Let Me Upgrade You: Impact of Mobile Genetic Elements on Enterococcal Adaptation and Evolution

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ABSTRACT Enterococci are Gram-positive bacteria that have evolved to thrive as both commensals and pathogens, largely due to their accumulation of mobile genetic elements via horizontal gene transfer (HGT). Common agents of HGT include plasmids, transposable elements, and temperate bacteriophages. These vehicles of HGT have facilitated the evolution of the enterococci, specifically *Enterococcus faecalis* and *Enterococcus faecium*, into multidrug-resistant hospital-acquired pathogens. On the other hand, commensal strains of *Enterococcus* harbor CRISPR-Cas systems that prevent the acquisition of foreign DNA, restricting the accumulation of mobile genetic elements. In this review, we discuss enterococcal mobile genetic elements by highlighting their contributions to bacterial fitness, examine the impact of CRISPR-Cas on their acquisition, and identify key areas of research that can improve our understanding of enterococcal evolution and ecology.

KEYWORDS enterococci, horizontal gene transfer, mobile genetic elements, pheromone-responsive plasmid, phage, transposon, virulence, antibiotic resistance, CRISPR

Enterococci, commensal bacteria that make up less than 0.1% of the healthy intestinal microbiota (1), have continuously faced selective pressure for genes that provide beneficial adaptations, facilitating coevolution with eukaryotes since the early Paleozoic era (2). Around the 1960s, it was discovered that, in addition to being resident members of the human microbiota (3, 4), the enterococci colonize the gastrointestinal (GI) tracts and guts of other animals and insects (5, 6), and are members of food (7), plant (8), soil (9), and water ecosystems (10). Since the 1980s, enterococci have emerged as opportunistic pathogens, causing hospital-acquired urinary tract, wound, endocarditis, and bloodstream infections (11, 12). Antibiotic therapies can cause multidrug-resistant (MDR) enterococcal expansion in the intestine as other resident microbes are depleted, resulting in life-threatening infections due to enterococcal translocation across the intestinal barrier into the bloodstream (13, 14). MDR enterococci, including vancomycin-resistant enterococci (VRE), cause over 54,000 infections in the United States annually (15).

Studies on enterococcal epidemiology (discussed comprehensively in reference 16) reflect that enterococci emerged as a major cause of nosocomial infections in two distinct phases. During the late 1970s, *Enterococcus faecalis* strains dominated the clinical isolate pool (17). Since the early 2000s, however, vancomycin-resistant *Enterococcus faecium* isolates became more prevalent than *E. faecalis* (18, 19). Recently, health care systems of many countries, including those in North America and Europe, have witnessed increasing levels of VRE infections (20). According to the surveillance data from the National Healthcare Safety Network, VRE are responsible for 3% of all reported nosocomial infections (21). Several reports indicate that the risk of VRE infections is particularly high among intensive care unit (ICU) and immunocompromised patients (20). Further, mortality rates due to VRE-mediated bacteremia are notably higher than those caused by vancomycin-susceptible enterococci (20).

Excessive exposure to antimicrobials used to sterilize hospital surfaces and broad-spectrum antibiotics has resulted in the rise of MDR *E. faecalis* and *E. faecium* (22). These

accessory resistance genes complement the enterococci's intrinsic resistance to cephalosporins, penicillins, and clinically relevant concentrations of aminoglycosides (17, 23). Considering the clinical significance of vancomycin-resistant enterococcal infections, several studies have focused on the ecology, evolution, and dissemination of vancomycin resistance genes (reviewed in reference 24). Clinical *E. faecalis* and *E. faecium* isolates resistant to the "last-line-of-defense" antibiotics, linezolid and daptomycin, have also been identified (23, 25, 26). While exploring adaptive traits of vancomycin-resistant *E. faecium* in immunocompromised patients, researchers noted that the emergence of linezolid or daptomycin resistance is associated with prolonged antibiotic exposure in these patients (27).

E. faecalis and *E. faecium* have adapted to nosocomial settings due to their ability to acquire mobile genetic elements (MGEs) through horizontal gene transfer (HGT). HGT facilitates acquisition of novel traits, including genes that promote virulence and antibiotic resistance (28–32). Mobile genes that increase clinical resilience are genetically similar to genes found in other bacterial genera, suggesting their broad distribution among pathogens (29, 33, 34). Clinical *E. faecium* strains have twice as many genes associated with MGEs than nonclinical strains (35), while some clinical isolates of *E. faecalis* are comprised of up to 25% MGEs (36). Transferable plasmids (mobilizable and conjugative) and bacteriophages play central roles in HGT of bacterial genes and transposons (37), driving bacterial evolution. Many MGEs encode antimicrobial resistance (AMR) genes, which we will describe in this review. Antimicrobials used to combat enterococcal infections, such as tetracycline, aminoglycosides, and erythromycin, were nullified due to the emergence of resistance genes that were rapidly disseminated among the enterococci via MGEs (38). This has necessitated updated treatment guidelines for MDR enterococcal infections and development of the new antibiotic treatment regimens, such as daptomycin and linezolid (39–41).

Despite their benefits to the bacteria, MGEs can adversely affect their growth and survival (42, 43). Plasmid maintenance requires synthesis of additional cellular macromolecules and can be a metabolic burden on the host, while conjugation requires the production of pili, secretion systems, and additional DNA replication proteins following induction. It is important to note that, often, these fitness costs exist initially, but over time, these costs are mitigated via adaptive evolution, and the benefits of accessory plasmid genes can outweigh the costs (44, 45). Additionally, it is possible that conjugative structures may expose the bacteria to virulent phages that are otherwise unable to penetrate bacterial cellular barriers (46). This phenomenon has been studied for the F pili of *Escherichia coli* and filamentous phages but has not been thoroughly explored in enterococci (47). MGEs like prophages and transposons can integrate into bacterial chromosomes and disrupt gene function (48, 49). Stressors and nutrients, such as Casamino Acids, various temperatures, and DNA-damaging antimicrobials, drive prophage induction leading to bacterial lysis (50, 51). Hence, bacteria have acquired defense systems to protect themselves from these foreign MGEs. Clustered regularly interspaced short palindromic repeats (CRISPR) and corresponding Cas proteins represent an adaptive bacterial genetic system that can prevent MGE acquisition (52).

In this review, we (i) summarize current knowledge of enterococcal MGEs, with a specific emphasis on *E. faecalis* and *E. faecium*, (ii) highlight the contributions of MGEs to enterococcal fitness within the scope of intestinal colonization and infection, (iii) discuss the impact of CRISPR-Cas on enterococcal MGE acquisition, and (iv) identify key areas of MGE research that, if explored, should improve our understanding of enterococcal evolution and ecology.

CONTRIBUTIONS OF PHAGE-MEDIATED HGT TO ENTEROCOCCAL GENOME DIVERSITY AND NICHE ADAPTATION

Bacteriophages (phages) are viruses that infect and replicate in bacteria and are the most abundant organisms on earth (53). Since the initial discovery of enterococcal phages almost a century ago (54), these phages have been isolated and characterized from diverse environments, including wastewater, livestock runoff, and the mammalian

intestine, with the most commonly studied enterococcal phages today being those that infect *E. faecalis* and *E. faecium* (55–57). Phages often lyse bacteria, but some phages (temperate phages) can integrate into the bacterial genome, an event termed lysogeny. Since phages can transfer DNA from cell to cell via transduction, phage-driven HGT can have a significant impact on host genetic composition and phenotypic characteristics as well as the surrounding microbial community (reviewed in references 58 and 59).

Phage-adapted replication and genome packaging mechanisms influence phage-mediated HGT. Upon infection, temperate phages integrate into the host genome and are maintained as prophages. Newly integrated prophages can supply genes which enhance host fitness, including antibiotic resistance, stress tolerance, and immunity against secondary infection by closely related phages (superinfection exclusion) (51, 60–62). In this scenario, the temperate phage is an MGE, whereupon integration, phage auxiliary genes bestow a benefit upon the host. HGT can also occur via specialized transduction when prophages excise specific bacterial DNA flanking the phage integration site and package it into progeny virions (59, 63). Additionally, generalized transduction occurs when nonphage, chromosomal DNA fragments are packaged into phage capsids (59, 63). The recently discovered process of lateral transduction occurs *in situ* prior to prophage excision, resulting in packing of up to several hundred kilobases of host DNA into infectious virions (64). The authors demonstrated that these *Staphylococcus aureus* prophages can integrate into a new host, carrying the DNA from the previous host. In each mode of transduction, host DNA-carrying phages can infect vulnerable hosts where the DNA can be integrated into the chromosome. Prophages can confer a competitive advantage to their host bacterium by switching to the lytic cycle and producing infective viruses which target phage-susceptible bacteria (65, 66). The shift from the lysogenic to lytic cycle can occur spontaneously or in response to UV radiation and antibiotics that induce DNA damage, triggering the bacterial SOS response (51, 67–71). Although the mobilization of phage-encoded virulence determinants and other factors are well studied in some Gram-positive pathogens such as *S. aureus* and group A *Streptococcus* (72–75), much remains unknown about the influence of prophages on enterococcal virulence and genetic plasticity.

The emergence of MDR enterococci makes it imperative to identify and characterize drivers of enterococcal evolution. With limited studies highlighting phage-mediated HGT in enterococci, we are just now appreciating potential contributions of phages to enterococcal evolution. Yasmin and colleagues demonstrated that temperate phages induced from clinical *E. faecalis* isolates are capable of intraspecies generalized transduction of antibiotic resistance genes and *gelE* encoding a metalloprotease implicated in virulence (76, 77). Enterococcal phages have also been shown to successfully transfer tetracycline and gentamicin resistance between enterococcal species via generalized transduction (78). Enterococci are not intrinsically resistant to tetracycline; enterococcal tetracycline resistance is common due to the distribution of the resistance genes *tetM* and *tetL* (23). While gentamicin monotherapy is not used to treat enterococcal infections, it is used synergistically with beta-lactams to achieve a 70% cure rate in susceptible isolates (23, 79). Other studies observed that the fitness and virulence of a probiotic *E. faecalis* strain were significantly enhanced when transduced by temperate phages from a pathogenic *E. faecalis* strain (80). Considering that enterococci regularly encounter prophage-inducing environmental stimuli in the GI tracts of animals, including bacterial signaling molecules (80), dietary components (50, 81), and sublethal antibiotic doses (51, 76), it is likely that temperate phage-mediated HGT can increase the distribution of pathogenic traits among enterococci.

Studies highlighting widespread prophage distribution among virulent enterococci underline the importance of prophage dynamics during enterococcal pathogenicity. The well-characterized clinical VRE isolate *E. faecalis* V583 harbors seven prophage-like elements. These elements, designated pp1 to pp6 and EfCIV583 (phage-related *E. faecalis* chromosomal island), constitute up to 10% of the V583 genome (36, 82). Upon

excision of these prophage-like elements, host cell lysis is not detected (51). Polylysogeny, the presence of more than one prophage-like element in the genome, has been reported in multiple clinical isolates of *E. faecalis* besides V583 (36, 76, 82), suggesting that *E. faecalis* prophages are more widely distributed than previously thought. Contrastingly, there are still many strains of *E. faecalis* that do not harbor multiple prophages, such as the common lab strain OG1RF. Pp2, a cryptic prophage, is a part of the core *E. faecalis* genome (82). Pp2 has yet to be shown to excise from the bacterial chromosome and produce infectious virions; however, pp2 tail proteins have been identified in the supernatant of *E. faecalis* OG1RF cultures (83). Interestingly, it was recently observed that while the presence of clinically relevant antibiotics altered the excision of six of seven prophages via the *E. faecalis* V583 SOS response, including DNA-damaging antimicrobials and various temperatures, only four of these prophages were able to form infectious virions (51). Additionally, studies have elucidated interaction between coinhabiting prophages, ranging from cooperation to interference. EfCIV583 relies on pp1 for induction, genome packaging, and transmission but interferes with pp1 infectivity (50, 51, 84). On the other hand, the presence of pp1 impedes pp4 excision, while pp6 excises only when both pp3 and pp5 are deleted from the chromosome (51). Using an innovative DNA sequencing-based "transductomics" approach, wherein deep sequencing identifies DNA originating from ultrapurified virus-like particles, such as excised prophages, it was discovered that pp1, pp5, and EfCIV583 are capable of transducing at relatively high frequencies, potentially through lateral transduction, a recently discovered form of HGT (64, 85). The authors observed that these *E. faecalis* V583-transducing phages not only carried bacterial DNA surrounding either the region of the prophage integration site but also packaged several additional regions, including transposase-carrying insertion sequence (IS) elements and the three rRNA operons (85). The authors did not report mobilization of antibiotic resistance genes. Using this technology, future studies can reveal virus-like particle-host interactions in complex communities like the intestine.

Although prophages are pervasive among MDR enterococci, only a limited number of studies have addressed their impact on enterococcal fitness and evolution. *E. faecalis* V583 prophages pp1, pp4, and pp6, harboring homologs of the *Streptococcus mitis* platelet binding phage tail proteins PblA and PblB, have been shown to be crucial for adhesion to human platelets *in vitro* and are speculated to contribute to the development of infective endocarditis (51). In another study, investigators established that pp5 is required for efficient biofilm formation, and its induction facilitates biofilm dispersal (80). These findings emphasize the potential influence of enterococcal prophages in biofilm-associated infections, such as endocarditis, central-venous-catheter infections, or implant infections. Further, the therapeutic potential of virulent phages and their lysins is diminished against prophage-carrying clinical isolates of VRE (86, 87). Additionally, *E. faecalis* V583 can release EfCIV583 to target and lyse competitor *E. faecalis* strains in the GI tract of mice (50). Collectively, these studies provide evidence of the influence of prophages in enterococcal physiology, pathogenicity, and adaptation in competitive ecosystems.

While prophages constitute a large part of the *E. faecalis* mobilome, there are still major gaps in the field. To fully comprehend the influence of prophages on enterococcal physiology and adaptation, future research needs to explore the genetic content, composition, and molecular epidemiology of prophages. As many prophage genes are hypothetical or of unknown function, characterization of such prophage genes in relation to virulence or adaptation would divulge how maintaining prophages can augment enterococcal pathogenicity and fitness. The generation of a transposon library in *E. faecalis* MMH594, an MDR isolate that harbors prophage-like elements, could be a powerful tool for such future studies (88). Another noticeable knowledge gap is the lack of prophage-mediated HGT studies in other species of *Enterococcus*. *E. faecium* is an equal, if not greater, burden on the hospital system due to AMR gene acquisition. The study of temperate phages among *E. faecium* strains is limited, potentially due to

the genetic intractability of the bacterium, although methodologies to overcome this are reported (30, 89–91). By expanding studies to include prophages of commensal enterococcal strains native to nonhuman hosts, we might elucidate mechanisms of temperate phage HGT relevant to hospital-adapted enterococci.

The fitness trade-offs of defending against phage infection versus allowing phage acquisition are complex, as lysogenic phages can act either as predators replicating within and lysing bacterial cells or as agents of HGT carrying novel genetic information. Investigation into the dynamics of temperate phage acquisition could provide insights into the complexity of microbial community interactions related to DNA transmission.

CONJUGATIVE AND BROAD-HOST-RANGE PLASMIDS OF ENTEROCOCCI

Plasmids are autonomously replicating, extrachromosomal genetic elements that are critical drivers of microbial adaptation and evolution via HGT. Although plasmids do not encode essential genes and can be a metabolic burden for the cell, they often provide beneficial genes that augment bacterial fitness in complex environments (92, 93). Plasmids are divided into narrow- or broad-host-range plasmids, referring to the specificity of the replication system encoded on the plasmid and the ability of the plasmid to be replicated within a single or multiple bacterial species.

PHEROMONE-RESPONSIVE PLASMIDS

Pheromone-responsive plasmids (PRPs) are narrow-host-range plasmids that are laterally transmitted via conjugation from plasmid-harboring donors to plasmid-free recipient cells (Fig. 1A). Briefly, recipient cells produce and secrete a chromosomally encoded, plasmid-specific conjugation peptide referred to as the pheromone, denoted with a "c" before the plasmid's name. When the donor cell senses the pheromone, downstream transcriptional regulators are activated and mediate the expression of the PRP conjugation genes (94). Highly conserved, large cell wall-anchored surface proteins called aggregation substances (AS) encoded by PRPs facilitate physical contact between donor and recipient cells (95). The operon in which the AS is encoded collectively produces mating channels between the cells, resulting in horizontal plasmid transfer via type IV secretion system (T4SS) conjugation machinery (95). The T4SS is also involved with integrative and conjugative elements conjugation (discussed later) (96). Since pheromones are constitutively expressed, self-induction of plasmid conjugation is prevented by the production of an inhibitory protein encoded on the PRP, denoted "i." PRP transfer has been thoroughly reviewed elsewhere (97–99).

Some PRPs encode antibiotic resistance genes and a wide variety of virulence factors (99). pHKK100, the first plasmid described to mediate vancomycin resistance, transfers via pheromone response (100). In addition to initiating PRP conjugation, AS also increases bacterial virulence by promoting adhesion to, internalization into, and survival within cultured human cells (101–105). PRPs also increase chromosomal diversity, resulting in transconjugants with hybrid donor-recipient genomes and the transfer of chromosomal virulence factors such as antibiotic resistance and the *E. faecalis* pathogenicity island (discussed later) (28, 31). To date, about 35 PRPs have been identified, but only in *E. faecalis* and *E. faecium* (28, 99), suggesting that PRPs are restricted to enterococci.

pCF10 is a well-studied PRP that encodes approximately 60 protein-coding genes that increase *E. faecalis* virulence. These genes include secreted proteins, transcription factors, and orthologs of UV and tetracycline resistance (98) that provide a selective advantage to pCF10-harboring cells. In the GI tract of germfree mice, rapid high-frequency pCF10 transfer was shown to be strongly dependent on the proximity of donor and recipient cells, but the transmission was not significantly impacted by the presence of a defined microbiota (106). Interestingly, the competitive advantage of pCF10 carriage in the intestine was distinct from antibiotic resistance or bacteriocin production and led to the speculation that the pCF10-encoded AS may influence bacterial fitness in the intestine (106). Although AS-mediated resistance to phagocytic killing has
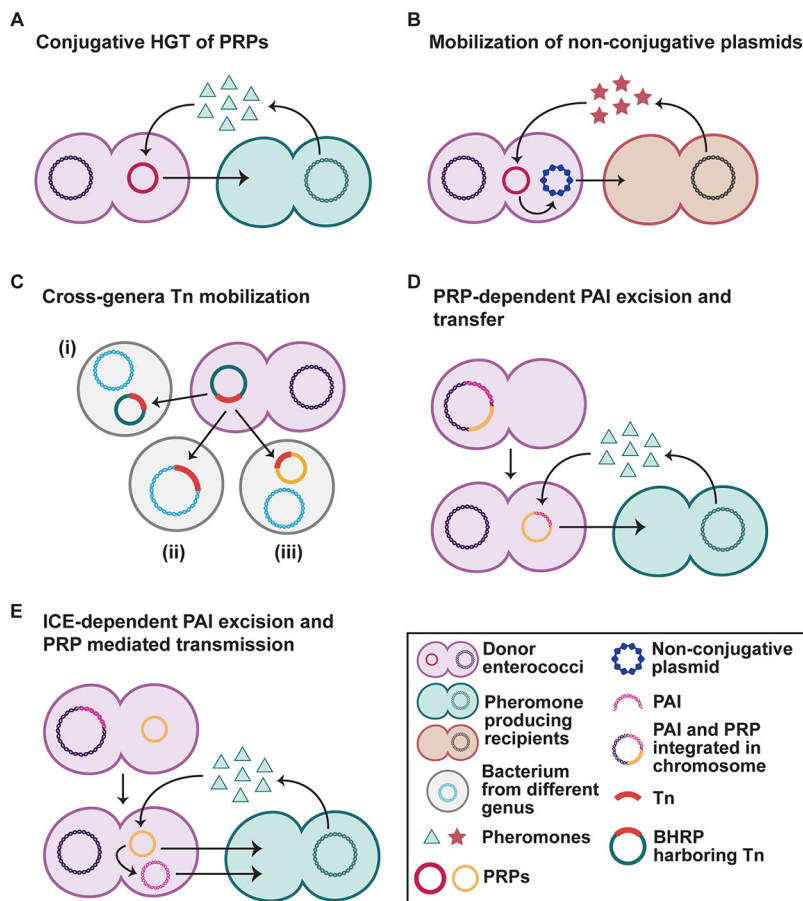
FIG 1 Simplified models for intra- and interspecies dissemination of enterococcal plasmids. (A) PRP replication and conjugation is initiated by donor cells sensing pheromones produced by plasmid-less recipient cells. (B) Upon sensing noncognate peptide signals produced by bacteria from a different genus, PRPs can facilitate mobilization of nonconjugative plasmids. (C) Plasmid-mediated Tn mobilization across genera. A plasmid carrying the Tn can enter the new host; the Tn can replicate independently (i) or integrate into the chromosome (ii) or another plasmid (iii) of the recipient if the plasmid is unable to replicate in the new host. (D and E) Two mechanisms of PAI movement between enterococcal strains. (D) PRP integration and excision can mobilize the PAI by integrating near PAI on the chromosome and excise the PAI upon excision. This PAI-PRP hybrid circularizes and can be conjugated to pheromone producing *E. faecalis* cells. (E) Alternatively, the PAI can function as an integrative conjugative element (ICE) and relies on the direct repeats flanking the PAI and products of PAI-encoded enzymes to excise from and recombine into chromosomes. Mobilization to PAI-less cells is thought to rely on PRP presence.

been suggested to benefit pCF10-carrying *E. faecalis* in an endocarditis model (105, 107), the contributions of AS in GI colonization are yet to be explored.

Understanding pheromone specificity is an expanding area of research. While some pheromones initiate replication and transfer of a specific plasmid, the PRP pMG2200 responds to synthetic cCF10 pheromone. This is because pMG2200 encodes genes identical to the replication and conjugation genes in pCF10, the cognate PRP for cCF10 (108). This phenomenon of pheromone cross talk has been documented for other pheromone-plasmid pairs (99). However, unlike pMG2200, it is unknown whether other PRPs that respond to cCF10, such as pHKK703 (109), pMB1 (110), and pAMS1 (111), encode identical PRP regulation genes and are therefore pCF10 chimeric derivatives. Future studies are necessary to determine *in vivo* cross talk between PRP pheromones and to determine genetic relatedness among plasmids that respond to the same pheromone.

The first identified PRP, pAD1 (112), follows typical PRP conjugation and encodes a plasmid addiction system to promote plasmid maintenance in the absence of selection (29, 113). The pAD1 toxin/antitoxin *par* locus encodes two convergently transcribed

mRNAs, RNA I and RNA II. The stable RNA I encodes the membrane-active peptide toxin, Fst, that interferes with bacterial cell membrane integrity (114), whereas RNA II is an unstable sRNA antitoxin that represses Fst production by binding to RNA I. Upon loss of pAD1, the antitoxin is rapidly degraded, while the more stable toxin kills the *E. faecalis* cell (115, 116). *E. faecalis* V583 harbors the PRP pTEF1, a plasmid with a high level of nucleotide similarity to pAD1, and encodes a locus identical to *par* (36). Similar toxin/antitoxin systems have been identified on plasmids and chromosomes of species from *Enterococcus*, *Lactobacillus*, and *Staphylococcus*, indicating that *par* is the prototype of a family of type I toxin/antitoxin systems (117).

A recent study focusing on the correlation between antimicrobial use and resistance in livestock found that pAD1 is present in 38% and 30% of *E. faecalis* isolated from retail beef and human clinical samples, respectively, suggesting a clinical implication to ingesting pAD1-contaminated samples (118). Enhanced virulence of pAD1-carrying *E. faecalis* strains in mice is attributed to the plasmid-encoded hemolytic cytolysin (119). Epidemiological analysis of an MDR *E. faecalis* outbreak showed that patients infected with a cytolytic strain were at a 5-fold greater risk of death regardless of treatment, and of the patients that died, 71% were infected with a cytolytic strain (11). However, the cytolytic activity of this strain was not confirmed to be a result of pAD1 carriage. Despite this, other studies have shown that there is increased virulence associated with pAD1, specifically due to the cytolysin's activity against multiple eukaryotic cells (120). It is hypothesized that lysing eukaryotic cells releases nutrients like cytochromes that are otherwise unavailable to noncytolytic bacteria, potentially allowing for increased survival through aerobic respiration (120, 121). Cytolytic *E. faecalis* strains also colonize the bloodstream significantly higher than noncytolytic strains in mice (11), supporting this theory. pAD1 also encodes UV resistance via *uvrA*. UV-resistant *E. faecalis* that was exposed to UV irradiation had a higher probability of developing spontaneous antibiotic resistance mutations than non-UV-resistant *E. faecalis* cells (122).

pPD1 is an *E. faecalis* PRP that encodes the bacteriocin Bac-21 (123). Bacteriocins are small cyclic peptides that are capable of killing or inhibiting growth of related bacteria by disrupting the proton motive force of the cytoplasmic membranes and energized membrane vesicles and are commonly produced by many lactic acid bacteria (124, 125). Bac-21 is a 70-amino-acid protein whose corresponding nucleotide sequence is identical to that of the PRP pMB2-encoded bacteriocin AS-48 (126). In the mammalian intestine, *E. faecalis* strains harboring pPD1 outcompete pPD1-deficient *E. faecalis* strains, suggesting that Bac-21 production confers a colonization benefit (127).

PRP conjugation has been documented within *E. faecalis* strains and between *E. faecalis* and *E. faecium*. The PRP pBRG1 is a vancomycin resistance (*vanA*)-carrying plasmid that can transfer from *E. faecium* to *E. faecalis* (128). There is no doubt that this intragenus transfer is concerning regarding the spread of antibiotic resistance genes. Although studies showing PRP transfer to nonenterococcal bacteria are limited, PRP transmission from *E. faecalis* to distant bacterial species, including *S. aureus* and *Streptococcus gordonii*, have been documented (129–131). Other than its cognate pheromone cAM373, the PRP pAM373 can also initiate conjugation in response to noncognate peptides produced by *S. gordonii* and *S. aureus* (132–134). Although cAM373 produced by *S. gordonii* and *E. faecalis* bear no structural resemblance, the *S. gordonii*-cAM373 could induce transfer of a lab-constructed, nonconjugative plasmid from *E. faecalis* into *S. gordonii in vitro* (133) (Fig. 1B). While PRPs do not appear to replicate efficiently in nonenterococcal hosts (28), the full breadth of PRP transmission and maintenance can only be realized if other bacterial genera that frequently coreside with enterococci in microbial communities are studied in this context.

While many enterococcal PRPs have been identified, not all are as well studied as the plasmids discussed above. Future studies focusing on PRP discovery, genetic diversity, and distribution will be critical to understanding their contributions to enterococcal fitness and pathogenicity. Over 900 *E. faecalis* and *E. faecium* genomes have been sequenced (135), but many of these genomes are deposited in public databases as draft genomes fragmented into multiple contigs. Draft genomes are useful for surveying

genomic composition of isolates; however, it cannot be assumed that the entire genome is resolved in these assemblies. The combined use of overlapping short and long high-throughput sequencing reads will allow gap closures in these draft genomes, likely resulting in novel plasmid discovery. Determining if PRPs are truly enterococci specific will clarify how the PRP system arose and why it seems to be rare or nonexistent in other genera and could aid in restricting the potential transmission of these MGEs.

BROAD-HOST-RANGE PLASMIDS

Mobile broad-host-range plasmids (BHRPs) often harbor multiple origins of replication. Host range can be further expanded if plasmid replication initiation and essential proteins are also present on the BHRP and are not dependent on host factors (136, 137). These features allow BHRPs to replicate in distantly related bacteria and serve as mediators of HGT across genera (137). However, it is important to note that some narrow-host-range plasmids contain multiple replication origins (137). BHRPs can be divided into incompatibility groups (Inc-groups). Inc-groups are defined as the failure of two coresident plasmids to be inherited without external selection; if introducing a second plasmid interrupts the inheritance of the first, they are incompatible (138). Inc18-family plasmids are common among the enterococci, staphylococci, and streptococci (139, 140). Inc18-family plasmid biology is comprehensively reviewed by Kohler et al. (136).

Inc18 plasmids have been a focus of interest because they frequently carry antibiotic resistance genes, including resistance to chloramphenicol, vancomycin, and macrolide-lincosamide-streptogramin (MLS) antibiotics (136, 141). Chloramphenicol was used for the treatment of VRE (142, 143), while MLS antibiotics were used as a growth promoter in agriculture (144). pAM830, an Inc18-type plasmid encoding the *vanA* transposon Tn*1546*, is responsible for the transfer of vancomycin resistance from enterococci to methicillin-resistant *S. aureus* (MRSA) (28). Genomic analyses of vancomycin-resistant MRSA strains suggest that either the entire pAM830 was transferred or pAM830 facilitated conjugative dissemination of only the *van* gene carrying Tn*1546*, which ultimately recombined with the chromosome or another plasmid within MRSA (145–147) (Fig. 1C). pAMβ1 and pIP501 are two of the most well-studied Inc18-type plasmids originally isolated from *E. faecalis* (148) and *Streptococcus agalactiae* (149). pIP501 confers resistance to erythromycin and chloramphenicol, while pAMβ1 carries resistance markers for lincosamides, streptogramin B, and erythromycin. Both of these two BHPs are capable of antibiotic resistance transmission to other Gram-positive bacteria, including *Listeria* spp., *Leuconostoc* spp., *Lactococcus* spp., and *Streptomyces lividans*, while pIP501 can even disseminate antibiotic resistance to the Gram-negative bacterium *E. coli* (28). Another *E. faecalis* plasmid, pRE25, sharing 30.5 kb of sequence fragment with pIP501 and harboring 12 antibiotic resistance markers, is capable of conjugal transfer into *Listeria innocua* and *Lactococcus lactis* (28). In addition to disseminating antibiotic resistance genes, conjugative transfer of multiresistance plasmids from other Gram-positive bacteria into enterococci has also been reported. An *L. monocytogenes* plasmid, pIP811, which confers resistance to chloramphenicol, erythromycin, and streptomycin, can be transferred to enterococci via conjugation (150). Lateral transmission of pAMβ1 from *Lactococcus* to *E. faecalis in vitro* and *in vivo* has also been demonstrated (151–153). Collectively, these studies raise concerns regarding cross-genera horizontal transfer, not only of antibiotic resistance markers but also of other plasmid-encoded virulence and environmental adaptation traits. The high sequence identity among BHPs indicates that such interspecies plasmid transmission influences gene content of plasmids across species. Therefore, identifying factors that facilitate BHRP transmission and investigating the molecular mechanism(s) of BHRP dissemination are critical for understanding how these plasmids facilitate genome plasticity and bacterial evolution.

OTHER MOBILE ELEMENTS

Transposable elements (TEs) are widely distributed in prokaryotic genomes, including enterococci. TEs are DNA fragments that move autonomously to new locations within and between DNA molecules present in a single cell; however, they rely on

conjugative plasmids and transducing phages for intercellular movement. All TEs encode a transposase, an enzyme that catalyzes excision and integration of TEs through transposition. Insertion of TEs can disrupt, activate, or silence gene functions (154). Broadly, TEs can be categorized into IS elements and composite transposons (Tns). IS elements are small MGEs composed of two inverted repeat sequences flanking the transposase open reading frame (ORF). In contrast, composite Tns consist of resistance genes or other adaptive traits bordered by a pair of IS elements.

The incorporation of TEs potentially facilitated the propagation of beneficial traits and genome plasticity in enterococci. TEs play a role in the shaping of the enterococcal genome through genomic rearrangements and recombination, thus facilitating *E. faecium* genome plasticity (155). Upon activation through cell stress, transposons can induce mutations through novel insertions that may allow for bacterial survival (141). Vancomycin resistance in *E. faecalis* V583 encoded by the *vanB* operon in Tn*1549* found on a PRP was likely acquired via HGT (36, 156). Conjugative transposition of *vanB* Tn*1549* from a plasmid results in transfer of antibiotic resistance between enterococci and human commensal bacteria in a germfree mouse model of GI colonization (157). Vancomycin resistance is associated with other transposons. Tn*1547* encodes the *vanB* operon flanked by IS16- and IS25-like elements and can integrate into plasmids and chromosomes (158). *E. faecium* Tn*5382* encodes the *vanB2* operon and has been identified in multiple clinical isolates (159). Tn*1546* encodes the *vanA* operon and has been identified in *E. faecalis*, *E. faecium*, as well as in other species, including *Enterococcus gallinarum* and *Enterococcus casseliflavus* (160). Apart from vancomycin, a gentamicin resistance-encoding transposon, Tn*5281*, is present on the PRP pBEM10 and is widely distributed among *E. faecalis* and *E. faecium* isolates from different geographical locations (161–164). The DNA sequence and genetic organization of Tn*5281* are similar to those of other Tns such as Tn*4001* and Tn*4031*, found in *S. aureus* and *Staphylococcus epidermidis*, respectively. This similarity suggests that these elements are closely related, possibly as a result of interspecies transfer (164, 165). Together, these studies indicate that transposon-encoded antibiotic resistance potentiates rapid and widespread dissemination and diversification among *Enterococcus* species and, more broadly, other bacterial species.

Pathogenicity islands (PAIs) are large chromosomal genetic elements that are horizontally transmitted among Gram-positive and Gram-negative bacteria (166). The *E. faecalis* PAI encodes nearly 150 genes, including virulence factors that vary between strains (82, 167–170). Examples include a cytolysin, a virulence-associated surface protein encoded by *esp*, and an AS (171, 172). The PAI is enriched in pathogenic *E. faecalis* strains and is excised at a rate of 1 in 1,000 cells (171). Evidence suggests that PAI intra- and interspecies mobilization is dependent on the PRPs pTEF1 and pTEF2 of *E. faecalis* V583 (173). These PRPs could facilitate PAI transmission in two ways. Manson and colleagues demonstrated that pTEFs integrate into the *E. faecalis* V583 chromosome in close proximity to the PAI, and subsequent excision of plasmid-PAI hybrids potentially allows for PAI movement between bacterial cells (31) (Fig. 2D). The authors also showed that the *E. faecalis* V583 pTEFs can mobilize random segments of chromosomal DNA, including vancomycin resistance, multilocus sequence type (MLST) markers, and capsule genes, indicating that plasmid-mediated HGT is a major driving force behind the evolution of MDR enterococci (31). In contrast, the *E. faecalis* UW3114 PAI is capable of precise excision and circularization and can undergo horizontal intra- and interspecies transfer with the help of a PRP (173) (Fig. 1E). It is suggested that the *E. faecalis* UW3114 PAI functions similarly to an integrative conjugative element (ICE), relying on the direct repeats flanking the PAI and phage-related excisionase and integrase genes to excise from and recombine into bacterial chromosomes (173). Nucleotide sequence identity exists at one end of the PAI with conjugation-related structural genes of enterococcal PRPs pAM373 and pAD1 (174), indicating that part of the PAI may have evolved from the integration of a PRP into the chromosome. A region present on the PAI has 87% nucleotide identity to a transfer origin in pAD1 (171), further supporting this theory. Although a number of PAI ORFs remain
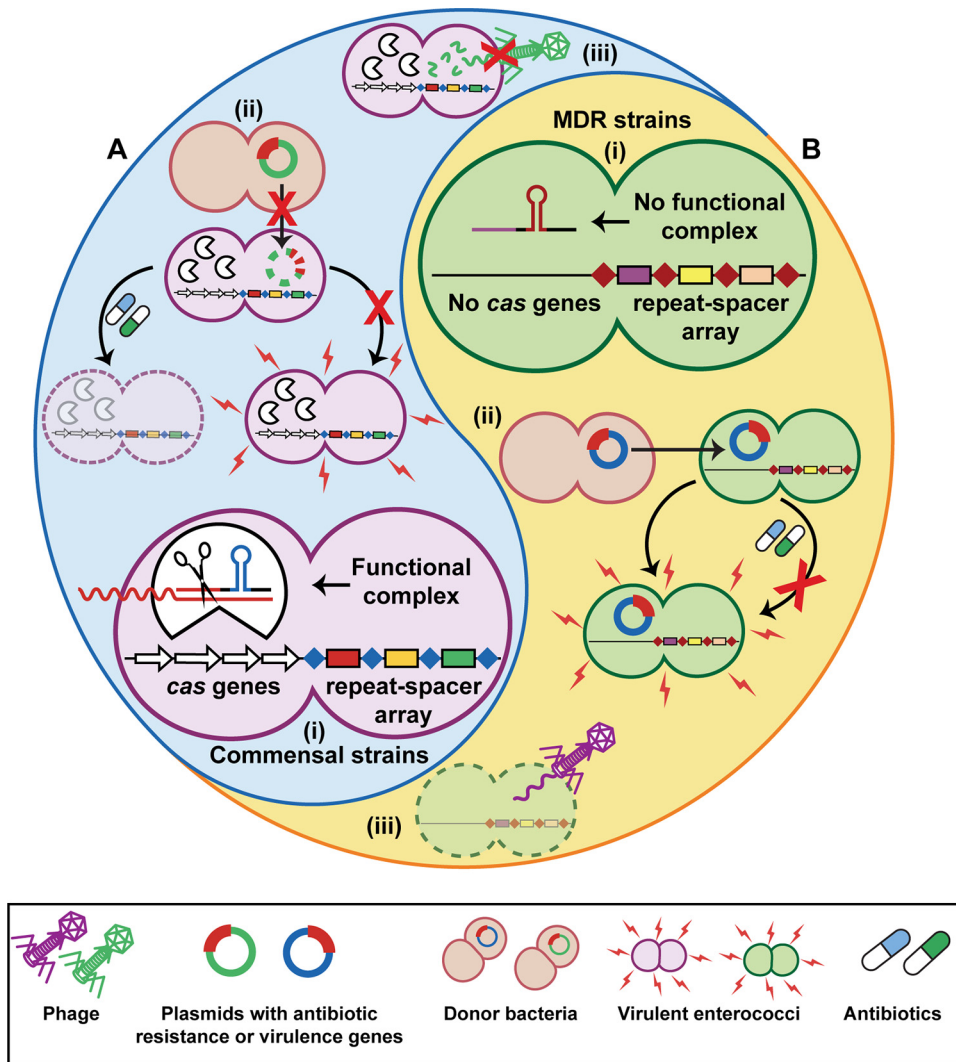
FIG 2 CRISPR-Cas is a fitness balance for enterococci. (A and B) Commensal strains typically harbor a functional CRISPR-Cas system (A), while MDR clinical isolates do not (B). Generally, commensal strains carry the *cas* genes and repeat spacer array, resulting in a fully functional system (A, i), while MDR strains contain only the orphan CRISPR2 repeat-spacer array (B, i). The presence of functional CRISPR-Cas results in reduced plasmid transfer, preventing transfer of virulence or antibiotic resistance genes (A, ii); however, CRISPR-Cas also blocks phage infection and killing of a host bacterium (A, iii). (B, ii) On the other hand, plasmid transfer is not blocked in MDR strains, allowing them to gain accessory genes. (B, iii) This trade-off makes MDR strains sensitive to phage infection.

uncharacterized, the absence of these genes from certain human isolates suggests that the products of these genes could facilitate the commensal to pathogen transition of the enterococci. Additionally, tracing the evolutionary origins of enterococcal PAIs will provide further enlightenment as to how enterococci become successful pathogens.

CRISPR AND HGT

Many bacteria have established barriers to combat HGT (50, 51). One such defense system is CRISPR-Cas, an adaptive immune system that utilizes an RNA-guided nuclease to block acquisition of MGEs in a sequence-dependent manner (52). CRISPR-Cas systems are present throughout enterococcal species, most frequently in commensal isolates (175, 176). CRISPR loci consist of several direct repeat regions separated by variable spacers, which are sequences derived from foreign DNA which serve as adaptive memory units for identifying invading nucleic acid targets. Identified spacers match phage, plasmid, and nonkin chromosomal sequences (177, 178). CRISPR interference is

adaptive, and new targeting spacers can be acquired as invading DNA threats are encountered (52, 179). However, spacer acquisition is tightly regulated and occurs at very low rates (180). Still, this may provide a benefit to cells over other phage protection mechanisms like surface receptor modification, as alteration of these receptors is known to increase antibiotic susceptibility (141, 181).

These repeat-spacer arrays are in close proximity to the *cas* genes (177). The *cas* genes encode a large and diverse family of proteins which interact with the transcribed spacers to form the CRISPR-Cas complex (182). Unique *cas* genes allow CRISPR systems to be divided into three major types, types I, II, and III, defined by the presence of *cas3*, *cas9*, and *cas10*, respectively (183). Type IV CRISPR has been identified but is much less common (184). CRISPR types are further divided into subtypes based on additional signature genes or gene variants (183). CRISPR classification and mechanisms of action have been thoroughly reviewed elsewhere (183, 184).

To date, type II-A and type I-C CRISPR loci have been found in *Enterococcus* (175). Type II-A CRISPR-Cas systems are significantly more common in commensal *Enterococcus* strains than in clinical isolates, and the lack of functional CRISPR-Cas is correlated with acquired antibiotic resistance in *E. faecalis* (176, 185, 186) (Fig. 2A and B). The type II-A subtype is broken down further into four systems within *Enterococcus*, CRISPR1-Cas, CRISPR2, CRISPR3-Cas, and CRISPR4. Of enterococcal genomes screened thus far, CRISPR1-Cas and CRISPR2 seem the most predominant (176, 187–189). CRISPR1-Cas is the most commonly identified functional CRISPR system in *E. faecalis*, with spacers varying by strain (176, 178, 187, 190, 191). CRISPR1-Cas has also been identified in *E. faecium*, primarily in isolates without antibiotic resistance genes, and in *Enterococcus hirae* (176, 187).

Many MDR *E. faecalis* strains lack functional CRISPR-Cas systems (176, 186). The CRISPR2 locus is nonfunctional, consisting only of the orphan repeat-spacer array and no *cas* genes (176, 192, 193). CRISPR2 is nearly ubiquitous within *E. faecalis* and common among *E. faecium* (176, 187, 189). It is theorized that CRISPR2 is maintained because of a self-preservation mechanism within the locus itself, using terminal repeats within the CRISPR array to prevent loss of the terminal spacer (182). Additionally, in the presence of *cas9*, the CRISPR2 locus becomes functional for defense (194). CRISPR systems are detected less frequently in other disease-associated species like *E. gallinarum* and *E. casseliflavus* (191).

Lack of CRISPR systems is associated with detergent and chlorohexidine resistance, increased biofilm production, and bacteriocin activity (186, 195). Conversely, the presence of a functional CRISPR-Cas locus correlates with a lack of virulence genes in both *E. faecalis* and *E. faecium* (176, 185, 191) (Fig. 2A). The *E. faecalis* oral commensal strain OG1RF contains both functional CRISPR1-Cas and an orphaned CRISPR2 locus, while the hospital-acquired strain V583 harbors only the CRISPR2 orphan locus (192, 193). This correlation between the absence of functional CRISPR-Cas in hospital-adapted strains indicates a fitness cost for harboring CRISPR-Cas; in order to protect against phage infection and/or plasmid acquisition, CRISPR-Cas is needed, which may not benefit enterococcal strains when adapting to environments outside the GI tract such as the hospital setting (Fig. 2B).

In addition to the above type II-A CRISPR systems, a type I-C CRISPR locus has been identified in three pathogenic strains of *Enterococcus cecorum*, a poultry pathogen, and predicted to be functional in two strains (175). This CRISPR type is rare in enterococci but commonly found in streptococci (175). Commensal strains of *E. cecorum* harbor the more typical type II-A CRISPR arrangement (175). The presence of a type 1-C locus in pathogenic strains is unusual, as it defies the established pattern of disease-associated strains being less likely to contain a functional CRISPR system (176, 185, 186, 191).

Although CRISPR-Cas was identified as an antiphage system, CRISPR also guards against invading MGEs. *E. faecalis* type II CRISPR-Cas blocks antibiotic resistance encoding plasmid conjugation *in vivo* (196, 197). Additional known targets of enterococcal CRISPR-Cas include the PRPs pTEF1 and pTEF2, phages, and prophages (176, 178). No spacers have been identified against conjugative transposons; the *tet*(M) tetracycline

resistance gene, commonly carried by Tn*916* and its relatives, is present in strains with functional CRISPR-Cas (176). Many spacers have been found in *E. faecalis* and *E. hirae*, which correspond to bacterial chromosomal sequences (178, 187). Specifically, spacers have been identified with homology to the *E. faecalis* V583 *ref35B* gene, potentially encoding an antisense ncRNA (178, 198). It is unknown if CRISPR-Cas systems target PAIs. Many spacers have yet to be matched with a target sequence, likely due to the lack of available MGE sequences in GenBank (178, 199).

Current research describes the ramifications of harboring a CRISPR-Cas system as it relates to the pathogenic potential of enterococci while also inspiring future research into the context of CRISPR-Cas in multispecies environments. Known spacers targeting the chromosome of non-CRISPR-harboring bacteria, in addition to the high abundance of CRISPR-positive strains in environments like endodontic biofilms, warrant investigations into how CRISPR might modulate polymicrobial interactions (186, 187). Experimentation on the role of CRISPR within a multispecies microbial community could provide insight into the complex nature of microbial interactions in various niches.

CONCLUSIONS

The recent accessibility of next-generation sequencing technologies has increased our knowledge of the role of MGEs in enterococcal biology. The discovery that MGEs can constitute 25% of the *E. faecalis* V583 genome opened the door to a multitude of studies elucidating the role of the mobilome in enterococcal evolution (36). By studying *E. faecalis* and *E. faecium* as model enterococci, it is becoming clear that the enterococcal mobilome drives environmental adaptation and niche colonization (Fig. 1). Similar studies in other species, including *E. gallinarum* and *E. casseliflavus*, are necessary to answer questions regarding the overall impact of MGEs on enterococcal evolution. Obtaining more complete genome sequences of these understudied species is a key first step. Closing genomes reveals plasmid carriage, prophage presence, and the distribution of transposable elements within the genome (200, 201). By comparing the mobilomes of *E. faecalis* and *E. faecium* to other species, we can resolve many of the questions regarding the role of MGEs in virulence and ecological competition in addition to better understanding the MGE host range.

Prophage contribution to enterococcal evolution is understudied. Even though *E. faecalis* V583 prophages are associated with virulence and interbacterial competition, the ecological and evolutionary impact remains underexplored. Studies focusing on single prophage-harboring strains of *E. faecalis* should reveal distinct prophage benefits. Homologs of the *E. faecalis* V583 prophages have been identified in other strains, but similarities and differences among these prophages are yet to be elucidated (76, 202). Prophage-mediated HGT and its contributions to virulence have been studied in other bacteria, such as *S. aureus*. Examples include pathogenicity and genomic island transfer between strains (203–205), gene regulation upon prophage excision and integration (206), and DNA transduction (207, 208). Similar studies in enterococci will further contribute to our understanding of the role of coevolution between phages and their hosts and the implications of undiscovered and uncharacterized prophages on enterococcal environmental adaptation.

Few studies have assessed the ability of the enterococci to share MGEs like plasmids with other genera (133). While PRPs have been extensively studied in *E. faecalis*, more work should focus on the ability of other bacterial species to induce the pheromone-responsive conjugation system through secreted peptides, especially since other bacteria such as staphylococci are known to produce a variety of small, secreted peptides (209). Alternatively, it is possible that PRPs will fail to replicate or will be subjected to CRISPR-Cas-targeted degradation in the new host, nullifying the hypothesis that PRPs can be maintained outside the enterococci (99, 130). Finally, chromosomal conformation capture (Hi-C) technology with deep sequencing that estimates physical proximity between DNA molecules (210, 211) could potentially be leveraged to track lateral dissemination

of enterococcal prophages, plasmids, and PRPs within complex multispecies communities (212, 213).

Finally, the role of CRISPR-Cas systems in controlling enterococcal HGT has not yet been thoroughly characterized. While identified spacers indicate that CRISPR-Cas is able to target the genome of non-CRISPR-harboring strains (178), the circumstances upon which targeting of nonhost genomes may occur remains unclear. Further, contributions of CRISPR-Cas-mediated restriction of phage predation or MGE movement and subsequent impact on microbial community dynamics should be assessed in the context of polymicrobial environments, such as the GI tract. High-throughput sequencing could be leveraged to match enterococcal CRISPR spacers to their source in order to provide additional context on the role of CRISPR-Cas and identify common threats that enterococci face while also providing insight on spacer acquisition and retention mechanisms.

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