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# Resistance in Vancomycin-Resistant Enterococci

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## Introduction

The genus *Enterococcus* consists of facultative Gram-positive cocci that have been isolated from a variety of animals, plants and environmental sources. Although fifty-eight species have been described to date, *Enterococcus faecalis* and *Enterococcus faecium* are responsible for the majority of human infections (1). These organisms are frequently found as normal members of the gastrointestinal microbiota, but may become opportunistic pathogens, especially in the critically ill and immunocompromised patient population. Enterococci are known to cause a variety of infections, including skin and soft tissue infections, urinary tract infections, device infections, bloodstream infections, and infective endocarditis (2). Taken together, from 2015 to 2017, enterococci were the second leading cause of healthcare-associated infections overall, and the leading cause of central line associated bloodstream infections in long-term acute care hospitals and on oncology units (3).

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Complicating matters is the emergence of antibiotic resistance. Early experience in treating infective endocarditis with penicillin monotherapy revealed that a subset of streptococci (now known to be enterococci since the genus *Enterococcus* would not be formally established until 1984 (4)) displayed an inherent tolerance to the action of this drug (5). The advent of combination therapy with aminoglycosides improved cure rates from approximately 40 to 88%, at the cost of increased complexity and toxicity of the regimens (6). As new therapies entered the clinical space, however, enterococci have responded with a diverse array of intrinsic and acquired resistance determinants that continue to present therapeutic dilemmas to physicians.

VRE have been identified by the Centers for Disease Control and Prevention as a serious threat, leading to at least 5,400 estimated deaths and over $500 million in excess healthcare costs annually, as of 2017 (7). Resistance to newer antibiotics, including daptomycin and oxazolidinones, continues to emerge (8), while microbiological and clinical data guiding the most effective use of these agents remains to be resolved (9, 10). Thus, an understanding of emergent mechanisms of resistance in enterococci can provide insights into the best treatment approaches for these opportunistic pathogens, and help to guide the physician in making rational therapeutic decisions at the patient bedside.

## From commensal to a formidable clinical challenge

The origins of the enterococci can be traced back some 400 million years ago, to the appearance of the first terrestrial land animals (11). It is likely that these ancestral enterococci emerged from the water in the gastrointestinal (GI) tract of their hosts, as members of this genus are able to tolerate high concentrations of bile acids and possess a diverse set of genes involved in the metabolism of carbohydrates. To survive in this new environment, enterococci also developed a rugged adaptability, including a tolerance to elevated temperatures and high salt concentrations, and resistance to killing by a variety of chemical disinfectants (12). These same traits have enabled enterococci to colonize the human GI tract and survive in the modern hospital environment.

More recently, the beginning of the antibiotic era and the widespread use of antibiotics in both clinical practice, animal husbandry and agriculture has shaped the evolutionary trajectory of enterococci, particularly in relation to drug resistance. These factors have driven both the sequential emergence of *E. faecalis* in the 1970s and then vancomycin-resistant *E. faecium* in the late 1980s. Additionally, the selective antibiotic pressure has driven the continued evolution of resistance to newer antimicrobials (13). Although β-lactam antibiotics have long been the backbone of therapy for serious enterococcal infections, it was observed that minimum inhibitory concentrations (MICs) for these agents were at least an order of magnitude higher than those for streptococci, and combination therapy with aminoglycosides was needed to achieve reliable bactericidal effect. Activity across classes of β-lactam antibiotics also varies, with the aminopenicillins (such as ampicillin) having the greatest potency, followed by ureidopenicillins, penicillin G, and imipenem (14, 15). Most cephalosporins, as monotherapy, have no activity. This intrinsic resistance can be traced, in part, to the penicillin binding proteins (PBPs) which construct the peptidoglycan layer that surrounds the enterococcal cell. Functionally, PBPs can be divided into two classes. The

type A bifunctional enzymes are capable of performing both the transglycosylation and transpeptidation reactions needed to elongate and crosslink peptidoglycan chains. In contrast, type B monofunctional transpeptidases catalyze only peptide crosslinking (16). Both *E. faecalis* and *E. faecium* produce six PBPs, three class A and three class B, each of which have varying affinities for β-lactams (17, 18). The primary determinant of reduced susceptibility to β-lactam antibiotics is the low affinity class B enzyme PBP5 in *E. faecium* (19). This phenotype is conferred via a combination of factors, including alterations in PBP5 gene expression and a mosaic of changes in the amino acid sequence of the enzyme, which appear to influence the conformation of the active site and the resultant affinity for β-lactams (20–22). The presence of the resistance alleles encoding PBP5 variants has been associated with hospital-adapted strains of *E. faecium*, and may be one of several factors that allowed these isolates to thrive in the healthcare setting (23).

The orthologue in *E. faecalis* is PBP4, which is required for cephalosporin resistance but, in general, does not confer resistance to aminopenicillins (17, 24). Penicillin-resistant, ampicillin-susceptible *E. faecalis*, and fully ampicillin-resistant isolates, have been described. The mechanistic basis for these resistance phenotypes appears to be related to amino acid substitutions, which remodel the PBP4 active site, and promoter mutations, which increase expression of the gene (25, 26). Ampicillin susceptibility may not reflect concomitant susceptibility to penicillin, piperacillin or imipenem, and this assumption should be made with caution, especially when the latter compounds are used for treatment of deep-seated infections when isolates display elevated ampicillin MICs, although still within the susceptible range (25, 27).

Resistance to cephalosporins relies on the contribution of multiple proteins, though the full scope of this intrinsic resistance has not yet been elucidated. The presence of PBP4 (or PBP5 in *E. faecium*) is necessary, but not sufficient, for elevated cephalosporin MICs, and the class B enzyme appears to work in concert with one of two class A PBPs (PonA or PbpF) to synthesize the cell wall in the presence of drug (17). Additionally, two stress response systems, the CroRS two component system and IreK eukaryotic-like serine/threonine kinase, and MurAA (a cytosolic enzyme which catalyzes the first committed step in peptidoglycan biosynthesis) are required for cephalosporin resistance in *E. faecalis* (28–31).

Aminoglycosides are not usually active at generally achievable concentrations against enterococci as monotherapy, limited by poor uptake of the antibiotic into the cytoplasm. In addition, several commonly encoded aminoglycoside modifying enzymes (AME) confer resistance to various clinically available aminoglycosides. These include the 6'-acetyltransferase AAC(6')-Ii, which is an intrinsic property of *E. faecium* and inactivates tobramycin, sisomicin, kanamycin, and netilmicin, and the acquired phosphotransferase APH(3')-NIa present in many clinical enterococcal isolates that mediates resistance to kanamycin and amikacin (32, 33). As a result, only gentamicin and streptomycin are reliably active for synergistic use with β-lactams, which became the standard of care for enterococcal endocarditis for many decades. The emergence of high-level resistance to the aminoglycosides gentamicin and streptomycin (HLRAG) in the United States was first documented in 1983 (34). Isolates with high-level resistance, defined as growth in the

presence of 500 μg/mL of gentamicin, or 2,000 μg/mL of streptomycin, do not show synergism in combination with β-lactams. High level resistance to gentamicin is most commonly mediated by acquisition of a bifunctional AME, the AAC(6')-Ie-APH(2")Ia enzyme, though streptomycin retains synergistic activity in the presence of this enzyme (35). In the case of streptomycin, inactivation by an adenyltransferase abolishes synergy, as do mutations in the 30S ribosomal subunit; the latter allow the translation of RNA despite extremely high concentrations (> 128,000 μg/mL) of streptomycin (36).

Given the differential binding affinities of the enterococcal PBPs to β-lactams, Mainardi and colleagues noted that a combination of amoxicillin and cefotaxime was capable of saturating all major PBPs from *E. faecalis* with a synergistic effect (37). Subsequent studies confirmed this synergism with the combination of ampicillin and ceftriaxone in vitro, and in a rabbit model of infective endocarditis (38, 39). These observations, and the increasing frequency with which HLRAG was encountered in clinical isolates, eventually led to the clinical evaluation of dual β-lactam combinations for the treatment of *E. faecalis* infections (40). Of note, the limited in vitro data available for dual β-lactam combinations against ampicillin sensitive *E. faecium* indicated that only a few strains exhibited the synergistic phenotype (only 3/9 strains tested by time-kill curves) (41). Thus, the double β-lactam combination does not appear to be reliable for infections caused by *E. faecium.* Further, some enterococcal isolates possessing a beta-lactamase have been described, and such strains may not be readily recognized by the clinical microbiology laboratory, as enterococci do not release enzyme into the extracellular environment, and resistance may only be apparent at high inoculum (42).

As rates of high-level ampicillin resistance (>128 μg/mL) in *E. faecium* became increasingly common, the glycopeptide vancomycin began to see increased use. Vancomycin binds to the terminal two D-alanine residues of the pentapeptide moiety of peptidoglycan, inhibiting the transglycosylation and transpeptidation reactions and leading to an arrest of cell wall synthesis (43). Resistance arises via alteration of the terminal D-Ala-D-Ala, to either D-Ala-D-Ser (low-level resistance, 7-fold decrease in binding) or D-Ala-D-Lac (high-level resistance, 1,000-fold decrease in binding) (44). The metabolic machinery needed to carry out this substitution are encoded on the *van* operons, named by convention after the gene encoding the amino acid ligase (e.g., VanA, VanB). Intrinsic resistance among enterococci can be seen in *E. gallinarum* and *E. casseliflavus*, which carry the *vanC* operons (C1 and C2) on the chromosome and display low level resistance to glycopeptides (MICs 2-32 μg/mL) (45, 46). Among clinical isolates, acquired vancomycin resistance due to *vanA*, frequently found on the Tn *1546* transposon in association with plasmids, predominates. *vanB*-mediated resistance has been associated with a different conjugative transposon (Tn*5382*, sometime referred to as Tn*1549*), and is less common among clinical isolates of enterococci, although geographic variation of the circulating clones may influence local frequencies (47–49).

Gene clusters coding for vancomycin resistance are common in nature, and the source of the *van* genes identified in current enterococcal isolates is likely a soil bacteria of the genus *Paenibacillus* (50). The emergence of vancomycin resistance in clinical strains of enterococci was first reported in England in 1986, with resistance subsequently reported from other countries in Europe and the United States (51). In Europe, VRE reservoirs were

identified in livestock animals and in humans in the community. The niches of VRE were related to the widespread use of the glycopeptide avoparcin as a growth promoter in animal husbandry (52). In the US, avoparcin was not approved for agricultural use, and VRE were largely limited to the hospital setting, without widespread dissemination among healthy humans or livestock (53). Subsequent to the ban of avoparcin use in 1995 in Europe, the frequency of VRE isolated from animals began to fall, with data from Denmark showing a decline in chickens from a peak of 72.7% at the time of the ban to 5.8% in 2000 (54). In contrast, in US hospitals, rates of vancomycin resistance have remained relatively stable, with approximately 80% and 7-10% of *E. faecium* and *E. faecalis* isolates reported as resistant, respectively; although overall numbers of infections due to VRE have declined from 2012 to 2017, this is likely as a result of infection control measures (3, 7).

## Mapping multi-drug resistance

The advent of whole genome sequencing has led to further advances in the understanding of the population dynamics of enterococci and the spread of VRE. In *E. faecalis*, older analyses using a multilocus sequence type (MLST) based strategy described a diversity of sequence types without a particular host specificity, as both human and animal isolates had a uniform distribution among the clonal complexes identified (55). These studies also identified recombination as an important contributor to the evolution and population structure of *E. faecalis*, and unlike *E. faecium*, did not point to the emergence of a specific hospital adapted lineage, though some hospital associated clonal clusters were more likely to carry resistance determinants (56, 57). Initial genomic studies, though limited by the numbers of isolates studied, supported the observations that *E. faecalis* lacks a distinct division into hospital adapted clades (58). In contrast, a larger study of 515 isolates from primarily the United Kingdom (UK) and United States identified three distinct lineages (termed L1, L2, and L3), which the authors postulated could represent hospital-associated lineages of *E. faecalis* (59). Approximately 90% of the vancomycin-resistant *E. faecalis* identified in the study clustered into one of these three lineages, although the lineages themselves were a mix of vancomycin resistant and susceptible isolates. Strains belonging to L1-L3 were also enriched for aminoglycoside, chloramphenicol, macrolide, and tetracycline resistance determinants, as compared to non-lineage isolates. Further studies, with a larger number of more geographically diverse isolates, will be needed to firmly establish the existence of hospital adapted lineages in *E. faecalis.*

The population structure of *E. faecium* is more clearly defined, likely due to the importance of vancomycin-resistant *E. faecium* as healthcare-associated pathogens and concomitant surveillance studies to track their spread. Typing of *E. faecium* using MLST suggested a number of strains from healthcare associated infections formed a related group named Clonal Complex 17, though evolutionary relationships were difficult to resolve due to high rates of recombination (52). Initial genomic studies confirmed this broad division of *E. faecium* into two distinct lineages, a hospital-adapted clade A and human commensal clade B (60). Lebreton and colleagues described a further split of clade A isolates into epidemic hospital isolates (clade A1) and strains of animal origin (clade A2), and using a molecular clock analysis placed the bifurcation of this line approximately 80 years ago, or around the time antibiotics were introduced into clinical practice (61). There is significant genetic

diversity between clade A and clade B (average nucleotide identity of 93.9-95.6%), suggesting that a speciation event may be ongoing, potentially driven by adaptation, and subsequent isolation, of clade A1 strains in the hospital environment (58, 62). Subsequent studies have continued to add isolates and refine the population structure, though the majority originate from the United States and Europe, and may not reflect the true global diversity of the species. A large study of clinical isolates from hospitals across the UK and Ireland, and a study from Latin America, were not able to resolve a distinct animal associated clade A2, suggesting that these isolates may have been early branching points of clade A whose distribution on the phylogenetic tree may be impacted by recombination events (63, 64). A subsequent study from the UK including over 1,400 *E. faecium* genomes from livestock, wastewater, and human sources, supports the division into human commensal, animal-associated, and hospital-associated clades, and the authors noted limited transfer of genes and resistance determinants between strains of human and livestock origin (65). Strains collected from wastewater treatment plants belonged to all three groups, highlighting the potential for strain to strain contact, and gene transfer, in this setting, and the need for effective sanitation in low resource settings to combat the spread of resistance.

In addition to understanding the evolution of a multidrug-resistant pathogen, features of each clade may provide insight into GI colonization. While strains from both clade A and clade B were able to establish persistent GI colonization when introduced individually in a mouse model, when given together, strains from the commensal clade B were able to outcompete those from the hospital-adapted clade A (66). Thus, understanding the population structure and selective pressures driving the evolution of VRE can inform potential prevention and control strategies in the hospital setting.

## VRE and the microbiome

The microbiota of the human GI tract consists of over 100 cultivable species and many more that rely on symbiotic relationships with members of the larger microbial community or human host for growth (67). While well adapted to the human GI tract, enterococci generally comprise a small fraction of the microbial diversity under normal conditions. These healthy microbial communities limit the ability of multi-drug resistant bacterial strains, such as VRE, to establish a foothold in the colon, a phenomenon known as colonization resistance (68). Risk factors for VRE colonization include features that either directly or indirectly lead to disruption of the normal microbial flora, including antibiotic use, hospitalization, discharge to a long-term care facility, or dialysis (69–71). Clinical studies evaluating the duration of carriage report a median time to VRE clearance after hospital discharge from 2 to 4 months, consistent with a reconstitution of the colonic flora, though prolonged carriage may result from continued perturbation (71, 72).

Recent investigations have begun to probe both the host and microbial mechanisms behind colonization resistance. From the host standpoint, defense of the GI tract relies upon physical traits, such as stomach pH and the intestinal mucous barrier, as well as antimicrobial peptides of the innate immune system secreted into the luminal interior (68, 73). The GI tract of healthy individuals is largely composed of obligate anaerobes belonging to the *Firmicutes* and *Bacteroidetes*, which survive through the metabolism of carbohydrates

from the host diet (74). Restriction of this regular supply of dietary fibers, a situation that may occur in hospitalized or critically ill patients when enteral feeding is suspended or significantly altered, can induce the microbiota to turn to the host mucin layer for an energy source (75). This phenomenon results in thinning of an important GI protective barrier, allowing pathogenic organisms to gain proximity to the host epithelium and potentially translocate into the bloodstream to cause disease. In the clinical setting, loss of a diverse intestinal flora after antibiotic administration with subsequent domination by VRE was a precipitating event that predicted subsequent bacteremia in hospitalized patients with neutropenia (76, 77). The microbial community also influences immune mediated colonization resistance. Antimicrobial peptides (AMPs) such as RegIIIY, an antibacterial lectin secreted by murine epithelial and Paneth cells with activity against Gram-positive organisms including VRE, are produced via stimulation of Toll-like receptors by lipopolysaccharide (LPS) from intestinal Gram-negative bacteria (78). In a murine GI colonization model, depletion of Gram-negative commensals via the administration of broad-spectrum antibiotics promoted VRE colonization, a phenotype that could be rescued through exogenous administration of LPS (79).

Microbial communities in the intestinal lumen can also provide protection against VRE colonization independent of the influence on host response. High relative abundance of the genus *Barnesiella* was associated with a resistance to domination of the murine microbiota by VRE, even in mouse knockout strains deficient in the signaling mediators necessary for activation of the innate immune response and production of AMPs such as RegIIIY (80). Moreover, patients who developed VRE colonization after allogeneic-hematopoietic stem cell transplantation were more likely to have reduced levels of anaerobic *Barnesiella* in the pre-transplant microbiota, suggesting a role for this genus in preventing VRE domination in the clinical setting, although the specific mechanism of this effect is not known (80). In addition, some members of the microbiota are able to produce compounds with a direct inhibitory effect on VRE. Bacteriocins are a class of bacterial derived AMPs that can have narrow or broad range activity against other bacterial species and may be involved in competition for resources in an ecological niche (81). Commensal strains of *E. faecalis* carrying a pheromone-responsive plasmid defective for conjugation encoding the enterococcal Bacteriocin-21 were able to colonize and clear the GI tract of mice dominated by VRE (82).

Highlighting the complexity of the interactions leading to microbiota derived colonization resistance, the anaerobic commensal, *Blautia producta*, was found to produce a novel lantibiotic with similarities to nisin capable of killing VRE in vitro, but was unable to provide protection in the mouse intestine when given by itself (83). A synergistic consortium of four different bacterial species was required to prevent VRE domination in the GI tract of ampicillin-treated mice (84). Production of β-lactamases by the Gram-negative anaerobes *Bacteroides sartorii* and *Parabacteroides distasonis* provided protection for the ampicillin susceptible *B. producta*, while *Clostridium bolteae* was associated with engraftment and persistence of *B. producta* in the perturbed colonic flora. Thus, commensal members of the microbiota play a role in colonization resistance, but even small changes from the pressure of broad-spectrum antibiotics can alter the balance needed for protection.

Adaptations by enterococci that promote intestinal colonization may also be tied to antibiotic resistance. Upon exposure to certain bile acids present in the mammalian GI tract, *E. faecium* was observed to undergo a morphotype switch from distinct diplococci to long chains (85). This phenotype was associated with an increase in biofilm formation (dependent on the activity of the autolysin AtlA) and colonic aggregation seen in the GI tract of VRE colonized mice. In a serial passage experiment designed to identify the genes that contribute to this phenotype, mutations were observed in genes encoding proteins of the LiaFSR and YycFG stress response systems, which have been implicated in resistance to daptomycin (a lipopeptide antibiotic similar to AMPs) in clinical enterococcal isolates (see section on daptomycin resistance below) (86, 87). It is conceivable that mutations in genes modulating the cell envelope stress response, which could allow for increased survival in the face of host secreted and microbiota derived AMPs, may also prime intestinal pathogens to resist attack by antibiotics with similar mechanisms or bacterial targets. Indeed, daptomycin resistance in enterococcal isolates from patients without exposure to the drug has been reported (88, 89), though more research is needed to identify if exposure to host derived AMPs can induce cross-resistance to currently used therapeutics.

## Antibiotic resistance in the new millennium

The intrinsic and ever expanding repertoire of acquired resistance determinants in enterococci have necessitated the progressive development of new strategies to meet clinical needs. Despite the introduction of novel compounds with VRE activity, enterococcal infections in the setting of multi-drug resistance remain a clinical challenge. Here, we provide an overview of the mechanisms of resistance to newer agents that are often employed in treating severe enterococcal infections.

### Daptomycin

Daptomycin (DAP) is a lipopeptide antibiotic with in vitro bactericidal activity against vancomycin-resistant *E. faecalis* and *E. faecium.* Indeed, DAP has become first-line therapy for VRE, in particular for *E. faecium* (90). DAP was originally isolated as a mix of natural products from *Streptomyces roseosporus*, and consists of a 13-amino acid peptide core with a fatty acyl tail made by a non-ribosomal peptide synthetase complex (91). In the active form of the drug, this cyclic core binds a calcium ion, leading to an amphipathic molecule with a positively charged surface and a hydrophobic tail (92). DAP is then able to insert into the Gram-positive bacterial membrane in a phosphatidylglycerol (PG) dependent manner, in which the positively charged surface interacts with the negatively charged PG headgroups, and the hydrophobic tail anchors amongst the lipid acyl chains (93). The next steps in the mechanism of action are more poorly understood, but the DAP-calcium complex appears to equilibrate and oligomerize across the outer and inner membrane leaflets (94). Initial studies found that DAP treatment led to membrane disruption and ion leakage suggesting pore formation (95), but this appears to be a late phenomenon in DAP treated cells, and is likely not the primary mechanism of action. Mueller and colleagues have shown that DAP is capable of rigidifying the bacterial membrane, sequestering fluid lipids and leading to the dissociation of membrane bound enzymes that are important for cell envelope biogenesis

and peptidoglycan synthesis (96). The exact membrane or cellular target by which DAP exerts its bactericidal action is an area of intensive research.

DAP resistance arises in both *E. faecalis* and *E. faecium*, and though the genetic pathways implicated in resistance share similarities between the strains, the molecular mechanisms that underlie resistance appear to have important differences (90). The unifying features of resistance to DAP involve two sets of mutations that work in concert to bring about high-level resistance to the antibiotic. The first involves changes in two component signaling systems that activate the cell envelope stress response (inducing tolerance to the antibiotic), while the second alters enzymes important for phospholipid metabolism and result in a full resistance phenotype. In *E. faecalis*, these changes lead to redistribution of membrane phospholipid microdomains away from the division septum, potentially as a “diversion” tactic to protect the sensitive biosynthetic machinery located at the septum where cell division occurs (97). In *E. faecium*, major changes in membrane architecture are not observed in DAP resistant strains (87). Instead, DAP resistance-associated mutations appear to influence cell surface charge similar to the “repulsion” mechanism proposed for *Staphylococcus aureus.*

Three major two component regulatory systems have been implicated in enterococcal DAP resistance to date, including LiaFSR, YycFG, and YxdJK (86, 87, 98). Using whole genome sequencing across a variety of DAP resistant *E. faecium* strains of clinical origin, the LiaFSR (for Lipid II Interacting Antibiotics) operon was implicated as the major pathway associated with resistance (87). This system is conserved among medically important members of the *Firmicutes*, and consists of a sensor histidine kinase (LiaS), its cognate response regulator (LiaR), and a predicted transmembrane regulatory protein (LiaF) (99). In the presence of cell membrane stress, LiaS activates the system by phosphorylating LiaR, which induces oligomerization and increases its DNA binding affinity upstream of target genes (100). DAP resistance is tied to mutations leading to changes in LiaR which mimic phosphorylation, or alterations of LiaF which appear to activate the system (101, 102). Clinically, these changes can have important consequences, since they may lead to tolerance (lack of bacterial killing, even at 5x MIC of DAP) despite only minor changes in MIC (e.g., from 1 to 4 μg/mL, previously in the “susceptible” category) (103). Indeed, clinical failures of DAP have been associated with this scenario (101), and isolates with DAP MICs in the 3 to 4 μg/mL range are associated with mutations in the LiaFSR system (104).

Critical among the genes regulated by LiaR is an operon encoding three proteins, LiaXYZ, of which LiaX sits at the fulcrum of the enterococcal stress response. LiaX possesses two predicted domains, an N-terminal α-helical domain capable of binding DAP and the human cathelicidin LL-37 (a cationic AMP of the innate immune system), and a C-terminal domain of β-pleated sheets that appears to function in a regulatory role (105). A frameshift mutation resulting in premature truncation of the C-terminal domain of LiaX obtained from an experimental evolution model of DAP resistance in *E. faecalis* was sufficient to activate the LiaFSR response, bring about redistribution of phospholipid microdomains, and increase virulence in a *C. elegans* model related to resisting attack by host AMPs (105, 106). Further, purified N-terminus of LiaX was able to confer protection to DAP susceptible *E. faecalis*

strains, but not to strains lacking the response regulator LiaR or *S. aureus*, suggesting a specific role for LiaFSR signaling in enterococcal DAP resistance.

The YycFG two component system is an essential regulator of cell wall homeostasis, known to be active in modulating peptidoglycan synthesis, cell wall remodeling and autolysin expression (107). It appears to be the second most frequent pathway to DAP resistance in *E. faecium*, and it is important to note that isolates with mutations in *yycFG* do not appear to demonstrate the same synergistic interaction between DAP and β-lactams (the see-saw effect), as is seen in LiaFSR mediated DAP resistance (87).

YxdJK is a two component system that controls a network of ATP Binding Cassette (ABC) transporters involved in bacitracin resistance (108). In *E. faecalis* strains lacking the LiaR response regulator, a putative activating mutation of the YxdK sensor kinase was associated with elevated DAP MICs. Indeed, deletion of the YxdJ response regulator was sufficient to revert strains to DAP susceptibility (98). In *E. faecium*, the YxdJK system (ChtRS) has been associated with modulation of tolerance to the cationic disinfectant chlorhexidine (109).

## Lipoglycopeptides

The lipoglycopeptide antibiotics are built around a glycopeptide core similar to vancomycin, with the addition of a hydrophobic substituent that serves to anchor the antibiotic molecule to the target cell. Telavancin, dalbavancin, and oritavancin are the currently available antibiotics in this class, though there are significant differences in their activity against VRE (110). The glycopeptide core of telavancin and dalbavancin binds preferentially to peptidoglycan precursors ending in D-Ala-D-Ala, and, as a result, they do not exhibit clinically significant activity against enterococci with *vanA* mediated vancomycin resistance (111). These agents may test susceptible against *vanB*-mediated resistance in vitro, since, similar to teicoplanin, they do not appear to be inducers of expression the *vanB* gene cluster. However, mutations may arise that lead to constitutive activation of the *vanB* operon and expression of resistance against these compounds (112, 113).

Oritavancin, in contrast, retains in vitro activity against VRE exhibiting both *vanA* and *vanB* resistance. This compound has an expanded set of interactions with peptidoglycan precursors, extending to the L-lysine in the third position of both the pentadepsipeptide and amino acid cross bridge, which appears to increase its affinity for D-Ala-D-Lac (114). In addition, the 4'-chlorobiphenylmethyl side chain allows for an increased binding affinity to lipid II, disrupts the transglycosylation reaction needed for peptidoglycan extension, and can compromise the membrane integrity of target bacteria (115). The multiplicity of the modes of action for this compound lead to potent bactericidal activity. Despite these potential advantages, the optimal setting and dosing strategy for the use of oritavancin against VRE in clinical practice, especially in the setting of daptomycin resistance or treatment failure, remains unclear. Reduced susceptibility to oritavancin upon serial passage in the laboratory has been documented in both vancomycin-susceptible and resistant isolates of *E. faecalis* and *E. faecium*, with MIC increases from 4 to 32 fold (116). The mechanistic bases behind these increases are not well understood. Overexpression of the *vanA* operon, with the exclusive production of D-Ala-D-Lac termini, was associated with a 16-fold increase in MIC (117). Expression of *vanZ*, a gene encoding a protein of unknown function also present in

the *vanA* operon and known to mediate teicoplanin resistance, was also able to independently increase the MIC of oritavancin by 8-fold (117). Cross-resistance to oritavancin was also found to develop in two clinical *E. faecium* isolates exposed in vitro to simulated exposures of DAP at 12 mg/kg (118). Whole genome sequencing revealed changes in LiaS, the sensor kinase of the LiaFSR system, in one isolate, and a mutation in a bacitracin resistance transporter gene in the second isolate. While further work is needed to characterize the contributions of these systems to increasing oritavancin MICs, these results suggest caution is needed when using oritavancin as salvage therapy for DAP-resistant VRE infections.

## Oxazolidinones

Linezolid and tedizolid are the two clinically available compounds in the oxazolidinone class, and linezolid is the only antibiotic with US Food and Drug Administration approval for the treatment of VRE bacteremia. These compounds act at the bacterial ribosome, binding to the A site and preventing the docking of the aminoacyl-tRNA complex, thus inhibiting the synthesis of the polypeptide chain (119, 120). Tedizolid exhibits greater potency than linezolid, with MICs from four to eight-fold lower, due to a hydroxymethyl modification of the oxazolidinone ring and a fourth D-ring moiety not present in linezolid (121). Mutational resistance arises from changes in the 23S rRNA (most commonly the substitutions G2505A and G2576U) that alter the binding site for both antibiotics, leading to decreased affinity for the drug and higher MICs. Enterococci possess multiple copies of each 23S rRNA gene, thus the number of mutated alleles present in a strain determines the ultimate efficacy of the drug, as the bacteria will have a mixed population of “sensitive” and “resistant” ribosomes (122). Under selective pressure, resistant alleles may recombine to replace wild type alleles in the genome, leading to progressively fewer ribosomes susceptible to inhibition, and loss of drug activity (123). In the setting of recombination, this gene-dosage effect can lead to the rapid emergence of resistance. In a comparison of an *E. faecalis* laboratory strain and its recombination deficient mutant under linezolid selection, emergence of the first 23S rRNA mutation did not differ between the strains (124). Subsequent mutations at alternate alleles, however, occurred in the wild-type strain six passages before the recombination deficient mutant. The potential for emergence of resistance in this manner may be a consideration when using oxazolidinones for VRE infections with high inoculum. Resistance related to mutations in the genes encoding the ribosomal proteins L3 and L4 has been described in enterococci, though the specific contribution to the resistance phenotype is not known (125). In other Gram-positive cocci, changes in ribosomal proteins occur concomitantly with 23S rRNA mutations, and may represent compensatory mutations which mitigate a potential fitness defect (126).

Transmissible resistance to oxazolidinones is more worrisome, given the potential for horizontal gene transfer to lead to the wide dissemination of resistance. Two main transmissible determinants of oxazolidinone resistance have been characterized, *cfr* and *optrA*, with a third *poxtA* recently described in a clinical methicillin-resistant *S. aureus* isolate and animal isolates of enterococci from China and Tunisia (127, 128). Cfr, for chloramphenicol-florfenicol resistance, is an enzyme that methylates the adenine nucleotide at position 2503 of the 23S rRNA and leads to resistance to oxazolidanones in addition to

phenicols, pleuromutilins, and streptogramin A. The gene was first isolated in Germany from *Staphylococcus sciuri* recovered from a bovine mastitis infection, in association with a plasmid that was transferable to *S. aureus* laboratory strains (129). The first *cfr* positive clinical isolate of *E. faecalis* was described in 2012 (130), and the gene has since been reported from both animal and clinical isolates recovered across the globe (131). While tedizolid exhibits lower MICs than linezolid against *cfr* positive strains in vitro, tedizolid showed decreased efficacy in a mouse peritonitis model against a strain of *E. faecium* with *cfr(B)*, as compared to either linezolid or DAP (132). Both *optrA* (oxazolidinone phenicol transferable resistance) and *poxtA* (phenicol oxazolidinone and tetracyclines) encode proteins with homology to the ATP binding subunit of ABC transporters, and appear to be associated with IS*1216* mobile elements (133–135). Initially thought to be part of a drug efflux system, it was later shown that this class of ABC-F family proteins confer resistance via a mechanism of ribosomal protection (136). As is implied by their name, each determinant provides protection against a different spectrum of antibiotics active at the ribosome, and, in vitro, *optrA* is associated with a greater fold-change in MIC for oxazolidinones. These genes appear to be widespread amongst animal enterococcal isolates, potentially linked to phenicol use in veterinary medicine. Although the *optrA* gene was the most commonly identified transmissible oxazolidinone resistance determinant in *E. faecalis* strains resistant to linezolid from a wide survey of clinical isolates, the overall resistance mediated by these genes remains low (131).

## New generation tetracyclines

Resistance to the tetracycline class of antibiotics is common in enterococci, and is primarily mediated through one of two mechanisms, drug efflux via efflux pumps typically carried on plasmids (*tet*(K), *tet*(L)) and target protection at the ribosome mediated by genes on mobile elements such as Tn*916* (*tet*(M), *tet*(O), *tet*(S)) (8). The newer compounds to enter the market, including the glycylcycline tigecycline, the aminomethylcycline omadacycline, and the synthetic eravacycline, were designed to retain activity in the setting of common tetracycline resistance determinants, and offer potential options for the treatment of VRE infections. The agent with the most clinical experience, tigecycline, is approved for use in intra-abdominal infections and skin and soft tissue infections for vancomycin-susceptible isolates of *E. faecalis*, and shows in vitro activity against VRE of both species (137, 138). Due to low serum concentrations, this antibiotic has typically been used only for intra-abdominal infections or as a part of combination therapy in recalcitrant enterococcal bacteremia and infective endocarditis.

Resistance to tigecycline has emerged with clinical use. The primary mechanism of resistance appears to involve mutations in the S10 protein of the 30S ribosomal subunit (139). These mutations cluster into an extended loop of the protein, which protrudes near the tigecycline binding site on the 16S rRNA, potentially leading to decreased access or binding to the ribosome (140). While tigecycline is a less efficient substrate for both efflux pumps and ribosomal protection proteins, the presence of the genes encoding both the *tet*(L) efflux pump and *tet*(M) protection factor was associated with resistance in clinical isolates of *E. faecium* (141). An analysis of gene expression suggested that both increases in transcription and an expansion of gene copy number were responsible for the phenotype. Further, in an

experimental evolution model performed with *E. faecalis* under tigecycline exposure in a bioreactor, antibiotic exposure led to the excision and amplification of *tet*(M) in association with a Tn*916* transposon, leading to an expansion of gene copy number and inducing high rates of conjugative transfer of resistance, as well as increased expression of *tet*(M), with the concurrent emergence of S10 protein mutations (142). Thus, proliferation of traditional tetracycline resistance determinants may play a role in the emergence of tigecycline resistance. Mutations in the *rpsJ* gene encoding the S10 protein, or in the 16S rRNA itself, also appear to influence the susceptibility of eravacycline and omadacycline (143, 144), and the role of these agents in the clinical treatment of VRE infections remains to be defined.

## Summary

Since the emergence of VRE in the late 1980s, clinicians have faced major challenges when treating patients infected with these problematic pathogens. Despite possessing a relatively low virulence potential, the intrinsic tolerance to common broad-spectrum antimicrobials and an enduring ability to adapt have allowed enterococci to leave behind their roots as a commensal of the gastrointestinal tract and evolve into a leading cause of healthcare-associated infections. Unfortunately, the factors predisposing to enterococcal infections often leave the most vulnerable patient populations at highest risk. Innovation has brought new therapeutics with activity against VRE to the patient bedside, but this has not slowed the emergence of novel mechanisms of resistance. Thus, new strategies are needed to win the evolutionary arms race against these organisms.

A deep understanding of the molecular mechanisms of resistance may offer insights into how to disarm VRE. The identification of the two component signaling systems responsible for activating the stress response may allow for the development of new compounds, which block the adaptive response and re-sensitize resistant bacteria, breathing new life into currently available therapeutics. Research into the complex interactions of the microbiome can lead to pre- or probiotics that prevent colonization or restore a colonization resistant flora after antibiotic treatment. The emergence of phage therapy may also permit developing therapeutic interventions capable of evolving alongside the pathogen itself. Until these novel options enter the clinical arena, it will be up to physicians to make the most efficient use of currently available drugs against these multidrug-resistant organisms.

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## Synopsis

Serious infections due to vancomycin-resistant enterococci (VRE) have historically proven to be difficult clinical cases, requiring combination therapy and management of treatment-related toxicity. Despite the introduction of new antibiotics with activity against VRE to the therapeutic armamentarium, significant challenges remain. An understanding of the factors driving the emergence of resistance in VRE, the dynamics of gastrointestinal colonization and microbiota mediated colonization resistance, and the mechanisms of resistance to the currently available therapeutics will permit clinicians to be better prepared to tackle these challenging hospital-associated pathogens.

**Key Points**

- Vancomycin resistant enterococci (VRE) are a leading cause of healthcare associated infections, and the emergence of resistance to most available antibiotics makes treatment a major clinical challenge.
- The sturdiness and genomic plasticity of VRE have led to the adaptation of a hospital-associated clade of *E. faecium* with multiple drug-resistance determinants.
- A healthy gastrointestinal microbiota can provide resistance to VRE colonization, but antibiotic use can disrupt this protective flora and leave vulnerable patients at risk for subsequent infection.
- New antibiotics with activity against VRE have entered clinical practice, but an understanding of the clinical role and mechanisms of resistance to these compounds is crucial to optimize their use in challenging infections.