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REVIEW

The Enterococcus: a Model of Adaptability to Its Environment

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SUMMARY The genus *Enterococcus* comprises a ubiquitous group of Gram-positive bacteria that are of great relevance to human health for their role as major causative agents of health care-associated infections. The enterococci are resilient and versatile species able to survive under harsh conditions, making them well adapted to the health care environment. Two species cause the majority of enterococcal infections: *Enterococcus faecalis* and *Enterococcus faecium*. Both species demonstrate intrinsic resistance to common antibiotics, such as virtually all cephalosporins, aminoglycosides, clindamycin, and trimethoprim-sulfamethoxazole. Additionally, a remarkably plastic genome allows these two species to readily acquire resistance to further antibiotics, such as high-level aminoglycoside resistance, high-level ampicillin resistance, and vancomycin resistance, either through mutation or by horizontal transfer of genetic elements conferring resistance determinants.

KEYWORDS *Enterococcus*, antibiotic resistance, horizontal gene transfer

INTRODUCTION

Enterococci are leading causes of health care-associated infections (HAIs) globally, in particular urinary tract, soft tissue, and device-associated infections. Multidrug resistance is common, which prolongs hospitalization time, increases treatment cost, and increases the risk of treatment failure and death. In the past few decades, our knowledge of enterococcal biology, ecology, virulence, and genetics has steadily increased. However, there are still important questions about these pathogens that remain to be solved, in particular how to effectively treat multidrug-resistant strains. In this review, we present a general overview of the genus *Enterococcus*, the clinically

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relevant species, their mechanisms of infection and antibiotic resistance, the state of the art in treatment, and challenges and perspectives for the future.

BASIC MICROBIOLOGY

The enterococci are ubiquitous Gram-positive bacteria that have been isolated from soil, surface waters, and seawater; in association with plants; in fermented food products; as part of the gut microbiota of both vertebrates and invertebrates; and as causative agents of human disease (1–13). They have a low-GC genome content of about 34 to 45%, a genome size ranging from 2.3 to 5.4 Mb with 2,154 to 5,107 predicted genes, and a genus core genome with the number of genes ranging from 605 to 1,037 depending on the data set and criteria used for analysis (14, 15). The enterococcal pangenome is larger and reflects the highly plastic nature of their genomes and particular niche adaptations.

The term "entérocoque" was first coined by Thiercelin in 1899, when he described gut commensal bacteria with the ability to become pathogenic (16). Due to morphological and some biochemical similarities, the enterococci were considered part of the genus *Streptococcus* (17, 18) and classified as group D streptococci until the mid-1980s. Although four separated branches of streptococci were identified, the pyogenic streptococci, the viridans streptococci, the lactic streptococci, and the enterococcus (19), the term "enterococcus" was more considered a placeholder name for Gram-positive cocci isolated from the gut/feces rather than a monophyletic group. As Sherman stated in 1938, "'The enterococcus' as this term is commonly used among bacteriologists, has about as much biological meaning as *the bear*" (20). However, based on a detailed analysis of biochemical and culture characteristics, in 1970, Kalina (21) proposed that the so-called enteric streptococci should be placed in a genus of their own, the *Enterococcus*. It was only in 1984 that the formal proposal of the genus *Enterococcus* became more accepted (22), and it appeared as a properly recognized genus separated from the streptococci in an editorial addendum to the 1986 edition of *Bergey's Manual of Systematic Bacteriology* (23). The genus *Enterococcus* has to date 58 described species with valid publications (according to compiled information from the List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.net/enterococcus.html#r]) (24). The family *Enterococcaceae* was first proposed by Ludwig and collaborators (25) in 2009 based on 16S rRNA gene similarity and originally comprised *Enterococcus*, *Vagococcus*, *Tetragenococcus*, and *Melissococcus*. Other presumptive genera within the *Enterococcaceae* are *Catellicoccus* (26) and *Pilibacter* (27); however, the precise phylogenetic position of *Tetragenococcus*, *Melissococcus*, *Catellicoccus*, and *Pilibacter* is not clear due to the limited number of species in each genus that have been described and sequenced and the observation that *Melissococcus* and at least one species of *Tetragenococcus* may branch within *Enterococcus* (15, 28–30). The *Enterococcaceae* are in the order *Lactobacillales* with other families of medical and economic importance, like the *Lactobacillaceae* and the *Streptococcaceae*, class *Bacilli* in the phylum *Firmicutes*. The number of predicted *Lactobacillales*-specific clusters of orthologous genes that appear to be essential for these bacteria is 567 (31).

SPECIES DIFFERENTIATION AND LABORATORY DIFFERENTIATION

Enterococci are non-spore-forming ovoid bacteria (22) that exist individually or as pairs, chains, or groups. They are chemo-organotrophic facultative anaerobes with homofermentative metabolism, with lactic acid as the predominant end product of carbohydrate fermentation (29).

Different selective media have been tested for the isolation and identification of enterococci; however, there are no definitive biochemical tests to differentiate *Enterococcus* from other Gram-positive catalase-negative cocci. Most enterococci are oxidase and catalase negative, salt tolerant (as high as 6.5%), resistant to 40% bile, esculin hydrolytic, and able to grow in the presence of sodium azide (up to 0.4%). In addition to the above-described characteristics, all described and tested species produce β-glucosidase; leucine arylamidase; acid from the sugars D-fructose, galactose, β-gentiobiose,

glucose, lactose, maltose, D-mannose, ribose, trehalose, cellobiose, and *N*-acetylglucosamine; and the glycosides salicin, methyl β-D-glucoside, amygdalin, and arbutin. In general, enterococci are urease negative and do not produce acid from D-arabinose, erythritol, D- and L-fucose, methyl α-D-xyloside, and L-xylose; these metabolic characteristics have been used in the development of commercial testing kits. Growth occurs at between 10°C and 45°C, with optimal growth for most species at 35°C to 37°C (32). The enterococci are remarkably resistant to desiccation (32). Only two enterococcal species are reported to be mobile: *Enterococcus gallinarum* and *E. casseliflavus*/*E. flavescens* (33–36).

As early as 1919, Orla-Jensen (discussed in reference 37) proposed the separation of *Streptococcus faecalis* and *Streptococcus faecium* into two different species based on the ability of the former to tolerate potassium tellurite and produce black colonies. Additional biochemical tests, such as testing of the ability to reduce tetrazolium salts to the chromogenic formazan in the presence of glucose, were introduced along the way to improve species identification (38–42). A widely used system for classification and differentiation of enterococci was introduced by Lancefield in a seminal paper in 1933 based on serological groups (43). In this paper, the enteric streptococci were part of antigenic group D, and her classification system is still in use to differentiate *Enterococcus* from most *Streptococcus* species.

If grown on horse blood agar, enterococci can be alpha-, beta-, or nonhemolytic and form 1- to 2-mm colonies with a wet appearance (44). Based on their metabolic capabilities, different selective culture media have been developed for the isolation of enterococci; these selective media frequently contain bile salts, sodium azide, antibiotics, and esculin or tetrazolium salts. Not all enterococcal species are able to grow in these selective media, but the most clinically relevant species grow well. Most clinical testing for enterococcal identification includes catalase testing, pyrrolidonyl arylamidase/pyrrolidonyl-aminopeptidase (PYR) testing and a bile esculin hydrolysis test. Commercial kits have been developed to standardize and optimize the detection of enterococci in the clinical setting, all requiring previous isolation and culture of the organisms, potentially delaying diagnosis. Additionally, accurate differentiation between species in species groups is not always achieved based on phenotypic tests only (45).

The identification of enterococci to the species level has clinical relevance due to the antibiotic resistance profiles of the different pathogenic enterococci. Since the introduction of molecular techniques into clinical microbiology laboratories, improved species identification and expedited testing options have been developed; these techniques are also useful for epidemiology and surveillance and in the diagnosis of difficult cases. Molecular diagnosis techniques are gaining popularity; however, in resource-limited regions, they are still not widely in use in the clinical microbiology laboratory. Molecular-based methods have the potential advantages of increased diagnostic accuracy, providing information about antimicrobial resistance, and reduced time and cost compared to traditional cultivation and phenotypic testing.

Among the newer systems for classification and identification of enterococci are matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), nucleic acid amplification tests (NAATs), peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH), and multilocus sequence typing (MLST).

MALDI-TOF MS-based identification is a powerful, fast, and reliable method that is starting to gain traction more broadly for routine detection in clinical microbiology laboratories for species identification (46, 47). The clinical use of MALDI-TOF MS-based methods allows for rapid identification of enterococci directly from blood culture bottles, potentially reducing the time to antimicrobial treatment initiation (48). MALDI-TOF MS has a high sensitivity, being able to identify about 94% of isolates to the species level, including differentiating between closely related species (49, 50); additionally, it could potentially be useful for antibiotic resistance profiling, for instance, for detection of the presence of *van* genes, although it is not yet in use in clinical practice (51, 52).

NAAT methods are based on PCR amplification and subsequent sequencing or

array/hybridization or real-time PCR amplification (53) of one or more genes that are useful for organism identification to the genus or species level and equally important to detect antimicrobial resistance genes. Different genes have been used for diagnostic and phylogenetic purposes. 16S rRNA gene sequencing is commonly used to identify bacterial species and allows discrimination of enterococci to the species level (45, 54–56); however, differentiating from species within a species group, such as the *E. faecium* group, can be less accurate (49, 57, 58). Several other genes have been proposed to help differentiate enterococcal species, such as *ddl* (D-alanine:D-alanine), *atpA* (ATP synthase), *groES* and *groEL*, *sod* (superoxide dismutase), and *tuf* (elongation factor Tu) (58–63), although, to our knowledge, no systematic comparison of the specificities and sensitivities of different genes has been done. Multiplexed real-time PCR permits testing for more than one gene, allowing the simultaneous determination of the species and potential antibiotic resistance genes (64), and genus- and species-specific assays have been developed, aimed at rapid detection (65). PNA-FISH targeting species-specific rRNA allows for rapid detection of the presence of enterococci from blood culture bottles. These tests allow differentiation of *E. faecalis*, *E. faecium*, and other less-common enterococcal species (66). Commercial clinically approved systems have been developed based on the different technologies described above, but a detailed description of commercial testing methods is beyond the scope of this review.

MLST provides strain identification and has been used to study molecular epidemiology and also to study outbreaks (67, 68), largely replacing pulsed-field gel electrophoresis (PFGE) analysis because of higher reproducibility and easier implementation. Recently, a new iteration to improve resolution has been implemented by performing core genome MLST (cgMLST), which expands the number of genes from 7 or so housekeeping genes to up to 1,423 (69) and is more cost-effective to implement than whole-genome sequencing (WGS) and average nucleotide difference analysis (70). In a study comparing MLST versus WGS for 495 clinical *E. faecium* isolates plus 11 reference genomes, the authors found high discrepancy between the two methods, and they mostly attributed these differences to a lack of robustness of MLST due to a high degree of recombination between isolates (71). Bayesian analysis of population structure (BASP) is a method that improves identification of deep-branching lineages and recombination and is more robust than MLST-based studies using DNA sequence or molecular marker data (72).

CLINICALLY SIGNIFICANT SPECIES AND LESS-COMMON SPECIES

Enterococci are considered commensal organisms of the human gastrointestinal tract; however, they can also be pathogenic, mostly linked to HAIs, commonly causing urinary tract infection (UTI), bacteremia, endocarditis, burn and surgical site wound infections, abdomen and biliary tract infections, and infection of catheters and other implanted medical devices. In most surveys, enterococci are the third most common cause of native valve endocarditis, after *Staphylococcus aureus* and viridans streptococci (73, 74). In humans, *E. faecalis* and *E. faecium* are the most abundant enterococcal species. All *Lactobacillales* comprise less than 1% of the gut microbiota in adults with westernized diets (75, 76). In Hadza hunter-gatherers and in a group of rural Papua New Guineans, there seems to be an enrichment for enterococci (77, 78).

MacCallum and Hastings (79) first reported a putative enterococcal infection in 1899, describing a case of endocarditis and offering a detailed description of the isolated bacteria, which they dubbed *Micrococcus zymogenes*. At around the same time, Thiercelin (16) described round commensal enteric bacteria (an entérocoque) capable of causing diarrheal disease and septicemia. Other early reports describe infections caused by *Streptococcus/Enterococcus faecalis* as the causative agent of endocarditis, puerperal fever, wound infections in First World War soldiers, bacteremia, and fever (80–84). Interestingly, the early literature also describes attempts at curing infections prior to the broad introduction of antibiotics by preparing a vaccine from the patient's own fecal contents, which successfully cleared the symptoms (83). The incidence of enterococcal infections has been increasing steadily since the late 1970s (13, 85, 86). In both Europe

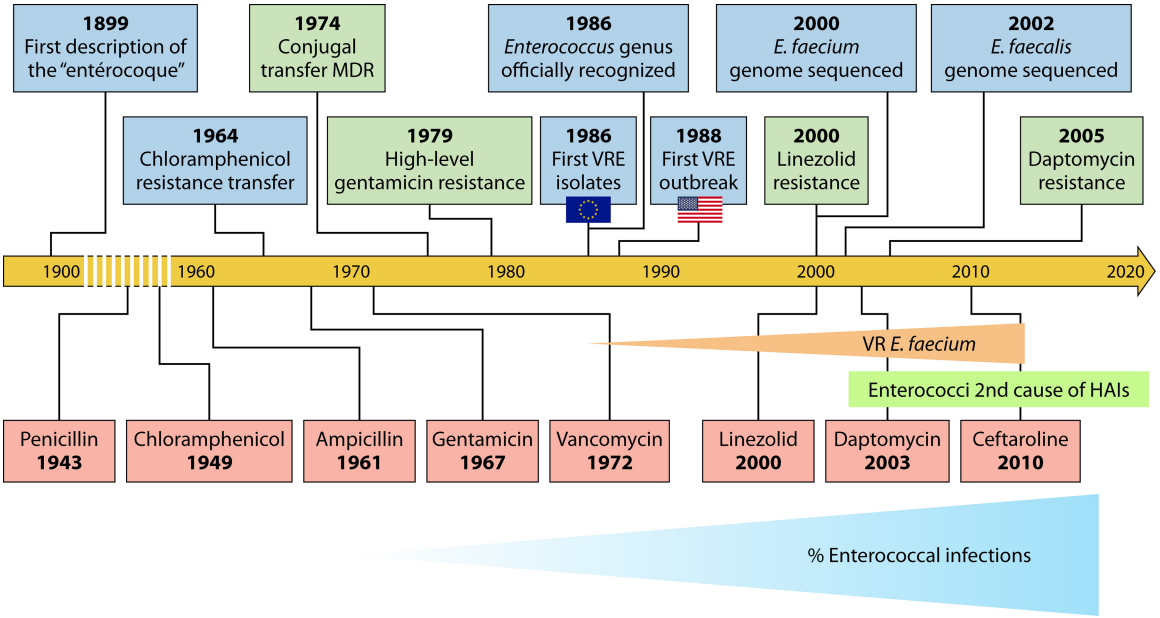

FIG 1 Timeline of relevant events in the history of enterococci as human pathogens (blue rectangles), appearance of antibiotic resistance (green rectangles), and antibiotic clinical debut (red rectangles). The timeline begins in 1899 with the first formal description of putative enterococci, as round enteric bacteria. The timeline then jumps to 1964 to the first description of the transfer of chloramphenicol resistance, only 15 years after its clinical introduction. Similar stories occurred for aminoglycosides and glycopeptides. Since the late 1980s, the prevalence of vancomycin-resistant (VR) *E. faecium* has been increasing, as has the overall percentage of enterococcal HAIs. Resistance to the newest introduced antibiotics, linezolid and daptomycin, emerged very rapidly after their clinical introduction, but the majority of enterococci remain susceptible. MDR, multidrug resistance.

and the United States, enterococci at the genus level are the 2nd most common pathogens associated with HAI, *E. faecalis* was the 5th most frequently isolated organism from catheter-associated urinary tract infections (CAUTIs) and third for central line-associated bloodstream infections (CLABSIs), and *E. faecium* was the 11th and 5th, respectively (87–89).

The success of enterococci in establishing themselves as HAI agents is partly due to their intrinsic resistance to many antimicrobials and their capacity to acquire new resistance traits. The most prevalent species in HAIs is *E. faecalis*, which is more virulent than *E. faecium* but with less-dramatic levels of intrinsic and acquired antimicrobial resistance. Historically, *E. faecalis* has been isolated in about 50.3% of all enterococcal HAIs; however, there is an increasing trend for *E. faecium*-caused infections, mostly associated with the rise of vancomycin- and β-lactam-resistant *E. faecium* strains (90). Roughly 10% of *E. faecalis* isolates are vancomycin resistant, compared to 80% of *E. faecium* isolates (89). Together, *E. faecalis* and *E. faecium* cause about 75% of all typed enterococcal infections (89). A timeline highlighting the major events in the establishment of enterococci as important HAI agents is shown in Fig. 1.

Nontyped enterococci, including nontyped *E. faecium* and *E. faecalis* and all other non-*faecalis* non-*faecium* enterococci (OE), comprise about 24.6% of all enterococcal infections (89); however, the percentage of OE is not reported separately from nontyped putative *E. faecium* and *E. faecalis* infections. The incidence of infections caused by OE has been on the increase; cases of OE bacteremia in U.S. hospitals ranked 10th among HAIs in the period comprising 2011 to 2014 (89), compared to 11th in the period comprising 2009 to 2010 (91). Species such as *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. hirae*, *E. mundtii*, *E. avium*, and *E. raffinosus* have been associated with human infection mostly in people with concurrent hematological malignancies, neutropenia, and previous corticosteroid treatment (92). *E. durans*, *E. hirae*, and *E. mundtii* belong to the *E. faecium* species group (29), suggesting that the capacity to become pathogenic was present in the shared common ancestor of this group. *E. gallinarum* and *E. casseliflavus* have intrinsically

low-level resistance to vancomycin, which could be a potential treatment problem if rates of infections caused by these organisms continue to rise (93, 94). A recent study by Manfredo Vieira and colleagues (95) implicates *E. gallinarum* in the induction of autoantibodies linked to autoimmune disease after translocation from the gut to the liver in mice with autoimmune susceptibility and proposed that a similar mechanism could occur in people with autoimmune diseases such as lupus erythematosus, suggesting a new role for enterococci in human health. *E. pallens*, *E. gilvus*, and *E. raffinosus* belong to the same species groups (14, 15, 29). *E. pallens* has been associated with spontaneous peritonitis in patients with liver cirrhosis and has been isolated from ascites fluid, so far limited to 4 cases reported in Quebec Province, Canada (96, 97). The importance of this organism as a human pathogen is yet to be determined. *E. gilvus* was isolated as part of mixed infections with *E. faecium* and *E. casseliflavus* from the bile of a patient with cholecystitis (96). Because most infections caused by OE occur in severely ill patients with other comorbidities, it is difficult to establish the mortality rate of bacteremia caused by these organisms (98).

VIRULENCE

The enterococci are not highly virulent organisms, and the success of *E. faecalis* and *E. faecium* as pathogens in the hospital setting is primarily related to their survival capabilities in a hostile antimicrobial-rich environment. That said, several traits in both species have been linked with their pathogenic potential and ability to cause disease. These include the ability to evade the immune system; the capacity to attach to host cells, the extracellular matrix (EM), and inert materials, such as a variety of medical devices; and the ability to form biofilms that make them more resistant to antibiotic killing and phagocytic attack (99). Virulence factors are more evident in *E. faecalis*, perhaps explaining its still leading role in enterococcal infections.

Many proteins have been described as part of the virulence repertoire of pathogenic enterococci.

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are surface elements that help enterococci to adhere to host tissues, helping in the initiation of infection (100).

In *E. faecalis*, MSCRAMM genes are found in most strains and are expressed *in vivo* during human infection (101). One of the best-characterized MSCRAMMs is Ace, a collagen-binding protein (102) that enhances early heart valve colonization, suggesting an important role in the early establishment of endocarditis (103).

MSCRAMM genes are enriched in clinical isolates of *E. faecium*, and genes of this family present in the genome were more abundant in endocarditis isolates (104). In *E. faecium*, Acm (a collagen-binding protein) is the best-characterized MSCRAMM. The *acm* gene is primarily present in health care-associated isolates (present in 99% of analyzed isolates), although one study found that it was disrupted by a transposon in commensal isolates, becoming nonfunctional (105).

Pilin gene clusters (PGCs) are present in both *E. faecalis* and *E. faecium* and encode LPxTG-like motif surface proteins that are responsible for the assembly of long filamentous structures extending from the surface, called pili. Like the MSCRAMMs, pili can function as adhesins (106). In *E. faecalis*, the *ebp* (endocarditis- and biofilm-associated pilus) PGC is associated with initial adherence and biofilm formation and has been implicated in the pathogenesis of endocarditis and UTI (107). In *E. faecium*, the role of the pilum is not entirely clear; however, there seem to be differential regulation of the PCG and differential assembly of pilus proteins between clinical isolates and commensal strains (108, 109).

Cytolysin (Cyl) (also called hemolysin), encoded by the $cylL_L$ and $cylL_S$ genes, contributes to virulence in *E. faecalis* infections. Cyl is a secreted two-peptide lytic protein that damages host cells and promotes infection. It also has bacteriocin activity, damaging other Gram-positive organisms (110–112). The cytolysin operon is normally located on mobile elements such as conjugative plasmids or within the pathogenicity

TABLE 1 Antimicrobial resistance in enterococci

Antimicrobial class (agents)	Representative resistance gene(s)/operon(s)	Mechanism of resistance
Aminoglycosides (gentamicin, kanamycin)	*aac-2′-aph-2″-Ie*, *aph-3′-IIIa*	Modification of the aminoglycoside
β-Lactams	*pbp4* (*E. faecalis*), *pbp5* (*E. faecium*)	Reduced affinity for the antibiotic
Chloramphenicol	*cat*	Acetylation of chloramphenicol
Clindamycin	*lsa*(A)	Putative efflux
Daptomycin	*liaFSR*	Alteration in membrane charge and fluidity
Erythromycin	*ermB*	Ribosomal methylation
Fluoroquinolones	*gyrA*, *parC*	Modifications in quinolone resistance-determining region
Glycopeptides	*vanA*, *vanB*, *vanD*, *vanM*	Modified peptidoglycan precursors terminating in D-lactate
	vanC, *vanE*, *vanG*, *vanL*, *vanN*	Modified peptidoglycan precursors terminating in D-serine
Oxazolidinones	rRNA genes	Mutations reducing affinity
	cfr	Methylation of 23S rRNA
Rifampin	*rpoB*	Point mutations reducing affinity
Streptomycin	*ant-6*	Modification of streptomycin
Tetracyclines	*tet*(L)	Efflux
	tet(M)	Ribosomal protection
Tigecycline	*tet*(L), *tet*(M)	Increased expression

island (PAI) and is often found in association with aggregation substance genes (113). Aggregation substance is a pheromone-induced surface protein that plays dual roles in mating pair formation during conjugation and virulence. It is involved in vegetation formation in infective endocarditis, extracellular matrix adherence, and phagocytosis protection, and it potentiates the pathogenic effect of Cyl (113–115).

Another virulence factor that increases the ability of *E. faecalis* to cause disease is gelatinase (GelE), a matrix metalloproteinase that hydrolyzes gelatin, collagen, and other proteins. Gelatinase plays a role in the development of endocarditis (116) and inhibits complement-mediated responses (117). *gelE* is cotranscribed with *sprE*, a gene encoding a serine protease; together, the two genes contribute to virulence (118). The expression of both genes is under the control of the *fsr* locus, a master regulator which also plays a role in biofilm formation, the expression of surface proteins, and metabolism (119). The cell wall-associated enterococcal surface protein (Esp) (120) contributes to cell adhesion in both *E. faecalis* and *E. faecium*, playing a role in urethral colonization (121) and endocarditis (122) and promoting biofilm formation (121, 123, 124). However, by itself, Esp is neither necessary nor sufficient to successfully establish infection and is not present in all clinical isolates. The phosphotransferase system (PTS) genes encode transmembrane proteins that participate in sugar intake. Diversification of the PTS allows enterococci to use a broad variety of sugars as carbohydrate sources and better adapt to changing environments. The PTS can act as part of the general stress response (125), as virulence factors helping the enterococci to colonize and survive within the host (126), and in biofilm and endocarditis development (127).

Genes encoding several of these virulence factors are often colocated in PAIs or mobile elements, facilitating their spread between isolates. PAIs are large elements that can be acquired by horizontal transfer and confer virulence to bacterial pathogens (128, 129). Several in-depth reviews of enterococcal virulence are recommended (130–133).

ANTIMICROBIAL SUSCEPTIBILITY AND INTRINSIC MECHANISMS OF RESISTANCE

E. faecalis and *E. faecium* are characterized by their reduced susceptibility to many agents that are quite active against streptococci and staphylococci. A list of antimicrobial agents to which enterococci are resistant is included in Table 1. Among the β-lactams, they are intrinsically resistant to virtually all cephalosporins (with possible exceptions being ceftaroline and ceftobiprole, which have *in vitro* activity against *E. faecalis*), antistaphylococcal penicillins, and aztreonam (134). *E. faecalis* strains can be susceptible *in vitro* to carbapenems, but there are few clinical data supporting the use of these agents for treatment of human infections. Enterococci are intrinsically susceptible to vancomycin but resistant to clindamycin, trimethoprim-sulfamethoxazole, and clinically achievable concentrations of aminoglycosides. They are intrinsically suscep-

tible to tetracyclines and erythromycin, although acquired resistance to these agents is widespread (except for tigecycline) (135, 136). The newer agents linezolid, tedizolid, daptomycin, televancin, and oritavancin are active against enterococci, and the pristinamycin combination quinupristin-dalfopristin is active against *E. faecium* only. Fluoroquinolones have activity against enterococci, although ciprofloxacin's MICs are borderline for non-urinary-tract infections, and fluoroquinolone resistance is common in clinical *E. faecium* strains (137). In the clinical setting, ampicillin remains the treatment of choice for susceptible strains in patients who can tolerate this agent.

Enterococcal resistance to β-lactams is attributable to the expression of a low-affinity penicillin-binding protein (PBP) designated PBP4 in *E. faecalis* and PBP5 in *E. faecium* (138–140). Strains from which these *pbp* genes have been deleted exhibit reduced MICs for active β-lactams and reductions into the susceptible range for β-lactams that have poor activity against wild-type strains (141, 142). Many enterococcal strains also exhibit tolerance to the bactericidal activity of the active β-lactams, with minimal bactericidal concentrations greatly exceeding MICs (143). This tolerance has clinical significance in the treatment of endocarditis, with cure rates with β-lactam antibiotics alone being approximately 40% (144). The addition of streptomycin or gentamicin to an active β-lactam results in bactericidal synergism *in vitro* and yields clinical cure rates exceeding 70% (145). Experiments performed by Moellering and Weinberg (146) in the 1970s attributed this synergism to increased streptomycin penetration into the cell in the presence of penicillin or vancomycin, implying that the killing activity was provided by the aminoglycoside, once it achieved entry into the cell, facilitated by the cell wall-active agent. More recently, clinical data indicate that outcomes of *E. faecalis* endocarditis treatment are equivalent with combinations of ampicillin, which is active against *E. faecalis*, and ceftriaxone, which is not (147, 148). Although the mechanism for this apparent clinical synergism is not clear at present, it has been postulated that the combination of the two antibiotics inhibits all the *E. faecalis* PBPs more effectively than either antibiotic alone (149).

Resistance to clinically achievable concentrations of aminoglycosides has been attributed to the poor penetration of these agents through the enterococcal cell envelope (146). The reason for this poor penetration is not clear, but it has been postulated that enterococcal metabolism is essentially anaerobic, precluding aminoglycoside transport across the cytoplasmic membrane, which is an oxygen-dependent process. Clindamycin resistance in *E. faecalis* is attributable to the *lsa*(A) gene, which is believed to encode an ABC superfamily of proteins that confers resistance to lincosamides, pleuromutilins, and streptogramin A antibiotics from the cell (150). Resistance to trimethoprim-sulfamethoxazole in enterococci is an *in vivo* phenomenon. *In vitro*, wild-type enterococci appear to be susceptible to this combination, but trimethoprim-sulfamethoxazole is not effective in treating enterococcal infections in animal models. This appears to be due to the capacity of enterococci to absorb folate from the environment, thereby bypassing the steps toward folate synthesis blocked by the combination (151). There are no compelling clinical data on the effectiveness of trimethoprim-sulfamethoxazole in the treatment of human enterococcal infections.

ACQUIRED ANTIMICROBIAL RESISTANCE

Resistance to β-Lactams

As noted above, enterococci are intrinsically resistant to most β-lactams, being susceptible to only a limited number of penicillins (ampicillin, mezlocillin, penicillin, and piperacillin). Resistance to these penicillins is achievable through two mechanisms. The first, and least important, is the production of β-lactamase (152). A number of strains and some outbreak strains of *E. faecalis* that produce β-lactamase have been reported. Molecular analysis shows that in all cases, this β-lactamase is identical to that produced by *S. aureus*, in some cases within genetic regions identical to that of the *S. aureus* β-lactamase transposon Tn*551* (153). The *S. aureus* β-lactamase is a narrow-spectrum enzyme that is active only against the penicillins that happen to have activity against *E. faecalis*. Expression of the β-lactamase in *E. faecalis* differs from that in *S.*

aureus in that β-lactamase transcription is not inducible by exposure to β-lactam agents, and it appears that the enzyme remains membrane bound. The consequence of these two differences is that expression stays at a low level and does not confer significant resistance with a standard inoculum (154). With a high inoculum, however, animal studies suggest that enterococcal β-lactamase production compromises β-lactam therapy but can be counteracted by the addition of a β-lactamase inhibitor (155). Reports of β-lactamase production in strains of *E. faecium* are quite rare, and the strains expressing it have not been extensively analyzed.

High-level penicillin resistance in *E. faecium* is due to the expression of low-affinity PBP5 (142). Some resistant strains have been shown to express increased quantities of PBP5, although this has not been the most frequent mechanism of resistance. The most common mechanism is through a mutation in the *pbp5* gene leading to amino acid substitutions in or near the active site of the enzyme (142, 156, 157). Molecular epidemiological data suggest that highly ampicillin-resistant strains fall into relatively few lineages that have spread widely, largely in hospitals, causing clinical infections and colonization of patients exposed to a variety of antibiotics (158). In many centers, rates of high-level ampicillin resistance in *E. faecium* exceed 70% (89).

Higher-level resistance to penicillins in *E. faecalis* is a much rarer event than in *E. faecium*. In one instance (138), increased expression of low-affinity PBP4 was implicated, but other cases have implicated amino acid changes within the enzyme itself. A recent report showed that reduced susceptibility in *E. faecalis* appeared due to the combination of increased expression of PBP4 (resulting from an adenine deletion upstream of the promoter sequence) and an alanine-to-tyrosine substitution adjacent to the active site (159). A second amino acid substitution was present in the N-terminal region of the protein but did not contribute to resistance. The mutated enzyme had a lower melting temperature, suggesting that it was less stable, offering a possible explanation as to why such mutant enzymes appear to be rare. In this case, the patient from whom the strain was isolated had been exposed to several years of treatment with aminopenicillins for a prosthetic knee infection.

The fact that deletion of PBP4 or PBP5 results in β-lactam susceptibility of *E. faecalis* and *E. faecium*, respectively, indicates that these proteins are required for resistance (141, 142). They are not, however, sufficient for resistance, since other proteins that are required for resistance expression have been found. In *E. faecalis*, the CroRS regulatory locus is required for cephalosporin resistance, as is a serine-threonine eukaryote-like kinase, IreK (also known as Stk) (160, 161). The presence of genes for two of the three *E. faecalis* class A PBPs (*ponA* and *pbpZ*) is also required for resistance to cephalosporins in *E. faecalis* (141). Deletion of the equivalent class A PBPs in *E. faecium* also results in increased cephalosporin susceptibility, but the susceptibility is restricted to certain cephalosporins (cefepime and ceftriaxone) that have a common side chain (141, 162). It is also an unstable phenotype that converts at a high frequency back to cephalosporin resistance, which in some cases is influenced by the *E. faecium* version of Stk (163). Cephalosporin resistance in this instance was also associated with the expression of a protein found associated with PBP5 by affinity chromatography, which has been designated P_5AP (penicillin binding protein 5-associated protein) (163). In an *E. faecium* strain in which *pbp5* was deleted, resistance to ampicillin emerged through the activity of an L,D-transpeptidase insensitive to inhibition by penicillins (but susceptible to carbapenems) (164). There is still much to be learned about how the low-affinity PBPs interact with their substrates and with β-lactam antibiotics and about the combination of cell wall synthesis proteins that leads to resistance in enterococci.

Resistance to Glycopeptides

The glycopeptide antibiotic vancomycin remained virtually universally active against *E. faecalis* and *E. faecium* for nearly three decades after its clinical introduction. In the early 1980s, strains began to emerge, first in Europe and then in the United States, that expressed inducible, high-level resistance to vancomycin and the more recently introduced antibiotic teicoplanin (165, 166). Resistance was attributable to the acquisition of

operons that altered the nature of peptidoglycan precursors, substituting a D-lactate for the terminal D-alanine in the UDP-MurNac pentapeptide (167, 168). In the process of establishing the peptide cross-link essential for cell wall stability, the terminal D-alanine is removed from the chain to provide the energy for the transpeptidation reaction. Vancomycin binds to the terminal D-alanine of the cell wall precursor, preventing PBP access (vancomycin, because of its large size, also interferes somewhat with the adjacent transglycosylation reaction). Vancomycin binds to pentapeptide stems terminating in D-lactate with a roughly 1,000-fold-lower affinity than it does to those terminating in D-alanine and therefore is not an effective inhibitor of cell wall synthesis in these strains.

The first glycopeptide resistance operon that was described was the *vanA* operon (167), and this remains the most commonly encountered operon in the clinical setting. The operon consists of seven genes whose combined purpose is to replace the glycopeptide-susceptible pentapeptide terminating in D-Ala-D-Ala with a glycopeptide-resistant pentadepsipeptide precursor terminating in D-Ala-D-Lac. *vanS* encodes a transmembrane sensor kinase that is involved in detecting glycopeptides in the environment and phosphorylating VanR, whereby VanR is converted from a repressor of operon transcription to an activator (169). VanR regulates 3 downstream genes: *vanH*, *vanA*, and *vanX*. VanH is a dehydrogenase that reduces pyruvate to D-lactate, and VanA is a ligase that binds a D-alanine to the newly formed D-lactate to form a D-Ala-D-Lac depsipeptide (167), which is then ligated to the UDP-MurNAc tripeptide peptidoglycan precursor by the cellular adding enzyme. *vanX* encodes the VanX amidase, whose purpose is to cleave D-Ala-D-Ala, thereby reducing cellular quantities of D-Ala-D-Ala that can be used to create vancomycin-susceptible peptidoglycan precursors (170). Two additional genes that are not essential for glycopeptide resistance expression are included in the operon. *vanY* is a carboxypeptidase that cleaves the terminal D-alanine from cellular pentapeptide precursors, further reducing vancomycin-susceptible precursors (171). The final gene is *vanZ*, which encodes a protein of unknown function that contributes to resistance to the glycopeptide teicoplanin (172).

VanC-type vancomycin resistance operons, first described as intrinsic components of *E. gallinarum* and *E. casseliflavus*, produce peptidoglycan precursors terminating in D-Ala-D-Ser (93, 173). They encode a (serine) racemase (VanT), a D-Ala-D-Ser ligase (VanC), a combined dipeptidase-carboxypeptidase(VanXY), and the products of the regulatory genes *vanR* and *vanS*. The *vanG* operon has an additional carboxypeptidase, an analogue of VanW from the *vanB* operon, and an additional regulatory gene (*vanU*) (174).

There have been nine glycopeptide resistance operons described over the past few decades (Fig. 2). They fall into two general categories: those that replace the terminal D-Ala with a D-lactate (*vanA*, *vanB*, *vanD*, and *vanM*) (175–177) and those that replace the terminal D-Ala with a D-serine (*vanC*, *vanE*, *vanG*, *vanL*, and *vanN*) (94, 178–180). As opposed to the D-Lac-type operons, the operons encoding proteins that result in precursors terminating in D-Ser confer relatively lower levels of resistance to vancomycin but remain susceptible to teicoplanin. The mechanisms of the D-Lac operons all confer resistance to vancomycin and teicoplanin, although the *vanB* operon is not induced by the presence of teicoplanin, so strains in which the induction mechanism is intact will appear susceptible to teicoplanin (181). Clinical experience using this agent to treat VanB-type vancomycin-resistant enterococci (VRE) indicates that treatment failure is common, due to the emergence of strains with constitutive expression of the operon (182). The *vanC* operons confer resistance to vancomycin but not teicoplanin (173).

The *vanA* operon is carried by the Tn*3* family transposon Tn*1546* (167), which can be located on the chromosome or on transferable plasmids. The *vanB* operon is most commonly carried by Tn*5382* (also referred to in some publications as Tn*1549*) (183), a Tn*916* family element that also may be incorporated into the chromosome or a plasmid. The *vanC* operons are intrinsic to *E. casseliflavus* and *E. gallinarum* (94), which are rare causes of human infection (173). The remainder of the operons are found rarely,

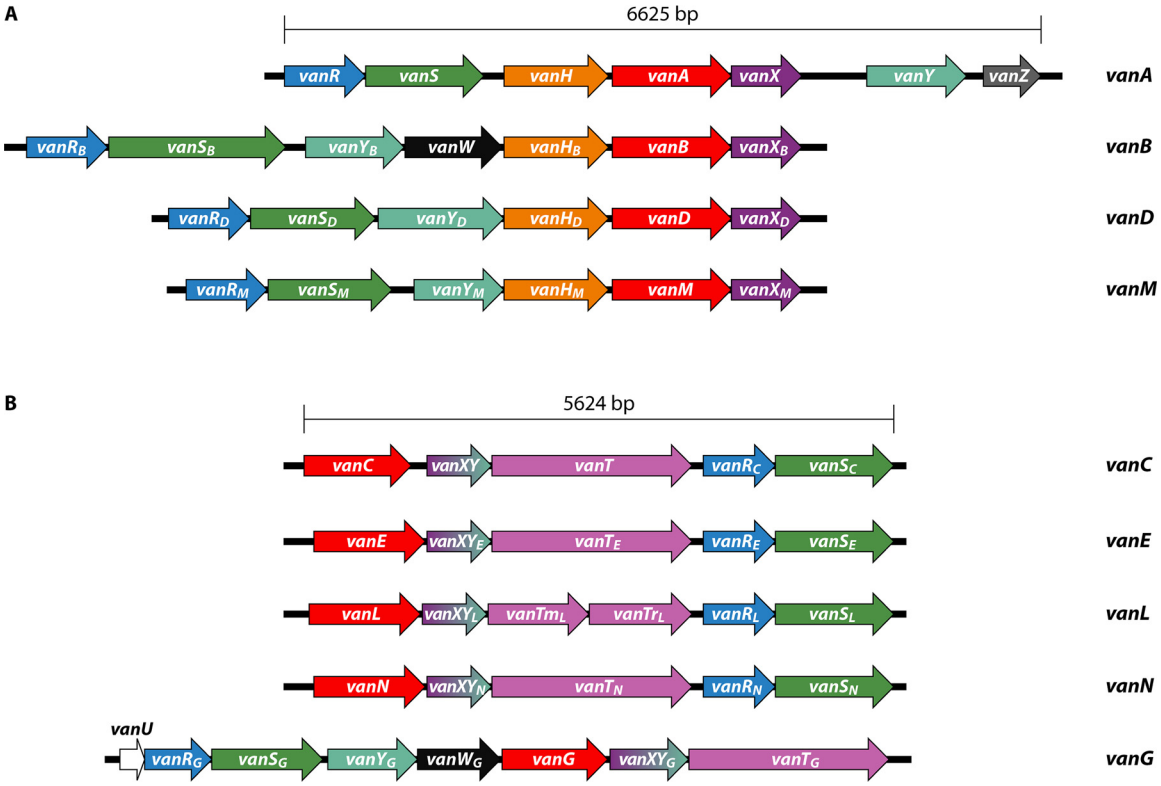

FIG 2 Depictions of known glycopeptide resistance operons. (A) The four glycopeptide resistance operons that yield peptidoglycan precursors terminating in D-Ala-D-Lac. Arrows reflect the directions of transcription and relative sizes of the open reading frames. (B) The five glycopeptide resistance operons that yield peptidoglycan precursors terminating in D-Ala-D-Ser. See the text for descriptions of the open reading frame roles.

although local outbreaks of some of them have been reported. The *vanN* and *vanG* operons have been shown to be transferable, with *vanG* being found within different integrative and conjugative element (ICE)-type elements (184).

Resistance to Aminoglycosides

As noted above, enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides. Aminoglycosides are useful for achieving bactericidal synergism in combination with cell wall-active agents, which is important in the treatment of enterococcal endocarditis (144, 146). Since the clinical utility of these combinations has been recognized, strains that have expressed high levels of resistance to aminoglycosides have emerged (MICs of >500 μg/ml for gentamicin and >2,000 μg/ml for streptomycin) (185). This level of resistance is due to the expression of aminoglycoside-modifying enzymes and negates the synergistic benefit of the combinations in the clinical setting. The gene encoding the most common enzyme conferring resistance to gentamicin (and other aminoglycosides except streptomycin) is *aac-6′-Ie-aph-2″*, classically found within Tn*4001* in staphylococci and other variants in enterococci (186, 187). In some studies, this enzyme has been the exclusive cause of high-level gentamicin resistance in enterococci (188). Expression of a second phosphotransferase [APH(2″)-Ic] has been associated with lower gentamicin MICs (ca. 256 μg/ml) but still negates ampicillin-aminoglycoside synergism. Such isolates may not be detected by clinical microbiology laboratories using concentrations of 500 or 1,000 μg/ml to screen for high-level resistance (189). Resistance to streptomycin in enterococci is most commonly encoded by the *ant-6* gene (190). Very high levels of streptomycin resistance have also been attributed to ribosomal mutations (185). Finally, intrinsic resistance to kanamycin and tobramycin in *E. faecium* is attributable to chromosomally encoded AAC(6′)-Ii (191).

Resistance to Fluoroquinolones

Ciprofloxacin and levofloxacin have marginal activity against enterococci, and their use is restricted to the treatment of urinary tract infections due to susceptible strains. Moxifloxacin is more potent against Gram-positive bacteria than the other two but still exhibits only intermediate activity versus enterococci (192). High-level resistant strains have been shown to contain mutations in both *gyrA* and *parC* (193, 194). Some strains have mutations in only *parC*, suggesting that this topoisomerase may be the primary target of fluoroquinolones in enterococci. There has been suggestion in some studies that efflux pumps are also involved in enterococcal fluoroquinolone resistance, but specific efflux pumps have not been identified (195).

Resistance to Linezolid

Linezolid remains broadly active against both *E. faecalis* and *E. faecium* (196). Resistance frequently occurs through mutations in the rRNA genes. *E. faecium* has six such ribosomal genes, while *E. faecalis* has four, and the level of resistance expressed depends upon the number of these genes that contain the relevant mutations (197). Once a single such mutation occurs, continued selective pressure by linezolid has been associated with "gene conversion," in which further genes acquire the same mutation through homologous recombination with the mutated gene. Conversely, if there remains a single such wild-type gene, then gene conversion can lead to restoration of susceptibility in the absence of antibiotics (198), suggesting that there is some selective disadvantage to these mutations in the absence of selective pressure. Resistance due to changes in ribosomal proteins L3, L4, and L22 appears to be extremely rare.

Enterococci can also develop resistance to linezolid through acquisition of the *cfr* or *cfr*(B) gene (199), which encodes a methyltransferase that modifies A2503 in bacterial 23S rRNA. This enzyme confers resistance to a variety of antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, as well as decreased susceptibility to the 16-membered macrolides spiramycin and josamycin. Cfr is commonly plasmid encoded and transferable and has been associated with outbreaks of linezolid resistance in a variety of Gram-positive species (200). Finally, plasmid-mediated resistance has also been attributed to the acquisition of *optRA*, which encodes a putative ABC transporter (201).

Resistance to Daptomycin

Daptomycin is a cyclic lipopeptide that acts by interacting with the cytoplasmic membrane in the presence of physiological concentrations of calcium, resulting in a variety of alterations in cell membrane characteristics. It is a cationic peptide whose first attraction to the cell membrane is through its interaction with phosphatidylglycerol. In the presence of physiological concentrations of calcium, daptomycin aggregates and then inserts into the membrane. This membrane insertion is followed by the transition of phospholipids and daptomycin to the inner leaflet of the membrane. The result includes ion leakage, which may result from daptomycin "pores" in the membrane or through a "lipid extraction effect," whereby lipids aggregate in areas on the membrane surface and are then extracted. Temporary water channels may be formed by this extraction, with associated ion leakage.

Resistance to daptomycin occurs through a variety of mutations that have different effects depending on the species. In *E. faecalis*, resistance is associated with a movement of membrane phospholipids away from the septum, which may divert daptomycin from the septum. In *E. faecium*, resistance is associated with repulsion of daptomycin from the cell membrane due to changes in membrane phospholipids, similar to that seen in resistant strains of *S. aureus* (202). Mutations resulting in daptomycin resistance are commonly identified in the *liaFSR* operon, which encodes a 3-component regulatory pathway involved in the response to cell membrane stress. Daptomycin-resistant *E. faecium* strains that have mutations in the *liaFSR* system also exhibit synergism between ampicillin and daptomycin (203).

The clinical importance of this synergism remains to be established. Although overall rates of daptomycin resistance remain low, the risk of acquiring daptomycin resistance during therapy is substantial.

Resistance to Tetracyclines

Tetracycline resistance in enterococci is quite prevalent and frequently mediated by a ribosomal protection mechanism mediated by *tet*(M), which is most often carried by conjugative transposons (CTns) related to Tn*916* (204, 205). Efflux-mediated resistance mechanisms encoded by genes such as *tet*(L) are also present in enterococci (205). Tigecycline is a minocycline derivative that is broadly active because it is not susceptible to most tetracycline resistance mechanisms, including Tet(M) and Tet(L). Despite this characteristic, *E. faecium* strains have been reported in which resistance to tigecycline has been tentatively attributed to the overexpression of plasmid-mediated *tet*(L) and *tet*(M) genes (206).

GENOME PLASTICITY

As a major cause of hospital-acquired infections, enterococci have become an important problem in clinical practice. Their abilities to survive in the environment and tolerate disinfectants, their intrinsic antimicrobial resistance, and their remarkable genome plasticity have helped to establish these organisms as frequent inhabitants of hospitals and other health care facilities. The majority of clinical isolates of both *E. faecalis* and *E. faecium* generally lack the adaptive immunity CRISPR-Cas loci (207), suggesting that the selective pressure faced by these organisms induced a trade-off of losing some protection from potentially harmful invading DNA versus gaining the ability to rapidly evolve new traits. Indeed, it has been shown that in strains with functional CRISPR-Cas loci and the innate immunity restriction-modification system, there was a 4-log reduction in the acquisition of pheromone-responsive plasmids (208). Horizontal gene transfer has played a key role in the evolution and success of clinical isolates of *E. faecalis* and *E. faecium*.

The most frequently reported mechanism for foreign DNA acquisition in enterococci is via conjugation, with less information about the role of phage-mediated transduction, although there is evidence that phages could be another important mechanism used by enterococci to share genes even between different species (209, 210). Contrary to streptococci, natural transformation has never been observed (211).

Conjugative Transposable Elements

Conjugation is the process of genetic material transfer from a donor to a recipient cell involving complex machineries encoded by mobilization (MOB) genes and mating pair formation (MPF) genes (212). Conjugation occurs via conjugative plasmids and conjugative transposons (or integrative conjugative elements). Conjugative plasmids encode the proteins required for their transfer from a donor to a recipient cell, during conjugation. The first report of conjugative plasmid transfer in the enterococci came from the observation of multiple-antibiotic-resistance transfer in *E. faecalis* by Jacob and Hobbs (213). Most clinical isolates carry plasmids and transposable elements that commonly encode antibiotic resistance factors, virulence factors, and bacteriocins (210, 211). Plasmids can harbor transposons that are capable of cotransfer and integration in the chromosome by site-specific recombination or by homologous recombination. Ten different plasmid families have been described in enterococci based on their replication initiation genes, but this might be only a small fraction of the actual diversity of enterococcal plasmids (214). Different types of plasmids are found in the two most relevant clinical species. In *E. faecalis*, pheromone-responsive plasmids (PRPs) are widely distributed and are a major source of antimicrobial resistance transfer, having high efficiency, with transfer rates of about 10^{-1} transconjugant cells per donor (215). PRPs have a narrow host range; the pheromones that induce plasmid transfer are heptapeptides or octapeptides chromosomally encoded by lipoprotein genes (derived from the signal peptide) and are released into the medium by the future recipient cell, which

does not carry a PRP. PRPs encode specific receptors for a given pheromone; the formation of a mating pair is mediated by plasmid-encoded aggregation substance, which facilitates donor-recipient contact and is also a virulence factor, as discussed above (216–218). PRPs have a complex regulation (for excellent reviews, see references 219 and 220). Two of the best-studied families of PRPs in *E. faecalis* are pCF10 and pAD1, both of which have clinical relevance. pCF10 plasmids are mostly vehicles for antibiotic resistance genes (215, 219), whereas pAD1 plasmids carry cytolysin, bacteriocins, hemolysins, and UV light resistance (218, 220–222). Remarkably, PRPs are also able to mobilize large chromosomal regions (up to 857 kb) via the formation of a plasmid-chromosome cointegrate (223). Non-pheromone-responsive plasmids (NPRPs) conferring resistance to macrolides, aminoglycosides, and glycopeptides also occur in *E. faecalis* and can coexist with PRPs; these plasmids show a broader host range than PRPs (224, 225). Hybrid plasmids, derived from multiple plasmid recombination events, are a potential problem in the spread of multidrug resistance. pRE25 was identified from an *E. faecalis* food isolate, and it carries resistance to 12 antimicrobials and has a broad host range (226). It was subsequently determined that it was widespread in *E. faecium* isolates (227). Inc18-PRP hybrid plasmids are documented as disseminators of *vanA* resistance between *E. faecalis* strains (228).

In *E. faecium*, there is no evidence that a system such as the PRP of *E. faecalis* is largely used. There are a few reports about PRP-like systems (229, 230), but newer literature has not provided strong evidence for the widespread use of this mechanism in *E. faecium* strains. Transfer of large regions of the chromosome has also been observed in *E. faecium*, although the mechanism is different from what has been observed in *E. faecalis* because it is not mediated by PRPs (231–233). Interestingly, the *pbp5* gene has been shown to be transferable as part of large chromosomal regions, and *pbp5* horizontal gene transfer might be relevant in the acquisition of β-lactam resistance in clinical strains (233, 234). Two of the most prevalent plasmid types are the Inc18 group and the pRUM family (227). These plasmids commonly use as a maintenance mechanism a toxin-antitoxin system that ensures plasmid survival even in the absence of antibiotic selection (235). pRUM-like plasmids have a narrow host range, mostly confined to the *E. faecium* species complex (236); carry resistance to erythromycin, chloramphenicol, streptomycin, and streptothricin; and can carry *vanA* resistance (218, 228). Inc18 plasmids such as pAMβ1 and mosaic plasmids such as Inc18/pRUM show a high degree of shuffling, and they are a frequent finding in clinical isolates but are also present in sewage and animal isolates and are able to disseminate *vanA* glycopeptide resistance (228, 237).

Transposable elements constitute the majority of mobile genetic elements (MGEs) present in enterococcal genomes (Fig. 3) (238–240). CTns have a broad host range and can cross between different species and genera and even transfer between Gram-positive and Gram-negative bacteria. CTns are mobile elements that possess the genetic information to mediate their own transfer within cells and between cells and are also able to comobilize other plasmids, transposons, and large chromosomal fragments and induce chromosomal deletions by excision (231, 241, 242). The first conjugative transposon known to carry antibiotic resistance was identified in *E. faecalis* in 1981 by Clewell and colleagues (224, 243). The authors described a chromosomally located element named Tn*916* that carried tetracycline resistance and was able to mobilize via transposition to either the chromosome of a recipient cell or a conjugative plasmid. Incorporation of the transposon into a conjugative plasmid increases its frequency of transfer. Conjugative transposons can also facilitate the transfer of chromosomal genes even in the absence of the transfer of the transposon itself (204). After the description of Tn*916*, other conjugative transposons have been described in enterococci, mostly associated with resistance to macrolide-lincosamide-streptogramin B (MLS_B) and, importantly, with glycopeptide resistance (*vanB2* type) (235).

Insertion Sequence Elements and Tn*3*-Like Transposons

Insertion sequences (ISs) are the most basic transposable elements, carrying only

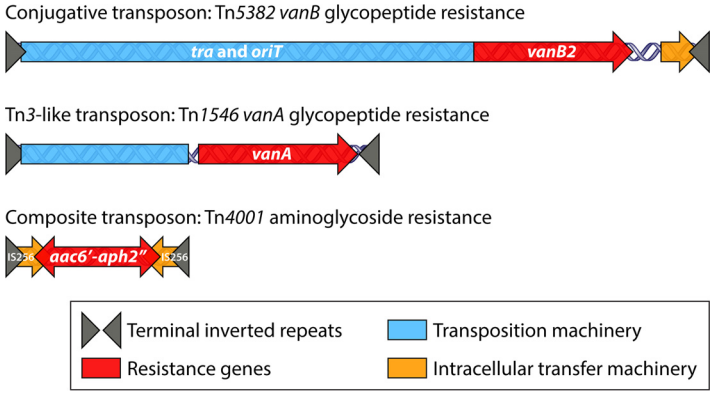

FIG 3 Transposable elements in enterococci. The cartoon depicts the three kinds of transposable elements found in enterococci: conjugative transposons, such as Tn*5382* carrying the *vanB2* operon; insertion sequence elements (IS) in the Tn*3* family, such as Tn*1546* carrying *vanA* resistance; and composite transposons, such as Tn*4001*, for high-level aminoglycoside resistance. These three types of transposable elements can exist either in the chromosome or in plasmids as part of larger mobilizable elements.

information necessary for their own transposition. However, ISs can form composite transposons by flanking resistance or pathogenesis genes, moving between replicons by a replicative transposition mechanism. This kind of transposable elements has been linked to high-level gentamicin resistance, such as Tn*5281* or (in one instance) the *vanB1* glycopeptide resistance element Tn*1547* (244–246). Tn*3*-like transposons move intracellularly within or between replicons (i.e., chromosome to chromosome or chromosome to plasmid) via a replicative mechanism mediated by a transposase and a resolvase. Tn*3*-like transposons mostly reside within conjugative plasmids and are associated with MLS_B resistance and with high-level glycopeptide resistance (*vanA* type) (167, 247).

Reports of antibiotic resistance transfer between strains of enterococci go back as early as 1964 for chloramphenicol resistance (248). Conjugal plasmid transfer of multiple antibiotic resistance determinants has been documented in enterococci since the 1970s (213, 249). In both *E. faecalis* and *E. faecium*, vancomycin resistance is mostly disseminated by non-pheromone-responsive plasmids; these plasmids can carry conjugative transposons but also nonconjugative transposons, such as the vancomycin resistance element Tn*1546* hosting the *vanA* operon (250, 251). The first report of a vancomycin-resistant enterococcal outbreak appeared in 1988 and reported VRE recovered from patients in England since 1986 (both *E. faecalis* and *E. faecium*) (252). *E. faecalis* strain V583 was the first vancomycin-resistant strain reported from the United States (253).

POPULATION BIOLOGY

MLST schemes have been used extensively to understand the epidemiology and population structure of the two major enterococcal pathogens (67, 68) More recently, WGS methods have also been incorporated, and we have a fairly good picture about how these organisms emerged as important human pathogens and the challenges that they present.

E. faecalis strains have limited phylogenetic diversity in their core genome, with average nucleotide identity (ANI) values of 97.7% to 99.5%; in contrast, the shared gene content of these strains is more variable (70.95% to 96.5%) (254). This variability is due to genome size variation attributed to gains in genetic material via horizontal gene transfer; up to 25% of the genome in *E. faecalis* strains can comprise mobile elements acquired by horizontal gene transfer events, showing an even greater degree of recombination than *E. faecium* (223, 240). The genome sizes of different strains reflect this genome plasticity, with a probable minimal genome size of 2.74 Mb and genome sizes as large as 3.36 Mb (254). The phylogeny of *E. faecalis* does not show a significant

association of strains based on their origin (hospital isolates, strains able to colonize hospitalized and nonhospitalized people, and animal isolates) and does not have a clear clade structure (72), although certain clonal complexes (CCs), such as CC2, CC16, and CC87, are more associated with multidrug-resistant strains and are enriched for mobile elements, and CC2 and CC87 are almost exclusively identified from HAIs (255). Clinical isolates tend to have larger genome sizes and carry more exogenously acquired DNA, such as transposons, ISs, plasmids, and phages, than commensal strains. *E. faecalis* clinical isolates are enriched for a PAI of approximately 150 kb, which harbors virulence factors and other traits that contribute to better adaptation to the host and contains different types of mobile elements. The *E. faecalis* PAI shows heterogeneity between isolates, including within the same genetic lineage, which indicates that different regions from the PAI can be mobilized independently (256–258). The differences observed in gene content and the prevalence of the PAI in clinical strains suggest the evolution of niche adaptation, suggesting that in the future, clearly separated subpopulations of *E. faecalis* could appear (259). Raven and colleagues (260) found limited international dissemination of *E. faecalis*, with local clonal expansion of dominant lineages.

In contrast to *E. faecalis*, the population structure of *E. faecium* is more complex and shows a clearer separation between clinical and commensal isolates (261, 262). *E. faecium* forms two distinct clades: clade A, which mostly comprises isolates from animal, environmental (clade A2), and clinical (clade A1) origins, and clade B, which mostly comprises isolates obtained from nonhospitalized people (commensal) (254, 261, 263). The ANI in the core genome between clades A and B ranges between 93.9 and 96% (239, 254). The accepted cutoff for bacterial species designation is an ANI of $>$94% (264). *E. faecium* clades A and B could be the result of a speciation process, which in the future will lead to two separate species, but there is still gene flow between the two groups, as evidenced by the finding of hybrid clade AB isolates (239, 263). The lack of competition between two populations is also a factor to determine if those two populations comprise two different species (265). Clade A1 isolates tend to have larger genomes that are enriched in mobile elements, importantly related to antibiotic resistance and carbohydrate utilization (263). Recombination plays a predominant role over mutation for the diversification of the species (266). Up to 38% of the genome can be of foreign origin (267). Clinical isolates have a larger genome than commensal strains and animal isolates (263). Interestingly, clade A1 strains have gained predominance in the clinical setting only since the 1980s. Earlier isolates do not cluster with clade A1 (158), perhaps due to their increased evolvability and capacity to gain new traits.

The split between clades A1 and A2 has been calculated to have occurred roughly 80 $\pm$ 30 years ago (263), probably several years before antibiotics started to be broadly used in human health and agriculture but well within the "antibiotic era." A recent study with a large data set (495 isolates) challenges the subdivision of clade A into the A1 and A2 subgroups, instead suggesting a clonal expansion of clade A strains in the clinical setting (71); the original description of clades A1 and A2 was performed using a data set of 58 isolates, which perhaps explains the discrepancies. The same authors (71) also noticed that in their data set, about the same proportions of clade B isolates were recovered from the community setting as from the health care setting, in accordance with what was reported by Lebreton et al. (263), suggesting that clade B strains are also part of the HAI pool. Studies with larger data sets and broader geographic and ecological sampling would help better our understanding of the *E. faecium* population structure.

Using *Escherichia coli* and *Bacillus anthracis* mutation rates to calculate the divergence time of human commensal strains and clinical isolates, Galloway-Peña and collaborators determined that the divergence of the two clades occurred about 1 million to 300,000 years ago (261), Interestingly, the smaller time estimate coincides with new data pushing back the evolutionary history of our species, *Homo sapiens*, to about 300,000 years ago (268), perhaps showing the split of a human-

associated clade as early as the dawn of our species. In another study based on the frequency of mutations to fosfomycin resistance, the divergence between the commensal clade (B) and the clinical and animal clade (A) was dated at 3,000 years ago, significantly later than the previous estimate (263). In this work, the authors proposed that the split between the animal clade and the human commensal clade coincided with an "increasing insulation between the flora of humans and animals, which likely stemmed from increased urbanization, increased domestication of animals providing restricted and specialized diets" (263). However, this interpretation is somewhat problematic because even if it is true that 3,000 years ago humans were establishing large urban centers, there was little insulation of animals and humans, and domestication of major animal groups occurred earlier and was not a one-time event; moreover, it occurred independently in different geographic areas and along a large time span and included phylogenetically unrelated species (269, 270). For many domestic species, bidirectional gene flow between the domestic species and their wild-type counterparts existed for a long time (271). Moreover, 3,000 years ago, humans and their domestic animals were already populating the entire globe, except for Antarctica, implying that the separation of clades A and B occurred in a very specific geographic place in the world and that later clade B isolates were introduced to other human populations worldwide and clade A isolates were introduced to their domestic animals. Although it is still not completely clear what drove *E. faecium* into its actual population structure, it is clear that there are two well-differentiated populations (clades A and B) occupying mostly nonoverlapping niches.

A wealth of information has been accumulated in the past two decades about the population biology of *E. faecalis* and *E. faecium*, but little is known about the population structure of the non-*faecium* non-*faecalis* enterococci.

TREATMENT OF ENTEROCOCCAL INFECTIONS

The cornerstones of antimicrobial therapy of enterococcal infections have been those β-lactams that demonstrate *in vitro* activity (predominantly ampicillin but also penicillin and piperacillin) and vancomycin. Therapy with a single such agent is generally adequate for routine infections for which bactericidal therapy is not required (skin and soft tissue infections, urinary tract infections, surgically drained intra-abdominal infections, and intravenous [i.v.] line-associated bloodstream infections). For those infections for which bactericidal therapy is optimal (endocarditis, osteomyelitis, and meningitis), traditional therapy has included an active β-lactam or vancomycin in combination with either streptomycin or gentamicin. Cure rates for enterococcal endocarditis predating the use of combination therapy were approximately 40% with penicillin alone (144). Combining penicillin with streptomycin elevated cure rates to 70% or higher (144, 145), and while there are no randomized controlled trials to reliably define cure rates, most studies using combinations report cure rates exceeding 70% (272).

The use of aminoglycosides to synergize with cell wall-active agents in the treatment of endocarditis was followed by the emergence of strains expressing aminoglycoside-modifying enzymes that resulted in high-level resistance to streptomycin or gentamicin and negated the *in vitro* synergism observed against strains that do not express these enzymes (185). Cure rates for monomicrobial therapy of endocarditis caused by strains expressing high-level aminoglycoside resistance appear to approximate the dismal results observed before synergistic therapy was used, necessitating alternative treatment strategies (272).

Mainardi and colleagues (149) first reported *in vitro* synergism between ampicillin and cefotaxime against *E. faecalis*, showing that *in vitro* susceptibility to either agent was enhanced in the presence of the other. They hypothesized that this synergism was due to the more complete inhibition of all the enterococcal PBPs by the combination of agents. These findings were later supported by Gavaldà and colleagues (273), whose animal studies showed similar synergisms between ampi-

cillin and ceftriaxone. Subsequent human studies confirmed the effect of the combination of ampicillin and ceftriaxone against ampicillin-susceptible *E. faecalis*, whether or not the causative strains expressed high-level aminoglycoside resistance (147, 148, 274, 275). It is important to recognize that these studies are observational and not randomized, like all the data supporting the use of penicillin-aminoglycoside combinations. These results have led to a change in the consensus recommendations from a range of societies, including the American Heart Association and the Infectious Diseases Society of America, for the treatment of endocarditis (276), suggesting that ampicillin-ceftriaxone combination therapy is a reasonable treatment option for endocarditis due to high-level aminoglycoside-resistant strains and as a reasonable alternative for strains without high-level aminoglycoside resistance, for example, in patients with compromised renal function for whom the risks of renal damage from the aminoglycoside are significant.

The optimal duration of therapy for enterococcal endocarditis is 4 to 6 weeks, with no studies available to help distinguish between these two durations. Olaison and colleagues (277) reported a 78-patient study suggesting that the aminoglycoside component of the combination regimen could be discontinued after 15 days without a change in the cure rate but with beneficial effects for renal function. Retrospective data also suggest that one can achieve cure rates comparable to those for native valve disease in enterococcal prosthetic valve endocarditis caused by susceptible strains (272).

More recently, an impressive Danish study (278) showed that a regimen consisting of 17 days of intravenous treatment for endocarditis caused by one of four species (*S. aureus*, streptococci, *E. faecalis*, or coagulase-negative staphylococci) followed by a roughly equivalent number of days of active oral therapy was noninferior to a full course of i.v. therapy. A total of 97 patients with *E. faecalis* endocarditis (39 with prosthetic valve endocarditis) were included in that study, with results being essentially equivalent to the overall results for the combined endpoint and for each of the component endpoints. Caveats include that the patients had to be clinically stable at the time of randomization, that valve replacement surgery prior to a switch to oral therapy was permitted, and that the study was not blind. This study has the potential to have a major impact on costs for endocarditis patients whose condition has been stabilized after an initial 2 weeks of intravenous therapy or after surgical repair of an infected valve.

Of course, there are patients who are unable to tolerate β-lactam antibiotics or who are infected with *E. faecium* strains that are resistant to all β-lactams and vancomycin and produce aminoglycoside-modifying enzymes. In contrast to the 1990s, when there were no effective therapies for highly resistant strains, we now have several alternatives with *in vitro* activity against resistant strains. The first of these to become clinically available was the pristinamycin combination quinupristin-dalfopristin. This combination was synergistically active versus *E. faecium* strains lacking the *erm* macrolide resistance gene (but not *E. faecalis*). Although it was shown to be an effective therapy in the treatment of vancomycin-resistant enterococcal infections, its use was associated with vein inflammation and significant myalgias (279). Linezolid, an oxazolidinone antibiotic available in both i.v. and oral forms, was next licensed and has proven to be consistently active *in vitro* against resistant enterococci. Limitations on its use include bone marrow suppression when administered for more than 2 weeks (280) and an increased risk of serotonin syndrome occurring in patients being treated with selective serotonin reuptake inhibitors (SSRIs) (281). It is also strictly bacteriostatic, although it has been shown to be as effective in the treatment of bacteremias as more-bactericidal agents (282), and there have been instances of success in treating enterococcal endocarditis (283).

Daptomycin is a cyclic lipopeptide approved in 2003 (284). It is bactericidal against many enterococcal strains. Originally approved for a dose of 4 mg/kg of body weight/day, pharmacodynamic analyses indicated that higher doses would be needed for many strains (285). At present, physicians are using up to 8 to 10 mg/kg per day. One

recent study (282) comparing daptomycin to linezolid for the treatment of enterococcal bacteremia showed linezolid to be superior to daptomycin at a dose of 6 mg/kg/day or lower but that the two regimens were equivalent when daptomycin was administered in a dose of >6 mg/kg/day.

Some *E. faecium* strains with higher MICs of daptomycin exhibit reduced MICs when exposed to a combination of daptomycin and a β-lactam antibiotic (to which the strains are resistant). The mechanism(s) underlying this apparent synergism remains unclear, although in some instances, greater binding of daptomycin to the cell membrane was seen in the presence of ampicillin (286). Other data suggest that such synergism occurs in strains with mutations of the *liaFSR* locus (203). Compelling clinical data to support the improved efficacy of these combinations against resistant strains are not available.

There remain more questions than answers for the treatment of serious multiresistant enterococcal infections. The Gram-Positive Committee of the Antimicrobial Resistance Leadership Group, an NIH-funded clinical trial consortium, recently outlined several unmet needs in the treatment of enterococcal infections, including the role of combination therapy with β-lactams for the treatment of enterococcal bloodstream infection and osteomyelitis, the role of combination β-lactam therapy against vancomycin-resistant enterococci, the optimal length of therapy for vancomycin-resistant enterococcal bloodstream infection, the optimal therapy for vancomycin-resistant enterococcal endocarditis, and the optimal therapy for vancomycin-resistant enterococcal infection caused by strains with elevated daptomycin MICs (287).

CONCLUSIONS

Enterococci, and particularly the clinically prevalent enterococcal species *E. faecalis* and *E. faecium*, continue to be important nosocomial pathogens. They are hearty species capable of surviving in important biological niches, such as the human gastrointestinal tract, and under stringent environmental conditions, facilitating their spread in institutions. Their broad spectrum of intrinsic resistance and tolerance to the bactericidal activity of many agents, combined with their prodigious ability to acquire resistance to available antibiotics, present ongoing therapeutic challenges to clinicians worldwide. They also express an increasing variety of virulence characteristics that promote colonization and infection. Moreover, their well-developed ability to acquire novel determinants for both resistance and virulence has kept them ahead of the many attempts to control the damage that they inflict on patients in our health care systems. Comprehensive strategies to contain their spread, limit their virulence, and eliminate them from infected sites will be required to prevent them from seriously limiting our ability to successfully treat a variety of serious diseases.

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