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# Review

# Pathogenic enterococci: new developments in the 21st century

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**Abstract.** Enterococci, traditionally viewed as Gram-positive commensal bacteria inhabiting the alimentary canals of humans and animals, are now acknowledged to be organisms capable of causing life-threatening infections in humans, especially in the nosocomial environment. The existence of enterococci in such a dual role is facilitated, at least in part, by its intrinsic and acquired resistance to virtually all antibiotics currently in use. Beginning with the initial identification of a 'streptococci of fecal origin' in the late 19th century, enterococci have been studied for over a century now. A number of comprehensive reviews during this time have addressed various aspects of enterococci, including classification, biology, virulence, antibiotic resistance and so on. This review specifically addresses the important advances in the field of enterococcal research that have occurred since the beginning of the 21st century. Most notable among these developments have been the insights into enterococcal genomes and pathogenicity.

**Key words.** Enterococci; microbial pathogenesis; virulence; nosocomial; antibiotic resistance; pathogenicity; gut microflora.

## Introduction

Enterococci are Gram-positive bacteria that occur singly, in pairs or short chains. They are present in the colon of all humans in numbers as high as $10^8$ colony-forming units (CFUs) per gram of feces and are recognized as facultative anaerobes with an optimum growth temperature of 35 °C [1, 2]. The enterococci are intrinsically rugged organisms endowed with traits such as growth in a temperature range between 10 and 45 °C, growth in broth containing 6.5 % NaCl or pH of 9.6 and ability to survive at 60 °C for 30 min [3]. Since the establishment of a separate genus, *Enterococcus*, to then include the streptococcal species *S. faecalis and S. faecium* [4], the number of enterococcal species has expanded to 23 with inclusion of new members based on DNA-DNA reassociation studies, 16S ribosomal RNA (rRNA) sequencing and whole cell protein (WCP) analysis [5].

The medical importance of the enterococci far outweighs the relatively insignificant proportion (less than 1 %) of the total adult human intestinal microflora they represent. *Enterococcus* species now rank among the leading causes of nosocomial infections [6], and estimates have placed the cost of curing the ~800,000 cases of enterococcal infections each year in the United States alone at around $0.5 billion. The vast majority of infection-derived clinical isolates belong to the species *E. faecalis*, while *E. faecium* remains the species exhibiting a disproportionately greater resistance to multiple antibiotics [7–10].

Vancomycin has been used as the drug of last resort in the treatment of Gram-positive bacterial infections, especially those caused by enterococci. The increasing occurrence of vancomycin-resistant enterococci, however, poses a serious problem, not only in the treatment of enterococcal infections, but also because it carries with it an increased risk of horizontal transfer of this resistant determinant to other vancomycin-susceptible species [11]. The recent isolation of vancomycin-resistant *Staphylo-*

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*coccus aureus* in the United States is alarming in this regard [12, 13].

As noted above, this review will specifically focus on new developments that have occurred since the beginning of this century in the field of enterococcal research and will be limited to a discussion of the enterococcal genome, the first *E. faecalis* pathogenicity island, virulence determinants in enterococci, animal models of infection and antimicrobial resistance mechanisms. For a comprehensive overview of all aspects of enterococci the reader is referred to the recently published book on this subject [14].

## The enterococcal genomes

Contemporary molecular biology has revolutionized our ability to study biological systems. The sequencing and annotation of bacterial genomes continue at a furious pace with 84 bacterial genomes completely sequenced to date and 152 in the process of being sequenced [15]. The increasing prevalence of multiple-drug resistant organisms such as enterococci in the clinical setting has demanded efforts to identify novel targets for generation of new antibiotic therapies. Sequencing the bacterial genome offers an avenue for the identification of molecular targets unique to each species and subsequently spur the development of novel antimicrobials.

Sequencing of the enterococcal genomes to gain insight into their genetic makeup and better understand the biology of this organism was therefore undertaken in the late 1990s, when increasing enterococcal infections and dwindling treatment options caught the attention of concerned health care professionals and scientists alike. The sequencing of the *E. faecalis* genome was undertaken by the Institute for Genomic Research (TIGR), and that of the *E. faecium* genome by the Joint Genome Institute of the Department of Energy in the United States. The genome sequence of *E. faecalis* strain V583 was recently completed [16] and will be discussed first, followed by the limited sequence information available from a yet-to-be fully assembled and annotated *E. faecium* genome.

## The genome of *E. faecalis* V583

*E. faecalis* strain V583 represented the first vancomycin-resistant clinical isolate reported in the United States [17] and exhibited the VanB phenotype. The circular chromosome of V583 comprised 3,218,031 bp with an average G + C content of 37.5%. A total of 3182 open reading frames (ORFs) with an average ORF size of 889 bp was inferred from the sequence, with 1760 of these ORFs showing similarity to known proteins. Also among the protein coding genes were 221 of unknown function, 495 with conserved hypothetical functions and 706 with no database matches. Strikingly unique to this genome was the fact that over 25% of the genome was made up of mobile and/or exogenously acquired DNA which included a number of conjugative and composite transposons, a pathogenicity island (described later in more detail), integrated plasmid genes and phage regions, and a high number of insertion sequence (IS) elements.

The vancomycin-resistant phenotype of V583 appeared to be encoded within a previously unknown mobile genetic element that bore some degree of resemblance to the *E. faecalis vanB* vancomycin-resistance conjugative transposon Tn*1549* [18]. Although these two elements shared identity within the region responsible for synthesis of modified peptidoglycan precursors terminating in D-lactate, divergence from the rest of the Tn*1549* element was apparent in V583 due to the presence of multiple insertions, deletions and rearrangements, coupled with low sequence similarity between conserved genes. The atypical trinucleotide content of the V583 conjugative transposon-like element suggested that this Tn*1549*-like element was likely to have been acquired by lateral gene transfer, perhaps mediated by flanking Tn*916*-like genes. A locus encoding homologs of the VncRS two-component signal transduction system presumably associated with vancomycin-tolerance in *Streptococcus pneumoniae* [19, 20] was also observed in V583 to be associated with Tn*916*-like genes and flanked by IS*256* elements. The precise role of this element in vancomycin tolerance, however, remains to be established.

Three IS elements, IS*Ef1*, IS*256* and IS*1216*, were the most common among 38 insertion sequence elements arranged in two distinct clusters on the V583 genome. One of these clusters appeared to be associated with the enterococcal pathogenicity island and the other within a region of atypical trinucleotide composition encoding most of the pantothenate biosynthesis steps. Seven regions presumably derived from integrated phages from other low-GC Gram-positive bacteria encoded potential virulence determinants, including multiple homologs of *Streptococcus mitis* proteins involved in binding to human platelets and a gene product involved in heme biosynthesis.

The V583 genome revealed, in addition to the chromosome, three circular plasmids, pTEF1 (66320 bp), pTEF2 (57660 bp) and pTEF3 (17963 bp). Plasmids pTEF1 and pTEF2 were structurally similar to the pheromone-responsive plasmids pAD1 and pCF10, respectively, and pTEF3 belonged to the pAM$\beta$1 family of broad host range plasmids [21–23]. Analysis of the V583 sequence revealed five sex pheromones encoded within lipoprotein signal peptides, and an additional 76 predicted lipoproteins some of which could potentially represent pheromone precursors. A novel pheromone inhibitor in pTEF2 also suggested the existence of a wide variety of different players in the sex pheromone signaling system of *E. fae-*

*calis*. In addition to the resident plasmids in V583 the genome also revealed three chromosomal regions that appeared to be remnants of integrated plasmids, emphasizing the contribution of plasmids to evolution of the enterococcal genome.

While over 85% of the V583 ORFs had their highest matches to other sequenced low-GC Gram-positive organisms, there was essentially no large-scale gene synteny between the V583 genome and that of any sequenced low-GC bacterium. This lack of synteny was explained as likely due to numerous mobile genetic elements that could have contributed to multiple rearrangements within the V583 genome. Consistent with the natural habitat of the enterococci being the GI tract was the finding that the enterococcal genome had 35 probable PTS-type sugar transporters involved in the acquisition and fermentation of nonabsorbed sugars in the GI tract. This high number of sugar transporters was comparable to that in *Listeria* and substantially more than in any other sequenced organism [16]. Also higher than in any other sequenced bacterium were the 14 predicted metal ion P-type ATPases, which along with cation homeostasis mechanisms likely contribute to the remarkable resistance of *E. faecalis* to pH, salt, metal ions and desiccation.

A comprehensive genome-wide analysis identified 134 putative surface-exposed proteins that might be associated with colonization or virulence. Included among these were proteins with potential choline or integrin binding motifs as well as those with a consensus LPxTG motif for cell wall anchoring by sortases [24]. Interestingly, almost one-half of the putative surface proteins contained stretches of nucleotide repeat motifs within the coding or promoter region which could support phase variation by a slippage mechanism [16]. A summary of the ORFs within the *E. faecalis* genome is graphically shown in figure 1.

## The *E. faecium* genome

Sequencing of the *E. faecium* genome was undertaken as a collaborative project involving the University of Texas Houston Health Sciences Center, and performed by the Joint Genome Institute of the US Department of Energy (http://www.jgi.doe.gov/). The sequenced strain was ATCC BAA-472 (also referenced in the literature as TEX16, TX0016), a 1992 isolate from the blood of a patient with infective endocarditis [25, 26]. The genome size for this strain was estimated to be around 2.9 Mb, slightly smaller than the 3.2 Mb genome of *E. faecalis* strain V583. The *E. faecium* sequencing project highlighted the capabilities of high-throughput nucleotide sequencing by accomplishing the feat of sequencing the entire *E. faecium* genome in a single day. The draft version

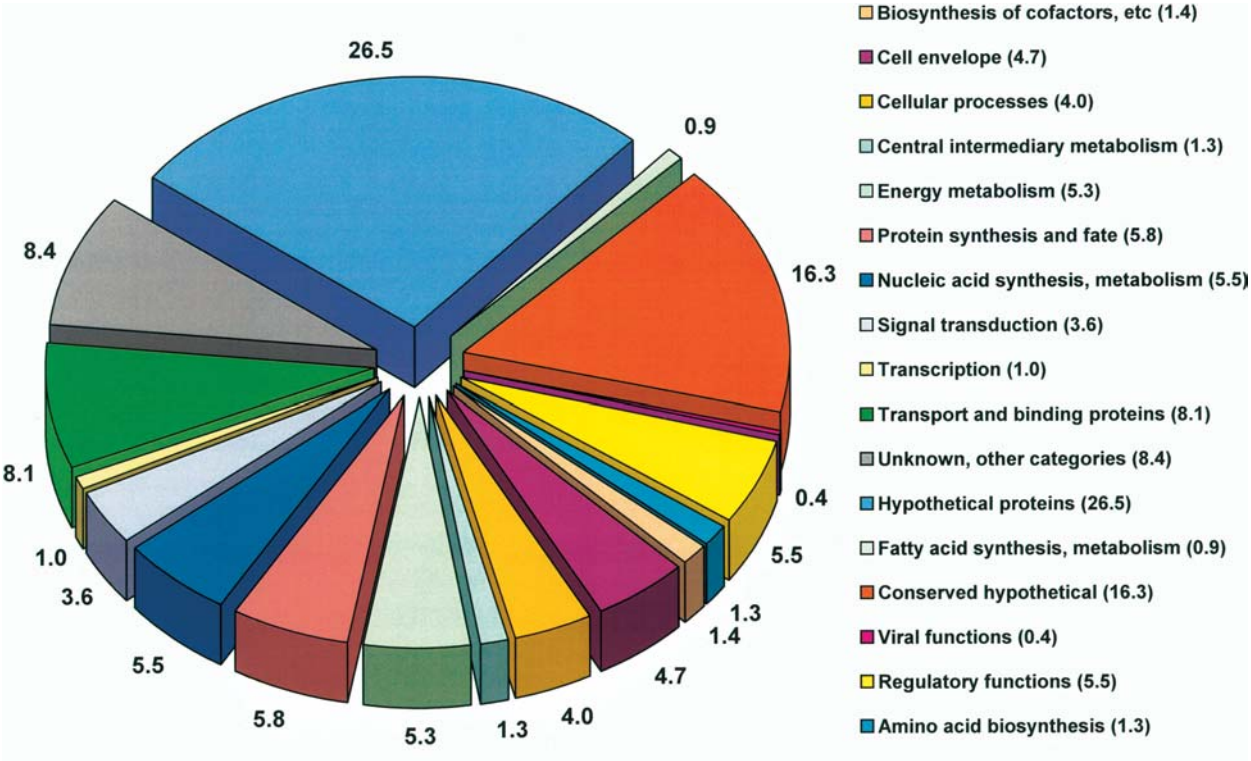



Figure 1. Pie chart showing the percentage of genes in the *E. faecalis* V583 genome that are represented in each of the families listed in the legend. Numbers adjacent to each pie piece and the gene family in the legend denote the percentage values. Adapted from [16].

of the sequence files are available at the following web link http://genome.ornl.gov/microbial/efae/ The data available at this site consist of 2,928,706 bp in 300 contigs of 20 reads or greater, with an inferred 37.8% G + C content and 3309 potential protein-coding genes. Presently these raw data are being finished for final presentation by the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC). The data so far assembled and annotated consist of 365 contigs with an average contig size of 4710 bp and representing a 11 × coverage of the genome. These data can be accessed at the link http://hgsc.bcm.tmc.edu/microbial/Efaecium/

## The *E. faecalis* pathogenicity island

Virulence genes of bacterial pathogens may be encoded on the chromosome or on extrachromosomal elements such as plasmids and bacteriophages. It is widely recognized now that virulence is often multifactorial and coordinately regulated with virulence genes often clustered on the genome in distinct regions termed pathogenicity islands (PAIs) [27]. PAIs are thus associated with virulent lineages, frequently absent or modified in less virulent nonpathogenic strains of the same or closely related species and contain large contiguous blocks of virulence genes. The recognition of PAIs is an important element in the evolution of bacterial pathogens through horizontal spread of virulence genes, similar to the horizontal transfer mediated by plasmids, bacteriophages and transposons.

The first enterococcal PAI [28] was identified in the genome of a multiple-antibiotic resistant strain of *E. faecalis* (MMH594) that caused a hospital ward outbreak in the mid 1980s [29]. The PAI, which varied only marginally in composition between strains tested, was approximately 150 kb in size, encoded 129 ORFs and possessed virtually all of the hallmarks of a PAI [27], including large size, terminal duplication at the target site, significantly variant G + C content of 32.2% compared with the chromosomal average of 37.5%, and the presence of genes that encoded transposases, transcriptional regulators and proteins with known or potential roles in virulence or adaptation and survival in different environments.

The inferred functional roles of the various ORFs encoded within the *E. faecalis* pathogenicity island are depicted in figure 2. Interestingly, almost one-half of the genes represented those encoding hypothetical or conserved hypothetical functions. Among virulence traits encoded within the *E. faecalis* PAI were the cell surface-associated protein Esp, the secreted toxin cytolysin and aggregation substance. Although there were no genes on the PAI encoding antibiotic resistance markers, genes that appeared to encode a DNA-damage-inducible protein, an AraC-like transcriptional regulator, a conjugated bile acid hydrolase, components of the phosphotransferase (PTS) system and a Gls24-like starvation-inducible protein were present. The PAI also encoded 18 ORFs for which no function could be predicted. The role(s) of these genes in bacterial survival or proliferation in the unique environment encountered in the hospital, or at sites of infection, remains to be explored.

A novel feature identified in the *E. faecalis* pathogenicity island was the ability to modulate virulence of the organism by selective high-frequency deletion of specific regions from within the PAI. This feature was discovered by careful comparison of sequences comprising the PAI

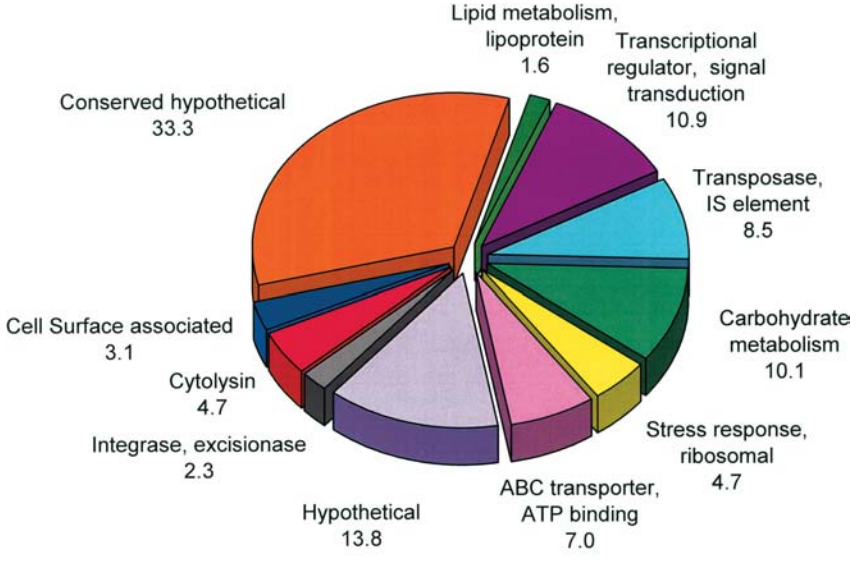
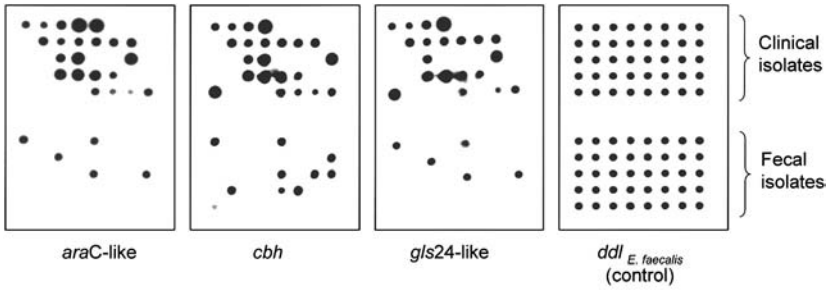



Figure 2. Pie chart showing the percentage of genes in the *E. faecalis* MMH594 PAI [28] that are represented in each of the categories listed.

in strain MMH594, with that of similar regions existing within the genomes of the vancomycin-resistant serial isolates V583 and V586 [17]. Although both V583 and V586 exhibited what appeared to be identical pulse field gel electrophoretic banding patterns, alignment and comparison of nucleotide sequence from the regions encoding the cytolysin and Esp functions revealed the deletion of a 17-kb region in strain V583 compared with V586. The missing segment was identified to be the result of a high-frequency spontaneous excision from within the PAI by an as yet unknown mechanism. The frequency of excision was estimated by quantitative polymerase chain reaction (PCR) to be around 1 in $10^3$ cells, a rate similar to that observed in modulation of virulence in other bacterial species by phase variation [30]. In addition, comparison of PAI sequences between V583, V586 and MMH594 revealed a high degree of sequence identity, with exceptions being the presence or absence of multiple IS elements. The insertion and excision of these IS elements likely promotes homologous recombination among similar elements and plays an important role in the ability of the organism to maintain the plasticity of the genome. This notion has been further reinforced after the genome sequence of V583 has highlighted the role played by mobile elements in the evolution of this organism [16].

Southern hybridization analysis was employed to independently test whether new traits not previously associated with known roles in enterococcal disease pathogenesis, but found in the PAIs were truly components of a PAI and not randomly interspersed chromosomal genes. A panel of 40 clinical isolates from widely dispersed geographical sites, and 40 fecal isolates from healthy volunteers, were examined by hybridization for the presence of the *araC*-like regulator, the stress-inducible *gls24* homolog and the inferred conjugated bile acid hydrolase (*cbh*), as representatives of potential new virulence factors. Clinical isolates were observed to be significantly enriched for all three genes: *ara*C-like ($P < 0.001$), *cbh* ($P < 0.01$) and *gls*24-like ($P < 0.001$) as shown in figure 3, supporting a role for these gene products in enterococcal pathogenesis and validating the genetic element as a PAI.

While the origin of this PAI remains speculative at this point, at least one-third of this PAI appeared to have evolved from integration into the chromosome of a pAD1- or pAM373-like conjugative plasmid sequence [28]. This feature was evident from extensive nucleotide sequence identity that existed at the 5′ end of the PAI with contiguous conjugation-related structural genes of the pheromone-responsive plasmids. Remnants of conjugation-efficient plasmid genes within the PAI suggested the possibility of transfer of the PAI by a conjugative process. The only transfer-related genes present, however, were those specifying a TraG-like protein (unknown function) and a region with 87% identity at the nucleotide level to a second transfer origin (*ori*T) recently identified in pAD1 [23]. The prospect for mobilization of the PAI from a donor to recipient strain was examined by standard filter mating experiments, which revealed that while the $Erm^R$ and $Gn^R$ determinants could be transferred from MMH594b (donor, derivative of MMH594 with the PAI tagged with a $Cm^R$ determinant) to FA2-2 (plasmid-free recipient, lacking the pathogenicity island) at frequencies of $5.02 \times 10^{-6}$ and $2.6 \times 10^{-8}$ per donor cell, respectively, there was no detectable transfer of the $Cm^R$ marker into the recipient. These experiments supported the observa-




| Gene (role) | Number positive | | P (chi-square) |
|---|---|---|---|
| | Clinical | Fecal | |
| *ara*C-like (transcriptional regulator) | 22 | 5 | 0.001 |
| *cbh* (bile acid hydrolase) | 22 | 9 | 0.01 |
| *gls*24-like (stress induced) | 20 | 5 | 0.001 |

Figure 3. Dot blot hybridization analysis to probe for PAI sequences in clinical versus stool isolates of *E. faecalis*. DNA from each of 40 infection-derived and 40 stool isolates from diverse geographical regions was spotted on a Zeta Probe membrane and hybridized to $^{32}$P-labeled DNA probes for three representative genes present on the PAI. A significant enrichment for these genes is evident among infection-derived isolates as listed. As a control probe to verify equivalent loading of DNA, the *E. faecalis*-specific *ddl* gene specifying D-Ala:D-Ala ligase was used [120].

tion that transfer of the PAI occurred more on an evolutionary scale rather than on a readily detectable laboratory scale [28].

The discovery of a number of ORFs within the PAI, with as yet unknown function, but which are absent from a non-infection-derived isolate and rare in fecal isolates, implicated their contribution to enterococcal survival in the hospital, or in the process of disease transmission or pathogenesis. This PAI may thus serve as a useful marker for detecting unusually pathogenic *E. faecalis* strains. Rapid identification of such strains could trigger enhanced infection control procedures within a hospital ward and help in curtailing intrahospital spread, such as frequently occurs in ward outbreaks. In addition to known virulence traits, the PAI revealed a number of genes which may serve as novel targets around which new therapeutic interventions may be designed.

The identification of an *esp* homolog in *E. faecium* fueled speculation as to the existence of a PAI in this species as well. Willems et al. [31, personal communication] have explored this possibility in some detail and determined that a 25-kb DNA segment surrounding the *esp* gene in an *esp*-positive *E. faecium* strain was not present in the genome of the *esp*-negative strain ATCC BAA-472 [31]. While the organization and composition of the regions flanking *esp* in *E. faecium* exhibited little similarity to that of *E. faecalis* [28], its absence in at least one other *E. faecium* strain suggested the possibility of a PAI in *E. faecium* as well. This aspect is currently under investigation in a collaborative effort between our laboratory and that of Willems et al.

## Enterococcal virulence determinants

Enterococci are well suited for inhabiting the mammalian gastrointestinal tract and for the most part exist as commensals in harmony with the host as well as other coresident gut flora. Perturbations in the dynamics of this host-commensal relationship, such as promoted by antibiotic treatment, host injury or diminished host immunity, could allow these intestinal bacteria to gain access to extraintestinal host sites and cause infection. Another mechanism by which the enterococci can deviate from their commensal behavior is through the acquisition of new traits that allow the bacterium to overcome host defenses and colonize new niches. The latter argument is gaining ground after the identification of the *E. faecalis* PAI, which clearly highlights genetic differences between infection-derived and commensal strains [28]. A number of studies over the years have addressed the issue of enterococcal virulence and the identification of enterococcal virulence factors [3, 32–38]. Most prominent among these virulence determinants have been the surface adhesins Esp and aggregation substance (AS), MSCRAMM Ace, secreted toxin cytolysin, secreted proteases gelatinase and serine protease, enterococcal capsule, cell wall polysaccharides and extracellular superoxide. Each of these virulence factors will be discussed briefly in the following sections with respect to recent developments.

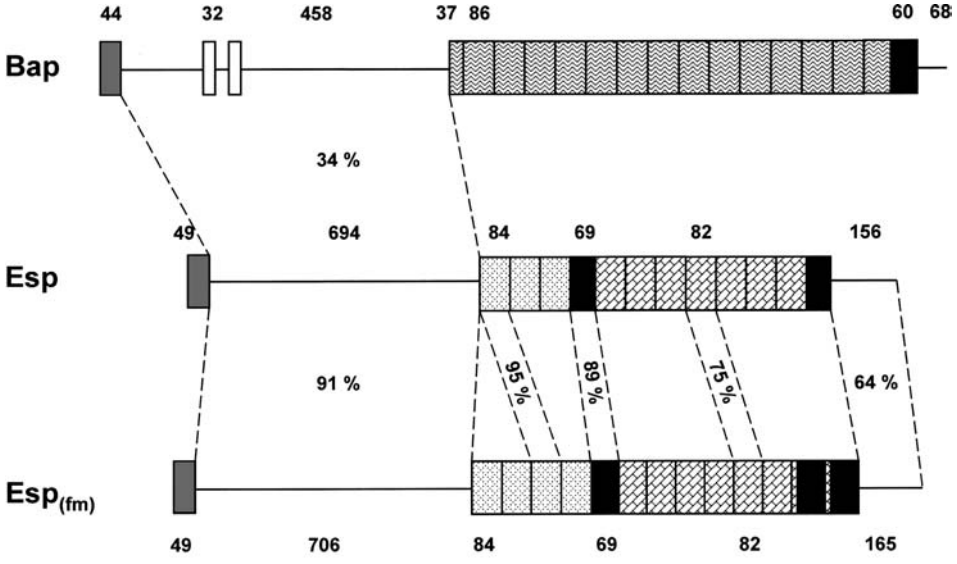



Figure 4. Schematic representation of the similarities between surface protein Esp from *E. faecalis* (Esp), *E. faecium* ($Esp_{(fm)}$) and the biofilm-associated protein Bap from *S. aureus.* The repeat domains in Esp are represented by shaded boxes as follows: A repeats, ▒; B repeats, ■ and C repeats, ▨. Amino acid residues within each domain or repeat unit are denoted by the adjacent numerals. Regions sharing identity are shown connected by dotted lines and enclosing the percentage identity values.

## Enterococcal surface protein, Esp

Enterococcal surface protein, Esp, was identified initially in a highly virulent gentamicin- resistant bacteremia *E. faecalis* isolate [36]. This protein possesses characteristic structural features including large, highly conserved repetitive blocks as shown in figure 4. Mature Esp from the *E. faecalis* strain MMH594 consists of 1873 amino acids. The core region consisting of repeat units made up about 50% of the protein and has a unique architecture made up of distinct tandem repeating units. The first repeating unit located downstream of the N-terminal domain consists of three 84-residue repeats specified by nearly identical 252-nucleotide tandem repeats (A repeats). Seven nearly identical 246 nucleotide tandem repeating units (C repeats), encoding reiterations of an 82-amino acid sequence, are flanked by the B repeats, which share 74% sequence identity at the amino acid level. The highly conserved multiple repeat structure allows for expression of alternate forms that differ in the number of repeat units as a result of interrepeat recombination [36].

Esp exhibits global structural similarity to the *Streptococcus pyogenes* R28 [39], *Streptococcus agalactiae* Rib [40, 41], C alpha protein [42] and to the *Staphylococcus aureus* biofilm-associated protein, Bap [43]. This similarity is restricted to a highly conserved region within the C repeat units of the Esp protein to corresponding regions within the group A and B streptococcal proteins, while the similarity with Bap is limited to the nonrepeat N-terminal region.

Infection-derived *E. faecalis* isolates were enriched for the *esp* gene [36], and an *esp* homolog was recently reported in *E. faecium* isolates [44–46]. The variant *esp* gene was significantly enriched ($P < 0.0001$) among epidemic vancomycin-resistant *E. faecium* isolates (VREF) that are genetically distinct from nonepidemic VREF obtained from hospitals on three continents [45]. A similar study in the United Kingdom detected *esp* in over 60% of vancomycin-resistant and vancomycin-sensitive clinical isolates, but not in environmental isolates [46]. A screening study of enterococcal virulence factors also identified the *esp* homolog to be enriched among clinical *E. faecium* isolates compared with food or starter isolates [44]. In a recent study, the *esp* gene was found to be significantly associated more with ampicillin-resistant than ampicillin-sensitive strains of *E. faecium* ($P < 0.001$), regardless of the isolation site [47].

Bap was described recently from *S. aureus* and bears an appreciable level of structural and sequence similarity to the Esp protein [43]. In this species it was enriched among isolates that were highly capable of forming biofilms and adhering to abiotic surfaces. Incidentally, Esp was recently shown to be involved in biofilm formation as well. Toledo-Arana et al. [48] found a significant correlation between the presence of Esp and the ability of *E. faecalis* to form biofilms on polystyrene, with 93.5% of tested *esp*-positive isolates forming a biofilm. None of the *esp*-negative isolates tested in this study were capable of forming biofilms. The presence of Esp did not, however, promote the adhesion of *E. faecalis* to other medically relevant substrates such as silicone rubber, fluoroethylene-propylene or polyethylene in another study [49]. In evaluating the adhesion of enterococcal strains to two types of urinary catheter materials, Joyanes et al. [50] concluded that *E. faecalis* showed greater adherence to these abiotic surfaces compared with *E. faecium.* Adherence was further shown not to be related to bacterial surface hydrophobicity, hemolysin or gelatinase production. An investigation of adhesive properties of *E. faecalis* strains to intestinal Int-407 and Girardi heart cell lines in vitro similarly revealed no role for Esp in promoting adhesion to these cell types [51].

The role of Esp in colonization and persistence of *E. faecalis* in an animal model of ascending urinary tract infection was evaluated by comparing an Esp-positive strain of *E. faecalis* to its isogenic Esp-deficient mutant [52]. Groups of CBA/J mice were challenged transurethrally with $10^8$ CFU of either the parent or mutant strain and bacteria enumerated in the urine, bladder and kidneys 5 days post-infection. Significantly higher numbers of bacteria were recovered from the bladder and urine of mice challenged with the Esp-bearing parent strain than from mice challenged with the Esp-deficient mutant, pointing to a role for Esp as a virulence factor in this infection model. These results suggested that Esp may serve to promote bacterial adhesion to bladder epithelium through specific components of the bladder wall such as mucin or uroplakin. This hypothesis is currently under investigation in our laboratory. The observations of a role for Esp in forming biofilms on abiotic surfaces and in urinary tract infections support a multifunctional role for Esp.

## Aggregation Substance (AS)

AS, a pheromone-inducible surface protein of *E. faecalis*, has been shown to mediate adhesion in vitro to cultured renal tubular cells [53], and to augment internalization of *E. faecalis* by cultured human intestinal epithelial cells [54, 55]. In vivo, aggregation substance may contribute to the pathogenesis of enterococcal infection through a number of mechanisms [10]. Among a number of different functions ascribed to AS in addition to promoting cell-cell contact are adhesion to host cells, adhesion to extracellular matrix (ECM) proteins, increased cell surface hydrophobicity, increased vegetation size in experimental endocarditis and contribution to resistance to killing by PMNs [35, 53, 56–58]. Recent studies have focused on understanding the role of AS in enterococcal ad-

hesion, internalization and transmigration across enterocytes, on identification of specific regions of AS that trigger cell-cell aggregation and in host immune responses.

Aggregation substance contains two RGD motifs, also found in fibronectin, which are commonly associated with integrin-binding proteins [53, 59]. In addition to the conjugative transfer function, the occurrence of these RGD motifs implies a role of the AS in eukaryotic cell binding. Indeed, earlier studies have demonstrated the ability of AS to bind renal epithelial cells via the RGD motif [53]. *E. faecalis* strain OG1X transformed with the plasmid pAM721 to constitutively express AS bound to colonic (HT 29, HT 29/1 and T84) and duodenal (Hutu 80) cells in vitro at significantly higher levels than the parent strain OG1X lacking AS [35]. Interestingly, the adherence of *E. faecalis* to HCT-8 cells originating from the ileum was independent of AS. In the same study, the investigators also evaluated the potential invasive role of aggregation substance in promoting the internalization and translocation of enterococci from the luminal to the basolateral side of the intestinal epithelial cells. The *E. faecalis* strain expressing AS was internalized by the colonic and duodenal cells at levels significantly higher than the AS negative strain, while no difference was observed with the ileal cells. In vitro, AS did not promote the translocation of enterococci across an intact intestinal epithelial cell layer [35]. A different study with the same isogenic pair showed a significantly higher adhesion of AS-positive cells to colonic mucosa from both normal colon and colon from patients with Crohn's disease, as compared with the AS-negative strain [60].

Expression of AS also leads to significantly increased adhesion to ECM proteins fibronectin, thrombospondin, vitronectin and collagen type I but not to laminin or collagen type IV, compared with an AS-negative isogenic strain. Constitutive expression of AS on the cell surface allows *E. faecalis* to adhere to immobilized fibronectin at 5-fold greater amounts compared with *S. aureus* Cowan cells and 30-fold higher than *Streptococcus bovis* [56]. Using a nisin-inducible expression system for controlled expression of AS at the cell surface of *E. faecalis* and *Lactococcus lactis*, AS expression was associated with increased internalization of *E. faecalis* by HT-29 enterocytes and of *L. lactis* by HT-29 and Caco-2 enterocytes [54]. Transposon insertion mutagenesis was used as a tool for identifying the domains of aggregation substance potentially crucial for in vivo virulence and led to the identification of a N-terminal variable region as responsible for mediating aggregation. In the same study, the authors also showed that merely an increase in the cell surface hydrophobicity did not trigger aggregation [57].

Aggregation substance being a surface-exposed protein, several studies have been directed towards studying the role of AS in host immunity. AS was shown to promote opsonin-independent binding of *E. faecalis* to PMNs via a $\beta$2 integrin [59], and also promote adherence as well as internalization of *E. faecalis* by macrophages in a concentration- and time-dependent manner via an interaction with the CD11b/DC18 integrin [58]. Enterococci expressing AS were also found to resist phagocytosis significantly better than an isogenic AS-negative strain by inhibition of the respiratory burst [(production of reactive oxygen species (ROS)] in the macrophages [58].

In vivo studies on the role of aggregation substance in endocarditis have revolved around the rabbit endocarditis model. Using the purified N-terminal region of AS lacking the signal sequence, it was demonstrated that prior immunization of rabbits with this purified AS fragment did not impart protection against subsequently induced enterococcal endocarditis [61]. This suggested that AS may not be involved in early establishment of the vegetation and that its primary role in virulence may be immune system evasion as shown by macrophage and PMN binding studies [58, 59].

## Collagen-binding adhesin, Ace

Ace is a collagen-binding MSCRAMM on enterococci and is structurally and functionally related to the staphylococcal Cna adhesin [62]. Ace is ubiquitous among commensal and pathogenic isolates of *E. faecalis* [63], is apparently expressed during infection in humans and human-derived antibodies to Ace can block adherence to extracellular matrix proteins in vitro [34, 64]. An intragenic probe of the *ace* gene showed specific hybridizations to all *E. faecalis* isolates tested and was suggested as a tool for speciating enterococci [63]. X-ray crystallographic analysis of Ace has recently been reported [65].

Ace was previously shown to mediate binding to an extracellular matrix component, type I collagen [62]. Nallapareddy et al. recently showed that in *E. faecalis* strain, OG1RF, Ace mutant binds collagen types I and IV and laminin at significantly lower levels compared with wild type [34]. However, this binding was only observed when *E. faecalis* cells were cultured at 46 °C and not at 37 °C [64]. Employing anti-Ace antibodies, Ace was detected in 90% of enterococcal endocarditis patient sera samples, suggesting that *ace* is expressed in vivo. The host factors that may be regulating *ace* expression in vivo have not been identified yet.

An *ace* homolog, designated *acm*, was recently identified in *E. faecium* [66] and exhibits the same kind of domain architecture as Ace. While Acm only exhibits similarity to the Ace protein within the A domain, it shows much greater similarity to the *S. aureus* collagen-binding adhesin, Cna, within domains A and B. Functionally, Acm was shown to be the primary adhesin responsible for the ability of *E. faecium* to bind collagen. The same study also found that although 32 of 32 *E. faecium* isolates

tested harbored an *acm* gene, a number of them did not exhibit a collagen-binding phenotype. Consistent with the nonadherent phenotypes is the identification of deletions and mutations within the *acm* gene and its putative promoter regions [66].

## Cytolysin

The *E. faecalis* cytolysin [33, 67] lyses a broad range of eukaryotic and prokaryotic cells, is usually plasmid encoded [68] and enhances the virulence of *E. faecalis* in animal models [33, 69–71]. More recently, the cytolysin operon was detected as a component of the *E. faecalis* pathogenicity island in close proximity to the *esp* gene [28].

Expression of cytolysin, which is known to contribute to enterococcal virulence, was found to be regulated by a novel two-component regulatory system via a quorum-sensing mechanism [37]. The cytolysin system is unique in that it is not regulated by a conventional two-component signal transduction system consisting of a histidine kinase and a response regulator. The regulatory system instead consists of two open reading frames, *cylR1* and *cylR2*, encoding an apparent transmembrane protein of unknown function (CylR1) and a helix-turn-helix DNA-binding protein (CylR2), respectively. By using transcriptional fusions to a promoterless $\beta$-galactosidase gene, it was shown that the fully processed and activated cytolysin subunit CylLs alone induces transcription from the cytolysin promoter pL in the presence of the two regulatory proteins CylR1 and CylR2. Transcription of the regulatory genes *cylR1* and *cylR2* remain unaffected by exposure to CylLs. The same study also demonstrated that the autoinduction of the cytolysin operon occurrs when sufficient inducer (CylLs) accumulates in the extracellular milieu. In planktonic brain heart infusion (BHI), culture induction was observed at cell densities of $10^7$ CFU or higher [37].

## Gelatinase, serine protease and the Fsr regulator

A locus, *fsr*, with sequence similarity to components of the staphylococcal *agr* locus [72], positively regulates the expression of gelatinase and serine protease (encoded by *gelE* and *sprE*, respectively) in *E. faecalis* OG1RF [73, 74]. The *agr*-like locus comprises three genes, which have been designated as *fsrA*, *fsrB* and *fsrC*, for *E. faecalis* regulator. Downstream of *fsrC* are two ORFs, *gelE* coding for gelatinase (a metalloprotease) and *sprE* coding for a serine protease. In a limited epidemiological study *fsr* was detected in 12 of 12 (100%) endocarditis isolates tested as compared with 10 of 19 (53%) stool isolates [75]. A similar study identified a 23.9-Kb deletion encompassing the *fsrA*, *fsrB* and 5′ end of *fsrC* genes, which was responsible for the gelatinase-negative phenotype in 79% of 33 clinical *E. faecalis* urine isolates [76]. Mutants with insertion disruptions in each of the three regulatory genes within this locus were significantly attenuated in a mouse peritonitis model compared with the parent strain [77]. Northern blot analyses revealed an absence of *gelE* and *sprE* transcripts in *fsrA*, *fsrB* and *fsrC* mutants [74], confirming a regulatory role of the *fsr* genes in the expression of *gelE* and *sprE*. In vivo studies with the *gelE* mutant, the gelatinase-positive *sprE* mutant and the *fsr* mutants showed delayed killing in a mouse peritonitis model [73, 74]. FsrB was predicted to be a membrane protein with multiple transmembrane segments and responding to GBAP (gelatinase biosynthesis activation pheromone) in a quorum-sensing manner. A deletion in *fsrB* resulted in decreased virulence as compared with the wild-type OG1RF in a rabbit endophthalmitis model [78]. In both the *Caenorhabditis elegans* killing and mouse peritonitis, model system *fsrB* deletion mutants were attenuated compared with parent strains [32]. In another similar study, *fsrA, fsrC*, *gelE* and *sprE* mutants were also found to be attenuated in their ability to kill *C. elegans,* although *gelE* and *sprE* mutants were attenuated to a lesser extent [79].

Characterization of the *fsr* locus revealed the cotranscription of *fsrA, fsrB* and *fsrC*, with the transcription of *fsrA* under the control of its own promoter and the transcription of *fsrB* and *fsrC* being *fsrA* dependent and under the control of the *fsrB* promoter. Genes *gelE* and s*prE* were also confirmed to be cotranscribed, with transcription being regulated by the *gelE* promoter [77].

## Cell wall carbohydrate and capsular polysaccharide

Because of their complexity and ability to confer resistance to phagocytosis, bacterial capsular components have a crucial role to play in the pathogenic process by evading the host immune system. Antigenicity of these components also makes them attractive candidates for developing antibacterial vaccines. An operon-encoding synthesis of capsular polysaccharide of the type most commonly expressed by clinical isolates of *E. faecalis* was recently identified [80]. Compositional analysis of the purified CW carbohydrate fractions showed it to be comprised of glycerol phosphate, glucose and galactose residues. The *cps* operon was organized as a cluster of 11 ORFs designated *cps*A-*cps*K and found to specify the enterococcal type-specific carbohydrate. Insertional inactivation of the genes in the operon yielded isogenic *cps*-negative mutants with enhanced susceptibility to phagocytic killing in vitro and compromised ability to persist in mouse regional lymph nodes [80].

A second capsular polysaccharide present on the surface of both *E. faecalis* and *E. faecium* was also purified and

chemically characterized [81]. The purified carbohydrate fraction was compositionally and immunologically distinct from that described above [80], and antibodies to this fraction showed opsonic activity in a neutrophil-mediated phagocytosis assay. The protective efficacy of these antibodies was demonstrated in a subsequent study using a mouse infection model, suggesting the possibility that these antibodies may be useful for prevention of enterococcal infections [82].

A gene cluster encoding a rhamnose-containing polysaccharide termed enterococcal polysaccharide antigen (*epa*) has been identified and characterized by Xu et al. [83]. The genetic determinant encoding this polysaccharide was a set of 17 contiguous genes labeled the *epa* gene cluster. Disruptions in two of the genes, *orfde4* and *orfde6*, resulted in a statistically significant delay in killing in a mouse peritonitis model [83], suggesting the possible role of this polysaccharide as a virulence factor.

## Extracellular superoxide

Enterococci are unique in their ability to produce substantial amounts of superoxide, a trait that appears to show more correlation with *E. faecalis* isolates from the bloodstream. The biochemical pathway for the production of superoxide by *E. faecalis* was recently characterized [84], as was the demonstration of superoxide production by wild-type *E. faecalis* strains in the rat colon. Chinese hamster ovary cells and HT-29 enterocytes showed increased DNA damage after incubation with wild-type *E. faecalis* OG1RF, which produces superoxide, whereas an isogenic mutant attenuated for superoxide production did not [38]. A similar study demonstrated hydroxyl radical production by *E. faecalis* colonizing the rat intestine [85]. These results point to *E. faecalis* being a potent source of oxidative stress on the intestinal epithelium, and perhaps a role for superoxide production in bacterial translocation across the epithelium or contribution to chromosomal instability associated with intestinal polyps and colorectal cancer.

## Infection models to evaluate enterococcal virulence

The earliest reported animal model of infection with enterococci was that by MacCallum and Hastings [86], who demonstrated the lethality of human-derived enterococci in mice and rabbits when injected intraperitoneally or intravenously at high doses. The same study also exposed the limitations of animal models by showing variations in susceptibility by different animal species. It is therefore important to select a model appropriate for the questions to be answered and to interpret the results with caution. The traditional models used to assess enterococcal virulence have been the murine peritonitis [73, 87], a rabbit or rodent endocarditis [88–90], and the murine orogastric feeding models [91–93]. These models have been complemented by a number of in vitro studies that have been performed using cultured enterocytes, macrophages or epithelial cells to examine issues such as adherence, invasion, translocation and intracellular survival of enterococci [35, 58].

The peritonitis model has proven useful to compare the relative lethality of isogenic mutants, but provides little information on the pathogenesis of infection or the mechanisms which lead to death. The endocarditis model employs in vivo catheterization to cause valvular damage. In addition to affording comparison of lethality between strains, this model also allows morphological and histological examination of the vegetation and monitoring of bacteremia. The murine orogastric feeding model remains the method of choice to evaluate intestinal colonization and overgrowth. In this model, colonization by enterococcus with levels as high as $10^{10}$ CFU (colony-forming units) per gram of cecum can be achieved after the bacteria are orally administered following antibiotic pretreatment. This model also affords the ability to assess translocation across the intestinal epithelium, allowing subsequent detection of bacteria in the blood, spleen, liver and mesenteric lymph nodes [93].

A rabbit endophthalmitis model was developed to study enterococcal infection [94, 95] and had several advantages over other models mentioned earlier. In this model an extremely low inoculum of as little as 10 colony-forming units could be used to set up an infection within the eye, which is an immune-privileged site. The course of infection can be monitored over a period of 3–5 days by relatively noninvasive techniques used in clinical ophthalmology. The model circumvents the inherent difficulty in using immunocompromised animals while allowing the examination in real time of physiological functions such as inflammation and loss of retinal function.

An intravenous mouse infection model has been developed by Gentry-Weeks et al. to closely mimic human enterococcal bloodstream infections [96]. Unlike the murine intraperitoneal infection model in which death of infected mice was rapid and within 24–48 h post-infection, mortality in the refined model was delayed and occurred between 7 and 10 days post-infection. The delayed mortality allowed the 'virulence ranking' of *E. faecalis* isolates based on their ability to cause death, and the model may prove useful to evaluate strains which exhibit subtle differences in virulence.

A *C. elegans* model for evaluating Gram-positive bacterial virulence factors, including that of enterococci, was recently developed [32]. In this nonvertebrate model it was found that both *E. faecalis* and *E. faecium* could persist in the *C. elegans* intestinal tract, although only *E. fae-*

*calis* proved lethal to the organism. While the mechanisms of *E. faecalis* lethality in this model remain to be established, the model proved useful in identification of a potentially new virulence factor (ScrB, a sucrose hydrolase) and confirmed that two previously characterized virulence factors, Cyl and FsrB, also play a role in *C. elegans* killing. In contrast, AS, a well-characterized virulence factor in mammalian infections did not appear to be a virulence factor in this model system. These results once again highlight the importance of choosing an appropriate model system to evaluate enterococcal virulence and the need for cautious interpretation of results.

## Antimicrobial resistance mechanisms

Enterococci are intrinsically resistant to most antimicrobial agents used to treat infections in humans. Prominent among these agents are β-lactamase-resistant penicillins, cephalosporins and low levels of aminoglycosides typically used to treat infections caused by other Gram-positive cocci [97]. Of greater concern has been the ability of enterococci to acquire resistance to antimicrobials, facilitated at least in part by transfer of plasmids and transposons or chromosomal exchange [10]. Enterococci are endowed with elegant mechanisms of genetic exchange through which they have acquired high-level resistance to β-lactams, aminoglycosides and glycopeptides [98, 99]. The acquisition of resistance to most antimicrobial agents currently in use and the intrinsic ruggedness of the organism confer upon enterococci a select advantage for persistence in healthcare settings [100].

The intrinsic low-level resistance of enterococci to the β-lactam antibiotics has been recognized for a long time [101], as well as the fact that *E. faecium* in general is less susceptible to this class of antibiotics compared with *E. faecalis*. High-level resistance to penicillins occurs mainly by two known mechanisms: overproduction of an altered penicillin-binding protein (PBP5) with low affinity towards penicillins or structural alterations within PBP5 that further reduce susceptibility to inhibition by penicillins [102]. β-Lactamase production by enterococci is rare [103], and genetic evidence suggests that enterococci may exhibit this phenotype through acquisition of the β-lactamase determinant from *S. aureus* [104]. More recently, a novel mechanism of β-lactam resistance that circumvents the DD-transpeptidation reaction that occurs during the final stage of peptidoglycan synthesis was reported in *E. faecium* [105]. In this laboratory mutant lacking a *pbp5* gene, resistance occurred due to peptidoglycan cross-links resulting from β-lactam-insensitive LD-transpeptidation.

A low level of intrinsic resistance to aminoglycosides is attributed to the inability of the drug to cross the enterococcal cell membrane. Consequently, the clinical regimen of combination therapy calls for administration of an aminoglycoside along with a cell wall-active antibiotic. The latter serves to increase uptake of the aminoglycoside and augments the action of both antibiotics. High-level resistance to aminoglycosides is common among enterococci and occurs through the production of a spectrum of aminoglycoside-modifying enzymes [106]. Most notable of these are the aminoglycoside acetyltransferases, phosphotransferases and nucleotidyltransferase. Aminoglycoside resistance associated with a change in ribosome structure has not been reported except for streptomycin, where resistance arises through a change in the 30S ribosomal subunit structure [107].

Glycopeptide resistance in enterococci occurs through the acquisition of transposable genetic elements (VanA, VanB, VanD, VanE and VanG phenotypes) or a nontransferable chromosomal determinant (VanC) [108, 109]. Among the five phenotypes of acquired glycopeptide resistance that have been documented in enterococci, VanA and VanB remain the most globally widespread and prevalent. Resistance results from the production of peptidoglycan precursors with reduced binding affinity for glycopeptides and is encoded by complex clusters of *van* genes [110]. The prototype *vanA* element is Tn*1546*, a 10.8-kb transposon that carries a cluster of seven genes controlling the switch to a cell wall composition from pentapeptide terminating in D-Ala-D-Ala to one ending in D-Ala-D-Lac and mediates high-level inducible resistance to both vancomycin and teicoplanin [111]. The *vanB* cluster encodes inducible resistance to vancomycin but not to teicoplanin and is disseminated by large transposons such as Tn*1547* and Tn*5382* [112, 113]. The VanC phenotype is chromosomally encoded in certain motile enterococci, constitutively expressed [114] and results in modified pentapeptides terminating in D-Ala-D-Ser. The *vanD* operon mediates moderate-level resistance to both vancomycin and teicoplanin and is chromosomally encoded [115]. The *vanE* and *vanG* clusters cause low-level resistance to vancomycin and are believed to be inducible [116]. Much of the recent work in the area of glycopeptide resistance has centered on understanding the origins and modes of dissemination of the various resistance determinants [117, 118] and in evaluating alternatives to treat vancomycin-resistant enterococcal infections [119].

## Conclusions and future directions

Enterococci have clearly emerged from long being considered harmless bacteria languishing in the intestinal tract to medically important multiple antibiotic-resistant nosocomial pathogens. The debate continues over whether serious enterococcal infections arise from one's own indigenous flora or from exogenously acquired strains. However, epidemiological studies clearly show the exis-

tence of clonality among outbreak isolates and support the notion that a subset of virulent lineages with greater propensity to cause disease exist and are often responsible for infections of epidemic proportions. It is also clear that antibiotic resistance is just one of many traits that virulent enterococci possess as compared with commensal isolates. The *E. faecalis* genome sequence as well as identification of the PAI in this species have highlighted numerous genes encoding protein products of unknown function and provided compelling evidence for genetic differences between commensal and infection-derived isolates. It is hoped that as we begin to unravel the role of many of these gene products in the near future by employing functional genomic approaches, we will come up with new tools to combat serious enterococcal infections.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