Siderophore vaccine conjugates protect against uropathogenic Escherichia coli urinary tract infection

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Uropathogenic Escherichia coli (UPEC) is the primary cause of uncomplicated urinary tract infections (UTIs). Whereas most infections are isolated cases, 1 in 40 women experience recurrent UTIs. The rise in antibiotic resistance has complicated the management of chronic UTIs and necessitates new preventative strategies. Currently, no UTI vaccines are approved for use in the United States, and the development of a highly effective vaccine remains elusive. Here, we have pursued a strategy for eliciting protective immunity by vaccinating with small molecules required for pathogenesis, rather than proteins or peptides. Small iron-chelating molecules called siderophores were selected as antigens to vaccinate against UTI for this vaccine strategy. These pathogen-associated stealth siderophores evade host immune defenses and enhance bacterial virulence. Previous animal studies revealed that vaccination with siderophore receptor proteins protects against UTI. The poor solubility of these integral outer-membrane proteins in aqueous solutions limits their practical utility. Because their cognate siderophores are water soluble, we hypothesized that these bacterial-derived small molecules are prime vaccine candidates. To test this hypothesis, we immunized mice with siderophores conjugated to an immunogenic carrier protein. The siderophore–protein conjugates elicited an adaptive immune response that targeted bacterial stealth siderophores and protected against UTI. Our study has identified additional antigens suitable for a multicomponent UTI vaccine and highlights the potential use of bacterial-derived small molecules as antigens in vaccine therapies.

vaccine conjugate | urinary tract infection | siderophore | UPEC | iron acquisition

Both the physical and financial burdens of urinary tract infections (UTIs) are staggering. Half of all women experience a symptomatic UTI in their lifetime (1). And of those women, almost half suffer a recurrence within the next year (1). In the United States, where the annual societal cost of UTI is likely underestimated at $3.5 billion (2), 4 million women have UTIs continuously (3). Uropathogenic Escherichia coli (UPEC) is a subclass of extraintestinal pathogenic E. coli (ExPEC) and is the etiological agent for 80% of all uncomplicated UTIs (1). In 2006, there were 11 million physician visits, over 1.7 million emergency room visits, and 479,000 hospitalizations of both men and women in the United States for UTI (2, 4). Altogether, these estimates place the United States for UTI first among kidney and urologic diseases in terms of total cost. UTIs occur in bacteria, not commonly ExPEC (5), contaminate the periurethral area and traverse the urethra to colonize the bladder and its underlying epithelium, causing cystitis (6, 7). If left untreated, UPEC may ascend the ureters and establish a secondary infection in the kidney parenchyma, causing pyelonephritis. At this juncture, UPEC can elicit serious complications, including renal scarring, septicemia, and death.

UTIs are routinely treated with antibiotic therapy, including trimethoprim–sulfamethoxazole (TMP–SMX) and ciprofloxacin. Women experiencing at least two UTIs per year are frequently given antibiotics prophylactically (8). Not surprisingly, the rates of resistance to these antibiotics in UPEC strains have steadily risen over the past few decades. In the United States, Canada, and elsewhere, ~10–25% of uncomplicated UTI isolates are resistant to TMP–SMX (9–11). This trend is forcing physicians to reach for more expensive and sometimes less effective drugs to treat UTIs (10, 12, 13). Even more troubling is the rise in multidrug resistance among UPEC strains, as a recent international study found that over 10% of E. coli cystitis isolates are resistant to at least three different classes of antimicrobial agents (14). These trends challenge the prescription choices of physicians to address shifting microbial susceptibilities (15).

To compound the danger of antibiotic resistance, there are no currently licensed vaccines in the United States to combat recurrent UTIs in women. In Europe, two vaccines against UTIs called SolcoUrovac and Uro-Vaxom are licensed for use in women with recurrent UTIs (16). SolcoUrovac is a vaginal suppository containing 10 heat-killed UPEC strains that provides relatively poor protection in the absence of frequent administration (17). Uro-Vaxom is an oral capsule containing a lyophilized mixture of membrane proteins from 18 UPEC isolates that is expected to be taken daily. Although this vaccine offers protection against UPEC, its success is limited due to toxicity and poor adherence to the daily regimen (18). Due to these drawbacks, no vaccines are licensed for use in the United States (19). Given the paucity of effective vaccines, the increasing rate of UPEC antibiotic resistance, the decline in novel antibiotic scaffolds, and the need to reduce healthcare expenditures, new therapeutic strategies to manage UTIs must be explored.

Previous work using unbiased genomic and proteomic screens identified bacterial targets that are expressed in vivo by UPEC during UTIs in women, reside on the surface of the bacterium, are immunogenic, and carry out a critical function for survival of E. coli in the host (20). Six bacterial iron acquisition system proteins met all criteria. These findings are supported by a rich history of genomic, proteomic, and genomic studies. From these efforts, we evolved the hypothesis that small iron-chelating compounds called siderophores can be used as vaccine antigens. These siderophores are not produced by common UTI pathogens, such as UPEC (5), yet are expressed in the bladder and its underlying epithelium, providing a strategic advantage to the bacteria. By using small iron-chelating compounds called siderophores as vaccine antigens, vaccination allows the immune system to discriminate UTI pathogens from commensal bacteria and to respond to them with an immune response (21). In our studies, small molecules are used as immunogens instead of the more commonly studied proteins, which have practical limitations for vaccine development.

Significance

Urinary tract infections (UTIs) are primarily caused by uropathogenic Escherichia coli (UPEC), and 1 in 40 women experience chronic UTIs during their lifetime. The antibiotic courses required to treat infections promote antibiotic resistance, and current vaccine options offer limited protection. We have pioneered a strategy using small iron-chelating compounds called siderophores as vaccine antigens. These siderophores are not produced by commensal bacteria and are required for UTI. The siderophore vaccines reported here are easy to formulate and reduce bacterial burdens in a murine model of UTI. This report highlights the untapped resource of bacteria-specific small molecules as potential vaccine antigens and provides a proof of principle for incorporating these compounds into multicomponent vaccines for the prevention of bacterial infections.


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transcriptomic, and proteomic studies that have also identified iron acquisition systems as prime anti-UTI targets (21–27).

Iron is an essential cofactor in many biological processes, including DNA synthesis, electron transfer, and central metabolism (28). Iron acquisition is generally required for bacterial growth during infection (28, 29). One facet of innate immunity, coined “nutritional immunity,” restricts bacterial infections by limiting access to critical metal cofactors (28, 30). The mammalian host limits intracellular and freely circulating iron by sequestering it in proteins such as lactoferrin, transferrin, ferritin, and hemoglobin (31). Notably, the primary site of UPEC infection, the bladder, has lower iron levels than serum (32). Thus, it is not surprising that over 14 gene clusters implicated in iron acquisition have been identified as important virulence factors in UPEC strains (33–37); these gene clusters encode up to four siderophore biosynthesis and uptake systems as well as receptors for the acquisition of heme, ferric citrate, and ferrous iron. Of the many classes of siderophores, UPEC strains typically encode at least three of the following siderophores: yersiniabactin (Ybt), aerobactin (Aer), enterobactin (Ent), and the glucosylated Ent, salmochelin (Glc-Ent) (38, 39).

Bacterial iron acquisition is a natural target of the host immune system. For example, serum albumin and lipocalin-2 bind and inactivate Ent (40, 41). To evade host immunity, pathogenic E. coli strains typically encode a combination of Ybt, Aer, and Glc-Ent siderophores, which are not recognized by host defenses (39). By evading host defenses to secure nutrient iron, Ybt, Aer, and Glc-Ent serve as urovirulence factors (33, 36). Notably, Ybt and Aer are more prevalent among pathogenic E. coli strains than commensal isolates (39). Moreover, previous studies that systematically assessed the use of surface-exposed iron receptors as potential vaccine antigens found that two of the stealth siderophore receptors, those that recognize Ybt and Aer, protect against UTI (20, 42, 43). The hydrophobic nature of these outer-membrane receptors, however, makes these antigens insoluble in water, complicating the purification and formulation of the vaccines. Here, we examine the potential use of the small-molecule siderophores Ybt and Aer as protective vaccine antigens that could bolster the efficacy of other immunoprotective strategies.

**Results**

By virtue of the confirmed importance of Ybt and Aer in uropathogenesis (33–37), the proven efficacy of their receptors in experimental vaccines (20, 42), their increased prevalence among pathogenic E. coli (39), and amenable biochemical features, we hypothesized that Ybt and Aer could represent valid vaccine candidates. Because the siderophores are small (<564 Da) and unlikely to be immunogenic, Ybt and Aer were conjugated to cationized BSA (cBSA), an immunogenic carrier protein that has aminoethyl-capped carboxylic acids (44–46). The positive surface charge of cBSA increases vaccine binding to immune cells, and the aminoethyl modifications improve the coupling reaction by both eliminating carboxylic acids and providing additional primary amines on the carrier protein (44–46). Both Ybt and Aer have carboxylic acid moieties; thus, standard amide coupling conditions were used to prepare the cBSA–siderophore conjugates using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as the coupling reagent (SI Appendix, Fig. S1 A and B). cBSA incubated with EDC in the absence of siderophore was used as a negative control.

Liquid chromatography and tandem mass spectrometry on trypsin-digested cBSA–siderophore conjugates confirmed linkage of the siderophores to cBSA. For the cBSA–Aer conjugate, peptide fragment ions supporting the linkage of Aer to Lys266, aminoethyl-Asp314, and aminoethyl-Glu337 were detected (SI Appendix, Fig. S2 A–C and Tables S1–S3). For cBSA–Ybt, peptide ions supporting the linkage of Ybt to aminoethyl-Asp272 and Lys337 were detected (SI Appendix, Fig. S2D and Tables S4 and S5). Altogether, these data confirm at least two conjugation sites on cBSA for each siderophore.

To assess the efficacy of vaccination with the stealth siderophore conjugates, mice were immunized intranasally with 10 μg of vaccine conjugate prepared in 20 μL of PBS (SI Appendix, Fig. S1C). Three vaccine groups were examined along with a cBSA control, including cBSA–Ybt, cBSA–Aer, or a 1:1 mixture of cBSA–Ybt:cBSA–Aer, which was composed of 5 μg of each conjugate. Previous reports have shown that intranasal vaccination provides the most consistent protection in murine UTI vaccine studies (20, 42, 47). Therefore, mice were boosted intranasally with 20 μL of PBS containing 2.5 μg of vaccine conjugate 7 and 14 d postimmunization. UPEC strain HM69, a strain recently isolated from a patient with uncomplicated cystitis, was selected for challenge because it encodes Ent, Ybt, and Aer (38). On day 21 postimmunization, mice were transurethrally inoculated with 10⁶ colony-forming units (cfus) of HM69, and after 48 h, the bacterial burdens in the urinary tract were quantified.

Vaccination with cBSA–Ybt reduced bacterial burden by 12-fold in the urine (P = 0.04) and 10-fold in the kidneys (P = 0.01), whereas cBSA–Aer reduced bacterial burden by 19-fold (P = 0.02) in the urine (Fig. 1 A–C). Coimmunization with 1:1 cBSA–Ybt:Aer also decreased bacterial burdens in the urine by 14-fold (P = 0.3) and, most dramatically, reduced bacterial burden in the kidneys by 126-fold (P = 0.002) (Fig. 1 A–C). Altogether these data demonstrate that the siderophore–protein conjugates significantly reduce the bacterial burden in experimental UTI, particularly disseminating to the kidneys. At the time of sacrifice, a subset of the kidneys and bladders from infected mice were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Five-micrometer histologic sections were randomized and blindly scored for neutrophilic inflammation by a board-certified veterinary pathologist. Scores were semiquantitative and ranged from 0 (no inflammation) to 3 (severe inflammation) (SI Appendix, Fig. S3). Vaccination with cBSA–Ybt, cBSA–Aer, and 1:1 cBSA–Ybt:cBSA–Aer significantly reduced pyelonephritis and overall inflammation scores (Fig. 1 D–F).

To ascertain whether the vaccine specifically targets Ybt and Aer, three isogenic mutants were generated; E. coli HM69 ybtS::can (ybtS) and iucA::kan (iucA) have the Ybt and Aer biosynthetic machinery disrupted, respectively. In addition, both biosynthetic operons were disrupted in a ybtS–iucA double mutant. We hypothesized that if the vaccines specifically targeted Ybt or Aer during infection, then infection with each siderophore biosynthesis mutant would result in the corresponding vaccine-escape phenotype. Mice were vaccinated with either cBSA or cBSA–Ybt (SI Appendix, Fig. S1C) and then transurethrally challenged with ybtS. After 48 h, there were no significant differences in the bacterial burdens of ybtS between cBSA- and cBSA–Ybt-vaccinated mice (Fig. 2A and SI Appendix, Fig. S4A). Similar experiments were conducted by vaccinating mice with cBSA–Aer and then challenging with iucA, as well as by covaccinating with 1:1 cBSA–Ybt:cBSA–Aer and then challenging with ybtS–iucA (Fig. 2B and C and SI Appendix, Fig. S4B and C). In all instances, no significant differences were identified between the vaccinated groups and the cBSA control mice (Fig. 2 and SI Appendix, Fig. S4), indicating that the vaccines elicit an immune response specifically targeting Ybt and Aer during UTI.

The unexpected differences in kidney colonization for iucA and ybtS–iucA mutants (Fig. 2B and C) compared with the cBSA–Aer and 1:1 cBSA–Ybt:cBSA–Aer-vaccinated and infected with WT HM69 (Fig. 1C) could be due to disparities between the effects of genetically disrupting intracellular siderophore biosynthesis and immunologically targeting extracellular siderophore activity. In the case of iucA (Fig. 2B) and cBSA–Aer (Fig. 1C), it is possible that the immune response does not inactivate Aer-mediated iron acquisition as well as genetically disrupting Aer biosynthesis. Whereas in the case of ybtS–iucA (Fig. 2C) and 1:1 cBSA–Ybt:cBSA–Aer (Fig. 1C), ybtS–iucA may colonize the kidneys better than WT in 1:1 cBSA–Ybt:cBSA–Aer-vaccinated mice because of increased virulence or Ent production in ybtS–iucA. Studies have shown that disrupting siderophore biosynthesis impacts central metabolism, which could
Vaccination with siderophores reduces bacterial burdens and inflammation in the urine and kidneys during UTI. CBA/J mice were vaccinated with cBSA, cBSA-Ybt, cBSA-Aer, or 1:1 cBSA-Ybt:cBSA-Aer. At 21 d after the first immunization, mice were transurethrally inoculated with $10^7$ cfu of wild-type HM69. After 48 h, cfus in the (A) urine, (B) bladder, and (C) kidneys were enumerated by serial dilution and plating on LB agar. Limit of detection was $10^2$ cfu/mL of urine or cfu/g of organs. Bladders and kidneys were fixed, embedded in paraffin, sectioned, and H&E stained. Tissue sections were randomized and blindly scored in inflammation in the (D) bladder, and (E) kidneys. The pathology scores in (E) and (F) were combined to quantify overall inflammation in (D). Significance was calculated using a one-tailed Mann–Whitney test. In all panels, bars represent the median; in A–C, n ≥ 38; D–F, n ≥ 8.

![Figure 1](image)

**Fig. 1.** Vaccination with siderophores reduces bacterial burdens and inflammation in the urine and kidneys during UTI. CBA/J mice were vaccinated with cBSA, cBSA-Ybt, cBSA-Aer, or 1:1 cBSA-Ybt:cBSA-Aer. At 21 d after the first immunization, mice were transurethrally inoculated with $10^7$ cfu of wild-type HM69. After 48 h, cfus in the (A) urine, (B) bladder, and (C) kidneys were enumerated by serial dilution and plating on LB agar. Limit of detection was $10^2$ cfu/mL of urine or cfu/g of organs. Bladders and kidneys were fixed, embedded in paraffin, sectioned, and H&E stained. Tissue sections were randomized and blindly scored for inflammation in the (D) bladders and (E) kidneys. The pathology scores in (E) and (F) were combined to quantify overall inflammation in (D). Significance was calculated using a one-tailed Mann–Whitney test. In all panels, bars represent the median; in A–C, n ≥ 38; D–F, n ≥ 8.

**Fig. 2.** Protection provided by siderophore vaccines requires the biosynthesis of bacterial stealth siderophores. CBA/J mice were vaccinated according to the protocol but transurethrally inoculated with the corresponding siderophore biosynthesis mutant. (A) cBSA–Ybt-vaccinated mice were inoculated with HM69 ybtS, (B) cBSA–Aer-vaccinated mice were inoculated with HM69 iucA, and (C) 1:1 cBSA–Ybt:cBSA–Aer-vaccinated mice were inoculated with HM69 ybtS:iucA for each experiment. cBSA was used as the negative control (dark gray bar). In all instances, mice were transurethrally inoculated with $10^5$ cfus of the indicated strain. After 48 h, cfus in the urine and kidneys were enumerated by serial dilution and plating on LB agar. Statistical analyses using a one-tailed Mann–Whitney test identified no significant differences between negative control and siderophore-vaccinated groups (0.2336 < P < 0.4761). Limit of detection was $10^2$ cfu/mL of urine or cfu/g of kidneys; bars represent the median; and n ≥ 14.

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protein, immunization with Ybt and Aer no longer protected against UTI (Fig. 3A and B and SI Appendix, Fig. S6A), revealing that Ybt and Aer require cBSA to elicit a protective immune response.

To fully address whether the siderophores are acting as haptons, we next vaccinated the mice with cBSA unconjugated to Ybt, Aer, or a 1:1 mixture of Ybt:Aer. We hypothesized that if the siderophores are acting as haptons, then protection will be lost when the mice are vaccinated with cBSA and unconjugated siderophore. The mice were immunized with 0.75 μg of siderophore plus 10 μg of cBSA on day 0 and boosted with 0.19 μg of siderophore plus 2.5 μg of cBSA on days 7 and 14 postimmunization (SI Appendix, Fig. S1C). On day 21 postimmunization, mice were transurethrally inoculated with 10⁶ cfu of HM69, and after 48 h, the bacterial burdens in the urinary tract were quantified. In a small pilot experiment, bacterial burdens were diminished in the urine and kidneys (Fig. 3C and D and SI Appendix, Fig. S6B) following the same trend as the conjugated vaccine (Fig. 1), indicating that the siderophores are not acting as haptons and do not require conjugation to cBSA to protect against pyelonephritis.

Because Ybt and Aer require cBSA as an immunogen to elicit a protective immune response and bacterial production of the targeted siderophore, we hypothesized that protection is mediated by the adaptive immune system. Because the highly cationic surface charge of cBSA interferes with analysis by ELISA (51), we examined this notion using our murine model of ascending UTI. Mice were immunized once with 10 μg of cBSA, cBSA–Ybt, cBSA–Aer, or a 1:1 mixture of cBSA–Ybt:cBSA–Aer and then challenged transurethrally with E. coli HM69 7 d postimmunization. Challenging shortly after immunization precludes the development of an effective adaptive immune response (52). After 48 h, the bacterial burdens in the urinary tract were enumerated, and as predicted, no protection was observed (Fig. 3E and F and SI Appendix, Fig. S6C), suggesting that protection is mediated by an adaptive immune response.

Discussion

These findings provide a significant conceptual advance in which bacterial-derived small molecules have been successfully formulated in a vaccine regimen that reduces both inflammation and the bacterial burden in the kidneys and urine in a murine model of UTI (Fig. 1). To protect against pyelonephritis, the bacteria used for challenge must encode the targeted stealth siderophores (Fig. 2). The siderophores do not need to be conjugated to the immunogen to elicit protection, indicating that despite their small molecular weights (481 Da and 564 Da), Ybt and Aer are large enough to stimulate a protective immune response (Fig. 3A–D). In addition, successful vaccination with siderophores requires repeated co-administration with the immunogen cBSA over the course of 3 wk (Fig. 3E and F). Altogether, these data suggest that the cBSA-siderophore conjugates elicit an adaptive immune response that specifically targets pathogens encoding stealth siderophores. We propose that Aer and Ybt are suitable for inclusion in multicomponent vaccines due to their amenable biochemical features, specificity for pathogenic bacteria compared with commensals or organisms, and synergistic protection in the kidneys.

We have been unable to confirm, however, that the protective immune response is antibody-mediated, as we do not detect siderophore-specific antibodies by direct or sandwich ELISAs or ELISpot using the cBSA-conjugated siderophores as capture antigens. Because cBSA interferes with ELISAs (51) and the siderophores are not suited for adsorption to ELISA plates, we have also probed for anti-siderophore antibodies by direct and sandwich ELISAs with Ybt and Aer conjugated to cholera toxin or BSA; neither revealed siderophore-specific antibodies. It is possible that these carrier proteins are poorly haptenized or antisiderophore antibodies do not recognize the siderophores in the context of these carrier proteins. Despite our best efforts, we have been unable to identify siderophore-specific antibodies, however the data presented suggest that the vaccine is specific (Fig. 2) and acts in a time frame...
consistent with adaptive immunity (Fig. 3 E and F). It is possible that siderophore-specific antibodies are either absent or present and effective but below the limit of detection. Nonetheless, the utility of small molecules formulated for use as vaccine antigens is intriguing.

Previous publications describing the use of siderophores in vaccines against human pathogens primarily focus on eliciting an antibody response and either did not observe protection against infection or did not use an animal model to evaluate the effectiveness of the vaccine. The first report describing the development of monoclonal antibodies (MAbs) against a siderophore targeted pseudobactin, the siderophore of the plant pathogen *Pseudomonas putida*. These MAbs were primarily used for studying iron acquisition in the plant pathogen and were not developed for therapeutic applications (53). Shortly thereafter, another group elicited mouse MAbs against ferric Aer (FeAer) and demonstrated that these antibodies restricted *E. coli* growth in calf serum (54). These FeAer-specific MAbs were useful for detecting Aer-producing *Enterobacteriaceae* but did not provide protection in three tested models of infection (55, 56). A lethal i.p. infection model with *E. coli* and *Klebsiella pneumoniae* was used to assess the protection provided upon either passive or active immunization of Swiss mice (55). In both cases, vaccination did not significantly improve the survival of the mice despite the confirmed presence of anti-FeAer antibodies. The same vaccine preparation was also used to assess protection in a passive transmucosal immunization chick model challenged with *E. coli*, and despite the presence of Aer-specific antibodies, the chicks succumbed to infection similar to the unvaccinated controls (55). The last published study regarding siderophore vaccine preparation successfully elicited vibriobactin-specific antibodies in mice, but the protective effects of these antibodies were never reported (57). Building upon these studies, we have shown that vaccination with siderophores is protective in a mouse model of ascending UTI. The success of the vaccine strategy described here compared with previous publications using siderophores may be due to the intranasal immunization route and an animal model that better recapitulates natural infection.

It has been documented that vaccination with outer-membrane proteins up-regulated during iron limitation protects against *E. coli* infections (43). Specifically, our laboratory investigated the immunoprotective properties of the outer-membrane protein receptors for Ybt and Aer: namely, FyuA and IutA, respectively (20, 42). Immunization with FyuA resulted in a 1.99 logs decrease in the bacterial burden in the kidneys (urine counts were not reported), and immunization with IutA reduced bacterial burden in the urine, bladder, and kidneys by 1.3, 1.0, and 0.9 logs, respectively. Studies reported here recapitulate these findings with the exception of the protection observed in the bladder with the IutA vaccine. In our extensive experience with developing UTI vaccines, we have found that protection in the bladder is more difficult to achieve in our murine model than protection in the kidneys, likely due to low levels of antigen-specific antibody detected in the bladder (20, 42). This may be due to technical issues implicit in a murine model of UTI or the lower abundance of antibodies in the urine compared with the sera (20, 58–60). Alternatively, the lack of protection in the bladder in response to targeting Aer-mediated iron acquisition could be due to the different immune response elicited by vaccination with the low-molecular mass siderophores compared with the proteinaceous receptors.

Building upon these prior studies, we now show that vaccination with siderophores protects against pyelonephritis in a mouse model of ascending UTI and circumvents the biochemical challenges associated with using outer-membrane proteins as antigens (Fig. 1). Moreover, Aer and Ybt may be suitable for a multicomponent vaccine due to their amenable biochemical features, specificity for pathogenic bacteria over commensal organisms, and synergistic protection in the kidneys. The practical utility of cBSA as an immunogen in human vaccines remains to be evaluated in future studies. Combining Aer and Ybt with other protective antigens such as adhesins, toxins, and other iron receptors may prove to be a successful vaccination strategy (19). Adding Aer and Ybt to such multicomponent vaccine preparations should prove simple due to their amenable biochemical properties and their ability to elicit protection without being conjugated to a carrier protein (Fig. 3D).

Notably, highly evolved iron acquisition strategies, including Aer and Ybt, are critical virulence factors for ExPEC and other pathogenic species within the *Enterobacteriaceae* (61, 62). Therefore, by defining the protective efficacy of two stealth siderophores prevalent among pathogenic *E. coli* (39), we have demonstrated that Aer and Ybt are valuable antigens that have not heretofore been included in any UTI vaccines and, more generally, exemplify the untapped resource of bacteria-specific small molecules as potential vaccine antigens.

Online Methods

Descriptions of the bacterial strains, culture methods, and recombinering; cBSA–siderophore conjugation; tandem mass spectrometry; and histologic scoring are in SI Appendix, SI Materials and Methods.

Vaccination Procedures. All mouse studies were conducted in accordance with protocol 00005052 approved by the Institutional Animal Care and Use Committee at the University of Michigan. This protocol is in compliance with the National Institutes of Health guidelines for the humane use and care of laboratory animals.

Vaccination was performed as previously described (SI Appendix, Fig. S1C) (20). All vaccine doses were prepared in advance from 2 mg/mL stocks. Aliquots were flash-frozen and stored at −80 °C until the time of vaccination. Briefly, 6–7-wk-old CBA/J mice were intranasally immunized with 20 μL of vaccine conjugate (10 μL/nare). On day 0, the mice received 10 μg of the appropriate vaccine conjugate (0.5 mg/mL in PBS, pH 7.4). On days 7 and 14, mice were boosted with 2.5 μg of vaccine conjugate (0.125 mg/mL in PBS, pH 7.4). Mice were challenged, as described below, on day 21.

One exception to this protocol was for vaccinations that used unconjugated siderophores. When PBS pH 7.4 or unconjugated cBSA was used as controls, mice were immunized with 0.75 μg siderophore (37.5 μg/mL in PBS pH 7.4) and boosted with 0.19 μg of siderophore (9.4 μg/mL in PBS pH 7.4). The amounts of siderophores used were calculated based on the maximum amount of siderophore the mice could potentially receive from the cBSA–vaccine conjugates. A second exception to the above protocol was when mice were transurethrally challenged 1 wk after the initial immunization. No boosters were given to assess the requirement for adaptive responses to elicit protection.

Murine Model of Ascending UTI. A previously described murine model of ascending UTI was used to assess vaccine efficacy (20, 58). Unless noted otherwise, female CBA/J mice were transurethrally challenged with 50 μL of 4 × 10⁹ cfu/mL of E. coli Hm69 resuspended in PBS, resulting in an inoculum of 10⁶ cfu per mouse. The inoculum was delivered using a sterile 0.28-mm inner-diameter polyethylene catheter connected to an infusion pump (Harvard Apparatus) over a period of 20 s. Infection progressed for 48 h, after which bacterial burdens in urine as well as bladder and kidney homogenates were enumerated. Tissues were homogenized in PBS with a GLH homogenizer (Omni International) and plated on LB agar using an Autoplate 4000 spiral plater (Spiral Biotech). A QCount automated plate counter (Spiral Biotech) was used to enumerate colony counts on the plates.

Statistical Analysis. Unpaired, nonparametric analysis was performed using the Mann–Whitney test calculated in GraphPad Prism 6.01 for Windows. Results were considered significant if the P value was less than or equal to 0.05.

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