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Putative Surface Proteins Encoded within a Novel Transferable Locus Confer a High-Biofilm Phenotype to *Enterococcus faecalis*†

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Enterococci are opportunistic pathogens and among the leading causes of nosocomial infections. *Enterococcus faecalis*, the dominant species among infection-derived isolates, has recently been recognized as capable of forming biofilms on abiotic surfaces in vitro as well as on indwelling medical devices. A few bacterial factors known to contribute to biofilm formation in *E. faecalis* have been characterized. To identify additional factors which may be important to this process, we utilized a Tn*917*-based insertional mutagenesis strategy to generate a mutant bank in a high-biofilm-forming *E. faecalis* strain, E99. The resulting mutant bank was screened for mutants exhibiting a significantly reduced ability to form biofilms. One mutant, P101D12, which showed greater than 70% reduction in its ability to form biofilms compared to the wild-type parent, was further characterized. The single Tn*917* insertion in P101D12 was mapped to a gene, *bee-2*, encoding a probable cell wall-anchored protein. Sequence information for the region flanking *bee-2* revealed that this gene was a member of a locus (termed the *bee* locus for *b*iofilm *e*nhancer in *e*nterococcus) comprised of five genes encoding three putative cell wall-anchored proteins and two probable sortases. Contour-clamped homogeneous electric field gel and Southern hybridization analyses suggested that the *bee* locus is likely harbored on a large conjugative plasmid. Filter mating assays using wild-type E99 or mutant P101D12 as a donor confirmed that the *bee* locus could transfer conjugally at high frequency to recipient *E. faecalis* strains. This represents the first instance of the identification of a mobile genetic element conferring biofilm-forming property in *E. faecalis*.

Biofilm formation is a dynamic process involving the attachment of bacteria to a biotic or abiotic surface followed by growth and maturation (2). Structurally, biofilms consist of single- or multispecies microbial communities encased in an extracellular polymeric matrix, which is mainly composed of carbohydrates. Existence as a biofilm confers a significant survival advantage to bacteria, increasing their resistance to stressful environmental conditions (7), rendering them several-fold more resistant to antimicrobial agents (4), and assisting them in evading the host immune system more effectively (45) than the planktonic cells. Thus, while an ability to form biofilms in itself may not be considered a virulence trait, since many nonpathogenic bacteria form biofilms, existence as biofilms has been found to facilitate survival and persistence of pathogens in the host (11). The Centers for Disease Control now estimates that 65% of human infections may be biofilm related (27). The pathogenesis of diseases like infective endocarditis, infectious kidney stones, and lung infections in cystic fibrosis has been attributed to biofilms (24).

Enterococci play dual roles in human ecology. They exist as commensals in the gastrointestinal tract or manifest themselves as opportunistic pathogens, and they are among the leading causes of nosocomial infections, causing bacteremia, urinary tract infections, and endocarditis (9, 30). Enterococci have been found to form biofilms on several medical devices implanted in patients, such as central venous catheters, urinary catheters, intrauterine devices, and prosthetic heart valves (3). A few factors contributing to the process of enterococcal biofilm formation have been identified, including the cell surface-localized enterococcal surface protein Esp (36, 41), the two-component quorum-sensing signal transduction system Fsr (12, 26), the secreted metalloprotease GelE (12, 16, 21), the sugar-binding transcriptional regulator BopD (13), and an autolysin and the enterococcal polysaccharide antigen Epa (21).

In an attempt to identify additional factors that may influence the process of biofilm formation in *Enterococcus faecalis*, a Tn*917* mutant bank was generated in a high-biofilm-forming *E. faecalis* strain, E99. The region flanking the single Tn*917* insertion in eight mutants which showed a significant reduction in the ability to form biofilms was characterized, and the transposon was found to be inserted within the open reading frames (ORFs) or the intergenic regions of a novel gene cluster, which we designate as the *bee* (*b*iofilm *e*nhancer in *e*nterococcus) locus. In this study, we further characterized mutant P101D12, in which *bee-2* was inactivated by a single Tn*917* insertion. We show that the *bee* locus is likely carried on a large conjugative plasmid and is transferred at high frequency to recipient *E. faecalis* strains by a conjugal mating process, resulting in the enhancement of biofilm formation by the transconjugants.

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† Supplemental material for this article may be found at http://jb.asm.org/.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. faecalis* strain E99 was isolated from the urine of a patient at the Veterans Administration hospital in Arkansas (provided by K. T. Madhusudhan). OG1RF (5) and JH2SS (14, 42), plasmid-free *E. faecalis* strains, were used as recipients for the filter mating assays. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was used as a host

TABLE 1. *E. faecalis* strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. faecalis strains		
E99	Clinical isolate	This study
P101D12	E99 with a Tn*917* insertion in *bee-2*, Erm^r	This study
BLG1	P101D12 complemented with pBLG1 expressing *bee-2*, Spc^r	This study
BLG2	P101D12 complemented with pBLG2 expressing *bee-3*, Spc^r	This study
BLG12	P101D12 complemented with pBLG12 expressing *bee-2* and *bee-3*, Spc^r	This study
OG1RF	Plasmid free, Rif^r, Fus^r	5
JH2SS	Plasmid free, Str^r, Spc^r	14, 42
POTC-1, POTC-2, and POTC-3	Transconjugants derived from P101D12 and OG1RF mating, Rif^r, Fus^r, Erm^r	This study
POJTC-1, POJTC-2, and POJTC-3	Transconjugants derived from POTC-2 and JH2SS mating, Str^r, Spc^r, Erm^r	This study
IG9, IIB3, and IIE7	Transconjugants derived from E99 and OG1RF mating, Rif^r, Fus^r	This study
Tn*917* mutant strains		
P105H2	Tn*917* insertion in *srt-1*	This study
P101A12	Tn*917* insertion in *srt-1*	This study
P101D12	Tn*917* insertion in *bee-2*	This study
P76D4	Tn*917* insertion in *bee-1*	This study
P94C6	Tn*917* insertion in *bee-1*	This study
P110B1	Tn*917* insertion in *bee-3*	This study
P15D1	Tn*917* insertion upstream of *srt-1*	This study
P77E12	Tn*917* insertion upstream of *srt-1*	This study
Plasmids		
pAT28	Shuttle vector, Spc^r	44
pTV1-OK	Temperature-sensitive plasmid containing Tn*917*, Kan^r, Erm^r	10
pBLG1	pAT28 containing full-length *bee-2* downstream of the *aphA-3* promoter, Spc^r	This study
pBLG2	pAT28 containing full-length *bee-3* downstream of the *aphA-3* promoter, Spc^r	This study
pBLG12	pAT28 containing both *bee-2* and *bee-3* downstream of the *aphA-3* promoter, Spc^r	This study

for plasmid purifications. A list of all of the plasmids and *E. faecalis* strains used in this study is shown in Table 1. *E. faecalis* was cultured in Trypticase soy broth (TSB) supplemented with 0.5% glucose. Antibiotics (Sigma, St. Louis, MO) used for selection included kanamycin (25 μg/ml) for E99, kanamycin (1,000 μg/ml) and erythromycin (10 μg/ml) for E99(pTV1-OK), and erythromycin (10 μg/ml) for all of the Tn*917* mutants. Strain OG1RF was selected using rifampin (25 μg/ml) and fusidic acid (10 μg/ml), whereas streptomycin (250 μg/ml) and spectinomycin (250 μg/ml) were used for JH2SS. Spectinomycin at 500 μg/ml for *E. faecalis* and 150 μg/ml for *E. coli* was used for the selection of strains containing pAT28-based constructs in the complementation assay. No significant differences were found in the rates of growth for the mutant and transconjugants compared to the growth rate of the wild-type strain in medium with or without appropriate antibiotics.

Biofilm assay. The biofilm assay on the Tn*917* mutants was performed using flat-bottom polystyrene microtiter plates (Corning Inc., Corning, NY) as described previously (36, 41). The assay was done independently in triplicate, with 12 replicates per strain per assay. Statistical significance was tested using Student's *t* test. Wherever appropriate, multigroup comparisons were made by analysis of variance (ANOVA) using Tukey's test.

DNA manipulations. Electrocompetent *E. coli* or *E. faecalis* (33) cells were prepared as described previously and transformed with specific plasmids using a Gene Pulser unit (Bio-Rad Laboratories, Hercules, CA). Nucleotide sequence information was obtained using an ABI3730 capillary sequencer at the Oklahoma Medical Research Foundation (Oklahoma City, OK). Takara LA *Taq* polymerase (TaKaRa Biomedicals, Shiga, Japan) was used for all PCR amplifications meant for cloning. Oligonucleotide primer sequences are listed in the supplemental material.

Generation of a Tn*917* mutant library in E99. Plasmid pTV1-OK containing transposon Tn*917*, a temperature-sensitive origin of replication, and a kanamycin resistance determinant was used for performing mutagenesis (10). *E. faecalis* strain E99 was transformed with pTV1-OK by electroporation, and the transformants were selected on brain heart infusion (BHI) agar plates containing kanamycin (1,000 μg/ml) and erythromycin (10 μg/ml). E99(pTV1-OK) was grown at 30°C for 10 h, and appropriate dilutions were plated on prewarmed BHI agar plates supplemented with erythromycin at 10 μg/ml. The plates were further incubated at the nonpermissive temperature of 42°C for 48 h. The loss of plasmid pTV1-OK was confirmed by streaking the mutant colonies on BHI agar plates containing kanamycin (1,000 μg/ml). To generate a mutant bank in E99, 10,000 individual mutant colonies were archived. In order to confirm the presence of a single Tn*917* insertion in the selected mutants, Southern analysis was performed on genomic DNA (2 μg) restricted with HindIII and EcoRI, employing a Tn*917*-specific probe (Fig. 1) generated using primers Tn*917*-1 and Tn*917*-2.

Characterization of the regions flanking the Tn*917* insertion in P101D12. Genomic DNA (2 μg) from P101D12 was restricted with EcoRI (New England Biolabs Inc., Beverly, MA), and the resulting fragments were self-ligated at 16°C using T4 DNA ligase (Promega, Madison, WI). To map the Tn*917* insertion, an

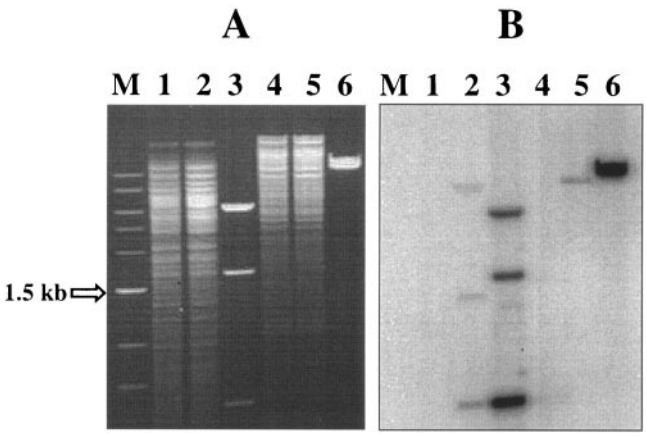

FIG. 1. The presence of a single Tn*917* insertion within the *bee* locus in mutant strain P101D12 was confirmed by Southern analysis using a Tn*917*-specific probe (a 1.9-kb internal region of Tn*917* containing two HindIII restriction sites) as described previously (10). (A) Genomic DNA obtained from wild-type strain E99 and mutant P101D12 was restricted with HindIII (lanes 1 and 2) and EcoRI (lanes 4 and 5). (B) Southern hybridization was performed subsequently with the Tn*917* probe obtained by using primers Tn*917*-1 and Tn*917*-2. The HindIII (lane 3)- and EcoRI (lane 6)-restricted plasmid pTV1-OK was used as the positive control. For size reference, a 1-kb DNA ladder (New England Biolabs, Beverly, MA) was used (lane M).

inverse PCR amplification was performed using a Takara LA PCR kit (Panvera Corp., Madison, WI) and outward-facing Tn*917*-specific primers SLT-L and SLT-R. The amplified fragment was purified from a 0.8% low-melting-point agarose gel using a Wizard Preps DNA purification system (Promega, Madison, WI), and the regions flanking the Tn*917* insertion were sequenced with primers SLT-L and SLT-R. Homology searches were performed by BLAST analyses using National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and The Institute for Genomic Research (http://www.tigr.org) databases.

Dot blot analysis. Dot blot analysis was performed on DNA isolated from 40 *E. faecalis* isolates. E99 DNA was used as the positive control. DNA from each strain was denatured in 0.4 N sodium hydroxide to a final concentration of 1 μg/ml and spotted onto a Zeta-probe GT genomic-tested blotting membrane (Bio-Rad, Hercules, CA) by placing 100 μl of sample into each well across the row of a 96-well dot blot apparatus (Bio-Rad, Hercules, CA). The membrane was rinsed thrice with Tris-EDTA buffer, pH 8.0. The DNA was then cross-linked to the membrane using a UV cross-linker (UVP Inc., Upland, CA). The membrane was probed at high stringency with the 1.4-kb *bee* locus-specific PCR product obtained from E99 DNA using primers P101D12-1 and P101D12-2 and radiolabeled with a Radprime DNA labeling system (Invitrogen, Carlsbad, CA).

Complementation of the transposon mutant. To complement the biofilm defect in P101D12, 0.99-kb, 1.8-kb, and 2.6-kb regions (corresponding to full-length *bee-2*, full-length *bee-3*, and both *bee-2* and *bee-3*, respectively) were amplified from E99 DNA using primer pairs E99-1/E99-3R, E99-7L/E99-4R, and E99-1/E99-4R. Primers E99-1 and E99-7L contained the recognition sequence for restriction endonuclease SacI, whereas primers E99-3R and E99-4R contained the recognition sequence for XbaI. The amplified products were restricted with SacI and purified from a 0.8% low-melting-point agarose gel. Primers Aph-1 (with an EcoRI recognition sequence) and Aph-2 (with a SacI recognition sequence) were used to amplify a 358-bp fragment containing the *aphA-3* promoter (28) from the plasmid pTCV-*aphA3*. The amplification product was subsequently restricted with SacI, purified from a 0.8% low-melting-point agarose gel, and ligated to the SacI-restricted gene fragments using T4 DNA ligase (Promega, Madison, WI) at 16°C. One microliter of each of the ligation mixes was used in PCR amplification reactions with primer pairs Aph-1/E99-3R and Aph-1/E99-4R to enrich for the ligated *aphA-3* and *bee-2*, *aphA-3* and *bee-3*, and *aphA-3* and *bee-2–bee-3* products. All of the amplified products were then restricted with EcoRI and XbaI, purified from a low-melting-point agarose gel, and subsequently ligated to EcoRI/XbaI-restricted shuttle vector pAT28 (44) to generate plasmids pBLG1, pBLG2, and pBLG12. To verify the plasmid constructs, nucleotide sequence information was obtained from plasmids pBLG1, pBLG2, and pBLG12 using primer Aph-1. Mutant strain P101D12 was transformed with pBLG1, pBLG2, or pBLG12 to generate strains BLG1, BLG2, and BLG12. The abilities of the complemented strains to form biofilms were assessed using the 96-well-plate biofilm assay described above. Multigroup comparisons were made by ANOVA using Tukey's test to assess statistical significance.

RNA isolation and RT-PCR analysis. Total RNA was isolated by a method described previously (32). Residual genomic DNA contamination was removed using a DNA-free DNase treatment and removal kit (Ambion, Austin, TX). Reverse transcription was performed using a Superscript first-strand synthesis system for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendations.

Filter mating assays. Filter mating was carried out as described previously (6) with a donor/recipient ratio of 1:10. Overnight cultures of donor (0.5 ml; P101D12 or POTC-2) and recipient (4.5 ml; OG1RF or JH2SS) strains were mixed, and the cells were collected on a 0.2-μm cellulose nitrate membrane filter (Whatman International Ltd., Maidstone, England). The filter was then placed on a BHI agar plate bacteria side down and incubated at 37°C for 16 h. The cells from the filter and plate were recovered and resuspended in 1 ml BHI broth, and appropriate dilutions were plated on BHI agar plates containing suitable antibiotics. For the P101D12 (donor) and OG1RF (recipient) mating, the transconjugants were selected using rifampin, fusidic acid, and erythromycin. One of the transconjugants from the P101D12-OG1RF mating, POTC-2, served as the donor for the secondary filter mating, with JH2SS as the recipient. The transconjugants arising from the POTC-2 and JH2SS mating were selected using spectinomycin, streptomycin, and erythromycin. The transfer frequency was expressed as the number of transconjugants per recipient.

The filter mating between wild-type E99 as the donor and OG1RF as the recipient strain was carried out essentially as described above. However, due to the absence of a suitable antibiotic resistance marker for the transconjugants, appropriate dilutions of the bacterial cell suspension were plated on BHI agar plates containing rifampin and fusidic acid. This antibiotic selection allowed the recipient OG1RF as well as the transconjugants to grow. Individual colonies were picked, and an increase in the ability to form biofilms was then used as a screen for transconjugants. One hundred and eighty individual colonies were inoculated into the wells of 96-well plates containing TSB supplemented with 0.5% glucose. The crystal violet binding assay was then used to identify transconjugants that showed an enhanced ability to form biofilms compared to control strain OG1RF (recipient). After the initial identification of the transconjugants in a single-well assay, the biofilm assay was performed in 12 replicates per strain thrice independently to confirm the initial observation. The presence of the *bee* locus in three transconjugants identified by this screen was confirmed by PCR with primers P101D12-1 and P101D12-2 (data not shown) as well as by contour-clamped homogeneous electric field (CHEF) gel and Southern analysis using a *bee* locus-specific probe (see Fig. 7).

CHEF gel analysis. CHEF gel analysis was performed as described previously (1, 22, 40) with some modifications. A portion of the agarose plug containing total DNA from each strain was cut with a sterile razor and restricted with I-ceuI (New England Biolabs, Beverly, MA) at 37°C for 16 h. The plugs were washed in 1 ml dilute Tris-EDTA for 1 h at 37°C, then carefully loaded into the wells of a 1% pulsed-field certified agarose gel (Bio-Rad Laboratories, Hercules, CA) in 0.5× TBE (45 mM Tris HCl, 45 mM boric acid, 1 mM EDTA), and electrophoresed using a CHEF DRII device (Bio-Rad Laboratories, Hercules, CA) with the pulse time ramped from 5 to 120 s at 150 V for 40 h. The gels were then stained with ethidium bromide and photographed using a UVP gel documentation system (UVP Inc., Upland, CA) before the DNA was transferred to Zeta-probe GT genomic-tested blotting membrane by capillary or vacuum blotting. Southern hybridization analysis was then performed sequentially using either a 1.9-kb Tn*917*-specific probe generated using primers Tn*917*-1 and Tn*917*-2, a 1.4-kb *bee* locus-specific probe generated using primers P101D12-1 and P101D12-2, or a 1-kb probe specific for *E. faecalis* 23S rRNA genes generated using primers EF23sFor and EF23sRev.

Nucleotide sequence accession number. The DNA sequence reported in this article has been deposited in the GenBank nucleotide sequence database under accession number DQ137124.

RESULTS

Effect of glucose on the ability of *E. faecalis* strain E99 to form biofilms. Glucose in the growth medium has been found to influence the ability of *E. faecalis* strains to form biofilms (13, 26, 36) such that the presence of 0.5% (wt/vol) or greater amounts of glucose is required for biofilm enhancement. To assess if glucose is similarly necessary for E99 to form biofilms, we used the crystal violet binding assay described in Materials and Methods. In agreement with previous reports, E99 formed significantly more biofilms in the presence of 0.5%, 0.75%, and 1% glucose than in the presence of 0.25% glucose ($P < 0.05$) (data not shown).

Tn*917* mutagenesis and characterization of the *bee* locus. Tn*917* mutagenesis identified eight mutants (Table 1) with significantly (>70%) reduced biofilm-forming ability compared to parent strain E99. In all of these mutants, the single Tn*917* insertion mapped to the *bee* locus, and mutant P101D12 was chosen for further characterization. The presence of a single Tn*917* insertion in P101D12, that within *bee-2*, was confirmed by inverse PCR and Southern analysis using a Tn*917*-specific probe (Fig. 1). The wild-type strain E99 (Fig. 1, lanes 1 and 4) was used as a negative control, and the purified plasmid pTV1-OK (Fig. 1, lanes 3 and 6) was used as a positive control. The *bee* locus (Fig. 2A) is comprised of three structural genes, designated *bee-1*, *bee-2*, and *bee-3*, which encode putative cell wall-anchored proteins, and two genes, *srt-1* and *srt-2*, which encode probable sortases. The deduced amino acid sequences of Bee-1, Bee-2, and Bee-3 revealed the presence of the conserved LPxTG consensus sequence (20) commonly found in gram-positive cell wall-anchored surface proteins at their C termini. The 1,083-residue Bee-1 protein revealed some interesting structural features upon BLASTP analysis.

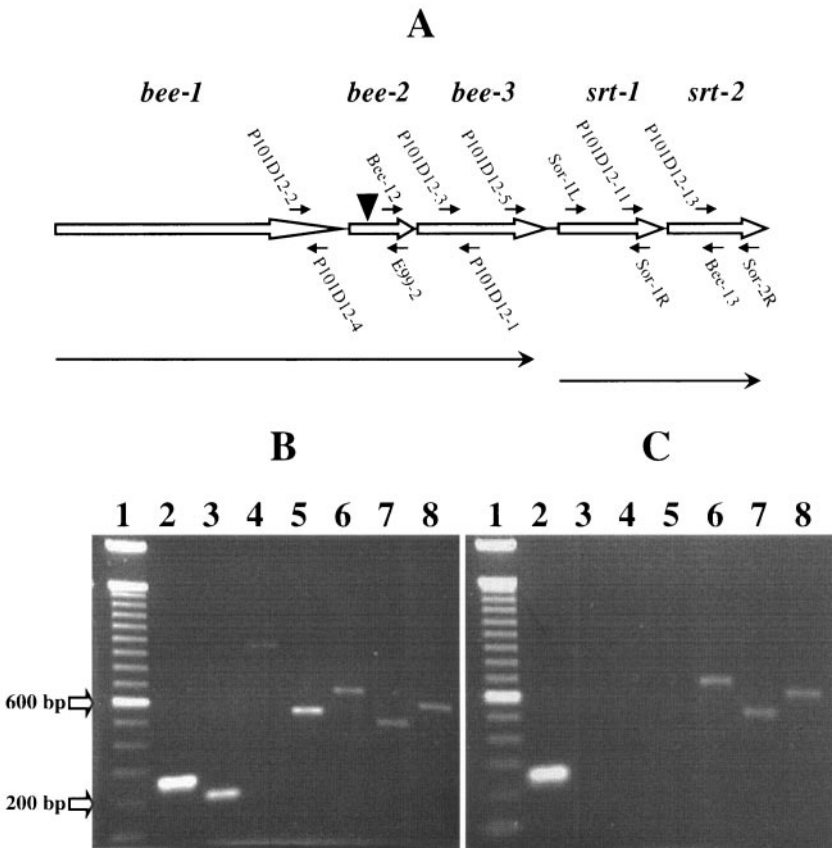

FIG. 2. RT-PCR analyses to assess the expression and transcriptional linkage of genes within the *bee* locus in the wild-type strain E99 and mutant P101D12. (A) Schematic of the *bee* locus. The *bee* locus is comprised of three genes, *bee-1*, *bee-2*, and *bee-3*, encoding putative cell wall-anchored proteins, downstream of which are two ORFs, *srt-1* and *srt-2*, encoding putative sortase enzymes. The locations of various primers used for the RT-PCR analysis are indicated. The position of the Tn*917* insertion (▼) in mutant P101D12 is also indicated. Line arrows indicate putative transcriptional units. (B) Total RNA extracted from E99 was reverse transcribed using random hexamers and reverse transcriptase. An aliquot of the cDNA was then amplified by PCR with gene-specific primers. Primers P101D12-2/P101D12-4 (lane 2), P101D12-3/P101D12-1 (lane 3), P101D12-2/E99-2 (lane 4), Bee-12/P101D12-1 (lane 5), Sor-1L/Sor-1R (lane 6), P101D12-13/Sor-2R (lane 7), and P101D12-11/Bee-13 (lane 8) yielded 259-, 203-, 954-, 532-, 650-, 485-, and 581-bp amplification products, respectively. This implies that the genes *bee-1*, *bee-2*, and *bee-3* may be cotranscribed as one transcriptional unit, with *srt-1* and *srt-2* cotranscribed independently. (C) Total RNA extracted from mutant strain P101D12 was used for RT-PCR. Primers P101D12-2/P101D12-4 (lane 2) yielded an expected 259-bp amplification product, indicating that *bee-1* was being transcribed. However, no amplification products were obtained when primer pairs P101D12-3/P101D12-1 (lane 3), P101D12-2/E99-2 (lane 4), or Bee-12/P101D12-1 (lane 5) were used for amplification, suggesting that Tn*917* insertion had abrogated not only the expression of *bee-2* but also that of the downstream gene *bee-3*. Sor-1L/Sor-1R (lane 6), P101D12-13/Sor-2R (lane 7), and P101D12-11/Bee-13 (lane 8) yielded 650-, 485-, and 581-bp amplification products, respectively, implying that the Tn*917* insertion in *bee-2* did not abrogate expression of *srt-1* and *srt-2*. For size reference, a 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used (lane 1).

While no repeat motifs were discernible, the region extending from residues 729 to 908 was identified by the conserved domain database search (18) to bear a low degree of similarity to the B domain of collagen-binding protein from *Staphylococcus aureus* (E = 1e−04) and *Bacillus subtilis* (E = 3e−05) (15, 35). The primary sequence of these collagen adhesins consists of a nonrepeat, collagen-binding A region followed by one to four 23-kDa B region repeats. The B domain has been suggested to serve as a "stalk" that projects the A regions from the bacterial cell surface to facilitate bacterial adherence to collagen. Residues 343 through 487 of Bee-1 also revealed a low degree of similarity (E = 6e−08) to the von Willebrand factor type A domain (47) that has been shown to bind to a variety of ligands, including collagen, laminin, and glycosaminoglycans.

While the 243-residue Bee-2 protein did not reveal any unique features or conserved domains except for putative signal sequence and the conserved LPxTG motif, the deduced 495-residue Bee-3 protein revealed a low degree of similarity to a conserved domain within outer membrane proteins from *Leuconostoc mesenteroides*.

The deduced amino acid sequences of both Srt-1 and Srt-2 revealed a high level of similarity to the conserved domains within sortase-type enzymes that cleave surface proteins at the LPxTG motif between threonine and glycine and catalyze the formation of an amide bond between the carboxyl group of threonine and the amino group of the cell wall cross-bridges (19). Table 2 lists the closest homologs of the predicted proteins encoded by the genes in the *bee* locus that were revealed using the BLASTP algorithm.

Gene expression and transcriptional linkage analysis in E99 and P101D12. Primer pairs P101D12-2/P101D12-4, P101D12-3/P101D12-1, P101D12-2/E99-2, and Bee-12/P101D12-1 were

TABLE 2. Homologies of the predicted proteins encoded by the *bee* locus genes determined by BLASTP (NCBI, NLM)

Protein	Length (amino acids)	BLASTP hits	Accession no.	Organism	% Identity	% Similarity	Expect (E) value
Bee-1	1,083	Predicted OMP[a]	ZP_00062640	*Leuconostoc mesenteroides*	25	38	2e−48
Bee-2	243	Predicted OMP	ZP_00062638	*Leuconostoc mesenteroides*	33	48	4e−17
Bee-3	495	Predicted OMP	ZP_00062638	*Leuconostoc mesenteroides*	39	54	3e−80
Srt-1	398	Sortase	EAN10600	*Enterococcus faecium*	50	71	2e−75
Srt-2	373	Sortase	EAN10837	*Enterococcus faecium*	45	66	2e−75

[a] OMP, outer membrane protein.

used to detect gene transcripts corresponding to *bee-1*, *bee-3*, *bee-1* and *bee-2*, and *bee-2* and *bee-3*, respectively. As shown in Fig. 2B, all of the PCR amplifications yielded products of expected sizes: 259 bp (lane 2), 203 bp (lane 3), 954 bp (lane 4), and 532 bp (lane 5), respectively. This suggested that *bee-1*, *bee-2*, and *bee-3* may be transcriptionally linked. No amplification product was detected with primers P101D12-2 and Sor-1R or with P101D12-5 and Sor-1R, implying that *srt-1* is not transcriptionally linked to *bee-3*. However, as shown in Fig. 2B, PCR amplifications using the primer pairs Sor-1L/Sor-1R, P101D12-13/Sor-2R, and P101D12-11/Bee-13 yielded expected 650-bp (lane 6), 485-bp (lane 7), and 581-bp (lane 8) products, respectively, implying that *srt-1* and *srt-2* are expressed and likely cotranscribed. Reverse transcription reactions performed in the absence of reverse transcriptase to control for residual genomic contamination did not yield any amplification products.

RT-PCR analysis was also performed on RNA from the mutant P101D12 to investigate the effect of the Tn*917* insertion on the expression of *bee-2* and the genes downstream. As shown in Fig. 2C, primer pairs P101D12-3/P101D12-1 (lane 3), P101D12-2/E99-2 (lane 4), and Bee-12/P101D12-1 (lane 5) failed to yield amplification products, suggesting that the Tn*917* insertion in *bee-2* had abrogated the expression of *bee-2* as well as the downstream gene *bee-3*. Expected 259-bp (Fig. 2C, lane 1), 650-bp (Fig. 2C, lane 6), 485-bp (Fig. 2C, lane 7), and 581-bp (Fig. 2C, lane 8) products were obtained using primers P101D12-2/P101D12-4, Sor-1L/Sor-1R, P101D12-13/Sor-2R, and P101D12-11/Bee-13, indicating that the Tn*917* insertion did not abrogate the expression of the upstream gene *bee-1* or the two downstream putative sortase genes *srt-1* and *srt-2*.

Complementation of P101D12. In order to confirm that the decrease in the biofilm-forming ability of mutant strain P101D12 was due to the insertion of Tn*917* in the *bee* locus and not due to any other polar effect of the insertion, complementation studies were performed. RT-PCR analysis of strain P101D12 indicated that the insertion of Tn*917* in *bee-2* had abrogated the expression of the downstream gene *bee-3* but not that of genes *srt-1* and *srt-2*. P101D12 was therefore transformed with shuttle vector pAT28 (44) alone (vector control) or pAT28 harboring the full-length *bee-2* gene, the full-length *bee-3* gene, or both the *bee-2* and *bee-3* genes downstream of a constitutive *aphA-3* promoter (28) to generate strains BLG1, BLG2, and BLG12, respectively. As shown in Fig. 3, strains BLG1 and BLG2 formed significantly more biofilms than the mutant strain P101D12 containing vector alone (ANOVA; Tukey's test, $P < 0.05$). A significantly higher biofilm-forming ability of BLG12 (which was complemented with both *bee-2* and *bee-3*) compared to BLG1 (which was complemented with only *bee-2*) and BLG2 (which was complemented with only *bee-3*) was also observed (ANOVA; Tukey's test, $P < 0.01$).

Transfer of the *bee* locus to other *E. faecalis* strains. The nucleotide sequence downstream of the probable sortase gene *srt-2* revealed the presence of genes encoding putative transposase and resolvase (data not shown), suggesting that the *bee* locus could be present on a mobile genetic element and thereby could horizontally transfer to other *E. faecalis* strains. Strain P101D12, which had a Tn*917* insertion in *bee-2*, was filter mated with *E. faecalis* strain OG1RF. The absence of *bee* locus genes in recipient OG1RF was confirmed using Southern hybridization analysis. Erythromycin resistance encoded by Tn*917* was used as a marker for selection of the transconjugants. Transconjugants arose at a frequency of 4×10^{-2}/recipient (3.2×10^{-1}/donor). As shown in Fig. 4A, I-ceuI-restricted DNA from three randomly chosen transconjugants, POTC-1 (lane 1), POTC-2 (lane 2), and POTC-3 (lane 3); the donor, P101D12 (lane 4); and the recipient, OG1RF (lane 5), was separated using CHEF gel electrophoresis and analyzed by Southern hybridization using 23S rRNA genes (Fig. 4B) and Tn*917*-specific probes (Fig. 4C). Four bands from the transconjugants as well as P101D12 and OG1RF hybridized to the 23S rRNA gene probe, indicating that these were chromosomal fragments. However, the Tn*917*-specific probe hybridized to a distinctly smaller band with an apparent

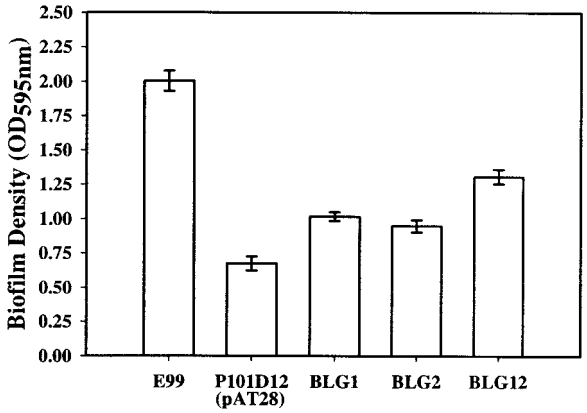

FIG. 3. Biofilm formation by wild-type strain E99, mutant P101D12 (pAT28), and complemented strains BLG1 (P101D12 transformed with pAT28 harboring *bee-2* downstream of the *aphA-3* promoter), BLG2 (P101D12 transformed with pAT28 harboring *bee-3* downstream of the *aphA-3* promoter), and BLG12 (P101D12 transformed with pAT28 harboring *bee-2* and *bee-3* downstream of the *aphA-3* promoter). The *y* axis represents the optical density (OD) of dissolved crystal violet measured at 595 nm. The error bars represent the mean ± standard error.

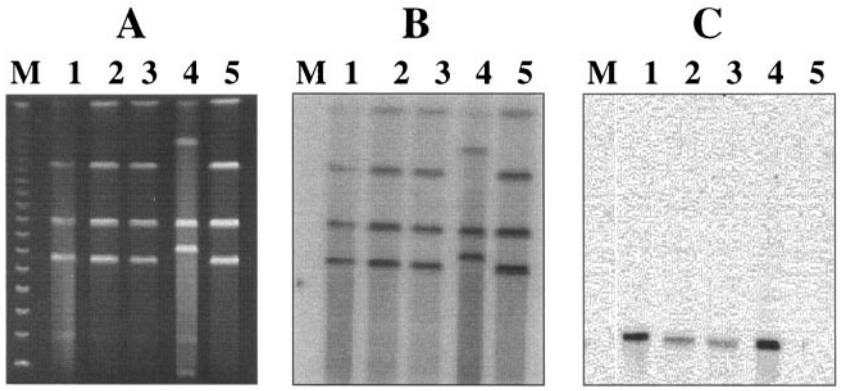

FIG. 4. CHEF gel and Southern hybridization analysis of transconjugants obtained from filter mating experiments employing mutant P101D12 as the donor and OG1RF as the recipient. (A) CHEF gel of I-ceuI-restricted DNA from three primary transconjugants, POTC-1, POTC-2, and POTC-3 (lanes 1, 2, and 3, respectively); donor strain P101D12 (lane 4); and recipient strain OG1RF (lane 5). (B and C) Also shown are Southern hybridization analyses of I-ceuI-restricted DNA from the transconjugants (lanes 1 to 3), donor strain P101D12 (lane 4), and recipient strain OG1RF (lane 5) using a 23S rRNA gene probe (B) and a Tn*917*-specific probe (C). Bacteriophage lambda concatemers were used as molecular size markers (lane M; New England Biolabs, Beverly, MA).

size of ~80 kb in the transconjugants and the donor strain P101D12 (Fig. 4C, lanes 1, 2, 3, and 4). This band was absent in recipient strain OG1RF (Fig. 4C, lane 5), implying that the *bee* locus is not located on the chromosome but likely harbored on a large conjugative plasmid that has transferred from the donor to the recipients.

The possibility of secondary transfer of the *bee* locus to another *E. faecalis* recipient strain, JH2SS, was investigated using one of the primary transconjugants (POTC-2) from the P101D12 and OG1RF mating as the donor. JH2SS also lacked *bee* locus genes, as was confirmed by Southern hybridization analysis. The secondary transfer frequency for the *bee* locus was found to be similar to the primary transfer frequency at 3.8×10^{-2}/recipient (2.92×10^{-1}/donor). As shown in Fig. 5A, I-ceuI-restricted DNA from three randomly chosen transconjugants, POJTC-1 (lane 1), POJTC-2 (lane 2), and POJTC-3 (lane 3); the donor, POTC-2 (lane 4); and the recipient, JH2SS (lane 5), was separated by CHEF gel electrophoresis and analyzed by Southern hybridization using 23S rRNA genes (Fig. 5B) and Tn*917*-specific probes (Fig. 5C). Four bands from the transconjugants as well as POTC-2 and JH2SS hybridized to the 23S rRNA gene probe, indicating that they were chromosomal. Similar to that observed with the P101D12/OG1RF transconjugants, the Tn*917*-specific probe hybridized to a distinct smaller band with an apparent size of ~80 kb in transconjugants POJTC-1, POJTC-2, and POJTC-3 and donor strain POTC-2 (Fig. 5C, lanes 1, 2, 3, and 4). This band was absent in recipient strain JH2SS (Fig. 5C, lane 5).

In order to investigate whether the acquisition of the *bee* locus was accompanied by an enhancement in the biofilm-forming ability of the transconjugants, the wild-type E99 strain was filter mated with OG1RF. Although no antibiotic resistance marker was available for the *bee* locus, based on our previously observed *bee* locus transfer frequencies of ~4 transconjugants per 100 recipient cells, we screened a total of 180 colonies comprised of both recipient OG1RF as well as transconjugants by the crystal

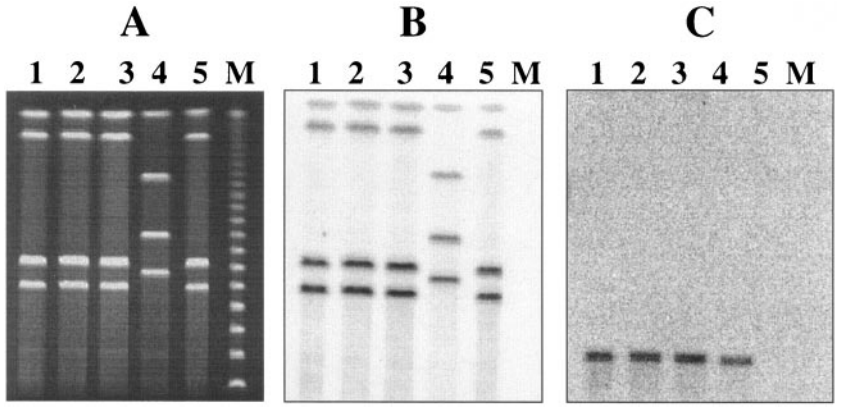

FIG. 5. CHEF gel and Southern hybridization analysis of transconjugants obtained from filter mating experiments employing primary transconjugant POTC-2 as the donor and JH2SS as the recipient. (A) I-ceuI-restricted DNA from three secondary transconjugants, POJTC-1, POJTC-2, and POJTC-3 (lanes 1, 2, and 3, respectively); donor POTC-2 (lane 4); and recipient JH2SS (lane 5). (B and C) Also shown are Southern hybridization analyses of I-ceuI-restricted DNA from the transconjugants (lanes 1 to 3), donor strain POTC-2 (lane 4), and recipient strain JH2SS (lane 5) using a 23S rRNA gene probe (B) and a Tn*917*-specific probe (C). Bacteriophage lambda concatemers were used as molecular size markers (lane M; New England Biolabs, Beverly, MA).

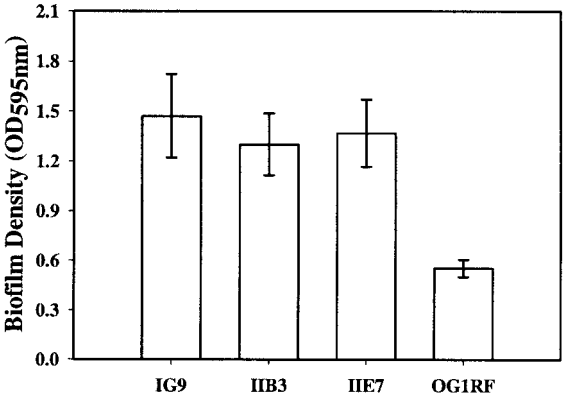

FIG. 6. Biofilm assay on three transconjugants, IG9, IIB3, and IIE7, obtained by filter mating wild-type E99 with *E. faecalis* strain OG1RF. Crystal violet binding was used to measure the 24-h biofilm densities of IG9, IIB3, IIE7, and parent strain OG1RF grown in TSB supplemented with 0.5% glucose. The error bars represent the mean ± standard error.

violet binding biofilm assay as described in Materials and Methods. As shown in Fig. 6, three transconjugants, IG9, IIB3, and IIE7, which showed an enhancement in biofilm-forming ability compared to parent strain OG1RF (ANOVA; Tukey's test, $P < 0.01$), were identified. PCR on genomic DNA prepared from IG9, IIB3, and IIE7 with primers P101D12-1/P101D12-2 revealed the presence of a 1.4-kb region corresponding to the 3′ end of *bee-1*, full-length *bee-2*, and the 5′ end of *bee-3*. In addition, CHEF gel followed by Southern hybridization analysis of I-ceuI-restricted DNA from E99 (Fig. 7, lane 1), IG9, IIB3, IIE7 (Fig. 7, lanes 2, 3, and 4), and OG1RF (Fig. 7, lane 5) using a *bee* locus probe revealed the presence of the expected extrachromosomal band with an apparent size of ~80 kb in E99 and the transconjugants. These results are in agreement with those obtained from CHEF gel and Southern hybridization analyses of transconjugants obtained using P101D12/OG1RF and POTC-2/JH2SS mating pairs (Fig. 4 and 5).

In separate experiments, we also compared the biofilm-forming abilities of transconjugants POTC-1, POTC-2, and POTC-3 to recipient strain OG1RF using the crystal violet binding assay. As expected, owing to the Tn*917* insertion within *bee-2*, no significant difference in the biofilm-forming abilities of these strains was noted. These results provide strong evidence that the biofilm phenotype imparted to transconjugants IG9, IIB3, and IIE7 (obtained using E99 as the donor and OG1RF as the recipient) is attributable to the *bee* locus and not a result of cotransfer of some other unknown element.

Detection of the *bee* locus in other *E. faecalis* strains. Dot blot hybridization was used to assess the presence of the *bee* locus in a collection of archived *E. faecalis* isolates. A 1.4-kb region corresponding to the 3′ end of *bee-1*, full-length *bee-2*, and the 5′ end of *bee-3* was used as a probe for the high-stringency hybridization. Out of a total of 40 randomly selected *E. faecalis* isolates that were screened, this gene cluster was detected in only 2 geographically unrelated clinical isolates (data not shown). DNA from wild-type strain E99 was spotted as a positive control for the hybridization assay.

DISCUSSION

Surface proteins play an important role in the multistep and multifactorial processes of biofilm formation. The ability of bacterial cells to persist as a biofilm poses a therapeutic challenge by limiting the antimicrobial treatment options available. In *E. faecalis*, very few surface-associated proteins, including aggregation substance (8), Esp (31), and Ace (29), have been extensively characterized. Notably, several studies have shown that expression of Esp enhances biofilm formation in *E. faecalis* (21, 37, 41). Aggregation substance was found to influence biofilm formation by promoting bacterial cell-cell interaction due to positive cooperativity (46). However, the genome of *E. faecalis* strain V583 (25) reveals the presence of 41 putative cell

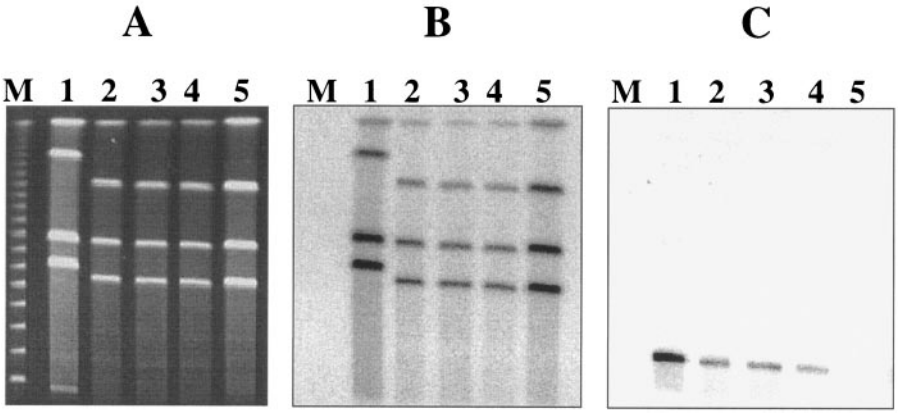

FIG. 7. CHEF gel and Southern hybridization analysis of transconjugants obtained from filter mating experiments employing wild-type E99 as the donor and OG1RF as the recipient. (A) I-ceuI-restricted DNA from donor E99 (lane 1); three transconjugants, IG9, IIB3, and IIE7 (lanes 2, 3, and 4, respectively); and recipient strain OG1RF (lane 5). (B and C) Also shown are Southern hybridization analyses of I-ceuI-restricted DNA from donor strain E99 (lane 1), the transconjugants (lanes 2 to 4), and recipient strain OG1RF (lane 5) using a 23S rRNA gene probe (B) and a *bee* locus-specific probe (C). Bacteriophage lambda concatemers were used as molecular size markers (lane M; New England Biolabs, Beverly, MA).

wall-anchored proteins and three sortase-like enzymes. Seventeen of these 41 putative cell wall-anchored proteins were found to contain tandem immunoglobulin-like folds commonly found in staphylococcal MSCRAMMs (34). While the functions of most of these proteins are presently unknown, it is possible that some of them may play a role in biofilm formation.

In this study, we identified a novel gene cluster, the *bee* locus, comprising genes encoding three putative cell surface-anchored proteins, Bee-1, Bee-2, and Bee-3, and two sortase-like enzymes, Srt-1 and Srt-2. This locus is not present in the *E. faecalis* genome strain V583 (25). The deduced protein sequences of Bee-1, Bee-2, and Bee-3 revealed the presence of a signal sequence at the amino termini and an LPxTG motif followed by a hydrophobic domain at the C termini. Thus, there is strong evidence to support the hypothesis that Bee-1, Bee-2, and Bee-3 are anchored to the bacterial cell wall by a sortase-dependent mechanism. Furthermore, BLASTP analyses revealed that the proteins Bee-1, Bee-2, and Bee-3 bear extensive similarities to predicted cell wall-anchored proteins of unknown functions from *Leuconostoc mesenteroides* (Table 2).

The *bee* locus includes two ORFs in tandem, downstream of *bee-3*, that encode putative sortases. This kind of sortase-substrate clustering might represent an independent functional unit encoding cell surface-associated proteins along with sortase enzymes exclusively dedicated to the cell wall anchoring of those proteins. This hypothesis is supported by the observation that the Tn*917* mutant P101A12, which has a Tn*917* insertion in *srt-1* (Table 1), is positive for the expression of enterococcal surface protein, Esp, as detected by enzyme-linked immunosorbent assay using antibodies specific to the N-terminal domain of Esp (data not shown). Studies have identified genes encoding sortase enzymes juxtaposed with genes encoding their substrates in the genomes of other gram-positive organisms, including *E. faecalis*, *Streptococcus agalactiae*, and *Corynebacterium diphtheriae* (17, 23, 34, 43). Interestingly, immunogold electron microscopy revealed that some of the surface-exposed proteins encoded at these loci formed pilus-like structures extending from the bacterial cell surface in *S. agalactiae* and *C. diphtheriae* (17, 43). Similarly, a gene cluster comprised of three genes encoding surface-anchored proteins of unknown function is present immediately upstream of three sortase-like proteins in the genome of virulent *Streptococcus pneumoniae* (39). It remains to be seen whether the *bee* locus similarly represents a gene cluster involved in the expression of pilus-like structures extending from the cell surface in *E. faecalis*.

The nucleotide sequence flanking the Tn*917* insertion in at least eight mutants that were attenuated in the ability to form biofilms revealed that Tn*917* had inserted within different regions of the ORFs or the intergenic regions of the *bee* locus. The identification of several independent mutants with decreased ability to form biofilms, all of which had an insertion in the *bee* locus, suggested that the observed phenotype was specifically due to the inactivation of one or more genes within the *bee* locus rather than any other nonspecific polar effect of the insertion. However, RT-PCR analysis of RNA purified from the mutant P101D12, which had an insertion in gene *bee-2*, revealed that the Tn*917* insertion not only interfered with the expression of *bee-2* but also had a polar effect on the expression of the downstream gene *bee-3*. Complementation in *trans* with either *bee-2* or *bee-3* alone significantly increased the ability of P101D12 to form biofilms. Complementation of the mutant strain with a plasmid constitutively expressing both *bee-2* and *bee-3*, however, further enhanced the biofilm-forming ability of P101D12, suggesting that both Bee-2 and Bee-3 may be important for biofilm formation. The exact mechanism by which these two putative cell wall-associated proteins may enhance biofilm formation remains to be resolved. Interestingly, complementation of the defect in *trans* did not result in restoration of biofilm-forming ability to wild-type levels. Although the reasons for this remain unclear at this time, it is possible that overexpression of the Bee proteins driven by a heterologous promoter leads to an incorrect surface display disturbing the optimal stoichiometric interaction between these proteins at the cell surface.

Sequence information downstream of *srt-2* revealed the presence of genes putatively encoding transposition functions. A site-specific recombinase belonging to the resolvase family (exhibiting 80% identity and 92% similarity to a resolvase identified in *Staphylococcus epidermidis* strain RP62A; GenBank ID 57854758) and a probable transposase (with 59% identity and 79% similarity to the transposase found on *Staphylococcus aureus* transposon Tn*552*, which encodes β-lactam resistance; GenBank ID 33390965) suggested the location of the *bee* locus on a mobile genetic element such as a transposon. Filter mating experiments, however, revealed that this locus is able to transfer to recipient *E. faecalis* strains at high frequencies, typical for conjugative plasmids. To determine the nature of the genetic element harboring the *bee* locus, we employed a CHEF gel approach previously used to examine genome diversity in enterococci (22) and to assign a chromosomal location to the *vanG* operon in *E. faecalis* (1). The enzyme I-ceuI is an intron-encoded endonuclease that specifically recognizes unique sequences present within *rrn* operons (22). *E. faecalis* strains were postulated to contain four *rrn* operons based on mapping experiments (22), and this was subsequently confirmed by the complete genome sequence of strain V583 (25). CHEF gel analysis of I-ceuI-digested DNA from *E. faecalis* followed by Southern hybridization analysis with 23S rRNA gene probes would therefore be expected to identify the four chromosomal fragments. Our Southern hybridization results with a 23S rRNA gene probe, shown in Fig. 4, 5, and 7, clearly identify these chromosomal fragments from donors, recipients, and transconjugants. Additionally, the banding pattern of the I-ceuI fragments also distinguishes between donor and recipient strains, such that in all cases described, it is clear that the transconjugants (obtained from independent mating experiments) were derived from the recipient. Probes specific to either the *bee* locus or Tn*917* hybridized to the same extrachromosomal band with an apparent size of ~80 kb (Fig. 4, 5, and 7). These results therefore support our argument that the *bee* locus is harbored on a large extrachromosomal element with an apparent size of ~80 kb. Our results showing high-frequency transfer of this element in filter mating experiments strongly suggest that the *bee* locus is harbored on a conjugative plasmid. No transconjugants were detected when mating experiments were performed in broth, suggesting that the conjugative plasmid may not be pheromone responsive.

It is not surprising that the *bee* locus genes were detected in only two other strains among our archived isolates. While it is possible that a higher frequency of occurrence of this locus may become evident upon screening more isolates, the probability remains that this locus is a recent acquisition in *Enterococcus faecalis*. The latter possibility is strengthened by the observation that the *bee* locus is likely carried on a large conjugative plasmid. A number of recent comparative genomic hybridization studies (38, 39) have revealed extensive genetic heterogeneity, even among the same serotype, and the absence of a number of gene clusters among closely related strains, suggesting that they are absent or significantly divergent in these strains. Interestingly, a majority of the loci that differed in these strains encoded surface proteins or were related to pathogenesis (39), suggesting that such differences may contribute to differences in virulence. The existence of a *bee* locus on a conjugative element with an ability to transfer at high frequency and confer on recipients a high-biofilm phenotype may define the emergence of *E. faecalis* strains ideally suited to persist and disseminate virulence traits in the nosocomial environment.

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