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

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Summary

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

Results

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

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

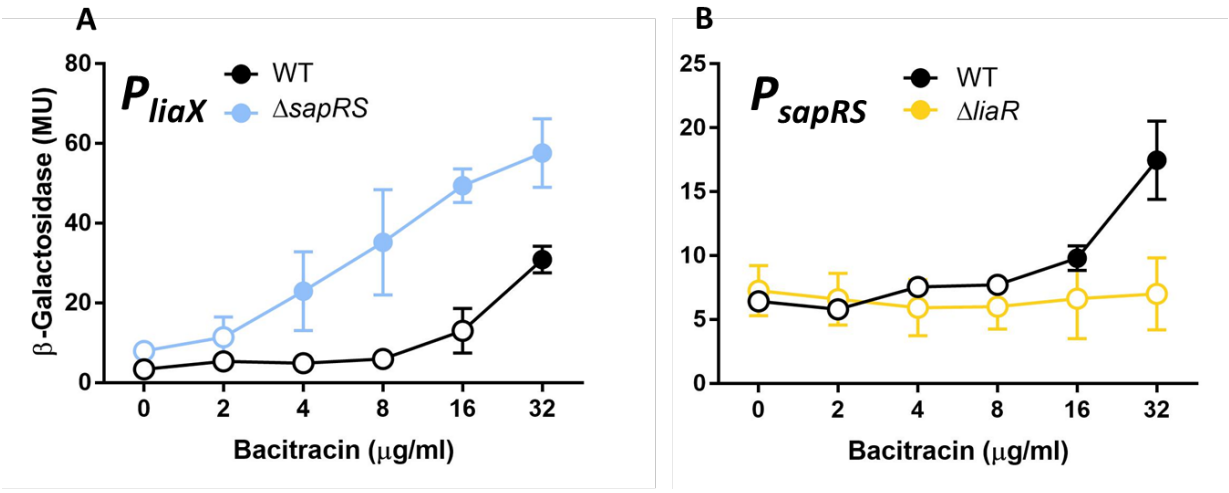

Figure 1. The LiaFSR operon responds to bacitracin exposure and induces the expression of both *liaXYZ* and *sapRS*, with the absence of *sapRS* resulting in the hypersensitivity of the *liaXYZ* promoter. Cells of *E. faecalis* JH2-2 wild-type or isogenic deletion strains harbouring a P_{liaX}-*lacZ*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

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

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

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

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

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

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

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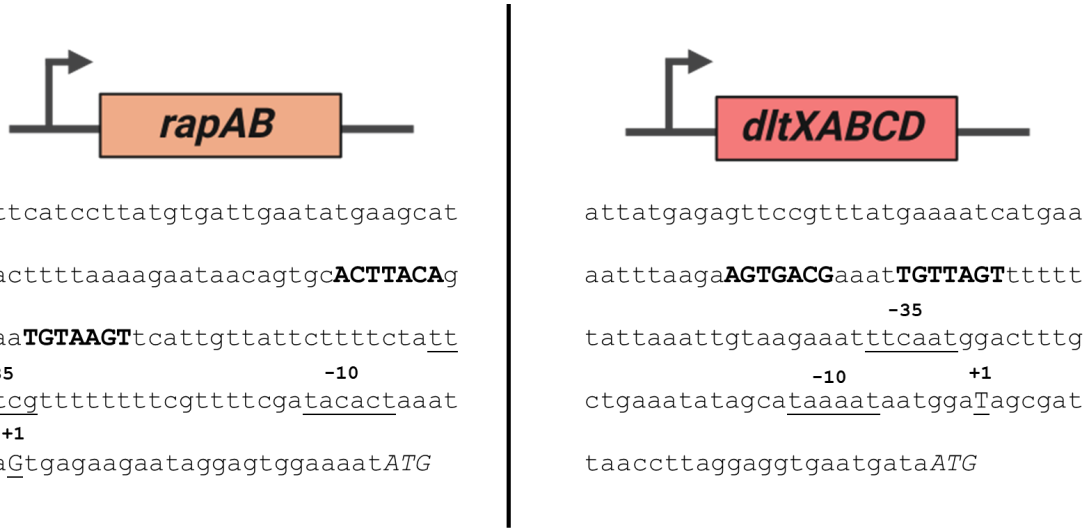

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Figure 2. Schematic of the promoter regions of both *rapAB* and *dltABCD*. The sequence 150 base pairs upstream of the ATG start codons are shown. The proposed binding site for SapR on both the *rapAB* and *dltABCD* promoters is in bold and capitalised,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

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

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

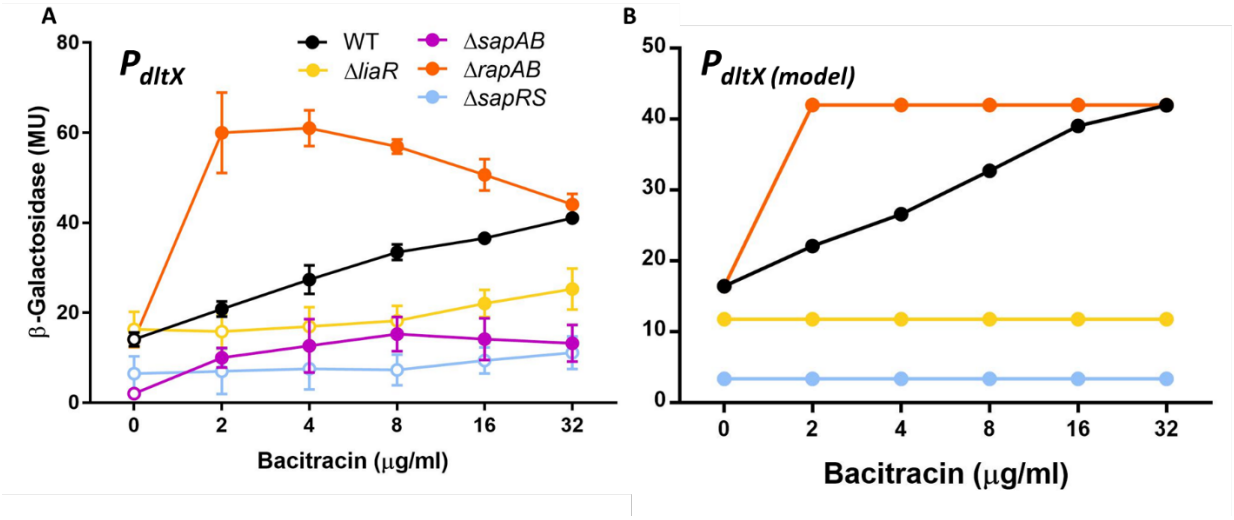

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Figure 3. Induction of the resistance operon *dltXABCD* by bacitracin. Panel A indicates the experimental induction of *dltX*. Cells of *E. faecalis* harbouring a *PdltX-lacZ* transcriptional fusion were grown to exponential phase and challenged with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

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

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

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

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

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

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

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

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

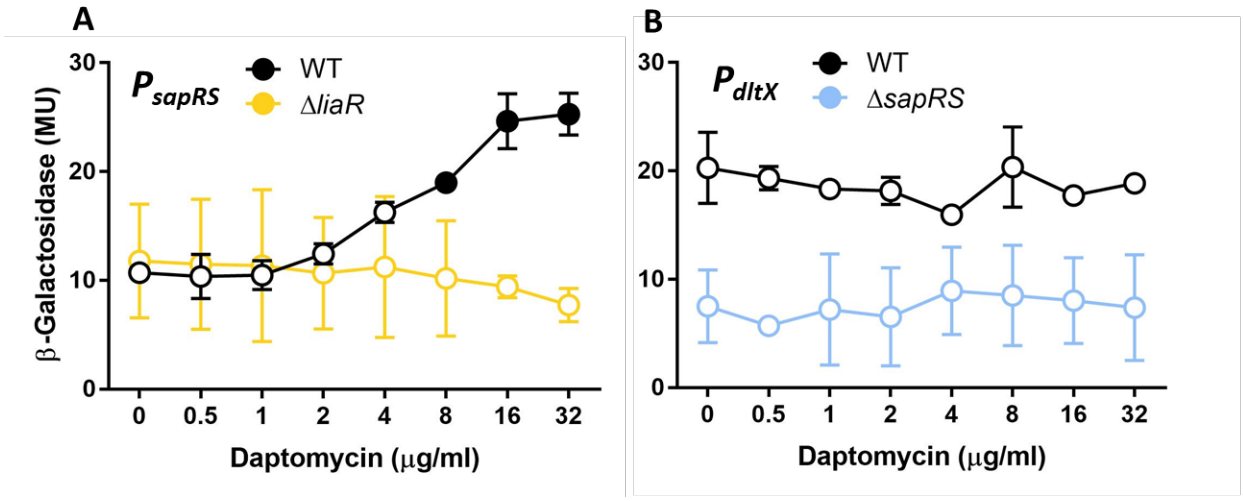

Figure 4. Exposure to daptomycin results in the induction of *sapRS* but does not induce the expression of *dltXABCD*. Cells of *E. faecalis* harbouring a P_{sapRS}-*lacZ* (panel A) or a P_{dltX}-*lacZ* (panel B) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations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

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

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

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

The network components vary in importance during interactions with antimicrobial producer strains. As stated above, 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[51–54]. In such environments, *E. faecalis* interacts with other microbes and must defend itself against antimicrobial producing bacteria. Therefore, we next 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 utilised 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[55], bacitracin producer *Bacillus lichenformis* ATCC10716[56], nisin-A producer *Lactococcus lactis* NZ9000[57] and nisin-P producer *Streptococcus gallolyticus* AB39[58]. Each producer was spotted

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

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

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

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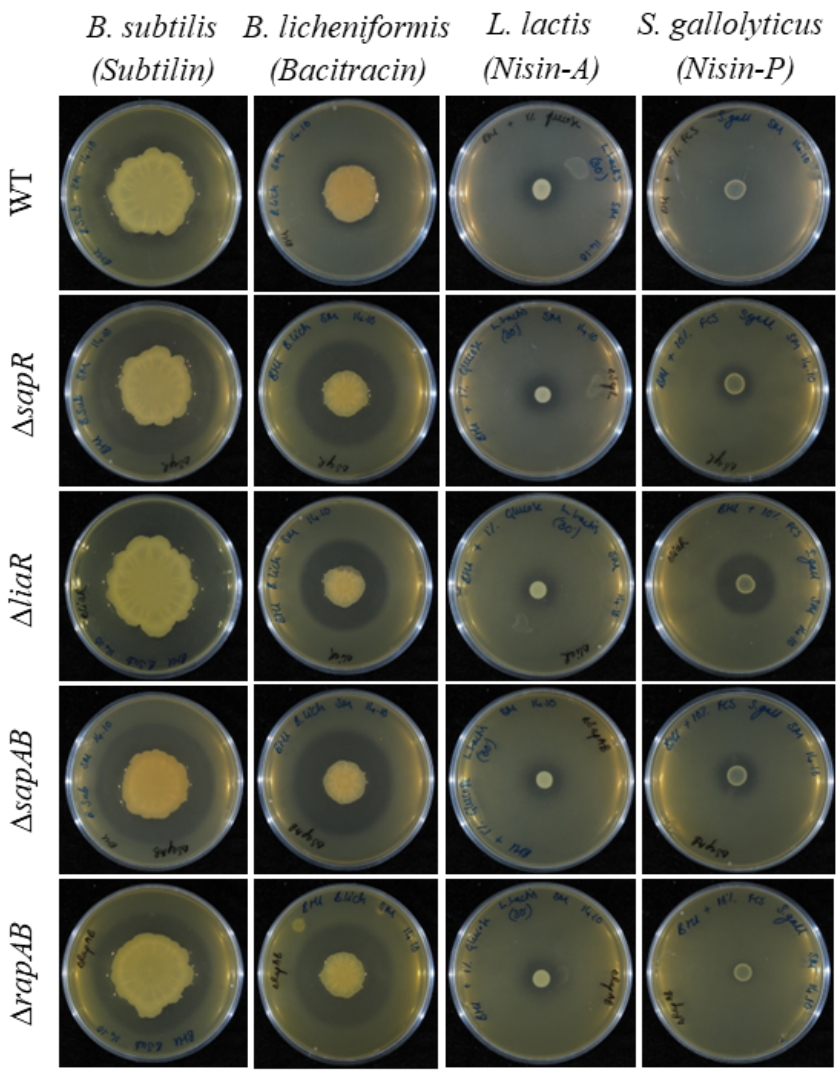

Figure 5. ***E. faecalis*** **deletion strains show differential zones of inhibition in deferred antagonism tests against antimicrobials produced by other firmicute bacteria.** The antimicrobial producer strains indicated at the top were grown overnight and then adjusted to an OD_{600} of 0.5 with fresh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

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

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.

Discussion

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

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

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

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

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

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

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

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

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

413

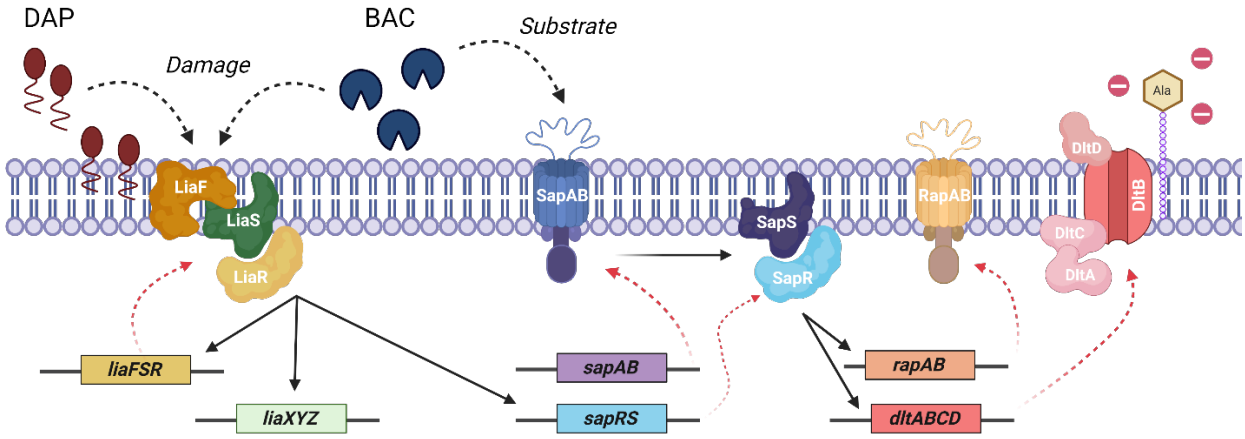

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Figure 6. Proposed model of the antibiotic resistance network. Schematic illustrating the genes and proteins involved in antibiotic detection and resistance, with transcriptional regulation and translation demonstrated by black and red arrows respectively. 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,

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

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

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

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

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

470

471 # Experimental Procedures

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

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

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

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

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

β-Galactosidase assays. For quantitatively assessing induction of *lacZ* reporter constructs in *E. 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)[82]. 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[34,83], 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.

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.

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

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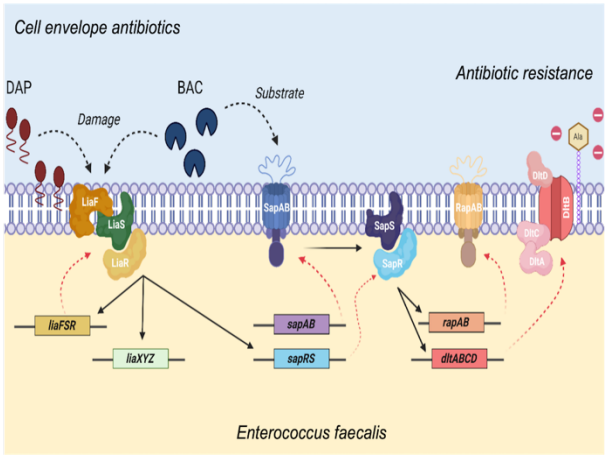
579

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 *dtlXABCD*
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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Supplementary Material

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

Table S2. Primers used in this study.

Table S3. Parameter values used in the mathematical model.

Table S4. Experimental numerical data for Figures 1, 3 and 4.

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