Regulatory interactions between daptomycin and bacitracin responsive pathways coordinate the cell envelope antibiotic resistance response of *Enterococcus faecalis*

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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. To better understand the regulation of DAP resistance and how this relates to mutations observed in DAP-resistant clinical isolates of enterococci, we here explored the interplay between these two regulatory pathways. Our results show that SapR regulates an additional resistance operon, *dltXABCD*, a known DAP resistance determinant, and show that LiaFSR regulates the expression of *sapRS*. This regulatory structure places SapRS target genes under dual control, where expression is directly controlled by SapRS, which itself is up-regulated through LiaFSR. The network structure described here shows how *E. faecalis* coordinates its response to cell envelope attack and can explain why clinical DAP resistance often emerges via mutations in regulatory components.

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

44 # Introduction

45 The rise of antibiotic resistant bacteria is one of the greatest current threats to public health, resulting in
46 670,000 infections a year and 33,000 deaths in Europe alone (Organisation for Economic Co-operation
47 and Development, 2020). Of these infections, the "ESKAPE" organisms (Zhen *et al.*, 2019)
48 (*Enterococci* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,
49 *Pseudomonas aeruginosa*, *Enterobacter* spp.) have driven the rising number of nosocomial and
50 antibiotic-resistant infections in the past decade. Of these bacteria, the enterococci are the second most
51 common causative agent of nosocomial infections in the US; including bacteraemia, endocarditis and
52 urinary tract infections (Miller *et al.*, 2016; Chiang *et al.*, 2017; Said *et al.*, 2021). The two species most
53 frequently isolated, *Enterococcus faecalis* and *Enterococcus faecium*, remain a major infection-control
54 challenge, particularly in healthcare settings.

55 Enterococci became recognised as important nosocomial pathogens due to their high level of intrinsic
56 resistance to several antimicrobials (Arias and Murray, 2012) (e.g. penicillin, ampicillin and
57 cephalosporins) and their capacity to acquire further resistance determinants. One such acquired
58 resistance is to the glycopeptide antibiotic vancomycin, which occurs through plasmid acquisition and
59 was first reported in the 1988 (Leclercq *et al.*, 1988; Uttley *et al.*, 1989), 30 years after vancomycin was
60 introduced for clinical use (Levine, 2006). Despite the molecular mechanisms of vancomycin resistance
61 in enterococci being well understood today (Courvalin, 2006), infections by vancomycin resistant
62 enterococci (VRE) still result in serious health and economic impacts (Puchter *et al.*, 2018) and are an
63 increasing problem worldwide.

64 One of the last-resort antibiotics used to treat these VRE infections is the lipopeptide antibiotic
65 daptomycin (DAP) (Sauermann *et al.*, 2008). Disappointingly, within two years of clinical introduction
66 of the drug in 2003, DAP-resistant enterococcal isolates were reported (Munoz-Price *et al.*, 2005), and
67 in contrast to vancomycin resistance, this occurred through a subtle chromosomal change based on the
68 mutations in genes *liaF*, *cls* and *gdpD* (Palmer *et al.*, 2011; Arias *et al.*, 2011; Tran *et al.*, 2013; Reyes
69 *et al.*, 2015). LiaF is a transmembrane protein involved in monitoring the integrity of the cell envelope

70 and responding to damage (Tran *et al.*, 2016), whereas Cls (cardiolipin synthase) and GdpD (glycerol-
71 phosphodiester phosphodiesterase) are both involved in phospholipid metabolism (Arias *et al.*, 2011).
72 Of these, mutation of *liaF* has been proposed to be the first pivotal event towards DAP resistance (Miller
73 *et al.*, 2013; Tran *et al.*, 2013). However, the exact role of the *liaF* mutations in DAP resistance so far
74 remains unclear.

75 LiaF is part of a three-component regulatory system, LiaFSR, which is important amongst the
76 Firmicutes for coordinating the cell envelope stress response (CESR) against antimicrobial-induced
77 damage (Kuroda *et al.*, 2003; Jordan *et al.*, 2006; Martínez *et al.*, 2007; Suntharalingam *et al.*, 2009).
78 The system is comprised of LiaF and a conventional two-component system (TCS): the sensor kinase
79 LiaS and the response regulator LiaR (Jordan *et al.*, 2006; Schrecke *et al.*, 2013; Wolf *et al.*, 2010).
80 LiaF is an inhibitor of LiaS, maintaining the sensor kinase in an inactive conformation (Jordan *et al.*,
81 2006). Rather than detecting a specific antimicrobial compound, LiaFSR responds to cell envelope
82 damage, although the exact stimulus is unknown (Wolf *et al.*, 2012). Upon sensing this damage, LiaF
83 releases LiaS, which is then able to phosphorylate LiaR to induce the expression of the system's target
84 operon, *liaXYZ* (Khan *et al.*, 2019; Miller *et al.*, 2013). The *liaXYZ* cluster is thought to be involved in
85 sensing and binding antimicrobials at the cell surface to provide resistance (Khan *et al.*, 2019a).

86 To add to this complexity, the Lia system does not exist in isolation, but is just one of many TCSs
87 involved in monitoring cell envelope integrity, each responding to their own individual stimuli and
88 activating a unique set of target genes. The CroRS system, unique to the enterococci, is the main
89 regulator required for cephalosporin resistance (Comenge *et al.*, 2003), whereas the VicKR (YycFG)
90 system is essential across the low-CG Gram positives and is involved in regulating cell division, lipid
91 biosynthesis, biofilm and virulence (Winkler and Hoch, 2008). An additional element of the network is
92 the serine/threonine kinase IreK and the phosphatase IreP, involved in maintaining cell wall integrity
93 by potentially regulating peptidoglycan biosynthesis and metabolism (Kristich *et al.*, 2007a; Kristich *et*
94 *al.*, 2011; Iannetta *et al.*, 2021).

95 A further TCS involved in monitoring the cell envelope is EF0926/27, which we have now renamed
96 SapRS (Sensor of Antimicrobial Peptides), identified in our previous work (Gebhard *et al.*, 2014).
97 SapRS is part of a bacitracin (BAC) resistance network comprised of the histidine kinase SapS, the
98 response regulator SapR and the Bce-like ABC transporters: SapAB (EF2752/51) and RapAB
99 (Resistance against Antimicrobial Peptides) (EF2050/49) (Gebhard *et al.*, 2014). The use of the sensory
100 transporter SapAB to control the activity of SapRS implements a 'flux-sensing' mechanism to regulate
101 BAC resistance, as was shown for the homologous system in *Bacillus subtilis* (Fritz *et al.*, 2015). In
102 brief, upon exposure, BAC binds to its membrane-associated target molecule, undecaprenol-
103 pyrophosphate (UPP), blocking the dephosphorylation and recycling of UPP in the lipid II cycle of cell
104 wall biosynthesis (Storm and Strominger, 1974; Storm, 1974). The sensory ABC transporter SapAB,
105 based on biochemical and structural evidence from the *B. subtilis* system, forms a sensory complex with
106 SapRS (Dintner *et al.*, 2014; George and Orlando, 2023). In the absence of BAC, the transporter
107 maintains the histidine kinase, SapS, in an 'OFF' state (Koh *et al.*, 2021). Upon detection of BAC-UPP
108 complexes, SapAB switches its role from repressor to activator of SapS, resulting in kinase
109 autophosphorylation (Koh *et al.*, 2021). SapS can then phosphorylate SapR, which in turn induces the
110 production of the resistance transporter RapAB. RapAB, again based on evidence from its *B. subtilis*
111 homologue, frees UPP from the inhibitory grip of BAC using a target protection mechanism (Kobras *et*
112 *al.*, 2020), allowing dephosphorylation of UPP and continuation of cell wall synthesis, rendering the
113 cell resistant to BAC. The equivalent system in *B. subtilis* is a self-contained module, involving only a
114 single transporter (BceAB) and TCS (BceRS) (Ohki *et al.*, 2003) with no known further genes involved
115 in its signalling or resistance mechanism. However, in *E. faecalis*, we observed that the TCS SapRS is
116 itself transcriptionally induced by BAC, however we had not identified the regulatory system that
117 controls this expression (Gebhard *et al.*, 2014).

118 Interestingly, recent work by the Arias lab demonstrated that when experimentally evolving *E. faecalis*
119 for DAP resistance in a Δ*liaFSR* background, mutations were observed in the sensory transporter
120 SapAB (Prater *et al.*, 2021, 2019), suggesting a functional link between the Sap and Lia systems. In *B.*
121 *subtilis*, the Lia system is known to be one of the main components of BAC resistance (Mascher *et al.*,

122 2003; Rietkötter *et al.*, 2008), but there is currently no evidence for a role of LiaFSR in response to
123 BAC in *E. faecalis*. Neither is there any indication of a role for the Sap system in responding to DAP
124 exposure. However, this recent evidence suggested an interplay between both the Sap and Lia systems
125 and that, potentially, both systems may be contributing to DAP resistance.

126 In accordance with the need to deepen our knowledge of the CESR in *E. faecalis,* in this study we
127 sought to examine the potential interplay between the Sap and Lia systems and investigate the
128 involvement of further genes associated with this regulatory network. Using gene deletions, analysis of
129 promoter activity and mutation of putative regulator binding sites, we provide evidence of the activation
130 of LiaFSR signalling in response to BAC and describe a direct functional link between the Lia and Sap
131 systems by demonstrating the regulation of *sapRS* by LiaR in response to antibiotic exposure. We
132 further identify the *dltXABCD* operon, a known determinant of DAP resistance (Prater *et al.*, 2019), as
133 a target of SapRS regulation. Through a detailed analysis of promoter activation in response to DAP
134 exposure, we show that the same regulatory network also controls the response of *E. faecalis* to this
135 antibiotic. The complex regulatory architecture of LiaFSR controlling expression of *sapRS*, which in
136 turn controls a set of BAC and DAP resistance genes appears unique to enterococci. It may allow
137 sensitive and robust changes in gene expression through 'priming' of the SapRS response via LiaFSR-
138 dependent upregulation of SapRS levels and offers an explanation why mutations in DAP-resistant
139 clinical isolates and experimentally evolved strains so often are found in these regulatory genes.

140

141 # Results

142 **LiaFSR responds to bacitracin and controls expression of *sapRS*.** As mentioned above, from
143 previous work we had identified increased expression of *sapRS* under exposure to BAC or mersacidin
144 (Gebhard *et al.*, 2014). Interestingly, we also had shown that *sapRS* was not autoregulated, in contrast
145 to other two component systems such as CroRS and LiaFSR (Comenge *et al.*, 2003; Jordan *et al.*, 2006),
146 and regulation did not depend on either SapAB or RapAB (Gebhard *et al.*, 2014). We therefore here
147 first aimed to identify the regulator of *sapRS*. For possible candidates, we considered potential

148 regulators that are common amongst the Firmicutes and are known to respond to BAC. The most
149 probable candidate appeared to be LiaFSR, often referred to as the 'master regulator' of the CESR in
150 *Bacillus subtilis* (Mascher *et al.*, 2003) and recently shown to have a possible functional link to the Sap
151 system (Prater *et al.*, 2021, 2019).

152 To test if LiaFSR might regulate *sapRS* expression, we first had to establish if LiaFSR was able to
153 respond to BAC treatment. It is well established that *liaX* is under the control of LiaFSR (Khan *et al.*,
154 2019), and therefore we exposed *E. faecalis* harbouring a P_{liaX}-*lacZ* transcriptional fusion to increasing
155 levels of BAC, as a readout for LiaFSR activity. The results showed a ~10-fold increase in P_{liaX} activity
156 at 32 μg ml^{-1} BAC compared with untreated cells (Fig. 1A, black line), indicating that LiaFSR can
157 indeed respond to BAC exposure in *E. faecalis* albeit weakly. Addition of higher concentrations of BAC
158 significantly impacted cellular growth and was therefore not used to test promoter induction.

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

167 **The bacitracin-response of LiaFSR is masked by the primary resistance determinant, RapAB.** A
168 surprising feature of the BAC-induced LiaR activity was the high concentration required to trigger a
169 response, as *liaX* expression only significantly increased at 32 μg ml^{-1}. One possible explanation for
170 this might be that the response was masked by other components of the BAC stress response in *E.*
171 *faecalis*, for example RapAB, which plays an active role in the removal of BAC from UPP (Gebhard *et*
172 *al.*, 2014), protecting the cell from damage. In *B. subtilis*, it was observed that the RapAB equivalent,
173 BceAB, was the primary response to protect the cell from BAC exposure and masked the response of
174 the Lia system (Radeck *et al.*, 2016). To prevent the induction of the SapRS-dependent BAC resistance

175 genes of *E. faecalis*, including *rapAB* (Gebhard *et al.*, 2014), and thus remove potential interference
176 with Lia signalling, we therefore introduced the P_{liaX}-*lacZ* transcriptional fusion into the Δ*sapRS*
177 background. Compared to the wild type, the expression of the *liaX* promoter in response to BAC
178 challenge of the deletion strain showed increased sensitivity, significantly inducing expression from 4
179 μg ml^{-1} BAC and reaching overall higher activities (Fig. 1A, blue line). This showed that the weak
180 response of Lia signalling to BAC in the wild type was indeed due to masking effects of the resistance
181 genes controlled by SapRS, implying the presence of multiple layers of protection, similar to those
182 observed in *B. subtilis* (Radeck *et al.*, 2016). These data also provided further indication of physiological
183 links between the SapRS- and LiaFSR-dependent components of the CESR in *E. faecalis*.

184 To test the physiological implications of LiaR controlling *sapRS* expression, the deletion strains were
185 tested for their minimal inhibitory concentration (MIC) of BAC (Table 1). Consistent with our previous
186 results (Gebhard *et al.*, 2014), deletion of the known BAC resistance operon *rapAB* and the regulatory
187 operons *sapRS* and *sapAB* led to a two- to four-fold decrease in MIC. Deletion of *liaR* only had a minor,
188 if any, effect on MIC, with 8-16 μg ml^{-1} BAC required to prevent its growth, compared to 16 μg ml^{-1}
189 for the wild type. This mild phenotype was consistent with LiaR acting as an inducer of the main BAC-
190 responsive TCS, SapRS, rather than directly inducing the resistance genes. LiaR activity thus likely acts
191 to amplify the response, but some resistance gene expression still occurs from basal levels of SapRS.
192 This is addressed in more detail in the following section. Complementation of BAC sensitivity in the
193 *liaR*-deleted strain with an ectopic copy of the gene driven by the native promoter of the *liaFSR* operon
194 (i.e. a P_{liaF}-*liaR* construct) unfortunately was unsuccessful. It had been shown previously for the LiaFSR
195 system of *B. subtilis* that its function is exquisitely sensitive to the stoichiometry of the three proteins
196 (Schrecke *et al.*, 2013). It is therefore likely that a similar situation exists in *E. faecalis* and that we were
197 not able to re-create the exact cellular level of LiaR needed for the regulatory system to function
198 correctly. Complementation of reporter gene induction could not be tested, because all plasmids
199 available for this study carry the same replication origin for *E. faecalis* and could therefore not be
200 maintained in combination.

201 **A LiaR binding site is present in the *sapRS* promoter and required for induction.** To confirm that
202 the regulation of *sapRS* was directly due to LiaR activity, particularly in light of the lack of
203 complementation of the resistance phenotype, we searched the promoter sequence upstream of the *sapR*
204 start codon for a potential LiaR binding site. The promoter contained sequences with a good match to
205 consensus -10 and -35 elements with conventional spacing (Fig. 1C, underlined). LiaR of *E. faecalis*
206 has been shown to have a complex mechanism of promoter recognition, involving a primary consensus
207 binding sequence and adjoining secondary recognition sequence, and activation involves a dimer to
208 tetramer transition (Davlieva *et al.*, 2015). This is further complicated by the consensus sequence having
209 only four, evenly spaced, conserved residues: T-N_4-C-N_4-G-N_4-A, with degeneracy observed between
210 different LiaR targets (Davlieva *et al.*, 2015). Searching the *sapR* promoter for a LiaR binding site
211 revealed the existence of two such sequences, positioned directly next to each other and each with only
212 a single mismatch to the consensus (Fig. 1C, bold and white-on-black letters). From this, it was not
213 possible to predict which was the most likely candidate binding site, or if these two sequences might
214 represent the previously described primary and secondary recognition sequences.

215 To test this experimentally, we generated two new P_{sapR}-*lacZ* fusions by DNA synthesis, one in which
216 two bases of the promoter-distal putative binding sites had been mutated, and one where two positions
217 of the promoter-proximal binding site had been changed (Fig. 1C, coloured letters). When either of
218 these constructs was introduced into wild-type *E. faecalis* and assayed for induction by BAC, no
219 response could be detected at any tested antibiotic concentrations (Fig. 1D). These data strongly suggest
220 that the identified sequences indeed constitute the LiaR binding site and are likely the pair of binding
221 sites required for the primer-to-tetramer transition mechanism of LiaR-dependent promoter activation.
222 This further confirmed direct regulation of *sapRS* expression by the LiaFSR system.

223 **SapRS controls the expression of the *dltABCD* operon.** In *B. subtilis*, both the Lia and Bce systems
224 act as self-contained modules, controlling the regulation of only a single resistance operon each, *liaIH*
225 and *bceAB* respectively, each encoded adjacently to its regulatory operon on the chromosome (Ohki *et*
226 *al.*, 2003; Mascher *et al.*, 2003; Wolf *et al.*, 2010). However, the direct interaction between Lia and Sap
227 signalling demonstrated above suggests that the regulatory setup in *E. faecalis* is much more complex.

228 This is further supported by evidence from the literature, with the indication that the Lia system has a
229 larger operon than just itself and *liaXYZ* (Khan *et al.*, 2019). There is also a proposal that Lia contributes
230 to DAP resistance through the regulation of *dltXABCD* (*dlt*), although this is likely an indirect effect
231 also involving the *yvcRS* operon (Prater *et al.*, 2021, 2019), which encodes the sensory transporter
232 SapAB that works together with SapRS in signalling of BAC stress, as explained above. Evidence for
233 the link between LiaFSR, SapAB-SapRS and the *dlt* operon comes from a study evolving *E. faecium*
234 for DAP resistance in the absence of LiaFSR. Suppressor mutations occurred in the *sapAB* (*ycvRS*)
235 operon and correlated with an increase in *dlt* transcription when measured by qPCR and an increase in
236 cell surface charge (Prater *et al.*, 2021, 2019). Dlt is responsible for the D-alanylation of teichoic acids
237 on the bacterial cell surface, resulting in a decrease in the negative charge of the cell envelope (Peschel
238 *et al.,* 1999), a mechanism commonly involved in DAP resistance amongst the low-CG bacteria (Tran
239 *et al.,* 2015). Moreover, the *dltABCD* operon is located directly adjacent to *sapAB* on the chromosome,
240 further supporting a functional link between the genes. To expand our understanding of the Lia/Sap
241 regulatory network and how a BAC resistance system might be linked to DAP resistance, we therefore
242 next sought to investigate the contribution of LiaFSR and SapRS to *dlt* regulation.

243 To this end, we first constructed a transcriptional P_{dltX}-*lacZ* fusion to test *dlt* induction by BAC. The
244 results showed that in wild-type JH2-2 carrying the fusion, increasing BAC concentrations led to a
245 dose-dependent increase in *dlt* expression, resulting in ~4-fold higher activity at 32 µg ml^{-1} compared
246 to untreated conditions (Fig. 2A, black line), showing that *dlt* expression is induced in response to BAC
247 in *E. faecalis*. When we tested the response of the reporter in strains carrying deletions of either *sapRS*
248 or *sapAB,* the results showed a decrease in basal activity in both strains and a complete loss of the
249 promoter's BAC response in Δ*sapR* (Fig. 3A, blue and magenta lines). Loss of SapAB still resulted in
250 induction compared with uninduced cells, but overall activities were considerably lower than in the
251 wild-type strain. This indicated that SapRS was essential for *dlt* expression in response to BAC, but a
252 residual amount of *dlt* induction remained in the *sapAB* deletion. This is consistent with the less direct
253 regulatory role of SapAB, i.e. via controlling SapRS signalling activity and not the target genes directly.
254 While SapAB is required to activate SapRS signalling, it does not influence the expression of *sapRS*

255 (Gebhard *et al.*, 2014), which is instead controlled by LiaFSR as we have shown above. Thus, in a
256 *sapAB*-deleted strain, the cellular amount of SapRS will still increase in response to BAC due to LiaR
257 activity, leading to higher basal activity of the TCS, which can explain the weak induction of P_{dltX} -*lacZ*
258 observed in this strain.

259 Overall, the behaviour of the *dltX* promoter was very similar to that of the previously identified SapR
260 target, the *rapAB* operon (Gebhard *et al.*, 2014). A P_{rapA}-*lacZ* fusion responded to BAC challenge with
261 a five-fold induction of activity (from 23 to 112 Miller Units), while this activity was reduced by 80%
262 in a *sapS*-deleted strain, and low expression but observable BAC-induction was observed in the *sapAB*
263 deletion background (Gebhard *et al.*, 2014). The similar behaviour of the two promoters was consistent
264 with both being controlled by the same regulator.

265 **A SapR binding site is present in the *dltXABCD* promoter and required for induction.** To confirm
266 that *dlt* operon control was due to direct activation by SapRS, we again inspected the sequence of the
267 promoter region upstream of the first gene of the operon, *dltX*. This revealed a putative SapR binding
268 site, similar to the one we had previously identified in the *rapAB* promoter (Gebhard *et al.*, 2014) and
269 in agreement with the consensus sequence for BceR-like regulators in Firmicutes bacteria, i.e. an
270 inverted repeat around a central ACA-N_4-TGT motif (Dintner *et al.*, 2011) (Fig. 3). We next constructed
271 a reporter gene fusion where the putative SapR binding site was mutated at one position in each half of
272 the core motif (Fig. 3B, coloured letters). When wild-type *E. faecalis* carrying this reporter construct
273 was challenged with BAC, induction was completely abolished (Fig. 3C), showing that the putative
274 binding site was indeed essential for promoter activation and that control of *dlt* operon expression was
275 likely a direct activity of SapR.

276 Because we had shown above that LiaR regulates *sapRS* expression, we next aimed to find out if
277 deletion of *liaR* also influenced *dlt* expression. When we tested the response of the *dltX* promoter fusion
278 to BAC in a strain carrying deletion of *liaR*, we observed that deletion of *liaR* resulted in a noticeably
279 weaker amplitude of *dlt* expression in response to BAC, being ~2-fold lower than the wild-type strain
280 at 32 μg ml^{-1} (Fig. 2A, yellow line). In addition, the sensitivity of the *dlt* promoter response was much

281 lower in the *liaR* deletion compared with the wild-type response, with significant activation over
282 baseline occurring at 16 μg ml^{-1} rather than at 2 μg ml^{-1} in the wild type. However, there was no effect
283 on basal activity of *dlt*, in contrast to deletion of *sapR* and *sapAB*. These data are consistent with LiaR
284 regulating *sapRS* expression, and SapRS being the actual regulator of *dlt* expression in response to
285 BAC.

286 **The primary bacitracin resistance determinant, RapAB, provides a negative feedback on the**
287 **wider regulatory network.** As we had observed before with the *liaX* and *sapRS* promoters, we also
288 wanted to investigate if the protection provided by the SapRS-target RapAB was dampening the
289 expression of *dlt* in response to BAC. To examine this, we tested the response of *dlt* expression in the
290 absence of *rapAB.* In a *ΔrapAB* background, the *dlt* promoter demonstrated markedly increased
291 sensitivity, and a much stronger response to BAC, resulting in a ~3-fold increase in expression at 2 μg
292 ml^{-1} when compared to the wild-type (Fig. 2A, orange line). This response demonstrated the presence
293 of a layered protection with RapAB activity moderating *dlt* expression.

294 Overall, these findings were rather surprising, as the signalling pathway appeared remarkably complex
295 to result in a relatively simple outcome, i.e. inducing the expression of two resistance genes in response
296 to an antibiotic. To test if our understanding of the regulatory pathway was plausible, we therefore
297 developed a representative mathematical model to see if this would reproduce the behaviour we had
298 observed in the experiments. At the core, this model was based on a simplified form of the flux-sensing
299 mechanism described previously for the *B. subtilis* BceRS-BceAB system (Fritz *et al.*, 2015), which
300 was then expanded upon to reproduce the hypothesised network structure investigated here. In brief,
301 the model considered BAC binding to UPP to form BAC-UPP complexes with the on-rate dependent
302 on the BAC concentration. These complexes then drive transport activity of, in this case, SapAB,
303 according to a soft-switch type functional response (see Methods), which the model translates into
304 activation of SapRS and thus *dlt* expression. Importantly, SapRS activity also drives expression of the
305 resistance transporter operon, in this case *rapAB*, and production of RapAB leads to a reduction in
306 formation of UPP-BAC complexes due to the target protection activity of the transporter (Fritz *et al.*,
307 2015; Kobras *et al.*, 2020). This creates the negative feedback loop that is characteristic of the flux-

308 sensing mechanism and leads to the gradual response behaviour of the output promoters (Fig. 2B, black
309 symbols). To adapt this model to the Lia-Sap regulatory pathway of *E. faecalis*, we additionally
310 considered the activity of the Lia system. This was modelled to also respond to BAC-UPP complexes
311 in a switch like manner representing the generation of cellular damage caused by these complexes. The
312 model then linked the Lia and Sap components by modulating the SapRS signalling output (i.e. *dlt* and
313 *rapAB* expression) according to Lia activity, driven by BAC. The mathematical details of the model are
314 explained in the Methods section. Fitting to experimental data was performed by matching differential
315 activity of SapRS against both *rapAB* and *dlt* recorded at different BAC concentrations.

316 This model accurately reflected the behaviour of our experimental strains, depicting the same gradual
317 response to BAC of the *dltX* promoter in wild-type *E. faecalis* (Fig. 2B, black symbols), as well as the
318 hypersensitive response in the *rapAB* deletion strain, where the negative feedback from RapAB-driven
319 removal of BAC was missing (orange symbols). Importantly, the model gave the same complete loss
320 of *dltX* activity when *sapRS* was deleted (blue symbols) as observed experimentally, as well as the
321 normal basal level activity but loss of BAC-dependent induction when *liaR* was deleted (yellow
322 symbols). To simplify the previous comprehensive model of signalling by Bce-like systems, we here
323 considered the sensory complex of SapAB and SapRS as a single agent in the network. While this
324 allowed us to test the new connection to Lia-dependent signalling and the additional target operon *dlt*
325 without overly complicating the model, the model now does not differentiate between SapAB and
326 SapRS activities separately. Therefore, the *sapAB* deletion strain from the experimental data was not
327 specifically considered in the theoretical analyses.

328 Overall, the close agreement between theoretical and experimental data strongly suggested that our
329 reconstruction of the regulatory pathway and connection between the Lia and Sap systems was
330 theoretically plausible and that no further major players were needed to explain the behaviour of the
331 *dltX* target promoter in the various mutant backgrounds analysed here.

332

333 **The LiaFSR-SapAB-SapRS network responds to daptomycin challenge.** We had now established
334 a sequential order of *dlt* regulatory control: in response to BAC, LiaFSR induces the expression of
335 *sapRS*, and in turn SapRS, activated by its sensory transporter SapAB (Gebhard *et al.*, 2014), induces
336 the expression of *dlt*. As described in the introduction, the Lia system of *E. faecalis* is primarily known
337 for its contribution to DAP resistance in clinical strains (Arias *et al.*, 2011), and as we showed above,
338 only makes a minor contribution to BAC resistance. Moreover, increased expression of *dlt* is a known
339 cause of DAP resistance (Prater *et al.*, 2019). It was therefore surprising to find that *dlt* regulation was
340 separated from LiaFSR activity by the BAC-responsive SapAB-SapRS regulatory system. We were
341 particularly intrigued by this network structure, because the mode of action of DAP makes it very
342 unlikely to be recognised by SapAB, and thus DAP exposure should not trigger SapRS activation.

343 To dissect the expected differences in sensory perception of the two antibiotics, their mode of action
344 needs to be considered. BAC forms a complex with UPP, which acts as the input for SapRS signalling
345 via SapAB (Kobras *et al.*, 2020); at the same time, BAC induces cell envelope damage, triggering
346 LiaFSR activation (Wolf *et al.*, 2012). DAP, on the other hand, forms a tripartite complex with Lipid II
347 and phosphatidyl-glycerol in the membrane (Grein *et al.*, 2020), which leads to dissociation of
348 components of the cell wall biosynthetic machinery from the membrane and thus inhibits cell wall
349 synthesis (Müller *et al.*, 2016). Eventually, DAP also causes membrane depolarisation (Müller *et al.*,
350 2016), although it has not yet been shown if this is also a direct consequence of the tripartite complex
351 formation. The DAP-induced cell envelope damage activates the LiaFSR response (Hachmann *et al.*,
352 2009; Wecke *et al.*, 2009). However, because DAP does not bind to UPP or the sugar-pyrophosphate
353 moiety of Lipid II, which is the common feature of substrates for BceAB-type transporters (George *et*
354 *al.*, 2022; Kobras *et al.*, 2020), it is not expected to be recognised by SapAB to trigger SapRS signalling.
355 To understand the rationale of directly linking Lia and Sap signalling in *E. faecalis* to control a known
356 DAP-resistance operon (*dlt*), we therefore set out to investigate how each of the network nodes responds
357 to this second antibiotic.

358 The first step in the signalling cascade is the activation of P_{sapR} by LiaFSR signalling. When we exposed
359 wild-type *E. faecalis* carrying the P_{sapRS}-*lacZ* transcriptional fusion to increasing concentrations of DAP,

360 a gradual activation was observed from 1 μg ml^{-1} to an approximately four-fold maximal activation at
361 concentrations above 8 μg ml^{-1} of the antibiotic (Fig. 4A, black line). Consistent with LiaR being the
362 sole regulator of the *sapR* promoter, deletion of *liaR* completely abolished promoter induction, while
363 deletions of *sapRS* or *sapAB* had no effect (Fig. 4A, coloured lines).

364 Next, we tested the response of the P_{dltX}-*lacZ* transcriptional fusion, identified as a SapRS target above,
365 to increasing DAP concentrations. This reporter also showed a dose-dependent response to the antibiotic
366 from 1 μg ml^{-1}, with an approximately three-fold maximal activation observed at 4 μg ml^{-1} DAP (Fig.
367 4B, black line). Similarly to the BAC response, and consistent with the direct regulatory control of the
368 promoter by the SapAB-SapRS system, loss of either of their encoding operons reduced the basal
369 activity of the *dlt* promoter and abolished or greatly reduced the DAP response (Fig. 4B, light blue and
370 purple lines). The slight remaining induction in the *sapRS*-deleted strain is discussed further below.
371 Deletion of *liaR* only abolished the DAP response but did not affect the basal activity (Fig. 4B, yellow
372 line), consistent with the corresponding BAC-data and the indirect effect of Lia signalling on *dlt* operon
373 expression. Overall, these data closely reflected the behaviour of the regulatory network in response to
374 BAC, with the exception of the lack of negative feedback provided by RapAB, which is addressed
375 below. These results also provided experimental proof that the Sap signalling system was indeed a
376 component of the Lia-dependent control of DAP resistance gene expression in *E. faecalis*, even though
377 DAP did not belong to its expected substrate spectrum.

378 In this light, it was intriguing that deletion of *sapAB* abolished induction of the *dlt* promoter. Bce-type
379 transporters, such as SapAB, generally activate signalling of their cognate two-component system in
380 the presence of a suitable substrate (Staroń *et al.*, 2011; Gebhard *et al.*, 2014; Gebhard and Mascher,
381 2011). We had therefore considered that SapAB might be dispensable for DAP signalling. Recent data
382 from cryo-EM structures of the BceAB-BceS complex from *B. subtilis*, however, had indicated that
383 transporter and histidine kinase can mutually affect each other's activity (George and Orlando, 2023),
384 which may suggest that in *E. faecalis* the physical presence of SapAB is required for optimal SapS
385 activity, even in the absence of a substrate. Another curious observation in the data was the slight DAP
386 response, albeit at a much-reduced level, which was still apparent in the *sapRS* deletion background

387 (Fig. 4B, light blue line). This was entirely unexpected, if SapR was indeed the sole regulator of *dltX*,
388 and is also in contrast to the BAC response of the promoter, where no induction remained after deletion
389 of *sapRS*. These data suggest that yet another regulatory pathway in the cell, one that responds to DAP
390 but not BAC, contributes to the control of *dlt* operon expression.

391 Earlier we had shown that *rapAB* deletion had a negative effect on the BAC response, resulting from
392 the removal of the antibiotic and therefore the stimulus from the cell. As DAP is not considered a
393 suitable substrate for BceAB-family transporters, it was not expected that RapAB could remove DAP.
394 Consistent with this, no effect on regulation of either the LiaR-target P_{sapR}, or the SapR target P_{dltX} was
395 observed in the *rapAB*-deleted strain (Fig. 4A&B, orange lines). Nevertheless, the regulatory network
396 structure described here places the *rapAB* operon, which is a known direct target of SapR (Gebhard *et*
397 *al.*, 2014), also under control of a DAP-responsive signalling pathway. We therefore tested a previously
398 constructed P_{rapA}-*lacZ* reporter construct (Gebhard *et al.*, 2014) for its response to DAP challenge.
399 Indeed, the results showed that the *rapA* promoter responded with an approximately three-fold induction
400 to DAP, and the effects of the different gene deletions were very similar to what we had observed for
401 the *dlt* promoter (Fig. 4C). The response amplitude was lower than the five-fold induction reported
402 previously for BAC challenge of the same reporter (Gebhard et al, 2014), which can likely be explained
403 by the amplifying effect of SapAB-SapRS signalling with BAC, but not DAP. Importantly, no partial
404 induction of P_{rapA} was observed in the *sapRS* deleted strain, suggesting that the additional, as yet
405 unidentified, regulatory control of the *dlt* operon implied by the data shown in Fig. 4B must be specific
406 to that target promoter.

407 **The regulatory network contributes to DAP resistance of *E. faecalis*.** Finally, to test if all regulatory
408 components are required to protect *E. faecalis* against DAP, the deletion mutants were tested for their
409 MIC of the antibiotic. For all deletions of regulatory components, a two- to four-fold reduction in MIC
410 from 16 mg ml^{-1} in the wild type to 4-8 mg ml^{-1} in the deletion strains was observed (Table 1). This was
411 consistent with their contribution to *dlt* operon expression. While for BAC resistance, LiaR played a
412 lesser role, for DAP resistance no such difference could be detected between the Lia and Sap
413 components of the network. This is consistent with the BAC response involving an active amplification

414 of signalling by the SapAB-SapRS component, whereas the DAP response cannot be amplified due to
415 lack of substrate recognition by the transporter. Also consistent with the transcriptional reporter data
416 and inability of BceAB-type transporters to recognise DAP, loss of RapAB had no significant effect on
417 DAP resistance (Table 1). Unfortunately, despite numerous attempts, we could not construct a *dlt*
418 operon deletion without causing significant polar effects. Therefore, the precise contribution of the Dlt
419 system to DAP resistance, e.g. relative to other LiaFSR targets, could not be determined here.

420

421 **Discussion**

422 In this study, we aimed to expand our understanding of the response of *E. faecalis* to cell envelope
423 targeting antibiotics and investigate a potential functional link between the damage-sensing LiaFSR
424 signalling pathway and the BAC-responsive SapAB-SapRS system. Our findings revealed the
425 transcriptional regulation of the BceRS-type TCS SapRS by LiaFSR. In addition, we also demonstrated
426 the regulation of the DAP resistance operon *dltXABCD* by SapRS. Even though the Sap system had
427 been shown previously to control BAC resistance and possess a very narrow inducer range (Gebhard *et*
428 *al.*, 2014), our results showed that the regulatory interplay between Lia and Sap signalling allows the
429 entire network to respond to both BAC and DAP challenge. Susceptibility testing showed that within
430 this network, protection against BAC was primarily provided by the SapRS-controlled transporter
431 RapAB, while DAP resistance required the full complement of regulatory components. This work
432 therefore offers an explanation for previous observations of suppressor mutations in *sapAB* when a
433 LiaR-deficient strain of *E. faecalis* was evolved for DAP resistance (Prater *et al.*, 2021, 2019). A
434 working model for the proposed regulatory network structure is presented in Figure 5.

435 LiaFSR is well-known to control DAP resistance in *E. faecalis*, and mutations that lead to constitutive
436 activation of Lia signalling are commonly observed in resistant clinical isolates (Arias *et al.*, 2011;
437 Miller *et al.*, 2013). While evidence is available that *dlt* operon over-expression leads to DAP resistance
438 (Prater *et al.*, 2019), work to date has mostly focussed on the primary LiaFSR target, the *liaXYZ* operon
439 (Davlieva *et al.*, 2015). The mechanism of resistance provided by this operon is not yet fully understood,

440 but LiaX is thought to directly counteract the antibiotic and somehow mitigate its effects on the
441 cytoplasmic membrane (Khan *et al.*, 2019). Of the two known resistance determinants, one therefore is
442 a direct target of LiaFSR signalling (*liaXYZ*), while the other (*dltXABCD*) is regulated via an expanded
443 network involving the SapAB-SapRS system, as shown here. The reasons for this are not yet clear.

444 The situation is further complicated by our observation that some induction of *dlt* expression in response
445 to DAP exposure still occurred in the *sapRS*-deleted strain (Fig. 4B), suggesting that yet another, so far
446 unidentified regulator contributes to *dlt* regulation. Importantly, this response was specific to DAP,
447 because no residual activation after BAC exposure was observed in the same genetic background (Fig.
448 2A). In *B. subtilis*, extracytoplasmic function (ECF) sigma factors play a crucial role in the cell envelope
449 stress response, including control of the *dlt* operon (Helmann, 2016). In *E. faecalis,* to our knowledge,
450 to date no ECF has been shown to be involved, although at least two putative ECF sigma factor/anti-
451 sigma factor pairs are present in the genome, with homology to SigV and YlaC in *B. subtilis* (Casas-
452 Pastor *et al*., 2021). SigV of *E. faecalis* has been shown to contribute to lysozyme resistance (Le Jeune
453 *et al.*, 2010) While SigV does not seem to control the *dlt* operon, at least in response to lysozyme (Le
454 Jeune *et al.*, 2010), further investigations are needed to explore the contribution of this sigma factor and
455 YlaC to the extended cell envelope stress response network in this organism.

456 The second target of the Lia-Sap signalling pathway is the operon for the BAC resistance transporter
457 RapAB. Consistent with our current understanding of transporters of the BceAB-type, RapAB could
458 protect the cell from BAC, but not from DAP, as seen in the MIC values (Table 1). BceAB-type
459 transporters recognise antimicrobial peptides that bind to either UPP or the UPP-sugar moiety of lipid
460 II (George and Orlando, 2023; Kobras *et al.*, 2020). DAP binds in a fundamentally different manner,
461 forming a tripartite complex with lipid II and phosphatidyl-glycerol (Grein *et al.*, 2020), and thus does
462 not match the substrate spectrum of a transporter like RapAB. In close agreement with the susceptibility
463 data, we could also show that the target protection mechanism, by which RapAB removes BAC from
464 the cell, results in a negative feedback effect on the network's response to BAC, whereas no such effect
465 was observed during DAP exposure (Figs. 2 and 4). This raises the question why the *rapAB* operon was
466 placed under control of a regulatory network that responds to DAP challenge, and indeed we could

467 show that the *rapA* promoter itself is also significantly induced by this non-substrate antibiotic (Fig. 4).
468 One explanation could be that this is simply an unintended consequence of the network structure, which
469 may have primarily evolved to respond to antibiotics from which RapAB can protect. Alternatively, the
470 answer may be found in the natural habitats of *E. faecalis* with high densities of competing micro-
471 organisms, e.g. the soil or animal gut. Here, it may be an effective protection strategy to use a regulatory
472 network that can respond to multiple different input antibiotics and respond by increased expression of
473 resistance genes that can protect from a range of antibiotic mode-of-actions.

474 The regulatory network we describe here provides such a structure: it responds to two inputs, BAC and
475 DAP, and it controls two outputs, RapAB and DltXABCD production. As stated above, of these inputs,
476 BAC is most effectively counteracted by RapAB, while DAP is counteracted by changes in surface
477 charge resulting from Dlt activity. Particularly given that LiaFSR signalling can directly lead to the
478 activation of another DAP resistance operon, *liaXYZ*, it is surprising that the *dlt* operon is not simply
479 also a direct LiaR-target. And moreover, why is the Lia system brought into the network to provide
480 DAP-responsiveness to SapAB-SapRS signalling when one of the main SapR targets, *rapAB*, cannot
481 protect the cell from this antibiotic? While we cannot yet answer this question, a speculative explanation
482 for the convergence of Lia and Sap signalling within the same network might be found if we consider
483 *E. faecalis* in its natural habitat.

484 As mentioned above, in an environment with high population density and diversity combined with
485 scarcity of nutrients, it is plausible to expect that multiple species capable of antimicrobial production
486 would secrete their inhibitory substances to gain a competitive advantage. The Lia-Sap regulatory
487 network might equip *E. faecalis* with the means of reacting to one antimicrobial in a way that primes it
488 to respond to further drugs. In this scenario, LiaFSR is the first level of the response. This system
489 responds to the broadest substrate range, because its stimulus is cell envelope damage rather than a
490 specific compound. Its activity then primes the more drug-specific Sap system to respond. If the
491 encountered drug is a substrate for this system (here exemplified by BAC), SapAB will amplify the
492 response through increasing SapS activity (Dintner *et al.*, 2014; Gebhard *et al.*, 2014; George and
493 Orlando, 2023). The resulting high levels of RapAB will remove the antibiotic and thus dampen both

494 the Lia and Sap responses through the negative feedback, preventing excess activation (Fig. 2) (Fritz *et*
495 *al.*, 2015). If the encountered antibiotic is not a RapAB substrate (here exemplified by DAP), the
496 network responds sensitively on all levels (Fig. 4). Up-regulation of *rapAB* in this case may be an
497 accident, or it could prepare the cell for the arrival of a suitable substrate, in which case its resistance
498 would be instantly available. The additional input from an as-yet-unidentified DAP-responsive system
499 on *dlt* expression might provide a further means of tuning the network response to the balance of
500 antibiotics encountered.

501 To test this proposal, data are required on how the network responds to mixtures of various inducers
502 but also in mixed microbial communities *E. faecalis* might naturally encounter. While such a
503 comprehensive investigation is beyond the scope of the present study, we have carried out initial tests
504 studying the inhibition of *E. faecalis* and derived mutants by a set of known antibiotic producers. The
505 details of these experiments are presented in Supplemental File S2. In brief, we grew the different *E.*
506 *faecalis* strains in agar overlays on plates that had been pre-grown with spots of four antimicrobial
507 producing strains: the subtilin producer *Bacillus subtilis* ATCC6633 (Banerjee and Hansen, 1988), BAC
508 producer *Bacillus lichenformis* ATCC10716 (Johnson *et al.*, 1945), nisin A producer *Lactococcus lactis*
509 NZ9000 (Kuipers *et al.*, 1998) and nisin P producer *Streptococcus gallolyticus* AB39 (Aldarhami *et al.*,
510 2020). While we cannot confirm that each of these strains produced their expected antimicrobial under
511 the chosen growth conditions, each producer caused the appearance of an inhibition zone in the *E.*
512 *faecalis* overlay, showing that some antimicrobial compounds were indeed secreted. In these
513 antagonism assays, the broad importance of the role played by LiaFSR in response to a diversity of
514 antimicrobials was well demonstrated, because deletion of *liaR* resulted in increased sensitivity against
515 all tested antimicrobial producers, except for *L. lactis* NZ9000. In contrast, components of the Sap
516 system only contributed to protection from the BAC producer *B. lichenformis* ATCC10716 and the
517 subtilin producer *B. subtilis* ATCC6633. The only antimicrobial producer against which neither of the
518 genes appeared to give a protective effect was the nisin-A producer *L. lactis* NZ9000. However, as
519 stated we cannot currently ascertain whether the antimicrobial produced under these conditions was
520 indeed nisin A, or whether *E. faecalis* uses alternative resistance mechanisms against nisin A not

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

525 The regulatory wiring we have uncovered here between the LiaFSR and the Bce-type SapAB-SapRS
526 systems, resulting in the control of the *dltXABCD* and *rapAB* resistance operons, is remarkably complex
527 compared with other Firmicutes bacteria. For example, in *B. subtilis* LiaFSR controls its own regulon,
528 comprised solely of *liaIH* and its own encoding genes (Wolf *et al.*, 2010). In *S. aureus*, the LiaFSR
529 homologue VraRS has been shown to regulate the expression of multiple genes involved in cell wall
530 biosynthesis (Wu *et al.*, 2020), but has not been observed to regulate a BceRS-type TCS. Bce-type
531 systems in most Firmicutes bacteria either solely control their own transporter, as found with BceAB-
532 RS in *B. subtilis* (Ohki *et al.*, 2003), or can be involved with other Bce-type systems, such as the BraRS-
533 VraDE (Hiron *et al.*, 2011) and GraRS-VraFG (Meehl *et al.*, 2007) systems in *S. aureus*. In some cases,
534 Bce-type systems can also control additional genes, for example the GraRS TCS has also been shown
535 to regulate the *dlt* operon (Kraus *et al.*, 2008; Cheung *et al.*, 2014; Ledger *et al.*, 2022). However, to our
536 knowledge there have been no prior reports of direct regulatory relationships that hardwire the Lia and
537 Bce-type pathways together.

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

544

545 **Experimental Procedures**

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

558 *E. faecalis* was transformed by electroporation as previously described(Cruz-Rodz and Gilmore, 1990).
559 *E. coli* was transformed by heat-shock of $CaCl_2$ competent cells, followed by 1 hour recovery time
560 (Hanahan and Glover, 1985). Growth was measured as optical density at 600 nm (OD_{600}) on a the
561 Biochrom™ Novaspec Pro Spectrophotometer using cuvettes with 1 cm light path length or in 96-well
562 plates with 100 μL culture volumes on a Spark®Microplate reader (Tecan).

563 **Construction of plasmids and genetic techniques.** All primer sequences used for cloning are listed in
564 Table S2 in the supplementary material. Transcriptional promoter fusions to *lacZ* in *E. faecalis* were
565 constructed in the vector pTCVlac (Poyart and Trieu-Cuot, 1997). All fragments were cloned via the
566 EcoRI and BamHI sites of the vector. Additionally, vectors with the mutated binding sites for LiaR and
567 SapR were synthesized and sub-cloned into pTCVLac by GeneArt (ThermoFisher Scientific Inc.,
568 Darmstadt). Constructs for unmarked deletions in *E. faecalis* were cloned into pLT06 (Thurlow *et al.*,
569 2009). For each gene or operon to be deleted, 700- to 1000-bp located immediately before the start
570 codon of the first gene ("up" fragment) and after the stop codon of the last gene ("down" fragment)
571 were amplified. The primers were designed to create a 17- to 20-bp overlap between the PCR products

572 (Table S2), facilitating the fusion of the fragments by PCR overlap extension (Ho *et al.,* 1989) and were
573 subsequently cloned into the NcoI and BamHI site of the vector pLT06.

574 Gene deletions were performed as previously described (Thurlow *et al.,* 2009). Briefly, following
575 transformation of the parent strain with the temperature-sensitive vector pLT06, overnight cultures were
576 grown at 30° containing chloramphenicol and reinoculated 1:1000 into 10 mL BHI the next morning.
577 Cells were then grown at 30° for 2.5 hours, followed by increasing to 42° for a further 2.5 hours to force
578 single-site integration. Cells were then serially diluted onto BHI agar containing chloramphenicol and
579 X-Gal and incubated at 42°. Blue colonies growing at 42°C were screened for the targeted integration
580 using PCR with primers flanking the site of integration. Positive clones were then serially passaged for
581 two days from overnight culture in BHI medium with no selection at 30° to allow a second site
582 recombination event. Cultures were then serially diluted on to MM9-YEG agar (Kristich *et al.,* 2007b)
583 containing 10 mM *p*-chloro-phenylalanine for counter-selection and X-Gal at 37°C. The resulting white
584 colonies were screened for the deletion of the target genes by PCR. All cloned constructs were checked
585 for PCR fidelity by Sanger sequencing, and all created strains were verified by PCR using the primers
586 given in Table S2.

587 **Antimicrobial susceptibility assays.** For antibiotic susceptibility assays, minimum inhibitory
588 concentrations (MICs) were determined by broth dilution assays. Colonies of *E. faecalis* were
589 suspended in sterile Phosphate Buffered Saline (PBS) to 0.5 McFarland standard turbidity and diluted
590 1:1,000 in a total volume of 1.5 ml BHI medium. For assays with daptomycin, the medium was
591 supplemented with 500 mM $CaCl_2$. Of the inoculated medium, 100 ml aliquots were added to wells
592 containing two-fold serial dilutions of the antibiotic. After 24 h incubation at 37°C, growth was
593 determined by measuring optical density (OD_{600}) on a Spark®Microplate reader (Tecan). The MIC was
594 scored as the lowest antibiotic concentration where no growth was observed following subtraction of
595 the OD_{600} of a well containing sterile medium.

596 **β-Galactosidase assays.** For quantitatively assessing induction of *lacZ* reporter constructs in *E.*
597 *faecalis*, exponentially growing cells (OD_{600} = 0.4-0.5) inoculated 1:250 from overnight cultures in BHI

medium were exposed to different concentrations of bacitracin for 1 h or daptomycin for 30 minutes. Cells were harvested via centrifugation and stored at -20°C. β-Galactosidase activities were assayed in permeabilised cells and expressed in Miller units (MU) (Gebhard *et al.*, 2006). For this, cells were resuspended in 1 ml Z-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 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, two volumes were taken: 200 µl and 400 µl cells made up to 1 mL each with Z-buffer. This volume corresponds to the 'volume of cells' in the Miller Unit (MU) equation below. Following this, 20 µl 0.1% (w/v) SDS and 40 µl chloroform were added and vortexed for 5 seconds, then rested for 5-10 minutes. Reactions were started by adding 200 µl *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg mL^{-1} in Z-buffer) and incubated at room temperature until yellow colouration was observed. If no colour change was visible, the reaction was incubated for 20 minutes. Reactions were stopped by adding 500 µl 1M Na_2CO_3, and the time recorded, which corresponds to the 'time' in the Miller Unit (MU) equation below. Absorbance at 420 nm (A_{420}) was then read. MUs were calculated using the following equation:

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

Computational modelling. Rather than directly modelling the temporal dynamics of the regulatory network as done for the *B. subtilis* Bce system (Fritz *et al.*, 2015; Piepenbreier *et al.*, 2020), we chose to focus on the (meta) stable state reached in response to challenge with a given bacitracin concentration. The quantities modelled are the concentrations of bacitracin [*bac*], UPP [*UPP*], UPP-bound bacitracin [*UPPbac*], and the effective activities of SapRS [*SapRS*], RapAB [*RapAB*], LiaX [*Lia*], and DltXABCD [*Dlt*]. For the modelling, we made repeated use of a soft-switch sigmoid type function

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

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

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

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

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

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

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

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

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

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

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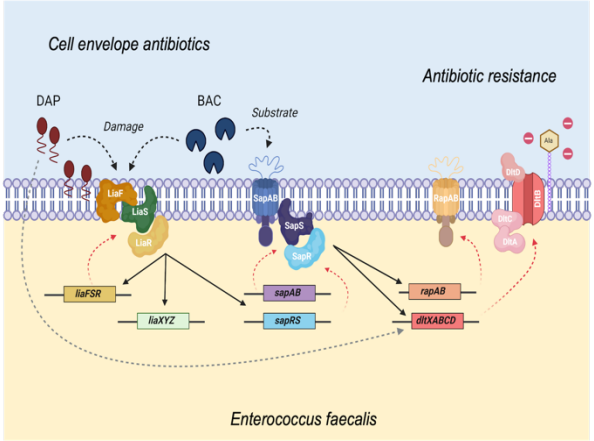
Author contributions

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

Graphical abstract



Abbreviated summary

This study explored a regulatory network controlling resistance against daptomycin and bacitracin in *Enterococcus faecalis*. We show that resistance gene expression for both antibiotics is controlled by two regulatory systems, LiaFSR and SapRS, working in sequence and together processing two stimuli. Convergence of this network on the *dltXABCD* target operon, known to be associated with daptomycin resistance may explain why clinical resistance can emerge via regulatory mutations.

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860

861 # Figure legends

862 **Figure 1. The LiaFSR system responds to bacitracin exposure and induces the expression of**
863 **both *liaXYZ* and *sapRS*.** Cells of *E. faecalis* JH2-2 wild type or isogenic deletion strains harbouring a
864 P_{liaX}-*lacZ* (a) or P_{sapRS}-*lacZ* (b) transcriptional fusion were grown to exponential phase and challenged
865 with increasing concentrations of bacitracin. b-galactosidase activity, expressed as Miller units (MU),
866 was assayed following 1h incubation. (c) Schematic of the promoter region upstream of *sapR*. The
867 start codon is shown as ATG and putative -10 and -35 promoter elements are underlined. The two
868 proposed LiaR binding sites are indicated in bold capitals, black on white, and white on black,
869 respectively. Mutations introduced in the first and second binding site are shown in orange and brown,
870 respectively. (d) P_{sapRS}-*lacZ* transcriptional fusions carrying the wild-type promoter sequence or the
871 mutations in the first (LiaR*) or second (LiaR**) binding site of the promoter were introduced into
872 wild-type *E. faecalis* and assayed as before. The colour code corresponds to that used in panel c.
873 Please note the data for the native promoter is identical between panels b and d. Results are means and
874 standard deviations for three biological repeats. The significance of induction relative to untreated
875 cells was calculated for each strain by a two-way ANOVA test. Significance is indicated by a filled
876 symbol ($p < 0.05$); unfilled symbols represent no significant increases compared to uninduced
877 conditions. Numerical data for these graphs is provided in Table S4.

878

879 **Figure 2. Induction of the resistance operon *dltXABCD* by bacitracin.** Panel A indicates the
880 experimental induction of *dltX.* Cells of *E. faecalis* harbouring a *PdltX-lacZ* transcriptional fusion
881 were grown to exponential phase and challenged with increasing concentrations of bacitracin. Beta-
882 galactosidase activity, expressed as Miller units (MU), was assayed following 1h incubation in wild
883 type (WT) and deletion strain backgrounds. Results are means and standard deviations for three
884 biological repeats The significance of induction relative to untreated cells was calculated for each
885 strain by a one-tailed, pairwise *t*-test analysis. Significance is indicated by a filled symbol ($p < 0.05$),

886 unfilled symbols represent no significant increases compared to uninduced conditions. Panel B
887 indicates the mathematical model of *dltX* induction in the *E. faecalis* strains indicated in panel A.
888 Numerical data for these results is provided in Table S4.

889

890 **Figure 3. A putative SapR binding site in the *dltX* promoter is required for bacitracin-**
891 **responsive promoter induction.** (a, b) Schematics of the promoter regions of the known SapR target
892 *rapAB* (a) and the newly identified target *dltXABCD* (b). The start codons are shown as ATG, and
893 putative -10 and -35 promoter elements and likely +1 position are underlined. The known SapR
894 binding site in P_{rapA} and the proposed site in P_{dltX} are shown in bold. Mutations introduced in the
895 proposed binding site are shown in pink. (c) The P*dltX-lacZ* transcriptional reporter and a derivative
896 carrying the two mutations in the putative SapR binding site from panel b were introduced into wild-
897 type *E. faecalis.* Exponentially growing cells were challenged with increasing concentrations of
898 bacitracin. β-galactosidase activity, expressed as Miller units (MU), was assayed following 1h
899 incubation. Results are means and standard deviations for three biological repeats. The significance of
900 induction relative to untreated cells was calculated for each strain by a two-way ANOVA test.
901 Significance is indicated by a filled symbol ($p < 0.05$); unfilled symbols represent no significant
902 increases compared to uninduced conditions. Numerical data for these graphs is provided in Table S4.

903

904 **Figure 4. The Daptomycin-response of the regulatory network.** *E. faecalis* cells harbouring P_{sapRS}-
905 *lacZ* (a), P_{dltX}-*lacZ* (b) or P_{rapAB}-*lacZ* (c) transcriptional fusions were grown to exponential phase and
906 challenged with increasing concentrations of daptomycin. b-galactosidase activity, expressed as
907 Miller units (MU), was assayed following 30 minutes incubation in wild type (WT) or deletion strain
908 backgrounds, as indicated. Results are means and standard deviations for three biological repeats. The
909 significance of induction relative to untreated cells was calculated for each strain by a two-way
910 ANOVA test. Significance is indicated by a filled symbol ($P < 0.05$), unfilled symbols represent no

911 significant increases compared to uninduced conditions. Numerical data for these graphs is provided
912 in Table S4.

913

914 **Figure 5. Proposed model of the antibiotic resistance network.** Schematic illustrating the genes and
915 proteins involved in antibiotic detection and resistance. Transcriptional regulation of genes is shown by
916 black straight arrows, translation of genes to proteins is shown by dashed red arrows. Exposure to
917 bacitracin (BAC) causes damage to the cell envelope, which acts as the stimulus for the removal of
918 repression by LiaF, triggering the activation of LiaS. LiaS activity leads to phosphorylation of LiaR,
919 which in turn induces the expression of its target promoters: *liaFSR*, *liaXYZ* and *sapRS*. Simultaneously,
920 BAC is also detected by the SapAB/SapS sensory complex, leading to an amplification of the signalling
921 response and via SapR. SapR then induces the expression of the resistance genes, *rapAB* and *dltXABCD,*
922 which remove BAC from UPP and reduce the positive charge of the cell envelope, respectively.
923 Conversely, exposure to daptomycin (DAP) again results in cell envelope damage, which triggers the
924 phosphorylation of LiaR and induces the expression of *sapRS*. DAP is not recognised by SapAB and
925 no amplification of the signal occurs. The LiaR-induced increase in SapRS production is nevertheless
926 sufficient to increase basal SapRS activity, again leading to the induction of *rapAB* and *dltXABCD*. Of
927 these, only the Dlt system is thought to counteract DAP action. The observed DAP-responsive increase
928 of P_{dltX} activity in the absence of SapRS by an as-yet-unidentified additional regulatory system is
929 indicated by a grey, dashed arrow and a question mark.

930

931 # Tables

932 Table 1. Bacitracin and daptomycin sensitivity of *E. faecalis* strains

	Minimal inhibitory concentration (MIC; μg ml^{-1}) [a]	
Strain or genotype	Zn^{2+}-Bacitracin	Ca^{2+}-Daptomycin
JH2-2	16	16
JH2-2 Δ*sapRS*	4-8	4
JH2-2 Δ*sapAB*	4-8	4-8
JH2-2 Δ*rapAB*	4-8	8-16

JH2-2 Δ*liaR*	8-16	4-8
JH2-2 Δ*liaR*/P_{liaF}-*liaR*	8	4-8

933 [a] Results are from three independent repeat experiments. Where the MIC values differed between
934 repeats, the range of obtained results is reported.

935

936 # Supplementary Material

937 **Table S1.** Vectors, plasmids and strains used in this study.

938 **Table S2.** Primers used in this study.

939 **Table S3.** Parameter values used in the mathematical model.

940 **Table S4.** Experimental numerical data for Figures 1-4.

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

942 **File S2.** Description, methods and results of interaction studies between antibiotic producing strains and *E.*
943 *faecalis* wild type and deletion strains.

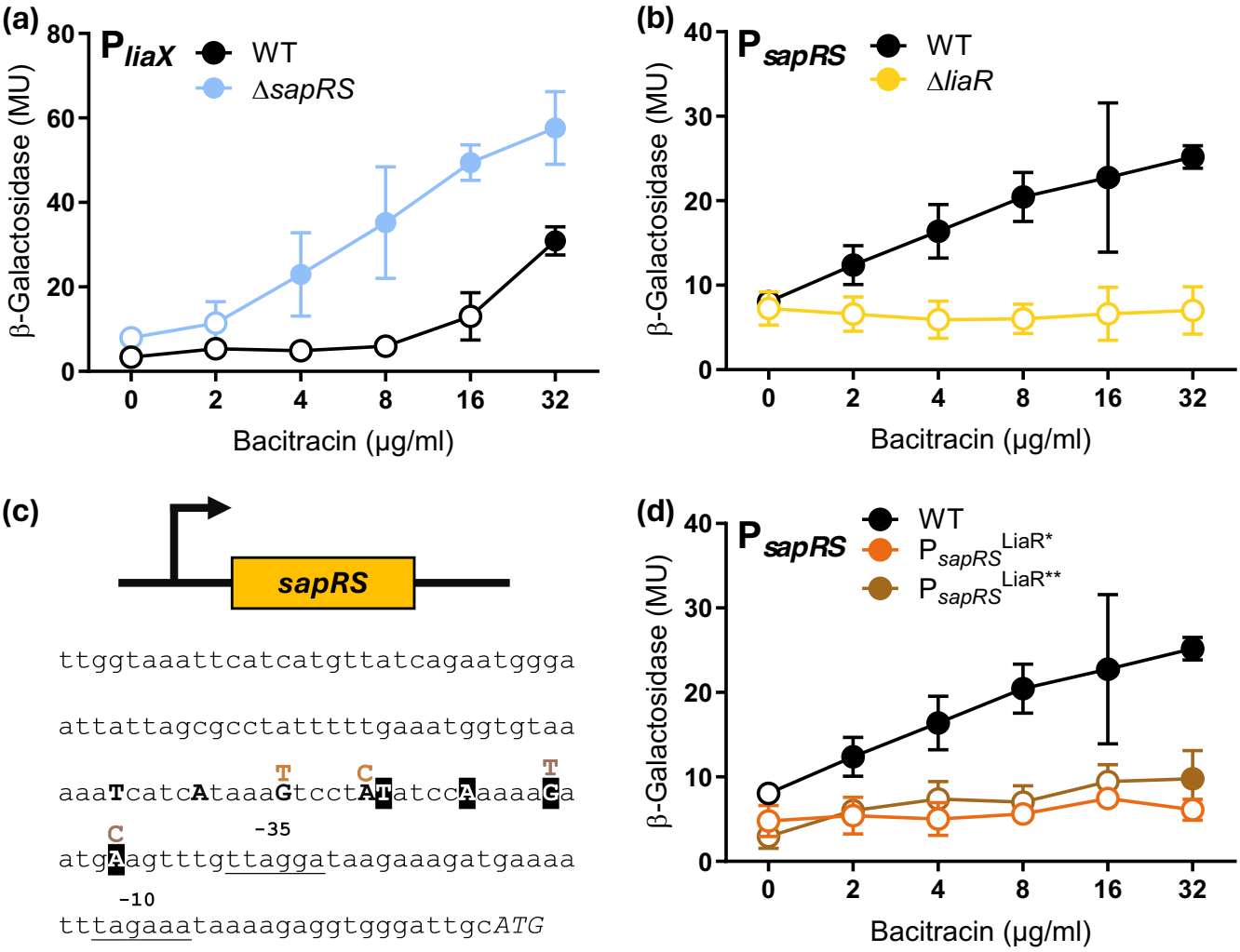

Figure 1. The LiaFSR system responds to bacitracin exposure and induces the expression of both *liaXYZ* and *sapRS*. Cells of *E. faecalis* JH2-2 wild type or isogenic deletion strains harbouring a P_{liaX}-*lacZ* (a) or P_{sapRS}-*lacZ* (b) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. β-galactosidase activity, expressed as Miller units (MU), was assayed following 1h incubation. (c) Schematic of the promoter region upstream of *sapR*. The start codon is shown as ATG and putative -10 and -35 promoter elements are underlined. The two proposed LiaR binding sites are indicated in bold capitals, black on white, and white on black, respectively. Mutations introduced in the first and second binding site are shown in orange and brown, respectively. (d) P_{sapRS}-*lacZ* transcriptional fusions carrying the wild-type promoter sequence or the mutations in the first (LiaR*) or second (LiaR**) binding site of the promoter were introduced into wild-type *E. faecalis* and assayed as before. The colour code corresponds to that used in panel c. Please note the data for the native promoter is identical between panels b and d. 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 two-way ANOVA test. Significance is indicated by a filled symbol ($p < 0.05$); unfilled symbols represent no significant increases compared to uninduced conditions. Numerical data for these graphs is provided in Table S4.

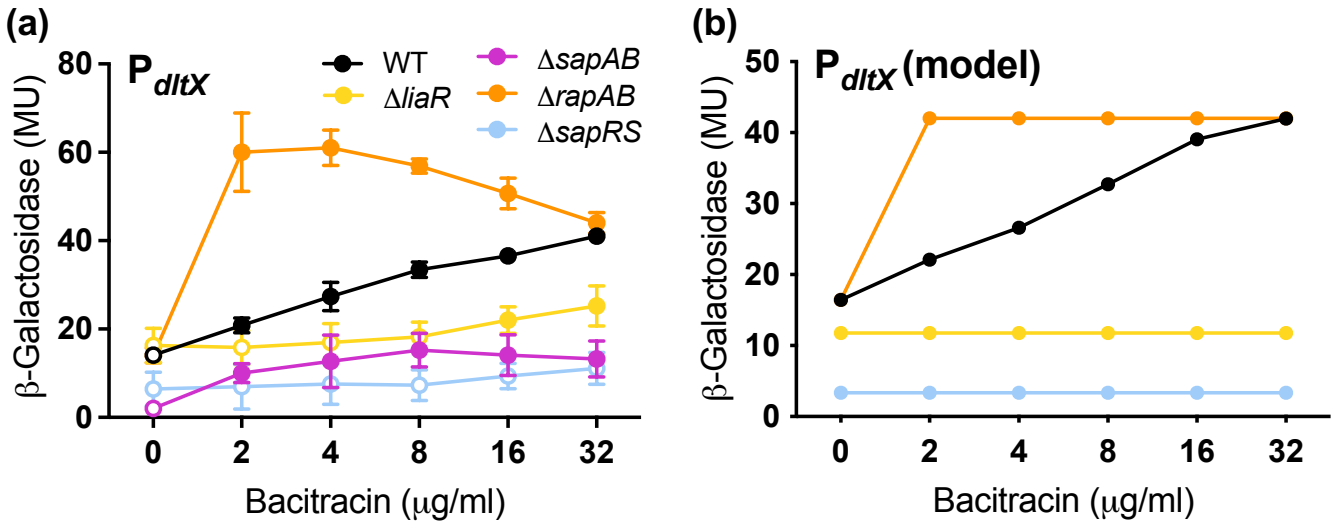

Figure 2. Induction of the resistance operon *dltXABCD* by bacitracin. Panel A indicates the experimental induction of *dltX.* Cells of *E. faecalis* harbouring a *PdltX-lacZ* transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. Beta-galactosidase 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. Numerical data for these results is provided in Table S4.

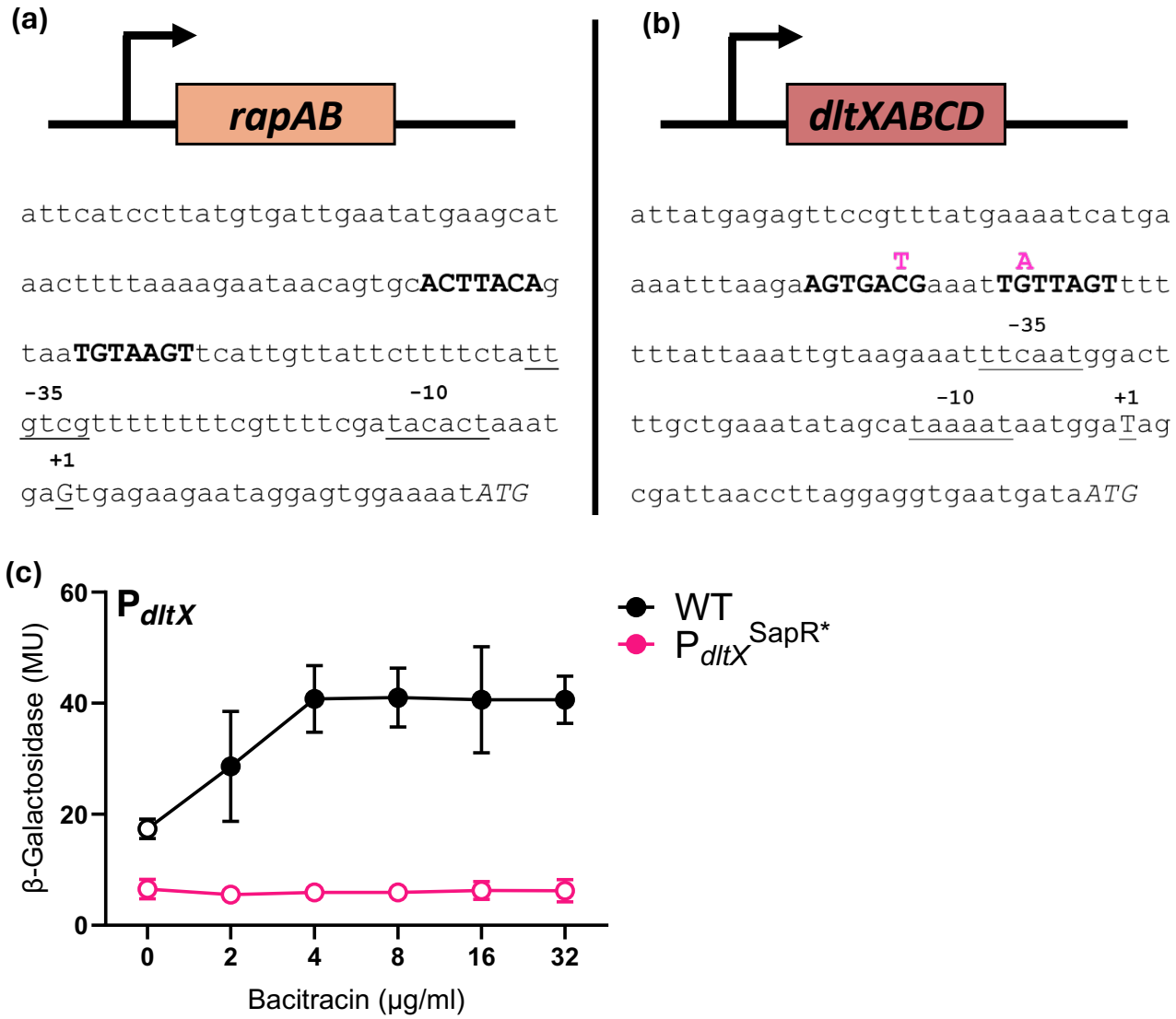

Figure 3. A putative SapR binding site in the *dltX* promoter is required for bacitracin-responsive promoter induction. (a, b) Schematics of the promoter regions of the known SapR target *rapAB* (a) and the newly identified target *dltXABCD* (b). The start codons are shown as ATG, and putative -10 and -35 promoter elements and likely +1 position are underlined. The known SapR binding site in P_{rapA} and the proposed site in P_{dltX} are shown in bold. Mutations introduced in the proposed binding site are shown in pink. (c) The P*dltX*-*lacZ* transcriptional reporter and a derivative carrying the two mutations in the putative SapR binding site from panel b were introduced into wild-type *E. faecalis.* Exponentially growing cells were 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 two-way ANOVA test. Significance is indicated by a filled symbol ($p < 0.05$); unfilled symbols represent no significant increases compared to uninduced conditions. Numerical data for these graphs is provided in Table S4.

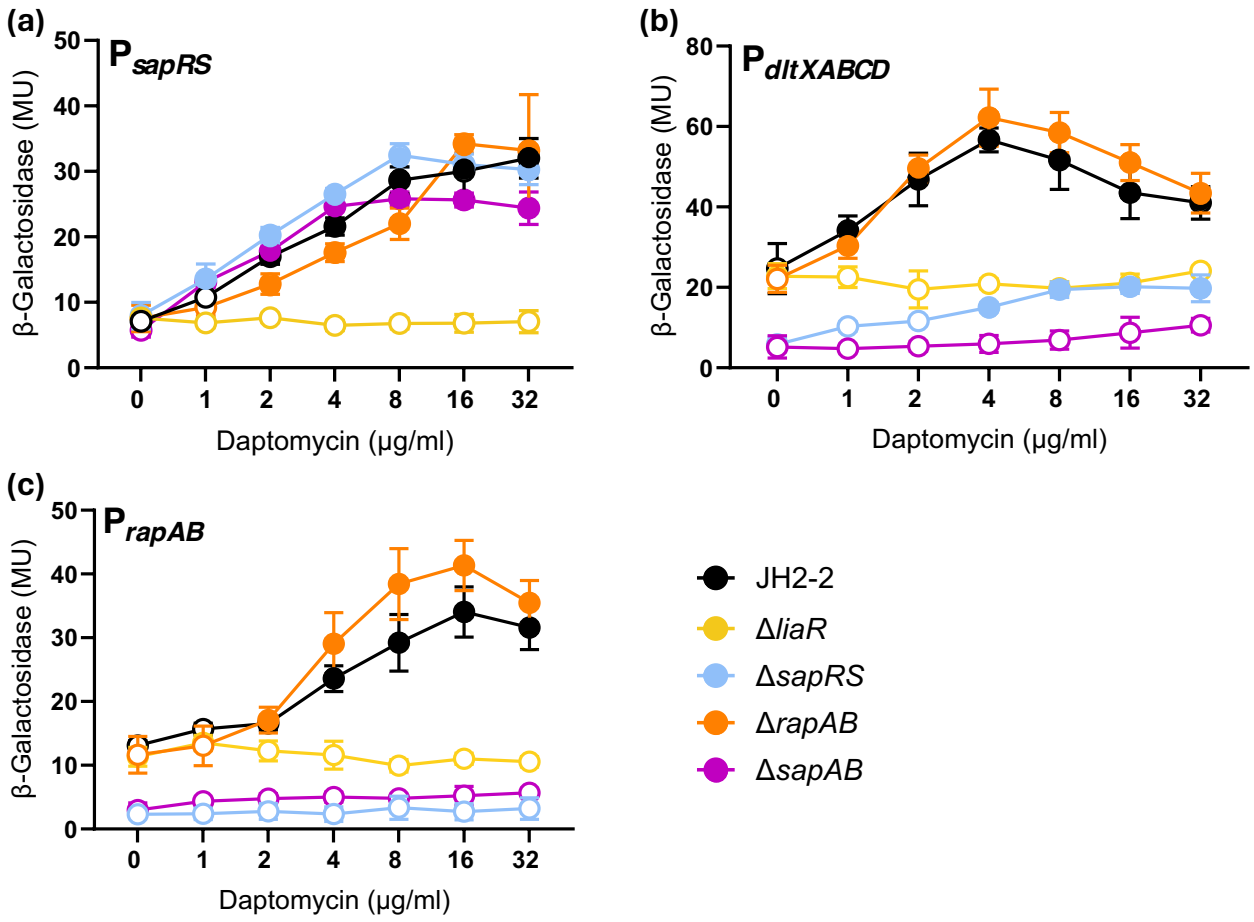

Figure 4. The Daptomycin-response of the regulatory network. *E. faecalis* cells harbouring P_{sapRS}-*lacZ* (a), P_{dltX}-*lacZ* (b) or P_{rapAB}-*lacZ* (c) transcriptional fusions were grown to exponential phase and challenged with increasing concentrations of daptomycin. β-galactosidase activity, expressed as Miller units (MU), was assayed following 30 minutes incubation in wild type (WT) or deletion strain backgrounds, as indicated. 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 two-way ANOVA test. Significance is indicated by a filled symbol ($P < 0.05$), unfilled symbols represent no significant increases compared to uninduced conditions. Numerical data for these graphs is provided in Table S4.

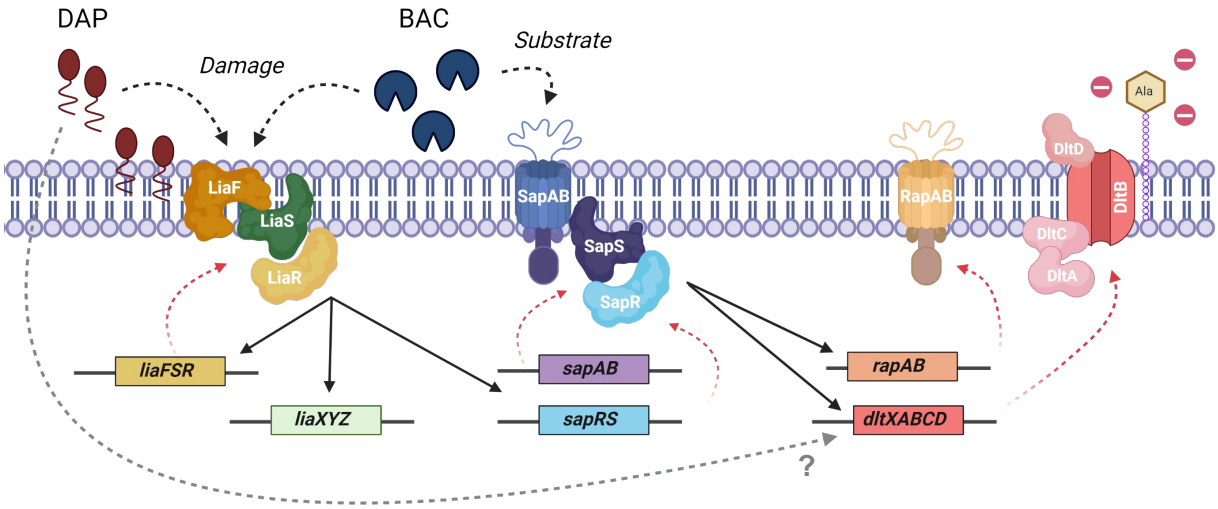

Figure 5. Proposed model of the antibiotic resistance network. Schematic illustrating the genes and proteins involved in antibiotic detection and resistance. Transcriptional regulation of genes is shown by black straight arrows, translation of genes to proteins is shown by dashed red arrows. Exposure 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: liaFSR, liaXYZ and sapRS. Simultaneously, BAC is also detected by the SapAB/SapS sensory complex, leading to an amplification of the signalling response and via SapR. SapR then induces the expression of the resistance genes, rapAB and dltXABCD, which remove BAC 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. DAP is not recognised by SapAB and no amplification of the signal occurs. The LiaR-induced increase in SapRS production is nevertheless sufficient to increase basal SapRS activity, again leading to the induction of rapAB and dltXABCD. Of these, only the Dlt system is thought to counteract DAP action. The observed DAP-responsive increase of PdltX activity in the absence of SapRS by an as-yet-unidentified additional regulatory system is indicated by a grey, dashed arrow and a question mark.

File S2: Description, methods and results of interaction studies between antibiotic producing strains and *E. faecalis* wild type and deletion strains.

Results:

The network components vary in importance during interactions with antimicrobial producer strains. As stated in the main manuscript, we were surprised by the complexity of the regulatory pathway controlling what in other Firmicutes bacteria is a fairly straightforward response to antibiotic challenge, where each regulatory system controls its own resistance genes. To shed some light on the reasons for the complexity of signalling in enterococci, we considered the environments these bacteria can be found in. *E. faecalis* is a common member of many natural microbial communities, such as in soil and water or the gastrointestinal tract of humans and animals. There, the enterococci reside within the small and large intestine and represent up to 1% of the faecal flora (Lorian, 1994; Sghir *et al.*, 2000; Tendolkar *et al.*, 2003; Eckburg *et al.*, 2005). In such environments, *E. faecalis* interacts with other microbes and must defend itself against antimicrobial producing bacteria. Therefore, we wanted to assess the role of the individual components of the resistance network in protecting *E. faecalis* from antimicrobial activity produced by potential competitor bacteria. To do this, we used deferred antagonism assays to simulate relevant environmental pressures from other microbes the enterococci may encounter. We used four antimicrobial producing strains: the subtilin producer *Bacillus subtilis* ATCC6633 (Banerjee and Hansen, 1988), bacitracin producer *Bacillus lichenformis* ATCC10716 (Johnson *et al.*, 1945), nisin-A producer *Lactococcus lactis* NZ9000 (Kuipers *et al.*, 1998) and nisin-P producer *Streptococcus gallolyticus* AB39 (Aldarhami *et al.*, 2020). Each producer was spotted onto a plate, and the antimicrobial was allowed to accumulate over multiple days. This was followed by the addition of an overlay containing the different *E. faecalis* strains to assess their sensitivity against the produced compounds based on the size of the zone of inhibition (Figure S1). Please note that we cannot confirm that each of these strains produced their expected antimicrobial under the chosen growth conditions. However, each producer caused the appearance of an inhibition zone in the *E. faecalis* overlay, showing that some antimicrobial compounds were indeed secreted.

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

In contrast, when testing the *E. faecalis* strains against the bacitracin producer *B. lichenformis* ATCC10716, deletion of *sapR, sapAB* and *rapAB* showed similar increased sensitivity, consistent with their contribution to the bacitracin resistance network that we have examined in this study and previously (Gebhard *et al.*, 2014). Although also presenting increased sensitivity compared with the wild type, deletion of *liaR* resulted in a marginally smaller zone of inhibition compared with the other deletion strains. This is in line with our data presented above, showing the Lia system has a more indirect role in controlling the bacitracin response by regulating *sapRS* expression.

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

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

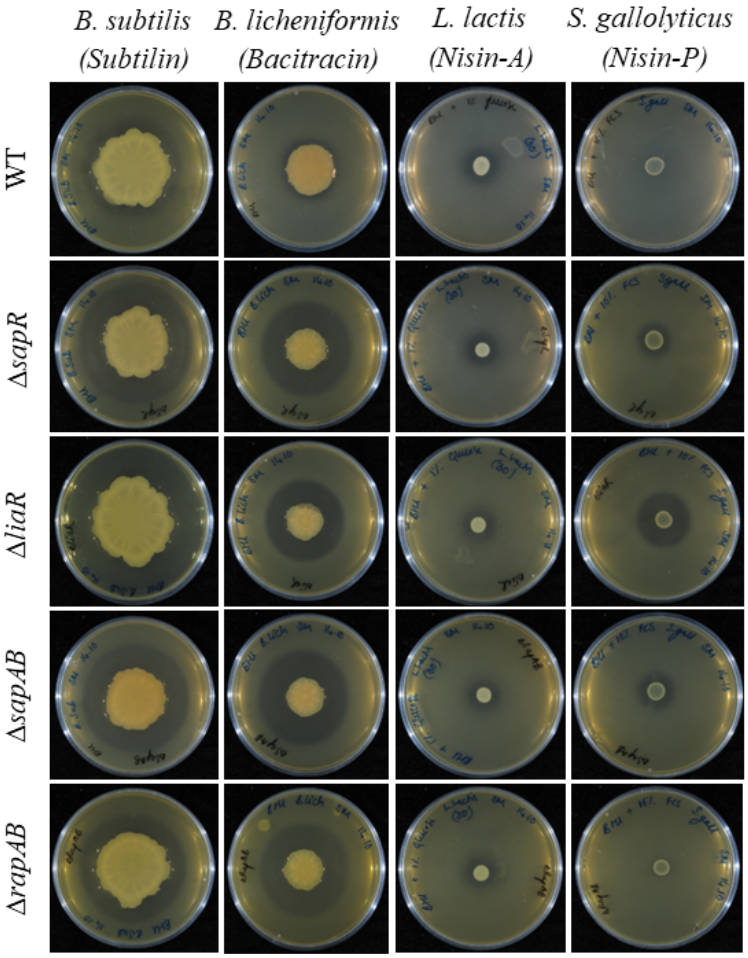

Figure S1. *E. faecalis* deletion strains show differential zones of inhibition in deferred antagonism tests against antimicrobials produced by other firmicute bacteria. The antimicrobial producer strains indicated at the top were grown overnight and then adjusted to an OD_{600} of 0.5 with fresh media. 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.

Experimental Procedures

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

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Table S1. Vectors, plasmids and strains used in this study.

Vectors	Description	Source
pTCVlac	*E.coli*-Gram positive shuttle vector for transcriptional fusions to lacZ; erm^r, kan^r	Poyart and Trieu-Cuot, 1997
pLT06	Vector for creation of unmarked deletions in *E. faecalis*: cm^r	Thurlow *et al.,* 2009

Plasmids	Description	Source
pSMMTCV01	pTCVlac containing the transcriptional P_{dltX}-lacZ fusion	This study
pSMMTCV02	pTCVlac containing the transcriptional P_{sapRS}-lacZ fusion from	This study
pSMMTCV05	pTCVlac containing the transcriptional P_{liaXYZ}-lacZ fusion	This study
pSMMTCV18	pTCVlac containing the transcriptional P_{rapAB}-*lacZ* fusion	
pLWTCV01	pTCVlac containing the transcriptional P_{ddltX}^{SapR*}-lacZ fusion with mutated binding site	This study
pLWTCV02	pTCVlac containing the transcriptional P_{sapRS}^{LiaR*}-lacZ fusion with mutated binding site	This study
pLWTCV03	pTCVlac containing the transcriptional P_{sapRS}^{LaiR*}-lacZ fusion with mutated binding site	This study
PSMMLT06-03	pLT06 containing the deletion construct for Δ*liaR*	This study
PSMMLT06-04	pLT06 containing the deletion construct for Δ*sapRS*	This study

Bacterial Strains - *E.coli*	Description	Source
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
MC1061	*F- Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 λ- e14- mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2(r-m+)*	Laboratory stock

Bacterial Strains - *E. faecalis*	Description	Source
JH2-2	Laboratory strain, plasmid-free; rif^r; fs^r	Jacob *et al.*, 1975
Δ*rapAB*	JH2-2 carrying an unmarked deletion of the operon	Gebhard *et al.*, 2014
Δ*sapAB*	JH2-2 carrying an unmarked deletion of the *sapAB* operon	Gebhard *et al.*, 2014
Δ*liaR* (SGF09)	JH2-2 carrying an unmarked deletion of *liaR*	This study
Δ*sapRS* (SGF11)	JH2-2 carrying an unmarked deletion of the *sapRS*	This study

SGF01	JH2-2 carrying pSMMTCV01; kan^r	This study
SGF24	JH2-2 carrying pSMMTCV02; kan^r	This study
SGF13	JH2-2 carrying pSMMTCV05; kan^r	This study
SGF82	JH2-2 carrying pSMMTCV18; kan^r	This study
SGF101	JH2-2 carrying pLWTCV01; kan^r	This study
SGF102	JH2-2 carrying pLWTCV02; kan^r	This study
SGF103	JH2-2 carrying pLWTCV03; kan^3	This study
SGF17	Δ*sapRS* carrying pSMMTCV01; kan^r	This study
SGF98	Δ*sapRS* carrying pSMMTCV02; kan^r	This study
SGF34	Δ*sapRS* carrying pSMMTCV05; kan^r	This study
SGF96	Δ*sapRS* carrying pSMMTCV18; kan^r	This study
SGF04	Δ*rapAB* carrying pSMMTCV01; kan^r	This study
SGF100	Δ*rapAB* carrying pSMMTCV02; kan^r	This study
SGF95	Δ*rapAB* carrying pSMMTCV18; kan^r	This study
SGF05	Δ*sapAB* carrying pSMMTCV01; kan^r	This study
SGF99	Δ*sapAB* carrying pSMMTCV02; kan^r	This study
SGF97	Δ*sapAB* carrying pSMMTCV18; kan^r	This study
SGF18	Δ*liaR* carrying pSMMTCV01; kan^r	This study
SGF25	Δ*liaR* carrying pSMMTCV02; kan^r	This study
SGF94	Δ*liaR* carrying pSMMTCV18; kan^r	This study

Table S2. Primers used in cloning

Primers	Sequence[1]	Description
P_{dltX}fwd	AATTTgaattcGATTTTCTTTCCGCCGATGG	Used to amplify 400 bases upstream of the *dltX* start codon
P_{dltX}Rev	AATTTggatccGTTTTCATTATCATTCACCTCC	Used to amplify 400 bases upstream of the *dltX* start codon
P_{sapRS}fwd	AATTTgaattcGGTTAGGACTCTGCCG	Used to amplify 400 bases upstream of the *sapRS* start codon
P_{sapRS}Rev	AATTTggatccGCCATGCAATCCCACC	Used to amplify 400 bases upstream of the *sapRS* start codon
P_{liaXYZ}Fwd	AATTTgaattcGGATGATCGTACTAATG	Used to amplify 400 bases upstream of the *liaXYZ* start codon
P_{liaXYZ}Rev	AATTTggatccCTTTCATGGATATTGC	Used to amplify 400 bases upstream of the *liaXYZ* start codon
*liaR*_KO_Fwd_BF	AATTTgaattcACGGTAATTATGAAAGTCCAG	Used to amplify 800-1000 bases upstream of the *liaR* start codon
*liaR*_KO_Rev_BF	AATTT**CTGATATTTA**CATTACTTTGATCACTTGCAG	Used to amplify 800-1000 bases upstream of the *liaR* start codon including an overhang complementary to the 'after gene' fragment.
*liaR*_KO_Fwd_AG	AATTT**CAAAGTAATG**TAAATATCAGATAAATCC	Used to amplify 800-1000 bases downstream of the *liaR* stop codon including an overhang complementary to the 'before gene' fragment.
*liaR*_KO_Rev_AG	AATTTggatccGTTTCTTCTTTAAACCAT	Used to amplify 800-1000 bases downstream of the *liaR* stop codon
*sapRS*_KO_Fwd_BF	AATTTgaattcGAATGGTTGGCGATTTTCGG	Used to amplify 800-1000 bases upstream of the *sapRS* start codon
*sapRS*_KO_Rev_BF	AATTT**CCTACTTTTTA**CATGCAATCCCACCTTTCTA	Used to amplify 800-1000 bases upstream of the *sapRS* start codon including an overhang complementary to the 'after gene' fragment.
*sapRS*_KO_Fwd_AG	AATTT**GGATTGCATG**TAAAAAGTAGGTCTATCAGC	Used to amplify 800-1000 bases downstream of the *sapRS* stop codon including an overhang complementary to the 'before gene' fragment.
*sapRS*_KO_Rev_AG	AATTTggatccCGTAAATAAACTATTTACGCT	Used to amplify 800-1000 bases downstream of the *sapRS* stop codon
*liaR*_KO_confirmation_Fwd	AATTT GTT CTC ATA TCC TTA CTA ATA GG	Used to amplify upstream of the 'before gene' fragment of *liaR*
*liaR*_KO_confirmation_Rev	AATTT CAT TCT TTG TTC CTC CTT CAC	Used to amplify downstream of the 'after gene' fragment of *liaR*
*sapRS*_KO_confirmation_Fwd	AATTT CAA CCT TCA TGT TTG CTA ATG	Used to amplify upstream of the 'before gene' fragment of *sapRS*
*sapRS*_KO_confirmation_Rev	AATTT C ATC CCA CCA CTG TCA TTG	Used to amplify downstream of the 'after gene' fragment of *sapRS*

[1] Start and stop codons are underlined and overhangs are bolded for clarity. Restriction enzyme sites are in lower case.

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