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Horizontal Gene Transfer and the Genomics of Enterococcal Antibiotic Resistance

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Summary

Enterococci are Gram-positive bacteria that normally colonize gastrointestinal tracts of humans and animals. They are of growing concern because of their ability to cause antibiotic resistant hospital infections. Antibiotic resistance has been acquired, and has disseminated throughout enterococci, via horizontal transfer of mobile genetic elements. This transmission has been mediated mainly by conjugative plasmids of the pheromone-responsive and broad host range incompatibility group 18 type. Genome sequencing is revealing the extent of diversity of these and other mobile elements in enterococci, as well as the extent of recombination and rearrangement resulting in new phenotypes. Pheromone-responsive plasmids were recently shown to promote genome plasticity in antibiotic resistant *Enterococcus faecalis*, and their involvement has been implicated in *E. faecium* as well. Further, incompatibility group 18 plasmids have recently played an important role in mediating transfer of vancomycin resistance from enterococci to methicillin resistant strains of *S. aureus*.

Introduction

Enterococci, once considered harmless commensals of the gastrointestinal tract of humans and animals, have emerged over the last 30 years as important hospital pathogens. *Enterococcus faecalis* and *E. faecium* are the species most associated with hospital-acquired infections, and strains possessing acquired resistance to the last-line antibiotic vancomycin are common [1]. The ability of *E. faecalis*, *E. faecium,* and likely other enterococci to acquire mobile genetic elements encoding traits such as antibiotic resistance has contributed to their emergence as leading hospital pathogens, and antibiotic resistance and virulence traits have accumulated in lineages associated with hospital infection outbreaks, such as the *E. faecium* clonal complex (CC) 17 and *E. faecalis* CC2 [2,3]. *E. faecalis* V583, a CC2 strain, possesses ~620 kilobases (kb) of novel coding potential as compared to the smaller genome of the non-clinical isolate *E. faecalis* OG1RF, with most of the difference being accounted for by mobile genetic elements [4,5]. Plasmids are abundant in the enterococci, as illustrated by the finding of 1–7 plasmids in 88 out of 93 *E. faecium* isolates [6]. They comprise a substantial fraction of the auxiliary genome, and are responsible for much of the horizontal gene transfer that has allowed antibiotic

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and virulence traits to converge in hospital adapted lineages. Remarkably different plasmid types occur in *E. faecalis* and *E. faecium* despite their close phylogenetic relationship [6,7]. Since both species can be found in the human gastrointestinal tract, this may be evidence of niche isolation, perhaps at the microscale.

We recently deposited draft sequence of 28 enterococcal genomes into GenBank, including 16 *E. faecalis*, 8 *E. faecium*, 1 *E. gallinarum*, and 3 *E. casseliflavus* strains [8]. These strains represent clinical, environmental and animal isolates collected over the past century. The structure and diversity of the mobile elements that confer antibiotic resistance in these strains are being catalogued in ongoing studies. The role of transposons in conferring antibiotic resistance in enterococci, and mechanisms of enterococcal antibiotic resistance, were recently reviewed [9]. This review focuses on the vectors of horizontal transmission of most of the antibiotic resistance that has emerged in genomic studies of the enterococci, much of which has been mediated by transmissible or conjugative plasmids, and on their role in enterococcal genome plasticity. Not only have mobile genetic elements been important in the acquisition and dissemination of antibiotic resistance in the enterococci, in an even more alarming development, broad host range plasmids have conveyed vancomycin resistance to strains of methicillin-resistant *Staphylococcus aureus* (MRSA), and it appears that this transmission is occurring repeatedly in epidemiologically independent events.

Pheromone-responsive plasmids are highly adapted for efficient horizontal gene transfer

Pheromone-responsive plasmids mainly have been described in *E. faecalis*. These are rapid disseminators of antibiotic resistance and other traits throughout the species *faecalis*, but appear to be less common outside of that range. Because of the wide variety of gastrointestinal tracts colonized by enterococci, ranging from insects to man [10,11], it is tempting to speculate that this facile gene exchange mechanism evolved to shuttle niche specialization traits as *E. faecalis* strains from prey comingled with *E. faecalis* strains from predators, allowing *E. faecalis* as a species to readily adapt to the dietary habits and other peculiarities of particular hosts. The efficient transfer of pheromone-responsive plasmids within the species *faecalis* stems from their novel exploitation of *E. faecalis* cellular processes (for excellent and thorough reviews of model pheromone-responsive plasmids pCF10 and pAD1, see [12,13]). Two such plasmids were identified in the genome sequence of the prototype multidrug resistant CC2 *E. faecalis* strain, V583, and named pTEF1 and pTEF2 [5]. Similar plasmids are common in draft genomes recently released [8].

The pheromones that induce efficient plasmid transfer are chromosomally encoded within genes for lipoprotein signal peptides. These hydrophobic segments are proteolytically cleaved from lipoprotein precursors and released as linear septa- or octapeptides into the medium or environment. The post-translational processing of signal peptides to generate pheromones is catalyzed by chromosomally encoded proteins including the membrane metalloprotease, Eep [12,14]. Structures of five pheromones have been determined (cCF10, cAD1, cAM373, cPD1, cOB1) [15]. Based on the inferred lipoproteins encoded within the V583 genome, 76 additional pheromones are predicted [5].

Pheromone-responsive plasmids encode specific receptors for the cognate pheromone. For example, pCF10 senses cCF10 (of amino acid sequence LVTLVFV) [13]. A key response to the pheromone is effective donor-recipient pair formation, which is mediated by a plasmid-encoded, donor cell-specific adhesin termed aggregation substance (*e.g.*, PrgB) [12,13]. To prevent competing, non-effective donor-donor aggregation, pCF10 and other pheromone-responsive plasmids encode a cognate quenching peptide (for example, the cCF10 structural analog iCF10, of amino acid sequence AITLIFI), that competitively interacts with a

pheromone-responsive transcriptional repressor (e.g., PrgX), preventing induction of aggregation substance [12–14]. To further suppress autoaggregation, a plasmid-encoded membrane protein is also produced by the donor cell (e.g., PrgY) that sequesters autoexpressed cCF10 after Eep processing [12–14]. The combined activities of PrgY and iCF10 suppress sensing of endogenously produced pCF10 to the point that increases in the cCF10 pheromone in the environment, stemming from production by a candidate recipient, can be detected. Thus, pheromone-responsive plasmids are dependent upon peptide signal sensing, with conjugative functions induced by pheromone-producing recipient cells in proximity.

Pheromone-responsive plasmids confer a number of auxiliary traits on *E. faecalis*. They provide accessory genes encoding bacteriocin and cytolysin production, ultraviolet resistance, and antibiotic resistance, among others (Table 1). Vancomycin resistance is conveyed less commonly by pheromone-responsive plasmids. A *vanB*-encoding pheromone-responsive plasmid was recently isolated from a Japanese outbreak of *E. faecalis* infection, and its sequence reported (pMG2200; [16]). Pheromone-responsive plasmids have recently been reported to promote chromosomal diversification in *E. faecalis*, generating transconjugants possessing donor-recipient hybrid genomes, and mobilizing all chromosomally encoded traits queried, including vancomycin and tetracycline resistances [17], as discussed further below.

Pheromone-responsive plasmids have rarely been described in other enterococcal species. A few have been reported in *E. faecium* that either encode vancomycin resistance (pBRG1; [18]; pHKK100; [19]), or facilitate co-transfer of a non-conjugative vancomycin resistance plasmid into recipients (pHKK703; [20]), indicating that pheromone-responsive plasmids promote vancomycin resistance dissemination in *E. faecium*. To our knowledge, transfer of these plasmids has been evaluated for *E. faecalis,* but not *E. faecium* recipients, thus the efficacy of these plasmids in disseminating antibiotic resistances among *E. faecium* populations is less clear. These experiments and sequencing of pBRG1, pHKK100, pHKK703 for comparison to *E. faecalis* pheromone-responsive plasmids will yield information on their role in *E. faecium* ecology.

E. faecalis harboring pheromone-responsive plasmids can detect and respond to pheromone signals produced by other bacterial genera as well, promoting the interspecies spread of antibiotic resistance genes [21]. Production of *E. faecalis* aggregation substance-inducing signals was observed for *S. aureus*, *Streptococcus gordonii* and *E. hirae* [22]. The *S. aureus* chromosome encodes a lipoprotein (CamE) with little sequence identity to *E. faecalis* lipoproteins that is processed and exhibits cAM373 pheromone activity [23]. The staphylococcal plasmid pSK41 also has been found to encode a lipoprotein (TraH) that, when processed, has cAD1 activity [24].

Pheromone-responsive plasmids do not appear to replicate or become established efficiently in non-enterococcal hosts. However, a cAM373-like pheromone produced by *S. gordonii* Challis facilitated the pAM373 mobilization of a co-resident, non-conjugative erythromycin resistance plasmid from *E. faecalis* donors into *S. gordonii* Challis recipients, and this occurred at a 1000-fold greater efficiency from *E. faecalis* donors exposed to *gordonii*-cAM373 (Figure 1, [21]). Production of *S. gordonii*-cAM373 was dependent upon an Eep homologue in that host [21], suggestive of a role for Eep in pheromone processing in non-enterococcal species.

Non-pheromone dependent conjugative plasmids as transmitters of antibiotic resistance across genus boundaries

Broad host range conjugative plasmids also occur in enterococci. Of these, the most thoroughly characterized are those of the incompatibility group 18 (Inc18). The Inc18 classification is based on specific plasmid maintenance functions, including replication and post-segregational

killing systems [25]. Because of the observed modularity and high rate of recombination between enterococcal plasmids, it has been suggested that these should be re-classified based on their replication initiation genes [7]. Inc18-type plasmids are particularly abundant in *E. faecium* [6]. The most common plasmid maintenance system on these plasmids is a toxin-antitoxin post-segregational killing system, which insures the persistence of plasmid containing cells in a population even in the absence of direct antibiotic selection [26]. The toxin-antitoxin systems are termed ω-ε-ζ and *axe-txe* [9].

Two of the most well-characterized Inc18-type plasmids are pAMβ1, which was originally identified in *E. faecalis* [27], and pIP501, which was originally identified in *Streptococcus agalactiae* [28]. Replication of these plasmids occurs by a theta mechanism which requires two plasmid-encoded elements: a rate-limiting replication protein [29], and a short origin located down stream of the *rep* gene [30]. They are maintained at less than 10 copies per cell [30]. pAMβ1 encodes resistance to macrolides (such as erythromycin), lincosamides and streptogramin B (the MLS antibiotics) [27,31], and pIP501 encodes additionally resistance to chloramphenicol [28]. Both of these plasmids can disseminate antibiotic resistance to other Gram-positive bacteria, including *Streptomyces lividans*, *Leuconostoc* spp., *Listeria* spp.*,* and *Lactococcus* sp. [32–35]. pIP501 has also been shown to be transferable to the Gram-negative bacterium, *Escherichia coli* [32]. The ability of these plasmids to transfer from cell to cell involves a number of plasmid-encoded transfer (*tra*) genes [36]. The transfer components and how they interact within the cell have been studied [37], but the function of most proteins implicated in transfer remain to be elucidated. Plasmid transfer machinery may be co-opted by co-resident plasmids which are mobilizable, but lack the genes for the type IV secretion apparatus.

Sequence analysis of plasmids, such as *E. faecalis* plasmid pRE25, has shown that they are not static structures, but rather recombine to form derivatives with hybrid replication and maintenance functions (Table 1), conveying multiple antibiotic resistances. The 50 kb plasmid pRE25 carries genes that confer resistance to 12 different antibiotics. Within pRE25, 30.5 kb of sequence appears to be derived from pIP501, and other regions appear to originate from a staphylococcal plasmid [38]. In a recent large study of *E. faecium* plasmid diversity, pRE25 was found to be widespread. Plasmids more closely related to pIP501 and pAMβ1 were present to a lesser extent [6]. pRE25 can transfer by conjugation into *Listeria innocua* and *Lactococcus lactis*, indicating it is capable of broad host range transmission of resistance and other genes [38]. A plasmid sharing about 12 kb of pRE25 sequence, including antibiotic resistance genes, pKL0018, has been identified in the fish pathogen *Lactococcus garvieae* [39]. This raises the possibility that composite plasmids carrying resistance to multiple antibiotics can rapidly spread among multispecies communities.

Another plasmid type found to be common among *E. faecium* strains is typified by pRUM [6]. This plasmid encodes resistance to erythromycin, chloramphenicol, streptomycin and streptothricin, with resistances to the latter two being encoded by genes that are highly conserved with those found on Tn*5405* and identified in staphylococci and *Campylobacter* [40]. The conservation of the arrangement of these genes among different elements present in both Gram-positive and Gram-negative bacteria suggests facile, broad host range horizontal transmission.

Dissemination of vancomycin resistance among enterococci and beyond

Key mediators of vancomycin resistance in *E. faecalis* and *E. faecium* are pheromone-independent plasmids, such as pHTβ (and its derivatives pHTα and pHTγ), and pMG1 [41–43]. These plasmids are large (>63 kb) and efficiently transfer among *E. faecalis* strains [44]. Understanding transfer mechanisms of these plasmids is of interest as the pHT plasmids are

known to carry the Tn*1546* transposon [41–44], a non-conjugative class II transposable element of the Tn*3* family of transposons that conveys the *vanA* operon [9]. The host range of these plasmids is unknown.

Tn*1546* has also been found to reside on broad host range plasmids of the Inc18 class. The first known plasmid identified as carrying the Tn*1546 vanA* transposon, pIP816, was isolated from an *E. faecium* strain in France in 1986 and recently sequenced [45]. The nucleotide sequence revealed identities between the pIP816 replication region and that of pAMβ1. Since 2002, ten transfers of vancomycin resistance from enterococci to methicillin resistant strains of *S. aureus* have been documented in the US [46]. An Inc18-type plasmid has been associated with most of the transfers of Tn1546 to MRSA. The prototype of these plasmids, pAM830, shares extensive sequence identity with plasmids pIP501 and pAMβ1 by Southern hybridization [47]. This type of plasmid appears to have been responsible for mediating an outbreak of such transfers in the Detroit area, likely accounting for 7/10 VRSA strains [48]. This development is of special concern as vancomycin is a last-line antibiotic for the treatment of MRSA infections, which are increasingly common in both the hospital and the community.

The factors that promote transfer of Inc18 plasmids and the resistances they convey are not well understood (Figure 2). Attempts to recreate these transfers in the lab have met with limited success [47,49,50]. Research is beginning to explore the basis for how and why vancomycin-resistant enterococci and MRSA co-colonize wounds and other sites providing an opportunity for transfer to occur. Factors such as diabetes and prior antibiotic treatment appear to play a role [51].

Enterococcal plasmids as mediators of genome plasticity

A mechanism for chromosome-to-chromosome gene transfer in *E. faecalis* was recently elucidated [17]. Chromosome-to-chromosome transfer of antibiotic resistance and virulence markers had been inferred and observed in several enterococcal species (for a discussion of this, see [17]). Using *E. faecalis* strain V583 as a donor, and placing a selectable tetracycline resistance marker at various points within its pathogenicity island (PAI), it was initially noted that the PAI transferred from the V583 donor to recipient strain OG1RF at a rate of about 10^{-10}. Importantly, this transfer only occurred when either of the 2 pheromone responsive type plasmids, pTEF1 or pTEF2, were present in the donor strain. It was observed that the inferred PAI excisionase and integrase genes were not involved in the transfer, so transfer of other regions of the chromosome was examined. It was found that a selectable marker placed anywhere around the circumference of the chromosome could be transferred if pTEF1 or pTEF2 were present in the cell. Moreover, transfer did not occur if plasmid transfer functions were specifically deleted, including the cis-acting origins of transfer, *oriT*. From the V583 genome sequence, 10 highly conserved copies of IS256 were identified, 6 on the chromosome, 2 on pTEF1, 1 on pTEF2, and 1 on pTEF3. Using a PCR strategy, it was shown that in a 10 ml culture, all possible recombinations across the IS256 copies could be identified – that is, the plasmids integrated into the chromosome at every IS256 site, which presumably served as the point of initiation of conjugal transfer of chromosomal genes. The extent of donor chromosome transferred was observed to vary, but the largest transfer observed was 857 kb, or over 25% of the V583 genome [17]. Traits that were mobilized into the OG1RF recipient included a capsule locus, a vancomycin resistance transposon, the PAI, and even MLST markers creating a double locus variant of the parental strain in a single event [17]. These results demonstrate that pheromone-responsive plasmids are a significant driver of *E. faecalis* genome plasticity. Conjugative transfer of chromosomal determinants has also been reported among *E. faecium* [52], and from *E. faecium* donors to *E. faecalis* recipients [53]. It remains to be determined if a similar mechanism contributes to *E. faecium* genome diversity, and what role these transfers have in interspecies interactions.

Clustered, regularly interspaced short palindromic repeats (CRISPR) defense against the entry of foreign DNA has been the subject of considerable interest lately, and was recently reviewed [54,55]. The genome sequence of *E. faecalis* OG1RF revealed that this strain possesses two CRISPR loci, one with CRISPR-associated genes (*cas*) genes (CRISPR1-*cas*), and the other an orphan locus lacking *cas* genes (CRISPR2) [4]. CRISPR loci have been shown in other bacteria to confer resistance to plasmid and phage entry in a manner analogous to acquired immunity [56,57]. This immunity depends on the presence of specific target-derived spacer sequences, the intervening repeat palindromes, and nuclease activity encoded by the *cas* genes [54,55]. *E. faecalis* V583 shares only the orphan CRISPR2 locus with OG1RF, and lacks CRISPR1-*cas* [4,58]. The absence of CRISPR-*cas* in V583 may have reduced the barrier to entry of foreign elements, resulting in the convergence and accumulation of 6 plasmids or plasmid remnants, 7 phage or phage remnants, and over 40 IS elements [3,5], while OG1RF natively lacks plasmids [4]. The interrelationships of enterococcal CRISPR defense, plasmids, and genome plasticity are the subject of ongoing investigation.

Conclusion

Elucidation of a large number of enterococcal genome sequences reveals that many of the traits that have resulted in the development of multidrug resistant, hospital adapted strains are either plasmid encoded, or are transferred by plasmid mobilization of chromosomal sequences [17]. Plasmids are widespread in *E. faecalis* and *E. faecium* [6,7], and mediate the efficient transfer of resistance, virulence and other adaptive traits throughout the genus and beyond. Not only have they played an important role in the development of highly hospital adapted strains, they are now moving resistances to last line drugs, such as vancomycin, into the staphylococci. A recent study found evidence for a reservoir of novel antibiotic resistance determinants encoded by the human gut microbial community [59]. As erstwhile commensals, enterococci are uniquely positioned to tap into that reservoir and serve as a pivot point for the future dissemination of antibiotic resistance as well.

Acknowledgments

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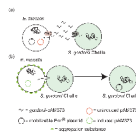
Figure 1.
Model for mobilization of antibiotic resistance from *E. faecalis* (donors) to *S. gordonii* Challis (recipients) in response to *gordonii*-cAM373 pheromone [21,62]. (a) *S. gordonii* Challis cells produce a signal, *gordonii*-cAM373, that is detected by *E. faecalis* cells carrying pAM373 via the plasmid-encoded membrane protein, TraC. (b) Conjugative functions are induced on pAM373 by the presence of *gordonii*-cAM373, leading to mating pore formation and mobilization of a non-conjugative erythromycin resistance plasmid. The result of this interaction is erythromycin-resistant *S. gordonii* Challis. In the absence of pAM373, no transfer of erythromycin resistance from *E. faecalis* to *S. gordonii* Challis occurs (not shown; [21]). Note that a simplified model for pheromone excretion and uptake is shown.
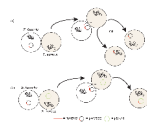
Figure 2.
Possible scenarios which allowed for dissemination of vancomycin resistance to MRSA from VRE. Co-colonization of patients with VRE and MRSA allowed for the transfer of pAM830 which carried the transposon (Tn*1546*) encoding genes associated with high level glycopeptide resistance. Several outcomes appeared to have occurred based upon the analysis of the resultant VRSA strains. It would appear that: (a) only Tn*1546* may have been transferred during the conjugation process instigated by pAM830 and recombined on the chromosome of the *S. aureus* recipient or the complete plasmid was transferred to the *S. aureus* recipient and was stably maintained; (b) the plasmid carrying Tn*1546* was not completely transferred to the recipient cell or incompatibility issues were encountered between pAM830 and existing plasmids in the MRSA strain and the transposon was found in the recipient strain on a native plasmid.

Table 1

Selected enterococcal plasmids with sequenced genomes.

Plasmid[1]	Size[1]	Rep family[2]	Host[1]	Antibiotic resistance[3]	Other traits[3]	Accession number[1]	Reference[3]
Pheromone-responsive plasmids:							
pCF10	67.7	9	E. faecalis	Tet(M)	AS, UV	AY855841	[60]
pAD1	59.3	9	E. faecalis	--	AS, Cyt, UV	**	[61]
pAM373	36.8	8	E. faecalis	--	AS	AE002565	[62]
pMG2200	106.5	-	E. faecalis	Van(B)	AS, Bac, UV	AB374546	[16]
pBEE99	80.6	-	E. faecalis	--	AS, Bac, Bee, UV	GU046453	[63]
Broad host range plasmids:							
pAMβ1	27.8	1	E. faecalis	MLS		GU128949	[27]
pIP816	34.6	1	E. faecium	Van(A)		AM296544	[45]
pRUM	24.9	17	E. faecium	Cm, Sp, Sm, MLS		AF507977	[64]
pIP501	30.6	1	S. agalactiae	Cm, MLS		**	[65]
pAM830	45.0	-	E. faecalis	MLS, Van(A)		**	[8,47]
pRE25	50.2	1, 2	E. faecalis	Cm, MLS		X92945	[38]
Unknown host range:							
pHTβ	63.7	U	E. faecium	Van(A)		AB183714	[42]
pMG1	65.1	-	E. faecium	Gm, Km		AB206333	[44]

[1] From NCBI Entrez Genomes (http://www.ncbi.nlm.nih.gov/sites/genome); GenBank accession numbers are shown. Plasmid size is shown in kilobases (kb), and for pAD1, pIP501, and pAM830 was extracted from references.

** accession number for complete plasmid is not available; readers are directed to references for more information.

[2] From recently defined classification system [7]. U, unique Rep family; -, not typed in previous study [7].

[3] Antibiotic resistance and other traits were extracted from references. --, no known antibiotic resistance traits encoded. Tet(M), tetracycline resistance; Cm, chloramphenicol resistance; Gm, gentamicin resistance; Km, kanamycin resistance; MLS, macrolide, lincosamide, streptogramin B resistance; Van(A)/Van(B), vancomycin resistance; AS, aggregation substance; UV, ultraviolet resistance; Bee, biofilm enhancer in *Enterococcus*, Cyt, cytolysin; Bac, bacteriocin.