Novel regulatory logic in the antibiotic resistance response of *Enterococcus faecalis* against cell envelope targeting antibiotics

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Summary

Enterococcal infections frequently show high levels of antibiotic resistance, including to cell envelope-acting antibiotics like daptomycin (DAP). While we have a good understanding of the resistance mechanisms, less is known about the control of such resistance genes in enterococci. Previous work unveiled a bacitracin resistance network, comprised of the sensory ABC transporter SapAB, the two-component system (TCS) SapRS and the resistance ABC transporter RapAB. Interestingly, components of this system have recently been implicated in DAP resistance, a role usually regulated by the TCS LiaFSR. We here explored the interplay between these two regulatory pathways. Our results show the regulation by SapR of an additional resistance operon, *dltXABCD,* and show that LiaFSR regulates the expression of *sapRS*, placing SapRS target genes under dual control: *dltXABCD* expression depends on both antibiotic-induced cellular damage (LiaFSR) *and* the presence of a substrate drug for the sensory transporter (SapAB). We further show that this network protects *E. faecalis* from antimicrobials produced by potential competitor bacteria, providing a potential rationale for the evolution of this regulatory strategy. The network structure described here can explain why clinical DAP resistance often emerges via mutations in regulatory components, which may ultimately lead to the discovery of new therapeutic targets.

Keywords: antimicrobial peptides, cell envelope stress, antimicrobial resistance, two-component system, signalling

42 # Introduction

43 The rise of antibiotic resistant bacteria is one of the greatest current threats to public health, resulting in
44 670,000 infections a year and 33,000 deaths in Europe alone[1]. Of these infections, the "ESKAPE"
45 organisms[2] (*Enterococci* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*
46 *baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) have driven the rising number of nosocomial
47 and antibiotic-resistant infections in the past decade. Of these bacteria, the enterococci are the second
48 most common causative agent of nosocomial infections in the US; including bacteraemia, endocarditis
49 and urinary tract infections[3–5]. The two species most frequently isolated, *Enterococcus faecalis* and
50 *Enterococcus faecium*, remain a major infection-control challenge, particularly in healthcare settings.

51 Enterococci became recognised as important nosocomial pathogens due to their high level of intrinsic
52 resistance to several antimicrobials[6] (e.g. penicillin, ampicillin and cephalosporins) and their capacity
53 to acquire further resistance determinants. One such acquired resistance is to the glycopeptide antibiotic
54 vancomycin, which occurs through plasmid acquisition and was first reported in the 1988[7,8], 30 years
55 after vancomycin was introduced for clinical use[9]. Despite the molecular mechanisms of vancomycin
56 resistance in enterococci being well understood today[10], infections by vancomycin resistant enterococci
57 (VRE) still result in serious health and economic impacts[11] and are an increasing problem worldwide.

58 One of the last-resort antibiotics used to treat these VRE infections is the lipopeptide antibiotic
59 daptomycin (DAP)[12]. Disappointingly, within 2 years of clinical introduction of the drug in 2003, DAP-
60 resistant enterococcal isolates were reported[13], and in contrast to vancomycin resistance, this occurred
61 through a subtle chromosomal change based on the mutations in genes *liaF*, *cls* and *gdpD*[14–17]. LiaF is
62 a transmembrane protein involved in monitoring the integrity of the cell envelope and responding to
63 damage[18], whereas Cls (cardiolipin synthase) and GdpD (glycerol-phosphodiester phosphodiesterase)
64 are both involved in phospholipid metabolism[14]. Of these, mutation of *liaF* has been proposed to be the
65 first pivotal event towards daptomycin resistance[15,19]. However, the exact role of the *liaF* mutations in
66 DAP resistance so far remains unclear.

67 LiaF is part of a three-component regulatory system, LiaFSR, which is important amongst the
68 Firmicutes for coordinating the cell envelope stress response (CESR) against antimicrobial-induced
69 damage[20–23]. The system is comprised of LiaF and a conventional two-component system (TCS): the
70 sensor kinase LiaS and the response regulator LiaR[20,24,25]. LiaF is an inhibitor of LiaS, maintaining the
71 sensor kinase in an inactive conformation[20]. Rather than detecting a specific antimicrobial compound,
72 LiaFSR responds to cell envelope damage, although the exact stimulus is unknown[26]. Upon sensing this
73 damage, LiaF releases LiaS, which is then able to phosphorylate LiaR to induce the expression of the
74 system's target operon, *liaXYZ*[19,27]. The *liaXYZ* cluster is involved in sensing and binding antimicrobials
75 at the cell surface to provide resistance[27].

76 To add to this complexity, the Lia system does not exist in isolation, but is just one of many TCSs
77 involved in monitoring cell envelope integrity, each responding to their own individual stimuli and
78 activating a unique set of target genes. The CroRS system, unique to the enterococci, is the main
79 regulator required for cephalosporin resistance[28], whereas the VicKR (YycFG) system is essential
80 across the low-CG Gram positives and is involved in regulating cell division, lipid biosynthesis, biofilm
81 and virulence[29]. An additional element of the network is the serine/threonine kinase IreK and the
82 phosphatase IreP, involved in maintaining cell wall integrity by potentially regulating peptidoglycan
83 biosynthesis and metabolism[30–32].

84 A further TCS involved in monitoring the cell envelope is EF0926/27, which we have now renamed
85 SapRS (Sensor of Antimicrobial Peptides), identified in our previous work[33]. SapRS is part of a
86 bacitracin resistance network comprised of the histidine kinase SapS, the response regulator SapR and
87 the Bce-like ABC transporters: SapAB (EF2752/51) and RapAB (Resistance against Antimicrobial
88 Peptides) (EF2050/49)[33]. The use of the sensory transporter SapAB to control the activity of SapRS
89 implements a 'flux-sensing' mechanism to regulate bacitracin resistance, as was shown for the
90 homologous system in *Bacillus subtilis*[34]. In brief, upon exposure, bacitracin binds to its membrane-
91 associated target molecule, undecaprenol-pyrophosphate (UPP), blocking the dephosphorylation and
92 recycling of UPP in the lipid II cycle of cell wall biosynthesis[35,36]. The sensory ABC transporter SapAB,
93 based on biochemical evidence from the *B. subtilis* system, forms a sensory complex with SapRS[37]. In

94 the absence of bacitracin, the transporter maintains the histidine kinase, SapS, in an 'OFF' state[38]. Upon
95 detection of bacitracin-UPP (BAC-UPP) complexes, SapAB switches its role from repressor to activator
96 of SapS, resulting in kinase autophosphorylation[38]. SapS can then phosphorylate SapR, which in turn
97 induces the production of the resistance transporter RapAB. RapAB, again based on evidence from its
98 *B. subtilis* homologue, frees UPP from the inhibitory grip of bacitracin using a target protection
99 mechanism[39], allowing dephosphorylation of UPP and continuation of cell wall synthesis, rendering the
100 cell resistant to bacitracin. The equivalent system in *B. subtilis* is a self-contained module, involving
101 only a single transporter (BceAB) and TCS (BceRS)[40] with no known further genes involved in its
102 signalling or resistance mechanism. However, in *E. faecalis*, we observed that the TCS SapRS is itself
103 transcriptionally induced by bacitracin, however we had not identified the regulatory system that
104 controls this expression[33].

105 Interestingly, recent work by the Arias lab demonstrated that when experimentally evolving *E. faecalis*
106 for DAP resistance in a Δ*liaFSR* background, mutations were observed in the sensory transporter
107 SapAB[41,42], suggesting a functional link between the Sap and Lia systems. In *B. subtilis*, the Lia system
108 is known to be one of the main components of bacitracin resistance[43,44], but there is currently no
109 evidence for a role of LiaFSR in response to bacitracin in *E. faecalis*. Neither is there any indication of
110 a role for the Sap system in responding to DAP exposure. However, this recent evidence suggested an
111 interplay between both the Sap and Lia systems and that, potentially, both systems may be contributing
112 to resistance against bacitracin and daptomycin.

113 In accordance with the need to deepen our knowledge of the CESR in *E. faecalis,* in this study we
114 sought to examine the potential interplay between the Sap and Lia systems and investigate the
115 involvement of further genes associated with this regulatory network. Utilising mutagenesis and
116 analysis of promoter activity, we provide evidence of the activation of LiaFSR signalling in response
117 to bacitracin and describe a direct functional link between the Lia and Sap systems by demonstrating
118 the regulation of *sapR* by LiaR in response to antibiotic exposure. We also show the contribution of an
119 additional resistance operon involved in response to bacitracin and unravel a differential response of
120 the network between bacitracin and daptomycin treatment. Our data show that interplay between SapRS

121 and LiaFSR signalling effectively implements a logic 'AND' gate, whereby expression of SapR target
122 genes is under dual control and requires two separate signalling inputs. These observations also explain
123 the occurrence of mutations in both regulatory systems during clinical and experimental emergence of
124 DAP resistance in enterococci found previously, thus providing a compelling model for the complex
125 CESR network in *E. faecalis*.

126

127 # Results

128 **LiaFSR controls expression of SapRS.** As mentioned above, from previous work we had identified
129 increased expression of *sapRS* under exposure to bacitracin or mersacidin[33]. Interestingly, we also
130 showed that *sapRS* was not autoregulated, in contrast to other two component systems such as CroRS
131 and LiaFSR[20,28], and regulation did not depend on either SapAB or RapAB[33]. We therefore here first
132 aimed to identify the regulator of *sapRS*. For possible candidates, we considered potential regulators
133 that are common amongst the Firmicutes and are known to respond to bacitracin. A potential candidate
134 was LiaFSR, deemed the 'master regulator' of the CESR in *Bacillus subtilis*[43] and recently shown to
135 have a possible functional link to the Sap system[41,42]. Because of this evidence, we first aimed to
136 examine the relationship between these two systems.

137 Firstly, we had to test if LiaFSR was able to respond to bacitracin treatment. It is well established that
138 *liaX* is under the control of LiaFSR[27], and therefore we exposed *E. faecalis* harbouring a P_{liaX}-*lacZ*
139 transcriptional fusion to increasing levels of bacitracin, as a readout for LiaFSR activity. The results
140 showed a ~10-fold increase in *liaX* expression at 32 μg ml^{-1} bacitracin compared with untreated cells
141 (Fig. 1A, black line), indicating that LiaFSR can indeed respond to bacitracin exposure in *E. faecalis.*

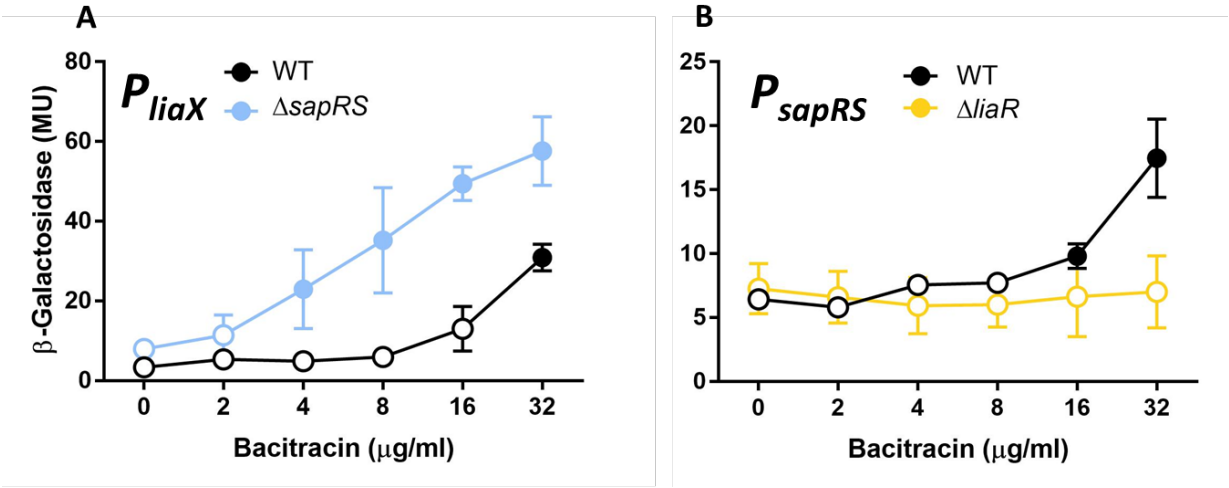

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Figure 1. The LiaFSR operon responds to bacitracin exposure and induces the expression of both *liaXYZ* and *sapRS*, with the absence of *sapRS* resulting in the hypersensitivity of the *liaXYZ* promoter. Cells of *E. faecalis* JH2-2 wild-type or isogenic deletion strains harbouring a P_{liaX}-*lacZ* (panel A) or P_{sapRS}-*lacZ* (panel B) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. β-galactosidase activity, expressed as Miller units (MU), was assayed following 1h incubation. Results are means and standard deviations for three biological repeats. The significance of induction relative to untreated cells was calculated for each strain by a one-tailed, pairwise *t*-test analysis. Significance is indicated by a filled symbol ($p < 0.05$); unfilled symbols represent no significant increases compared to uninduced conditions.

143

144 Following this, we tested if LiaR can regulate the expression of *sapRS*. To do this, we monitored the
145 response of a transcriptional P_{sapRS}-*lacZ* fusion to increasing bacitracin. The results showed that in wild-
146 type *E. faecalis* carrying the transcriptional *sapRS-lacZ* fusion, bacitracin concentrations of 16 μg ml^{-1}
147 or higher led to significant induction of the reporter, with a three-fold increase compared to uninduced
148 cells at 32 μg ml^{-1} (Fig. 1B, black line). Deletion of *liaR* in the reporter strain resulted in a complete loss
149 of *sapRS* induction, with expression remaining at basal levels. This indicated that LiaR indeed regulates
150 the expression of *sapRS* in response to bacitracin exposure, presenting first evidence of a direct
151 functional link between the Lia and Sap regulatory systems.

152 A surprising feature of this LiaR regulation was the high concentration required to induce a response,
153 as *liaX* expression only significantly increased at 32 μg ml^{-1}. One possible explanation for this might
154 be that the response was masked by other components of the bacitracin stress response in *E. faecalis*,
155 for example RapAB, which plays an active role in the removal of bacitracin from UPP[33], protecting the

156 cell from damage. In *B. subtilis*, it was observed that the RapAB equivalent, BceAB, was the primary
157 response to protect the cell from bacitracin exposure and masked the response of the Lia system[45]. To
158 prevent the induction of the main bacitracin resistance genes of *E. faecalis*, controlled by SapRS[33] and
159 thus remove potential interference with Lia signalling, we therefore introduced the P_{liaX}-*lacZ*
160 transcriptional fusion into the Δ*sapRS* background. Compared to the wild type, the expression of the
161 *liaX* promoter in response to bacitracin challenge of the deletion strain showed increased sensitivity,
162 significantly inducing expression from 4 μg ml^{-1} bacitracin and reaching overall higher activities (Fig.
163 1A, blue line). This showed that the weak response of Lia signalling to bacitracin in the wild type was
164 indeed due to masking effects of the resistance genes controlled by SapRS, implying the presence of
165 multiple layers of protection, similar to those observed in *B. subtilis*[45], as well as physiological links
166 between the SapRS- and LiaFSR-dependent components of the CESR in *E. faecalis*.

167 **SapRS controls the expression of the *dltABCD* operon.** To expand our understanding of the Lia/Sap
168 regulatory network, we next considered if other genes may also be under its control. Currently, the only
169 known gene under SapRS regulation is the resistance ABC-transporter operon *rapAB* [33]. In *B. subtilis*,
170 both the Lia and Bce systems act as self-contained modules, controlling the regulation of only a single
171 resistance operon each, *liaIH* and *bceAB* respectively, each encoded adjacently to its regulatory operon
172 on the chromosome [24,40,43]. However, the regulatory setup in *E. faecalis* seems to be much more
173 complex. This is supported by evidence from the literature, with the indication that the Lia system has
174 a larger operon than just itself and *liaXYZ* [27]. There is also a proposal that Lia contributes to DAP
175 resistance through the regulation of *dltXABCD* (*dlt*), although there is no evidence to date that Lia is a
176 direct regulator of this operon. Dlt is responsible for the D-alanylation of teichoic acids (TA) on the
177 bacterial cell surface, resulting in a decrease in the negative charge of the cell envelope[46], a mechanism
178 commonly involved in DAP resistance amongst the low-CG bacteria[47].

179 Interestingly, recent evidence has suggested a regulatory link between SapRS and *dlt*. When evolving
180 *E. faecium* for DAP resistance in the absence of LiaFSR, mutations occurred in the *sapAB* homologue,
181 *yvcRS*. These mutations correlated with an increase in *dlt* transcription when measured by qPCR and
182 resulted in an increase in cell surface charge[41,42]. In addition, the *dltABCD* operon is located directly

183 downstream from *sapAB* on the chromosome, suggesting a possible functional link between the genes.
184 Moreover, the promoter region of *dlt* contains a putative SapR binding site, similar to that of the *rapAB*
185 promoter (Fig. 2). This body of evidence therefore led us to investigate the contribution of LiaFSR and
186 SapRS to *dlt* regulation.

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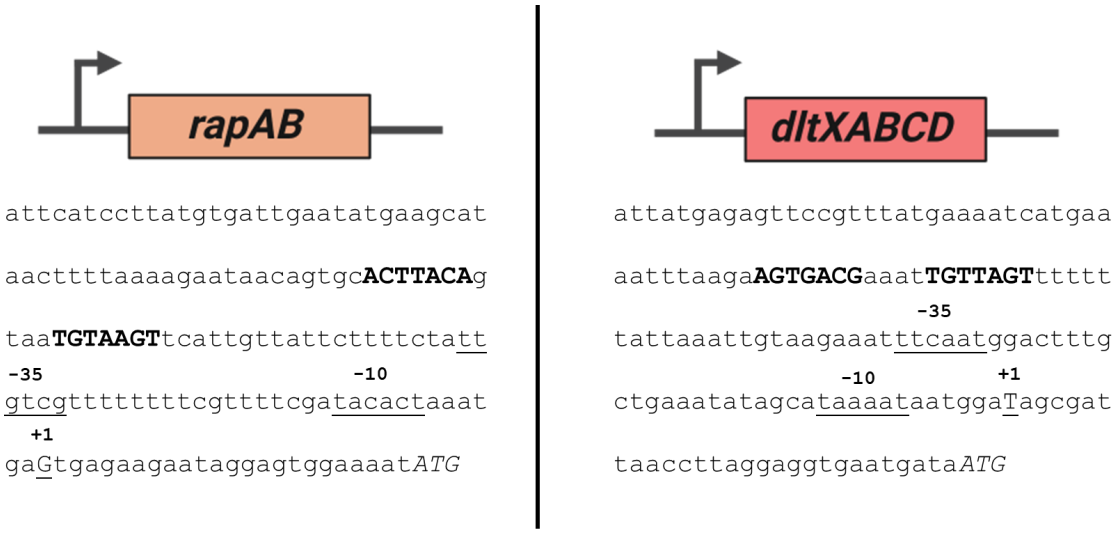

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Figure 2. Schematic of the promoter regions of both *rapAB* and *dltABCD*. The sequence 150 base pairs upstream of the ATG start codons are shown. The proposed binding site for SapR on both the *rapAB* and *dltABCD* promoters is in bold and capitalised, and the likely -10 and -35 elements are underlined. The experimentally confirmed transcriptional start sites are capitalised and underlined (+1) and the translational start site is shown in italicised capitals[33,84].

189

190 To this end, we first constructed a transcriptional P_{dltX}-*lacZ* fusion to test *dlt* induction by bacitracin.
191 The results showed that in wild-type JH2-2 carrying the fusion, increasing bacitracin concentrations led
192 to a dose-dependent increase in *dlt* expression, resulting in ~4-fold higher activity at 32 μg ml^{-1}
193 compared to untreated conditions (Fig. 3A, black line), showing that *dlt* expression is indeed induced
194 in response to bacitracin in *E. faecalis*. When we tested the response of the reporter in strains carrying
195 deletions of either *sapRS* or *sapAB,* the results showed a decrease in basal activity in both strains and a
196 complete loss of the promoter's bacitracin response in Δ*sapR* (Fig. 3A, blue and magenta lines). Loss
197 of SapAB still resulted in induction compared with uninduced cells, but overall activities were
198 considerably lower than in the wild-type strain. This indicated that SapRS was essential for *dlt*

199 expression in response to bacitracin, but a residual amount of *dlt* induction remained in the *sapAB*
200 deletion. This is consistent with the less direct role of SapAB in signalling, i.e. via controlling SapRS
201 activity and not the target genes directly. The residual bacitracin response observed in the *sapAB* deleted
202 strain can likely be explained by increased production of SapRS due to LiaFSR signalling, which would
203 be expected to lead to increased basal activity of SapRS and thus the *dlt* promoter.

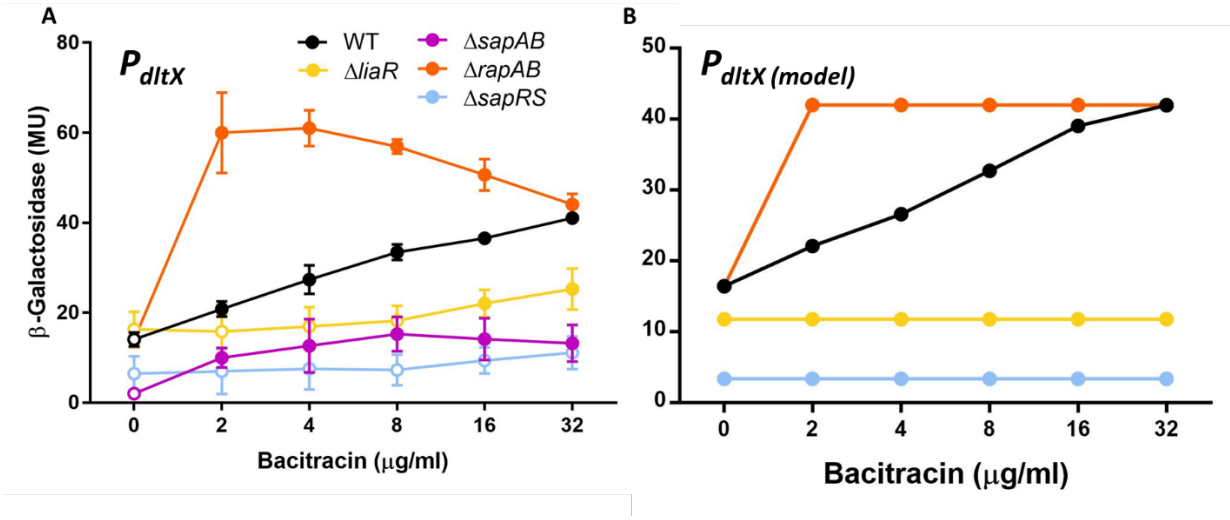

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Figure 3. Induction of the resistance operon *dltXABCD* by bacitracin. Panel A indicates the experimental induction of *dltX.* Cells of *E. faecalis* harbouring a *PdltX-lacZ* transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. Beta-galactosidase activity, expressed as Miller units (MU), was assayed following 1h incubation in wild type (WT) and deletion strain backgrounds. Results are means and standard deviations for three biological repeats The significance of induction relative to untreated cells was calculated for each strain by a one-tailed, pairwise *t*-test analysis. Significance is indicated by a filled symbol ($p < 0.05$), unfilled symbols represent no significant increases compared to uninduced conditions. Panel B indicates the mathematical model of *dltX* induction in the *E. faecalis* strains indicated in panel A.

205

206 As we had shown above that LiaR regulates *sapRS* expression, we next aimed to find out if deletion of
207 *liaR* also influenced *dlt* expression. When we tested the response of the *dltX* promoter fusion to
208 bacitracin in a strain carrying deletion of *liaR*, we observed that deletion of *liaR* resulted in a noticeably
209 weaker amplitude of *dlt* expression in response to bacitracin, being ~2-fold lower than the wild-type
210 strain at 32 μg ml^{-1} (Fig. 3A, yellow line). In addition, the sensitivity of the *dlt* promoter response was
211 much lower in the *liaR* deletion compared with the wild-type response, with significant activation over

212 baseline occurring at 16 μg ml^{-1} rather than at 2 μg ml^{-1} in the wild type. However, there was no effect
213 on basal activity of *dlt*, in contrast to deletion of *sapR* and *sapAB*. These data are consistent with LiaR
214 regulating *sapRS* expression, and SapRS being the actual regulator of *dlt* expression in response to
215 bacitracin.

216 As we had observed before with the *liaX* and *sapRS* promoters, we also wanted to investigate if the
217 protection provided by the SapRS-target RapAB was dampening the expression of *dlt* in response to
218 bacitracin. To examine this, we tested the response of *dlt* expression in the absence of *rapAB.* In a
219 *ΔrapAB* background, the *dlt* promoter demonstrated markedly increased sensitivity, and a much
220 stronger response to bacitracin, resulting in a ~3-fold increase in expression at 2 μg ml^{-1} when compared
221 to the wild-type (Fig. 3A, orange line). This response demonstrated the presence of a layered protection
222 with RapAB activity moderating *dlt* expression.

223 Overall, these findings were rather surprising, as the signalling pathway appeared remarkably complex
224 to result in a relatively simple outcome, i.e. inducing the expression of two resistance genes in response
225 to an antibiotic. To test if our understanding of the regulatory pathway was plausible, we therefore
226 developed a representative mathematical model to see if this would reproduce the behaviour we had
227 observed in the experiments. At the core, this model was based on a simplified form of the flux-sensing
228 mechanism described previously for the *B. subtilis* BceRS-BceAB system[34], which was then expanded
229 upon to reproduce the hypothesised network structure investigated here. In brief, the model considered
230 bacitracin binding to UPP to form UPP-bacitracin complexes with the on-rate dependent on the
231 bacitracin concentration. These complexes then drive transport activity of, in this case, SapAB,
232 according to a soft-switch type functional response (see Methods), which the model translates into
233 activation of SapRS and thus *dlt* expression. Importantly, SapRS activity also drives expression of the
234 resistance transporter operon, in this case *rapAB*, and production of RapAB leads to a reduction in
235 formation of UPP-bacitracin complexes due to the target protection activity of the transporter[34,39]. This
236 creates the negative feedback loop that is characteristic of the flux-sensing mechanism and leads to the
237 gradual response behaviour of the output promoters (Fig. 3B, black symbols). To adapt this model to
238 the Lia-Sap regulatory pathway of *E. faecalis*, we additionally considered the activity of the Lia system.

239 This was modelled to also respond to UPP-bacitracin complexes in a switch like manner representing
240 the generation of cellular damage caused by these complexes. The model then linked the Lia and Sap
241 components by modulating the SapRS signalling output (i.e. *dlt* and *rapAB* expression) according to
242 Lia activity, driven by bacitracin. The mathematical details of the model are explained in the Methods
243 section. Fitting to experimental data was performed by matching differential activity of SapRS against
244 both *rapAB* and *dlt* recorded at different bacitracin concentrations.

245 This model accurately reflected the behaviour of our experimental strains, depicting the same gradual
246 response to bacitracin of the *dltX* promoter in wild-type *E. faecalis* (Fig. 3B, black symbols), as well as
247 the hypersensitive response in the *rapAB* deletion strain, where the negative feedback from RapAB-
248 driven removal of bacitracin was missing (orange symbols). Importantly, the model gave the same
249 complete loss of *dltX* activity when *sapRS* was deleted (blue symbols) as observed experimentally, as
250 well as the normal basal level activity but loss of bacitracin-dependent induction when *liaR* was deleted
251 (yellow symbols). A *sapAB* deletion strain was not specifically considered as the model did not
252 differentiate between SapAB and SapRS activities and thus both strains would have been
253 mathematically identical. This close agreement between theoretical and experimental data strongly
254 suggested that our reconstruction of the regulatory pathway and connection between the Lia and Sap
255 systems was theoretically plausible and that no further major players were needed to explain the
256 behaviour of the *dltX* target promoter in the various mutant backgrounds analysed here.

257

258 **Daptomycin induces the expression of *sapRS* but not *dltABCD*.** We had now established a sequential
259 order of *dlt* regulatory control; in response to bacitracin, LiaFSR induces the expression of *sapRS*, and
260 in turn SapRS, activated by its sensory transporter SapAB, induces the expression of *dlt*. However,
261 treatment with bacitracin made it difficult to separate out the individual contributions to regulation by
262 LiaFSR and SapRS, as both signalling systems respond to this antibiotic. Bacitracin forms a complex
263 with UPP, which acts as the input for SapRS signalling via SapAB[39]; at the same time, bacitracin
264 induces cell envelope damage, triggering LiaFSR activation[26]. To allow us to differentiate between the
265 contribution made by each regulator to the network, we instead required an antibiotic that would only

266 trigger signalling by one of the systems. To this end, we turned to daptomycin (DAP), which activates
267 the Lia system by causing damage to the cell envelope[48,49]. However, it does not bind to UPP or the
268 sugar-pyrophosphate moiety of Lipid II, which is the common feature of substrates for BceAB-type
269 transporters[39]. Indeed, the tripartite complex of DAP with Lipid II and phosphatidyl glycerol in the
270 membrane[50] is unlikely to be recognised by SapAB, and thus DAP exposure should not trigger SapRS
271 activation, allowing us to study the specific impact of LiaFSR signalling in the regulatory pathway.

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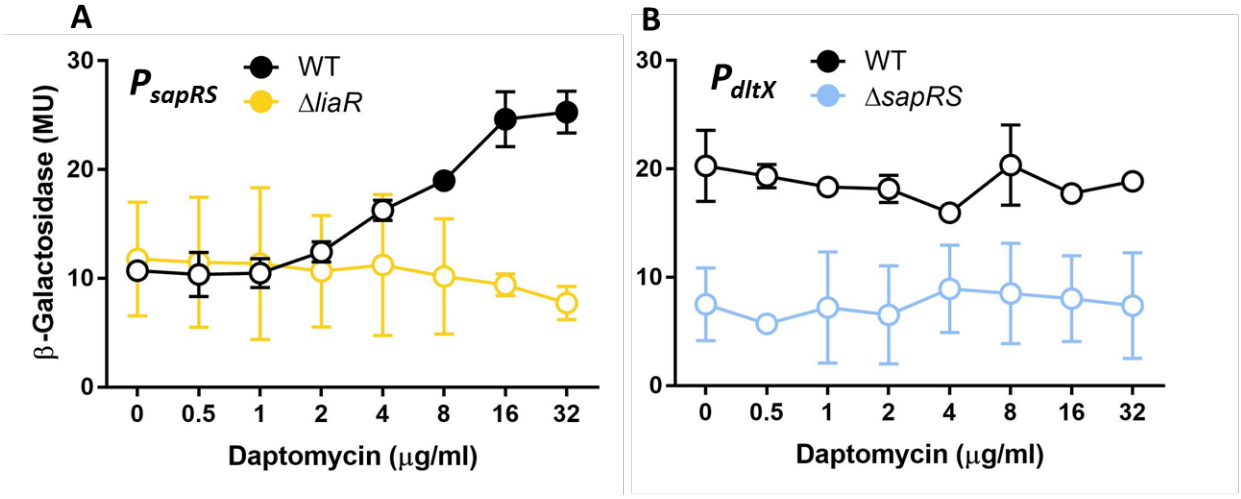

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Figure 4. Exposure to daptomycin results in the induction of *sapRS* but does not induce the expression of *dltXABCD*. Cells of *E. faecalis* harbouring a P_{sapRS}-*lacZ* (panel A) or a P_{dltX}-*lacZ* (panel B) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of daptomycin. Beta-galactosidase activity, expressed as Miller units (MU), was assayed following 30 minutes incubation in wild type (WT) and deletion strain backgrounds indicated. Results are means and standard deviations for two biological repeats. The significance of induction relative to untreated cells was calculated for each strain by a one-tailed, pairwise *t*-test analysis. Significance is indicated by a filled symbol ($P < 0.05$), unfilled symbols represent no significant increases compared to uninduced conditions.

274

275 To investigate the effect of DAP exposure on LiaFSR and SapRS signalling and target gene expression,
276 we again utilised transcriptional promoter fusions. The P_{sapRS}-*lacZ* transcriptional fusion (LiaFSR
277 target) in the wild-type background showed a ~2-fold induction upon DAP treatment when compared
278 to unexposed levels (Fig. 4A). As we had seen for bacitracin-induction, deletion of *liaR* again

279 completely abolished *sapRS* activation, showing the induction of *sapRS* by DAP was occurring through
280 LiaR regulation. This showed that DAP exposure indeed triggered LiaFSR signalling.

281 To test how DAP induction was propagated further down the regulatory pathway, we next exposed the
282 P_{dltX}-*lacZ* transcriptional fusion (SapRS target) to increasing DAP concentrations. Surprisingly, in wild-
283 type *E. faecalis*, we found daptomycin treatment did not result in any induction of *dlt*, with expression
284 remaining at basal levels across all tested concentrations (Fig. 4B). As seen for the bacitracin challenge,
285 deletion of *sapRS* resulted in a decrease in basal expression, from 20 MU to 7.5 MU, and again, there
286 was no induction of the *dlt* promoter in response to daptomycin exposure. This indicated that although
287 *sapRS* transcription was induced in response to daptomycin treatment, remarkably, expression of its
288 target gene *dlt* remained at basal levels. This discovery suggested a differential response in the
289 signalling cascade dependent on the inducer, indicating that bacitracin and daptomycin might be
290 differentially sensed by SapRS, as further explained in the discussion.

291 **The network components vary in importance during interactions with antimicrobial producer**
292 **strains.** As stated above, we were surprised by the complexity of the regulatory pathway controlling
293 what in other Firmicutes bacteria is a fairly straightforward response to antibiotic challenge, where each
294 regulatory system controls its own resistance genes. To shed some light on the reasons for the
295 complexity of signalling in enterococci, we considered the environments these bacteria can be found
296 in. *E. faecalis* is a common member of many natural microbial communities, such as in soil and water
297 or the gastrointestinal tract of humans and animals. There, the enterococci reside within the small and
298 large intestine and represent up to 1% of the faecal flora[51–54]. In such environments, *E. faecalis* interacts
299 with other microbes and must defend itself against antimicrobial producing bacteria. Therefore, we next
300 wanted to assess the role of the individual components of the resistance network in protecting *E. faecalis*
301 from antimicrobial activity produced by potential competitor bacteria. To do this, we utilised deferred
302 antagonism assays to simulate relevant environmental pressures from other microbes the enterococci
303 may encounter. We used four antimicrobial producing strains: the subtilin producer *Bacillus subtilis*
304 ATCC6633[55], bacitracin producer *Bacillus lichenformis* ATCC10716[56], nisin-A producer *Lactococcus*
305 *lactis* NZ9000[57] and nisin-P producer *Streptococcus gallolyticus* AB39[58]. Each producer was spotted

306 onto a plate, and the antimicrobial was allowed to accumulate over multiple days. This was followed
307 by the addition of an overlay containing the different *E. faecalis* strains to assess their sensitivity against
308 the produced compounds based on the size of the zone of inhibition (Fig. 5).

309 Firstly, we tested the *E. faecalis* strains against the antimicrobial subtilin, produced by *B. subtilis*
310 ATCC6633. Of the components under investigation in this study, the LiaFSR system appeared to
311 contribute most strongly to resistance against subtilin, as the Δ*liaR* mutant displayed the largest increase
312 in zone of inhibition compared to the wild type. The deletions of *sapR*, *sapAB* and *rapAB* also displayed
313 an increased zone of inhibition compared to the wild type, but to a lesser extent than Δ*liaR*. This
314 therefore suggests they play a lesser role in subtilin resistance.

315 In contrast, when testing the *E. faecalis* strains against the bacitracin producer *B. lichenformis*
316 ATCC10716, deletion of *sapR, sapAB* and *rapAB* showed similar increased sensitivity, consistent with
317 their contribution to the bacitracin resistance network that we have examined in this study and
318 previously[33]. Although also presenting increased sensitivity compared with the wild type, deletion of
319 *liaR* resulted in a marginally smaller zone of inhibition compared with the other deletion strains. This
320 is in line with our data presented above, showing the Lia system has a more indirect role in controlling
321 the bacitracin response by regulating *sapRS* expression.

322

323

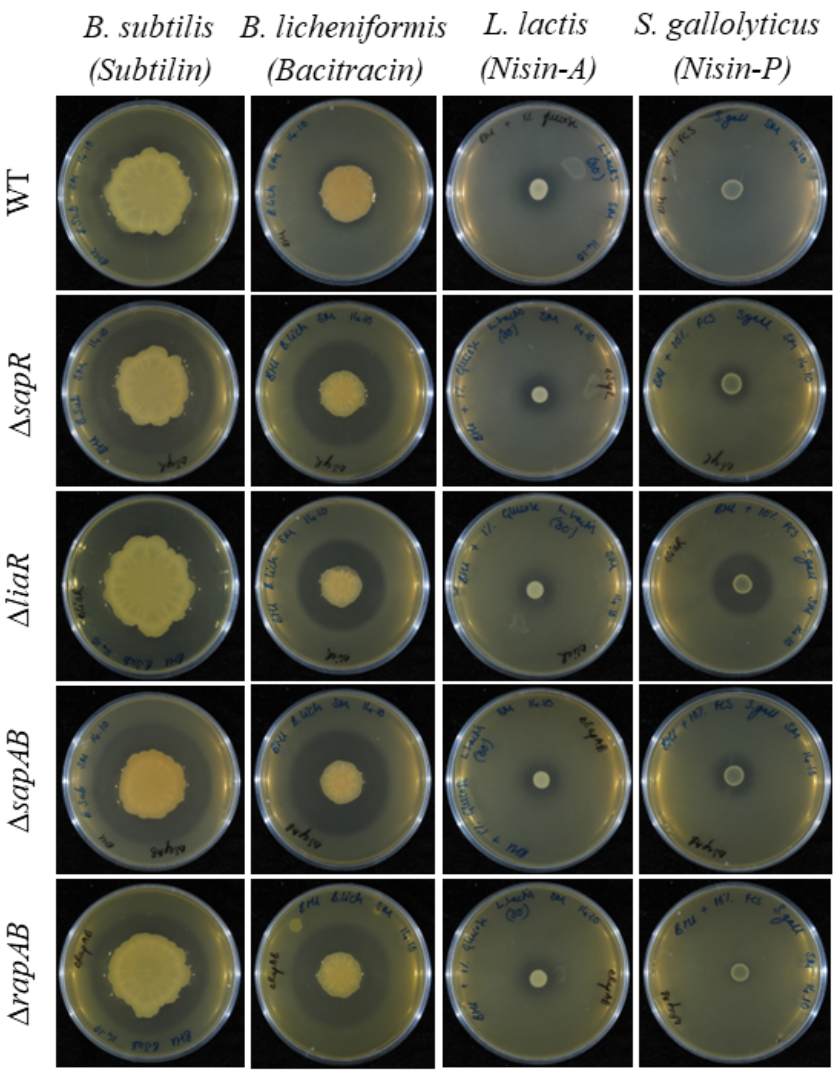


Figure 5. *E. faecalis* deletion strains show differential zones of inhibition in deferred antagonism tests against antimicrobials produced by other firmicute bacteria. The antimicrobial producer strains indicated at the top were grown overnight and then adjusted to an OD_{600} of 0.5 with fresh media. Aliquots (5ul) from each producer strain was then spotted and incubated at 25°C for 36-45 hours to allow antimicrobial accumulation. Overnight cultures of the *E. faecalis* strain indicated on the left were then added as a soft agar overlay. Plates were incubated at 25°C for 24 hours and zones of inhibition in the *E. faecalis* lawn were used to assess susceptibility to antimicrobials produced by the central strain.

We next tested the *E. faecalis* mutants against the nisin producers, *L. lactis* NZ9000 and *S. gallolyticus* AB39. Although there was some inhibition of wild type *E. faecalis* by the nisin-A producer *L. lactis*, there was no difference in sensitivity between the wild type and deletion strains. This suggests either that the genes of our regulatory pathway do not contribute to resistance against this antibiotic, or that

330 under the chosen conditions the inhibitory activity of *L. lactis* NZ9000 is primarily due to a different
331 antimicrobial than nisin-A. In contrast, the nisin-P producer *S. gallolyticus AB39*, despite having no
332 inhibitory effect on wild type *E. faecalis*, was able to strongly inhibit growth of the *liaR* deletion strain.
333 Deletions of both *sapR* and *sapAB* also resulted in increased sensitivity but to a lesser degree than *liaR*
334 deletion. Deletion of *rapAB* had little to no effect compared to the wild type, suggesting that the other
335 target genes of the regulatory pathway but not the RapAB transporter are responsible for nisin P
336 resistance.

337 These findings indicate that in response to different antimicrobials, the various members of this
338 resistance network have differing roles to play and also differ in their relative importance. In response
339 to subtilin and nisin-P, LiaR clearly plays a very important role in resistance, but it is a less important
340 component in response to bacitracin exposure. This reflects LiaR's role as a key regulator in the cell
341 envelope stress response more widely, but with mostly a moderating role in response to bacitracin. The
342 *sapR*, *sapAB* and *rapAB* deletions all present with very similar effects in response to the antimicrobials,
343 reflecting the interdependent functional relationship between the three components. The notable
344 exception to this is RapAB in the context of nisin-P, where the transporter does not appear to contribute
345 to protection of the cell.

346

347 **Discussion**

348 In this study, we aimed to expand our understanding of the cell envelope stress response of *E. faecalis*
349 using the previously described bacitracin resistance network as our foundation. Our findings revealed
350 the regulation of the BceRS-type TCS SapRS via the damage sensor, LiaFSR. In addition, we also
351 demonstrated the regulation of the resistance operon *dltXABCD* via SapRS. This work describes a direct
352 functional link between the Lia and Sap signalling systems in *E. faecalis*, which can explain previous
353 observations of suppressor mutations in *sapAB* when a LiaR-deficient strain was evolved for DAP
354 resistance[41,42]. Moreover, through creation of deletion strains and analysing promoter fusions, we have
355 revealed the presence of a differential network response through the damage-sensing LiaFSR and drug-

356 sensing SapAB-SapRS systems, leading to *dlt* expression in response to bacitracin, but not daptomycin.
357 This discovery implies there is distinct control on subsections of the network depending on the inducer.

358 To understand the mechanisms of this differential control, the mode-of-actions of both antibiotics must
359 be considered. Bacitracin is a peptide antibiotic, which forms a complex with undecaprenyl
360 pyrophosphate (UPP), an intermediate of the Lipid II cycle[35,36]. The formation of this complex prevents
361 the dephosphorylation of UPP and slows cell wall formation[59,60]. In contrast, daptomycin is a
362 lipopeptide antibiotic which forms a tripartite complex between lipid II and the membrane phospholipid
363 phosphatidyl glycerol[50]. The formation of this complex leads to a loss of membrane potential and cell
364 death. The similarity between both antibiotics is the formation of cell envelope damage, the known
365 stimulus for the activation of the "damage sensor" LiaFSR. However, they differ in their mechanism of
366 action with bacitracin preventing cell wall synthesis and daptomycin directly affecting cell membrane
367 integrity.

368 Since both antibiotics cause cell envelope damage, both lead to induction of LiaR target genes (Figures
369 1&3), including *sapRS*. SapAB, the sensory transporter that controls SapRS activity, however, so far
370 has been shown to be activated by only two antibiotics, bacitracin and mersacidin [33], both of which bind
371 to lipid II cycle intermediates[35,61,62]. This is consistent with the current working model for BceAB-type
372 transporters, to which SapAB belongs, recognising the complex between a peptide antibiotic and a lipid
373 II cycle intermediate as their substrate[39]. Importantly, the type of antibiotics to which BceAB-type
374 transporters respond all interact with the lipid II cycle intermediates via at least the pyrophosphate group
375 (e.g., bacitracin) or the sugar-pyrophosphate moiety of Lipid II (e.g. the lantibiotics, including
376 mersacidin)[62,63]. In the case of bacitracin, it is therefore the presence of bacitracin-UPP complexes that
377 triggers SapAB activity, leading to SapRS activation via the transport flux-sensing mechanism
378 demonstrated for the *B. subtilis* system[34,38]. Thus, the presence of bacitracin triggers both Lia and Sap
379 signalling simultaneously, inducing the expression of *dlt*.

380 In contrast, DAP interacts with its cellular target in a fundamentally different way, forming a tripartite
381 complex between Lipid II and phosphatidyl glycerol (PG)[50]. It therefore would not be expected to form
382 the type of complexes that could act as substrate for SapAB[39], and Sap signalling is not activated.

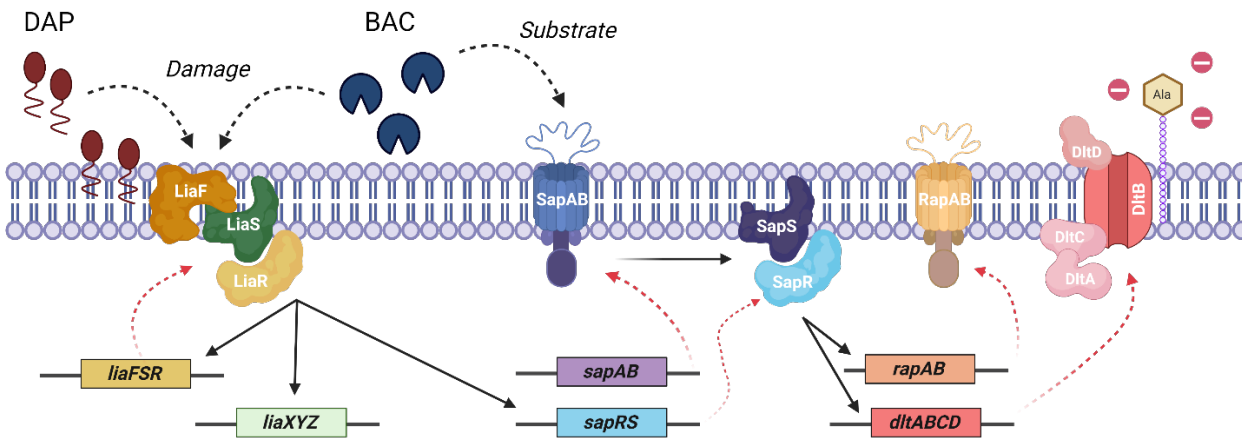
383 BceAB-type transporters have been shown to repress histidine kinase activity in the absence of
384 substrate[38]. Thus, the lack of a SapAB substrate likely leads to the transporter maintaining SapS in an
385 'OFF' conformation, preventing activation of SapR target genes, i.e. *dlt.* Such a mechanism can explain
386 why in the presence of DAP, when the LiaFSR system activates expression of the *sapRS* operon, no
387 induction of *dlt* expression was observed, because SapAB – unable to detect the tripartite DAP/lipid
388 II/PG complex - would have prevented SapRS signalling.

389 Taken together, these findings indicate the presence of a logic 'AND' gate, whereby two inputs (the
390 damage sensing input provided by LiaFSR and the drug-sensing input provided by SapAB) are needed
391 to obtain the output, which is the induction of *dlt* expression (Figure 6). This mode of regulation offers
392 an explanation for the mutations observed previously in *sapAB* when evolving for DAP resistance in
393 the absence of *liaR*[41,42]. A gain-of-function mutation in *sapAB* would allow the activation of Sap
394 signalling in the absence of a substrate and induce *dlt* expression, essentially overriding the 'AND' gate
395 and providing DAP resistance. This working model also explains the low but statistically significant
396 induction of *dlt* expression by bacitracin we observed in the absence of *sapAB* (Fig. 3). The mutant still
397 possesses SapRS and thus bacitracin-challenge leads to activation of *sapRS* expression via LiaR. This
398 in turn is likely to cause an increase in basal activity of SapRS-signalling and thus moderate *dlt*
399 activation, even if the full activation via SapAB is missing. A similar effect has been described
400 previously for the second known SapRS target, *rapAB*, where deletion of *sapAB* resulted in low
401 promoter activity of *rapAB*, but with a small level of residual bacitracin response remaining[33].

402 The regulatory wiring we have uncovered here between the LiaFSR and the Bce-type SapAB-SapRS
403 systems, resulting in the control of the *dltXABCD* and *rapAB* resistance operons, is remarkably complex
404 compared with other Firmicutes bacteria. For example, in *B. subtilis* LiaFSR controls its own regulon,
405 comprised solely of *liaIH(G)* and its own encoding genes [24]. In *S. aureus*, the LiaFSR homologue VraRS
406 has been shown to regulate the expression of multiple genes involved in cell wall biosynthesis[64], but
407 has not been observed to regulate a BceRS-type TCS. Bce-type systems in most Firmicutes bacteria
408 either solely control their own transporter, as found with BceAB-RS in *B. subtilis*[40], or can be involved
409 with other Bce-type systems, such as the BraRS-VraDE[65] and GraRS-VraFG[66] systems in *S. aureus*. In

410 some cases, Bce-type systems can also control additional genes, for example the GraRS TCS has also
411 been shown to regulate the *dlt* operon[67–69]. However, to our knowledge there have been no prior reports
412 of direct regulatory relationships that hardwire the Lia and Bce-type pathways together.

413



414

Figure 6. Proposed model of the antibiotic resistance network. Schematic illustrating the genes and proteins involved in antibiotic detection and resistance, with transcriptional regulation and translation demonstrated by black and red arrows respectively. Exposure to bacitracin (BAC) causes damage to the cell envelope, which acts as the stimulus for the removal of repression by LiaF, triggering the activation of LiaS. LiaS activity leads to phosphorylation of LiaR, which in turn induces the expression of its target promoters: its own, *liaXYZ* and *sapRS*, indicated by the black arrows. Simultaneously, bacitracin is also detected by SapAB. Communication between SapAB and SapS is indicated by a faded arrow, resulting in the activation of SapS and phosphorylation of SapR. SapR then induces the expression of the resistance genes, *rapAB* and *dlt,* which remove bacitracin from UPP and reduce the positive charge of the cell envelope, respectively. Conversely, exposure to daptomycin (DAP) again results in cell envelope damage, which triggers the phosphorylation of LiaR and induces the expression of *sapRS*. However, daptomycin is not recognised by SapAB, therefore SapS is kept in an 'OFF' conformation and SapR is not phosphorylated. SapR is therefore unable to induce the expression of *rapAB* and *dlt*.

415

416 Our findings reported here show that although the enterococci possess the same regulators as *S. aureus*
417 and *B. subtilis*, evolution has taken these components down different routes. A potential reasoning
418 behind *E. faecalis* maintaining this intricate setup could be that having *dlt* and *rapAB* under Sap control,
419 and *sapRS* itself under Lia regulation, may provide the cell with a layered response. Under this premise,

420 when the cell is experiencing a mild bacitracin attack, signalling in the Lia-Sap pathway is likely to be
421 dominated by the Sap system, which responds to the presence of UPP-bacitracin complexes, even if
422 those do not yet cause cellular damage. Sap signalling then activates both *rapAB* and *dltXABCD*. Of
423 these, RapAB provides the main target protection mechanism, especially at lower antibiotic
424 concentrations, whereas expression of *dlt* is likely of minor importance, based on evidence from *B.*
425 *subtilis*[39,45]. This is consistent with our observations in *E. faecalis,* where the strength of *rapAB*
426 induction is much higher than that of *dlt*: at 4µg/mL bacitracin, *rapAB* expression reaches >100 MU [33],
427 compared to *dlt*, which reaches a maximum of 25 MU (Fig. 3). However, when the antibiotic threat
428 begins to exceed the protection level RapAB can provide, damage to the cell occurs and the Lia system
429 is activated. LiaFSR is then able to boost the signalling cascade by increasing *sapRS* expression. We
430 see this in the absence of *rapAB*, which mimics an extreme failure of target protection, leading to a
431 drastic increase in *dlt* expression (Fig. 3). Thus, the regulatory structure between Lia and Sap signalling
432 in *E. faecalis* allows a second line of defence to be mounted if the primary resistance system is no longer
433 sufficient to fully protect the cell.

434 The genes discussed here are all part of the core genome of *E. faecalis* and thus contribute to intrinsic,
435 rather than acquired drug resistance in this bacterium. To better understand the evolutionary context
436 that appears to have given rise to the unusually complex regulation of the resistance genes, we
437 considered the broader scope of this network - was it able to protect the enterococci from antimicrobial
438 challenge from potential competitors in their natural environments? The importance of the role played
439 by LiaFSR in the cell envelope stress response was well demonstrated by the antagonism assays, in
440 which deletion of *liaR* resulted in increased sensitivity against all tested antimicrobial producers, except
441 for *L. lactis* NZ9000 (nisin-A). In contrast, components of the Sap system only contributed to protection
442 from the bacitracin producer *B. lichenformis* ATCC10716 and the subtilin producer *B. subtilis*
443 ATCC6633. The only antimicrobial producer against which neither of the genes appeared to give a
444 protective effect was the nisin-A producer *L. lactis* NZ9000. However, we cannot currently differentiate
445 whether this was because *L. lactis* did not produce sufficient nisin-A under the chosen growth conditions
446 to detect differences, or because *E. faecalis* uses alternative resistance mechanisms against nisin-A not

447 controlled by either the Lia or Sap systems. Overall, it appears that the regulatory network studied here
448 plays a key role in protecting *E. faecalis* from antimicrobial peptide producing bacteria that it would
449 realistically encounter in its natural habitats, offering an explanation for the existence of such a complex
450 regulatory strategy.

451 One of our most striking results was that daptomycin challenge, although able to activate Lia signalling,
452 was unable to activate the Sap system, potential mechanistic reasons for which are explained above.
453 This was a surprising finding, as *dlt* is known to respond to daptomycin in other Firmicutes, e.g. *S.*
454 *aureus* [70–74]. The lack of *dlt* expression in response to DAP may, however, explain the sensitivity to this
455 antibiotic observed throughout the *Enterococcus* genus and its effectiveness when treating VRE
456 infections[75]. This is despite the bacteria being in possession of an effective resistance mechanism, except
457 this is not induced upon DAP challenge. It is then plausible to see why clinical DAP resistance could
458 result from overriding of the molecular controls investigated here to trigger high *dlt* operon expression,
459 specifically via mutations in genes encoding the components of the LiaFSR system[19], or experimentally
460 in *sapAB* as suppressor mutations of a LiaR-deficient strain[41,42]. DAP was introduced for treatment of
461 enterococci infections in 2003[12] and the first resistant isolates were identified already in 2005[13]. It may
462 be that the rapid emergence of DAP resistance is in part derived from a dysregulation of *dlt* expression
463 by such mutations of regulatory components.

464 Overall, our findings contribute to an increasing understanding of the regulatory network protecting *E.*
465 *faecalis* from cell envelope attack and provide insights into the regulatory cascade that is involved in
466 controlling resistance gene expression in response to cell envelope targeting antibiotics. Our findings
467 also provide valuable context in which to better understand the emergence of DAP resistance in clinical
468 environments. Ultimately, a system-wide understanding of antimicrobial resistance regulation may lead
469 to identification of an 'Achilles' heel' within the network and identify new therapeutic targets.

470

471 **Experimental Procedures**

472 **Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this study are listed
473 in Table S1 in the supplementary material. *E. coli* MC1061 was used for cloning with pTCVlac, and
474 strain DH5α was used for all other cloning. *E. coli*, *Bacillus licheniformis* and *Bacillus subtilis* were
475 routinely grown in lysogeny broth (LB) at 37°C with agitation (200 rpm). *Lactococcus lactis* was grown
476 routinely in M17 supplemented with 0.5% lactose at 30°C without agitation. *Enterococcus faecalis and*
477 *Streptococcus gallolyticus were* grown routinely in brain heart infusion (BHI) at 37°C without agitation,
478 with media for the latter being supplemented with 5% (v/v) foetal bovine calf serum. Solid media
479 contained 15 g l^{-1} agar. Selective media contained chloramphenicol (10 µg ml^{-1} for *E. coli*; 15 µg ml^{-1}
480 for *E. faecalis*), kanamycin (50 µg ml^{-1} for *E. coli*; 1000 µg ml^{-1} for *E. faecalis*), spectinomycin (100
481 µg ml^{-1} for *E. coli*; 500 µg ml^{-1} for *E. faecalis*). For blue-white screening, 5-bromo-4-chloro-3-indolyl-
482 β-d-galactopyranoside (X-Gal) was used at 120 µg/ml. Bacitracin was supplied as the Zn^{2+} salt. All
483 media for experiments with daptomycin were supplemented with 50 µM $CaCl_2$.

484 *E. faecalis* was transformed by electroporation as previously described[76]. *E. coli* was transformed by
485 heat-shock of $CaCl_2$ competent cells, followed by 1 hour recovery time[77]. Growth was measured as
486 optical density at 600 nm (OD_{600}) on a the Biochrom™ Novaspec Pro Spectrophotometer using cuvettes
487 with 1 cm light path length or in 96-well plates with 100 µL culture volumes on a Spark®Microplate
488 reader (Tecan).

489 **Construction of plasmids and genetic techniques.** All primer sequences used for cloning are listed in
490 Table S2 in the supplementary material. Transcriptional promoter fusions to *lacZ* in *E. faecalis* were
491 constructed in the vector pTCVlac[78]. All fragments were cloned via the EcoRI and BamHI sites of the
492 vector. Constructs for unmarked deletions in *E. faecalis* were cloned into pLT06[79]. For each gene or
493 operon to be deleted, 700- to 1000-bp located immediately before the start codon of the first gene ("up"
494 fragment) and after the stop codon of the last gene ("down" fragment) were amplified. The primers
495 were designed to create a 17- to 20-bp overlap between the PCR products (Table S2), facilitating the
496 fusion of the fragments by PCR overlap extension[80] and were subsequently cloned into the NcoI and
497 BamHI site of the vector pLT06. Gene deletions were performed as previously described[79]. Briefly,
498 following transformation of the parent strain with the temperature-sensitive vector pLT06, overnight

499 cultures were grown at 30° containing chloramphenicol and reinoculated 1:1000 into 10 mL BHI the
500 next morning. Cells were then grown at 30° for 2.5 hours, followed by increasing to 42° for a further
501 2.5 hours to force single-site integration. Cells were then serially diluted onto BHI agar containing
502 chloramphenicol and X-Gal and incubated at 42°. Blue colonies growing at 42°C were screened for the
503 targeted integration using PCR with primers flanking the site of integration. Positive clones were then
504 serially passaged for two days from overnight culture in BHI medium with no selection at 30° to allow
505 a second site recombination event. Cultures were then serially diluted on to MM9-YEG agar[81]
506 containing 10 mM *p*-chloro-phenylalanine for counter-selection and X-Gal at 37°C. The resulting white
507 colonies were screened for the deletion of the target genes by PCR. All cloned constructs were checked
508 for PCR fidelity by Sanger sequencing, and all created strains were verified by PCR using the primers
509 given in Table S2.

510 **Antimicrobial susceptibility assays.** For antibiotic susceptibility assays, minimum inhibitory
511 concentrations (MICs) were determined by broth dilution assays in BHI medium in 96-well plates,
512 containing serial two-fold dilutions of antibiotic. Colonies of *E. faecalis* were suspended in sterile
513 Phosphate Buffered Saline (PBS) to 0.5 McFarland standard turbidity and diluted 1:1,000 in a total
514 volume of 100µL. After 24 h incubation at 37°C, growth was determined by measuring optical density
515 (OD_{600}) on a Spark®Microplate reader (Tecan). The MIC was scored as the lowest antibiotic
516 concentration where no growth was observed following subtraction of the OD_{600} of a well containing
517 sterile medium.

518 **β-Galactosidase assays.** For quantitatively assessing induction of *lacZ* reporter constructs in *E.*
519 *faecalis*, exponentially growing cells (OD_{600} = 0.4-0.5) inoculated 1:250 from overnight cultures in BHI
520 medium were exposed to different concentrations of bacitracin for 1 h or daptomycin for 30 minutes.
521 Cells were harvested via centrifugation and stored at -20°C. β-Galactosidase activities were assayed in
522 permeabilised cells and expressed in Miller units (MU)[82]. For this, cells were resuspended in 1 ml Z-
523 buffer (8.04 g Na_2HPO_4*$7H_2O$, 2.76 g NaH_2PO_4*H_2O, 0.123 g $MgSO_4$*$7H_2O$ and 5 mL 1M KCl in 495
524 mL dH_2O, pH 7). The samples were adjusted to OD_{600} = 0.5 in a 1 ml volume of Z-buffer and from this,
525 two volumes were taken: 200 µl and 400 µl cells made up to 1 mL each with Z-buffer. This volume

526 corresponds to the 'volume of cells' in the Miller Unit (MU) equation below. Following this, 20 µl
527 0.1% (w/v) SDS and 40 µl chloroform were added and vortexed for 5 seconds, then rested for 5-10
528 minutes. Reactions were started by adding 200 µl *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg
529 mL^{-1} in Z-buffer) and incubated at room temperature until yellow colouration was observed. If no colour
530 change was visible, the reaction was incubated for 20 minutes. Reactions were stopped by adding 500
531 µl 1M Na_2CO_3, and the time recorded, which corresponds to the 'time' in the Miller Unit (MU) equation
532 below. Absorbance at 420 nm (A_{420}) was then read. MUs were calculated using the following equation:

533 $$Miller\ Units\ (MU) = \frac{A420 * 1000}{Time\ (minutes) * volume\ of\ cells\ (in\ ml) * OD600}$$

534 **Computational modelling.** Rather than directly modelling the temporal dynamics of the regulatory
535 network as done for the *B. subtilis* Bce system[34,83], we chose to focus on the (meta) stable state reached
536 in response to challenge with a given bacitracin concentration. The quantities modelled are the
537 concentrations of bacitracin [*bac*], UPP [*UPP*], UPP-bound bacitracin [*UPPbac*], and the effective
538 activities of SapRS [*SapRS*], RapAB [*RapAB*], LiaX [*Lia*], and DltXABCD [*Dlt*]. For the modelling,
539 we made repeated use of a soft-switch sigmoid type function

540 $$f_y(\mathrm{x}; y_{\mathrm{base}}, y_{\mathrm{max}}, \mathrm{x}_0, \mathrm{k}) = f_{\mathrm{max}}\left(1 + \left(\frac{y_{\mathrm{max}}}{y_{\mathrm{base}}} - 1\right) e^{-k(x-x_0)}\right)^{-1},$$

541 where x is the input quantity and y the output which varies between y_{base} and y_{max}, with x_0 controlling
542 the switching threshold and k setting the sharpness of the transition. The equations of state for the model
543 are then simply

544 $$k_{on}([Dlt])([bac] - [UPPbac])[UPP] - k_{off}([RapAB])[UPPbac] = 0$$

545 $$[Lia] = f_{Lia}([UPPbac])$$

546 $$[SapRS] = f_{SapRS}([Lia])$$

547 $$[Dlt] = f_{dlt}([SapRS])$$

548 $$[RapAB] = f_{RapAB}([SapRS])$$

549 We modelled the binding and off rates for the UPP / bacitracin interaction as being linearly proportional
550 to [*Dlt*] and [*RapAB*], respectively, with Dlt reducing bacitracin binding and RapAB increasing the off-
551 rate. The biological reasoning for this is explained in the Results section.

552 For [*bac*], actual values from the experimental work were used, and the switching parameters for [*Dlt*]
553 and [*RapAB*] as functions of [*SapRS*] were determined by fitting actual activity levels observed
554 experimentally of these quantities for different bacitracin levels. The remaining parameters were fitted
555 to achieve a description of experimental results for the wild-type strain by the model output. The values
556 for each parameter are given in Table S3. Solving the equations of state for a given input bacitracin
557 level [*bac*] yielded predictions for [*UPP*] and [*UPPbac*], which in turn drive the response curves plotted
558 in Figure 3B.

559 **Antagonism assays on solid media.** A plate-based assay was utilised to measure growth inhibition
560 between antibiotic producer strains and *E. faecalis* strains. For each species, cultures of the
561 antimicrobial-producing bacteria were grown overnight in the respective growth medium and
562 temperatures stated above and adjusted to $OD_{600} = 0.5$. Aliquots (5 µl) from each producer culture were
563 spotted onto the centre of a BHI agar plate and incubated at room temperature (20-25°C) for 3-5 days
564 to allow accumulation of antimicrobial products. Overnight cultures of *E. faecalis* strains were grown
565 at 37°C in BHI medium, inoculated 1:100 into fresh medium and grown to OD = 0.5. Next, 3 mL of
566 liquid BHI soft agar (7.5 g/L, 50°C) were inoculated with 30 µL culture. The soft agar was then poured
567 onto the plate containing the pre-grown antimicrobial-producing strains and left to dry. The plate was
568 then incubated overnight at room temperature to allow *E. faecalis* to grow to visualise the zone of
569 inhibition. Results were recorded photographically using a PowerShot G camera attached to a lightbox.

570 **Data accessibility.** The numerical data underpinning the results shown in Figures 1, 3 and 4 are
571 available in Table S4. The *Mathematica* file for the model is provided in the supplementary material.

572

573 # Acknowledgements

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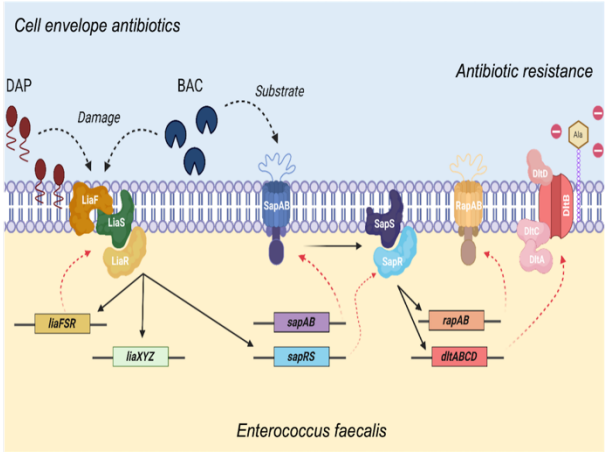
Author contributions

SMM, SG, TR and GF contributed to the conception of the study; SMM and SG designed the experimental work; SMM carried out all data acquisition; SMM, TR and SG carried out data analysis and interpretation; SMM, SG, TR and GF wrote the manuscript.

Graphical abstract



Abbreviated summary

This study explored a regulatory network controlling resistance against daptomycin and bacitracin in *E. faecalis*. We show that the *dltXABCD* operon, which can protect against both antibiotics, is controlled by two regulatory systems, LiaFSR and SapRS. Strikingly, this strategy allows *dtlXABCD* expression only in response to bacitracin, but not daptomycin, potentially explaining the natural DAP-sensitivity of *E. faecalis* and why clinical resistance can emerge via regulatory mutations.

593 # References

594 1. Organisation for Economic Co-operation and Development. Antimicrobial Resistance.
595 Tackling the Burden in the European Union. *Eur Cent Dis Prev Control*. 2020:1-20.
596 https://www.oecd.org/health/health-systems/AMR-Tackling-the-Burden-in-the-EU-OECD-
597 ECDC-Briefing-Note-2019.pdf.

598 2. Zhen X, Lundborg CS, Sun X, Hu X, Dong H. Economic burden of antibiotic resistance in
599 ESKAPE organisms: a systematic review. *Antimicrob Resist Infect Control*. 2019;8:137.
600 doi:10.1186/s13756-019-0590-7

601 3. Miller WR, Murray BE, Rice LB, Arias CA. Vancomycin-Resistant Enterococci: Therapeutic
602 Challenges in the 21st Century. *Infect Dis Clin North Am*. 2016;30(2):415-439.
603 doi:10.1016/j.idc.2016.02.006

604 4. Chiang H-Y, Perencevich EN, Nair R, *et al*. Incidence and Outcomes Associated With
605 Infections Caused by Vancomycin-Resistant Enterococci in the United States: Systematic
606 Literature Review and Meta-Analysis. *Infect Control Hosp Epidemiol*. 2017;38(2):203-215.
607 doi:10.1017/ice.2016.254

608 5. Said MS, Tirthani E, Lesho E. *Enterococcus Infections*. Rochester General Hospital: StatPearls
609 Publishing, Treasure Island (FL); 2021. http://europepmc.org/abstract/MED/33620836.

610 6. Arias CA, Murray BE. The rise of the Enterococcus: Beyond vancomycin resistance. *Nat Rev*
611 *Microbiol*. 2012;10(4):266-278. doi:10.1038/nrmicro2761

612 7. Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-Mediated Resistance to Vancomycin and
613 Teicoplanin in *Enterococcus faecium*. *N Engl J Med*. 1988;319(3):157-161.
614 doi:10.1056/NEJM198807213190307

615 8. Uttley AHC, George RC, Naidoo J, *et al*. High-level vancomycin-resistant enterococci causing
616 hospital infections. *Epidemiol Infect*. 1989;103(1):173-181. doi:DOI:
617 10.1017/S0950268800030478

618 9. Levine DP. Vancomycin: A History. *Clin Infect Dis*. 2006;42(Supplement_1):S5-S12.
619 doi:10.1086/491709

620 10. Courvalin P. Vancomycin Resistance in Gram-Positive Cocci. *Clin Infect Dis*.
621 2006;42(Supplement_1):S25-S34. doi:10.1086/491711

622 11. Puchter L, Chaberny IF, Schwab F, Vonberg R-P, Bange F-C, Ebadi E. Economic burden of
623 nosocomial infections caused by vancomycin-resistant enterococci. *Antimicrob Resist Infect*
624 *Control*. 2018;7(1):1. doi:10.1186/s13756-017-0291-z

625 12. Sauermann R, Rothenburger M, Graninger W, Joukhadar C. Daptomycin: A Review 4 Years
626 after First Approval. *Pharmacology*. 2008;81(2):79-91. doi:10.1159/000109868

627 13. Munoz-Price LS, Lolans K, Quinn JP. Emergence of Resistance to Daptomycin during
628 Treatment of Vancomycin-Resistant *Enterococcus faecalis* Infection. *Clin Infect Dis*.
629 2005;41(4):565-566. doi:10.1086/432121

630 14. Arias CA, Panesso D, McGrath DM, *et al*. Genetic Basis for In Vivo Daptomycin Resistance
631 in Enterococci. *N Engl J Med*. 2011;365(10):892-900. doi:10.1056/NEJMoa1011138

632 15. Tran T, Panesso D, Mishra Nagendra N, *et al*. Daptomycin-Resistant *Enterococcus faecalis*
633 Diverts the Antibiotic Molecule from the Division Septum and Remodels Cell Membrane
634 Phospholipids. *MBio*. 2013;4(4):e00281-13. doi:10.1128/mBio.00281-13

635 16. Reyes J, Panesso D, Tran TT, *et al*. A *liaR* Deletion Restores Susceptibility to Daptomycin and
636 Antimicrobial Peptides in Multidrug-Resistant *Enterococcus faecalis*. *J Infect Dis*.
637 2015;211(8):1317-1325. doi:10.1093/infdis/jiu602

638 17. Palmer K, Daniel A, Hardy C, Silverman J, Gilmore M. Genetic Basis for Daptomycin
639 Resistance in Enterococci. *Antimicrob Agents Chemother*. 2011;55(7):3345-3356.
640 doi:10.1128/AAC.00207-11

641 18. Tran TT, Miller WR, Shamoo Y, Arias CA. Targeting cell membrane adaptation as a novel
642 antimicrobial strategy. *Curr Opin Microbiol*. 2016;33:91-96.

643 doi:https://doi.org/10.1016/j.mib.2016.07.002

644 19. Miller C, Kong J, Tran TT, Arias CA, Saxer G, Shamoo Y. Adaptation of *Enterococcus*
645 *faecalis* to daptomycin reveals an ordered progression to resistance. *Antimicrob Agents*
646 *Chemother*. 2013;57(11):5373-5383. doi:10.1128/AAC.01473-13

647 20. Jordan S, Junker A, Helmann J, Mascher T. Regulation of LiaRS-Dependent Gene Expression
648 in *Bacillus subtilis*: Identification of Inhibitor Proteins, Regulator Binding Sites, and Target
649 Genes of a Conserved Cell Envelope Stress-Sensing Two-Component System. *J Bacteriol*.
650 2006;188(14):5153-5166. doi:10.1128/JB.00310-06

651 21. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. Two-component system
652 VraSR positively modulates the regulation of cell-wall biosynthesis pathway in
653 *Staphylococcus aureus*. *Mol Microbiol*. 2003;49(3):807-821.
654 doi:https://doi.org/10.1046/j.1365-2958.2003.03599.x

655 22. Martínez B, Zomer AL, Rodríguez A, Kok J, Kuipers OP. Cell envelope stress induced by the
656 bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. *Mol*
657 *Microbiol*. 2007;64(2):473-486. doi:https://doi.org/10.1111/j.1365-2958.2007.05668.x

658 23. Suntharalingam P, Senadheera MD, Mair RW, Levesque CM, Cvitkovitch DG. The LiaFSR
659 system regulates the cell envelope stress response in *Streptococcus mutans*. *J Bacteriol*.
660 2009;191(9):2973-2984. doi:10.1128/JB.01563-08

661 24. Wolf D, Kalamorz F, Wecke T, *et al*. In-Depth Profiling of the LiaR Response of *Bacillus*
662 *subtilis*. *J Bacteriol*. 2010;192(18):4680-4693. doi:10.1128/JB.00543-10

663 25. Schrecke K, Jordan S, Mascher T. Stoichiometry and perturbation studies of the LiaFSR
664 system of *Bacillus subtilis*. *Mol Microbiol*. 2013;87(4):769-788.
665 doi:https://doi.org/10.1111/mmi.12130

666 26. Wolf D, Domínguez-Cuevas P, Daniel R, Mascher T. Cell Envelope Stress Response in Cell
667 Wall-Deficient L-Forms of *Bacillus subtilis*. *Antimicrob Agents Chemother*.

668 2012;56(11):5907-5915. doi:10.1128/AAC.00770-12

669 27. Khan A, Davlieva M, Panesso D, *et al*. Antimicrobial sensing coupled with cell membrane
670 remodeling mediates antibiotic resistance and virulence in *Enterococcus faecalis*. *Proc Natl*
671 *Acad Sci*. 2019;116(52):26925-26932. doi:10.1073/pnas.1916037116

672 28. Comenge Y, Quintiliani R, Li L, *et al*. The CroRS Two-Component Regulatory System Is
673 Required for Intrinsic β-Lactam Resistance in *Enterococcus faecalis*. *J Bacteriol*.
674 2003;185(24):7184-7192. doi:10.1128/JB.185.24.7184-7192.2003

675 29. Winkler ME, Hoch JA. Essentiality, bypass, and targeting of the YycFG (VicRK) two-
676 component regulatory system in gram-positive bacteria. *J Bacteriol*. 2008;190(8):2645-2648.
677 doi:10.1128/JB.01682-07

678 30. Kristich C, Wells C, Dunny G. A eukaryotic-type Ser/Thr kinase in *Enterococcus faecalis*
679 mediates antimicrobial resistance and intestinal persistence. *Proc Natl Acad Sci*.
680 2007;104(9):3508-3513. doi:10.1073/pnas.0608742104

681 31. Kristich C, Little J, Hall C, Hoff J, Low D. Reciprocal Regulation of Cephalosporin Resistance
682 in *Enterococcus faecalis*. *MBio*. 2011;2(6):e00199-11. doi:10.1128/mBio.00199-11

683 32. Iannetta AA, Minton NE, Uitenbroek AA, *et al*. IreK-Mediated, Cell Wall-Protective
684 Phosphorylation in *Enterococcus faecalis*. *J Proteome Res*. 2021;20(11):5131-5144.
685 doi:10.1021/acs.jproteome.1c00635

686 33. Gebhard S, Fang C, Shaaly A, *et al*. Identification and characterization of a bacitracin
687 resistance network in *Enterococcus faecalis*. *Antimicrob Agents Chemother*. 2014.
688 doi:10.1128/AAC.02111-13

689 34. Fritz G, Dintner S, Treichel SN, *et al*. A New Way of Sensing: Need-Based Activation of
690 Antibiotic Resistance by a Flux-Sensing Mechanism. *MBio*. 2015;6(4):e00975-15.
691 doi:10.1128/mBio.00975-15

692 35. Storm DR. Mechanism of Bacitracin Action: A Specific Lipid-Peptide Interaction. *Ann N Y*

693 *Acad Sci*. 1974;235(1):387-398. doi:https://doi.org/10.1111/j.1749-6632.1974.tb43278.x

694 36. Storm DR, Strominger JL. Binding of bacitracin to cells and protoplasts of Micrococcus
695 lysodeikticus. *J Biol Chem*. 1974;249(6):1823-1827. doi:10.1016/s0021-9258(19)42861-5

696 37. Dintner S, Heermann R, Fang C, Jung K, Gebhard S. A sensory complex consisting of an
697 ATP-binding cassette transporter and a two-component regulatory system controls bacitracin
698 resistance in *Bacillus subtilis*. *J Biol Chem*. 2014;289(40):27899-27910.
699 doi:10.1074/jbc.M114.596221

700 38. Koh A, Gibbon MJ, Van der Kamp MW, Pudney CR, Gebhard S. Conformation control of the
701 histidine kinase BceS of *Bacillus subtilis* by its cognate ABC-transporter facilitates need-based
702 activation of antibiotic resistance. *Mol Microbiol*. 2021;115(1):157-174.
703 doi:https://doi.org/10.1111/mmi.14607

704 39. Kobras C, Piepenbreier H, Emenegger J, Sim A, Fritz G, Gebhard S. BceAB-Type Antibiotic
705 Resistance Transporters Appear To Act by Target Protection of Cell Wall Synthesis.
706 *Antimicrob Agents Chemother*. 2020;64(3):e02241-19. doi:10.1128/AAC.02241-19

707 40. Ohki R, Giyanto, Tateno K, *et al*. The BceRS two-component regulatory system induces
708 expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. *Mol Microbiol*.
709 2003;49(4):1135-1144. doi:https://doi.org/10.1046/j.1365-2958.2003.03653.x

710 41. Prater A, Mehta H, Kosgei A, *et al*. Environment Shapes the Accessible Daptomycin
711 Resistance Mechanisms in *Enterococcus faecium*. *Antimicrob Agents Chemother*.
712 2019;63(10):e00790-19. doi:10.1128/AAC.00790-19

713 42. Prater A, Mehta H, Beabout K, *et al*. Daptomycin Resistance in *Enterococcus faecium* Can Be
714 Delayed by Disruption of the LiaFSR Stress Response Pathway. *Antimicrob Agents*
715 *Chemother*. 2021;65(4):e01317-20. doi:10.1128/AAC.01317-20

716 43. Mascher T, Margulis NG, Wang T, Ye RW, Helmann JD. Cell wall stress responses in
717 *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol Microbiol*.

718 2003;50(5):1591-1604. doi:https://doi.org/10.1046/j.1365-2958.2003.03786.x

719 44. Rietkötter E, Hoyer D, Mascher T. Bacitracin sensing in *Bacillus subtilis*. *Mol Microbiol*.
720 2008;68(3):768-785. doi:https://doi.org/10.1111/j.1365-2958.2008.06194.x

721 45. Radeck J, Gebhard S, Orchard PS, *et al*. Anatomy of the bacitracin resistance network in
722 *Bacillus subtilis*. *Mol Microbiol*. 2016;100(4):607-620. doi:10.1111/mmi.13336

723 46. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F. Inactivation of the *dlt* Operon in
724 *Staphylococcus aureus* Confers Sensitivity to Defensins, Protegrins, and Other Antimicrobial
725 Peptides. *J Biol Chem*. 1999;274(13):8405-8410. doi:10.1074/jbc.274.13.8405

726 47. Tran T, Munita J, Arias C. Mechanisms of drug resistance: daptomycin resistance. *Ann N Y*
727 *Acad Sci*. 2015;1354(1):32-53. doi:https://doi.org/10.1111/nyas.12948

728 48. Wecke T, Zühlke D, Mäder U, *et al*. Daptomycin versus Friulimicin B: In-Depth Profiling of
729 *Bacillus subtilis* Cell Envelope Stress Responses. *Antimicrob Agents Chemother*.
730 2009;53(4):1619-1623. doi:10.1128/AAC.01046-08

731 49. Hachmann A., Angert E, Helmann J. Genetic Analysis of Factors Affecting Susceptibility of
732 *Bacillus subtilis* to Daptomycin. *Antimicrob Agents Chemother*. 2009;53(4):1598-1609.
733 doi:10.1128/AAC.01329-08

734 50. Grein F, Müller A, Scherer KM, *et al*. Ca^{2+}-Daptomycin targets cell wall biosynthesis by
735 forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids.
736 *Nat Commun*. 2020;11(1):1455. doi:10.1038/s41467-020-15257-1

737 51. Tendolkar PM, Baghdayan AS, Shankar N. Pathogenic enterococci: new developments in the
738 21st century. *Cell Mol Life Sci C*. 2003;60(12):2622-2636. doi:10.1007/s00018-003-3138-0

739 52. Eckburg P, Bik E, Charles B, *et al*. Diversity of the Human Intestinal Microbial Flora. *Science*
740 *(80-)*. 2005;308(5728):1635-1638. doi:10.1126/science.1110591

741 53. Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J. Quantification of Bacterial Groups

742 within Human Fecal Flora by Oligonucleotide Probe Hybridization. *Appl Environ Microbiol*.
743 2000;66(5):2263-2266. doi:10.1128/AEM.66.5.2263-2266.2000

744 54. Lorian V. Colonization resistance. *Antimicrob Agents Chemother*. 1994;38(7):1693.
745 doi:10.1128/AAC.38.7.1693

746 55. Banerjee S, Hansen JN. Structure and expression of a gene encoding the precursor of subtilin,
747 a small protein antibiotic. *J Biol Chem*. 1988;263(19):9508-9514.
748 doi:https://doi.org/10.1016/S0021-9258(19)76571-5

749 56. Johnson B, Anker H, Meleney F. Bacitracin: A New Antibiotic Produced by a Member of the
750 *B. subtilis* Group. *Science (80-)*. 1945;102(2650):376-377. doi:10.1126/science.102.2650.376

751 57. Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. Quorum sensing-controlled gene
752 expression in lactic acid bacteria. *J Biotechnol*. 1998;64(1):15-21.
753 doi:https://doi.org/10.1016/S0168-1656(98)00100-X

754 58. Aldarhami A, Felek A, Sharma V, Upton M. Purification and characterization of nisin P
755 produced by a strain of *Streptococcus gallolyticus*. *J Med Microbiol*. 2020;69(4):605-616.
756 doi:https://doi.org/10.1099/jmm.0.001170

757 59. Storm DR, Strominger JL. Complex Formation between Bacitracin Peptides and Isoprenyl
758 Pyrophosphates. *J Biol Chem*. 1973;248(11):3940-3945. doi:10.1016/s0021-9258(19)43823-4

759 60. Economou N, Cocklin S, Loll P. High-resolution crystal structure reveals molecular details of
760 target recognition by bacitracin. *Proc Natl Acad Sci*. 2013;110(35):14207-14212.
761 doi:10.1073/pnas.1308268110

762 61. Cudic P, Kranz J, Behenna D, *et al*. Complexation of peptidoglycan intermediates by the
763 lipoglycodepsipeptide antibiotic ramoplanin: Minimal structural requirements for
764 intermolecular complexation and fibril formation. *Proc Natl Acad Sci*. 2002;99(11):7384-
765 7389. doi:10.1073/pnas.102192099

766 62. Staroń A, Finkeisen DE, Mascher T. Peptide antibiotic sensing and detoxification modules of

767 *Bacillus subtilis*. *Antimicrob Agents Chemother*. 2011;55(2):515-525.
768 doi:10.1128/AAC.00352-10

769 63. Gebhard S, Mascher T. Antimicrobial peptide sensing and detoxification modules: Unravelling
770 the regulatory circuitry of *Staphylococcus aureus*. *Mol Microbiol*. 2011;81(3):581-587.
771 doi:10.1111/j.1365-2958.2011.07747.x

772 64. Wu S, Lin K, Liu Y, Zhang H, Lei L. Two-component signaling pathways modulate drug
773 resistance of *Staphylococcus aureus* (Review). *Biomed Rep*. 2020;13(2):5.
774 doi:10.3892/br.2020.1312

775 65. Arii K, Kawada-Matsuo M, Oogai Y, Noguchi K, Komatsuzawa H. Single mutations in BraRS
776 confer high resistance against nisin A in *Staphylococcus aureus*. *Microbiologyopen*.
777 2019;8(11):e791. doi:https://doi.org/10.1002/mbo3.791

778 66. Meehl M, Herbert S, Götz F, Cheun A. Interaction of the GraRS Two-Component System with
779 the VraFG ABC Transporter To Support Vancomycin-Intermediate Resistance in
780 *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2007;51(8):2679-2689.
781 doi:10.1128/AAC.00209-07

782 67. Ledger EVK, Mesnage S, Edwards AM. Human serum triggers antibiotic tolerance in
783 *Staphylococcus aureus*. *Nat Commun*. 2022;13(1):2041. doi:10.1038/s41467-022-29717-3

784 68. Cheung AL, Bayer AS, Yeaman MR, *et al*. Site-specific mutation of the sensor kinase *graS* in
785 *Staphylococcus aureus* alters the adaptive response to distinct cationic antimicrobial peptides.
786 *Infect Immun*. 2014;82(12):5336-5345. doi:10.1128/IAI.02480-14

787 69. Kraus D, Herbert S, Kristian SA, *et al*. The GraRS regulatory system controls *Staphylococcus*
788 *aureus* susceptibility to antimicrobial host defenses. *BMC Microbiol*. 2008;8(1):85.
789 doi:10.1186/1471-2180-8-85

790 70. Bayer AS, Mishra NN, Cheung AL, Rubio A, Yang S-J. Dysregulation of *mprF* and *dltABCD*
791 expression among daptomycin-non-susceptible MRSA clinical isolates. *J Antimicrob*

792 *Chemother*. 2016;71(8):2100-2104. doi:10.1093/jac/dkw142

793 71. Yang S, Kreiswirth BN, Sakoulas G, *et al*. Enhanced Expression of *dltABCD* Is Associated
794 with the Development of Daptomycin Nonsusceptibility in a Clinical Endocarditis Isolate of
795 *Staphylococcus aureus*. *J Infect Dis*. 2009;200(12):1916-1920. doi:10.1086/648473

796 72. Cafiso V, Bertuccio T, Purrello S, *et al*. *dltA* overexpression: A strain-independent keystone of
797 daptomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Int J Antimicrob Agents*.
798 2014;43(1):26-31. doi:https://doi.org/10.1016/j.ijantimicag.2013.10.001

799 73. Mishra NN, Bayer AS, Weidenmaier C, *et al*. Phenotypic and Genotypic Characterization of
800 Daptomycin-Resistant Methicillin-Resistant *Staphylococcus aureus* Strains: Relative Roles of
801 *mprF* and *dlt* Operons. *PLoS One*. 2014;9(9):e107426.
802 https://doi.org/10.1371/journal.pone.0107426.

803 74. Bertsche U, Weidenmaier C, Kuehner D, *et al*. Correlation of Daptomycin Resistance in a
804 Clinical *Staphylococcus aureus* Strain with Increased Cell Wall Teichoic Acid Production and
805 d-Alanylation. *Antimicrob Agents Chemother*. 2011;55(8):3922-3928.
806 doi:10.1128/AAC.01226-10

807 75. Turnidge J, Kahlmeter G, Cantón R, MacGowan A, Giske CG. Daptomycin in the treatment of
808 enterococcal bloodstream infections and endocarditis: a EUCAST position paper. *Clin*
809 *Microbiol Infect*. 2020;26(8):1039-1043. doi:10.1016/j.cmi.2020.04.027

810 76. Cruz-Rodz AL, Gilmore MS. High efficiency introduction of plasmid DNA into glycine
811 treated *Enterococcus faecalis* by electroporation. *Mol Gen Genet MGG*. 1990;224(1):152-154.
812 doi:10.1007/BF00259462

813 77. Hanahan D, Glover DM. DNA cloning: a practical approach. *DNA cloning a Pract approach*.
814 1985;1:109-135.

815 78. Poyart C, Trieu-Cuot P. A broad-host-range mobilizable shuttle vector for the construction of
816 transcriptional fusions to β-galactosidase in Gram-positive bacteria. *FEMS Microbiol Lett*.

817 1997;156(2):193-198. doi:10.1111/j.1574-6968.1997.tb12726.x

818 79. Thurlow L, Chittezham TV, Hancock L. Capsular Polysaccharide Production in *Enterococcus*
819 *faecalis* and Contribution of CpsF to Capsule Serospecificity. *J Bacteriol*. 2009;191(20):6203-
820 6210. doi:10.1128/JB.00592-09

821 80. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap
822 extension using the polymerase chain reaction. *Gene*. 1989;77(1):51-59.
823 doi:https://doi.org/10.1016/0378-1119(89)90358-2

824 81. Kristich CJ, Chandler JR, Dunny GM. Development of a host-genotype-independent
825 counterselectable marker and a high-frequency conjugative delivery system and their use in
826 genetic analysis of *Enterococcus faecalis*. *Plasmid*. 2007;57(2):131-144.
827 doi:https://doi.org/10.1016/j.plasmid.2006.08.003

828 82. Gebhard S, Tran SL, Cook GM. The Phn system of Mycobacterium smegmatis: a second high-
829 affinity ABC-transporter for phosphate. *Microbiology*. 2006;152(11):3453-3465.
830 doi:https://doi.org/10.1099/mic.0.29201-0

831 83. Piepenbreier H, Sim A, Kobras CM, *et al*. From Modules to Networks: a Systems-Level
832 Analysis of the Bacitracin Stress Response in *Bacillus subtilis*. *mSystems*. 2020;5(1):e00687-
833 19. doi:10.1128/mSystems.00687-19

834 84. Allen D. Transcriptional regulation of the *dlt* operon in *Enterococcus faecalis* and further
835 characterization of a *dltA* mutant. 2008.

836

837 # Supplementary Material

838 **Table S1.** Vectors, plasmids and strains used in this study.
839 **Table S2.** Primers used in this study.
840 **Table S3.** Parameter values used in the mathematical model.
841 **Table S4.** Experimental numerical data for Figures 1, 3 and 4.

842 **File S1.** Mathematical model file for the application *Mathematica*