-4 in Δ*psdRS* strain, (E) BP1-4 Δ*bceRS* strain, (F) PB1-4 in WT, (G) PB1-4 in

933 Δ*bceRS* strain and (H) PB1-4 in Δ*psdRS* strain. Black and grey bars, induction with bacitracin

934 and nisin, respectively; white bars, non-induced controls.

935

936 **Figure S2. Unravelling the roles of different promoter elements in RR-promoter**

937 **specificity.** (A and B) Schematic of chimeric promoters derived from P$_{bceA}$ and P$_{psdA}$,

938 respectively. Composition of each chimeric promoter is indicated as follows: M, main binding

939 site; L, linker; S, secondary binding site. MBS and SBS from P$_{bceA}$ and P$_{psdA}$ are indicated as

940 in Fig.1. (C to H) Activities of chimeric promoters compared with WT promoters in different

941 genetic backgrounds of *B. subtilis*. Transcriptional *lacZ* fusions of WT promoters (P$_{bceA}$ and

942 P$_{psdA}$) as well as different sets of chimeras from (A) and (B) were integrated at *amyE* locus in

943 *B. subtilis* WT strain, Δ*psdRS* strain (TMB1462) and Δ*bceRS* strain (TMB1460). Promoter

944 activities were measured by β-galactosidase assay as described for Fig. 2. (C) P$_{bceA}$-derived

945 chimeras in WT, (D) P$_{bceA}$-derived chimeras in Δ*psdRS* strain, (E) P$_{bceA}$-derived chimeras in

946 Δ*bceRS* strain, (F) P$_{psdA}$-derived chimeras in WT, (G) P$_{psdA}$-derived chimeras in Δ*bceRS* strain

947 and (H) P$_{psdA}$-derived chimeras in Δ*psdRS* strain. Black bars and grey bars represent samples

948 induced with bacitracin and nisin, respectively, while white bars stand for non-induced
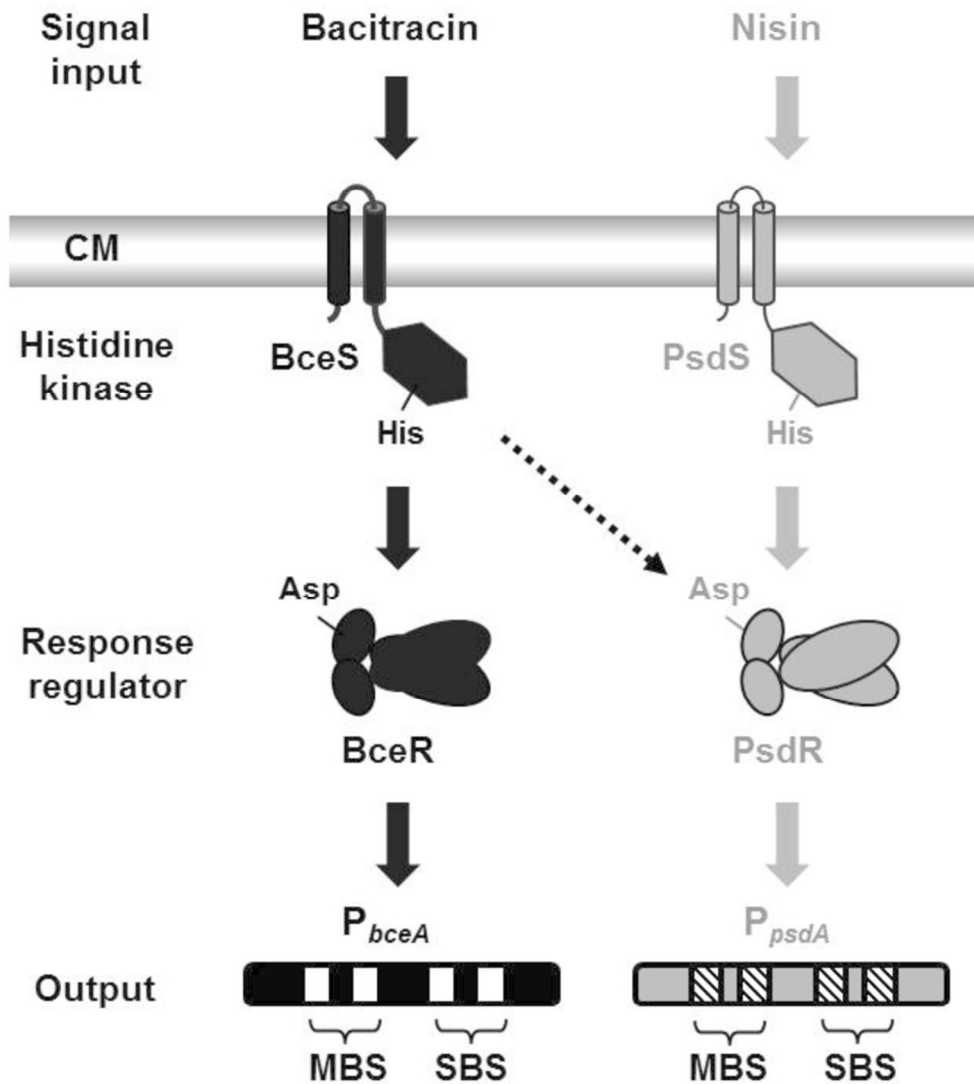
949 controls.

Figure 1. Model of signal transduction pathways of two Bce-like systems after induction with corresponding AMPs in Bacillus subtilis. The TCSs Bce and Psd and their inducing antibiotics as signal inputs are highlighted black and grey, respectively. For reasons of simplicity, the ABC transporters of both systems are not shown. Solid arrows indicate the signal transduction pathway within one system, while cross-regulation between BceS and PsdR is highlighted by the dotted arrow. On each promoter, MBS representing for the main binding site and SBS representing for the secondary binding of Bce-like RRs are filled with white on bceA promoter and slashes on psdA promoter. CM, cell membrane.

85x94mm (300 x 300 DPI)
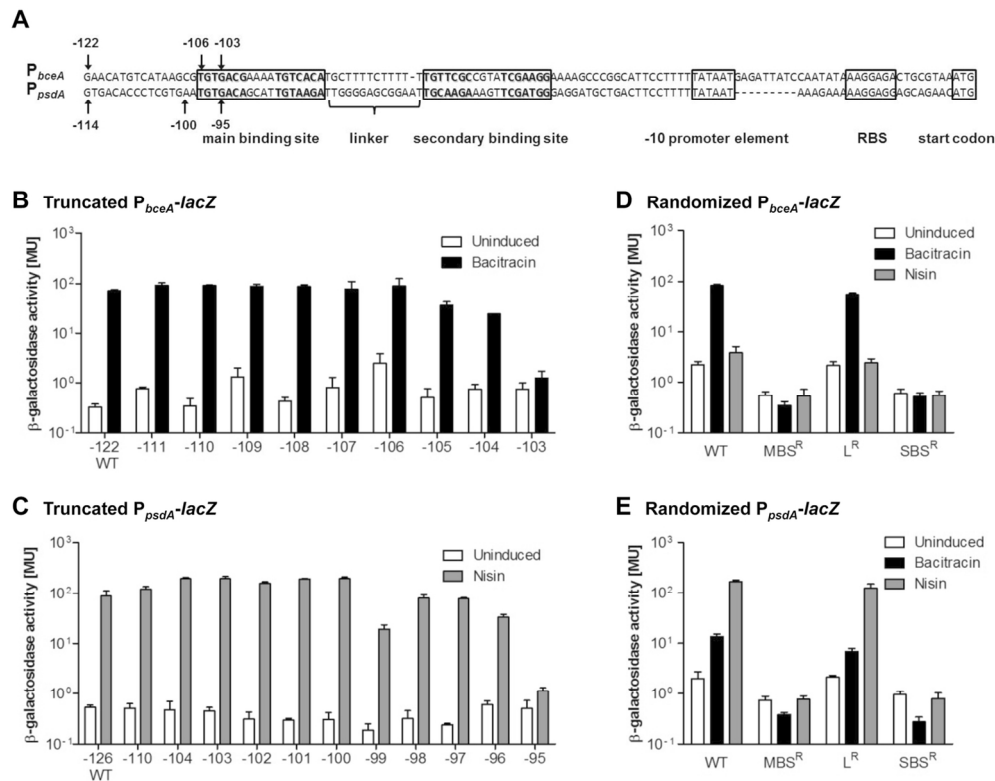
Figure 2. Functional analysis of bceA and psdA promoters of B. subtilis. (A) DNA sequence alignment of the bceA promoter and the psdA promoter. Different motifs are framed and annotated underneath the DNA sequence. Important positions on each promoter are marked with arrows according to the start codon of the corresponding regulated gene. Half binding sites of Bce-like RRs on each promoter are emphasized in bold face. Activities of (B) truncated constructions of the bceA promoter (from -122: +82 to -103: +82) and (C) truncated constructions of the psdA promoter (from -126: +30 to -95: +30) according to the start codon of regulated genes. Activities of (D) PbceA mutants and (E) PpsdA mutants with MBSR (main binding site random mutation), LR (linker random mutation) and SBSR (secondary binding site random mutation) are compared with the corresponding WT promoters. All promoter constructions were fused to lacZ and introduced into amyE locus of B. subtilis 168. Cultures growing exponentially in LB were challenged with Zn2+-bacitracin 30 µg ml-1 (black bars) or nisin 2 µg ml-1 (grey bars) for 30 min, comparing with the non-induced condition (white bars). β-galactosidase activities are expressed in Miller Units (MU) (Miller, 1972) and results are shown as the mean plus standard deviation of three biological replicates. A log scale is applied for reasons of clarity.
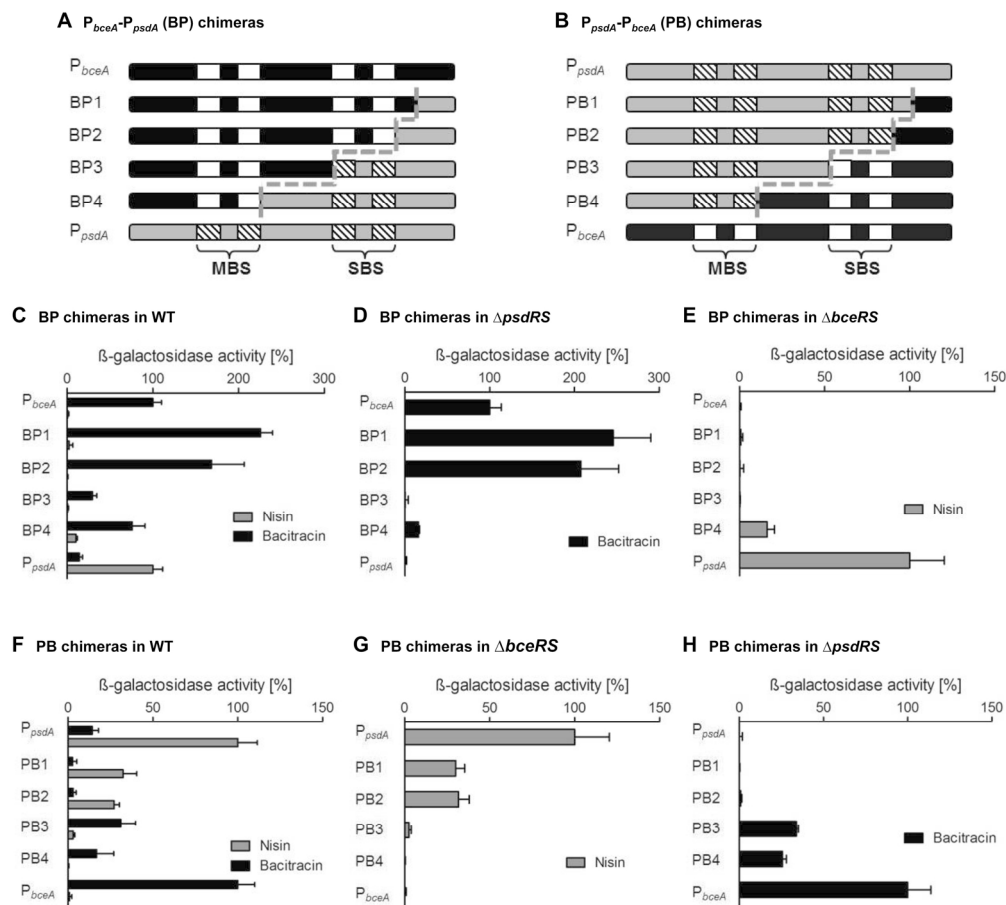
124x98mm (300 x 300 DPI)

Figure 3. Functional studies of chimeric promoters derived from PbceA ("B") and PpsdA ("P"). Schematic of series of chimeric promoters (A) BP1-4, bceA promoter fragments (black) with gradual substitutions of 3' region by increased corresponding parts of psdA promoter (grey) and (B) PB1-4 vice versa are compared with WT PbceA and PpsdA. The MBS and SBS of PbceA and PpsdA are represented as in Fig.1. Grey dashed lines indicate the fusion points of each chimera. (C to H) Activities of chimeric promoters compared with WT promoters in different genetic backgrounds of B. subtilis. Transcriptional lacZ fusions of WT promoters (PbceA and PpsdA) as well as different sets of chimeras (BP1-4 and PB1-4) were integrated at the amyE locus of B. subtilis wildtype (WT), ΔpsdRS strain (TMB1462) and ΔbceRS strain (TMB1460). Promoter activities were measured by β-galactosidase assay as described in Fig. 2. (C) BP1-4 in WT, (D) BP1-4 in ΔpsdRS strain, (E) BP1-4 ΔbceRS strain, (F) PB1-4 in WT, (G) PB1-4 in ΔbceRS strain and (H) PB1-4 in ΔpsdRS strain. For reasons of clarity, the values of promoter activities induced by bacitracin are represented as % relative to the native PbceA, while the values of promoter activities induced by nisin are represented as % relative to the native PpsdA promoters. Both wild type promoters are set to 100% after subtraction of the uninduced promoter activities. The original data sets corresponding to Fig. 3 are provided in Fig. S1. Black and grey bars, induction with bacitracin and nisin, respectively.
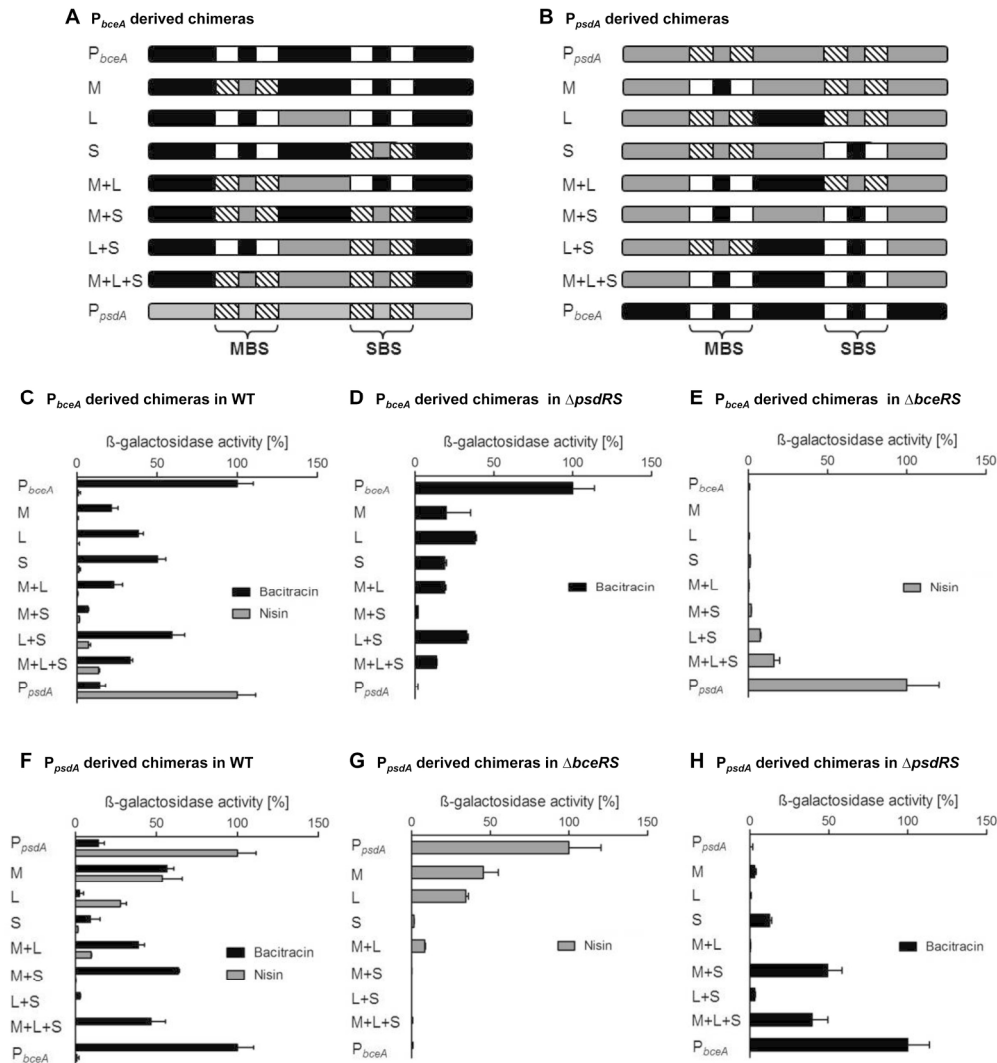
173x161mm (300 x 300 DPI)

Figure 4. Unravelling the roles of different promoter elements in RR-promoter specificity. (A and B) Schematic of chimeric promoters derived from PbceA and PpsdA, respectively. Composition of each chimeric promoter is indicated as follows: M, main binding site; L, linker; S, secondary binding site. MBS and SBS from PbceA and PpsdA are indicated as in Fig.1. (C to H) Activities of chimeric promoters compared with WT promoters in different genetic backgrounds of B. subtilis. Transcriptional lacZ fusions of WT promoters (PbceA and PpsdA) as well as different sets of chimeras from (A) and (B) were integrated at amyE locus in B. subtilis WT strain, ΔpsdRS strain (TMB1462) and ΔbceRS strain (TMB1460). Promoter activities were measured by β-galactosidase assay as described for Fig. 2. (C) PbceA-derived chimeras in WT, (D) PbceA-derived chimeras in ΔpsdRS strain, (E) PbceA-derived chimeras in ΔbceRS strain, (F) PpsdA-derived chimeras in WT, (G) PpsdA-derived chimeras in ΔbceRS strain and (H) PpsdA-derived chimeras in ΔpsdRS strain. Black bars and grey bars represent samples induced with bacitracin and nisin, respectively. Data representation as described for Fig. 3; original data sets are provided in Fig. S2.
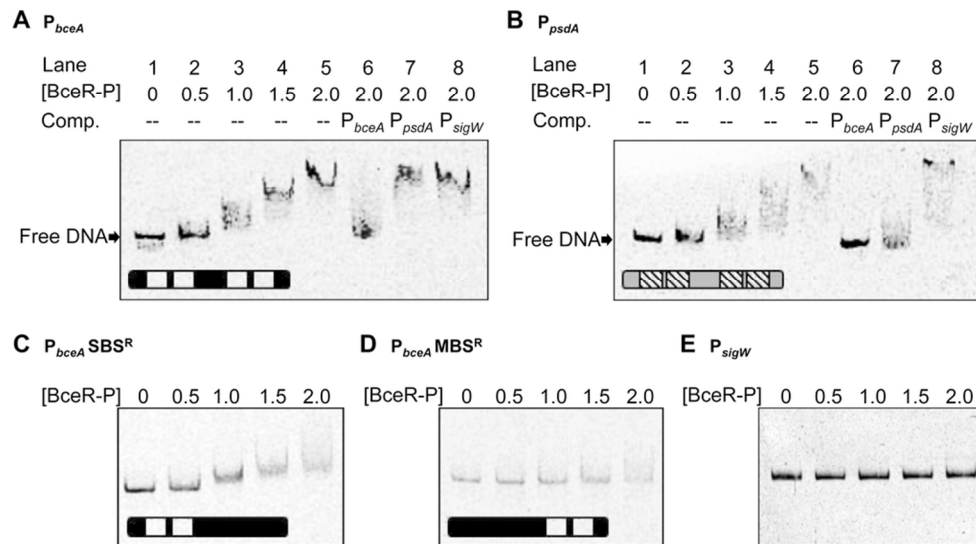
200x215mm (300 x 300 DPI)

Figure 5. In vitro binding of BceR-P to PbceA and PpsdA. Increasing concentrations of phosphorylated 10×His-BceR were incubated with 30 fmol of different 6FAM-labeled promoter DNA fragments as follows: (A) PbceA from -122 to +82, (B) PpsdA from -126 to +30, (C) PbceA SBSR (SBS inactivated), (D) PbceA MBSR (MBS inactivated), and (E) PsigW as a negative control. Schematics of bceA-like promoters and corresponding mutants are shown in the lower left corner of each gel. The concentrations of phosphorylated BceR are indicated above the gel by [BceR-P] in μM. 900 fmol of unlabelled competitor (comp.) DNA fragments containing PbceA, PpsdA and PsigW were added for lanes 6, 7 and 8, respectively, in (A) and (B).
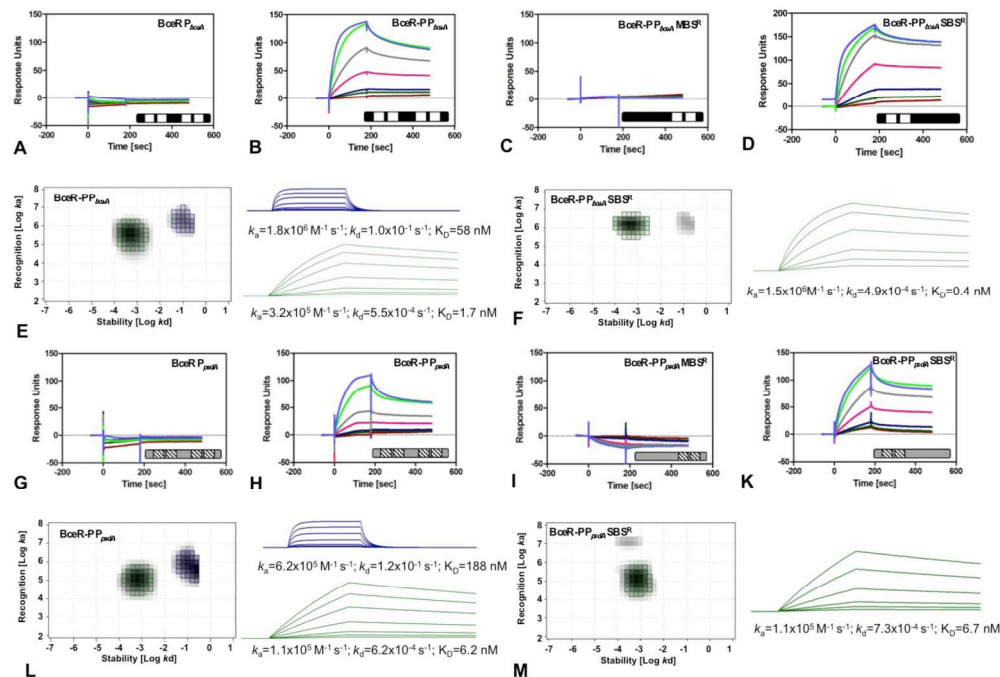
88x49mm (300 x 300 DPI)

Figure 6. Surface plasmon resonance spectroscopy of and IM analysis BceR-P binding within the PbceA and PpsdA promoter region. (A) BceR binding to PbceA, (B) BceR-P binding to PbceA, (C) BceR-P binding to PbceA MBSR (MBS inactivated), and (D) BceR-P binding to PbceA SBSR (SBS inactivated), (E) IM and in silico sensorgrams of BceR binding to PbceA,; (F) IM and in silico sensorgrams of BceR binding to PbceA SBSR (SBS inactivated), (G) BceR binding to PpsdA, (H) BceR-P binding to PpsdA, (I) BceR-P binding to PpsdA MBSR, (K) BceR-P binding to PpsdA SBSR), (L) IM and in silico sensorgrams of BceR binding to PpsdA, and (M) IM and in silico sensorgrams of BceR binding to PpsdA SBSR (SBS inactivated). SPR sensorgrams: 0.2 nM (red line), 0.5 nM (brown line), 1 nM (dark blue line), 2.5 nM (magenta line), 5 nM (green line), 7.5 nM (lime green line), and 10 nM (blue line), respectively, of each of purified BceR or BceR-P was passed over the chip. The sensorgrams show each one representative example of three independently performed experiments. IM analyses: the blue spots in the IMs represent the fast ON/fast OFF interaction, which corresponds to the SBS, the green spots the slow ON/slow OFF interaction corresponding to the higher affine MBS. The respective calculated sensorgrams are shown in the same colors. The calculated overall affinities (KD), as well as the ON (ka) and OFF (kd) rates, are indicated below the respective in silico sensorgram. The grey shapes of the IM peaks represent the weighing factors meaning the darker the grey scale, the stronger the contribution.
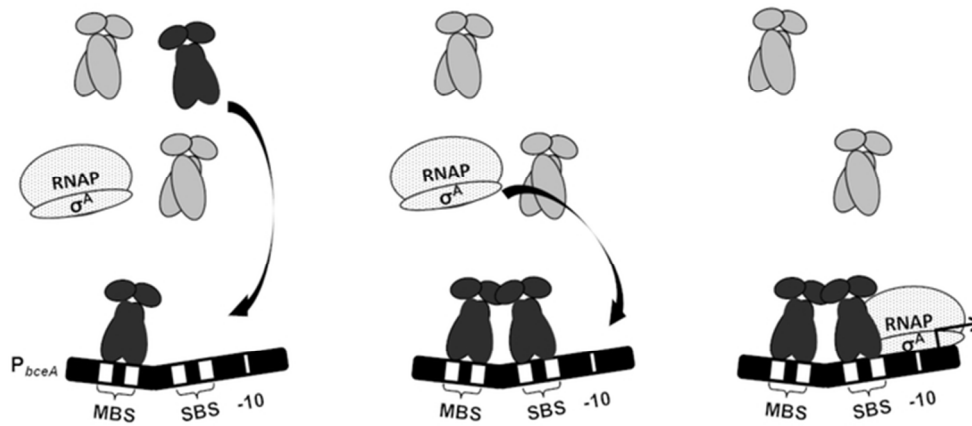
170x117mm (300 x 300 DPI)

Figure 7. Model of the specific transcriptional activation of PbceA by BceR and RNA polymerase. Initially, a BceR dimer (black), but not a PsdR dimer (grey) preferentially binds to the MBS of PbceA. This interaction then facilitates the binding of a second BceR dimer to the SBS directly upstream of the -10 element of PbceA. This second binding event then mediates the binding of the σA subunit of the RNA polymerase holo-enzyme to the promoter region to ultimately initiate transcription. Presumably, the structure of the DNA is altered by the linker region between two binding sites (DNA bending). See discussion for details.

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