Enterococci are among the first microbial colonizers of the infant gastrointestinal tract and, in healthy adults, they are part of the commensal intestinal microbiota (1). However, since the middle to late 1970s, enterococci have emerged as opportunistic pathogens and major causes of health-care-associated infections, most notably urinary tract infections (UTIs) and bacteremia, second only to *Staphylococcus* spp. in the hospital setting (2–5). Among cases of enterococcal endocarditis, *Enterococcus faecalis* has been recognized as the most abundant species, accounting for more than 90% of isolates from this infection (2, 3, 6). Treatment of enterococcal endocarditis has been clinically challenging due to the emergence of strains resistant to commonly used antibiotics, i.e., aminoglycosides, although more recent use of ampicillin in combination with ceftriaxone seems to have alleviated this concern (7). The associated mortality remains high, with rates between 9 and 15% in Europe and the United States, respectively (8, 9).

Adherence to and colonization of host tissue components, such as platelets and the extracellular matrix (ECM), are abilities that facilitate the early steps toward the development of infective endocarditis. To date, a variety of factors involved in the pathogenesis of experimental *E. faecalis* aortic valve infection have been identified; these include MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins such as the adhesin to collagen, *Ace*, and the endocarditis and biofilm-associated pili, *Ebp* (10–12).

Fibronectin is a glycoprotein consisting of two protein chains of approximately 250 kDa each, covalently linked by disulfide bonds, that is present in soluble forms in blood plasma and other body fluids. An insoluble, fibrillar form of fibronectin is present in the ECM and is known to support bacterial adherence (13). A recent study from Torelli et al. reported the presence of a fibronectin-binding protein in *E. faecalis* JH2-2, designated enterococcal fibronectin-binding protein A (*EfbA*) (14). Similar to the pneumococcal adherence and virulence factor A (*PavA*) of *Streptococcus pneumoniae*, *EfbA* displays an unconventional structure for adhesins in that it lacks an N-terminal signal sequence for targeting the protein to the Sec system and a C-terminal LPXTG cell wall anchorage motif (14–16). Homologous proteins lacking these motifs include Fbp54 of *Streptococcus pyogenes* (17), FbpA of *Streptococcus gordoni* (18), the *Streptococcus mutants* SmFnb (19), and the group B streptococcus SrbA (20), collectively referred to as PavA-like proteins, several of which were shown to be involved in bacterial adherence to fibronectin and/or to mediate virulence in experimental models (15–21).

*E. faecalis* JH2-2 *EfbA* consists of an N-terminal predicted domain involved in fibronectin binding (FbpA; amino acids 4 to 429), followed by a domain of unknown function (Duf814; amino acids 448 to 533) often observed in association with FbpA. *EfbA* was shown to be important in experimental urinary tract infection. Here, we expressed and purified the *E. faecalis* OG1RF *EfbA* and confirmed that this protein binds with high affinity to immobilized fibronectin, collagen I, and collagen V. We constructed an efbA deletion mutant and demonstrated that its virulence was significantly attenuated (*P* < 0.0006) versus the wild type in a mixed inoculum rat endocarditis model. Furthermore, efbA deletion resulted in diminished ability to bind fibronectin (*P* < 0.0001) and reduced biofilm (*P* < 0.0001). Reintroduction of efbA into the original chromosomal location restored virulence, adherence to fibronectin, and biofilm formation to wild-type levels. Finally, vaccination of rats with purified recombinant *EfbA* protein protected against OG1RF endocarditis (*P* = 0.008 versus control). Taken together, our results demonstrate that *EfbA* is an important factor involved in *E. faecalis* endocarditis and that rEfbA immunization is effective in preventing such infection, likely by interfering with bacterial adherence.
the role of EfbA in the pathogenesis of *E. faecalis* infective endocarditis has not previously been reported.

In the present study, we generated a nonpolar *efbA* deletion mutant of *E. faecalis* OG1RF, restored the gene in its original chromosomal location, and evaluated the ability of these derivatives to infect damaged heart valves in a rat model, adhere to immobilized fibronectin, and form biofilm. Finally, we provide evidence that EfbA is a protective antigen that could be used as a vaccine to combat *E. faecalis* experimental endocarditis.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in the present study are described in Table 1. Unless otherwise specified, *E. faecalis* were cultured at 37°C in brain heart infusion (BHI; Difco Laboratories) broth and agar or in BHI supplemented with 40% horse serum (BHIS) (Sigma, St. Louis, MO). *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth and agar (Difco Laboratories). The concentrations of the antibiotics used for selection were as follows: for *E. faecalis*, erythromycin (10 μg/ml), fusidic acid (25 μg/ml), and gentamicin (200 μg/ml); and for *E. coli*, ampicillin (100 μg/ml) and gentamicin (25 μg/ml). Enterococcosel agar (EA; Difco Laboratories) supplemented with rifampin at 100 μg/ml was used to recover bacteria from tissue homogenates.

**Construction of isogenic mutants.** *E. faecalis* OG1RF isogenic mutants were constructed using pHOU1, a vector that contains a mutated *pheS* allele conferring susceptibility to *p*-chloro-phenylalanine (p-Cl-Phe) (22). To generate an *efbA* deletion mutant, two fragments encompassing the upstream (507 bp) and downstream (522 bp) DNA region flanking *efbA* were amplified by PCR using the primer pair Up_efbF/Up_efbR and Down_efbF/Down_efbR (see Table S1 in the supplemental material), respectively. The fragments were subsequently fused together by overlap extension PCR and the product cloned into BamHI/PstI-digested pHOU1, giving pTX5707. After transformation into electrocompetent *E. faecalis* CK111 using standard procedures (23, 24), pTX5707 was transferred to *E. faecalis* OG1RF by filter mating. Single-crossover integration was confirmed by PCR amplified with the primers Up_efbF and Down_efbR (see Table S1 in the supplemental material). To distinguish between the parent and the complemented strain, a silent mutation (TTG) was introduced into the leucine codon TTA corresponding to amino acid position 79 using the primers SmutF and SmutR (see Table S1 in the supplemental material). Leucine is the most abundant amino acid in EfbA (60 of 570 amino acids), and codon usage was as follows: 25 TTA, 13 CTT, 9 CTA, 6 TTG, 5 TCG, and 2 CTG. After digestion with BamHI and PstI, the PCR product was cloned into pHOU1, giving pTX5708. The construct was then electrotransformed into *E. faecalis* CK111, transferred to TX5707 via conjugation and processed as described above. The resulting *efbA* complemented strain was named TX5708. Sequencing of PCR products amplified using primers EfbOutF and EfbOutR and pulsed-field gel electrophoresis (25) were performed to confirm the deletion and restoration of *efbA* in TX5707 and TX5708. In addition, excision of the pHOU1 derivatives was confirmed by absence of growth on BHI-gentamicin agar plates.

**Growth characteristics.** Growth of the OG1RF derivatives in BHIS or tryptic soy broth supplemented with 0.25% glucose (TSBG) was monitored as described elsewhere (11).

**Heterologous expression, purification of recombinant EfbA and binding to ECM proteins.** N-terminal 6×His-tagged recombinant EfbA was produced in *Escherichia coli* BL21(DE3) harboring the plasmid pET19b-EfbA. The construct was generated by amplifying the full-length *efbA* gene from OG1RF using the primer pair pET_efbF and pET_efbR after introduction into Ndel/BamHI-digested pET19b (see Table S1 in the supplemental material). BL21(DE3) cultures were grown at 37°C to the exponential phase, and protein overexpression was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactosidase). Purification of rEfbA by affinity chromatography was carried out using His GraviTrap columns (GE Healthcare) according to the protocol of the manufacturer. The columns were washed with washing buffer (20 mM sodium phosphate [pH 7.4], 500 mM NaCl, and 45 mM imidazole), and then the protein was eluted in elution buffer (20 mM sodium phosphate [pH 8.0], 500 mM NaCl, and 500 mM imidazole). The protein was desalted in 50 mM Tris-HCl buffer (pH 8.0) and 5% glycerol using PD-10 desalting columns (GE Healthcare).
Healthcare), and the concentration was determined by the BCA method (Pierce); rEfbA was stored at −70°C until used.

Concentration-dependent binding of rEfbA to fibronectin and adherence to various ECM proteins was assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (26). Briefly, Immulon 2B microwell plates (medium binding; Thermo Scientific, Woburn, MA) were coated overnight at 4°C with ECM proteins at 1 µg/well. The surfaces of the wells were blocked with 2% bovine serum albumin (BSA) at room temperature for 1 h and washed with phosphate-buffered saline (PBS [pH 7.4]), followed by the addition of different concentrations of rEfbA. After incubation for 2 h at room temperature, the plates were rinsed thrice with PBS-T (PBS with 0.05% Tween 20), and bound protein was detected by incubation with anti-His, monoclonal antibodies (GE Healthcare) and alkaline phosphatase-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories); signal was detected using p-nitrophenyl phosphate (Sigma). The absorbance at 405 nm was measured with a microplate reader (Thermo Scientific).

**Binding of *E. faecalis* to immobilized fibronectin.** Overnight cultures in BHIS of *E. faecalis* were collected by centrifugation and then adjusted to an optical density at 600 nm (OD600) of 1.0 in PBS. A portion (100 µL) of the bacterial suspension was inoculated into Immulon 2B microwell plates coated overnight with 20 µg of fibronectin or BSA (control)/ml, as previously described (27). After 2 h of incubation at room temperature, the plates were washed with PBS to remove unbound bacteria, and adherent cells were fixed with Bouin’s fixation solution (Sigma-Aldrich, St. Louis, MO). Each well was then rinsed with PBS and stained with 1% (wt/vol) crystal violet for 30 min at room temperature. Adherent cells were dissolved in an ethanol-acetone solution (80 and 20%, respectively), and the absorbance (OD570) was measured using a microplate reader (Thermo Scientific). To calculate the adherence of *E. faecalis* cells to fibronectin, the OG1RF value was set at 100% and binding of TX5707 and TX5708 was expressed as percentage of binding relative to OG1RF.

**Biofilm formation assay.** The biofilm assay was performed as described previously (11). *E. faecalis* strains were cultured overnight in TSBG at 37°C and diluted to an OD600 of 0.05 in TSBG. Portions (200 µL) of the bacterial suspension were inoculated into 96-well polystyrene microtiter plates (BD Biosciences), followed by incubation for 24 h at 37°C. Biofilm density was determined by crystal violet staining (11).

**Rat infective endocarditis model.** Animal experimental procedures were approved by the Animal Welfare Committee, University of Texas Health Science Center—Houston, and performed in accordance with institutional guidelines. A mixed infection competition assay was performed as we previously published (10). Briefly, groups of 7 to 11 catheterized Sprague-Dawley rats, weighing 200 to 300 g, were infected with a BHIS-grown mixture of TX5707 and either OG1RF or TX5708 (mixed in a 1:1 ratio, estimated by the OD600 and verified by CFU counts) via the tail vein, 24 h after catheter placement. To determine the CFU present in the vegetations, animals were euthanized at 48 h postinfection, hearts were aseptically removed, and the aortic valves containing vegetations were excised, weighed, and homogenized in saline solution. Serial dilutions were plated onto EA-plus-rifampin (100 µg/ml) plates. The ratio of TX5707 and OG1RF or TX5708 was determined by hybridizing colony lysates under high-stringency conditions with intragenic probes of efbA and ace to confirm *E. faecalis* (10).

**Antibody titers and in vivo protection against *E. faecalis* OG1RF infection in the rat endocarditis model.** The immunization protocol was adapted from our previous publication (10). For active immunization, a group of 10 Sprague-Dawley rats, weighing 200 to 300 g, received a dose of 100 µg/rat of rEfbA suspended in Freund complete adjuvant (FCA) at week 1, followed by another dose of 100 µg of rEfbA/rat in Freund incomplete adjuvant (FICA) at 3 and 5 weeks. The control group (n = 36) received FCA-FICA sham immunization at the same frequency. At week 7, rats were implanted with a catheter across the aortic valves. After 24 h, the animals were inoculated via the tail vein using BHIS-grown *E. faecalis* OG1RF (2.7 × 10⁸ CFU for the rEfbA immunized rats and 2 × 10⁸ CFU for the sham-immunized rats) and sacrificed 48 h later, and the aortic valves processed as described above for CFU. At 24 h prior to infection with *E. faecalis* OG1RF, the presence of anti-EfbA antibody titers in the sera from vaccinated and sham-immunized rats was assessed by ELISA according to a previously described protocol (28).

**Statistical analysis.** Statistical comparisons were performed by paired Student t test and by Mann–Whitney test using GraphPad Prism (v4.00 for Windows; GraphPad Software, San Diego, CA). The Fisher exact test was used to compare the numbers of infected versus noninfected animals in the active immunization experiments. Differences were considered significant at a P ≤ 0.05.

**RESULTS**

Characterization of EfbA in *E. faecalis* OG1RF. A previous study showed that EfbA is a PavA-like fibronectin-binding protein localized to the outer cell surface of *E. faecalis* strain JH2-2 and functions as a bacterial adhesin to immobilized fibronectin (14). *E. faecalis* OG1RF encodes a 570-amino-acid protein that is 99% identical to JH2-2 EfbA, with five nonconserved residues; three changes (at positions 231, 259, and 361) are in the predicted N-terminal domain and another (position 453) is in the Duf814 domain (see Fig. S1 in the supplemental material). To confirm the function of the predicted homologue protein in *E. faecalis* OG1RF, the full-length OG1RF EfbA was expressed as histidine-tagged fusion protein in *E. coli*, purified using Ni-NTA affinity chromatography (see Fig. S2 in the supplemental material), and tested for the ability to bind immobilized human fibronectin using a microtiter plate-based assay. rEfbA showed concentration-dependent binding to fibronectin and low affinity to BSA (Fig. 1). The binding was saturable at high concentrations of rEfbA with a calculated Kd value of 0.58 pM (Fig. 1). As seen in Fig. 2, we detected high affinity of rEfbA to collagen I and collagen V (Fig. 2). Little binding to laminin, mucin, heparin, hyaluronic acid, collagen IV, and fibrinogen was observed (Fig. 2).

**Importance of efbA in a mixed infection endocarditis model.** To test whether efbA contributes to *E. faecalis* ability to infect heart valves, we constructed a nonpolar efbA deletion mutant (TX5707) and a derivative strain in which the efbA gene was restored in its

![FIG 1 Binding of rEfbA to immobilized fibronectin detected by ELISA. Immuno 2B microwell plates coated with fibronectin at 1 µg/well were incubated with increasing concentrations of rEfbA. Binding of rEfbA to BSA or fibronectin was detected using anti-His, tagged antigens. The data points represent the means ± the standard deviations from three independent experiments each with eight technical replicates.](http://iai.asm.org/)
The growth kinetics of the deletion mutant and the restored \( efbA \) strain were similar to that of the parental strain OG1RF in broth cultures (BHIS and TSBG) (see Fig. S3 in the supplemental material). As shown in Fig. 3A, the mean percentages of total CFU from rats inoculated with an equal suspension of BHIS-grown OG1RF and TX5707 were 75% versus 25% (see Fig. S4 in the supplemental material), respectively, at 48 h postinfection, therefore showing a clear advantage of the parental strain compared to the \( efbA \) deletion mutant (\( P < 0.0006 \)). In a similar experiment, we used a bacterial suspension that contained 59% TX5707 and 41% TX5708 and, again, significantly higher numbers of the EfbA-expressing strain (TX5708) were recovered than the EfbA-deficient mutant (Fig. 3B and see Fig. S5 in the supplemental material) (\( P < 0.0086 \)), confirming that \( efbA \) contributes to this endovascular infection.

**Role of EfbA in fibrinogen binding and biofilm formation.** In order to further elucidate whether the effect of \( efbA \) deletion on \( E. \ faecalis \) pathogenicity might be via its ability to bind to fibrinogen, we examined whole-cell binding of wild-type OG1RF and the \( efbA \) deletion mutant to immobilized fibrinogen. As shown in Fig. 4A, deletion of \( efbA \) caused a 44% decrease in fibrinogen adherence compared to the wild type (\( P < 0.0001 \)). As expected, the \( efbA \) reconstituted strain showed fibrinogen binding similar to that of OG1RF.

Next, we examined whether the attenuated virulence phenotype of the \( efbA \) deletion mutant correlated with biofilm formation. As seen in Fig. 4B, the density of the biofilm produced by TX5707 showed a modest but significant reduction (15%) compared to the wild type (\( P < 0.0001 \)). Restoration of the biofilm formation capacity was observed in the in situ \( efbA \)-reconstituted strain.

**In vivo protection against \( E. \ faecalis \) OG1RF rat endocarditis.** The data described above demonstrated that EfbA play an important role in the development of infective endocarditis by \( E. \ faecalis \). We therefore reasoned that vaccination with rEfbA might be protective against \( E. \ faecalis \) infection in the rat endocarditis model. After active immunization with three doses of 100 \( \mu \)g of rEfbA in FCA-FICA, we found that sera from vaccinated rats (\( n = 8 \)) contained high levels of anti-EfbA titers (1:12,800), whereas the sera from sham-immunized animals (\( n = 8 \)) were not reactive to EfbA (Fig. 5A), therefore demonstrating active production of anti-EfbA antibodies. Upon challenge with BHIS-grown OG1RF, 95% of the 36 sham-immunized animals developed \( E. \ faecalis \) endocarditis with a mean of 4.8 log\(_{10} \) CFU recovered from vegetations (Fig. 5B). Immunization with rEfbA was effective in reducing the number of infected animals to 60% (Fisher exact test; \( P = 0.015 \) versus sham immunized) and the bacterial burden recovered from vegetation to a mean of 2.1 log\(_{10} \) CFU (\( P = 0.008 \) versus sham immunized). These data therefore demonstrated that active immunization of rats with rEfbA protects from \( E. \ faecalis \) colonization of aortic valves.
DISCUSSION

Enterococci, particularly *E. faecalis* and, to a lesser extent, *E. faecium*, are the third most common pathogens isolated from native and prosthetic valve endocarditis worldwide (3). Enterococcal infection of the cardiac endothelium is believed to be a result of bacteremia with subsequent bacterial colonization of ECM components of the damaged heart tissues with subsequent formation of infected thrombotic vegetations (consisting of microbes, platelets, fibrin, and host cells) (29,30).

Fibronectin is an important component of the extracellular matrix (ECM) of the cardiac endothelium and is exposed when this tissue is injured. In addition, fibronectin binds to fibrin and platelets, thereby contributing to the thrombogenicity of surfaces (31). Adherence to fibronectin by Gram-positive bacteria has been shown to be an important virulence factor that facilitates host colonization and supports disease progression (32–34). This ability is mediated by fibronectin-binding MSCRAMMs exhibiting typical structural features of cell wall anchored proteins, as well as anchorless but surface-located adhesins and invasins (35, 36).

A previous report characterized EfbA, a PavA-like fibronectin-binding protein of *E. faecalis* JH2-2, and demonstrated that it plays a major role in *in vitro* adherence to ECM proteins and supports urinary tract infections in a murine model (14). However, to date, no studies have demonstrated the involvement of *E. faecalis* fibronectin-binding proteins in the pathogenesis of infective endocarditis. Thus, the above observations led us to undertake the examination of the potential role of EfbA in the development of *E. faecalis* endocarditis.

**FIG 4** Effect of efbA deletion on *E. faecalis* OG1RF adherence to fibronectin and biofilm formation. (A) Whole-cell binding of *E. faecalis* OG1RF, TX5707, and TX5708 to immobilized fibronectin. BHI-grown cells were adjusted to an OD600 of 1 before inoculation in wells coated with 20 μg of fibronectin. Bars represent the adherence percentage compared to the wild-type strain OG1RF (defined as 100%). The data are presented as means ± the standard deviations of at least three independent experiments. (B) Biofilm formation with overnight cultures of OG1RF, the deficient EfbA mutant strain TX5707, and the reconstituted derivative strain TX5708. Horizontal lines show the means of three independent experiments, each performed with eight technical replicates. Statistical analysis was performed using a Student t test.

**FIG 5** Experimental endocarditis model in rEfbA-immunized rats. Rats were immunized with a dose of 100 μg of EfbA, followed by two booster immunizations at weeks 3 and 5. (A) Antibody titers detected by ELISA in eight EfbA-immunized rats and in the pooled sera of eight sham-immunized animals prior to infection with *E. faecalis* OG1RF. Each line depicts the anti-EfbA titers for each antibody dilution tested. (B) Two weeks after the last vaccine administration, a catheter was placed across the aortic valves of the rats and left for 24 h prior to challenge with 2.7 × 10⁸ CFU of *E. faecalis* OG1RF. Catheterized sham-immunized rats were inoculated with 2 × 10⁸ CFU of *E. faecalis* OG1RF. Black triangles and circles represent OG1RF cells recovered from the vegetations of EfbA-immunized and sham-immunized rats, respectively. Horizontal lines indicate the geometric means. A value of 0 depicts an uninfected animal. The means of log₁₀ CFU from vegetations were compared by unpaired t test.
present study and investigate whether EfbA contributes to *E. faecalis* OG1RF endocarditis in rats with catheter-induced aortic vegetations.

First, we confirmed that EfbA in OG1RF is a fibronectin-binding protein by showing that it adhered to immobilized human fibronectin in a concentration-dependent manner. EfbA supported adherence to other host ECM proteins such as collagen I and collagen V and modest levels of binding to laminin, mucin, heparin, hyaluronic acid, collagen IV, and fibrinogen.

To assess the role of EfbA in the pathogenicity of experimental endocarditis, we used a mixed inoculum model in which OG1RF and TX5707 (OG1RFΔefbA) were inoculated intravenously at a ratio of approximately 1:1. Deletion of *efbA* resulted in significant attenuation in the ability of the isogenic mutant strain to outcompete the parental OG1RF. Restoration of *efbA* in TX5707 resulted in increased colonization of the heart valves back to OG1RF levels, therefore indicating that *efbA* is important in this endovascular infection. To the best of our knowledge, this is the first demonstration that a fibronectin-binding protein contributes to the pathogenesis of *E. faecalis* infective endocarditis. Although another role, including an intracellular function, for *efbA* or EfbA cannot be excluded, we suggest that the difference in virulence of TX5707 is likely due to a reduced ability of the mutant to adhere to fibronectin at the site of damage, since the *efbA* deletion had no effect on growth but resulted in lower adherence to immobilized fibronectin compared to the wild-type cells, and this ability was restored in the *in situ* complemented strain TX5708.

Together with attachment to and colonization of host surfaces, the capacity to form biofilm is considered important for enterococcal infections [37, 38]. Interestingly, a study that evaluated the origin of 163 *E. faecalis* isolates in relation to biofilm production showed that endocarditis isolates are associated with greater biofilm densities than nonendocarditis isolates [39]. Our results showed that the *efbA* deletion mutant had a significant reduction in its biofilm-forming capacity, therefore demonstrating that the EfbA fibronectin-binding protein contributes to the biofilm phenotype. Taken together, with the data on whole-cell binding to fibronectin, these results are consistent with the idea that the abrogation of EfbA from the cell surface of *E. faecalis* OG1RF causes reduced attachment/maintenance of adherence to aortic valves or to the sterile vegetation created by the catheter during infective endocarditis.

Emergence of multi-antibiotic-resistant enterococci causing endocarditis poses a clinical challenge [7, 40]. In this regard, there is increasing interest in the use of alternatives approaches to current antibiotic strategies such as the development of novel immunotherapies targeting proteins that are important for pathogenicity [41, 42]. MSCRAMMs, which are factors ubiquitously present in traditional and opportunistic Gram-positive pathogens, have been used as a target to develop vaccine candidates against *E. faecalis* [10, 43–45]. Three proteins have been identified thus far as potential targets against *E. faecalis* endocarditis. Singh et al. demonstrated that passive and active immunization against *Ace* diminished the bacterial counts recovered from aortic valve vegetations and protected against *E. faecalis* cardiac valve infection [10]. IgG Fab fragments against the aggregation substance AS were shown to confer partial protection against enterococcal endocarditis [45]. In addition, passive immunization using a monoclonal antibody raised against the major component of *E. faecalis* pili, EbC, significantly prevented the establishment of rat endocardi-
Panesso 18. 12. 18.
Kristich 17. 18. 12.
Kawabata 10. 17. 12.
Mu Holmes 27. 25. 25.


