Supporting Information

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SI Text

Verification of Transconjugants. To collect evidence that transfer of the inserted marker within the PAI in fact involved transfer of the PAI as intended (as opposed to some unanticipated recombinational rescue of the tetracycline resistance marker by a mobile element in the donor), three regions of the PAI, known to be absent in OG1RF (1), were sampled in each transconjugant by PCR. Primers were designed to identify regions near the three points used to drive plasmid integration. These included an intergenic region between EF0482 and EF0483, the *cbh* gene (EF0521), and EF0592. All transconjugants, but not recipient controls, were found to possess all three regions of the PAI tested. Further, to determine whether insertion occurred in the OG1RF chromosome at the *attPAI* locus known for PAI insertion in V583 (see Fig. 1), PCR primers were designed to read into the PAI from the chromosome on both sides of *attPAI*. Whereas the amplification in the recipient OG1RF results in generation of an 893-bp fragment using primers that flank *attPAI*, a large insertion within *attPAI* will exceed the length that can be amplified by Taq polymerase and no product will be obtained. For each of the transconjugants tested, no product was obtained, suggesting either insertion of the PAI as anticipated, or deletion of the *attPAI*. To prove PAI insertion, primers were designed to read outward from the ends of the PAI and were used in combination with those complementary to points within the chromosome on either side of *attPAI*. For each transconjugant, identical products were obtained, indicating that in all cases, the PAI was present at the *attPAI* within OG1RF, and all were present in the same orientation as in the donor strain V583. Additionally, PCR was used to confirm that p3TET was still integrated within the *cbh* gene as in the original donor. PCR using plasmid-specific primers was also used to detect the presence of any of the three plasmids native to the V583 donor. The plasmids pTEF1 and pTEF3 were not detected in any of the transconjugants; however, products specific for plasmid pTEF2 were positive in 3 of the 12 transconjugants.

Determination of Donor DNA Boundaries in Transconjugants. Comparison of chromosome sequences showed that PAI donor V583 and recipient OG1RF share extensive gene synteny both 5′ and 3′ to the conserved PAI attachment site. To determine the boundaries of PAI-flanking donor DNA in the transconjugants, 50 kb either side of the PAI were queried by long-template PCR by using primers shown in Table S1 and determining the presence of restriction polymorphisms unique to donor or recipient. Primers pairs were used to amplify 10 overlapping LT-PCR products of ≈10 kb each. Each amplicon was digested with a restriction enzyme selected to distinguish donor from recipient DNA (Fig. S5 and Table S4). For each transconjugant class tested, the restriction profiles of PCR products B, C, D, E, F, G, H, I, and J (Fig. S5) were identical to donor V583, and distinct from OG1RF, showing that ≈30 kb or more donor sequence flanked each side of the PAI. Restriction digestion of PCR product A of TC1, TC4, TC5, and TC12 also showed identity to V583. For TC3, PCR amplicon A exhibited the OG1RF digestion pattern, indicating that the crossover for this transconjugant occurred between A and B, whereas the others extended beyond the range tested. All five transconjugants yielded an amplicon J with restriction profile identical to V583. Thus, in four of the five transconjugants profiles, at least 100 kb of V583 donor chromosomal DNA was acquired in addition to the 138 kb that comprises the pathogenicity island and, in all cases, this DNA appears to be contiguous. With one of the boundaries of the DNA acquired by transconjugant TC3 fixed within PCR amplicon A or between amplicons A and B, it could be calculated to have acquired ≈40-kb of donor sequence left of the PAI as illustrated (Fig. S5) and >50 kb right of the PAI, corresponding to a total of at least 225 kb of donor DNA, including the 138-kb PAI.

Consistent with the results of primer walking either side of the PAI in the transconjugants, comparative genome hybridization data indicated that the donor/recipient crossover in TC12 occurred in the 107 kb between EF0184 and EF0302 on the left side of the PAI as shown in Fig. S5 (which includes prophage 1; Fig. 4) and between EF1175 and EF1329 on the other side. The other four transconjugants, like OG1RF, were negative for prophage 01, meaning the left border of acquired DNA occurred within the 120 kb between EF0355 and EF0479. To the right of the island as illustrated (Fig. S5), the first ORF unique to the donor V583 and absent in recipient OG1RF is EF0723, and all five transconjugants were positive for this gene. Based on transconjugant genes found by CGH to be common to the donor and absent in the recipient, the right-hand boundaries were determined as follows: TC1 (EF0823–EF0931), TC3 (EF0723–EF0728) TC4 (EF0967–EF1051), and TC5 (EF0967–EF1051).

To more precisely define the DNA boundaries on both sides of the PAI, the software program MUMmer 3.0 was used to align the V583 genome sequence with that published for strain OG1RF (2) to identify polymorphisms. PCR primers (Table S1) were designed at ≈10-kb intervals to amplify DNA sequences containing SNPs, within the regions of probable DNA recombination for the five transconjugants as identified by CGH. The resulting PCR products were sequenced to identify whether the DNA was from the chromosome of the donor or recipient strain. In all cases, SNP analysis supported the integration of contiguous sequences of donor DNA.

Role of RecA in Transfer. In Hfr-type DNA transfer, RecA-mediated homologous recombination in the recipient is often required for rescue and integration of the acquired DNA into the recipient chromosome. To detect a possible RecA requirement for the acquisition of chromosomal DNA by an *E. faecalis* recipient, the rate of transfer of the tetracycline resistance marker inserted in the PAI *cbh* gene of the V583*cbh*::pJMM33 donor, into either the *recA*-deficient strain UV202 (3, 4), or the wild-type parental strain JH2-2, was compared. Matings were performed three times in triplicate. No tetracycline-resistance transconjugants were detected in the UV202 recipient, whereas transfer into JH2-2 occurred at a rate of $1.6 \times 10^{-10} \pm 1.97 \times 10^{-10}$ per donor, comparable with that seen with the OG1RF recipient. This shows that acquisition of the PAI by a *rec*-independent mechanism, as would be predicted to occur in ICE element movement, does not occur within the limits of detection, supporting a model where homologous recombination is the main mechanism of recovery of the PAI.

To test the role of RecA in mediating the formation of a cointegrate between the mobilizing plasmid and the donor chromsome, *rec*A itself was deleted, as described in *Materials and Methods*, in three independent isolates of the donor strain V583*cbh*::pJMM33. The ability of resident pTEF1 or pTEF2 to integrate and mobilize the chromosome was then assessed in triplicate matings. Again, no tetracycline-resistant transconjugants were recovered, indicating that the main mechanism of plasmid integration into the chromosome under the conditions tested was RecA-dependent.

Conjugation Versus Transformation and Transduction. We initially hypothesized, based on conserved excisionase and integrase genes, and terminal duplications, that the PAI would transfer between *E. faecalis* strains by a conjugative ICE-like mechanism. Therefore, initial transfer experiments used a conjugation format. Because ICE-like transfer did not appear to occur, and in one putative transconjugant (TC12; Fig. 5) a putative phage was observed to have been acquired, we conducted additional experiments to detect possible DNA transfer by other mechanisms. To determine whether the presence of extracellular DNA was involved in transfer, V583*cbh*::pJMM33 was mixed with OG1RF in the presence of DNase. Mating experiments were set up in the usual manner, except that the donor/recipient cell mixture was resuspended in 250 μl of 300 μg/mL DNase I before spread plating onto nonselective BHI agar. No difference was noted when comparing transfer frequency in the presence or absence of DNase (Table 2), indicating that extracellular DNA is not needed for transfer.

To test whether a phage may be involved in transfer, a 25-mL broth culture of V583*cbh*::pJMM33 was grown overnight and filtered through a 0.45-μm filter. To the filtrate, 25 mL of fresh BHI broth was added, and inoculated with the recipient strain OG1RF. After overnight growth in the conditioned medium, potential transductants were selected on plates containing rifampicin, fusidic acid, and tetracycline. However, after 48 h, no colonies were observed. This experiment was repeated three times, yielding negative results on each occasion.

To determine whether other plasmid transfer functions were involved in the movement of chromosomal DNA or whether functions encoded within the PAI or other chromosomally embedded plasmid remnant contributed, double deletions of the putative pTEF1 and pTEF2 plasmid relaxase genes and the plasmid TraG-homologs were constructed in V583*cbh*::pJMM33 (pTEF1)(pTEF2*efb0049::Spc*). Relaxase binds and nicks a cognate *oriT* sequence, initiating the transfer process (5). A putative pTEF1 relaxase (EFA0025) was identified that possesses 65% identity to the experimentally identified relaxase of pAD1, which belongs to a new class that lacks a 3-histidine motif (6). Interestingly, two proteins with 99% identity to the pAD1 relaxase are present in the chromosome of V583, one within the PAI (EF0505) and the second (EF0025) within an area thought to comprise a region of integrated plasmid. The relaxase of pTEF2 (*efb0030*) is 99% identical at the protein level to that of pCF10 (PcfG), and there are no other homologs in the strain. Allelic exchange was used to delete 721-bp of the 786-bp gene *efa0025* and 1,552-bp of the 1,686-bp gene *efb0030* in the strain V583*cbh*::pJMM33 (pTEF1)(pTEF2*efb0049::Spc*). This strain contains a tetracycline marker integrated in the *cbh* gene of the PAI. The resulting mutant lacking both pTEF1 and pTEF2 relaxases, termed V583*cbh*::pJMM33 (pTEF1*Δefa0025*)(pTEF2*efb0049::Spc,Δefb0030*), was mated with OG1RF. No transconjugants were obtained in independent triplicate experiments.

Another function essential for transfer of pheromone-responsive plasmids is TraG (7). A putative TraG on pTEF1, EFA0030, has 82% identity and 88% similarity to Orf53, a TraG homolog found on pAD1 demonstrated to be essential for conjugative transfer of the plasmid (6). A putative TraG homolog was also identified on pTEF2, EFB0025, has 99% homology at the protein level to PcfC (8), a membrane-bound putative ATPase related to the coupling proteins of Gram-negative T4S machines. Both TraG homologs were deleted by allelic exchange, deleting 2,137 bp of *efa0030* and 1,731-bp of *efb0025*, and generating strain V583*cbh*::pJMM33(pTEF1*Δefa0030*) (pTEF2*efb0049::spc,Δefb0025*). Mating experiments using the double TraG-homolog deletion mutant strain as a donor and OG1RF as a recipient were performed in triplicate independent experiments, but no transfer of the chromosomally integrated tetracycline resistance marker was detected (Table 4).

SI Materials and Methods

Generation of *E. faecalis* Insertional Mutants. Insertion of selectable markers was generally achieved by single crossover integration. Primers used to amplify and clone a portion of a gene of interest to target recombination are listed in Table S1. Amplification products were restricted and ligated into the corresponding sites of either p3TET or p3CAT insertion vectors (9) and electroporated into *E. faecalis* strain V583 (10). Tetracycline-resistant transformants were screened by PCR (Table S1) to confirm plasmid integration. This methodology was used to integrate p3TET into V583 chromosomal loci *ef0479* (*int*), *ef0521* (*cbh*), *ef0590*, *ef2686* (*elrA*), *ef1854*, *ef2507*, and the integenic region between *ef2484* and *ef2485*. The same approach was used to integrate p3CAT into *efb0050*.

Generation of *E. faecalis* Deletion Mutants. Deletions of the PAI excisonase, *oriT*, and TraG-like homolog, and the *oriT*, relaxase, and TraG-like homologs on both pTEF1 and pTEF2 were generated by splicing by overlap extension PCR (SOEing), essentially as described (11) using internal primers (Table S1) to create the complementary overlap, keep deletions in frame, and incorporate an EagI restriction site into the sequence to aid in subsequent identification of deletion mutants. The SOE PCR product was cloned into plasmid pLT06. Desired constructs were introduced into *E. faecalis* V583, and allelic exchange was performed essentially as described (12), generating deletions *Δef0480exc, ΔoriT, Δef0503, Δefa0025*, *Δefa0030*, *Δefb0025*, *Δefb0030*, pTEF1*ΔoriT,* and pTEF2*ΔoriT* (Table S3). To integrate a selectable marker into pTEF2, plasmid pJMM73a was constructed from pLT06 by first generating a SOE PCR product containing overlapping sequence of *efb0049* into which an EagI site was inserted and ligating this into plasmid pLT06. A spectinomycin resistance marker was amplified from plasmid pAT28 (13) by using primers containing EagI restriction sites. Plasmid pJMM73a was digested with EagI and the EagI-digested PCR fragment encoding spectinomycin resistance ligated into the construct. Allelic exchange was then performed to integrate the spectinomycin resistance marker into the gene locus *efb0049* of pTEF2.

Comparative Genome Hybridization. The gene content of transconjugants, donors, and recipients was compared by using Affymetrix GeneChip, SLARE1 (8). Criteria used to make Absent/Present calls: Log2 [strain average signal/row average signal] ≥ −1.5; Log2 [strain average signal/V583 average signal] ≥ −2; and Log2 [strain average signal/average signal for V583 Present calls] ≥ −3. Microarray data have been deposited in the ArrayExpress database with the series accession number E-MEXP-2135.

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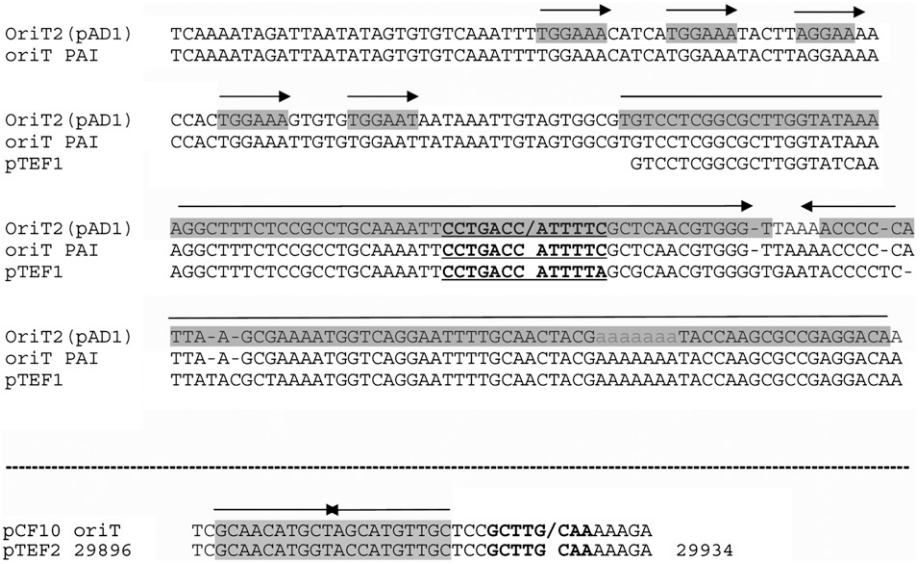

Fig. S1. Alignment of pTEF1 and pTEF2 origins of transfer with their closest homologs. The *oriT2* of pAD1 has a large inverted repeat sequence (indicated by arrows and highlighted in gray) located adjacent to a series of five repeats of 6 bp, with 5-bp spacings (indicated by arrows and highlighted in gray). The putative *ori*T of pTEF1 shares 97% similarity at the nucleotide level with *oriT2*; however, the five 6-bp repeats 5′ of the large inverted repeat are absent. pTEF2 contains a 38-nt DNA stretch that has 94% identity to the *oriT* of pCF10. Experimentally proven *oriT* nick sites of pAD1 and pCF10 are indicated by (/), and surrounding nucleotides are in bold.

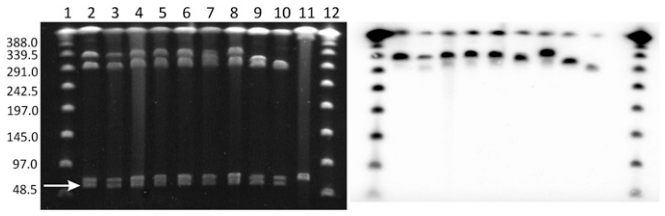

Fig. S2. Retransfer of the *E. faecalis* pathogenicity island. SfiI macrorestriction patterns of TC9 transconjugants (*Left*) and corresponding blot (*Right*) hybridized with an internal probe to the transfered *tetM*. Lane 1, lambda DNA concatamers; lane 2, primary transconjugant donor strain TC9; lanes 3–10, secondary transconjugant strains; lane 11, recipient strain OG1SSp; lane 12, lambda DNA ladder standard. Plasmid pTEF2 is indicated by an arrow and is present in the donor and all transconjugants.

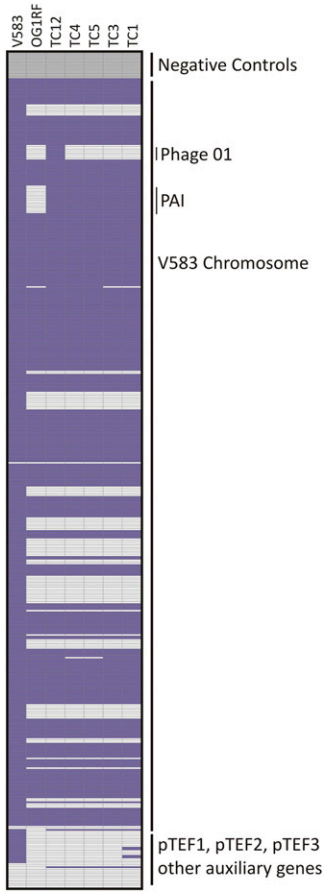

Fig. S3. Identification of transferred genes by comparative genomic hybridization. Donor, recipient, and transconjugant strain chromosomes were compared by hybridization to a custom Affymetrix microarray, designed mainly to be complementary to V583-encoded ORFs. Absent genes (light gray or white) and present (violet) calls for *E. faecalis* strains: V583 (donor and positive control), OG1RF (recipient), and representatives of the five transconjugant macrorestriction profiles TC12, 4, 5, 3, and 1. Probe sets are ordered from top to bottom according to their occurrence in the *E. faecalis* V583 chromosome.

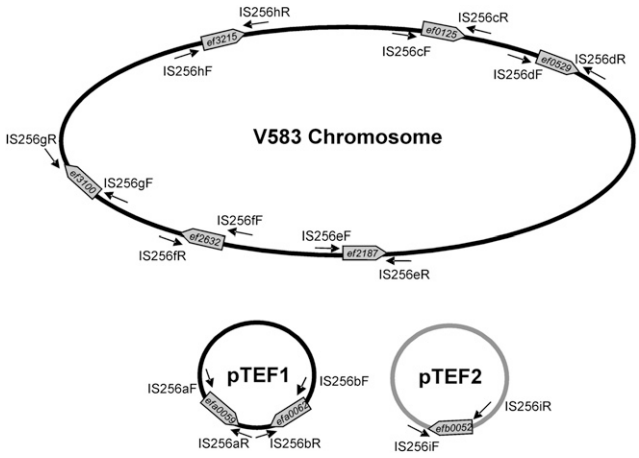

Fig. S4. Schematic of primer design for detecting recombination between IS256 elements on pTEF1, pTEF2, and the chromosome of V583. IS256 copies are labeled IS256a–i, with the ORF designation of the transposase and orientation of transcription indicated within each copy. Flanking arrows indicate the designation and orientation of PCR primers complementary to unique sequences that flank each IS256 copy.

Fig. S5. Identification of restriction fragment length polymorphisms (RFLP) in sequences flanking PAI insertions in transconjugants. Schematic of the chromosome flanking the pathogenicity island, indicating the position and designation of long-template PCR (LT-PCR) products used for RFLP analysis. Amplified fragments of ≈10 kb size (*A–J*) were restricted with SspI (amplicons *A, E, F, H*, and *J*), XmnI (*B*), HaeII (*C*), StyI (*D* and *I*), or EaeI (*G*) and compared with donor and recipient controls to identify the origin of polymorphisms. Fragment sizes for each PCR product are listed in Table S2.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)
Table S4 (DOC)