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Pathogenicity of Enterococci

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Introduction

Enterococci are hardy, gram-positive cocci that are common residents of the gastrointestinal tracts of nearly all land animals, including humans. While a core member of the microbiome, they are also capable of causing a variety of severe infections, most often among antibiotic-treated hospitalized patients with perturbed intestinal microbiota. Here we present an overview of the pathogenicity of enterococci, and discuss the most prominent features of this hospital-associated pathogen.

The origin of the term “enterococcus” dates to the end of the 19th century when Thiercelin described a saprophytic gram-positive coccus of intestinal origin capable of causing infection (1, 2). In the same year, a very detailed picture of enterococcal pathogenesis was reported by MacCallum and Hastings (3), who isolated and characterized an organism (now believed to be a cytolytic strain of *Enterococcus faecalis*) from a lethal case of acute endocarditis. Termed *Micrococcus zymogenes* by them, bacteria recovered from the blood and cardiac tissue of the patient and inoculated into pure culture were able to recapitulate elements of endocarditis in animals, satisfying Koch’s Postulates and establishing *Enterococcus* as a bona fide pathogen of humans and animals. From these early descriptions, the paradigm of enterococci as a commensal opportunist has been established. More than a century later, enterococci are widely recognized as leading hospital pathogens (4, 5).

Infections occurring mainly among hospitalized patients, caused by microbes of the genus *Enterococcus* (most notably *E. faecalis* and *Enterococcus faecium*) include urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis (6). Enterococci are now the third most common nosocomial pathogen, causing 14% of hospital-acquired infections (HAI) in the United States between 2011 and 2014, an increase from 11% in 2007 (4, 7). In addition to nosocomial infections, enterococci are responsible for 5–20% of community-acquired endocarditis (8).

This review focuses on the mechanisms by which enterococci cause human disease, with particular attention to advances in the field since the last edition of this text (9). Additional

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perspectives and information may be found in the complete volume on enterococcal biology (10).

Biology and Characteristics

Enterococci can be easily isolated from a wide range of hosts, including invertebrates, insects, and mammals (Figure 1). This host range highlights the ability of enterococci to survive a diversity of innate host defenses in the gut (11). In humans, enterococci are one of the earliest colonizers of the infant gut, and as a result are core members of the intestinal microbiome (12, 13). They are also common among commensal microbiota of domesticated and wild animals, and can cause infections in these animals as well (14–16). Due to their widespread occurrence among humans and animals, they are readily isolated from the environments inhabited by those hosts, often associated with plants, in soil and in water (17, 18). Because of their numerous auxotrophies, enterococci persist in the environment but do not likely proliferate to high numbers there.

Enterococci were formerly classified as group D streptococci (19) but were given genus status in 1984 (20) based on nucleic acid hybridization studies that showed a more distant relationship to the streptococci. The genus *Enterococcus* contains over 50 species, and appears to have branched from its last common ancestor approximately 425 million years ago (21). Members of this genus are endowed with intrinsic properties that confer the ability to survive host defenses and compete in the intestinal tract, and then persist and spread in the natural environment or hospital, leading to colonization of new hosts. Among these traits are the ability to grow over wide-temperature and pH ranges, survive desiccation, and grow in the presence of 6.5% NaCl and 40% bile salts (17).

Antibiotic Resistance

The challenge of disease caused by enterococci is compounded by their limited susceptibility to antibiotics, due to both intrinsic and acquired antibiotic resistances. Enterococci are intrinsically resistant to cephalosporins, aminoglycosides, lincosamides and streptogramins (6, 22). Intrinsic resistance of enterococci undoubtedly positions them well to acquire additional resistances on mobile genetic elements. In the antibiotic-treated patient, enterococci accumulate to high numbers (23), and co-exist in intimate association with other overtly antibiotic-resistant microbes, the precise environment to enhance the probability of contact between enterococci and other species harboring new resistances on mobile elements, leading to element acquisition.

Because enterococci are not killed by many β -lactams, new approaches to treatment were sought. The discovery that aminoglycosides were synergistic with β -lactams and could achieve cidal activity (24), resulted in combination therapy becoming the standard of care. However, only a few years later, aminoglycoside resistance in enterococci was reported (25), and found to be due to the acquisition of a plasmid-borne resistance factor. High-level resistance to aminoglycosides is conferred by aminoglycoside-modifying enzymes, especially a bifunctional enzyme (26), and mobile elements conferring this trait have spread throughout the enterococci (27). Further compromising combination therapy, enterococci that expressed a β -lactamase have been identified (28), but these remain surprisingly rare.

Vancomycin was introduced in the late 1950s (29), and because of its limited spectrum and intravenous dosing, received limited application in hospitalized patients with β -lactam allergies and infections due to organisms with other antibiotic resistances (30). Vancomycin was found to be effective against aminoglycoside-resistant enterococci (31), and became an leading alternative therapy in the late 70's and early 80's (32). However, vancomycin resistance emerged in the mid-80's – first in Europe (33, 34), and then in the United States (35). Enterococci resistant to vancomycin are now globally disseminated, with as much as 80% of *E. faecium* isolates being resistant to vancomycin in some hospitals (4, 36).

The rapid rise of vancomycin resistance in enterococci at the beginning of the 21st Century raised alarms, as it represented the loss of an important bactericidal, last line therapy. Daptomycin was shown to be effective against vancomycin-resistant enterococci (VRE) (37), but its value was found to be limited by the rate of daptomycin-resistance development during the course of treatment (38–40). Resistant isolates had mutations in several genes that affect cell membrane composition and charge, including the cardiolipin synthase *cls*, glycerophosphoryldiester-phosphodiesterase *gdpD*, and the stress-sensing response component *liaF* (41, 42). Confounding a precise understanding of its mechanism of action, *Staphylococcus aureus* was observed to alter different pathways in achieving daptomycin resistance (43). Furthermore, resistance conferring mutations derived in enterococci *in vitro* only partially overlap with those that arise *in vivo*, potentially because of differences in lipid incorporation from the environment into the enterococcal membrane (44). Lipidomic studies of daptomycin-resistant strains showed differences in lipid profiles compared to sensitive strains, particularly in phosphatidylglycerols, cardiolipins and glycolipids (45).

Linezolid, the first oxazolidinone antibiotic, was approved by the FDA in 2000, and its introduction into clinical use was quickly followed by the emergence of linezolid-resistant VRE strains in the US in 2001 (46), and in the UK in 2002 (47). Sequencing of the resistant isolates revealed a G2576U mutation in the 23S ribosomal RNA subunit (48). This mutation was also seen in resistant organisms isolated from a gnotobiotic mouse model of infection (49), where its occurrence was found to depend on the dose of antibiotic that reached the intestine. A linezolid-resistant isolate from Thailand subsequently was shown to have acquired the *cfr* methyltransferase on a plasmid, leading to methylation of position A2503 in the 23S rRNA and causing high-level resistance (50).

A third antibiotic that has found utility in treating vancomycin resistant enterococci is the glycylicycline tigecycline. It has been used successfully, alone and in combination with other antibiotics, to treat VRE infection, but resistance has also emerged. A study of 73 tigecycline non-susceptible *E. faecium* and *E. faecalis* isolates collected from 2007–2015 found mutations in various efflux pumps that were identified and associated with tigecycline resistance (51). Serially passaged *E. faecium in vitro* in the presence of tigecycline selected for mutations in ribosomal protein subunit *rpsJ*, but this mutation has not yet been functionally validated (51, 52). Nonetheless, enterococci appear able to evolve resistance to antibiotics of all classes that have so far been introduced to practice.

Environmental persistence

As implied by the clonality of infection, epidemiological studies highlight person-to-person transmission of endemic strains within the hospital (53–55) implying that enterococci persist for extended periods in the hospital environment. Transmission occurs through contact with healthcare personnel, and inanimate objects such as bedrails, nursing station keyboards, hospital drapery, and ear-probe thermometers (56). Enterococci are unusually resistant to common antiseptics and disinfectants, as well UV radiation, starvation and desiccation (21, 57, 58). The ability to survive nutrient poor environments, as well as desiccation has led some to speculate that enterococci may enter a viable but non-culturable state as an adaptation to poor growth conditions (59–61), but details of such a mechanism have yet to be elucidated.

Studies have examined the environmental ruggedness mainly of *E. faecalis* (62–70), which develops tolerance to otherwise lethal levels of bile salts and detergents, such as sodium dodecyl sulfate (SDS), if first subcultured in the presence of sub-lethal levels (62). This phenomenon likely contributes to the ability of enterococci to survive cleaning regimens employed in most hospitals as part of infection control programs. Regulatory systems govern the adaptive response to environmental insults (71, 72). In a study by Hancock and Perego (71), inactivation of a response regulator, designated RR06, led to an increased sensitivity to heat stress (growth at 46°C), as well as detergent stress (0.003% SDS). Le Breton et al. (72) showed that another response regulator, RR10, negatively regulates the heat shock proteins DnaK and GroEL. Inactivation of the *rr10* gene resulted in acid-sensitivity, but also enhanced survival at 50°C.

To identify the genetic contributors to this intrinsic ruggedness, a spectrum of enterococcal species, including clinical isolates as well as species never reported to be associated with human infection, were compared for resistances to chemical compounds as well as environmental stresses (21). All enterococci were found to be intrinsically much more resistant to most insults than other related microbes, indicating that many of the underlying traits were acquired as the genus branched from its ancestors. The two enterococcal species that are most pathogenic to humans, *E. faecalis* and *E. faecium*, showed the greatest resistance to desiccation and starvation (21). These two species also show high levels of resistance to the common hospital disinfectants chloroxylenol and chlorhexidine. The molecular mechanisms that contribute to these phenotypes were narrowed to a set of 126 genes that distinguished enterococci from ancestors (21). Additionally, the two-component system ChtRS was recently identified as an important regulator of chlorhexidine resistance in *E. faecium* (73).

Pathogenic Mechanisms

For enterococci to cause disease several barriers must first be overcome. An initial barrier is the ability to overcome colonization resistance provided by competing microbes, and host defenses such as gastric acid and bile, and colonize the intestinal tract. From this reservoir the bacteria can amplify in number and spread to sites vulnerable to infection. A basic prediction from such a model is that the probability of infection should be a function of the intestinal burden of bacteria in the gut reservoir – the more bacteria, the greater the

probability of contamination of a potential infection site in numbers large enough to overcome host defenses. Indeed, colonization of the GI tract has been shown to be directly associated with risk of infection (23, 74). Infection occurs when enterococci overwhelm host defenses, replicate at rates that exceed clearance, and when pathologic changes result through direct toxin activity, or indirectly by bystander damage from the inflammatory response (75, 76).

Human Colonization

Enterococci are core but numerically minor members of the human microbiome (77). The occurrence of 2 species, *E. faecalis* and *E. faecium*, in similar abundance, suggests that neither evolved in humans, but rather likely entered the primate lineage early and from organisms consumed from lower in the food chain. Both *E. faecalis* and *E. faecium* are found in relative abundance in human feces (10^5 - 10^7 organisms per gram) (78), but typically represent less than 1% of the total microbial population (17). Colonization resistance (79) is a term applied to the challenge an organism faces in colonizing a stable, established community. It is a main mechanism of innate defense of the gastrointestinal tract. Colonization and proliferation of hospital-adapted lineages of enterococci is usually associated with antibiotic-induced disruption of the community structure (80). Therefore, effectively managing the human microbiome in health and disease represents a theoretically promising strategy for preventing hospital infection.

A primary barrier to invading the gut consortium and colonization by orally-acquired microbes is gastric pH, which is inhospitable for most microorganisms, including enterococci. Compromise of this barrier enhances the oral acquisition of enterococci from a contaminated environment. In the intensive care unit setting in particular, patients are placed on H₂-receptor antagonists as prophylaxis for treating stress ulcers; the consequence of this action results in a pH increase from pH 2 to pH 3.5–5.3, depending on the antagonist employed (81). In a study by Basaran et al. (82), H₂-receptor antagonists were shown to promote colonization of the small bowel, as well as translocation of enteric bacteria from the intestinal tract to extraintestinal tissues.

Several studies have examined the ability of enterococci to tolerate acidic pH (64, 83). Flahaut et al. (64) demonstrated that exposure of *E. faecalis* to a sub-lethal pH level (pH 4.8) for 15–30 minutes protected the organism to a normally lethal challenge at pH 3.2, suggesting an adaptive response to changing acid stress. Suzuki and colleagues (83) showed that an *E. faecalis* mutant defective in F₁-F₀ H⁺-ATPase activity was unable to grow at pH<6. The H⁺-ATPase is used to regulate the cytoplasmic pH of *E. faecalis* by proton extrusion, and this enzyme has been shown to be activated at low pH (83). Teng et al. (84) identified a two-component regulatory system, termed EtaRS, which appears to be involved in acid stress response and virulence. Inactivation of the response regulator, EtaR, results in increased acid sensitivity and decreased virulence in a murine peritonitis model.

Colonization resistance is also imposed by the stability of a mature complex community of gut microbes. The use of broad-spectrum antibiotics, many of which possess little or no anti-enterococcal activity, destabilizes this community by removing susceptible members (85–87), opening opportunities for invasion by new organisms. In a study designed to examine

the persistence and density of colonization by vancomycin-resistant *E. faecium* in a murine model, Donskey et al. (88) showed that suppression of the anaerobic microflora by the use of antimicrobials that target this microbial population resulted in increased colonization density and prolonged VRE persistence. This observation highlights the importance of the anaerobic flora in suppressing enterococcal growth within the intestinal microenvironment. Several other studies have also examined factors that lead to overgrowth of enterococci, especially VRE, in the GI tract of hospitalized patients. Treatment with broad-spectrum antibiotics, especially cephalosporins and metronidazole to which enterococci are intrinsically resistant, leads to clearance of commensals and overgrowth of enterococci (80, 89). In extreme cases, hospitalized patients treated with these antibiotics develop a near monoculture of VRE in their intestine, and are at high risk for enterococcal bacteremia and other enterococcal infections (80). Factors that limit the outgrowth of *E. faecium* in the antibiotic perturbed gut include reduced expression of the host innate immune factor RegIII γ , which results indirectly from the loss of gram-negative members of the gut microflora (90). RegIII γ production relies on signaling through TLR-5 and IL-22, and supplementation of TLR agonists has been shown to restore RegIII γ expression and suppress *E. faecium* growth (90). In mice, the IL-22 signaling pathway and in particular the IL-22 receptor has been shown to be required for *E. faecalis* colonization resistance. *Il22ra1*^{-/-} mice were observed to be particularly susceptible to *E. faecalis* overgrowth and subsequent systemic dissemination (91). This restriction of *E. faecalis* outgrowth is controlled in part by fucosylated glycans, which are downregulated in *Il22ra1*^{-/-} mice. Oral supplementation of fucosylated glycans was shown to promote diversity of the gut microbiota and restore the growth restriction of *E. faecalis* (91).

In addition to antibiotic and host immune factors, hospital-adapted strains of enterococci are endowed with properties that allow them to colonize microniches that are likely less suitable for colonization by commensal strains. For example, some hospital-associated strains of *E. faecalis* contain an additional bile salt hydrolase (92). *E. faecalis* also produces extracellular superoxide (93) leading to DNA damage in colonic epithelial cells (94) and chromosomal instability (95), potentially relating to local inflammation, changes in the nutritional properties of the mucosa, and development of colorectal cancer. Enterococci also have been associated with inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC). Enterococcal abundance, particularly of *E. faecium*, is higher in fecal samples from CD patients as compared to healthy controls (96, 97). Increased *E. faecalis* abundance and anti-*E. faecalis* antibodies have also been observed in UC patients (98). Germ-free IL-10 knockout mice colonized with *E. faecalis* develop chronic IBD-like symptoms (99), highlighting the bacteria's ability to perpetuate gut inflammation. A subset of virulence factors in *E. faecalis*, including the metalloprotease GelE, enterococcal polysaccharide antigen Epa, and cell surface-associated lipoprotein Lgt, were shown to influence induction of IBD in these mice (99).

Enterococci must compete with other microbes in the gut for space, binding sites and nutrients. Enterococci have long been known to be prolific producers of bacteriocins, many of which are encoded by mobile elements (100, 101). Plasmid pPD1, harbored by some *E. faecalis* strains, encodes a bacteriocin that inhibits other *E. faecalis* strains, eventually leading under some conditions to strain replacement *in vivo* (102). Importantly, the

bacteriocin-expressing strain was able to reduce the intestinal load of VRE in a co-colonization study, highlighting the potential therapeutic value of such agents.

The apparently hasty accretion of mobile elements by enterococci affects their ability to compete in surprising ways. It was recently shown that commensal *E. faecalis* are able to induce self-killing of the vancomycin-resistant strain V583, through the production of the pheromone peptide cOB1 (103). This pheromone induces a cascade of events that involves expression of conjugation-associated functions on a pheromone-responsive plasmid resident within V583, that then engage in a lethal crosstalk with a gene or genes in another mobile genetic element in the chromosome of V583, inducing death of V583 by an as yet unknown mechanism.

The ability to obtain nutrients within the competitive environment of the gut is an important aspect of enterococcal colonization efficiency (104). Within *E. faecium*, the two main clades A and B differ in the availability of carbohydrate utilization pathways. Strains in clade A1, which includes most infection-associated strains, express more transporters and metabolic pathways for the utilization of carbohydrates derived from the gut mucosa, as opposed to the diet, such as mucins and epithelial cell glycosidic surface decorations (104). Inactivation of a PTS system related to this ability reduced gut colonization in an antibiotic-treated mouse model (104, 105). Clade B strains do not possess this pathway (104), highlighting ecological differences between pathogenic and commensal strains. Meanwhile, clade B commensal *E. faecium* strains outcompete clade A strains in the murine intestinal tract in the absence of antibiotics (106).

Antagonisms within the gut community also influence colonization. Among commensals, *Barnesiella* species have been observed to be antagonistic to enterococcal colonization (107). Colonization resistance to VRE could also be artificially effected with a four-species cocktail containing *Bacteroides sartorii*, *Parabacteroides distasonis*, *Clostridium bolteae*, and *Blautia producta* (108). To colonize and establish residence within the gut, then, multidrug-resistant strains of enterococci must successfully navigate the hazards of innate host defenses, and enter a destabilized consortium lacking overt antagonists.

Translocation from the gut into the bloodstream

Enterococcal overgrowth in the colon increases the chance, by simple numeric probability, of dissemination into the bloodstream, and contamination of other body sites in numbers large enough to establish infection (Figure 2). Mechanisms that contribute to enterococcal translocation are still being discovered. In some cases, enterococci may be phagocytosed by intestinal epithelial cells, dendritic cells, or other tissue-resident leukocytes, and transported across the intestinal wall to the underlying lymph system. Failure to kill the phagocytosed organism could then lead to abscess formation in reticuloendothelial organs, and systemic spread (109). Gentry-Weeks et al. (110) showed that *E. faecalis* possesses the ability to survive for up to 72 hours in peritoneal macrophages. Wells et al. (111, 112) induced intestinal *E. faecalis* overgrowth by antibiotic treatment, and observed organisms adhering to epithelial surfaces of the ileum, cecum, and colon. They also showed that enterococci possess the ability to translocate from the intestinal lumen into the mesenteric lymph nodes, liver, and spleen (111, 112).

Bacteria also diffuse through the gut barrier at low levels, and enterococci may gain access to the bloodstream that way. Inert particles the size of bacteria, and without any special properties, penetrate the gut mucosal barrier (113) – special invasive properties do not appear to be essential for at least some level of translocation into the bloodstream. In a healthy, immune competent host, innate defenses are well equipped to eliminate such invaders. So long as the microbes leaking into the bloodstream and occurring in the lymphatics are easily killed, such as the predominating anaerobes in the healthy gut community, there is little ill effect. However, because enterococci persist in phagocytic cells, their extraintestinal accumulation may more readily lead to infection, especially in an immune compromised or disease-stressed patient.

A variety of enterococcal factors have been examined for role in colonization and translocation out of the gastrointestinal tract. Olmsted et al. (114) found a plasmid-encoded surface protein, aggregation substance, to promote internalization of *E. faecalis* by cultured intestinal epithelial cells (HT-29). To further dissect the functional domains of aggregation substance involved in internalization by HT-29 cells, Waters et al. (115, 116) examined the various domains of aggregation substance to determine their roles in this process. Surprisingly, the aggregation domain, and not the N-terminal RGD motif of aggregation substance, was found to be important for internalization by HT-29 cells (116). An additional finding from this study was the apparent requirement for a cofactor (likely lipoteichoic acid), as expression of aggregation substance in the binding substance mutant INY3000 failed to efficiently internalize. In a subsequent study it was shown that the amino-terminal aggregation domain of the protein could bind efficiently to purified LTA, as well as LTA purified from INY3000, suggesting that another cell component other than LTA was responsible for the cooperative internalization of *E. faecalis* by HT-29 cells (115).

Bacteremia

Enterococcal bloodstream infections (BSI) are associated with a high level of mortality. In a recent large survey study of Canadian hospitals (117), the incidence of enterococcal BSI of hospitalized patients was 6.9 per 100,000, with most cases due to *E. faecalis* (4.5 per 100,000). Incidence of enterococcal BSI increased between 2008–2014 (9–14 per 100,000) in Switzerland (118), suggesting that enterococcal BSI are on the rise. Overall mortality from nosocomial enterococcal BSI is quite high, ranging from 25–50% (119, 120).

Most cases of enterococcal BSI are thought to result from translocation of enterococci from the gut into the bloodstream. Other routes of infection include along intravenous lines, endocarditis, urinary tract infections and other abscesses (109, 121, 122). The risk factors for mortality associated with enterococcal bacteremia include severity of illness (based on APACHE II scores), patient age, and use of third-generation cephalosporins or metronidazole (123). Huycke et al. (54) found that patients infected with hemolytic, gentamicin-resistant *E. faecalis* strains had a five-fold increased risk for death within three weeks compared to patients with nonhemolytic, gentamicin-susceptible strains. Moreover, mode of treatment was not associated with outcome, discounting the direct contribution of aminoglycoside resistance. In another study, Caballero-Granado et al. (124) analyzed the clinical outcome, including mortality, for bacteremia caused by *Enterococcus* spp. with and

without high-level gentamicin resistance. Mortality associated with high-level gentamicin resistance (29%) was not significantly different from gentamicin-susceptible strains (28%). Taken together, these two studies suggest that high-level aminoglycoside resistance itself does not explain clinical outcome, and that the presence of other factors, such as the *E. faecalis* cytolysin (hemolysin) appear to contribute enhanced lethality. In support of the latter, the *E. faecalis* cytolysin has been shown to result in acute toxicity in a number of animal models. Cytolysin significantly lowers the LD50 of the infecting strain for mice (125–127). As will be discussed later, the cytolysin also contributes to the acute toxicity of lupine endocarditis and endophthalmitis models (128, 129), and also contributes to nematode killing in *C. elegans* (130).

Urinary Tract Infection

In a nosocomial population surveyed between 2011 and 2014, enterococci were the most commonly isolated gram-positive bacteria from catheter-associated urinary tract infections (CAUTI), with over 20,000 cases reported to the CDC National Healthcare Safety Network between 2011 and 2014 (4). Of these cases, just over 50% were caused by *E. faecalis*, followed by “other *Enterococcus* species” (~30%) and *E. faecium* (~20%). Most worrisome was the fact that ~85% of *E. faecium* isolates were vancomycin-resistant, with the fraction of resistant isolates increasing each year (4).

A few studies have been aimed at understanding the interaction of enterococci with uroepithelial tissue (131, 132). Kreft et al. (132) showed a potential role for the plasmid-encoded aggregation substance in the adhesion of enterococci to renal epithelial cells. *E. faecalis* harboring the pheromone-responsive plasmid pAD1, or various isogenic derivatives, were better able to bind to the LLC-PK cultured pig renal tubular cell line than plasmid-free bacteria. Their findings also showed that a synthetic peptide containing the fibronectin motif, Arg-Gly-Asp-Ser, could inhibit binding. This structural motif mediates the interaction between fibronectin and eukaryotic surface receptors of the integrin family (133). Guzman and coworkers (131) analyzed the ability of strains of *E. faecalis* isolated from either urinary tract infections or endocarditis to adhere to urinary tract epithelial cells and to the Girardi heart cell line. UTI isolates adhered to urinary tract epithelial cells *in vitro*, whereas strains from endocarditis adhered efficiently to the Girardi heart cell line, suggesting that environmental adaptations can facilitate interactions with host tissues. Shankar et al. (134) showed that the *E. faecalis* Esp surface protein co-localizes in the bladder, but not the kidneys, in an ascending urinary tract infection model, suggesting that the protein has tissue specificity for bladder epithelium. Tomita et al. (135) showed that highly adherent enterococcal UTI strains recognize the extracellular matrix proteins fibronectin, laminin, and collagen types I, II, IV, and V. One of these strains, AS14, was subjected to transposon mutagenesis with Tn916 and mutants with altered collagen IV and laminin binding were identified. Out of 14 single transposon insertion mutants, 13 mapped to the recently identified Ace protein (136–140). The level of adherence of mutants to collagen IV and laminin was 1 to 2 orders of magnitude lower than the wild-type strain, but the level of adherence to fibronectin remained the same as that of the wild-type strain. The collagen binding proteins Ace (*E. faecalis*) and Acn (*E. faecium*) were originally identified because of their homology to the amino terminal end of Cna, the collagen binding protein from *S.*

aureus (138, 139). The ability of most *E. faecalis* Ace-positive strains to adhere to collagen and laminin was highly dependent on growth temperature, with binding occurring at 46°C, but not at 37°C (136). No plausible explanation existed for this unusual binding phenotype, until observations by Tomita et al. (135) showed that gelatinase-positive *E. faecalis* strains adhered poorly to collagen and laminin due to the ability of the protease to cleave these proteins. Shiono et al. (141) showed that some *E. faecalis* clinical isolates adhere efficiently to human bladder carcinoma cells, as well as to human bladder epithelial cells, and that these interactions could be inhibited by pretreating the bacteria with fibronectin or trypsin.

More recently, several studies have identified a pilus in *E. faecalis* (140, 142–145), and have shown that Ebp pili are important for adhesion to the urinary epithelium and urinary catheters through the binding of fibrinogen released into the bladder during catheterization (146–148). In a mouse model of CAUTI, *E. faecalis* infection could be blocked by the administration of antibodies raised against the tip subunit of the Ebp pili, EbpA (149). It is hoped that these antibodies could potentially be used to prevent infection in catheterized patients without the use of antibiotics.

Endocarditis

Of the infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging (8, 150). Enterococci account for between 10–20% of all cases of IE (151, 152), with *E. faecalis* causing a majority of cases. In IE, bacterial colonization of the endocardium leads to vegetations within the tissue and a biofilm-like matrix surrounding the bacteria, which is difficult to penetrate either by the immune system or antibiotics (153–155).

Several virulence factors have been identified as playing a major role in the pathogenesis of enterococcal IE, with adhesion to host tissues by cell surface adhesins among the best characterized. In *E. faecalis*, aggregation substance (AS) represents a class of large, pheromone-inducible surface proteins involved in mediating cell-cell adhesion as a first step in conjugal plasmid transfer (156). These proteins also appear to be key players in both attachment to heart tissue and the development of biofilm during IE. Three highly-related AS proteins have been well studied in this regard, including Asa1, Asc10 and Asp (155, 157, 158). All three proteins appear to aid in cell-cell adhesion, and also contribute to the exopolymeric matrix that accumulates within the biofilm.

The EfaA antigen is another adhesin that was originally identified as expressed in serum but not broth culture (159), and database homology searches revealed extensive sequence similarity with several streptococcal adhesins. Shepard and Gilmore (160) showed that of all the suspected *E. faecalis* virulence factors, EfaA resulted in the most dramatic increase in expression when comparing serum-grown versus broth-grown *E. faecalis*. It is now known that EfaA forms part of a putative ABC-type transporter specific for manganese, along with EfaB and EfaC. As manganese is tightly sequestered within the mammalian host, it is not unexpected that *E. faecalis* grown in serum would require the induction of a high-affinity manganese transporter, EfaABC. The induction of the *efaCBA* operon occurs at relatively low manganese levels, as under high manganese levels the product of the *efaR* gene, a manganese responsive transcriptional regulator, represses transcription from the *efaCBA*

operon (159). More recently, EfaR inactivation has also been shown to impair the ability of *E. faecalis* to form biofilms, survive inside macrophages, and tolerate oxidative stress (161).

Pili encoded by the Ebp gene cluster in *E. faecalis* have also been shown to be important for both adherence and biofilm formation in IE (162). High titers of antibodies against the EbpABC proteins in patients with enterococcal IE have been demonstrated, indicating that they are expressed at high levels within the host during infection (162). Other important virulence factors in *E. faecalis* IE include the metalloprotease GelE (163), collagen-binding adhesin Ace (164), fibronectin-binding protein EfbA (165), cytolysin (128) and PTS permease BepA (166). A review of the many virulence factors that play a role in *E. faecalis* endocarditis was recently published (167).

In *E. faecium*, less is known regarding specific virulence factors and the role that they play in IE. However, the enterococcal surface protein Esp and the collagen-binding protein Acm have been shown to be important for the pathogenesis of *E. faecium* IE in a rat model of infection (168, 169). In infected animals, *E. faecium* strains that lack either Esp or Acm were recovered at lower numbers than their wild-type counterparts. Antibodies against both proteins have also been detected in patients diagnosed with *E. faecium* IE, and pretreatment of bacteria with anti-Acm antibodies derived from an infected patient was shown to reduce the ability of the bacteria to bind to collagen (162, 168). It seems that targeting the adhesive and biofilm development properties of enterococci, in addition to treatment with antibiotics, may be a promising method of treating enterococcal IE in the clinic.

Surgical site infections

Enterococci are common causes of surgical site infection at all anatomical sites (170), including the eye (129, 171). The eye affords a unique opportunity to visualize the evolution of such an infection using readily available equipment. Small numbers of organisms injected into the vitreous of a rabbit (129) or mouse (172), can be directly studied using an ophthalmoscope or slit lamp, and declining organ function can be measured quantitatively by electroretinography (173). Enterococci rapidly proliferate at this site, with a 10 μ l injection of enterococci suspended in PBS remaining as a light-refracting bead. After a few hours, the small bead acquires a coat of fibrin. About 12 hours later, the micro vessels in the retina begin to dilate, and subsequently turn white as they become coated with adherent neutrophils. After about 24 hours, neutrophils can be seen streaming from vessels in the optic nerve head to the infection nidus. These various measurement parameters allow various steps in the pathogenesis of infection to be dissected, and the roles of various traits expressed by the bacterium to be defined. In this model, the enterococcal cytolysin makes a profound contribution to organ destruction, which is not salvaged by otherwise effective antimicrobial or anti-inflammatory therapy (173). In contrast, identical infections initiated with isogenic cytolysin knockout strains resolved completely with antimicrobial and anti-inflammatory therapy, with little loss of organ function. Using this model, GelE and SprE proteases, both controlled by the Fsr quorum-regulated system, were observed to make measurable contributions to virulence in the rabbit model of endophthalmitis (174).

Root canal failure

Due in part to its resistance to many disinfectants and antiseptics, *E. faecalis* is an important cause of root canal failure (175, 176). This is particularly true in cases of chronic and persistent periradicular lesions leading to root canal failure, where *E. faecalis* was isolated in 24–77% of cases (176). Genetic analysis of *E. faecalis* strains isolated from infected root canals suggests that these strains, while not clonal, are highly related and may share additional traits that lead to efficient colonization of the oral cavity (177, 178). Calcium hydroxide, a common disinfectant used within the root canal, has been shown to be ineffective at killing *E. faecalis* on its own (179), as have other disinfectants such as EDTA and citric acid (180, 181). It appears that the bacteria can resist starvation within the root canal for long periods of time, and are then able to be resuscitated by serum that enters the canal from the alveolar bone and periodontal ligament (59). Provided with an adequate food supply, *E. faecalis* may utilize proteases such as SprE and GelE, and the collagen binding protein Ace, to bind to dentin and form biofilms (182). Once the infection is established, the most efficient treatments include prolonged washing of the canal with sodium hypochlorite or chlorhexidine solution (176). Using chlorhexidine or sodium hypochlorite washes during the initial procedure are thought to be the best options for preventing *E. faecalis* infection of the root canal and preventing failure.

Immune evasion

For enterococci to maintain an infection, they must successfully evade both specific and nonspecific host defense mechanisms. Other gram-positive pathogens possess attributes that allow them to survive in the host in spite of powerful nonspecific host defenses mediated primarily by professional phagocytes, i.e. neutrophils, monocytes, and macrophages. These factors include antiphagocytic polysaccharide capsules, antiphagocytic surface proteins, such as the Group A streptococcal M protein, and various secreted toxins with direct phagocytic cell toxicity. The most important cell type for clearing bacteria during the early innate immune response to enterococcal infection is neutrophils, with complement and antibody-mediated killing also playing a role (183). Neutropenic mice are less able to clear *E. faecium* from their bloodstream as compared to wild-type counterparts, and show higher inflammatory cytokine levels after intraperitoneal infection (184).

Numerous studies have characterized the host response to enterococcal infection (183, 185–187). *E. faecalis* have been found to be able to actively cleave the complement protein C3, mediated by the metalloprotease GelE, leading to reduced phagocytosis and higher rates of bacterial survival (188). In a case report, Bottone and colleagues (189) isolated three highly mucoid encapsulated strains of *E. faecalis* from patients with urinary tract infections. These mucoid isolates appeared to persist longer in the mouse following intraperitoneal injection compared to non-mucoid controls, but crude mortality was not changed, suggesting a role in preventing or delaying phagocytic clearance (190). Once phagocytosed, *E. faecalis* are able to resist intracellular killing in both polymorphonuclear leukocytes (PMNs) and macrophages (110). In PMNs, opsonized bacteria are more readily killed than unopsonised cells, and aggregation substance appears to limit opsonization leading to survival within phagosomes (191). Recent studies examining *E. faecalis* survival in macrophages showed that phagosomes containing *E. faecalis* did not acidify or fuse with autophagosomes, leading

to greater bacterial survival (192). *E. faecalis* is also able to survive the oxidative burst within macrophages, which is in part mediated by the production of catalase (112, 193). Survival within macrophages may also allow for bacterial translocation across the intestinal epithelium, leading to dissemination of *E. faecalis* throughout the body (112).

Several studies have examined the diversity of cell wall polysaccharides produced by *E. faecalis* (194–197). Based on genetic and serologic data, *E. faecalis* capsular polysaccharides fall into four serogroups (197, 198). Antibodies to *E. faecalis* capsular polysaccharides do however cross-protect across the species boundary (196), as antibodies to *E. faecalis* capsular antigens are reactive with some *E. faecium* capsular antigens and mediate opsonophagocytosis. The genetic basis for *E. faecalis* capsular biosynthesis is encoded by the *cpsC-K* operon (194, 199). In addition to capsular antigens, Murray and coworkers have identified another cell wall polysaccharide, called the enterococcal polysaccharide antigen, whose synthesis is encoded by the *epa* locus (200–202). This pathway appears to be widespread and conserved within *E. faecalis* and it has been designated the *E. faecalis* group antigen (194), analogous to the streptococcal group antigens. Antibodies to the Epa polysaccharide have also been shown to promote opsonophagocytic clearance by neutrophils (200), but this prospect remains controversial (196). A recent study showed that inactivation of a predicted wall teichoic acid biosynthetic gene in *E. faecalis* caused differences in wall teichoic acid and Epa composition, and led to increased susceptibility to complement-mediated killing by neutrophils (203). Finally, enterococcal cell surface-associated polysaccharides have been found to impact GI tract colonization and infection in a variety of settings (99, 195, 204–207). The extent to which enterococcal surface-associated polysaccharides could be used as potential vaccines awaits further study.

Tissue Damage

Following adhesion to host cell surfaces and evasion of the host immune response, the last step in the pathogenesis of enterococcal infection is the production of pathologic changes in the host. Such changes can be induced by the host inflammatory cascade or by direct damage as a result of secreted toxins or proteases, as well as the production of toxic metabolic byproducts. Each of these mechanisms has been observed in studies of *E. faecalis* pathogenesis.

Indirect Tissue Damage

Enterococcal lipoteichoic acid (LTA), also known as the Group D streptococcal antigen, has been implicated in a variety of biological processes (204, 208–211). Some properties ascribed to LTA include modulation of the host immune response as well as mediating the adherence of enterococci to host cells. Bhakdi et al. (212) found LTA from enterococci to be as inflammatory as lipopolysaccharide (LPS) of gram-negative bacteria, although more recent findings dispute this claim (213). Strains of *E. faecalis* defective in enterococcal binding substance (which is at least partially derived from LTA), and also defective in the protein adhesin aggregation substance, did not induce clinical signs of illness when injected intraventricularly into rabbits (214). While EBS⁺AS⁻ or EBS⁻AS⁺ strains caused signs of illness and pericardiac inflammation, all rabbits injected with the EBS⁺AS⁺ strain developed illness and died. Surprisingly little inflammation was observed in rabbits injected with the

EBS⁺AS⁺ strain, despite the lethality observed. The authors state that such observations are consistent with the presence of a superantigen. The presence of LTA (EBS) and AS together may mediate effects on the host immune response that differ from those seen when either component acts alone.

Direct Tissue Damage

The enterococcal cytolysin and two proteases, a zinc metalloprotease (gelatinase) and a serine protease, are secreted factors well suited to contribute to disease severity (126, 128, 129, 215, 216). The enterococcal cytolysin is well established in several independent labs and animal models (126, 128, 129). The presence of the cytolysin has also been shown to promote the appearance of *E. faecalis* in the bloodstream (217). The genetics and biology of the enterococcal cytolysin have been reviewed elsewhere (218, 219). The toxin is genetically and structurally related to the lantibiotic family of bacteriocins, but diverges from this family in that it possesses both bacteriocin activity as well as toxicity towards a variety of mammalian cell types. The cytolysin operon is typically encoded on large pheromone-responsive plasmids, but has also been shown to reside within an *E. faecalis* pathogenicity island (134). The operon consists of six genes, designated *cylL_L*, *cylL_S*, *cylM*, *cylB*, *cylA*, and *cylI*, with each gene product playing an essential role in toxin synthesis, modification, secretion, activation, and immunity. Transcription of the operon is repressed by the products of a divergent operon encoding *CylR1* and *CylR2*, and repression is alleviated by the accumulation of fully processed *CylL_S*, which serves as a quorum sensing molecule to prime the synthesis of more toxin components as cell density increases (220).

The most direct and quantitative evidence for pathologic damage attributable to the cytolysin was obtained using a rabbit model of endophthalmitis (129). This model was selected because a robust infection can be established with as few as 10 organisms due to the natural aberrations in the intraocular immune response. This limited response provides the offending bacterium an opportunity to adapt to *in vivo* growth conditions and environmental cues. Moreover, highly sensitive and quantitative measurements of the evolution of disease can be made. A role for the cytolysin in tissue pathology was unambiguously demonstrated both by a reduction in β -wave response (ERG), and complete destruction of retinal architecture 24 hours post-infection (129).

Because of its broad protease specificity, the enterococcal gelatinase may also play a measurable role in systemic disease (127), as well as in a model of dental caries using germ-free rats (215). In the germ-free rat model, Gold et al. (215) showed that a proteolytic (Gel+) strain exhibited cariogenic activity, whereas three nonproteolytic strains exhibited little cariogenicity. Dupont et al. (127) showed a reduced LD50 for mice injected with gelatinase-producing (Gel+) strains, and these findings were subsequently confirmed using isogenic strains defective in protease production (216). On the same transcript as the *geE* gene resides the *sprE* gene, which encodes a serine protease. Both proteases have been shown to play important roles in *C. elegans* nematode killing (221), lupine endophthalmitis (222), and murine peritonitis (216). As discussed previously, gelatinase has been shown to be important in *E. faecalis* biofilm formation (223, 224), and also appears to play a role in the degradation of the host extracellular matrix proteins collagen and laminin (135).

Both gelatinase and the SprE serine protease are regulated at the transcriptional level by a quorum sensing signal transduction system termed *fsr* (225). The *fsr* locus encodes a response regulator, FsrA, which is thought to be activated by phosphorylation at a conserved aspartyl residue by phosphotransfer from a histidine kinase, FsrC. The kinase is thought to undergo autophosphorylation upon sensing the accumulation of a peptide lactone quorum molecule (226), which is encoded at the carboxy-terminus of FsrB. Most if not all the phenotypic effect of inactivating the *fsr* locus resides in the lack of protease production. While initially the only genes thought to be transcriptionally controlled by activated FsrA were *fsrB/C* and *gelE/sprE*, various studies have suggested that additional genes are controlled by the *fsr* locus (221, 222, 227). More recently a third locus under the control of FsrA has been identified (228); this locus encodes the antimicrobial peptide Enterocin O16 (229).

There is some indication that clinical isolates of *E. faecalis* may be enriched for the proteolytic trait, as greater than 50% of isolates from both endocarditis and other clinical sources exhibited gelatinase activity, whereas only 27% of community fecal isolates possessed this trait (230). Studies that have explored the presence of *fsr* genes and *gelE* in epidemiology have found widely differing results (231–234). In addition to differences in sample size and setting, this could be because a large percentage of gelatinase-negative clinical isolates contain a 23.9-kb chromosomal deletion that results in the deletion of both *fsrA* and *fsrB*, and yields a null protease phenotype (235). As this represents a defined deletion, it is tempting to speculate why *E. faecalis* would delete these important virulence factors. Analogous to the description of the *E. faecalis* pathogenicity island, which is known to be modulated through the insertion and deletion of genetic information (92), the organism may simply choose to modulate its virulence for fear of overtly offending its host.

While *E. faecium* appears to lack both proteolytic and cytolytic activities, it has been shown to encode a hyaluronidase (236), which appears to be enriched in non-stool isolates. Hyaluronidase activity has yet to be demonstrated in these isolates, but it is conceivable that this trait is emerging within the *E. faecium* population. An *E. faecium* pathogenicity island has also been described, which contains an ortholog of the *E. faecalis* *esp* gene (237, 238). Apart from the *esp* gene and an *araC*-like transcriptional regulator, none of the remaining ORF's resemble those present in the *E. faecalis* pathogenicity island, and the nature of this element remains largely unknown.

Toxic Metabolites

In addition to secreted proteins, *E. faecalis* and *E. faecium* have also been shown to produce toxic oxygen metabolites that can cause cell or organ damage (94, 239). Using a worm model, Moy et al. (239) demonstrated that *E. faecium* produces hydrogen peroxide at levels that cause cellular damage. *E. faecium* transposon insertion mutants were identified which altered *C. elegans* killing activity and displayed altered levels of hydrogen peroxide production. Mutation of an NADH oxidase-encoding gene eliminated nearly all NADH oxidase activity and reduced hydrogen peroxide production and killing, whereas mutation of a gene encoding an NADH peroxidase resulted in enhanced levels of hydrogen peroxide and more rapid nematode killing. Depending on the culture conditions, *E. faecium* can produce

hydrogen peroxide at varying levels, and this production appears to correlate inversely with nematode survival.

According to one study, the vast majority (87/91) of *E. faecalis* strains tested produce superoxide (O_2^-), whereas *E. faecium* isolates (5/13) did so less frequently (240). The study authors speculated that membrane-damaging effects of oxygen radicals might potentiate cellular damage to nearby intestinal epithelial cells. In proof, it was shown that the production of extracellular superoxide and hydrogen peroxide damages colonic epithelial cell DNA (94). The answer to why *E. faecalis* produces extracellular oxygen radicals stems from the fact that this oxygen radical is a byproduct of an incomplete respiratory chain. *E. faecalis* is capable of reconstituting a cytochrome complex in the presence of exogenous heme (241), enabling it to effectively respire. The fact that enterococci generate a byproduct that damages its host appears to be an unintended consequence of incomplete respiration.

Comparative Genomics of *E. faecalis* and *E. faecium*: Emergence of Pathogenic, Resistant Lineages

With the development and widespread use of next-generation sequencing and comparative analysis of bacterial genomes, a great deal has been learned about the genetic makeup of pathogenic *E. faecalis* and *E. faecium* strains. The first enterococcal genome to be completely sequenced was a vancomycin-resistant *E. faecalis* strain isolated from an HIV-infected patient (35, 242). The genome of this isolate, termed V583, made apparent the importance of mobile genetic elements in the development of *E. faecalis* into a hospital-adapted pathogen (35, 242). When compared to a commensal-like *E. faecalis* strain, OG1RF (243), which harbors no known functional mobile elements, V583 was found to possess a genome more than 25% larger as the result of harboring three plasmids, a pathogenicity island (PAI) (92, 243), and several integrated prophages (242).

First identified in the multiple drug-resistant strain MMH594 (92), the PAI has been shown to be widely distributed among hospital-adapted, pathogenic strains of *E. faecalis*. It encodes the virulence factors cytolysin and aggregation substance (AS), ESP, and a variety of other elements believed to play a role in pathogenesis (92, 244). Overall, the trend of increased mobile genetic element content among multidrug-resistant, hospital-adapted strains holds true within *E. faecalis*, and is strongly correlated with the absence of a functional CRISPR-Cas system (245).

The population structures of *E. faecalis* and *E. faecium* have been widely studied, initially by MLST and related techniques (246–249), and more recently by comparing sequences of entire genomes of many isolates of both species (104, 250, 251) (Figure 3). These studies revealed a pronounced clade structure within the *E. faecium* species. The two main branches, clades A and B, are so divergent from one another that they approach the limits for designation as separate species (Figure 3A) (104, 248). Most hospital-adapted *E. faecium* isolates belong to clade A, and closely resemble strains isolated from agricultural and companion animals. Non-hospital associated human fecal isolates of *E. faecium*, on the other hand, mainly belong to clade B, and appear to have diverged from clade A strains several thousand years ago (104, 248, 252–254).

In contrast to the pronounced clade structure of *E. faecium*, the population structure of *E. faecalis* is less discrete (Figure 3B) (250, 251). A study of *E. faecalis* mainly derived from Great Britain revealed three dominant lineages, named L1, L2, and L3 (Figure 3B) (250). However, these lineages are fairly closely related, compared to the A and B clades of *E. faecium*. This finding supports previous work comparing a smaller set of *E. faecalis* genomes (253).

Animal Models to Study Pathogenic Mechanisms

Several animal models have been developed to study different aspects of enterococcal pathogenesis. Using both invertebrate and vertebrate host organisms, many important discoveries have been made in identifying the roles of specific virulence factors in enterococcal disease. Model organisms have also been employed in the discovery of novel antibacterial compounds. Here we summarize major advances in the establishment of relevant models of enterococcal disease; a more complete examination of models of enterococcal infection has been presented recently in two excellent reviews (255, 256).

Invertebrates

Invertebrate models of enterococcal infection have been very useful due to the ability to use large numbers of organisms in each experiment, and because they are known to be natural hosts of enterococci in the environment. *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* have all been used in different infection contexts, largely to examine the effects of virulence factors on lethality of the whole organism (130, 257, 258). It has been observed that there is good correlation between virulence factors in invertebrate hosts and in mammalian infection models (130, 221). Invertebrates, particularly *C. elegans*, have also proven to be very fruitful for high-throughput screening for novel anti-enterococcal compounds, as they allow for identification of bactericidal compounds with low host toxicity, as well as host immunomodulatory molecules that impact the course of infection (259).

Vertebrates

Several infection models have been established in small mammals for many of the most important enterococcal diseases. Two of the earliest infection models were developed for enterococcal IE, first in rabbits by MacCallum and Hastings in the first description of enterococcal disease (260), and later modified by Garrison and Freedman (261). A rat model of infection was developed by Santoro and Levison in the 1970s (262). Both of the rabbit and rat IE models involve the insertion of a catheter into the heart valve followed by administration of bacteria. The introduction of a foreign body allows for initial adherence of enterococcal cells, resulting in a robust infection of the cardiac tissue. The determination of many important virulence factors for endocarditis have been shown using these animal models (128, 157, 255).

Intestinal colonization by *E. faecalis* and *E. faecium* has been studied in antibiotic-treated mice in order to model antibiotic treatment in hospitalized human patients. Generally, mice are treated with high concentrations of broad-spectrum antibiotics, particularly

spectinomycin or metronidazole, to allow enterococcal invasion into the microbiota. Kommineni and colleagues were able to obtain stable, long-term colonization of an introduced *E. faecalis* strain into an intact mouse microbiota through long-term administration of the bacteria in drinking water (102). Recently, studies examining the localization of *E. faecalis* within the intestinal tract have been performed in germ-free mice, allowing for direct visualization of bacteria in contact with intestinal tissue (263). How closely this localization resembles that of enterococci in a fully colonized animal has yet to be determined.

Mice have also been used with great success to study bacteremia and peritonitis caused by *E. faecalis* and *E. faecium* (126, 264). Urinary tract infection models, both catheter-associated and endogenous, have been established in female mice and were instrumental in identifying virulence factors, particularly adhesins, in enterococcal pathogenesis (134, 147, 265, 266). Finally, to study postoperative infections, a highly tractable rabbit endophthalmitis model was established by Stevens and colleagues in 1992 (267). This model involves the direct placement of small numbers of bacteria into the vitreous, directly behind the lens, where the evolution of infection can be easily observed by direct ophthalmoscopy as well as slit lamp biomicroscopy. This allows the influence of a number of virulence traits on the host/microbe dynamic to be assessed in real time (129).

From one of the first descriptions of enterococcal infection in 1899 (3) to the understanding of the role of virulence factors in disease and identification of novel antimicrobials, animal models have proven to be extremely important in the study of enterococcal pathogenesis, and their further development will continue to provide insight into diseases caused by enterococci.

Conclusions and Perspectives

Enterococci are unusually well adapted for survival and persistence in a variety of adverse environments, including inanimate surfaces in the hospital environment and at sites of infection. This intrinsic ruggedness undoubtedly played a role in providing opportunities for enterococci to interact with other overtly drug-resistant microbes and acquire additional resistances on mobile elements. The rapid rise of antimicrobial resistance among hospital-adapted enterococci has rendered nosocomial infections a leading therapeutic challenge. Having added about a quarter of a genome of additional DNA conveyed by mobile elements, there are undoubtedly many more properties that have been acquired that help enterococci persist and spread in the hospital setting, and cause disease that have yet to be defined. Much remains to be learned about these ancient and rugged microbes.

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Figure 1.

Animal hosts that have been associated with enterococcal colonization. A simplified tree of life, with blue shading indicating animals from which enterococci have been isolated.

Corresponding geologic periods are indicated on the left. Reproduced with permission from (11).

Figure 2. Routes of dissemination of enterococci from the intestinal reservoir of a hospitalized patient. Bacteria from the intestine can seed infections throughout the body and contaminate surfaces, leading to patient-to-patient spread. Reproduced with permission from (122).

A

B

Figure 3.

Population structure of *Enterococcus faecium* and *Enterococcus faecalis* based on comparisons of whole genomes. (A) RAxML tree of 1,344 single copy core genes found in 73 *E. faecium* genomes, showing the clade structure of the *E. faecium* population. Reproduced with permission from (104). (B) Phylogeny of 515 *E. faecalis* isolates based on 1,293 conserved core genes found in 99% of isolates. The L1, L2, and L3 lineages are

highlighted with red, purple, and turquoise lines, respectively. Reproduced with permission from (250).

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Table 1.

Enterococcal virulence factors

Virulence factor	Description	Reference
Cytolysin ^a	Hemolysin/bacteriocin, lyses a broad range of eukaryotic and gram-positive cells	(128, 129, 218, 268)
Gelatinase ^a	Secreted zinc metalloprotease	(216, 221, 222)
Serine Protease ^a	Secreted serine protease	(216, 221, 222)
Hyaluronidase ^b	Degrades hyaluronic acid	(236)
Esp ^{a,b}	Cell wall anchored protein, enhances biofilm formation and colonization of bladder epithelium	(92, 237)
Aggregation substance ^{a,b}	Cell wall anchored protein, involved in conjugation and adhesion to eukaryotic cells	(128, 132, 214, 269)
Acc ^{a,b}	Cell wall anchored protein, collagen binding protein	(135, 136, 138, 139)
Epa ^{a,b}	Enterococcal polysaccharide antigen, anti-phagocytic cell wall polysaccharide	(200)
Cps ^{a,b}	Capsular polysaccharide, anti-phagocytic cell wall polysaccharide	(194, 196)
LTA ^{a,b}	Lipoteichoic acid, enterococcal group antigen, binding substance for conjugation	(214, 270)
Toxic metabolites ^{a,b}	Reactive oxygen species: extracellular superoxide, hydrogen peroxide	(94, 239)

^aVirulence factors identified in *Enterococcus faecalis*^bVirulence factors identified in *Enterococcus faecium*