The PavA-like Fibronectin-Binding Protein of Enterococcus faecalis, EfbA, Is Important for Virulence in a Mouse Model of Ascending Urinary Tract Infection

Riccardo Torelli, Pascale Serror, Francesca Bugli, Francesco Paroni Sterbini, Ada Rita Florio, Annarita Stringaro, Marisa Colone, Elena De Carolis, Cecilia Martini, Jean-Christophe Giard, Maurizio Sanguinetti, and Brunella Posteraro

Enterococcus faecalis is an established nosocomial pathogen, yet the pathogenesis of enterococcal infections, particularly of urinary tract infections (UTIs), remains to be fully elucidated. Fibronectin-binding proteins have been identified as potent adhesins in pathogenic Gram-positive cocci. Here, we characterized EfbA, which is encoded by the enterococcal orthologue of Streptococcus pneumoniae pavA. Similar to PavA, the anchorless EfbA protein was localized to the enterococcal cell outer surface and bound to immobilized human fibronectin. In addition to abrogated EfbA expression, deletion of the efbA gene eliminated EfbA from the cell surface and drastically reduced the enterococcal cell binding to immobilized fibronectin. The ΔefbA deletion mutant was highly attenuated vs wild-type in a murine ascending UTI model, consistent with an increased tropism for the kidney relative to the bladder. These results provide the first evidence that EfbA of E. faecalis plays a role in UTIs, probably contributing to the pathogenesis in this site.

Enterococcus faecalis is an opportunistic pathogen that contributes to a large number of nosocomial infections worldwide [1]. In the United States, approximately 12% of hospital-acquired infections are caused by Enterococcus species [2], many of which are linked with resistance against multiple antibiotics [3], compromising effective therapy. Among hospitalized patients, E. faecalis is a common cause of urinary tract infections (UTIs) that most often lead to bacteremia, which is in turn associated with a high mortality rate [4].

Bacterial adherence is an important step in the process of disease that facilitates colonization of and translocation across the mucosal barrier, which eventually results in subcellular dissemination within the host [4]. Like other Gram-positive bacteria (e.g., Staphylococcus aureus), E. faecalis is loaded with surface-exposed adhesins that mediate binding to human receptors or to various components of the extracellular matrix (ECM) and thus are called adhesins of the microbial surface component–recognizing adhesive matrix molecules (MSCRAMM) type [5]. These are cell wall–anchored surface proteins that have characteristic immunoglobulinlike folds [6].

In general, the function of E. faecalis cell surface structures has been poorly investigated, even with respect to UTIs [7–9]. In an ascending UTI model, the presence of enterococcal surface protein, encoded by an acquired gene, was shown to increase the persistence of bacteria in the urinary bladders of mice [7], whereas Kau et al [9] demonstrated that E. faecalis has
greater tropism for the kidneys. Using a similar model, the MSCRAMM adhesion of collagen of \textit{E. faecalis} (Ace) was recently identified as a putative virulence factor involved in colonization of the renal tissue [10].

In Gram-positive bacteria, adhesive properties have also been demonstrated for a newer class of surface proteins [11] that lack a typical signal sequence and the LPXTG cell wall anchorage motif [12]. One such protein is pneumococcal adherence and virulence factor A (PavA) from \textit{Streptococcus pneumoniae}, which was not only shown to mediate pneumococcal binding to immobilized fibronectin but also identified as a pneumococcal virulence factor. Deficiency in PavA reduces the ability of pneumococci to adhere to or invade human epithelial and endothelial cells in vitro and attenuates virulence of pneumococci in mice infection models [13–15]. Another fibronectin-binding protein, Fbp54 from \textit{Streptococcus pyogenes}, which shows 67% homology to PavA, also lacks localization features common to surface proteins of Gram-positive bacteria [16]. Notably, this protein was shown to be protective against the streptococcal surface proteins of Gram-positive bacteria [16]. Notably, this protein was shown to be protective against the streptococcal infection.

In the present study, we report the first characterization of a PavA-like fibronectin-binding protein in \textit{E. faecalis}, encoded by the EF1249 locus, which was termed enterococcal fibronectin-binding protein A (EfbA). We found that EfbA, an isogenic deletion mutant of the \textit{E. faecalis} strain JH2-2 [18], was abrogated in EfbA production thus resulting in a strongly diminished ability of the strain to bind to immobilized human fibronectin. This mutant was also attenuated in a mouse model of ascending UTI, providing strong evidence that EfbA plays a role in the pathogenesis of enterococcal UTIs.

**METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. \textit{Enterococcus faecalis} strains included the wild-type JH2-2 and its derivatives and 15 fresh isolates recovered from clinical specimens (endocarditis, urine, urinary catheters, and wounds) at the Università Cattolica del Sacro Cuore of Rome, Italy. The strains were grown at 37°C in brain heart infusion (BHI) broth (Fluka), BHI plus 40% horse serum (Sigma) (BHI-S) broth, or, for some experiments, M17 medium supplemented with 0.5% glucose (GM17) and with erythromycin, when required. \textit{Escherichia coli} strains were grown at 37°C in Luria–Bertani broth containing chloramphenicol (10 μg/mL) and ampicillin (50 μg/mL).

**Construction of efbA Mutant and Complemented Strains**

To construct a ΔefbA deletion mutant of \textit{E. faecalis} JH2-2, 2 DNA fragments corresponding to the chromosomal DNA regions upstream (including the start codon and the 5’ part of the coding sequence) and downstream (including the 3’ part of the gene and the stop codon) of ef1249 (Figure 1) were polymerase chain reaction (PCR) amplified from JH2-2 genomic DNA using primers designed on the basis of the genome sequence of \textit{E. faecalis} V583 strain [19] with flanking restriction sites (see Supplementary Table 1). After digestion with SalI, the 2 PCR products were self-ligated in order to create a copy of the EF1249 gene deleted for an internal fragment of 1481 base pairs (approximately 86%); digested by appropriate restriction enzymes; cloned into plasmid pMAD, a thermosensitive pE194ts-based delivery vector system [20]; and finally

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<th>Table 1. Bacterial Strains and Plasmids Used in This Study</th>
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<tr>
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Abbreviations: Amp, ampicillin; bgaB, \textit{Bacillus stearothermophilus} gene encoding a thermostable β-galactosidase; Em, erythromycin; Fus, fusidic acid; Rif, rifampin.

* Superscripts “r” and “ts” indicate resistance and temperature-sensitivity, respectively.
transformed into electrocompetent cells of *E. coli* Top10 (Invitrogen), as described previously [21]. The recombinant plasmid, pMAD–ΔEF1249, was then electroporated into JH2-2 cells. Gene replacement was performed via a double-crossover event by a method based on the conditional replication of the pMAD plasmid [22], in which transformants were grown at the permissive temperature (30°C) on GM17 plates with erythromycin (100 μg/mL) and then shifted to the nonpermissive temperature (42°C) in the presence of erythromycin to select single-crossover integrants. Plasmids excision by a second recombinant event was stimulated by growing integrants for 4 hours at 30°C, followed by overnight incubation at 42°C without erythromycin to select an *ef1249* double-crossover mutation. Successful targeted mutation of *ef1249* in strain ΔefbA (Table 1) was confirmed by PCR, sequencing, and Southern blot analysis (results not shown).

For complementation, the entire *ef1249* region was amplified from JH2-2 genomic DNA (see Supplementary Table 1 for primers) and cloned in pMAD [10]. The resulting plasmid, pMAD–EF1249, was confirmed by sequencing and then electroporated into the ΔefbA deletion mutant to create the efbA complemented strain (Table 1).

**Expression, Purification, and Immunoblot Analysis of Recombinant EfbA**

Expression and purification of EfbA was performed as described previously [23]. Briefly, the entire EfbA coding region was PCR amplified from JH2-2 DNA using primers properly designed to generate a PCR product with *SalI* and *HindIII* sites at their respective 5’ and 3’ ends (see Supplementary Table 1). This product was digested with *SalI* and *HindIII*, cloned into pQE31 (Qiagen), and transformed into *E. coli* M15 (pREP4).
(Qiagen). Induction of N-terminally xHIs-tagged EfbA expression was obtained with isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mM. The recombinant EfbA (rEfbA) protein was purified by nickel column chromatography using Ni-NTA Agarose (Qiagen), and protein concentration was determined using the bicinchoninic acid protein assay (Pierce Chemical). After visualization on a sodium dodecyl sulfate 10% polyacrylamide gel, the eluted rEfbA was dialyzed against phosphate-buffered saline (PBS) and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare) by electroblotting. To detect rEfbA, blots were blocked with 4% skim milk (Sigma) in PBS and 0.05% Tween 20 (PBS-T), incubated at 37°C for 2 hours with 1:1000 dilution of anti-(H)5 mouse antibodies (Penta-His Antibody; Qiagen). The blots were then washed with PBS-T and incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-conjugated goat antimouse secondary antibodies (Sigma) diluted 1:10000 in PBS-T. Antibody binding was detected using 3’, 3’, 5’, 5’-tetramethylbenzidine liquid substrate (Sigma) according to the manufacturer’s instructions.

Quantitative Real-Time Reverse Transcription PCR
To test efba gene expression levels in JH2-2 and in the ΔefbA deletion mutant during growth in BHI and BHI-S at 37°C, total RNA was extracted from exponential-phase enterococcal cultures with an RNeasy minikit (Qiagen), which includes an RNase-free DNase treatment step to eliminate DNA. Quantitative real-time reverse transcription PCR (RT-PCR) was performed in an iCycler iQ system (Bio-Rad Laboratories), using rpoB as the normalization gene [24], with primers (see Supplementary Table 1) that were designed to produce amplicons of similar lengths using Beacon Designer 7 software (Premier Biosoft International). The relative messenger RNA (mRNA) expression level of the target gene in each sample was calculated using the comparative cycle-threshold method [25].

Enzyme-Linked Immunosorbent Assay (ELISA) and Whole-Cell ELISA
Binding of rEfbA to collagen types I and IV (human placenta; Sigma), fibronectin (human plasma; Enzyme Research Laboratories), and laminin (human placenta; Sigma) was measured essentially as described elsewhere [26]. High-binding microtiter plate (Immuno 2 HB; Corning) wells were coated overnight at 4°C with 10 µg/mL of each ECM protein, and bovine serum albumin (BSA; Sigma) was used as a negative control. After washing the wells and blocking the remaining protein-binding sites by 1% BSA and 0.1% Tween 20 in PBS (blocking buffer), amounts of up to 0.5 µg of rEfbA diluted in blocking buffer were added to the wells and incubated at 37°C for 2 hours. The wells were then washed with 0.1% Tween 20 to remove unbound protein, and bound rEfbA was detected by the mouse anti-(H)5 primary antibody and goat antimouse secondary antibody conjugated to HRP.

Binding of E. faecalis strains to the immobilized ECM proteins (all 20 µg/mL) was determined by whole-cell ELISA, using a previously described protocol [27] with some modifications. Briefly, bacteria were collected from overnight cultures in BHI-S, washed with PBS (pH 7.4), and resuspended in PBS to an optical density at 600 nm (OD600) of 0.5, and 100 µL was added to wells of a microtiter plate and allowed to bind overnight at 4°C. The wells were washed and subsequently blocked with blocking buffer for 1 hour at 37°C. Binding of E. faecalis strains was assayed by incubation for 1 hour at 37°C with an antienterococcus serum, which was raised in rabbit against formalin-killed whole cells of the E. faecalis strain ΔefbA (Table 1). Bound antibodies were detected by incubation with an HRP-conjugated goat antirabbit immunoglobulin G (Millipore).

For both types of ELISA, the absorbance was measured at 450 nm with an ELISA reader.

Immunofluorescence and Immunelectron Microscopy
Immunofluorescence staining was performed as described previously [24]. Briefly, whole cells of an enterococcal exponential-phase culture in BHI-S were fixed with PBS containing 4% paraformaldehyde for 5 minutes, then incubated overnight with a polyclonal antiserum against rEfbA (which was generated in mouse by routine immunological techniques) diluted 1:500 in PBS–0.5% BSA. After washing with PBS, bound antibodies were detected by incubation for 1 hour with Alexa 488–coupled antimouse antibodies (Sigma) diluted 1:200 in PBS–0.5% BSA. After the cells were washed with PBS and resuspended in 100 µL of PBS, a 15-µL sample was applied to a glass slide, air dried, and heat fixed. Analysis was performed with a confocal microscope (Olympus Fluoview IX70). Preimmune antiserum from the same animal was used as a negative control, whereas the rabbit antienterococcus serum (see above) was included as a positive control.

For immunoelectron microscopy studies, enterococci were harvested by centrifugation from exponential-phase cultures in BHI-S and washed with PBS. Ultrathin sections were obtained according to a well-developed procedure [28]. Immunogold labeling was performed as described previously [29], using the anti-EfbA serum (1:100 dilution) or preimmune antiserum (1:100 dilution) followed by 10-nm gold goat antimouse immunoglobulin G (1:20 dilution) (Sigma). Samples were examined in a Philips 208 transmission electron microscope (FEI Company).

Mouse UTI Model of Virulence
The mouse UTI infection experiments were performed by following the protocol described by Singh et al [30] and already adopted by us [10], under the approval of the Università
Cattolica del Sacro Cuore of Rome Institutional Animal Use Committee. Briefly, groups of 15 female BALB/c mice weighing 20–25 g each were transurethrally challenged with approximately $1 \times 10^4$ colony-forming units (CFUs) of wild-type JH2-2 and its derivatives (Table 1). At 48 hours after infection, bladder and kidney pair cultures were prepared from mice that had been humanely killed to determine the recovered CFUs from organ homogenates.

Statistical Analysis
Statistical comparisons were performed with GraphPad Prism software (version 4.03 for Windows). Differences were considered significant when $P < .05$.

RESULTS
Characterization of EfbA, a Novel E. faecalis Cell-Surface Protein
In silico search of the genome sequence of E. faecalis strain V583 [19] revealed a 1713–base pair gene, ef1249, which encodes a 570 amino acid residue protein showing 51% identity (71% similarity) to S. pneumoniae PavA [13]. The analysis of 15 clinical isolates of E. faecalis for the presence of ef1249 by using PCR amplification with primers designed to amplify the complete coding region demonstrated that ef1249 was found in all (not shown). The putative EF1249 protein is composed of an N-terminal fibronectin-binding domain (Pfam PF05833), followed by a conserved domain of unknown function including the conserved motif (D/E)X(W/Y)XH (Du814). EF1249, designated EfbA because of its inferred role in the enterococcal fibronectin binding, contains 60 Leu residues, 48 Lys residues, and 45 Glu residues. The high preponderance of Leu residues was previously noted not only for PavA [13] but also for S. pyogenes Fbp54 [16, 31]. Similar to PavA, EfbA had no conventional leader sequence required for protein export via the general secretory pathway and no described motif involved in the localization of cell surface-exposed proteins (eg, LPXTG). According to the genetic structure of the locus and the predicted sites of transcriptional start and termination of efbA, RT-PCR analysis of E. faecalis JH2-2 RNA allowed us to obtain an expected-size PCR product corresponding to an approximately 1.7 kb mRNA (Figure 1).

Full-length recombinant N-terminally x6His-tagged EfbA protein was purified from E. coli and was shown to bind avidly to human fibronectin immobilized onto microtiter plate wells (Figure 2). Binding of rEfbA to fibronectin was saturable, and maximal binding levels were dependent upon the amounts of fibronectin immobilized (not shown). By contrast, we detected significantly reduced binding to immobilized collagen type I, collagen type IV, and laminin ($P < .0001$) (Figure 2).

Recent work aimed at identifying parameters that promote E. faecalis adherence to ECM components demonstrated that growth in 40% horse serum elicited adherence to fibronectin (and fibrinogen) but not to elastin and BSA [32]. To test whether EfbA is a serum-inducible protein, we examined the...
$efbA$ expression in *E. faecalis* JH2-2 after growth at 37°C in BHI broth or the same medium supplemented with horse serum at the above cited concentration (BHI-S). At 4 different time points—early exponential ($OD_{600} = 0.3$ after 4 hours), exponential ($OD_{600} = 0.8$ after 8 hours), early stationary ($OD_{600} = 1.0$ after 12 hours), and stationary ($OD_{600} \geq 1.0$ after 16 hours)—phases of growth—cells were harvested, and transcription of $efbA$ was analyzed by real-time RT-PCR. In the BHI-S–grown cells, transcripts of $efbA$ were detected in all phases of growth, and the expression levels increased significantly at the stationary phase (Figure 3). In the BHI-grown cells, the $efbA$ expression amounts observed at 4, 8, and 12 hours were respectively 9.1-, 6.4-, and 2.1-fold of that observed at the 0-hour time point.

Next, to prove that EfaA is a surface-exposed protein, localization of EfbA was determined via immunogold labeling experiments using a specific mouse antiserum. The results demonstrated that EfbA was present on the cell surface of JH2-2 cells grown in BHI-S; ultrathin sections of these specimens showed relatively uniform distribution of the gold particles around the bacterial cell surface (Figure 4A and B). Accordingly, immunofluorescence studies using the anti-EfbA serum confirmed the presence of EfbA on the cell surfaces of both strains of JH2-2 and the reconstituted mutant (Figure 4C). No surface localization of EfbA was detected in the $\Delta efbA$ deletion mutant cells (Figure 4A–C).

**Effect of EfbA Gene Deletion on Adherence of *E. faecalis* to Fibronectin**

To assess the role of EfbA in the interaction of *E. faecalis* cells with ECM molecules, we investigated the ability of wild-type JH2-2 and its isogenic strain $\Delta efbA$ to bind to fibronectin and the other ECM protein components (Figure 5). Whole-cell ELISA analysis in which fibronectin, laminin, collagen type I, collagen type IV, and BSA (negative control) were immobilized showed that the $\Delta efbA$ cells displayed significantly reduced binding only to fibronectin (8.7-fold; $P < .0001$), suggesting that EfbA promotes specific adherence of *E. faecalis*.
cells to fibronectin. As expected, complementation of the ΔefbA deletion mutant with plasmid pMAD-EF1249 resulted in restoration of the fibronectin adherence phenotype to levels equal to that of the wild-type strain (Figure 5).

Attenuation of E. faecalis Virulence in the Mouse Model of Ascending UTIs

To test the effect of deletion on the ability of E. faecalis to cause UTIs, we used a mouse model of ascending infection to compare JH2-2 and the ΔefbA deletion mutant for virulence. Mice (n = 15) were infected via intraurethral catheterization with 1 × 10⁴ CFUs of each enterococcal strain, and at 48 hours, the mean (geometric) CFUs recovered from kidney pairs and bladders were enumerated. As shown in Figure 6, the log₁₀ CFUs (per gram of tissue) of bacteria recovered from the kidneys infected by JH2-2 were significantly higher than that recovered from the kidneys infected by the ΔefbA strain (3.8 × 10⁶ vs 2.6 × 10⁵, respectively; P = .002). Again, a comparison of the mean log₁₀ CFUs of bacteria recovered from the bladders showed that the differences between JH2-2 and the ΔefbA mutant (0.65 log₁₀; P = .01) were statistically significant, but to a lower extent. These results are consistent with an increased tropism of E. faecalis for the kidney relative to the bladder, thereby indicating that the efbA gene contributes significantly to ascending UTIs.

DISCUSSION

Fibronectin acts as a molecular bridge for the adherence of several Gram-positive cocci, including streptococci and staphylococci [33], and it is also considered a signal transduction trigger leading to bacterial host invasion [34]. Whereas cell-wall attached fibronectin-binding proteins are recognized as important multifunctional virulence factors of S. aureus [35], Enterococcus faecium [27], and Streptococcus pyogenes [36], anchorless but surface-exposed adhesins (and invasins) have emerged as a new class of virulence factors, particularly for S. pyogenes and S. pneumoniae [11].

We described the first characterization of an E. faecalis fibronectin-binding adhesin, which does not possess a signal sequence or an LPXTG-mediated membrane anchor but is localized on the bacterial surface. This protein, named EfbA, was identified in E. faecalis by sequence homology with PavA...
from *S. pneumoniae*, a well-known anchorless but surface-located protein that is essential for pneumococcal adherence and virulence [13–15].

Initial studies on the *E. faecalis* adhesion to ECM proteins classified this organism as nonadherent to fibronectin (and fibrinogen) [37], but stressful environmental conditions (ie, after growth at 46°C or in presence of serum) were then shown to increase the binding of *E. faecalis* to collagen type I, collagen type IV, and laminin [38]. This conditional adhesion phenotype, for which bacteria grown in broth under conventional in vitro conditions do not adhere efficiently to the different ECM components, underscored an apparently stringent regulation of the expression of *E. faecalis* adhesins and thus complicated the identification of these proteins [6]. Finally, Nallapareddy and Murray [32] demonstrated the enhancing effect of serum on adherence of *E. faecalis*. It is known that MSCRAMMs and cell-wall anchor family proteins [6] were not differentially regulated or downregulated during growth of *E. faecalis* in human urine in vitro [41]. Similar to PavA, EfBA may be a key virulence determinant of enterococci under in vivo conditions and hence contribute to the pathogenesis of UTIs. Nonetheless, the role of EfBA in the *E. faecalis* pathogenicity remains to be deciphered.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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