Antibody-Based Therapy for Enterococcal Catheter-Associated Urinary Tract Infections

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ABSTRACT  Gram-positive bacteria in the genus Enterococcus are a frequent cause of catheter-associated urinary tract infection (CAUTI), a disease whose treatment is increasingly challenged by multiantibiotic-resistant strains. We have recently shown that E. faecalis uses the Ebp pilus, a heteropolymeric surface fiber, to bind the host protein fibrinogen as a critical step in CAUTI pathogenesis. Fibrinogen is deposited on catheters due to catheter-induced inflammation and is recognized by the N-terminal domain of EbpA (EbpANTD), the Ebp pilus's adhesin. In a murine model, vaccination with EbpANTD confers significant protection against CAUTI. Here, we explored the mechanism of protection using passive transfer of immune sera to show that antisera blocking EbpANTD-fibrinogen interactions not only is prophylactic but also can act therapeutically to reduce bacterial titers of an existing infection. Analysis of 55 clinical CAUTI, bloodstream, and gastrointestinal isolates, including E. faecalis, E. faecium, and vancomycin-resistant enterococci (VRE), revealed a diversity of levels of EbpA expression and fibrinogen-binding efficiency in vitro. Strikingly, analysis of 10 strains representative of fibrinogen-binding diversity demonstrated that, irrespective of EbpA levels, EbpANTD antibodies were universally protective. The results indicate that, despite diversity in levels of fibrinogen binding, strategies that target the disruption of EbpANTD-fibrinogen interactions have considerable promise for treatment of CAUTI.

IMPORTANCE  Urinary catheterization is a routine medical procedure, and it has been estimated that 30 million Foley catheters are used annually in the United States. Importantly, placement of a urinary catheter renders the patient susceptible to developing a catheter-associated urinary tract infection, accounting for 1 million cases per year. Additionally, these infections can lead to serious complications, including bloodstream infection and death. Enterococcus strains are a common cause of these infections, and management of enterococcal infections has been more difficult in recent years due to the development of antibiotic resistance and the ability of strains to disseminate, resulting in a major threat in hospital settings. In this study, we developed an antibiotic-sparing treatment that is effective against diverse enterococcal isolates, including vancomycin-resistant enterococci, during catheter-associated urinary tract infections.

It is estimated that 20% to 50% of all hospitalized patients receive a urinary catheter (1, 2), placing them at risk for developing a catheter-associated urinary tract infection (CAUTI) (3). Short-term urinary catheterization increases the risk of developing CAUTI and other complications up to 80%, and prolonged catheterization can increase the risk to 100% (4–6). CAUTI is the most common cause of health-care-associated infection (HAI) worldwide, accounting for 40% of all HAIs (7, 8), and often leads to secondary bloodstream infection, with a 7-day mortality rate of more than 30% (7, 9–11). Current guidelines recommend antibiotic treatments lasting 7 to 14 days to prevent CAUTI (8, 12); however, control of CAUTIs has become a major challenge due to the development and dissemination of antibiotic resistances among the bacteria that cause HAI (9, 10).

A prominent example comes from bacteria in the genus Enterococcus, which have emerged as a leading cause of HAI and CAUTI (13, 14). Enterococcal HAI isolates are most commonly Enterococcus faecalis and Enterococcus faecium, whose ability to withstand heat, UV radiation, and aseptic solutions (14–16) allows them to persist in the hospital ecology (14, 16). Treatment is increasingly challenging because of their intrinsic and acquired resistance to recently introduced antibiotics (15–17), and vancomycin-resistant enterococci (VRE) along with multiply resistant enterococci (MRE) strains are now common in CAUTI (18). Complete recovery from VRE infection may be prolonged and in some cases requires >3 years (19). Currently, 30% of all enterococcal HAI isolates are resistant to vancomycin (20), leading the Centers for Disease Control (CDC) to classify VRE as a serious threat, recommending continuous monitoring and the development of new therapeutic strategies (20).

New and improved treatment strategies will come from unraveling the host and bacterial factors that shift enterococci from commensalism to a pathogenic lifestyle. In this regard, we have recently found that urinary catheterization in both mice and hu-
In this study, we evaluated the potential of EbpA\textsubscript{NTD}-based immunotherapies for treatment of human CAUTI. The contribution of EbpA to CAUTI pathogenesis caused by a broad range of \textit{E. faecalis} and \textit{E. faecium} clinical isolates, the contribution of fibrinogen binding to biofilm formation on catheters recovered from human CAUTI, and the efficacy of EbpA\textsubscript{NTD}-based immunotherapy for treatment of CAUTI caused by a diverse collection of enteroococcal clinical isolates were examined. Our results indicate that EbpA\textsubscript{NTD}-based immunotherapy is broadly effective and suggest that this approach would be effective for other enteroococcal infections where fibrinogen is present.

RESULTS

\textit{E. faecalis} colocalizes with fibrinogen during human CAUTI. To explore the role of the \textit{E. faecalis}-fibrinogen interaction in human CAUTI, we examined the distribution of fibrinogen and \textit{E. faecalis} on catheters recovered from human CAUTI. The catheters were obtained from patients undergoing both urological and nonurological procedures who developed an \textit{E. faecalis}-positive urine culture prior to or after the placement of the catheter. Catheters were removed at different indwelling times and were immediately treated with fixative and then analyzed by fluorescence microscopy to assess patterns of fibrinogen deposition and enterooccal binding. This examination revealed both extensive but nonuniform distribution of fibrinogen (anti-Fg; Fig. 1) and extensive colonization of the catheter by \textit{E. faecalis} (anti-\textit{E. faecalis} [anti-group D]) (Fig. 1). Furthermore, \textit{E. faecalis} localized only to regions with deposited fibrinogen (MERGE, Fig. 1), consistent with a role for fibrinogen in promoting \textit{E. faecalis} adherence and biofilm formation on catheters.

Passive transfer of Ebp\textsubscript{ANTD} antibodies prevents CAUTI. Vaccination with EbpA or EbpA\textsubscript{NTD} protects mice from CAUTI by \textit{E. faecalis} OG1RF (23). Protection correlates with the development of serum antibody that can block EbpA-fibrinogen binding (23), suggesting an effector role for blocking antibody in protection. This hypothesis was tested by passive transfer of antisera to naive mice that were then implanted with catheters and challenged with \textit{E. faecalis}. Mice received either 1 intraperitoneal (i.p.) dose of antisera at 4 h prior to catheter implantation and infection (ci) or doses at 12 and 4 h prior to ci (Fig. 2A). Antiserum was from mice immunized with EbpA or with EbpA\textsubscript{NTD} or was serum from mice mock vaccinated with phosphate-buffered saline (PBS). Treated mice were then implanted with catheters and challenged with \textit{E. faecalis} OG1RF. After 24 h postinfection (hpi), urine samples were collected, and mice were sacrificed to harvest bladders and catheters (Fig. 2A). Titration of bladder homogenates and urine indicated that anti-EbpA antibodies were present following treatment with sera from immunized mice but not but not following treatment with sera from mock-immunized mice and that their levels were higher in the mice that received 2 doses (Fig. 2B and C). Treatment with either anti-Ebp\textsubscript{Full} or anti-Ebp\textsubscript{ANTD} antisera significantly reduced the mean bacterial burdens in the bladder (Fig. 2D) and catheter (Fig. 2E) of the treated mice by ~3 logs compared to mice receiving serum from mock-vaccinated mice (\(P < 0.005\)). Anti-Ebp\textsubscript{Full} and anti-Ebp\textsubscript{ANTD} treatments were equally effective, and those mice that received a second dose of immune sera were more efficiently pro-

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**FIG 1** \textit{E. faecalis} colocalized with Fg on human urinary catheters. Urinary catheters with an indwelling time of 18 h (A), 24 h (B and C), 8 days (D), or 9 days (E) were recovered from patients with an enterooccal UTI. The presence and distribution of bacteria and fibrinogen were assessed by immunofluorescence using antibody staining to detect fibrinogen (anti-Fg; green) and \textit{E. faecalis} (anti-group D; red). As a negative control, a piece of the catheter was incubated with the secondary antibody only to assess background fluorescence.
tected, with a reduction of mean bladder and catheter titers of ~4 logs compared to control mice ($P < 0.0005$). These data show that the serum antibody targeting EbpA NTD is the immune effector conferring protection in EbpA-immunized mice.

**Catheterization facilitates release of antibodies into the bladder and urine.** Serum antibody typically is not present at high concentrations in the bladder or urine. However, catheterization induces bladder inflammation, suggesting that, similarly to the release of fibrinogen (21, 23), catheter-induced inflammation also promotes the release of serum antibody into the bladder lumen. To assess this, we compared the levels of release of anti-EbpA NTD antibodies into the bladders of uncatheterized and catheterized mice at several time points prior to and after catheter implantation. Titers were also determined following challenge with *E. faecalis* OG1RF (timeline, Fig. 3A). While anti-EbpA NTD antibody was present in the serum of both catheterized and uncatheterized mice, antibody was detected in urine only following implantation of catheters (Fig. 3B) and was present at the earliest time point tested (4 h postcatheterization; Fig. 3B). Following infection, anti-EbpA NTD antibodies were detected in bladder homogenates from both catheterized and uncatheterized mice; however, titers were lower by ~2 logs in the uncatheterized group (Fig. 3B). Taken together, these data show that catheterization and not infection is the major factor resulting in the release of serum antibody into the bladder.

**Anti-EbpA NTD antibody reduces titers of a preexisting infection.** The therapeutic potential of anti-EbpA NTD antibodies was explored by testing whether passive transfer of immune serum could reduce bacterial titers of a preexisting CAUTI. Mice were implanted with catheters and challenged with *E. faecalis* OG1RF, and infected mice were treated at 12 hpi with a single i.p. dose of 100 or 200 µl of a selected antiserum (timeline, Fig. 3C), including anti-EbpA Full, anti-EbpA NTD, anti-EbpA CTD sera, serum from PBS-mock-vaccinated mice, or an antiserum (anti-*Streptococcus* group D antigen) that recognizes lipoteichoic acid (LTA), an abundant but unrelated surface antigen. At 24 h post-antibody treatment, mean bladder (Fig. 3D) and urine (Fig. 3E) CFUs were significantly reduced by treatment with anti-EbpA Full or anti-

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**FIG 2** Passive immunization with anti-EbpA Full and anti-EbpA NTD antibodies prevents *E. faecalis* CAUTI. Mice ($n = 10$) were given a dose of 100 µl of PBS sera or 100 µl of anti-EbpA Full or anti-EbpA NTD with a titer of 1 × 10$^7$. (A) Experimental timeline. Sac, time of mouse sacrifice. (B and C) Detection of EbpA Full and EbpA NTD antibodies in bladder (B) and in urine (C) analyzed by diluting each sample 1:100 before serial dilution. (D and E) Prophylaxis treatment performed using 1 dose at 4 h prior infection (D) or 2 doses at 4 h prior infection and 12 h postinfection (hpi) (E). Values represent means ± SEM. The Mann–Whitney U test was used for mouse experiments; $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; ns, values were not statistically significantly different. The horizontal bar represents the median value. The horizontal broken line represents the limit of detection (LOD) of viable bacteria. Animals that lost the catheter were not included in this work.
Ebp\textsuperscript{NTD} sera but not by treatment with anti-EbpA\textsuperscript{CTD}, mock sera, or anti-group D antigen antisera (P < 0.0005). Therapeutic efficacies were more pronounced in mice treated with the higher doses of EbpA\textsuperscript{Full}, anti-EbpA\textsuperscript{NTD}, anti-EbpA\textsuperscript{CTD}, or anti-group D antibody was administered intraperitoneally (i.p.) at 12 h postinfection (hpi) (anti-EbpA\textsuperscript{Full}, anti-EbpA\textsuperscript{NTD}, anti-EbpA\textsuperscript{CTD}, and anti-group D antibody titers of 1 × 10\textsuperscript{7}). (D and E) Following 24 h of infection, bacterial burdens in bladder tissue (D) or recovered from catheters (E) were quantitated as the number of CFU recovered. Values represent means ± SEM. The Mann-Whitney U test was used; P < 0.05 was considered statistically significant. *, P < 0.05; **, P < 0.005; ***, P < 0.0005; ns, values were not statistically significantly 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.

**E. faecalis infection does not confer protection against subsequent infection.** Hospitalized patients with an enterococcal infection developed antibodies that recognized the Ebp pilus and its individual structural subunits (24, 25). However, it has not been demonstrated that these antibodies are protective against a subsequent infection. To assess this, mice were implanted with catheters and infected with E. faecalis OG1RF or were mock infected with PBS. To maximize the immune response and to prevent expulsion of the catheter and the resulting loss of infection, mice were not manipulated for the first 14 days p.i. Then, starting on day 14, urine and blood samples were collected every 7 days or as otherwise indicated (timeline, Fig. 4A) to assess levels of bacterial CFU (Fig. 4B) and production of anti-E. faecalis and anti-EbpA\textsuperscript{NTD} antibodies (see Fig. S1A and B in the supplemental material). At day 35, mice were treated with vancomycin (0.5 g/liter in drinking water) for 10 days and the clearance of CAUTI was monitored by assessment of urine CFU, which became undetectable by day 56.
At that time (Fig. 4A), mice were implanted with an additional catheter and were rechallenged with the original *E. faecalis* OG1RF (n = 15) (A); infection was followed by measuring the bacterial burden in urine (B). After antibiotic treatment, mice were infected and bacterial burdens in bladder tissue or recovered catheters were quantitated as the number of CFU recovered. (D to G) Mice were immunized with mock serum (n = 5), serum from infected mice (n = 15 [D and E] or n = 10 [F and G]), anti-EbpANTD antibodies (n = 15 [D and E] or n = 5 [F and G]), or diluted anti-EbpANTD antibodies (n = 5). Following 24 h of infection, bladders (D and F) and catheters (E and G) were harvested and bacterial burdens were quantified. Values represent means ± SEM. The Mann-Whitney U test was used; *P < 0.05; **, P < 0.005; ***P < 0.0005; ns, values were not statistically significantly 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.

(Fig. 4B). At that time (Fig. 4A), mice were implanted with an additional catheter and were rechallenged with the original *E. faecalis* strain (OG1RF). When examined at 24 hpi, mean CFU levels in bladder and mean CFU levels on catheters were not significantly different between mice previously infected by *E. faecalis* and those that were mock infected (Fig. 4C), despite the fact that the former group developed antibodies against *E. faecalis* and EbpANTD (see Fig. S1A and B). However, the antibody titers were ~3 logs lower than those developed in EbpANTD-vaccinated mice (see Fig. S1A and B) or in rabbits conventionally vaccinated with *E. faecalis* or EbpACTD (26). Furthermore, in contrast to serum from EbpANTD-vaccinated mice, passive transfer of serum collected from infected mice neither prevented (Fig. 4D) nor treated (Fig. 4E) *E. faecalis* CAUTI and was no more effective than serum.
from mice mock vaccinated with PBS (Fig. 4D and E). Since it seemed likely that this failure to provide protection was correlated with the relatively low serum anti-EbpANTD titers that had developed during infection versus active vaccination, serum from EbpANTD-vaccinated mice was diluted to a titer equivalent to that obtained by infection (1 \times 10^{-4}) (see Fig. S1C). The diluted serum from vaccinated mice was unable to protect against infection and failed to reduce bladder (Fig. 4F) and catheter (Fig. 4G) titers any better than serum from infected mice or from mice mock vaccinated with PBS. This result confirms that the anti-EbpANTD antibody concentration is crucial for protection and suggests that anti-EbpANTD antibodies generated in infected hospitalized patients may not be protective against a subsequent infection.

The EbpA sequence is highly conserved across multiple enterococcal species. An ideal antivirulence therapeutic target should be conserved in carriage and sequence across many pathogenic strains and species. In this regard, the Ebp pilus has been reported to be widely distributed across the members of the genus Enterococcus (24, 27). To extend these observations, we examined a diverse collection of 55 isolates to assess the presence and conservation of the Ebp pilus operon in clinical strains (see Table S1 in the supplemental material). This analysis revealed that all three genes that comprise the Ebp pilus were present in all 55 enterococcal genomes analyzed (Fig. 5A; see also Fig. S2A). Expanding this analysis to a collection of genome sequences available in public databases representing multiple enterococcal species, a hidden Markov model was used to search for EbpA homologous sequences. A total of 480 peptides matching EbpA were identified from eight Enterococcus species, including E. faecalis, E. faecium, E. gallinarum, E. saccharolyticus, E. mundtii, E. hirae, E. casseliflava-
vus, and E. flavescens. After filtering for unique sequences and removal of sequences with low coverage (<80%) of the EbpA open reading frame were performed, the resulting 137 unique peptide and nucleotide sequences were aligned (Fig. 5B). The resulting gapped alignment shows that the sequences representing the first ~100 amino acids of EbpA, comprising mainly the signal sequence, are highly divergent between species of Enterococcus, although they are generally well conserved within species (e.g., E. faecalis peptide sequences share 99.6% average pairwise identity in the first 100 amino acids compared to 46.8% average pairwise identity in the first 100 amino acids in all Enterococcus EbpA peptide sequences). In contrast, the sequences of the EbpA N-terminal domain (NTD) and C-terminal domain (CTD) are well conserved across the members of the Enterococcus genus, with an average pairwise identity of 73.7%, including 100% peptide sequence identity of the EbpA NTD MIDAS motif (Fig. 5B) that is required for binding to fibrinogen (23). This high degree of sequence similarity predicts conservation of the protective epitopes recognized by anti-EbpA<sup>NTD</sup> antibodies raised against EbpA of E. faecalis OG1RF.

### Surface-expressed EbpA correlates with fibrinogen binding among enterococcal strains.

Conservation of EbpA epitopes predicts that an EbpA antiserum raised against one strain will recognize EbpA expressed by diverse strains. This prediction was tested using antisera raised against EbpA of E. faecalis OG1RF and a panel of clinical strains isolated from the urinary tract (UT), the bloodstream, and the gastrointestinal tract (GIT), including representatives of E. faecalis, E. faecium, E. gallinarum, and VRE, as well as several unclassified enterococcal isolates (see Table S1 in the supplemental material). Expression of cell surface EbpA was evaluated by enzyme-linked immunosorbent assay (ELISA) following in vitro culture under conditions known to promote Ebp pilus expression in OG1RF (static culture in brain heart infusion [BHI] medium for 18 h at 37°C). It was found that the EbpA antiserum detected surface-expressed EbpA in all strains tested (see Fig. S2A); however, there was a considerable range in the amount of EbpA that was detected across the panel of isolates (see Fig. S2B). To evaluate whether this range reflected differences in the efficiency by which the antiserum recognized EbpA from different strains versus differences in the levels of Ebp pilus expression, the panel of isolates was tested for fibrinogen binding. This analysis also revealed a range of binding efficiencies of the various isolates (see Fig. S2C). However, there was a positive (r = 0.5385) and significant (P < 0.0001) correlation between the detected levels of surface-expressed EbpA and the levels of fibrinogen bound (Fig. 5C). Correlation between detection and function suggests that there is heterogeneity in Ebp expression between isolates rather than differential abilities of the anti-EbpA antiserum to detect heterologous EbpA proteins. Taken together, these data show that EbpA is a ubiquitous factor expressed among enterococcal strains and species that it has a conserved antigenic profile.

EbpA<sup>NTD</sup>-based immunotherapies can prevent and treat CAUTI caused by diverse enterococcal isolates. The conservation of EbpA epitopes suggests that EbpA<sup>NTD</sup>-based immunotherapies would be effective against CAUTI caused by a wide range of enterococcal strains and species. To test this, mice were immunized with the OG1RF EbpA<sup>NTD</sup>-based vaccine using a standard protocol (100 μg purified EbpA<sup>NTD</sup> emulsified in Freund’s complete adjuvant, with booster immunizations corresponding to the original dose on weeks 4 and 8) or were subjected to mock vaccination with PBS in adjuvant, as described previously (23). Mice were challenged by a panel of strains selected to have high levels of surface-expressed EbpA and of fibrinogen binding that also reflected clinical diversity, including a UTI isolate (EC#1), a VRE blood isolate (EC#25), and an E. faecium GIT isolate (EC#30) (see Table S1 in the supplemental material) (Fig. 5A; see also Fig. S2A and B). Efficacy of protection was benchmarked against infection by E. faecalis OG1RF. Four weeks after the second boost, mice were implanted with catheters and challenged with 2 × 10<sup>7</sup> CFU of the indicated strains. Analyzed at 24 hpi, it was found that all tested strains were able to colonize the bladder and the catheter (Fig. 6A). Furthermore, EbpA<sup>NTD</sup> vaccination significantly reduced bladder and catheter burdens versus the levels seen with mock-vaccinated mice for all strains examined (Fig. 6A).

Examined next was whether passive transfer of anti-EbpA<sup>NTD</sup> antibodies could treat CAUTI by reducing bacterial titers of an ongoing infection. For this analysis, the number of clinical strains tested was expanded to include those showing (i) high EbpA expression and high fibrinogen binding levels (EC#1, EC#25, EC#30, EC#31, and EC#37); (ii) low EbpA expression and low fibrinogen binding levels (EC#15, EC#43, and EC#49); and (iii) a low EbpA expression level but a high fibrinogen binding level (EC#29 and EC#32) (Fig. 5C; see also Fig. S2A and B in the supplemental material). As before, these strains represented UTI, blood, and GIT isolates. Mice were catheterized and challenged with 2 × 10<sup>7</sup> CFU of the indicated strains; then, mice received 1 dose of 200 μL of anti-EbpA<sup>NTD</sup> or PBS (mock)-vaccinated serum at 2 hpi. Examined at 24 hpi, all strains that highly expressed EbpA were found to have colonized the bladder and catheter with CFU levels equivalent to or higher than those measured for E. faecalis OG1RF (Fig. 6B and C) and that treatment with EbpA<sup>NTD</sup> antibodies reduced bacterial titers by ~3 to 4 logs compared to the levels seen with sera from control mice subjected to mock treatment with PBS (Fig. 6B and C). Those strains that expressed low levels of EbpA <i>in vitro</i> were also able to cause CAUTI and to colonize the bladder and catheter (Fig. 6D and E); however, their colonization was not as efficient (10<sup>6</sup> to 10<sup>7</sup> CFU/mL) as seen with OG1RF and the strains expressing high levels of EbpA (10<sup>7</sup> to 10<sup>8</sup> CFU/mL). Analysis of two strains (EC#29 and EC#32) that showed anomalous binding behavior <i>in vitro</i> (high levels of binding of Fg with only low-level expression of EbpA) revealed that they were also able to colonize the bladder and catheter (10<sup>7</sup> to 10<sup>8</sup> CFU/mL). However, despite their low levels of EbpA expression <i>in vitro</i>, treatment with anti-EbpA<sup>NTD</sup> antibodies was an effective therapy, reducing catheter and bladder CFUs ~1 to ~1.5 logs compared to PBS mock treatment serum controls (Fig. 6B and C). Overall, these data show that EbpA<sup>NTD</sup>-based immunotherapies have efficacy against a broad range of isolates.

### DISCUSSION

In this study, we showed that passive immunization with anti-EbpA<sup>NTD</sup> antibodies is effective both as a prophylactic and as a therapy for the reduction of bacterial titers in murine CAUTI model and that the level of protection correlated with the concentration of anti-EbpA<sup>NTD</sup> antibodies. More importantly, this therapy was also protective against a broad range of urinary tract, bloodstream, and GIT enterococcal clinical strains, which included E. faecalis, E. faecium, and VRE. Our observation that, during human CAUTI, E. faecalis colocalized with Fg deposited onto the mucosa was confirmed by electron microscopy (Fig. 6A). Therefore, anti-EbpA antibodies could also eradicate E. faecalis from the bladder and catheter and could therefore be used to prepare a vaccine that would prevent CAUTI. This hypothesis needs to be tested in a mouse model.
FIG 6 EbpANMD-based vaccine and anti-EbpANMD antibody treatment prevented and reduced bacterial titers of different enterococcal isolates. (A) Mice were immunized and received two booster immunizations with doses of 100 μg EbpANMD. Four weeks following the final immunization, mice were implanted with catheters and challenged with 2 × 10^7 CFU of E. faecalis OG1RF (n = 5) or with the indicated clinical enterococcal strains (n = 5). Following 24 h of infection, bacterial burdens in bladder tissue or recovered catheters were quantitated as the number of CFU recovered. (B to E) To assess the therapeutic effect of anti-EbpANMD antibodies against enterococci-infected mice, antibodies were administered intraperitoneally 12 hpi (anti-EbpANMD antibody titer of 1 × 10^7). Enterococcal strains were divided into two groups: (i) those that highly expressed EbpA and bound to Fg (B and C; n = 5) and (ii) those that did not express EbpA (n = 10 [EC#15 and EC#43] and n = 15 [EC#49]) or bind to Fg (D and E; n = 15) under in vitro conditions. Bladder (B) and catheter (C) bacterial burdens were quantified as described below. Values represent means ± SEM. The Mann-Whitney U test was used; P < 0.05 was considered statistically significant. *, P < 0.05; **, P < 0.005; ***, P < 0.0005; ns, values were not statistically significantly 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.
a urinary catheter recapitulated results from the murine CAUTI model and from ex vivo analyses with catheters removed from infected humans (28, 29) and suggests that EbpANTD-based immunotherapies can be directly translatable to treatment of human disease.

Since it has been shown that EbpA is required for infection in the murine model (23) and that mutation of the EbpA MIDAS motif blocks both Fg binding and virulence (23, 30), it is likely that EbpA–Fg interactions represent a key step in CAUTI pathogenesis by directing initial adherence to the catheter surface. This is supported by studies showing colocalization of catheter Fg and bound enterococci (22, 23). Thus, the most parsimonious model for protection would be via a steric mechanism where antibodies that block binding to Fg prevent this initial attachment event. However, since passive immunization could act therapeutically against an established infection, the mechanism of protection may be more complex. Attachment and biofilm formation may be dynamic, involving multiple cycles of attachment and detachment. Thus, when detached, enterococci may become susceptible to antibodies that block reattachment. Alternatively, a multifactorial mechanism could involve an EbpA-dependent opsonic activity or an unknown EbpA activity in a domain adjacent to its Fg binding site. Therefore, it will be of great interest to characterize the basis of EbpANTD-mediated protection.

On the basis of our bioinformatic analysis, we found that EbpANTD was highly conserved among diverse enterococcal species, suggesting that multiple enterococcal lineages use a common mechanism of CAUTI pathogenesis. This would explain the effectiveness of the EbpANTD immunotherapies observed in the present study. Other enterococcal species distributed across different clades of the Enterococcus genus phylogeny (31, 32) have been found to cause human infections, suggesting that these diverse species may also have common traits for colonization, and virulence, which may include EbpA.

Supporting the hypothesis of common mechanisms was the overall correlation between EbpA expression in vitro and Fg binding across a panel of 55 strains. However, there were exceptions, including two strains with a low level of EbpA expression but a high level of fibrinogen binding. That these atypical strains colocalizing Fg and EbpA were inoculated from a single bacterial colony grown on BHI agar plates. Unless otherwise specified, E. faecalis and S. aureus were grown overnight on brain heart infusion broth (BHI) (BD Company) and were inoculated from a single bacterial colony grown on BHI agar plates. Liquid cultures were grown statically at 37°C for 18 h or as otherwise indicated. Bacterial strains are listed in Table S1 in the supplemental material.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Unless otherwise specified, E. faecalis strain OG1RF and its derivatives and enterococcal strains were grown overnight on brain heart infusion broth (BHI) (BD Company) and were inoculated from a single bacterial colony grown on BHI agar plates. Liquid cultures were grown statically at 37°C for 18 h or as otherwise indicated. Bacterial strains are listed in Table S1 in the supplemental material.

**General cloning techniques.** Bacterial genomic DNA (gDNA) was isolated using a Wizard genome DNA purification kit (Promega Corp.). Primers were purchased from Integrated DNA Technology. Phusion High Fidelity DNA polymerase was purchased from New England Biolabs and used according to the methods described by the manufacturer.

**Antibodies used in this study.** The primary antibodies used in the study were rabbit anti-Streptococcus group D antigen (anti-E. faecalis lipoteichoic acid) (Lee Laboratories) (26) and mouse anti-EbpAFull, mouse anti-EbpANTD, and mouse anti-EbpACTD (generated in this study). The secondary antibodies used in the study were IRDye 800CW donkey anti-goat (catalog no. 926-32213) and IRDye 680LT goat anti-rabbit (catalog no. 926-68021) antibodies from Li-Cor Biosciences and horseradish per-
oxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antisera from KPL.

**Human urinary catheter analysis.** The human urinary catheter was collected per the study approval from the Washington University School of Medicine Internal Review Board (approval #201410058). After collection, the catheter was cut into 10-cm-long segments and the segments were fixed with formalin for 1 h and washed (3 times) with PBS. For analysis, the first 10 cm of the catheter’s tip was blocked by overnight incubation at 4°C with 1.5% bovine serum albumin (BSA)–0.1% sodium azide–PBS (BB) and then washed three times for 5 min each time with PBS-T (PBS containing 0.05% Tween 20). A 15-ml solution containing goat anti-human fibrinogen and rabbit anti-Streptococcus group D (1:500) antisera in dilution buffer (PBS with 0.05% Tween 20, 0.1% BSA, and 0.5% methyl α-D-mannopyranoside) was added, and the reaction mixture was incubated at room temperature for 2 h. Then, it was washed (3 times) for 5 min each time with PBS-T and incubated with donkey anti-goat IRDye 800CW (diluted 1:10,000) in dilution buffer for 45 min at room temperature. Following an additional 3 washes with PBS-T, catheters were examined for infrared signal using an Odyssey Imaging System (Li-Cor Biosciences). Autofluorescence was assessed in nonimplanted catheters and catheters not incubated with the primary antibody and was minimal.

**Mouse catheter implantation and infection.** The mice used in this study were 6-week-old female wild-type C57BL/6Ncr mice purchased from Charles River Laboratories. Mice were subjected to transurethral implantation and inoculated as previously described (34). Mice were anesthetized by inhalation of isoflurane and implanted with a 5-mm length of platinum-cured silicone catheter. When indicated, mice were infected immediately following catheter implantation with 50 μl of E. coli K10262 (diluted 1:10,000) in dilution buffer for 45 min at room temperature. Following an additional 3 washes with PBS-T, catheters were examined for infrared signal using an Odyssey Imaging System (Li-Cor Biosciences). Antibody titers in the bladder and catheters, as described above. For the passive immunization experiments, the specified antiserum was administered intraperitoneally 1 day before experimentation, and mice were anesthetized by inhalation of isoflurane and then given 5 mg of BW (bacterial protein). Following a 30 min wait, mice were injected intraperitoneally with 50 μl of the bacterial solution (diluted 1:10,000) in dilution buffer for 45 min at room temperature. Following an additional 3 washes with PBS-T, catheters were examined for infrared signal using an Odyssey Imaging System (Li-Cor Biosciences). Active and passive immunization of mice. Mice were immunized as previously described (23). Groups of 10 mice were used for each antibody dose. One hundred micrograms of Ebpa proteins or PBS was emulsified with either Freund’s complete adjuvant (first vaccination) or Freund’s incomplete adjuvant (boosts). Mice were vaccinated intramuscularly and then with the same dose at 4 and 8 weeks. Mouse sera were collected prior to immunization and every week after to determine the antibody response. At 4 weeks after the second boost, mice were implanted with catheters and challenged with –2 × 10⁷ CFU of E. faecalis OGI1RF or clinical enterococcal isolates. Mice were sacrificed at 24 hpi to determine bacterial titers in the bladder and catheters, as described above. For the passive immunization experiments, the specific antisera was administered intraperitoneally 1 day before experimentation, and mice were anesthetized by inhalation of isoflurane and then given 5 mg of BW (bacterial protein). Following a 30 min wait, mice were injected intraperitoneally with 50 μl of the bacteria (diluted 1:10,000) in dilution buffer for 45 min at room temperature. Following an additional 3 washes with PBS-T, catheters were examined for infrared signal using an Odyssey Imaging System (Li-Cor Biosciences).

**Expression of Ebpa in the cell surface of enterococcal strains.** Surface expression of Ebpa by clinical and laboratory enterococcal strains was detected by whole-cell ELISA as previously described (23). Bacterial strains were grown for 12 h in BH broth, washed (3 times) with PBS, and normalized to an OD₆0₀ of 0.5. Then, bacterial cells were washed and resuspended with 50 mM carbonate buffer (pH 9.6) containing 0.1% sodium azide and 100 μl of the each bacterial solution was used to coat Immulon 4HB microtiter plates overnight at 4°C. Plates were washed with PBS-T to remove unbound bacteria and blocked for 2 h with BB followed by washes performed with PBS-T. Ebpa surface expression was detected using mouse anti-Ebpa antibody, which was diluted 1:100 in dilution buffer before serial dilutions were performed as described above. A 100-μl volume was added to the plate, and the reaction mixture was incubated for 2 h. After the incubation, the plates were washed with PBS-T followed by 1 h of incubation with HRP-conjugated goat anti-rabbit antisera (1:2,000) and then with PBS-T. Detection was performed using a TMB substrate reagent set. The reaction mixtures were incubated, developed, and measured as described above. As an additional control, rabbit anti-Streptococcus group D antisera was used to verify that whole cells of all strains were bound to the microtiter plates at similar levels.

**Whole-bacterial binding to fibrinogen.** To determine whether enterococcal strains adhere to immobilized fibrinogen as previously described (23), Immulon 4HBX microplates were coated overnight at 4°C with 100 μg/ml of human fibrinogen that was free of plasminogen and von Willebrand factor (Enzyme Research Laboratory). The plates were blocked for an hour with BB, followed with PBS washes (performed 3 times for 5 min each time). Bacterial strains were grown for 12 h in BH broth, were normalized to an OD₆0₀ of 0.5, and then were 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 performed using 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 washes performed with PBS-T, and were then blocked overnight at 4°C with BB, and PBS-T washes were performed. Then, plates were incubated for an hour at room temperature with rabbit anti-Streptococcus group D antigen antisera (1:500). Plates were washed with PBS-T, incubated with Odyssey secondary antibody (goat anti-rabbit IRDye 680LT, diluted 1:10,000 in dilution buffer) for 45 min at room temperature, and washed with PBS-T. As a final step, the plates were scanned for infrared signal using an Odyssey imaging system (Li-Cor Biosciences).
**EbpA sequence analysis.** Using the amino acid sequence of *E. faecalis* OG1RF as a query, we identified homologs of EbpA in the UniProtKB database using the PHMMER webserver (42, 43). Nucleotide sequences of the genes encoding matches were then retrieved from the Ensembl database (44) using their unique identifiers. Homologous gene sequences were then filtered to remove duplicate nucleotide sequences and nucleotide sequences which did not cover >80% of the OG1RF gene sequence. Amino acid sequences of the unique EbpA homologs were then aligned using the MAFFT algorithm (45) and the BLOSUM62 scoring matrix, and a corresponding codon-based alignment of the nucleotide sequences was constructed using PAL2NAL (46). The percent identity of these alignments was then visualized using sliding windows of 20 amino acids and 60 nucleotides for the protein and gene sequences, respectively.

**Statistical analyses.** Data from multiple experiments were pooled. Two-tailed Mann-Whitney *U* tests were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA) for all comparisons described for CAUTI experiments. Antibody titration, expression of EbpA, and Digestive and Kidney Diseases (NIDDK) (P50-DK0645400).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01563-16/-/DCSupplemental.

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