Enterococcus faecalis rnjB Is Required for Pilin Gene Expression and Biofilm Formation[▽]

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Pili in Gram-positive bacteria play a major role in the colonization of host tissue and in the development of biofilms. They are promising candidates for vaccines or drug targets since they are highly immunogenic and share common structural and functional features among various Gram-positive pathogens. Numerous publications have helped build a detailed understanding of pilus surface assembly, yet regulation of pilin gene expression has not been well defined. Utilizing a monoclonal antibody developed against the *Enterococcus faecalis* major pilus protein EbpC, we identified mutants from a transposon (Tn) insertion library which lack surface-exposed Ebp pili. In addition to insertions in the *ebp* regulon, an insertion in *ef1184* (*dapA*) significantly reduced levels of EbpC. Analysis of in-frame *dapA* deletion mutants and mutants with the downstream gene *rnjB* deleted further demonstrated that *rnjB* was responsible for the deficiency of EbpC. Sequence analysis revealed that *rnjB* encodes a putative RNase J2. Subsequent quantitative real-time PCR (qRT-PCR) and Northern blotting demonstrated that the *ebpABC* mRNA transcript level was significantly decreased in the *rnjB* deletion mutant. In addition, using a reporter gene assay, we confirmed that *rnjB* affects the expression of the *ebpABC* operon. Functionally, the *rnjB* deletion mutant was attenuated in its ability to produce biofilm, similar to that of an *ebpABC* deletion mutant which lacks Ebp pili. Together, these results demonstrate the involvement of *rnjB* in *E. faecalis* pilin gene expression and provide insight into a novel mechanism of regulation of pilus production in Gram-positive pathogens.

Enterococcus faecalis, a normal commensal of the human gastrointestinal tract, is also an opportunistic pathogen and a major cause of nosocomial infections. *E. faecalis* is one of many Gram-positive pathogens recently discovered to possess surface pili. These Gram-positive pili are distinct from Gram-negative pili in their structure and mechanism of assembly (35, 42). Pilus expression has been closely associated with virulence in multiple human pathogens, including group A *Streptococcus* (33), group B *Streptococcus* (24), and *Corynebacterium diphtheriae* (43). In *E. faecalis*, the biofilm-associated pili (Ebp) are considered to be among its major virulence factors and play an important role in biofilm formation and the development of endocarditis (35). Mutations in *ebp* structural genes have been shown to significantly reduce *E. faecalis* biofilm formation *in vitro* (35) and to decrease the ability of *E. faecalis* to form vegetations in a rat endocarditis model (20). The Ebp pilus also plays a role in murine urinary tract infection (UTI), as was demonstrated using an ascending UTI model (40), which provides further evidence for the importance of pili in bacterial infection.

Three genes encoding the *E. faecalis* pilus proteins (*ebpA*, -*B*, and -*C*) are located in the same operon (35). Ebp pili are formed by the cross-linking of all three pilus proteins (35). EbpR, encoded by the gene upstream of *ebpABC*, is a transcription activator of the *ebpABC* genes. It has been demonstrated that deletion of *ebpR* leads to reduced levels of the *ebpABC* mRNA, as evaluated by quantitative real-time PCR (qRT-PCR), and of pili, as evaluated by Western blotting (5). Many environmental factors affect pilus production, including culture medium (tryptic soy broth [TSB] versus brain heart infusion [BHI]), serum, and bicarbonate (6). In a recent study, Bourgogne et al. demonstrated that the addition of bicarbonate to culture media enhanced the expression of the *ebpR* and *ebpABC* locus (6). In general, however, the genetic regulation mechanism of pilin gene expression remains poorly understood.

In the present study, we utilize a monoclonal antibody (MAb) developed against EbpC (the major pilus unit), to identify mutants that lack Ebp pili. Three gene insertion mutants identified from the screen were transposon (Tn) insertions in *ebpR*, *ebpA*, and the *dapA/rnjB* locus. Characterization of in-frame *dapA* deletion mutants and mutants with the downstream *rnjB* gene deleted demonstrated that *rnjB*, not *dapA*, is required for *ebp* operon gene expression as well as biofilm formation.

MATERIALS AND METHODS

Strains, plasmids, growth media, and chemicals. The bacterial strains and plasmids used in this study are listed in Table 1. Brain heart infusion (BHI) broth

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TABLE 1. Strains and plasmids used in this study

Strain (GenBank accession no.) or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. faecalis		
OG1RF	Wild-type strain, Fus^r Rif^r, p-Cl-Phe^r	34
OG1RF Tn library	OG1RF Tn insertion mutants	17
TX5608 (Δ*ebpABC* mutant)	OG1RF *ebpABC* deletion mutant	35
Δ*rnjB* mutant	OG1RF *rnjB* in-frame deletion	This study
E. coli		
XL1-Blue	Cloning host	Stratagene
EC1000	Cloning host, provides RepA in *trans*	25
Plasmids		
pQE30:rEbpC	Recombinant EbpC expression vector	35
pMSP3535	Nisin-inducible expression vector, Em^r	8
pCJK47	Delivery plasmid with *pheS** counterselectable marker	8
pMSP:*rnjB*	*rnjB* cloned into pMSP3535	This study
pTEX5585	P$_{ebpA}$::*lacZ* fusion in pTCV-*lacZ*	6
pTEX5586	P$_{ebpR}$::*lacZ* fusion in pTCV-*lacZ*	5
pCJK47:*dapA*	Intermediate construct carrying *dapA*-flanking sequence	This study
pCJK47:*rnjB*	Intermediate construct carrying *rnjB*-flanking sequence	This study

and tryptic soy broth without glucose (TSB) were purchased from Difco Laboratories (Detroit, MI). All chemicals were purchased from Sigma (St. Louis, MO). Oligonucleotides used in this study were purchased from Invitrogen (La Jolla, CA) and are listed in Table 2.

Development of anti-EbpC MAbs. Recombinant EbpC (rEbpC) was produced in *Escherichia coli* as previously described by Nallapareddy et al. (35). To generate antibodies, BALB/c mice were immunized with the rEbpC protein using standard techniques (21). The splenocytes were collected and fused with SP2/O mouse myeloma cells as previously described (21). Monoclonal antibodies from single-cell clones were evaluated for binding specificity to rEbpC and to native antigen via enzyme-linked immunosorbent assays (ELISAs) and whole-cell ELISAs, respectively (19, 37) followed by kinetic screening for highest-affinity clones using surface plasmon resonance (SPR) as previously described (11).

Flow cytometry analysis. Aliquots equivalent to an optical density at 600 nm (OD$_{600}$) of 0.2 of *E. faecalis* cells in BHI medium were harvested and washed twice with phosphate-buffered saline (PBS) before resuspension in 100 μl of 1% bovine serum albumin (BSA) in PBS with 5 μg/ml anti-EbpC MAb 69 at 25°C for 1 h. This was followed by secondary labeling using a phycoerythrin-conjugated goat anti-mouse IgG (Jackson Immunoresearch, PA). The bacterial cells were fixed with 1% paraformaldehyde and analyzed with a BD FACSCalibur flow cytometer (BD Biosciences, CA).

Whole-cell ELISA library screen. The Tn insertion library in a 96-well format (17) kindly provided by D. A. Garsin was inoculated into 96-well U-bottom microplates (Greiner Bio-one, NC) containing 150 μl of BHI broth per well. Bacteria were cultured for 24 h before centrifugation and subsequently washed in 200 μl of PBS. Cells were then resuspended in 100 μl of 50 mM carbonate buffer, pH 9.6, and were used to coat Microlon 600 ELISA microtiter plates (Greiner Bio-one, NC) at 37°C for 2 h. Cells were washed 3 times with PBS-0.2% Tween 20 (PBST) and then incubated with 5% dry milk in PBST to block nonspecific binding. Anti-EbpC MAb 69 at 5 μg/ml in 5% dry milk in PBST was added to each well and incubated at 37°C for 1 h. The wells were washed 3 times with PBST, followed by the addition of 100 μl of a 1:3,000 dilution of goat anti-mouse IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch). After 1 h of incubation at 37°C, the wells were washed 3 times with PBST. Tetramethylbenzidine (TMB) substrate was added, and the mixture was incubated at room temperature for 10 min. The absorbance of each well was measured at an OD$_{450}$.

Extraction of cell wall-associated proteins and Western blotting. Surface protein extracts from *E. faecalis* strains were prepared using mutanolysin as described previously with minor modifications (35). Briefly, the *E. faecalis* cells grown to stationary phase were harvested and washed with 0.02 M Tris-HCl (pH 7.0), 0.01 M MgSO$_4$ buffer and resuspended in 1/10 volume of this buffer containing 100 μM phenylmethylsulfonyl fluoride (PMSF). Following the addition of mutanolysin to a final concentration of 10 U/1 OD$_{600}$ of cells, tubes were incubated at 37°C for 1 h in a rotating shaker. After centrifugation at 12,000 ×

g for 10 min, the supernatants were concentrated by lyophilization, and aliquots were stored at −70°C. Equal amounts of total protein from mutanolysin extracts were loaded on 4% to 12% NuPAGE Novex bis-Tris gels (Invitrogen) under reducing conditions in MOPS (morpholinepropanesulfonic acid) buffer and transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) according to the manufacturer's protocol. Membranes were then probed with anti-EbpC MAb followed by HRP-conjugated goat anti-mouse IgG antibody and developed using the TMB substrate system (KPL).

Construction of in-frame deletion mutants. *E. faecalis* OG1RF in-frame deletion mutants were obtained using the PheS counterselection markerless exchange system (22). First, ~1-kb fragments of OG1RF chromosomal DNA flanking target genes were PCR amplified using the primer pairs listed in Table 2. The two amplicons for each gene were then simultaneously cloned into the counterselectable plasmid pCJK47 (22). The resulting plasmid was transformed into the conjugative donor strain CK111 (22) by electroporation. Blue colonies were used to conjugate with the recipient strain, OG1RF. Transconjugant colonies were selected for the integration of the plasmid and then cultured on MM9YEG agar supplemented with 10 mM *p*-chloro-phenylalanine. PCR analyses using primer pairs outside the targeted gene were performed on chromosomal DNA to identify mutant versus wild-type sequence at the target locus. PCR products were sequenced to confirm gene deletions.

Complementation analysis. The nisin-inducible *E. faecalis* expression vector pMSP3535 was applied in the complementation analysis according to the protocol of Bryan et al. (8). Briefly, a 1,699-bp fragment containing the *rnjB* gene and its ribosome binding site was PCR amplified from *E. faecalis* OG1RF and cloned under the control of the nisin promoter of the shuttle vector pMSP3535. Both the empty vector and the resulting plasmid were transformed into the *rnjB* deletion mutant. Nisin was added to the culture media at 25 ng/ml to induce exogenous expression of the *rnjB* gene. The presence of EbpC on the cell surface after nisin induction was determined by flow cytometry.

Northern blotting. An amount of cells grown in BHI to exponential phase that was equivalent to an OD$_{600}$ of ~1.0 was collected for RNA extraction. Total RNA samples were extracted and blotted using standard methods and a Nucleo-Spin RNA II kit (Machery-Nagel GmbH & Co., Duren, Germany). Eight micrograms of RNA was analyzed by Northern blotting with ^{32}P-radiolabeled oligonucleotides specific for the 5′ untranslated region (5′-UTR) of *ebpA* (CAGTTAAATTAGAATTGCCTAGCACG), *ebpC* (GTCGTCGGTATGACCGTTATCA), or 23S rRNA (CGCCCTATTCAGACTCGCTTT). RNA levels were quantified using a Storm phosphorimager (GE Health Care), and the approximate sizes of the RNAs were determined by comparing their migration distances with those of the two rRNAs.

qRT-PCR. An amount of cells grown in BHI to exponential phase that was equivalent to an OD$_{600}$ of ~1.0 was collected for RNA extraction. Total RNA and cDNA were prepared using methods provided by the manufacturer. Quantitative real-time PCR (qRT-PCR) on cDNA was performed using a SYBR

TABLE 2. Primers used in this study

Construction or test and primer or probe	Sequence
dapA deletion construction	
1184del-1F	5′-GCGCGGATCCTGAGGTTATTGGCGTCTAAG-3′
1184del-1R	5′-GGCCCTGCAGCCTATTCCCTCCCGAGTATA-3′
1184del-2F	5′-AAAACTGCAGAAGAACGTGTAATCATAGAGAG-3′
1184del-2R	5′-GCCGGAATTCTAAATCGCCTTCTTCAATTC-3′
1184delUpF	5′-TAAGTCAAAAGTCAATGGAT-3′
1184delDnR	5′-AATCATAATATTTTCGGCCG-3′
rnjB deletion construction	
1185del-1F	5′-GCGCGGATCCTGGTTACGCCGTTTCAAGAATC-3′
1185del-1R	5′-GGCCCTGCAGATTTTTTCCATTTTCACGAACGCC-3′
1185del-2F	5′-AAAACTGCAGTTCGTCCTGATCAAAGCAGG-3′
1185del-2R	5′-GCCGGAATTCTCAGTTGCTTGCTCTTCTAAAC-3′
1185delUpF	5′-ATATAGAAGTGAAACAAGCAGATGC-3′
1185delDnR	5′-TTCACGAATCAAACGGCTCA-3′
ebpA qRT-PCR	
Forward primer	CAACAACACCAGGGCTTTTTG
Reverse primer	ACCGGACCAGTCAACGACTAAG
ebpB qRT-PCR	
Forward primer	CGTACAGGCGGCAAGTCTTT
Reverse primer	AGGTATTCCCCCGCTTGATTT
ebpC qRT-PCR	
Forward primer	GCGGCACACTAAAATTCGTTTA
Reverse primer	GTCGTCGGTATGACCGTTATCA
23S rRNA qRT-PCR	
Forward primer	GTGATGGCGTGCCTTTTGTA
Reverse primer	CGCCCTATTCAGACTCGCTTT
dapA cloning	
Forward primer	GCGCGGATCCGGAGGGAATAGGATGGA
Reverse primer	CCGGCTCGAGTTAAATCTCTAACGTGC
rnjB cloning	
Forward primer	GCGCGGATCCGGTGAAAAGAGTGAG
Reverse primer	CCGGCTCGAGCTATGCGTTATTTTGG
Northern blot probes	
ebpA 5-UTR probe	CAGTTAAATTAGAATTGCCTAGCACG
ebpC probe	GTCGTCGGTATGACCGTTATCA
23S rRNA probe	CGCCCTATTCAGACTCGCTTT

green PCR master mix kit and an ABI7900 real-time PCR system (Applied Biosystems). The expression of 23S rRNA, *ebpA*, *ebpB*, and *ebpC* was analyzed using primer pairs listed in Table 2. For each primer set, a reference curve was established using the genomic DNA purified from wild-type OG1RF cells. The amounts of gene transcripts (ng/ml) obtained for *ebpA*, *ebpB*, and *ebpC* were normalized with the amount of 23S rRNA transcript.

β-Galactosidase assay. Cells containing the pTCV-lac vector or transcriptional fusions were grown overnight in BHI broth with kanamycin (1,000 μg/ml). Each was inoculated into BHI broth with kanamycin at a starting OD_{600} of 0.05 and was harvested at mid-log phase and early stationary phase. For each strain, 10^9 cells were washed and resuspended in 0.5 ml Z buffer (60 mM Na_2HPO_4, 40 mM $NaHPO_4$, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), mixed with a 0.25-ml volume of 0.1-mm-diameter zirconia beads (BioSpec Products, Bartlesville, OK), and disrupted using a minibeadbeater for 1 min. The cell lysates were centrifuged at 13,600 rpm for 1 min to remove cell debris before being used in a β-galactosidase assay. The β-galactosidase assay was performed according to the protocol of Bourgogne et al. (6), with some modifications. Dilutions of cell lysate were incubated with *o*-nitrophenyl-β-D-galactoside (ONPG) until a yellow color developed. The reaction was stopped by the addition of 0.5 volume of 1 M Na_2CO_3, and the absorbance of each well was measured at an OD_{420}. The total protein content of each cell lysate was determined using a standard bicinchoninic acid (BCA) assay to normalize samples as described previously (31). The β-galactosidase units of the samples corresponded to an OD_{420}/protein concentration in mg/ml.

Biofilm formation assay. A biofilm density detection assay was carried out as described previously by Mohamed et al. (32) with some modifications. Briefly, *E. faecalis* strains were grown overnight at 37°C before being diluted 1:100 into 10 ml TSBG (TSB plus 2% glucose) and cultured at 37°C in a Microtest 96-well tissue culture plate (BD Biosciences) for 24 h. The supernatant culture was carefully removed by gentle aspiration. The plate was washed four times with 200 μl PBS. The adherent cells were fixed with Bouin fixative for 30 min, washed two

times with PBS, and then stained with 1% crystal violet for 30 min. The stain was rinsed and solubilized in ethanol-acetone (80:20). The absorbance of each well was measured at an OD_{595}. Each assay was performed in quadruplicate in three independent experiments.

RESULTS

Detection of surface EbpC through the use of an anti-EbpC monoclonal antibody. Using an anti-EbpC MAb, we were able to detect the presence of EbpC on the cell surfaces of most *E. faecalis* OG1RF cells grown under standard laboratory conditions in all growth phases (Fig. 1). The specificity and affinity of MAb 69 coupled with flow cytometry analysis provided a sensitive method to quantitatively evaluate the presence of EbpC on cell surfaces, and our results demonstrate that a higher percentage of cells express pili than previously reported using microscopy and polyclonal antibody labeling (35). Mean fluorescence intensity (MFI) in each growth phase was compared to that of an *ebpABC* deletion mutant control (Fig. 1). The result demonstrated that the surface display of EbpC is not growth phase related under these *in vitro* growth conditions. This observation provides evidence that EbpC is readily displayed in the *E. faecalis* population, which allowed us to consider the feasibility of a screen strategy to identify mutants deficient in surface pili.
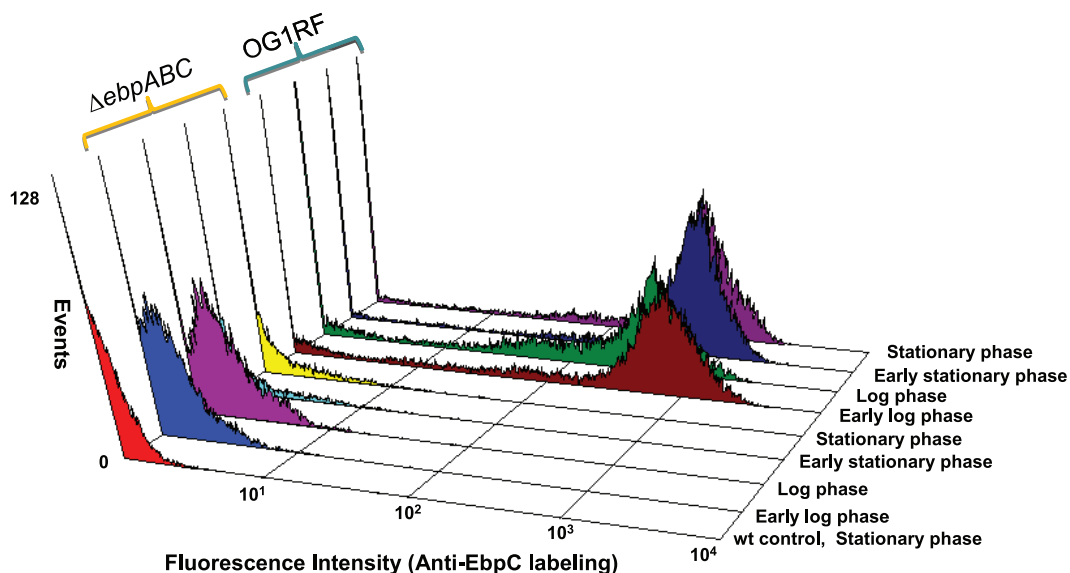
FIG. 1. Cell surface binding of anti-EbpC MAb 69 to *E. faecalis*. Wild-type OG1RF (wt) and *ebp* deletion mutant (Δ*ebpABC*) cells grown in BHI medium to early exponential phase, exponential phase, early stationary phase, and stationary phase were labeled with anti-EbpC MAb 69 and a secondary antibody-phycoerythrin conjugate, and 10,000 cells were analyzed by flow cytometry. Wild-type OG1RF cells grown to stationary phase and labeled with secondary antibody only (wild-type control) served as a negative control.

Identification of EbpC-deficient *E. faecalis* mutant strains. In an effort to identify the genetic factors that contribute to *E. faecalis* pilus gene expression and/or pilus assembly, we developed a whole-cell ELISA-based screening method using the anti-EbpC MAb 69. The 540-member Tn insertion mutant library used in the screen contains *E. faecalis* mutants that cover approximately one-fourth of all nonessential genes (17). Anti-EbpC MAb 69 was used to label cells from the insertion mutant library. Mutants which displayed the lowest levels of anti-EbpC MAb binding were selected. Figure 2 depicts the distribution of the ELISA signals of library members. Four mutants which repeatedly generated the lowest ELISA signals included those with insertions in *ebpR* (also known by its systematic name *ef1090*), *ebpA* (*ef1091*), *ef1184*, and *ef1579* (Fig.

2). *ebpR* is a positive transcriptional regulator for the *E. faecalis ebpABC* operon (5), and thus its disruption would have a negative effect on EbpC expression. *ebpA* is the first gene of the *ebpABC* operon (35), and its disruption would have a polar effect on downstream genes, including *ebpC*. Of greatest interest was the identification of *ef1184* and *ef1579*, representing *E. faecalis dapA*, which encodes a dihydrodipicolinate synthase, and *lexA*, which encodes a LexA repressor enzyme. Neither gene has been previously associated with pilus gene expression or pilus assembly in *E. faecalis*. In this study, we focus on the *ef1184* insertion mutant.

To confirm the whole-cell ELISA results, we analyzed each identified mutant strain using flow cytometry analysis with anti-EbpC MAb 69 staining (Fig. 3A to E). The *ef1184* insertion
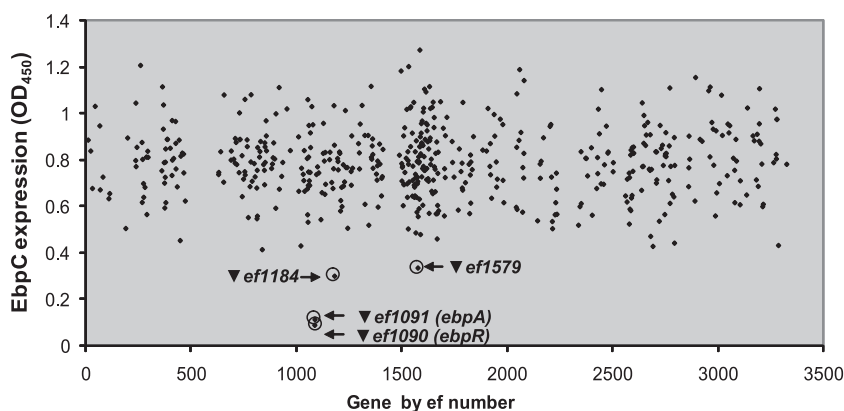

FIG. 2. Identification of genes involved in the surface expression or regulation of EbpC. Each *E. faecalis* gene has a systematic name beginning with "*ef*," and the 540 clones in the Tn library are represented by the number in their name on the *x* axis. Relative whole-cell ELISA signals are represented on the *y* axis. Each clone was labeled with our anti-EbpC antibody followed by a goat anti-mouse IgG-HRP conjugate and developed with the TMB substrate. Tn insertions in *ef1090* (*ebpR*), *ef1091* (*ebpA*), *ef1184*, and *ef1579* (as indicated by arrows) were identified in two independent evaluations as clones which produced the lowest ELISA signal.
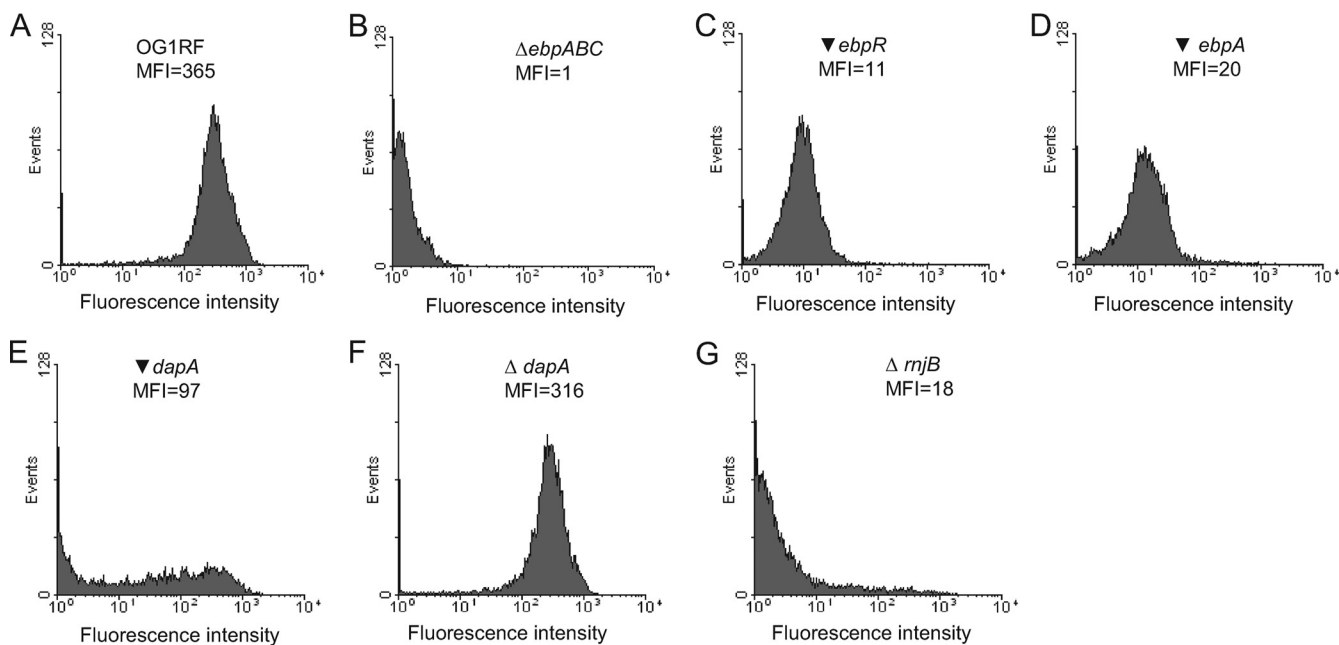
FIG. 3. Flow cytometry analysis of surface-exposed EbpC in *E. faecalis* wild-type and mutant strains. Each population was labeled with anti-EbpC MAb 69. Mean fluorescence intensities are compared in the OG1RF (A), Δ*ebpABC* (*ebpABC* deletion) (B), *ebpR* (*ef1090* insertion mutant) (C), *ebpA* (*ef1091* insertion mutant) (D), *ef1184* (*dapA* insertion mutant) (E), Δ*dapA* (*dapA* deletion mutant) (F), and Δ*rnjB* (*rnjB* deletion mutant) (G) strains. The MFI value of each sample is indicated. ▼, Tn insertion mutant.

mutant showed an MFI that was 27% that of wild-type OG1RF. In comparison, *ebpR* and *ebpA* insertion mutants had MFIs of 3 and 5% of that for OG1RF, and a complete deletion of the *ebpABC* genes (35) resulted in an MFI of 0.3% of that of the wild type. Thus, the level of surface-exposed EbpC is reduced in the three insertion mutants. The decrease in surface-displayed EbpC in these insertion mutants was further confirmed by Western blotting (Fig. 4). Ladders of high-mo-
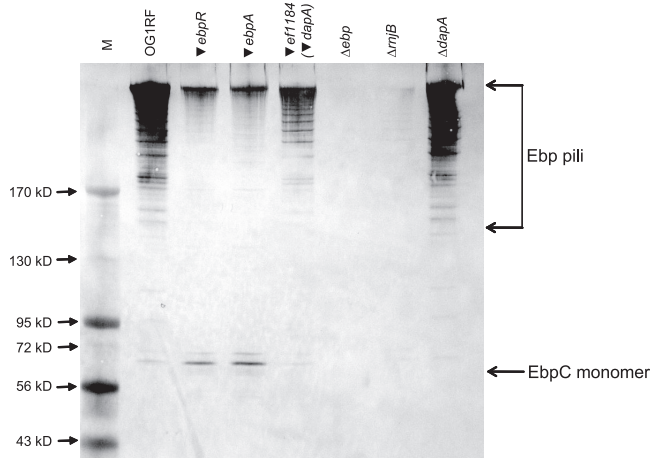

FIG. 4. Western blotting results of Ebp pilus formation of the wild-type OG1RF and mutant strains. Mutanolysin surface extractions were probed with anti-EbpC MAb 69. Lanes (left to right): EZ-run protein marker (M), OG1RF, *ebpR* mutant, *ebpA* mutant, *ef1184* mutant, Δ*ebpABC* mutant, Δ*rnjB* mutant, and Δ*dapA* mutant. High-molecular-weight Ebp pilus polymers and EbpC monomers are indicated. ▼, Tn insertion mutant.

lecular-weight covalently linked EbpC were observed in cell wall extract fractions of wild-type OG1RF and to a lesser extent in the *ef1184* insertion mutant, reflecting the 3- to 4-fold decrease seen in fluorescence intensity by flow cytometry of the *ef1184* mutant population compared to that of the wild type. Surface extracts of both the *ebpA* and *ebpR* insertion mutants exhibited a single low-molecular-weight band likely representing the EbpC monomer (molecular mass, 65 kDa), indicating the deficiency of pilus polymer formation in these two mutants. All together, ELISA, flow cytometry, and Western analyses indicated that the insertion in *ef1184* caused a reduction in the level of surface-exposed EbpC.

Deletion of *rnjB* reduces the level of surface-exposed EbpC. Sequence analysis of the 3.7-kb gene locus containing *ef1184* predicted the presence of three complete open reading frames, *asd* (*ef1183*), *dapA* (*ef1184*), and *rnjB* (*ef1185*). No transcriptional terminator-like sequence can be identified in between the open reading frames, suggesting that they are transcribed together as one polycistronic mRNA (Fig. 5A). This gene cluster organization is similar to that of a *B. subtilis* diaminopimelate operon which contains four genes, *asd*, *dapG*, *dapA*, and *rnjB* (previously *ymfA*) (Fig. 5A) (12). Both *asd* (encodes an aspartate-semialdehyde dehydrogenase) and *dapA* (encodes a dihydrodipicolinate synthase) are involved in the synthesis of the peptidoglycan precursor diaminopimelate, while the gene product of *rnjB* in *B. subtilis* was identified as a novel endoribonuclease, RNase J2 (16). BLASTN search using the sequence of the OG1RF *ef1183-ef1185* locus revealed >99% identity among all 23 known *E. faecalis* genomes, which clearly demonstrates the ubiquitous presence of this cluster among *E. faecalis* strains.

The Tn*917* insertion site in the *dapA* (*ef1184*) insertion mutant was located at +169 bp in the *dapA* gene (17). A polar
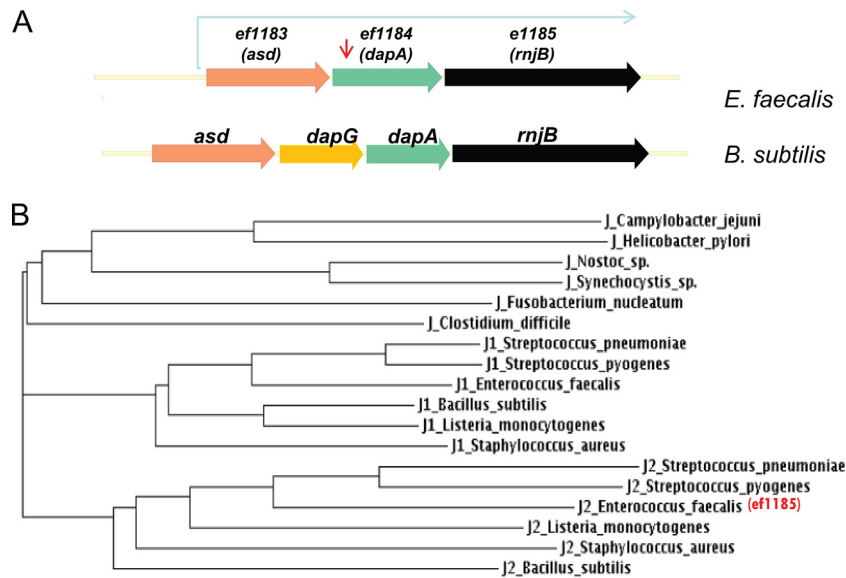
FIG. 5. *E. faecalis rnjB* encodes an RNase J2 ortholog. (A) Comparison of the *E. faecalis* OG1RF *ef1183-ef1185* operon with the *B. subtilis asd-rnjB* operon. The red arrow indicates the Tn insertion site in the *ef1184* mutant (*dapA* insertion mutant). (B) Phylogram of six RNase J1s and six RNase J2s from the bacterial order *Bacilli* and several other bacterial RNase Js based on multiple-sequence alignment generated by ClustalW2 with default parameters.

effect of the Tn insertion in the *dapA* gene may also alter the expression of the downstream *rnjB* gene. To determine which gene is responsible for the reduced EbpC surface display seen in the insertion mutant, we constructed two in-frame deletion mutants with either *dapA* (Δ*dapA* mutant) or *rnjB* (Δ*rnjB* mutant) from the wild-type OG1RF strain deleted. Both mutants are viable, indicating that *dapA* and *rnjB* are not essential genes for *E. faecalis*. The EbpC surface display of each mutant was analyzed by flow cytometry. Interestingly, the Δ*rnjB* mu-

tant exhibited a low level of EbpC on the cell surface (5% of the wild-type MFI) (Fig. 3G). In contrast, the Δ*dapA* mutant showed a level of anti-EbpC labeling similar to that of wild-type OG1RF (Fig. 3F). To confirm the importance of the *rnjB* gene in altering levels of surface-exposed EbpC, the gene was cloned into the nisin-inducible expression vector pMSP3535 and introduced into the Δ*rnjB* mutant. As shown in Fig. 6, the in *trans* addition of *rnjB* in the Δ*rnjB* mutant partially restored levels of surface-exposed EbpC (Fig. 6D and E).
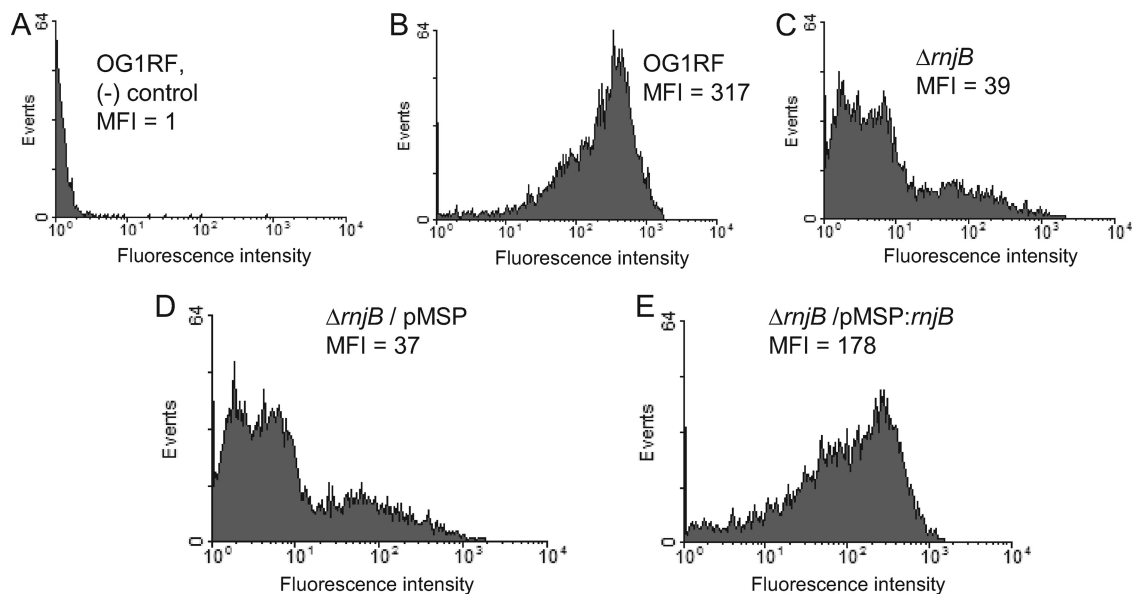

FIG. 6. Complementation of the *rnjB* deletion in OG1RF. Flow cytometry analysis of OG1RF (wild type) (B), the Δ*rnjB* mutant (C), the Δ*rnjB* mutant with the vector control (D), and the Δ*rnjB* mutant with the *rnjB* complement vector (E). Cells grown to the stationary phase in BHI medium with 25 ng/ml nisin were labeled with anti-EbpC MAb 69 followed by a secondary antibody-phycoerythrin conjugate. Mean fluorescence intensities are compared to that of the control, OG1RF with secondary antibody only (A).
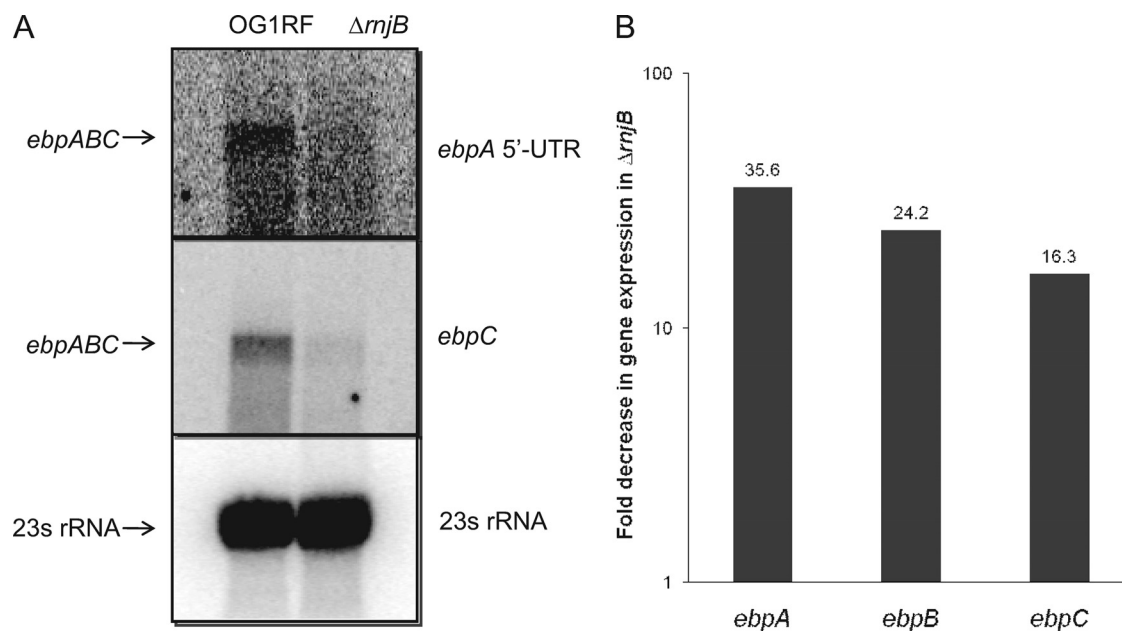
FIG. 7. Comparison of transcript levels of *ebp*-related genes in wild-type OG1RF and the Δ*rnjB* mutant. (A) Northern blot of RNA isolated from mid-log-phase wild-type OG1RF and Δ*rnjB* cells. Blots were hybridized with ^{32}P-labeled 5′-UTR–*ebpA* (top panel), *ebpC* (middle panel), and 23S rRNA (loading control) (bottom panel) probes. (B) qRT-PCR analysis of RNA isolated from mid-log-phase wild-type OG1RF and Δ*rnjB* cells. Each column represents the gene of interest shown in the *x* axis. The fold decrease in gene expression in the Δ*rnjB* mutant corresponds to the ratio of the transcript level of each gene in the wild-type strain to that of each gene in the Δ*rnjB* mutant. The level of each gene transcript is tested in triplicate and normalized using 23S rRNA transcript levels.

The effect of *rnjB* on pili was further confirmed by Western blotting. As shown in Fig. 4, ladders of high-molecular-weight bands representing the Ebp pilus polymers were detected in the cell wall extractions of both wild-type OG1RF and the Δ*dapA* mutant probed with anti-EbpC MAb, which demonstrated that pilus formation is not affected by the deletion of *dapA*. In comparison, these high-molecular-weight bands are absent in cell wall extracts of the Δ*rnjB* mutant, similar to what occurs in the *ebpABC* deletion mutant (Fig. 4). This indicates that the formation of the EbpC-containing pilus is attenuated in the Δ*rnjB* mutant. Considering all data together, we conclude that *rnjB* somehow regulates the level of surface-exposed EbpC and that the loss of EbpC on the cell surface of the *dapA* Tn insertion mutant is the result of its polar effect on the downstream *rnjB* gene.

As an initial step toward characterizing the function of RnjB, we carried out sequence comparisons using BLASTP and ClustalW. Multiple sequence alignments (not shown) clearly showed that RnjB belongs to the RNase J family. Many Gram-positive bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *E. faecalis*, and *Listeria monocytogenes*, contain two RNase J family members. *E. faecalis* RnjB is a member of the RNase J2 cluster (Fig. 5B). The orthology of *E. faecalis* RnjB and *B. subtilis* RnjB is also supported by their presence in similar operons.

***rnjB* affects *ebpABC* gene expression.** The effect of *rnjB* on surface-displayed EbpC may occur at the RNA level and affect processes such as transcriptional regulation and RNA processing, or it may occur at the protein level and affect processes such as translation, transportation, and pilus assembly. To start

to elucidate the mechanism of the *rnjB* effect on EbpC surface display, we explored the steady-state mRNA levels for *ebp* genes. Figure 7A shows the results of a Northern blot analysis of RNAs isolated from wild-type OG1RF and the Δ*rnjB* mutant. Both an *ebpA* 5′-UTR probe and an *ebpC* probe detect an ~7-kb mRNA in the wild-type OG1RF RNA blot, which presumably represents the polycistronic *ebpABC* mRNA (Fig. 7A). In comparison, the intensity of that band is strongly reduced in the Δ*rnjB* mutant RNA blot probed with either probe (Fig. 7A). These results clearly demonstrate that the *ebpABC* transcript level is greatly decreased in the Δ*rnjB* mutant compared to that of wild-type OG1RF. qRT-PCR analysis further demonstrated that the steady-state mRNA levels of *ebpA*, *ebpB*, and *ebpC* were, respectively, 36-, 24-, and 16-fold higher in OG1RF than in the Δ*rnjB* mutant (Fig. 7B). Thus, the expression of all three *ebp* pilin genes is affected by *rnjB* at the mRNA level.

We next evaluated the effect of *rnjB* on an *ebpA*::*lacZ* transcriptional fusion. pTEX5585 is a pTCV-lac-derived vector containing the *ebpA* promoter, the 5′-UTR, and the first 41 nucleotides (nt) of the coding region upstream of a LacZ coding region (6). As shown in Fig. 8A, LacZ expression from this reporter in OG1RF is 20-fold higher than in the Δ*rnjB* mutant at both log phase and stationary phase. Since *ebpA* is known to be regulated by EbpR, we also analyzed a similar *ebpR*::*lacZ* transcriptional fusion. Figure 8 shows that *ebpR*::*lacZ* expression is not affected by Δ*rnjB* and thus that the effect on *ebpA*::*lacZ* is specific. Furthermore, this result shows that the effect of *rnjB* on *ebpABC* expression is not an indirect effect of higher *ebpR* transcription.
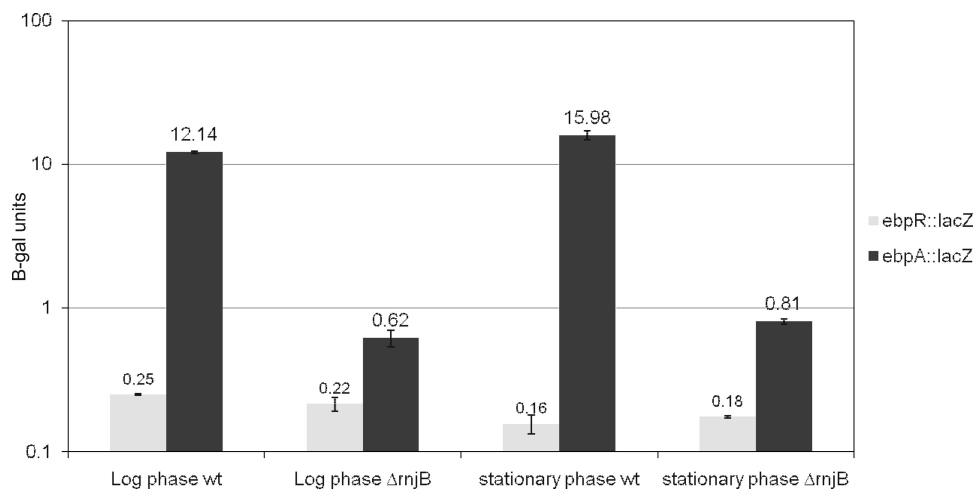
FIG. 8. *rnjB* affects the expression of an *ebpA*::*lacZ* reporter but not an *ebpR*::*lacZ* reporter. Wild-type OG1RF and the Δ*rnjB* mutant containing either P_{ebpR}::*lacZ* or P_{ebpA}::*lacZ* were grown in BHI and collected at log phase and stationary phase for β-galactosidase (B-gal) assay. The y axis represents the β-galactosidase units (OD_{420}/total protein concentration in mg/ml). Error bars represent the standard errors of the measurements from three individual cultures.

rnjB is essential for *E. faecalis* biofilm formation. To evaluate the functional impact of the *rnjB* deletion on virulence-related phenotypes, we evaluated the in-frame deletion mutant to monitor differences in the abilities of the strains to form biofilm on a polystyrene surface. Utilizing a polystyrene biofilm assay, it has been previously demonstrated that *E. faecalis* Ebp pili are required for biofilm formation (35). Figure 9 demonstrates that, compared to the wild type, the Δ*rnjB* mutant is attenuated in biofilm formation, a result similar to that of the *ebpABC* deletion mutant. In contrast, the *dapA* deletion mutant demonstrated biofilm formation similar to that of the wild type, consistent with our findings that the loss of the pilus phenotype of the *dapA* Tn insertion mutant is a result of its polar effect on *rnjB* expression.
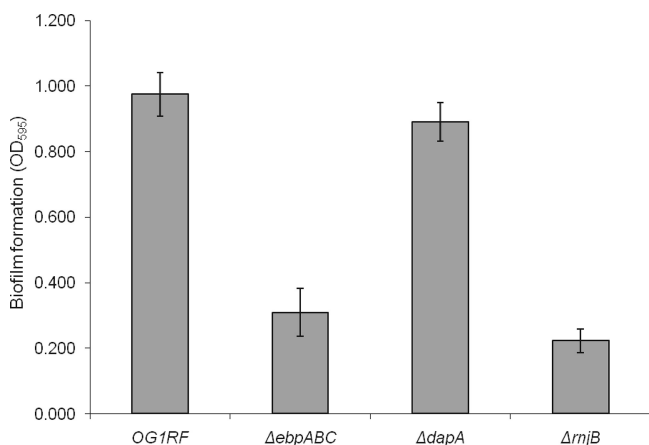

FIG. 9. Biofilm formation of OG1RF gene deletion mutants. Cells grown for 24 h on polystyrene microtiter plates were evaluated for biofilm formation, expressed as a mean crystal violet absorbance of a biofilm biomass in four independent microtiter wells. Error bars represent the standard errors of the means.

DISCUSSION

Numerous recent publications have identified pili as key players in the virulence of many Gram-positive bacteria (28, 39). Among them, the Ebp pilus of *E. faecalis* has been shown to play an important role in bacterial adhesion, biofilm formation, and pathogenesis in animal models (35, 40). In this study, we report that the expression of the *E. faecalis ebpABC* operon is greatly reduced by the deletion of *E. faecalis rnjB*, which encodes the RNase J2. This finding demonstrates for the first time an association between *E. faecalis* pilin gene expression and RNA processing.

Most studies on Gram-positive mRNA processing have been carried out with *Bacillus subtilis* (13). Two enzymes recently identified in *B. subtilis*, RNase J1 and RNase J2, have been shown to possess both endoribonuclease and 5′-to-3′ exoribonuclease activities (16, 29). *B. subtilis* RNase J1 is an essential enzyme, and cells depleted of RNase J1 have multiple defects (14–16, 44). In contrast, the *B. subtilis* RNase J2 is not essential. In fact, a complete deletion does not have a known growth phenotype (26), and no endogenous substrates for RNase J2 have been thoroughly characterized. Both RNase J enzymes of *B. subtilis* have clear orthologs in *E. faecalis* (BLAST scores of $<10^{-200}$ and 10^{-145}) (4, 36). We show that *E. faecalis rnjB* is not an essential gene for growth. This finding is similar to what was found for *B. subtilis*, in which RNase J2 is nonessential, but different from what was found for *Streptococcus pyogenes*, in which RNase J2 is essential (10). The discovery that *E. faecalis* RNase J2 plays a significant role in pilin gene expression provides an excellent phenotype which is easily monitored by flow cytometry, whole-cell ELISA, Western blotting, or *lacZ* fusion analysis as a tool to facilitate research in understanding Gram-positive RNase J2 function and activity.

Regulation of pilus surface display may occur during transcription, translation, protein export, or pilus assembly. In this study, we found that mRNA levels of all three pilin genes were significantly lower in the Δ*rnjB* mutant than in the wild type, as

analyzed by Northern blotting and qRT-PCR. These results suggest that *rnjB* regulates Ebp pili at the RNA level. We propose three hypotheses regarding the mechanism of this regulation process. First, RNase J2 may enhance the *ebp* promoter activity directly or indirectly. Second, RNase J2 may degrade a small inhibitory RNA which functions to inhibit the *ebpABC* transcript by annealing to the 5'-UTR region of the *ebpABC* mRNA. Third, RNase J2 may regulate the *ebpABC* operon directly by processing and stabilizing the *ebpABC* transcript, which is similar to the observed role of *B. subtilis* RNase J1 in 16S rRNA maturation (7). Since the *ebpA::lacZ* fusion contains only the first 118 nt of the *ebpABC* mRNA, RNase J2 would need to act there. However, probing a Northern blot with a probe for the 5'-UTR of *ebpA* did not detect such a processing event, making this mechanism less likely. However, this possibility cannot be ruled out until the 5' end of *ebpABC* mRNA is determined to be the same in the wild type and *rnjB* deletion mutant.

It is also worthwhile to note that in the *ef1183-ef1185* gene cluster, the *rnjB* gene, which codes for the RNase J2 ortholog, is located downstream of two genes encoding key enzymes in the biosynthesis of *meso*-diaminopimelic acid (mDAP) and L-lysine, essential components for the synthesis of the peptidoglycan cell wall. In plants, the *dapA* gene product, DHDPS, is solely responsible for lysine biosynthesis and is strongly inhibited by lysine (3), whereas in bacteria, these enzymes are less responsive or insensitive to lysine inhibition (10). In contrast, it has been reported that this *E. coli* DHDPS expression is regulated by mDAP, suggesting the involvement of this enzyme in bacterial cell wall synthesis (2). In *Serratia marcescens*, regulation of the *dapA* gene cluster has been associated with swarming motility, cell envelope architecture, and cell attachment ability (41). Regulation of the *E. faecalis dapA* gene or the *ef1183-ef1185* cluster has not been addressed before, but it is reasonable to hypothesize that factors regulating *dapA* affect the downstream *rnjB* gene and thereby affect pilus surface expression. This is consistent with our observation that the Tn insertion in *dapA* exhibited downregulation of pilus formation in this study. Similar gene loci with identical gene organizations are present in many other Gram-positive bacteria, including *B. subtilis*, *Listeria innocua*, and *Lactobacillus salivarius*, suggesting a common mechanism for the regulation of RNase J2. Understanding the regulation of this gene cluster might provide insight into the association of lysine synthesis or cell wall formation with expression of *E. faecalis* RNase J2 and, subsequently, with formation of pili.

The whole-cell ELISA screening method enabled us to identify genes affecting *E. faecalis* protein surface display. Using larger Tn libraries in future efforts, such as the one recently generated by Kristich et al., may allow for the discovery of additional genes involved in the pilin display process (23). The differences in pilus formation seen by Western blotting and flow cytometry between the *dapA* Tn insertion, the *dapA* inframe deletion, and the *rnjB* in-frame deletion mutants emphasize the requirement for making in-frame deletion mutants for a complete understanding of such Tn library screening results, especially when a polar disruption may be involved.

As demonstrated by our flow cytometry and Western blot results, pilus formation in the *dapA* insertion mutant was only partially inhibited. This suggests that the Tn mutant still allows

for partial expression of *rnjB*. Promoter prediction of the 300-bp sequence upstream of the *rnjB* gene using BPROM software identified the presence of a putative promoter region for *rnjB* (−35 box, TGGAGC 233 bp upstream of the start codon, and −10 box, GGCTAAAATT 208 bp upstream of the start codon), suggesting the possibility of an independent *rnjB* transcript in addition to the *asd dapA rnjB* polycistronic transcript. This could account for the observation of partial expression of pili in the *dapA* insertion mutant as observed by Western blotting (Fig. 4), since *rnjB* could still be independently transcribed.

The finding that *E. faecalis rnjB* is required for pilin gene expression impacts two previously independent fields of Gram-positive-bacterial research. Discovery of pili in Gram-positive bacteria and their association with bacterial virulence has stimulated research of this key virulence factor in many important Gram-positive pathogens, including *E. faecalis* (35), *C. diphtheriae* (43), *S. pyogenes* (1), *Streptococcus agalactiae* (27, 38), and *S. pneumoniae* (18). Separately, the field of mRNA decay in Gram-positive bacteria has benefited recently from the discovery of RNase J1 and J2, which play a significant role in the maturation and degradation of RNAs (9, 16, 30). The identification of *rnjB* as a mediator of pilin gene expression through our whole-cell library screen is the first association of RNase J2 with Gram-positive pilin gene expression.

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