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EbpA vaccine antibodies block binding of *Enterococcus faecalis* to fibrinogen to prevent catheter-associated bladder infection in mice

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Abstract

Enterococci bacteria are a frequent cause of catheter-associated urinary tract infections, the most common type of hospital-acquired infection. Treatment has become increasingly challenging because of the emergence of multiantibiotic-resistant enterococcal strains and their ability to form biofilms on catheters. We identified and targeted a critical step in biofilm formation and developed a vaccine that prevents catheter-associated urinary tract infections in mice. In the murine model, formation of catheter-associated biofilms by *Enterococcus faecalis* depends on EbpA, which is the minor subunit at the tip of a heteropolymeric surface fiber known as the endocarditis- and biofilm-associated pilus (Ebp). We show that EbpA is an adhesin that mediates bacterial attachment to host fibrinogen, which is released and deposited on catheters after introduction of the catheter into the mouse bladder. Fibrinogen-binding activity resides in the amino-terminal domain of EbpA (EbpA^{NTD}), and vaccination with EbpA and EbpA^{NTD}, but not its carboxyl-terminal domain or other Ebp subunits, inhibited biofilm formation in vivo and protected against catheter-associated urinary tract infection. Analyses in vitro demonstrated that protection was associated with a serum antibody response that blocked EbpA binding to fibrinogen and the formation of a fibrinogen-dependent biofilm on catheters. This approach may provide a new strategy for the prevention of catheter-associated urinary tract infections.

INTRODUCTION

Catheter-associated urinary tract infections (CAUTIs) are the most common cause of hospital-acquired infections with the incidence of conversion from sterile urine to bacteriuria occurring at the rate of 3 to 10% per day (1–3). Furthermore, 3% of all patients with chronic indwelling urinary catheters will develop bacteremia within 30 days (4), and virtually all patients will develop an infection once the catheter has been in place >30 days (1–3). Drug

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SUPPLEMENTARY MATERIALS

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References (52–54)

resistance has become a critical concern for treatment of CAUTIs, particularly for infections caused by Gram-positive bacteria in the genus *Enterococcus*, which account for 15% of all CAUTIs (5). Because of their tolerance to heat, aseptic solutions, and intrinsic antibiotic resistance, enterococci have been difficult to control in the hospital environment (6–10). Of concern, their intrinsic resistances have been augmented by the emergence of strains resistant to nearly all antibiotics commonly used in treatment, including vancomycin. Treatment now has few options and often requires frequent removal and replacement of the catheter (1, 3, 11). Thus, the development of alternative therapies and prophylactic strategies is required.

A limiting factor for the development of new therapies has been the relatively poor understanding of the molecular details of enterococcal CAUTI pathogenesis. However, the ability of *Enterococcus faecalis* to cause CAUTIs is known to derive from its capacity to form biofilms on catheters, which allows the bacterium to persist in the bladder despite a robust inflammatory response (12–14). In animal models of CAUTIs, a long hair-like extracellular fiber known as the endocarditis-and biofilm-associated pilus (Ebp) has been shown to contribute to both biofilm formation and disease (15–18). A member of the sortase-assembled pilus family, the Ebp pilus is a heteropolymer composed of three subunits: the major shaft subunit (EbpC) and the minor sub-units at the base (EbpB) and tip (EbpA) of the fiber (19, 20). The enzyme sortase C (SrtC) catalyzes the formation of isopeptide bonds between EbpA to EbpC and among the EbpC subunits that make up the shaft and finally between the EbpC shaft and EbpB. Sortase A (SrtA) covalently attaches the mature fiber to peptidoglycan of the bacterial cell wall via EbpB (19, 20). In a murine model of CAUTIs, *E. faecalis* mutants lacking the EbpA tip protein are highly attenuated, as are strains that express a mutant EbpA containing a point mutation in the metal ion-dependent adhesion site (MIDAS) motif located in the von Willebrand factor A (vWA) domain of EbpA (18). In the same mouse model in the absence of the implanted catheter, *E. faecalis* is highly attenuated (18). Thus, on the basis of its location at the fiber tip and its importance in CAUTI pathogenesis, we hypothesized that EbpA is an adhesin that promotes attachment to the catheter surface. Paradoxically, when tested in vitro before implantation, wild-type *E. faecalis* (which expresses EbpA) was not able to adhere to the catheter and thus was unable to form a biofilm (fig. S1A). Thus, the nature of EbpA-host receptor interactions in catheter-associated biofilm formation and whether this interaction could be targeted for the development of new therapeutics are unknown.

Here, we elucidated how *E. faecalis* exploits the host inflammatory response caused by catheter implantation to establish and persist during CAUTIs. We found that host fibrinogen is released into the bladder upon catheterization as part of the host inflammatory response and subsequently accumulates in the bladder and becomes deposited on the implanted catheter. We discovered that EbpA's N-terminal domain mediated binding to fibrinogen. This resolved the paradoxical finding that the catheter was required for CAUTIs, even though enterococcus was unable to bind to the catheter material in vitro when grown in human urine. The N-terminal domain of EbpA is composed of a MIDAS-containing vWA domain, which is important for adhesion to extracellular matrix (ECM) proteins, and a fibrinogen-binding SdrG-like domain. The fibrinogen-binding interaction was found to be

crucial during *E. faecalis* CAUTIs and was abolished by a point mutation in the MIDAS motif. Further, we found that *E. faecalis* uses fibrinogen for growth, thus enhancing biofilm formation on the catheter. On the basis of these findings, we developed an EbpA-based vaccine that protected mice from *E. faecalis* infection by inhibiting the interaction between EbpA and fibrinogen.

RESULTS

The MIDAS motif in EbpA is necessary for *E. faecalis* biofilm formation

We first investigated the role of EbpA in biofilm formation using a tryptic soy broth supplemented with 0.25% glucose (TSBG) standard culture medium and an in vitro polyvinyl chloride (PVC) coverslip biofilm assay. We measured biofilm formation of a well-characterized collection of *E. faecalis* mutants constructed in the OG1RF strain (18, 19) that lacked either EbpA (EbpA, EbpAB, or EbpABC SrtC), lacked the ability to attach the pilus and other sortase substrates to the cell wall (SrtA), or expressed EbpA with an altered MIDAS motif designated as EbpA^{AWAGA}, based on alanine substitution mutations of three residues predicted to coordinate a metal ion (underlined) in the five-residue motif (Asp³¹⁵-Trp-Ser³¹⁷-Gly-Ser³¹⁹). These EbpA-altered mutants were compared to a mutant that expresses EbpA on the cell surface in the absence of the pilus shaft (EbpC) (19) and to wild-type *E. faecalis* OG1RF. All mutants lacking a wild-type surface-attached EbpA were defective for biofilm formation compared to *E. faecalis* OG1RF when analyzed after 48 hours of incubation under static conditions (Fig. 1A). This defect was attributed to the function of EbpA because the mutants lacking only EbpA or having the point mutations in the EbpA MIDAS motif (EbpA^{AWAGA}) were as defective in forming biofilms as any of the other mutants (EbpA, Fig. 1A). In contrast, formation of an EbpA-dependent biofilm did not require the major shaft protein (EbpC) (Fig. 1A). These data indicate that like CAUTI pathogenesis (18), EbpA facilitates biofilm formation by a mechanism that requires its MIDAS motif; however, the mechanism by which EbpA facilitates TSBG PVC biofilm formation is unknown.

The N-terminal domain of EbpA is required for fibrinogen binding

The typical function of a MIDAS motif in most proteins is to coordinate protein-protein interactions involved in cellular adherence (21). Thus, we hypothesized that it likely serves a similar function in EbpA. The Ebp pilus has been implicated in both collagen and fibrinogen binding (16), suggesting that these ECM proteins are possible receptors for EbpA. We investigated this hypothesis by comparing wild-type *E. faecalis* OG1RF to a pilus-deficient mutant (EbpABC SrtC) and an EbpA MIDAS mutant (EbpA^{AWAGA}) for their ability to bind to surfaces coated with collagen I or fibrinogen using an enzyme-linked immunosorbent assay (ELISA). This analysis revealed that whereas no strain had a defect for binding to collagen I (Fig. 1B), the loss of pili or presence of point mutations in the EbpA MIDAS motif significantly reduced binding to fibrinogen ($P < 0.0005$) (Fig. 1C). A computational search for known conserved domains predicted that the N-terminal domain of EbpA (200 to 577 amino acids) contains a region (99.96% confidence) that resembles blood clotting factor, which includes the MIDAS-containing vWA domain (Fig. 1D). Because blood clotting function is a well-studied feature of fibrinogen-binding proteins such as ClfB,

we hypothesized that the N-terminal domain of EbpA is implicated in fibrinogen binding. To test this, genetic constructs encoding truncated EbpA corresponding to its N-terminal (EbpA^{NTD}, residues 115 to 577) and C-terminal (EbpA^{CTD}, residues 594 to 1077) domains were designed (Fig. 1D). Each truncated version was found to be stable when expressed in *Escherichia coli* (fig. S2). Purified EbpA^{CTD} and EbpA^{NTD} were compared to wild-type EbpA (EbpA^{Full}) and the defective MIDAS EbpA^{AWAGA} (fig. S2) for their ability to bind to fibrinogen, as measured by ELISA. To detect all species of EbpA, we used a polyclonal mouse anti-EbpA^{Full} that recognized in similar level Full, NTD, and CTD EbpA proteins (fig. S3). Consistent with our model, we found that EbpA^{NTD} bound to fibrinogen at a level equivalent to EbpA^{Full}, whereas neither EbpA^{CTD} nor EbpA^{AWAGA} demonstrated any ability to bind to fibrinogen (Fig. 1E).

***E. faecalis* OG1RF interacts with released fibrinogen in the catheterized mouse bladder**

Urinary catheterization is associated with inflammation and edema even in the absence of infection (12, 22–24). Given that fibrinogen is a prominent component of inflammatory exudates (25, 26), we investigated whether fibrinogen might be present in the mouse bladder when catheterized. For this experiment, a 5-mm platinum-cured silicone tube was transurethrally implanted into the mouse bladder lumen to mimic catheterization. Paraffin-embedded sections of bladder tissue were prepared from mice that were sacrificed at 3, 6, 9, and 24 hours after catheter implantation. The sections were stained for fibrinogen (anti-fibrinogen antiserum), uroplakin III (anti-UpIII to delineate the superficial umbrella cells) (27), and 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Analysis of nonimplanted bladders by immunofluorescence microscopy revealed an intact urothelium with no detectable fibrinogen at any time point. However, upon catheter implantation, fibrinogen was detected in the lumen of the bladder at 3 and 6 hours after catheter implantation, although the urothelium appeared intact. By 9 and 24 hours after catheter implantation, the urothelium appeared compromised with significant accumulation of fibrinogen (Fig. 2A). In addition, direct immunofluorescence staining of recovered catheters as early as 1 hour after catheter implantation revealed extensive deposition of fibrinogen, which increased in a time-dependent fashion (Fig. 2B).

To further understand the interaction between *E. faecalis* and fibrinogen during CAUTIs, implanted animals were then challenged with 1×10^7 colony-forming units (CFU) of *E. faecalis* OG1RF, and 24 hours after implantation, catheters and bladder tissue were recovered and stained for fibrinogen and *E. faecalis* (goat anti-fibrinogen and rabbit anti-streptococcal group D antigen, respectively). Examination by immunofluorescence microscopy revealed that the wild-type strain colocalized with fibrinogen on both the catheter and the urothelium (OG1RF; Fig. 3, A and B). In contrast, the mutant lacking pili (EbpABC SrtC) and a MIDAS motif mutant (EbpA^{AWAGA}) were not detected on either surface (Fig. 3B). These data reveal that EbpA-fibrinogen interactions are a critical component of *E. faecalis* CAUTIs.

Fibrinogen is a key element of *E. faecalis* OG1RF growth and biofilm formation in urine

To determine the importance of EbpA-fibrinogen interactions on catheter biofilm formation in the bladder, biofilm formation was examined on catheters in vitro in the presence of

human female urine. Paradoxically, *E. faecalis* OG1RF grew poorly (0.5 log from the initial inoculum $\sim 5 \times 10^5$ CFU/ml) in urine (Fig. 4A) and failed to form catheter-associated biofilms even when supplemented with glucose as an additional energy source (fig. S1B). Because urine is the principal host component that *E. faecalis* is exposed to during CAUTIs, this raised the question whether *E. faecalis* actively replicates during murine CAUTIs or if the disease is the result of nonreplicating persistence of the original inoculum. This was tested using a method that monitors the segregation of a plasmid that cannot replicate at in vivo temperature (28). In actively dividing cells, the nonreplicating plasmid is asymmetrically passed to daughter cells and is lost from the population over time. This analysis revealed that the percentage of bacterial cells maintaining the plasmid during CAUTIs dropped to nearly undetectable levels by 24 hours after infection in the bladder (fig. S4), as would be expected for rapidly dividing cells. In vitro, when urine was supplemented with fibrinogen, we found that *E. faecalis* OG1RF consumed the fibrinogen to grow (Fig. 4, E and F) and formed catheter-associated biofilms (Fig. 4, A and B), thus resolving the paradox. BSA or casamino acids also supported growth (Fig. 4, A, C, and D), but only fibrinogen was capable of promoting biofilm formation on catheters (Fig. 4G). Furthermore, similar to CAUTI biofilm formation in vivo (Fig. 3B), the ability to form biofilms in vitro (96-well polystyrene microplates) in fibrinogen-supplemented urine was lost in the mutant in which the MIDAS motif of EbpA was rendered nonfunctional (EbpA^{AWAGA}; Fig. 4, H and I).

Immunization with EbpA^{NTD} protected mice from *E. faecalis* CAUTIs

The data above revealed that the interaction between EbpA^{NTD} and fibrinogen was a critical component of catheter biofilm formation by *E. faecalis*. Thus, we hypothesized that an intervention that blocks this interaction would be protective against development of *E. faecalis* CAUTIs. Therefore, we investigated the efficacy of immunizing mice with purified EbpA^{Full} emulsified in Freund's complete adjuvant at doses ranging from 0.3 to 100 μ g, with booster immunizations corresponding to the original dose on weeks 4 and 8. We found that immunization with EbpA^{Full}, but not phosphate-buffered saline (PBS) with adjuvant, generated high titers of anti-EbpA antibodies as early as 2 weeks after immunization (fig. S5A). This immunogenic response was long-lasting and was still vigorous when examined 13 weeks after vaccination with titers correlating with EbpA dose (fig. S5B). Four weeks after the second boost, mice were implanted with catheters and challenged with 2×10^7 CFU of *E. faecalis* OG1RF. We found that even at the lowest EbpA dose, vaccination significantly reduced bacterial burdens in both the bladder and catheter by ~ 4 logs compared to control mice receiving only PBS with adjuvant ($P < 0.0005$) (Fig. 5, A and B). Furthermore, direct immunofluorescence staining of the recovered catheters revealed that whereas *E. faecalis* colocalized with fibrinogen deposition in PBS control mice, vaccination with EbpA^{Full} in adjuvant reduced bacterial accumulation to undetectable levels (Fig. 5C).

To examine whether protection correlated with fibrinogen binding, additional mice were vaccinated with pilus subunits EbpB and EbpC and the EbpA^{CTD} and EbpA^{NTD} truncated versions. We found that all proteins produced a strong immunogenic response (fig. S6, A to D and I), lasting through the time of the experiment (fig. S6, E to H and J). Upon challenge, immunization with EbpB and EbpC and the EbpA^{CTD} failed to provide protection, with

bacterial burdens in bladders and catheters similar to the PBS control. However, immunization with EbpA^{NTD} was as effective as EbpA^{Full} in reducing bladder and catheter bacterial burdens ($P < 0.0005$) (Fig. 5, D and E). To confirm this result, we tested the binding of purified EbpA variant proteins to immobilized human fibrinogen in the presence of antisera from EbpA^{CTD}-, EbpA^{Full}-, or EbpA^{NTD}-vaccinated mice. We found that sera from EbpA^{Full}- and EbpA^{NTD}-vaccinated mice effectively blocked the interaction in contrast to sera from EbpA^{CTD}-vaccinated mice, which had no effect (Fig. 5F). Together, these data implicate a protective antibody response that targets a critical EbpA-fibrinogen interaction that is required for subsequent formation of catheter biofilm formation and disease.

DISCUSSION

Here, we have identified an essential step in the pathogenesis of *E. faecalis* CAUTIs and exploited this knowledge for the development of a new therapeutic that prevents disease in a murine model (fig. S7). We have shown that this bacterium, via its Ebp pilus, can persist by taking advantage of the host inflammation response and the associated deposition of fibrinogen to enhance biofilm formation and growth. Previously, we have shown that interleukin-1 α (IL-1 α) and IL-6 were elevated during catheter implantation and that these inflammatory cytokines were further increased about twofold when *E. faecalis* was present (12). These cytokines have been shown to trigger the expression of fibrinogen in the liver and its release into the circulatory system (29–32), where it subsequently may be able to leak into the bladder because of tissue damage caused by the catheter (fig. S7). The importance of inflammation during CAUTI pathogenesis is illustrated by the fact that in the absence of catheter-induced inflammation, *E. faecalis* OG1RF is cleared from bladders within a few days (14), possibly due to nutritional deprivation, immune surveillance, or both. We discovered that growth of *E. faecalis* OG1RF in urine requires a protein source (fig. S7). Proteinuria is induced upon catheterization but also as the result of renal damage, chronic kidney disease, and diabetes (33–35), which could make patients susceptible to infection (36–39). Although *E. faecalis* CAUTIs induces neutrophil infiltration in the bladder, the infection is not cleared (12). Thus, activation of the immune response provides a milieu that supports *E. faecalis* growth, whereas biofilm formation and/or other pathogenic mechanisms may allow *E. faecalis* to simultaneously evade the immune response. Analysis of the role of fibrinogen in nutrient acquisition and in inflammation may further elucidate these mechanisms.

EbpA contributed to biofilm formation, if standard TSBG medium was used, on several different materials including PVC, polystyrene, and silicon, but not under urine conditions. Under urine conditions, biofilm formation required both EbpA and the presence of fibrinogen. Thus, differences in biofilm formation were mostly dependent on the media and not the surface of the catheter. Accordingly, biofilm formation in vitro in TSBG did not accurately reflect the requirements for CAUTIs in vivo. These results also explain our previous findings in which genes required for biofilm formation in TSBG in vitro were not required in forming biofilms on catheters in vivo, arguing that the TSBG assay is not readily translatable to CAUTIs (14, 40). They also highlight that in vitro efforts to study factors important for biofilm formation in vivo require an assay that most closely mimics conditions

encountered in vivo. Whereas EbpA is involved in biofilm formation in the standard TSGB assay, the biofilms formed are not robust and are markedly improved using fibrinogen-supplemented urine. Furthermore, it has been shown that pH can influence biofilm formation by the fungus *Aureobasidium pullulans* when PVC surfaces are used (41). It is possible that pH-mediated differences in surface charge can explain EbpA's differential contribution to biofilm formation in TSGB versus fibrinogen-supplemented urine. However, this phenomenon has to be further characterized. Together, these observations validate that the fibrinogen-supplemented urine biofilm assay more accurately reproduces in vivo CAUTI conditions and should be of utility for further analysis of enterococcal biofilm formation in disease. Fibrinogen is a complex glycoprotein comprising two sets of disulfide-bridged A α , B β , and γ chains (32). It is thus possible that EbpA may recognize fibrinogen via either its carbohydrate or its peptide moieties. Given that MIDAS-containing vWA domains mediate protein-protein interactions (42), we predict that the EbpA-fibrinogen interaction will also be a protein-protein interaction.

We also show that the EbpA-fibrinogen interaction is a vulnerable step that is sensitive to therapeutic intervention. It remains to be determined if this approach will also be protective for humans. However, bladder catheterization of humans does result in inflammation (22, 43–45), and fibrinogen has been linked to multiple human inflammatory diseases where it functions not only to protect tissues but also as an important feedback regulator of the immune response (25).

It has been shown that hospitalized patients develop antibodies that recognize Ebp pili, although there is no evidence that these antibodies are protective in humans (15). A recent study showed that a monoclonal antibody against EbpC confers protection on rats against *E. faecalis* endocarditis (46). In our mouse model of CAUTIs, we tested EbpC as a vaccine candidate and found that it did not confer protection against *E. faecalis* CAUTIs. Only EbpA and more specifically EbpA^{NTD} were protective against CAUTIs. This difference emphasizes that it is essential to understand the underlying molecular mechanisms of host-pathogen interactions made by *E. faecalis* to properly intervene in a specific disease. A comparison of CAUTI pathogenesis uncovered in this study with endocarditis may reveal why EbpC was protective in the latter but not the former. Here, we elucidated that the molecular basis of protection by the EbpA vaccine was due to the disruption of an interaction that is critical in pathogenesis, between EbpA and fibrinogen. The mechanism of protection induced in response to EbpC vaccination for endocarditis remains to be determined. If our discovery could be translated to clinical application, it would offer a prophylactic option to prevent *E. faecalis*-mediated CAUTIs. However, development of such a technology will require significant resources to investigate whether translation to humans is possible and economically feasible. Additionally, although EbpA^{NTD} is highly conserved, this study was focused on EbpA from *E. faecalis*; therefore, a broad panel of different enterococcal strains would need to be tested before clinical translation.

Our finding that protection is only associated with EbpA^{NTD} suggests that the Ebp pilus has evolved a decoy strategy to sequester its vulnerable EbpA^{NTD} from immune surveillance during CAUTIs. It is likely that the identification of this vulnerability will provide insight into the pathogenesis of other *E. faecalis* catheter-related diseases and will reveal general

mechanisms applicable to catheter infections caused by other bacterial pathogens that can also be exploited for the development of effective therapies.

MATERIALS AND METHODS

Study design

For experiments involving mice, we used 6-week-old female mice, and 10 mice were used for each treatment because in our experience 10 mice is the minimum number of mice needed to observe statistically significant differences per treatment. We used an optimized murine model of *E. faecalis*-mediated CAUTIs that faithfully reproduces the pathogenesis of human disease, by trans-urethral implantation of a 5-mm platinum-cured silicone tube into the bladder lumen. For immunohistochemistry of mouse bladder and immunofluorescence staining of the catheter, implanted and infected mice were randomly taken from the 10 mice treated group. Then, bladder and catheters were immunostained for *E. faecalis* and fibrinogen to visualize colocalization. For the vaccination experiments, we monitored the production of antibodies against Ebp proteins in treated mice; blood samples were taken from each mouse every week for the time period of the experiment and evaluated by ELISA. For the in vitro urine growth and biofilm experiments, we collected and pooled urine from at least three human female donors between 20 and 35 years of age who were healthy and excluding those with kidney disease, a current UTI, or those undergoing antibiotic treatment. All assays were done at least three times in triplicate. This study was not blinded.

Bacterial strains and growth conditions

Unless otherwise specified, *E. faecalis* strains OG1RF and its derivatives were grown overnight on Brain Heart Infusion (BHI) broth (BD Company) supplemented with rifampin (25 µg/ml) (Sigma-Aldrich) and fusidic acid (25 µg/ml) (Sigma-Aldrich) and were inoculated from a single bacterial colony grown on BHI agar plates supplemented with rifampin and fusidic acid. Liquid cultures were grown statically at 37°C for 18 hours. *E. coli* strains were grown in LB broth or agar (Becton, Dickinson and Company) supplemented with ampicillin (100 µg/ml) and kanamycin (100 µg/ml). Bacterial strains are listed in table S1.

General cloning techniques

Bacterial genomic DNA (gDNA) was isolated with the Wizard Genome DNA purification kit (Promega Corp.). Plasmid DNA was purified with the Wizard Plus SV Minipreps DNA Purification System (Promega Corp.). Primers were purchased from Integrated DNA Technologies. Phusion High-Fidelity DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs and used according to the methods described by the manufacturers. DNA fragments generated by polymerase chain reaction (PCR) or restriction digestion were purified by QIAquick PCR or Gel Extraction kit (Qiagen Inc.). Plasmids were transformed into *E. coli* TOP10 or M15 (pREP4). All constructs were confirmed by DNA sequence analysis of the inserts. Plasmids and primers used in this study are listed in tables S2 and S3, respectively.

Construction of plasmids for the expression of 6xHis-EbpA^{Full}, 6xHis-EbpA^{AWAGA}, and 6xHis-EbpA^{NTD} proteins

DNA fragments containing *ebpA* and *ebpA*^{NTD} were amplified by PCR from gDNA of *E. faecalis* OG1RF (SJH-1994). A fragment containing *ebpA*^{AWAGA} was amplified from an *E. faecalis ebpA*^{AWAGA} chromosomal MIDAS motif mutant (SJH-2001) with primers ALFM01 and ALFM02. The full amino acid sequence of EbpA was analyzed to detect structural domains. The EbpA signal sequence was predicted to be composed of amino acids 1 to 34 with SignalP4.1 server. Additionally, we deduced that the vWA domain was located within residues 200 to 385. On the basis of the flexibility and hydrophobicity analyses of EbpA, we determined that starting from amino acid 115 would result in a soluble protein that would not disturb the vWA domain. Amplified genes were close to full length, starting from 115 until 1072 amino acids, missing C-terminal hydrophobic and positively charged domains. The resulting PCR products were inserted into pQE-30Xa between its Bam HI and Nhe I restriction sites. The resulting plasmids pSJH-687 (*ebpA*) and pSJH-688 (*ebpA*^{AWAGA}) were used to transform *E. coli* M15 (pREP4) to create strains SJH-2610 and SJH-2611, respectively. ALFM51 and ALFM52 were used to obtain an *ebpA*^{NTD} fragment with pSJH-2610 as a template. The whole plasmid was amplified except the C-terminal domain (encoding amino acid residues 594 to 1072). The resulting fragment was digested Eco RV and then inserted into pQE-30Xa by ligation with Blunt/T4 ligase master mix, creating plasmid pSJH-689.

Purification of 6xHis-EbpA^{Full}, 6xHis-EbpA^{AWAGA}, and 6xHis-EbpA^{NTD} proteins

To overexpress proteins, *E. coli* M15 (pREP4) containing plasmid pSJH-687, pSJH-688, or pSJH-689 were cultured in 5 liters of fermentor vessel containing Super Broth at 37°C. When an OD₆₀₀ (optical density at 600 nm) of 4.0 was attained, cultures were induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside and incubated for an additional 1.5 hours. Cells were harvested by centrifugation for 10 min at 4°C. The pellets were suspended in 1× PBS with 250 mM NaCl and disrupted by sonication (Fisher, sonic dismembrator). The lysates were cleared by centrifugation at 18,600g for 30 min at 4°C and filtered with a 0.22-μm GP Millipore Express Plus membrane (SCGPT02RE, Millipore Inc.). EbpA proteins were purified by chromatography with a Talon cobalt affinity column (Clontech Inc.).

Fractions containing EbpA or EbpA^{AWAGA} were pooled and subjected to additional purification by hydrophobic interaction chromatography (PHE15 source; GE Healthcare). Fractions containing EbpA or EbpA^{AWAGA} were pooled and dialyzed against 20 mM tris (pH 8.0), followed by a chromatography with a Source 15Q column (GE Healthcare). For further purification of EbpA^{NTD} protein, fractions from the Talon column were pooled and dialyzed against 20 mM tris (pH 8.0), followed by chromatography over an SP Sepharose Fast Flow column (GE Healthcare). Fractions containing EbpA^{NTD} were pooled and dialyzed against 20 mM tris (pH 8.0) followed by a chromatography with a Source 15Q column (GE Healthcare). EbpA proteins were concentrated with an Amicon Ultra cell with YM-10 filter membrane (10,000 molecular weight cutoff; EMD Millipore Inc.).

Purification of 6xHis-EbpA^{CTD}, 6xHis-EbpB, and 6xHis-EbpC proteins

Overexpression and purification of these proteins were done by following the protocol used by Nielsen *et al.* (18).

Coverslip biofilm assay

E. faecalis strains were grown overnight, and then the cultures were diluted to OD₆₀₀ of 0.2 in BHI broth, followed by 1:100 dilution in tryptic soy broth supplemented with 0.25% glucose (TSBG). Biofilm assay was performed as previously described (40). Bacterial cells were allowed to attach to the polyvinyl chloride coverslips for 48 hours at 37°C under static conditions. Coverslips were washed with sterile water and then stained with 0.5% crystal violet (Sigma-Aldrich) for 10 min at room temperature. Excess dye was removed by rinsing with sterile water, and the coverslips were allowed to dry at room temperature. Biofilms were then dissolved with 500 µl of 33% acetic acid (Fisher Scientific), and the absorbance (OD₅₉₅) was measured with a microplate reader (Molecular Devices). Experiments were performed independently in triplicate with three coverslips per condition per experiment.

Urine biofilm assay on silicon catheters and 96-well polystyrene plates

E. faecalis strains were grown overnight, and then the cultures were diluted to an OD₆₀₀ of 0.2 in BHI broth. The diluted culture was centrifuged and washed (three times) with 1× PBS, followed by 1:100 dilution in urine supplemented with indicated source of protein or in TSBG as a standard control. Bacterial cells were allowed to attach to the silicon catheters (1 cm, Nalgene 50 silicon tubing, Brand Products) or 96-well polystyrene plates (Greiner CELLSTAR) for 48 hours at 37°C under static conditions. Silicon catheters and 96-well polystyrene plate were washed with sterile water and then stained with 0.5% crystal violet for 10 min at room temperature. Excess dye was removed by rinsing with sterile water, and the coverslips were allowed to dry at room temperature. Biofilms were then dissolved with 500 or 200 µl of 33% acetic acid for silicon catheters or 96-well polystyrene, respectively, and the absorbance (OD₅₉₅) was measured with a microplate reader (Molecular Devices). Experiments were performed independently in triplicate with three coverslips per condition per experiment. Urine was pooled from three healthy female donors, clarified by centrifugation, filter-sterilized, and adjusted to pH 6.5 before use.

E. faecalis OG1RF growth in urine

Bacterial growth from overnight cultures was normalized to an OD₆₀₀ of 1.0 in BHI broth. Bacterial cells were then washed (three times) with 1× PBS to remove traces of BHI broth, followed by 1:1000 dilution into undiluted pooled female urine (adjusted to pH 6.5), and, when indicated, supplemented with Fg, BSA, or casamino acids. Bacterial growth was monitored by quantifying CFU. To examine the consumption of Fg by *E. faecalis*, bacteria were grown in the presence of Fg and incubated for 24 hours at 37°C, and samples were then analyzed by negative staining and transmission electron microscopy as below.

Transmission electron microscopy

Cells were fixed in 4% paraformaldehyde–0.5% glutaraldehyde in 100 mM Pipes–0.5 mM MgCl₂ (pH 7.2) for 1 hour at 4°C. Cells were absorbed to grids, washed in distilled water,

and stained with 1% uranyl acetate–1.6% methylcellulose. Excess liquid was gently removed and grids were allowed to air dry. Cells were analyzed on a JEOL 1200EX transmission electron microscope at an accelerating voltage of 100 kV. Images were acquired with an XR80M-B 8-megapixel charge-coupled device camera system (Advanced Microscopy Techniques Corp.).

Antibodies used in this study

Primary antibodies—Goat anti-fibrinogen (Sigma-Aldrich); mouse anti-uropod III (Research Diagnostics); rabbit anti-*Streptococcus* group D antigen (Lee Laboratories); and mouse anti-EbpA^{Full}, mouse anti-EbpA^{NTD}, mouse anti-EbpA^{CTD}, anti-EbpC, and anti-EbpB were generated in this study; rabbit anti-EbpA^{CTD} was made from C-terminal domain (EbpA^{CTD}) (18).

Secondary antibodies—Alexa Fluor 488–labeled donkey anti-goat, Alexa Fluor 594–labeled donkey anti-mouse, Alexa Fluor 647–labeled donkey anti-rabbit, IRDye 800CW donkey anti-goat, and IRDye 680LT goat anti-rabbit were used. Alexa Fluor secondary antibodies were purchased from Invitrogen Molecular Probes, and IRDye conjugate secondary antibodies were from LI-COR Biosciences.

Whole bacterial binding to collagen I and fibrinogen

To determine whether *E. faecalis* strains adhere to immobilized collagen I and fibrinogen, we adapted the method described by Nallapareddy *et al.* (16). Immulon 4 HBX flat-bottom microplates (Thermo Fisher) were coated overnight at 4°C with human collagen I (100 µg/ml) (BD Biosciences) and human fibrinogen free from plasminogen and von Willebrand factor (Enzyme Research Laboratory). The plates were blocked for an hour with 5% milk in PBS, followed with PBS washes (three times for 5 min). Bacteria strains were grown overnight in BHI broth, normalized to an OD₆₀₀ of 1.0, and then washed and resuspended in PBS. A total of 100 µl of bacteria was added to the coated wells and incubated for an hour at 37°C, followed by PBS washes with the wash function of a microplate reader (ELX405 Select CW, BioTek Instruments) to remove the unbound bacteria. Next, bacterial cells were fixed with formalin for 20 min at room temperature, followed by three washes with PBS containing 0.05% Tween 20 (PBS-T). Then, the plates were blocked overnight at 4°C with 5% milk PBS-T (PBS-T5M), followed by three washes with 1% milk PBS-T (PBS-T1M). After the washes, the plates were incubated for an hour at room temperature with rabbit anti-*Streptococcus* group D antigen antisera (1:500). Plates were washed with PBS-T1M, incubated with the Odyssey secondary antibody (goat anti-rabbit IRDye 680LT, diluted 1:10,000) for 45 min at room temperature, and washed with PBS-T (three times). As a final step, the plates were scanned for infrared signal with the Odyssey Imaging System (LI-COR Biosciences).

EbpA protein binding to fibrinogen

Purified EbpA^{WT}, EbpA^{AWAGA}, EbpA^{NTD}, and EbpA^{CTD} were tested for Fg binding. Plates were coated with Fg, blocked, and washed as described above. When indicated, EbpA proteins were incubated with sera from vaccinated mice or with rabbit anti-EbpA^{CTD} (18) for an hour at room temperature before the fibrinogen-EbpA binding assay. EbpA proteins

were incubated with Fg for an hour at room temperature. Mouse anti-EbpA^{Full} was used as primary antibody to detect bound EbpA. The use of anti-EbpA^{Full} for detection of Full, NTD, and CTD EbpA proteins was validated by ELISA and shown to recognize all of these proteins at similar levels (fig. S6). Similarly, it was confirmed that anti-EbpA^{NTD} and anti-EbpA^{CTD} specifically recognized NTD or CTD, respectively, and both recognized EbpA^{Full}. Goat anti-mouse or anti-rabbit IRDye secondary antibodies were used, and analyses were done with Odyssey Imaging System (LI-COR Biosciences) as described above.

Determination of antibody responses in mice

Each protein (10 µg) was used to coat Immulon 4 HBX flat-bottom microplates overnight at 4°C. Plates were washed (three times) using PBS with 0.05% Tween 20 (PBS-T) to remove unbound protein. The plates were blocked for 2 hours with PBS containing 1.5% BSA and 0.1% sodium azide and washed (three times) with PBS-T. Serum samples from vaccinated mice were diluted 1:100 in dilution buffer (PBS with 0.05% Tween 20, 0.1% BSA, and 0.5% methyl α-D-mannopyranoside) before serial dilutions. Then, diluted samples were added into the plate and incubated for 2 hours at room temperature. After the incubation, the plates were washed (three times) with PBS-T, followed by a 1-hour incubation with horseradish peroxidase-conjugated goat anti-mouse antisera (1:2000), and then washed (three times) with PBS-T. Detection was performed with the TMB Substrate Reagent Set (BD #555214). The reactions were incubated for 5 min to let color to develop and then stopped by the addition of 1.0 M sulfuric acid. The absorbance was determined at 450 nm.

Bioinformatic analyses

Initial amino acid sequence analyses for domain structure of EbpA protein were done by BLAST and DELTA-BLAST (47, 48). Molecular modeling of structures used resources available from the PHYRE2 server (49).

Mouse catheter implantation and infection

Mice used in this study were 6-week-old female wild-type C57BL/6Ncr mice purchased from the National Cancer Institute. Mice were transurethrally implanted and inoculated as previously described (14). Mice were anesthetized by inhalation of isoflurane and implanted with a 4- to 5-mm lengths of platinum-cured silicone catheter. When indicated, mice were infected immediately after catheter implantation with 50 µl of $\sim 1 \times 10^7$ or 2×10^7 CFU of bacteria in $1 \times$ PBS directly into the bladder by transurethral inoculation as previously described (50). To extract the catheters and organs, mice were sacrificed at desired time points by cervical dislocation after anesthesia inhalation, and the bladders were aseptically harvested. Subsequently, the silicone implant was retrieved from the bladder when present. All studies and procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Immunization of mice

Immunization protocol was adapted from the method described by Langermann *et al.* (51). Groups of 10 mice were used for each vaccine dose. EbpA proteins or PBS was emulsified with either Freud's complete adjuvant (first vaccination) or Freud's incomplete adjuvant

(boosts). Mice were vaccinated intramuscularly with the various doses indicated in the test (time 0) and then with the same dose at 4 and 8 weeks. Mouse sera were collected before and every week after time 0 to determine the antibody response. At 4 weeks after the second boost, mice were implanted with catheters and challenged with $\sim 1 \times 10^7$ CFU of *E. faecalis* OG1RF. Mice were sacrificed 24 hours after infection to determine bacterial titers in the bladder and catheters, as described above.

Deposition of fibrinogen in implanted catheters

Catheters were transurethrally implanted into the mouse bladder as described above. Nonimplanted catheters were used as a control for any autofluorescence. Catheters were retrieved from implanted mouse bladder at specified time points (3, 6, 9, and 24 hours), washed with PBS, fixed with formalin for 20 min, and then washed with PBS. Catheters were blocked with PBS-T5M overnight at 4°C, washed with PBS-T1M (three times for 5 min), and incubated at room temperature for 2 hours with primary antibody (goat anti-fibrinogen; 1:500). The catheters were then washed with PBS-T1M (three times for 5 min), incubated with the Odyssey secondary antibody (donkey anti-goat IRDye 800CW; diluted 1:10,000) for 45 min at room temperature, and washed with PBS-T (three times). As a final step, the catheters were scanned for infrared signal with the Odyssey Imaging System (LI-COR Biosciences).

Histopathology and immunofluorescence

Bladders were fixed in neutral buffered formalin for 1 to 2 hours at room temperature and dehydrated in 70% ethanol overnight at 4°C. Fixed bladders were embedded in paraffin, sectioned, and mounted on slides. All sections were deparaffinized with xylene (two times for 10 min), rehydrated with isopropanol (three times for 5 min), and washed with water for 5 min. Bladder antigens were retrieved by boiling the section in 10 mM Na-citrate for 30 min and washed in water for 5 min, followed by washes with PBS (three times for 5 min). The sections were then blocked with 1% BSA and 0.3% Triton X-100 in PBS for 1 hour and incubated with primary antibodies (1:100) overnight at 4°C, followed by three washes with PBS. Next, sections were incubated with the secondary antibodies (1:500) for 1 hour at room temperature, followed by three washes with PBS. Sections were then counterstained with Hoechst dye specific for DNA (1:20,000 in PBS). The sections were analyzed by epifluorescence microscopy on a Zeiss Axioskop 2 MOT Plus microscope.

Statistical analyses

Data from multiple experiments were pooled. Two-tailed Mann-Whitney *U* tests were performed with GraphPad Prism 5 software (GraphPad Software) for all comparisons described in CAUTI experiments. Biofilm formation and binding assay were analyzed with a paired *t* test to evaluate the significance of differences. Values represent means $\pm$ SEM derived from at least three independent experiments. **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; ns, differences not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. EbpA^{NTD} is critical for biofilm formation and fibrinogen recognition

(A) *E. faecalis* strains with deletions () of Ebp pilus subunits and Gram-positive bacterial assembly proteins were evaluated for biofilm formation in a standard in vitro polyvinyl chloride coverslip assay after 48 hours by staining with crystal violet. The EbpA MIDAS motif mutant was designated as EbpA^{AWAGA}. (B and C) Adherence of the indicated whole bacterial strains to (B) collagen I (Col I)-coated or (C) fibrinogen (Fg)-coated surfaces was assessed by ELISA using a rabbit anti-group D streptococcal antibody. (D) EbpA domain structure, as predicted by PHYRE2 software (47). S, signal sequence; CWSS, C-terminal cell wall sorting signal; vWA, von Willebrand factor A domain containing a MIDAS motif. Shown below the figure are the regions included in the indicated EbpA subdomain proteins. (E) ELISA assay to quantitate binding of the indicated purified proteins to immobilized fibrinogen using a mouse anti-EbpA^{Full} antisera. All assays used human collagen I and fibrinogen. Data represent means $\pm$ SEM derived from at least three independent experiments with differences between mean values evaluated for significance using a paired *t* test: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; ns (differences not significant), *P* > 0.05.

Fig. 2. Time course of fibrinogen release and deposition on catheters implanted in mouse bladders

Mice were implanted with catheters and challenged by 1×10^7 CFU *E. faecalis* OG1RF. (A and B) At the indicated time points after infection, bladder tissue (A) and catheters (B) were recovered and subjected to analysis by immunofluorescence using antibody staining to detect fibrinogen (anti-Fg; green). Samples were costained with antibody to detect uroplakin III (anti-UpIII; red) or with DAPI (blue) to delineate the urothelium and cell nuclei, respectively (representative images). The white broken line separates the bladder lumen (L) from the urothelium surface (U).

Fig. 3. Colocalization of *E. faecalis* EbpA with fibrinogen in catheter-implanted mouse bladders Catheter-implanted mice were challenged with the indicated *E. faecalis* OG1RF (infected) or were unchallenged (uninfected). (A and B) Bladder tissue (A) and catheters (B) were analyzed after 24 hours of implantation by immunofluorescence using antibody staining to detect fibrinogen (anti-Fg; green) and *E. faecalis* (anti-group D; pink). Antibody staining for uroplakin III (anti-UpIII; red) or with DAPI (blue) delineated the urothelium and cell nuclei, respectively. (A) Mice were infected with *E. faecalis* OG1RF (wild type) (representative images). (B) Mice were infected with *E. faecalis* OG1RF (wild type) or by mutants that did not express pili (Δ EbpABC Δ SrtC) or that expressed EbpA with a defective MIDAS motif (EbpA^{AWAGA}). Bacterial EbpA colocalization with fibrinogen was compared to that for mock-infected mice (PBS control) (representative images). The white broken line separates the bladder lumen (L) from the urothelium surface (U).

Fig. 4. *E. faecalis* OG1RF biofilm formation in human urine requires fibrinogen

Bacterial growth was determined by the number of CFU after 24 hours (or otherwise indicated) of culture in human urine alone or in urine supplemented with the indicated concentrations of fibrinogen (Fg), bovine serum albumin (BSA), or casamino acids (CA). (A) Cultures were inoculated to an initial density of $\sim 5 \times 10^5$ CFU/ml. (B to D) Growth curves in urine over a range of concentrations of fibrinogen (B), BSA (C), or CA (D). (E) Examination of uninoculated fibrinogen-supplemented urine by negative staining with 1% uranyl acetate and electron microscopy revealed lattice-like structures that were consumed after 24 hours of culture with *E. faecalis*. Scale bar, 500 nm. (F) Biofilm formation in vitro on 1-cm silicon catheters in urine supplemented with fibrinogen and BSA. Scale bar, 500

nm. (**G** to **I**) Biofilm formation in a standard 96-well polystyrene plate assay comparing wild-type (OG1RF) and MIDAS mutant (EbpA^{AWAGA}) strains in fibrinogen-supplemented (**H**) or either BSA- or CA-supplemented urine (**I**). Biofilm formation in a standard growth medium (TSGB) was included for comparison. Values represent means $\pm$ SEM derived from at least three independent experiments with differences between mean values evaluated for significance using a paired *t* test: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; ns, *P* > 0.05. Human urine was pooled from three healthy female donors, clarified by centrifugation, and adjusted to pH 6.5 before use.

Fig. 5. EbpA adhesin-based vaccine protects mice from *E. faecalis* CAUTIs

Mice were immunized and received two booster immunizations with the indicated doses of the various Ebp proteins (EbpB, EbpC, EbpA^{Full}, EbpA^{NTD}, and EbpA^{CTD}). Four weeks after the final immunization, mice were implanted with catheters and challenged with 1×10^7 CFU of *E. faecalis* OG1RF. After 24 hours of infection, bacterial burdens in bladder tissue (**A** and **D**) or recovered catheters (**B** and **E**) were quantitated as the number of CFU recovered. (**C**) The presence and distribution of bacteria and fibrinogen were assessed in catheters recovered from three mice taken randomly in the indicated treatment groups by immunofluorescence staining using antibody staining to detect fibrinogen (anti-Fg) and *E. faecalis* (anti-group D). (**F**) Ability of sera recovered from immunized mice to block binding of purified EbpA proteins to a fibrinogen-coated surface was evaluated by ELISA. Mouse anti-EbpA^{Full} was used to detect all species of EbpA. Rabbit anti-EbpA^{CTD} was used

as a negative control. Rabbit anti-fibrinogen was used to block fibrinogen before adding the proteins. Values represent means $\pm$ SEM. Mann-Whitney U test was used for mouse experiments and paired t test for binding assays. $P < 0.05$ was considered statistically significant. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$; ns, values were not statistically different. The horizontal bar represents the median value. The horizontal broken line represents the limit of detection of viable bacteria. Animals that lost the catheter were not included in this work.