Role of the Emp Pilus Subunits of *Enterococcus faecium* in Biofilm Formation, Adherence to Host Extracellular Matrix Components, and Experimental Infection

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Enterococcus faecium is an important cause of hospital-associated infections, including urinary tract infections (UTIs), bacteremia, and infective endocarditis. Pili have been shown to play a role in the pathogenesis of Gram-positive bacteria, including *E. faecium*. We previously demonstrated that a nonpilated $\Delta empABC::cat$ derivative of *E. faecium* TX82 was attenuated in biofilm formation and in a UTI model. Here, we studied the contributions of the individual pilus subunits EmpA, EmpB, and EmpC to pilus architecture, biofilm formation, adherence to extracellular matrix (ECM) proteins, and infection. We identified EmpA as the tip of the pili and found that deletion of *empA* reduced biofilm formation to the same level as deletion of the *empABC* operon, a phenotype that was restored by reconstituting *in situ* the *empA* gene. Deletion of *empB* also caused a reduction in biofilm, while EmpC was found to be dispensable. Significant reductions in adherence to fibrinogen and collagen type I were observed with deletion of *empA* and *empB*, while deletion of *empC* had no adherence defect. Furthermore, we showed that each deletion mutant was significantly attenuated in comparison to the isogenic parental strain, TX82, in a mixed-inoculum UTI model ($P < 0.001$ to 0.048), that reconstitution of *empA* restored virulence in the UTI model, and that deletion of *empA* also resulted in attenuation in an infective endocarditis model ($P = 0.0088$). Our results indicate that EmpA and EmpB, but not EmpC, contribute to biofilm and adherence to ECM proteins; however, all the Emp pilins are important for *E. faecium* to cause infection in the urinary tract.

Enterococcus faecium, a common human commensal, is currently one of the more problematic causes of hospital-associated (HA) infections in the United States, accounting for approximately 38% of clinical enterococcal isolates (1). The infections caused by *E. faecium*, which include urinary tract infections (UTIs), bloodstream infections, and endocarditis, among others, represent a major therapeutic challenge due to the typical resistance of this species to multiple antibiotics (2). Several lines of evidence, including molecular epidemiological studies based on multilocus sequence typing (MLST) (3–5) and comparative whole-genome analyses (6, 7), indicate that *E. faecium* strains that cause outbreaks and infections in hospitalized patients (HA clade or subclade A1) are considerably different from those forming part of the normal microbiota of healthy individuals (community-associated [CA] clade or clade B). Indeed, HA clade strains, compared to CA clade strains, are associated with increased presence, differential expression, and/or carriage of functional forms of genes encoding putative or confirmed virulence determinants that can participate in the adhesion of *E. faecium* to host tissues, including Acm (adhesin of collagen from *E. faecium*) (8), Esp (enterococcal surface protein) (9), other MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) (10, 11), and proteinaceous surface structures known as pili (10).

Pili in Gram-positive bacteria consist of covalently linked pilin subunits, usually encoded by two or three genes arranged in an operon, that extend from the surface of the cell and are assembled by a class C sortase (12, 13). The genome of *E. faecium* TX16 (DO) contains four pilus-encoding gene clusters predicted to form four distinct pilus-like structures (10, 14–16). Indeed, the conditional

expression of two distinct types of pili was demonstrated in an *E. faecium* hospital-acquired bloodstream isolate (15). In addition, differential assembly of the pilin proteins encoded by one of these pilin clusters, the pilin gene cluster 1, has been observed between a bloodstream isolate and a community-derived stool isolate, with the latter strain displaying only the pilin proteins anchored to the cell wall, not those associated with pilus fibers (17).

Another of these pilus clusters, pilin cluster 3, also known as the *empABC* operon (previously *ebpABC_{fm}*) (18), is enriched in isolates of clinical origin (11), and we had previously shown that in the endocarditis-derived *E. faecium* strain TX82, allelic replacement of *empABC* ($\Delta empABC::cat$) affected primary attachment and biofilm formation (18). In addition, we found that the

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$\Delta empABC::cat$ mutant was significantly attenuated, compared to the parental strain TX82, in an experimental model of UTI (18). Furthermore, an epidemiological study found a significant association between the degree of biofilm formation and the presence of the *empABC* operon (19). The *empABC* operon is composed of three genes that are cotranscribed and that encode the structural subunit proteins EmpA, EmpB, and EmpC; *bps*, encoding the class C sortase, is downstream of the *empABC* operon, separated by a predicted strong transcriptional terminator and shown to be transcribed independently (10, 18). EmpC has been demonstrated to be the major pilin (10, 15, 18), while EmpA and EmpB are predicted to be incorporated into the fiber as minor components (18). However, the role of individual Emp pilus subunits in pilus-associated functions has not been explored previously. In this study, we investigated the contributions of each of the subunits of Emp to pilus architecture, biofilm formation, adherence to components of the host extracellular matrix (ECM), and infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Relevant characteristics of the bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains, used for cloning experiments, were cultured at 37°C in Luria-Bertani (LB) (Becton, Dickinson [BD], Franklin Lakes, NJ) broth or agar. *Enterococcus* strains were routinely grown at 37°C using brain heart infusion (BHI; BD) broth or agar or tryptic soy broth (BD) supplemented with 0.25% (vol/vol) glucose (TSB-G). Ampicillin at 100 µg/ml and gentamicin at 25 µg/ml were used for selection in *E. coli*, while gentamicin at 200 µg/ml was used for enterococci. Enterococcosel agar (BD) supplemented with vancomycin (6 µg/ml) was used to grow the bacteria recovered from the animal experiments. Growth characteristics of TX82, its *emp* deletion derivatives, and the *empA* reconstituted strain were assessed in BHI broth by measuring the optical density at 600 nm (OD₆₀₀) and by determining the number of CFU on BHI agar, as previously described (18).

Construction of markerless deletions of *empA*, *empB*, and *empC* genes and generation of an *empA* reconstituted strain (restoration of the *empA* gene in its native location). Nonpolar deletions of the individual genes encoding the pilus subunits EmpA, EmpB, and EmpC of *E. faecium* TX82 (Fig. 1 and Table 1) were constructed using a previously described system (20), based on the pHOU1 vector (21) carrying the *pheS*⁺ allele that confers susceptibility to *p*-chloro-phenylalanine (20, 21). Briefly, a fragment upstream and a fragment downstream of the genes to be deleted were amplified using the primers listed in Table S1 in the supplemental material. The two fragments flanking each gene were fused together by crossover PCR, cloned into pGEM or TOPO vectors, and subcloned into pHOU1 (Table 1). The recombinant pHOU1 plasmids were propagated in *E. coli* EC1000 and transferred to *Enterococcus faecalis* CK111 by electroporation (Table 1). Double-crossover homologous recombination was achieved by first transferring the plasmids into *E. faecium* TX82 through filter mating with *E. faecalis* CK111 carrying the recombinant plasmids, followed by culturing the gentamicin-resistant *E. faecium* colonies that integrated the plasmid on minimal medium 9–yeast extract–glucose (MM9YEG) medium supplemented with 10 mM *p*-chloro-phenylalanine. Deletion of the genes was detected by PCR and confirmed by sequencing, with verification of the correct background by pulsed field-gel electrophoresis (PFGE).

For generation of the *empA* reconstituted strain (Fig. 1 and Table 1), a 4,309-kb fragment that encompasses the region from bp 439 upstream to bp 480 downstream of the *empA* gene was amplified from TX82 (a single silent mutation [Fig. 1; see Table S1 in the supplemental material] was introduced into the fragment, within the *empB* coding region, to differentiate this reconstituted strain from TX82) and cloned into the pHOU1 vector. Once the fragment was cloned into pHOU1, the approach described above for the generation of the deletions was followed to intro-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>E. faecium</i>		
TX82	Endocarditis isolate; Amp ^r Van ^r	8
TX5645	TX82 $\Delta empABC::cat$; allelic replacement of <i>empABC</i> with <i>cat</i> (chloramphenicol acetyltransferase) gene; Chl ^r	18
TX6138	TX82 $\Delta empA$; <i>empA</i> markerless deletion mutant	This study
TX6139	TX82 $\Delta empB$; <i>empB</i> markerless deletion mutant	This study
TX6154	TX82 $\Delta empC$; <i>empC</i> markerless deletion mutant	This study
TX6152	TX6138:: <i>empA</i> ; TX82 $\Delta empA$ with <i>empA</i> reconstituted <i>in situ</i> in the chromosome	This study
<i>E. faecalis</i> CK111	OG1Sp <i>upp4</i> ::P23 <i>repA4</i> ; conjugative donor that allows pHOU1 replication	20
<i>E. coli</i>		
TG1	<i>E. coli</i> host strain used for routine cloning	
DH5 α	<i>E. coli</i> host strain used for routine cloning	
EC1000	<i>E. coli</i> host strain for cloning of RepA-dependent plasmids	43
Plasmids		
pGEM-T Easy	Plasmid used for initial cloning of PCR fragments; Amp ^r	Promega
Blunt II-TOPO	Plasmid used for initial cloning of PCR fragments; Kan ^r	Invitrogen
pHOU1	Conjugative donor plasmid that carries Gen ^r and the counterselectable <i>pheS</i> ⁺ gene	21
pQE30	Expression vector	Qiagen
pTEX5636	3,192-bp fragment from TX16 <i>empA</i> (encoding mature EmpA without signal peptide or cell wall anchor domain) cloned into pQE30 expression vector	This study

^a Amp, ampicillin; Chl, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Van, vancomycin. Superscript “r” designates resistance.

duce *in situ* the *empA* gene into the chromosome of the $\Delta empA$ strain (Table 1, TX6138).

Reverse transcriptase PCR (RT-PCR). Total RNA from TX82 and the panel of deletion mutants was isolated from cells grown in TSB-G to mid-logarithmic phase (OD₆₀₀, ~0.6). A 5-ml volume of the cultures was mixed with 10 ml of RNA protect reagent (Qiagen, Hilden, Germany), harvested, and resuspended in 0.9 ml of TRIzol reagent (Ambion-Thermo Scientific, Waltham, MA). Cells were disrupted by bead beating for 1 min, twice, with cooling on ice in between (BioSpec Products, Bartlesville, OK). RNA extraction and purification were performed using a PureLink RNA extraction kit (Ambion) in accordance with the manufacturer’s instructions. cDNA was synthesized using a SuperScriptIII First-Strand synthesis system (Invitrogen, Carlsbad, CA). Primers, targeting an intragenic region of *empC* (see Table S1 in the supplemental material), were used to evaluate *empC* expression. Expression of the gene encoding gyrase A (*gyrA*) of *E. faecium* was used as an internal control (see Table S1), as

FIG 1 Schematic representation of the *emp* operon of *E. faecium* TX82, its deletion derivatives, and the *empA* reconstituted strain. The *emp* operon consists of three genes, *empA*, *empB*, and *empC*, encoding the pilin subunits; *bps*, located after a predicted transcriptional terminator (indicated with a lollipop) downstream of *empC*, encodes a class C sortase. The genes deleted from each mutant are indicated, and the silent mutation introduced in the *empA* reconstituted strain is indicated with an asterisk.

previously described (18). A PCR in the absence of reverse transcriptase was used to ensure the absence of genomic DNA in the RNA samples.

Antibodies against EmpA. Previously generated goat polyclonal antibodies were used to detect EmpC (10), while rabbit polyclonal antibodies against EmpA were generated in this study. The DNA sequence corresponding to amino acids 30 to 1093 of EmpA from *E. faecium* strain TX16 (see Table S1 in the supplemental material) was cloned into the pQE30 (N-terminal His₆ tag fusion) expression vector (10), generating the plasmid pTEX5636 (Table 1). Expression of recombinant EmpA (rEmpA) was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and protein purification was performed by nickel affinity chromatography using His-GraviTrap columns (GE Healthcare, Uppsala, Sweden). Protein concentrations were determined by absorption spectroscopy at 280 nm using calculated molar absorption coefficient values (22). Rabbit immunization with rEmpA was done by following a preapproved protocol and guidelines of the Animal Welfare Committee of the University of Texas Health Science Center at Houston. In brief, 1 mg of rEmpA in Freund's complete adjuvant (FCA) was subcutaneously injected at multiple sites into a New Zealand White male adult rabbit (~3 kg) on day 1. Booster doses prepared in emulsified Freund's incomplete adjuvant (FIA) were given subcutaneously at multiple sites on days 21 and 42. The animal was anesthetized prior to blood collection via cardiac puncture, followed by euthanasia. Anti-EmpA antibody titers in rabbit sera were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (23) with some modifications. Briefly, 96-well plates (Immulon 4HBX; Thermo Fisher Scientific, Waltham, MA) coated overnight with 1 μg of rEmpA in 50 mM carbonate-bicarbonate buffer, pH 9.6, were used to test the rabbit sera (serial dilutions from 1:200 to 1:25,600). Detection was performed with peroxidase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) and 1-Step PNPP substrate (Thermo Fisher Scientific). The highest serum dilution with an absorbance at 405 nm of ≥0.10 at 3 min after addition of the substrate was established as the antibody titer (23).

Immunoelectron microscopy. Immunoelectron microscopy to study Emp pilus architecture in TX82 and its *emp* deletion mutants was performed as previously described (24), with minor modifications. Cells were grown in TSB-G to exponential phase, harvested by centrifugation, and then washed with 0.1 M NaCl. Immunogold labeling was performed using the anti-EmpA and anti-EmpC antibodies described above, followed by 18-nm-gold-particle-conjugated goat anti-rabbit IgG (1:20 dilution of 1 mg/ml; Jackson ImmunoResearch Laboratories) for EmpA staining or

12-nm-gold-particle-conjugated donkey anti-goat IgG (1:20 dilution; Jackson ImmunoResearch Laboratories) for EmpC staining. Samples were viewed with a JEOL 1400 transmission electron microscope.

WC ELISA. Surface display of EmpA and EmpC, using TX82, the panel of *emp* deletion mutants, and the *empA* reconstituted strain, was measured by whole-cell (WC) ELISA, by use of a previously described methodology (25, 26). Rabbit and goat polyclonal antibodies (1:5,000 dilution of a 1-mg/ml concentration) against EmpA (described above) and EmpC (generated in a previous report [10]), respectively, were used as primary antibodies, while alkaline phosphatase-conjugated F(ab')₂ fragment goat anti-rabbit IgG and donkey anti-goat IgG (1:5,000 dilution; Jackson Immuno Research Laboratories) were added to the respective wells as secondary antibodies. Detection was performed by measuring the absorbance at 405 nm with a microplate reader (Thermo Fisher Scientific), after the addition of 1-Step PNPP substrate (Thermo Fisher Scientific).

Dot blot analysis. Dot blot analysis was performed as described by Konto-Ghiorgi et al. (27) with slight modifications. In brief, bacteria were grown in TSB-G to an OD₆₀₀ of 0.6, harvested, and washed twice with Tris-buffered saline (TBS). The nitrocellulose membrane was spotted with approximately 2.3×10^6 CFU or 2.3×10^5 CFU and then blocked with 5% skim milk in TBS. EmpC was detected with the goat anti-EmpC polyclonal antibodies (1:2,500 dilution of a 1-mg/ml concentration), followed by incubation with peroxidase mouse anti-goat IgG (H+L) (1:20,000 dilution; Jackson Immuno Research Laboratories). Detection was performed using the Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

In vitro biofilm formation assay. An *in vitro* biofilm formation assay was performed as previously described (26). In brief, *E. faecium* strains were grown overnight at 37°C in TSB-G broth and then diluted in the same medium to an OD₆₀₀ of 0.1, before inoculation into polystyrene plates (BD). Bacteria were allowed to grow for 24 h under static incubation at 37°C. The plates were gently washed three times with phosphate-buffered saline (PBS), followed by treatment with Bouin's fixative (Sigma-Aldrich Co., St. Louis, MO). After the fixative was removed, the bacterial cells were washed with PBS and stained with 1% crystal violet solution (Sigma-Aldrich Co). Ethanol-acetone (80:20) to solubilize the dye and a microplate reader (Thermo Fisher Scientific) were used to measure the absorbance at 570 nm.

ECM binding assay. Adherence to immobilized fibrinogen and collagen type I was assayed using a crystal violet-based staining method as described previously (28), with some modifications. In brief, Immulon

FIG 2 Immunoelectron microscopy analysis of pilus architecture in *E. faecium* TX82 and its deletion derivatives. Cells were grown to mid-log phase in TSB-G and stained with anti-EmpA and/or anti-EmpC antibodies. α , anti. (A) TX82 stained with anti-EmpA (18 nm); (B) TX82 stained with anti-EmpC (12 nm); (C) TX82 stained with anti-EmpA (18 nm; arrows marked "A") and anti-EmpC (12 nm; arrow marked "C"); (D) $\Delta empA$ mutant stained with anti-EmpA (18 nm); (E) $\Delta empA$ mutant stained with anti-EmpC (12 nm); (F) $\Delta empB$ mutant stained with anti-EmpA (18 nm); (G) $\Delta empB$ mutant stained with anti-EmpC (12 nm); (H) $\Delta empC$ mutant stained with anti-EmpA (18 nm); (I) $\Delta empC$ mutant stained with anti-EmpC (12 nm). Scale bars, 0.2 μ m.

4HBX 96-plate wells (Thermo Fisher Scientific) were coated overnight with 10 μ g/ml of the ECM proteins, followed by the blocking of unbound sites with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The *E. faecium* strains grown for 16 h at 37°C in BHI broth were collected by centrifugation, washed twice with PBS, and resuspended in 1% BSA in PBS to an OD₆₀₀ of 1.0. The bacteria were allowed to bind at 37°C for 2 h to the ECM-coated wells; this was followed by two washes with PBS to remove unbound cells. Detection of the adherent cells was performed as described above for the biofilm formation assay.

Mouse UTI model. *E. faecium* TX82, its *emp* deletion derivatives, and the *empA* reconstituted strain were grown for 24 h in BHI broth, harvested, and resuspended to the same OD₆₀₀ in 0.9% saline solution. The bacterial suspensions, estimated to contain 10⁸ CFU, were diluted 1:10 and mixed together at an approximately 1:1 ratio (TX82 with each deletion mutant and the $\Delta empA::empA$ mutant with the $\Delta empA$ mutant). In addition, serial dilutions of the inoculum were made in 0.9% saline and plated to determine the actual numbers of CFU. Six-week-old female ICR mice were inoculated via intraurethral catheterization with the mixed bacterial suspension (approximately 10⁶ CFU of each strain), and 48 h after infection, the animals were euthanized and CFU counts were determined from kidneys and bladders by plating tissue homogenates onto Enterococcosel agar (BD) with vancomycin (6 μ g/ml), as previously described (29). All colonies that grew (up to 47 CFU/organ) were picked into

the wells of microtiter plates containing BHI broth plus 15% glycerol, grown overnight, and then replica plated onto Hybond-N+ membranes placed on BHI agar. After overnight growth on the membranes, colonies were lysed to perform high-stringency hybridization (30), using intragenic DNA probes of *ddl* and the corresponding *emp* gene. Hybridization results were used to generate the percentage of each strain recovered from the organs.

Rat infective endocarditis model. Aortic valve endocarditis was induced in male Sprague-Dawley rats by placement of a catheter across the valve, according to a previously published method (23). Twenty-four hours after catheter placement, TX82 or the $\Delta empA$ deletion mutant (TX6138) grown for 24 h at 37°C in BHI broth and resuspended in saline was inoculated (intravenously via tail vein) into 16 rats. In addition, the inoculum was plated onto BHI agar to determine the actual numbers of CFU inoculated into the rats. The animals were euthanized 48 h after bacterial inoculation, and their hearts were aseptically removed. Establishment of bacterial endocarditis was confirmed at autopsy by evidence of vegetations formed around aortic valves and correct placement of the catheter across the valve. Vegetations together with the aortic valve were excised, weighed, and homogenized in 1 ml saline. The homogenized tissue was plated on Enterococcosel agar (BD) with 6 μ g/ml of vancomycin, and colonies were counted after 48 h of incubation at 37°C to determine the number of CFU per gram of vegetation. All the animal experi-

FIG 3 Surface display of EmpA and EmpC by *E. faecium* TX82, its deletion derivatives, and the *empA* reconstituted strain. Surface display of EmpA (A) and EmpC (B) detected by WC ELISA using polyclonal antibodies against EmpA and EmpC, respectively. Bars represent the mean $\pm$ standard deviation of the percentage of absorbance measured at 405 nm for each strain compared to that for TX82, from at least two independent experiments representing 10 wells per strain. Differences between TX82 and its derivatives were analyzed using a *t* test; *P* values are indicated on the figure. (C) Dot blot analysis of whole bacteria (approximately 2.3×10^6 CFU or 2.3×10^5 CFU of TX82 and its derivatives) using polyclonal antibodies against EmpC.

ments described in this study were performed according to protocols used previously in our laboratory (18, 23, 29, 31) and in accordance with pre-approved protocol and guidelines of the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Statistical analyses. GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA) was used for statistical analyses. An unpaired *t* test was used to evaluate differences between the strains in the ELISA and biofilm formation assays. Analysis of variance (ANOVA) with Bonferroni's multiple-comparison posttest was used to compare the results from the adherence assays. Differences in the lengths of pilus fibers produced by TX82 and the $\Delta empA$ mutant were analyzed using an unpaired *t* test. A paired *t* test was used to compare the percentage of bacteria recovered in kidneys and bladders with the percentage of bacteria inoculated into the mice, while the Mann-Whitney test was used to analyze the differences in the log₁₀ number of CFU of TX82 and that of the $\Delta empA$ deletion mutant recovered from vegetations.

RESULTS AND DISCUSSION

E. faecium has rapidly emerged as a very important cause of hospital-associated infections (1). Different surface proteins, including pili, have been implicated in enterococcal pathogenesis (2, 32). The *empABC* operon is a three-gene locus (Fig. 1) that encodes a prototypical pilus structure consisting of a backbone pilin subunit, EmpC, and two minor accessory pilins, EmpA and EmpB (15, 18). In a previous study, we found that allelic replacement of the *empABC* operon from the endocarditis-derived isolate, TX82, led to significant attenuation in biofilm formation and in a mouse model of UTI (18); however, the role of the individual Emp pilus

subunits to pilus-related functions has not yet been explored. Here, we sought to investigate the contribution of EmpA, EmpB, and EmpC to pilus architecture, biofilm formation, and adherence to components of the ECM and to the ability of *E. faecium* to cause infection. To address the function of each Emp pilus subunit, we individually deleted each gene of the *emp* operon from the endocarditis-derived *E. faecium* isolate TX82 ($\Delta empA$, TX6138; $\Delta empB$, TX6139; $\Delta empC$, TX6154) (Fig. 1 and Table 1). Deletions were designed to be unmarked and nonpolar to avoid affecting the remaining pilin genes or the downstream gene, *bps*, that codes for a class C sortase. We also reconstituted *in situ* the *empA* gene into the chromosome of the $\Delta empA$ deletion strain (TX6138) to generate strain TX6152 (Fig. 1 and Table 1). Equivalent growth kinetics measured by OD₆₀₀ (see Fig. S1 in the supplemental material) and comparable numbers of CFU (data not shown) at all the time points tested were observed between the panel of deletion mutants, the *empA* reconstituted strain, and the parental strain.

EmpA localizes at the tip of the fiber, while EmpC is distributed along the length of the pilus shaft. We analyzed pilus architecture in TX82 and its deletion derivatives by immunoelectron microscopy using antibodies generated against rEmpA and rEmpC proteins (EmpB localization was not studied since a previous study showed that the homolog of this subunit in *E. faecalis*, *ebpB*, was barely detectable by microscopy or WC ELISA [25]). EmpA is a von Willebrand factor A domain-containing protein predicted to be the tip pilin. As observed in Fig. 2A and C, EmpA

FIG 4 Contribution of each Emp pilin subunit to biofilm formation. Cells grown for 24 h in TSB-G were analyzed for biofilm formation using a crystal violet-based assay. Median values measured at 570 nm, interquartile ranges, and minimum and maximum values (whiskers) from three independent experiments representing 30 wells per strain are indicated. A *t* test was used to compare biofilm density values between TX82 and its derivatives; *P* values are indicated on the figure.

localized at the tip of pilus-like structures; however, it was also abundantly seen on the surface, apparently not forming part of pilus polymers, on TX82 cells (Fig. 2A). In agreement with previous studies (10, 15, 18), our microscopy results confirmed EmpC as the major pilin, forming the backbone of the Emp pili (Fig. 2B, C, and G). As expected, the anti-EmpA antibody did not stain the $\Delta empA$ deletion strain (Fig. 2D). In contrast, in the $\Delta empB$ deletion strain, EmpA was observed on the bacterial surface and at the tip of pilus polymers (Fig. 2F), while in the $\Delta empC$ deletion strain, EmpA was seen only on the bacterial surface (not forming part of pilus polymers) (Fig. 2H). Importantly, deletion of *empA* or *empB* did not abrogate pilus polymerization, as pilus-like structures were seen with the anti-EmpC antibody on the $\Delta empA$ and $\Delta empB$ strains (Fig. 2E and G); as expected, the anti-EmpC antibody did not stain the $\Delta empC$ deletion strain (Fig. 2I).

EmpA is important for wild-type length of the pilus fiber. Our electron microscopy experiments also provided evidence that

the tip subunit, EmpA, is important for determining the wild-type length of the pilus fiber. We measured the pilus lengths from 97 and 85 pilus fibers from TX82 and the $\Delta empA$ deletion strain, respectively, and found that deletion of the *empA* gene caused a significant increase in the length of the pilus fibers (Fig. 2E) compared to that of TX82 cells (Fig. 2B). The average length of pili produced by the $\Delta empA$ deletion strain was $1.22 \pm 0.76 \mu\text{m}$ (mean $\pm$ standard deviation) compared to $0.69 \pm 0.38 \mu\text{m}$ for TX82 (median for $\Delta empA$, $1.03 \mu\text{m}$, and for TX82, $0.69 \mu\text{m}$, $P < 0.0001$) (see Fig. S2 in the supplemental material). This finding is opposite to the shorter FimP fibers observed in *Actinomyces oris* when the tip subunit, *fimQ*, was deleted (33). In addition, we observed that deletion of *empA* caused a reduction in the number of pili per bacterial cell, which is in accordance with previous findings (33). In *Streptococcus agalactiae*, deletion of *pilA*, the first gene of the operon encoding the pilin adhesin, was associated with longer pilus fibers due to an increase in transcription of the downstream gene, *pilB*, encoding the shaft (27, 34). We observed only a slight increase in *empC* mRNA levels in the *empA* and *empB* deletion strains, compared to those in TX82, which suggests that increased expression of *empC* in the $\Delta empA$ deletion strain is probably not the sole cause of increased length of the pilus fibers in *E. faecium* (Fig. S3). In contrast, in other Gram-positive organisms, the levels of the shaft subunit have been implicated in the regulation of pilus length (35). While the underlying mechanism for the tip subunit's control of pilus length remains to be determined, this result suggests important differences in the regulation of pilus assembly between enterococci and other Gram-positive bacteria.

Surface display of EmpA and EmpC by TX82, its isogenic *emp* deletion mutant derivatives, and the *empA* reconstituted strain. We next investigated the surface display of EmpA and EmpC by the single *emp* deletion mutants and the *empA* reconstituted strain in comparison to that by the parental strain, TX82, and the previously generated *empABC* operon deletion with *cat* gene allelic replacement ($\Delta empABC::cat$) (18). WC ELISA, using our anti-EmpA antisera, revealed that this subunit is abundantly present on the surface of TX82 at mid-exponential phase (Fig. 3A). EmpC pilin was also displayed profusely on the surface of TX82 (Fig. 3B), as previously demonstrated (18). Consistent with an earlier report (18), we did not detect EmpA or EmpC on

FIG 5 Contribution of each Emp pilin subunit to ECM adherence. Adherence levels of *E. faecium* TX82, its *emp* deletion derivatives, and the *empA* reconstituted strain to immobilized fibrinogen (A) and collagen type I (B) are shown. Bars represent the mean $\pm$ standard deviation of the percentage of adherence of each strain compared to that of TX82 (defined as 100%) from four independent experiments representing 29 wells per strain. Statistical analysis was performed using ANOVA with Bonferroni's posttest; *P* values are indicated on the figure.

FIG 6 Attenuation of *empA*, *empB*, and *empC* deletion mutants in a mouse UTI model, using a mixed inoculum. Mice were infected with a mixed bacterial suspension of TX82 and the $\Delta empA$ deletion strain (A), $\Delta empB$ deletion strain (B), and $\Delta empC$ deletion strain (C) or the *empA* reconstituted strain ($\Delta empA::empA$) and $\Delta empA$ deletion strain (D). Horizontal lines represent the mean of the percentage of total bacteria inoculated into each mouse and recovered 48 h after infection in kidneys and bladders. In some instances, no bacteria were recovered (two in panel A [bladder] and one in panel C [kidney]). Results were analyzed using a paired *t* test; *P* values are indicated on the figure.

the surface of the strain in which the *emp* operon had been replaced with *cat* ($\Delta empABC::cat$) (Fig. 3A and B). In addition, and as anticipated, the $\Delta empA$ and $\Delta empC$ deletion strains did not express EmpA or EmpC on the surface, respectively (Fig. 3). By WC ELISA, we observed that deletion of *empC* and, to a minor extent, deletion of *empB* caused a significant reduction in EmpA surface display compared to that of TX82 cells ($P < 0.0001$ and $P = 0.0070$, respectively) (Fig. 3A). In addition, EmpC levels on the surface of the $\Delta empA$ deletion strain were significantly diminished ($P < 0.0001$), while deletion of the *empB* gene did not affect surface display of the shaft pilin subunit ($P = 0.8404$) (Fig. 3B). No significant differences in EmpA and EmpC levels between TX82 and the *empA* reconstituted strain were observed, indicating restoration of pilin protein levels on the surface of the reconstituted strain ($P = 0.7275$ and $P = 0.4409$, respectively) (Fig. 3A and B).

Since pili were shown to be longer in the $\Delta empA$ deletion strain than in TX82, which is inconsistent with the reduced levels of

EmpC seen on this strain by WC ELISA (Fig. 3B), we further evaluated the levels of EmpC by whole bacteria by an immunodot assay. As observed in Fig. 3C, a slight increase in EmpC (not a decrease, as shown by WC ELISA), consistent with a slight increase at the mRNA level (see Fig. S3 in the supplemental material), was observed in the $\Delta empA$ deletion mutant compared to that of TX82 and the *empA* reconstituted strain. It is possible that the longer pili on the $\Delta empA$ deletion strain are more fragile and detach from the bacterial surface during the processing by WC ELISA. Furthermore, we observed a modest decrease in EmpC levels in the $\Delta empB$ deletion strain, probably due to the release of pilus fibers, consistent with its role as a pilus anchor (Fig. 3C) (25). These results, together with our electron microscopy studies, suggest that all the subunits of the pili are important for correct integrity of pilin fibers on the surface of *E. faecium* cells.

EmpA is the main component of Emp pili that mediate bio-film formation. In a previous study, Sillanpää et al. showed that

FIG 7 Effect of *empA* deletion in a rat model of infective endocarditis. A monoinfection experiment was done using inocula of 10^9 CFU of TX82 or the $\Delta empA$ deletion strain. Data are expressed as $\log_{10}$ CFU/g of bacteria recovered 48 h after infection from vegetations (horizontal lines represent the geometric mean CFU/g). Results were analyzed using the Mann-Whitney test; the *P* value is indicated on the figure.

allelic replacement of the *empABC* operon significantly affected biofilm, with a 75% reduction in biofilm formation in the $\Delta empABC::cat$ strain relative to that of TX82 (18). Here, we investigated the contribution of each Emp pilus subunit to biofilm formation. As shown in Fig. 4, deletion of *empA* reduced biofilm formation to the same extent as observed with the deletion of the operon, indicating that the tip subunit, EmpA, is the main component of the Emp pili mediating biofilm formation ($P < 0.0001$). In addition, when *empA* was reconstituted *in situ*, biofilm formation was restored ($P > 0.05$). Surprisingly, we found the major backbone pilin, EmpC, to be dispensable for this process ($P > 0.05$), which is consistent with abundant EmpA seen on the surface of the $\Delta empC$ deletion strain (Fig. 2H), while deletion of *empB* led to a significant decrease in biofilm formation ($P < 0.0001$) (Fig. 4). These results indicate that EmpA and, to a lesser extent, EmpB play important roles in biofilm formation, even when the EmpC pilin backbone is absent.

EmpA and EmpB are important for adherence to ECM proteins. Adherence to ECM proteins is proposed to be the first step in the infection process of *E. faecium* (36). Zhao et al. previously demonstrated that strain TX82 shows high levels of adherence to fibrinogen, collagen type I, and fibronectin, by use of a sensitive radioactive assay (36). Since pili in other Gram-positive organisms have been implicated in binding to ECM proteins (27, 37), including recent reports that demonstrated that EbpA of *E. faecalis* mediates adherence to host fibrinogen (26, 38), known to be exposed/released after trauma (38), our hypothesis was that Emp pili contributed to the adherence capacity of TX82. We investigated the involvement of each Emp pilus subunit to the adherence of *E. faecium* TX82 to fibrinogen and collagen type I. As shown in Fig. 5, deletion of *empA* and *empB* significantly reduced fibrinogen ($P < 0.001$) and collagen I ($P < 0.05$ and $P < 0.01$, respectively) adherence compared to that in TX82, suggesting that these two subunits contribute to the adherence capacity of TX82. The *empA* reconstituted derivative showed almost wild-type (WT) levels of adher-

ence ($P > 0.05$), and the $\Delta empC$ deletion strain showed no attenuation in binding; in fact, its fibrinogen binding ability was increased in comparison to that of TX82 ($P < 0.01$) (Fig. 5). In other Gram-positive organisms, including *Corynebacterium diphtheriae*, deletion of the gene encoding the shaft subunit also did not affect the adherence phenotype, although pilus assembly was abolished (39). The fact that deletion of *empC* showed an increase in binding is intriguing; one possibility is that in the absence of EmpC, other cell wall-associated proteins, including EmpA, may have a closer or tighter interaction with components of the ECM.

The three pilin subunits encoded by the *emp* operon are important in a mouse model of UTI. In order to elucidate the role of the individual Emp pilin subunits in infection, we evaluated each of the deletion strains in a mouse model of UTI. We found that after 48 h of infection, each of the deletion mutants was significantly attenuated in kidneys ($P = 0.028$ to $P = 0.048$) and in bladders ($P < 0.0001$ to $P = 0.0124$) in comparison to that of TX82 (Fig. 6A to C). These results indicate that EmpA, EmpB, and EmpC are all required for full virulence of *E. faecium* TX82 in the UTI model. It is worth mentioning that even though the *empC* deletion strain showed no attenuation in our *in vitro* assays (biofilm [Fig. 4] and ECM adherence [Fig. 5]), our *in vivo* model results demonstrate the importance of this subunit in the virulence of *E. faecium* (Fig. 6C). We also evaluated the *empA* reconstituted strain ($\Delta empA::empA$) versus the $\Delta empA$ deletion mutant and found significantly higher percentages of the reconstituted strain, in kidneys ($P = 0.0153$) and bladders ($P < 0.0008$), than of the *empA* deletion strain (Fig. 6D), confirming the involvement of EmpA in *E. faecium* pathogenesis in UTI.

Importance of EmpA in an endocarditis model. EmpA has a von Willebrand factor type A domain (VWA) with a metal ion-dependent adhesion site (MIDAS) motif. Given the demonstrated role of tip pilin proteins containing VWA domains in cell adhesion and pathogenesis (27, 37, 40, 41) and our result that implicates EmpA in biofilm formation, ECM adherence, and UTI, our hypothesis is that this subunit plays an important role in endovascular infection. As shown in Fig. 7, we found that after monoinfection of TX82 and the $\Delta empA$ deletion mutant in a rat endocarditis model, the $\log_{10}$ numbers of CFU of bacteria recovered from the vegetations were significantly lower in the rats inoculated with the *empA* deletion strain than in those inoculated with TX82 ($P = 0.0088$) (Fig. 7), suggesting that EmpA is also important in the pathogenesis of endocarditis.

Considering that the *empABC* operon is found in the majority of isolates of clinical origin (96% to 100%, depending on the clinical source) but is also frequently found in nonclinical isolates (73% and 77% of fecal and animal isolates, respectively, harbor the *emp* operon) (11), Emp pili represent an excellent target for vaccine development. In addition, success in preventing endocarditis infection by a monoclonal antibody that targets the Ebp pilus of *E. faecalis* has been demonstrated (42). Furthermore, it was recently shown that immunization with EbpA, the counterpart of EmpA in *E. faecalis*, provides protection against catheter-associated bladder infections in mice (38). Our finding that EmpA is important for *E. faecium* biofilm formation, ECM adherence, and infection suggests that this pilin subunit could be a potential target for the development of alternative therapeutic approaches to counteract this multidrug-resistant pathogen.

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REFERENCES

1. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S, for the National Healthcare Safety Network (NHSN) Team and Participating NHSN Facilities. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. *Infect Control Hosp Epidemiol* 34:1–14. <http://dx.doi.org/10.1086/668770>.
2. Arias CA, Murray BE. 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* 10:266–278. <http://dx.doi.org/10.1038/nrmicro2761>.
3. Top J, Willems RJ, Blok H, de Regt M, Jalink K, Troelstra A, Goorhuis B, Bonten M. 2007. Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. *Clin Microbiol Infect* 13:316–319. <http://dx.doi.org/10.1111/j.1469-0691.2006.01631.x>.
4. Willems RJ, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *mBio* 3:e00151–12. <http://dx.doi.org/10.1128/mBio.00151-12>.
5. Leavis HL, Bonten MJ, Willems RJ. 2006. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 9:454–460. <http://dx.doi.org/10.1016/j.mib.2006.07.001>.
6. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS. 2013. Emergence of epidemic multi-drug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* e00534–13. <http://dx.doi.org/10.1128/mBio.00534-13>.
7. Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE. 2012. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. *PLoS One* 7:e30187. <http://dx.doi.org/10.1371/journal.pone.0030187>.
8. Nallapareddy SR, Singh KV, Murray BE. 2006. Construction of improved temperature-sensitive and mobilizable vectors and their use for constructing mutations in the adhesin-encoding *acm* gene of poorly transformable clinical *Enterococcus faecium* strains. *Appl Environ Microbiol* 72:334–345. <http://dx.doi.org/10.1128/AEM.72.1.334-345.2006>.
9. Willems RJ, Homan W, Top J, van Santen-Verheul M, Tribe D, Manzioros X, Gaillard C, Vandenbroucke-Grauls CM, Mascini EM, van Kregten E, van Embden JD, Bonten MJ. 2001. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. *Lancet* 357:853–855. [http://dx.doi.org/10.1016/S0140-6736\(00\)04205-7](http://dx.doi.org/10.1016/S0140-6736(00)04205-7).
10. Sillanpää J, Nallapareddy SR, Prakash VP, Qin X, Hook M, Weinstock GM, Murray BE. 2008. Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*. *Microbiology* 154:3199–3211. <http://dx.doi.org/10.1099/mic.0.2008/017319-0>.
11. Sillanpää J, Prakash VP, Nallapareddy SR, Murray BE. 2009. Distribution of genes encoding MSCRAMMs and pili in clinical and natural populations of *Enterococcus faecium*. *J Clin Microbiol* 47:896–901. <http://dx.doi.org/10.1128/JCM.02283-08>.
12. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. 2006. Pili in Gram-positive pathogens. *Nat Rev Microbiol* 4:509–519. <http://dx.doi.org/10.1038/nrmicro1443>.
13. Danne C, Dramsi S. 2012. Pili of Gram-positive bacteria: roles in host colonization. *Res Microbiol* 163:645–658. <http://dx.doi.org/10.1016/j.resmic.2012.10.012>.
14. Hendrickx AP, van Wamel WJ, Posthuma G, Bonten MJ, Willems RJ. 2007. Five genes encoding surface-exposed LPXTG proteins are enriched in hospital-adapted *Enterococcus faecium* clonal complex 17 isolates. *J Bacteriol* 189:8321–8332. <http://dx.doi.org/10.1128/JB.00664-07>.
15. Hendrickx A, Bonten MJ, van Luit Asbroek M, Schapendonk CM, Kragten AHM, Willems RJ. 2008. Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate. *Microbiology* 154:3212–3223. <http://dx.doi.org/10.1099/mic.0.2008/020891-0>.
16. Qin X, Galloway-Peña JR, Sillanpää J, Roh J, Nallapareddy SR, Chowdhury S, Bourgogne A, Choudhury T, Muzny DM, Buhay CJ, Ding Y, Dugan-Rocha S, Liu W, Kovar C, Sodergren E, Highlander S, Petrosino JF, Worley KC, Gibbs RA, Weinstock GM, Murray BE. 2012. Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. *BMC Microbiol* 12:135. <http://dx.doi.org/10.1186/1471-2180-12-135>.
17. Hendrickx AP, Schapendonk CM, van Luit-Asbroek M, Bonten MJ, van Schaik W, Willems RJ. 2010. Differential PilA pilus assembly by a hospital-acquired and a community-derived *Enterococcus faecium* isolate. *Microbiology* 156:2649–2659. <http://dx.doi.org/10.1099/mic.0.041392-0>.
18. Sillanpää J, Nallapareddy SR, Singh KV, Prakash VP, Fothergill T, Ton-That H, Murray BE. 2010. Characterization of the *ebp_{fm}* pilus-encoding operon of *Enterococcus faecium* and its role in biofilm formation and virulence in a murine model of urinary tract infection. *Virulence* 1:236–246. <http://dx.doi.org/10.4161/viru.1.4.11966>.
19. Almohamad S, Somarajan SR, Singh KV, Nallapareddy SR, Murray BE. 2014. Influence of isolate origin and presence of various genes on biofilm formation by *Enterococcus faecium*. *FEMS Microbiol Lett* 353:151–156. <http://dx.doi.org/10.1111/1574-6968.12418>.
20. Kristich CJ, Chandler JR, Dunne GM. 2007. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of *Enterococcus faecalis*. *Plasmid* 57:131–144. <http://dx.doi.org/10.1016/j.plasmid.2006.08.003>.
21. Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA. 2011. The *hyl_{Efm}* gene in pHyl_{Efm} of *Enterococcus faecium* is not required in pathogenesis of murine peritonitis. *BMC Microbiol* 11:20. <http://dx.doi.org/10.1186/1471-2180-11-20>.
22. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 4:2411–2423. <http://dx.doi.org/10.1002/pro.5560041120>.
23. Singh KV, Nallapareddy SR, Sillanpää J, Murray BE. 2010. Importance of the collagen adhesin Ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. *PLoS Pathog* 6:e1000716. <http://dx.doi.org/10.1371/journal.ppat.1000716>.
24. Chang C, Mandlik A, Das A, Ton-That H. 2011. Cell surface display of minor pilin adhesins in the form of a simple heterodimeric assembly in *Corynebacterium diphtheriae*. *Mol Microbiol* 79:1236–1247. <http://dx.doi.org/10.1111/j.1365-2958.2010.07515.x>.
25. Sillanpää J, Chang C, Singh K, Montealegre MC, Nallapareddy SR, Harvey BR, Ton-That H, Murray BE. 2013. Contribution of individual Ebp pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. *PLoS One* 8:e68813. <http://dx.doi.org/10.1371/journal.pone.0068813>.
26. Montealegre MC, La Rosa SL, Roh JH, Harvey BR, Murray BE. 2015. The *Enterococcus faecalis* EbpA pilus protein: attenuation of expression, biofilm formation, and adherence to fibrinogen start with the rare initiation codon ATT. *mBio* 6:e00467–5. <http://dx.doi.org/10.1128/mBio.00467-15>.
27. Konto-Ghiorgi Y, Mairey E, Mallet A, Duménil G, Caliot E, Trieu-Cuot P, Dramsi S. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. *PLoS Pathog* 5:e1000422. <http://dx.doi.org/10.1371/journal.ppat.1000422>.
28. Wolz C, McDéviat D, Foster TJ, Cheung AL. 1996. Influence of agr on fibrinogen binding in *Staphylococcus aureus* Newman. *Infect Immun* 64:3142–3147.
29. Singh KV, Nallapareddy SR, Murray BE. 2007. Importance of the *ebp* (endocarditis- and biofilm-associated pilus) locus in the pathogenesis of *Enterococcus faecalis* ascending urinary tract infection. *J Infect Dis* 195:1671–1677. <http://dx.doi.org/10.1086/517524>.
30. Singh KV, Coque TM, Weinstock GM, Murray BE. 1998. In vivo testing of an *Enterococcus faecalis* *efaA* mutant and use of *efaA* homologs for species identification. *FEMS Immunol Med Microbiol* 21:323–331. <http://dx.doi.org/10.1111/j.1574-695X.1998.tb01180.x>.

31. Kemp KD, Singh KV, Nallapareddy SR, Murray BE. 2007. Relative contributions of *Enterococcus faecalis* OG1RF sortase-encoding genes, *srtA* and *bps* (*srtC*), to biofilm formation and a murine model of urinary tract infection. *Infect Immun* 75:5399–5404. <http://dx.doi.org/10.1128/IAI.00663-07>.
32. Hendrickx APA, Willems RJL, Bonten MJM, van Schaik W. 2009. LPxTG surface proteins of enterococci. *Trends Microbiol* 17:423–430. <http://dx.doi.org/10.1016/j.tim.2009.06.004>.
33. Wu C, Mishra A, Yang J, Cisar JO, Das A, Ton-That H. 2011. Dual function of a tip fimbrillin of *Actinomyces* in fimbrial assembly and receptor binding. *J Bacteriol* 193:3197–3206. <http://dx.doi.org/10.1128/JB.00173-11>.
34. Dramsi S, Caliot E, Bonne I, Guadagnini S, Prévost MC, Kojadinovic M, Lalioui L, Poyart C, Trieu-Cuot P. 2006. Assembly and role of pili in group B streptococci. *Mol Microbiol* 60:1401–1413. <http://dx.doi.org/10.1111/j.1365-2958.2006.05190.x>.
35. Swierczynski A, Ton-That H. 2006. Type III pilus of corynebacteria: pilus length is determined by the level of its major pilin subunit. *J Bacteriol* 188:6318–6325. <http://dx.doi.org/10.1128/JB.00606-06>.
36. Zhao M, Sillanpää AJ, Nallapareddy SR, Murray BE. 2009. Adherence to host extracellular matrix and serum components by *Enterococcus faecium* isolates of diverse origin. *FEMS Microbiol Lett* 301:77–83. <http://dx.doi.org/10.1111/j.1574-6968.2009.01806.x>.
37. Izoré T, Contreras-Martel C, Mortaji El L, Manzano C, Terrasse R, Vernet T, Di Guilmi AM, Dessen A. 2010. Structural basis of host cell recognition by the pilus adhesin from *Streptococcus pneumoniae*. *Structure* 18:106–115. <http://dx.doi.org/10.1016/j.str.2009.10.019>.
38. Flores-Mireles AL, Pinkner JS, Caparon MG, Hultgren SJ. 2014. EbpA vaccine antibodies block binding of *Enterococcus faecalis* to fibrinogen to prevent catheter-associated bladder infection in mice. *Sci Transl Med* 6:254ra127. <http://dx.doi.org/10.1126/scitranslmed.3009384>.
39. Mandlik A, Swierczynski A, Das A, Ton-That H. 2007. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. *Mol Microbiol* 64:111–124. <http://dx.doi.org/10.1111/j.1365-2958.2007.05630.x>.
40. Whittaker CA. 2002. Distribution and evolution of von Willebrand/ integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. *Mol Biol Cell* 13:3369–3387. <http://dx.doi.org/10.1091/mbc.E02-05-0259>.
41. Nielsen HV, Guiton PS, Kline KA, Port GC, Pinkner JS, Neiers F, Normark S, Henriques-Normark B, Caparon MG, Hultgren SJ. 2012. The metal ion-dependent adhesion site motif of the *Enterococcus faecalis* EbpA pilin mediates pilus function in catheter-associated urinary tract infection. *mBio* 3:e00177-12. <http://dx.doi.org/10.1128/mBio.00177-12>.
42. Pinkston KL, Singh KV, Gao P, Wilganowski N, Robinson H, Ghosh S, Azhdarinia A, Sevik-Muraca EM, Murray BE, Harvey BR. 2014. Targeting pili in enterococcal pathogenesis. *Infect Immun* 82:1540–1547. <http://dx.doi.org/10.1128/IAI.01403-13>.
43. Leenhouts K. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 253:217–224. <http://dx.doi.org/10.1007/s004380050315>.