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

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ABSTRACT

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*), homologue of the *Streptococcus pneumoniae pavA*, in the genome of the 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, as truncation of this region caused a 2.4-fold decrease ($p<0.05$) in *E. faecium* TX82 adhesion to fibronectin. Deletion of *fnm* resulted in a significant reduction ($p<0.001$) in the ability of the mutant TX6128 to bind fibronectin compared to the wild-type strain; reconstitution *in situ* of *fnm* in the deletion mutant strain restored adherence. In addition, the Δ*fnm* mutant was highly attenuated *vs* TX82 ($p\leq0.0001$) in a mixed inoculum rat endocarditis model. Taken together, these results demonstrate that Fnm affects *E. faecium* fibronectin adherence and is important in the pathogenesis of experimental endocarditis.

INTRODUCTION

Bacterial adherence to host tissues and extracellular matrix (ECM) proteins is a critical step in the process of infection as 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 the host-microbe interactions (2). Host ligands include ECM components e.g., fibronectin, collagen and laminin as well as molecules also present in blood including fibrinogen and vitronectin (2).

Generally reported as a well-adapted commensal of the gastrointestinal tract of humans and animals, *E. faecium* has emerged over the last three decades as one of the leading cause of hospital-associated diseases including urinary tract infections, bacteremia, intra-abdominal infections and endocarditis (3-5). The rising incidence of multi-antibiotic resistant nosocomial infections caused by *E. faecium* has led to the inclusion of these organisms in the list of "no ESKAPE" (*E. faecium*, *S. 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 healthcare-associated *E. faecium* are vancomycin resistant (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 contributes to *E. faecium* pathogenesis.

Fibronectin (Fn) is a large multi-domain dimeric glycoprotein found in body fluids and in the ECM (7); targeting of Fn by several pathogens was 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 the reported streptococcal and staphylococcal Fn-binding proteins are characterized by the presence of an N-terminal signal sequence, which is needed for exporting 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 (35-40 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 for prototypic Fn-binding proteins (15, 16). Nonetheless, PavA was shown to be present on the surface of *S. pneumoniae* and to exhibit binding to immobilized Fn (14). Notably, isogenic *pavA* deletion mutants were highly (approximately 10^4 fold) attenuated in virulence in a mouse sepsis model, suggesting a direct role for PavA in pneumococcal pathogenesis (14). Attenuation 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 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 systemic spread of pneumococci (19).

PavA homologues have been identified in other streptococci including the *Streptococcus gordonii* FbpA (20), the *Streptococcus pyogenes* Fbp54 (21, 22) and the *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 of *Enterococcus faecalis*, and demonstrated that the derived recombinant protein binds to immobilized Fn and plays a role in the pathogenesis of urinary tract infections (UTIs) (25).

The present work was initiated to identify and study the PavA homologue of *E. faecium* and its contribution to Fn adherence and in the context of infection. Here, we show that Fnm, encoded by the *E. faecium* homologue of *S. pneumoniae pavA*, is a 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 demonstrated that Fnm is important in the pathogenesis of experimental endocarditis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains and plasmids used in this study are listed in Table 1. Enterococci were routinely grown at 37°C in Brain Heart Infusion (BHI) (Difco Laboratories) broth and agar, or in M17 broth (Difco Laboratories), unless otherwise indicated. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) (Difco Laboratories) broth and agar. 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 ampicillin 100 µg/ml and gentamicin 25 µg/ml.

DNA techniques. *E. faecium* genomic DNA was isolated from a single colony after overnight growth in BHI broth, as previously described (26) . Plasmids were isolated from *E. coli* using the 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 (Table S1). DNA fragments were purified by agarose gel

electrophoresis and the Wizard SV Gel and PCR clean-up system columns (Promega Corporation). Restriction endonucleases (New England BioLabs, United Kingdom) and T4 DNA ligase (New England BioLabs, United Kingdom) were used according to the manufacturer's recommendations. The DNA sequencing service was provided by Genewiz Inc, NJ, USA.

Construction of an *fnm* deletion mutant and its reconstitution. Mutants were generated using pHOU1, a *pheS**-based counterselection system, as previously described (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 PCR reactions using the primers pairs fnmUpF/fnmUpR and fnmDownF/fnmDownR (Table S1), respectively. The two fragments were then fused together by Splicing by Overlap Extension (SOE)-PCR. The resulting 1722 bp product was digested with the restriction enzymes BamHI and SphI and 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 primers FbpOutF/FbpOutR (Table S1). pTX6128 was electroporated into *E. faecalis* CK111 using standard procedures (29, 30) before transferring to *E. faecium* TX82 by filter matings. The mating mixture was cultivated on BHI plates containing gentamicin and erythromycin to detect single cross-over integrants and then replated onto MM9YEG media containing *p*-chloro-phenylalanine (*p*-Cl-Phe) (7mM) to select for vector excision. Excision of pTX6128 was confirmed by absence of growth on BHI-gentamicin plates; colonies lacking *fnm* were detected by PCR and one of these was designated TX6128.

For restoration of *fnm* in TX6128, the *fnm* gene with its native promoter was amplified by PCR with primers fnmUpF/ fnmDownR (Table S1). Following digestion with BamHI and SphI, the resulting 3180 bp fragment was cloned into pHOU1, giving pTX6155 which, after confirming the insert by sequencing, was electroporated into *E. faecalis* CK111, transferred to *E. faecium* TX6128 and then processed as above. The resulting *fnm* reconstituted strain was named TX6155.

Deletion and restoration of the *fnm* gene in TX6128 and TX6155 were confirmed by sequencing the PCR product amplified using primers FbpOutF/FbpOutR. In addition, the strain identities were confirmed by pulsed-field gel electrophoresis according to a previously described method (31).

Whole cell fibronectin binding assays. *E. faecium* TX82, TX6128 and TX6155 cells were harvested by centrifugation from exponential phase cultures in BHI supplemented with 40% sera (BHIS) and the concentration adjusted to an 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 washing three times with PBS, a volume of 100 µl of cell suspension was added. Plates were incubated for 2 h at RT. After 3 washes with PBS to remove unbound cells, cells were fixed with Bouin's fixation solution (Sigma-Aldrich Co., St. Louis, MO) for 30 min at RT. Each well was then washed with PBS and stained with 1% (weight/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 percent of binding relative to OG1RF (defined as 100%).

Expression and purification of Fnm and its truncated derivatives. DNA regions corresponding to full length Fnm or to the truncated derivatives Fnm_{1-295}, Fnm_{1-414}, and $Fnm_{416-535}$ were amplified from TX82 genomic DNA using specific primers (listed in Supplementary Table 1), which introduced NdeI and BamHI restriction sites. The fragments were cloned in-frame into the plasmid pET19b and the overexpression constructs transformed into *E.coli* BL21(DE3). Cultures were grown to exponential phase and protein expression induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. Purification of histidine-tagged recombinant Fnm (rFnm) and its derivatives by Ni-NTA chromatography was carried out using His GraviTrap columns (GE Healthcare), following 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 BCA method (Pierce). Recombinant proteins were stored at -70°C until used.

Generation of polyclonal antibodies and purification of antigen specific IgGs. Polyclonal rabbit antibodies against rFnm were raised at Bethyl Laboratories (Montgomery, USA), and rFnm specific antibodies were purified using CnBr-sepharose 4B coupled with rFnm as described elsewhere (32). Eluted antibodies were neutralized immediately using 0.1M TRIS (pH 8.0), dialyzed extensively against PBS, and concentrations were determined using an IgG molar absorption coefficient value of 210000 M^{-1} C^{-1} and a molecular mass of 150000 Da.

Enzyme-linked immunosorbent assay (ELISA). Binding of rFnm to ECM proteins was measured as described previously (33). Medium binding Immulon 2B microwell plates (Thermo

171 Scientific, Woburn, MA) were coated with ECM proteins (1µg/100µl) dissolved in 50mM
172 carbonate buffer and incubated overnight at 4°C. Wells were blocked with 2% BSA at RT for 1h,
173 washed with PBS, followed by the addition of various concentrations of recombinant proteins.
174 After incubation for 2 h at RT, plates were washed three times with PBS-T (PBS with 0.05%
175 Tween-20) and binding of full length rFnm and truncated versions to ECM was detected by
176 incubation with anti–His monoclonal antibodies (GE Healthcare) and alkaline phosphatase-
177 conjugated anti-mouse IgG antibodies (Jackson Immunoresearch Laboratories); *p*-nitrophenyl
178 phosphate (Sigma) was used for signal detection. The absorbance at 405 nm was then determined
179 with a microplate reader (Thermo Scientific, Waltham MA).

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

189 **Experimental endocarditis and urinary tract infection models.** The animal experimental
190 procedures were carried out in accordance with the institutional policies stipulated by the Animal
191 Welfare Committee, University of Texas Health Science Center at Houston. Aortic valve
192 endocarditis was induced in white Sprague-Dawley rats according to a previously published
193 method (35). Briefly, catheterized rats were inoculated with a mixture of TX82 and TX6128

(approximately 1:1 ratio, determined by absorbance at 600 nm) intravenously, via the tail vein, 24 h after the catheter placement. The inocula were then serially diluted and plated to determine the actual colony forming units (CFUs) and percentage of each strain. At 48 h post-infection, 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-microtiters plates containing BHI broth, grown overnight, replica plated onto BHI agar and transferred onto 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 percentage of wild type and mutant colonies recovered from aortic valves.

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

Statistical analysis. Statistical comparisons were performed by paired student *t* test using the Graph Pad Prism version 4.00 for Windows (GraphPad Software, San Diego,CA). Differences were considered significant at a $p \leq 0.05$.

RESULTS

Genetic organization of *fnm* of *E. faecium* TX82. 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). Fig.1 depicts the genomic organization of the *pavA* homologue in TX82 (HMPREF9522_00874). It is 1707 bp in length, 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 and it contains an N-terminal region designated by InterPro (http://www.ebi.ac.uk/interpro/) as a homologue of the fibronectin-binding protein A N-terminus (PF05833; 4-431 aa) of *S. gordonii* (FbpA) followed by a domain of unknown function (Duf814; 451-535 aa) (Fig.1, expanded segment). Based on the predicted function, we designated this protein as 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 anchorage motif at the C-terminal (14). This protein shares 52% identity and 70% similarity to the *S. pneumoniae* PavA (Figure 2). In addition, Fnm is 49-70% identical and 68-84% similar to the *E. faecalis* EfbA (25), *S. pyogenes* Fbp54 (39), *S. gordonii* FnBpA (20), Group B streptococci SfbA (24) and the *S. mutans* SmFnB (23) (Figure 2).

Recombinant Fnm protein of *E. faecium* 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 (Figure 3A). Significant amounts of rFnm also bound to laminin and type V collagen and intermediate levels of rFnm binding were seen to wells coated with fibrinogen, collagen I or heparan sulphate (Figure 3B). Little to no binding was observed to transferrin, BSA, heparin, hyaluronic acid, mucin or collagen IV (Figure 3B).

238 In order to investigate the functional domains of the protein required for Fn binding, truncated
239 versions of rFnm (1-295 aa, 1-414 aa, 414-568 aa) were generated in *E. coli*, purified and tested
240 for their ability to bind to Fn (Figure 4A). The truncated proteins $rFnm_{1-295,}$ containing part of the
241 *in silico* designated N-terminal Fn-binding domain, and $rFnm_{414-535}$, corresponding to only the C-
242 terminal Duf814 domain, showed significantly less ($p<0.05$) binding to Fn compared to full-
243 length rFnm (Figure 4B), albeit some binding remained. However, a protein containing the
244 extended N-terminal FbpA domain (aa 1-414) bound to Fn at similar levels compared to the full-
245 length rFnm. These data demonstrate that the N-terminal domain alone is sufficient for wild-
246 type level Fn -binding. The finding differs from earlier reports on PavA of *S. pneumoniae*, where
247 the C-terminus truncated proteins did not show any binding to Fn (14).

248 To confirm the role of Fnm in Fn-binding of *E. faecium* TX82 cells, we constructed an in-frame
249 markerless *fnm* mutant (TX6128) and a knocked-in (reconstituted) strain in which the gene was
250 restored in its native site into the chromosome of the *fnm* deletion mutant (TX6155). Compared
251 to TX82, TX6128 and TX6155 exhibited similar growth rates in BHIS at 37°C (data not shown).
252 Subsequent whole cell binding to immobilized Fn showed that the deletion mutant displayed
253 29% reduced binding ($p<0.001$) compared to TX82 (Figure 5A). The remaining ability of the
254 deletion mutant to bind Fn could be attributed to redundancy of fibronectin-binding proteins in
255 *E. faecium*, including the WxL domain-containing proteins SwpA-C, LwpA-C andDufA-C (26).
256 Reconstitution of *fnm* in TX6155 restored the wild type phenotype.

257 **Fnm is expressed on the surface of *Enterococcus faecium*.** Next, we examined whether Fnm is
258 expressed on the surface of *E. faecium*. For this, we generated cell wall extracts by mutanolysin
259 digestion from TX82, TX6128 and TX6155 cells. As serum has been shown to act as a biological
260 cue that elicits adherence to 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 from exponential phase and stationary phase of growth. Immunoblotting results using anti-Fnm specific antibodies showed that Fnm was found only in the cell wall extract of the wild-type strain TX82 and the *fnm* reconstituted strain TX6155 while it was not detected in the cell wall extract of the deletion mutant TX6128 (Figure 6). In addition, it was evident that Fnm expression was increased in the presence of 40% horse serum and expression levels were higher in the stationary phase compared to exponential phase of growth (Figure 6). There was no detectable level of Fnm expression in the absence of serum, which suggests 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 the 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, catheterized rats were injected intravenously through the tail vein with an inoculum mixture of TX82 and TX6128. CFU determination showed that the administered inoculum mix consisted of 47% wild type cells (2.9 X 10^9 CFU) and 53% (3.4 X 10^9 CFU) *fnm* deletion mutant cells, respectively. As shown in Figure 7, TX6128 was significantly attenuated *vs*. the wild type TX82 ($p \leq 0.0001$ by paired student *t* test). This suggests a clear advantage of TX82 over the *fnm* deletion mutant and indicates a role of Fnm in the pathogenesis of infective endocarditis. With a mixed inoculum containing equal numbers of CFUs 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).

As the Fnm-homolog protein of *E. faecalis* (EfbA) has been shown to contribute to UTI in a murine model (25), we tested whether Fnm plays a role in the pathogenicity of UTI caused by *E.*

faecium TX82. Mice were inoculated via an intraurethral catheter with a mixture consisting of 53% of TX82 cells and 47% of TX6128 cells. However, as shown in Figure S1, both TX82 and TX6128 numbers were recovered at similar rates from mice kidneys (p=0. 6544) or bladders (p =0.0562), indicating the TX6128 was not attenuated *vs* the wild-type strain.

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 the endocarditis and biofilm associated pili Ebp_{fm} (42), the enterococcal surface protein Esp (43), the serine–glutamate repeat-containing protein A SgrA (44) and the WxL proteins (26) . In addition, we identified a significant association between collagen adherence and presence of a functional *acm* gene encoding an adhesin of collagen in clinical versus community isolates (45, 46). Furthermore, Acm was found to be important in the pathogenesis of infective endocarditis (47). A report on *E. faecium* adherence to Fn demonstrated an increased ability of clinical isolates, especially endocarditis-derived nosocomial isolates, to bind to Fn compared to nonclinical isolates (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 may 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

306 protein as it lacks a secretory sequence, identifiable Fn-binding repeats and a conventional
307 LPXTG anchoring motif (Figure 1). Fnm shares high similarity to PavA and other homologous
308 proteins found in streptococci and in *E. faecalis* (Figure 2), suggesting a conserved function,
309 which might be different from its role as an adhesin. They all contain a large N-terminal domain
310 annotated as a Fn-binding domain ("FbpA") followed by a C-terminal Duf814 domain. While the
311 Duf814 has yet to be characterized, it often has been reported in association with the FbpA
312 domain, including in archeal and eukaryotic adhesins (50). As all the PavA homologues lack
313 conventional secretory and anchorage sequences, the existence of a novel yet-to-be determined
314 mechanism of secretion and cell-surface association have been postulated (14). Bacterial cell
315 surface association, despite the absence of a putative signal sequence and a cell wall anchor
316 motif, has also been exhibited by other virulence-associated proteins such as the streptococcal
317 surface dehydrogenase, surface enolase of *S. pyogenes*, and the pneumococcal α-enolase (51-53).
318 It is also possible that this protein has another function, perhaps intracellular, with the adherence
319 phenotype being a "moonlighting" function, as suggested for Gnd (51-54).

320 In our work here, we found that full-length recombinant Fnm binds to Fn in a concentration
321 dependent manner and also binds to other host ECM proteins such as laminin and collagen V.
322 (Figure 3). Previous reports on the *S. pneumoniase* PavA demonstrated that the C-terminal 189
323 residues were essential for protein binding to Fn (14). However, Courtney et al. showed that the
324 N-terminal region of Fbp54 is responsible for the majority of the binding of *S. pyogenes* to Fn
325 (39). For Fnm of *E. faecium*, we mapped the principle Fn-binding domain to the N-terminal half
326 of the protein. However, there was modest binding of $Fnm_{416-535}$ to immobilized Fn, suggesting
327 that the C-terminal Duf814 domain could also mediate adherence, although reduced (Figure 4).

328 The ability of bacteria to sense and finely regulate the production of virulence factors is

329 important in their transition from being a colonizer to a pathogen and for avoidance of the host
330 immune defense (55). Several enterococcal adhesins have been shown to be conditionally
331 expressed in response to environmental stress, including growth at 46°C or presence of serum
332 (56, 57). A study that examined the adherence phenotype of diverse *E. faecalis* strains to ECM
333 proteins after *in vitro* growth under mimicking physiological conditions demonstrated that serum
334 promotes enterococcal binding to Fn (58). In keeping with these data, we showed that Fnm is
335 found in cell wall extracts of *E. faecium* TX82 grown in BHI supplemented with 40% horse
336 serum but not when grown in BHI only (Figure 6). These results suggest that Fnm is not
337 constitutively expressed by *E. faecium* but is elicited under certain conditions, where serum may
338 serve as a signal to induce the production of Fnm on the cell surface (40). Inactivation of *fnm*
339 was associated with a significant reduction ($p<0.001$) in the fibronectin binding of *E. faecium*
340 TX82 (Figure 5), consistent with the absence of this adhesin from the cell surface. It would be of
341 interest to examine human sera for the presence of antibodies against Fnm to provide evidence of *in vivo*
342 expression in man.
343 Infective endocarditis, a disease that involves bacterial infection of heart valves and/or the inner
344 surface of the heart chamber (endothelium), is often initiated by a damage of the endothelium
345 that disrupts the integrity of the aortic valves and exposes underlying tissues and ECM, including
346 Fn (59). Enterococci may adhere directly to the site of damage or to sterile thrombotic
347 vegetations, consisting of fibrin and platelets, leading to infected vegetations. As enterococci are
348 the third most common etiological cause of this disease (60), we hypothesized that Fnm could
349 play a role in the formation of *E. faecium* vegetations on the aortic valves. Deletion of *fnm* from
350 *E. faecium* TX82 genome resulted in significant attenuation in the ability of the isogenic mutant
351 TX6128 to compete with the wild-type in infection of catheter-induced vegetations, therefore
352 demonstrating that Fnm is an important factor in the experimental model of endocarditis caused

by *E. faecium*. Reintroduction of *fnm* in 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 currently under investigation in our laboratory. Interestingly, we have observed a similar "bimodal" pattern with one or two animals showing the reverse of the others with several other enterococcal mutants previously tested (35, 47, 61).

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

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 *E. faecium* binding to Fn and to a wide range of ECM molecules. Deletion of *fnm* diminished the *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. Taken together, our report contributes to the understanding of the fundamentals of interaction between *E. faecium* and their host.

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376

377 **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	(62)
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 counter selectable marker; Gen^R	(27)
pTX6128	pHOU1 derivative carrying a 1719 bp BamHI/SphI fragment for deletion of *fnm*	This study
pTX6155	Vector for reconstitution of *fnm* containing a 3180 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 the 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

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

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FIGURE LEGENDS

Figure 1. Diagrammatic representation of the genetic organization and domain mapping of Fnm of *Enterococcus faecium* TX82. 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 region spans from amino acid 4 to 431; the Duf814 domain (domain of unknown function; 451-535 aa) is commonly found in association with the FbpA domain.

Figure 2. Alignment of the *E. faecium* TX82 Fnm amino acid sequence with other reported PavA homologue proteins. EfbA, *E. faecalis*; Fbp54, *S. pyogenes;* PavA, *S. pneumoniae;* FnBpA, *S. gordonii;* SfbA, Group B Streptococci*;* SmFnB, *S. mutans* . Residues conservation is indicated by shades of blue. Highly conserved sequences are shown in dark blue; low consensus is indicated in light blue. The red and the green lines under the residues indicate the FbpA domain and the Duf814, respectively. The alignment was generated using the MUSCLE program, applying the default settings.

Figure 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. ECM proteins (1µg/well) were coated on to ELISA plates and incubated with various concentrations of rFnm. Binding of rFnm to ECM proteins was detected using anti-His tagged antibodies. Data points represent mean ± standard deviation.

Figure 4. (**A)** Domain structure of truncated rFnm polypeptides and (**B)** their binding to immobilized fibronectin as detected by ELISA. Equimolar concentrations of full length or truncated versions of rFnm were tested for reactivity to immobilized fibronectin (1µg/well).

596 **Figure 5.** Binding of of *E. faecium* TX82, TX6128 and TX6155 cells to fibronectin. Fibronectin
597 (20 µg/well) was immobilized on a microtiter plate, and BHIS-grown TX82, TX6128 and
598 TX6155 *E. faecium* cells were added to the wells and allowed to adhere for 2 h. Adherent
599 bacteria were detected by crystal violet staining. Experiments were performed three times each
600 with eight technical replicates; values represent means ± standard deviation. * $p<0.05$; ns, non
601 significant. Binding to fibronectin of TX6128 and TX6155 was expressed relative to the
602 adherence ability of *E. faecium* TX82 (defined as 100%).

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

608 **Figure 7.** Attenuation of a nonpolar *fnm* deletion mutant in a mixed inoculum infection model of
609 rat endocarditis. Rats were sacrificed at 48 hours post infection and bacteria recovered from
610 vegetations. Horizontal lines indicate means ($p\leq0.0001$ by paired *t* test) for percentages of
611 bacteria in the aortic valves *versus* percentages in the inoculum.