Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits

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The *Enterococcus faecalis* pathogenicity island (PAI) encodes known virulence traits and >100 additional genes with unknown roles in enterococcal biology. Phage-related integration and excision genes, and direct repeats flanking the island, suggest it moves as an integrative conjugative element (ICE). However, transfer was observed not to require these genes. Transfer only occurred from donors possessing a pheromone responsive-type of conjugative plasmid, and was invariably accompanied by transfer of flanking donor chromosome sequences. Deletion of plasmid transfer functions, including the *cis*-acting origin of transfer (*oriT*), abolished movement. In addition to demonstrating PAI movement by a mechanism involving plasmid integration, we observed transfer of a selectable marker placed virtually anywhere on the chromosome. Transfer of this selectable marker was observed to be accompanied by chromosome-chromosome transfer of vancomycin resistance, MLST markers, and capsule genes as well. Plasmid mobilization therefore appears to be a major mechanism for horizontal gene transfer in the evolution of antibiotic resistant *E. faecalis* strains capable of causing human infection.

antibiotic resistance | horizontal gene transfer | plasmid mobilization

Horizontal gene transfer has contributed to the evolution of enterococci into leading causes of hospital-acquired infection (1, 2). The first *Enterococcus faecalis* genome sequenced, vancomycin resistant strain V583, consisted of >25% mobile elements (3), including a large pathogenicity island (3, 4). How these elements were acquired into the chromosome is unknown. Movement of the VanB vancomycin resistance element is known to involve transfer of large donor chromosome segments (5). V583 expresses an antiphagocytic capsule that varies among *E. faecalis* strains (6, 7), and despite lacking hallmarks of a mobile element, the operon is widely distributed within the species in a pattern consistent with horizontal transfer (8).

The *E. faecalis* PAI encodes 129 predicted ORFs, including known virulence traits Esp, cytolysin, and aggregation substance (4). The presence of phage-related integration and excision proteins, homologs of plasmid conjugation functions, and terminal direct repeats, suggest that it moves as an integrative conjugative element (ICE). A previous study observed chromosome-chromosome transfer of the *esp* gene by a conjugation-like process; however, it was not determined whether the *esp* was encoded within a pathogenicity island (9). A second study observed transfer of a 27.7-kb segment of the PAI, including the *esp* and cytolysin genes (10).

Because the V583 PAI possessed the hallmarks of an ICE and evidence had emerged for its movement, it was of interest to determine whether it transferred as an ICE. It was of further interest to determine whether its transfer related to the spread of other virulence and resistance traits. We report transfer of the PAI by a mechanism independent of ICE functions and further show horizontal transfer of all regions of the *E. faecalis* chromosome, including a vancomycin resistance transposon, the capsule locus, and alleles used for multilocus sequence typing.

Results

***E. faecalis* PAI Transfer.** To detect transfer and directionality, a selectable tetracycline resistance marker was inserted into three sites spanning the PAI (Fig. 1). Insertions were confirmed by PCR, and the resulting strains were used as donors. Plasmid-free strain OG1RF (11, 12) served as the recipient. Transconjugants were selected on tetracycline for the transferred marker, and rifampicin and fusidic acid for the recipient background. All matings were performed at least three times. Irrespective of selectable marker location, transconjugants were obtained at $\approx 10^{-10}$ per donor (Table 1). No gradient of transfer was noted and, unexpectedly, insertional inactivation of the putative integrase gene did not affect transfer. Transconjugants from six independent mating experiments were verified to possess the recipient capsule locus by PCR (Table S1), confirming their OG1RF origin. Each strain was also verified as having (*i*) the tetracycline resistance marker in the PAI as in the donor, and (*ii*) the entire PAI as a contiguous element inserted into the known chromosomal *attPAI* locus (*SI Text*).

Characterization of Transferred Element. Transconjugants were analyzed by pulsed-field electrophoresis (Fig. 2). All insertions occurred within the recipient SfiI restriction fragment that harbors *attPAI* (4), as verified by PCR. Insertions occurred in five size classes (classes A–E; Fig. 2), not precisely as expected for ICEs (13). Because ends of the PAI were present and fused to *attPAI*, variation was either internal (*E. faecalis* PAIs are known to vary in a modular way; ref. 14) or the result of donor DNA flanking the PAI. PAI contiguity and lack of internal variation was verified in each case by overlapping long template PCR amplification (Table S1), implying variation in flanking sequences.

Role of Putative PAI ICE-Like Genes. Because of imprecise transfer and because the integrase was not required, it was of interest to determine whether inferred PAI ICE-like excisionase and an *oriT* homolog contributed to transfer. An in-frame deletion of the excisionase (*ef0480*) in strain V583*cbh*::pJMM33 resulted in a donor that transferred the PAI at a rate similar to the parental (Table 1). A PAI *oriT*-like sequence with 99% identity to *oriT2* of pAD1 (15) (Fig. S1) was also deleted in V583*cbh*::pJMM33. Again, PAI transfer was unaffected (Table 1). Finally, an in-frame deletion in a TraG-like gene (*ef0503*) 98% identical to *orf53* in pAD1 and previously shown essential for conjugal transfer (16) also had no

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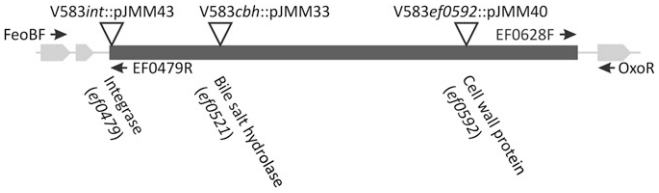

Fig. 1. *E. faecalis* pathogenicity island showing tetracycline resistance marker integration within the *int* gene (orf EF0479), the choline bile salt hydrolase *cbh* gene (orf EF0521), or distal orf EF0592 encoding a surface protein of unknown function. The location and direction of primer pairs (Table S1) used to amplify the left and right PAI junctions are indicated.

effect (Table 1), collectively supporting a PAI transfer mechanism different from that of ICEs (13).

Exclusion of Other PAI Functions in Transfer. To exclude other hypothetical or unrecognizable PAI functions as mediating transfer, OG1RF transconjugants possessing the entire pathogenicity island (TC3, 4, 5, 7, and 12) were assessed for the ability to serve as donors. None was capable of transferring the PAI-resident tetracycline resistance marker to *E. faecalis* OG1SSp (17) (Table 2). The absence of detectable PAI transfer between the OG1RF transconjugant and OG1SSp suggests that the original PAI transfer involved V583-encoded functions external to the pathogenicity island (and beyond the acquired donor DNA flanking the PAI in the transconjugants tested).

Plasmid pTEF2 cotransferred with the pathogenicity island in three of the original transconjugants, implicating its possible involvement. Therefore, one profile A strain harboring pTEF2 (TC1) (Fig. 2), and the profile C strain with pTEF2 (TC9), were tested as donors. As shown in Table 2, marker transfer occurred in both cases. Retransfer was also not precise (Fig. S2). Hybridization confirmed that the *tet*M marker was present on the chromosome in a PAI-associated SfiI fragment. Interestingly, the transconjugants differing from the donor appeared to be similar to PFGE profiles C and E (Fig. 2), suggesting variation by discrete mechanisms, such as the excision of a phage or other mobile element. Second-generation transconjugants of TC1 also revealed PFGE patterns varying from the donor and appearing similar to PFGE profiles B and E seen originally on PAI transfer into OG1RF (Fig. 2).

Table 1. Effect of PAI mutation/marker location on frequency of transfer

Donor strain	Insertion site/genotype	Transfer frequency (per donor)
V583*int*::pJMM43	Integrase gene	2.9 (± 1.9) × 10^{-10}
V583*cbh*::pJMM33	Conjugated-bile salt hydrolase	5.4 (± 3.8) × 10^{-10}
V583*ef0592*::pJMM40	Large cell-wall associated protein	6.1 (± 2.1) × 10^{-10}
V583*cbh*::pJMM33*	Conjugated-bile salt hydrolase	2.5 (± 2.0) × 10^{-10}
V583*cbh*::pJMM33Δ*exc*	Conjugated-bile salt hydrolase, deleted excisionase	8.1 (± 1.7) × 10^{-10}
V583*cbh*::pJMM33Δ*oriT*	Conjugated-bile salt hydrolase, deleted *oriT*	2.9 (± 4.1) × 10^{-10}
V583*cbh*::pJMM33Δ*ef0503*	Conjugated-bile salt hydrolase, deleted *traG*	1.9 (± 2.2) × 10^{-10}

*In the presence of 300 μg/mL of DNase, see *SI Text*.

Determination of the Boundaries of DNA Transferred. V583 donor gene content in transconjugants of all five PFGE profiles (TC1, TC3, TC4, TC5, and TC12) was determined by using a custom V583 antisense microarray (8). All transconjugants were confirmed to possess each PAI orf (EF0479–EF0628) as well as additional donor-specific genes (Fig. S3). For strain TC1, all ORFs on plasmid pTEF2 were present. Strain TC12 also contained ORFs EF0302–EF0355, which correspond to putative prophage 01 in the donor, raising the prospect of phage involvement in transfer. Sequences flanking the PAI were further characterized by detecting restriction polymorphisms and then single nucleotide polymorphisms (SNPs) to localize points of crossover more precisely. In the transconjugants analyzed, between 38 and 156 kb of DNA was acquired left of the PAI, and 108–563 kb of DNA was acquired right of the PAI, corresponding to a total length of transferred DNA of 285–857 kb, or 9–27% of the donor chromosome (Table 3).

Transfer of Non-PAI Donor DNA. Because PAI transfer was not precise and did not involve PAI-encoded functions, it was of interest to determine whether there was anything specific to the PAI, or whether PAI transfer had been observed only because that was what was selected. To determine whether other regions of the V583 genome could transfer, a collection of selectable marker insertions spanning the chromosome (18) was used. These mutants possessed tetracycline marker insertions into each nonessential response regulator of V583 (18). Each of the tetracycline resistance markers was observed to transfer (Fig. 3). Additional marker insertions in the *elrA* gene (encoding an internalin-like gene product; refs. 19 and 20) and *EF1852* (a component of a putative PTS system) also transferred. Rates of transfer varied from 1.1×10^{-5} to 9.1×10^{-11} per donor.

Transfer of the native vancomycin resistance operon in the V583 chromosome was not detected by using direct vancomycin selection. Vancomycin resistance is known to be inefficient for direct selection (21). The chromosomal region encoding vancomycin resistance, in fact, transferred at a frequency of 1.98×10^{-8} per donor, using as a surrogate, transfer of the tetracycline marker inserted within the vancomycin resistance operon in the *vanR* response regulator (RR11). Additionally, transfer of the tetracycline resistance marker inserted into RR01, 87 kb from RR11, occurred at a frequency of 3.29×10^{-10} per donor. Of the RR01 transconjugants selected with tetracycline, 30% also expressed vancomycin resistance on counter selection.

The observation of very high rates of marker transfer from some locations raised the question of whether regionally specific events were occurring (e.g., the *tetM* markers of RR08 and V583*ef1852*::pJMM61 are only 10 kb apart; however, the transfer rate varies by 3 orders of magnitude; Fig. 3). To test whether the products of transfer from regions where transfer occurred at high rates differed from that seen elsewhere, DNA of eight RR08 transconjugants was digested with the enzyme I-CeuI which recognizes a conserved site unique to the 23S rRNA operon of bacterial genomes and cleaves only chromosomal DNA (22, 23). Hybridization of the *tetM* probe to PFGE resolved I-CeuI restriction fragments showed that it was plasmid-borne in six transconjugants, whereas it occurred in the chromosome in two. In the six where *tetM* hybridized to a plasmid band, four size variations were observed. In contrast, similar experiments on 10 transconjugants from the lower frequency mating of donor V583*ef1852*::pJMM61 showed the marker to be on the chromosome in each case. Therefore, at least some of the elevated RR08 transfer rate stemmed from plasmid rescue of the marker without apparent need for additional recombination in the recipient.

Tetracycline resistance markers in RR16 and RR18 transferred at the highest rates (1.1×10^{-5} per donor). Five of 10 RR16 transconjugants tested possessed the replication genes of pTEF2 as determined by PCR, implying the presence of a potentially functional plasmid. Total DNA from the 10 transconjugants was therefore restricted with SfiI, which cleaves the chromosome five

Table 2. Rates of secondary PAI transfer to OG1SSp

Donor strain	Donor type	Transfer frequency
TC7	PFGE profile A	ND $<4.4 \times 10^{-11}$
TC3	PFGE profile B	ND $<7.9 \times 10^{-11}$
TC4	PFGE profile C	ND $<1.0 \times 10^{-11}$
TC5	PFGE profile D	ND $<6.4 \times 10^{-11}$
TC12	PFGE profile E	ND $<3.2 \times 10^{-11}$
TC1	PFGE profile A* + pTEF2	$2.9 (\pm 1.6) \times 10^{-9}$
TC9	PFGE profile C + pTEF2	$2.7 (\pm 1.2) \times 10^{-9}$

ND, Not detected over limit of detection indicated.
*As defined in Fig. 2.

times and linearizes pTEF2. After electrophoretic separation, the DNA was hybridized with probes generated to (*i*) the *tetM* gene (TetM-FW and TetM-RV; Table S1), (*ii*) the replication protein encoding gene of pTEF1 (repA1F and repA1R) and (*iii*) the replication protein encoding gene of pTEF2 (repA2F and repA2R). All three probes hybridized to the same plasmid band in each of the five transconjugants, although the size of the plasmid bands differed—138 kb in one strain, 163 kb in three transconjugants, and 220 kb in one strain. This observation indicated that the tetracycline resistance marker had been rescued by an event involving recombination with both plasmids. The five remaining RR16 transconjugants were positive only for pTEF1. In those strains, the *tet*M probe hybridized to three different sized pTEF1-positive plasmids of ≈76 kb (two transconjugants), 85 kb (two transconjugants), or 101 kb. pTEF3 was not observed in transconjugants.

Because transfer of marker insertions in the PAI did not generate such plasmid products, it was of interest to determine the extent that plasmid rescue may have skewed presumed chromosome-chromosome transfer rates in other matings. Ten transconjugants each from donors RR01, RR02, RR03, RR04, RR06, RR09, RR10, RR11, RR13, RR15, RR16, RR17, and RR18 were screened by colony blot for the presence of the replication functions of either pTEF1 or pTEF2 (*repA* gene homologs *efa0001* and *efb0001*), as first evidence of the possibility of plasmid rescue. Of the 130 transconjugants, 28 contained pTEF1 *repA*, whereas 30 contained pTEF2 *repA*; only the 5 RR16 transconjugants noted above possessed both pTEF1 and pTEF2 *repA* genes. A total of 79 transconjugants were plasmid-free, ruling out recombinational rescue and plasmid transfer as seen in RR16 and RR18 experiments (Table S2). With the exceptions of transconjugants from RR16 and RR18 donors as noted above, 40–100% of the transconjugants from each of the other transfers completely lacked pTEF1 or pTEF2, meaning that the rates of marker transfer noted in Fig. 3 closely approximate chromosome-chromosome transfer of the tetracycline resistance marker, within a <2.5-fold range of uncertainty.

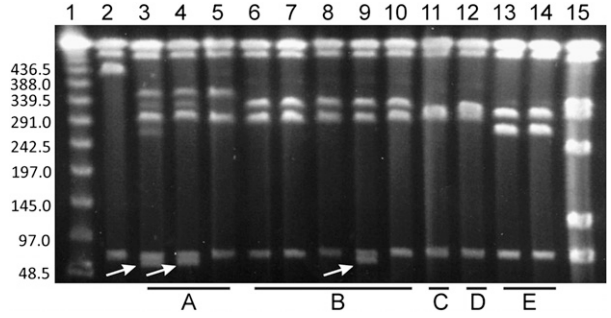

Fig. 2. SfiI macrorestriction patterns of *E. faecalis* PAI transconjugants. Lane 1, lambda DNA ladder standard; lane 2, recipient strain OG1RF; lanes 3–14, transconjugant strains TC1, TC2, TC7, TC4, TC6, TC8, TC9, TC10, TC3, TC12, TC5, and TC11, respectively; lane 15, donor strain V583. PFGE pattern groupings are indicated below the gel. Plasmid pTEF2, 57.7 kb in size and present in 3 of the 12 transconjugants, is indicated by arrows.

Role of pTEF1 and pTEF2 in Chromosomal DNA Transfer. The observations (*i*) that transfer of the PAI from an OG1RF transconjugant into a second recipient only occurred when pTEF2 was present (Table 2); (*ii*) that inactivation of putative PAI functions related to transfer did not affect transfer rate (Table 1); and (*iii*) that pTEF2 transferred along with the PAI in 3 of the first 12 transconjugants examined (Fig. 2) implied that pTEF2 may be involved in PAI transfer (and, perhaps, also in transfer of other regions of the chromosome). To determine whether both pTEF1 and pTEF2 were capable of mediating chromosome-chromosome marker transfer (in the latter case using pTEF2Cm with selectable chloramphenicol resistance gene integrated into plasmid gene *efb0050*) in the absence of functions encoded by the other mobile elements present in V583 [e.g., PAI or phage (all *E. faecalis* strains harbor phage 02, which appears to be part of the core genome; ref. 8)], the plasmids were individually introduced into OG1RF*erlA*::pJMM42 possessing the tetracycline marker arbitrarily inserted into the *erlA* gene. Transfer of the chromosomal tetracycline resistance marker from the OG1RF background occurred from either pTEF1 or pTEF2Cm-harboring donors at a similar rate of 4.1×10^{-9} and 4.3×10^{-9} per donor, respectively (Table 4). To determine whether pTEF1 also mediated bona fide chromosome-chromosome transfer, eight transconjugants from the OG1RF*elrA*::pJMM42(pTEF1) mating were digested with I-CeuI, separated by PFGE, and hybridized with the *tetM* probe. In each case, the *tetM* marker was located on a chromosomal fragment of the transconjugant.

To prove the converse, that resident conjugative plasmids were necessary for transfer of the PAI from strain V583, a derivative of this strain cured of all plasmids (strain V19) was obtained from Axel Hartke (University of Caen). The tetracycline marker again was integrated into the *cbh* gene of the PAI in V19, and the resulting plasmid-free strain V19*cbh*::pJMM33 was tested in conjugation experiments. Transfer of tetracycline resistance was not observed in nine independent mating experiments.

Role of Plasmid Transfer Determinants in the Movement of Chromosomal DNA. To determine whether a mechanism similar to that known for *Escherichia coli* Hfr donors (24) accounted for chromosome-chromosome transfer of *E. faecalis* genes, the putative *oriT* of pTEF1 and pTEF2 were deleted. The *oriT* of both plasmids was identified based on extensive nucleotide identity with experimentally identified origins of transfer on plasmids pAD1 and pCF10 (Fig. S1). Allelic exchange was used to delete 39 bp of the 40-bp *oriT* of pTEF2Sp and delete the 169-bp pTEF1 *oriT* in the strain V583*cbh*::pJMM33(pTEF1)(pTEF2*efb0049::Spc*), yielding V583*cbh*::pJMM33(pTEF1Δ*oriT*)(pTEF2*efb0049::Spc*Δ*oriT*). Using this donor in triplicate matings, no transfer of the chromosomal tetracycline resistance marker was detected (Table 4). In control experiments, deletion of the putative *oriT* from pTEF1 and pTEF2 also prevented transfer of the plasmids themselves.

Additional experiments showed that pTEF1 and pTEF2 plasmid transfer functions TraG and relaxase were essential, as described in *SI Text*. Transfer was further found to be insensitive to the presence of 300 μg/mL DNase and was not affected by sterile filtrates of donor cultures (*SI Text*). Finally, transconjugants were only recovered when *recA* was functional in both donors and recipients (*SI Text*).

Contribution of Horizontal Marker Transfer to the Generation of Diversity Within *E.* faecalis. Because selection for transfer of the tetracycline resistance marker inserted into RR01 resulted in transconjugants harboring also the unselected vancomycin resistance operon, it was of interest to determine what other nonselected genes of interest had transferred in the above experiments. eBURST analysis of enterococci implied substantial horizontal mixing of MLST markers (25), and horizontal spread of the

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Table 3. Size of DNA regions transferred from donor to recipients

Trans-conjugant	Limits of transferred DNA: First SNP identical to donor	Limits of transferred DNA: Last SNP identical to donor	Minimal size of transferred DNA, kb	Percentage of chromosome transferred
TC1	346440	855190	508.8	15.8
TC3	406434	691160	284.7	8.8
TC4	336350	968399	632.0	19.6
TC5	346440	926691	580.3	18.0
TC12	288799	1146312	857.5	26.6

E. faecalis capsule operon was inferred from its distribution (8). It was therefore of interest to determine whether genes related to these also transferred. The large amount of donor DNA acquired by transconjugant TC12 (857 kb) suggested the prospect that flanking donor DNA may have changed the MLST type of the recipient. Sequencing of the *gdh* MLST locus showed that TC12 had, in fact, possessed the donor locus and was now, by MLST analysis, a single locus variant of the recipient strain. The tetracycline resistance marker inserted into RR06 positioned the selectable marker close to two MLST loci. Ten transconjugants from this donor were therefore analyzed. Nine transconjugants were found to be identical to the recipient; however, one was observed to be a double locus variant, containing donor versions of both *gdh* and *yip*. This observation shows that plasmid-mediated mobilization of chromosomal DNA contributes to the MLST diversity of the *E. faecalis* species, including the generation of double locus variants in a single step, and single locus variants possessing 25% of the genome derived from horizontal transfer.

To determine whether transfer of chromosomal markers could result in the conversion of transconjugants from one capsule type to another, tetracycline resistance marker insertions were made in proximity to the capsule operon. Specifically, the selectable marker was placed within *ef2507* encoding an unknown hypothetical protein (V583*ef2507*::pJMM79). Using this strain as a donor, transfer of the chromosomal tetracycline resistance marker was observed to occur at a rate of 1.51×10^{-10} in one experiment. Three transconjugants were tested for transfer of the type 2 capsule operon into OG1RF by using the *cps*-specific primers described (Table S1), and one transconjugant was found to possess the entire *cps* operon *cpsA-cpsK* as found in the donor.

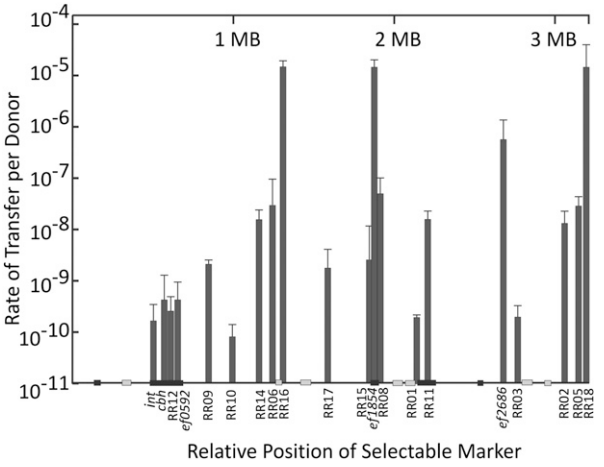

Fig. 3. Rate of transfer of tetracycline resistance from various points in the V583 chromosome. The relative location of tetracycline resistance marker insertions within the chromosome are indicated on the horizontal axis with scale shown above. Large regions of acquired/mobile DNA that occur naturally in the V583 donor chromosome are identified by small boxes along the lower horizontal axis (putative phage shown in light gray; other mobile elements such as the pathogenicity island, black).

Evidence for Plasmid Cointegrate Formation. The observation of mobilization of an antibiotic resistance marker from any point within the chromosome at rates that showed no obvious gradient of transfer (Fig. 3), combined with the evidence that transfer could be effected by either pTEF1 or pTEF2, suggested that pTEF1 and pTEF2 are capable of insertion at multiple points around the chromosome, possibly as the result of recombination across shared repeated sequences or IS element-mediated cointegrate formation. Ten highly conserved (> 98% identical) copies of IS256 are present in the genome of V583 (six on the chromosome, two on pTEF1, one on pTEF2, and one on pTEF3), indicating recent movement. This observation suggests the potential for cointegrate formation by *recA*-dependent or -independent means, placing a plasmid *oriT* in *cis* with the chromosomal marker (as is known for F integration into the *E. coli* chromosome; ref. 26). To test this reasoning, PCR primers complementary to unique DNA sequences flanking IS256 copies on the chromosome, and on pTEF1 and pTEF2 (Table S1 and Fig. S4) were used to amplify potential recombination products that could occur upon recombination, using DNA from a 10-mL V583 culture ($\approx 1 \times 10^{10}$ cells). PCR products corresponding to the predicted sizes were obtained by using the forward primer from each plasmid IS element (IS256aF, IS256bF, and IS256iF; Fig. S4), in combination with the reverse primer from each chromosomal IS element (IS256cR, IS256dR, IS256eR, IS256fR, IS256gR, and IS256hR). PCR products were sequenced to confirm the hybrid nature of the amplified DNA. PCR products were also obtained by using the forward primers from the pTEF1 IS elements (IS256aF and IS256bF) and the reverse primer of the pTEF2 IS element (IS256iR). Therefore, in a relatively small population, all possible IS256-related cointegrants appear to occur, creating the potential for transfer of chromosomal genes from virtually any point.

Discussion

Our original hypothesis was that, based on its structure, the *E. faecalis* PAI was capable of movement as an ICE. We observed transfer of the entire PAI at similar rates, irrespective of the location of the tetracycline resistance marker as would be predicted (13). However, the integrase, excisionase, and PAI-resident putative conjugation genes did not contribute detectably to transfer. Moreover, PAI transfer was not precise. Cotransfer of DNA flanking the PAI suggested a more general mechanism. It was indeed observed that resistance markers placed around the entire chromosome could be transferred, and this transfer depended on the integration of either one of two resident putatively pheromone responsive plasmids, pTEF1 or pTEF2.

Coburn et al. (10) reported transfer of portions of the PAI into a recipient where rescue by a resident plasmid with sequence identity to genes within the PAI appears to have occurred. Efficient rescue may have precluded their detection of lower rates of transfer of the entire element. Oancea et al. (9) reported movement of genes known to occur on the PAI between enterococcal strains by an unknown mechanism. We believe our findings explain these observations.

We observed chromosome-chromosome transfer of as much as 857 kb of donor DNA. Plasmid-associated transfer of chromosomal DNA between *E. faecalis* strains was inferred in 1978 by Franke and colleagues (27), who observed transfer of selectable markers in the presence of coresident transmissible plasmids, but not from a plasmid-free donor. Francois et al. (28) observed transfer of *tetS* from *E. faecalis* BM4210 to JH2-2 and OG1RF only in the presence of conjugative plasmid, pIP825, and acquisition of the 40-kb *tetS* element was associated with movement of large pieces of the chromosome. ICE have also been implicated in

Table 4. Role of plasmids and plasmid functions in chromosomal DNA transfer

Donor strain	Description	Transfer frequency
OG1RF*elrA*::pJMM42	Tc^R	ND ($<5.1 \times 10^{-11}$)
OG1RF*elrA*::pJMM42(pTEF1)	Tc^R, Gm^R; contains pTEF1	4.1 (± 1.1) $\times 10^{-9}$
OG1RF*elrA*::pJMM42(pTEF2Cm)	Tc^R, Cm^R; contains pTEF2Cm	4.3 (± 2.8) $\times 10^{-9}$
OG1RF*elrA*::pJMM42(pTEF1Δ*oriT*)	Tc^R, Gm^R; contains pTEF1 *oriT* deletion	ND ($<6.0 \times 10^{-11}$)
OG1RF*elrA*::pJMM42(pTEF1Δ*efa0025*)	Tc^R, Gm^R; contains pTEF1 relaxase deletion	ND ($<7.7 \times 10^{-11}$)
OG1RF*elrA*::pJMM42(pTEF1Δ*efa0030*)	Tc^R, Gm^R; contains pTEF1 TraG-homolog deletion	ND ($<5.6 \times 10^{-11}$)
V19*cbh*::pJMM33	Tc^R	ND ($<4.1 \times 10^{-11}$)
V583*cbh*::pJMM33(pTEF2*efB0049::Spc*,Δ*oriT*)(pTEF1 Δ*oriT*)	Tc^R, Sp^R; *oriT* deletions in both pTEF1 and pTEF2	ND ($<3.3 \times 10^{-11}$)
V583*cbh*::pJMM33(pTEF2*efB0049::Spc*,Δ*efB0025*) (pTEF1Δ*efA0030*)*	Tc^R, Sp^R; *TraG-homolog* deletions in both pTEF1 and pTEF2	ND ($<1.8 \times 10^{-11}$)
V583*cbh*::pJMM33(pTEF2*efB0049::Spc*,Δ*efB0030*) (pTEF1Δ*efA0025*)*	Tc^R, Sp^R; *relaxase* deletions in both pTEF1 and pTEF2	ND ($<3.6 \times 10^{-11}$)

ND, not detected.
*See *SI Text*.

transfer of chromosomal DNA in *E. faecalis* (29). Tn925 (a homolog of the prototype ICE, Tn916) can mobilize chromosomal genes in *E. faecalis*, resulting in nonselected transfer of carbohydrate fermentation genes and chromosomal antibiotic resistance markers (29). Transfer of large regions of chromosomal DNA has also been observed in *E. faecium* (30, 31). Rice et al. (31) noted transfer of a low-affinity *pbp5* in *E. faecium* at rates of $\approx 10^{-9}$ transconjugants per recipient. Regions of the chromosome within which *pbp5* inserted were heterologous, generating transconjugants with altered PFGE patterns (31). McAshan et al. (30) observed that transfer of vancomycin resistance genes from *E. faecium* was associated with the acquisition of fragments of DNA from 145 to 277 kb by the recipient (30). Also in *E. faecium*, it was noted that the mechanism of Tn*5385* transfer involved recombination between flanking donor sequences with homologous sequences in the recipient (32). MLST studies have found identical alleles in genetically diverse *E. faecalis* isolates (25); because of inferred high rates of recombination in *E. faecium*, eBURST is of limited value for ancestry determination (33).

The role of plasmids in the transfer of chromosomal DNA has been well documented in Gram-negative bacteria, with the F plasmid of *E. coli* being the prototype (34–36). In Gram positives, an integrated sex factor in *Lactococcus lactis* transfers chromosomal DNA at frequencies inversely correlated to the distance from the transfer origin (37, 38). In *Streptococcus agalactiae,* chromosomal segments as large as 334 kb appear to have been transferred, potentially through mobilization by conjugative elements (39).

We observed transfer of chromosomal DNA only in the presence of one of the two endogenous putatively pheromone-responsive, conjugative plasmids in the V583 donor. Introduction of either plasmid into a plasmid-free strain also mobilized chromosomal DNA. The fact that deletion of *oriT* abrogated chromosome-chromosome transfer, and that an *oriT* only functions in *cis*, shows that plasmid/chromosome cointegrate formation is necessary for chromosomal DNA transfer in *E. faecalis* as tested. The chromosomal tetracycline resistance marker was sometimes found rescued by one of the endogenous plasmids (pTEF1 or pTEF2). Thus, recombination between the chromosome and plasmid allows for mobilization of chromosomal DNA via an Hfr-like mechanism, or after excision, via an F′-like mechanism. Analysis of RR08 transconjugants from our study shows that both types can occur when selecting for transfers from the same chromosomal locus. The rate of resistance marker transfer appears to depend on local recombination frequencies, and whether rescue as a transmissible, independently replicating plasmid is likely.

Transposable elements facilitate cointegrate formation by both *recA*-dependent and *recA*-independent mechanisms (40). In *Rhizobium* species, it has been shown that the genome is in constant flux, with many of the possible products of recombination between repeated elements existing (41, 42). Interestingly in *Staphylococcus epidermidis*, it has been noted that the bacterial insertion sequence element IS256 occurs preferentially and is abundant in nosocomial isolates, and it has been hypothesized that it may be involved in the adaptation of the genome of clinical isolates (43). IS256 was examined arbitrarily in the present study, and all predicted recombinations were found. The *E. faecalis* V583 genome contains a total of 38 IS elements, and there are three additional regions where plasmid remnants have integrated. It is known that large chromosomal deletions result from recombination between heterologous Tn916-like elements in enterococci (44) and that the integrase from some Tn916-like elements can promote circularization with termini derived from heterologous transposons (45). Collectively, these observations imply that there are many other recombinants in the population that were undetected.

The high pathogenicity island in *E. coli* appears to have been acquired in two steps (46). First, it was introduced into *E. coli* by phage-, plasmid-, or ICE-mediated transfer. It then appears to have spread within the species by mobilization by conjugative plasmids. Our results indicate that this model can be extended to movement of the *E. faecalis* PAI, although we cannot exclude PAI transfer via an ICE-type mechanism in some environment other than that tested here.

We believe we have proven a mechanism of transfer that accounts for numerous past observations relating to the emergence of *E. faecalis* among the vanguard of hospital pathogens with increased virulence and antibiotic resistance. We observed transfer not only of plasmid-mediated movement of the *E. faecalis* PAI, but also MLST markers, a capsule locus, and antibiotic resistance determinants. This work highlights the importance of the unique pheromone responsive class of plasmids in enterococci to the evolution of this important hospital pathogen.

Materials and Methods

Bacterial Strains. Bacterial strains and plasmids used in this study are listed in Table S3. *E. faecalis* strains were grown at 37 °C in Brain Heart Infusion (BHI) without aeration or on BHI agar [1.4% (wt/vol)]. Antibiotics used for selection included ampicillin (50 μg/mL), rifampicin (50 μg/mL), fusidic acid (25 μg/mL), streptomycin (1,000 μg/mL), spectinomycin (250 μg/mL), and chloramphenicol (12 μg/mL for *E. coli* and 20 μg/mL for *E. faecalis*), and tetracycline (15 μg/mL). Details of strain and plasmid construction are provided in *SI Text*.

Conjugation. Because of the low frequency of transfer, mating experiments were performed by using the entire surface of agar plates. Briefly, 500 μl of overnight culture of donors and recipients were individually pelleted and washed (due to the slow growth of *E. faecalis* UV202, where required, it was

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used at a 500 μl of UV202 to 2.5 μl of donor strain ratio). Washed cells were resuspended with 125 μl of BHI, mixed, and spread onto BHI agar. To test whether extracellular DNA was involved in transfer, 300 μg/mL DNase was added to the mating before spreading. After 24 h at 37 °C, plates were scraped and cells resuspended in 1 mL of BHI. One hundred microliter aliquots were plated on BHI agar with appropriate selection and incubated for 48 h. Transfer rates were calculated as transconjugants per donor cell.

To test for phage involvement, 25 mL of V583*cbh*::pJMM33 overnight growth was pelleted and the supernatant was sterilized by passage through a 0.45-μm filter. The filtrate was diluted 2-fold with fresh medium, and the conditioned medium was inoculated with OG1RF. After overnight incubation, cells were harvested, resuspended in 2 mL of fresh BHI, and plated with tetracycline selection.

Characterization of Transconjugants. PCR was used to confirm strain identity by amplifying donor and recipient-specific genes and to detect site-specific integration of the PAI. All primer sequences used in this study are listed in Table S1. Standard PCR used Taq DNA polymerase (New England Biolabs) and long-template PCR used the Expand Long Template PCR kit (Roche), both performed according to manufacturer's instructions (Table S4). Pulsed-field gel electrophoresis of transconjugant genomes digested with I-CeuI, SfiI, or SmaI was performed as described (47). Radiolabeled probes were prepared by incorporation of [α-^{32}P]dCTP-labeled deoxynucleotides (Perkin-Elmer) using Ready-To-Go DNA labeling beads (GE).

Multi-Locus Sequence Typing. An established set of *E. faecalis* MLST primers were used for amplification and sequencing as described (http://efaecalis.mlst.net; ref. 48) and are listed in Table S1. The seven genes evaluated for MLST of *E. faecalis* are *aroE, gdh, gki, gyd, pstS, xpt*, and *yqiL*.

Nucleotide Sequence Analysis. Sequencing was performed by the Massachusetts General Hospital DNA Sequencing Core and assembled using the Seqman program (DNAstar). Open source program MUMmer 3.0 (49) (www.cs.jhu.edu/~genomics/MUMmer) was used for alignment of the V583 and OG1RF genomes and detection of SNPs.

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