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The rise of the *Enterococcus*: beyond vancomycin resistance

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Abstract

The genus *Enterococcus* includes some of the most important nosocomial multidrug-resistant organisms, and these pathogens usually affect patients who are debilitated by other, concurrent illnesses and undergoing prolonged hospitalization. This Review discusses the factors involved in the changing epidemiology of enterococcal infections, with an emphasis on *Enterococcus faecium* as an emergent and challenging nosocomial problem. The effects of antibiotics on the gut microbiota and on colonization with vancomycin-resistant enterococci are highlighted, including how enterococci benefit from the antibiotic-mediated eradication of Gram-negative members of the gut microbiota. Analyses of enterococcal genomes indicate that there are certain genetic lineages, including an *E. faecium* clade of ancient origin, with the ability to succeed in the hospital environment, and the possible virulence determinants that are found in these genetic lineages are discussed. Finally, we review the most important mechanisms of resistance to the antibiotics that are used to treat vancomycin-resistant enterococci.

Enterococci have been known for more than a century for their role as a common cause of endocarditis¹, a disease that is fatal without effective antimicrobial therapy. The enterococci are Gram-positive, facultatively anaerobic oval cocci that form chains of various lengths; they are sturdy and versatile, with a particular ability to survive under harsh conditions (including high salt concentrations) and at a wide range of temperatures (from 10 °C to >45 °C). The first description of an enterococcal infection — namely, infective endocarditis — dates from 1899 (REF. 1), and enterococci were subsequently shown to cause a range of infections in the community setting (including pelvic infections, neonatal infections and urinary tract infections (UTIs)), as well as infective endocarditis. However, in spite of their pathogenic potential, enterococci generally display low levels of virulence, as evidenced by their presence as natural colonizers of the gastrointestinal (GI) tract in most humans and animals and by the fact that they have been used safely for decades as probiotics in humans and farm animals.

Both microbial and host factors can contribute to the conversion of a second-rate pathogen into a first-rate clinical problem. For the enterococci, such factors appear to include their inherent ability to resist antimicrobial agents (for example, clindamycin, cephalosporins and aminoglycosides), their capacity to acquire and disseminate determinants of antibiotic resistance (for example, vancomycin resistance gene clusters) and their malleable genomes, which may contribute to their adaptation to harsh environments (including hospitals) and increase the ability of certain lineages to colonize the GI tract and/or disseminate outside the bowel. Moreover, the increasing number of patients who are hospitalized in critical care units and are immunosuppressed, mechanically compromised (by catheters, for example) and receiving multiple antimicrobial agents favours the ability of multidrug-resistant organisms such as enterococci to cause disease. In this Review, we discuss the factors that may have contributed to the rise of enterococci as nosocomial pathogens, with an emphasis on the epidemiology and pathogenesis of infections by these species, and on mechanisms of resistance to the most relevant anti-enterococcal agents used in clinical practice. For a more detailed discussion of the clinical and therapeutic aspects of enterococcal infections, the reader is directed to other recent reviews^{2,3}.

The epidemiology of enterococcal infections

In hospitals in the United States, enterococci are the second most common organisms recovered from catheter-associated infections of the bloodstream and urinary tract, and from skin and soft-tissue infections^{2,4}. Hospital-associated enterococcal infections in the United States have emerged in two distinct waves. The first wave began in the late 1970s and was associated with the introduction of third-generation cephalosporins⁵; during this era, *Enterococcus faecalis* accounted for 90–95 % of clinical enterococcal isolates. We are now in the midst of the second wave, caused by *Enterococcus faecium*, which is much more frequently resistant to vancomycin and ampicillin than *E. faecalis*. This wave has increased steadily since the early 1990s in hospitals in the United States, but is now also present in other parts of the world^{4,6} and is associated with the increased use of vancomycin and broad-spectrum antibiotics. Indeed, the number of infections with vancomycin-resistant enterococci (VRE) in US hospitals increased from 9,820 in 2000 to 21,352 in 2006 (REF. 7); furthermore, *E. faecium* is now almost as common a cause of nosocomial infections as *E. faecalis*⁴. This change in species is of paramount clinical importance, as *E. faecium* is by far the more difficult of the two species to treat. For example, in the United States, the percentage of *E. faecium* isolates that were resistant to vancomycin rose from 0 % before the mid 1980s to more than 80 % by 2007 (REF. 2); by contrast, only ~5 % of *E. faecalis* isolates are vancomycin resistant⁴.

Multidrug-resistant enterococci are currently less of a problem outside the United States. The initial reports of VRE in Europe in the late 1980s were of organisms that were mostly colonizers of the GI tract of animals and humans in the community. Indeed, a strong correlation between the use of avoparcin, a glycopeptide antibiotic that was used in animal feed, and the emergence of VRE in Europe led to the ban of this compound from animal husbandry in 1996 (REF. 8). Although a decrease in the prevalence of VRE in animals in Europe was initially observed after the avoparcin ban, subsequent surveillance has revealed an increase in nosocomial ampicillin- and/or vancomycin-resistant enterococcal infections over the past decade. For example, in the Netherlands, the average number of invasive ampicillin-resistant enterococcal infections per hospital increased from ~10 infections in 1999 to ~50 in 2005 (REF. 9). By 2007, vancomycin resistance among clinical enterococcal isolates from Europe varied from >30 % in countries such as Greece and Ireland to less than 1 % in Scandinavian countries⁸, although a recent alarming report from Sweden documented an approximately fourfold increase in infections by VRE in the period 2007–2009 compared with 2000–2006 (REF. 10). In Latin America, a multicentric prospective study in four

countries found that the majority (~78 %) of enterococcal infections are still caused by ampicillin- and vancomycin-susceptible *E. faecalis*, although the remaining ~22 % are caused by genetic lineages of multidrug-resistant *E. faecium* that are similar to those observed in the United States¹¹. In Asia, the number of infections by vancomycin-resistant *E. faecium* is still low, although outbreaks have been documented¹².

Colonization of the GI tract

Enterococci are common inhabitants of the GI tract of humans and other animals as well as that of insects and nematodes. Although these species normally constitute a small proportion of the gut microbiota¹³, an important first step towards nosocomial enterococcal infection seems to be increased density of colonization of the GI tract (FIG. 1). The exposure of hospitalized patients to antibiotics (for example, cephalosporins and some penicillins — that is, piperacillin–tazobactam — with activity against Gram-negative bacteria and Gram-positive species excluding *E. faecium*) results in substantial changes in the gut microbiota that facilitate colonization of the GI tract by VRE^{14,15}. Pioneering work using mouse models of intestinal colonization¹⁶ has provided considerable insights into the mechanisms by which VRE gain the upper hand in the gut after antibiotic exposure. These studies found that lipopolysaccharide and flagellin of Gram-negative bacteria, including anaerobes, stimulate (via interactions with Toll-like receptors)^{16,17} the production of REGIII γ by Paneth cells; REGIII γ is a C-type lectin with antimicrobial activity against Gram-positive bacteria, including VRE¹⁶. Depletion of the Gram-negative microbiota by antibiotics decreases the production of REGIII γ and thus facilitates overgrowth of VRE^{16,17} in the GI tract. This highlights the role of the intestinal microbiota in modulating colonization by multidrug-resistant organisms (FIG. 2). Indeed, the use of the antibiotics metro-nidazole, neomycin and vancomycin in mice allows VRE to become the predominant intestinal species and to remain dominant for up to 2 months after the antibiotic regimen is discontinued¹⁵. Extending this work to humans, it was shown that invasion of the bloodstream of hospitalized patients by VRE is preceded by VRE becoming the predominant species in the GI tract of these patients¹⁵. In addition to the intestinal changes, the vancomycin resistance and the high minimal inhibitory concentrations of piperacillin and cephalosporins displayed by ampicillin-resistant *E. faecium* allow these organisms to survive in the gut of patients who are being treated with these antibiotics.

Nosocomial transmission of VRE

Enterococci are ‘tough bugs’ that can survive for long periods on environmental surfaces, including medical equipment, bed rails and doorknobs¹⁸ (FIG. 3). They are tolerant to heat, chlorine and some alcohol preparations¹⁸, which may help explain why these organisms are widely disseminated in the hospital setting. There are several risk factors for developing a nosocomial VRE infection: close physical proximity to patients infected or colonized with VRE; a long period of hospitalization; multiple courses of antimicrobials; hospitalization in long-term facilities, surgical units or intensive-care units; solid organ and bone marrow transplantation; co-morbidities such as diabetes, renal failure or haemodialysis; and the presence of a urinary catheter¹⁹.

An understanding of the transmission dynamics of enterococci in the hospital environment is pivotal for infection control. Hospitalized patients often receive antimicrobials that increase the density of VRE in the GI tract, in turn facilitating the spread of these organisms. A mathematical model²⁰ of transmission drew parallels between VRE transmission and the transmission of a vector-borne infection such as malaria. In this model, health care personnel play the part of the mosquito, carrying VRE on their hands from patients who are VRE positive to those who are VRE negative, and to their surroundings, and there is a potential to transmit the pathogens with each contact. Transmission can be amplified depending on how

many patients have contact with the ‘vector’ (REF. 20) and correlates with the density of VRE in patient stools. This concept highlights the importance of curtailing the chain of transmission through active surveillance and contact precautions for infected and colonized individuals, through implementation of strict hand hygiene practices for health care workers, through judicious use of antimicrobials (via programmes of antibiotic stewardship) and through aggressive environmental cleaning methods^{19,21}.

Population genetics of enterococci

Early studies on the molecular epidemiology of *E. faecalis*²² and *E. faecium*²³ using pulsed-field gel electrophoresis (PFGE) showed the spread of single clones to different US states. Multilocus sequence typing (MLST), which is based on allelic differences in seven housekeeping genes and is preferred for analyses of ancestral relationships, helped establish that enterococcal isolates recovered from hospitalized patients often cluster in specific groups (designated clonal complexes) that are different from those of commensal isolates or isolates recovered from animals. Indeed, the majority of hospital-derived isolates of *E. faecalis* cluster in two clonal complexes, CC2 and CC9 (REFS 24,25), and the increased frequency of isolation of *E. faecium* worldwide is due to the presence of a polyclonal subpopulation (particularly MLST sequence type 17 (ST17), ST18, ST78 and ST192, which were previously designated clonal complex CC17)^{6,26}; recent comparisons of available genome sequences support the concept of a hospital-associated clade that is genetically distinct from most commensal isolates from animals and humans^{6,27–29}.

In a recent comparison of 100 core genes from available *E. faecium* genomes²⁹, it was found that most (91) of these genes split into two groups that differ by 3.5–4 %, confirming that there are indeed two ancestral ‘clades’ of *E. faecium*. The ‘hospital-associated’ group contains most of the *E. faecium* clinical isolates that have been sequenced, although it also contains isolates of community origin; the ‘community-associated’ group consists almost entirely of isolates from the community (from both humans and animals). In addition to the differences between the genes of the two clades, molecular-clock analysis estimated that these groups diverged from each other at least 300,000 years ago. As previously reported, insertion sequence 16 (IS16)²⁷ and the gene encoding the ampicillin-resistant penicillin-binding protein 5 (*pbp5R*) are consistently found in the hospital-associated strains, whereas the gene encoding the ampicillin-sensitive PBP5 (*pbp5S*) is present in the community-associated clade³⁰.

One of the main challenges in the analysis of entero-cocci is the enormous plasticity of their genomes. In both *E. faecalis* and *E. faecium*, acquired elements can account for up to 25 % of the genome^{28,31–37}. Moreover, in a series of elegant experiments, it has been shown that conjugation (bacterial mating) between enterococci can produce transconjugant strains with hybrid genomes; that is, large fragments (up to 800 kb) of chromosomal DNA can be transferred from a donor strain to a recipient strain and can result in changes in the MLST pattern³⁸. The transfer depends on the presence of pheromone-responsive plasmids that can insert into the chromosome, presumably by recombination between homologous sequences on the plasmid and chromosome³⁸ (reviewed in REFS 33,39). Evidence of recombination in nature was obtained by a recent analysis which noted that in some of the analysed strains, all of the core genes are from one clade, but in the majority of analysed strains, between one and eight genes have been replaced with the corresponding gene from the other clade²⁹. In one strain, which was considered in this analysis to be a true hybrid strain, 26 of the 92 genes analysed belong to the community-associated group, whereas the rest belong to the hospital-associated clade²⁹.

Another important component in the evolution of multidrug-resistant strains of hospital-associated entero-cocci may be their lack of CRISPR (clustered regularly interspaced short

palindromic repeats) elements, which provide bacteria with a defence system against incoming DNA. Indeed, the presence of antibiotic resistance determinants is inversely correlated with the presence of CRISPRs in *E. faecalis*, and most isolates from hospital-associated clonal complexes lack CRISPRs⁴⁰.

Enterococcal pathogenesis

The rise of enterococci as nosocomial pathogens was initially thought to have resulted entirely from the selective advantage provided by their resistance to antibiotics. However, it is likely that other virulence determinants are also involved in the success of these microorganisms in the hospital setting. Research into the mechanisms by which enterococci cause disease has yielded important insights into their biology. Unlike streptococci and staphylococci, most enterococci do not produce a set of potent pro-inflammatory toxins, but they are equipped with many genes encoding adhesion proteins that may mediate adherence to host tissues, consistent with their pathogenic role in infective endocarditis. An extended review of all the potential pathogenic factors identified in enterococci is beyond the scope of this Review, and here we concentrate on those that have been shown to have an important role in experimental mammalian models (TABLE 1).

Secreted factors

Several proteins that are secreted into the extracellular medium have been implicated in enterococcal virulence. Haemolysin–cytolysin (Cyl) is a toxin produced by ~30 % of *E. faecalis* strains and is encoded on pheromone-responsive plasmids or pathogenicity islands. Cyl is secreted extracellularly as two structural subunits (CylL-L and CylL-S) and then proteolytically activated^{41,42}. Cyl can lyse red blood cells from humans, horses and rabbits, but not sheep or cows, and can also lyse some human white blood cells⁴³. *E. faecalis* strains expressing *cyl* are more virulent in various animal models than isogenic strains without *cyl*⁴³.

Other important secreted factors include the proteases gelatinase (GelE) and extracellular serine proteinase (SprE). GelE seems to mediate virulence through effects such as degradation of host tissues and modulation of the host immune response⁴⁴. It has an important role in clearing misfolded proteins⁴⁵ and participates in the activation of autolysin, a peptido-glycan-degrading enzyme⁴⁶, which leads to the release of extracellular DNA and the formation of a biofilm. The genes encoding these proteases in *E. faecalis* are regulated by the Fsr quorum sensing system⁴⁷, which is homologous to the Agr system of staphylococci (involved in regulating the expression of several virulence factors in *Staphylococcus aureus*) and has been shown to affect the pathogenesis of some *E. faecalis* infections^{48,49}. Mutants lacking GelE show a marked decrease in biofilm formation⁵⁰, a decrease in translocation across T84 intestinal cells⁵¹, attenuated virulence in peritonitis, endocarditis, endophthalmitis and *C. elegans* models^{52–56}, and reduced adherence to dental roots⁵⁷. It should be noted that Cyl and GelE are seen equally in isolates recovered from clinical infections and in those from stools of healthy individuals, illustrating that various putative enterococcal virulence determinants can be also found in strains colonizing the GI tract of healthy individuals⁵⁸.

Cell surface determinants

One group of *E. faecalis* surface proteins that has been extensively characterized is the family of aggregation substance proteins (AS proteins). These proteins are encoded by pheromone-responsive plasmids that often also harbour antibiotic resistance genes. AS proteins cause clumping of *E. faecalis* cells and mediate high-frequency transfer of plasmid DNA in liquid media. These proteins increase *E. faecalis* binding to cultured renal epithelial

cells, survival within polymorphonuclear neutrophils and internalization by intestinal cells⁵⁹, and they affect the pathogenesis of experimental endocarditis (presumably by favouring the formation of large bacterial aggregates on the cardiac valve)^{60,61}. The enterococcal surface proteins Esp and Esp_{fm}, encoded by genes that seem to have been acquired within a pathogenicity island, are commonly found in clinical isolates, are anchored to the cell wall, affect bio-film formation⁶² and have a role in experimental UTI and/or endocarditis models^{63–65}.

Enterococcal MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) are considered to be important elements in the early stages of infection, as they may bind components of the host extracellular matrix. Although many putative adhesins have been described in both *E. faecalis* and *E. faecium*, the most extensively studied are the collagen adhesins Ace in *E. faecalis* and Acm in *E. faecium*. Both are cell wall-anchored adhesins that contain LPXTG-like motifs (as do AS proteins and Esp), have immunoglobulin-like folds⁶⁶ and have considerable similarity with the *S. aureus* collagen adhesin (Cna) (there is a 62 % overall similarity between Acm and Cna)⁶⁷. They have both been shown to affect the pathogenesis of enterococcal infections *in vivo*. *E. faecalis* Ace is a collagen and laminin adhesin that is conditionally expressed after growth in collagen or serum⁶⁸ and binds collagen in a specific manner referred to as the ‘collagen hug’ (REF. 69), in which Ace appears to embrace the collagen molecule after initial docking. An *ace* deletion mutant is substantially attenuated in models of endocarditis⁷⁰ and UTIs^{71,72} compared with the parental strain, and antibodies specific for the collagen-binding domain of Ace protect rats from endocarditis⁷⁰. *E. faecium* Acm binds collagen, and an *acm* deletion mutant is attenuated in experimental endocarditis⁷³. Community-associated strains (isolates from animal and human stools), unlike hospital-associated strains, often have an *acm* pseudogene and rarely express Acm or adhere to collagen. Thus, Acm may have a role in the increased ability of members of the hospital-associated *E. faecium* clade to cause disease⁷³.

Another enterococcal surface protein, ElrA (entero-coccal leucine-rich-repeat-containing protein), is a cell wall-associated protein from the WxL family. An *E. faecalis* *elrA* deletion mutant has attenuated virulence in a mouse peritonitis model and cannot infect macrophages (this is associated with a decrease in the interleukin-6 response)⁷⁴.

Some enterococcal proteins with LPXTG-like motifs and immunoglobulin-like folds are tethered into pili, which are filamentous structures protruding from the cell surface^{75,76}. Similar pili that are present in other Gram-positive organisms have an important role in infection and colonization of host tissues⁷⁷, and typically contain two or three pilin subunits that are encoded in loci which also harbour one or more pilus-specific sortase genes⁷⁵. Following assembly of the pilin subunits by the pilus-specific sortase, a housekeeping sortase then attaches the pilus to the cell wall. Ebp (endocarditis and biofilm-associated pili) pili are ubiquitous in *E. faecalis*; their expression is repressed by the Fsr system⁷⁸ and activated by RnjB, an RNase of the J2 family⁷⁹. Ebp pili are important for biofilm formation and for the pathogenesis of experimental endocarditis and UTIs⁸⁰. Only one other pilus locus, designated biofilm enhancer in *Enterococcus* (*bee*), has been found in *E. faecalis*, but this locus is rare⁸¹, whereas *E. faecium* often harbours four or more putative pilus loci⁸², two of which have been confirmed to produce pili⁷⁶. One of these loci has homology to *E. faecalis* Ebp and also affects biofilm formation and the pathogenesis of mouse UTIs⁸³.

Polysaccharides are important components of the Gram-positive cell surface and have a crucial role in pathogenesis, mediating evasion of phagocytosis by polymorphonuclear neutrophils and stimulating cytokine production⁸⁴. Some *E. faecalis* strains, especially those belonging to hospital-associated clonal clusters, have a capsular polysaccharide locus (*cps*) containing 8–9 genes. Isolates that are designated polysaccharide type A and type B lack *cps*

genes, and it was shown that the opsonic antibodies used to classify type A isolates actually recognize lipoteichoic acid (LTA)⁸⁵. Type C and D polysaccharides mask LTA and confer resistance to complement-mediated opsonophagocytosis^{86,87}.

Enterococcal polysaccharide antigen (Epa), a 37 kDa cell wall antigen, is recognized by sera from most patients with serious *E. faecalis* infections⁸⁸. Epa is a rhamnose-containing polysaccharide, biosynthesis of which is encoded by the *epa* locus (containing 16 genes), and it is postulated to be cell wall associated⁸⁹. Disruption of the *epa* cluster decreases biofilm formation and translocation across an enterocyte monolayer⁹⁰, increases susceptibility to polymorphonuclear neutrophil-mediated killing⁹¹, and causes attenuation in mouse peritonitis and UTI models⁹².

Enterococcal cell membrane glycolipids and LTA⁹³ also appear to be important in pathogenesis. An *E. faecalis* mutant that is deficient in the glycolipid α -diglycosyl diacylglycerol (DGLcDAG) shows reduced adherence to enterocytes and reduced biofilm formation and was cleared faster from the bloodstream of mice than wild type *E. faecalis*⁹⁴.

Other influences on virulence

Non-pheromone-responsive, large transferable plasmids, 150–250 kb in size, are common among *E. faecium* clinical isolates^{11,95,96} and have a role in virulence⁹⁷. Transfer of one of these megaplasmids from a clinical strain (*E. faecium* str. TX16(DO)) to a commensal *E. faecium* strain increases the virulence of the commensal in experimental mouse peritonitis⁹⁷. Similarly, transfer of a megaplasmid from a different *E. faecium* clinical strain increases the ability of a laboratory strain (*E. faecium* str. D344SRF) to colonize the GI tract of mice⁹⁸. The precise role of specific genes carried by these plasmids in virulence or colonization is unclear, but recent evidence suggests that one gene, *hylEfm* (encoding a family 84 glycosyltransferase), which is frequently referred to as a virulence factor, is not the main mediator of the increased virulence that is conferred by the megaplasmids⁹⁹, indicating that other genes on these plasmids may offer a competitive advantage.

Some stress response proteins are also important for virulence. Gls24 is one such enterococcal protein, mediating resistance of *E. faecalis* to bile salts; interestingly, Gls24-specific immune serum protects mice against a lethal *E. faecalis* challenge in the peritonitis model, and a *gls24* mutant is attenuated in experimental endocarditis¹⁰⁰. Similar proteins in *E. faecium* have also been shown to be important in mouse peritonitis¹⁰¹. In addition, *E. faecalis* produces three types of peroxidase that detoxify reactive oxygen species (the mediators of bacterial killing by phagocytes): NADH peroxidase (Npr)^{102,103}, Ahp (an alkyl hydroxyperoxide reductase system) and thiol peroxidase (Tpx). Tpx is the most relevant peroxidase for protection in the phagocyte environment, and a *tpx* deletion mutant is attenuated in mouse peritonitis¹⁰³.

Mechanisms of antibiotic resistance

Enterococci are intrinsically resistant to several antibiotics and also readily accumulate mutations and exogenous genes that confer additional resistance. The acquisition of resistance genes often occurs by conjugation using pheromone-responsive plasmids (encoding the AS proteins mentioned above) and their high-frequency transfer between *E. faecalis* isolates¹⁰⁴, conjugative plasmids with a broad host range, or conjugative transposons with the potential to carry multiple antibiotic resistance genes¹⁰⁵.

The challenges in the treatment of enterococcal infections have been apparent since the early days of penicillin use, in the 1940s, when penicillin monotherapy was noted to be successful for infective endocarditis caused by streptococci¹⁰⁶ and staphylococci, but not

enterococci¹⁰⁷. As opposed to their effect against most streptococci, β -lactam antibiotics such as penicillins are not bactericidal against many strains of enterococci; moreover, tolerance to penicillins (that is, lack of killing despite growth inhibition) can be rapidly elicited in some strains by intermittent exposure to the antibiotics¹⁰⁸. The combination of a penicillin and streptomycin (an aminoglycoside) was empirically found to be curative in patients with enterococcal endocarditis and was shown to produce an synergistic bactericidal effect *in vitro*¹⁰⁹ (that is, a 3 log₁₀ decrease overall and a 2 log₁₀ decrease versus penicillin alone 24 hours after exposure); this antibiotic combination became the standard treatment for enterococcal endocarditis by the 1950s. Many of the modern nosocomial *E. faecium* isolates are resistant to ampicillin and vancomycin, and also have high-level resistance to aminoglycosides (which eliminates the effectivity of antibiotic combinations (synergism)). This is one of the most pressing issues currently facing clinicians in hospitals worldwide, as other therapeutic options are not reliable, have toxic side effects or have not been tested in prospective randomized clinical trials. In this section, we discuss the major mechanisms of antibiotic resistance that adversely affect the treatment of enterococcal infections (FIG. 4).

β -lactam resistance

Penicillin (or ampicillin) alone or with an aminoglycoside was the cornerstone for treatment of enterococcal infections for more than half a century. Ampicillin resistance, which is rare in *E. faecalis*, occurs in ~90 % of modern-day hospital-associated *E. faecium* isolates. *S. aureus* commonly uses β -lactamase to overcome penicillin, but this mechanism is rarely seen in enterococci, although there were outbreak strains of *E. faecalis* in the 1990s¹¹⁰ that produced a β -lactamase identical to the staphylococcal enzyme. The presence of β -lactamase is not a therapeutic challenge, as it is inhibited by β -lactamase inhibitor combinations (such as ampicillin–sulbactam), but it may pose a diagnostic challenge, as strains test positive for resistance only at high inocula. The mechanism responsible for ampicillin resistance in *E. faecium* is the production of PBP5, which has low affinity for the penicillins (and perhaps is overproduced)¹¹¹. The PBP5-encoding gene in ampicillin-resistant strains, *pbp5R*, is found in *E. faecium* isolates in the hospital-associated clade and differs by ~5 % from *pbp5S*, the gene in ampicillin-susceptible strains in the community-associated clade³⁰. In the laboratory, *pbp5R* can transfer between bacterial cells, at times along with a transposon (Tn5382) that carries vancomycin resistance genes¹¹², to *E. faecium* isolates that are more susceptible to ampicillin, probably by a similar mechanism to that used for the transfer of large chromosomal fragments between *E. faecalis* strains³⁸. The crystal structure of PBP5 indicates that specific amino acid differences may be responsible for resistance by interfering with the architecture of the active site or with β -lactam binding^{113,114}. High-level resistance to ampicillin emerged in hospitals in the United States in the 1970s to 1980s¹¹⁵, and it was into this ampicillin-resistant *E. faecium* background that vancomycin resistance first emerged in the United States. Although in Europe vancomycin resistance was initially predominant in ampicillin-susceptible strains that are now recognized as belonging to the community-associated *E. faecium* clade, a similar pattern of emergence of resistance — first to ampicillin and then to vancomycin — has subsequently been observed in hospital-associated *E. faecium* from Europe⁸.

Aminoglycoside resistance

Although enterococci exhibit intrinsic resistance to low to moderate levels of aminoglycosides, the uptake of these highly polar molecules¹¹⁶ can be enhanced by the addition of cell wall-active agents, including penicillins and vancomycin¹⁰⁹.

However, the occurrence of acquired high-level resistance to all available aminoglycosides eliminates the potential for synergistic treatments. High-level resistance to streptomycin results from ribosomal mutations, which usually change the S12 ribosomal protein, or the

acquisition of an aminoglycoside nucleotidyltransferase, ANT(3'')-Ia¹¹⁷ or ANT(6')-Ia¹¹⁸. High-level resistance to gentamicin is usually the consequence of a transposon that encodes the bifunctional aminoglycoside-modifying enzyme AAC(6')-Ie-APH(2'')-Ia, which confers resistance to all commercially available aminoglycosides except streptomycin. Aminoglycoside-modifying enzymes catalyse the covalent modification of amino and hydroxyl groups within the aminoglycoside molecule, markedly decreasing the binding affinity between the antibiotic and the bacterial ribosome¹¹⁹. *E. faecium* is intrinsically resistant to synergistic treatments using tobramycin owing to the ubiquitous AAC(6') (which confers resistance to tobramycin)¹¹⁶, and a 16S ribosomal RNA methyltransferase enzyme, EfmM, has also recently been linked to intrinsic aminoglycoside resistance in this species¹²⁰.

Glycopeptide resistance

In the past, vancomycin was the usual alternative to ampicillin for infections caused by ampicillin-resistant enterococci or in patients with severe allergy to β -lactams. The emergence of high-level resistance to glycopeptides in enterococci in 1986 (REFS 121,122) was surprising, as vancomycin had been used for decades without the emergence of resistance. However, it is now clear that vancomycin resistance is common in nature¹²³. Indeed, the members of several Gram-positive genera, such as *Lactobacillus*, *Leuconostoc* and *Pediococcus*¹²⁴, are inherently resistant to glycopeptides, as these organisms produce peptidoglycan precursors with decreased affinity for vancomycin; a similar resistance is seen in *Enterococcus gallinarum* and *Enterococcus casseliflavus*. However, the actual origin of the genes responsible for high-level vancomycin resistance in enterococci has been linked to soil *Paenibacillus* spp.¹²⁵.

The mechanism of vancomycin resistance has been extensively reviewed previously^{126,127}. Glycopeptide resistance involves two pathways: replacement of the terminal D-Ala of peptidoglycan precursors with D-lactate (D-Lac), which produces high-level resistance, or with D-Ser, which produces low-level resistance; and prevention or destruction of precursors that end in D-Ala-D-Ala by specific D, D-dipeptidases and carboxypeptidases. The D-Lac replacement eliminates one of the five hydrogen bonds that would be established between vancomycin and the D-Ala-D-Ala termini, resulting in an almost 1,000-fold decrease in affinity¹²⁷. Replacement of D-Ala by D-Ser also decreases the binding affinity of glycopeptides for peptidoglycan precursors, although to a lesser degree. Enterococcal strains with induced or constitutively expressed vancomycin resistance operons have reduced fitness *in vitro* and decreased dissemination among mice compared with uninduced isogenic strains¹²⁸.

Resistance to quinupristin-dalfopristin

Quinupristin-dalfopristin was the first drug approved by the US Food and Drug Administration (FDA) for the treatment of VRE infections¹²⁹. This antibiotic is a mix of two semisynthetic streptogramins. Like the macrolides and lincosamides, quinupristin-dalfopristin inhibits protein synthesis by interacting with the 50S ribosomal subunit and produces a synergistic effect by direct interaction of both compounds with a single nucleotide (A2062) at the peptidyltransferase centre, leading to a significant conformational change that results in bactericidal activity¹³⁰. Most *E. faecalis* isolates are resistant to dalfopristin owing to the activity of Lsa, a predicted ATP-binding protein¹³¹. Several resistance mechanisms reduce the activity of quinupristin-dalfopristin against *E. faecium*, including drug modification, inactivation and efflux through virginiamycin acetyltransferase (Vat)(virginia-mycin is another streptogramin mixture that is similar to quinupristin-dalfopristin), virginiamycin B lyase (Vgb) and the ATP-binding cassette protein macrolide-streptogramin resistance protein (MsrC), respectively^{132,133}. The *erm* genes, encoding

enzymes that methylate 23S rRNA and cause resistance to macrolides, lincosamides and quinupristin (the MLS_B phenotype; quinupristin is a streptogramin B antibiotic), result in decreased bactericidal activity of quinupristin–dalfopristin in experimental endocarditis¹²⁹.

Linezolid resistance

Linezolid is the second of the two compounds that are approved by the FDA to treat VRE and is used worldwide. This oxazolidinone is bacteriostatic and inhibits protein synthesis by interfering with the placement of the aminoacyl tRNA at the A site of the bacterial ribosome^{134,135}. Resistance to linezolid is still rare but has been documented in enterococcal outbreaks and even sporadically in patients who have never received the antibiotic. The most common mechanism of resistance found in enterococci involves G2576T mutations in genes encoding domain V of the 23S rRNA (position 2576 refers to the nucleotide position originally assigned to rRNA genes in *Escherichia coli*). This mutation interferes with the positioning of crucial nucleotides in the linezolid-binding site¹³⁴. As *E. faecium* and *E. faecalis* have six and four copies of the rRNA genes, respectively, the levels of resistance correlate with the number of mutated alleles¹³⁵. Intracellular recombination between gene copies also seems to have a role in the amplification of mutated rRNA genes, as bacteria that are deficient in recombinase A exhibit a delay in such amplification^{136,137}. Interestingly, the linezolid resistance that is found in some clinical isolates of staphylococci, mediated by the acquired gene *cfr* (which encodes a methyltransferase that modifies position A2503 of the 23S rRNA), may have been transferred from an *Enterococcus* species¹³⁸.

Daptomycin resistance

Daptomycin, a lipopeptide antibiotic, has potent *in vitro* bactericidal activity against enterococci (including ampicillin- and vancomycin-resistant *E. faecium*) and is commonly used in the United States and other countries to treat VRE infections (although it is not approved by the FDA for this indication). Recently, whole-genome sequencing of a daptomycin-susceptible and a daptomycin-resistant *E. faecalis* clinical isolate recovered from a patient before and after daptomycin therapy, respectively, showed that simultaneous mutations in two genes are sufficient to cause resistance¹³⁹. One gene, mutation of which appears to be essential for resistance to occur, encodes a membrane protein, LiaF, that is predicted to be a member of a three-component regulatory system (LiaFSR) involved in the cell envelope stress-sensing response to antibiotics. The second gene encodes a protein of the glycerophosphoryl diester phosphodiesterase family and probably has a role in metabolism of membrane phospholipids¹³⁹. Comparative analysis of the resistant and susceptible isolates revealed that the resistant mutant has alterations in the ultrastructure of the cell membrane and cell wall, and that daptomycin is less able to depolarize the cell membrane of the resistant isolate than that of the susceptible isolate. *In vitro* selection studies of the sequenced isolate *E. faecalis* str. V583 have suggested a role in daptomycin resistance for genes encoding cardiolipin synthase, a phospholipid synthesis enzyme¹⁴⁰.

Conclusions

The enterococci are perhaps the most striking examples of organisms that, historically, were regarded as second-rate pathogens (when compared with *Staphylococcus aureus*, group A streptococci and pneumococci, for example) but which, over the past few decades, have become one of our most challenging nosocomial problems. This change in status is in part due to the increased use of antibiotics in hospitals worldwide: antibiotics can cause changes in the gut microbiota of patients, and these changes cause subsequent alterations in the local immune system. Enterococci take advantage of this phenomenon and ‘conquer’ the prized niche of the GI tract, and this niche is the primary source of organisms that then cause enterococcal infections. Although hospital-associated clones of *E. faecium* are intrinsically

different in their core genome from strains in the community-associated clade, the malleability of enterococcal genomes has almost certainly contributed to the rise of these organisms as important nosocomial pathogens. Thus, the anticipated advances in enterococcal genomics should include the discovery of important clues about the evolution of enterococci and the delineation of the important differences between community-associated and nosocomial enterococci, as well as differences in the nosocomial clade. *In vivo* screening for the activation of specific genes in different models of infection¹⁴¹ and for the role of small RNAs¹⁴², non-coding RNA and anti-sense RNA¹⁴³ is one of the approaches that may provide clues to understanding these differences.

Within the genus *Enterococcus*, *E. faecium* has emerged as the most therapeutically challenging organism. In this species, many of the differences between hospital- and community-predominant clones arose long before the modern-day hospital existed³⁴. However, acquisition of antibiotic resistance genes, and probably other genes, has escalated in recent years. At least in *E. faecalis*, this acquisition has occurred most significantly in lineages that lack the CRISPR defence system against incoming foreign DNA, and these are the very lineages that contain the majority of hospital-associated strains. The selective advantage conferred by antibiotic resistance can be enormous, particularly in the hospital setting, where there is such widespread antibiotic use.

Because enterococci have shown the ability to develop resistance to essentially every antibiotic used against them, novel approaches are needed. Future studies should further dissect the mechanism by which enterococci can colonize the GI tract and should then focus on ways to reduce or prevent this colonization. Strategies, including immunological approaches, to prevent infection in the face of colonization should be explored. In addition, the elucidation of mechanisms of resistance to the newer anti-enterococcal antibiotics such as daptomycin should yield important insights for the development of future therapeutic options. Additional new strategies, perhaps phage-based or a combined therapeutic–immunological approach, should also be considered.

For the foreseeable future, however, the enterococci will remain an important nosocomial problem. Thus, continued study of enterococcal biology and genetics is warranted and will undoubtedly contribute to our understanding of bacterial evolution, as well as yield insights into the complex interactions between the host and the resident bacterial flora that is a common source of nosocomial infections.

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Glossary

Probiotics	Microorganisms such as bacteria and yeasts that, when ingested, are thought to provide a beneficial effect to the host
Third-generation cephalosporins	β -lactam compounds with broad-spectrum activity against Gram-positive cocci (except enterococci) and many Gram-negative bacteria. These antibiotics include ceftazidime, ceftriaxone, cefotaxime and cefpodoxime

Lipopolysaccharide	An important component of the outer membrane of Gram-negative organisms, composed of a membrane lipid (lipid A) and a polysaccharide chain
Flagellin	A protein that forms the flagellum of Gram-negative bacteria
Toll-like receptors	Receptors that are found on and in eukaryotic cells and recognize molecular patterns which are shared by bacterial pathogens
Lectin	A protein that binds to carbohydrate moieties
Clonal complexes	Groups of bacterial isolates, as derived from multilocus sequence typing (MLST) analysis. A clonal complex usually includes isolates that differ from one another at only one of the seven loci analysed by MLST
Clade	A group of bacterial isolates that are genetically related and probably share a common ancestor
Pheromone-responsive plasmids	Plasmids that are known to respond to a peptide pheromone produced by a recipient cell, which initiates the mating process
Aggregation substance proteins	A family of surface-localized proteins that are encoded by pheromone-responsive plasmids. These proteins mediate binding of donor bacterial cells to recipients cells
LPXTG-like motifs	Specific amino acid sequences (X refers to any amino acid) that are found in surface proteins and are necessary for the specific attachment of the protein to the cell wall peptidoglycan
Immunoglobulin-like folds	Structural domains of immunoglobulins, consisting of two sheets of antiparallel strands that form a sandwich-like structure. These folds are shared by some bacterial surface proteins
Pseudogene	A gene that has lost its ability to be expressed or to be functional
Pili	Hair-like projections that are present on the bacterial surface
Pilin subunits	Proteins that form the pilus
Sortase	An enzyme for which the main function is to attach surface proteins to the cell wall peptidoglycan or to other proteins after recognition of an LPXTG motif
Opsonic	Referring to the ability of a moiety to increase phagocytosis of an invading pathogen
Lipoteichoic acid	An important structure of the cell wall of Gram-positive bacteria. It is composed of cell membrane-anchored teichoic acids (chains of ribitol phosphate)
β-lactam antibiotics	Naturally produced antimicrobials encompassing several classes, including penicillins, cephalosporins, cephamycins, monobactams and carbapenems, all of which contain a β -lactam ring

Streptogramins

Naturally occurring antibiotics that are produced by soil bacteria and inhibit protein synthesis. A mixture of the streptogramins quinupristin and dalbapristin is currently available for clinical use and is approved by the US Food and Drug Administration for the treatment of vancomycin-resistant *Enterococcus faecium* infections

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Figure 1. The crucial role of the gastrointestinal tract in enterococcal infection and spread

Enterococci from the gastrointestinal tract can access the bloodstream by moving across the intestinal lining and passing through the liver. When in the bloodstream, these organisms can reach the heart and then potentially cause infective endocarditis. Faecal contamination of the environment (which can then be a source for colonization of other patients) and of the patient's skin (the main source of infections of the urinary tract and of intravenous catheters) frequently occurs.

Figure 2. The effects of antibiotic administration on the gastrointestinal microbiota and the emergence of vancomycin-resistant enterococci

a. In the absence of antibiotics, mouse intestinal epithelial cells and Paneth cells produce the C-type lectin REGIII γ , which has antimicrobial activity against Gram-positive bacteria (purple). The production of REGIII γ is triggered by the presence of Gram-negative bacteria (pink); their MAMPs (microorganism-associated molecular patterns), such as the outer-membrane lipopolysaccharide (in the intestinal lumen) and flagellin (in subepithelial tissues), are recognized by pattern recognition receptors such as Toll-like receptor 4 (TLR4) and TLR5, respectively. **b.** Antibiotic administration leads to a reduction in the Gram-negative bacteria, which decreases REGIII γ production by intestinal epithelial cells and Paneth cells. **c.** Enterococci take advantage of this reduction in REGIII γ secretion to become the dominant members of the gut microbiota. IL-22, interleukin-22. Figure is modified, with permission, from REF. 144 © (2010) American Society for Clinical Investigation.

Figure 3. Major routes of nosocomial transmission of vancomycin-resistant enterococci

The main risk for colonization and subsequent nosocomial infection with vancomycin-resistant enterococci (VRE) include close physical proximity to patients who are infected or colonized with VRE (or to the rooms of these patients); a long period of hospitalization; hospitalization in long-term facilities, surgical units or intensive-care units; the presence of a urinary catheter; and the administration of multiple courses of antibiotics. Many antibiotics increase the density of VRE organisms in the gastrointestinal tract, which, in turn, facilitates the spread of these organisms through faecal contamination of the hospital environment, including inanimate objects and the hands of health care workers and visitors. Enterococci can survive for long periods on environmental surfaces, including medical equipment, toilets, bed rails and doorknobs, and are tolerant to heat, chlorine and some alcohol preparations. IV, intravenous.

Figure 4. Main mechanisms of enterococcal antibiotic resistance

Enterococci are intrinsically resistant to several antibiotics and can acquire mutations and exogenous genes that confer resistance to additional drugs. The main mechanisms of antibiotic resistance are shown. In *Enterococcus faecium*, resistance to ampicillin occurs through the production of penicillin-binding protein 5 (PBP5), which has low affinity for β -lactams. Enterococci exhibit intrinsic low-level resistance to aminoglycosides such as streptomycin or gentamicin owing to low uptake of these highly polar molecules. High-level resistance results from the acquisition of aminoglycoside-modifying enzymes or, for streptomycin, can result from ribosomal mutations that result in altered target binding. Resistance to the glycopeptide vancomycin occurs through a well-characterized mechanism of reduced vancomycin-binding affinity, involving alterations in the peptidoglycan synthesis pathway. Resistance of *Enterococcus* spp. to the streptogramin quinupristin–dalfopristin (Q–D) involves several pathways, including drug modification (by virginiamycin acetyltransferase (Vat)), drug inactivation (through virginiamycin B lysase (Vgb)) and drug efflux (via the ATP-binding cassette protein macrolide streptogramin resistance protein (MsrC)). Resistance to the oxazolidinone linezolid is rare, but the most common pathway involves mutation in the 23S ribosomal RNA ribosome-binding site. Resistance of *E. faecalis* to the lipopeptide daptomycin has been shown to involve altered interactions with the cell membrane and requires the membrane protein LiaF and enzymes involved in phospholipid metabolism, such as a member of the glycerophosphoryl diester phosphodiesterase family (GdpD) and cardiolipin synthase (Cls).

Table 1Enterococcal determinants that have been shown to cause virulence phenotypes *in vivo*

Determinant	Possible function and role
Secreted factors	
Cyl	<ul style="list-style-type: none"> Increases virulence of <i>E. faecalis</i> after intraperitoneal inoculation in mice⁴³ Role in experimental endophthalmitis¹⁴⁵ Lysis of red blood cells⁴³, retinal cells, polymorphonuclear neutrophils and macrophages
GelE	<ul style="list-style-type: none"> Role in experimental endocarditis^{55,56}, peritonitis⁵² and endophthalmitis⁵⁴ Role in clearing misfolded proteins from the bacterial cell⁶¹ Affects translocation across intestinal epithelial cells (<i>in vitro</i>)⁵¹ Activates autolysin and biofilm formation⁴⁶ Affects adherence to dental roots⁵⁷ Regulated by the Fsr quorum sensing system⁴⁷
Cell surface determinants and their formation	
AS proteins	<ul style="list-style-type: none"> Promote transfer and survival in neutrophils for some plasmids and affect internalization of <i>E. faecalis</i> by epithelial cells⁶¹ Role in <i>E. faecalis</i> experimental endocarditis⁶⁰
Esp (<i>E. faecalis</i>) and Esp _{rim} (<i>E. faecium</i>)	<ul style="list-style-type: none"> Affect biofilm formation⁶² Role in experimental UTIs and/or endocarditis^{63–65}
Ace (<i>E. faecalis</i>) and Acm (<i>E. faecium</i>)	<ul style="list-style-type: none"> Role in experimental endocarditis and UTIs^{70–72} Mediate adherence to collagen (Acm), and to collagen and laminin (Ace)⁶⁸
ElrA	<ul style="list-style-type: none"> Role in experimental peritonitis (<i>E. faecalis</i>)⁷⁴ Affects the ability to infect macrophages⁷⁴
Ebp (<i>E. faecalis</i>) and Ebp _{rim} (<i>E. faecium</i>) proteins	<ul style="list-style-type: none"> Form pili^{75,83} and play a part in experimental endocarditis and/or UTIs^{75,80} Affect biofilm formation^{75,83} Mediate adherence to platelets, fibrinogen and collagen (<i>E. faecalis</i>)¹⁴⁶
SrtA	<ul style="list-style-type: none"> Role in catheter-associated UTIs¹⁴⁷ Important for biofilm formation¹⁴⁷
<i>epa</i> cluster	<ul style="list-style-type: none"> Affect biofilm formation⁸⁸ and translocation across intestinal epithelial cells⁸⁹ Role in experimental peritonitis and UTIs^{89,92} Affect bacterial cell susceptibility to killing by polymorphonuclear neutrophils⁹⁰
DGlcDAG glycolipid	<ul style="list-style-type: none"> Role in experimental bacteraemia⁹⁴ Affects biofilm formation and adherence to intestinal epithelial cells⁹⁴
Other factors	

Determinant	Possible function and role
Megaplasms (E. faecium)	<ul style="list-style-type: none"> Affect colonization of the mouse gastrointestinal tract⁹⁸ Role in experimental peritonitis⁹⁷
Gls24 (E. faecalis), and GlS20 and GlS33 (E. faecium)	<ul style="list-style-type: none"> Role in experimental peritonitis¹⁰¹ and, for GlS24, also in endocarditis¹⁰⁰
Peroxidases such as Tpx (E. faecalis)	<ul style="list-style-type: none"> Role in experimental peritonitis¹⁰³ Protection within the phagocyte environment¹⁰³
MsrAB (E. faecalis)	<ul style="list-style-type: none"> Role in experimental UTIs¹⁴⁸ Affect survival in peritoneal macrophages¹⁴⁸
PerA (E. faecalis)	<ul style="list-style-type: none"> Role in experimental peritonitis¹⁴⁹ Role in biofilm formation, and affects survival within macrophages <i>in vitro</i>
Bop	<ul style="list-style-type: none"> Contributes to prolonged mouse bacteraemia and biofilm formation¹⁵⁰
SigV	<ul style="list-style-type: none"> Role in mouse UTIs and experimental mouse bacteraemia¹⁵¹

Ace, adhesin of collagen from *E. faecalis*; Acn, adhesin of collagen from *E. faecium*; AS, aggregation substance; Bop, biofilm on plastic surfaces; Cyl, haemolysin–cytolysin; DGLcDAG, α -diglycosyl diacylglycerol; Ebp, endocarditis- and biofilm-associated pili; Ebp_{fm}, *E. faecium* Ebp; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; ElrA, enterococcal leucine-rich-repeat-containing protein; *epa*, enterococcal polysaccharide antigen; Esp, enterococcal surface protein; Esp_{fm}, *E. faecium* Esp; GelE, gelatinase; MsrAB, methionine sulfoxide reductase AB; PerA, pathogenicity island-encoded regulator; SigV, extracytoplasmic function σ -factor; SrtA, sortase A; Tpx, thiol peroxidase; UTI, urinary tract infection.