

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

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4                    Sali M. Morris<sup>1</sup>, Georg Fritz<sup>2</sup>, Tim Rogers<sup>3</sup>, Susanne Gebhard<sup>1\*</sup>

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6                    <sup>1</sup>Life Sciences Department, Milner Centre for Evolution, University of Bath, Claverton  
7        Down, Bath, BA2 7AY, United Kingdom; <sup>2</sup>School of Molecular Sciences, The University of  
8        Western Australia, WA 6009 Crawley, Australia; <sup>3</sup>Department of Mathematical Sciences,  
9                    University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom

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11                    \*Corresponding author. Phone: +44 1225 386421; E-mail: [s.gebhard@bath.ac.uk](mailto:s.gebhard@bath.ac.uk);

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21

## 22 **Summary**

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

38

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

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## 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<sup>1</sup>. Of these infections, the “ESKAPE”  
45 organisms<sup>2</sup> (*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<sup>3-5</sup>. 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<sup>6</sup> (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<sup>7,8</sup>, 30 years  
55 after vancomycin was introduced for clinical use<sup>9</sup>. Despite the molecular mechanisms of vancomycin  
56 resistance in enterococci being well understood today<sup>10</sup>, infections by vancomycin resistant enterococci  
57 (VRE) still result in serious health and economic impacts<sup>11</sup> 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)<sup>12</sup>. Disappointingly, within 2 years of clinical introduction of the drug in 2003, DAP-  
60 resistant enterococcal isolates were reported<sup>13</sup>, and in contrast to vancomycin resistance, this occurred  
61 through a subtle chromosomal change based on the mutations in genes *liaF*, *cls* and *gdpD*<sup>14-17</sup>. LiaF is  
62 a transmembrane protein involved in monitoring the integrity of the cell envelope and responding to  
63 damage<sup>18</sup>, whereas Cls (cardiolipin synthase) and GdpD (glycerol-phosphodiester phosphodiesterase)  
64 are both involved in phospholipid metabolism<sup>14</sup>. Of these, mutation of *liaF* has been proposed to be the  
65 first pivotal event towards daptomycin resistance<sup>15,19</sup>. 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<sup>20-23</sup>. The system is comprised of LiaF and a conventional two-component system (TCS): the  
70 sensor kinase LiaS and the response regulator LiaR<sup>20,24,25</sup>. LiaF is an inhibitor of LiaS, maintaining the  
71 sensor kinase in an inactive conformation<sup>20</sup>. Rather than detecting a specific antimicrobial compound,  
72 LiaFSR responds to cell envelope damage, although the exact stimulus is unknown<sup>26</sup>. 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*<sup>19,27</sup>. The *liaXYZ* cluster is involved in sensing and binding antimicrobials  
75 at the cell surface to provide resistance<sup>27</sup>.

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<sup>28</sup>, 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<sup>29</sup>. 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<sup>30-32</sup>.

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<sup>33</sup>. 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)<sup>33</sup>. 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*<sup>34</sup>. 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<sup>35,36</sup>. The sensory ABC transporter SapAB,  
93 based on biochemical evidence from the *B. subtilis* system, forms a sensory complex with SapRS<sup>37</sup>. In

94 the absence of bacitracin, the transporter maintains the histidine kinase, SapS, in an ‘OFF’ state<sup>38</sup>. Upon  
95 detection of bacitracin-UPP (BAC-UPP) complexes, SapAB switches its role from repressor to activator  
96 of SapS, resulting in kinase autophosphorylation<sup>38</sup>. 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<sup>39</sup>, 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)<sup>40</sup> 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<sup>33</sup>.

105 Interestingly, recent work by the Arias lab demonstrated that when experimentally evolving *E. faecalis*  
106 for DAP resistance in a  $\Delta liaFSR$  background, mutations were observed in the sensory transporter  
107 SapAB<sup>41,42</sup>, 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<sup>43,44</sup>, 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

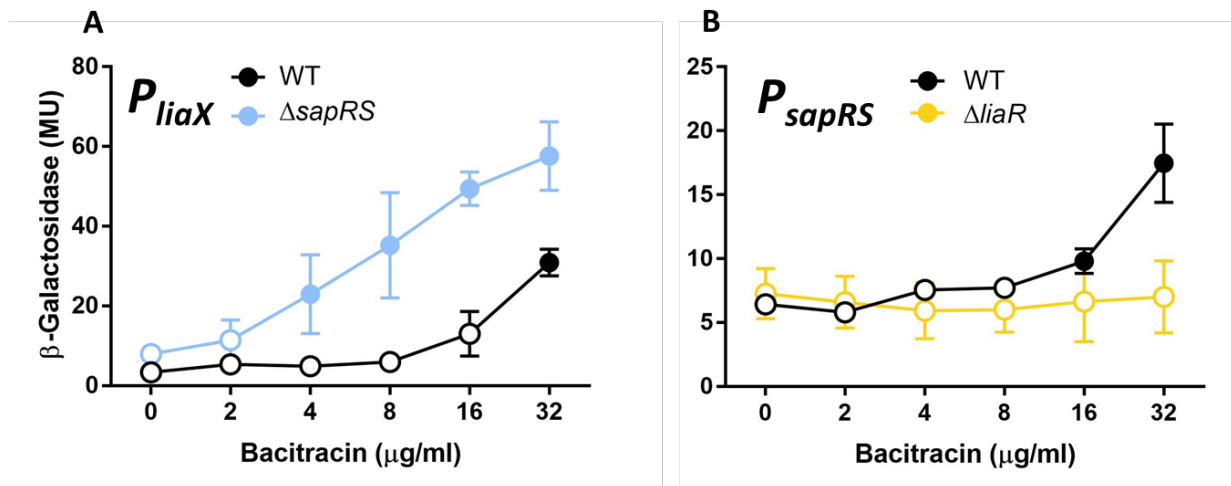
121 and LiaFSR signalling effectively implements a logic ‘AND’ gate, whereby expression of SapR target  
122 genes is under dual control and requires two separate signalling inputs. These observations also explain  
123 the occurrence of mutations in both regulatory systems during clinical and experimental 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<sup>33</sup>. Interestingly, we also  
130 showed that *sapRS* was not autoregulated, in contrast to other two component systems such as CroRS  
131 and LiaFSR<sup>20,28</sup>, and regulation did not depend on either SapAB or RapAB<sup>33</sup>. 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*<sup>43</sup> and recently shown to  
135 have a possible functional link to the Sap system<sup>41,42</sup>. 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<sup>27</sup>, 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  $\mu\text{g ml}^{-1}$  bacitracin compared with untreated cells  
141 (Fig. 1A, black line), indicating that LiaFSR can indeed respond to bacitracin exposure in *E. faecalis*.



142

**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.  $\beta$ -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  $16 \mu\text{g ml}^{-1}$   
147 or higher led to significant induction of the reporter, with a three-fold increase compared to uninduced  
148 cells at  $32 \mu\text{g ml}^{-1}$  (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  $32 \mu\text{g ml}^{-1}$ . 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<sup>33</sup>, 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<sup>45</sup>. To  
158 prevent the induction of the main bacitracin resistance genes of *E. faecalis*, controlled by SapRS<sup>33</sup> and  
159 thus remove potential interference with Lia signalling, we therefore introduced the *P<sub>liaX-lacZ</sub>*  
160 transcriptional fusion into the  $\Delta$ *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  $\mu\text{g ml}^{-1}$  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*<sup>45</sup>, as well as physiological links  
166 between the SapRS- and LiaFSR-dependent components of the CESR in *E. faecalis*.

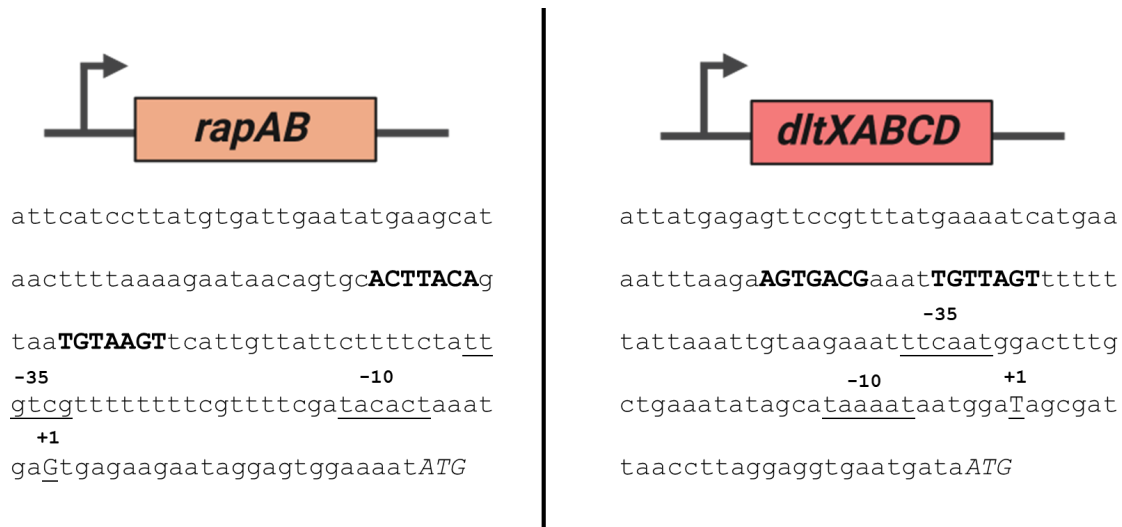
167 **SapRS controls the expression of the *dltABCD* operon.** To expand our understanding of the Lia/Sap  
168 regulatory network, we next considered if other genes may also be under its control. Currently, the only  
169 known gene under SapRS regulation is the resistance ABC-transporter operon *rapAB*<sup>33</sup>. 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<sup>24,40,43</sup>. 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*<sup>27</sup>. 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<sup>46</sup>, a mechanism  
178 commonly involved in DAP resistance amongst the low-CG bacteria<sup>47</sup>.

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 *ycvRS*. These mutations correlated with an increase in *dlt* transcription when measured by qPCR and  
182 resulted in an increase in cell surface charge<sup>41,42</sup>. 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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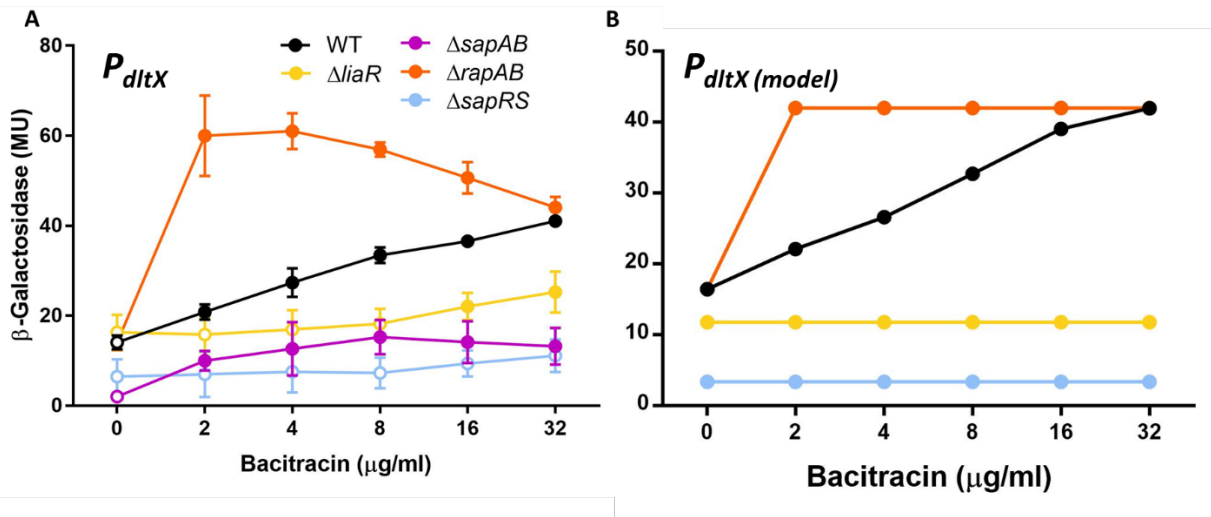


**Figure 2. Schematic of the promoter regions of both *rapAB* and *dltABCD*.** The sequence 150 base pairs upstream of the ATG start codons are shown. The proposed binding site for SapR on both the *rapAB* and *dltABCD* promoters is in bold and capitalised, and the likely -10 and -35 elements are underlined.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

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  $\mu\text{g ml}^{-1}$   
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  $\Delta sapR$  (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.



204

**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  $\mu\text{g ml}^{-1}$  (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  $16 \mu\text{g ml}^{-1}$  rather than at  $2 \mu\text{g ml}^{-1}$  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  $\Delta$ *rapAB* 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  $2 \mu\text{g ml}^{-1}$  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<sup>34</sup>, 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<sup>34,39</sup>. 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.

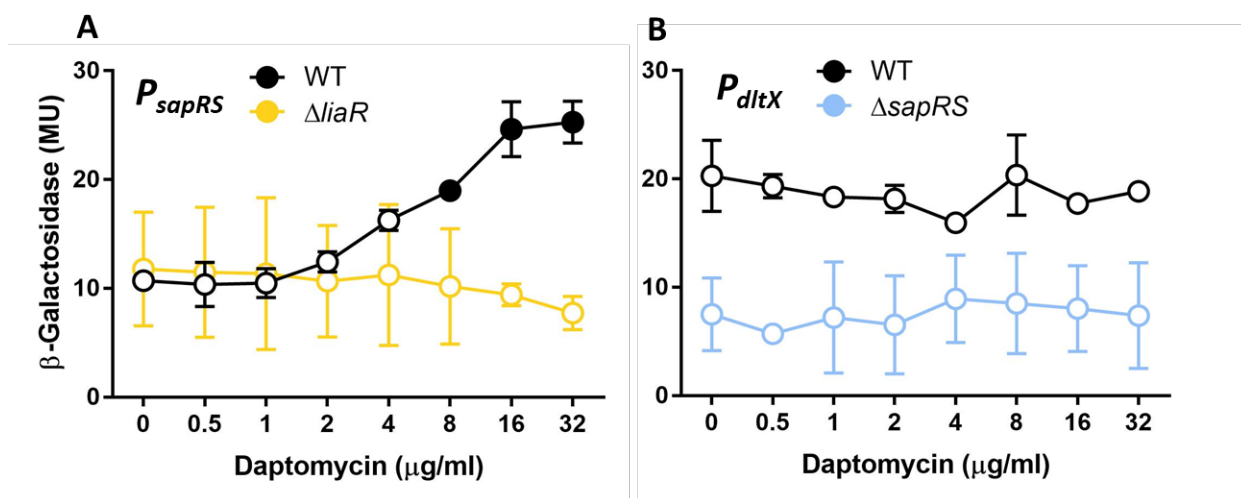
245 This model accurately reflected the behaviour of our experimental strains, depicting the same gradual  
246 response to bacitracin of the *dltX* promoter in wild-type *E. faecalis* (Fig. 3B, black symbols), as well as  
247 the hypersensitive response in the *rapAB* deletion strain, where the negative 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<sup>39</sup>; at the same time, bacitracin  
264 induces cell envelope damage, triggering LiaFSR activation<sup>26</sup>. 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<sup>48,49</sup>. 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<sup>39</sup>. Indeed, the tripartite complex of DAP with Lipid II and phosphatidyl glycerol in the  
270 membrane<sup>50</sup> 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.

272



273

**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<sub>sapRS</sub>-lacZ* (panel A) or a *P<sub>dltX</sub>-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<sub>sapRS</sub>-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<sub>dltX</sub>-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<sup>51-54</sup>. 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<sup>55</sup>, bacitracin producer *Bacillus licheniformis* ATCC10716<sup>56</sup>, nisin-A producer *Lactococcus*  
305 *lactis* NZ9000<sup>57</sup> and nisin-P producer *Streptococcus gallolyticus* AB39<sup>58</sup>. Each producer was spotted

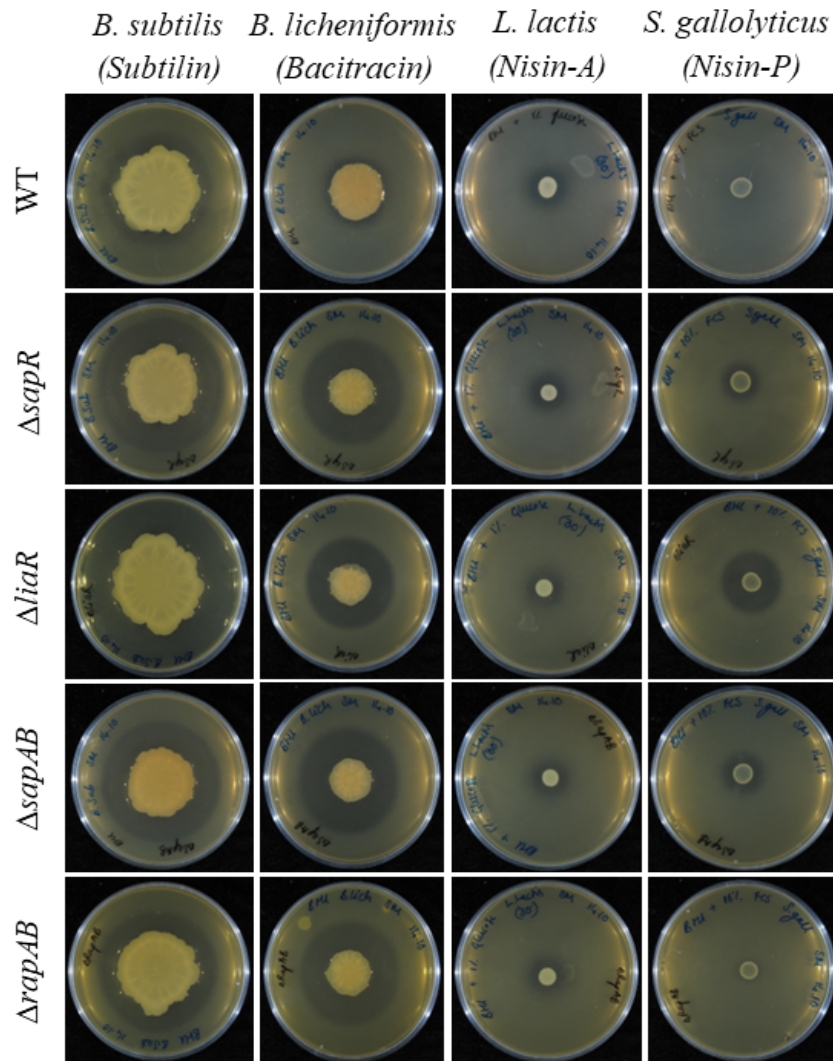
306 onto a plate, and the antimicrobial was allowed to accumulate over multiple days. This was followed  
307 by the addition of an overlay containing the different *E. faecalis* strains to assess their sensitivity against  
308 the produced compounds based on the size of the zone of inhibition (Fig. 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  $\Delta liaR$  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  $\Delta liaR$ . 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<sup>33</sup>. 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



324

**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<sub>600</sub> 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.

325

326 We next tested the *E. faecalis* mutants against the nisin producers, *L. lactis* NZ9000 and *S. gallolyticus*  
327 AB39. Although there was some inhibition of wild type *E. faecalis* by the nisin-A producer *L. lactis*,  
328 there was no difference in sensitivity between the wild type and deletion strains. This suggests either  
329 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<sup>41,42</sup>. 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<sup>35,36</sup>. The formation of this complex prevents  
361 the dephosphorylation of UPP and slows cell wall formation<sup>59,60</sup>. In contrast, daptomycin is a  
362 lipopeptide antibiotic which forms a tripartite complex between lipid II and the membrane phospholipid  
363 phosphatidyl glycerol<sup>50</sup>. 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<sup>33</sup>, both of which bind  
371 to lipid II cycle intermediates<sup>35,61,62</sup>. 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<sup>39</sup>. 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)<sup>62,63</sup>. 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<sup>34,38</sup>. 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)<sup>50</sup>. It therefore would not be expected to form  
382 the type of complexes that could act as substrate for SapAB<sup>39</sup>, and Sap signalling is not activated.

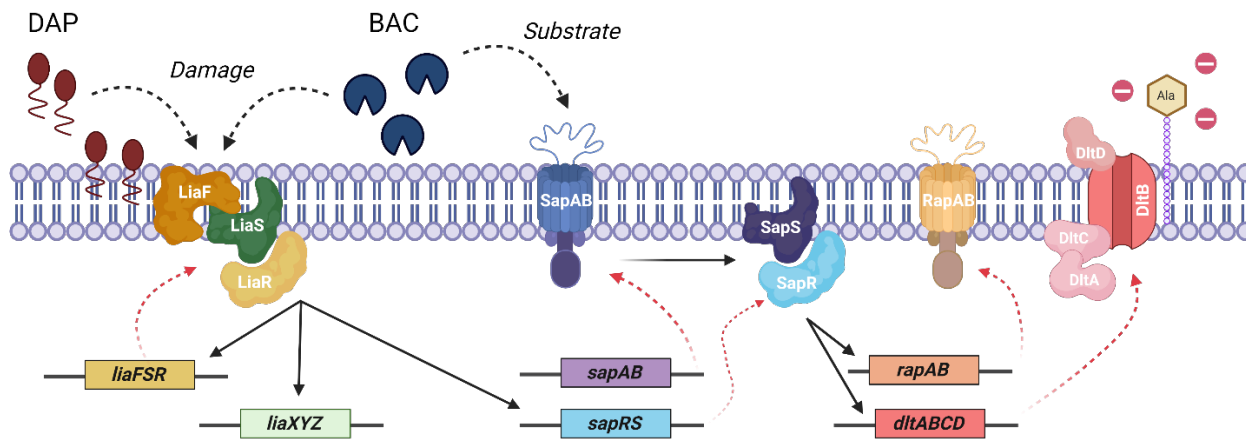
383 BceAB-type transporters have been shown to repress histidine kinase activity in the absence of  
384 substrate<sup>38</sup>. Thus, the lack of a SapAB substrate likely leads to the transporter maintaining SapS in an  
385 ‘OFF’ conformation, preventing activation of SapR target genes, i.e. *dlt*. 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*<sup>41,42</sup>. 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<sup>33</sup>.

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<sup>24</sup>. In *S. aureus*, the LiaFSR homologue VraRS  
406 has been shown to regulate the expression of multiple genes involved in cell wall biosynthesis<sup>64</sup>, 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*<sup>40</sup>, or can be involved  
409 with other Bce-type systems, such as the BraRS-VraDE<sup>65</sup> and GraRS-VraFG<sup>66</sup> 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<sup>67-69</sup>. 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*<sup>39,45</sup>. 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<sup>33</sup>,  
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*<sup>70-74</sup>. 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<sup>75</sup>. 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<sup>19</sup>, or experimentally  
460 in *sapAB* as suppressor mutations of a LiaR-deficient strain<sup>41,42</sup>. DAP was introduced for treatment of  
461 enterococci infections in 2003<sup>12</sup> and the first resistant isolates were identified already in 2005<sup>13</sup>. 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 DH5 $\alpha$  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 galloyticus* 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<sup>-1</sup> agar. Selective media contained chloramphenicol (10  $\mu$ g ml<sup>-1</sup> for *E. coli*; 15  $\mu$ g ml<sup>-1</sup>  
480 for *E. faecalis*), kanamycin (50  $\mu$ g ml<sup>-1</sup> for *E. coli*; 1000  $\mu$ g ml<sup>-1</sup> for *E. faecalis*), spectinomycin (100  
481  $\mu$ g ml<sup>-1</sup> for *E. coli*; 500  $\mu$ g ml<sup>-1</sup> for *E. faecalis*). For blue-white screening, 5-bromo-4-chloro-3-indolyl-  
482  $\beta$ -d-galactopyranoside (X-Gal) was used at 120  $\mu$ g/ml. Bacitracin was supplied as the Zn<sup>2+</sup> salt. All  
483 media for experiments with daptomycin were supplemented with 50  $\mu$ M CaCl<sub>2</sub>.

484 *E. faecalis* was transformed by electroporation as previously described<sup>76</sup>. *E. coli* was transformed by  
485 heat-shock of CaCl<sub>2</sub> competent cells, followed by 1 hour recovery time<sup>77</sup>. Growth was measured as  
486 optical density at 600 nm (OD<sub>600</sub>) on a the Biochrom<sup>TM</sup> Novaspec Pro Spectrophotometer using cuvettes  
487 with 1 cm light path length or in 96-well plates with 100  $\mu$ L culture volumes on a Spark<sup>®</sup> 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<sup>78</sup>. 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<sup>79</sup>. 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<sup>80</sup> and were subsequently cloned into the NcoI and  
497 BamHI site of the vector pLT06. Gene deletions were performed as previously described<sup>79</sup>. 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<sup>81</sup>  
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<sub>600</sub>) on a Spark<sup>®</sup> Microplate reader (Tecan). The MIC was scored as the lowest antibiotic  
516 concentration where no growth was observed following subtraction of the OD<sub>600</sub> 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<sub>600</sub> = 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)<sup>82</sup>. For this, cells were resuspended in 1 ml Z-  
523 buffer (8.04 g Na<sub>2</sub>HPO<sub>4</sub>\*7H<sub>2</sub>O, 2.76 g NaH<sub>2</sub>PO<sub>4</sub>\*H<sub>2</sub>O, 0.123 g MgSO<sub>4</sub>\*7H<sub>2</sub>O and 5 mL 1M KCl in 495  
524 mL dH<sub>2</sub>O, pH 7). The samples were adjusted to OD<sub>600</sub> = 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  $\mu$ l  
527 0.1% (w/v) SDS and 40  $\mu$ l chloroform were added and vortexed for 5 seconds, then rested for 5-10  
528 minutes. Reactions were started by adding 200  $\mu$ l *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (4 mg  
529 mL<sup>-1</sup> 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  $\mu$ l 1M Na<sub>2</sub>CO<sub>3</sub>, 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:

$$533 \quad \text{Miller Units (MU)} = \frac{A_{420} * 1000}{\text{Time (minutes)} * \text{volume of cells (in ml)} * OD_{600}}$$

534 **Computational modelling.** Rather than directly modelling the temporal dynamics of the regulatory  
535 network as done for the *B. subtilis* Bce system<sup>34,83</sup>, 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

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

541 where *x* is the input quantity and *y* the output which varies between  $y_{\text{base}}$  and  $y_{\text{max}}$ , 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

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

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

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

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

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

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  $\mu$ l) 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  $\mu$ L 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

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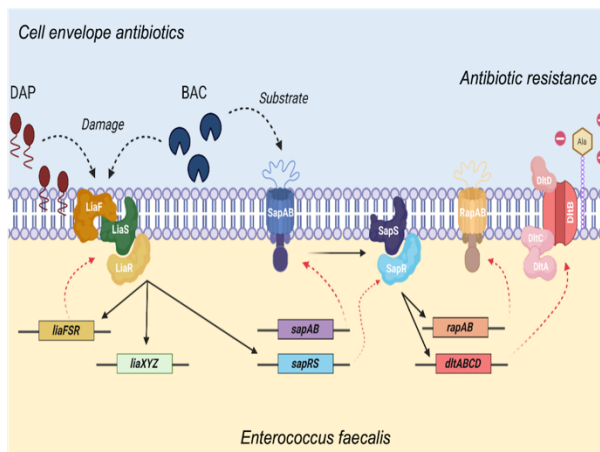
579

## 580 Author contributions

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

584

## 585 Graphical abstract



586

## 587 Abbreviated summary

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

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## 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*