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**THE MECHANISM OF RESISTANCE TO
CONJUGATED OLIGOELECTROLYTES IN
*ENTEROCOCCUS FAECALIS***

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INTERDISCIPLINARY GRADUATE SCHOOL
SCElse
2018

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OLIGOELECTROLYTES IN *ENTEROCOCCUS FAECALIS***

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Interdisciplinary Graduate School
SCElse

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Abstract

The growing problem of antibiotic resistance in bacteria, along with the dearth of new antibiotics, has led to the search for alternative antimicrobial therapeutic agents. Since the primary reason for the quest for novel antibiotics is to stay one step ahead of the emergence of resistance, knowledge on how bacteria become resistant to a new antimicrobial agent is necessary to facilitate the development of new antimicrobials.

Oligophenylenevinylene conjugated oligoelectrolytes (COEs) have π -conjugated backbones and terminal ionic pendant groups. They have a high affinity for the lipid bilayer and spontaneously intercalate into the membrane. The ability of phenylenevinylene COEs to perturb bacterial membranes varies according to the number of aromatic rings and the type of ionic fragment. It was observed that the COEs with pyridinium cationic groups, i.e. COE1-3Py, were more inhibitory than when tetraalkylammonium groups were present (i.e. COE1-3C). In this study, the antibacterial mechanism of these molecules was investigated along with the mechanism of resistance.

By exposing *Enterococcus faecalis* OG1RF to increasing concentrations of COE1-3C or COE1-3Py, we were able to experimentally evolve resistant strains EFC3C and EFC3Py, respectively. Whole genome sequencing of resistant isolates revealed mutations in the *liaFSR* three component regulatory system. Specifically, EFC3Py had a mutation in *liaR*, while EFC3C had a mutation in *liaF*. Fatty acid analysis of EFC3C and EFC3Py revealed an increase in the levels of cis-vaccenic acid and cyclopropane, both of which are associated with increase in membrane order. Transcriptomic analysis of wild type *E. faecalis* treated with COE1-3C and COE1-3Py indicated that the compounds induced a general stress response along with changes in the expression of membrane transporters. In the EFC3C and EFC3Py strains, gene expression analysis revealed that most of the genes induced or repressed in EFC3C were also altered in EFC3Py. In both mutants, EFC3C and EFC3Py, transporters of the ATP binding cassette superfamily were the most significantly induced or repressed genes compared to the wild type. Overall, the significantly upregulated and downregulated transporter genes were generally involved in transport of ferrichrome, lipoproteins export, amino acids,

riboflavin, mannitol/fructose, oligopeptide and choline. In addition, cell envelope response regulon (LiaFSR), *dlt* operon and penicillin binding proteins (PBPs) were also differentially expressed. The mutations in the *liaFSR* regulon correlate to the resistance to COEs as confirmed by the deletion and complementation studies.

Collectively, the data suggest that the COEs target bacterial membranes and disrupt membrane function by membrane perturbation. The transcriptomic response of *E. faecalis* to conjugated oligoelectrolytes revealed the mechanisms adopted to tolerate the membrane stress elicited by COEs. Additionally, COE1-3C and COE1-3Py appear to be promising antimicrobials against *E. faecalis* and that there is a high barrier to the emergence of severely resistant strains constrained by biological limits of membrane adaptation that can occur in *E. faecalis*.

1 Introduction

1.1 *Enterococcus faecalis* OG1RF – Overview

In 1899, Thiercelin proposed the name “*Enterocoque*” to describe a gram positive diplococcus of intestinal origin that he had observed (Thiercelin, 1899; Thiercelin, 1899). These facultative anaerobes belong to the phylum Firmicutes with low GC-content (Lebreton *et al.*, 2014). They are commensal bacteria that inhabit the gastrointestinal tract of humans, animals and insects (Van Tyne and Gilmore, 2014). In humans, they occur in the mucosal layer of the small intestine and its epithelial crypts (Sekirov *et al.*, 2010). They can survive in a broad range of environments, such as animals, plants, water, soil and food (Castillo-Rojas *et al.*, 2013). This is due to their ability to tolerate a wide range of temperatures (5⁰C to 50⁰C), osmotic stress and alkaline or acidic environments (Ramsey *et al.*, 2014; Huycke *et al.*, 1998). Of all the enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly associated with human enterococcal infections, with *E. faecalis* involved in 80-90% of human endodontic infections (Fisher and Phillips, 2009; Huycke *et al.*, 1998). *E. faecium* and *E. faecalis* grow in a pH range of 4.6 - 9.9 and can survive even in the presence of 40% (w/v) bile salts. In addition, the cation homeostasis of *E. faecalis* is considered to enable it tolerate 6.5% sodium chloride as well as salts, metals and desiccation (Ramsey *et al.*, 2014).

E. faecalis requires a number of amino acids (such as isoleucine, serine, methionine, histidine, tryptophan, arginine, leucine, valine and glutamine) and vitamins (riboflavin, folic acid, pantothenate and biotin) for its growth (Lebreton *et al.*, 2014). *E. faecalis* can perform aerobic respiration in the presence of heme, although they are neither able to synthesize heme nor do they possess porphyrin rings required for cytochrome bd, which is necessary for respiration (Baureder and Hederstedt, 2012; Frankenberg *et al.*, 2002).

These commensals, which are also a major cause of nosocomial infections, are associated with urinary tract infections, bacteremia, endocarditis, endodontic infections and meningitis of immunocompromised patients (Fisher and Phillips, 2009). This could be attributed to them being ‘tough’ organisms, which could

survive for prolonged period on hospital equipment, railings of hospital bed or door knobs (Arias and Murray, 2012). The increasing number of enterococcal infections led to the inclusion of *E. faecium* by the Infectious Disease Society of America (IDSA) as a part of the ESKAPE pathogens, including *E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Brown, 2015).

One of the main issues with the treatment of enterococcal infections is that they are either intrinsically tolerant or resistant to many antibiotics under clinical use (Kristich *et al.*, 2014). This argues for the continued search for new antibiotics, with novel mechanisms of action, to treat enterococcal infections.

However, in order for enterococci to behave as pathogens, they must express factors which help them adhere to the host tissue, initiate a host immune response and secrete toxins to damage the tissues. Some of these virulence factors include aggregation substances, gelatinase, cytolysin, hyaluronidase, lipoteichoic acids, cell surface adhesins and capsular polysaccharides among others (Jett *et al.*, 1994). The aggregation substances of *E. faecalis* may be associated with the internalization of the bacteria by intestinal epithelium (Sava *et al.*, 2010). Enterococcal surface protein (Esp) is an important virulence factor associated with urinary tract colonization. Mutants deficient in D-alaninated lipoteichoic acids (LTA) show reduced tendency to form biofilms and adherence to epithelial cells, apart from resistance to antimicrobial peptides. Gelatinase, a protease which hydrolyzes hemoglobin, collagen and gelatin, has been associated with enterococcus associated endocarditis (Gutschik *et al.*, 2009). By correlating the rise of *Enterococcus* spp. as nosocomial pathogens with their pathogenesis, developing treatments against these virulence factors could help in resolving the antimicrobial resistance associated with them. For example, reducing the biofilm formation or expression of virulence factors in *Enterococcus* could serve as another approach to treat drug resistant infections (Coverlet *et al.*, 2013).

1.2 Overview of the mechanisms of action of conventional antibiotics

Antibiotics can be classified according to the bacterial cellular function or component they target in order to kill the bacteria. The different mechanisms of action include: inhibition of DNA synthesis, RNA synthesis, cell wall synthesis and protein synthesis. Antibiotics such as quinolones form a complex with the topoisomerases (creates a break in the double stranded DNA and reseals the strand during DNA synthesis); thereby inhibiting the DNA replication (Chopra *et al.*, 2002). Rifamycin (Rif) inhibits RNA transcription by binding close to the active site of the β -subunit of the RNA polymerase. Mutations in this region on the *rpoB* gene encoding the β -subunit of the RNA polymerase are associated with resistance to rifamycin (Aubry-Damon *et al.*, 1998). Among the protein synthesis inhibitors, the mechanism of action is essentially involved in initiation, elongation and termination. During the initiation step, the 50S subunit is inhibited by antibiotics such as orthosomycins avilamycin (Avn), evernimicin (Evn) and thiostrepton (Ths) while the 30S subunit is inhibited by edeine (Ede), kasugamycin (Ksg), thermorubin (Thb) and pactamycin (Pct). The elongation process is the other target for classes of antibiotics such as aminoglycosides, chloramphenicol, fusidic acids, lincosamides, macrolides, streptogramins, tetracyclines and oxazolidinones. While termination is inhibited by blasticidin S (BlS), chloramphenicol (Cam), puromycin (Pmn) and sparsomycin (Spr). Ribosome recycling is inhibited by fusidic acid (Wilson, 2014). Another bacterial structure disrupted by some antibiotics is the cell wall (Chopra *et al.*, 2002). For example, β -lactam antibiotics block the cross – linking of peptidoglycan units, while glycopeptide antibiotics bind to the peptidoglycan units and inhibit transglycosylation and PBP activity. Antibiotics such as bacitracin and fosfomycin inhibit the transport and synthesis of the peptidoglycan units respectively.

1.3 Mechanism of resistance against antibacterial agents

Bacteria can inherently show resistance to particular antibiotics or acquire resistance via horizontal gene transfer or mutations in their genomic DNA. The inherent resistance could be due to the bacteria's structural or functional characteristics. For example, in Gram negative bacteria, some of the antibiotics

are capable of permeating via nonspecific channels, porins, through the outer membrane which is absent in the Gram positive bacteria. Reduction in the production of these channels, or mutations in genes associated with the regulation of porin expression, have led to clinically relevant resistance to carbapenems in enterobacteriaceae and *Pseudomonas* spp. (Blair *et al.*, 2015). Alternatively, some Gram negative bacteria may overexpress efflux pumps to aid in transporting the drug out of the cell. Another resistance mechanism involves alteration of the bacterial target site, thereby interrupting its action, such as is seen with rifampicin resistance as described above. Bacteria may also modify antibiotics by synthesizing enzymes to add chemical groups and thereby prevent their binding to target sites. For example, aminoglycoside antibiotics have free hydroxyl and amide groups that are modified by addition of nucleotides, acyl and phosphate groups (Blair *et al.*, 2015). This rise in drug resistance mechanisms has limited the efficacy of currently available antimicrobials. Limited number of antibiotics with novel mechanisms of action has added to this problem of antimicrobial resistance (AMR).

1.4 Current status of antibiotics

Even with the development of innovative approaches to antibiotic discovery, including target-based, high throughput screening methods and the mass screening of novel metabolites from uncultured microorganisms, no new classes of antibiotics were introduced since the introduction of quinolones in 1962 until after 2000 (Ling *et al.*, 2015, Walsh, 2003). Antibiotics approved by the FDA between 2010 and 2015 include ceftazidime/avibactam (2015), tedizolid (2014), dalbavancin (2014), oritavancin (2014), ceftolozane/tazobactam (2014), telavancin (2013) and ceftaroline (2010). Antibiotics approved by the FDA between 2010 and 2015 include ceftazidime/avibactam (2015), tedizolid (2014), dalbavancin (2014), oritavancin (2014), ceftolozane/tazobactam (2014), telavancin (2013) and ceftaroline (2010). Tedizolid (2014) has been reported to be effective against *Staphylococcus aureus* and enterococci which are less sensitive to vancomycin, daptomycin or linezolid but not against *optrA* resistance carrying MRSA strains (Barber *et al.*, 2016; van Harten *et al.*, 2017). Oritavancin (2014) shows *in vitro*

activity against VREs but clinical data is not available (Balli *et al.*, 2014, Arias *et al.*, 2010). (Ma *et al.*, 2016). Vancomycin resistant *S. aureus* and *van-A* expressing vancomycin resistant enterococci are partially sensitive to telavancin (2013), while they are resistant to dalbavancin (2014) (Hurdle *et al.*, 2011). Ceftazidime/avibactam (2015) and ceftolozane/tazobactam (2014) are combination antibiotics of cephalosporins and β -lactamases. Ceftazidime-avibactam resistance has been observed in patients infected with *Klebsiella pneumoniae* carbapenemase producing strains after 10-19 d of treatment (Shields *et al.*, 2017). Similarly, resistance was observed in isolates of *Pseudomonas aeruginosa* collected ten years before the introduction of this drug combination (Winkler *et al.*, 2015). Ceftolozane/tazobactam has *in vitro* activity with *Pseudomonas* species but resistance has been reported (van Duin and Bonomo, 2016). AMR has been described for every class of antibiotic and antimicrobial discovery has not kept pace with microbial adaptation, highlighting the need for new antimicrobials with activity against drug resistant strains or that do not elicit AMR. There have been other promising antimicrobial compounds in the early stage of development that target Gram positive bacteria, such as teixobactin (targets lipid II and lipid III), sortase transpeptidase inhibitors (targets sortases), lipoteichoic acid inhibitors, nisin derived lipopeptides but further research is needed to inspect their scope in the clinical settings (van Harten *et al.*, 2017).

1.5 Antimicrobial peptides as alternatives to conventional antibiotics

Cationic antimicrobial peptides (CAMPs) are an ancient class of host-defense antimicrobial agents (Peschel and Sahl, 2006). Some of the classic examples of naturally occurring CAMPs are LL-37 and human β defensins (hBDs) (Nakatsuji and Gallo, 2012). Bacteria adopt several resistance mechanisms against CAMPs, which mainly include disrupting AMP attachment to the target, antimicrobial peptide insertion, pore formation and membrane permeability (Hancock and Sahl, 2006).

The cell-wall associated teichoic acid polymers render a strong anionic property to the bacterial membrane surface. In *S. aureus*, this negative charge is

neutralized by the addition of D-Alanine to teichoic acid by the products encoded by the *dlt* operon, thereby attenuating the charge based interaction between positively charged CAMP and the microbial envelope (Figure 1.1). The inactivation of *dlt* operon makes *S. aureus* sensitive to defensins, protegrins and other antimicrobial peptides. Similarly, *S. aureus*, *E. faecalis* and other Gram positive bacteria make their membrane surface more positively charged by adding L-Lysine to the phosphatidyl glycerol via *mprf* (multiple peptide resistance factor). Conversely, Gram negative bacteria increase the negative charge on their membrane surface by modifications in the Lipid A moiety of the LPS. Other resistance mechanisms adopted by bacteria against CAMP include production of efflux pumps, enzymatic hydrolysis of antimicrobial peptides, biofilm formation and development of a protective capsule (Hancock and Sahl, 2006).

Figure 1.1 D-Alanylation of lipoteichoic acids (LTA) and cell wall associated teichoic acids (WTA) results in neutralizing the negative charge of teichoic acids. This reduces the charge based interaction with the cationic antimicrobial peptides. Figure reproduced from (Maria-Neto *et al.*, 2015).

The lactic acid bacteria (LAB) produced bacteriocin, nisin A, is the most widely used antimicrobial peptide used as a food preservative (Allen *et al.*, 2014). Nisin targets both Lipid II and operates as a pore forming compound

(Cotter *et al.*, 2013). It has been shown that changes in membrane fluidity, cell wall modifications and F_0F_1 ATPase may be involved in cell envelope stress response against nisin in *L. monocytogenes* (Bergholz *et al.*, 2013).

The potential for gramicidin as an effective antimicrobial agent was subsequently discovered in 1939 (Urry, 1971). It was later found to be toxic when administered in bacterially infected mice to treat systemic infections and hence was used as topical agent only to treat wounds during the World War II (Van Epps, 2006). It specifically kills Gram positive bacteria through the formation of a cation selective channel in the bacterial membrane. Due its systemic toxicity, its use is restricted to topical infections (Liou *et al.*, 2015).

Polymyxins were discovered in 1947, within this group of antibiotics, polymyxin B and colistin have been used as therapeutics worldwide (Falagas *et al.*, 2005). They are cationic polypeptides that are currently administered for the treatment of gram negative pathogen associated infections (Poirel *et al.*, 2017). Their preferential charge based interaction with the LPS of the outer membrane of the Gram negative bacteria results in increased permeability of the membrane, leakage of cell constituents and eventually, cell death (Falagas *et al.*, 2005; Storm *et al.*, 1977).

There has been some success over the past two decades in designing antimicrobial peptides that could be prescribed in a clinical setting. However, to date, only four cationic peptides have reached phase 3 clinical efficacy trials. Among these, only two CAMPs have demonstrated good activity: pexiganan (a synthetic analogue of frog peptide, magainin) and omaganan (a cattle derived indolicidin variant CP266) (Hancock and Sahl, 2006; Hancock and Lehrer, 1998). Pexiganan was developed for treatment of diabetic foot ulcers, but was denied FDA approval for medical use as it failed to show any distinct benefits compared to good wound management. Similarly, omaganan was not approved for medical use, due to the study being underpowered. Currently, additional phase 3-b trials are on the way for omaganan based on the observation of an improvement in treated volunteers in the previous trial (Hancock and Sahl, 2006; Hancock and Lehrer, 1998).

One possible disadvantage associated with CAMPs is that they share features with eukaryotic nuclear localization signals, thus creating a need to evaluate toxicity associated with peptides, these include apoptosis and mast cell degranulation. Due to this problem of systemic toxicity, the use of CAMPs is generally restricted to topical applications rather than systemic use, as is observed in the case of cationic peptides such as gramicidin S and polymyxin B which are currently used as topical creams and solutions (Hancock and Sahl, 2006). Secondly, antimicrobial peptides are prone to proteolysis, which renders them unsuitable for *in vivo* applications (Hancock and Sahl, 2006). Finally, the high cost of manufacturing peptides by solid phase synthesis makes these compounds expensive, e.g. \$100-\$200 per gram (Hancock and Sahl, 2006).

1.6 Daptomycin

The antimicrobial peptide Daptomycin is a cyclic lipopeptide antibiotic produced by the soil-bacterium *Streptomyces roseosporus*. It consists of a cyclic depsipeptide attached to a decanoyl fatty acid chain (Pogliano *et al.*, 2012). The hydrophilic depsipeptide unit is comprised of 13 amino acids, including 3 D-amino acids (D-Alanine, D-Serine, D-Asparagine) and 3 uncommon amino acid groups (ornithine, kynurenine and (2S,3R)-3-methylglutamic acid) (Debono *et al.*, 1988). Daptomycin is active against a wide variety of Gram positive pathogens, including Methicillin resistant *Staphylococcus aureus* (MRSA), Methicillin resistant *Staphylococcus epidermis* (MRSE) and Vancomycin resistant enterococci (VRE) (Pogliano *et al.*, 2012).

Daptomycin is an anionic molecule, which in the presence of calcium ions (Ca^{2+}) forms a Ca^{2+} -Daptomycin complex, which behaves as ‘de facto cationic antimicrobial peptide’ with an increased affinity for anionic phospholipids such as phosphatidylglycerol (PG) of the bacterial membranes (Jung *et al.*, 2017; Scott *et al.*, 2007; Hachmann *et al.*, 2011). However, it should be noted that the exact molecular mechanism of resistance exhibited by Gram positive pathogens to daptomycin is not completely understood and indeed, appears to vary in different organisms. For example, in *Bacillus subtilis*, mutations associated

with phosphatidylglycerol synthase (*pgsA*) and cell envelope stress response regulon (*liaFSR* operon) lead to increased resistance to DAP (Hachmann *et al.*, 2011). In contrast, genes associated with cell surface charge(*dlt*), lysylation of PG (*mprf*), two component systems (TCS) such as *vraSR* and *yycFG* (*walKR*), cardiolipin synthase (*cls*) and phospholipid synthase *pgsA* are involved in resistance against this antibiotic in *S. aureus*.(Mishra *et al.*, 2014; Bertsche *et al.*, 2013; Cui *et al.*, 2009; Camargo *et al.*, 2008; Tran *et al.*, 2015; Peleg *et al.*, 2012). Daptomycin is a last resort antibiotic for complex enterococcal infections. The mechanisms of resistance in enterococci involve changes in cell envelope stress response system (*liaFSR*) and phospholipid metabolism (*gdpD* and *cls*) in *E. faecalis* and *liaSR*, *yycFG* (two-component system involved in sensing cell envelope stress) and cardiolipin synthase (*cls*) in *E. faecium* (Munita *et al.*, 2015; Reyes *et al.*, 2015; Tran, Panesso, Gao, *et al.*, 2013; Diaz *et al.*, 2014; Arias *et al.*, 2011; Reyes *et al.*, 2015).

Two recent reports on the mechanism of daptomycin resistance in *B. subtilis* and *S. aureus* have shown the lipid based interaction of these bacteria with daptomycin. In *B. subtilis*, it has been shown that daptomycin exposure results in a substantial rearrangement of fluidic lipid domains that eventually lead to rapid detachment of membrane associated lipid II and phospholipid synthases (Müller *et al.*, 2016). Recently, it has also been shown that supplementation of fatty acids into the membrane of *E. faecalis* serves to protect the cell membrane from daptomycin. This effect is independent of, but related to, the function of the LiaFSR system, where LiaFSR influences the ability of the bacteria to utilize fatty acids to increase tolerance to membrane damaging agents (Harp *et al.*, 2016).

1.7 Conjugated Oligoelectrolytes

Conjugated oligoelectrolytes are π -conjugated polymers with terminal ionic groups. Conjugated oligoelectrolytes (COE) are a class of compounds defined by conjugated repeat phenylene vinylene backbone with terminal ionic pendant groups. The word ‘conjugated’ refers to the alternating double and single bonds, which provide the molecule certain electrical and optical properties.

Because their charge distribution and hydrophobicity mimics that of phospholipids, these amphiphiles have a strong affinity with microbial membranes (Garner *et al.*, 2010). Upon spontaneously inserting into the membrane, some COEs have been shown to have antimicrobial activity in a clinically interesting range and are particularly active against Gram positive bacteria (Hinks *et al.*, 2015). The antimicrobial function of COEs has been convincingly related to specific structural features as seen for two of the most potent classes of COEs identified to date. Both compounds have a backbone of three aromatic units and differ only in the terminal charge moiety with COE1-3C(1,4-bis(4'-(N,N-bis(6'''-(N,N,N-trimethylammonium)hexyl)amino)-styryl)benzene tetraiodide) being terminated by a tetraalkylammonium ion and termination of COE1-3-PY with a pyridinium ion. While the antimicrobial effect of these compounds has been demonstrated, no studies have been conducted on their mechanism of action. Such mechanistic understanding of the mode of action is necessary to direct synthetic design of more potent analogues.

Antimicrobial susceptibility assays showed that COEs had a higher minimum inhibitory concentration against Gram negative bacteria (*Escherichia coli* K12 and *Shewanella oneidensis* MR-1) than for Gram positive bacteria (*E. faecalis* OG1RX and *Bacillus megaterium*) (Yan *et al.*, 2015). COEs disrupt the bacterial membranes by hydrophobic mismatch. Some molecules such as the five-ringed COE1-5C perturb the lipid bilayer by positive hydrophobic mismatch, i.e. the length of molecules (≈ 5 nm) is greater than the lipid bilayer (≈ 4 nm). In contrast, the three-ringed COE1-3C initiates a negative mismatch with the lipid bilayer, where the molecule is smaller than the lipid bilayer (Hinks *et al.*, 2015)

COE-1-5C is a five ringed COE molecule that has been found to improve the butanol tolerance of *E. coli* by hydrophobic interaction with the acyl chains of phospholipids. Mechanisms associated with the fortification of the lipid bilayer by COE1-5C have been found to have similarity to hopanoids and sterols. They induce phase changes in the membranes, which fortify the lipid bilayer to maintain the lamellar phase after solvent exposure (Hinks *et al.*, 2015). UV-vis absorption and cyclic voltammetry studies with COE, 4, 4'-bis(4'-(N, N-bis

(6'' - (N, N, N trimethylammonium) hexyl) amino)-styryl) stilbene tetraiodide (known as DSSN+) and phospholipid bilayers has shown that the changes in the membrane charge permeability depend not only on the incorporation of COEs in the membrane but also the interaction of COE with the phospholipid bilayer and the other additives in the membrane. Phospholipid bilayers with negatively charged additives, cholic acid, which promotes alkyl chain disorder showed an increased COE uptake and charge permeability in comparison to the unmodified membrane bilayers. In contrast, the addition of choline, a positively charged molecule did not change the COE uptake in comparison to the bilayers in the absence of additives. However, the addition of cholesterol at low levels led to enhanced membrane charge permeability with no change in COE incorporation into the bilayer (Jahnke *et al.*, 2015).

The ability of COEs to mimic the activity of outer membrane cytochromes in extracellular electron transport (EET) was studied in *S. oneidensis*. DSSN+ was found not to function in the same capacity as outer membrane cytochrome proteins (MtrC and OmcA), but could increase the current output in the knockout, which was less than that observed for the wild type. It was found that its activity was independent of the flavin derivatives produced by *Shewanella* (Wang *et al.*, 2014). DSSN+ has been shown to increase the outer membrane permeability of *E. coli* by quantifying the release of periplasmic enzyme alkaline phosphatase (ALP) and cytoplasmic enzyme β -galactosidase. Since the conditions mimic extracellular electron transfer (EET), it suggests that COEs enhanced the electron transfer across *E. coli* cell membranes via increased permeabilization of the outer membrane (Catania *et al.*, 2016). It has already been observed that increasing permeability of the outer membrane using chitosan, ethylenediaminetetraacetic acid (EDTA) and polyethyleneimine (PEI) improved electron transfer across *P. aeruginosa* (Liu *et al.*, 2012).

The antimicrobial properties of COEs have been studied with *E. faecalis* and its ability to target the membrane has been described (Hinks, *et al.*, 2015). The ability of COEs to elicit resistance in *E. faecalis* has not been described yet, studying this would provide more insights into the mechanism of action of these molecules and strategies to overcome the resistance.

1.8 Enterococci and multidrug resistance

Enterococci show an intrinsically high level of resistance to antibiotics such as the cephalosporins, clindamycin and have also been found to be tolerant to clinically achievable concentrations of aminoglycosides and vancomycin (Figure 1.2) (Lebreton *et al.*, 2014). Multi-drug resistant enterococci are more difficult to treat due to their resistance to clinically prescribed antimicrobials. This has led doctors to use daptomycin, a FDA approved lipopeptide antibiotic, as the last resort antibiotic for treatment of severe multi-drug resistant enterococci infections. Daptomycin is known to preferentially interact with division septum of *E. faecalis* and in resistant strains it fails to localize in these regions (Tran, *et al.*, 2013). Similarly, as discussed earlier the other cationic antimicrobials also interact with the bacteria at lipid domains localized at discrete locations in the membrane. Dispersion of these domains would lead to disruption of the functions of proteins localized in this region and eventually leading to cell death (Rashid *et al.*, 2016).

Figure 1.2 Antimicrobial resistance mechanisms adopted by *Enterococcus* spp. against different classes of antibiotics. The figure is reproduced from (Arias and Murray, 2012)

1.9 *E. faecalis* and fatty acid composition

The different bacterial lipid classes and their organization play important roles in dictating the physiology, virulence, signaling, secretion pathways and resistance to antibiotics of *E. faecalis* (Rashid *et al.*, 2017). It has been shown that by supplementing *E. faecalis* with fatty acids found in bile or serum enables it to tolerate SDS, daptomycin and high levels of bile (Harp *et al.*, 2016). To better understand how *E. faecalis* responds to membrane stress, it is important to understand how it regulates membrane lipid metabolism and the primary membrane components of growing cells.

In *E. faecalis*, the major fatty acids are palmitic acid (C_{16:0}) and *cis*-vaccenic acid (C_{18:1; Δ11}) in its exponential phase at 37⁰ C (Teixeira *et al.*, 1983, Saito *et al.*, 2014). The cyclopropane fatty acid content increases in the membrane of *E. faecalis* as they enter stationary phase (Saito *et al.*, 2014; Jungkind and Wood, 1974). In *E. coli* it was observed that this increase was due to the expression of RpoS synthesized during the stationary phase. This activates the promoter of cyclopropane synthase (Wang and Cronan, 1994).

E. faecalis and *E. faecium* cell membranes primarily consist of phosphatidylglycerol (PG), cardiolipin (DPG), lysyl-phosphatidyl glycerol (LPG) and glycerophospho-diglycodiacylglycerol (GP- DGDAG) (Mishra *et al.*, 2012). The diacylglycerol of these lipids mentioned above have shorter chain fatty acids (C₁₄ and C₁₆) at position 2 and longer chain fatty acids (C₁₈ and C₁₉) at position 1. The C₁₉ cyclopropane fatty acid is distributed at a position 1 similar to its precursor, octadecenoic acid (C₁₈) (Fischer, 1990). Added to this, *E. faecalis* can utilize exogenous fatty acids present in the host or environment and this can enhance resistance to membrane damaging agents (Saito *et al.*, 2014). As a commensal organism, *E. faecalis* has access to the fatty acids present in the bile and serum and these may help the bacterium in surviving exposure to antimicrobial peptides. It is currently unknown how incorporation of the exogenous fatty acids into the membrane of enterococci impacts other metabolic process and de novo fatty acid biosynthesis (Harp *et al.*, 2016). However, it is clear that changes in the fatty acid composition of

membrane lipids or the class of lipids present affects the interaction of the membrane with antibiotics, and hence, their potency.

1.10 *E. faecalis* and cyclopropane fatty acids

In other organisms, various factors affect the synthesis of cyclopropane fatty acids, such as low pH, poor oxygen levels and aeration temperature (Jungkind and Wood, 1974). Cyclopropane fatty acid synthesis is a post-synthetic modification of phospholipids in the bacterial membrane bilayer and does not involve any free fatty acids or intermediates of phospholipid synthesis. Only the cyclopropanization step is thought to occur as a post-synthetic modification of phospholipids. Studies with cyclopropane synthase isolated from *Clostridium butyricum* have shown that it transfers the methylene group from *S* – adenosyl-L-methionine (SAM) to a monounsaturated fatty acid residue of a phospholipid in the lipid bilayer (Jungkind and Wood, 1974). The presence of cyclopropane fatty acids versus the *cis*-unsaturated fatty acids would lead to a more rigid membrane due to its higher melting point (Zhu *et al.*, 2010).

Several studies described in this chapter point towards how *E. faecalis* could alter its physiology, virulence and genetic response to the changes in its environment. The bacterial membrane acts as barrier between the cell and its surroundings by creating a transmembrane gradient. Thus, membrane lipids and proteins are attractive targets for antimicrobial therapy. By targeting the membrane, the gradient maintained by it and the functions of the various lipid components associated with the lipid bilayer will also be disrupted (Figure 1.3).

Figure 1.3 Components of the bacterial membrane and different roles in which they are involved. A) Exosome vesicle formation, B) membrane bound receptors, C) primary messengers, D) lipid domain and protein interaction, E) proton gradient and energetics, F) physical barrier and G) as second messengers. The figure is reproduced from (Epand et al., 2016)

1.11 Aims and Objectives

There is a growing urgency to identify and validate novel antimicrobial compounds for use in controlling infections by pathogens of concern such as *E. faecalis*, which displays intrinsically high levels of antibiotic resistance. Part of the validation and optimization of new antimicrobial compounds is based on determination of the mechanism of action of those potential therapeutics, which enable structural modification to optimize activity against the target species as well as to minimize toxicity to the infected host. The objective of this study was to understand the antimicrobial mechanism of two membrane intercalating conjugated oligoelectrolytes COE1-3Py and COE1-3C against *E. faecalis*. The specific aims of this project include:

A) **Aim 1:** Development of *in vitro* evolved resistance to COEs in *E. faecalis* and characterization of the mutations involved by whole genome sequencing.

B) **Aim 2:** Characterize the transcriptomic response of *E. faecalis* wild type and *in vitro* adapted strains in response to treatment with COEs.

C) **Aim 3:** Determine the role of genes associated with resistance to COEs and their role in the modification of membrane lipid profiles.

1.12 Overview of Experimental Approaches:

Adaptive Evolution: In these experiments, the bacteria were transferred from the culture with the highest concentration of conjugated oligoelectrolytes (COEs) in which the bacteria grew into fresh medium containing different concentrations of COEs. This was continued on a daily basis, until no further increase in the MIC was observed.

Whole genome sequencing of adapted strains: The adapted strains were sequenced to identify mutations in comparison to the sequence of unadapted wild type strains.

Transcriptome analysis: The differentially expressed genes in the mutants were identified in comparison to the wild type strains. In addition, the differential expression on addition of the conjugated oligoelectrolytes was also studied in the mutants in comparison to the wild type.

Fatty acid analysis: Since COEs are known to interact with the lipid bilayer, a fatty acid analysis of the adapted and wild type was done to understand the changes at the acyl chain level of phospholipids.

Construction of isogenic knockout strains: Mutants were constructed by replacement of the functional genes in the wild type with the mutations observed in the adapted strains. This would reveal the role of the mutation on the resistance to the molecules.

2 Membrane adaptation limitations in *Enterococcus faecalis* underlie sensitivity and the inability to develop significant resistance to conjugated oligoelectrolytes

2.1 Introduction

Antimicrobial resistance (AMR) is of an increasingly global concern. To stave off an impending and much feared post-antibiotic era, a high level discussion was convened by the UN General Assembly in September 2016 to secure global implementation strategies to combat AMR (Laxminarayan *et al.*, 2016). Antibiotic discovery was prolific between 1940 and 1960 during which time all of the major classes of antibiotics in clinical use today were discovered. As antibiotic discovery tapered off, progress proceeded mainly through stereochemical modifications to existing antibiotics (Walsh, 2003; Walsh and Wencewicz, 2014). Even with the application of innovative approaches to antibiotic discovery, including target-based high-throughput methods and the mass screening of novel metabolites from uncultured microorganisms, no new classes of antibiotics were introduced since the introduction of quinolones in 1962 until after 2000 (Ling *et al.*, 2015; Walsh, 2003). Between 2010 and 2015, some of the FDA approved antibiotics include ceftazidime/avibactam (2015), tedizolid (2014), dalbavancin (2014), oritavancin (2014), ceftolozane/tazobactam (2014), telavancin (2013) and ceftaroline (2010). While ceftaroline is a novel cephalosporin, drugs such as ceftazidime/avibactam and ceftolozane/tazobactam are a combination of existing classes of antibiotics namely, cephalosporin and β -lactamase inhibitors (Ma *et al.*, 2016). The lipoglycopeptides dalbavancin, oritavancin and televancin are similar to vancomycin in that they target the cell wall precursor lipid II (van Harten *et al.*, 2017). AMR has been described for every class of existing antibiotics and antimicrobial discovery has not kept pace with microbial adaptation, highlighting the need for new antimicrobials with activity against drug resistant strains or which do not elicit AMR. Many promising antimicrobial compounds in the early stage of development, such as teixobactin (targets lipid II and lipid

III) , sortase transpeptidase inhibitors (targets sortases), lipoteichoic acid inhibitors , nisin derived lipopeptides all target the cell envelope to some degree although further research is needed to fully appreciate their clinical worth (van Harten *et al.*, 2017).

Antimicrobial peptides (AMP) are components of the innate immune system that target the cell envelope of microbes and whose use as therapeutic agents has been explored in the recent years. However, due to issues with stability, toxicity and high manufacturing costs (Pachón-Ibáñez *et al.*, 2017) no modified AMP has received approval for clinical use, although a few AMP and their derivatives have reached the pre-clinical stage (Li *et al.*, 2017).

Conjugated oligoelectrolytes (COE) are a class of compounds defined by repeat phenylene-vinylene units with terminal ionic-pendant groups. Because their charge distribution and hydrophobicity mimics that of phospholipids, these amphiphiles have a strong affinity for microbial membranes (Garner *et al.*, 2010). Upon spontaneously inserting into microbial membranes, some COEs have antimicrobial activity in a clinically applicable range and are particularly active against Gram positive organisms (Hinks *et al.*, 2015).

The antimicrobial function of COEs have been related to specific structural features and the two most potent antimicrobial structures amongst two related classes of COEs have a backbone of three aromatic repeat units and differ only in the terminal charge moiety with COE1-3C (1,4-bis(4''-(N,N-bis(6'''-(N,N,N-trimethylammonium)hexyl)amino)-styryl)benzene tetraiodide) being terminated by a tetraalkylammonium group and COE1-3Py with a pyridinium ion (Yan *et al.* 2016) (Figure 2.1). While the antimicrobial effect of these compounds has been demonstrated, and the mode of action is hypothesized to be through the membrane disrupting properties of COEs, no studies have been conducted to confirm the mechanism of action (Hinks, *et al.*, 2015; Yan *et al.*, 2016). Such information is vital as a molecular level understanding of COE interactions with membranes is necessary to direct synthetic design towards more potent analogues of this emerging class of compounds. Additionally, there is no existing knowledge about the emergence or potential mechanism of resistance against this class of antimicrobials.

Figure 2.1 Structure of COE1-3C and COE1-3Py.

Enterococcus faecalis is a Gram positive bacterium that is part of the healthy intestinal flora of humans (Wurster *et al.*, 2016) but is implicated in recurring urinary tract infections. *E. faecalis* poses a clinical challenge as it has intrinsic resistance (encoded by chromosomal elements) and acquired resistance (via uptake of exogenous transposons or pheromone responsive plasmids carrying antibiotic resistant genes) to a number of antimicrobials

Here, the interaction of COE1-3C and COE1-3Py with *E. faecalis* cell membranes was explored. In particular, limited resistance to COE1-3C and COE1-3Py developed and the accompanying changes in fatty acid composition and lipidomic profile were surveyed to understand natural adaptations to COEs. Standard MIC tests were performed to determine if COE resistance confers cross-resistance to other membrane disrupting antibiotics. Additionally, assays were performed using model membranes to assess lipid composition dependent membrane-binding and the resulting membrane perturbations caused by these compounds. Whole genome sequencing of resistant isolates identified mutations in genes associated with the regulation of cell envelope stress

response in *E. faecalis* OG1RF, suggesting that the primary mechanism of resistance is through membrane reorganization to modify the uptake of COEs.

2.2 Materials and Methods

2.2.1 Conjugated oligoelectrolytes

COE1-3C(1,4-bis(4''-(N,N-bis(6'''-(N,N,N-trimethylammonium)hexyl)amino)-styryl)benzene tetraiodide) and COE1-3Py ((1,4-bis(4''-(N,N-bis(6'''-(pyridinium)hexyl)amino)-styryl)benzene tetraiodide)) were synthesized as described by Garner *et al.*, 2010 and Yan *et al.*, 2016, respectively.

2.2.2 Minimum Inhibitory Concentration (MIC) Testing

MIC tests were conducted using broth microdilution methods as based on a method reported by Wiegand *et al.*, 2008. Experiments were carried out in triplicate and compounds were two-fold serially diluted in Brain Heart Infusion (BHI) broth. Growth in each of the wells was assessed after 18 h incubation at 37°C. The minimum inhibitory concentration (MIC) was defined here as the lowest concentration of antibiotic that inhibited visible growth as observed with the unaided eye (Wiegand *et al.*, 2008).

2.2.3 Development of *in vitro* resistance to COEs in *E. faecalis* OG1RF

COE1-3C and COE1-3Py resistant strains of *E. faecalis* OG1RF were generated by serial passage in BHI medium containing increasing concentrations of either COE1-3C or COE1-3Py in triplicate. On day 1, the optical density (OD₆₀₀) of *E. faecalis* OG1RF was adjusted to 0.1, then cultures were exposed daily to two fold increases in COE concentrations beginning at 0.5 x MIC until it reached a maximum MIC beyond which it did not increase any further. On a given day, the culture with the highest concentration of COE that showed visible growth of *E. faecalis* OG1RF after 24 h incubation was used as the inoculum for the subsequent day and incubated at both the concentration of COE from which it was taken and at a two-fold increment above that concentration. Serial passaging was continued over 14 d to until no further increase in resistance was observed. Daily aliquots were recovered from the serial passage cultures for each of the experiments and stored at -80°C. Each isolate collected from these experiments was passaged 3 – 4 times in BHI medium free of COE and the

MIC of the tolerant strains was determined to ensure the resistant phenotype was maintained. The resulting strains resistant to COE1-3C and COE1-3Py were designated EFC3C and EFC3Py, respectively.

Figure 2.2 Illustration of *in vitro* passing methodology of *Enterococcus faecalis* OG1RF in increasing concentration of COEs over a 14-day period led to development of COE1-3C and COE1-3Py resistant strains, namely EFC3C and EFC3Py, respectively. Here, MIC_{wt} refers to the minimum inhibitory concentration of the wild type.

2.2.4 Cross resistance studies of EFC3C and EFC3Py

The EFC3C and EFC3Py strains were tested for cross resistance to the other COE and also to daptomycin (A.G. Scientific Inc.). The cross resistance studies were performed by determining the MIC of daptomycin using broth microdilution tests as described above. Concentrations of daptomycin ranged between 1.23 and 315.84 μM .

2.2.5 Whole genome sequencing of EFC3C and EFC3Py

Genomic DNA was harvested from overnight cultures of EFC3C and EFC3Py in BHI broth using an Invitrogen Pure Link DNA Mini kit. DNA was

sequenced on an Illumina MiSeq (Illumina, CA, USA) with 300 bp paired-end sequences and sequences were assembled using CLC Genomics Workbench 8.0. The sequenced genomes were mapped and annotated against the *E. faecalis* OG1RF reference genome (NC_017316) from the NCBI database. The threshold variant frequency was set at 35%. The synonymous mutations were omitted from further analysis and structural variations (such as deletions, insertions, inversions and translocation) were confirmed manually on the mapping track as described (Rashid et al., 2017).

2.2.6 Extraction of lipids from *E. faecalis* OG1RF, EFC3C and EFC3Py

E. faecalis OG1RF wild type, EFC3C and EFC3Py were grown for 12 h in BHI at 37°C. Overnight suspensions were centrifuged at 9000 g for 2 min and the cell pellets of all three *E. faecalis* strains were weighed and subjected to the Bligh and Dyer method of whole cell lipid extraction (Bligh and Dyer, 1959). The total lipid extract from the organic phase was dried and resuspended in 1:1 (v/v) chilled chloroform: methanol solvent to a final concentration of 10 mg/ml.

2.2.7 Fatty acid methyl ester (FAME) analysis

Five replicates of *E. faecalis* OG1RF wild type, EFC3C and EFC3Py were grown for 12 h in BHI at 37°C. Overnight suspensions were centrifuged at 9000 g for 2 min and the cell pellets of all three *E. faecalis* strains were weighed. Fatty acid analyses were carried out by the Identification Service of DSMZ, Braunschweig, Germany.

2.2.8 Effect of fatty acid supplementation on *E. faecalis* OG1RF COE toxicity

E. faecalis OG1RF wild type was grown overnight in BHI at 37°C. The overnight culture was grown to the mid-log phase in BHI with palmitic acid (10 µg/ml), *cis*-vaccenic acid (10 µg/ml) or *cis*-9, 10-methyleneoctadecanoic acid (12.5 µg/ml), respectively at 37°C. The fatty acid concentrations were chosen based on growth assays with the fatty acids and solvent controls. The conditioned mid-log phase culture was subjected to MIC tests in BHI medium

modified with the appropriate fatty acid whose effect on sensitivity to COEs was of interest.

2.2.9 Membrane binding assays by quartz crystal microbalance-dissipation measurements

QCM-D experiments with a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) were conducted in order to characterize the interaction between the test compounds and a supported lipid bilayer with 30% bacterial lipid extract.

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipids in chloroform and bacterial lipid extracts dissolved in a 1:1 ratio of chloroform and methanol were dried at a 70:30 (DOPC:bacterial lipid extract) mass ratio using nitrogen gas and dissolved in 10 mM Tris buffer solution [pH 7.5] with 150 mM NaCl at 1 mg/mL lipid concentration. The lipids were extruded using an Avanti Mini-Extruder with 50 nm pore filter and the prepared vesicles were approximately 70 nm. The vesicles were diluted to 0.2 mg/mL in 10 mM Tris buffer solution [pH 7.5] with 250 mM NaCl. For the formation of SLB using vesicle fusion method, the baseline QCM-D signals of Δf and ΔD were stabilized using a 10 mM Tris buffer solution [pH 7.5] with 150 mM NaCl. Following stabilization, 10 mM Tris buffer solution [pH 7.5] with 250 mM NaCl were added and 0.2 mg/mL lipid vesicles in 250 mM NaCl Tris buffer were added. The vesicles were washed with Tris buffer, followed by buffer exchange against 150 mM NaCl Tris buffer.

The QCM-D technique monitors changes in resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, piezoelectric quartz crystal sensor chip as functions of time, which reflect the acoustic mass and viscoelastic properties, respectively, of an adsorbate on the surface. The sensor chip had a fundamental frequency of 5 MHz and a sputter-coated, 50 nm thick layer of silicon dioxide (model no. QSX 303, Q-Sense AB). The experimental data was collected at the third ($n = 3$), fifth ($n = 5$), seventh ($n = 7$), ninth ($n = 9$) and eleventh ($n = 11$) odd overtones using the QSoft software program (Q-Sense AB) and the data was normalized according to the overtone number. All presented data was collected at the fifth overtone.

2.2.10 Model membrane permeabilization assay by electrochemical impedance spectroscopy

Gold electrodes supplied by SDx Tethered Membranes (Roseville, NSW) were coated with 10% benzyl-disulphide-bis-tetraethyleneglycolmonophytane tethering groups and 90% benzyl-disulphide-bis-tetraethyleneglycol spacer lipids in ethanol. The assembled TethaPlate (SDx Tethered Membranes, Roseville, NSW) contained six wells with 2.1 mm² electrodes per cartridge and was used to deposit lipids for the tethered lipid bilayer membranes (tBLM) formation. The subsequent assembly of the lipid bilayer was performed from the addition of 8 µL of 3 mM of diphytanyl diether phosphatidyl choline (DPEPC) and glycerol diphytanyl ether (GPDE) at 70:30 molar ratio mixed with bacterial lipid extract at appropriate SDx lipid:bacterial lipid extract mass ratio. After 2 min incubation, the flow cell was rapidly rinsed with 3 x 100 µL of 10 mM Tris buffer solution [pH 7.5] with 150 mM NaCl, resulting in the spontaneous formation of tBLM. Following the formation of tBLM, 100 µL of samples were injected into the wells. TethaPod (SDx Tethered Membranes) and tethaPATCH (SDx Tethered Membranes) were used as membrane conductance and capacitance reader and potentiostat connectivity, respectively. All measurements were collected and analyzed using tethaQUICK software (SDx Tethered Membranes).

2.3 Results and Discussion

2.3.1 MIC of COE1-3C and COE1-3Py against *E. faecalis* OG1RF

Both COE1-3C and COE1-3Py inhibit the growth of *E. faecalis* at 2 and 1 µM, respectively (Table 2.1). The lower MIC of COE1-3Py can likely be attributed to the substitution of the trialkylammmonium group of COE1-3C with the pyridinium ion (Yan *et al.*, 2016).

TABLE 2.1 Minimum inhibitory concentrations of COE1-3C, COE1-3Py and daptomycin

Strain	Description	Growth conditions	MIC (μ M)		
			COE1-3C	COE1-3Py	Daptomycin
<i>E. faecalis</i> OG1RF	Wild type	BHI	2	1	1.23
EFC3C^a	COE1-3C resistant	BHI	8	1	9.87
EFC3Py^b	COE1-3Py resistant	BHI	8	16	9.87
DAP 21^c	Daptomycin resistant	BHI	2	1	78.96
DAP 22^d	Daptomycin resistant	BHI	2	1	78.96
<i>E. faecalis</i> OG1RF	Wild type	BHI + palmitic acid C _{16:0} (10 μ g/mL)	2	1	ND
<i>E. faecalis</i> OG1RF	Wild type	BHI + <i>cis</i> -vaccenic acid C _{18:1} ω 7c (10 μ g/mL)	8	2	ND
<i>E. faecalis</i> OG1RF	Wild type	BHI + <i>cis</i> -9,10-methyleneocta-decanoic acid C _{19:0} cyclo ω 8c (12.5 μ g/mL)	8	4	ND

^a COE1-3C resistant strains with in frame deletion in *liaF* at position 179 and a substitution in the intergenic region between *treB* (PTS family trehalose porter, IIBC component) and *gloA6* (lactoylglutathione lyase). ^b COE1-3Py resistant strains with a non-synonymous substitution at position 97 in the *liaR* gene. ^c DAP21 strains have mutations in cardiolipin synthase 1 (*cls1*; RF10364), a gene encoding a putative chaperone protein regulated by the *liaFSR* operon (RF11464) and hypothetical membrane protein (RF11507) (Rashid *et al.*, 2017). ^d DAP22 strains have mutations in gene encoding a putative chaperone protein regulated by the *liaFSR* operon (RF11464) and hypothetical membrane protein (RF11507) along with mutations in cardiolipin synthase 2 (*cls2*; RF11324) and a putative metal-dependent HD-domain-containing hydrolase (RF11901) (Rashid *et al.*, 2017). ND – Not determined.

2.3.2 Resistance of *E. faecalis* to EFC3C and EFC3Py to COE1-3C and COE1-3Py

By serial passage of *E. faecalis* in incrementally increasing concentrations of COEs, it was possible to produce mutants that were resistant to COE1-3C (EFC3C, MIC = 8 μ M) and COE1-3Py (EFC3Py, MIC = 16 μ M), achieving a four and 16 fold increase in MIC, respectively (Table 2.1). EFC3Py was cross resistant to COE1-3C but there was no cross resistance between EFC3C and COE1-3Py. This suggests a different mechanism of action between the two COEs used here, which is likely to be related to the differences in their molecular structure. These differences in activity may either indicate that they have different affinities for different lipid classes or the difference in activity could be the result of some nuanced arrangement of their acyl chains in the cell membrane. These results support the suggestion that subtle differences in molecular structure lead to different modes of action. Understanding such interactions is of scientific importance and critical for the subsequent modification and optimization of antimicrobial agents. The four and 16 fold increase in MIC between the wild type and the adapted strains for COE1-3C and COE1-3Py, respectively, is relatively small compared to that which occurs for other compounds. For example, the MIC for *in vitro* evolved daptomycin resistant strains represents a 256 - 512 fold increase in MIC compared to that of sensitive strains (Rashid *et al.*, 2017, Palmer *et al.*, 2011). In our study we observed an 8 fold increase in cross resistance to daptomycin.

Previous *in vitro* evolution studies with daptomycin (DAP) and resistant variants of *E. faecalis* V583 showed “step pattern” of increase in DAP resistance over a 15 d period, wherein some of the isolates showed a peak in daptomycin resistance on the 14th day (Palmer *et al.*, 2011). In our study, the MIC of COEs plateaued on day 5 and day 12 and for COE1-3C and COE1-3Py without observing further changes in the MIC values after subsequent passaging in the COE for the remaining period (Figure 2.3). The inability of *E. faecalis* to adapt significantly to COE is an interesting and desirable feature of this class of compounds to be explored in further detail.

Figure 2.3 *In vitro* passing of *Enterococcus faecalis* OG1RF in increasing concentration of COEs over a 14 d period led to development of COE1-3C (blue) and COE1-3Py (green) resistant strains, EFC3C and EFC3Py, respectively.

2.3.3 The genetic basis for EFC3C and EFC3Py resistances differ

To confirm that the observed differences in the MIC of COE1-3C and COE1-3Py elicited a unique resistance in EFC3C and EF 3CPy respectively, whole genome sequencing was performed. The genomes of EFC3C and EFC3Py harbored mutations associated within *liaFSR*, a three component system. LiaFSR homologous systems involved in cell envelope associated stress response are widespread in the *Firmicutes* except in *Lactobacillus* and *Clostridium* (Jordan *et al.*, 2006; Kuroda *et al.*, 2003; Martínez *et al.*, 2007; Suntharalingam *et al.*, 2009; Jordan *et al.*, 2008). Sequencing of the EFC3C strain revealed an in frame deletion of an isoleucine codon at position 179 in *liaF*, a negative regulator of LiaR in the LiaFSR system as well as a single nucleotide substitution in the intergenic region of *treB* (PTS family trehalose porter, IIBC component) and *gloA6* (lactoylglutathione lyase). In contrast, a non-synonymous substitution at position 97 was found in *liaR* for EFC3Py. Additional SNPs were also identified in EFC3Py, including a, MerR family transcriptional regulator, site specific tyrosine recombinase XerD and ATP-binding protein region of a multidrug ABC transporter (Appendix C1). However, when strains collected during the course of *in vitro* passing harboring mutations exclusively in these genes were tested, their MICs were

identical to the wild type *E. faecalis* and hence it was concluded that the *xerD* and *merR* genes did not play a role in resistance to COE1-3C and COE1-3Py. Since the *liaFSR* operon is known to be associated with resistance to daptomycin, we tested for daptomycin cross resistance. The MIC of daptomycin for *E. faecalis* serially passaged in the presence of daptomycin *in vitro* was reported to plateau at 512 µg/ml, representing a 256 fold increase in tolerance compared to the wild type (Palmer *et al.*, 2011). In this study (Table 2.1), EFC3C and EFC3Py were mildly resistant to daptomycin at a concentration of 9.87 µM, which is around 8 fold higher than the wild type (MIC= 1.23 µM). MIC of COEs against daptomycin resistant strains DAP - 21 and DAP - 22 was also determined. DAP-21 and DAP-22 were adapted from the same *Enterococcus faecalis* OG1RF stock used to streak the plate from which colonies were picked for the COE adaptation work. DAP-21 and DAP-22 were passaged in the presence of daptomycin (AG Scientific) in Brain Heart Infusion (BHI) broth (Acumedia, Neogen) until the MIC values reached a maximum at 78.96µM (Rashid *et al.*, 2017). The results show that the highly daptomycin resistant strains (MIC=78.96µM) do not show increased resistance to either of the COEs, where they show MICs of 2 and 1µM for COE1-3C and COE1-3CPy, respectively. Similarly, the evolved COE resistant mutants also show low levels of DAP resistance (MIC= 9.87 µM) but do not achieve the high level of DAP resistance associated with the DAP 21 and DAP 22 strains. Trans *et al.*, (2013) have shown that the presence of an in-frame deletion at position 177 of *liaF* is associated with a daptomycin resistance of 16 µg/mL. In this study, it was observed that a similar daptomycin resistance at 16 µg/mL was observed with the *liaF* mutation at position 179. During the *in vitro* evolution of resistance to daptomycin in enterococcus, mutations were also observed in other genes, such as cardiolipin synthases (*cls*) 1 and 2 (Rafi *et al.*, 2017). Similarly, V583 showed a daptomycin resistant phenotype on passaging at higher concentrations of DAP after mutations appeared in *cls* and hypothetical membrane associated proteins (Palmer *et al.*, 2011). The lack of mutations in the cardiolipin synthases or genes responsible for higher levels of daptomycin resistance may be the reason for the lower levels of cross resistance to daptomycin in EFC3C and EFC3Py. This suggests that perhaps the mechanisms of resistance for the two classes of compounds are somewhat

different, although there is some role of *liaFSR* in mediating low levels of DAP resistance as well as resistance to the COEs. While the precise role of *liaFSR* in daptomycin resistance is unknown, it is hypothesized that this effect is mediated by as a consequence of loss of *liaFSR* function

2.3.4 Fatty acid profiles of EFC3C, EFC3Py and *E. faecalis* wild type

The membrane fatty acid profiles of EFC3C, EFC3Py and the wild type were determined using fatty acid methyl ester (FAME) analysis. It has been shown that the membranes of stationary phase cells of *E. faecalis* OG1RF are primarily composed of palmitic acid, *cis*-vaccenic acid and *cis*-9,10-methyleneoctadecanoic acid (Teixeira *et al.*, 1983; Saito *et al.*, 2014). In the COE adapted mutants, the same dominant fatty acids were observed, although their relative contributions were significantly different from the wild type *E. faecalis* (Table 2.2).

Both EFC3C and EFC3Py showed a difference in the level of C_{19:0 cyclo_ω8c} in the membrane, with an increase of 62.8% and 38.18 %, respectively ($p \leq 0.001$, $n=5$), in comparison to the wild type. The fatty acid analysis also revealed a 5.8% and 8.2% decrease in C_{16:0} ($p \leq 0.001$ for EFC3C and EFC3Py, $n=5$) (Figure 2.4) when compared with the wild type. There was a 5.65% decrease in the level of C_{18:1 ω 7c} in EFC3C ($p \leq 0.001$, $n=5$) and 6.15% increase in this fatty acid was observed in EFC3Py ($p \leq 0.001$, $n=5$) in comparison to the wild type. The data suggest that cyclopropane fatty acids, which are implicated in general membrane associated stress (Chang and Cronan, 1999; Grogan and Cronan, 1997), were responsible for COE resistance in general (and hence explains the cross resistance between COE1-3C and COE-3Py) but that C_{18:1 ω 7c} is found to specifically mediate COE1-3Py resistance. Cyclopropane fatty acids are implicated in membrane stress and it is thought they improve the stability of the membrane under extreme conditions such as high osmotic pressure and high temperature, while also reduces the permeability to toxic compounds (Poger and Mark, 2015). However, the absolute changes in fatty acids here are of low abundance, to confirm that the observed fatty acid changes are able to bring about COE resistance we performed further tests.

Table 2.2 Percentage of total membrane content determined by fatty acid analysis

Fatty acid	% of total membrane content*		
	<i>E. faecalis</i> wild type	EFC3C	EFC3Py
C_{10:0}	0.000	0.05±0.05	0.016 ± 0.04
C_{12:0}	0.292±0.24	0.186±0.25	0.204 ± 0.28
C_{14:0}	3.55±0.27	3.614±0.46	3.464 ± 0.42
C_{15:1} ω8c	0.062±0.12	0.000	0.074 ± 0.10
C_{16:0}	40.066±1.03	37.734±1.55 ****	36.776 ± 0.66 ****
C_{17:0} cyclo	ND	0.1025±0.21	0.042 ± 0.09
C_{17:1} ω8c	1.242±1.55	0.855±1.71 **	0.000
C_{18:1} ω7c	33.848±0.88	31.934±1.61 ****	35.934 ± 1.43 ****
C_{18:0}	5.654±0.70	5.262±0.43	5.452 ± 0.32
C_{18:1} ω9c	0.066±0.13	0.000	0.000
C_{19:1} ISO I	0.44±0.06	0.406±0.05	0.362 ± 0.06
C_{19:0} cyclo ω8c	7.37±0.56	12±0.69****	10.184 ± 0.37****
C_{19:0} 10 methyl	0.824±0.07	1.312±0.28	0.978 ± 0.20
C_{20:2} ω6,9c	0.000	0.046±0.10	0.000
C_{20:1} ω7c	0.398±0.08	0.514±0.06	0.588 ± 0.10
C_{16:1} ω7c/15 ISO 2OH	3.922±0.30	4.072±0.26	3.998 ± 0.25
C_{15:0} iso 2OH/16:1ω7c	1.092±0.08	1.1±0.08	1.11 ± 0.05
C_{17:1} iso/ anteï B	0.834±0.11	0.682±0.06	0.62 ± 0.05
Uneluted peak	0.306±0.08	0.274±0.09	0.202 ± 0.13

*The values are shown as averages ± standard deviations of five different replicates of *E. faecalis* OG1RF, EFC3C and EFC3Py. Significant differences between the mutants EFC3C and EFC3Py in comparison to the wild type was calculated using two-way ANOVA and Tukey's multiple comparisons test-and corrected p-values indicated with ** $p \leq 0.01$ and *** $p \leq 0.001$. Unknown peak eluted between C_{14:0} and C_{16:1} ω7c/15 ISO 2OH

Figure 2.4 Percentage changes in the three dominant fatty acids (>10% each of the total fatty acid content) determined as a percentage of total membrane content; shown as averages $\pm$ standard deviations of five biological replicates of *E. faecalis* OG1RF, EFC3C and EFC3Py. Significant differences between the mutants EFC3C and EFC3Py in comparison to the wild type was calculated using two-way ANOVA and Tukey's multiple comparisons test-and corrected p-values indicated with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$

2.3.5 Effect of fatty acid supplementation to *E. faecalis* on sensitivity to COEs

It has been shown that manipulating the fatty acid content of the growth medium can be used to alter the membrane composition (Harp *et al.*, 2016; Saito *et al.*, 2014). Therefore, *E. faecalis* cultures were grown in the presence of different fatty acids to determine if changes in the membrane fatty acids would alter the MIC of COE1-3C or COE1-3Py. *E. faecalis* OG1RF does not possess genes for β -oxidation as observed by homology searches with the existing genomic sequences of *E. faecalis* (Harp *et al.*, 2016). This suggests *E. faecalis* cannot degrade fatty acids from the medium and it has been shown that these bacteria can incorporate exogenous fatty acids into its membrane (Saito *et al.*, 2014; Harp *et al.*, 2016).

Supplementing palmitic acid into the growth medium did not increase the susceptibility of *E. faecalis* to either COE1-3C or COE1-3Py (Table 2.1). This is in line with the FAME analysis data which shows a decrease in palmitic acid suggesting that supplementation of this fatty acid would not influence the resistance against COEs. Supplementation with *cis*-vaccenic acid increased the MIC of COE1-3C and COE1-3Py in the wild type by two fold and four fold, respectively (Table 2.1). This was in agreement with the fatty acid measurements of EFC3Py, which suggests an association of increased *cis*-vaccenic acid with tolerance to higher concentration of COE. Even though EFC3C shows a decrease in *cis*-vaccenic acid (Figure 2.4), on the addition of this fatty acid to the wild type OG1RF increases the resistance of the membrane COEs (Table 2.1). Finally, the addition of *cis*-9, 10-methyleneoctadecanoic acid (Table 2.1) also increased the MIC of both the compounds by four fold. This supports the influence of increase in the levels of the cyclopropane fatty acids on the antimicrobial action of COEs.

It has been shown that the presence of cyclopropane fatty acids versus the *cis*-unsaturated fatty acids would lead to a more rigid membrane due to its higher melting point (Zhu *et al.*, 2010). Cyclopropane fatty acids moderate the packing and order of the lipid bilayer, thereby decrease the fluidity of the lipid bilayer (Poger and Mark, 2015). This suggests that changes in membrane order associated with cyclopropane fatty acids could affect COE resistance. Recently, it has been shown that supplementation of exogenous fatty acids into the membrane of *E. faecalis* serves to protect the cell membrane from daptomycin (Saito *et al.*, 2014; Harp *et al.*, 2016). These results suggest that subtle changes to membrane fatty acid compositions are associated with daptomycin and COE resistance.

2.3.6 Physical basis of COE resistance

Both *cis*-vaccenic acid and cyclopropane fatty acids in general are likely to alter the physical and chemical properties of the membrane. One possible method whereby FA changes could influence the MIC of COEs is by excluding them

from the membrane, alternatively reordering may also accommodate favorable packing of the COEs in the membrane.

To gain insight into the physicochemical interactions driving the antibacterial activity of the COE compounds against bacterial cell membranes, quartz crystal microbalance-dissipation (QCM-D) experiments were conducted to evaluate membrane binding to supported lipid bilayers (SLBs) containing defined model phospholipids or more complex mixtures of bacterial lipid extracts. The QCM-D results demonstrated that both COE1-3C and COE1-3PY exhibited preferential binding to bacterial lipid SLBs over the model phospholipid compositions, as indicated by larger frequency shifts which are proportional to the amount of adsorbed mass (Appendix A). Interestingly, the kinetics of membrane binding were quite different for the two compounds. COE1-3C binding to the bacterial lipid SLBs exhibited monotonic adsorption kinetics, whereas COE1-3Py had more complex, two-step interaction kinetics, suggesting that the interaction involves some degree of membrane perturbation. This observation would be in agreement with the increased hydrophobicity, the main driving force for COE uptake, of COE1-3Py (Yan *et al.*, 2015; Hinks *et al.*, 2014). An additional finding of the QCM-D measurements is that the two compounds showed minimal interactions with SLBs composed of the mammalian cell membrane-mimicking phosphatidylcholine lipids alone, supporting that compound binding shows some selectivity for lipid components found in microbial membranes.

To further assess the effect of COE binding on membrane permeability, we conducted electrochemical impedance spectroscopy (EIS) measurements using a tethered lipid bilayer membrane platform. In direct agreement with the QCM-D measurements, addition of COE1-3Py caused concentration-dependent membrane leakage by pore formation and/or lysis, as evidenced by increased conductivity across the model membranes (Appendix A). A marked transition in membrane permeabilization occurred around 1-3 μM . In contrast, the addition of COE1-3C led to markedly reduced measurement responses in the EIS experiments, which is also consistent with monotonic binding observed in the QCM-D measurements and indicates only minor effects on membrane

permeability. Interestingly, membrane extracts from mutants that are resistant to COEs showed a modified, molecule-specific response to COE treatment. For example, extracts from the EFCPy strain exhibited increased porosity upon treatment with COE-3Py but the response to COE1-3C was negligible. Likewise, membrane extracts from EFC3C were preferentially permeabilized by COE1-3C but not COE1-3Py. Thus, there was a specific membrane response to each of the COEs and this is supported by the genetic and fatty acid data (Table 2.1 and Figure 2.4). It is counter intuitive, however, that membrane mutants appear less tolerant to COEs in that their membranes become more easily perturbed by the compound they have been desensitized too. This could be interpreted as cells being sensitized to initiate a response to these molecules quickly. Further evidence of a differential response to COEs comes from the way EFC3C and EFC3Py accumulate COEs in the membrane. EFC3C, appears to preferentially absorb COEs compared to the wild type whilst EFC3Py appears to exclude COEs, in particular COE1-3Py. Overall, COE1-3C accumulates to a lesser degree in the membrane than does COE1-3Py which is in agreement with the QCMD analysis. These findings are interesting and seem to be driven by uptake. It is easy to imagine that the response to COE1-3Py, which accumulates more readily in the membrane, is to exclude it. Alternatively, it seems counter intuitive that an adaptation exists that results in greater accumulation of a toxin. However, the EIS analysis points to a milder degree of perturbation from COE1-3C indicating that its more well tolerated in the membrane, it is easy to imagine therefore that a more energetically favorable response is to ensure it is accommodated in the membrane in an ordered manner, additional uptake is merely a side effect of this.

2.3.7 Conclusions

It has been shown that two structurally related compounds, COE1-3C and COE1-3Py, which differ only in the presence of a terminal pyridinium group have markedly different membrane perturbing effects on *E. faecalis* and that this is apparent from the different genetic and fatty acid adaptations to each of these COEs. Furthermore, significant resistance to these compounds was not developed in *E. faecalis*. These findings are of significance from a molecular design point of view as they confirm only minor structural modifications can lead to very different antimicrobial effects. To understand such interactions in fine detail will lead to the design and synthesis of a new class of potent antimicrobials which do not elicit drug resistance.

3 Transcriptomic response of *Enterococcus faecalis* OG1RF, EFC3C and EFC3Py to conjugated oligoelectrolytes

3.1 Introduction

Enterococci are commensal to the gut microflora of humans and animals (Van Tyne and Gilmore, 2014); they are also found in water, soil and vegetables (Creti *et al.*, 2004; Burgos *et al.*, 2009). The two most clinically relevant species associated with enterococci infections include *Enterococcus faecalis* and *Enterococcus faecium* (Facklam R, 2002). These are opportunistic pathogens with intrinsic or acquired resistance to several antibiotics (Leavis *et al.*, 2006). A number of novel antimicrobial agents have recently been developed against Gram positive bacteria, which are also effective against enterococci strains. Some of these antimicrobials which are in their early stages of development include lipid II targeting molecules (derivatives of nisin, teixobactin and analogues of vancomycin), sortase inhibitors, lipoteichoic acid and teichoic acid inhibitors among others (van Harten *et al.*, 2017). However, resistance has already been observed against daptomycin, the drug of last resort for enterococci infections, which is under clinical use (Diaz *et al.*, 2014; van Harten *et al.*, 2017). With the emergence of antibiotic resistance to existing antibiotics, there is a need to develop new drugs which can tackle the resistance mechanisms in bacteria.

Conjugated oligoelectrolytes (COEs) have a π -conjugated aromatic backbone rings with terminal ionic pendant groups (Thomas *et al.*, 2013). They have been shown to spontaneously intercalate into the lipid bilayer owing to their amphiphilic properties (Hinks, Poh, *et al.*, 2015). Whole genome sequencing of *E. faecalis* strains EFC3C and EFC3Py that had developed resistance to these COEs indicated that these strains have mutations in *liaFSR*, a three component signal transduction system. LiaFSR systems are known to respond to different stimuli in different organisms but recently it has been known to be associated with daptomycin resistance in enterococci (Munita *et al.*, 2012; Suntharalingam

et al., 2009). The LiaSR system is involved in sensing and responding to cationic antimicrobial peptides (cAMP) in *Bacillus subtilis* (Jordan *et al.*, 2006). In *Streptococcus pneumoniae*, LiaRS senses cell envelope associated damage. In another Gram positive bacterium, *Listeria monocytogenes*, LiaR is associated with nisin resistance (Bergholz *et al.*, 2013; Eldholm *et al.*, 2010). A *liaR* deletion from a daptomycin resistant strain of *E. faecalis* (carrying mutations in *liaF*, glycerophosphoryldiester-phosphodiesterase *gdpD* and cardiolipin synthase *cls*) has been found to restore the localization of cardiolipin domains to the septum (Reyes *et al.*, 2015). However the direct effect of *liaFSR* on lipid metabolism or membrane lipid composition remains unknown.

To understand how the membrane associated proteins mediate the resistance to COEs, the changes in the gene expression profiles of EFC3C and EFC3Py (COE resistant strains), as well as the wild type *E. faecalis* were investigated using a global transcriptomic approach. Based on this analysis, it was observed that several membrane associated transporters, pH stress, *dlt* operon, PBP 1 and PBP3, *liaFSR* regulon and its downstream targets were affected in the EFC3C and EFC3Py strains. Furthermore, the COE treatment on wild type strains led to differential regulation of primary and secondary transporters in the membrane.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture medium

The bacterial strains (Table 3.1) used in this study were cultured in Brain Heart Infusion (BHI) broth, overnight at 37⁰ C under static conditions.

Table 3.1 List of bacterial strains used in this study and their characteristics

Strain Name	Genotypic characteristics	Source
<i>Enterococcus faecalis</i> OG1RF	wild type	(Dunny <i>et al.</i> , 1978)
<i>E. faecalis</i> EFC3C	COE1-3C resistant strains with an in frame deletion in <i>liaF</i> at position 179 and a substitution in the intergenic region between <i>treB</i> (PTS family trehalose porter, IIBC component) and <i>gloA6</i> (lactoylglutathione lyase).	This study
<i>E. faecalis</i> EFC3Py	COE1-3Py resistant strains with a non-synonymous substitution at position 97 in the <i>liaR</i> region of EFC3Py and mutations in MerR family transcriptional regulator, site specific tyrosine recombinase XerD and ATP-binding protein region of a multidrug ABC transporter	This study

3.2.2 Sample preparation for RNA extraction

Overnight grown bacterial cultures were inoculated into 5 ml of BHI medium and grown at 37⁰ C without shaking. COE1-3C and COE1-3Py were added to the culture at the minimum inhibitory concentration for each strain at an OD_{600nm} of 0.4. COE1-3C and COE1-3Py were used to treat EFC3C, EFC3Py and wild type at their MIC concentrations in triplicate for 15 min (Table 3.2). The cell pellet from 1.8 ml of this treated culture was used for RNA extraction as described in the next section.

Table 3.2 List of samples and treatment conditions used for transcriptomic analysis

Treatment conditions	List of strains and treatment concentration		
	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
	OG1RF	EFC3C	EFC3Py
	wild type		
Untreated control	0 μ M	0 μ M	0 μ M
COE1-3C	2 μ M	8 μ M	8 μ M
COE1-3Py	1 μ M	1 μ M	16 μ M

3.2.3 RNA Extraction

Total RNA was extracted using the moBio Ultraclean microbial extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol. The isolated RNA was treated with TURBO DNA-free™ kit (Invitrogen Ambion) to remove any traces of DNA. The RNA was purified after DNase treatment using RNA Clean XP bead suspension (Agencourt Bioscience). The purified RNA was eluted with nuclease-free water (Invitrogen Ambion). The total RNA quantity and purity was determined using a NanoDrop spectrophotometer (Thermo Scientific, DE, USA) and Qubit® RNA HS Assay Kit by Qubit® 2.0 Fluorometer (Life Technologies) according to the manufacturer's protocol.

3.2.4 Determination of RNA concentration and RNA integrity number (RIN)

The integrity of RNA was measured using a 2200TapeStation system (Agilent Technologies) with RNA Analysis ScreenTape (Agilent Technologies). The use of tapestation system enables determination of the RNA integrity number (RIN), an indicator of integrity of RNA in the sample (Schroeder *et al.*, 2006)

and a minimum RIN value of 8 was used. The minimum concentration of RNA was ≥ 40 ng/ μ l and DNA content was $< 10\%$.

3.2.5 RNA Sequencing

Library preparation was performed according to Illumina's TruSeq Stranded mRNA protocol with the following modifications: The oligo-dT mRNA purification step was omitted and instead, 200 ng of total RNA were directly added to the Elution2-Frag-Prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely dual barcoded with Illumina's TruSeq HT RNA barcoded adapters to allow pooling of libraries for sequencing. The finished libraries were quantitated using Invitrogen's Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent). Library concentrations were then normalized to 4 nM and validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol, and Illumina's PhiX control library as standard. The libraries were then pooled at equimolar concentrations and sequenced on an Illumina HiSeq2500 sequencer in rapid mode at a read-length of 100 bp paired-end.

3.2.6 Differential expression analysis using EdgeR

The quality of Illumina pair-ended reads was first assessed using FastQC (Andrews, 2010). Ribosomal RNA reads were discarded using the SortMeRNA software (Kopylova *et al.*, 2012) and the remaining non-rRNA sequences were mapped against the OG1RF reference genome accession number NC_017316.1 using Burrows-Wheeler Alignment software (Li and Durbin, 2009). Aligned data was sorted with SAMTools (Li *et al.*, 2009) and HTSeq was used to extract counts (Anders *et al.*, 2015). Differential expression (DE) analysis was performed using the EdgeR pipeline (Robinson *et al.*, 2010). Briefly, counts were normalized to total library size, followed by dispersion estimation and differential expression test. For DE gene detection, a false discovery rate (FDR) of 0.05 was chosen as the threshold.

3.3 Results and Discussion

3.3.1 Transcriptomic Analysis

To investigate how the mutations in *liaF* and *liaR* identified in strains EFC3C and EFC3Py, respectively, mediate resistance to COEs, a transcriptomic approach was undertaken. Using this approach, the change in gene expression profiles of the wild type as well as the COE resistant mutants were compared in the presence and absence of COE1-3C or COE1-3Py. The individual responses are detailed in the sections below.

Overall, the fold change in gene expression of wild type (COE1-3C or COE1-3Py treated), EFC3C (untreated) and EFC3Py (untreated) was measured in comparison to the untreated wild type. The gene expression in EFC3Py and EFC3C exposed to either COE1-3C or COE1-3Py was studied in comparison to untreated EFC3Py and EFC3C, respectively. Differential sequence analysis using EdgeR revealed that gene expression in the wild type strain differed for 36 and 51 genes upon treatment with COE1-3C and COE1-3Py, respectively (Figure 3.4 and Figure 3.5). Compared to the wild type, 255 genes in EFC3Py and 106 genes in EFC3C were differentially expressed in the absence of the COEs (Figure 3.6 and Figure 3.7). A significant difference in the transcriptomic response to each of the COEs in the mutants was observed. Upon treatment with COE1-3Py, 136 genes were differentially regulated in EFC3Py (Figure 3.8), whereas in contrast only two genes were differentially regulated upon addition of COE1-3C. In EFC3C, only 1 and 5 genes showed altered expression when treated with COE1-3Py and COE1-3C, respectively, in comparison to untreated EFC3C. Those genes that were altered in expression, ≥ 4 and ≤ -4 , was considered for the Table B-1 – B-8 and Figures 3.2 – 3.8 in order to focus on the highly differentially expressed transcripts.

For wild type cells treated with COE1-3C or COE1-3Py, there were 7 genes with similar changes in expression, ≥ 4 and ≤ -4 , although they also differed in 13 and 18 genes, respectively. The transcriptomic response of *in vitro* evolved COE resistant strains revealed that 95.3% of the differentially expressed genes in EFC3C were differentially expressed in EFC3Py. Interestingly, EFC3Py had

an additional 60 genes that were differentially expressed, that were not altered in EFC3C.

Genes were functionally annotated based on the KEGG BRITE hierarchy (Figures. 3.4 -3.8). Based on the functional annotations, it was observed that the genes that were affected were predominantly related to: drug resistance, signal transduction, membrane transporters, cell communication and metabolism. The genes that could not be categorized into KEGG pathways were assigned as unclassified. Around 10% of the genes associated with the response to COE treatment and adaptation were associated with membrane transport function in all comparisons. Hence, TransporterDB was used to categorize the differentially expressed genes involved in transporter activity according to the substrates, family of transporters and transporter class (Elbourne *et al.*, 2017). It should be noted that this also included genes not necessarily classified as membrane transporters according to KEGG Orthology. Membrane transporters of lipoprotein and antimicrobial peptide efflux pumps were downregulated in EFC3Py, EFC3C, COE1-3C treated *E. faecalis* wild type and COE1-3Py treated *E. faecalis* wild type (Figure. 3.3). However, the multidrug efflux pump was significantly upregulated in COE1-3C and COE1-3Py treated EFC3Py (Figure 3.2(A)). The iron-hydroxamate transporter was upregulated in EFC3C, EFC3Py and COE1-3Py treated EFC3Py (Figure 3.2 (A)). The membrane transporters affected, in terms of being induced or repressed, were from the ATP-binding Cassette (ABC) Superfamily (Figure 3.2 (B) and Figure.3.3 (B)). ABC transporters are ubiquitous membrane proteins that couple adenosine triphosphate (ATP) hydrolysis to the translocation of diverse substrates across cell membranes (Locher *et al.*, 2002). For example, EfrCD, a multidrug efflux pump was found to be highly expressed in EFC3Py. These transporters are discussed in detail below.

Figure 3.1 The number of differentially expressed genes based on RNA-seq data between (a) wild type (Wt) treated with COE1-3Py (WC) or COE1-3C (WD), (b) EFC3C (DCt) vs. EFC3Py (CCt), (c) EFC3C treated with COE1-3C (DD) and EFC3C treated with COE1-3Py (DC) and (d) EFC3Py treated with COE1-3C (CD) and EFC3Py treated with COE1-3Py (CC).

Figure 3.2 The number and types of transporter associated genes, based on the (A) transporter substrate and (B) transporter family that were upregulated in *E. faecalis* OG1RF strains in relation to COE treatment or in COE resistant mutants. Transporter genes were identified based on analysis using TransporterDB (Elbourne *et al.*, 2017).

Figure 3.3 The number and types of transporter associated genes, based on the (A) transporter substrate and (B) transporter family that were downregulated in *E. faecalis* OG1RF strains in relation to COE treatment or in COE resistant mutants. Transporter genes were identified based on analysis using TransporterDB (Elbourne et al., 2017).

3.3.2 The response of *E. faecalis* wild type to COE1-3C and COE1-3Py treatment

The transcriptomic analysis of *E. faecalis* OG1RF wild type treated with COE1-3C or COE1-3Py at their minimum inhibitory concentration showed a differential expression of 36 and 51 genes, respectively. Approximately 30 genes for both treatments could not be classified according to the KEGG Orthology or were identified to be hypothetical genes. In response to COE1-3C treatment, genes associated with signal transduction and metabolism were either induced or repressed.

Figure 3.4 (a) Distribution of differentially expressed genes in COE1-3C treated *Enterococcus faecalis* wild type (Ef) based on KEGG Orthology (KO). The differentially expressed genes which did not fall into any KEGG pathway were assigned as uncharacterized. (b) Dot plot representing all of the genes categorized according to the KEGG pathways and their fold change.

Figure 3.5 Distribution of differentially expressed genes in COE1-3Py treated *Enterococcus faecalis* wild type (Ef) based on KEGG Orthology (KO). The differentially expressed genes which did not fall into any KEGG pathway were assigned as uncharacterized. (b) Dot plot representing all of the genes categorized according to the KEGG pathways and their fold change.

Adenylosuccinate synthetase (OG1RF_10304) was downregulated by 15 fold after treatment with COE1-3C (Table B-1). In *E. coli*, this enzyme, encoded by *purA*, has been demonstrated to be involved in the synthesis of adenosine monophosphate (AMP) from inosine monophosphate or IMP (He and Zalkin, 1994; Sun *et al.*, 2011). It was shown that deletion of *purA* reduced acid resistance and it was involved in an ATP dependent DNA damage repair system (Sun *et al.*, 2011). This gene was observed to be downregulated in *E. faecalis* V583 upon treatment with the macrolide antibiotic erythromycin (Aakra *et al.*, 2005). In *E. faecalis*, based on studies with a deletion mutant of genes involved in (p)ppGpp synthesis, it was suggested that increased levels of this alarmone could affect *de novo* pyrimidine synthesis genes including *purA* (Gaca *et al.*, 2013). Based on these observations, it is suggested that reduced expression of *purA* is a consequence of stress induced by COE1-3C.

Of the changes in gene expression, those associated with transporter function were one of the major groups of genes, based on KEGG analysis, that were differentially expressed. Transporters associated with multidrug resistance

(MDR transporters) are generally classified into two major categories (a) primary transporters of the ABC superfamily and (b) secondary transporters which use a proton motive force or sodium motive force to export drugs across the membrane (Bolhuis *et al.*, 1997). It was found that in the presence of COE1-3C, the activity of 7 of the ABC family of membrane transporters (Figure 3.3(b)) associated with lipoprotein transport and antimicrobial peptide efflux (Figure 3.3(a)) were repressed. In COE1-3C, the secondary transporters in the Resistance/Nodulation/Cell Division (RND) family (OG1RF_10301 and OG1RF_12269) were downregulated and COE1-3Py induced the Na⁺/H⁺ antiporter (OG1RF_10288 and OG1RF_10369) (Table B-1 and Table B-2). This suggests there may be an increased proton gradient after treatment with these conjugated oligomers. Similarly, the riboflavin transporter family was upregulated by 7.5 fold when wild type was treated with COE1-3Py.

Genes that were exclusively induced in *E. faecalis* wild type treated with COE1-3Py were associated with carbon metabolism, signal transduction and transporter activity (Table B-2). Catalase (OG1RF_11314), which is associated with virulence, and is often transcribed under oxidative stress conditions (Frankenberg *et al.*, 2002), also was induced. Genes coding for a Na⁺/H⁺ antiporter (OG1RF_10288 and OG1RF_10369), which is known to function under low pH conditions (Kakinuma, 1987), were upregulated in the presence of COE1-3Py. Similarly, induction of a glutamate: GABA antiporter (OG1RF_10368 and OG1RF_11810), involved in the conversion of glutamate to GABA, was observed. This antiporter is associated with reduced membrane potential and helps in maintaining pH homeostasis in enteric bacteria (Krulwich *et al.*, 2011). Overall, the genetic responses of *E. faecalis* to COEs suggests that cells induce a general stress response that includes the repression of a significant number of genes associated with secondary transport functions such as RND (drug/ proton transporter) in COE1-3C and upregulation of Na⁺/H⁺ antiporter in COE1-3Py.

3.3.3 Transcriptomic profiles of EFC3C and EFC3Py in comparison to untreated wild type *E. faecalis*

Both EFC3Py and EFC3C were less susceptible to COE1-3C in comparison to the wild type (Chapter 2, Table 2.1). In contrast, EFC3Py was less sensitive to COE1-3Py, while EFC3C remained sensitive. To understand the adaptation of these spontaneous mutants to COEs, the gene expression profiles of the untreated mutants EFC3C and EFC3Py were compared with the untreated wild type. Of the genes induced or repressed in EFC3Py, 49.59% were similarly changed in EFC3C (Figure 3.1 b). This suggests that these genes are associated with the resistance to COEs in general but the remaining 50.41% of genes expressed in EFC3Py may be specifically associated with COE1-3Py resistance. The genes expressed in common between EFC3Py and EFC3C were associated with KO categories of amino acid metabolism, carbohydrate metabolism, glycan biosynthesis and metabolism as well as lipid metabolism, membrane transport, signal transduction and translation (Table B-5).

Figure 3.6 (a) Distribution of differentially expressed genes in EFC3Py in comparison to the *E. faecalis* wild type (Ef) based on KEGG Orthology (KO). The differentially expressed genes which did not fall into any KEGG pathway were assigned as uncharacterized. (b) Dot plot representing all of the genes categorized according to the KEGG pathways and their fold change.

Figure 3.7 (a) Distribution of differentially expressed genes in EFC3C in comparison to the *E. faecalis* wild type (Ef) based on KEGG Orthology (KO). The differentially expressed genes which did not fall into any KEGG pathway were assigned as uncharacterized. (b) Dot plot representing all of the genes categorized according to the KEGG pathways and their fold change.

3.3.4 The role of differential expression of various transporter families in EFC3C and EFC3Py in comparison to the untreated wild type

ABC transporters are associated with the transport of different types of substrates ranging from small ions, sugars, amino acids, vitamins to proteins and complex sugars (Davidson *et al.*, 2008). They are typically been associated with osmotic stress, pathogenesis, transport of lipids and export of biomolecules during differentiation (Lubelski *et al.*, 2007). In both mutants, EFC3C and EFC3Py, transporters of the ATP binding cassette superfamily were the most significantly induced or repressed genes compared to the wild type (Figures 3.2 b and 3.3 b). In EFC3Py, genes associated with the transport of amino acids via ABC transporters were highly upregulated with a fold change ranging from 8.95 to 11.48 (Figure 3.2). This was closely followed with a nearly 5 fold increase in spermidine/putresine transport in comparison to the wild type. Both EFC3C and EFC3Py showed increased expression in riboflavin transporters. Genes encoding Type IV Secretory Pathway Family and citrate-Mg²⁺:H⁺ (CitM) Citrate-Ca²⁺:H⁺ (CitH) Symporter (CitMHS) Family were also induced in EFC3Py. In contrast, the sugar specific PTS was exclusively induced in EFC3C.

Based on the changes in the gene expression, EFC3Py showed increased expression of amino acid transporters (Table B-5), while EFC3C showed increased expression in iron-hydroxamate uptake (Table B-4). Overall, the significantly upregulated and downregulated transporter genes were generally involved in transport of lipoproteins export, amino acids, riboflavin, mannitol/fructose, oligopeptide and choline are discussed below.

A putative glutamine ABC superfamily ATP binding cassette transporter (OG1RF_10895 - OG1RF_10898) was upregulated in EFC3Py. Glutamate uptake by *Bacillus subtilis* has been shown to be associated with butanol stress, where the glutamate was converted to proline by anabolic or osmoadaptive biosynthesis routes (Mahipant *et al.*, 2017). Proline, glutamate or glycine betaine, are known as osmoprotectants (Robert *et al.*, 2000; Khudyakov *et al.*, 2012). This raises the possibility that osmotic stress resistance mechanisms may protect EFC3Py and EFC3C from the effects of the COEs and this could be tested by supplementing their growth medium with such osmoprotectants.

It was observed that enzymes involved in lactose catabolism (OG1RF_10434) and glucose 1, 6-bisphosphate production (OG1RF_10682) were repressed in EFC3Py. Both EFC3C and EFC3Py showed reduced expression of 6-phospho-beta-glucosidase (OG1RF_11199) (Figure 3.5), which catalyzes a reaction $6\text{-phospho-beta-D-glucosyl-(1,4)-D-glucose} + \text{H}_2\text{O} = \text{D-glucose} + \text{D-glucose 6-phosphate}$ (Kanehisa and Goto, 2000), whose final product enters glycolysis. In this study, the binding protein component of the ABC type Fe^{3+} -hydroxamate transport system (OG1RF_12351) was induced in EFC3C and EFC3Py. Additionally, the ferrichrome ABC transporter substrate-binding protein (OG1RF_10136) and iron ABC transporter permease (OG1RF_12353) were upregulated in EFC3C. After exposure to COE1-3Py, the substrate binding protein (OG1RF_10136) and ATP binding protein (OG1RF_10137) of ferrichrome ABC transporter were also upregulated. Similarly, the iron ABC transporter operon (OG1RF_12351, OG1RF_12352, OG1RF_12353 and OG1RF_12354) was induced. Preliminary studies on the effect of supplementation of 2 μM heme to *E. faecalis* OG1RF showed that there was an increase in resistance to COE1-3Py in EFC3Py (data not shown). By delving further into the effect of iron on the levels of fumarate, succinate, cytochrome *bd* and lactate, it may be possible to determine the effect of COEs on the whether it affects the respiratory mechanism in the mutants. Or if it is a general stress response to membrane damage by COEs.

3.3.5 Differential regulation of cell envelope stress response related regulons and stimulons in untreated EFC3C and EFC3Py

The cell envelope stress response is orchestrated by signal transduction systems and regulons, which respond to cell wall targeting agents and other envelope perturbing conditions by stimulating target genes. Despite the diversity in the cell envelope stress response stimulon among Gram positive bacteria, two systems are prominently associated with such stress responses are the *dlt* operon and penicillin binding proteins (PBPs) (Jordan *et al.*, 2008). The transcriptomic profile of EFC3C and EFC3Py showed downregulation of genes involved in lipoteichoic acid synthesis, *dltA-D* (OG1RF_12109 - OG1RF_12112) in both EFC3C and EFC3Py (Table B-3). The regulation of the D-Ala esterification of the lipoteichoic acids varies in different bacteria

according to the environmental conditions (McCormick *et al.*, 2011). It has been previously shown that decreased lipoteichoic acid production and the concomitant decreased cell wall hydrophobicity in *E. faecalis* AR01/DGVS mutants was associated with increased resistance to monolaurin (Dufour *et al.*, 2007). However, previous studies with COE and OG1RF Δ *dltA-D* mutants showed no difference in susceptibility to COE1-3C and COE1-3Py in *dlt* mutants of *E. faecalis* OG1X in comparison to the *E. faecalis* OG1X wild type (Hinks *et al.*, 2015). Therefore, a change in *dlt* operon expression (Table B-3) implies that COEs induce a general cell envelope associated stress response in *E. faecalis*.

The transcriptomic analysis also revealed that the penicillin binding protein 1A (OG1RF_10417), associated with peptidoglycan biosynthesis, was downregulated in both EFC3C and EFC3Py (Table B-3). PBP1 depletion improves the efficacy of killing by DAP in MRSA (Berti *et al.*, 2016). In the case of EFC3Py, the cell division protein FtsI or PBP3 (OG1RF_12158) was also downregulated (Table B-5). Normally, FtsI or PBP3 are localized at the septal ring during division and inactivation of FtsI may result filamentation in bacteria (Fontana *et al.*, 2000; Weiss *et al.*, 1999; Wissel and Weiss, 2004). Together, the transcriptomic data presented here, showing reduced expression of the *dlt* operon, *pbp1* and *pbp3* strongly suggest that the COEs are inducing cell envelope stress via membrane perturbation in *E. faecalis*.

Whole genome sequencing of EFC3C and EFC3Py revealed mutations associated with the LiaFSR operon. In recent years, the role of this operon in daptomycin resistance in enterococci has been demonstrated (Reyes *et al.*, 2015; Tran, Panesso, Mishra, *et al.*, 2013; Arias *et al.*, 2011). LiaFSR three component system consists of response regulator LiaR, which acts as the transcriptional repressor or activator of target genes namely *liaFSR* operon and *yvlB* operon (consisting of *yvlD*, *pspC* and *yvlB*) (Davlieva *et al.*, 2015). The mutations observed in the DNA sequencing were observed in the RNA sequencing data with *liaF* downregulated at -5.32 and -9.43 in EFC3C and EFC3Py, *liaS* downregulated at -4.88 and -10.27 and *liaR* was downregulated in EFC3Py at -8.98. In EFC3Py, *yvlD*, *pspC* and *yvl* were significantly

repressed, -92.16, -102.71 and -215.68, respectively (Table B-3), while EFC3C showed a fold change of -28.47, -32.26 and -64.19, respectively, for the same genes (Table B-3). In *S. mutans* and *S. pneumoniae*, *hrcA* (heat shock response regulator) has a LiaR binding motif in its promoter, suggesting it is regulated by this three component system (Eldholm *et al.*, 2010; Shankar *et al.*, 2015). HrcA in turn negatively regulates the production of GroEL and DnaK proteins, which are involved in responding to environmental stress response. Interestingly, the *hrcA-grpE-dnaK* operon (OG1RF_11076 to OG1RF_11078) was also downregulated in EFC3Py (Figure 3.7). The altered expression of GroEL and DnaK could lead to pronounced susceptibility to stress and thereby reducing cell survival. Alternatively, this can be a severe fitness cost incurred by the LiaFSR mutations, which can be addressed in the future work on the influence of LiaFSR mutations on the relative fitness with the unadapted strains. It is known that DnaK participates in acid stress tolerance via biogenesis of ATPase or by acting as a chaperone to this complex (Lemos *et al.*, 2001). These ATPases help in providing energy to most of the ABC transporter proteins involved in transport (Zhong and Tai, 1998). Studies demonstrating the acid tolerance or susceptibility and ATPase assays of EFC3C and EFC3Py will help in understanding the effect of this downregulation on the antimicrobial action of COEs.

3.3.6 Upregulation of riboflavin transporters in EFC3C and EFC3Py

In the absence of a tricarboxylic acid cycle and oxidative phosphorylation, *E. faecalis* undergoes aerobic respiration via cytochrome bd, NADH dehydrogenases and quinones (Keogh *et al.*, 2017). Cytochrome bd formation requires the presence of heme (Ramsey *et al.*, 2014). Since the iron transporters are upregulated, it could be hypothesized that EFC3C and EFC3Py favor an aerobic respiration. In the absence of oxygen (anaerobic conditions), fumarate, nitrite, ferric, carbon dioxide and sulphite could serve as electron acceptors. The NADPH:quinone (or fumarate) reductase reduces fumarate to succinate (Ramsey *et al.*, 2014). Fumarate reductase was however found to be repressed in EFC3Py. Riboflavin has been found to be essential for its synthesis (Ramsey *et al.*, 2014). Among the five genes upregulated in common to EFC3Py and

EFC3C, a riboflavin transporter (OG1RF_11260) was induced by 4.71 and 5.31 fold, respectively. Here the downregulation of fumarate reductase suggests that either riboflavin transport is upregulated to increase the activity of this enzyme or serves as an extracellular electron acceptor due to a repressed fumarate reductase. This is based on a recent study on the role of riboflavin as an extracellular electron transporter in *E. faecalis* biofilms (Zhang *et al.*, 2014). Further studies involving supplementing riboflavin in the growth medium of the mutants and measuring the level of fumarate and succinate will help in identifying the role of this vitamin in the mutants.

3.3.7 The effect of COE1-3Py on the differential expression of genes in EFC3Py

The response of EFC3Py to COE1-3Py differed by 3 genes, while 44 genes were common to both. The peptide ABC transporter substrate-binding protein was downregulated upon treatment with COE1-3Py. This could be seen as an effect of the stress response to COEs. Similar to EFC3Py, ABC transporters associated with iron-hydroxamate, zinc, phosphonate, lipid A, cation and some multidrug efflux ABC transporters were induced after exposure to COE1-3Py (Table B-6). The genes encoding enzymes such as conjugal transfer protein TraX, phosphoesterase, polysaccharide lyase, peptidase M23 and cobalamin synthesis were upregulated along with an adhesion protein and ribosomal protein. Transporters such as PTS fructose/mannose/sorbose transporters, manganese/zinc and phosphonate were repressed. In the previous comparison of EFC3PC and EFC3Py with wild type *E. faecalis*, the probable role of COE as an extracellular electron transport was discussed. In the context of manganese transporters, extracellular electron transfer in enterococci biofilms is enhanced by the addition of iron in comparison to supplementation of other metals such as magnesium, manganese or heme (Keogh *et al.*, 2017). OG1RF_12366, one of the five genes encoding the lipoprotein classified as a transport and binding protein was downregulated in the presence of erythromycin in a vancomycin resistant strain of *E. faecalis* V583 (Aakra *et al.*, 2005). Altered transport of this operon, consisting of OG1RF_12366 to OG1RF_12368 was suggested to represent a general stress response mechanism

(Aakra *et al.*, 2005). Here, the drug efflux transporter encoded by OG1RF_11766 and OG1RF_11767 was induced 787 and 699 fold, respectively. The product of these genes has been associated with the efflux of fluoroquinolones in *E. faecalis* and was involved in fluorescent substrates such as ethidium and Hoechst 33342 (Hürlimann *et al.*, 2016). The strong induction of this multidrug efflux transporter could be associated in reducing the accumulation of the COEs or accumulation of toxic substances from the environment owing to the membrane perturbation induced by COE1-3Py.

Figure 3.8 (a) Distribution of differentially expressed genes in EFC3Py treated with COE1-3Py based on KEGG Orthology (KO). The differentially expressed genes which did not fall into any KEGG pathway were assigned as uncharacterized. (b) Dot plot representing all of the genes categorized according to the KEGG pathways and their fold change.

3.3.8 The effect of COE1-3Py on the differential expression of genes in EFC3C

Treatment of EFC3C with COE1-3Py significantly altered the expression of four genes (Table B-7). COE1-3Py treatment of EFC3C led to changes in genes previously observed to be affected in EFC3Py untreated or COE1-3Py treated cells. For example, genes associated with lactose catabolism (OG1RF_10434) and spermidine transporters (OG1RF_10993) were induced in the untreated EFC3Py mutant while the divalent ion transporter (OG1RF_11677) was downregulated in COE1-3Py treated EFC3Py. Thus the lack of significant number of changes in gene expression may reflect the inability of EFC3C to develop resistance to COE1-3Py.

3.3.9 The effect of COE1-3C on gene expression in EFC3Py and EFC3C

The multidrug ABC transporter encoded by OG1RF_11766 and OG1RF_11767 was significantly induced after treatment of EF3Py with COE1-3C (Table B-8). In the case of EFC3C, there is no significant change in the response to COE1-3C.

3.3.10 Summary

The RNA-seq was highly useful in understanding the response of *E. faecalis* wild type, EFC3C and EFC3Py to COEs. The two molecules COE1-3C and COE1-3Py exert similar effects although COE1-3Py seems to exert a stronger effect than COE1-3C considering the number of transcriptome level changes in the mutants. The mechanism of action seems to be similar with both the molecules affecting the expression of multidrug transporters, general membrane stress response and osmotic shock associated proteins. EFC3C and EFC3Py that developed resistance to COEs after serial passage were found to downregulate the cell envelope stress response regulon (*liaFSR* and other genes regulated by *liaR* such as *pspC* and *hrcA*). The observations also indicate that EFC3C and EFC3Py may have an altered cell envelope owing to changes in their *dlt* operon and penicillin binding proteins (PBP). Additionally, there was also significant induction of iron-hydroxamate, glutamate uptake, choline binding protein and

riboflavin in the mutants. In conclusion, *E. faecalis* OG1RF responds to cell envelope stress induced by COEs by inducing or repressing a broad range of genes associated with the production of transporters and their binding proteins. Furthermore, many genes involved in general cell envelope associated stress were also affected.

4 Phenotypic and gene expression profiling of mutations in the *liaFSR* regulon associated with *Enterococcus faecalis* OG1RF's adaptation to COEs

4.1 Introduction

In order to survive in a complex environment, bacteria modulate their gene expression in response to environmental variables such as osmotic stress, temperature, pH and nutrient availability to optimize their fitness (Mascher, 2006). The bacterial cell envelope functions to maintain the cell integrity and growth and mediates solute transport by recognizing and responding to these environmental cues via signal transduction systems. These signaling systems comprise sensing modules (such as chemotaxis receptors, serine/threonine kinases, histidine-protein kinases and anti- σ factors) along with their corresponding response regulators or sigma factors (Mascher *et al.*, 2006). Many antibiotics of clinical importance target the cell envelope and these may activate signaling systems such as extracytoplasmic functioning σ factors or two component systems (TCS) (Jordan *et al.*, 2008).

Conjugated oligoelectrolytes (COEs) are amphipathic molecules with π -conjugated structural units and terminal ionic pendant groups. They have a high affinity for the lipid bilayer and spontaneously intercalate into the membrane. The ability of phenylenevinylene COEs to perturb bacterial membranes varies according to the number of aromatic rings and the type of terminal ionic group. It was observed that the COEs with three aromatic groups and terminal pyridinium cationic groups, i.e. COE1-3Py, were more inhibitory than COEs with terminal tetraalkylammonium groups (COE1-3C) (Yan *et al.*, 2016). In this study, the mechanism of adaptation adopted by *E. faecalis* OG1RF that was passaged in increasing concentrations of COEs was examined (Chapter 2). For two strains, EFC3C and EFC3Py that developed resistance to COE1-3Py and COE1-3C, respectively, mutations were found in the *liaFSR* operon, a three component cell envelope stress sensing system. Resistance to COE1-3C and COE1-3Py involved a deletion of Ile at position 179 in *liaF* (in EFC3C) and a substitution of valine with isoleucine at position 97 of *liaR* (in EFC3Py). The gene expression profiles of EFC3C and EFC3Py showed changes in genes

encoding cell wall proteins, cell envelope stress associated signal transduction systems, iron transporters, osmotic stress and membrane associated primary and secondary transporters.

LiaFSR is a three component system that is conserved in Gram positive bacteria and that functions as a damage sensing and signal transducing system (Munita *et al.*, 2012). ‘lia’ refers to lipid II interacting antibiotics because it was identified by treating *Bacillus subtilis* with antibiotics that interfered with lipid II and cell wall synthesis, e.g. nisin, bacitracin, vancomycin and ramoplanin (Mascher *et al.*, 2003). It includes a transmembrane histidine kinase (LiaS), cytosolic response regulator (LiaR) and a membrane bound negative regulator (LiaF). LiaF interacts with LiaS to maintain the LiaFSR regulon in an “OFF” state. In the presence of a membrane stress, LiaS responds by phosphorylating LiaR, which in turn leads to the transcription of operons with LiaR binding regions (Davlieva *et al.*, 2015). *In silico* analysis shows that three genes in *E. faecalis* (Figure 4.1), are regulated by LiaR, *yvlD* (putative integral membrane protein), *pspC* (phage shock protein C) and *yvlB* (putative regulator of stress) (Miller *et al.*, 2013).

Figure 4.1 Schematic representation of the *liaFSR* operon and downstream genes homologous to proteins in *Streptococcus mutans*. The figure is reproduced from (Miller *et al.*, 2013).

LiaR, a master regulator of the LiaFSR system (Figure 4.1) has been implicated in the development of resistance to cell membrane targeting cationic antimicrobial peptides (CAMP) (Reyes *et al.*, 2015). CAMPs target anionic lipids such as phosphatidyl glycerol (PG) and cardiolipin (CL) in the membrane. In *Enterococcus* spp., LiaR responds to cell membrane damage induced by daptomycin via redistribution of the cardiolipin microdomain (Tran *et al.*, 2013). Recently, it has been shown that supplementation of the growth medium with exogenous fatty acids results in their incorporation into the membrane of *E. faecalis* and this change in the membrane lipid composition protects the cell membrane from daptomycin (Saito *et al.*, 2014). In *Streptococcus mutans*, LiaFSR has been shown to induce genes involved in peptidoglycan and membrane protein synthesis along with cell envelope associated proteases and chaperones in addition to having an influence on the cell shape or morphology of *S. mutans* (Suntharalingam *et al.*, 2009). Based on a study on the role of LiaFSR in responding to bacitracin mediated stress in *Bacillus subtilis*, it has been shown that this system rapidly senses cell envelope perturbing stress signals and switches off once the stressor is removed (Kesel *et al.*, 2013).

In this chapter, the direct influence of specific mutations in *liaF* and *liaR* in COE resistance was investigated. This involved generating mutations in wild type *E. faecalis* that recapitulate the mutations observed in the evolved strains, EFC3C and EFC3Py. Complementation of the mutations was undertaken and the phenotypes of the mutants were compared to the wild type for planktonic and biofilm growth as well as sensitivity to COEs.

4.2 Materials and Methods

4.2.1 Conjugated oligoelectrolytes and other antibiotics

COE1-3C (1,4-bis(4''-(N,N-bis(6'''-(N,N,N-trimethylammonium)hexyl)amino)-styryl)benzene tetraiodide) and COE1-3Py ((1,4-bis(4''-(N,N-bis(6'''-(pyridinium)hexyl)amino)-styryl)benzene tetraiodide)) were synthesized as described by Garner *et al.*, 2010 and Yan *et al.*, 2016, respectively.

4.2.2 Bacterial strains and culture media

E. faecalis strains (Table 4.1) were cultured in Brain Heart Infusion (BHI) broth at 37°C under static conditions. Stellar™ cells were grown in Luria Bertani (LB) broth or agar 37°C with shaking at 200 rpm. Competent cells were prepared in 4X BGYT medium (76 g BHI, 20 g yeast extract, 8 g glucose and 400 ml of 1 M Tris (pH 7.0) in 1 L). The 4X medium was diluted to 2X and used to recover the competent cells after electroporation. Antibiotics were supplemented at the following concentrations when required: Stellar™ cells were supplemented with kanamycin at 50 µg/ml or 750 µg/ml of erythromycin and *E. faecalis* strains were supplemented with kanamycin at 500 µg/ml or 25 µg/ml of erythromycin.

Table 4.1 List of strains used in this study

Strain	Description	Growth media
<i>E. faecalis</i> OG1RF	wild type;	BHI
<i>E. faecalis</i> EFC3C	COE1-3C resistant <i>E. faecalis</i>	BHI
<i>E. faecalis</i> EFC3Py	COE1-3Py resistant <i>E. faecalis</i>	BHI
EFC3Py:: <i>liaR</i>	EFC3Py derivative complemented with pAL1 carrying wild type <i>liaR</i>	BHI + 500 µg/ml Kan
<i>E. faecalis</i> OG1RF <i>liaF</i> _{Δ11e179}	<i>E. faecalis</i> OG1RF derivative with an in-frame deletion in isoleucine at position 179	BHI
<i>E. faecalis</i> OG1RF <i>liaF</i> _{Δ11e179} :: <i>liaF</i>	<i>E. faecalis</i> OG1RF <i>liaF</i> _{Δ11e179} derivative complemented with pAL1 carrying wild type <i>liaR</i>	BHI + 500 µg/ml Kan

4.2.3 Chromosomal inactivation of *liaF* and *liaR*

To create in frame deletions of *liaF* in *E. faecalis* OG1RF, 800 bp upstream and downstream of the isoleucine at position 179 was amplified from *E. faecalis* EFC3C using the primer pairs *liaF*_INF_F and *liaF*_INF_R (Table 4.2). These primers were used to amplify the *liaF* gene with 15 bp homologous to the ends of the linearized vector. The PCR reaction was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc (NEB)). *EcoRI* and *HindIII* restriction endonucleases (New England Biolabs, Inc. (NEB)) were used to generate a linearized form of the temperature sensitive vector, pGCP213 (Nielsen *et al.*, 2012), and to digest the amplified *liaF* PCR product. CloneAmp HiFi PCR Premix was used to create a clone of *liaF* in temperature sensitive shuttle vector pGCP213. The PCR fragment was cloned in pGCP213 using the In-Fusion[®] HD PCR Cloning Kit (Clontech) (Figure 4.3). This In-Fusion[®] reaction mixture was used to transform Stellar[™] Competent Cells. The transformants were selected by growth at 37°C on LB plates supplemented with 750 µg/ml of erythromycin (Erm). pGCP213_ *liaF*_{Δ179} (Figure 4.2 (a)) derived from the transformed cells were purified using the Purelink Plasmid extraction kit. The deletion constructs pGCP213_ *liaF*_{Δ179} were sequenced using vector specific universal primers M13F(-20) and M13R(-20) to verify the gene insertion (Table 4.2).

Table 4.2 List of primers used to construct the plasmids and checking the gene product after transformation.

Primers	S Sequence	Description
<i>liaF</i> _INF_F	gatatctgcagaattcccgaagc cgatccatttt	Primer used during PCR amplification of <i>liaF</i> from EFC3C.
<i>liaF</i> _INF_R	taccgagctcgatccatattggtt caggagtttctgt	Primer used during PCR amplification of <i>liaF</i> from EFC3C.
<i>liaR</i> _INF_F	gatatctgcagaattcgagaacg tgttgcggc	Primer used during PCR amplification of <i>liaR</i> from EFC3Py.

Table 4.2 List of primers used to construct the plasmids and checking the gene product after transformation (continued).

Primers	Sequence	Description
<i>liaR_INF_R</i>	tgattacgccaagctctgccgcca aaacgtcct	Primer used during PCR amplification of <i>liaR</i> from EFC3Py.
<i>liaFcomp_INF_F</i>	gcttgatatcgaatttaggcgacc aagtgattat	Primer used during PCR amplification of <i>liaF</i> from OG1RF.
<i>liaFcomp_INF_R</i>	tagaactagtggatctcaagcata atctggaacatcatatggataagc gtaatctggaacatcgtatgggtat acacggatcacctcaac	Primer used during PCR amplification of <i>liaF</i> from OG1RF.
<i>liaRcomp_INF_F</i>	gtgaggaactgaattcgtgatcaa agtaatgtta gtggatgacc	Primer used during PCR amplification of <i>liaR</i> from OG1RF.& to check gene insertion
<i>liaRcomp_INF_R</i>	acctgttgaatgatctgcagttaag cataatctggaacatcatatggata agcgtaatctggaacatcgtatgg gtatttggttaaaccatg	Primer used during PCR amplification of <i>liaR</i> from OG1RF and to check gene insertion.
M13F(-20)	gtaaaacgacggccagt	Forward primer to check insertion in pGCP123
M13F(-40)	gttttccagtcacgac	Forward primer to check insertion in pGCP213
M13R-pUC (-26)	caggaaacagctatgac	Reverse primer to check insertion in pGCP213 & pGCP123

The *liaF* _{Δ lle179} mutant was created by transformation of *E. faecalis* OG1RF with the deletion construct pGCP213_*liaF* _{Δ lle179} by electroporation. The transformed OG1RF colonies were isolated by growth at 30°C on 25 µg/ml of erythromycin. Selection of *E. faecalis* OG1RF for chromosomal integration of the plasmid

was ensured by passaging at 42°C in LB broth with 25 µg/ml erythromycin. The subsequent step involved selection of *E. faecalis* OG1RF for excision of plasmid by passaging at 30°C without erythromycin. The erythromycin sensitive colonies were confirmed for in frame deletions in *liaF* by sequencing of the PCR amplified gene.

The *liaR*_{V97I} from EFC3Py was amplified using the primers *liaR*_INF_F and *liaR*_INF_R (Table 4.2). The same procedure as described above was used to drive the pGCP213_*liaR*_{V97I} (Figure 4.2 (b)) construct from the Stellar™ Competent Cells. The insertion of *liaR*_{V97I} in pGCP213_*liaR*_{V97I} construct was verified by sequencing with universal primers M13F (-20) and M13R (-20). This was followed by transformation of *E. faecalis* OG1RF using the method as described above.

4.2.4 Complementation of wild type *liaF* and *liaR* genes into the mutants

The *liaF* gene, along with its promoter, was amplified from *E. faecalis* OG1RF with primers *liaF*comp_INF_F and *liaF*comp_INF_R (Table 4.2) with an extension of 15 bp homologous to the plasmid pGCP123 (Nielsen *et al.*, 2012) using CloneAmp HiFi PCR Premix. The PCR fragment was cloned in pGCP123 using the In-Fusion HD PCR Cloning Kit (Clontech) (Figure 4.3) to construct pGCP123_*liaF* (Figure 4.2 (c)). The plasmid was transformed in Stellar™ competent cells and selected at 37°C on 50 µg/ml of kanamycin. The plasmid sequence was verified by sequencing. The *E. faecalis* OG1RF *liaF*_{Δlle179::}*liaF* mutant was transformed with pGCP123_*liaF* and selected on 500 µg/ml kanamycin plates.

pAL1, which is derived by inactivating the chloramphenicol resistance gene in pABG5 (Kline *et al.*, 2009; Granok *et al.*, 2000) was used for the complementation of mutant with wild type *liaR*. Since the exact location of the native promoter of *liaR* was unknown, it was placed under the control of *rofA*. This promoter is induced under static incubation at 37°C. The wild type *liaR* gene was cloned as fusion to include a hemagglutinin (HA) peptide tag for future studies to characterize its expression (not in the scope of this chapter). The coding sequence of *liaR* was amplified from wild type *E. faecalis* OG1RF with *liaR*comp_INF_F and *liaF*comp_INF_R (Table 4.2, Figure 4.4) and inserted into the *EcoRI* and *PstI* digested pAL1 plasmid. The plasmid

pAL1_*liaR* (Figure 4.2) was constructed using the In-Fusion[®] HD PCR Cloning Kit (Clontech) and it was derived from the transformed Stellar[™] competent cells selected on agar plates with 50 µg/ml of kanamycin at 37°C. The plasmid sequence was verified by sequencing. EFC3Py cells were transformed with pAL1_*liaR* and isolated on 500 µg/ml kanamycin plates.

4.2.5 Preparation of *E. faecalis* OG1RF competent cells

E. faecalis was grown at 37⁰ C for 5-7 h statically with or without antibiotics in 50 ml of BGYT supplemented with 0.5 M sucrose and 25 µg/ml rifampicin with glycine at a range of concentrations between 3-7%. A control culture used the same medium without glycine. These cultures were inoculated with 25 µl of the starter culture and grown at 37⁰ C for 15-18 h for no more than 18 h. Based on the OD₆₀₀, the cultures showing 50-70% growth in comparison to 0% glycine control were diluted to OD₆₀₀ 0.05-0.08 in BGYT+ 0.5 M sucrose +antibiotic + x% glycine (where x was the concentration with 50-70% growth) and incubated for 1 h. After the incubation period, cultures were chilled on ice for 1 h and pelleted at 6000 g for 15 min. The cells were washed twice in 10 ml of chilled electroporation buffer and finally resuspended in 2ml of it. The electroporation buffer was prepared to a volume of 50 ml with 0.5 M sucrose, 10% glycerol and 32.5 ml of sterile distilled water. Approximately 100 µl of cells were aliquoted into tubes, frozen on dry ice or ethanol and stored at -80⁰C

Figure 4.2 Plasmid map of (a) pGCP213_liaR for replacement of *liaR* from wild type by homologous recombination - pGCP213 carrying *liaR* from EFC3Py and *Erm^R* encoding erythromycin resistance (b) pAL1_liaR for complementation of EFC3Py - pAL1 carrying *liaR* gene from *E. faecalis* OG1RF with a HA tag, under control of the *rofA* promoter and kanamycin resistance gene, *Kan^R*, (c) pGCP213_liaFΔIle179 for replacement of *liaF* from wild type OG1RF by homologous recombination - pGCP213 carrying *liaF*_{ΔIle179} from EFC3C and *Erm^R* encoding erythromycin resistance and (d) pGCP123_liaF for complementation of *E. faecalis* OG1RF *liaF*_{ΔIle179}::*liaF* - pGCP123 carrying *liaF* gene under a constitutive promoter from *E. faecalis* OG1RF with a HA tag and *Kan^R* encoding for Kanamycin resistance

Figure 4.3. In-Fusion® Cloning kit protocol. This kit requires a 15 bp overlap at the terminal ends of the linearized construct and gene of interest. In-Fusion® enzyme helps in fusing the vector and insert by recognizing the 15 bp region at their terminal end to obtain the desired construct. This is then transformed into Stellar™ competent cells and spread on an LB plate with an appropriate antibiotic. The figure is reproduced from the instruction manual of In-Fusion® Cloning kit (Raman and Martin, 2014).

4.2.6 Transformation of *E. faecalis* by electroporation

Less than 300 ng of DNA was used to electroporate 50 µl of electrocompetent bacterial cells in a sterile disposable cuvette (BIORAD, USA) with an interelectrode distance of 1 mm. The mixture was placed on ice prior to electroporation using Gene Pulser™ apparatus (BIORAD, USA) with the settings 1.6-1.75 kV, 200 ohm, 25 µF and a time constant of 4.0-4.7. Cells were resuspended in 250 µl of 2X BGYT medium and incubated at 37°C to allow for plasmid replication or at 42°C to prevent plasmid replication for 1-2 h. The cells were subsequently plated on BHI agar supplemented suitable antibiotics.

The plates were incubated at 30°C for pGCP213 and 37°C for pGCP123/pAL1 to observe the transformants.

4.2.7 Minimum Inhibitory Concentration (MIC) tests

MIC tests were conducted using broth microdilution methods as previously described (Wiegand *et al.*, 2008). Experiments were carried out in triplicate and compounds were diluted in a two-fold serial dilution in BHI broth. Growth in each of the wells was assessed after 18 h incubation at 37°C. The MIC was defined here as the lowest concentration of antibiotic that inhibits the visible growth of the tested isolate as observed with the unaided eye (Wiegand *et al.*, 2008).

4.2.8 Evaluating the effect of *liaF* and *liaR* mutations on the growth kinetics

Strains were grown overnight in BHI medium with antibiotics (where necessary) at 37°C under static conditions. The cultures were subsequently diluted to 1:1000 and growth was monitored as a measure of optical density at 600 nm over 18 h period using Tecan Infinite[®] 200 PRO spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

4.2.9 Biofilm measurement of *liaFSR* mutants

Cultures of strains (listed in Table 4.2) were inoculated at a CFU of 3×10^6 into 96 well flat bottom transparent microtiter plates (Thermo Scientific, Waltham, MA, USA) and incubated under static conditions for 24 h at 37°C. The supernatants were discarded and the microtiter wells were washed twice with PBS. A solution of 0.1% w/v crystal violet (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the surface adherent bacteria by incubation at 4°C. After 30 min incubation, the plate was washed twice with 1X PBS and the remaining crystal violet was solubilized with 200 µl/well at a 4:1 ratio of ethanol and acetone for 45 min at room temperature. The absorbance was measured at 595 nm using a Tecan Infinite[®] 200 PRO spectrophotometer.

4.3 Results and Discussion

4.3.1 Construction of the *liaFSR* mutants

The plasmid construct pGCP213_*liaF*_{Δ11e179} replicates in *E. faecalis* OG1RF in the presence of erythromycin at 30 °C in BHI medium. By changing the temperature to 42°C, in the presence of erythromycin, the temperature sensitive

plasmid cannot replicate and must integrate into the *E. faecalis* chromosome by single cross-over recombination to maintain erythromycin resistance. In the absence of erythromycin, the integrated plasmid can be resolved, resulting in either restoration of the wild type genome or leaving the mutated gene behind. Isolates of *E. faecalis* OG1RF carrying the *liaF*_{Δlle179} gene replacement were confirmed by sequencing. This strain was subsequently transformed with pGCP123_iaF for complementation of the mutation. While generating the *liaF*_{Δlle179} gene replacement strain was successful, it was not possible to generate the *liaR* mutation. Therefore, due to time constraints, the EFC3Py mutant was used for complementation with the wild type copy of *liaR* expressed from pAL1_iaR.

Figure 4.4 Determination of the size of the insert after PCR amplification from OG1RF (a) *liaF* insert (1606 bp) after using primers *liaF*_INF_F and *liaF*_INF_R (b) *liaR* insert (736 bp) after using primers *liaR*comp_INF_F and *liaR*comp_INF_R to amplify the insert from wild type OG1RF. A 1 Kb DNA ladder with size of bands indicated in bp was used to verify the size of the insert

4.3.2 Growth kinetics of *liaFSR* mutants

The growth of wild type OG1RF, EFC3C, EFC3Py, *E. faecalis* OG1RF *liaF*_{Δlle179}, *E. faecalis* OG1RF *liaF*_{Δlle179}::*liaF* and EFC3Py::*liaR* was measured

as OD_{600nm} vs time (Figure 4.5, Figure 4.6). The growth of wild type OG1RF, EFC3C, EFC3Py, *E. faecalis* OG1RF *liaF*_{Δlle179}, *E. faecalis* OG1RF *liaF*_{Δlle179::liaF} and EFC3Py::*liaR* was measured as OD_{600nm} vs time. The growth profiles of *E. faecalis* OG1RF *liaF*_{Δlle179}, *E. faecalis* OG1RF *liaF*_{Δlle179::liaF} and EFC3C as determined by one-way ANOVA were not statistically different from the wild type (Figure 4.5). The significant reduction in growth observed for the EFC3Py (evolved *liaR* mutant) with the *liaR* complementation plasmid (Figure 4.6) was unexpected based on previous observations where deletion of *liaR* in *E. faecium* had no effect on growth (Reyes *et al.*, 2015). These changes could be verified by studying the expression of *liaR* in the complemented strain in comparison to the wild type. Further experiments involving the transformation of EFC3Py with an empty vector, as well as testing a specific *liaR* mutant with the complementation vector, will help to clarify these observations.

Figure 4.5 Comparison of the growth of *Enterococcus faecalis* OG1RF wild type with *liaF* mutants and EFC3C as well as the complemented strains. The optical density (OD₆₀₀) represents the average of six biological replicates.

Figure 4.6. Comparison of the growth of *Enterococcus faecalis* OG1RF wild type with EFC3Py as well as the complemented strains. The optical density (OD₆₀₀) represents the average of six biological replicates.

4.3.3 Influence of *liaF* and *liaR* mutations on sensitivity to cell envelope interacting agents

The different strains used in this study were screened for resistance to five antibiotics (Table 4.3). With the exception of *E. faecalis* OG1RF *liaF*_{Δ*lle179*}::*liaF*, all of the strains tested showed an 8 fold increase in the MIC for nisin in comparison to the wild type. The increased susceptibility to nisin upon complementation with a functional *liaF* in *E. faecalis* OG1RF *liaF*_{Δ*lle179*} suggests that the sensitivity of *E. faecalis* OG1RF *liaF*_{Δ*lle179*} was due to the *liaF* mutation. A similar behavior was observed with vancomycin, where a 2 fold reduced susceptibility was observed in the *liaF* complemented strain in comparison to the *E. faecalis* OG1RF *liaF*_{Δ*lle179*}. EFC3Py showed a 4 fold increase in resistance to penicillin in comparison to the wild type. Complementation with the functional *liaR*, there was a 2 fold reduction in the MIC, suggesting that it may be partially rescued.

In contrast, there was no difference in the MIC for valinomycin for any of the strains compared to the wild type. The lack of effect may be related to the specific mode of action of this compound, where valinomycin is an antibiotic that disrupts the membrane potential of the bacteria and thereby kills the cell (Bhattacharyya *et al.*, 1971). Hence, the lack of change in the MICs for the

various COE resistant mutants suggests that LiaFSR does not alter the cells such that the membrane potential becomes easier to disrupt.

The MIC of daptomycin against the EFC3C, EFC3Py, EFC3Py::*liaR* and OG1RF *liaF*_{ΔIle179} mutant increased 8 fold in comparison to the wild type (Table 2.1 and Table 4.3). Complementation of *liaF* in the *liaF*_{ΔIle179} mutant subsequently reduced the resistance to daptomycin back to wild type levels. This experiment shows that *liaF*_{ΔIle179} does confer daptomycin resistance that can be rescued by complementation. In contrast, complementation of the EFC3Py mutant with *liaR* did not restore the wild type sensitivity to daptomycin. Therefore, while *liaF*_{ΔIle179} appears to be associated with daptomycin resistance, the *liaR* may not be associated with the resistance to DAP. By doing knockout studies on the other mutations observed in EFC3Py, we can understand the resistance to daptomycin. Furthermore, expression studies should be conducted to confirm if *liaR* is produced from the complementation vector under the conditions used here.

From the antimicrobial susceptibility assays (Table 2.1 and Table 4.3), it appears that the deletion of the isoleucine residue at position 179 is associated with COE1-3C resistance. The EFC3Py and EFC3Py::*liaR* strains showed a 4 fold and 8 fold increase in MIC against COE1-3C, suggesting that the presence of *liaR* and ABC transporter associated mutations, respectively, enables them to resist the antimicrobial activity of this compound. In contrast, the mutation in *liaR* alone appears to contribute to resistance against COE1-3Py in EFC3Py, as seen by the 16 fold increase in MIC with the mutant. The complemented strain was found to be susceptible to COE1-3Py in the presence of the functional *liaR*, suggesting that LiaR activity is linked to resistance to COE1-3Py.

Table 4.3 Minimum Inhibitory Concentration of different antibiotics against the different strains used in this study

Strain	NIS (µg/ml)	VAN (µg/ml)	PEN (µg/ml)	VAL (µg/ml)	COE 1-3C (µM)	COE 1- 3Py (µM)	DAP (µM)
<i>E. faecalis</i> OG1RF	256	2	2	> 128	2	1	1.23
<i>E. faecalis</i> EFC3Py	>2048	4	8	> 128	8	16	9.87
EFC3Py:: <i>liaR</i>	>2048	4	4	> 128	16	1	9.87*
<i>E. faecalis</i> OG1RF <i>liaF</i> _{Δ<i>lle179</i>}	>2048	4	2	> 128	4	1	9.87
<i>E. faecalis</i> OG1RF <i>liaF</i> _{Δ<i>lle179</i>} :: <i>liaF</i>	256	2	2	> 128	2	1	1.23

*data based on test with OG1RF with functional *liaR* but other mutations similar to EFC3Py (mentioned in Chapter 3, Table 3.1)

4.3.4 The effect of *liaF* and *liaR* mutations on biofilm growth

Enterococcal biofilm formation is associated with increased resistance to antimicrobials and phagocytosis in urinary tract infections (UTI) and endocarditis (Miller *et al.*, 2013). In many Gram positive bacteria, carbohydrate metabolism influences biofilm formation (Mohamed and Huang, 2007). For example, in *E. faecalis*, supplementation of 1% glucose in TSB has been found to increase biofilm formation in comparison to unsupplemented TSB (Pillai *et al.*, 2004). In this study, the influence of *liaF* and *liaR* mutations on biofilm formation was studied (Figure 4.7). The two strains that showed a significant change in biofilm formation were OG1RF *liaF*_{Δ*lle179*} (p < 0.01) and EFC3Py (p

< 0.05) (Figure 4.7). The OG1RF *liaF* $_{\Delta lle179}$ showed a slight increase in biofilm formation (OD₅₉₅ 0.6) compared to the wild type *E. faecalis* (OD₅₉₅ 0.5) (Figure 4.7). Complementation of *liaF* in the OG1RF *liaF* $_{\Delta lle179}$ mutant showed a significant reduction in the biofilm formation (OD₅₉₅ 0.3) and this may be due to over expression of *liaF* from the complemented vector, although this should be experimentally tested in the future. Surprisingly, EFC3Py also showed a reduction in biofilm formation compared to the parent strain. It should be noted that loss of LiaSR in *S. mutans* was associated with a reduction in biofilm formation (Li *et al.*, 2002) and hence is consistent with the results for the EFC3Py strain. Further work is necessary to determine if this effect is specific for the *liaR* mutation in that strain or if it is associated with the background mutations in this strain, which include mutations in a gene associated with the MerR family of transcriptional regulators, the site specific tyrosine recombinase *xerD* and an ATP-binding protein region of a multidrug ABC transporter in EFC3Py.

Figure 4.7 Biofilm growth of wild type, *liaF* and *liaR* mutants of *E. faecalis* after 24 h growth in TSB under static conditions at 37°C. The bars show the average of two biological replicates and three technical replicates for each strain. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$.

In conclusion, the results to date show that the *liaFSR* pathway is directly related to the cell envelope stress response of *E. faecalis* associated with treatment with COEs (COE1-3C and COE1-3Py). The MIC tests with the strains collected during the first few days of the *in vitro* passaging of OG1RF in

COE1-3Py also show an absence of resistance to COE1-3Py. These strains possessed mutations in MerR, XerD and multidrug ABC transporter except in *liaR* (data not shown). Similarly, complementation with a functional *liaR* revealed a similar increased susceptibility to COE1-3Py in EFC3Py. It is possible that the phenotypes tested here, such as changes in biofilm formation or resistance to daptomycin, could be associated to the other mutations in EFC3Py (Table 4.3 and Figure 4.7).

4.3.5 Summary

Collectively, the data presented here suggest that the LiaFSR three component regulatory system responds to membrane associated damage induced by COE1-3C and COE1-3Py, although the genetic response and mechanism for how this system regulates the stress response remains unknown. By studying the change in expression of these genes and the transcriptomic profiles of mutants in these genes, it may be possible to correlate to some of the phenotypes observed above with their expression or the LiaFSR controlled regulon.

5. Conclusions

The present work has demonstrated the ability of conjugated oligoelectrolytes (COEs) to function as antimicrobials by targeting the cell envelope in *Enterococcus faecalis* OG1RF. This organism is known to be tolerant to a wide range of stressors such as pH, alkaline stress, bile, detergents and few antimicrobials (Ramsey *et al.*, 2014). Despite this inherent ability to adapt to a number of factors that affect membrane integrity, the COEs are none the less, highly active against *E. faecalis* and the evolved mutants only show a marginal increase in their MIC. This suggests that it is difficult for *E. faecalis* to develop high levels of resistance to these compounds, in contrast to more traditional antibiotics such as vancomycin, where high levels of resistance are observed.

Differences in the mechanism of COE1-3C and COE1-3Py

Quartz crystal microbalance-dissipation (QCM-D) experimental measurements were performed to study the adsorption of COE1-3C and COE1-3Py on lipid bilayers. While the COE1-3C was found to exhibit monotonic adsorption kinetics, COE1-3Py showed a complex two step interaction kinetics. This indicated that COE1-3C had less of an impact on membrane perturbation than COE1-3Py. This difference in interaction of the two molecular structures suggests that this is the reason for a lower MIC of COE1-3Py compared to COE1-3C.

Resistance

Several laboratory studies have shown that membrane interacting agents tend to show reduced development of resistance, although the increased clinical use of such antibiotics could accelerate the selection for resistant strains (Hurdle *et al.*, 2011). To determine if *E. faecalis* can evolve resistance to COEs and to understand how that resistance may be mediated, COE resistant derivatives of OG1RF, EFC3C and EFC3Py were developed by *in vitro* passaging in increasing concentrations of COE1-3C and COE1-3Py, respectively. The differences in the toxicity of MIC of wild type and the mutants is small in comparison to other compounds. For example, a 256 - 512 fold increase in

resistance to daptomycin (via *in vitro* evolution) was observed compared to that of sensitive strains (Rashid *et al.*, 2017, Palmer *et al.*, 2011). This high level of resistance was associated with the accumulation of mutations in the *liaFSR* operon and cardiolipin synthases (Tran *et al.*, 2013). The inability of *E. faecalis* to adapt significantly to COE is an interesting and desirable feature of this class of compounds and supports their further development as novel antimicrobial agents for clinical applications.

EFC3C and EFC3Py are cross-resistant to daptomycin but daptomycin resistant strains are sensitive to COEs

The mutations observed in EFC3C and EFC3Py were associated with cross resistance to daptomycin but the *in vitro* evolved daptomycin resistant strains studied by Rashid *et al.* (2017), did not evoke resistance to COEs. Mutations in the *liaF* as observed in the EFC3C strain were sufficient to evoke daptomycin associated resistance (Tran *et al.*, 2013). The results from this study suggest that upregulation of a multidrug ABC transporter was sufficient to elicit daptomycin resistance (Figure 4.2), although further work will be needed to confirm this hypothesis. To do so, this gene should be deleted in the wild type background and tested for its sensitivity to daptomycin and the COEs.

Role of membrane composition on resistance to COEs

The increase in the levels of *cis*- vaccenic acid and cyclopropane fatty acids in EFC3Py is an adaptation to accommodate lesser COE1-3Py molecules. While COE1-3C has a milder effect on membrane permeability, the change in cyclopropane levels in EFC3C and EFC3Py could represent a general adaptation to COEs.

The difference in the uptake of the COE1-3Py and COE1-3C in the adapted strains EFC3C and EFC3Py suggest that there is a difference in the membrane lipid composition of these mutants in comparison to the wild type. This change could not be correlated to any changes in the gene expression of lipid synthesis or cardiolipin synthesis at the fold change ≥ 4 in the transcriptome data of the mutants. Measurement of the levels of phosphatidylglycerol (PG), cardiolipin (DPG) and lysyl-phosphatidyl glycerol (LPG) in the membrane extracts of these mutants could help in understanding the role of specific lipids on the

resistance to COEs. This is based on previous data suggesting the preferential interaction of COEs with liposomes rich in DPG (Hinks, *et al.*, 2015).

Further studies to assess the membrane fluidity profiles could be done by measuring the fluidic domains using dyes sensitive to changes in membrane fluidity such as Laurdan (Müller *et al.*, 2016). Recent studies showing the protective effect of the uptake of fatty acids against daptomycin in *E. faecalis* from its growth medium (Saito *et al.*, 2014), suggest that the changes observed in the work presented here in the membrane fatty acids could also be associated with daptomycin resistance apart from the *liaFSR* mutations.

Correlating gene expression level changes in EFC3C and EFC3Py to adaptation to COEs

Since *liaFSR* is a three component cell envelope stress sensing system in *E. faecalis*, a transcriptomic analysis was done (as discussed in Chapter 3) to observe any changes in the gene expression level associated with these mutations and under the influence of COEs. The transcriptomic response of *E. faecalis* wild type to COEs suggests that these cells exhibit a membrane stress response that includes the repression of a significant number of genes associated with secondary transporters such as the RND (drug/proton transporter) in COE1-3C and upregulation of Na⁺/H⁺ antiporter in COE1-3Py. The significant changes in the transporter activity, primary (ABC transporters) or secondary transporters, suggests that *E. faecalis* responds to conjugated oligomers by altering its membrane transporter activity.

From the biophysical data in Chapter 2 it was concluded that the lipid bilayer of EFC3Py had a reduced tendency to allow insertion of COEs into the membrane. This behavior was also observed in the transcriptomic response of EFC3Py to COE1-3Py and COE1-3C, wherein a multidrug efflux pump was upregulated significantly (Table B-6 and Table B-7).

Similarly, it was observed that the genes that were differentially regulated in EFC3C were a subset of genes affected in EFC3Py, suggesting that this could be the reason for the EFC3C's sensitivity to COE1-3Py. Moreover, the fold changes in the gene expression levels were higher in EFC3Py, suggesting its interaction with the lipid bilayer was greater than for COE1-3C as observed by the bio-physical analysis.

Surprisingly, there were no changes at the transcriptional level in the fatty acid synthesis pathways for the strain EFC3C or EFC3Py, in contrast to the changes in membrane fatty acid composition as observed based on the FAME analysis. This would suggest that the cells may primarily make changes in the membrane fatty acids in response to COEs by post-transcriptional responses. This is supported by the observation that cyclopropanization occurs as a post-synthetic modification of phospholipids (Jungkind and Wood, 1974).

The *dltABCD* operon encodes proteins responsible for the alanylation of lipoteichoic acids and wall associated teichoic acids of Gram positive bacteria (McCormick *et al.*, 2011; Hancock and Sahl, 2006). The downregulation of the *dlt* operon has been shown to lead to increased sensitivity to cationic antimicrobial peptides by increasing the negative charge on the membrane (Maria-Neto *et al.*, 2015). However, this downregulation has also been correlated to increased resistance to membrane damaging agents such as monolaurin and its derivative dodecyl glycerol in *E. faecalis* AR01/DGVS and *Staphylococcus mutans*, respectively (Dufour *et al.*, 2007; Brissette *et al.*, 1986). This suggests that there may be a correlation between the *dlt* mutations and membrane associated damage, which is the reason for the observed changes in these genes.

The strains EFC3Py and EFC3C that developed resistance to COEs after serial passage also possessed changes in the expression profile of the cell envelope stress response regulon (*liaFSR* and other downstream genes such as *pspC* and *hrcA*). This suggests that the bacteria sense the damage induced by COEs and adapt to it by downregulation of the membrane damage sensing regulon of *liaFSR*.

The induction of iron-hydroxamate, glutamate uptake, choline binding protein and riboflavin in the mutant are probably consequences of the membrane associated damage. By targeting the membrane, COEs disturb the membrane gradient by increasing its permeability (Chapter 2) and the functions of the various membrane proteins associated with the lipid bilayer is affected as seen in Chapter 3.

Contribution of the *liaFSR* operon to COE resistance

E. faecalis OG1RF strains carrying mutations in *liaF* and *liaR* homologous to EFC3C and EFC3Py were constructed and those mutants were complemented

with the functional *liaF* and *liaR* genes. The MIC tests with other antibiotics showed the in frame deletion in *liaF*, where a single isoleucine residue was deleted, was associated with a 2 fold increase in resistance to COE1-3C and a 4 fold increase in resistance to nisin. The non-synonymous substitution at position 97 in the *liaR* gene was associated with 16 fold increase in resistance to COE1-3Py and this mutation did not confer cross resistance to COE1-3C. Further studies on the gene expression level changes in these mutants and the complemented strains and characterizing their ability to form biofilms will improve our understanding of how these genes and associated pathways function and confer resistance to these novel COE molecules.

Role of mutation in the contingency locus in *liaF* on response to COE1-3C

The deletion of isoleucine residue at position 179 in *liaF* increased the resistance to COE1-3C. Such deletion of isoleucine residues in *liaF* from position 177 to 182 has been associated with stress response in *E. faecalis*. Such codon slippage in sequences of cell envelope stress regulators is used to modulate the response of *E. faecalis* to cell envelope stressors (Miller *et al.*, 2013). For example, R712 is a vancomycin resistant clinical isolate of *E. faecalis* was found to develop resistance to daptomycin due to a deletion in isoleucine at position 177 in *liaF*.

In summary, COE1-3Py and COE1-3C are antimicrobials that function by increasing membrane permeability and thereby induce membrane damage associated stress. They evoke a low level of resistance in the *in vitro* evolved strains EFC3C and EFC3Py. The resistant strains adapt by regulating their the signal transduction systems, *dlt* operon, PBPs, heat shock proteins, iron transporters, lipoproteins and drug efflux pumps. The difference in resistance towards COE1-3Py in EFC3C could be attributed to the absence of expression of a multi-drug resistance associated transporter. Future studies on the membrane fluidity and supplementation of substrates of the above mentioned transporters may help in correlating their influence on toxicity to COEs. Since COEs are highly specific for bacterial membranes as shown by biophysical studies, they may be useful to explore as a new class of antimicrobials in the future.

6 Future Work

The results from this work have helped to determine the role *liaFSR* in mediating resistance to membrane interacting molecules such as COEs. COEs, as such evoke a transcriptomic response which could be categorized as a general stress response and significantly affected the expression of membrane transporters. In order to elucidate the interaction of COEs with the membrane a few suggestions for future work have been discussed below.

Studying the membrane lipid distribution in the mutants using FM4-64 staining

The cytoplasmic membrane of *E. faecalis* OG1RF is primarily composed of phosphatidylglycerol (PG), cardiolipin (CL), lysyl-phosphatidylglycerol (LPG), glycerophospho-diglucosyl-diacylglycerols (GPDGDAG) in addition to diacylglycerol (DAG) and triacylglycerol (TAG) species (12). FM4-64 is a non-specific lipophilic membrane stain (53) which shows twice the fluorescence intensity in the septal region in comparison to other regions as observed previously in *E. faecalis* S613 and *Bacillus subtilis* (53, 64). FM4-64 has previously shown differences in staining patterns in response to changes in phospholipid content but was unaffected by redistribution of cardiolipin domains in *E. faecalis* (53). The daptomycin resistant isolate S613 Δ *liaF177*, with redistributed cardiolipin domains, did not display any delocalization of FM4-64 from the septum while the S613 Δ *liaF177gdpD170cls61* mutant showed a “heterogenous” localization of FM4-64 (19). This change was suggested to be representative of global structural changes in membrane architecture and correlated to changes in the level of phospholipid (decrease in CL and PG) in S613 Δ *liaF177gdpD170cls61* (53). It will be interesting to observe the membrane lipid distribution in the EFC3C and EFC3Py mutants to determine if there are global changes in lipid structure of their membranes.

Correlating role of lipid changes and transporter activity

Lipidomic studies done with EFC3C and EFC3Py have shown that the CL levels of EFC3Py had reduced significantly in comparison to wild type. By isolating the membrane transporters from the EFC3C or EFC3Py and including them in the liposomes prepared from wild type, EFC3C or EFC3Py lipid extracts and measuring the ATPase activity, we would be able to identify if the changes in the lipid profile of the wild type and mutants could affect the activity of the transporters. Since the ATPase activity has been observed to decrease in the presence of reduced CL levels, it would help to test the hypothesis that COE has a membrane dependent interaction as a result of which these proteins could be up or down regulated. Such proteoliposome has been previously used to measure the ATPase activity of ABC transporters in liposomes of *E. coli* lipid extracts, DOPC/DOPG/ Cardiolipin (CL) (Galián *et al.*, 2011).

Transporter mutant library screening

Screening *E. faecalis* strains with mutations in the genes encoding these transporters to observe the antimicrobial effect of COEs will help in understanding the role of these proteins in regulating the stress response to these molecules. COE1-3 molecules fluoresce at 560-566 nm. Determining if the RFP-tagged FtsZ or other cell wall proteins are colocalized with COE1-3C or COE1-3Py could help in understanding if there is any preference for COEs with the lipid bilayer regions in which these membrane proteins are localized.

Impact of *liaF* mutation on *E. faecalis* wound infection model

The *E. faecalis* wound infection model described by Chong *et al.*, (2017) could be used to observe the contribution of LiaF on the fitness of OG1RF by co-infecting wounded mice with OG1RF and OG1RF *liaF*_{Δ11e179}. Further, if the mutation does contribute to increased fitness *in vivo*, the immune response of the host to the OG1RF *liaF*_{Δ11e179} mutant vs the wild type could be assessed in terms of cytokine, macrophages and neutrophil measurement. In 2004, vancomycin resistant clinical isolate of *E. faecalis* (S613) and daptomycin resistant variant of S613 (named as R712) were isolated from a patient before and after daptomycin therapy. The R712 strain had a deletion of an isoleucine

at position 177 in *liaF*, cardiolipin synthase (*cls*) and glycerophosphodiesterase (*gdpD*) associated mutations (Miller *et al.*, 2013). By the above wound infection model, it would help us correlate the contribution of such an in-frame deletion of isoleucine in *liaF* on the fitness of OG1RF in such infections.

Appendix A

Figure A -1 QCM-D monitoring of COE interactions with supported lipid bilayers. Resonance frequency (Δf) and energy dissipation (ΔD) signals are presented as functions of time, which reflect the acoustic mass and viscoelastic properties, respectively. The baseline signals correspond to fabricated SLBs on silica-coated sensor chips, and 5 μ M COE compounds were added starting at $t = 5$ min under continuous flow conditions. All measurement shifts are relative to equivalent buffer conditions. Blue line- EFC3C; Green line - EFC3Py and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine).

Figure A-2 Electrochemical impedance spectroscopy (EIS) measurements using a tethered lipid bilayer membrane platform. Increasing concentrations of compound were added at the indicated time point. Control is standard tBLM lipid composition without bacterial cell lipid extract. Red – OG1RF wild type; Blue - EFC3C and Green - EFC3Py.

Appendix B

Table B-1 List of genes differentially expressed in *E. faecalis* OG1RF wild type upon treatment with COE1-3C.

Locus	Description	Fold change
OG1RF_10301	RND transporter	-5.02
OG1RF_10302	ABC transporter ATP-binding protein	-4.96
OG1RF_10303	ABC transporter permease	-4.99
OG1RF_10878	adhesin	5.36
OG1RF_11260	membrane protein	7.48
OG1RF_11384	permease	-5.54
OG1RF_11385	peptide ABC transporter ATP-binding protein	-6.20
OG1RF_11462	membrane protein	-4.82
OG1RF_11463	PspC family transcriptional regulator	-4.92
OG1RF_12269	RND transporter	-9.92
OG1RF_11185	molybdenum cofactor biosynthesis protein MoaB	-8.51
OG1RF_10527	peptide ABC transporter ATP-binding protein	-7.54
OG1RF_12267	permease	-6.16
OG1RF_12268	ABC transporter ATP-binding protein	-5.75

Table B-1 List of genes differentially expressed in *E. faecalis* OG1RF wild type upon treatment with COE1-3C (continued)

Locus	Description	Fold change
Signal Transduction		
OG1RF_11656	peptide ABC transporter permease	-7.30
OG1RF_11657	ABC transporter ATP- binding protein	-5.44
Hypothetical Proteins		
OG1RF_10304	hypothetical protein	-15.29
OG1RF_10353	hypothetical protein	-7.00
OG1RF_10354	hypothetical protein	-8.09
OG1RF_10531	hypothetical protein	-7.56
OG1RF_10532	hypothetical protein	-7.11
OG1RF_11751	hypothetical protein	-4.30
OG1RF_10659	hypothetical protein	-4.19
OG1RF_11252	hypothetical protein	-4.66
OG1RF_11464	hypothetical protein	-4.03

Table B-2 List of genes differentially expressed in *E. faecalis* OG1RF wild type on treatment with COE1-3Py

Locus	Description	Fold change
OG1RF_10288	sodium: proton antiporter	4.68
OG1RF_10367	decarboxylase	14.21
OG1RF_10369	sodium: proton antiporter	8.07
OG1RF_10526	permease	-6.05
OG1RF_11227	mandelate racemase	-6.25
OG1RF_11810	transporter	7.85
Overview		
OG1RF_11314	<i>katA</i> ; catalase	6.68
Hypothetical Protein		
OG1RF_10075	hypothetical protein	-4.04
OG1RF_12152	hypothetical protein	4.38
OG1RF_10659	hypothetical protein	-4.19
OG1RF_11252	hypothetical protein	-4.66
OG1RF_11464	hypothetical protein	-4.03
Nucleotide metabolism		
OG1RF_12279	allantoinase	-6.45

Table B-2 List of genes differentially expressed in *E. faecalis* OG1RF wild type on treatment with COE1-3Py (continued)

Locus	Description	Fold change
Cellular communication		
OG1RF_10368	amino acid permease	20.36
Carbohydrate metabolism		
OG1RF_10682	beta-phosphoglucomutase	-5.58
Signal transduction		
OG1RF_11938	fumarate reductase flavoprotein subunit	4.24
OG1RF_11185	molybdenum cofactor biosynthesis protein MoaB	-8.51
OG1RF_10527	peptide ABC transporter ATP-binding protein	-7.54
OG1RF_12267	permease	-6.16
OG1RF_12268	ABC transporter ATP-binding protein	-5.75

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs.

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
OG1RF_12235	protease	12.78	7.98
OG1RF_11260	membrane protein	4.71	5.37
OG1RF_11377	conjugal transfer protein TraX	-11.88	-4.36
OG1RF_10654	two-component system sensor histidine kinase	-10.39	-4.38
OG1RF_10707	DNA repair exonuclease	-12.77	-4.50
OG1RF_11074	magnesium- translocating P-type ATPase	-17.25	-4.71
OG1RF_11072	transcriptional regulator	-16.29	-5.12
OG1RF_10328	peptidase M23	-10.14	-5.17
OG1RF_11073	LysR family transcriptional regulator	-15.52	-5.29
OG1RF_12496	DNA-binding protein	-12.92	-5.43
OG1RF_12131	membrane protein	-10.51	-5.75
OG1RF_11569	membrane protein	-11.55	-5.79
OG1RF_10481	serine hydrolase	-12.75	-6.70

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs (continued).

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
OG1RF_12298	DNA-binding protein	-8.38	-7.40
OG1RF_11094	membrane protein	-7.43	-8.00
OG1RF_12067	tellurite resistance protein	-19.19	-8.03
OG1RF_11522	multidrug MFS transporter	-28.90	-9.24
OG1RF_12500	LytR family transcriptional regulator	-32.96	-10.80
OG1RF_10301	RND transporter	-11.14	-14.13
OG1RF_10302	ABC transporter ATP-binding protein	-11.37	-14.22
OG1RF_10303	ABC transporter permease	-10.98	-14.71
OG1RF_11385	peptide ABC transporter ATP- binding protein	-79.51	-24.34
OG1RF_11384	permease	-67.62	-24.34
OG1RF_12269	RND transporter	-94.34	-27.75
OG1RF_11462	membrane protein	-92.16	-28.47
OG1RF_12268	ABC transporter ATP-binding protein	-115.33	-31.39

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs (continued).

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
OG1RF_11463	PspC family transcriptional regulator	-102.71	-32.26
OG1RF_12267	permease	-139.87	-39.90
Amino acid metabolism			
OG1RF_10473	amidase	-16.37	-6.32
Carbohydrate metabolism			
OG1RF_11199	diacetylchitobiose-6- phosphate hydrolase	-12.80	-5.20
Glycan biosynthesis and metabolism			
OG1RF_10417	penicillin-binding protein 1A	-15.02	-4.39
Lipid metabolism			
OG1RF_11521	alkaline phosphatase	-18.88	-4.44
OG1RF_11570	glycerophosphoryl diester phosphodiesterase	-12.47	-7.06

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs (continued).

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
Membrane transport			
OG1RF_12351	iron ABC transporter substrate-binding protein	4.92	4.94
Metabolism of other amino acids			
OG1RF_12112	D-alanine-- poly(phosphoribitol) ligase subunit 1	-13.22	-5.96
OG1RF_12110	D-alanine-- poly(phosphoribitol) ligase subunit 2	-13.94	-6.39
Signal transduction			
OG1RF_12212	two-component system sensor histidine kinase	-10.27	-4.88
OG1RF_12211	LuxR family transcriptional regulator	-9.43	-5.32
OG1RF_12111	D-alanyl-lipoteichoic acid biosynthesis protein DltB	-13.88	-6.14

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs (continued).

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
Signal transduction			
OG1RF_12109	D-alanyl transfer protein DltD	-14.65	-6.63
OG1RF_11657	ABC transporter ATP-binding protein	-24.20	-10.80
OG1RF_11656	peptide ABC transporter permease	-33.58	-12.76
Translation			
OG1RF_10057	peptide ABC transporter substrate- binding protein	11.95	6.67
Hypothetical Proteins			
OG1RF_10492	hypothetical protein	8.12	4.01
OG1RF_11239	hypothetical protein	-8.85	-4.59
OG1RF_12328	hypothetical protein	-8.19	-4.66
OG1RF_10305	hypothetical protein	-7.64	-5.72
OG1RF_11093	hypothetical protein	-7.21	-7.37
OG1RF_11031	hypothetical protein	-14.21	-7.67
OG1RF_11032	hypothetical protein	-13.91	-8.00

Table B-3 List of genes differentially expressed that were common to both EFC3C and EFC3Py in comparison to the wild type in the absence of COEs (continued).

Locus	Description	Fold change in EFC3Py	Fold change in EFC3C
Hypothetical Proteins			
OG1RF_10354	hypothetical protein	-8.57	-8.69
OG1RF_11751	hypothetical protein	-18.00	-8.85
OG1RF_10353	hypothetical protein	-11.05	-10.30
OG1RF_11200	hypothetical protein	-32.92	-12.72
OG1RF_12066	hypothetical protein	-38.88	-13.80
OG1RF_10659	hypothetical protein	-56.58	-24.00
OG1RF_10304	hypothetical protein	-33.02	-24.97
OG1RF_10532	hypothetical protein	-71.91	-53.72
OG1RF_10531	hypothetical protein	-73.88	-56.24
OG1RF_11464	hypothetical protein	-215.68	-64.19
OG1RF_11252	hypothetical protein	-590.34	-195.04

Table B-4 Genes upregulated or downregulated only in the untreated mutant EFC3C compared to untreated *E. faecalis* wild type.

Locus	Description	Fold change in EFC3C
Membrane transport		
OG1RF_12353	iron ABC transporter permease	6.37
OG1RF_10136	ferrichrome ABC transporter substrate- binding protein	4.22
Carbohydrate metabolism		
OG1RF_11753	PTS maltose transporter subunit IIBC	-4.30

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type.

Locus	Description	Fold Change in EFC3Py
OG1RF_12481	choline-binding protein	11.09
OG1RF_10490	cell wall surface anchor protein	6.61
OG1RF_10485	cell wall surface anchor protein	4.99
OG1RF_10486	cell surface protein	4.64
OG1RF_10407	CRISPR-associated protein Csn2	4.28
OG1RF_12572	citrate transporter	4.15

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type. (continued)

Locus	Description	Fold Change in EFC3Py
OG1RF_10487	cell surface protein	4.08
OG1RF_10655	glucose transporter GlcU	-4.11
OG1RF_10836	nitrate ABC transporter permease	-4.12
	membrane protein	-4.45
	competence protein ComA	-4.58
OG1RF_10835	taurine ABC transporter ATP-binding protein	-4.64
OG1RF_11036	ATPase P	-4.78
OG1RF_11077	co-chaperone GrpE	-4.81
OG1RF_11383	NADPH:quinone reductase	-4.84
OG1RF_10516	acyltransferase	-5.26
OG1RF_10761	alpha/beta hydrolase	-5.26
OG1RF_11076	HrcA family transcriptional regulator	-5.33
OG1RF_10996	Cro/C1 family transcriptional regulator	-5.49
OG1RF_12210	potassium transporter Trk	-6.08
OG1RF_12536	two-component system sensor histidine kinase	-6.87

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type. (continued)

Locus	Description	Fold Change in EFC3Py
OG1RF_12535	two-component system response regulator	-7.54
OG1RF_11452	ABC transporter substrate-binding protein	-7.88
OG1RF_12071	transcriptional regulator	-8.27
OG1RF_12213	membrane protein	-8.98
OG1RF_10653	two-component system response regulator	-9.27
OG1RF_11908	virion core protein	-9.47
OG1RF_11793	chaperone protein ClpB	-10.38
OG1RF_11070	cell division protein FtsW	-11.75
OG1RF_11002	phosphoesterase	-12.55
OG1RF_11071	cell division protein FtsW	-17.83
Carbohydrate metabolism		
OG1RF_10297	mannitol-specific phosphotransferase enzyme IIA component	-4.59
OG1RF_10432	PTS fructose transporter subunit IIC	-4.64

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type. (continued)

Locus	Description	Fold Change in EFC3Py
OG1RF_10434	tagatose 1,6-diphosphate aldolase	-5.45
OG1RF_10298	mannitol-1-phosphate 5-dehydrogenase	-5.55
OG1RF_10682	beta-phosphoglucomutase	-6.08
Cellular communication		
OG1RF_10993	spermidine/putrescine ABC transporter substrate-binding protein	5.56
OG1RF_12366	peptide ABC transporter substrate-binding protein	5.51
OG1RF_10991	spermidine/putrescine ABC transporter permease	5.06
Folding, sorting and degradation		
OG1RF_10584	signal peptidase I	6.52
OG1RF_11078	molecular chaperone DnaK	-4.65
Glycan biosynthesis and metabolism		
OG1RF_12158	cell division protein FtsI	-4.08

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type. (continued).

Locus	Description	Fold Change in EFC3Py
Membrane transport		
OG1RF_10897	glutamine ABC transporter substrate-binding protein	11.48
OG1RF_10895	amino acid ABC transporter permease	9.61
OG1RF_10896	glutamine ABC transporter permease	8.95
OG1RF_10898	ABC transporter ATP-binding protein	8.42
Nucleotide metabolism		
OG1RF_10994	adenine deaminase	4.31
Signal transduction		
OG1RF_10760	membrane protein	-6.20
Translation		
OG1RF_10025	RpiR family transcriptional regulator	5.08

Table B-5 Genes upregulated or downregulated only in the untreated mutant EFC3Py compared to the untreated *E. faecalis* wild type. (continued)

Locus	Description	Fold Change in EFC3Py
Hypothetical protein		
OG1RF_10796	hypothetical protein	8.74
OG1RF_12152	hypothetical protein	7.91
OG1RF_10491	hypothetical protein	5.78
OG1RF_10860	hypothetical protein	4.38
OG1RF_10861	hypothetical protein	4.09
OG1RF_10482	hypothetical protein	-4.55
OG1RF_12166	hypothetical protein	-5.06
OG1RF_10445	hypothetical protein	-5.17
OG1RF_11254	hypothetical protein	-5.45
OG1RF_10446	hypothetical protein	-7.38
OG1RF_11910	hypothetical protein	-8.53
OG1RF_12471	hypothetical protein	-10.39

Table B-6 Differential gene expression of EFC3Py treated with COE1-3Py in comparison to the untreated strain.

Locus	Description	Fold change in EFC3Py
OG1RF_10838	manganese transporter	-5.86
OG1RF_10605	aldo/keto reductase	-4.28
OG1RF_11643	universal stress protein UspA	-4.08
OG1RF_12470	cobalamin synthesis protein CobW	4.12
OG1RF_10878	adhesin	4.40
OG1RF_11522	multidrug MFS transporter	4.61
OG1RF_10589	cation transporter	5.55
OG1RF_10328	peptidase M23	6.27
OG1RF_11002	phosphoesterase	6.56
OG1RF_11377	conjugal transfer protein TraX	6.73
OG1RF_10550	polysaccharide lyase	9.51
OG1RF_11767	multidrug ABC transporter ATP-binding protein	699.54

Table B-6 Differential gene expression of EFC3Py treated with COE1-3Py in comparison to the untreated strain(continued).

Locus	Description	Fold change in EFC3Py
OG1RF_11766	multidrug ABC transporter ATP-binding protein	787.22
Cellular communication		
OG1RF_12368	peptide ABC transporter permease	-5.16
OG1RF_12367	peptide ABC transporter permease	-4.76
OG1RF_12369	peptide ABC transporter ATP-binding protein	-4.55
OG1RF_12366	peptide ABC transporter substrate-binding protein	-4.09
Membrane transport		
OG1RF_11677	ABC transporter ATP-binding protein	-8.58
OG1RF_11678	membrane protein	-7.31
OG1RF_10684	PTS glucose transporter subunit IIABC	-7.12
OG1RF_10297	mannitol-specific phosphotransferase enzyme IIA component	-6.08
OG1RF_11679	manganese ABC transporter substrate-binding protein	-5.59

Table B-6 Differential gene expression of EFC3Py treated with COE1-3Py in comparison to the untreated strain.(continued)

Locus	Description	Fold change in EFC3Py
Membrane transport		
OG1RF_12479	PTS mannose transporter subunit IID	-4.73
OG1RF_12472	zinc-binding protein	4.78
OG1RF_12353	iron ABC transporter permease	5.94
OG1RF_12354	iron ABC transporter permease	6.02
OG1RF_12351	iron ABC transporter substrate-binding protein	7.96
OG1RF_10137	ferrichrome ABC transporter ATP-binding protein	8.07
OG1RF_12352	iron ABC transporter ATP-binding protein	8.44
OG1RF_10136	ferrichrome ABC transporter substrate-binding protein	13.42
Translation		
OG1RF_10021	PTS mannose transporter subunit IID	-4.35
OG1RF_10020	PTS alpha-glucoside transporter subunit IIBC	-4.28
OG1RF_12469	50S ribosomal protein L33	4.27

Table B-6 Differential gene expression of EFC3Py treated with COE1-3Py in comparison to the untreated strain. (continued)

Locus	Description	Fold change in EFC3Py
Hypothetical proteins		
OG1RF_10881	hypothetical protein	20.46

Table B-7 Differential expression of genes EF C3C treated with COE1-3Py in comparison to the untreated strain.

Locus	Description	Fold change in EFC3C
Cellular communication		
OG1RF_10993	spermidine/putrescine ABC transporter substrate-binding protein	4.77
OG1RF_10434	tagatose 1,6-diphosphate aldolase	5.08
Membrane transport		
OG1RF_11677	ABC transporter ATP- binding protein	-7.81
OG1RF_10432	PTS fructose transporter subunit IIC	5.47

Table B-8 Differential expression of genes EF C3Py treated with COE1-3C in comparison to the untreated EF C3Py.

Locus	Description	Fold change in EFC3Py
OG1RF_11766	multidrug ABC transporter ATP-binding protein	28.34
OG1RF_11767	multidrug ABC transporter ATP-binding protein	27.41

Appendix C

Table C-1 Single Nucleotide Polymorphisms in COE1-3C and COE1-3Py resistant strains EFC3C and EFC3Py

Gene locus	Description	Mutation associated with each strain	
		EFC3C	EFC3Py
OG1RF_12213	Membrane protein (<i>liaF</i>)	Ile179 (Deletion)	–
OG1RF_11256	Site-specific tyrosine recombinase XerD	–	Ala233Val (Substitution)
OG1RF_11765	MerR family transcriptional regulator	–	Val134_Asp135 InsValVal (Insertion)
OG1RF_11767	Multidrug ABC transporter ATP- binding protein	–	Thr188Asn (Substitution)
OG1RF_12211	DNA-binding response regulator (<i>liaR</i>)	–	Ala98Val (Substitution)

References

- Aakra Å, Vebø H, Snipen L, Hirt H, Aastveit A, Kapur V, *et al.* (2005). Transcriptional Response of *Enterococcus faecalis* V583 to Erythromycin. *Antimicrobial Agents and Chemotherapy* **49**:2246–2259.
- Anders S, Pyl PT, Huber W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)* **31**:166–169.
- Andrews S. (2010). FastQC A Quality Control tool for High Throughput Sequence Data. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. citeulike-article-id:11583827.
- Arias CA, Contreras GA, Murray BE. (2010). Management of multidrug-resistant enterococcal infections. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **16**:10.1111/j.1469-0691.2010.03214.x.
- Arias CA, Murray BE. (2012). The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Micro* **10**:266–278.
- Arias CA, Panesso D, McGrath DM, Qin X, Mojica MF, Miller C, *et al.* (2011). Genetic basis for in vivo daptomycin resistance in enterococci. *The New England journal of medicine* **365**:892–900.
- Balli EP, Venetis CA, Miyakis S. (2014). Systematic Review and Meta-Analysis of Linezolid versus Daptomycin for Treatment of Vancomycin-Resistant Enterococcal Bacteremia. *Antimicrobial Agents and Chemotherapy* **58**:734–739.
- Barber KE, Smith JR, Raut A, Rybak MJ. (2016). Evaluation of tedizolid against *Staphylococcus aureus* and enterococci with reduced susceptibility to vancomycin, daptomycin or linezolid. *The Journal of antimicrobial chemotherapy* **71**:152–155.
- Bergholz TM, Tang S, Wiedmann M, Boor KJ. (2013). Nisin Resistance of

Listeria monocytogenes Is Increased by Exposure to Salt Stress and Is Mediated via LiaR. *Applied and Environmental Microbiology* **79**:5682–5688.

Berti AD, Theisen E, Sauer J-D, Nonejuie P, Olson J, Pogliano J, *et al.* (2016). Penicillin Binding Protein 1 Is Important in the Compensatory Response of *Staphylococcus aureus* to Daptomycin-Induced Membrane Damage and Is a Potential Target for β -Lactam–Daptomycin Synergy. *Antimicrobial Agents and Chemotherapy* **60**:451–458.

Bertsche U, Yang S-J, Kuehner D, Wanner S, Mishra NN, Roth T, *et al.* (2013). Increased Cell Wall Teichoic Acid Production and D-alanylation Are Common Phenotypes among Daptomycin-Resistant Methicillin-Resistant *Staphylococcus aureus* (MRSA) Clinical Isolates Van Schaik, W (ed). *PLoS ONE* **8**:e67398.

Bhattacharyya P, Epstein W, Silver S. (1971). Valinomycin-induced uptake of potassium in membrane vesicles from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **68**:1488–1492.

Bligh EG, Dyer WJ. (1959). A Rapid Method of Total Lipid Extraction and Purification. *Canadian Journal of Biochemistry and Physiology* **37**:911–917.

Bolhuis H, van Veen HW, Poolman B, Driessen AJM, Konings WN. (1997). Mechanisms of multidrug transporters. *FEMS Microbiology Reviews* **21**:55–84.

Brissette JL, Cabacungan EA, Pieringer RA. (1986). Studies on the antibacterial activity of dodecylglycerol. Its limited metabolism and inhibition of glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans* BHT. *The Journal of biological chemistry* **261**:6338–6345.

Brown D. (2015). Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? *Nat Rev Drug Discov* **14**:821–832.

Burgos MJG, Lopez RL, Abriouel H, Omar N Ben, Galvez A. (2009). Multilocus sequence typing of *Enterococcus faecalis* from vegetable foods reveals two new sequence types. *Foodborne pathogens and disease* **6**:321–327.

Camargo ILB da C, Neoh H-M, Cui L, Hiramatsu K. (2008). Serial

Daptomycin Selection Generates Daptomycin-Nonsusceptible *Staphylococcus aureus* Strains with a Heterogeneous Vancomycin-Intermediate Phenotype . *Antimicrobial Agents and Chemotherapy* **52**:4289–4299.

Castillo-Rojas G, Mazari-Hiríart M, Ponce de León S, Amieva-Fernández RI, Agis-Juárez RA, Huebner J, *et al.* (2013). Comparison of *Enterococcus faecium* and *Enterococcus faecalis* Strains Isolated from Water and Clinical Samples: Antimicrobial Susceptibility and Genetic Relationships Ibekwe, AM (ed). *PLoS ONE* **8**:e59491.

Chang Y-Y, Cronan JE. (1999). Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. *Molecular Microbiology* **33**:249–259.

Chong KKL, Tay WH, Janela B, Yong AMH, Liew TH, Madden L, *et al.* (2017). *Enterococcus faecalis* Modulates Immune Activation and Slows Healing During Wound Infection. *The Journal of Infectious Diseases* **216**:1644–1654.

Chopra I, Hesse L, O'Neill AJ. (2002). Exploiting current understanding of antibiotic action for discovery of new drugs. *Journal of Applied Microbiology* **92**:4S–15S.

Comerlato CB, de Resende MCC, Caierão J, d'Azevedo PA. (2013). Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. *Memórias do Instituto Oswaldo Cruz* **108**:590–595.

Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, *et al.* (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *Journal of medical microbiology* **53**:13–20.

Cui L, Neoh H, Shoji M, Hiramatsu K. (2009). Contribution of *vraSR* and *graSR* Point Mutations to Vancomycin Resistance in Vancomycin-Intermediate *Staphylococcus aureus* . *Antimicrobial Agents and Chemotherapy* **53**:1231–1234.

Davidson AL, Dassa E, Orelle C, Chen J. (2008). Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems. *Microbiology and Molecular Biology Reviews* : *MMBR* **72**:317–364.

Davlieva M, Shi Y, Leonard PG, Johnson TA, Zianni MR, Arias CA, *et al.* (2015). A variable DNA recognition site organization establishes the LiaR-mediated cell envelope stress response of enterococci to daptomycin. *Nucleic Acids Research* **43**:4758–4773.

Diaz L, Tran TT, Munita JM, Miller WR, Rincon S, Carvajal LP, *et al.* (2014). Whole-Genome Analyses of Enterococcus faecium Isolates with Diverse Daptomycin MICs. *Antimicrobial Agents and Chemotherapy* **58**:4527–4534.

Dufour M, Manson JM, Bremer PJ, Dufour J-P, Cook GM, Simmonds RS. (2007). Characterization of Monolaurin Resistance in Enterococcus faecalis . *Applied and Environmental Microbiology* **73**:5507–5515.

van Duin D, Bonomo RA. (2016). Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Second-generation β -Lactam/ β -Lactamase Inhibitor Combinations. *Clinical Infectious Diseases* **63**:234–241.

Dunny GM, Brown BL, Clewell DB. (1978). Induced cell aggregation and mating in Streptococcus faecalis: evidence for a bacterial sex pheromone. *Proceedings of the National Academy of Sciences of the United States of America* **75**:3479–3483.

Elbourne LDH, Tetu SG, Hassan KA, Paulsen IT. (2017). TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. *Nucleic Acids Research* **45**:D320–D324.

Eldholm V, Gutt B, Johnsborg O, Brückner R, Maurer P, Hakenbeck R, *et al.* (2010). The Pneumococcal Cell Envelope Stress-Sensing System LiaFSR Is Activated by Murein Hydrolases and Lipid II-Interacting Antibiotics . *Journal of Bacteriology* **192**:1761–1773.

Epand RM, Walker C, Epand RF, Magarvey NA. (2016). Molecular mechanisms of membrane targeting antibiotics. *Biochimica et Biophysica Acta*

(BBA) - *Biomembranes* **1858**:980–987.

Van Epps HL. (2006). René Dubos: unearthing antibiotics. *The Journal of Experimental Medicine* **203**:259 LP-259.

Facklam R, Carvalho M TL. (2002). History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci. In: *n Gilmore M, Clewell D, Courvalin P, Dunny G, Murray B, Rice L (ed), The Enterococci*, pp. 1–54.

Falagas ME, Kasiakou SK, Saravolatz LD. (2005). Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clinical Infectious Diseases* **40**:1333–1341.

Fischer W. (1990). Bacterial Phosphoglycolipids and Lipoteichoic Acids. In: *Glycolipids, Phosphoglycolipids, and Sulfoglycolipids*, Kates, M (ed), Springer US: Boston, MA, pp. 123–234.

Fisher K, Phillips C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **155**:1749–1757.

Fontana R, Cornaglia G, Ligozzi M, Mazzariol A. (2000). The final goal: penicillin-binding proteins and the target of cephalosporins. *Clinical Microbiology and Infection* **6**:34–40.

Frankenberg L, Brugna M, Hederstedt L. (2002). Enterococcus faecalis Heme-Dependent Catalase. *Journal of Bacteriology* **184**:6351–6356.

Gaca AO, Kajfasz JK, Miller JH, Liu K, Wang JD, Abranches J, *et al.* (2013). Basal Levels of (p)ppGpp in Enterococcus faecalis: the Magic beyond the Stringent Response. *mBio* **4**:e00646-13.

Galián C, Manon F, Dezi M, Torres C, Ebel C, Lévy D, *et al.* (2011). Optimized Purification of a Heterodimeric ABC Transporter in a Highly Stable Form Amenable to 2-D Crystallization. *PLOS ONE* **6**:e19677.

Garner LE, Park J, Dyar SM, Chworos A, Sumner JJ, Bazan GC. (2010). Modification of the Optoelectronic Properties of Membranes via Insertion of

Amphiphilic Phenylenevinylene Oligoelectrolytes. *Journal of the American Chemical Society* **132**:10042–10052.

Granok AB, Parsonage D, Ross RP, Caparon MG. (2000). The RofA Binding Site in *Streptococcus pyogenes* Is Utilized in Multiple Transcriptional Pathways. *Journal of Bacteriology* **182**:1529–1540.

Grogan DW, Cronan JE. (1997). Cyclopropane ring formation in membrane lipids of bacteria. *Microbiology and Molecular Biology Reviews* **61**:429–441.

Gutschik E, Christensen N. (2009). Experimental Endocarditis in Rabbits. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology* **86B**:223–228.

Hachmann A-B, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD. (2011). Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. *Antimicrobial agents and chemotherapy* **55**:4326–4337.

Hancock REW, Sahl H-G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotech* **24**:1551–1557.

Harp JR, Saito HE, Bourdon AK, Reyes J, Arias CA, Campagna SR, *et al.* (2016). Exogenous Fatty Acids Protect *Enterococcus faecalis* from Daptomycin-Induced Membrane Stress Independently of the Response Regulator LiaR Vieille, C (ed). *Applied and Environmental Microbiology* **82**:4410–4420.

van Harten RM, Willems RJL, Martin NI, Hendrickx APA. (2017). Multidrug-Resistant Enterococcal Infections: New Compounds, Novel Antimicrobial Therapies? *Trends in Microbiology* **25**:467–479.

He B, Zalkin H. (1994). Regulation of *Escherichia coli* purA by purine repressor, one component of a dual control mechanism. *Journal of Bacteriology* **176**:1009–1013.

Hinks J, Poh WH, Chu JJH, Loo JSC, Bazan GC, Hancock LE, *et al.* (2015).

Oligopolyphenylenevinylene-Conjugated Oligoelectrolyte Membrane Insertion Molecules Selectively Disrupt Cell Envelopes of Gram-Positive Bacteria. *Applied and Environmental Microbiology* **81**:1949–1958.

Hinks J, Wang Y, Matysik A, Kraut R, Kjelleberg S, Mu Y, *et al.* (2015). Increased Microbial Butanol Tolerance by Exogenous Membrane Insertion Molecules. *ChemSusChem* **8**:3718–3726.

Hinks J, Wang Y, Poh WH, Donose BC, Thomas AW, Wuertz S, *et al.* (2014). Modeling Cell Membrane Perturbation by Molecules Designed for Transmembrane Electron Transfer. *Langmuir* **30**:2429–2440.

Hurdle JG, O'Neill AJ, Chopra I, Lee RE. (2011). Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat Rev Micro* **9**:62–75.

Hürlimann LM, Corradi V, Hohl M, Bloemberg G V, Tieleman DP, Seeger MA. (2016). The Heterodimeric ABC Transporter EfrCD Mediates Multidrug Efflux in *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy* **60**:5400–5411.

Huycke MM, Sahm DF, Gilmore MS. (1998). Multiple-Drug Resistant Enterococci: The Nature of the Problem and an Agenda for the Future. *Emerging Infectious Disease journal* **4**:239.

Jett BD, Huycke MM, Gilmore MS. (1994). Virulence of enterococci. *Clinical Microbiology Reviews* **7**:462–478.

Jordan S, Hutchings MI, Mascher T. (2008). Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiology Reviews* **32**:107–146.

Jordan S, Junker A, Helmann JD, Mascher T. (2006). Regulation of LiaRS-Dependent Gene Expression in *Bacillus subtilis*: Identification of Inhibitor Proteins, Regulator Binding Sites, and Target Genes of a Conserved Cell Envelope Stress-Sensing Two-Component System. *Journal of Bacteriology* **188**:5153–5166.

- Kakinuma Y. (1987). Sodium/proton antiporter in *Streptococcus faecalis*. *Journal of bacteriology* **169**:3886–3890.
- Kanehisa M, Goto S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research* **28**:27–30.
- Keogh D, Lam LN, Doyle LE, Matysik A, Pavagadhi S, Umashankar S, *et al.* (2017). Extracellular Electron Transfer Powers. *bioRxiv Cold Spring Harbor Laboratory*. doi:http://dx.doi.org/10.1101/130146.
- Kesel S, Mader A, Höfler C, Mascher T, Leisner M. (2013). Immediate and Heterogeneous Response of the LiaFSR Two-Component System of *Bacillus subtilis* to the Peptide Antibiotic Bacitracin. *PLOS ONE* **8**:e53457.
- Khudyakov JI, D’haeseleer P, Borglin SE, DeAngelis KM, Woo H, Lindquist EA, *et al.* (2012). Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, *Enterobacter lignolyticus*. *Proceedings of the National Academy of Sciences* **109**:E2173–E2182.
- Kline KA, Kau AL, Chen SL, Lim A, Pinkner JS, Rosch J, *et al.* (2009). Mechanism for Sortase Localization and the Role of Sortase Localization in Efficient Pilus Assembly in *Enterococcus faecalis*. *Journal of Bacteriology* **191**:3237–3247.
- Kopylova E, Noe L, Touzet H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics (Oxford, England)* **28**:3211–3217.
- Kristich CJ, Rice LB, Arias CA. (2014). Enterococcal Infection—Treatment and Antibiotic Resistance. In: *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, Boston: Massachusetts Eye and Ear Infirmary; 2014, pp. 1–63.
- Krulwich TA, Sachs G, Padan E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nat Rev Micro* **9**:330–343.
- Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. (2003).

Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Molecular Microbiology* **49**:807–821.

Laxminarayan R, Amábile-cuevas CF, Cars O, Evans T, Heymann DL, Hoffman S, *et al.* (2016). UN High-Level Meeting on antimicrobials—what do we need? *The Lancet* **388**:218–220.

Leavis HL, Bonten MJM, Willems RJL. (2006). Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Current Opinion in Microbiology* **9**:454–460.

Lebreton F, Willems RJL, Gilmore MS. (2014). Enterococcus Diversity, Origins in Nature, and Gut Colonization. In: Gilmore, MS, Clewell, DB, Ike, Y, & Shankar, N (eds), Boston.

Lemos JAC, Chen Y-YM, Burne RA. (2001). Genetic and Physiologic Analysis of the groE Operon and Role of the HrcA Repressor in Stress Gene Regulation and Acid Tolerance in *Streptococcus mutans*. *Journal of Bacteriology* **183**:6074–6084.

Li H, Durbin R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)* **25**:1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, *et al.* (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**:2078–2079.

Li J, Koh J-J, Liu S, Lakshminarayanan R, Verma CS, Beuerman RW. (2017). Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. *Frontiers in neuroscience* **11**:73.

Li Y-H, Lau PCY, Tang N, Svensäter G, Ellen RP, Cvitkovitch DG. (2002). Novel Two-Component Regulatory System Involved in Biofilm Formation and Acid Resistance in *Streptococcus mutans*. *Journal of Bacteriology* **184**:6333–6342.

- Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, *et al.* (2015). A new antibiotic kills pathogens without detectable resistance. *Nature* **517**:455–459.
- Locher KP, Lee AT, Rees DC. (2002). The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science (New York, NY)* **296**:1091–1098.
- Lubelski J, Konings WN, Driessen AJM. (2007). Distribution and Physiology of ABC-Type Transporters Contributing to Multidrug Resistance in Bacteria. *Microbiology and Molecular Biology Reviews : MMBR* **71**:463–476.
- Ma TK-W, Leung CB, Chow KM, Kwan BC-H, Li PK-T, Szeto CC. (2016). Newer antibiotics for the treatment of peritoneal dialysis-related peritonitis. *Clinical Kidney Journal* **9**:616–623.
- Mahipant G, Paemanee A, Roytrakul S, Kato J, Vangnai AS. (2017). The significance of proline and glutamate on butanol chaotropic stress in *Bacillus subtilis* 168. *Biotechnology for Biofuels* **10**:122.
- Maria-Neto S, de Almeida KC, Macedo MLR, Franco OL. (2015). Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1848**:3078–3088.
- Martínez B, Zomer AL, Rodríguez A, Kok J, Kuipers OP. (2007). Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. *Molecular Microbiology* **64**:473–486.
- Mascher T. (2006). Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. *FEMS microbiology letters* **264**:133–144.
- Mascher T, Helmann JD, Udden G. (2006). Stimulus Perception in Bacterial Signal-Transducing Histidine Kinases. *Microbiology and Molecular Biology Reviews* **70**:910–938.

- Mascher T, Margulis NG, Wang T, Ye RW, Helmann JD. (2003). Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Molecular Microbiology* **50**:1591–1604.
- McCormick NE, Halperin SA, Lee SF. (2011). Regulation of D-alanylation of lipoteichoic acid in *Streptococcus gordonii*. *Microbiology (Reading, England)* **157**:2248–2256.
- Miller C, Kong J, Tran TT, Arias CA, Saxer G, Shamoo Y. (2013). Adaptation of *Enterococcus faecalis* to Daptomycin Reveals an Ordered Progression to Resistance. *Antimicrobial Agents and Chemotherapy* **57**:5373–5383.
- Mishra NN, Bayer AS, Weidenmaier C, Grau T, Wanner S, Stefani S, *et al.* (2014). Phenotypic and Genotypic Characterization of Daptomycin-Resistant Methicillin-Resistant *Staphylococcus aureus* Strains: Relative Roles of *mprF* and *dlt* Operons Lowy, FD (ed). *PLoS ONE* **9**:e107426.
- Mohamed JA, Huang DB. (2007). Biofilm formation by enterococci. *Journal of medical microbiology* **56**:1581–1588.
- Müller A, Wenzel M, Strahl H, Grein F, Saaki TN V, Kohl B, *et al.* (2016). Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. *Proceedings of the National Academy of Sciences of the United States of America* **113**:E7077–E7086.
- Munita JM, Bayer AS, Arias CA. (2015). Evolving Resistance Among Gram-positive Pathogens. *Clinical Infectious Diseases* **61**:S48–S57.
- Munita JM, Panesso D, Diaz L, Tran TT, Reyes J, Wanger A, *et al.* (2012). Correlation between Mutations in *liaFSR* of *Enterococcus faecium* and MIC of Daptomycin: Revisiting Daptomycin Breakpoints. *Antimicrobial Agents and Chemotherapy* **56**:4354–4359.
- Nielsen H V, Guiton PS, Kline KA, Port GC, Pinkner JS, Neiers F, *et al.* (2012). The Metal Ion-Dependent Adhesion Site Motif of the *Enterococcus faecalis* EbpA Pilin Mediates Pilus Function in Catheter-Associated Urinary Tract Infection. *mBio* **3**:e00177-12.

- Pachón-Ibáñez ME, Smani Y, Pachón J, Sánchez-Céspedes J. (2017). Perspectives for clinical use of engineered human host defense antimicrobial peptides. *FEMS Microbiology Reviews* **41**:323–342.
- Palmer KL, Daniel A, Hardy C, Silverman J, Gilmore MS. (2011). Genetic basis for daptomycin resistance in enterococci. *Antimicrobial agents and chemotherapy* **55**:3345–3356.
- Peleg AY, Miyakis S, Ward D V, Earl AM, Rubio A, Cameron DR, *et al.* (2012). Whole Genome Characterization of the Mechanisms of Daptomycin Resistance in Clinical and Laboratory Derived Isolates of *Staphylococcus aureus* Ahmed, N (ed). *PLoS ONE* **7**:e28316.
- Pillai SK, Sakoulas G, Eliopoulos GM, Moellering RCJ, Murray BE, Inouye RT. (2004). Effects of glucose on fsr-mediated biofilm formation in *Enterococcus faecalis*. *The Journal of infectious diseases* **190**:967–970.
- Poger D, Mark AE. (2015). A Ring to Rule Them All: The Effect of Cyclopropane Fatty Acids on the Fluidity of Lipid Bilayers. *The Journal of Physical Chemistry B* **119**:5487–5495.
- Poirel L, Jayol A, Nordmann P. (2017). Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. *Clinical microbiology reviews* **30**:557–596.
- Raman M, Martin K. (2014). One solution for cloning and mutagenesis: In-Fusion® HD Cloning Plus. *Nat Meth* **11**. <http://dx.doi.org/10.1038/nmeth.f.373>.
- Ramsey M, Hartke A, Huycke M. (2014). The Physiology and Metabolism of Enterococci. In: Gilmore, MS, Clewell, DB, Ike, Y, & Shankar, N (eds), Boston.
- Rashid R, Cazenave-Gassiot A, Gao IH, Nair ZJ, Kumar JK, Gao L, *et al.* (2017). Comprehensive analysis of phospholipids and glycolipids in the opportunistic pathogen *Enterococcus faecalis*. *PLOS ONE* **12**:e0175886.

- Rashid R, Veleba M, Kline KA. (2016). Focal Targeting of the Bacterial Envelope by Antimicrobial Peptides. *Frontiers in Cell and Developmental Biology* **4**. doi:10.3389/fcell.2016.00055.
- Reyes J, Panesso D, Tran TT, Mishra NN, Cruz MR, Munita JM, *et al.* (2015). A liaR deletion restores susceptibility to daptomycin and antimicrobial peptides in multidrug-resistant *Enterococcus faecalis*. *The Journal of infectious diseases* **211**:1317–1325.
- Robert H, Le Marrec C, Blanco C, Jebbar M. (2000). Glycine Betaine, Carnitine, and Choline Enhance Salinity Tolerance and Prevent the Accumulation of Sodium to a Level Inhibiting Growth of *Tetragenococcus halophila*. *Applied and Environmental Microbiology* **66**:509–517.
- Robinson MD, McCarthy DJ, Smyth GK. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**:139–140.
- Saito HE, Harp JR, Fozo EM. (2014). Incorporation of Exogenous Fatty Acids Protects *Enterococcus faecalis* from Membrane-Damaging Agents Drake, HL (ed). *Applied and Environmental Microbiology* **80**:6527–6538.
- Sava IG, Heikens E, Huebner J. (2010). Pathogenesis and immunity in enterococcal infections. *Clinical Microbiology and Infection* **16**:533–540.
- Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, *et al.* (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC molecular biology* **7**:3.
- Sekirov I, Russell SL, Antunes LCM, Finlay BB. (2010). Gut Microbiota in Health and Disease. *Physiological Reviews* **90**:859 LP-904.
- Shankar M, Mohapatra SS, Biswas S, Biswas I. (2015). Gene Regulation by the LiaSR Two-Component System in *Streptococcus mutans*. *PLOS ONE* **10**:e0128083.
- Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, *et al.* (2017).

Emergence of Ceftazidime-Avibactam Resistance Due to Plasmid-Borne blaKPC-3 Mutations during Treatment of Carbapenem-Resistant Klebsiella pneumoniae Infections. *Antimicrobial Agents and Chemotherapy* **61**. doi:10.1128/AAC.02097-16.

Sun Y, Fukamachi T, Saito H, Kobayashi H. (2011). ATP Requirement for Acidic Resistance in Escherichia coli . *Journal of Bacteriology* **193**:3072–3077.

Suntharalingam P, Senadheera MD, Mair RW, Lévesque CM, Cvitkovitch DG. (2009). The LiaFSR System Regulates the Cell Envelope Stress Response in Streptococcus mutans . *Journal of Bacteriology* **191**:2973–2984.

Teixeira LM, Moss CW, Facklam RR. (1983). Gas-liquid chromatography of the fatty acids of Streptococcus faecalis with a fused silica capillary column. *FEMS Microbiology Letters* **17**:257–260.

Thiercelin ME. (1899). Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogène. *Compt rend Soc de biol* 51:269.

Thiercelin M. E. JL. (1899). Sur un diplococque saprophyte de l'intestin susceptible de devenir pathogene. *CR Soc Biol* **5**:269–271.

Thomas AW, Garner LE, Nevin KP, Woodard TL, Franks AE, Lovley DR, *et al.* (2013). A lipid membrane intercalating conjugated oligoelectrolyte enables electrode driven succinate production in Shewanella. *Energy & Environmental Science* **6**:1761–1765.

Tran TT, Munita JM, Arias CA. (2015). Mechanisms of Drug Resistance: Daptomycin Resistance. *Annals of the New York Academy of Sciences* **1354**:32–53.

Tran TT, Panesso D, Gao H, Roh JH, Munita JM, Reyes J, *et al.* (2013). Whole-Genome Analysis of a Daptomycin-Susceptible Enterococcus faecium Strain and Its Daptomycin-Resistant Variant Arising during Therapy. *Antimicrobial Agents and Chemotherapy* **57**:261–268.

Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, *et al.*

(2013). Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. *mBio* **4**:e00281-13-e00281-13.

Van Tyne D, Gilmore MS. (2014). Friend Turned Foe: Evolution of Enterococcal Virulence and Antibiotic Resistance. *Annual review of microbiology* **68**:337–356.

Urry DW. (1971). The Gramicidin A Transmembrane Channel: A Proposed π -sub(L,D)-sub Helix. *Proceedings of the National Academy of Sciences* **68**:672 LP-676.

Walsh C. (2003). Where will new antibiotics come from? *Nature reviews Microbiology* **1**:65–70.

Walsh CT, Wencewicz TA. (2014). Prospects for new antibiotics: a molecule-centered perspective. *J Antibiot* **67**:7–22.

Wang AY, Cronan JEJ. (1994). The growth phase-dependent synthesis of cyclopropane fatty acids in *Escherichia coli* is the result of an RpoS(KatF)-dependent promoter plus enzyme instability. *Molecular microbiology* **11**:1009–1017.

Weiss DS, Chen JC, Ghigo J-M, Boyd D, Beckwith J. (1999). Localization of FtsI (PBP3) to the Septal Ring Requires Its Membrane Anchor, the Z Ring, FtsA, FtsQ, and FtsL. *Journal of Bacteriology* **181**:508–520.

Wiegand I, Hilpert K, Hancock REW. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protocols* **3**:163–175.

Winkler ML, Papp-Wallace KM, Hujer AM, Domitrovic TN, Hujer KM, Hurless KN, *et al.* (2015). Unexpected challenges in treating multidrug-resistant Gram-negative bacteria: resistance to ceftazidime-avibactam in archived isolates of *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy* **59**:1020–1029.

- Wissel MC, Weiss DS. (2004). Genetic Analysis of the Cell Division Protein FtsI (PBP3): Amino Acid Substitutions That Impair Septal Localization of FtsI and Recruitment of FtsN. *Journal of Bacteriology* **186**:490–502.
- Wurster JI, Saavedra JT, Gilmore MS. (2016). Impact of Antibiotic Use on the Evolution of *Enterococcus faecium*. *The Journal of Infectious Diseases* **213**:1862–1865.
- Yan H, Catania C, Bazan GC. (2015). Membrane-Intercalating Conjugated Oligoelectrolytes: Impact on Bioelectrochemical Systems. *Advanced Materials* **27**:2958–2973.
- Yan H, Rengert ZD, Thomas AW, Rehmann C, Hinks J, Bazan GC. (2016). Influence of molecular structure on the antimicrobial function of phenylenevinylene conjugated oligoelectrolytes. *Chem Sci* **7**:5714–5722.
- Zhang E, Cai Y, Luo Y, Piao Z. (2014). Riboflavin-shuttled extracellular electron transfer from *Enterococcus faecalis* to electrodes in microbial fuel cells. *Canadian Journal of Microbiology* **60**:753–759.
- Zhong X, Tai PC. (1998). When an ATPase Is Not an ATPase: at Low Temperatures the C-Terminal Domain of the ABC Transporter CvaB Is a GTPase. *Journal of Bacteriology* **180**:1347–1353.