Dissecting an Antibiotic Resistance Network in Enterococcus faecalis

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Award date:
2023

Awarding institution:
University of Bath

Dissecting an Antibiotic Resistance Network in *Enterococcus faecalis*

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

August 2022

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Declaration of Authorship

This thesis includes a literature review (chapter 1), three results chapters (chapter 2 including parts A and B, chapter 3 and chapter 4) and a final conclusion. I am the sole author of each chapter aside from chapter 2, of which some data from the intended manuscript has been omitted for clarity and to highlight my own contributions. As such this chapter includes sections that were primarily penned by the first authors – the details of these contributions are outlined at the beginning of the chapter. In addition, a portion of the experimental work included in results chapter 3 include those completed by my collaborators. These contributions are likewise accredited in detail at the beginning of each relevant results chapter. A portion of the experimental work presented in chapter 4 was done by the second author, an undergraduate placement student under the supervision of myself. All the experimental work presented in results chapter 2 was completed by the submitting candidate.

“It is with passion, courage of conviction, and strong sense of self that we take our next steps into the world,

remembering that first impressions are not always correct.

You must always have faith in people. And most importantly,

you must always have faith in yourself.”

- *Elle Woods, Legally Blonde*

Acknowledgements

First and foremost, I would like to thank my supervisor, Professor Susanne Gebhard. You have been an excellent mentor - supportive, patient and kind. It is because of you that I am incredibly proud of this thesis and for all that you have done for me, I cannot thank you enough. I wish you all the best in Germany - your future students are incredibly lucky to have you.

To Dr James Horton, for always making me laugh and being a shoulder to cry on in the lab – we did a good job at convincing people we knew what we were doing. You are an excellent scientist and in you I have found a friend for life.

To Dr Jonathan Abrahams, who has had the patience of a saint and made me boundless cups of tea. Without you, this would never have been possible, and I can't thank you enough. Coming to Bath to do my PhD was good, but meeting you was even better. I'll see you in Oxford for the next chapter.

And finally, to my parents and my brother Chris, who have supported me in all my decisions, provided endless encouragement and have been there through all the good times and bad. I could not have done this PhD without you, so I dedicate all this work to you guys.

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Abstract

The emergence of antibiotic resistance is one of the most serious global threats to public health faced in the 21st century. At the forefront of these infections lie multidrug-resistant bacteria which can render antimicrobial treatment ineffective. *Enterococcus faecalis* has become recognised as important nosocomial pathogen due to a high level of intrinsic resistance to antimicrobials - however, our knowledge of these resistance pathways remains incomplete. In accordance with this requirement, the aims of this thesis were to deepen our knowledge of the *E. faecalis* cell-envelope stress response, to gain an understanding of the unique and complex setup of this antibiotic resistance network.

Our previous work has identified the existence of a bacitracin resistance module in *E. faecalis*, comprised of the ABC transporters, SapAB and RapAB, and the two-component system (TCS) SapRS. Recently, components of this network have been implicated in resistance to daptomycin, a role usually fulfilled by a second TCS LiaFSR. By unravelling the interplay between these two regulatory pathways, we demonstrate the existence of a logic 'AND' gate, whereby both antibiotic-induced cellular damage (LiaFSR) and the presence of a substrate drug for the network's sensory transporters (SapAB) are required to trigger expression of the SapR regulon.

To add to the complexity of this network, the Lia and Sap systems are just two of many TCS's involved in monitoring cell envelope integrity. The CroRS system is unique to the enterococci and is the main determinant of intrinsic β-lactam resistance. However, the genes within the CroR regulon remain unknown. Through experimental evolution, we were able to identify potential processes influenced by CroRS regulation. Firstly, upon evolving the *croRS* deletion for improved growth rate, we identified a potential role for CroRS in cell-envelope biosynthesis. Secondly, upon adaptation of the *croRS* deletion to increasing ampicillin concentration, we identified a potential role for CroRS in c-di-AMP metabolism.

As we gain a detailed understanding of the cell-envelope stress response within *E. faecalis*, this may ultimately lead to identifying an Achilles' heel within this network, enabling the repurposing of existing antimicrobials and the discovery of new therapeutic targets.

1. Literature Review

1.1 Background of the *Enterococcus* Genus

The term "entérocoque" was first used by Thiercelin in 1899, describing commensal gut bacteria that had the ability to become pathogenic (1). Despite originally being categorised within the *Streptococcus* genus due to biochemical and morphological similarities, the classification of "enterococcus" was considered more of a placeholder name for gram-positive cocci isolated from the gut (2). It was only in 1984, after detailed biochemical and culture analysis by Kalina (3), that the so-called enteric streptococci became its own genus, *Enterococcus*. Since then, the genus has over 50 species to date (4).

The *Enterococcus* genus is now defined as a group of low-CG, gram-positive, facultative anaerobes (5) that exist individually or as pairs, groups, or chains (6). Most enterococci are cytochrome c oxidase and catalase negative, salt tolerant, resistant to 40% bile, esculin hydrolytic, able to grow in the presence of sodium azide (up to 0.4%) (6) and at temperatures ranging from 10 – 45 °C (7). As a genus, they possess remarkably diverse genomes, ranging in size from size 2.3 to 5.4 Mb, and containing 605 to 1,037 genes within their core genus genome (8,9). Their large pangenome contains 2,154 to 5,107 predicted genes and reflects plastic nature of the genus, with enterococci being isolated from soil and water; plants and animals; vertebrates and invertebrates; and most recently, as the causative agents of disease (6,10–17).

In the human gastrointestinal tract, the enterococci reside within the small and large intestine, and represent 1% of the faecal flora, with *Enterococcus faecalis* and *Enterococcus faecium* being most commonly isolated (18–21). The intestinal microbiota contains a highly diverse range of microbes (21–23), and the administration of broad-spectrum antibiotics represents a large risk factor for the commensal-to-pathogen transition, which can precede an enterococcal infection (24). The treatment leads to the depletion of the intestinal commensals, in particular, the gram-negative Bacteroidetes (25–28). In addition to inhibiting pathogen growth directly via antimicrobial production, these Bacteroidetes can stimulate the production of RegIIIγ, a C-type lectin, from the Paneth cells lining the gut (29–31). RegIIIγ displays bactericidal activity by binding the peptidoglycan of the bacterial cell wall and forming pores (32). In the absence of this signalling, the enterococci can flourish, resulting in overgrowth.

Antibiotic treatment also leads to thinning of the mucin barrier which lines the epithelial surface, increasing the risk of microbes breaching the epithelium (33–35).

Within the *Enterococcus* genus, certain species can become pathogenic, arising from commensal strains and are a major cause of hospital-acquired infections (HAIs), being isolated in up to 17% of bloodstream infections in Europe in 2021 (36). These multidrug resistant lineages differ from commensal strains in multiple ways including having 25% larger genomes through the accumulation of mobile elements including phages, resistance genes and pathogenicity islands, and lacking CRISPR-Cas9 defences mechanisms (37–39). In humans, *E. faecium* and *E. faecalis*, are the most commonly isolated disease-related species, accounting for 75% of enterococci infections (40). Between them, *E. faecalis* and *E. faecium* cause 110,000 urinary tract infections, 25,000 cases of bacteraemia, 40,000 wound infections and 1,100 cases of endocarditis annually in the United States (41).

The more prevalent of the two is *E. faecalis*, which is more virulent than *E. faecium.* Its pathogenic strains often contain a pathogenicity island rarely found in *E. faecium* which contains genes involved in biofilm production and virulence (38). However, *E. faecalis* has lower levels of both intrinsic and acquired resistance compared to *E. faecium*, including vancomycin resistance, which has contributed to a decrease in survival rates for *E. faecium* infections in recent years (42). In addition to the threat posed by the *Enterococcus spp.*, they can also act as a reservoir for resistance, with recent evidence showing that co-colonisation with both vancomycin susceptible *S. aureus* and vancomycin resistant *E. faecalis* can enable the transfer of the VanA cluster to *S. aureus* during infections, subsequently resulting is vancomycin resistant *S. aureus* (43,44). As vancomycin is used as a drug of last resort in methicillin-resistant *Staphylococcus aureus* (MRSA) infections, this could potentially have a deep negative impact on the future treatment of MRSA. To combat the increasing levels of enterococcal infection, an array of antimicrobials are utilised (45). As a gram-positive bacterium, many of the antibiotics used to treat *E. faecalis* infections target the cell envelope, which we will be focussing on here.

1.2 The Bacterial Cell Envelope

The bacterial cell envelope is a complex and sophisticated multi-layered structure that serves to protect bacteria from their unpredictable environments, while allowing the movement of select nutrients into

the cell and waste products out. Most bacterial envelopes fall into one of two groups: gram-positive or gram-negative, based on differing staining characteristics as developed by Christian Gram in 1884 (46).

The gram-negative cell envelope is comprised of three principal layers: the outer membrane, the peptidoglycan cell wall, and the inner membrane. In contrast, the gram-positive cell envelope lacks an outer membrane, instead compensating with a much thicker peptidoglycan layer (Fig. 1). As *Enterococcus faecalis* is a gram-positive bacterium, this review will focus on the gram-positive cell envelope.

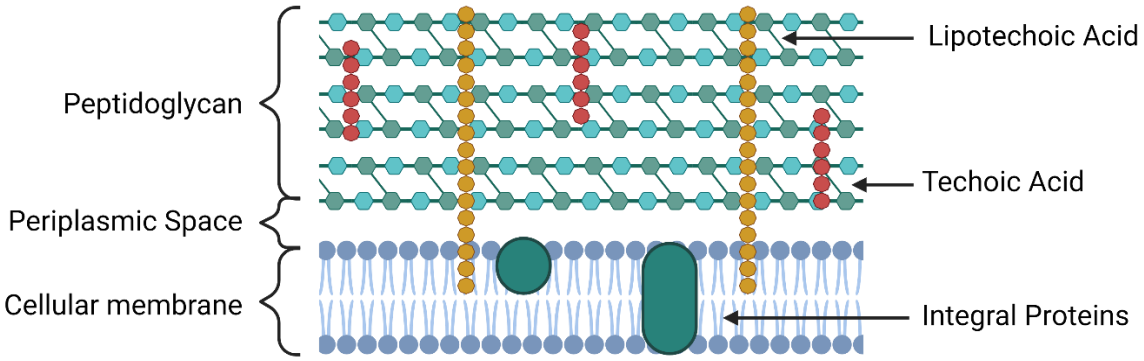

Figure 1. Structure of the gram-positive cell wall. Adapted from (47).

1.2.1 Cell Wall Synthesis

In order to maintain turgor pressure within the cell, bacteria have a rigid peptidoglycan layer, a structure comprised of two sugar derivatives: *N*-acetylglucosamine and *N*-acetylmuramic acid, which are connected via glycosidic covalent bonds, to produce a strand of repeating sugar residues (48). These long chains are synthesised adjacent to one another to form a sheet surrounding the cell and are connected through crosslinking of stem peptides attached to the disaccharide. For most of the enterococci, the stem peptide consists of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala and the presence of crosslinking forms a strong mesh like framework between the strands (49). Whilst the glycosidic bonds between the sugars give rigidity to the peptidoglycan sheet, the presence of this crosslinking between the sheets adds strength to the structure in both directions, resulting in an elastic stress-bearing structure (47).

Peptidoglycan synthesis occurs in three distinct stages (Fig. 2). Stage one occurs in the cytoplasm where the nucleotide precursors are synthesised: fructose-6-phosphate is converted into UDP-*N*-acetylglucosamine (UDP-*N*-GlcNAc) by the Glm enzymes (50) which in turn is converted to UDP-N-acetylmuramyl-pentapeptide (UDP-Mpp) by the Mur enzymes (MurA – MurF) (51). Adjacent to this reaction, the membrane-embedded undecaprenyl phosphate (UP) is also produced in the cytoplasm via the consecutive condensation reactions of farnesyl pyrophosphate (FPP) with eight isopentenyl pyrophosphates (IPP), catalysed by undecaprenyl phosphate synthase (UppS) (52). The resulting undecaprenyl pyrophosphate (UPP, C55-PP) is dephosphorylated by undecaprenyl pyrophosphate phosphatase (UppP) to produce undecaprenyl phosphate (C55-P) (53). Stage two of peptidoglycan synthesis occurs in the cytoplasmic membrane, where the enzyme phosphor-MurNAc-pentapeptide translocase (MraY) transfers the phosphor-MurNAc-pentapeptide moiety from UDP-Mpp to C55-P to yield uridine-monophosphate (UMP) and undercaprenyl-pyrophosphate-MurNAc-pentapeptide, also known as Lipid I (54).

After the synthesis of Lipid I, the glycosyltransferase MurG transfers a GlcNAc moiety from UDP-GlcNAc to Lipid I to produce undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)- GlcNAc, more commonly known as Lipid II (55–58). Lipid II is then transported from the inner membrane to the outer membrane by a flippase, where the final stage of peptidoglycan synthesis occurs via the action of penicillin-binding proteins (PBPs). The transglycosylase domain of these proteins polymerises the reaction between the sugar moieties of Lipid II to produce the glycan strands, whilst the transpeptidase domain catalyses the reactions between the stem peptides and interbridges of adjacent strands to ultimately result in the peptidoglycan layer (59,60). *Enterococcus faecalis* possess six PBPs, three class A, which are binfunctional transpeptidases/transglycolases and three class B, which are monofunctional transpeptidases. Interestingly, *E. faecalis* possesses PBP5, that appears sufficient for cross-linking even in the presence of β-lactams, therefore renders both *E. faecalis* and *E. faecium* intrinsically resistant to ampicillin.

1.2.2 Cell Wall Proteins

Threading throughout peptidoglycan layers are acidic components known as teichoic acids, long anionic structures composed of glycerol phosphate, glucosyl phosphate or ribitol phosphate repeats, connected by phosphate esters and typically containing sugars or D-alanine (61). There are two classes of teichoic acids: wall teichoic acids (WTAs), which are covalently attached to the muramic acid in the peptidoglycan; and lipoteichoic acids (LTAs), which are covalently attached to the head groups of the membrane phospholipids (62). As the phosphate groups are negatively charged, teichoic acids contribute to the negative electrical charge of the cell surface and play an important role in cation binding for transport into the cell (63).

1.2.3 The Enterococcal Polysaccharide Antigen (EPA)

All enterococci produce a rhamnose-containing polysaccharide, the enterococcal polysaccharide antigen (EPA). Expressed as an 18-gene cluster extending from *epaA* to *epaR,* these genes are involved in the generation, polymerisation, and export of this surface polysaccharide (64). The EPA is required for normal cellular growth, but also has a vital role in biofilm formation, virulence, colonisation, and resistance to antimicrobials (65–67). Like WTAs, the EPA is covalently bound to the peptidoglycan and is composed of rhamnose, *N*-acetylgalactosamine (GalNAc), galactose and GlcNAc (65,66,68). Covalently attached to this rhamnan backbone are teichoic acids, so-called “EPA decorations” (64). Whilst the backbone of the EPA is located deep in the cell wall, these decorations are exposed at the bacterial cell surface. Modification of the EPA with these decorations was found to be essential for virulence and resistance phenotypes (69).

1.2.4 The Cellular Membrane

The general structure of the cytoplasmic membrane is phospholipid bilayer, with each subunit composed of between two or four hydrophobic fatty acid tails and a hydrophilic glycerol phosphate head (70). As these phospholipids aggregate in aqueous environments, they naturally form a bilayer structure in which the fatty acids point inward towards each other to form a hydrophobic layer, whilst the hydrophilic portion points outwards to the external environment or to the cytoplasm (70). The cell membrane is also associated with a number of proteins: integral proteins, which penetrate the

hydrophobic core of the lipid bilayer, and peripheral proteins, which are attached to either the intracellular or extracellular surface of the lipid bilayer (71). The cell membrane plays three main roles; a permeability barrier, preventing leakage from the cell; an anchor for membrane associated proteins involved in transport, signalling and chemotaxis; and energy conservation, being the site of energy generation and the proton motive force.

Overall, the bacterial cell envelope is an essential yet vulnerable structure that provides the cell with an important sensory interface and molecular sieve, allowing the flow of information and transport of solutes. Because of the unique and essential nature of the components which make up the cell envelope, it is a target for numerous antibiotics.

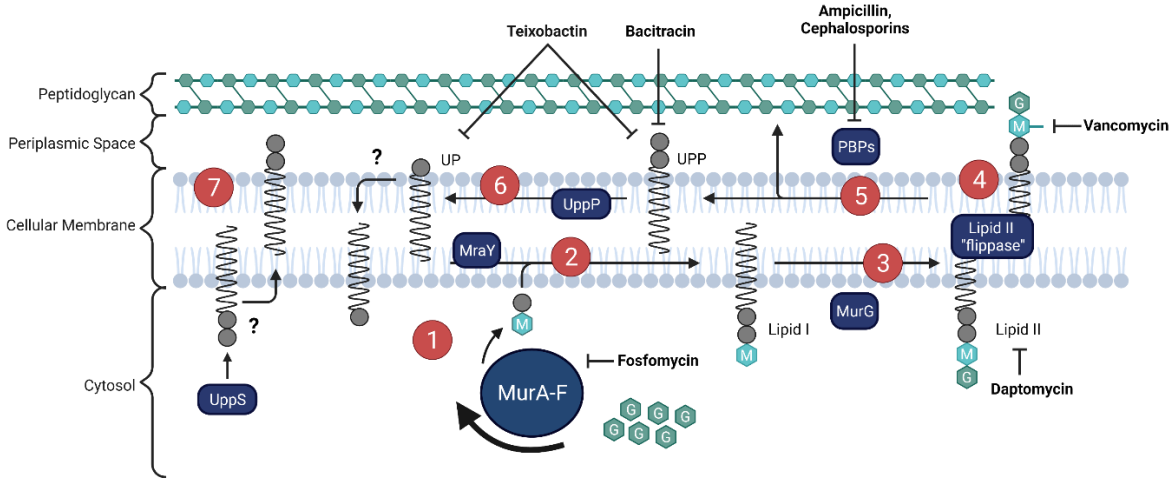

Figure 2. Schematic of the lipid II cycle and cell-wall targeting antimicrobials. 1) The MurA-F ligases catalyse the production of UDP-MurNAc-pentpeptide (M) from UDP-GlcNAc (G). 2) At the inner leaflet of the cytoplasmic membrane, the translocase MraY attaches UDP-MurNAc-pentapeptide to the lipid carrier undecaprenyl phosphate (UP), resulting in the formation of lipid I. 3) Subsequently, the transferase MurG attaches MurG UDP-GlcNAc to lipid I, resulting in the formation of lipid II. 4) Once formed, a flippase translocates lipid II to the outer leaflet of the membrane. 5) Penicillin binding proteins (PBP) are then able to add the subunits to the growing peptidoglycan cell wall, via crosslinking. 6) Upon the removal of the subunits, the lipid carrier is left its in pyrophosphate state (UPP), which is dephosphorylated back to UP and can be recycled to transport further subunits. The flipping of UP to the inner membrane is performed by an unknown mechanism. 7) Finally, to maintain the concentration of lipid carriers, UPP synthase (UppS) replenishes UPP on the cytoplasmic side of the membrane, which is subsequentially flipped to the outer membrane again, by an unknown mechanism. Antibiotics are in bold, and the component of the cycle they target is indicated by a T-shaped line. Adapted from (72).

1.3 Antimicrobials: Mechanisms and Resistance

1.3.1 Fosfomycin

Fosfomycin is a phosphonic antibiotic, discovered in 1969 being produced by certain types of *Streptomyces*. The World Health Organisation (WHO) considers fosfomycin critically important for human health, and it is included on the WHO's 'List of Essential Medicines' (73). Fosfomycin first enters the cell through the glycerophosphate transporters and works by inhibiting the action of MurA (UDP-N-acetylglucosamine-enolpyruvyltransferase), the enzyme that catalyses the first committed step of peptidoglycan synthesis (74). MurA catalyses the reaction between phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine, with the pyruvate moiety providing the linker that bridges the glycan and peptide portions of the peptidoglycan (75). Fosfomycin works by acting as a PEP analogue that covalently binds the active site of MurA, rendering the enzyme catalytically inactive (76).

Various mechanisms for fosfomycin resistance have been observed, particularly amongst gram-negative bacteria such as *E. coli*, including mutations in *murA*, conferring reduced affinity for fosfomycin and mutations in the chromosomal genes encoding fosfomycin transporters (77). In contrast, for gram-positive bacteria such as *Staphylococcus aureus*, the most prominent resistance mechanism is the emergence of plasmid-encoded enzymes which modify the antibiotic: FosA, FosB, FosC and FosX (77). Among these enzymes, FosB which catalyses the degradation of L-cysteine-fosfomycin, rendering the antibiotic harmless, is currently the only known fosfomycin resistance determinant observed in the *Enterococcus* spp. (78).

1.3.2 β-lactams

The discovery of a mouldy petri-dish in 1928 by Alexander Fleming led to the development of the world's first mass produced antibiotic, Penicillin G, which would go on to revolutionise modern medicine and herald the dawn of the antibiotic age. Penicillin is a β-lactam antimicrobial, named due to its possession of a β-lactam ring, which is able to act as an irreversible inhibitor of class A and class B PBPs, responsible for the cross-linking between the layers of peptidoglycan sheets. The presence of this β-lactam ring mimics the D-Ala-D-Ala portion of the peptide chain, the substrate for the PBPs, leading to irreversible blocking of the enzyme's active site and inactivation. This leads to a disruption of

peptidoglycan crosslinking, leading to an inevitable loss of cell wall strength and rigidity. This interruption of cell wall synthesis prevents peptidoglycan maturation, resulting in a weakened cell wall and ultimately, bacterial lysis.

Intrinsic tolerance of the enterococci to β-lactams is associated with the previously mentioned species-specific PBP gene *pbp5*, which encodes PBP4, an ortholog of PBP2a expressed by MRSA (79), and has a low binding affinity β-lactams (80,81). PBP4 is required for both the intrinsic resistance of the enterococci against ampicillin and cephalosporins, which are discussed in more detail below. However, the level of intrinsic resistance differs amongst the β-lactam antimicrobials, with penicillins (e.g. ampicillin) having the highest activity, carbapenems slightly lower, and cephalosporins the lowest (40).

1.3.3 Ampicillin

At its discovery, penicillin was a very effective antimicrobial, but only against gram-positive bacteria. This led to the development of ampicillin, an extended spectrum β-lactam, effective against both gram-positive and gram-negative bacteria. Despite the decreased susceptibility of *E. faecalis* to the β-lactams, as discussed above, ampicillin is currently the antibiotic of choice in the treatment of *E. faecalis* infections, as the low-level of intrinsic resistance is not enough to preclude the use of the antimicrobial. Increased levels of ampicillin resistance in *E. faecalis* are associated with overexpression of PBP4 or mutations in amino acid sequence of *pbp5*, predicted to have decreased affinity for β-lactams (82–84).

Another form of resistance is mediated by a β-lactamase which inactivates ampicillin through cleavage of the β-lactam ring (85). Originally described in staphylococci, resistance is passed on through an operon encoding *blaZ*, a β-lactamase; *blaR1*, a transmembrane sensor and signal transducer; and *blaI*, a repressor gene (86). Whilst common amongst the staphylococci, this mechanism of resistance remains relatively rare in *E. faecalis*.

A recent form of resistance to β-lactams to be discovered is through regulation of c-di-AMP levels in the cell. This second messenger has been termed the ‘essential poison’ due to both the lethal and essential nature of the signalling molecule (87) (*discussed in chapter four*). In bacteria which produce c-di-AMP, it has been shown to be essential due to its role in peptidoglycan synthesis (88). Conversely,

the overproduction of c-di-AMP has proven to be toxic, resulting from dysregulation of solute intake (89). Interestingly, it has recently been discovered that in many of the Firmicutes, higher c-di-AMP levels have been correlated with β-lactam resistance (90). This is thought to occur through c-di-AMP's regulation of cell wall composition, osmotic regulation, and/or controlling of PBP2a levels. In addition, recent work in *E. faecalis* has demonstrated that the complete loss or overaccumulation of c-di-AMP resulted in impaired antimicrobial tolerance, including tolerance to ampicillin (91).

1.3.4 Cephalosporins

Also β-lactam antimicrobials, the cephalosporins were discovered in 1945 being originally isolated from the fungus then known as *Cephalosporium* (92). Cephalosporins are divided into groups and can be modified to gain different properties. These groups are known as 'generations', generally resulting in higher activity against gram-negatives than the previous generation, but reduced activity against the gram-positives. However, the fourth generation represent broad-spectrum activity against both classes (93). Although related in structure to the penicillin's, with both containing a core β-lactam ring, the side chains and resulting configuration between members of each group are distinct (94).

The intrinsic resistance to cephalosporins is a well-known, but not completely understood, feature of the enterococci. Like ampicillin, cephalosporin resistance is associated with the decreased binding affinity to PBP4 (95). Interestingly however, deletion of *pbp5* results in a ~4,000-fold decrease in ceftriaxone susceptibility, a third generation cephalosporin, compared with only a four-fold decrease in ampicillin sensitivity (95). This suggests that PBP4 contributes differently to both ampicillin and ceftriaxone resistance.

In the presence of high concentrations of ceftriaxone, the transpeptidase module of all PBPs, except PBP5 are thought to be inactivated. As a class B PBP, PBP4 has monofunctional transpeptidase activity and must partner with a class A bifunctional glycotransferase to produce peptidoglycan (95), of which the enterococci possess three; PbpF, PonA and PbpZ (96). Whilst PbpZ was unable to provide transglycosylase activity, PbpF and PonA were shown to cooperate with PBP4 to permit growth in the presence of cephalosporins (95). As a result, the deletion of *ponA* or *pbpF* resulted in a loss of cephalosporin resistance, but the ampicillin MIC remained unchanged, the reason for this lack of change

however, remains unclear. This again suggests that resistance to both ampicillin and cephalosporin occurs via different mechanisms within the enterococci.

Regulatory pathways involved in the cell-envelope stress response (CESR) have also been associated with cephalosporin resistance. Among them is CroRS, named for its role in ceftriaxone resistance (97) which will be discussed in more detail later (*see section 1.4*). Interestingly, whilst the deletion of *croRS* resulted in a substantial decrease in cephalosporin resistance, the loss of CroR function was shown not to alter the expression of *pbp5* (97), suggesting this resistance phenotype occurs through a completely alternate mechanism. Another system which has been implicated is the serine/ threonine kinase IreK and its associated phosphatase IreP, with deletions of the latter in *E. faecalis* found to increase cephalosporin resistance (98). A third protein IreB, was found to negatively regulate cephalosporin resistance, and is the target of IreK and IreP (98). This pathway also appears to be cephalosporin specific, with no other changes in MICs identified. A gene which could potentially be under the control of the IreK signalling pathway is MurAA, which catalyses the first step of peptidoglycan synthesis (99). Deletion of *murAA* resulted in a loss of resistance to cephalosporins, despite the presence of the homologue MurAB. Interestingly, when expressed in *trans* from a vector, MurAA was able to restore the resistance phenotype in a Δ*irek* background, but not in deletions of *pbp5* or *croRS* (100). This implies that whilst *ireK* brings about resistance via MurAA, *croRS* and *pbp5* produce resistance via a different mechanism.

1.3.5 Vancomycin

Vancomycin is a glycopeptide antibiotic, produced by the actinomycete *Amycolatopsis orientalis* and first used to treat enterococcal infections in 1974 (101). Vancomycin acts by interfering with cell wall synthesis by binding to the terminal D-alanyl-D-alanine moiety of the pentapeptide precursors, NAM and NAG (102). This binding prevents transpeptidation via steric hindrance, weaking the cell wall and leading to cytolysis and death (102).

Acquired vancomycin resistance in *E. faecalis* first appeared in 1986, likely arising due to the use of the glycopeptide antibiotic avoparcin in agriculture, leading to cross resistance (103). Since then, multiple epidemics have plagued hospitals, and vancomycin resistant enterococci (VRE) are now

classified by the World Health Organisation as a 'superbug' (104). Resistance occurs through the amino acid change D-alanine-D-lactate in lipid II, which reduces the affinity for vancomycin by ~1000-fold. Originating from soil bacteria (105), nine vancomycin resistance clusters (*van*) have been observed within the enterococci (102), transferred by horizontal gene transfer, with *vanA* being most commonly isolated. The *vanA* cluster is under the control of two promoters: the first is responsible for expression of the TCS-encoding operon *vanRS* and the second is responsible for activating the resistance genes, *vanHAXYZ* (106). Firstly, VanS is a histidine kinase which responds to activation by glycopeptides and phosphorylates VanR, a response regulator, which in turn induces the expression of the resistance genes. Following this, *vanH* encodes a dehydrogenase, which catalyses the production of D-lactate from pyruvate. The gene *vanA*, which encodes a ligase, then catalyses the production of the depsipeptide D-alanyl-D-lactate. This depsipeptide is the incorporated at the terminus of the growing peptidoglycan in place of the normal D-ala-D-ala and included into the cell wall, preventing the binding of vancomycin. The remaining genes *vanXYZ*, work to remove D-Ala-D-Ala dipeptides and pentapeptides ending with D-Ala from the pool of precursors, preventing their incorporation into the cell wall and presenting as vancomycin targets.

1.3.6 Bacitracin

First isolated in 1943 by the bacteriologist Balbina Johnson, bacitracin is a peptide antibiotic, produced by the bacterium *Bacillus lichenformis* (107) and synthesised nonribosomally by a multienzyme complex composed of three subunits, BacA, BacB and BacC (108–110). The name is derived from the original patient the strain was isolated from, a young girl named Margaret Treacy, whose leg injury presented with a microbially-produced compound which showed antimicrobial activity (107). It received FDA approval for the treatment of bacterial infections in 1948, primarily as a topical treatment for infection in humans due to its high level of toxicity when taken orally. However, it has wide agricultural use, such as in the treatment of bacterial infections in both turkeys and chickens (111).

As a potent narrow spectrum antibiotic, bacitracin is primarily active against gram-positive bacteria, affecting the synthesis of the cell wall (112). Bacitracin exerts its bactericidal effect by the inhibition of peptidoglycan synthesis (113). It forms a complex with the lipid carrier, UPP (114), mediated by metal

ion, Zn^{2+} (115). As UPP acts as a carrier of a peptidoglycan subunits during cell wall synthesis, bacitracin prevents the dephosphorylation of UPP, inhibiting its conversion to UP. This in turn prevents the recycling of the lipid carrier, halting cell wall synthesis, resulting in cell death.

Enterococcus faecalis exhibits either high- and/or low-level resistance to bacitracin. The primary mechanism of high-level resistance is provided by the presence of the plasmid-borne *bcrABDR* cluster (116). The system is regulated by BceR, which acts as a bacitracin sensor and can induce the expression of *bcrABD*. The *bcrAB* genes encode for an ATPase and a six transmembrane helix permease, which together are proposed to mediate the active efflux of bacitracin, whilst *bcrD* encodes for a undecaprenol kinase/phosphatase that can convert UPP to UP (117). The overproduction of undecaprenol kinase/phosphatase increase the pool of UP, which can overcome the low levels of UPP when the pathway is blocked by bacitracin (118). Low-level resistance in *E. faecalis* comes from depleting the pool of UPP, the target of bacitracin. This action is performed by UppP, which decreases the efficiency of bacitracin by increasing the conversion of UPP to UP, a similar role to *bcrD*, discussed above, and *bcrC* in *Bacillus subtilis.* Mutants of UppP in *E. faecalis* lead to increased bacitracin sensitivity, due to the increased level of UPP and increased binding of the antibiotic (119). A further bacitracin resistance module has been discovered in *E. faecalis*, which is discussed later (*See section 1.4.4*).

1.3.7 Teixobactin

Isolated from the soil bacterium *Eleftheria terrae*, the so-called "resistance proof" teixobactin is a recently discovered depsipeptide antimicrobial (120) and the first with a novel mode of action to be discovered in over a decade. Potent against gram-positive bacteria, teixobactin targets the pyrophosphate-saccharide moiety found in both lipid II and lipid III (121), inhibiting both cell wall and teichoic acid biosynthesis (122). The presence of an outer membrane results in the innate resistance of the producer strain and other gram-negative bacteria.

Although no mechanism of resistance towards teixobactin has been found among the enterococci, *E. faecalis* demonstrates a remarkable level of tolerance to the antimicrobial. As tolerance precedes the development of resistance, it has become crucial to understand this tolerance further. Work by Darnell et al. has revealed the upregulation of CroRS, VicRK and LiaFSR *(further discussed in section 1.4)* in

response to teixobactin challenge (123). Further investigation demonstrated the deletion of *croRS* abolished this tolerance, suggesting the contribution of CroRS and/or its regulon to this phenotype. However, of the 219 genes found with the CroRS regulon, those responsible for tolerance are yet to be elucidated.

1.3.8 Daptomycin

Discovered in the late 1980s, daptomycin (DAP) is a lipopeptide antibiotic produced by *Streptomyces roseosporus* (124). DAP targets the cell membrane (CM), with insertion occurring preferentially at the septal division plane, requiring the presence of calcium ions to form a tripartite complex between the antibiotic, lipid II and the membrane phospholipid phosphatidyl glycerol (PG) (125). This complex is then able to span the membrane, influenced by another phospholipid, cardiolipin (CL), and forms a 'pore', which causes membrane distortion and ion leakage, resulting in a loss of membrane potential and ultimately cell death.

Resistance against DAP among the enterococci occurred in 2005, two years following the introduction of DAP into the clinic. Since then, three genes have been identified as being involved in DAP resistance, with mutations occurring in a sequential order (126). The first mutation occurs in the gene *liaF*, which is part of the three-component system LiaFSR, involved in regulating the cell envelope and discussed later (*see section 1.4*). In *E. faecalis*, deletion of an isoleucine at position 177 in LiaF results in a four-fold increase in DAP resistance, from 1 to 4 µg/mL (established clinical resistance breakpoint is 4 µg/mL) (127,128). Following this mutation, amino acid changes are observed in two other genes, both involved in phospholipid metabolism: *gpdD*, which encodes a glycerol-phosphodiester phosphodiesterase, and *cls*, which encodes cardiolipin synthase (Cls) (126).

It is thought that mutations in the LiaFSR system result in the redistribution of CL microdomains away from the septum, enabling in the diversion of DAP away from the septal plane (129), however the mechanism for this remains unclear. Resistance is then increased by altering the composition of the CM: PG content is reduced by *gdpD*, preventing the oligomerization of DAP in the membrane, and CL microdomains potentially 'trap' DAP, preventing it from reaching the inner leaflet (125). In contrast to

E. faecalis, resistance of *S. aureus* to DAP occurs through increasing the electrostatic charge of the cell surface, leading to repulsion, rather than diversion of the antibiotic (129).

In conclusion, *E. faecalis* has developed a number of complex mechanisms in order to overcome antimicrobial challenge, ensuring its own survival. The induction of resistance genes in response to antimicrobial exposure requires the intricate organisation of multiple signal transductions pathways. This coordinated response can be carried out by multiple regulators, including via two-component systems (TCSs). Frequently associated with antimicrobial resistance, as described above, TCSs are tasked with sensing of a specific stimuli and activating a response, maintaining the integrity of the cellular envelope.

1.4 Two-Component Systems involved in the Cell Envelope Stress Response

The monitoring and adapting to ever-changing environmental conditions are an essential aspect of bacterial survival. This involves detecting the presence of a stimulus and the transduction of this signal to inside the cell, a function which can be carried out by bacterial TCSs. TCSs have evolved to allow cells to respond to a wide variety of stimuli including nutrient deprivation, antibiotic challenge, host-pathogen interaction, osmotic shock and other stresses (130). The systems generally utilise the same molecular mechanism for signal conversion. Upon detection of the correct stimulus, this triggers the autophosphorylation of a histidine kinase, responsible for signal recognition. Once activated, phosphotransfer can then occur with the cognate response regulator. Response regulators are in general transcriptional regulators, and phosphorylation enables them to enter an active conformation in which they can either activate or repress their target genes.

The sequencing of the *E. faecalis* reference genome V583 revealed the presence of 17 TCSs based on homology searches and the presence of one orphan response regulator. These systems each respond to their own individual stimuli and activate their own unique set of target genes. Many of these TCSs are involved in monitoring the integrity of the cell wall, responding to alterations or dysfunctions of the envelope, and activating the appropriate counter measures to repair the damage and restore homeostasis (131).

1.4.1 LiaFSR

The three-component LiaFSR (lipid-II–interacting antibiotics) system is highly conserved and widespread amongst the Firmicutes, including LiaFSR in *Bacillus subtilis* and *Enterococcus faecalis*, VraRS in *Staphylococcus aureus* and CesSR in *Lactococcus lactis* (131–133). Amongst the phylum, two distinct groups exist based on the genomic context of the operon (134,135). Group I LiaFSR homologues are found adjacent to the genes within their regulon and present homology across the entire locus, such as the *lialH-(G)FSR* locus in *Bacillus subtilis*. In contrast, group II only show homology of the *liaFSR* locus and lack the *lialH* genes, as observed in *E. faecalis*. This genomic difference also represents a variation in the cellular role of these systems; whereas group I regulates its own expression and *liaIH*, group II systems represent the primary system involved in the cell-envelope stress response, possessing a much larger and complex regulon.

The operon is comprised of three genes: LiaF, LiaS and LiaR. Like a traditional TCS, LiaS is a histidine kinase, which is responsible for phosphorylating the response regulator LiaR. However, the system also possesses an additional component, LiaF, a membrane bound repressor protein, which acts as an inhibitor of LiaS (134). The system is known for being a "damage sensor", able to respond to perturbations in cell wall integrity caused by antimicrobial damage (131). The exact stimulus, however, that removes the inhibition of LiaF and allows the activation of LiaS remains unknown. In group A *Streptococcus*, the colocalization of LiaF and LiaS was found to reside within a microdomain, with LiaF found to have a crucial role in microdomain integrity (136). Cell envelope stress induced by antimicrobials resulted in disruption of the microdomain and activation of the LiaFSR system. Interestingly, deletion of the membrane phospholipid cardiolipin resulted in the loss of LiaS localisation, leading to LiaR activation, but the localisation of LiaF remained unaffected. In contrast, other evidence in *B. subtilis* suggests that Lia is responding to changes in lipid carrier pool sizes (137,138). However, the exact stimulus by which Lia detects cell envelope damage remains unknown.

When examining the LiaFSR system in *E. faecalis*, work by the Arias group has established the regulon of LiaR, which includes the *liaXYZ* operon (139). The protein LiaX has been shown to sense antimicrobial molecules and regulate changes in the cell membrane phospholipid content, which has

been shown to be particularly important in the development of daptomycin resistance (140). Interestingly, the two domains of LiaX have been demonstrated to serve different functions. Whilst the N-domain serves as an antimicrobial sentinel, the C-domain has been proposed to inhibit LiaFSR, potentially through the binding of LiaF and LiaS (139). In contrast to *liaX*, the role of the additional genes in the operon, *liaY* and *liaZ* are less well characterised. LiaY encodes a transmembrane protein harbouring a PspC domain, potentially involved in cell envelope stress adaption (141), whilst LiaZ also encodes a transmembrane protein with homology to bacterial holins involved in response to phage-mediated membrane damage (142). Interestingly, despite being surface exposed, LiaX itself lacks a transmembrane domain, therefore, it has been speculated that as *liaX* and *liaY* encode transmembrane proteins that interact with LiaX to contribute to the downstream signalling cascade (139).

1.4.2 CroRS

The CroRS (ceftriaxone resistance operon) system is unique to the enterococci and found to play a crucial role in the intrinsic β-lactam resistance of *E. faecalis,* with deletion of the operon found to result in a ~4,000-fold increase in ceftriaxone susceptibility (97). In addition, the individual deletion of *croR* resulted in bacitracin and vancomycin sensitivity, and the loss of teixobactin tolerance, as discussed above, indicating a more general role for the system in the cell-envelope stress response (130). The regulon of CroRS, however, remains elusive. CroR has been shown to directly bind to three promoters: its own, the extracellular factor, *salB* and the glutamine importer, *glnQHMP*. Whilst SalB has been demonstrated to have a role in maintaining cell wall integrity (143), the role of *glnQHMP* in cephalosporin resistance remains unclear. Additionally, recent work by Muller. et al (2019) has demonstrated, through RNA sequencing analysis, that CroR has 219 genes in its regulon. But again, further investigation is required to establish which genes give rise to the resistance phenotype. A similar system has been identified in *Listeria monocytogenes*, CesRK, which was shown to have a similar inducer spectrum to CroRS and, likewise, deletion resulted in β-lactam sensitivity. CroS has also been demonstrated to be phosphorylated in an IreK-dependent manner in response to cephalosporin challenge (99), indicating the potential for crosstalk between the systems.

1.4.3 VicRK

Also known as WalRK or YycFG, VicRK is a highly conserved TCS, shown to be essential to many of the Firmicutes (144). However, despite this degree of conservation and essentiality, the relative regulons of VicRK amongst the phylum display a huge amount of variation. Generally, the system is involved in the regulation of cell wall homeostasis, monitoring of membrane integrity and cellular division (145). In contrast to the LiaFSR system, which responds to cellular damage, VicRK appears to respond to normal cell wall metabolism, induced under regular growing conditions in *B. subtilis* (146). In *E. faecalis*, the operon includes VicRK and three other genes: *yycH*, *yycI*, *yycJ* (147). Whereas YycH and YycI act as repressors of VicK function, YycJ shares homology to an enzyme super-family containing metallo-β-lactamases (148). VicRK is also one of the few TCS which has been shown to utilise crosstalk to other regulatory pathways, i.e. with the PhoPR phosphate limitation TCS in *B. subtilis* (149,150). This observation implies that cross-communication is possible between TCSs and suggests there may be some level of redundancy or compensation present, which requires further investigation.

1.4.4 SapRS

The EF0926/27 TCS, which we have renamed SapRS (Sensor of antimicrobial peptides), was originally identified by Hancock and Perego in 2002 (130) via the sequencing of the *E. faecalis* V583 reference genome, although they were unable to identify a function for the operon. In 2014, work by Gebhard *et al.* classified SapRS as a BceRS-type TCS (151), a group of systems which regulate the expression of neighbouring BceAB-type ABC transporters (*discussed further in section 1.5*). These BceRS-type TCSs not only regulate their respective ABC-transporters, but they also rely on them for activation and sensing of their substrate (*discussed further in section 1.5*). SapRS is now known to be part of a bacitracin 'resistance module', which is discussed further below.

1.5 The Bacitracin Resistance Module

As discussed above, bacteria have developed numerous mechanisms by which to evade antimicrobial damage (*See section 1.3*). One mechanism includes the expression of specific ATP-binding cassette (ABC) transporters. One such group is the BceAB-type transporters, comprised of two proteins; one ATPase and one permease (152–154), the best understood example of which is BceAB in *B. subtilis*

(155). These transporters usually control the expression of BceRS-type TCS, which together form a 'resistance module' (155). Not only does the transporter provide the resistance aspect of these modules, but it also acts as the sensor for the activation of the TCS, as the sensor kinase alone cannot detect the substrate peptide (156). Without the presence of the substrate, the transporter maintains the sensor kinase of the system in an 'OFF' conformation, preventing its autophosphorylation (156).

Signalling within the Bce system in *B. subtilis* has been shown to be triggered by the transporter itself via a flux-sensing strategy, whereby BceAB is the sensor of the system and BceRS activity is determined by the rate of flux through the individual BceAB transporter. In this scenario, increasing BceAB expression reduces the load experienced by each transporter, resulting the negative feedback on its own expression via reducing BceS signalling. This mechanism means that signalling is directly proportional to transporter activity and the transporter has total control over the kinase activity, to autoregulate its own production through the TCS (137).

BceAB has recently been shown to bind bacitracin, which now suggests that the transporter is able to directly interact with the antimicrobial or at least the antimicrobial is part of the substrate the transporter recognises (157). The recognition of this complex demonstrates that this system uses a vacuum cleaner model, whereby BceAB recognises the UPP-bound-antimicrobial complex, and separates UPP from the antimicrobial and releases it into the extracellular space, essentially expelling the antimicrobial peptide via 'transport' by utilising ATP (157). Importantly, the type of antibiotics BceAB-type transporters respond to 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) (154). Overall, BceAB provides target protection to UPP by dislodging the antimicrobial from this cellular target.

In response to bacitracin, *B. subtilis* has been shown to upregulate the expression of three resistance modules: The BceAB system described above, the UPP phosphatase BcrC (*see section 1.3*), and the phage shock protein (Psp)-like LiaI and LiaH proteins. In contrast to the BceAB system described previously, which actively removes bacitracin from UPP, the phosphatase BcrC protects against bacitracin challenge by catalysing the dephosphorylation of UPP to UP, promoting the progression of

the lipid II cycle (158). The third system involves the *liaIH* operon, which is induced upon bacitracin damage by the TCS LiaFSR. The small membrane anchored protein LiaI recruits LiaH into static membrane-associated patches (134,159), however the role these patches play in protecting the cell remains unclear. Interestingly, it has been found that a hierarchy exists between these primary drug-sensing and secondary damage-sensing modules, whereby increased expression of the primary system, BceAB, reduced expression of the secondary layers, BcrC and LiaIH. Interestingly, in the absence of BceAB, BcrC demonstrated elevated expression, and upon the removal of both BceAB and BcrC, the increased expression of LiaIH was observed. This was termed 'active redundancy', where the absence of a primary system was compensated for by the increased expression of a secondary module (137).

The Bce system in *B. subtilis* is a self-contained module, involving only a single transporter and TCS with no known further genes. In contrast, the equivalent module in *E. faecalis* is comprised of a single BceRS-like TCS, but two BceAB-like transporters. The TCS involved in this bacitracin resistance is SapRS, identified in our previous work (151). SapRS is comprised of the histidine kinase SapS and the response regulator SapR. The Bce-like ABC transporters involved in the module are SapAB (EF2752/51) and RapAB (Resistance of Antimicrobial Peptides) (EF2050/49), which based on the investigations of BceAB, implement a 'flux-sensing' mechanism to regulate bacitracin resistance (151,160). SapAB so far has been shown to be activated by only two antibiotics, bacitracin and mersacidin (151), both of which bind to intermediates of the lipid II cycle, preventing peptidoglycan synthesis .

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 (113,161,162). The ABC transporter SapAB is potentially coupled with SapRS, with the four proteins forming a sensory complex, likewise to BceAB-RS (152). The constitutive formation of this complex allows the transporter to exert complete control its cognate kinase, maintaining SapS in an 'OFF' state. Upon detection of the bacitracin-UPP (BAC-UPP) complex, SapAB elicits a conformational change in SapS, resulting in kinase autophosphorylation (156). SapS can then phosphorylate SapR, which in turn, induces the expression of the resistance transporter RapAB. RapAB

can then break the interaction between bacitracin and UPP using ATP, releasing bacitracin back into the extracellular space and UPP can continue the cell wall cycle, rendering the cell resistant to bacitracin. However, within this system setup, a few questions remain unanswered:

a) Unlike BceRS, which is not regulated, SapRS, is under regulation. However, unlike other TCSs such as CroRS and LiaFSR, it is not under its own autoregulation, as its deletion still results in the activation of its own promoter (151). Therefore, this implies that transcription of *sapRS* is controlled from outside of the module, but regulator responsible for this remains unknown.

b) In *B. subtilis*, BceRS only controls the expression of *bceAB*. The binding site for SapR has been identified in the promoters of *sapAB* and *rapAB* based on similarity to the consensus binding site computationally characterised from the binding site in the *bceAB* promoter. However, unlike BceR, this site has also been identified in the promoter of *dltABCD*. The potential regulation of *dltABCD* by SapR however has not been investigated.

c) The resistance modules of *B. subtilis*, i.e. BceAB, BcrC and LiaIH, have been shown to display 'active redundancy' whereby the loss of a primary resistance mechanism can be compensated for by the increased expression of a secondary module. As similar modules exist in *E. faecalis*, such as the Sap and Lia systems, the same level of redundancy may potentially exist between the systems. This again however, remains unexplored.

d) In *B. subtilis* and *S. aureus*, the regulatory setup of the CESR in response to cell stress and antimicrobial challenge is well characterised. However, in *E. faecalis*, the picture is not quite so clear. This is in part due to the complexity of the network and the presence of an additional TCS unique to the enterococci, CroRS. Despite being named for its role in cephalosporin resistance, the genes responsible for the phenotype remain unclear, and there is a general lack of understanding of the role CroRS plays in

monitoring the integrity of the cell envelope. Therefore, an improvement in the understanding of the individual components and the CESR system overall is required.

1.6 Aims and Objectives

Enterococcus faecalis has become an infamous nosocomial pathogen, affecting people of all ages, and causing infections worldwide. As observed with many bacteria, there is a rising issue with treating these enterococcal infections due to the overuse of antibiotics and an ever-increasing level of antimicrobial resistance, leading to an increase in both morbidity and mortality. Whilst we have gained a deep level of understanding of the mechanisms involved in acquired resistances generated through plasmid acquisition i.e., vancomycin, our knowledge of the intrinsic resistance pathways within *E. faecalis* remains incomplete.

Whilst the Firmicutes maintain the same components to monitor their cell envelopes, such as LiaFSR and VicRK, they do not necessarily perform the same functions in differing bacteria. This demonstrates that evolution has taken these TCSs and other regulators down different routes to fit the specific needs of an individual bacterium. Therefore, despite knowing the role of these regulators in model organisms such as *B. subtilis*, this knowledge cannot be applied directly to other bacteria, such as the pathogens *S. aureus* and *E. faecalis*.

Previous work has identified a bacitracin resistance module within *B. subtilis*, comprised of a single ABC transporter, BceAB and TCS, BceRS. This work also led to the identification of a similar system setup in *E. faecalis*. However, in contrast to *B. subtilis*, *E. faecalis* possesses two BceAB-type ABC transporters, the sensor transporter SapAB and the resistance transporter, RapAB, in addition to the TCS, SapRS. Utilising this previous work as a foundation, we can gain a better understanding of not only this bacitracin network, but also gain an appreciation at a systems level of the response of the cell to antimicrobial stress and cell wall damage.

The aims of this work were to deepen our knowledge of the *E. faecalis* cell envelope stress response, to gain an understanding of the unique and complex setup of the system. Through this understanding, we would be able to appreciate both the signalling and potential compensation abilities of the antimicrobial

resistance pathways. Given the diminishing antimicrobial pipeline, it is crucial to develop a detailed understanding of how bacteria survive antimicrobial treatment as a means of identifying an Achilles' heel within the network, allowing both new strategies to be developed, and the repurposing of existing antimicrobials more effectively.

The aims were addressed in two sub-projects; the first, to expand our knowledge of the Sap and Lia systems, and the second, to gain an understanding of the CroRS system. Each aim is described in one of the following results chapters:

- Chapter 2 (Part A) expands our knowledge of the *E. faecalis* resistance module and describes a direct functional link between the Lia and Sap systems. We demonstrate the existence of a logic 'AND' gate whereby both antibiotic-induced cellular damage (LiaFSR) and the presence of a substrate drug for the network's sensory transporters (SapAB) are required to trigger target genes the SapR regulon.
- Chapter 2 (Part B) builds on the work performed in part A but does not contribute to the manuscript, through investigating the use of the Tobacco Hornworm (*Manduca sexta*) as an *E. faecalis* model host and adapting a CRISPR-Cas9 system for the creation of genetic mutations in *E. faecalis*.
- Chapter 3 expands our knowledge of the CroRS regulon through transcriptomic analysis and evolution experimentation. We demonstrate an important role for CroRS in the regulation of isoprenoid production and ultimately in cell wall biosynthesis.
- Chapter 4 builds on the work from the third chapter, by exploring the genes within the CroRS regulon responsible for β-lactam resistance. We observe that restoring adaptation to ampicillin in the absence of *croRS* results in suppressor mutations in genes involved in c-di-AMP production and propose a link between CroRS and c-di-AMP.

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This declaration concerns the article entitled:

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

Publication status (tick one)

Draft manuscript	X	Submitted		In review		Accepted		Published	

Publication details (reference)	

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

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Running title: Antibiotic resistance logic in *Enterococcus faecalis*

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

Data accessibility statement: The data that support the findings of this study are available in the supplementary material

2.1 Abstract

Enterococcal infections have become a significant public health burden, aggravated by high levels of acquired and intrinsic antibiotic resistance, including to cell envelope-acting antibiotics like daptomycin (DAP). While considerable efforts have been dedicated to understanding the mechanisms of resistance, far less is known about how the expression of such resistance genes is controlled in enterococci. Previous work unveiled a complex 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 fulfilled by a second TCS, LiaFSR. We therefore aimed to explore the interplay between these two regulatory pathways. Utilising transcriptional reporter gene fusions and gene deletion strains, we demonstrate the regulation of an additional resistance operon, *dltXABCD*, by SapR and show that LiaFSR regulates the expression of *sapRS*, placing SapRS target genes under dual control. Our results show that this strategy effectively implements a logic 'AND' gate, requiring both antibiotic-induced cellular damage (LiaFSR) and the presence of a substrate drug for the network's sensory transporters (SapAB) to trigger *dltXABCD* expression. Deferred antagonism assays showed that this network contributes to protecting *E. faecalis* from antimicrobials produced by potential competitor bacteria in relevant habitats, providing a potential rationale for the evolution of this regulatory strategy. The network structure described here offers an explanation for why DAP resistance can often emerge via mutations in regulatory components, which may ultimately lead to the discovery of new therapeutic targets.

2.2 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[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 determinant of cephalosporin resistance[28], whereas the VicKR (YycFG) system is essential across the low-CG Gram positives and is involved in 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 involved in 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, and propose how this can explain the occurrence of mutations in both regulatory systems during clinical and experimental emergence of DAP resistance in enterococci.

2.3 Materials and Methods

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) broth 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[45]. *E. coli* was transformed by heat-shock of $CaCl_2$ competent cells, followed by 1 hour recovery time[46]. 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 uL 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[47]. All fragments were cloned via the EcoRI and BamHI sites of the vector. The primers used are given in Tables S2 in the supplementary material.

Constructs for unmarked deletions in *E. faecalis* were cloned into pLT06[48]. 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[49] and were subsequently cloned into the NcoI and BamHI site of the vector pLT06. Gene deletions were performed as previously described[48]. 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[50] 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)[51]. 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], 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 [UPP-bac], and the effective activites 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

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

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

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

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

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

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.

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 the figure shown in Results.

Antagonism assays on solid media. A plate-based assay was utilised to measure growth inhibition between antibiotic producer strains and *E. faecalis* strains. Cultures of the antimicrobial-producing bacteria were grown overnight in the respective growth medium and temperatures stated above for each species 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 uL 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.

2.4 Results

2.4.1 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 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.*

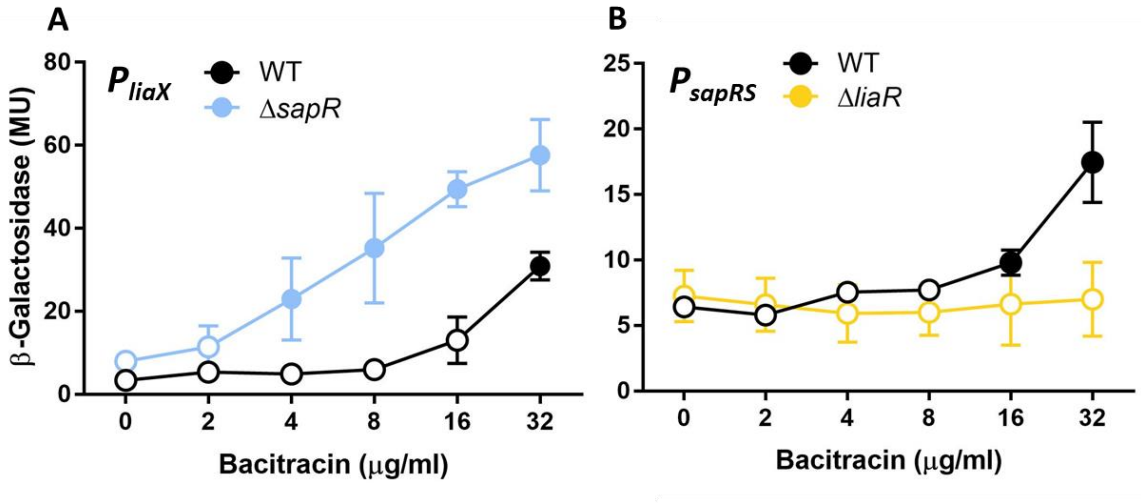

Figure 1. The LiaFSR operon responds to bacitracin exposure and induces the expression of both *liaXYZ* and *sapRS*, with the absence of *sapRS* resulting in the hypersensitivity of the *liaXYZ* promoter. Cells of *E. faecalis* JH2-2 harbouring a P_{liaX}-*lacZ* (panel A) or P_{sapRS}-*lacZ* (panel B) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. Beta-galactosidase activity, expressed as Miller units (MU), was assayed following 1h incubation. Results are means and standard deviations for three biological repeats. The significance of induction relative to untreated cells was calculated for each strain using a two-way ANOVA with a Sidak's post-hoc test. Significance is indicated by a filled symbol ($p < 0.05$); unfilled symbols represent no significant differences 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 *sapRS* 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}, corresponding to the MIC of the wild-type strain[33]. 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[52]. 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*[52], as well as physiological links between the SapRS- and LiaFSR-dependent components of the CESR in *E. faecalis*.

2.4.2 SapRS controls the expression of the *dltABCD* operon

To expand our understanding of the Lia/Sap regulatory network, we next wanted to consider other potential genes 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 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[53], a mechanism commonly involved in DAP resistance amongst the low-CG bacteria[54].

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

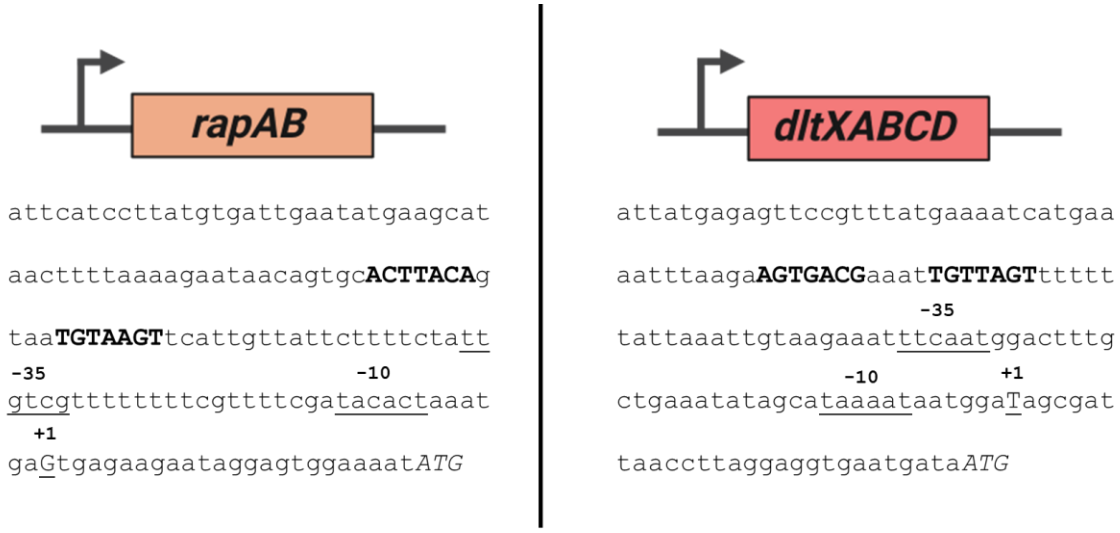

Figure 2. Schematic of the promoter regions of both *rapAB* and *dltABCD*. The sequence 150 base pairs upstream of the ATG start codons are shown. The proposed binding site for SapR on both the *rapAB* and *dltABCD* promoters is in bold and capitalised, and the likely -10 and -35 elements are underlined. The experimentally confirmed transcriptional start sites are capitalised and underlined (+1) and the translational start site is shown in italicised capitals[33,86].

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

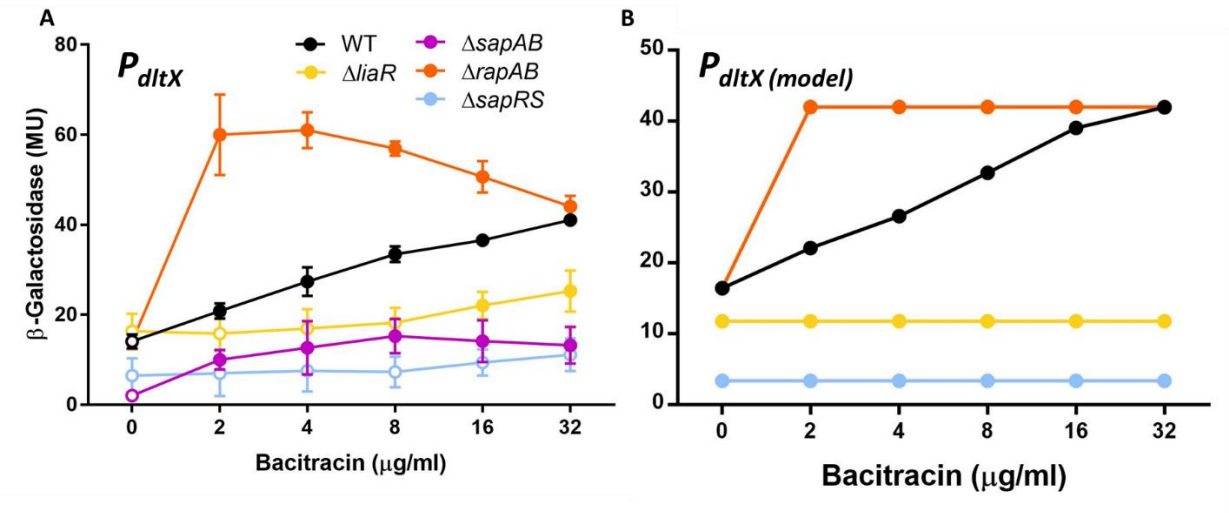

Figure 3. Induction of the resistance operon *dltXABCD* by bacitracin. Panel A indicates the experimental induction of *dltX*. Cells of *E. faecalis* harbouring a *PdltX-lacZ* transcriptional fusion were grown to exponential phase and challenged with increasing concentrations of bacitracin. Beta-galactosidase 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 a two-way ANOVA with a Sidak's post-hoc test. Significance is indicated by a filled symbol ($P < 0.05$), unfilled symbols represent no significant differences to uninduced conditions. Panel B indicates the mathematical model of *dltX* induction in the *E. faecalis* strains indicated in panel A.

As we had shown above that LiaR regulates *sapRS* expression, we next aimed to find out if deletion of *liaR* also influenced *dlt* expression. When we tested the response of the *dltX* promoter fusion to bacitracin in a strain carrying deletion of *liaR*, we observed that deletion of *liaR* resulted in a noticeably weaker amplitude of *dlt* expression in response to bacitracin, being ~2-fold lower than the wild-type strain at 32 μg ml^{-1} (Fig. 3A, yellow line). In addition, the sensitivity of the *dlt* promoter response was 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 (Fig 4). 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 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, 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. 3, 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.

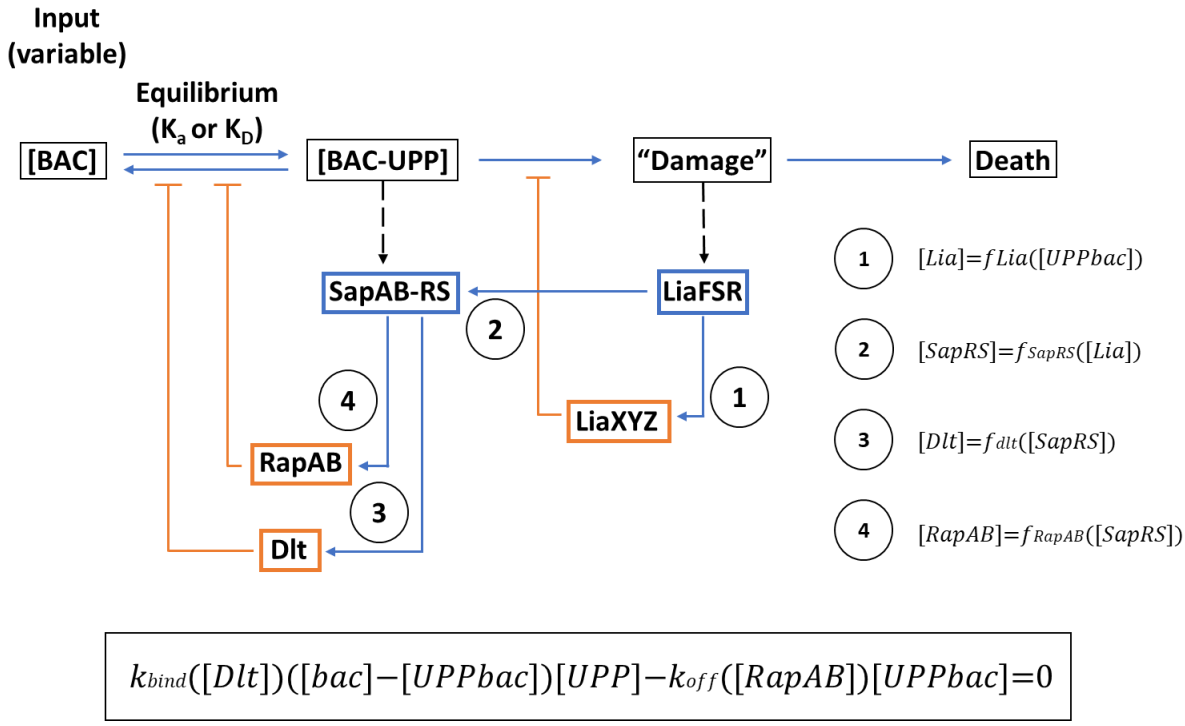

Figure 4. Mathematical reconstruction of the regulatory pathway based on the flux-sensing model previously described for the BceRS-BceAB system. The equations corresponding to the effective activities in the model are numbered, with sensory systems shown in blue and the effector systems shown in orange. As an input, the model considers bacitracin binding to UPP to form UPP-bacitracin (BAC-UPP) complexes, with the rate dependent on bacitracin concentration (BAC). As a result of the "damage" caused by these complexes, LiaFSR positively regulates the expression of *liaXYZ*, which has been modelled as a negative modifier of [BAC-UPP] related damage (1). Simultaneously, in response to this "damage", LiaFSR positively regulates the production of SapAB-RS, modelled as an amplification of the effect on *rapAB* and *dlt* (2). The production of *rapAB* reduces the formation of UPP-BAC complexes, increasing the OFF-rate of BAC binding to UPP (4), whilst *dlt* decreases the ON-rate of BAC binding (3). These quantities are then used to solve for the equation shown (black box).

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 correct and that no further major players needed to be considered to explain the behaviour of the *dltX* target promoter.

2.4.3 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). DAP exposure activates the Lia system by causing damage to the cell envelope[55,56]. 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]. The tripartite complex of DAP with Lipid II and phosphatidyl glycerol in the membrane[57] 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.

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. 5A). 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.

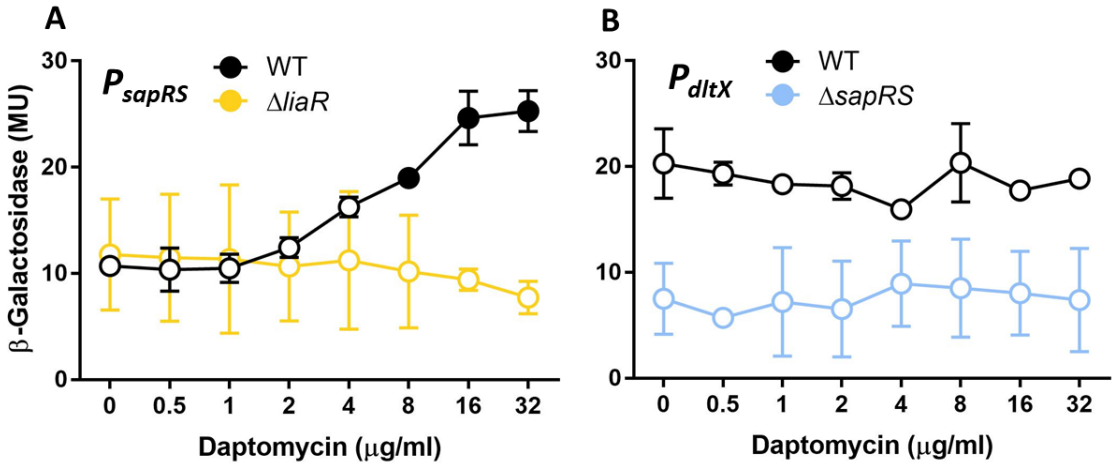

Figure 5. Exposure to daptomycin results in the induction of *sapRS* but does not induce the expression of *dltXABCD*. Cells of *E. faecalis* harbouring a P_{sapRS}-*lacZ* (panel A) or a P_{dltX}-*lacZ* (panel B) transcriptional fusion were grown to exponential phase and challenged with increasing concentrations 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 three biological repeats. The significance of induction relative to untreated cells was calculated for each strain by a two-way ANOVA with a Sidak's post-hoc test. Significance is indicated by a filled symbol ($P < 0.05$), unfilled symbols represent no significant differences to uninduced conditions.

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. 5B). 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: bacitracin or daptomycin. The mechanism by which we propose this difference arises is explained in the discussion.

2.4.4. 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 begin 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[58–61]. 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[62], bacitracin producer *Bacillus lichenformis* ATCC10716[63], nisin-A producer *Lactococcus lactis* NZ9000[64] and nisin-P producer *Streptococcus gallolyticus* AB39[65]. 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 (Fig. 6).

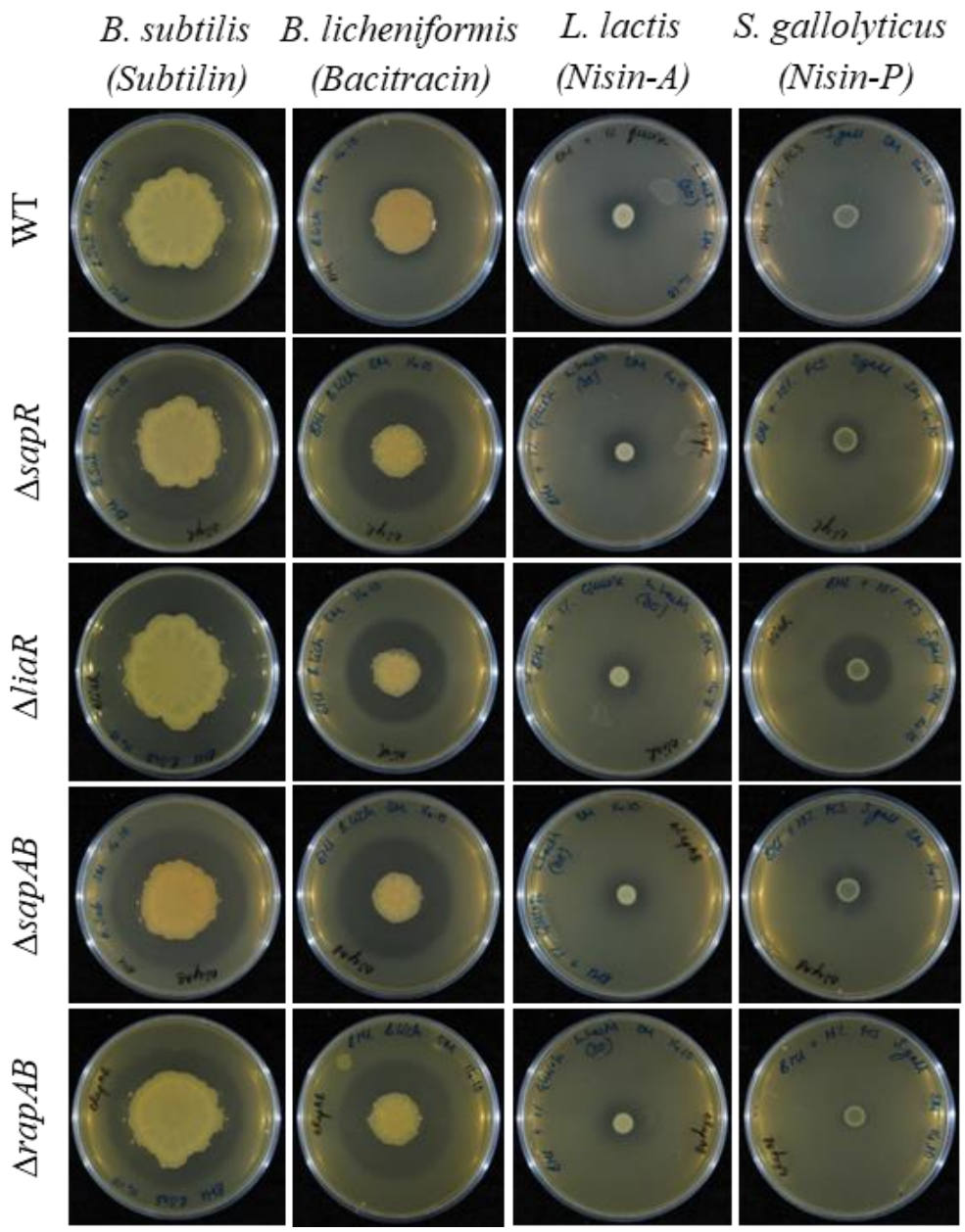

Figure 6. ***E. faecalis*** **deletion strains show differential zones of inhibition in deferred antagonism tests against antimicrobials produced by other firmicute bacteria.** The antimicrobial producer strains indicated at the top were grown overnight and then adjusted to an OD_{600} of 0.5 with fresh 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.

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[33]. 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.

2.5 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[66,67]. In contrast, daptomycin is a lipopeptide antibiotic which forms a tripartite complex between lipid II and the membrane phospholipid phosphatidyl glycerol[57]. 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 (Fig. 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[35,68,69]. 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)[69,70]. 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. It therefore does not form the type of complexes that could act as substrate for SapAB, 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 LiaFRS system activates expression of the *sapRS* operon, no induction of *dlt* expression was observed, because SapAB 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 (Fig. 7). 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].

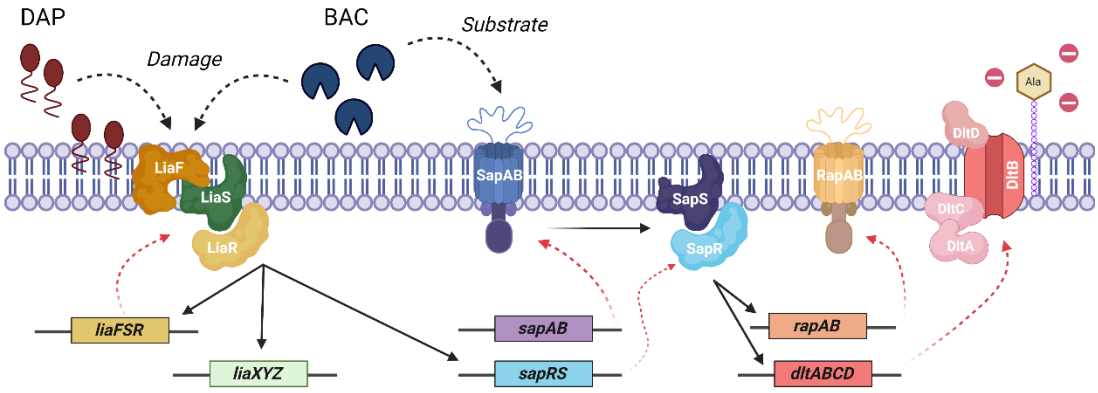

Figure 7. Proposed model of the antibiotic resistance network. Schematic illustrating the genes and proteins involved in antibiotic detection and resistance, with regulation and translation demonstrated by black and red arrows respectively. Upon exposure to bacitracin (BAC), this causes damage to the cell envelope, which acts as the stimulus for the removal of repression by LiaF, triggering the activation of LiaS. LiaS can then phosphorylate LiaR, which can in turn induce the expression of its target promoters; its own, *liaXYZ*, *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, which then phosphorylates SapR. SapR is then able to enter an active conformation and induce the expression of the resistance genes, *rapAB* and *dlt*, which remove bacitracin from UPP and reduce the positive charge of the cell wall respectively, resulting in bacitracin resistance. Conversely, in response daptomycin (DAP), this 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*.

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. For example, in *B. subtilis* LiaFSR controls its own regulon, comprised solely of *liaHI(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[71] but has not been

observed to regulate a BceRS-type TCS. Bce-type systems in most Firmicutes 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[72] and GraRS-VraFG[73] systems in *S. aureus*. In some cases, Bce-type systems can also control additional genes, for example the GraRS TCS has also been shown to regulate the *dlt* operon[74–76]. However, to our knowledge there have been no prior reports of direct regulatory relationships that hardwire the Lia and Bce-type pathways together.

Our findings reported here show that although the enterococci possess the same regulators as *S. aureus* and *B. subtilis*, evolution has taken these components down different routes. A potential reasoning behind *E. faecalis* maintaining this intricate setup could be that having *dlt* and *rapAB* under Sap control, 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*[39,52]. 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 and when 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 controlled by either the Lia or Sap systems. Overall, it appears that the regulatory network studied here plays a key role in protecting *E. faecalis* from antimicrobial peptide producing bacteria that it would realistically encounter in its natural habitats, offering an explanation for the existence of such a complex regulatory strategy.

One of our most striking results was that daptomycin challenge, although able to activate Lia signalling, was unable to activate the Sap system, potential mechanistic reasons for which are explained above. This was a surprising finding, as *dlt* is known to respond to daptomycin in other Firmicutes, e.g. *S. aureus* [77–81]. The lack of *dlt* expression in response to DAP may, however, explain the sensitivity to this antibiotic observed throughout the *Enterococcus* genus and its effectiveness when treating VRE infections[82]. This is despite the bacteria being in possession of a clearly rather effective resistance mechanism, except this is not induced upon DAP challenge. It is then plausible to see why clinical DAP resistance could result from overriding of the molecular controls investigated here to trigger high *dlt* operon expression, specifically via mutations in genes encoding the components of the LiaFSR system[19], or experimentally in *sapAB* as suppressor mutations of a LiaR-deficient strain[41,42]. DAP was introduced for treatment of enterococci infections in 2003[12] and the first resistant isolates were identified already in 2005[13]. It may be that the rapid emergence of DAP resistance is in part derived from a dysregulation of *dlt* expression by such mutations of regulatory components.

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

2.6 Acknowledgements

This work was supported in part by grant MR/N0137941/1 for the GW4 BIOMED MRC DTP, awarded to the Universities of Bath, Bristol, Cardiff and Exeter from the Medical Research Council (MRC)/UKRI. The authors gratefully acknowledge the Technical Staff within the Department of Biology and Biochemistry at the University of Bath for technical support and assistance in this work. We would also like to thank Mathew Upton for the gift of *Streptococcus gallolyticus*.

2.7 Supplementary

S1. Vectors, plasmids and strains utilised in this study.

Vectors	**Description**	**Source**
pTCVlac	*E.coli*-Gram positive shuttle vector for transcriptional fusions to lacZ; erm^r, kan^r	[47]
pLT06	Vector for creation of unmarked deletions in *E. faecalis*: cm^r	[48]

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
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*	[83]
MC1061	*F- Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 λ- e14-*	[84]

	mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2(r-m+)	

Bacterial Strains - *E. faecalis*	**Description**	**Source**
JH2-2	Laboratory strain, plasmid-free; rif^r; fs^r	[85]
Δ*rapAB*	JH2-2 carrying an unmarked deletion of the operon	[33]
Δ*sapAB*	JH2-2 carrying an unmarked deletion of the *sapAB* operon	[33]
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
SGF17	Δ*sapRS* carrying pSMMTCV01; kan^r	This study
SGF34	Δ*sapRS* carrying pSMMTCV05; kan^r	This study
SGF04	Δ*rapAB* carrying pSMMTCV01; kan^r	This study
SGF05	Δ*sapAB* carrying pSMMTCV01; kan^r	This study
SGF18	Δ*liaR* carrying pSMMTCV01; kan^r	This study
SGF25	Δ*liaR* carrying pSMMTCV02; kan^r	This study

S2. Primers utilised 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

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

*liaR*_KO_Fwd_BF	AATTTgaattcACGGTAATTATGAAAGTCCAG	Used to amplify 800-1000 bases upstream of the *liaR* start codon
*liaR*_KO_Rev_BF	AATTT**CTGATATTTAC**ATTACTTTGATCACTTGCAG	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**CAAAGTAATGT**AAATATCAGATAAATCC	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**GGATTGCATGT**AAAAAGTAGGTCTATCAGC	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*

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3. Chapter 2: Part B

3.1 Introduction

This appendix includes experimentation related to the first chapter but did not contribute to the manuscript. This includes utilisation of the *Manduca sexta* caterpillar for colonisation studies and the adaptation of a CRISPR-Cas9 system to create *E. faecalis* mutations.

3.1.1 Use of *Manduca sexta* to investigate *E. faecalis* gut colonisation

Enterococcus faecalis and *Enterococcus faecium* are common members of the gastrointestinal tract. However, both these enterococci can cause a number of infections, including of the urinary tract, wounds and heart valves, and are two of the most common causes of bloodstream infections, surpassed only by the staphylococci [1]. Due to the growing issue of antimicrobial resistance, these infections are rapidly becoming much more difficult to treat. Therefore, it has become imperative an understanding of the enterococci's ability to colonise is reached. In order to better understand this, both vertebrate and invertebrate models have been utilised [2]. Whilst mammalian models come with ethical issues, the usage of invertebrates such as the roundworm *Caenorhabditis elegans* and wax moth *Galleria mellonella* have gained popularity. This also includes the Tobacco Hornworm moth *Manduca sexta*, as a novel host model [3].

Tobacco Hornworms are most common in the southern United States, where they feed on plants and are considered pests [3]. They have been utilised as an insect model for several decades, giving important insights into research on hormonal regulation of development, metamorphosis, nicotine resistance, antimicrobial defences and bacterial colonisations - proving to be a versatile host model [4–6]. They have many advantages over other invertebrate models for microbiology research, such as the ability to be maintained at 37°, an available genome sequence and a sterile gastrointestinal tract [3]. The caterpillars also lack an adaptive immune system, providing an advantage over the common mouse model. This allows the uninterrupted study of the innate immunity and its protective antimicrobials.

In the manuscript, we identified a number of genes involved in antimicrobial resistance (AMR), namely, *sapRS, liaFSR, sapAB, rapAB* and *dltABCD.* Since 2014 and work by Mckelvery et al. in *Salmonella enteritidis*, it has been recognised that AMR genes may aid colonisation of the gastrointestinal tract [7]. It was found that deletion of genes related to AMR resulted in a significant decrease in colonisation compared to wild type and concluded that the mutation of specific AMR genes resulted in alterations to the cell membrane [7]. Work in *E. faecalis* has also suggested that AMR genes affect the integrity of the cell envelope and are key for colonisation [8–10]. Therefore, in this work, we aimed to optimise the use of the Tobacco Hornworm to investigate the contribution of the AMR genes we have identified on the colonisation ability of *E. faecalis*. To this end, we utilised a range of different sampling techniques (faecal or haemocoel collection) and alteration of the *E. faecalis* inoculation concentrations. Our results indicated difficulties in obtaining colonisation at the initial inoculum of 10^8 CFU across all three collection types. However, upon increasing the concentration to 10^9 CFU, we were able to detect the colonisation of wild type *E. faecalis* when obtained from haemocoel collection. This work demonstrates the suitability of *Manduca sexta* as a host model for studying *E. faecalis* colonisation.

3.1.2 Use of a novel CRISPR-Cas9 system to create genetic changes in *E. faecalis*

As both *Enterococcus faecalis* and *Enterococcus faecium* are important nosocomial pathogens, understanding the molecular mechanisms that underpin their antimicrobial resistance is critical in the development of treatment strategies. However, currently methods for genome editing in *E. faecalis* are sorely lacking, relying on allelic exchange and the successfully recombination of a temperature-sensitive vector. Not only is this a time-consuming process, which takes up to four weeks, it also yields a poor success rate. The practice first requires the successful cloning of the desired mutation into the temperature-sensitive plasmid pLT06[11]. Upon the transformation of *E. faecalis*, the process then involves antibiotic selection, screening for a single recombination event, the curing of the plasmid and screening for a second recombination event, resulting in a successful genome edit. To expand the genetic toolbox for the enterococci, a type II clustered regularly interspaced palindromic repeats (CRISPR) and its associated Cas9 protein have been developed to make genetic edits in *E. faecium* [12].

In order to generate mutations, the CRISPR targets a specific region of DNA, and double stranded breaks are introduced by the Cas9 protein. This acts as a form of defence for the bacteria against invading bacteriophages [13]. There are three distinct stages of this defence[14]:

i. **Adaptation**: When a virus invades a microbe, it inserts its DNA into the bacterial cell. A short DNA sequence is excised from this intruding DNA, known as a "spacer". This capture is performed by a bacterial CRISPR-associated endonuclease (Cas) protein, which will then incorporate the DNA into a specific site on the bacterial genome, known as a CRISPR locus. The insertion of multiple spacers enables the creation of a CRISPR array, which is a series of spacers between repeat sequences.

ii. **crRNA Processing**: The CRISPR array is transcribed into a crispr RNA (crRNA), which is a 17-20 nucleotide sequence complementary to the target DNA. In type II CRISPR-Cas systems, the maturation of the crRNA requires RNase III. The crRNA then hybridises with the tracrRNA, which serves as a binding scaffold for the Cas protein, to produce the guide RNA (gRNA). The Cas protein and gRNA together form the ribonucleoprotein complex.

iii. **Interference**: As the Cas is a non-specific nuclease, it is directed to a specific DNA locus by the guide RNA (gRNA), where it can make a double stranded break. The presence of a protospacer adjacent motif (PAM) in the genomic DNA is required for the binding and catalytic activity of the Cas protein. The site also allows the CRISPR-Cas to distinguish between self and foreign DNA. Upon secondary exposure to the virus, the ribonucleoprotein complex will be able to recognise the foreign sequence, enabling cleavage and degradation of the viral DNA.

The utilisation of CRISPR-Cas9 systems to generate mutations has been successfully demonstrated in eukaryotes[15] where the double-stranded break is repaired by non-homologous end joining (NHEJ). In contrast, no evidence of a NHEJ system has been found within *Enterococcus*, therefore, to survive the lethal effects of the CRISPR-Cas9 break, the enterococci rely on homologous recombination (HR) [16]. To exploit this, Maat et al. developed an approach to alter the genotype of *E. faecium* by introducing a vector, VDM1001, that contains both a protospacer, which targets a specific region of DNA, and the desired mutation flanked by the identical regions of DNA on the chromosome. Simultaneously, the

vector VPL3004 from *L. lactis* which carries the CRISPR-Cap9 machinery is introduced into the cell. Therefore, following the CRISPR-Cas9 induced DNA break, the cells can undertake a HR event, incorporating the mutation and escaping the CRISPR-Cas9 induced killing[17].

In this work, we aimed to adapt this system for use in *E. faecalis* to generate two individual mutations: the deletion of the resistance gene *dltA* and the creation of a point mutation in the response regulator *liaR* to replace the aspartic acid at position 191 with asparagine (D191N). We intended to delete *dltA* to disrupt the operon to confirm our findings regarding its regulation by *sapR*, examine any compensation with *rapAB* and analyse the feedback mechanisms via its absence. In contrast, the point mutation we aimed to create in *liaR* is known to cause the hyperactivation of the protein and has been found in clinically associated daptomycin resistant enterococci [18]. We intended to hyperactivate LiaR to confirm it regulates the transcription of *sapR*, test its effect on *dlt* expression and additionally investigate the effect of a clinical mutation on this regulatory pathway. We discovered, however, that despite being successful in creating genetic changes in *E. faecium*, the CRISPR-Cas9 system described here was unsuccessful in producing mutations in *E. faecalis* and will require further optimisation.

3.2 Methods and Materials

3.2.1 *Manduca sexta* Experimentation

Origin of the University of Bath colony of *Manduca sexta.* The University of Bath colony has been in continuous culture since 1978. Originally derived from Truman-Riddiford laboratories at the University of Washington, Seattle, the genetic stock has been without the addition of animals from elsewhere, to prevent the addition of genetic diversity into the colony. The animals date back to the ones originally collected in North Carolina 1976[19].

Caterpillar maintenance and *E. faecalis* culture conditions. *M. sexta* caterpillars were reared to the fifth larval instar under standardised conditions. They were maintained in 125 mL disposable cups on a wheat germ-based diet (table 1) at a constant temperature of 25°C with 50% humidity and 12 hours light/dark cycles [3]. Three days prior to *E. faecalis* inoculation, animals were shifted to a chlortetracycline, formaldehyde-free diet (table 1).

Table 1. Premix and diet cake recipes.

Premix		Diet Cake	
Amount	Ingredient	Amount	Ingredient
2.7kg	Wheatgerm	336g	Premix
1.26kg	Casein	1,770mL	Distilled water
1.08kg	Sucrose	22.5g	Agar
540g	Dried active yeast	4mL	Corn oil
360g	Wesson's salt mixture	4mL	Linseed oil
36g	Choline chloride	8mL	4% Formaldehyde
72g	Cholesterol	0.2g	Chlortetracycline
36g	Methyl paraben	0.2g	Vanderssant vitamins
54g	Sorbic acid	8g	Ascorbic acid

For inoculation, *E. faecalis* was grown overnight in 5 mL BHI medium. The OD_{600} of the culture was measured, followed by centrifugation at 5000 × *g* at 4°C for 10 minutes. The cells were then washed in 1 volume 1× phosphate buffered saline, (PBS, 137 mM NaCl. 2.7 mM KCl. 4.3 mM Na_2HPO) and resuspended to the required concentration to be fed to the animals. To determine the concentration of cells, *E. faecalis* optical densities were adjusted according to a standard determined by previous investigation using the V583 strain. The standard was determined by comparing the OD_{600} values and colony numbers of V583 following plating, to calculate the number of viable colonies per OD_{600}. The resulting standard used here was 0.6 x 10^9 CFU/ml per 1 OD_{600}. This was then used to calculate the volume of PBS required to produce the CFU/mL indicated. To verify the cells were at the required concentration, 100 uL of the solution was serially diluted and plated on to bile-esculin agar and colonies quantified after 24 hours.

Inoculation and treatment of the *Manduca* and sample collection. Suspensions of either *E. faecalis* laboratory strain JH2-2 or 0.85% sterile saline were washed and adjusted to the CFU/mL indicated. Following this, 10uL of suspension was fed directly into each caterpillar's mouth using a P10 pipette tip. Caterpillars were then held on their backs for 10 seconds to ensure the solution remained, replaced

into their housing cups and incubated at 37°C for a period of 1-3 days. During experimentation, animals were kept on a regular diet.

Prior to inoculation (day 0) and each day of the experiment (days 1-3), animals were weighed to assess their health status. Decreased weight compared to the previous day indicated illness and these animals were removed from the experiment [20]. Mortality and health were also assessed by daily observation. Larvae were scored as dead if they ceased responding to prodding, lost turgor and began to melanise [9].

To measure *E. faecalis* colonisation, two sample types were taken: faeces and haemocoel. Prior to faeces sample collection, the *Manduca* housing cups were cleaned daily to remove waste produced during overnight incubation. The caterpillars were then incubated a further three to four hours before sample collection, to produce an accurate time point. Faeces was collected from each caterpillar daily throughout the course of the experiment (days 1-3), with between one to five pellets being collected and weighed. The weight was then used to calculate the volume of sterile saline required to resuspend the faeces, to produce a ratio of one-part faeces to two parts saline. Prior to plating, the sample was vortexed and ground with an inoculation loop until homogenised. If difficulties with mixing occurred, samples were left in saline overnight at 4°C and resuspended the next morning. For haemocoel sampling, animals were first anesthetized on ice for 15 minutes prior to dissection. The sample was obtained by a ventral horizontal incision at the base of the abdomen and before the hindgut, then a vertical incision up the centre to the top of the abdomen, just before the thorax. The haemocoel was then removed with micro scissor and weighed. Sterile saline was added to achieve a 1:3 dilution of haemocoel to saline by weight. The sample was then thoroughly vortexed for 30 seconds and placed on ice. Following serial dilution, samples were plated on bile-esculin agar, incubated at 37°C for 12 hours and colonies counted.

3.2.2 CRISPR-Cas9 Experimentation

Bacterial strains and growth conditions. The *E. coli* strain EC1000[21] was grown in lysogeny broth (LB) medium at 37°C with shaking 200 rpm. *Lactobacillus lactis* MG1363 was grown in M17 broth supplemented with 0.5% w/v lactose. When required, antibiotics were used at the following

concentration: erythromycin 5 μg/mL for *L. lactis* and 50 μg/mL for *E. faecalis* and spectinomycin 200 μg/mL for *E. faecalis* and 100 μg/mL for *E. coli*. The plasmids VPL3004 and VDM1001 have been described previously [12].

Isolation of plasmids and bacterial transformation. Plasmid isolation of VDM1001 was performed using the Monarch® Plasmid Miniprep Kit according to the manufacturer's instructions (New England BioLabs). Isolation of VPL3004 from *L. lactis* was performed as follows: 2 mL of overnight culture was centrifuged at 16,000g for 1 minute and the resulting pellet resuspended in 200 μl lysis buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10 mM EDTA, 20% sucrose and 10 mg/ml lysozyme) and incubated at 55°C for 20 minutes. The protocol was then continued from the lysis stage of either the Monarch® Plasmid Miniprep Kit or alkaline lysis plasmid isolation.

Construction of the pVDM1001 CRISPR targeting vector and genetic modifications. To create the gRNA constructs, the pVDM1001 plasmid was implemented for the generation of a *dltA* deletion or a point mutation in *liaR* and modified via digestion with BsaI. For both individually, a 30-bp sequence, which did not align to another site on the *E. faecalis* JH2-2 genome, was identified for target recognition, immediately followed by the protospacer adjacent motif (PAM) NGG[22]. Primers of this sequence were then designed with additional nucleotides added for the necessary overhang ligation into the BsaI site in pVDM1001 (*liaR* D191N point mutation; SG1312 and SG1313, *dltA* deletion; SG1360 and SG1361). The nucleotide sequence of VDM1001 is available on NCBI Genbank with the accession number MN580666.

For the creation of the *dltA* deletion, the regions ~500bp upstream of the start codon of *dltA* (the "before" fragment") (SG1362 and SG1363) and ~500bp downstream of the STOP codon (the "after" fragment) (SG1364 and SG1365) were amplified individually. The reverse primer used to amplify the "before" sequence and the forward primer, used to amplify the "after" sequence, both contained the complementary regions to ensure the compatibility of the fragments once amplified. The fragments were then fused using nested primers by overlap extension PCR (SG1366 and SG1367) to produce a fragment containing an in-frame deletion by joining the start codon of *dltA* to the stop codon. The

resulting fragment was then cloned into pVDM1001 following digestion of the plasmid with SmaI and a blunt-end ligation, to create the VDM-Δ*dltA* repair construct.

For the creation of the point mutation in *liaR*, the forward primer was designed complementary to the region ~1kb upstream of desired point mutation. The reverse primer was designed to include the codon corresponding to the aspartic acid at position 191 in *liaR* and included the base change corresponding to the D191N substitution. The reverse primer also contained a substitution to create a synonymous mutation in the PAM site, to prevent the CRISPR targeting the region once inserted into the chromosome. The sequence was then amplified by PCR (SG1353 and SG1398). The fragment was then cloned into pVDM1001 following digestion of the plasmid with SmaI and a blunt-end ligation, to create VDM-*liaR-D191N* repair construct.

To create chromosomal mutation in *E. faecalis*, we first electroporated JH2-2 with VPL3004 and selected for transformants following 24hr incubation at 37° C on BHI with 50 μg/mL erythromycin. Presence of the plasmid was confirmed following isolation from JH2-2 and check primers were used to amplify a 900bp fragment of the plasmid, plus the size of inserted fragment (SG1314 and SG1315). Following confirmation, a positive colony containing VPL3004 was grown in BHI containing 50 μg/mL erythromycin and made competent to receive either VDM-Δ*dltA* or VDM-*liaR-D191N*. Following transformation with either vector, colonies were selected on 200 μg mL spectinomycin and 70 μg mL erythromycin and incubated for 48-72 hours at 30°. Resulting strains were replated to confirm growth on the presence of both erythromycin and spectinomycin.

3.3 Results

3.3.1 Sample collection from faeces produces inaccuracies in detecting colonisation

In order to utilise the *Manduca sexta* caterpillars as a model for establishing *E. faecalis* colonisation, we first aimed to verify the concentration of initial inoculum required to achieve reliable colonisation and produce a sensitive response. Therefore, the caterpillars were fed one of three concentrations: 10^8, 10^7 or 10^6 colony forming units per caterpillar, as indicated by the symbol shape on figure 1. We also utilised three independent cultures of wild-type *E. faecalis* JH2-2 (A, B and C), indicated by the symbol

colour shown in figure 1, as biological replicates. As a negative control, three caterpillars were fed a sterile saline solution, which resulted in no colonisation throughout the experiment (data not shown).

We first utilised faeces collection to detect gut colonisation (Fig. 1, panel A), as this would permit the tracking of the same animal over the course of three days, reducing biological variation. When performed as above, our results indicated no colonisation from faeces sample collected on day one following feeding (data not shown). We did, however, observe colonisation on day two, but only in a single caterpillar from WT-A, fed the initial inoculum of 10^8 CFU. On day 3, we observed colonisation of three caterpillars from the inoculated nine, but interestingly, these three were all inoculated from the same line, WT-A. The other independent wild-type cultures, B and C, failed to colonise across the three days. The results also showed the lack of a dose response depending on the initial inoculum, as on day three, the highest CFU/g was from the 10^7 CFU initial feed. These variable results found within faeces collection promoted us to investigate alternative forms of sample collection, to establish if this was a colonisation problem, or an issue with sample detection.

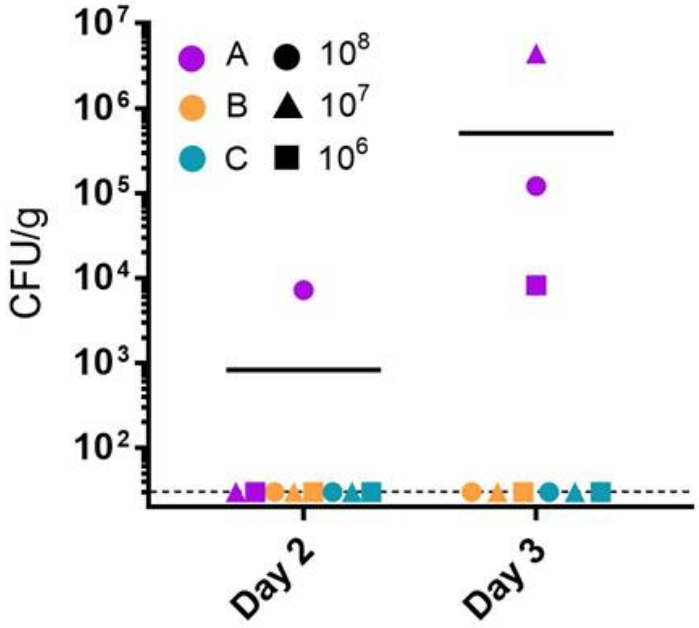

Figure 1. Validating the colonisation of *Manduca sexta* with *E. faecalis* from faecal samples. Following inoculation on day 0, faeces from 12 caterpillars were taken each day for a subsequent three days to monitor gut colonisation. Caterpillars which displayed below 30 CFU/g are indicated at the detection limit (dashed line). Each symbol represents a sample from a single caterpillar. Symbol shape represents initial inoculation concentration, with circles representing 10^8 CFU, triangles representing 10^7 CFU and squares representing 10^6 CFU. Symbol colour represents the independent line of wild type JH2-2 used for inoculation.

3.3.2 Increasing initial inoculum produces improved colonisation when detected via haemocoel dissection

Due to the large amount of variation present when utilising faecal collection, we next wanted to examine if extraction of the *Manduca sexta* haemocoel would produce reliable results. Due to the variability between the individual caterpillars we observed from the faeces sampling, we decided to use a single culture of wild-type *E. faecalis* as inoculum to reduce inconsistencies between results. In addition, we decided to inoculate all the Manduca with 10^8 CFU, to encourage successful colonisation. We also increased our sample size from three caterpillars to five to account for biological variation between the individual *M. sexta*.

The results demonstrated that much like the faeces, *E. faecalis* failed to colonise the gut on day one (data not shown). In contrast to the earlier experiment, on day two, three caterpillars presented colonisation out of the infected five that were dissected, averaging ~1,500 CFU/g (Fig. 2, panel A). However, the results from day three represented a decrease in CFU/g compared to day two, with three caterpillars displaying no colonisation. Overall, when comparing the outcome from both experiments, the haemocoel results were approximately several orders of magnitude lower than the faeces samples.

Once again, the variability within the results prompted us to reconsider our experimental methodology. Due to the overall low CFU/g obtained from the haemocoel extractions, we decided to increase the initial inoculum to 10^9 CFU, to encourage colonisation (Fig. 2, panel B). The results demonstrated colonisation across all three days, and an overall greater CFU/g compared with the 10^8 CFU inoculation. Again, however, there was a lack of incremental increase across the days, with an average decrease in CFU displayed between days one and two and a large amount of variation between individual caterpillars.

In summary, these data demonstrate the successful colonisation of the *Manduca sexta* intestinal tract with wild-type *E. faecalis.* It also illustrates the difficulties of the method, with large discrepancies displayed between independent *E. faecalis* cultures and individual caterpillars. Through this work however, we have identified the importance of inoculation concentration, with a higher initial CFU resulting in a greater rate of gut colonisation. We have also exemplified the importance of controlling for biological variation, in both the bacterial culture and the model organism. Further investigation returning to faeces sampling will be required to determine the optimal sample type suitable for detection the 10^9 CFU inoculation concentration. Overall, these data have contributed towards the optimisation of the Tobacco Hornworm as a model host for investing *E. faecalis* colonisation.

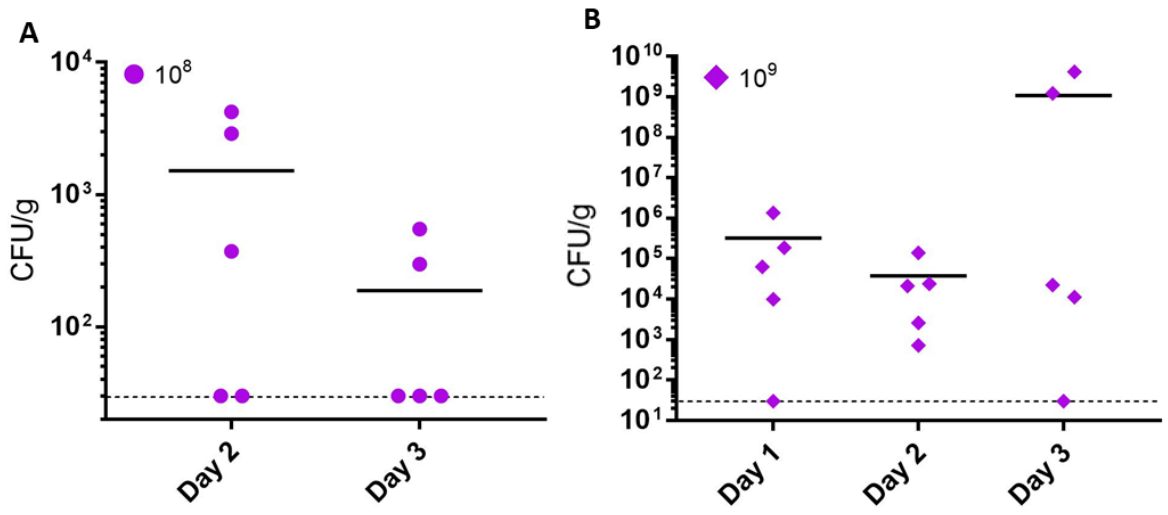

Figure 2. Validating the colonisation of *Manduca sexta* with *E. faecalis* from haemocoel collection following initial inoculation at 10^8 (panel A) or 10^9 (panel B) CFU. Following inoculation on day 0, five caterpillars were culled each day for the subsequent three days to monitor gut colonisation. Caterpillars which displayed below 30 CFU/g are indicated at the detection limit (dashed line). Each symbol represents a single caterpillar. Symbol shape represents initial inoculation concentration, with circles representing 10^8 CFU and diamonds representing 10^9 CFU.

3.3.3 Adaptation of a CRISPR-Cas9 system in *E. faecalis* requires different parameters from those for *E. faecium*

In this work, we set out to utilise the CRISPR-Cas9 genome editing system developed for *E. faecium* to create two *E. faecalis* mutants, firstly, the deletion of *dltA* and secondly, the substitution of D191N in LiaR to generate a hyperactive protein. While were unable to generate successful *E. faecalis* mutants using this methodology (recapped in Fig. 3), we have made steps to optimise the system for future use in *E. faecalis*.

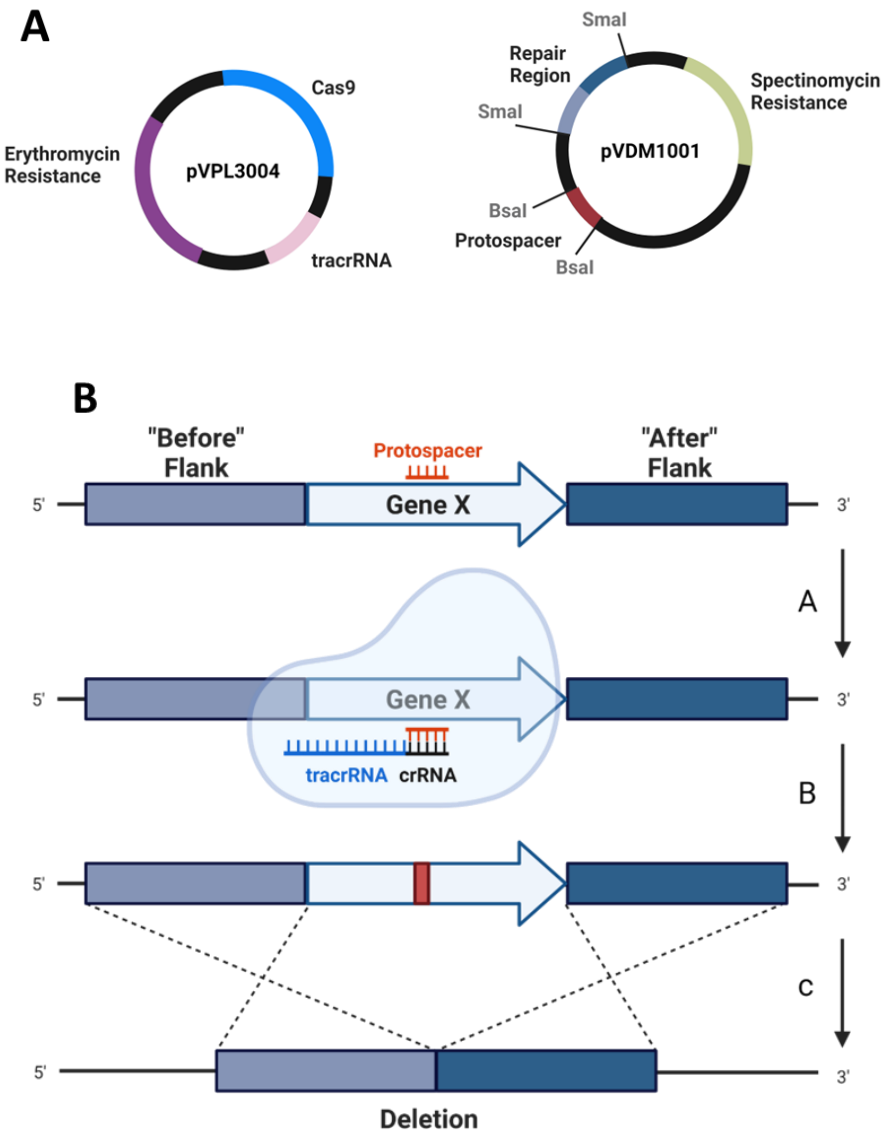

Figure 3. Schematic of the CRISPR-Cas9 method of genome editing. Panel A illustrates the plasmids used in the procedure. VPL3004 which contains the Cas9 sequence required to producer the CRISPR machinery and tracrRNA sequence, which allows the binding of the Cas9 protein (translucent) to the protospacer, which together form the gRNA, along with an erythromycin resistance marker. VDM1001 contains a repair region, in which we inserted either *dltA* or the *liaR-D191N* fragment for replacement. VDM1001 also contains the protospacer required for the targeting of a specific genomic sequence, along with a spectinomycin resistance marker. Panel B demonstrates the workflow of the protocol. Firstly, a region of ~30bp is selected to target a specific region of DNA where the change is desired. Primers are designed, annealed, and inserted into VDM1001 at the BsaI site (step A). The desired change is amplified and inserted into VDM1001 at the SmaI site. VPL3004 expresses the Cas9 machinery and tracrRNA, which binds to the gRNA at the targeted location (step B). Cas9 then causes a break in the DNA (red box), which is then repaired by non-homologous recombination. As the flanks are complementary to the repair region on VDM1001, that region is inserted to produce the necessary deletion or edit (step C). Adapted from[12].

Initially, we successfully generated the VDM-Δ*dlt* and VDM-*liaR-D191N* constructs, which included the insertion of the protospacer into the BsaI site of the vector and insertion of the repair region into the SmaI site. We also successfully transformed *E. faecalis* with pVPL3004, which contains the CRISPR-Cas9 machinery, originally derived from *Streptococcus pyogenes*[23]. We were, however, unable to transform *E. faecalis* containing VPL3004 with either VDM-Δ*dltA* or VDM-*liaR-D191N,* and following electroporation, one of two events occurred. The first event included no colonies present on BHI agar containing both erythromycin and spectinomycin, whereas the second demonstrated an equal number of colonies on both the plates electroporated plasmid DNA or the water control. Upon replating these colonies on to fresh BHI containing erythromycin and spectinomycin, no growth occurred, confirming these colonies as false positives.

As we had successfully introduced VPL3004 into JH2-2, enabling growth on erythromycin, the lack of growth on spectinomycin confirmed that the VDM1001 modified constructs had not been successfully introduced into JH2-2 containing VPL3004. We therefore aimed to establish if this was a specific VDM1001 issue, or if JH2-2 was unable to carry both VDM1001 and VPL3004 simultaneously. To test this, we firstly attempted to electroporate the unmodified VDM1001 plasmid into JH2-2 carrying VPL3004, and this, again, did not result in growth when selected on spectinomycin. This confirmed our modification to VDM1001 resulting in VDM-Δ*dltA* and VDM-liaR-*D191N* had not been the cause of this failure. We also electroporated VDM1001 containing the protospacer region, but not the modification region, to confirm that the lack of growth was not from the CRISPR-Cas9 machinery cutting the DNA, but then not successfully repairing it, but again, no growth occurred following electroporation. We then attempted to transform JH2-2 which did not contain VPL3004 with the unmodified VDM1001 plasmid, to confirm if the issue resulted in JH2-2 attempting to carry both plasmids. Again, however, no growth was observed when plated on to spectinomycin alone. This confirmed that the issue was directly related to the inability to transform *E. faecalis* with VDM1001.

Following this result and the presence of false-positive colonies, we then considered if the spectinomycin concentration used for selection was too low. To check this, we plated JH2-2 on to BHI

containing 50, 75, 100, 150 μg/mL spectinomycin, and confirmed no growth across the plates (data not shown). This therefore confirmed that antibiotic concentration was not an issue during the experiment.

We next considered if temperature was an issue, as VDM1001 is a temperature-sensitive plasmid, preferentially replicating at 30°. Therefore, following electroporation with either VDM-Δ*dltA* or VDM-*liaR-D191N*, plates were incubated at 28°, further slowing the growth of *E. faecalis* but giving VDM1001 increased time for replication, in the hopes of successfully producing the chromosomal modification. However, despite this lower temperature, we did not observe any growth following plating on to BHI containing erythromycin and spectinomycin. We also reattempted to transform the cells at 30° but increased the time of incubation, from over 2-3 days to 5-7 days. This did not result in the successfully transformation of the VDM1001 constructs.

Overall, despite the successful creation of the VDM1001 constructs and the successful transformation of *E. faecalis* with VPL3004, we were unable to transform JH2-2 with VDM1001 and unable to generate chromosomal mutations. This therefore demonstrates that different parameters are required for the use of this CRISPR-Cas9 system in *E. faecalis* compared to *E. faecium*.

3.4 Discussion

The work we have presented here aimed to optimise the use of *Manduca sexta* as an *E. faecalis* host model and the adaptation of a CRISPR-Cas9 system for use in *E. faecalis*.

Firstly, we aimed to optimise the use of the *Manduca sexta* as a model host for examining *E. faecalis* colonisation. To enable the tracking of the same animal over multiple days, we first monitored colonisation via faecal sampling. We also tested various initial inoculation concentration, 10^6, 10^7 and 10^8 CFU per caterpillar to establish the amount required to produce a sensitive response. Our findings demonstrated a lack of colonisation across the *M. sexta*, with only three animals displaying colonisation on the final day. This also indicated difficulties in colonisation dependent on the biological replicate of *E. faecalis* used, with only one of three lines colonising the animals across the experiment. To determine if this was a result of difficulties in sample technique or inoculation concentration, we next examined colonisation of the caterpillar haemocoel. Inoculating with 10^8 CFU alone, we again identified

difficulties in achieving consistent colonisation across the animals. Therefore, we increased the initial CFU to 10^9 per caterpillar. This alteration produced an improvement in the consistency of colonisation across the three days. Therefore, through the examination of different sample types and altering the initial inoculation concentration, we were able to recommend the utilisation of an initial inoculation concentration of 10^9 CFU. This allowed the detection of colonisation within the haemocoel across the subsequent three days of infection. Future work should revisit the collection of faecal samples, but utilising this higher inoculation concentration. This would permit the tracking of the same animal over the course of three days, reducing biological variation, which proved a large factor during this investigation. A further difficulty during this work included maintaining a consistent colonisation across the caterpillars. To counter this, we recommend the utilisation of a single culture of *E. faecalis* to be used per individual attempt of the experimentation as the variation produced from the caterpillars alone proved a substantial variable. To counter this variation, we also recommend a greater sample size per attempt should be used to produce an accurate depiction of colonisation. Biological repeats should then be performed to ensure reproducibility of results.

Once optimised, this technique could be used for measuring the colonisation abilities of *E. faecalis* mutants utilised in the manuscript: Δ*sapR*, Δ*liaR*, Δ*sapAB* and Δ*rapAB*. Interestingly, previous work has suggested a decrease in colonisation capacity in the absence of *sapRS* (Gebhard, personal communication). This indicates a role for *sapRS* in colonisation, potentially through the maintenance of cell envelope integrity. It would be interesting to speculate the same effect for the deletion of *dlt* or *rapAB*, as they are within the SapR regulon and potentially bring about this phenotype. In *Clostridioides difficile*, it has been shown that loss of *dlt* results in increased sensitivity to lysozyme, a host-produced antimicrobial peptide[24]. This enhanced lysozyme killing could potentially result in more efficient clearance of the bacterium from the gastrointestinal tract. With regards to our findings, could the decrease in D-alanylation of teichoic acids on the cell surface resulting from the loss of *dlt* regulation from SapR result in this decrease in colonisation capacity? Future experimentation should also be performed utilising Δ*sapAB* and Δ*rapAB* as they are part of the same regulatory system as *sapRS* to determine if the same effect is witnessed in the absence of either transporter. It would also be interesting

to test the colonisation ability of the *liaR* deletion, as it is the regulator of *sapRS* expression, to determine if a similar effect is observed. Additionally, work in *E. faecium* has demonstrated the increased expression of the LiaFSR system upon exposure to bile salts, which due to their amphipathic nature, can induce membrane damage and injure the bacteria that reside in the gastrointestinal tract. Overall, testing the ability of Δ*liaR* to colonise the tract of the *M. sexta* would provide key information about the role of LiaFSR in response to the intrinsic immune response. As observed during the antagonism assays, which tested the importance of LiaR in response to antimicrobials produced by other bacteria, we would expect a similar dramatic response in response to antimicrobials produced by the immune system. Overall, this work has provided a solid base towards establishing the use of *Manduca sexta* as a model host for *E. faecalis* colonisation.

Secondly, we sought to adapt a CRISPR-Cas9 system, originally developed to create mutations in *E. faecium,* for use in *E. faecalis*. Despite successful creation of the VDM1001 homologous repair constructs and the successful transformation of *E. faecalis* with the VPL3004 plasmid containing the CRISPR-Cas9 machinery, we were unable to generate a *dltA* deletion or point mutation in *liaR*. This failure we identified as an issue with the electroporation of the VDM1001 plasmid into JH2-2, despite multiple attempts at optimisation. The inability to introduce VDM1001 into *E. faecalis* was a surprising outcome, as the successful transformation of *E. faecium* with VDM1001 has been demonstrated previously[12] and as both bacteria belong to the same genus, we would not have expected major difference in the plasmid host range. The bacteria are, however, only distantly related within the *Enterococcus* genus and, as demonstrated phylogenetically, they share a lot of genetic differences [25]. In a study comparing the genomic features of the two, *E. faecalis* was found to have a larger genome and contain more core genes, indicating more complex gene networks that *E. faecium*[26]. In addition, it has been shown that mobile elements can cause a differential fitness cost to both *E. faecalis* and *E. faecium*, representing a difference in genetic machinery[27].

Regardless of these differences, however, VDM1001 was originally constructed via the modification of the pWS3 vector backbone[28]. The thermosensitive plasmid pWS3 is a derivative of the widely used Gram-positive thermosensitive plasmid pG$^+$host9 [29]. Therefore, as the origin of replication has been

carried down throughout these plasmids, the origin present in VDM1001 should be suitable for the successive replication of the plasmid in most Gram-positives, including *E. faecalis*. The use of pWS3 for genetic modification of *E. faecalis,* however, has not been reported. We therefore propose that VDM1001 may not be acceptable for use in *E. faecalis*. Whilst the potential replacement of the VDM1001 origin of replication with another confirmed for use in *E. faecalis* via cloning is a possibility, we suggest the use of a different vector.

Whilst the CRISPR-Cas9 system described here is designed to make permanent genetic changes, CRISPR interference (CRISPRi) takes advantage of a catalytically inactive Cas9 protein, which sterically blocks transcription elongation to control gene expression. While CRISPR-Cas9 technology for genetic modification is still lacking for *E. faecalis,* a CRISPRi system has previously been developed [30]. This system utilises the vector pGCP123 to result in the nisin-inducible knockdown of the CRISPR target. This vector contains a region for protospacer insertion, and a multicloning site, under the control of either T3 or M13 promoters [31]. As this vector has been successfully used in *E. faecalis,* we suggest evaluating the use of this vector in combination with VPL3004 to test its suitability in creating genetic modifications.

In future, a CRISPR-Cas9 system would allow the creation of the *dltA* deletion and the creation of a point mutation in *liaR*, in addition to other chromosomal modifications, allowing us to study the effect of these genetic changes and proving a vast improvement over the current system of allelic exchange.

In conclusion, this appendix has described the adaptation of two complex methodologies, with the goal of expanding our knowledge of a complex bacterium. The development of *Manduca sexta* as a model for studying *E. faecalis* colonisation would aid future investigations into understanding the mechanisms of *Enterococcal* establishment in the gut and pathogenesis. By understanding the genes important to colonisation, we can identify new ways to manage and treat these infections. In addition, the development of a CRISPR-Cas9 genome editing system for *E. faecalis* would allow direct chromosomal changes. This would allow targeted alterations of the *E. faecalis* genome, enabling genetic changes to be studied in *cis* rather than *trans*, removing issues related to copy number and genomic context. The system would not only ease the study of gene deletions, it would also enable the replication of genomic

environments and improve investigations into the effects of clinical mutations experimentally. Again, by having a deeper understanding of the genetic mechanisms involved in the development of antibiotic resistance observed in the clinic, this would enable to identification of genetic targets for future antimicrobial treatments.

3.5 Supplementary

Table S1. Primers utilised in this study.

Primer	Sequence	Description
SG1312	AAACATGTTTCAAACATTTTAGCAAAACTAGATGG	Protospacer sequence for LiaR D191N, Fwd
SG1313	AAAACCATCTAGTTTTGCTAAAATGTTTGAAACAT	Protospacer sequence for LiaR D191N, Rev
SG1314	GGGCGGTGATCACTGATGAATATA	VPL3004 check primer, Fwd
SG1315	ACCAATAATTCCTCAGTACCATCCAT	VPL3004 check primer, Rev
SG1353	[Phos]GGC GCG GGA GTT GCA TGA TTC AGT CAG TC	LiaR D191 Repair sequence, Fwd
SG1360	AAAC GGTGATGTCTATTTATCCCGCATTGGCGTTG	Protospacer sequence for *dltA* deletion, Fwd
SG1361	AAAAC AACGCCAATGCGGGATAAATAGACATCACC	Protospacer sequence for *dltA* deletion, Rev
SG1362	CCGATGGTCATTGGGATTTTGC	*dltA* Repair sequence, “before flank”, Fwd
SG1363	GGAAAATTCACATGTATAGCCGCCTCCTTAAAAC	*dltA* Repair sequence, “before flank”, Rev
SG1364	GCTATACATGTGAATTTTCCTCATATGATTCCC	*dltA* Repair sequence, “after flank”, Rev
SG1365	CTA AAT ATT TCT CCG CAC TCG	*dltA* Repair sequence, “after flank”, Rev
SG1366	GCCATTTATTTGCTAAGCC	*dltA* Repair sequence, nested, Fwd
SG1367	G GGT CCT GAA GAA ATA GTC	*dltA* Repair sequence, nested, Rev
SG1398	[Phos]GCCGCTTGGGTCCGATCATCGACATTTAGT	LiaR D191 Repair sequence, Rev

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4. CroRS controls central cell envelope biogenesis to mediate antimicrobial tolerance in *Enterococcus faecalis*

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Keywords: Antimicrobial resistance, two-component systems, cell envelope stress, experimental evolution

4.1 Introduction

The emergence of multidrug-resistant bacterial pathogens has rendered standard treatments ineffective, allowing infections to persist and spread. Significantly, antimicrobial tolerance (AMT), i.e., the ability of a bacterium to survive but not proliferate during antimicrobial exposure, has been shown to precede the development of bona fide antimicrobial resistance (AMR)[1]. Whilst resistance is defined as overcoming growth inhibition, tolerance represents the overcoming of antimicrobial killing, and as such, the mechanisms conferring these phenotypes are quite distinct[2]. AMT has been identified in a wide array of bacterial species to all bactericidal (cell-killing) antimicrobials, however, our understanding of the pathways and molecular mechanisms governing antimicrobial tolerance remains very limited[3,4,5].

Teixobactin represents a new class of antimicrobials with a unique chemical scaffold and lack of detectable resistance [6]. It has proven efficacy against multidrug-resistant pathogens such as enterococci, staphylococci and *Mycobacterium tuberculosis*[6]. Recent studies suggest the potency of teixobactin is due to its two-pronged attack on the cell envelope[7,8]. The unique enduracididine C-terminal headgroup of teixobactin specifically binds to the conserved pyrophosphate-saccharide moiety of cell wall precursors lipid II and lipid III[7]. Upon binding by the C-terminal headgroup to a lipid II molecule, the N-terminus coordinates the pyrophosphate of another lipid II molecule[7]. This configuration promotes the formation of a supramolecular fibrillar structure, displacing phospholipids and thinning the membrane [7]. Clustering of lipid II within this structure is thought to contribute to membrane disruption, generating a two-pronged action against cell wall synthesis and the cytoplasmic membrane to produce a highly effective compound[7]. Despite this, we have observed teixobactin tolerance in the opportunistic bacterial pathogen *Enterococcus faecalis*[9].

Enterococci are gram-positive bacterial commensals of the gastrointestinal tract of humans, animals and insects [10]. Their adaption to such harsh and varied ecological niches has resulted in a unique cell physiology[11,12]. *Enterococcus faecalis* and *Enterococcus faecium* are the most abundant enterococcal species in humans and are one of the leading causes of hospital-acquired infection[13]. In addition to teixobactin, *E. faecalis* also displays a remarkable intrinsic tolerance to vancomycin[9,14].

Bacterial stress response systems can act as determinants of antimicrobial resistance[15]. When bacteria are challenged with cell wall-acting antimicrobials they encounter a number of cellular stresses including oxidative stress and nutrient limitation, alongside cell-envelope stress[15]. These stresses elicit a variety of specific and highly regulated adaptive responses that not only protect bacteria from the offending stress but also promote changes in the cell that can impact innate susceptibility to antimicrobials[15]. Using whole-genome transcriptome profiling we have previously uncovered the two-component system CroRS as a critical regulator of antimicrobial tolerance in *Enterococcus faecalis*[9]. In addition, CroRS is also important for intrinsic resistance against cell wall antimicrobials such as cephalosporins and D-cycloserine[16]. Taken together, these studies demonstrate the crucial role of CroRS in controlling the molecular response of *E. faecalis* to antimicrobials. However, the precise architecture of the regulatory network that CroRS controls in response to antimicrobial attack requires elucidation. Identification of key genes regulated by CroRS in response to antimicrobials will aid in the understanding of the molecular mechanisms of antimicrobial tolerance in *E. faecalis*.

The aim of this study was to to identify key genes and pathways critical for CroRS-mediated antimicrobial tolerance. This was achieved by a two-pronged approach, on the one had by determining the teixobactin-induced CroRS regulon using RNA-seq analysis, and on the other to rescue the Δ*croRS* mutant phenotype using serial passaging to identify suppressor mutations. Here we report on the teixobactin-induced CroRS transcriptome and identify regulation of the isoprenoid biosynthesis pathway, essential for cell envelope biogenesis, as an important factor in CroRS-mediated antimicrobial tolerance in *E. faecalis*.

4.2 Materials and Methods

Bacterial growth/growth curves. All *E. faecalis* strains were routinely grown in brain heart infusion (BHI) broth or agar overnight at 37℃ with no aeration unless otherwise stated. All *Escherichia coli* strains were routinely grown in lysogeny broth (LB) and agar at 37℃ (200 r.p.m), unless otherwise stated. Cultures for RNA sequencing and optimization were grown as previously described[9]. Growth

was determined by optical density at 600 nm (OD_{600}). Teixobactin (TXB) stocks were made with dimethyl sulfoxide (DMSO) and stored at - 20℃.

Antimicrobial susceptibility assays. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations were determined as previously described[9]. Time-dependent kill assays were carried out to determine cell death kinetics over time as previously described[9].

RNA extraction and preparation of RNA samples for sequence analysis. *E. faecalis* JH2-2 and Δ*croRS* were grown in biological triplicate to mid-exponential phase (OD_{600} of 0.5) at 37℃, 130 r.p.m. Cultures were subsequently split to produce two sets of biological triplicates. One set was challenged with 0.5 μg/ml (0.25 × MIC) of TXB, while the other set were untreated controls. Cultures were grown for a further 1 h and harvested for RNA extraction. Total RNA was isolated using TRIzol-chloroform extraction as previously described[17]. RNA samples were purified using a Zymo RNA clean and concentrate kit as per the manufacturer's instructures. RNA concentration and integrity (RIN >8) was determined by bioanalyzer.

RNA sequencing and gene expression analysis. *(i) cDNA library preparation and sequencing of the E. faecalis JH2-2 and Δ*croRS* transcriptomes.* RNA libraries were prepped using the Zymo-Seq RiboFree Total RNA-Seq Library Kit. Sequencing was completed using an Illumina MiSeq (v3) system generating 150 bp paired-end reads. *(ii) Analysis of RNA sequencing data.* Adapter sequences were removed from raw fastq files using bbduk. Reads were aligned to the *E. faecalis* JH2-2 genome (GenBank accession numbers NZ_KI1518257.1 and NZ_KI1518256.1) using Bowtie2[18]. Statistical and principle-component analyses were performed using the Bioconductor DESeq2 package[19]. Parameters considered during analysis were the fold change (≥ 1.0-fold $\log_2$), the mean number of reads (>50), and the adjusted *P* value (P_{adj} <0.1). Genes were also annotated with the *E. faecalis* V583 reference (GenBank accession number NC_004668.1) gene homolog and ontology using KEGGRest. Gene annotations and ontology assignments were complemented with NCBI BLAST and literature searches when necessary. *(iii) Annotation of the differentially expressed genes*. Differentially expressed genes were assigned gene number annotations by matching the JH2-2 loci number to their respective homolog

and associated gene number using the *E. faecalis* V583 reference genome via NCBI BLAST, ensuring continuity with the existing literature. A script was developed to assign gene function descriptions and gene ontologies to differentially expressed genes by matching the V583 gene number of each differentially expressed gene to the fully annotated V583 genome using the KEGG Rest on R studios platform. The literature was consulted to assign gene functions and ontologies, when applicable. (iiii) *Hypergeometric testing to determine gene ontology enrichment.* Using a hypergeometric test, the significance of gene enrichment within each regulon was assessed. After assigning differentially expressed genes to KEGG pathways using KEGGRest in R Studio, the hypergeometric test was conducted by comparing the number of genes within the regulon to the total number of genes within the KEGG pathway, as described in the V583 genome. Subsequently, enriched genes were filtered for statistical significance ($P < 0.05$). Numerous genes belonged to multiple KEGG pathways and were arranged by KEGGRest according to their primary function.

Construction of the *liaX* promoter-*lacZ* reporter plasmid. The *liaX* transcriptional promoter fusion to *lacZ* in *E. faecalis* was constructed in the vector pTCVlac[20]. The *liaX* promoter fragment The *liaX* promoter DNA fragment was amplified with the primers 5'-AATTTGAATTCGGATGATCGTACTAATG-3' and 5'-AATTTGGATCCCTTTCATGGATATTGC-3'. The fragment was then inserted into pTCVlac via the EcoRI and BamHI sites.

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). 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:

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

Transmission electron microscopy. *E. faecalis* strains were inoculated from overnight culture at a starting OD_{600} of 0.05 and grown to mid-exponential phase (OD_{600} of 0.5, 1×10^8 CFU ml^{-1}) in fresh BHI broth. Afterwards, cell pellets were harvested by centrifugation (5000 × *g*, 4℃, 6 min) and suspended in primary fixative solution (5% glutaraldehyde in 0.1 M Cacodylate buffer) for 30 min at RT. The cells were washed three times with 0.1 M Cacodylate buffer, prior to the secondary fixation with 1% osmium and 1% potassium ferricyanide in 0.1M Cacodylate buffer for 30 min. The cells again were washed three times in 0.1M Cacodylate buffer and stored in the refrigerator for further processing. The fixed cells were washed with ddH_2O and dehydrated with ethanol at progressively increasing concentrations: 30 % ethanol for 5 min, 50 % ethanol for 5 min, 70 % ethanol for 5 min, and 95 % ethanol for 5 min, followed by three treatments with 100 % ethanol for 10, 15, and 20 min, respectively. The dehydration process was completed by two 15-min treatments with propylene oxide. The cells were treated for 2 h with a 1:1 mixture of resin and propylene oxide, then left in resin overnight before being fixed in fresh EmBed 812 resin and polymerized at 60℃ for 48 h. The Otago Micro and Nano Imaging (OMNI) Electron Microscopy centre at the University of Otago performed sample processing and TEM microscopy (Philips CM 100).

Serial passaging of Δ*croRS* for restored growth behaviour. To isolate suppressor mutants with improved fitness compared to the *croRS* deletion strain, we utilised a serial passaging method. Five independent overnight cultures were inoculated 1:1000 into 10 mL BHI medium and incubated at 37°

without agitation. Cultures were passaged every 48 hours under the same conditions. Cells were serially diluted and plated every three passages onto BHI and Bile-Esculin medium to ensure no contamination had occurred. The experiment was concluded upon visible confirmation of improved fitness, assessed as increased turbidity after overnight incubation, which appeared with 10 – 14 days.

4.3 Results

(The following transcriptomic data and analyses were produced by our collaborators in the Cook lab at the University of Otago, New Zealand.)

4.3.1 Whole-genome transcription profiling of the *E. faecalis* WT and Δ*croRS* mutant in the presence and absence of teixobactin

To obtain a holistic view of differential gene expression in the WT and Δ*croRS* strains (+/- teixobactin), four different gene expression profiles (> 1-fold $\log_2$) were generated by our collaborators following RNA sequencing analysis: Δ*croRS* vs WT untreated, Δ*croRS* vs WT treated, WT treated vs untreated and Δ*croRS* treated vs untreated. These four gene expression profiles were then fed into a filtering system to isolate the teixobactin-induced CroRS regulon. For a gene to be identified as a component of the teixobactin-induced CroRS regulon it had to meet four criteria: (1) differential expression in Δ*croRS* vs WT in the presence of teixobactin (2) differential expression in the WT in the presence vs absence of teixobactin, (3) no differential expression in the Δ*croRS* mutant in the presence vs absence of teixobactin, and (4) no differential expression in the Δ*croRS* vs WT in the absence of teixobactin.

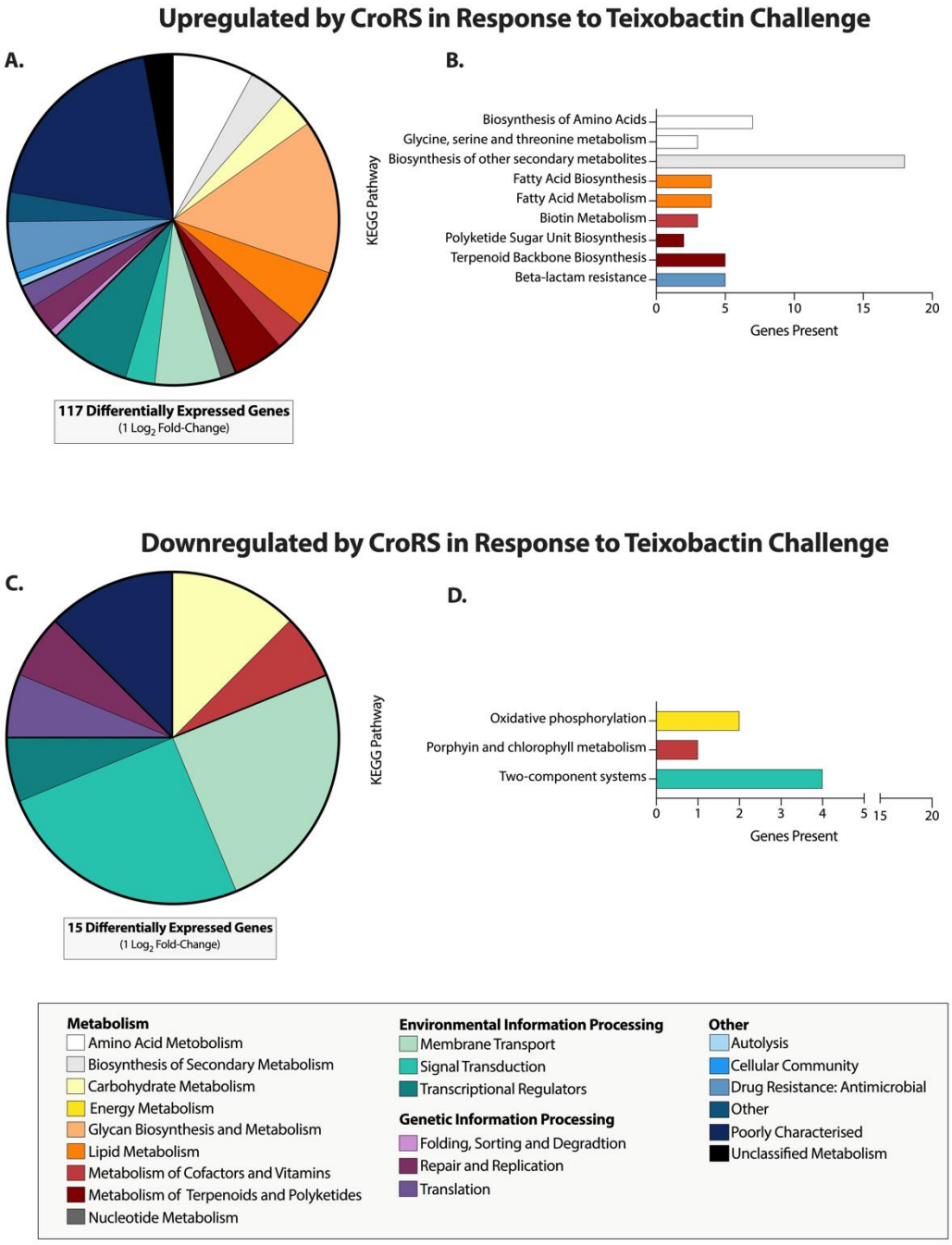


Figure 1. Functional classification and distribution of TXB-induced CroRS-regulated genes. Genes differently expressed (>1-fold $\log_2$) in the TXB-induced CroRS regulon were assigned to *E. faecalis* V583 KEGG ontologies. These pie charts represent the distribution of these ontologies up- and down-regulated by the TXB-induced CroRS regulon (A and C). In addition, hypergeometric testing was performed on the TXB-induced CroRS regulon to identify ontologies significantly (*p* value <0.05) up and downregulated (B and D).

Our collaborators first aimed to determine the TXB-induced CroRS regulon and to determine if CroRS was inducing a specific biological process in response to teixobactin by categorizing the relative

funtions of the genes identifed within the regulon into KEGG gene ontologies utlising the *E. faecalis* reference genome V583 (Fig. 1A&C). Subsequently, each ontology underwent hypergeometric testing to identify pathways significantly regulated by CroRS in response to teixobactin challenge (Fig. 1B&D). These results indicated that a total of 132 genes belonged to the teixobactin-induced CroRS regulon, of which 117 were upregulated and 15 downregulated. Of these genes, CroRS significantly upregulated pathways involved in both the biosynthesis of secondary metabolites and fatty acid metabolism (Fig. 1B). There was also an upregulation in genes involved terpenoid backbone synthesis. Terpenoids are essentially ubitious molecules within nature and are comprised of five-carbon isoprenoid isomers. One of the key pathways involved in the synthesis of these isoprenoid precurors is the mevalonate pahway, of which four genes were found to be downregulated in the absense of *croRS* in response to TXB (Table 1).

Interestingly, in response to teixobactin, CroRS also upregulated a number of a genes involved in β-lactam resistance, i.e. penicillin-binding proteins, as well as the biosynthesis and metabolism of amino acids (Fig. 1A&B). Conversely, teixobactin exposure also caused the CroRS-dependent downregulation of pathways involved in membrane transport, energy metabolism, and signal transduction (Fig. 1C&D).

4.3.2 CroRS significantly upregulates cell envelope biogenesis in response to antimicrobial stress

As observed in the transcriptome analysis, CroRS appears to upregulate genes involved in cell envelope biogenesis in response to antimicrobial stress. This was evidenced by the significantly reduced expression of genes involved in the metabolism of terpenoids and polyketides, the glycan biosynthetic pathway, and lipid metabolism in the *croRS* mutant compared to the wild type in the presence of TXB (Fig. 1; Table 1). These pathways all feed into the biogenesis of major cell envelope components such as peptidoglycan, cell wall polysaccharides, teichoic acids, and phospholipids, suggesting an important role for CroRS in maintaining cell wall synthesis during antibiotic attack.

In cell wall biogenesis, the first critical step is the synthesis of the essential isoprenoid lipid carrier undecaprenyl pyrophosphate (UPP). UPP serves as an anchor in the cell membrane for cell wall

precursors of peptidoglycan (lipid I and lipid II), wall teichoic acids (lipid III), and the enterococcal cell wall polysaccharide (Epa), to be used during envelope biogenesis[21]. Isoprenoids (including terpenoids) are an important group of metabolites in all living organisms and, in bacteria, act as essential building blocks for cell wall biosynthesis. The precursors to these isoprenoids can be synthesized using two main pathways; isopentenyl diphosphate (IPP) is synthesised by the MVA (mevalonate) pathway[22], and dimethylallyl diphosphate (DMAPP) is synthesised by the MEP (methylerythritol 4-phosphate, non-melvanoate) pathway[23]. The condensation of two IPP molecules and DMAPP by farnesyl diphosphate synthase (FppS), leads to the production of farnesyl diphosphate (FPP), at which point the isoprenoid pathway branches[24] (Fig. 2, adapted from [24]). The first branch results in the subsequent condensation of 8 IPP molecules with FPP by undecaprenyl pyrophosphate synthase (UppS) to yield the essential lipid carrier undecaprenyl pyrophosphate (UPP), needed for cell wall biosynthesis[21]. The second branch involves the sequential condensation of FPP with 4 IPP molecules by heptaprenyl diphosphate synthase (HppS) to produce heptaprenyl diphosphate (HepPP), an intermediate subsequently used in the synthesis of menaquinones[24]. Menaquinones can then act as electron carriers and generate a proton gradient across the cytosolic membrane, which is used in ATP production[25].

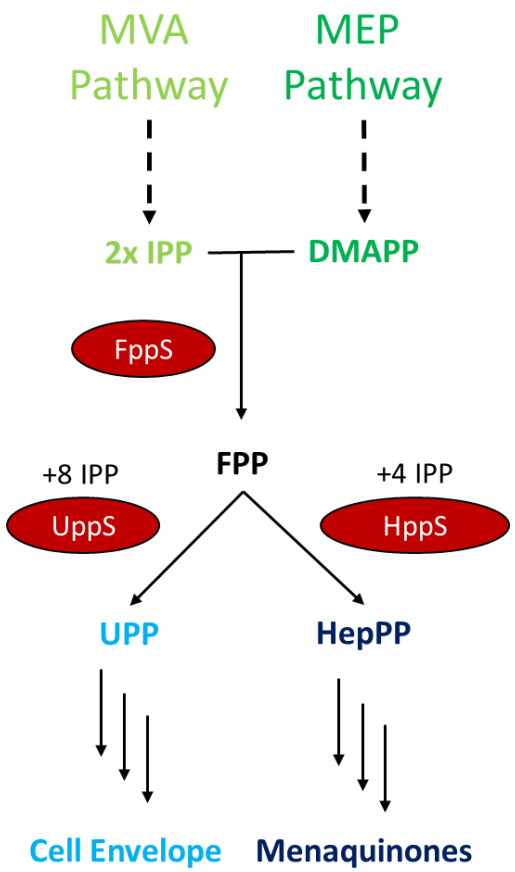


Figure 2. Schematic illustration of the biosynthesis of isoprenoids. The mevalonate pathway (MVA) results in the production of isopentenyl diphosphate (IPP), whilst the non-mevalonate pathway (MEP) results in the production of dimethylallyl diphosphate (DMAPP). Isoprenoid biosynthesis then in general begins with the condensation of the resulting DMAPP and two IPP molecules, carried out by farnesyl diphosphate synthase (FppS) to produce farnesyl diphosphate (FPP). The isoprenoid pathway then branches into two arms. The first results in the subsequent condensation of 8 IPP molecules with FPP, catalysed by UppS, yielding undecaprenyl diphosphate (UPP), an essential carrier in cell wall biosynthesis. On the other branch, the sequential condensation of FPP with 4 IPP molecules by heptaprenyl diphosphate synthase (HppS) produces heptaprenyl diphosphate (HepPP), an intermediate subsequently used in menaquinone biosynthesis.

Interestingly, in the Δ*croRS* mutant, four genes in the mevalonate pathway (EF0902, EF0903, EF1363, EF1364) were downregulated (-2.7 to -4.6 fold-$\log_2$) when compared to the WT in the presence of TXB (Table 1). In addition, undecaprenyl diphosphate synthase (UppS, EF2495) was also downregulated (-3.3 fold-$\log_2$) in the *croRS* mutant. These results suggest that in response to TXB expsoure, CroRS is able to increase isoprenoid production and thus likely cell envelope biogenesis.

As mentioned, the major components of the enterococcal cell wall include peptidoglycan, the Epa (enterococcal polysaccharide antigen) polysaccharide, and teichoic acids. Remarkably, genes involved in the biosynthesis of these components were also significantly downregulated in the *croRS* mutant in the presence of TXB compared to the wild type. Ten genes involved in peptidoglycan biosynthesis were downregulated (-1.9 to -5.4 fold-$\log_2$) in the *croRS* mutant compared to the WT in the presence of TXB (Table 1). Futhermore, seven genes involved in biosynthesis of the cell wall polysaccharide Epa were

downregulated (-1.4 to -2.9 fold-log_2) in the *croRS* mutant compared to WT in the presence of TXB. Likewise, seven genes involved in the biosynthesis of teichoic acids were downregulated (-1.5 to -5.4 fold-log_2) in the *croRS* mutant compared to WT. Therefore, in addition to isoprenoid synthesis, these findings suggest that CroRS also plays a role in regulating the synthesis of multiple essential cell wall components.

In addition to the cell wall, the cell membrane consitutes a significant portion of the bacterial cell envelope, with fatty acids representing their main component[26]. Interestingly, CroRS appeared to significantly upregulate fatty acid biosynthesis in response to TXB challenge (Fig. 1, Table 1), including the three genes EF2881 (*fabG*; 1.8 log_2), EF2882 (*fabD*; 1.4 log_2) and EF0283 (*fabF*; 1.6 log_2), all involved in the conversion of acetyl-CoA to fatty acids in Type II fatty acid biosynthesis[27]. This implied that CroRS can alter fatty acid synthesis, and thus likely membrane synthesis, in response to teixobactin exposure. Overall, these results suggest CroRS plays a key role in the altering the synthesis and composition of both cell wall and cell membrane in response to teixobactin challenge at multiple points in the biosynthetic pathways involved.

Table 1. Genes differentially expressed in the Δ*croRS* mutant compared to WT in the presence of TXB.

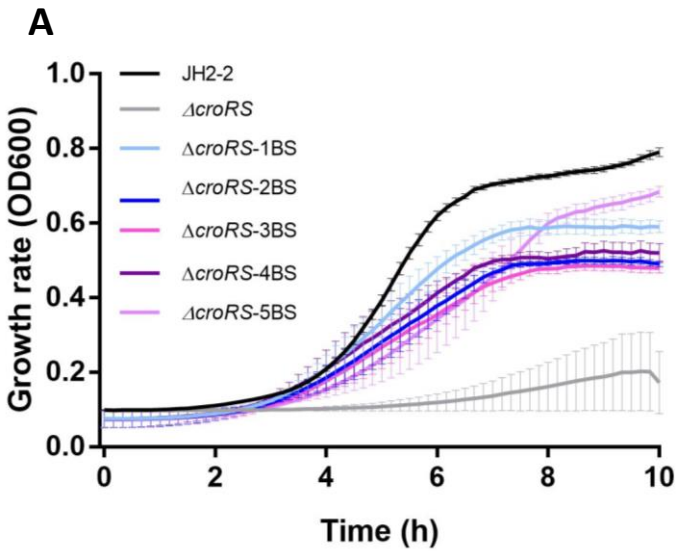
Pathway	Gene No.	Name	Log-fold change in expression
Terpenoid pathway	EF0902	*mvaK*	-2.9
	EF0903	*mvaD*	-3.7
	EF1363	*mvaS*	-4.4
	EF1364	*mvaE*	-4.6
UPP biosynthesis	EF2495	*uppS*	-3.3
PG biosynthesis	EF0746		-5.4
	EF0991	*pbpC*	-2.8
	EF1148	*pbp1A*	-2.6
	EF1300		-2.9
	EF1740	*pbp1B*	-2.2
	EF2585	*murT*	-3.3

	EF2586	*gatD*	-3.0
	EF2733	*murB*	-2.7
	EF2857	*pbp2B*	-4.8
	EF2860		-2.5
Epa polysaccharide	EF2170[#]	*epaX*	-2.1
	EF2171[#]		-2.8
	EF2192		-2.2
	EF2194		-2.6
	EF2195		-1.9
	EF2196	*epaC*	-1.9
	EF2197	*epaB*	-1.9
Teichoic Acids	EF0465		-2.0
	EF1212		-4.5
	EF1264		-1.5
	EF1569		-3.5
	EF1813		-3.4
	EF2703		-5.4
	EF3245		-5.2
Cell membrane			
Fatty acid synthesis	EF0283	*fabF-1*	-1.6
	EF2494	*cdsA*	-2.9
	EF2880	*fabF-2*	-1.5
	EF2881	*fabG*	-1.8
	EF2882	*fabD*	-1.4

4.3.3 Mutations in a heptaprenyl diphosphate synthase and the DNA-directed RNA polymerase delta subunit rescue impaired growth in the Δ*croRS* mutant

In addition to the transcriptome analysis, we utilised experimental evolution in a parallel approach aimed at understanding the physiological role of CroRS. The deletion of *croRS* results in a significant growth defect, with the strain displaying a longer lag phase and slower growth rate than the wild type.

Therefore to identify the cellular processes involving CroRS, we continually passaged the Δ*croRS* strain until the restoration of rapid growth was observed. This passaging resulted in the generation and isolation of five mutants (1BS – 5BS). These strains, Δ*croRS*-1BS to 5BS, demonstrated a decrease in length of the lag phase and an increase in growth rate compared to the Δ*croRS* parent strain (Fig. 3), however, these strains did not fully recover to wild-type levels (JH2-2), demonstrating a lower maximum density and a slower growth rate (Fig. 3).



B

Strain	Growth rate (h^{-1})
JH2-2	1.483 ± 0.041
Δ*croRS*	0.575 ± 0.132
Δ*croRS*-1BS	1.293 ± 0.023
Δ*croRS*-2BS	1.125 ± 0.016
Δ*croRS*-3BS	1.079 ± 0.070
Δ*croRS*-4BS	1.214 ±0.045
Δ*croRS*-5BS	0.949 ± 0.012

Figure 3. Growth curve of the evolved Δ*croRS* mutants 1BS – 5BS alongside the isogenic Δ*croRS* mutant and WT (JH2-2). Overnight cultures of *E. faecalis* JH2-2, Δ*croRS*, and Δ*croRS*-1BS to 5BS were diluted in fresh BHI and inoculated to an OD_{600} of 0.01 in 96-well plates. Cells were incubated at 37℃ with no aeration and growth was monitored as optical density (OD_{600}) with readings taken every 10 mins for 10 h (**A**). Results are presented in biological triplicate ± SD. The average growth rate was determined for each strain (**B**).

We next subjected the resulting strains to whole genome sequencing to identify the suppressor mutations responsible for the observed phenotype (Table 2). The evolved strain 1BS was found to contain a mutation in a putative autolysin, EF2367, which interestingly was also observed in 5BS, with the former obtaining an early STOP codon at amino acid position 18 and the latter gaining a missense mutation at amino acid position 482. In bacteria, the cell wall is a very dynamic structure, and in *Staphlococcus aureus*, the autolysin Atl is involved in the degradation of peptidoglycan during periods

of growth[28]. Whilst the effect of the missense mutation in 5BS remains unclear, the introduction of early STOP codon in 1BS strongly suggests a loss-of-function of the protein. Therefore, if EF2367 functions in a similar way to Atl in *S. aureus*, this suggests a decrease in peptidoglycan degradation contributes to restoration of the growth defect observed in the absense of *croRS*. The link between this autolysin and CroRS however remains unexplored.

In the evolved strain 3BS, we observed the acqusition of a missense mutation in the DNA-directed RNA polymerase subunit delta (*rpoE*, EF3238), and a missense mutation in the hypothetical protein EF0062 at amino acid position 1283. Interestingly, mutation of the hypothetical protein EF0062 was also observed in 5BS, with the same missense mutation occuring at exactly the same position. The function of this protein is unknown. The evolved strain 5BS also acquired a mutation in *pst2B*, a phosphate ABC transporter binding protein, gaining a missense mutation at amino acid position 221.

Most interestingly, however, three of five strains acquired a mutation in heptaprenyl diphosphate synthase (HppS). The strain 1BS, in addition to the autolysin mutation, acquired a deletion at base 52, resulting in a frameshift of *hppS*. Strikingly, in 2BS and 4BS, the mutation of *hppS* was the only SNP to occur. The mutation in 2BS occured at amino acid 189, and again resulted in a frameshift mutation, whilst in 4BS, mutation occured at amino acid position 31 and resulted in the introduction of an early STOP codon, with both mutations occuring earlier in the sequence than the observed 1BS mutation. The introduction of the frameshift mutations in 1BS and 2BS and STOP codon in 4BS are drastic mutations and most likely resulted in a complete loss-of-function of the protein. As discussed above, heptaprenyl diphosphate synthase is involved in the conversion of FPP generated in the mevalonate pathway to menquinoine, the electron acceptor in the electron transport chain in *E. faecalis*[24]. This result correlates with our observations of changes in the mevalonate in response to TXB challenge of the CroRS-regulon and suggests loss of CroRS has effects on cell wall biosynthesis also in the absense of antimicrobial challenge.

Table 2. Whole-genome sequencing of the evolved *croRS* mutants revealed mutations critical for improved growth.

Strain	Gene	SNP/ Indel	Codon	Mutation
1BS	Heptaprenyl diphosphate synthase *hppS* (EF2057)	216_225delAATAGCTGCA	Lys72	Frameshift
	N-acetylmuramoyl-L-amidase (EF2367)	52C>T	Gln18*	STOP gained
2BS	Heptaprenyl diphosphate synthase *hppS* (EF2057)	567T>A	Tyr189*	Frameshift
3BS	DNA-directed RNA polymerase subunit delta *rpoE* (EF3238)	575_576delTTinsAA	Val192Glu	Missense
	Hypothetical protein (EF0062)	3848A>T	Lys1283Ile	Missense
4BS	Heptaprenyl diphosphate synthase *hppS* (EF2057)	91G>T	Glu31*	STOP gained
5BS	Phosphate ABC transporter ATP-binding protein *pst2B* (EF1756)	661A>G	Thr221Ala	Missense
	N-acetylmuramoyl-L-amidase (EF2367)	482T>G	Val161Gly	Missense
	Hypothetical protein (EF0062)	3848A>T	Lys1283Ile	Missense

4.3.4 Antimicrobial susceptibility is intricately linked to the physiology of the *croRS* mutant

The physiology of the bacterial cell is severely compromised in the *croRS* mutant[29], and in addition to displaying a severe growth defect, the deletion of *croRS* also results in the increased sensitivity to cell-wall acting antimicrobials (Table 3). To determine if the restoration of the growth profile had also restored resistance to antimicrobials, antimicrobial susceptibility assays were carried out for the Δ*croRS*-1BS to 5BS mutants alongside the parental Δ*croRS* strain and the isogenic wild type. Interestingly, our findings showed that the improvement in growth had resulted in restoration of tolerance to teixobactin, vancomycin and bacitracin to wild-type levels in all mutants. Strangely however, despite the restoration of tolerance for both TXB and vancomycin, resistance levels of the

evolved strains had actually decreased below the concentration observed for the parent *croRS* strain, an observation that we cannot currently explain. The fact that mutants 2BS and 4BS, which both only carried single mutations in *hppS*, displayed near-complete recovery of tolerance to teixobactin and vancomycin showed that a disruption of HppS activity in the cell was sufficient to rescue tolerance in the Δ*croRS* background (Table 2).

In contrast, the resistance and tolerance to ceftriaxone increased ~8 to 256-fold across the five mutants, but did not improve completely to wild-type levels. For ampicillin, 1BS and 5BS had fully restored resistance, but the three other evolved strains demonstrated only a partial improvement. As stated above, mutants 1BS and 5BS contained multiple mutations, however, both contained mutations in a putative *N*-acetylmuramoyl-L-amidase cell autolysin (EF2367). Their complete restoration of ampicillin resistance thus suggests a functional role for this gene in β-lactam resistance.

Table 3. Antimicrobial susceptibilities of the Δ*croRS*-1BS to 5BS mutants compared to Δ*croRS* and wild-type (WT).

	Teixobactin		**Vancomycin**		**Bacitracin**		**Ceftriaxone**		**Ampicillin**	
Strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
WT	2	16 - 32	1	>128	16	≥64	>256	>256	0.5	0.5
Δ*croRS*	2	<4	0.25	<4	8 - 16	8 - 16	0.5	0.5	0.125	0.125
1BS	1	8 - 16	0.125	128	16	32	128	128	0.125	0.25 - 0.5
2BS	1	16	0.125	128	16	32	16	32	0.25	0.25
3BS	1 - 2	16	0.125	128	16	32	4	8	0.25	0.25
4BS	1	32	0.125	128	16	32	4	8	0.25	0.25
5BS	1	32	0.125	128	32	32	128	128	0.5	0.5

MIC and MBCs are given as μg/ml and are representative of at least biological triplicate

4.3.5 The cell wall stress response is activated in the *croRS* mutant but deactivated in the 4BS mutant K_a or K_d

Given the repeated indication from our data that CroRS is involved in controlling cell envelope biogenesis, and loss of this regulatory system leading apparent defects in maintaining cell envelope integrity, we aimed to determine the level of cell wall stress being experienced in the absense of *croRS*. In contrast to CroRS, which is unique to the enterococci, the LiaFSR three-component system is

commonly found amongst the Firmicutes. The LiaFSR system is known as a 'damage-sensor', responding fairly unspecifically to the cell envelope damage caused by antimicrobials and other cell envelope perturbing conditions [30]. Upon damage to the cell envelope, LiaF, which is an inhibitor of the LiaSR TCS, removes its inhibition of LiaS. LiaS is then able to activate LiaR via phosphorylation, which in turn activates expression of its target genes[30], including the *liaXYZ* operon, which is known to cause cell-wall remodelling in the presence of antimicrobials[31].

We therefore constructed a transcriptional P_{liaX}-*lacZ* fusion construct using the promoter region of *liaX*, to allow us to measure the activity of the LiaFSR regulatory system as a proxy for the level of cell envelope stress being experienced by the cell. By measuring the level of β-galactosidase activity, we observed a ~150-fold increase in P_{liaX}-*lacZ* expression in the *croRS* mutant compared to the WT in the absence of antimicrobial exposure (Fig. 4). This demonstrated that even in the absense of an antibiotic, the deletion of *croRS* resulted in a high level of cell envelope stress. Interestingly, when measuring the level of P_{liaX}-*lacZ* expression in the strains evolved for improved growth, the level of β-galactosidase activity had returned to had returned to near wild-type levels (Fig. 4), demonstrating the level of cell-wall stress had reduced, concomittantly with the improvement of the strains' growth behaviour.

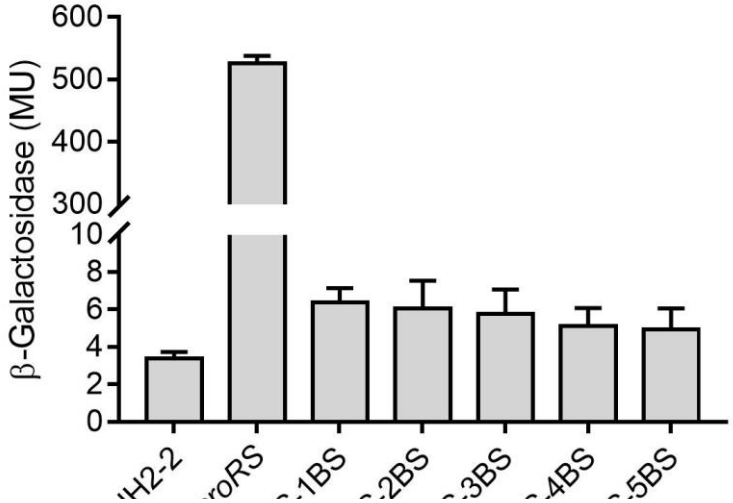


Figure 4. The deletion of *croRS* triggers a Lia-dependent response, which is then restored to wild-type levels in the evolved strains. The promoter region of LiaX was fused to *lacZ* and introduced into *E. faecalis* WT (JH2-2), the mutant strain Δ*croRS* and the Δ*croRS*-4BS strain evolved for faster growth. The resulting strains were then assayed for β-galactosidase activity, expressed in Miller units (MU). Results are the means ± SD for three biological replicates.

To further observe physiological changes in our strains, our collaborators in the Cook lab utilised transmission electron microscopy to visualise the cell morphology and cell envelope structures of the wild-type, Δ*croRS* and 4BS strains. They decided to focus on 4BS here, as it contained a single mutation in *hppS* and at an earlier amino acid position than 1BS and 2BS, with the introduction of a STOP codon at position 31, giving us the greatest confidence that this strain had a complete loss of function of this enzyme. Their observations revealed that compared to the wild-type, deletion of *croRS* resulted in an increase in cell size, and a reduction in the thickness of the cell envelope (Fig. 5 and 6). When analysing 4BS compared to the parent Δ*croRS* strain, a significant reduction in cell size was observed, comparable to wild-type cell size. They also observed an increase in cell envelope thickness in the 4BS mutant compared to Δ*croRS*, however this was not fully restored to wild-type levels (Fig. 5 and Fig. 6).

Overall, these findings demonstrate the drastic impact on the cell envelope resulting from the deletion of *croRS*. The absense of *croRS* causes visible and overt changes to cellular morphology and cell envelope thickness, and this is also reflected in the high level of intrinsic cell envelope stress experienced by the Δ*croRS* mutant. It was a striking observation that the mutation of a single gene was able to restore these phenotypes to near wild-type levels. The fact that this gene, *hppS*, was part of the isoprenoid metabolic pathway, which had also been identified as a physiological process controlled by CroRS, strengthened our findings that CroRS controls cell envelope biosynthesis in *E. faecalis*.

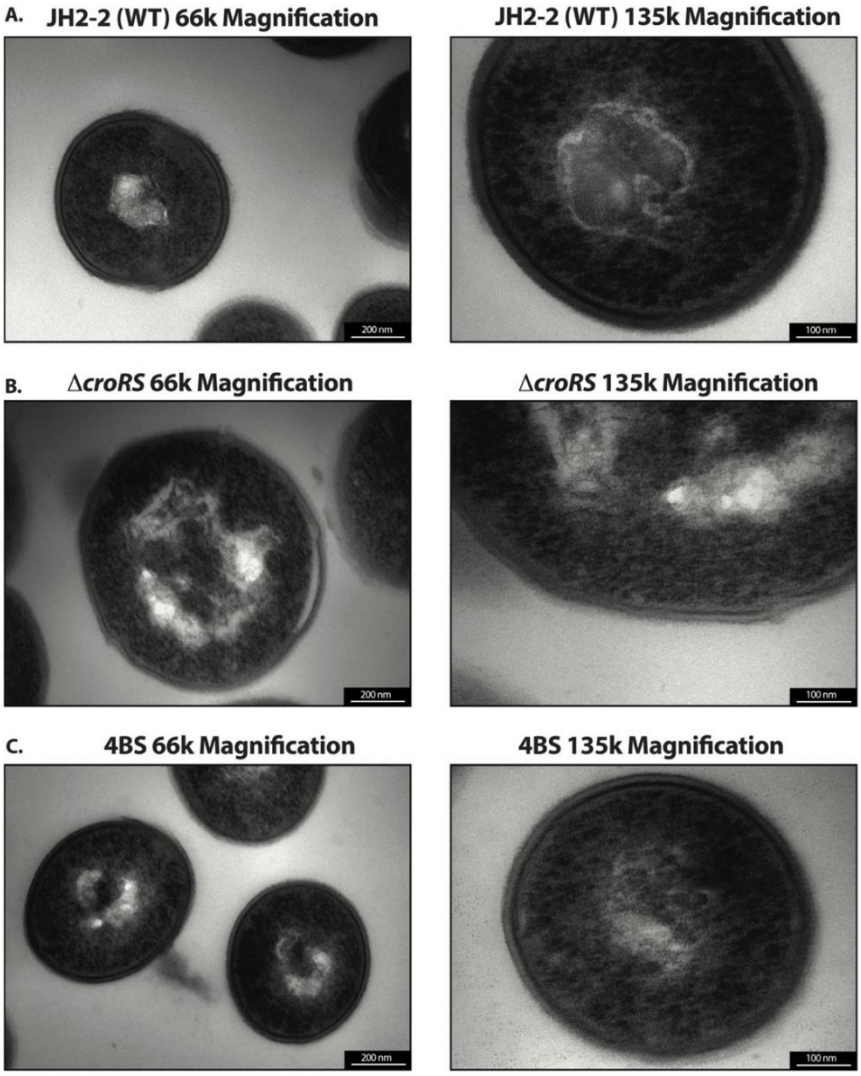


Figure 5. The *hppS* mutation in 4BS restores the abnormal cell morphology caused by CroRS deletion. Transmission electron microscopy of untreated mid-exponential phase (OD_{600} 0.5) *E. faecalis* strains JH2-2 (A), Δ*croRS* (B) and 4BS (C). TEM images captured at a magnification of either 66,000 × or 135,000 ×.

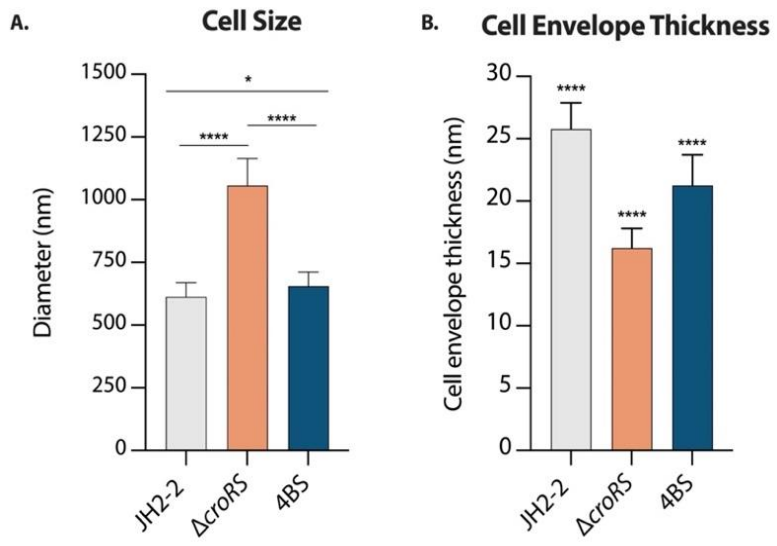


Figure 6. The *hppS* mutation in 4BS reduces cell size and increases cell envelope thickness to levels comparable to those of JH2-2 WT. (A) The diameter of the cell measures the distance from the edge of the cell to the other edge. (B) The cell envelope thickness was measured from the outer border of the cell membrane to the outer edge of the cell membrane at a magnification of 135,000 ×. A one-way ANOVA was used to determine statistical significance; *P* = **** <0.001 or *P* = * 0.5

4.4 Discussion

In this study, we aimed to identify the genes responsible for CroRS-mediated tolerence using genomewide expression profiling to determine the TXB-induced CroRS regulon. In parallel, we utilised experimental evolution to identify suppressor mutations which overcame the growth defect observed in the Δ*croRS* mutant.

Upon detailed examination of the TBX-induced CroRS regulon, we identified a remarkable number of genes involved in the synthesis of isoprenoids, peptidoglycan, teichoic acids and Epa, all essential for the bacterial cell wall, in addition to genes involved in regulating the cytoplasmic membrane, suggesting a role for CroRS in the generation and maintenance of the cell envelope. As the deletion of *croRS* results in a severe growth defect, parallel to the transcriptome results, we aimed to adapt Δ*croRS* for improved growth and identify the suppressor mutations responsible for the return of normal growth. Through this, we identified three of the five resulting strains contained a mutation in *hppS*, involved in the biosynthesis of the menaquinones, which occurred alongside other mutations in 1BS, but was the sole mutation observed in the strains 2BS and 4BS. We also further phenotyped the resulting evolved strains

and observed an increase in antibiotic resistance to full or near wild-type levels, a return to wild-type cell morphology and a reduction in cell envelope stress. Overall, these data support the hypothesis that CroRS controls the expression of genes involved in cell envelope biosynthesis via multiple pathways in response to antimicrobial stress.

From the findings of the transcriptome analysis, our collaborators observed a number of processes downregulated in the absense of *croRS* upon treatment with TXB. The main process affected was the biosynthesis of secondary metabolites, of which over 15 genes were upregulated by CroRS in response to TXB. However, the role of CroRS in the synthesis of secondary metabolites remains unexplored.

The results of the transcriptome analysis also identifed multiple genes involved in fatty acid synthesis and metabolism within the CroRS TXB-induced regulon. In *E. faecalis*, alterations in fatty acid composition of the cell membrane have been shown to affect cellular growth, morphology and antimicrobial tolerance[3]. This can be influenced by environmental availability of fatty acids as well as mutations in biogenesis pathways[3]. While it has been well documented that TXB's mode of action is through binding to the cell wall precursor lipid II, disrupting cell wall biosynthesis, a more recent observation is the formation of large TXB clusters on the cell membrane[8]. This is thought to aid in the physical separation of precursors from the cell wall biosynthesis machinery[7]. In addition, the slower formation of these clusters occurs on a timescale in which TXB kills bacteria[7]. Thus, the upregulation of Type II fatty acid biosynthesis by CroRS may contribute to TXB tolerance by mitigating the formation of these TXB clusters and potentially explain the observed loss of fatty acid metabolic regulation in the absense of *croRS*.

However, the most interesting processes identified in response to TXB both had five genes upregulated via CroRS – the biosynthesis of terpenoid backbones and β-lactam resistance. The analysis identified a large number of genes involved in multiple processes all contributing to cell wall synthesis, such as the biosynthesis of isoprenoid precursors, UPP and peptidoglycan. In particular, the analysis revealed four of the six genes involved in the MVA pathway were downregulated in the absense of *croRS* upon TXB exposure. These transcriptome data were the first indication of a role for CroRS in regulating cell wall biosynthesis, a result mirrored in the experimental evolution and discussed further below.

In parallel to observing the differential expression of cell wall biosynthesis genes in the transcriptome investigation, we repeatedly observed suppressor mutations in *hppS* when evolving for improved growth. Expression of this gene had remained constant during genomewide profiling, suggesting it is not itself a CroRS target. The presence of these *hppS* mutations not only led to restoration of the growth phenotype, but also to the restoration of antimicrobial tolerance to a number of cell-wall acting antimicrobials. HppS plays a key role in isoprenoid metabolism, whose synthesis generally begins with the condensation of dimethylallyl diphosphate (DMAPP) with two molecules of isopentenyl diphosphate (IPP) to produce farnesyl diphosphate (FPP)[24], which then can undergo conversion down one of two potential routes. The first route involves the production of HepPP from the condensation of FPP with four molecules of IPP by HppS, which produces heptaprenyl diphosphate (HepPP), as part of the biosynthesis of menaquinones. The second route involves the generation of UPP, via the condensation reaction between FPP and eight molecules of IPP, catalysed by UppS, to produce UPP, involved in the production of peptidoglycan[21]. We propose that the suppressor mutations identified in *hppS* result in the loss-of-function of the protein, which contains two aspartate-rich motifs (DDXXD), which are essential for *hppS* activity[32]. Whilst the introduction of the frameshift mutation or STOP codon in 2BS and 4BS, respectively, would prevent the translation of both motifs, the frameshift identified in 1BS would result in the loss of only the latter motif (Fig. 7). Regardless, in all three strains, a substantial loss of amino acid sequence would occur and likely prevent protein function. As HppS is involved in the conversion of FPP into HepPP, we propose a loss of function would lead to an increase in the conversion of FPP into UPP, essentially funnelling isoprenoids towards cell wall biosynthesis, to increase the production of peptidoglycan. This rerouting might then be responsible for the overcoming of the growth defect observed in the evolved strains.

Figure 7. **Schematic of the amino acid sequence of HppS indiating the relative position of the the supressor mutations identified in the evolved strains.** The strain and type of mutations observed (FS, frameshift; STOP, introduction of a STOP codon) is indicated above the sequence, with the amino acid positions highlighted in green. The location of the two highly conserved DXXDD motifs required for enzymatic activity are also shown (red, boxed).

The role of the other suppressor mutations identified in the evolved strains so far remains unclear, including those in *rpoE* (EF3238), *pst2B* (EF1756), the hypothetical protein EF0062 and an *N*-acetylmuramoyl-L-amidase autolysin (EF2367). For the latter, we observed that the strains which carried a mutation in this autolysin, 1BS and 5BS, displayed an increase in β-lactam resistance compared to the other evolved strains. This correlates with previous work, which has indicated a potential link between EF2367 and increased amoxicillin tolerance, and previous findings by Gilmore *et al* who have shown that EF2367 is important for cell fitness[33]. In *Streptococcus pneumoniae*, the inhibition of peptidoglycan synthesis by β-lactams triggers autolysin-induced cell death [34,35]. If the same mechanism applies in *E. faecalis*, the potential loss-of-function introduced by an early STOP codon in EF2367 could result in a reduction of β-lactam induced autolysin killing and could explain the increased β-lactam resistance observed in 1BS and 5BS. Interestingly however, EF2367 did not appear to be regulated by CroRS and its expression was not induced by antimicrobial stress (Table 3).

A second interesting gene mutated in the evolved strains was the DNA-directed RNA polymerase subunit delta *rpoE*. Deletion mutants of *rpoE* in *Bacillus subtilis* and *S. aureus* display an extended lag phase and defects in starvation-induced stationary phase survival[36,37]. As a subunit of the DNA-directed

RNA polymerase, it is likely that mutations in RpoE could have a profound effect on gene expression, a potential explanation for the effects observed in *B. subtilis* and *S. aureus* upon *rpoE* deletion. In contrast, the *rpoE* mutation observed in 3BS resulted in a reduced lag-phase. A potential explanation for this observation could be that the acquired missense mutation in 3BS, rather than causing a loss-of-function, may cause a gain-of-function in the protein, and could potentially result in the hyperactivation of RpoE, leading to an increase in gene expression to counter the effects of the deletion of *croRS*.

We also observed a T221A amino acid substitution in the phosphate ABC transporter subunit Pst2B. BLAST analysis showed that threonine 221 is a conserved residue in phosphate transporters across diverse bacterial species suggesting an important functional role. Previous analysis of the *E. faecium* homolog showed that an S199L amino acid substitution conferred protection against killing by vancomycin and chlorhexidine co-treatment [38]. This suggests the Pst2B T221A amino acid substitution may play a role in rescuing tolerance in the *croRS* deletion mutant.

In addition to restoring wild-type growth, the single *hppS* mutation was also able to reestablish tolerance to a number of antibiotics, reduce cell wall stress and restore normal cell morphology. The observed increased size of the Δ*croRS* strain may correlate with low cellular fitness, which includes factors such as growth rate and length of the lag-phase. As the deletion of *croRS* results in an increased lag-phase and decreased growth rate, we propose these factors contribute to the abnormal morphology of the *croRS* mutant. The increased size of the *croRS* mutant could also indicate an issue with osmoreguation and represent difficulties regulating turgor pressure in the absense of *croRS*[39]. Previous work has shown that CroR regulates the glutamine transporter *glnQ*[40], which plays a key role in regulating potassium and glutamate levels, the two most abundant solutes within the bacterial cell[41]. Potentially, in the Δ*croRS* mutant, there is a loss of *glnQ* regulation, leading to the dysregulation of intracellular solutes. This could potentially lead to a high level of osmotic pressure and an increase in cell volume, resulting in the observed increase in cell size. It has also been observed in *E. coli* that upon treatment with fosfomycin, which inhibits the MurA to MurF enzymes that catalyse the conversion of UDP-*N*-glucosamine to UDP-*N*-acetylmuramyl pentapeptide during the lipid II cycle, there was an increase in cell width [42]. Therefore, the increase in size we have observed with the deletion of *croRS* also could be

due to a loss of regulation of cell wall biosynthesis genes. The thinning of the cell wall in the Δ*croRS* mutant further adds to our hypothesis of CroRS having a role in regulating cell wall synthesis enzymes. A similar observation was made in *B. subtilis*, with the deletion of a PBP resulting in the reduction in cell envelope thickness [43]. We therefore propose that the absence of *croRS* results in the downregulation of genes involved in cell wall synthesis, and subseqently a reduction in peptidoglycan production. This then causes the observed growth defect of the Δ*croRS* mutant and morphological changes, such as the thinning of bacterial cell wall. Upon evolving for improved growth, resulting in the loss-of-function of HppS, we propose the production of menaquinoines from FPP is reduced with a concomittant overproduction of UPP from FPP. The increasing availability of the carrier molecule for cell envelope biogenesis then restores peptidoglycan production and ultimately, cell wall synthesis.

Overall, the combined results from both the transcriptomic data and evolution experimentation indicate an important role for CroRS in regulating the processes involved in the biosynthesis of the cell envelope. An improved understanding of the processes CroRS regulates will aid in uncovering the genes within the CroR regulon responsible for the resistance and tolerence pathways maintained by CroRS. An understanding of these tolerance strategies and their distribution in clinical settings will be fundamental to the development of more efficacious treatment regimens.

4.5 References

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Alteration of c-di-AMP levels is linked to restored β-lactam resistance in the absence of CroRS signalling in *Enterococcus faecalis*

Publication status (tick one)

Draft manuscript	X	Submitted		In review		Accepted		Published	

Publication details (reference)	

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Formulation of ideas: 90%

The experimental design and interpretation of data performed in this manuscript was predominantly the work of the first author (the candidate). Supervision and support was provided by co-author S. Gebhard (regular meetings to provide feedback and share ideas with the first author).

Design of methodology: 90%

The first author designed all the novel experiments included in the manuscript with guidance provided by the co-author S. Gebhard.

Experimental work: 30%

The evolution experimentation was performed by the candidate along with the sequence analysis. All other experimentation was performed by a co-author under the guidance and supervision of the candidate.

Presentation of data in journal format: 90%

The current version of the manuscript was completed by the first author. Rounds of editing/feedback were provided by co-author S. Gebhard.

Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed (typed signature)	Sali Morris	**Date**	11/09/22

5. Alteration of c-di-AMP levels is linked to restored β-lactam resistance in the absence of CroRS signalling in *Enterococcus faecalis*

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Running title: Restoration of β-lactam resistance in *E. faecalis*

Keywords: Antimicrobial resistance, two-component systems, cell envelope stress, experimental evolution

Data accessibility statement: The data that support the findings of this study are available in the supplementary material.

5.1 Abstract

Antimicrobial resistance is an every-increasing issue, amplified by the existence of the multidrug resistant bacteria, which render current antimicrobial agents ineffective. One such bacterium is *Enterococcus faecalis*, responsible for a number of hospital-acquired infections and possesses a remarkably high level of intrinsic antibiotic resistance, particularly to the β-lactams. Responsible for monitoring the integrity of the cell-envelope is a number of two-component systems, each tasked with detecting their own individual stimuli and each possessing their own unique regulon. One such system in *E. faecalis* is CroRS, known to play an important role in β-lactam resistance. Despite a number of genome profiling studies exploring the CroRS regulon, the exact genes responsible CroRS-induced for β-lactam resistance remain unclear. Therefore, to establish the role of CroRS in β-lactam resistance, we here utilised experimental evolution to restore resistance to ampicillin in the *croRS* deletion strain. By adapting three independent Δ*croRS* cultures to an approximately ten-fold increase in ampicillin concentration, we identified changes in enzymes controlling the synthesis and degradation of the second messenger c-di-AMP as the only common mutations within all the evolved lines. Quantification of c-di-AMP levels showed that deletion of *croRS* resulted in a marked increase in c-di-AMP level, while the evolved strains displayed a reduction in c-di-AMP levels to similar to the wild type. The decrease in second messenger level had also improved resistance to cell-wall acting antimicrobials. This suggested a physiological link between c-di-AMP levels and the degree of β-lactam resistance in *E. faecalis.*

5.2 Introduction

Known as the 'silent pandemic', antimicrobial resistance is an ever-increasing, worldwide issue, resulting in hundreds of thousands of deaths every year (1). At the forefront of these infections lie the multidrug-resistant (MDR) bacteria, rendering antimicrobials ineffective and increasing both the number and extent of infections (2,3). Such MDR bacteria include the vancomycin-resistant enterococci (VRE), for which the World Health Organisation has listed there is a "high priority" for new treatments (4).

In addition to this acquired glycopeptide resistance, the enterococci have become recognised as important nosocomial pathogens owing to their ability to gain resistance determinants from their environment and their high level of intrinsic resistance to several antimicrobials, particularly the β-lactams (5). This intrinsic β-lactam resistance is associated with the species-specific penicillin-binding protein (PBP) gene *pbp5*, encoding PBP4, which has a low binding affinity for ampicillin and cephalosporins (6). Elevated resistance in *E. faecalis* is often associated with overproduction of PBP4 or mutations in its amino acid sequence, predicted to lead to hyperactivity (7–9). Additionally, the enterococci possess a unique two-component system (TCS), CroRS, that is not present in other Firmicutes bacteria, and which has also been linked to β-lactam resistance. CroRS (ceftriaxone resistance operon) (10) is comprised of the histidine kinase, CroS, and its cognate response regulator, CroR, and is an important regulator of cell envelope stress, associated with antibiotic resistance and, more recently, antimicrobial tolerance (11). The TCS has been shown to play a significant role in the resistance to β-lactams, with its deletion resulting in a ~4000-fold increase in ceftriaxone sensitivity (10).

Many molecular mechanisms of antimicrobial resistance have been discovered, including mutations in drug targets, the activation of efflux pumps to remove the antibiotic and inactivation of the antimicrobial via enzymatic activity (12). However, since as early as 1944, it has been observed that bacteria are able to survive antibiotic treatment without acquiring resistance (13), a phenotype now referred to as 'tolerance'. Such tolerant bacteria display minimal inhibitory concentrations (MICs) that classify the microbe as sensitive, but nevertheless do not respond to antibiotic treatment (14). It is now becoming

increasingly clear that antimicrobial tolerance can facilitate the development of resistance (15). Interestingly, the CroRS TCS is not only important for true resistance, but has also been shown to play a substantial role in the intrinsic tolerance of *E. faecalis* to vancomycin and the 'resistance-proof' antibiotic teixobactin (16). Aiming to explore the mechanism underpinning this tolerance phenotype, work by Darnell and colleagues has uncovered the teixobactin-induced regulon of CroRS, which comprises ~200 genes (16). In addition, CroR has been shown to directly bind to the promoters of the secreted protein, SalB, and the glutamate transporter, GlnQ (10). These analyses have revealed a role for CroRS in maintaining cell envelope integrity through controlling genes involved in isoprenoid metabolism required for peptidoglycan synthesis (17). However, we still lack a complete understanding of how CroRS activity is linked to antimicrobial resistance.

Due to the striking increase in sensitivity to β-lactams observed upon *croRS* deletion, we here aimed to elucidate the role of CroRS in β-lactam resistance by utilising experimental evolution to restore ampicillin resistance in the *croRS* deletion strain. By adapting three independent *ΔcroRS* cultures to an approximately ten-fold increase in ampicillin concentration, we identified changes in enzymes controlling the synthesis and degradation of the second messenger c-di-AMP as the only common mutations within all the evolved lines. Quantification of c-di-AMP levels showed that deletion of *croRS* resulted in a marked increase in c-di-AMP level, while the evolved strains displayed a return to c-di-AMP levels similar to the wild type. The decrease in second messenger level had also improved resistance to cell-wall acting antimicrobials. This suggested a physiological link between activity of CroRS, c-di-AMP levels and the degree of β-lactam resistance in *E. faecalis.*

5.3 Materials and Methods

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplementary material. *Enterococcus faecalis* was routinely grown in brain heart infusion (BHI) (Merck) at 37° without agitation. Solid media contained 15g/L agar. Growth was measured as optical density at 600 nm (OD_{600}) on a Biochrom™ Novaspec Pro Spectrophotometer using cuvettes with 1 cm light path length, or in 96-well plates with 100 uL volumes on a Spark® Microplate reader (Tecan).

Experimental evolution. Adaptation of five independent lines of Δ*croRS* to ampicillin was performed over six weeks. Briefly, five independent overnight cultures were diluted 1:1000 into 10 mL BHI broth containing 0.05 μg/mL ampicillin and incubated at 37°C without agitation. After 48 hours, cultures were reinoculated into fresh BHI containing 0.05 μg/mL ampicillin and a sample was frozen at -80°C in 25% (w/v) glycerol for storage. After three successful passages, ampicillin concentration was increased to 0.1 μg/mL. The same procedure of three passages at the same concentration followed by a step-wise increase of ampicillin concentration was followed until cultures were adapted to 0.5 μg/mL. Cells were plated onto both BHI and bile esculin (Merck) solid media after each third passage to check for contamination.

Determination of minimal inhibitory concentration. Antimicrobial resistance of *E. faecalis* strains was determined using Etest® strips (bioMérieux) providing a concentration gradient from 256 to 0.016 μg/mL of vancomycin, ampicillin, daptomycin or ceftriaxone. Briefly, 5mL of Müller-Hinton (MH) (Merck) medium, supplemented with 20 mg/L of $CaCl_2$ and 10 mg/L $MgCl_2$, was inoculated 1:100 from an overnight culture and grown at 37°C without agitation to OD_{600} 0.6-0.8. Of this cell suspension 50 μL was then added to 5 mL molten MH soft agar (50°C, 0.75% (w/v) agar), mixed and overlaid on MH agar plates. After 15-20 minutes solidification, one Etest® strip was applied per agar plate. Results were recorded after 24 hr incubation at 37°C.

Growth curves. The growth rates of *E. faecalis* strains were assayed in 96-well plates. 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 100 μL BHI medium. Samples were incubated at 37°C under static conditions in a Spark® Microplate reader (Tecan). Optical density (OD_{600}) was measured every 10 minutes during 10 h incubation with 10 seconds of agitation occurring prior to each measurement.

Construction of plasmids and genetic techniques. The transcriptional promoter fusion P_{liaX}-*lacZ* in *E. faecalis* was constructed in the vector pTCVlac (18) by cloning via the EcoRI and BamHI sites of the vector. The primers used to amplify the *liaXYZ* promoter were SG0923 (5'-AATTTGAATTCGGATGATCGTACTAATG-3') and SG0924 (5'-AATTTGGATCCCTTTCATGGATATTGC-3')

β-Galactosidase assays. For quantitatively assessing induction of the P_{liaX}-*lacZ* reporter in *E. faecalis*, exponentially growing cells in BHI medium were prepared as previously described (19). B-Galactosidase activities were assayed in permeabilised cells and expressed in Miller units (MU) (20). Briefly, overnight cultures of *E. faecalis* were inoculated 1:250 in fresh BHI and grown to OD_{600} = 0.4-0.5 and subsequently treated with the antibiotic indicated. Cells were harvested via centrifugation and stored at -20°C. When required, 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), diluted 1:10 and absorbance at 600 nm was read. 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 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:

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

Genome assembly and SNP analysis. For the whole genome sequencing (WGS) of *E. faecalis* strains JH2-2, Δ*croRS*, 1AMP, 2AMP and 4AMP, a single colony was grown overnight in 2 mL of BHI media and subsequently centrifuged at 15 × g for 1 minute. The extraction of genomic DNA was performed using the GeneJET Genomic DNA Purification Kit (Thermo Scientific™). Illumina WGS of the strains was carried out by Microbial Genomics Sequencing Centre (MiGS, Pittsburgh, PA, USA) to a depth of ~60× coverage. Sequences were assembled *de novo* using Spades version 3.15.3, available at https://github.com/ablab/spades (21). For the ampicillin evolved strains, single nucleotide polymorphisms (SNPs) were called using Snippy version 4.5.0, available at

https://github.com/tseemann/snippy/, with default parameters (22), through the Cloud Infrastructure for Microbial Bioinformatics (CLIMB), using the Δ*croRS* sequence as a reference index. Gene function predictions were obtained using NCBI BlastN (23) against the *E. faecalis* JH2-2 sequence, which had been annotated using Prokka version 1.14.5, available at https://github.com/tseemann/prokka (24). If no annotation had been predicted, the sequence was searched against the NCBI database using BlastP.

Sequencing of whole populations. To obtain data on the frequency of SNPs and indels across the whole population of the evolved lineage, as opposed to a single final clone, the genomic extraction of whole populations of 1AMP adapted to the steps of 0.1 – 0.5 µg/mL ampicillin was performed. From the original freezer stock, 50 uL was inoculated into 5 mL of BHI and incubated at 37° with no agitation overnight. The OD of the culture was then measured, and a total of 4 OD was collected and subsequently centrifuged at 15 × g for 1 minute. Genomic extraction was then performed as described above and WGS was performed to a depth of ~200× coverage. SNP identification and frequency at each concentration was identified using Breseq version 0.37.0, in the 'polymorphism' mode with default parameters (25). The predicted SNP locations were determined against the reference sequence assembled from the *croRS* deletion strain as above. Gene function predictions were performed as above. All SNPs identified at each ampicillin concentration are included in Table S3. SNPs or indels which appeared at the first sequenced step and were stable in frequency over the course of the evolution experiment were excluded. Only SNPs present in >15% of the population were included in Figure 2 for clarity.

Quantification of c-di-AMP levels by ELISA assay. To determine the intracellular c-di-AMP concentration, cultures of *E. faecalis* were grown overnight in 10 mL of BHI medium, with 0.5 µg/µL ampicillin added to the samples indicated, and subsequently centrifuged at 5,000 × *g* for 10 minutes. Cell pellets were then weighed, and 4 mL of B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific) was added per gram of sample. Cells were then resuspended, incubated for 15 minutes at room temperature and the resulting lysate was centrifuged at 15,000 × g for 5 minutes. The supernatant was then collected and stored at -20°C until required. The c-di-AMP concentration was subsequently measured using the Cayman Chemical c-di-AMP ELISA kit according to the

manufacturer's instructions. To determine c-di-AMP concentration, a standard curve was produced with samples of known concentration (15 - 2,000 pg/mL) provided by the manufacturer (Cayman Chemical). The protein concentration of the B-PER™ lysate was determined using the Bio-Rad protein assay dye reagent concentrate against a bovine serum albumin (BSA) standard (0 - 1 µg BSA) following manufacturer's instructions. The cellular concentration of c-di-AMP was calculated by normalising the c-di-AMP concentration determined to the protein concentration in the lysate. The experiment was repeated in two to three biological replicates per strain, with two dilutions of each cell extract per assay. Each dilution was assayed in either duplicate or triplicate, resulting in four to six technical repeats per ELISA.

5.4 Results

5.4.1 Deletion of *croRS* results in increased sensitivity to cell wall-acting antibiotics

CroRS is a TCS that is well-known for its role in cephalosporin resistance. To establish the extent to which CroRS is involved in protection of the cell wall more broadly, we investigated the effect of numerous cell wall-acting antimicrobials upon *croRS* deletion. In line with previous work, we identified a substantial increase in the sensitivity to ceftriaxone in the *croRS* deletion strain (Table 1), with the MIC decreasing from >256 µg/mL to 0.5 µg/mL. Deletion of *croRS* also resulted in mildly increased sensitivity to vancomycin and daptomycin, with the MIC decreasing ~2.5-5 fold and ~4-8 fold, respectively. However, in contrast to the work by Comenge et al. (10), which identified no change in ampicillin sensitivity in the absence of *croRS*, we observed a ~10-fold decrease in ampicillin resistance upon *croRS* deletion. This difference could potentially be due to our use of Etest™ strips, compared to the broth dilution tests more commonly used in past studies (26). Overall, these results demonstrate the detrimental effects of loss of the CroRS regulatory system on *E. faecalis* when challenged with cell wall acting antimicrobials, particularly β-lactams.

Table 1. The minimal inhibitory concentrations (MICs) of *E. faecalis* strains.

	MIC (μg/μL)[1]			
Strain	Ampicillin	Ceftriaxone	Daptomycin	Vancomycin
JH2-2	0.5	>256	2 – 4	0.5 – 1
Δ*croRS*	0.05	0.5	0.05	0.19
1AMP	0.38	>256	0.05 – 1	0.19 – 0.25
2AMP	0.38 – 0.5	>256	0.19 – 0.38	0.19 – 0.25
4AMP	0.38 – 0.5	>256	0.19 – 0.38	0.094 – 0.125

[1]Results are recorded from three independent repeats; where a range of concentrations is given, results varied between replicates.

5.4.2 Adaptation to increasing ampicillin concentration restored resistance, but did not improve fitness

Upon observing an increase in ampicillin sensitivity in the absence of *croRS*, we decided to explore this phenotype further. We therefore sought to investigate the molecular basis for this increased ampicillin sensitivity via experimental evolution. For this, we serially passaged five independent lines of Δ*croRS* into increasing ampicillin levels. Over the course of six weeks, three of the lines survived, and the experiment was concluded upon the adaptation of the strains to the wild-type ampicillin MIC, 0.5 μg/mL. The resulting strains were named 1AMP, 2AMP and 4AMP.

Upon concluding the adaptation, we then aimed to determine: i) the ampicillin minimal inhibitory concentrations for the strains, ii) improvement in the MIC of other cell wall acting antibiotics and iii) effects on fitness through analysing growth rate. Overall, all three strains demonstrated a substantial decrease in ampicillin sensitivity, recording MIC values between 0.38 – 0.5 μg/mL (Table 1). This demonstrated a return to wild type level protection, and a ~10-fold increase compared to the original Δ*croRS* parent strain. In contrast to ampicillin, the three strains did not exhibit any change in sensitivity to daptomycin, and only a minor improvement was observed upon vancomycin challenge, demonstrating that the adaptation did not have a general effect on all cell-wall acting antimicrobials. Interestingly however, sensitivity to ceftriaxone, another β-lactam antimicrobial, had returned to >256

μg/mL, equivalent to the wild type. These results demonstrated that evolving the three independent Δ*croRS* lines to increasing ampicillin concentration had specifically restored resistance to β-lactams but not to other cell-wall targeting antimicrobials.

We next investigated if adaptation to ampicillin had produced any effect on the growth profile of the evolved strains. The deletion of *croRS* results in a severe general growth defect, displaying a longer lag phase and slower growth rate than the wild type (Fig. S1). In work carried out in parallel to this study, we had aimed to overcome this growth defect by serial passaging in the absence of antibiotic. This led to the establishment of wild-type growth kinetics, concomitant with a restoration of general antibiotic resistance (17). However, in the present study, only β-lactam resistance had increased, hinting at a more specific mechanism. We therefore wanted to test whether the serial passaging in ampicillin-containing media also had led to any improvement in growth rate in the absence of an antibiotic. Compared to the parent Δ*croRS* strain, all evolved strains showed a small improvement of growth, progressing to exponential phase sooner and presenting an increase in growth rate and maximum cell density (Fig. S1, Table S2). Compared to the wild type, however, 1AMP, 2AMP and 4AMP still presented with an increased lag phase before exponential growth (Fig. S1). Therefore, despite the restoration of ampicillin resistance, characteristics of wild-type growth had not fully returned. This signifies that passaging into increasing ampicillin concentration had exerted a specific selection pressure on the bacteria, regaining antibiotic resistance against β-lactams, but without resulting in restoration of general growth characteristics or a broad resistance spectrum.

5.4.3 The absence of *croRS* results in cell envelope stress, which is reduced by suppressor mutations

Alongside the severe growth defect, our previous work also reported an increase in cell envelope stress upon the deletion of *croRS* (17). Unlike CroRS, which is unique to the enterococci, the LiaFSR TCS is common amongst the Firmicutes and is known to respond to cell envelope damage (27). In enterococci, the LiaFSR system controls expression of the *liaXYZ* operon (28). Therefore measuring activation of *liaXYZ* expression using a transcriptional P_{liaX}-*lacZ* fusion can be used as a proxy for the level of stress being experienced by the cell. Our earlier work, as mentioned, had observed a drastically increased level of cell envelope stress in the Δ*croRS* strain based on P_{liaX}-*lacZ* activity, which was restored to wild-type levels in the strain evolved for fast growth rate (17). To determine whether the adaptation to ampicillin also had reduced the level of cell-wall stress, we therefore next measured the activity of the P_{liaX}-*lacZ* fusion in the 1AMP strain. As previously reported, our findings confirmed a dramatic increase in P_{liaX}-*lacZ* activity in the absence of *croRS*, increasing over 100-fold compared to wild-type levels (Fig. 1). Interestingly however, in 1AMP the activity of P_{liaX}-*lacZ* had returned to wild-type levels or even slightly lower, signifying this strain was no longer suffering from cell envelope stress. This was a surprising result, as it implied that despite neither restoring the growth profile nor the broad resistance

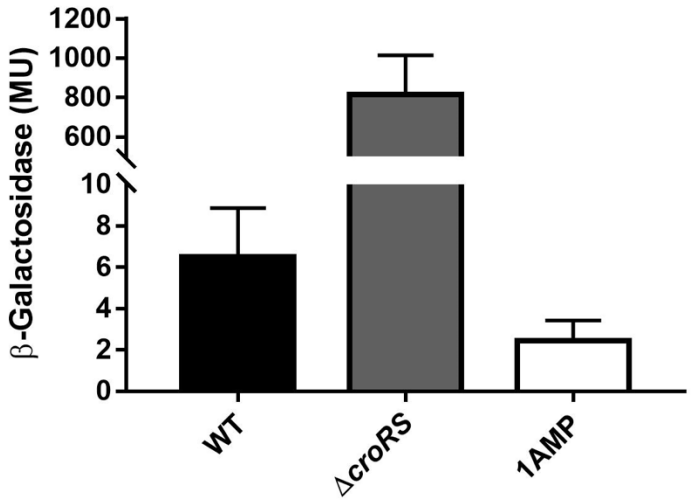

Figure 1. The deletion of *croRS* triggers a Lia-dependent response, which is then restored to WT levels in the evolved strains. The promoter region of *liaX* was fused to *lacZ* and introduced into *E. faecalis* WT (JH2-2), the mutant strain Δ*croRS* and the strain evolved for ampicillin resistance, 1AMP. The resulting strains were then assayed for β-galactosidase activity, expressed in Miller units (MU). Results are means plus standard deviations for three biological replicates.

spectrum, the suppressor mutations that returned β-lactam resistance to 1AMP had also fixed the envelope stress caused by the absence of *croRS*.

5.4.4 Adaptation to ampicillin results in mutations in genes involved in second messenger production

To better understand how the adaptation to ampicillin had led to the observed changes in phenotypes, we next want to identify the mutations acquired by the evolved strains via whole genome sequencing (WGS) of 1AMP, 2AMP and 4AMP. We found each strain contained numerous mutations, many of which related to cell wall functioning, implying a generic response to cell wall stress, unsurprising given the presence of ampicillin during the experiment. Amongst other changes, 1AMP had gained mutations in a putative N-acetylmuramoyl-L-alanine amidase, involved in the breakdown of the peptidoglycan cell wall (29) and in *bgsB*, a glycosyltransferase that affects cell wall composition (30) . In contrast, 2AMP had gained mutations in *epaI*, a component of the enterococcus polysaccharide antigen (EPA) and two mutations in *hsdS*, a restriction endonuclease subunit. In addition, the strain had also gained a mutation in the putative ABC transporter EF0057, proposed to be involved in zinc uptake (31). Interestingly, 4AMP also contained mutations in EF0057, although the role of this transporter in ampicillin resistance is not apparent. Furthermore, 4AMP contained a mutation in *ireB*, a substrate of the Ser/Thr protein kinase and phosphatase pair IreK and IreP (32), which is known to modulate CroRS activity (33). The loss of *ireB* is known to confer resistance to ceftriaxone (32), which could explain why a mutation in this gene appeared during our evolution experiment. Interestingly, 4AMP also acquired a mutation in *pbpC*, involved in peptidoglycan crosslinking, and whose mutation is known to play a role in ampicillin resistance (34). 1AMP and 4AMP also both contained mutations involved in carbon metabolism, *citG* and *pdh*, respectively. Strikingly however, the sequencing revealed that in addition to these individual genetic changes, the three resulting strains had all acquired mutations in the gene EF2413, annotated as an HD-domain containing protein.

In *B. subtilis*, *pgpH* is a phosphodiesterase capable of cleaving and degrading the second messenger c-di-AMP, is encoded next to *phoH* on the chromosome and contains seven transmembrane domains and an HD-domain (35,36). Although lacking a functional annotation in the *E. faecalis* JH2-2 genome, this

description also matched EF2413, which is also located next to *phoH* on the JH2-2 genome and possesses seven transmembrane domains and an HD-domain. Therefore, we concluded that EF2413 was likely a homologue of *B. subtilis pgpH*. Using the SMART database (37), we were able to predict the domain architecture of PgpH in *E. faecalis* and determine the relative location of the amino acid substitution in the evolved lines (Fig. 2). Whilst 2AMP and 4AMP had acquired mutations within the extracellular domain of the protein, gaining early terminations at amino acid locations 67 and 146, respectively, 1AMP had acquired a mutation within the seventh transmembrane helix, gaining a missense mutation at amino acid 456. However, based on this information alone, it is not clear whether these mutations are likely to cause a loss or gain of function of the protein.

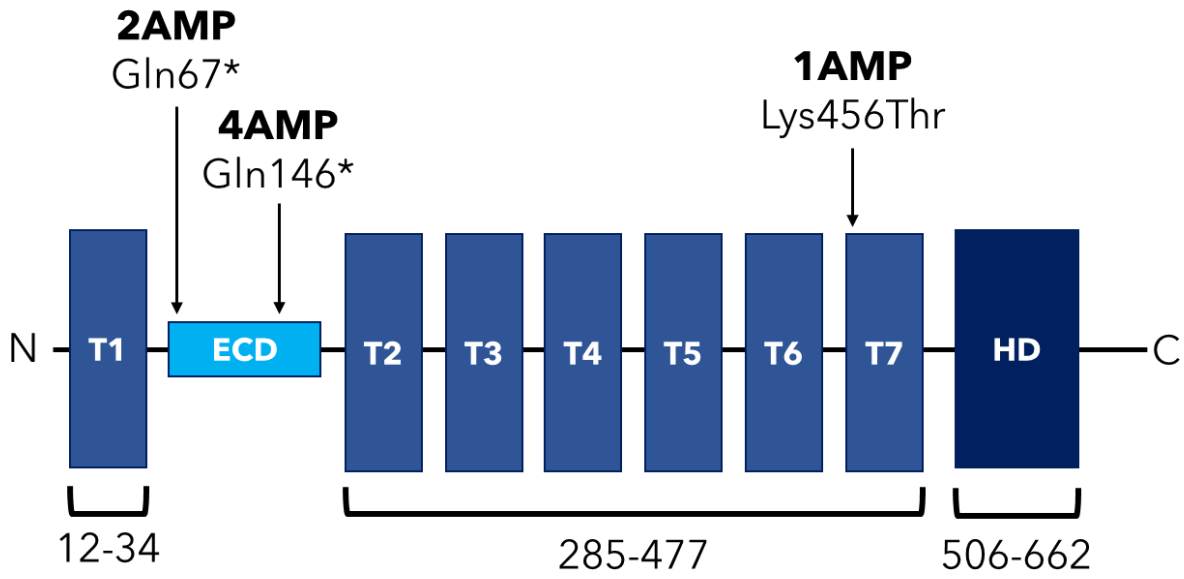

Figure 2. Schematic indicating the substitutions acquired in the phosphodiesterase PgpH. During adaptation to ampicillin, 1AMP, 2AMP and 4AMP all acquired mutations leading to amino acid substitutions or stop codons (*) in PgpH, the details and relative locations of each are indicated above the protein by the arrows. The seven transmembrane domains are indicated (TN), alongside the extracellular domain (ECD) and the HD-domain (HD). The domain architecture was predicted by the SMART database from the amino acid sequence. The coordinates of the relative amino acid positions for the transmembrane and HD-domains are indicated below the protein.

Strikingly, all three strains also possessed additional mutations related to c-di-AMP metabolism, as indicated in table 2. 1AMP contained a missense mutation in the c-di-AMP synthase, *cdaA* (38,39), and

both 2AMP and 4AMP contained mutations in *gdpP*, an additional phosphodiesterase involved in c-di-AMP degradation (40) ,with the introduction of a missense mutation or STOP codon respectively. 4AMP also contained a mutation in EF2156, a YbbR-like protein that has been linked to CdaA regulation in *B. subtilis* (41). Overall, these results indicated that to re-establish resistance to ampicillin in the absence of *croRS*, suppressor mutations consistently occurred in genes involved in the metabolism of c-di-AMP.

Table 2. Suppressor mutations acquired during adaptation of Δ*croRS* to 0.5 mg/mL ampicillin.

Strain	Gene	Locus Tag	SNP	Codon	Mutation	Annotation
1AMP	***cdaA***	**EF2157**	**C275T**	**Ala92Val**	**Missense**	**C-di-AMP synthase**
	Hypothetical Protein	EF2166	T763G	Phe255Val	Missense	-
	N-acetylmuramoyl-L-alanine amidase	EF2367	G331T	Gly11Trp	Missense	Autolysin
	pgpH	**EF2413**	**A1637C**	**Lys456Thr**	**Missense**	**Phosphodiesterase**
	atpA	EF2610	A881G	His294Arg	Missense	ATP Synthase F1 subunit α
	bgsB	EF2890	G11T	Gly4Val	Missense	Glycosyl transferase
	citG	EF3315	T678G	Phe226Leu	Missense	Triphosphoribosyl-dephospho-CoA synthase
2AMP	*hsdS*	N/A	451_543delAAinsGCT	Asn181Ala	Missense	Restriction endonuclease S subunit
	hsdS	N/A	G680A	Gly227Glu	Missense	Restriction endonuclease S subunit
	pgpH	**EF2413**	**C199T**	**Gln67***	**STOP gained**	**Phosphodiesterase**
	epaI	EF2190	C427T	Gln143*	STOP gained	Glycosyl transferase
	ABC transporter permease	EF0057	G7T	Glu3*	STOP gained	Part of a zinc uptake system
	gpdP	**EF0011**	**C1336T**	**Arg446***	**STOP gained**	**Phosphodiesterase**
4AMP	Hypothetical Protein	EF0773	303_307dupGACGT	Try103fs	Frameshift	-
	hsdS	N/A	G680A	Gly227Glu	Missense	Restriction endonuclease S subunit
	pbpC	EF0991	A1594C	Ile532Leu	Missense	Penicillin binding protein C
	ireB	EF1202	A149C	Aps50Ala	Missense	Part of the IreK system
	pdhB1	EF1354	T847G	Leu283Val	Missense	Pyruvate dehydrogenase complex

YbbR-like protein	EF2156	1030_1053dupCAACCAAGTACATCCAGTCAAGTA	Val351_Glu352insGlnProSerThrSerSerGlnVal	Insertion	Potential CdaA regulator
pgpH	**EF2413**	**C436T**	**Gln146***	**STOP gained**	**Phosphodiesterase**
feoB	EF0476	T1956G	Gly652Gly	Synonymous	Part of an iron uptake system
ABC transporter permease	EF0057	G7T	Glu3*	STOP gained	Part of a zinc uptake system
gpdP	***EF0011***	**C1718A**	**Ala573Asp**	**Missense**	**Phosphodiesterase**

[1]Hypothetical protein according to PROKKA annotation, mapped to *E. faecalis* V583.
[2]Locus tags are indicated by 'N/A' if no homologue was identified in *E. faecalis* V583.
[3]Genes directly involved in c-di-AMP metabolism are in bold.

5.4.5 Mutations in *cdaA* and *pgpH* occurred from the second stage of adaption

As we now had uncovered the mutations involved in restored ampicillin resistance, we aimed to determine the order in which these changes had appeared. To do this, we revisited the passages from each stage of ampicillin adaptation for the 1AMP strain. As explained in the methods section, during the experimental procedure each independent line was passaged three times at each ampicillin concentration, thus spending six to seven days at a specific concentration. Following the third passage, we assumed adaptation to the given concentration of ampicillin had occurred, and we froze the bacterial samples at this point, whilst simultaneously passaging the culture to the next increased ampicillin concentration. To analyse the order of acquisition of mutations and to assess the frequency of mutations at each ampicillin concentration step, these frozen cultures from the third passages of the 1AMP line were now subjected to sequencing and BreSeq analysis.

Surprisingly, these investigations demonstrated that the mutations observed in *cdaA* and *pgpH* both occurred from the second stage of adaption, at 0.2 μg/mL ampicillin, corresponding to a two-fold higher MIC compared to the *croRS* deletion (Fig. 3). These mutations were then maintained throughout the increasing ampicillin concentrations at 100% of the population. As the mutation in *pgpH* occurred early in 1AMP ampicillin adaptation and was observed at the end point for 1AMP, 2AMP and 4AMP, we propose this gene plays a key role in altering c-di-AMP levels to increase the resistance to β-lactams.

In contrast to *cdaA* and *pgpH*, mutations in *atpA*, *atpB* and *citG*, all occurred later during adaptation. Due to their respective roles in energy generation and carbon metabolism (42,43), we propose these latter changes acted to 'finetune' cellular metabolic functions. Mutation of these genes may potentially allow the adjustment of the cellular central metabolism to ensure cell survival upon increasing ampicillin exposure.

Occurring from the first step of adaption, we observed a fluctuation of mutations in the intergenic region upstream of *uvrA* (Fig. 3), a gene belonging to the nucleotide excision DNA repair pathway in *B. subtilis*, which functions in locating and excising bulky DNA lesions (44). The presence of mutations in DNA repair pathway genes is not uncommon in experimental evolution (45), as the dysregulation of

these genes can result in the further mutation of other genes to provide adaptation and ensure cell survival.

While the occurrence of multiple mutations at the same phase of the adaptation process makes it difficult to untangle the contribution of each mutation to the ampicillin phenotype, overall, our results indicate that mutations in c-di-AMP metabolism are a crucial first step in restoring ampicillin resistance in the absence of *croRS*.

5.4.6 Deletion of *croRS* results in increased c-di-AMP levels, which decreases through suppressor mutations

Our next goal was to determine the effect of the observed mutations in c-di-AMP metabolism on the cellular levels of the second messenger. Remarkably, compared to the wild type, we observed that deletion of *croRS* resulted in a ~6-fold increase in cellular c-di-AMP concentrations (Fig. 4). In the ampicillin-evolved strains 1AMP and 2AMP, the c-di-AMP level had returned to approximately wild-type concentrations. This was a striking result as it suggested a correlation between the cellular concentration of c-di-AMP and resistance to ampicillin in *E. faecalis*. To assess whether *E. faecalis* might use changes in c-di-AMP levels to actively respond to ampicillin exposure, we determined the cellular concentration of the second messenger following ampicillin challenge of the wild type. The results showed no significant change relative to untreated cells (Fig. 4), indicating that the changes observed in the Δ*croRS* strain were most likely a consequence of wider physiological changes in the cell upon deletion of *croRS* and not specifically linked to an ampicillin response.

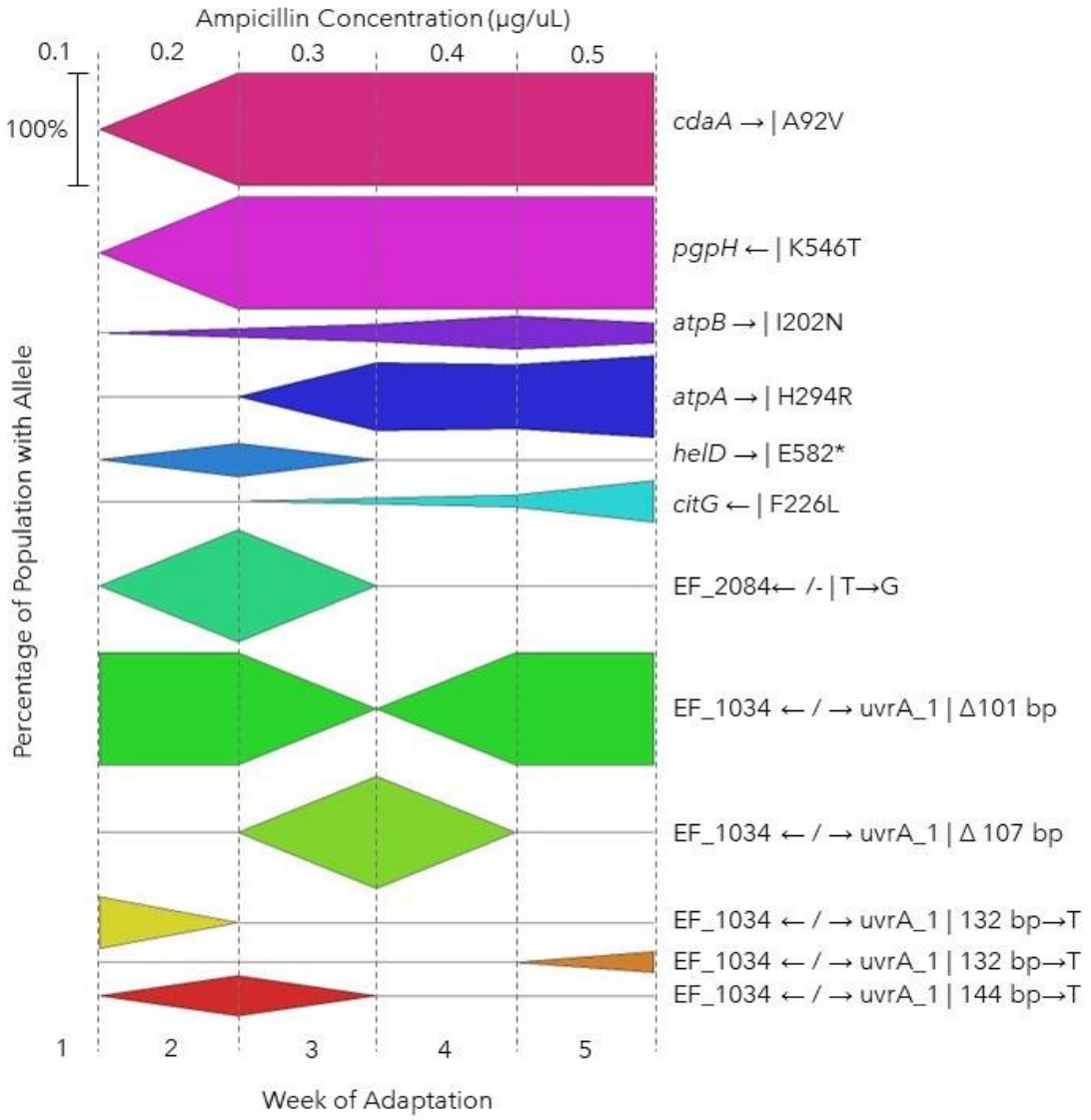

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Figure 3. Mutation of *cdaA* and *pgpH* occurs at the second stage of evolution for ampicillin resistance. The figure shows the evolutionary dynamics of twelve selected alleles associated with evolution of ampicillin resistance. The frequency of each mutation was quantified using Breseq and plotted as a function of time during the experiment on the lower *x*-axis. Dotted lines depict the stepwise changes in ampicillin concentration, which are labelled on the upper *x*-axis. The *y*-axis dimension of each symbol colour indicates the percentage of the population containing the SNP/Indel with a scale bar for 100% shown. Labels to the right give the details for each mutation, with gene orientation indicated by arrows. Changes in coding regions are given as the resulting amino acid substitution. Changes in intergenic regions are shown by arrows separated by a slash and base changes or deletions are given. The gene name is indicated where confirmed from the JH2-2 genome. Where the gene was unknown, the locus tag is given. Where no prediction was available, a dash is indicated (-). The rationale for selection of alleles to depict is explained in the Methods section, with a complete list of changes given in Table 2.

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Although we had observed increased levels of c-di-AMP in Δ*croRS*, this alone did not allow us to differentiate between whether c-di-AMP was high due to the absence of CroRS *per se*, or whether the changes in second messenger levels were related to the high cell envelope stress experienced by the Δ*croRS* strain (Fig. 1). To untangle this, we utilised one of the Δ*croRS* strains evolved earlier for faster growth mentioned above (17). This evolved strain, named 4BS, had a single SNP in the gene *hepS*, likely funnelling isoprenoid metabolism preferentially towards cell envelope biogenesis. Importantly, this mutation had also restored the cell envelope stress level, reported by the P_{liaX}-*lacZ* construct, to wild-type levels (17). Given that the 4BS strain did not possess mutations in genes involved in c-di-AMP metabolism, it provided an ideal opportunity to test for the potential reasons for the high second messenger concentrations in Δ*croRS*. Interestingly, 4BS showed the same reduction of c-di-AMP concentrations to wild-type levels as the ampicillin-evolved strains, despite its absence of mutations in relevant genes. Thus the degree of cell envelope stress and the cellular concentration of c-di-AMP appeared to be directly correlated in our strains, regardless of whether the evolution experiment had led to restoration of cell envelope biogenesis (4BS) or directly affected c-di-AMP metabolism (1AMP and 2AMP). Potential explanations for this are discussed in more detail below.

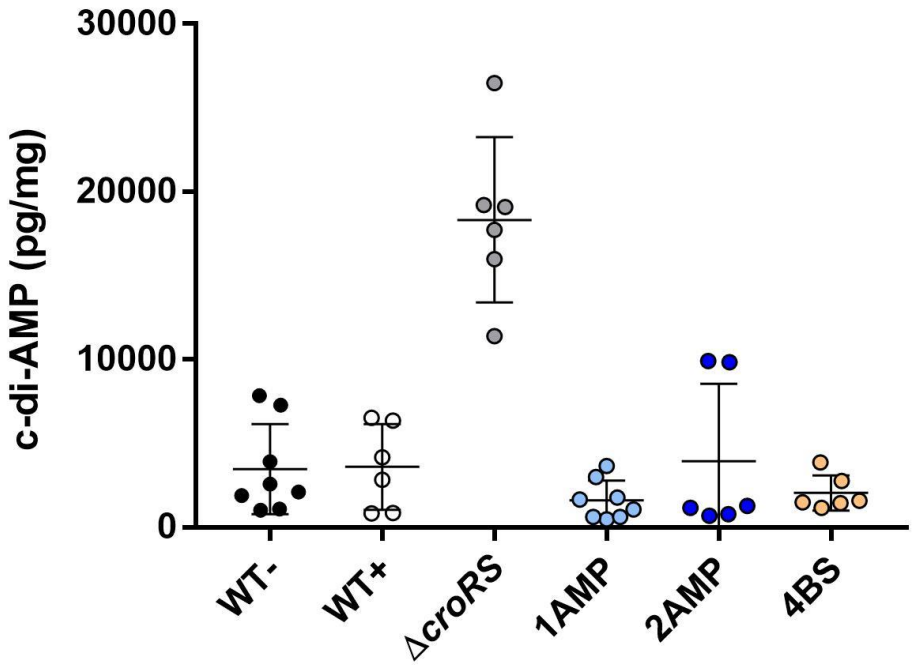

Figure 4. Deletion of *croRS* results in an increase in c-di-AMP, which is returned to wild-type levels in 1AMP and 2AMP. Cells of the *E. faecalis* strains indicated were grown overnight in BHI medium, cell extracts were prepared, and intracellular c-di-AMP levels were determined by ELISA. Nucleotide levels were normalised to protein concentration in the cell extracts and are expressed as pg c-di-AMP per mg protein. Data are plotted as individual data points from two to three independent experiments with two to three dilutions per cell extract and each dilution assayed in either duplicate or triplicate. Results are plotted together with means and standard deviations.

5.5 Discussion

In this study, we aimed to establish the role of the TCS CroRS in β-lactam resistance of *E. faecalis* by evolving three independent Δ*croRS* lines to a 10-fold increase in ampicillin concentration. Phenotyping revealed that adaptation had restored ampicillin resistance to near wild-type levels. We also observed that despite a reduction in cell envelope stress, there had been no major improvement in growth rate compared to the parent strain. Upon sequencing the evolved strains, we observed mutations in genes involved in regulating the concentration of c-di-AMP as the only commonality between all three lineages, which prompted us to investigate the cellular concentration of the secondary messenger. Surprisingly, we identified a ~6-fold increase in c-di-AMP in the absence of *croRS* compared to the

wild type, which the suppressor mutations acquired by strains 1AMP and 2AMP had returned to wild-type levels.

The observed mutations in genes linked to second messenger production to suppress the ampicillin-sensitivity of the *croRS* deletion strain suggested a link between CroRS signalling and c-di-AMP, however the exact relationship remains unclear. When comparing the strains evolved for ampicillin resistance against a previous Δ*croRS* derivative, strain 4BS, evolved for faster growth, we observed that under both selection pressures cell envelope stress, c-di-AMP levels and ampicillin resistance have all returned to normal levels. This implied that the routes *E. faecalis* takes to restore the effects of *croRS* deletion differ depending on the selection pressured applied, but lead to a similar outcome in terms of phenotype. This was interesting, because 4BS contained no mutations in *cdaA*, *pgpH* or *gdpP* , the genes responsible for c-di-AMP synthesis and degradation, and thus the restoration of wild-type second messenger levels in this strain must have been an indirect consequence of its suppressor mutation in *hepS*, affecting isoprenoid metabolism. To unpick the apparent link between the cellular stress experienced by the Δ*croRS* mutant, the increased c-di-AMP levels, and the degree of ampicillin resistance, we therefore considered the phenotypic differences between 4BS and 1AMP:

The parental strain Δ*croRS* is characterised by poor growth, a broad spectrum of sensitivity against cell envelope-active antibiotics, a high degree of cell envelope stress and increased concentrations of c-di-AMP. Evolution for fast growth in strain 4BS restored all of these phenotypes, whereas the mutations in 1AMP only restored β-lactam resistance, cell envelope stress and the c-di-AMP levels (Fig. 5). As stated above and in our previous study, the *hepS* mutation in 4BS likely restored a deficit in cell envelope biogenesis resulting from a shortage of undecaprenyl-pyrophosphate as the lipid shuttle for precursors needed extracellularly (17). In contrast, the only common mutations in the ampicillin-evolved strains all appeared to directly address the increase in c-di-AMP. The most plausible explanation for the different phenotypes then is that the root cause of the phenotypes of Δ*croRS* lies in its deficit in cell envelope biogenesis, with the elevated c-di-AMP levels one of its consequences. In turn, the increase in the second messenger concentration then appears to be the reason for the intrinsic cell envelope stress of the Δ*croRS* strain, as well as at least a partial cause of its β-lactam sensitivity.

This would explain why *hepS* mutation corrects all phenotypes, but mutations of the c-di-AMP metabolic genes only restore the two latter ones (Fig. 5, red vs blue lines). It should be noted that the c-di-AMP metabolic genes in 1AMP acquired their mutations early in the evolution experiment at the stage where a four-fold increase in ampicillin MIC had been achieved (Fig. 3). While we have no data on the c-di-AMP concentration in cells at that point, it is likely that the full restoration of ampicillin resistance to wild-type levels required acquisition of further mutations, e.g. the genes mentioned above for cell wall biosynthesis or central carbon metabolism. Nevertheless, our findings suggest that as in other Firmicutes bacteria, altering c-di-AMP levels is linked to cell envelope integrity with a specific connection to β-lactam resistance.

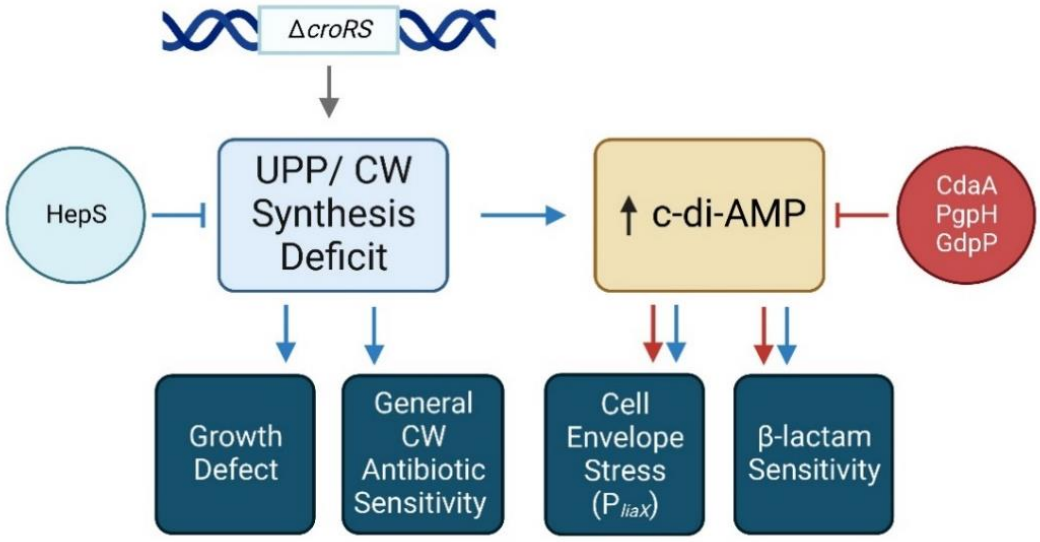

Figure 5. Model of the relationship between croRS and c-di-AMP. The deletion of *croRS* results in loss of regulation of several enzymes involved in the production of peptidoglycan precursors, which therefore results in a decrease in UPP synthesis, and a cell wall (CW) biosynthesis defect. This then results in a severe growth defect and increased sensitivity to cell-wall acting antimicrobials. Additionally, this deficit in cell wall biosynthesis also results in an increase in c-di-AMP levels. This increase in c-di-AMP levels then causes an increase in cell envelope stress, as measured through P_{liaX} expression, and increased β-lactam sensitivity. As explored in our previous work, the acquisition of a HepS mutation is able to restore UPP synthesis via the funnelling of isoprenids towards cell wall biosynthesis. This is able to restore wild-type growth, cell envelope stress, antibiotic resistance and c-di-AMP levels. Conversely, the work in this study has shown that mutations in CdaA, PgpH or GdpP are able to restore c-di-AMP levels, but not the cell wall biosynthesis defect. This results in a decrease in cell envelope stress and the restoration of β-lactam resistance, but the sensitivity to cell-wall acting antimicrobials and growth defect are not restored to wild-type levels.

Measuring the levels of c-di-AMP allowed some speculation on the potential effects of the mutations on both *cdaA*, *pgpH* and *gdpP*. From the data collected for 1AMP, the decrease in c-di-AMP likely was a result of a loss-of-function mutation in *cdaA*, thus reducing c-di-AMP synthesis. For the phosphatases, however, the effect was less clear. All three evolved strains contained mutations in *pgpH*, and both 2AMP and 4AMP contained mutations in both phosphatases, all of which were missense mutations or the introduction of a STOP codon. Surprisingly, in 2AMP, which contained a STOP codon in both *gdpP* and *pgpH*, the level of c-di-AMP was still lower compared to the parent strain. While we initially interpreted STOP codon mutations as a loss of function of the encoded protein, the drop in c-di-AMP levels in this strain may suggest that at least one of these mutations had in fact caused a gain of function. This could be conceivable for *gdpP*, where the STOP occurred late in the protein at amino acid position 446 of 658, in contrast to *pgpH*, where a STOP mutation was introduced early at amino acid position 67 of 732. Although the mutation in *gdpP* fell into the region of the protein predicted to contain the phosphodiesterase active site, it could potentially have led to a truncation that increased rather than decreased enzymatic activity. Without further biochemical evidence available, this has to remain speculation at this time, but will be an interesting question to explore in the future.

Since its discovery 10 years ago, c-di-AMP has been termed "the essential poison", due to it being both essential and toxic to many bacteria (30, 40). The second messenger has also been shown to have far-reaching consequences within the cell, most notably in osmoregulation and solute accumulation (47). Potassium (K^+) and glutamate are the most abundant cation and anion, respectively, in every living cell and require intricate balancing (48). In *B. subtilis*, c-di-AMP has been shown to play an important role in this balance via directly regulating intracellular K^+ concentration and maintaining osmoregulation within the cell. GlnQ is responsible for the transport of glutamine into the cell and is known in other Firmicutes to respond to the intracellular level of K^+ to regulate cellular solute accumulation (49). Therefore, c-di-AMP can indirectly control glutamate intake via regulating the activity of GlnQ. Strikingly, as mentioned in the introduction, CroR has been shown to bind the promoter of the glutamine transporter GlnQ (50) and therefore may well also play a role in osmoregulation in *E. faecalis*.

In addition to osmoregulation, c-di-AMP has also been implicated in a variety of other functions, such as peptidoglycan synthesis and β-lactam resistance (51). Whereas osmoregulation has been linked to the 'lethality' aspect of c-di-AMP accumulation, the 'essentiality' property comes from the role of the second messenger in peptidoglycan synthesis. Amongst the Firmicutes, the c-di-AMP synthase CdaA is frequently encoded in an operon with cell wall biosynthesis enzyme GlmM (49). It has now been identified in *S. aureus* that GlmM regulates c-di-AMP production via direct interaction with CdaA and that this affects peptidoglycan precursor synthesis (52). Furthermore, it was recently shown in *L. monocytogenes* that c-di-AMP accumulation impairs muropeptide synthesis by inhibiting K^+ transport and inhibiting the activity of Ddl, a ligase which catalyses the conjugation of two D-alanine molecules to form the D-alanyl-d-alanine dipeptide essential for cell wall biosynthesis (53). Our observation that loss of CroRS and the resulting defects in cell envelope biogenesis lead to an increase in cellular c-di-AMP levels suggests that similar functional links exist in *E. faecalis* as well.

There is currently a growing body of evidence which suggests a correlation between c-di-AMP levels and β-lactam resistance (54,55). For example, clinical β-lactam resistance in *S. aureus* has been identified frequently to be due to a loss-of-function mutation in *gdpP*, producing an increase in c-di-AMP levels (56). *B. subtilis* has also been reported to display increased β-lactam resistance when carrying a mutated *gdpP* gene (54). This indicates that cell wall changes may have occurred in strains with high c-di-AMP or, conversely, changes in osmoregulation provide protection against cell wall active compounds (57). As high levels of c-di-AMP are known to cause the dysregulation of osmolytes, it is plausible a link exists between osmotic balance of the cell and its sensitivity to cell wall active compounds. In this context, our findings were somewhat surprising in that the increase of c-di-AMP in the *ΔcroRS* strain was associated with a decrease in β-lactam resistance rather than an increase. Interestingly however, a similar phenotype has also been identified in *Listeria monocytogenes,* whereby a decrease in c-di-AMP contributed to β-lactam resistance (56). As stated above, for this second messenger it is essential the cell achieves the right balance and both too high and too low concentrations are detrimental. Therefore, our data are consistent with a model where *croRS* deletion leads to dysregulation of c-di-AMP, responding to some unknown factor linked to defects in cell envelope

biogenesis. Occurrence of suppressor mutations that reduce the c-di-AMP concentrations to physiological levels then might explain the observed restored resistance to ampicillin.

In conclusion, the findings reported here point towards an intricate relationship between cell envelope biosynthesis, c-di-AMP production and cell wall antibiotic resistance in *E. faecalis*, which relies on correct functioning of the CroRS regulatory system. It will be interesting to investigate further if there are direct regulatory links between two-component and second messenger signalling in enterococci and gain deeper mechanistic insights into those functional connections this work has revealed.

5.6 Supplementary

S1. Bacterial strains utilised in this study.

Bacterial Strains - *E. faecalis*	**Description**	**Source**
JH2-2	Laboratory strain, plasmid-free; rif^r; fs^r	(58)
Δ*croRS*	JH2-2 carrying an unmarked deletion of the operon	(59)
1AMP	Strain derived from the adaptation of Δ*croRS* to 0.5 μg/uL	This study
2AMP	Strain derived from the adaptation of Δ*croRS* to 0.5 μg/uL	This study
4AMP	Strain derived from the adaptation of Δ*croRS* to 0.5 μg/uL	This study
4BS	Strain derived from improved growth of Δ*croRS*	Todd, *in prep*

Table S2. The growth rates of *E. faecalis* strains.

Strain	Growth rate (h^{-1})
JH2-2	1.483 ± 0.041
Δ*croRS*	0.575 ± 0.132
1AMP -	0.0966 ± 0.711
2AMP -	1.13 ± 0.058
4AMP -	1.098 ± 0.017
1AMP +	1.13 ± 0.161
2AMP +	1.14 ± 0.094
4AMP +	1.185 ± 0.071

[1]Results are means from three independent cultures; standard deviation between replicates is indicated.

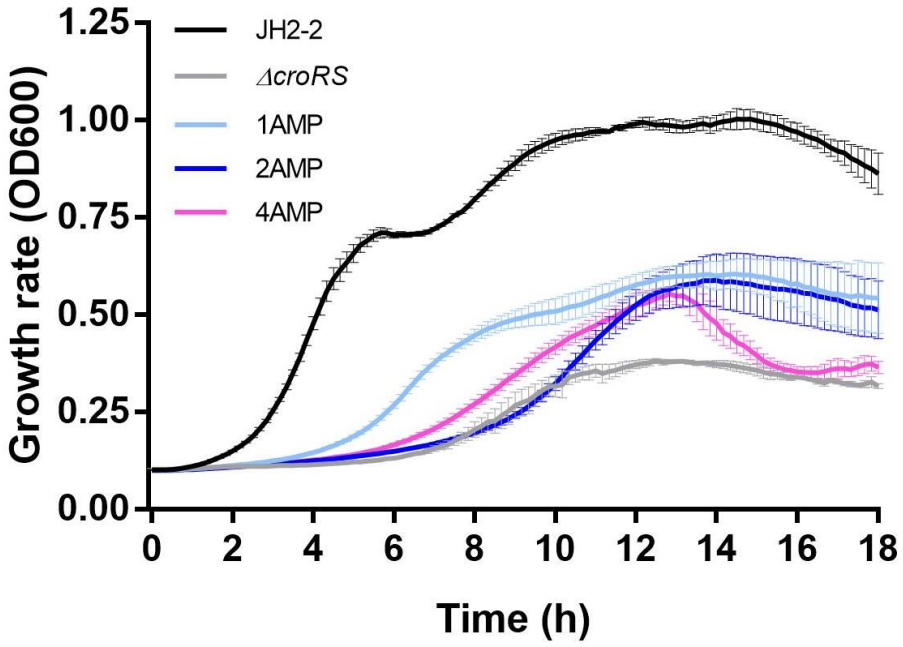

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Figure S1. Growth rates of ampicillin adapted strains. Overnight cultures of *E. faecalis* JH2-2, Δ*croRS* and 1AMP, 2AMP and 4AMP were diluted in fresh BHI and inoculated to an OD of 0.01 in 96-well plates. Cells were then incubated in a Tecan Spark and growth was monitored as optical density (OD_{600}) with readings taken every 10 minutes for 10 hrs. The results are taken from three independent replicates.

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Table S3. 1AMP mutations acquired at each stage of adaptation to ampicillin. Arrows in the Mutation Location column indicate the read direction of the gene the change occurs in. A forward slash ("/") Indicates an intergenic mutation; in these cases, arrows indicate the read direction of the nearest annotated gene(s). Percentage of population indicates the prevalence of a mutation at the end of each week of the evolution process.

Mutation Location	Distance from End of Contig	Change		% of Population with Mutation at Generation					Annotation
		Base(s)	Amino Acid	Gen1	Gen2	Gen3	Gen4	Gen5	
EF_1034[3] ← / → *uvrA_1* (WP_002387049[6])	5,653	Δ101 bp	-	100	100	Δ	100	100	EF_1034 hypothetical protein contains aminoglycoside phosphotransferase domain. *uvrA_1* encodes a subunit of the UvrABC DNA lesion recognition & repair system.
	5,653	Δ107 bp	-	-	-	100	-	-	
	5,647	Δ132 bp → T	-	45.9	-	-	-	-	
	5616	Δ144 bp → T	-	-	36.2	-	-	-	
	5628	Δ132 bp → T	-	-	-	-	-	18.3	
	5879	+96 bp	-	-	-	-	-	13.0	
	5861	+102 bp	-	6.8	-	-	-	-	
	5646	+96 bp	-	-	-	-	6.2	-	
pheT_2 (WP_002382113[6]) → / ← *glnP_2* (WP_002382114[6])	16479	T→G	-	100	100	100	100	100	*pheT* encodes phenylalanine tRNA ligase β subunit. *glnP_2* encodes a putative glutamine ABC transporter permease protein.
EF_2771[3] → / ← EF_1366[3]	26537	A→T	-	100	100	100	100	100	EF_2771 contains pfam TraX, responsible for amino-terminal acetylation of F-pilin subunits. EF_1366 contains DUF218.
WP_002357562[5] ←	49357	Δ72 bp	-	100	100	100	100	100	Encodes glucosaminidase domain-containing protein.
glpE_1 (WP_010773450[6]) → / → AAO80567[5]	3506	C→A	-	100	100	100	100	100	*glpE* is a Thiosulfate sulfurtransferase. Hypothetical protein encodes member of cell wall anchor family.
yttA (WP_002367468[6]) → / ← EF_3174[3]	14289	A→C	-	100	100	100	100	100	*yttA* encodes a putative membrane protein.

									EF_3174 is an NAD-dependant oxidoreductase.
/ ← *ltaS1_2* (WP_010773748[6])	241	C→T	-	100	100	100	100	100	Encodes lipoteichoic acid synthase 1.
lytG (WP_002366997[6]) ←	25349	C→A	G111W	100	100	100	100	100	Encodes exo-glucosaminidase.
bgsB[4] (WP_010706866[6]) ←	2458	C→A	G4V	100	100	100	100	100	Encodes α-monoglucosyldiacylglycerol synthase.
← *clsA_2*[1] (WP_002361214[6])	11	G→T	-	100	100	100	100	100	Encodes a major cardiolipin
	17	T→A	-	100	100	100	100	100	synthase.
clsA_2[1] (WP_002361214[6]) ←	985	C→A	L190F	-	-	-	-	6.0	
WP_002380514[5] →	3556	T→G	- F255V	100	100	100	100	100	Hypothetical protein contains MATE-like region, involved in multidrug and toxic compound extrusion.
23S Ribosomal RNA[4] ← / ← 16S	3284	Δ100 bp → GTT	-	100	100	100	100	100	
Ribosomal RNA[4]	3139	A→G	-	24.7	25.8	25.9	23.1	24.1	
EF_2084[3] ← / -[1]	83	A→T	-	92.2	92.1	93.5	92.3	100	Aligned sequence appears
	62	T→G	-	-	100	-	-	-	throughout bacterial kingdom
	50	T→C	-	5.7	5.7	5.4	5.7	-	but has no features identifiable
	20	T→C	-	-	-	-	5.6	-	through SMART database.
EF_1253[3]← / ← EF_1251[3]	5043	139 bp x2	-	46.9	40.8	46.0	50.6	47.9	EF_1253 contains an
	4760	G→T	-	-	-	-	12.7	-	uncharacterised ABC-type
	11684	T→A	-	5.5	-	-	5.9	-	transporter.
									EF_1251 is an uncharacterised hypothetical protein.
/ → *phoP_2*[1] (WP_002355963[6])	188	C→T	-	61.0	61.8	61.6	60.5	60.7	Encodes Alkaline phosphatase
	271	G→A	-	36.8	33.1	35.2	32.0	36.1	synthesis transcriptional
	275	G→T	-	36.8	33.1	35.2	31.9	36.1	regulatory protein.
	170	G→A	-	32.9	32.5	32.8	30.5	31.0	
	151	A→T	-	28.3	30.0	31.0	32.6	31.3	
	141	G→T	-	28.4	29.5	30.1	31.5	31.2	
	131	T→C	-	27.9	30.0	30.3	32.0	31.4	
galE_1 (WP_010706835[6]) ←	42999	G→A	I12I[2]	14.4	16.4	15.9	17.8	14.9	

	43143	A→G	S60S[2]	53.1	51.8	51.7	47.6	54.1	Encodes UDP-glucose 4-epimerase, involved in galactose metabolism pathway.
	43155	C→T	K64K[2]	53.3	53.2	52.5	46.4	54.5	
	43284	G→A	F107F[2]	50.2	54.6	54.9	50.5	58.0	
	43362	G→T	T133T[2]	48.9	56.9	53.1	50.6	50.3	
	43380	A→G	N139N[2]	49.7	55.3	53.9	51.1	49.5	
	43479	T→C	A172A[2]	49.3	51.4	50.0	51.1	47.3	
	43638	G→A	Y225Y[2]	-	6.2	6.1	6.7	9.8	
23S Ribosomal RNA[4] ←	2386	A→T	-	24.9	23.7	21.7	25.9	26.9	
	2444	C→T	-	25.9	25.0	22.7	25.5	28.2	
	2378	G→A	-	47.9	45.4	45.8	49.2	50.1	
/ → *acpS*[1] (WP_002379159[6])	128	T→C	-	61.6	62.6	64.2	64.0	66.8	Holo-[acyl-carrier-protein] synthase, essential in lipid synthesis.
	150	+A	-	7.3	10.3	13.3	10.3	8.8	
	151	T→G	-	6.5	9.1	8.5	7.6	7.7	
/ → EOT49873[1, 4]	421	A→C	-	44.4	41.6	40.4	46.3	43.3	IS-3 family transposase found in retroviruses and transposable elements
	362	G→T	-	46.8	42.6	43.2	48.1	42.5	
	187	A→G	-	44.2	44.8	42.7	45.1	40.1	
EOT49873[1, 4]→	474	A→G	S8S[2]	41.6	43.3	40.4	46.4	45.5	
	371	A→G	K93R	43.9	44.1	44.9	42.2	45.4	
	289	A→G	T120T[2]	42.6	47.6	42.5	40.9	46.1	
16S Ribosomal RNA[4] ←	326	T→C	-	24.1	26.3	21.4	25.7	25.1	
	1740	G→T	-	19.6	21.3	21.5	21.3	22.4	
pgpH (WP_002356744[6]) ←	24566	T→G	K546T	-	100	100	100	100	Encodes HD domain-containing protein, degrades cyclic-di-AMP.
cdaA (WP_002389035[6]) →	16549	C→T	A92V	-	100	100	100	100	Encodes cyclic-di-AMP synthase.
atpB (WP_002356551[6]) →	10677	T→A	I202N	-	7.5	16.1	29.8	18.5	Encodes ATP Synthase subunit A.
EF_0241[3] ← I ← *brnQ* (WP_002361550[6])	28833	A→T	-	11.1	-	-	-	-	EF_0241 hypothetical protein contains a heavy metal-binding domain. *brnQ* encodes a branched-chain amino acid transport system carrier protein.

EF_2684[3] → / → *elrD*[4] (WP_002362542[6])	3517	G→C	-	10.5	-	-	-	-	Both EF_2684 and *erlD* sequences contain WxL cell surface wall-binding domain
cydC[4] (EOT49687[6]) →	38305	A→T	I582F	8.2	-	-	-	-	Encodes Thiol reductant ABC
	38302	A→T	S583C	-	6.5	6.5	-	-	exporter subunit C
licC_5 (WP_002363934[6]) → / -[1]	70	T→A	-	5.6	5.3	6.2	9.4	-	Encodes permease IIC
	113	A→T	-	-	5.6	7.2	8.1	5.5	component
	110	A→T	-	7.1	-	6.7	5.5	-	
	68	C→A	-	-	7.0	-	6.7	-	
	80	G→T	-	-	-	-	5.9	-	
tRNA-arg[4] → / ← *tipA_1* (WP_002358872[6])	2216	A→T	-	5.7	-	5.7	-	-	*tipA_1* encodes HTH-type transcriptional activator.
EF_3075[3]	38054	A→T	T569T[2]	9.0	-	-	-	-	WxL cell surface wall-binding domain-containing protein
ypdA (WP_002354827[6]) ← / ← *metQ_3* (WP_002387408[6])	5829	C→G	-	7.4	-	-	-	-	*ypdA* encodes a sensor histidine kinase. *metQ_3* encodes D-methionine-binding lipoprotein.
AAO81449[5] → / ← *mhqD_1* (WP_002361880[6])	21739	C→A	-	5.7	-	-	-	-	Hypothetical protein encodes zinc-binding oxidoreductase. *mhqD_1* encodes putative hydrolase.
helD (EF_0933[6]) →	30849	G→T	E582*	-	29.5	-	-	-	Encodes DNA helicase IV.
arlS (WP_002363430[6]) → / -[1]	130	A→G	-	6.5	14.8	9.5	-	7.9	Encodes signal transduction
	150	C→A	-	7.6	14.7	9.9	-	7.9	histidine-protein kinase.
	127	T→C	-	6.5	14.7	9.5	-	7.9	
	140	C→A	-	6.5	14.5	9.1	-	7.6	
lipC (WP_002365717[6]) →	11448	C→G	I16M	-	13.9	-	-	-	Encodes spore germination lipase.
EF_1082[3] ←	8358	G→C	L46V	-	5.6	-	-	-	Uncharacterised hypothetical protein.
ebgA (AAO82413[6]) ←	7153	T→A	D666V	-	5.0	-	-	-	Encodes α subunit of evolved β-galactosidase
atpA (WP_002356555[6]) →	13168	A→G	H294R	-	-	60.7	57.6	73.3	Encodes ATP synthase subunit α
	13036	T→G	M250R	-	-	7.6	-	-	

WP_002360384[5] ←	15744	C→G	D174H	-	-	6.5	-	-	Uncharacterised hypothetical protein.
citG (WP_002379282[6])[1] ←	430	A→C	F226L	-	-	5	10.4	37.2	Encodes 2-(5’’-triphosphoribosyl)-3’-dephosphocoenzyme-A synthase
liaF[5] (WP_002381168[6]) ← / ← *greA* (WP_002355133[6])	17871	C→G	-	-	-	10.7	-	-	*liaF* encodes cell wall-active antibiotics response protein. *GreA* encodes a transcription elongation factor
lysM[5] (WP_002379724[6])[1] ←	17	A→C	H267Q	-	-	7.4	-	-	Encodes peptidoglycan-binding protein
ettA ←	40319	G→T	Q261K	-	-	6.1	-	-	Encodes energy-dependent translational throttle protein
EF_1137[3] ←	31183	G→T	S55I	-	-	-	13.4	-	Sequence contains pfam Intracellular septation protein (IspA).
rpoE →	23844	Δ99 bp	-	-	-	-	11.2	-	Encodes δ subunit of putative DNA-directed RNA polymerase
EF_0184[3] → / → *deoB*	59100	G→C	-	-	-	-	10.6	-	EF_1084 is an uncharacterised hypothetical protein. *deoB* encodes a phosphopentomutase.
ugpA ← / ← *msmE*	6855	C→G	-	-	-	-	9.4	-	*ugpA* encodes sn-glycerol-3-phosphatase transport system protein. *msmE* encodes a multiple sugar-binding protein
WP_002354864[6] ← / ← *rny*	8431	G→C	-	-	-	-	-	13.6	Hypothetical protein encodes a
	8426	T→A	-	-	-	13.2	-	-	TIGR00282 family
	8427	C→A	-	-	-	-	8.0	-	metallophosphoesterase.
	8437	T→A	-	-	-	6.7	-	-	*rny* encodes ribonuclease Y
AAO81950[5] ←	27304	C→G	T562T[2]	-	-	-	-	12.6	Encodes sensor histidine kinase
atpE_1 →	11048	C→T	L70F	-	-	8.7	7.3	10.7	Encodes ATP synthase subunit C

efrB[4] ←	24220	+15 bp	-	-	-	-	-	9.4	Multidrug efflux ABC transporter subunit
EF_2796[3] ← / ← EF_2797[3]	32145	A→T	-	-	-	-	-	6.4	EF_2796 sequence aligned by match to pfam PF03379. EF_2797 is unannotated hypothetical protein.
WP_002357954[5] ←	7273	G→T	S173R	-	-	-	-	5.1	Encodes *yutD* family protein.

[1]Mutation occurs within 1,000 bp of the start or end of a contig and may not be reliable. [2]Silent mutation.
[3]Hypothetical protein according to PROKKA annotation, mapped to *E. faecalis* V583.
[4]Hypothetical protein according to PROKKA annotation, since identified via NCBI BLAST.
[5]NCBI protein database accession number of closest aligned sequence (via NCBI BLAST) for hypothetical protein (according to PROKKA annotation). Sequence aligns only to multispecies entries or has no annotation in reference strain V583.
[6]NCBI protein database accession number of closest aligned sequence (via NCBI BLAST) to gene annotated by PROKKA.

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6. Final Conclusions

Enterococcus faecalis is a prominent nosocomial pathogen, associated with increasing antibiotic resistance and responsible for worldwide infections. Despite an in-depth knowledge of the mechanisms of acquired resistance, we still lack an understanding of the intrinsic mechanisms of antimicrobial resistance. Therefore, the aims of this study were to deepen our knowledge of the *E. faecalis* cell envelope stress response to gain an understanding of the complex setup of the system.

Throughout this work, I have demonstrated the role of the cell envelope stress response in protecting *E. faecalis* against antimicrobial damage. In chapter 2 (part A), I explored the bacitracin resistance network and discovered the regulation of SapRS by LiaR, uncovering an interplay between the Lia and Sap systems. Additionally, I also discovered the regulation of *dltABCD* by SapR, and showed its expression in response to bacitracin. I observed that the expression of *dlt* is under dual control, requiring damage to the cell envelope sensed by LiaFSR and the substrate-specific activation of SapAB – the effective implementation of a logic 'AND' gate. In chapter 2 (part B), I described two new methodologies for the study of *E. faecalis*; the use of *Manduca sexta* as a model host and the use of a CRISPR-Cas9 system to produce genetic mutations. My optimisation of the caterpillar model showed the successful colonisation of the *M. sexta* gut with *E. faecalis*, and I was able to recommend the use of faecal sampling and increased sample size for future work. Despite being unsuccessful in creating *E. faecalis* genetic mutations, I was able to pinpoint difficulties with the VDM1001 plasmid and provide a number of recommendations for future development.

One of the most surprising findings of this work was the observation that daptomycin was unable to induce *dlt* expression result, as is commonly observed throughout the Firmicutes. In *S. aureus, dlt* expression is induced by the GraRS TCS[1] which results in DAP resistance through a 'repulsion' mechanism[2], whereby the expression of *dlt* decreases the negative charge of the cell envelope, leading to the repulsion of cationic antimicrobials. This contrasts the mechanism in *E. faecalis*, which utilises a 'diversion' mechanism to relocate DAP's target binding away from the septum through phospholipid rearrangements [3]. This diversion occurs through the ordered progression of mutations in *LiaF*, *gdpP* and *cls*[4], with the latter shown to be responsible for phospholipid rearrangement in the presence of

DAP[5]. Therefore, the observed lack of *dlt* expression potentially explains the absence of a repulsion mechanism utilised by *E. faecalis*.

A similar diversion mechanism is also utilised by *E. faecium*[6]. However, recent work has demonstrated that *E. faecium* can utilise repulsion via *dlt* expression to become DAP resistant[7] and a study in Australasia reported mutations in *dltC* corresponding to DAP resistance[8]. We therefore propose that in *E. faecalis*, long-term DAP challenge results in mutations in *liaFSR*, *cls* and/or *gdpD* which result in a diversion mechanism. If the DAP challenge exceeds the limit which this protection provides or in the absence of a functional Lia system, mutations occur in *sapAB*, which result in *dlt* expression, and a repulsion mechanism is utilised. This mechanism would provide an additional level of protection to the cell and account for the complex network by which both LiaFSR and SapAB regulate *dlt* expression.

Future work should examine the relationship between Lia and Sap systems further. In addition to *dlt*, the potential of LiaR to induce the expression of *rapAB*, also within the SapR regulon should be examined. Through the utilisation of a P_{rapAB}-*lacZ* transcriptional fusion, it should be determined if the target protection potentially provided by LiaFSR extends to *rapAB*. In addition, as discussed in chapter 2, clinically observed mutations in LiaFSR are known to cause the hyperactivation of LiaR [4,9]. Through the construction of these clinical mutations, potentially through use of the CRISPR-Cas9 *E. faecalis* system, the effect of this hyperactivation on *dlt* expression could be examined. This would not only allow us to confirm the regulation of *dlt* by LiaR but would also allow us to determine if the presence of this LiaR mutation is able to override the logic 'AND' gate and induce *dlt* expression in the presence of DAP.

In chapter 3, together with collaborators, I explored the teixobactin-induced CroRS regulon, to understand the genes upregulated by this TCS in response to antibiotic challenge. Through transcriptome analysis, we showed a loss of induction in genes involved in cell wall synthesis in the absence of *croRS*. I validated these findings through a parallel approach by evolving the *croRS* deletion strain for improved growth, which demonstrated a consistent suppressor mutation in *hepS*. I proposed this mutation resulted in the funnelling of isoprenoid synthesis preferentially towards cell envelope synthesis, reducing the use of isoprenoids in menaquinone synthesis.

In a continuation of this work, in chapter 3 I again utilised experimental evolution, but this time by adapting strains of Δ*croRS* to increasing ampicillin concentrations. This work showed that in order to restore β-lactam resistance, suppressor mutations occurred in genes involved in c-di-AMP metabolism. I showed that these mutations were able to restore c-di-AMP levels to wild-type levels, along with a reduction in cell wall stress and the restoration of β-lactam resistance. Interestingly however, these c-di-AMP mutations were not able to restore generic resistance to a number of cell-wall acting antibiotics or restore wild-type growth.

It was an intriguing observation that upon both antibiotic and growth rate selection pressures, a range of suppressor mutations were observed. As the mutations in c-di-AMP metabolism occurred in the presence of ampicillin pressure and not in the growth-rate selection this suggests c-di-AMP regulation is intricately linked to β-lactam resistance. To investigate this further, future work should repeat the adaptation to ampicillin, but utilising the wild-type strain in place of Δ*croRS* to establish if the suppressor mutations in c-di-AMP metabolism are directly linked to the deletion of *croRS* or a generic response to increasing ampicillin exposure.

Interestingly however, a common mutation did occur upon both selection pressures, which was in the putative autolysin EF2367. The fact that this mutation appeared in both experiments suggests the enzyme is fundamentally associated with CroRS. As the enzyme is involved in the turnover of the bacterial cell wall, this adds to the hypothesis that *croRS* is involved in the regulation of enzymes tasked with the synthesis of the cell envelope. However, the transcriptome analysis of chapter 3 demonstrates however that EF2367 is not within the CroRS TXB-induced regulon. Whilst transcriptome analysis provides a good overall picture, the use of a transcriptional promoter fusion would allow more in-depth analysis of EF2367 expression in both the presence and absence of *croRS* and under differing antibiotic conditions.

The transcriptome analysis also revealed the down regulation of cell wall synthesis genes in the absence of *croRS*. Further analysis should investigate the expression of specific genes involved in cell wall synthesis, such as *mvaD*, a gene in the mevalonate pathway which was downregulated in the absence of *croRS*, to determine the exact regulation exerted by CroR on this process.

Our work into improving the growth phenotype of Δ*croRS* identified a mutation in *hppS*, a gene involved in isoprenoid synthesis. Interestingly, a similar experiment in *S. aureus* which aimed to improve the growth phenotype of a *cdaA* deletion, which displayed a growth defect, identified a *hppS* mutation[10]. Alongside our work, this result suggests an intricate triangle between CroRS, c-di-AMP and cell wall synthesis. Future work should aim to untangle this complex relationship and investigate the individual contributions by the observed *cdaA*, *pgpH* and *gpdP* mutations. However, as each adapted strain contains at least two mutations involved in secondary messaging, it is a possibility one mutation cannot occur without the other, due to the intricate balance of c-di-AMP, thereby preventing the construction of individual mutations.

Enterococcus faecalis is a complex and fascinating microorganism, able to survive in a range of environments through its highly versatile nature. It is this very feature which has allowed *E. faecalis* to become a notorious pathogen, able to sense and adapt to a range of adverse conditions, including antimicrobial exposure. My work has not only demonstrated the complex and intriguing nature of the antibiotic resistance network within *E. faecalis,* but has also made significant progress in our understanding of this intricate system – however, many questions still remain unanswered. As we make strides in our understanding, we get ever closer to uncovering an Achilles' heel within the network – a essential component, for which no redundancy exists. Such a target would allow the creation of new antimicrobials or the repurposing of existing treatments, enabling the successful management of *E. faecalis* infections and preventing thousands of deaths every year.

To anyone who chooses to work on this pathogen, I wish you the best of luck.

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