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The Fibronectin-Binding Protein Fnm Contributes to Adherence to Extracellular Matrix Components and Virulence of *Enterococcus faecium*

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The interaction between bacteria and fibronectin is believed to play an important role in the pathogenicity of clinically important Gram-positive cocci. In the present study, we identified a gene encoding a predicted fibronectin-binding protein of *Enterococcus faecium* (*fnm*), a homologue of *Streptococcus pneumoniae pavA*, in the genomes of *E. faecium* strain TX82 and all other sequenced *E. faecium* isolates. Full-length recombinant Fnm from strain TX82 bound to immobilized fibronectin in a concentration-dependent manner and also appeared to bind collagen type V and laminin, but not other proteins, such as transferrin, heparin, bovine serum albumin, mucin, or collagen IV. We demonstrated that the N-terminal fragment of Fnm is required for full fibronectin binding, since truncation of this region caused a 2.4-fold decrease ($P < 0.05$) in the adhesion of *E. faecium* TX82 to fibronectin. Deletion of *fnm* resulted in a significant reduction ($P < 0.001$) in the ability of the mutant, TX6128, to bind fibronectin relative to that of the wild-type strain; *in situ* reconstitution of *fnm* in the deletion mutant strain restored adherence. In addition, the Δ*fnm* mutant was highly attenuated relative to TX82 ($P \leq 0.0001$) in a mixed-inoculum rat endocarditis model. Taken together, these results demonstrate that Fnm affects the adherence of *E. faecium* to fibronectin and is important in the pathogenesis of experimental endocarditis.

Bacterial adherence to host tissues and extracellular matrix (ECM) proteins is a critical step in the process of infection, since it establishes the initial contact with the host. These interactions can facilitate translocation across the mucosal barrier and internalization into subcellular compartments, eventually leading to bacterial spread within eukaryotic cells (1). Particularly in Gram-positive pathogens, surface-exposed adherence molecules, such as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), are key players in host-microbe interactions (2). Host ligands include ECM components, e.g., fibronectin, collagen, and laminin, as well as molecules that are also present in blood, including fibrinogen and vitronectin (2).

Generally reported as a well-adapted commensal of the gastrointestinal tracts of humans and animals, *Enterococcus faecium* has emerged over the past 3 decades as a leading cause of hospital-associated diseases, including urinary tract infections (UTIs), bacteremia, intra-abdominal infections, and endocarditis (3–5). The rising incidence of multiantibiotic-resistant nosocomial infections caused by *E. faecium* has led to the inclusion of these organisms in the list of "no ESKAPE" (*E. faecium*, *Staphylococcus aureus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacteriaceae*) pathogens that pose a challenge to clinicians and threaten patient safety (6). In the United States, approximately 80% of health care-associated *E. faecium* strains are vancomycin-resistant enterococci (VRE), and more than 90% are ampicillin resistant (5). The frequent lack of an antibiotic regimen of proven efficacy has sparked an interest in understanding the molecular mechanisms that contribute to *E. faecium* pathogenesis.

Fibronectin (Fn) is a large multidomain dimeric glycoprotein found in body fluids and in the ECM (7). Targeting of Fn by several pathogens has been shown to be important in the establishment or dissemination of infection (8). Bacterial Fn-binding proteins were first discovered in *Staphylococcus aureus*, followed by *Streptococcus pyogenes* and many other Gram-positive and Gram-negative microbes (9–11). The majority of reported streptococcal and staphylococcal Fn-binding proteins are characterized by the presence of an N-terminal signal sequence, which is needed for export of the protein to the cell surface via Sec-dependent secretion, and an LPXTG motif at the C-terminal end for covalent anchoring to peptidoglycan. These proteins also possess specific signature repeat motifs (35 to 40 residues) in the C terminus, which mediate Fn binding (12, 13). *Streptococcus pneumoniae* also expresses another type of adhesin, known as the pneumococcal adherence and virulence factor A (PavA) protein (14), that lacks the above-mentioned features of prototypic Fn-binding pro-

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TABLE 1 Bacterial strains used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
DH5α	General cloning host	Stratagene
EC1000	Strain carrying the *repA* gene for replication of pHOU1	28
BL21(DE3)	Strain for recombinant protein expression	Life Technologies
E. faecium		
TX82	Nosocomial isolate (endocarditis); Amp^r Van^r Erm^r	59
TX6128	TX82Δ*fnm*; an *fnm* markerless deletion mutant	This study
TX6155	TX82Δ*fnm*::*fnm*; a derivative of TX6128 with *fnm* restored *in situ* in the chromosome	This study
E. faecalis CK111	Conjugative donor; provides *repA* in *trans* for pHOU1 replication	29
Plasmids		
pHOU1	Vector for allelic exchange in *E. faecium*; carries the *p*-chloro-phenylalanine counterselectable marker; Gen^r	27
pTX6128	pHOU1 derivative carrying a 1,719-bp BamHI/SphI fragment for deletion of *fnm*	This study
pTX6155	Vector for reconstitution of *fnm* containing a 3,180-bp fragment cloned into pHOU1	This study
pET19b	Vector for protein overexpression in *E. coli* containing an N-terminal histidine-tagged fusion; Amp^r	This study
pETFnm01	pET19b derivative carrying the construct for full-length Fnm protein overexpression; Amp^r	This study
pETFnm02	pET19b derivative carrying the construct for overexpression of Fnm_{1-295}; Amp^r	This study
pETFnm03	pET19b derivative carrying the construct for overexpression of Fnm_{1-414}; Amp^r	This study
pETFnm04	pET19b derivative carrying the construct for overexpression of $Fnm_{416-535}$; Amp^r	This study

[a] Amp^r, ampicillin resistant; Erm^r, erythromycin resistant; Gen^r, gentamicin resistant; Van^r, vancomycin resistant.

teins (15, 16). Nonetheless, PavA has been shown to be present on the surfaces of *S. pneumoniae* cells and to bind to immobilized Fn (14). Notably, isogenic *pavA* deletion mutants showed highly (approximately 10^4-fold) attenuated virulence in a mouse sepsis model, suggesting a direct role for PavA in pneumococcal pathogenesis (14). Attenuation of the virulence of PavA-deficient pneumococcal strains was also observed in a mouse meningitis model, and these strains also showed substantially reduced adherence to and internalization by epithelial cell lines (17). These results are consistent with the finding that PavA is important for the ability of pneumococci to escape phagocytosis and to induce adaptive immune responses (18). In addition, Kadioglu and colleagues demonstrated that PavA is required for successful colonization and long-term carriage on the murine nasopharynx and for the systemic spread of pneumococci (19).

PavA homologues have been identified in other streptococci; these include *Streptococcus gordonii* FbpA (20), *Streptococcus pyogenes* FBP54 (21, 22), and *Streptococcus mutans* SmFnB (23). A similar report showed that SfbA, a PavA homologue of group B streptococci, is important in the interaction of these bacteria with the blood-brain barrier endothelium and in the pathogenesis of neonatal meningitis (24). Recently, Torelli et al. identified EfbA, a PavA homologue in *Enterococcus faecalis*, and demonstrated that the derived recombinant protein binds to immobilized Fn and plays a role in the pathogenesis of UTIs (25).

The present work was initiated to identify and study the PavA homologue of *E. faecium* and its contribution to Fn adherence in the context of infection. Here we show that Fnm, encoded by the *E. faecium* homologue of *S. pneumoniae pavA*, is an Fn-binding protein. In addition, we evaluated the effect of the *fnm* deletion on the ability of *E. faecium* TX82 to bind to Fn, and we demonstrate that Fnm is important in the pathogenesis of experimental endocarditis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. Enterococci were routinely grown at 37°C in brain heart infusion (BHI) broth and agar, or in M17 broth (both from Difco Laboratories), unless otherwise indicated. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) broth and agar (Difco Laboratories). The following antibiotic concentrations were used for enterococci: ampicillin, 32 μg/ml; erythromycin, 10 μg/ml; gentamicin, 200 μg/ml. For *E. coli*, the concentrations used were 100 μg/ml ampicillin and 25 μg/ml gentamicin.

DNA techniques. *E. faecium* genomic DNA was isolated from a single colony after overnight growth in BHI broth, as described previously (26). Plasmids were isolated from *E. coli* using Wizard Plus SV MiniPreps system columns (Promega Corporation). Phusion DNA polymerase (New England BioLabs, United Kingdom) was employed for PCR amplification. Primers were purchased from Sigma-Aldrich (see Table S1 in the supplemental material). DNA fragments were purified by agarose gel electrophoresis and Wizard SV Gel and PCR Clean-Up system columns (Promega Corporation). Restriction endonucleases and T4 DNA ligase (both from New England BioLabs, United Kingdom) were used according to the manufacturer's recommendations. The DNA sequencing service was provided by Genewiz Inc., South Plainfield, NJ, USA.

Construction of an *fnm* deletion mutant and reconstitution of *fnm*. Mutants were generated using pHOU1, a *pheS**-based counterselection system, as described previously (27). Briefly, to construct an *fnm* deletion mutant of *E. faecium* TX82, the upstream DNA region flanking the *fnm* gene (672 bp) together with the initial 147 bp of the Fnm-encoding region and the 903-bp downstream flanking sequence were amplified by two independent PCRs using primer pairs fnmUpF/fnmUpR and fnmDownF/fnmDownR, respectively (see Table S1 in the supplemental material). The two fragments were then fused by SOE-PCR (splicing by overlap extension PCR). The resulting 1,722-bp product was digested with restriction enzymes BamHI and SphI and was cloned into similarly digested pHOU1 (27), giving pTX6128. pTX6128 was electroporated into *E. coli* EC1000, which supplies RepA in *trans* for its replication (28). Transformants were screened for the presence of the insert, and the fragment was sequenced using the primer pair FbpOutF/FbpOutR (see Table S1). pTX6128 was electroporated into *E. faecalis* CK111 using standard procedures (29, 30) before transfer to *E. faecium* TX82 by filter matings. The mating mixture was cultivated on BHI plates containing gentamicin and erythromycin for the detection of single-crossover integrants and was then replated onto MM9–yeast extract–glucose (MM9YEG) medium containing *p*-chloro-phenylalanine (*p*-Cl-Phe) (7 mM) to select for vector

excision. The excision of pTX6128 was confirmed by the absence of growth on BHI-gentamicin plates; colonies lacking *fnm* were detected by PCR, and one of these was designated TX6128.

For the restoration of *fnm* in TX6128, the *fnm* gene with its native promoter was amplified by PCR with primer pair fnmUpF/fnmDownR (see Table S1 in the supplemental material). Following digestion with BamHI and SphI, the resulting 3,180-bp fragment was cloned into pHOU1, giving pTX6155, which, after confirmation of the insert by sequencing, was electroporated into *E. faecalis* CK111, transferred to *E. faecium* TX6128, and then processed as described above. The resulting *fnm*-reconstituted strain was named TX6155.

The deletion and restoration of the *fnm* gene in TX6128 and TX6155, respectively, were confirmed by sequencing of the PCR product amplified with primer pair FbpOutF/FbpOutR. In addition, the identities of the strains were confirmed by pulsed-field gel electrophoresis according to a method described previously (31).

Whole-cell fibronectin binding assays. *E. faecium* TX82, TX6128, and TX6155 cells were harvested by centrifugation from exponential-phase cultures in BHI medium supplemented with 40% serum (BHIS), and the concentration was adjusted to an optical density (OD) at 600 nm of 1.0 in phosphate-buffered saline (PBS; pH 7.4). Immulon 2B microwell plates (Thermo Scientific, Woburn, MA) were coated overnight with 20 μg/ml of Fn. Bovine serum albumin (BSA) was used as a negative control. Wells were blocked with 2% BSA at room temperature (RT) for 1 h, and after three washes with PBS, a 100-μl volume of a cell suspension was added. Plates were incubated for 2 h at RT. After three washes with PBS to remove unbound cells, cells were fixed with Bouin's fixative solution (Sigma-Aldrich Co., St. Louis, MO) for 30 min at RT. Each well was then washed with PBS and was stained with 1% (wt/vol) crystal violet for 30 min at RT. Finally, adherent cells were dissolved in an ethanol-acetone solution (80% and 20%, respectively), and the absorbance at 570 nm was measured using a microplate reader (Thermo Scientific, Waltham, MA). Experiments were performed 3 times using 8 technical replicates each time. The adherence of TX6128 and TX6155 to Fn was expressed as the percentage of binding relative to the binding of *Enterococcus faecalis* OG1RF (defined as 100%).

Expression and purification of Fnm and its truncated derivatives. DNA regions corresponding to full-length Fnm or to its truncated derivative $Fnm_{1–295}$, $Fnm_{1–414}$, or $Fnm_{416–535}$ were amplified from TX82 genomic DNA using specific primers (listed in Table S1 in the supplemental material), which introduced NdeI and BamHI restriction sites. The fragments were cloned in frame into plasmid pET19b, and the overexpression constructs were transformed into *E. coli* BL21(DE3). Cultures were grown to exponential phase, and protein expression was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. Histidine-tagged recombinant Fnm (rFnm) and its derivatives were purified by Ni-nitrilotriacetic acid (NTA) chromatography using His GraviTrap columns (GE Healthcare) according to the manufacturer's instructions. The eluted proteins were desalted using PD-10 desalting columns (GE Healthcare). The protein concentration of each sample was determined by the bicinchoninic acid (BCA) method (Pierce). Recombinant proteins were stored at −70°C until use.

Generation of polyclonal antibodies and purification of antigen-specific IgGs. Polyclonal rabbit antibodies against rFnm were raised at Bethyl Laboratories (Montgomery, TX, USA), and rFnm-specific antibodies were purified using CnBr-Sepharose 4B coupled with rFnm as described elsewhere (32). Eluted antibodies were neutralized immediately using 0.1 M Tris (pH 8.0) and were dialyzed extensively against PBS, and concentrations were determined using an IgG molar absorption coefficient value of 210,000 M^{-1} cm^{-1} and a molecular mass of 150,000 Da.

Enzyme-linked immunosorbent assay (ELISA). The binding of rFnm to ECM proteins was measured as described previously (33). Medium binding Immulon 2B microwell plates (Thermo Scientific, Woburn, MA) were coated with ECM proteins (1 μg/100 μl) dissolved in 50 mM carbonate buffer and were incubated overnight at 4°C. Wells were blocked with 2% BSA at RT for 1 h and were then washed with PBS, followed by the addition of various concentrations of recombinant proteins. After incubation for 2 h at RT, plates were washed three times with PBS-T (PBS with 0.05% Tween 20), and the binding of full-length rFnm and truncated versions to ECM was detected by incubation with anti-His monoclonal antibodies (GE Healthcare) and alkaline phosphatase-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories); *p*-nitrophenyl phosphate (Sigma) was used for signal detection. The absorbance at 405 nm was then determined with a microplate reader (Thermo Scientific, Waltham, MA).

Extraction of CWA proteins with mutanolysin and immunoblot analysis. *E. faecium* strains were grown for 5 h or 16 h in BHI or BHIS broth, and cell wall-anchored (CWA) proteins were extracted with mutanolysin as described previously (34). After measurement of the protein concentrations by the BCA method (Pierce), equal amounts of mutanolysin-extracted proteins were resolved using SDS-PAGE gels under reducing conditions, and the proteins were transferred to nitrocellulose membranes. After blocking with 2% skim milk in PBS for 1 h, the membrane was probed with affinity-purified anti-rFnm antibodies, followed by horseradish peroxidase (HRP)-conjugated anti-goat IgG antibodies, and the signal was detected by using the SuperSignal West Pico chemiluminescent reagent (Thermo Scientific).

Experimental endocarditis and urinary tract infection (UTI) models. The animal experimental procedures were carried out in accordance with the institutional policies stipulated by the Animal Welfare Committee, University of Texas Health Science Center at Houston. Aortic valve endocarditis was induced in white Sprague-Dawley rats according to a method published previously (35). Briefly, catheterized rats were inoculated intravenously, via the tail vein, with a mixture of TX82 and TX6128 (at a ~1:1 ratio, determined by absorbance at 600 nm) 24 h after catheter placement. The inocula were then serially diluted and were plated to determine the actual CFU and percentage of each strain. At 48 h postinfection, animals were euthanized, hearts were aseptically removed, and the aortic valves were excised, weighed, and homogenized in 1 ml of saline solution. Serial dilutions were plated onto Enterococcosel Agar (EA) (Difco Laboratories) supplemented with 6 μg/ml vancomycin. Colonies were randomly picked into wells of 96-well microtiter plates containing BHI broth, grown overnight, replica plated onto BHI agar, and transferred to a filter overlaid on the BHI plate. The colonies were then lysed *in situ*, and the filters were hybridized under high-stringency conditions (36), using intragenic DNA probes of *fnm* and *ddl* (37) to calculate the percentages of wild-type and mutant colonies recovered from aortic valves.

For UTI infection experiments, 4- to 6-week-old female ICR mice (Harlan Laboratories) were used. The experiments were conducted according to the methodology previously adopted by Singh et al. (38).

Statistical analysis. Statistical comparisons were performed with a paired Student *t* test by using GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA). Differences were considered significant at a P value of ≤ 0.05.

RESULTS

Genetic organization of *E. faecium* TX82 *fnm*. Using the *S. pneumoniae* PavA sequence as the query for tBLASTn searches, we identified a homologous gene in the genome of *E. faecium* TX82. This gene was also found in all currently available *E. faecium* genome sequences (data not shown). Figure 1 depicts the genomic organization of the *pavA* homologue in TX82 (HMPREF9522_00874). It is 1,707 bp long and is flanked upstream by genes encoding a putative transposase and ABC transporters and downstream by a gene coding for a LysR-like transcriptional regulator. The putative protein consists of 568 amino acids (aa) and contains an N-terminal region identified by InterPro (http://www.ebi.ac.uk/interpro/) as a homologue of the N terminus (PF05833; aa 4 to

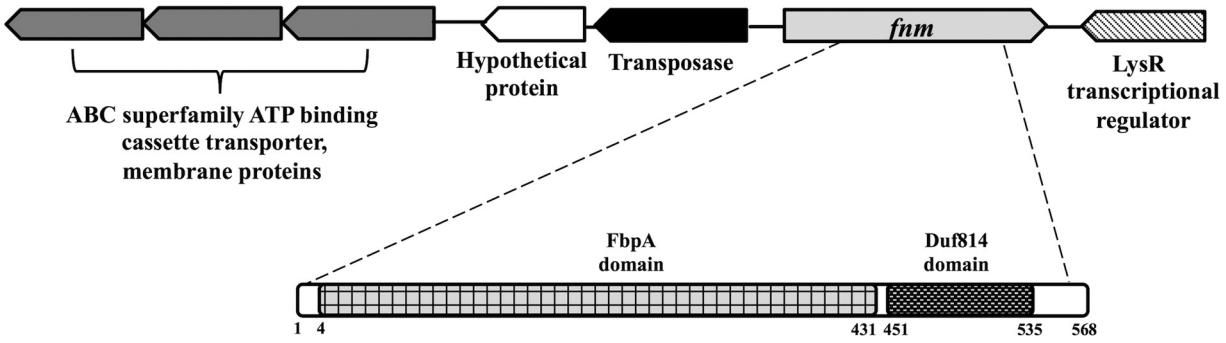

FIG 1 Diagram of the genetic organization and domain mapping of *Enterococcus faecium* TX82 Fnm. The *fnm* locus is flanked upstream by transposase- and ABC transporter-encoding genes and downstream by a gene coding for a LysR-like transcriptional regulator. The expanded segment shows the domain organization of Fnm. The annotated FbpA homologue domain spans the region from amino acid 4 to 431; the Duf814 domain (a domain of unknown function; aa 451 to 535) is commonly found in association with the FbpA domain.

431) of *S. gordonii* fibronectin-binding protein A (FbpA), followed by a domain of unknown function (Duf814; aa 451 to 535) (Fig. 1, expanded segment). Based on the predicted function, we designated this protein Fnm (fibronectin-binding protein of *Enterococcus faecium*). As with other reported PavA-like proteins, Fnm lacks an N-terminal signal sequence for exporting the protein and the typical Gram-positive LPXTG cell wall anchor motif at the C terminus (14). This protein shares 52% identity and 70% similarity with *S. pneumoniae* PavA (Fig. 2). In addition, Fnm is 49 to 70% identical and 68 to 84% similar to *E. faecalis* EfbA (25), *S. pyogenes* FBP54 (39), *S. gordonii* FnBpA (20), group B streptococcus SfbA (24), and *S. mutans* SmFnB (23) (Fig. 2).

The recombinant *E. faecium* Fnm protein binds to Fn and other components of the ECM. To determine if Fnm has the ability to bind to Fn, we expressed full-length Fnm as a histidine-tagged fusion protein in *E. coli* and purified it by Ni-NTA chromatography. The recombinant Fnm showed concentration-dependent binding to immobilized Fn, and this binding appeared to be saturated at concentrations of added rFnm above 5 pM (Fig. 3A). Significant amounts of rFnm also bound to laminin and type V collagen, and intermediate levels of rFnm binding to wells coated with fibrinogen, collagen I, or heparan sulfate were seen (Fig. 3B). Little to no binding to transferrin, BSA, heparin, hyaluronic acid, mucin, or collagen IV was observed (Fig. 3B).

In order to investigate the functional domains of the protein required for Fn binding, truncated versions of rFnm (comprising aa 1 to 295, aa 1 to 414, or aa 416 to 535) were generated in *E. coli*, purified, and tested for their abilities to bind to Fn (Fig. 4A). The truncated proteins $rFnm_{1–295}$, containing part of the *in silico*-designated N-terminal Fn-binding domain, and $rFnm_{414–535}$, corre-

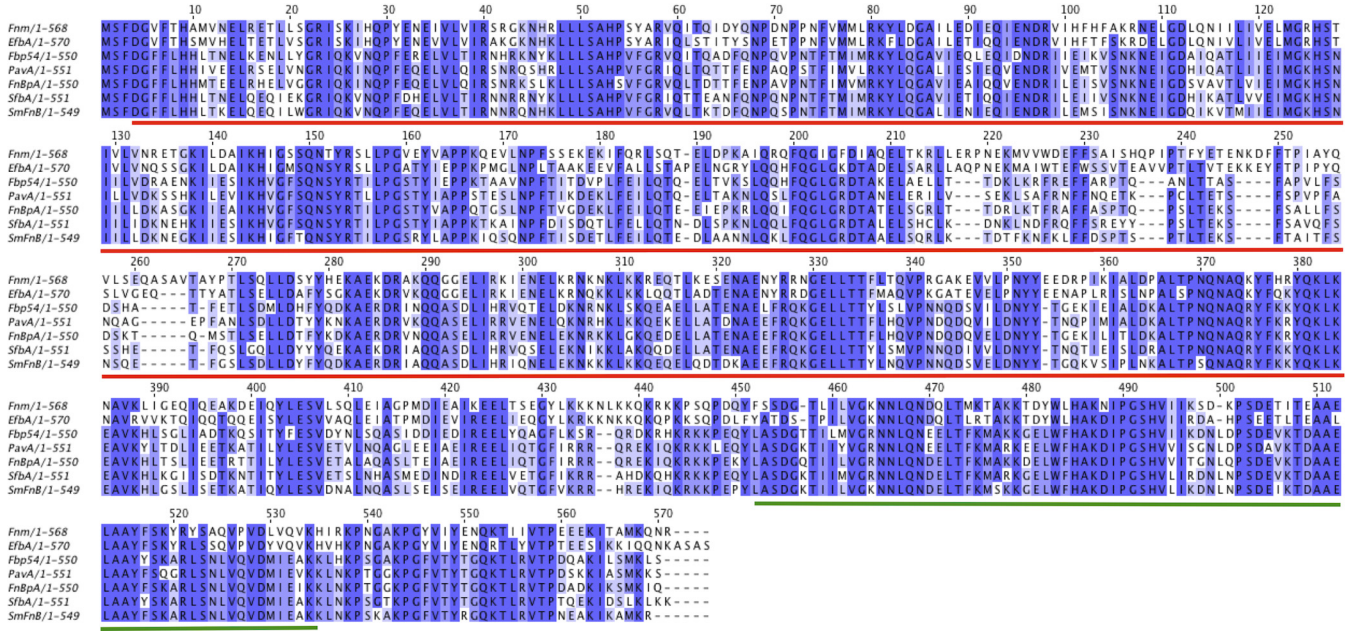

FIG 2 Alignment of the amino acid sequence of *E. faecium* TX82 Fnm with those of other reported PavA homologue proteins: *E. faecalis* EfbA, *S. pyogenes* FBP54, *S. pneumoniae* PavA, *S. gordonii* FnBpA, group B streptococcus SfbA, *S. mutans* SmFnB. Residue conservation is indicated by shades of blue. Highly conserved sequences are shown in dark blue; a low degree of consensus is indicated in light blue. The red and green lines under the residues indicate the FbpA and Duf814 domains, respectively. The alignment was generated using the MUSCLE program, by applying the default settings.

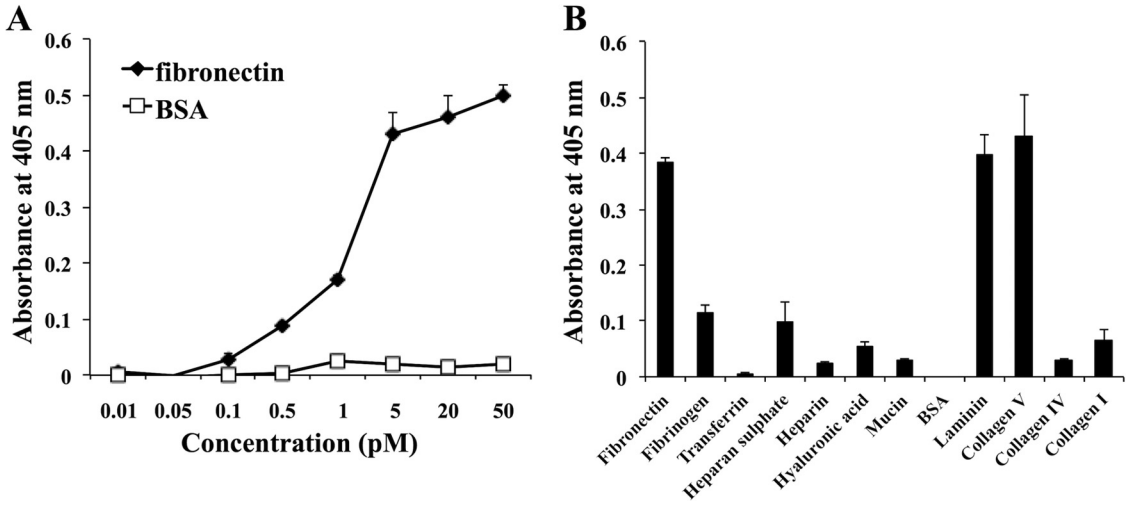

FIG 3 Binding of full-length rFnm to immobilized ECM components as detected by ELISA. (A) Concentration-dependent binding of rFnm to fibronectin. (B) Binding of rFnm to other immobilized ECM components. ELISA plates were coated with ECM proteins (1 μg/well), which were incubated with various concentrations of rFnm. The binding of rFnm to ECM proteins was detected using anti-His tag antibodies. Data represent means ± standard deviations.

sponding to the C-terminal Duf814 domain only, showed significantly less ($P < 0.05$) binding to Fn than did full-length rFnm (Fig. 4B), although some binding remained. However, a protein containing the extended N-terminal FbpA domain (aa 1 to 414) bound to Fn at levels similar to those of full-length rFnm. These data demonstrate that the N-terminal domain alone is sufficient for wild-type-level Fn binding. This finding differs from earlier reports on *S. pneumoniae* PavA, where the C-terminally truncated proteins showed no binding to Fn (14).

To confirm the role of Fnm in the binding of *E. faecium* TX82 cells to Fn, we constructed an in-frame markerless *fnm* mutant (TX6128) and a knocked-in (reconstituted) strain in which the gene was restored in its native site in the chromosome of the *fnm* deletion mutant (TX6155). TX6128 and TX6155 exhibited growth rates similar to that of TX82 in BHIS at 37°C (data not shown). In subsequent assays of whole-cell binding to immobilized Fn, the deletion mutant displayed a level of binding 29% lower ($P < 0.001$) than that of TX82 (Fig. 5). The remaining ability of the deletion mutant to bind Fn could be attributed to the redundancy of fibronectin-binding proteins in *E. faecium*, including the WxL domain-containing proteins SwpA-C, LwpA-C, and DufA-C (26). Reconstitution of *fnm* in TX6155 restored the wild-type phenotype.

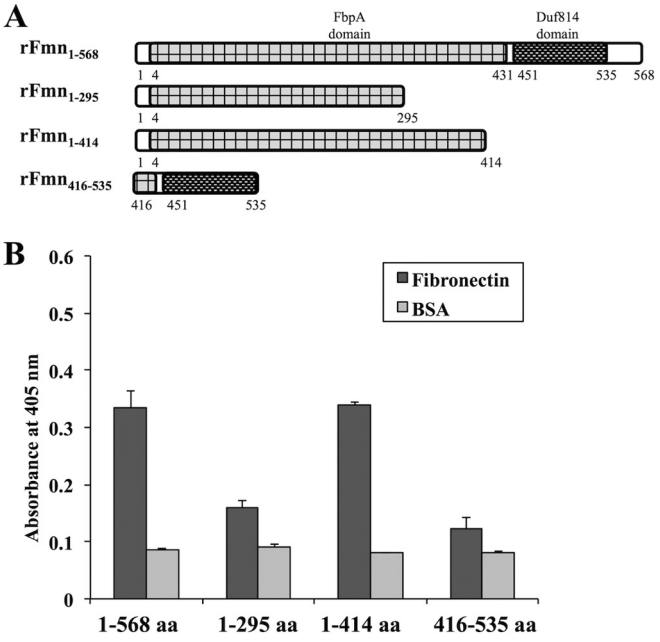

FIG 4 (A) Domain structures of truncated rFnm polypeptides. (B) Binding of truncated rFnm polypeptides to immobilized fibronectin as detected by ELISA. Equimolar concentrations of full-length rFnm or truncated versions of rFnm were tested for reactivity to immobilized fibronectin (1 μg/well).

Fnm is expressed on the surfaces of *Enterococcus faecium* cells. Next, we examined whether Fnm is expressed on the surfaces of *E. faecium* cells. For this purpose, we generated cell wall extracts by mutanolysin digestion of TX82, TX6128, and TX6155 cells. Since serum has been shown to act as a biological cue that elicits adherence to the extracellular matrix proteins of some other bacteria (40), *E. faecium* cells were grown in BHI broth in the presence or absence of horse serum at 37°C, and samples were collected at the exponential and stationary phases of growth. Immunoblotting using anti-Fnm specific antibodies showed that Fnm was found only in the cell wall extracts of the wild-type strain TX82 and the *fnm*-reconstituted strain TX6155; it was not detected in the cell wall extract of the deletion mutant TX6128 (Fig. 6). In addition, it was evident that Fnm expression was increased in the presence of 40% horse serum and that expression levels were higher in the stationary phase than in the exponential phase of growth (Fig. 6). No Fnm expression was detectable in the absence of serum, suggesting the possibility that stressful conditions may regulate the expression of Fnm protein *in vivo*.

Fnm contributes to the pathogenesis of infective endocarditis *in vivo*. Next, to determine whether a lack of Fnm translates into a reduced ability to cause infection, we used an established model of *E. faecium* endocarditis to test the *fnm* deletion mutant TX6128. For this purpose, catheterized rats were injected intrave-

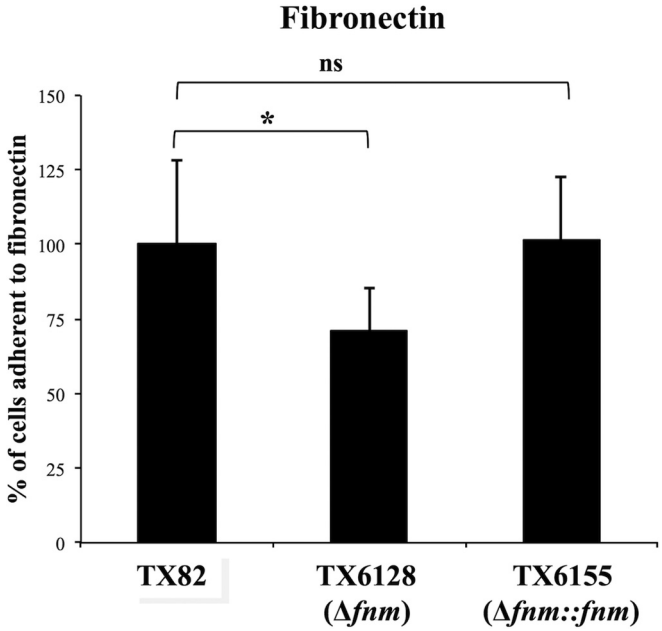

FIG 5 Binding of *E. faecium* TX82, TX6128, and TX6155 cells to fibronectin. Fibronectin (20 μg/well) was immobilized on a microtiter plate, and BHIS-grown *E. faecium* TX82, TX6128, and TX6155 cells were added to the wells and were allowed to adhere for 2 h. Adherent bacteria were detected by crystal violet staining. Experiments were performed three times each with eight technical replicates. Values are means ± standard deviations. *, $P < 0.05$; ns, not significant. The binding of TX6128 and TX6155 to fibronectin was expressed relative to the adherence ability of *E. faecium* TX82 (defined as 100%).

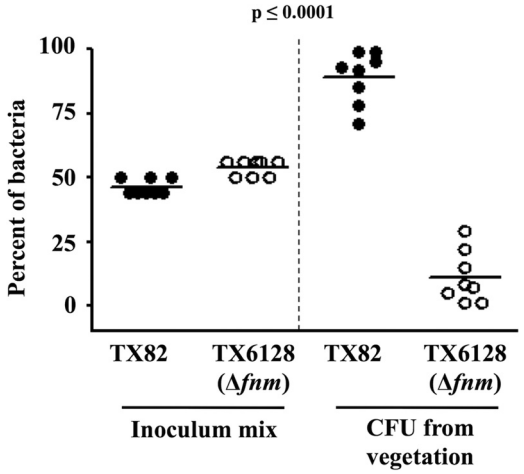

FIG 7 Attenuation of a nonpolar *fnm* deletion mutant in a mixed-inoculum infection model of rat endocarditis. Rats were sacrificed at 48 h postinfection, and bacteria were recovered from vegetations. Horizontal lines indicate mean percentages ($P \leq 0.0001$ by a paired *t* test) of bacteria in the aortic valves or in the inoculum.

nously, through the tail vein, with a mixture of TX82 and TX6128 as an inoculum. CFU determination showed that the mixed inoculum administered consisted of 47% wild-type cells (2.9×10^9 CFU) and 53% (3.4×10^9 CFU) *fnm* deletion mutant cells. As shown in Fig. 7, TX6128 was significantly attenuated relative to the wild-type strain, TX82 (P, ≤0.0001 by a paired Student *t* test). This suggests a clear advantage of TX82 over the *fnm* deletion mutant and indicates a role for Fnm in the pathogenesis of infective endocarditis. With a mixed inoculum containing equal numbers of CFU of TX6128 and TX6155, chromosomal reintroduction of *fnm* resulted in the restoration of the infective phenotype at wild-type levels in 40% of the animals ($n = 5$) (data not shown).

Because the Fnm homologue protein of *E. faecalis* (EfbA) has been shown to contribute to UTIs in a murine model (25), we tested whether Fnm plays a role in the pathogenicity of UTIs caused by *E. faecium* TX82. Mice were inoculated via an intraurethral catheter with a mixture consisting of 53% TX82 cells and 47% TX6128 cells. However, as shown in Fig. S1 in the supplemental material, TX82 and TX6128 were recovered at similar rates from mouse kidneys ($P = 0.6544$) or bladders ($P = 0.0562$), indicating that TX6128 was not attenuated relative to the wild-type strain.

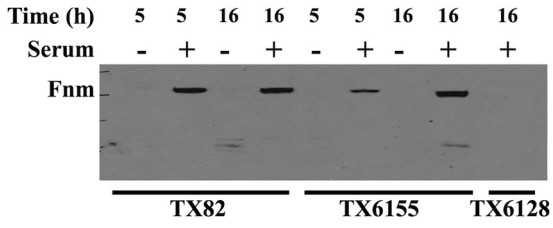

FIG 6 Western blot of cell wall-associated protein extracts. *E. faecium* strains were grown for 5 h (log phase) or 16 h (stationary phase) in BHI broth with or without 40% horse serum. CWA proteins were extracted by mutanolysin digestion and were immunoblotted with antibody against the rFnm protein. Only the sample collected from cells grown in BHIS for 16 h is shown for the deletion mutant TX6128, because all others were negative.

DISCUSSION

The process of bacterial infection is initiated by the interaction of microorganisms with components of host tissues. Previous studies have shown that *E. faecium* cells express a variety of surface proteins that can contribute to adhesion (41). These include an endocarditis- and biofilm-associated pilus protein (Ebp_{fm}) (42), an enterococcal surface protein (Esp) (43), serine–glutamate repeat-containing protein A (SgrA) (44), and the WxL proteins (26). In addition, we identified a significant association between collagen adherence and the presence of a functional *acm* gene, encoding an adhesin of collagen, in clinical but not community isolates (45, 46). Furthermore, Acm was found to be important in the pathogenesis of infective endocarditis (47). A report on the adherence of *E. faecium* to Fn demonstrated a greater ability of clinical isolates, especially endocarditis-derived nosocomial isolates, than of nonclinical isolates to bind to Fn (48). Also, in other Gram-positive bacteria, the presence of Fn-binding proteins on the surface was shown to play a critical role in the infection process (49). Thus, it is possible that interactions with Fn favor the initiation and establishment of *E. faecium* infections.

In the present study, homology-driven mining of the genome of *E. faecium* TX82 identified *fnm*, encoding a protein that exhibits considerable identity to the anchorless, but surface-exposed, fibronectin-binding PavA protein of *S. pneumoniae*. Like PavA, Fnm is an atypical Fn-binding protein in that it lacks a secretory sequence, identifiable Fn-binding repeats, and a conventional LPXTG anchoring motif (Fig. 1). Fnm shares high similarity with PavA and other homologous proteins found in streptococci and in *E. faecalis* (Fig. 2), suggesting a conserved function, which

might be different from its role as an adhesin. All the PavA homologues contain a large N-terminal domain annotated as an Fn-binding domain ("FbpA") followed by a C-terminal Duf814 domain. While Duf814 has yet to be characterized, it has often been reported in association with the FbpA domain, in archeal and eukaryotic as well as prokaryotic adhesins (11). Since all the PavA homologues lack conventional secretory and anchorage sequences, the existence of a novel, yet to be determined mechanism of secretion and cell surface association has been postulated (14). Bacterial cell surface association, despite the absence of a putative signal sequence and a cell wall anchor motif, has also been exhibited by other virulence-associated proteins, such as the streptococcal surface dehydrogenase, the surface enolase of *S. pyogenes*, and the pneumococcal α-enolase (50–52). It is also possible that this protein has another function, perhaps intracellular, and that the adherence phenotype is a "moonlighting" function, as suggested for Gnd (50–53).

In our work here, we found that full-length recombinant Fnm binds to Fn in a concentration-dependent manner and also binds to other host ECM proteins, such as laminin and collagen V (Fig. 3). Previous reports on *S. pneumoniae* PavA demonstrated that the C-terminal 189 residues were essential for the binding of protein to Fn (14). However, Courtney et al. showed that the N-terminal region of FBP54 is responsible for the majority of the binding of *S. pyogenes* to Fn (39). For *E. faecium* Fnm, we mapped the principal Fn-binding domain to the N-terminal half of the protein. However, there was modest binding of $Fnm_{416–535}$ to immobilized Fn, suggesting that the C-terminal Duf814 domain could mediate a reduced level of adherence (Fig. 4).

The ability of bacteria to sense and finely regulate the production of virulence factors is important in their transition from colonizers to pathogens and for avoidance of the host immune defense (54). Several enterococcal adhesins have been shown to be conditionally expressed in response to environmental stresses, including growth at 46°C or the presence of serum (34, 55). A study that examined the adherence phenotypes of diverse *E. faecalis* strains to ECM proteins after *in vitro* growth under conditions mimicking physiological conditions demonstrated that serum promotes enterococcal binding to Fn (40). In keeping with these data, we showed that Fnm is found in cell wall extracts of *E. faecium* TX82 grown in BHI medium supplemented with 40% horse serum but not in extracts of TX82 grown in BHI medium only (Fig. 6). These results suggest that Fnm is not constitutively expressed by *E. faecium* but is elicited under certain conditions, where serum may serve as a signal to induce the production of Fnm on the cell surface (40). Inactivation of *fnm* was associated with a significant reduction ($P < 0.001$) in the binding of *E. faecium* TX82 to fibronectin (Fig. 5), in accordance with the absence of this adhesin from the cell surface. It would be of interest to examine human sera for the presence of antibodies against Fnm in order to provide evidence of *in vivo* expression in humans.

Infective endocarditis, a disease that involves bacterial infection of heart valves and/or the inner surface of the heart chamber (endothelium), is often initiated by damage to the endothelium that disrupts the integrity of the aortic valves and exposes underlying tissues and ECM, including Fn (56). Enterococci may adhere directly to the site of damage or to sterile thrombotic vegetations, consisting of fibrin and platelets, leading to infected vegetations. Because enterococci are the third most common etiological cause of this disease (57), we hypothesized that Fnm could play a role in the formation of *E. faecium* vegetations on the aortic valves. Deletion of *fnm* from the genome of *E. faecium* TX82 resulted in significant attenuation of the ability of the isogenic mutant, TX6128, to compete with the wild type in the infection of catheter-induced vegetations, demonstrating that Fnm is an important factor in the experimental model of endocarditis caused by *E. faecium*. The reintroduction of *fnm* into its original chromosomal location resulted in a fully restored wild-type phenotype in 40% of the infected animals (data not shown); the genetic basis for the lack of restoration of Fnm function in all animals is under investigation in our laboratory. Interestingly, we have observed a similar "bimodal" pattern, with one or two animals showing the reverse of the results for the others, with several other enterococcal mutants tested previously (35, 47, 58).

In a recent investigation by Torelli et al., the Fnm homologue protein of *E. faecalis* JH2-2 (EfbA) was shown to contribute to pathogenesis in a murine model of ascending urinary tract infection, a finding consistent with an increased trophism for the kidneys and bladder (25). Interestingly, *fnm* deletion did not result in observable effects in the kidneys in a similar UTI model, suggesting that *E. faecium* Fnm does not contribute to this infection (see Fig. S1 in the supplemental material).

In summary, our study reports the identification and partial characterization of Fnm, an Fn-binding protein of *E. faecium* belonging to the PavA-like class of adhesins. We demonstrated that Fnm plays a key role in the binding of *E. faecium* to Fn and to a wide range of ECM molecules. Deletion of *fnm* diminished *in vitro* adherence to immobilized Fn and fibrinogen and resulted in significant attenuation of the ability of *E. faecium* TX82 to cause infective endocarditis *in vivo* in a rat model. Taking these findings together, our report contributes to the understanding of the fundamentals of interaction between *E. faecium* and its host.

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