Identification of the Outer Membrane Immunoproteome of the Uropathogen *Proteus mirabilis*: Insights into Virulence and Potential Vaccine Candidates

by

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DEDICATION

To Mom and Dad – Thank you for teaching me the value of education and instilling in me the belief that I could do and be whatever I want to in life. Your unwavering love and support mean more to me with each passing day.

To Cara – Thank you for being the best sister and friend I could ever hope for. I have always looked up to you and always will.

To Brent – Thank you for being my best friend, the love of my life, and my partner in all of life's adventures. I feel so lucky to have you as my husband, and I could not have done this without your love and support.

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ABSTRACT

Proteus mirabilis, a gram negative bacterium, represents a common cause of complicated urinary tract infections (UTIs) in catheterized patients or those with functional or anatomical abnormalities of the urinary tract. To systematically identify surface-exposed antigens, proteins in the outer membrane fraction of bacteria were separated by 2D gel electrophoresis and subjected to Western blotting with sera from mice experimentally infected with *P. mirabilis*. Proteins recognized by sera were identified by mass spectrometry. Thirty-seven antigens (including 24 outer membrane proteins) to which a humoral response had been mounted were identified; these antigens are presumably expressed during infection and therefore represent potential virulence factors. Six representative antigens were selected for further study. Of these antigens, three played no apparent role in pathogenesis, as strains with isogenic mutations were not attenuated in the mouse model of ascending UTI: a putative secreted 5'-nucleotidase (PMI0047), RafY (PMI0288), and FadL (PMI1810). However, two putative iron acquisition proteins, PMI0842 and PMI2596, both contribute to fitness in the urinary tract. The sixth antigen, ZnuB (PMI1150), was annotated as the inner membrane component of the high affinity zinc (Zn^{2+}) transport system ZnuACB. Components of this system have been shown to contribute to virulence in other pathogens; therefore, the role of ZnuACB in *P. mirabilis* was investigated by constructing a strain with an insertionally interrupted copy of *znuC*. The znuC::kan mutant was more sensitive to zinc limitation than wild type, was outcompeted by wild type in minimal medium, displayed

reduced swimming and swarming motility, and produced less *flaA* transcript and flagellin protein. Production of flagellin and swarming motility were restored by complementation with *znuCB in trans*. Swarming motility was also restored by the addition of Zn^{2+} to agar prior to inoculation. ZnuC offers a competitive advantage during urinary tract infection. Since we demonstrated a role for PMI0842, PMI2596, and ZnuC in UTI, we hypothesize that there is limited iron and zinc present in the urinary tract and that *P. mirabilis* must scavenge these ions to colonize and persist in the host.

CHAPTER I. Introduction

Proteus mirabilis, a causative agent of complicated urinary tract infections

General introduction to *P. mirabilis*

Proteus mirabilis is a motile, Gram negative bacterium. The genus *Proteus* is a member of the family *Enterobacteriaecae* (178, 179). The first description of *Proteus* bacteria was made in 1885 by Hauser, who named them for the character in Homer's Odyssey who 'has the power of assuming different shapes to escape being questioned' (81). This name is most likely in reference to the most distinguishing characteristic of *Proteus* species, swarming motility. Swarming motility is a specialized form of motility which involves the differentiation of bacterial cells into elongated forms that become covered with flagella; the cells then migrate across surfaces *en masse*. This characteristic behavior is described in more detail below. There are currently four recognized species of *Proteus*: *P. mirabilis*, *P. penneri*, *P. vulgaris*, and *P. myxofaciens* (179). These *Proteus* species are widely disseminated in the environment and are found in soil and water samples.

Three of the four species (all except *P. myxofaciens*) are also associated with opportunistic human infections. Of these three, *P. mirabilis* is most often isolated from infections; it is hypothesized that the reason may be a higher carriage rate of *P. mirabilis* in human intestines. *P. mirabilis* has been isolated from the respiratory tract, eyes, ears, throat, skin, and burns and is speculated to be a possible cause of gastroenteritis (41, 90,

178, 189). *P. mirabilis* is also a major cause of complicated urinary tract infection (UTI). In addition to its role as an opportunistic pathogen, there also appears to be a connection between infection with *P. mirabilis* and rheumatoid arthritis (54, 55, 184); it is proposed that subclinical urinary tract infection with *P. mirabilis* may lead to the development of cross-reactive antibodies.

Complicated UTI

As briefly mentioned above, *P. mirabilis* is a major causative agent of complicated UTI. Perhaps it is not surprising, given its identity as an opportunistic pathogen, that *P. mirabilis* is rarely associated with UTI in patients that are otherwise considered healthy; these types of infections, often referred to as community-acquired or uncomplicated UTIs, are caused by *Escherichia coli* in the vast majority of cases (204, 242). Instead, *P. mirabilis* is associated with complicated UTIs that occur in patients who have functional or anatomical abnormalities in the urinary tract or are undergoing catheterization (243, 244). Catheter-associated UTIs are the most common type of nosocomial infection, totaling over one million cases annually (225). Although complicated UTIs are often polymicrobial, *P. mirabilis* has been documented in as many as 44% of cases (244). Other bacterial species commonly detected in polymicrobial UTIs include *E. coli*, *Klebsiella pneumoniae*, *Providencia stuartii*, and *Morganella morganii*, as well as the other Proteus species *P. penneri* and *P. vulgaris* (149). Another important distinction between uropathogenic *E. coli* (UPEC) and *P. mirabilis*, besides the types of UTIs they cause, is that UPEC are a very specific subset of *E. coli*; however, it appears that all strains of *P. mirabilis*, regardless of isolate origin, are capable of causing UTI (176, 216).

UTIs typically occur in an ascending fashion. Bacteria colonize the periurethral area and gain access to the bladder through the urethra; in catheterized patients, colonization of the catheter provides direct access to the bladder. Once bladder colonization has occurred, bacteria can ascend the ureters to gain access to the kidneys. From there, the infection may spread into the bloodstream. *Proteus* UTIs are typically persistent infections that are difficult to treat (189). Complications of these infections include the development of bacteriuria, urinary tract obstruction, and the formation of bladder and kidney stones (209, 241, 244). In catheterized patients, *P. mirabilis* UTI can lead to catheter encrustation, which can subsequently result in obstruction of the flow of urine through the catheter. Once the infection has reached the kidneys, severe histological damage can occur. These infections, if left untreated, can result in sepsis (246).

The serious complications that can result from and the public health impact of *P. mirabilis* UTIs have resulted in vigorous research into the bacterial virulence factors that contribute to this disease (45, 90, 189). *P. mirabilis* HI4320 is a prototypical UTI isolate that was recovered from the urine of an elderly female nursing home patient with an indwelling catheter (149); recently, this strain was sequenced and annotated, which provided new insight into putative virulence factors encoded by this species (174). The development of a vaccine against *P. mirabilis* is also an active area of research. There is a defined population that would benefit from its development, namely, patients with known abnormalities of the urinary tract or those at the onset of long-term

catheterization. Considering the difficulty in treating these infections once they occur, a vaccine could have a major impact on patient care.

Swarming motility

As alluded to above, perhaps the most distinctive characteristic of *P. mirabilis* (and other *Proteus* species) is swarming motility. Swarming motility, simply defined, is "the movement of highly elongated and flagellated swarm cells across the surface of a solid medium in periodic cycles of movement and consolidation" (250). Considering the fact that this specialized form of motility was first reported by Hauser in 1885 (81), and has therefore been the subject of research for almost 125 years, our understanding of this behavior is still relatively poor.

P. mirabilis cultured in broth (referred to as vegetative swimmer cells) are typically 1-2 μm long; after inoculation onto solid agar medium, the cells undergo a drastic morphological change and they increase to 20-80 μm in length. As the shorter cells elongate, there is a corresponding increase in the number of flagella per cell; short cells typically have 1-10 flagella, but the number of flagella present on the elongated cells has been estimated to be between 500-5,000 (85). In addition, the rate of DNA replication remains constant even though septation is inhibited (69); thus, swarmer cells are polyploid. These morphological changes are referred to as the process of differentiation; differentiation is required for swarming motility. Swarming is typically thought about in terms of three stages: differentiation into swarmer cells, the migration of swarmer cells across a surface, and de-differentiation (or consolidation, the return to shorter cells). In addition, it is possible that recognition of an environmental cue is

necessary to begin differentiation. A defect at any stage of this process could result in either abnormal or a complete lack of swarming.

It is important to distinguish between swimming motility and swarming motility. Although both are forms of flagellar-mediated motility, they are quite different from each other. Swimming motility is thought of as an individual endeavor, while swarming requires movement of a group of bacteria (80). Individual swarmer cells do not have the ability to swarm by themselves (34). *Proteus* swarming is also different from the swarming motility observed in other types of bacteria; *Proteus* swarming seems to be uniquely regulated in temporal cycles (8). This temporal regulation is easily observed by monitoring swarming motility over the surface of agar plates, and is the cause of the characteristic bulls-eye pattern of swarming colonies.

Although early reports hypothesized that the formation of swarm cells was a response to the deterioration of nutritional conditions (akin to a stress response (152, 191)), it is now generally accepted as a normal part of the life cycle of *P. mirabilis*. Early reports also suggested that swarming was a negative chemotactic response (129), but later reports refuted those claims (249). Although the cues that initiate swarming are not completely understood, the inhibition of flagellar rotation and sensing of glutamine play a role (8, 9, 27, 30).

One of the reasons that swarming behavior is not better understood probably stems from that fact that much of what is known has been deduced from identifying strains with mutations that affect swarmer cell differentiation and migration (26-28, 30, 185). Swarming is a complex process that is apparently affected by many genes. Thus, it has not been easy to identify a common thread between many of these mutations.

Combining these vast amounts of data in a meaningful way has been challenging, and swarming motility is still an active area of research.

Pathogenesis during UTI

Discovery of much of the information in the following section has been made possible by the availability of a mouse model for ascending urinary tract infection. Most of this work was carried out using a modification of the protocol designed by Hagberg and colleagues (75). This procedure involves the sedation and temporary catheterization of mice; the inoculum is delivered through the catheter and introduced directly into the bladder. In general, most studies from our lab have utilized CBA/J mice; others have used BALB/c mice. This mouse model has been extremely valuable, especially since it recapitulates many important aspects of human infection. Bladders and kidneys of mice can be colonized. *P. mirabilis* can also spread from the tissues of the urinary tract to cause a systemic infection, as assessed by enumerating bacteria present in the spleen following transurethral inoculation. Additionally, stone formation in the urinary tract occurs, which is a hallmark of infection with *P. mirabilis* in human patients. In addition to an animal model, *in vitro* cell culture systems have also been utilized to characterize various virulence factors. Importantly, *P. mirabilis* is amenable to genetic manipulation; although not as straightforward as in *E. coli*, the generation of isogenic mutations is possible.

Flagella-mediated motility

When the annotation of the *P. mirabilis* HI4320 genome was completed, perhaps one of the most surprising findings was that all flagellum-related genes are located together within a single locus, which is highly unusual (174). Another interesting feature

is the copy of multiple genes encoding flagellin, *flaA* and *flaB*, which are located in direct proximity to each other (29). Normally, the *flaA* allele is expressed while *flaB* is silent (25). However, these genes can recombine, resulting in the formation of antigenically distinct flagella (25, 160). Considering that flagellin is strongly antigenic, it has been postulated that this recombination could contribute to immune evasion during infection.

The regulation of flagella-mediated motility in *P. mirabilis* appears to be similar to that in *E. coli*. That is, there appears to be a hierarchy of regulation composed of three classes (42, 217). Class I is comprised of FlhDC, the "master regulators" of flagellar motility. The FlhDC complex activates expression of Class II genes, which encode proteins necessary for basal body and hook formation, as well as FliA. Production of FliA, a transcription factor, results in the expression of the Class III genes, including flagellin.

The contribution of swarming motility to virulence of *P. mirabilis* remains a topic of great debate in the field (6, 7, 92, 143, 260). Interestingly, it appears that the expression of at least some virulence genes seems to be higher in swarmer cells than vegetative cells (10, 62, 239). However, *in vivo*, it appears that swarmer cells are in the minority (92, 257). It has been well documented that *P. mirabilis* can swarm across the surface of urinary catheters (102, 154, 194); therefore, it seems likely that this morphotype is encountered during human infection, at the very least during early stages.

It is difficult to apply classical genetic methods to distinguish between the contributions of swimming and swarming motility *in vivo*, since both types of motility are flagella-mediated. It is clear, however, that the production of flagella contributes to pathogenesis. A nonmotile mutant lacking flagella was generated by interrupting *flaD*,

which encodes the flagellar cap protein; this mutant synthesizes wild-type levels of FlaA but cannot assemble flagella (143). The FlaD$^-$ strain was significantly impaired in its ability to infect mice; this strain was recovered in numbers approximately 100-fold lower than the control strain (143). However, flagella do not appear to be absolutely necessary for infection, as a *P. mirabilis* strain lacking flagella has been isolated from a human UTI patient (260).

Fimbriae

A role for fimbriae in *P. mirabilis* pathogenesis was suggested 35 years ago when microscopic evaluation of renal tissue from infected rats revealed the presence of piliated bacteria (212). The strain of *P. mirabilis* used in the study was isolated from a patient experiencing UTI and produced two distinct types of fimbriae; one was 4 nm thick and the other was 7 nm thick (212). These fimbriae could be selected for based on culture technique; bacteria cultured to enrich for the 7 nm fimbria were more virulent in the rat model than bacteria cultured to enrich for the 4 nm think fimbria (212).

Historically, fimbriae of *P. mirabilis* were characterized by their hemagglutination (HA) properties and fell into two classes based on this activity: mannose-resistant/*Proteus*-like (MR/P) and mannose-resistant/*Klebsiella*-like (MR/K). As the names imply, HA activity of both types of fimbriae was resistant to mannose (*i.e.*, addition of mannose did not inhibit HA); fimbriae with MR/P activity resulted in agglutination of fresh (but not tannin-treated) erythrocytes, while fimbriae with MR/K activity resulted in agglutination of tannic acid-treated (but not fresh) erythrocytes. MR/K HA activity appeared to be associated with thin fimbriae (approximately 4-5 nm wide), and MR/P HA activity was associated with thicker fimbriae (7-8 nm wide) (167). A

collection of *P. mirabilis* strains was assessed for MR/P and MR/K HA activity; 100% of strains displayed both types of activity (1, 167). MR/P activity resulted in agglutination of erythrocytes from a broad spectrum of species; the majority of strains were able to agglutinate fowl, guinea pig, horse, sheep, and human erythrocytes (1, 167). The MR/P HA activity has been associated with the MR/P fimbriae encoded by *P. mirabilis* (discussed in detail below), while the fimbria(e) responsible for MR/K HA activity remains unclear.

Recently, the sequencing and annotation of *P. mirabilis* strain HI4320 revealed that a plethora of putative fimbriae are encoded in the genome; there are 17 chaperone-usher fimbrial operons and 13 additional orphan fimbrial genes (not associated with a complete operon) (174). Given that *P. mirabilis* can express more than one type of fimbriae at the same time (1, 21, 112, 144, 167, 212) and the large number of putative fimbriae encoded in the genome of at least one uropathogenic isolate (174), work characterizing *P. mirabilis* fimbriae must be performed with carefully designed controls.

To date, only five of these fimbriae have been characterized experimentally: mannose-resistant/*Proteus*-like (MR/P), uroepithelial cell adhesin (UCA, also called NAF for nonagglutinating fimbriae), ambient temperature fimbriae (ATF), *Proteus mirabilis* fimbriae (PMF), and *Proteus mirabilis* P-like fimbriae (PMP). These fimbriae are described in greater detail below.

MR/P

As described above, mannose-resistant/*Proteus*-like (MR/P) HA activity and associated fimbriae were first described and assessed in *P. mirabilis* by hemagglutination and microscopy (1, 167, 212). The fimbria associated with this activity was identified

and named the MR/P fimbria (21). Genes responsible for production of MR/P fimbriae are contained within two divergently transcribed segments, *mrpABCDEFGHJ* and *mrpI* (23).

Structural components of the fimbria are encoded on the *mrpABCDEFGHJ* transcript: MrpA is the major pilin (22, 23); MrpB is the fimbrial terminator (122); MrpE, MrpF, and MrpG are predicted to be minor pilins (23); and MrpH is the tip adhesin (119). MrpC and MrpD are the putative usher and chaperone, respectively, that aid in assembly and secretion of the fimbria (23). MrpJ, a regulatory protein that is not a structural component of the fimbriae (119), is described in more detail in the next section of this chapter (Reciprocal regulation of adherence and motility, below). MrpI is a recombinase that regulates expression of MR/P fimbriae. Downstream of *mrpA*, there is a putative stem-loop structure that is hypothesized to function as a transcriptional terminator or attenuator (22, 23); this structure would allow for production of greater amounts of MrpA (the major structural subunit) relative to the other proteins (including the minor subunits) that are encoded downstream on the same transcript.

As described above, *mrpI* and *mrpABCDEFGHJ* are divergently transcribed (23). Promoter sequences have only been found upstream of *mrpI* and *mrpA*, suggesting that genes *mrpABCDEFGHJ* are located on one transcript (23). The intergenic region between *mrpI* and *mrpA* contains an invertible element flanked by inverted repeats (255). This invertible element contains a canonical σ^{70} promoter that, when in the proper orientation, drives expression of *mrpA* (255). MrpI is the recombinase that controls switching of the invertible element (255). MrpI mediates bi-directional switching; that is, MrpI can change the promoter position both from ON to OFF (thereby shutting off

expression of MR/P fimbriae) and also from OFF to ON (thereby turning on fimbrial expression) (255). Furthermore, MrpI is the sole recombinase that performs these functions; an isogenic null mutant is incapable of changing promoter orientation (121). In contrast to *mrpA*, expression of *mrpI* is independent of invertible element orientation (255). The development of a PCR-based screen that differentiates between MR/P ON and MR/P OFF bacteria within a given population has proven a valuable tool for studying MR/P fimbrial expression and regulation (255). In addition, generation of the *mrpI* null mutant (which, again, is incapable of mediating switching of the invertible element) allowed the isolation of strains that had the promoter permanently in either the ON or the OFF position (the "locked on" or L-ON strain and the "locked off" or L-OFF strain, respectively) (121).

Strains of *P. mirabilis* with mutations in *mrpA*, *mrpB*, *mrpG*, and *mrpH* have contributed to understanding of the function of MR/P fimbriae and the roles of these individual components. MrpA, MrpG, and MrpH are required for the formation of fimbriae on the surface of the bacterial cell (21, 119, 125, 259). Perhaps it is not surprising, then, that most of these proteins are also required for MR/P HA activity (21, 119, 125, 259). MrpH, the tip adhesin, functions as the hemagglutinin (119); site-directed mutants of MrpH in lacking N-terminal cysteine residues (C66S and C128S), when expressed in *E. coli*, allowed formation of fimbriae but lacked HA activity (119). However, MrpH may not be the only source of MR/P HA activity in *P. mirabilis*; the MrpH mutant retained limited HA activity (119). In contrast, the MrpG mutant (which does not assemble fimbriae) lacked detectable HA activity (125). The reason for this discrepancy is unclear. The MrpB mutant produced significantly longer fimbriae than

wild type, yet displayed reduced HA activity (122); it was hypothesized that the longer fimbriae were more fragile and more prone to being sheared off the surface during sample preparation, resulting in the loss of the hemagglutinin (MrpH) (122). In addition, wild-type bacteria that overexpressed MrpB had significantly shorter fimbriae than wild type (122). These phenotypes are consistent with a role of MrpB as a fimbrial terminator protein, meaning that when MrpB is inserted into growing fimbriae, subunit addition is terminated.

MrpA mutants retained normal urease production, hemolytic activity, and adherence to exfoliated uroepithelial cells collected from healthy human volunteers (21, 259). However, adherence of an MrpA mutant to T24/83 (derived from a human bladder carcinoma) and HEp-2 (human laryngeal carcinoma) cells cultured *in vitro* was diminished significantly when compared with the adherence of wild type (188, 259). MrpA mutants retained normal swarming activity (21, 259), as did the L-ON strain (93). However, the L-OFF strain swarmed significantly more than both wild-type and L-ON strains (93).

MR/P fimbriae have also been implicated in autoaggregation (119, 188) and biofilm formation (93, 188). The L-ON strain developed mature biofilms faster than the wild-type and L-OFF strains (93). However, the ability to regulate MR/P expression is important in biofilm formation as well, since both L-ON and L-OFF strains ultimately produced thinner biofilms than the wild-type strain (93).

Initial studies revealed that sera from infected mice reacted strongly to MR/P fimbrial preparations, which indicates these fimbriae are expressed *in vivo* (20). The expression of MR/P fimbriae appears to be highly induced during infection (121, 255).

Culture under oxygen limitation *in vitro* induces MR/P expression (111). Since the urinary tract is proposed to be oxygen-limited (215), this condition could contribute to the upregulation of MR/P fimbriae observed *in vivo*. Many of the mutants described above have been assessed in the mouse model of UTI. The MrpA mutant of strain HI4320 was able to colonize mice, but was recovered in statistically significantly lower numbers than the wild-type strain from urine, bladders, and kidneys of infected mice (21). Therefore, MrpA is not absolutely required for infection, but contributes significantly to the ability of *P. mirabilis* to colonize the host. This mutant also resulted in significantly less severe renal damage than the wild-type strain, although this phenotype could be a function of the reduced colonization observed during infection (21). Not surprisingly, an MrpA mutant in another strain (Pr2921) was also outcompeted by wild type in the urinary tract during cochallenge and was significantly less infectious than wild type in an intravenous infection model (259). The MrpG mutant colonized mice significantly less than the wild-type strain (as assessed by quantitative culture of urine, bladders, and kidneys of infected mice) (125). The MrpH mutant colonized infected mice just as well as the wild-type strain during independent challenge but was outcompeted by wild type in urine, bladders, and kidneys during cochallenge (119); these data suggest MrpH is not required for colonization but contributes to the fitness of *P. mirabilis* during infection. It is interesting that the MrpH mutant was able to colonize mice just as well as wild type while MrpA and MprG mutants were not; these mutants all lack fimbriae on the surface (21, 119, 125, 259), so the reason for this phenotypic difference is not clear.

The L-ON and L-OFF mutants have also been utilized to assess the importance of MR/P fimbriae *in vivo*. There is no difference in colonization levels of L-ON, L-OFF,

and the wild-type strain during independent challenge (121). The L-OFF data correlate with MrpH mutant independent challenge data; that is, both experiments suggest that MR/P fimbriae are not required for infection. However, during cochallenge, L-OFF was significantly outcompeted by the wild-type strain in urine, bladders, and kidneys of infected mice (121), suggesting that MR/P fimbriae do, in fact, contribute a competitive advantage to *P. mirabilis* during infection. In another cochallenge experiment, the L-ON strain significantly outcompeted the L-OFF strain in bladders (but not kidneys) of infected mice (121). The L-ON strain also significantly outcompeted the wild-type strain in bladders (but not kidneys) during cochallenge (121). This particular result is somewhat surprising, considering that MR/P fimbriae are thought to be highly expressed *in vivo* by wild type (121, 255). Bacteria used for inoculation were cultured under conditions in which the wild-type cells would not have been synthesizing MR/P fimbriae (121); since the L-ON strain constitutively expresses MR/P fimbriae, perhaps the difference in MR/P expression at the time of inoculation was sufficient to affect the outcome of infection.

Despite differences in bladder colonization during infection, at least one study found that expression of MR/P fimbriae does not offer a quantitative advantage in adherence to bladder tissue *in vivo* (93). However, the presence of MR/P fimbriae may lead to qualitative changes in tissue binding; the L-ON strain was observed to adhere predominately to uroepithelial cells while the L-OFF strain adhered to lamina propria in areas where bladder cells had sloughed off, exposing layers which are not routinely exposed (93). MR/P fimbriae have also been proposed to facilitate binding to renal epithelium (199).

The *mrp* operon appears to be present in all isolates of *P. mirabilis* (23, 188). Additionally, MrpA from two clinical isolates were 100% identical, suggesting that the genes encoding MR/P fimbriae may be highly conserved (22, 259). The genome sequence of strain HI4320 revealed the presence of a second *mrp* operon, termed *mrp'*, immediately adjacent to *mrpI* (174). The *mrp'* operon is 66.7% identical to the *mrp* operon at the nucleotide level (174). However, there does not appear to be an invertible element in the promoter region of *mrp'*, suggesting this operon is not subject to regulation by MrpI (174). Interestingly, there are also two copies of *mrpA* in *P. mirabilis* Pr990 (259). The presence of more than one copy of the *mrp* operon (or at least *mrpA*, in the case of Pr990) in two clinical isolates of *P. mirabilis*, suggests this phenomenon could warrant further study. Zunino *et al.* speculated about the possibility of rearrangement of the two *mrpA* alleles (similar to what has been documented for flagellin genes *flaA* and *flaB*) (259); however, this hypothesis has not been experimentally evaluated. Furthermore, it has not yet been determined if the *mrp'* operon is expressed, results in production of fimbriae, or affects any aspect of *P. mirabilis* pathogenesis.

UCA (NAF)

Uroepithelial cell adhesin (UCA) was initially discovered in a uropathogenic isolate of *P. mirabilis* (strain HU1069) during a screen designed to identify outer membrane proteins that facilitated binding to uroepithelial cells (251). The identified UCA protein – the major structural subunit later designated UcaA – was purified to homogeneity for characterization; the purified protein retained the ability to bind to uroepithelial cells and, additionally, organized into long, flexible filaments with a diameter of 4 to 6 nm, consistent with the appearance of fimbriae (251).

N-terminal sequencing was performed on purified UCA. The sequence of the first 25 amino acids of the UCA protein displayed more similarity to the K99 pilus of *E. coli* (which mediates binding to intestinal epithelium) than to adhesins from uropathogenic *E. coli* (251). Subsequent determination of the sequence of the *ucaA* gene revealed that UcaA has the highest similarity to F17 and F111 fimbriae from bovine enterotoxigenic *E. coli* (33, 46). These findings led to postulation that UCA may function as a primary adhesin for *P. mirabilis* in the intestinal tract, although this hypothesis has not been experimentally tested (46, 251).

UCA fimbriae have since been renamed nonagglutinating fimbriae (NAF) to distinguish them from other *P. mirabilis* fimbriae that contribute to adherence (228). (Here, the two terms are used interchangeably based on their use in original literature since there is a lack of consensus in the field and both names are still employed.) Tolson and colleagues confirmed that what they designated the NAF subunit was identical to the previously identified UCA subunit based on N-terminal sequencing (228). Pre-incubation with monoclonal antibodies specific for NAF significantly reduced binding of *P. mirabilis* to HEp-2 and uroepithelial cells in vitro, providing additional evidence of a role for NAF in adherence to host cells (112, 228, 229).

A survey of a small collection of *P. mirabilis* strains revealed that all isolates produced UcaA (228). This conclusion was based on production of a protein of similar molecular weight to UcaA following the same purification technique used to isolate UcaA; protein bands were confirmed to be identical (or highly similar) to UcaA by N-terminal sequencing (228). UcaA was also purified from two *P. mirabilis* strains isolated from canine urine (33); N-terminal sequencing of UcaA from these canine isolates

revealed it as identical to that of the human isolate previously sequenced (33, 63, 251). UcaA appears to be present in 85-92% of *P. mirabilis* canine isolates (33, 63).

The binding properties of NAF were investigated further by using a thin layer chromatography approach to screen for NAF binding to a panel of commonly occurring glycolipid families that have been shown to mediate binding of other bacterial species (113). Purified NAF bound to asialo-GM_1, asialo-GM_2, and lactosyl ceramide (113). Binding to each glycolipid was concentration-dependent and was inhibited by treatment with monoclonal antibodies directed at NAF (113). In addition, binding of *P. mirabilis* to Madin-Darby canine kidney (MDCK) cells cultured *in vitro* was inhibited by treating the kidney cells with an antibody directed against asialo-GM_1 (113). However, no asialo-GM_1 has been detected in MDCK cells (113, 166); therefore, the anti-asialo-GM_1 antibody must be cross reactive with another glycolipid on the cell surface of MDCK cells. Interestingly, binding of *P. mirabilis* was not completely blocked by treatment with either anti-NAF or anti-asialo-GM_1 antibodies, suggesting other bacterial adhesins and host cell factors, respectively, are likely involved in the binding process.

Indeed, galectin-3 is an additional host factor to which NAF can bind (14). Galectin-3 was identified from and detected at the surface of MDCK cells, and galectin-3 purified from MDCK cells bound to purified NAF *in vitro* (14). Additionally, pre-treatment with an anti-galectin-3 antibody partially inhibited *P. mirabilis* binding to MDCK cells (14); therefore, binding of *P. mirabilis* to MDCK cells can be mediated through galectin-3.

UCA/NAF fimbriae do not appear to be responsible for the MR/K hemagglutinin activity of *P. mirabilis*; no hemagglutination was detected in *P. mirabilis* cultured for

optimal UCA/NAF expression or in *E. coli* expressing NAF during gain of function studies (46, 228, 251). Given data supporting a role for these fimbriae in adherence to host cells, it is surprising that their role in virulence has not been assessed. No such experiments have been reported to date, despite the availability of an animal model for *P. mirabilis* UTI.

ATF

Ambient temperature fimbriae (ATF) were named because they are expressed optimally during static culture in Luria broth at 23˚C (136). They are also expressed, to a lesser degree, during static and aerated culture in Luria broth at 37˚C, but not during culture in minimal medium, on agar plates, or at higher temperatures (such as 42˚C) (136). Expression of ATF does not correlate with hemagglutination (136, 258). A small collection of both clinical and non-clinical isolates have been screened for production of ATF; all strains tested positive (136, 137, 258). Original sequencing of the fimbrial gene cluster revealed the presence of only three genes: *atfA* (encoding a structural subunit), *atfB* (encoding a chaperone), and *atfC* (encoding a molecular usher) (137). However, the complete genome sequence of *P. mirabilis* HI4320 corrected an artifact of earlier cloning efforts and revealed the presence of an additional three genes in the operon. The complete gene cluster is now known to consist of *atfABCDEJ* (174).

A clinical isolate of *P. mirabilis* was used to generate an isogenic allelic replacement mutant that was incapable of producing ATF (258). The mutant maintained normal growth rate, hemolysis, urease activity, swarming motility, and hemagglutination properties (258). This mutant was also assessed in a mouse model of UTI. No significant difference in the colonization levels of the mutant and parent strain were observed during

either independent or cochallenge (258). Therefore, ATF do not appear to significantly contribute to the ability of *P. mirabilis* to colonize the urinary tract during experimental infection. Perhaps these results are not surprising, given the temperature regulation observed for ATF. It has been postulated that these fimbriae may play a role for adherence or survival of *P. mirabilis* outside the host.

PMF

Proteus mirabilis fimbriae (PMF) were identified from a crude fimbrial preparation of *P. mirabilis* strain HI4320 (19). Sequencing of the locus revealed the presence of five genes that appear to be contained in one operon: *pmfA* (the major structural subunit), *pmfC* (a putative usher), *pmfD* (a putative chaperone), *pmfE* (a putative minor subunit), and *pmfF* (the putative tip adhesin) (139). PMF appear to be widely distributed in both clinical and non-clinical isolates; either the *pmfA* gene or PmfA protein could be detected in every strain analyzed (19, 261).

Isogenic PmfA$^-$ mutants were constructed in two parent strains: HI4320 and Pr2921, another clinical isolate from a UTI patient (138, 261). Both PmfA$^-$ strains retained urease and hemolytic activity as well as normal swarming motility (138, 261). In addition, PMF are not responsible for the MR/K HA activity of *P. mirabilis* since PmfA$^-$ HI4320 retained it (138). PmfA$^-$ HI4320 adhered to exfoliated uroepithelial cells isolated from the urine of healthy human donors to the same degree as wild-type HI4320 (138). In contrast, PmfA$^-$ Pr2921 adhered significantly less than its parent strain to both exfoliated uroepithelial cells collected from urine and a cell line derived from a human bladder carcinoma (T24/83) cultured *in vitro* (261). The reason for this discrepancy in the role of PMF in adherence of the two isolates is not clear; perhaps strain Pr2921 lacks

other adhesins encoded by HI4320 and PMF consequently play a greater role in adherence of Pr2921 than HI4320.

During experimental UTI in a mouse model, PmfA$^-$ *P. mirabilis* HI4320 colonized the bladders of mice at levels statistically significantly lower than the wild-type strain (138); in contrast, kidney colonization was not affected by mutating *pmfA*. These data suggest that PMF may bind to receptors on the bladder epithelium *in vivo* but that perhaps other adhesins may be more important in adherence to renal tissue. PmfA$^-$ and wild-type Pr2921 were mixed together and used to infect mice in a cochallege experiment, which can be more sensitive at detecting subtle differences in fitness during infection. The PmfA$^-$ strain was outcompeted by the wild-type strain, to a statistically significant level, in both bladders and kidneys of infected mice (261). Again, it is unknown if a difference in the adhesins encoded by HI4320 and Pr2921 is responsible for the difference in kidney colonization. Perhaps PmfA$^-$ HI4320 would also be outcompeted in the kidney during cochallenge; this experiment has not been reported in the literature. Additionally, when mice were infected intravenously by tail vein injection, the PmfA$^-$ strain was recovered from kidneys of infected mice significantly less than the wild-type strain (261). However, bacteria in the bloodstream were not quantified at the time of sacrifice, so it is unclear if the PmfA$^-$ strain was attenuated for growth or survival in the bloodstream or specifically in its ability to gain access to or colonize the kidneys.

PMP

Proteus mirabilis P-like fimbriae were identified from a canine UTI-associated strain of *P. mirabilis* (33). The *pmpA* gene was found in 24 of 26 *P. mirabilis* strains tested for its presence. Most of these strains were isolated from canine urine or feces

(33). The genome sequence of strain HI4320 revealed that *pmpA* is also present in this clinical isolate from a human patient (174). Furthermore, the genome sequence revealed that *pmpA* is contained in a cluster of genes with other putative fimbrial proteins, including a chaperone and an usher (174). However, the prevalence of these genes in additional human isolates is currently unknown. In addition, the contribution of PMP to adherence or virulence has not been assessed.

Reciprocal regulation of adherence and motility

Sequencing of the entire MR/P fimbrial operon revealed the presence of the *mrpJ* gene (119). The 107-amino acid protein encoded by this gene, MrpJ, was predicted to be a transcriptional regulator based on its sequence (119). The presence of a transcriptional regulator following structural genes within a fimbrial gene cluster is not a common occurrence; thus, the function of MrpJ was investigated.

Interestingly, a strain of *P. mirabilis* HI4320 overexpressing MrpJ from a plasmid displayed reduced swimming and swarming motility compared to both the wild-type strain and a vector control (124). Elevated expression of MrpJ coincided with reduced expression of FlaA (flagellin, the major subunit of the flagellum) and reduced production of flagella, as assessed by Western blot and electron microscopy, respectively (124). Since MrpJ contains a putative helix-turn-helix domain, it was hypothesized that MrpJ reduces motility by acting at the transcriptional level, perhaps by downregulating expression of flagellin. Indeed, overexpression of MrpJ resulted in a reduction in *flaA* transcript (124). Furthermore, overexpression of MrpJ also resulted in reduced transcription of *flhDC*, the so-called master regulators of the flagellar cascade (124). These data suggest that MrpJ acts either directly on *flhDC* or a target upstream of *flhDC*.

Based on the results of electrophoretic mobility shift assays, MrpJ binds the *flhDC* promoter (172).

The genome sequence of *P. mirabilis* HI4320 revealed the presence of 14 MrpJ paralogues (174). Like *mrpJ*, 10 of these paralogues appear to be encoded within fimbrial operons; the remaining four are orphan genes (174). Alignment of the sequences of MrpJ and paralogues resulted in the identification of a conserved sequence: SQQQFSRYE (172). To investigate the importance of these conserved residues, a site-directed mutagenesis approach was undertaken; each of these residues in MrpJ was replaced with an alanine (172). All mutations within the conserved amino acids (with the exception of one) resulted in a loss of activity, which led to an increase in motility compared to wild-type MrpJ (172). Therefore, most of the residues in the conserved sequence contribute to the repression of motility. In light of the common features shared by MrpJ and its paralogues, the effect of each of the paralogues on motility was investigated. Similar to MrpJ, most of the paralogues, when overexpressed, led to a reduction in the production of FlaA and a repression of motility (172). In addition, at least one paralogue, UcaA, binds to the *flhDC* promoter, suggesting this activity may be a common mechanism shared by at least some MrpJ paralogues (172).

Since expression of MrpJ, a protein encoded in a fimbrial operon, represses motility, this protein represents a means of coordinating regulation of two seemingly opposing aspects of pathogenesis: adherence and motility. To investigate if this function is important during infection, a strain lacking MrpJ (Δ*mrpJ*) was generated and assessed for virulence in the mouse model of ascending UTI (124). Following cochallenge, Δ*mrpJ* was recovered in significantly lower numbers than the wild-type strain from bladders and

kidneys of infected mice (124). Thus, MrpJ offers a competitive advantage during infection. However, it is difficult to confidently interpret the data in that way for two reasons. First, Δ*mrpJ* had increased production of flagella, which could elicit a stronger host response (124). It is unclear if this would contribute to the out-competition observed *in vivo*; since the wild-type and Δ*mrpJ* strains were present together, they presumably were exposed to the same host response during infection, but this possibility cannot be ruled out. Second, MrpA levels were reduced in Δ*mrpJ* (124). The reason for this reduction is unclear; the mutation should not have a polar effect on MrpA expression (since MrpJ is downstream of MrpA), and experimental data confirmed that MrpJ expression does not affect transcription of the *mrp* operon (124). It is possible that deletion of *mrpJ* affects the stability of the *mrpABCDEFGHJ* transcript, but this hypothesis has not been experimentally tested. Regardless of the reason for it, lower levels of MrpA complicates the interpretation of the Δ*mrpJ* infection data since MrpA contributes to virulence (21, 259).

Again, as stated above, the coordinated regulation of adherence and motility may be important to pathogenesis; both aspects of pathogenesis are important during infection but have seemingly opposing functions. Perhaps it is not surprising, then, that there are other examples of coordinated expression of motility and adherence in bacteria. In *Bordetella pertussis*, the two component system BvgAS represses the flagellar regulon and activates transcription of adhesin genes (3). In *Vibrio cholerae*, mutations that affect motility directly feed back into the ToxR regulatory system and alter expression of the toxin co-regulated pilus (66). In uropathogenic *E. coli*, the PapX protein appears to function in much the same ways as MrpJ functions in *P. mirabilis*; PapX is encoded at

the end of the P fimbrial operon and represses motility by affecting the transcription of *flhD* (213). In fact, PapX acts as a functional homologue of MrpJ in *P. mirabilis*; expression of PapX in *P. mirabilis* resulted in decreased motility (124). Considering the high number of fimbriae encoded by *P. mirabilis*, it is not surprising that there are so many MrpJ paralogues in this strain (174). Teasing apart the function of each one may be difficult since they could possibly have overlapping or redundant function, and the exact function and mode of action of each paralogue has yet to be elucidated.

Toxins

Two toxins encoded by *P. mirabilis*, HpmA and Pta, have been characterized and are described below. In addition to HpmA and Pta, activity of the enzyme urease also contributes to tissue damage *in vivo*; urease is discussed in greater detail later in this chapter.

Hemolysin

Based on surveys of clinical isolates, 94-100% of *P. mirabilis* strains produce hemolytic activity (106, 207, 224). However, *P. mirabilis* strains lack the calcium-dependent hemolysin encoded by *hlyA* in *E. coli* and *hlyA* homologs in *P. vulgaris* and *M. morganii* (106, 248). The hemolytic activity encoded by *P. mirabilis* is calcium-independent and the protein responsible for this activity was named HpmA (for hemolysin of *Proteus mirabilis*) (106, 248). While initial reports identified this hemolytic activity as cell-associated (106, 207), later studies detected activity in cell-free preparations (207, 248); culture preparation and instability of secreted HpmA protein were proposed as reasons for this discrepancy. The genes encoding HpmB and HpmA are cotranscribed, and the promoter region of *hpmBA* contains a putative Fur-binding site

(236). However, expression of *hpmBA* does not appear to be affected by iron limitation (S. Himpsl and H.L.T. Mobley, unpublished data). HpmB is required for the secretion and activation of HpmA (236).

In addition to its hemolytic activity, HpmA also demonstrates cytotoxic activities against African green monkey kidney cells and human B-cell lymphomas, monocytes, bladder epithelium, and renal proximal tubular epithelial cells cultured *in vitro* (4, 145, 223). Interestingly, although hemolytic activity was detected in the supernatant, cytotoxic activity was only observed with total cell culture (not cell-free supernatant) (223). A strain of *P. mirabilis* with an interrupted copy of *hpmA* displayed neither the hemolytic nor the cytotoxic activity that was observed with the parent strain (223); these results suggest that the production of HpmA is required for both activities.

Fifty strains of *P. mirabilis* with a range of hemolytic activity were each injected intravenously into mice; a highly significant correlation between hemolytic titer and LD_{50} was observed (175). Additionally, an $HpmA^-$ mutant of *P. mirabilis* had a significantly higher LD_{50} than its parent strain when injected intravenously (223). However, in a mouse model of ascending urinary tract infection, there was no difference between the strains in terms of colonization or renal tissue damage (4, 143, 223).

Proteus toxic agglutinin

The recently described *Proteus* toxic agglutinin (Pta), an autotransporter with subtilisin-like serine protease activity, remains anchored at the bacterial surface (5). Pta is one of six putative autotransporters encoded in the genome of *P. mirabilis* strain HI4320 (174). It was identified as an outer membrane protein that reacts with sera from infected mice (165); see Chapter II. Similar to the widespread distribution of hemolysin,

pta was detected in every fecal and UTI isolate tested; however, expression of *pta* was detected only in urinary isolates (4).

Pta mediates autoagglutination of bacteria as well as toxicity against human bladder and kidney cells cultured *in vitro* (4, 5). Generally, secreted autotransporters have cytotoxic properties while membrane-bound autotransporters mediate adhesion or aggregation; Pta is the first characterized autotransporter to date that remains membrane-bound and exhibits both activities. Cytotoxicity, but not autoagglutination, requires serine protease activity (5). Pta is most active at alkaline pH (5), which is logical since the activity of the bacterial enzyme urease results in a local increase in urine pH during infection. Indeed, both transcription and protein levels of Pta are increased when bacteria are cultured in alkaline conditions (5). Temperature also affects transcript and protein level; culture at 37°C resulted in optimal levels of Pta (5). The addition of calcium, but not iron or magnesium, to the culture medium increased transcription of *pta* and possibly the stability of Pta.

HpmA and Pta both contribute to tissue damage observed in the bladder during experimental infection of mice, while renal damage appears to be mediated solely by Pta (4). In contrast to HpmA, Pta contributes to the ability of *P. mirabilis* to colonize the urinary tract; a strain with an interrupted copy of this gene was outcompeted by the wild-type strain during cochallenge and colonized the bladder, kidneys, and spleen at numbers significantly less than wild type during independent challenge (4, 5).

Urease

A major complicating factor associated with UTI caused by *P. mirabilis* is urolithiasis, or the formation of stones within the urinary tract. Stone production is a

result of the action of the bacterial enzyme urease. Urease catalyzes the hydrolysis of urea, liberating ammonia and carbon dioxide and increasing the pH of urine (141, 146, 147). As a result of the pH increase, normally soluble minerals precipitate, namely magnesium ammonium phosphate and calcium phosphate, leading to the formation of struvite and apatite crystals (72, 141, 146). Bacteria can be found within the matrix of the stones, where they are thought to be shielded from the effects of antibiotics and immunoglobulins (126). In addition, stones can obstruct the flow of urine, cause damage to host tissues, and serve as a nidus for other bacterial species to establish UTI.

The urease enzyme is a 250 kDa multimeric nickel metalloenzyme that is produced in the cytoplasm of bacteria (100, 148). The genes encoding *P. mirabilis* urease are *ureDABCEFG* (100, 101, 218). The active enzyme is a trimer of trimers, denoted $(UreABC)_3$, which is activated upon the insertion of nickel ions (147). UreC contains the nickel metallocenter (147). UreD, UreE, UreF, and UreG are accessory proteins that contribute to assembly of the active complex and insertion of the nickel ion into the metallocenter (147, 169). Mutation of *ureD*, *ureE*, or *ureG* resulted in production of an inactive enzyme lacking nickel ions (147, 169, 218); in contrast, mutation of *ureF* resulted in production of a functional enzyme, but with decreased activity (147). However, the exact mechanism of assembly is still unknown (147). The K_m value of the urease enzyme has been determined to be in the range of 13-60 mM urea; urea is present in human urine at concentrations up to 500 mM (37, 99). Despite such a high K_m, the enzyme is saturated with substrate in urine and operates at V_{MAX}.

Two regulators of urease transcription have been characterized: UreR and H-NS (histone-like nucleoid structuring protein). UreR is a member of the AraC family of

transcriptional regulators and contains both DNA- and urea-binding domains (162). The *ureR* gene is transcribed in the opposite direction of *ureDABCEFG*; UreR binds the promoters of *ureR* and *ureD* (47, 88, 226). Transcription of the structural genes of urease is urea-inducible (47, 88). However, UreR can bind even in the absence of urea (albeit with less affinity than in the presence of urea), suggesting that *P. mirabilis* is poised for a rapid response upon sensing of urea (47, 226). Whereas UreR acts as a positive regulator of urease activity and stimulates expression of the urease genes in the presence of urea, H-NS is a negative regulator that represses *ureR* transcription (44, 183). H-NS binds to the poly(A) tracts located in the intergenic region between *ureR* and *ureD* and induces a bend in the DNA that inhibits transcription of *ureR* (183). Interestingly, UreR and H-NS can displace each other from the target DNA under specific conditions (183).

A strain lacking urease was constructed and assessed in the murine model of ascending UTI (98). This mutant (containing an interruption in *ureC*) was negative for urease activity and colonized mice significantly less than the wild-type strain (97, 98). In addition, infection with the urease-negative strain resulted in less severe renal damage (97). Not surprisingly, no bladder or kidney stones were observed in mice infected with the urease-negative strain (97, 126). Given the complications that can arise from stone formation *in vivo*, it is not surprising that the enzyme responsible (urease) is considered a virulence factor.

Metal acquisition

Iron

Members of the family *Enterobacteriaceae*, to which *Proteus* belongs, typically produce siderophores of the catecholate, hydroxamate, and/or ferrioxamine type;

however, *Proteus* species do not produce any of these types of siderophores (59, 140). In contrast, *P. mirabilis* produces α-hydroxyisovaleric acid, which has been suggested to function in iron acquisition (59), although the importance of this compound in iron acquisition is debated in the field. *P. mirabilis* can also utilize a number of α-keto acids as siderophores (53). The production of α-keto acids results from the deamination of amino acids. It has long been recognized that *Proteus* species are capable of this type of reaction (83). However, a gene responsible for this activity, named *aad* for amino acid deaminase, was only identified and sequenced relatively recently (140). Surprisingly, the expression of *aad* is not under the regulation of the ferric uptake repressor Fur and is not affected by iron concentration (140); it was suggested by the authors that perhaps iron acquisition is not the only role of Aad. Although only six strains were analyzed, 100% of these *P. mirabilis* strains appeared to encode *aad* (140). Attempts to construct a strain of *P. mirabilis* lacking a function copy of *aad* were unsuccessful; since these attempts were made using methods previously reliable for mutant construction in *P. mirabilis*, it was hypothesized that Aad may be required for viability in *P. mirabilis* (140). Therefore, the role of this amino acid deaminase in virulence, or even its unequivocal role in iron uptake, has not been determined.

P. mirabilis can also utilize heme and hemin as iron sources (181). One outer membrane protein responsible for heme utilization, HmuR2, has been identified and offers a competitive advantage during ascending UTI in the mouse model (127). However, HmuR2 is not absolutely required for virulence since a strain lacking HmuR2 was able to colonize mice (127); this result is not unexpected, as many pathogens encode multiple iron acquisition systems (some of which are redundant) since iron is such an

important nutrient. *P. mirabilis* cannot utilize transferrin or lactoferrin as iron sources (181).

The genome sequence of *P. mirabilis* HI4320 revealed the presence of a large number of proteins which may play a role in iron acquisition (174). *P. mirabilis* encodes two seemingly complete siderophore synthesis operons. The presence of these operons was surprising, given the previously accepted findings that *P. mirabilis* does not produce siderophores. Current research in our lab is investigating the function of these operons; careful re-examination of siderophore synthesis has revealed that *P. mirabilis* does, in fact, produce siderophores (S. Himpsl and H. Mobley, unpublished data). The structure of these siderophores is under investigation. Additionally, signature-tagged mutagenesis studies identified several proteins that are implicated in iron uptake based on homology (38, 84); however, the function of most of these proteins remains unconfirmed.

Zinc

Much like iron, zinc is a required nutrient that is toxic at high levels. Therefore, its level must be carefully controlled (35). This control occurs mainly via the regulation and activity of import and export systems (79). Environmental conditions likely fall into one of three categories: limited zinc, excess zinc, and an intermediate level of zinc which is neither toxic nor limiting. Bacteria have transporters to deal with each of these scenarios; as zinc is assumed to be limited in the host, only zinc uptake systems will be addressed here (133, 254).

In *E. coli*, the acquisition of zinc under limiting conditions is achieved through the high-affinity zinc transport system ZnuACB (171). ZnuA is a periplasmic binding protein, ZnuB is an inner membrane protein, and ZnuC is a cytoplasmic ATPase. These

proteins are only produced under zinc limitation; their expression is under control of the zinc uptake repressor (Zur), which binds to the promoter region of *znuACB* and represses transcription when sufficient zinc is present in the cell (170).

When the cell encounters an environment with an intermediate level of zinc, it requires neither high-affinity importers nor exporters. Instead, zinc homeostasis is maintained through the function of low-affinity transporters. ZupT, the best characterized of these low-affinity transporters, has broad metal specificity; unlike ZnuACB, which is regulated by Zur, ZupT is expressed constitutively at low levels (70, 71). In addition to ZupT, zinc may be brought into the cell by other transporters; these transporters include PitA, an organic phosphate transporter, and CitM, a citrate transporter, in *E. coli* and *Bacillus subtilis*, respectively (24, 107).

Given that zinc is assumed to be limited in the host, it is perhaps not surprising that in recent years, zinc uptake systems have been shown to contribute to the virulence of many different pathogens (15, 40, 50, 67, 105, 117, 253). Of particular interest, UPEC requires the ZnuACB zinc transport system to attain maximal colonization of the urinary tract (196). These results suggest that perhaps the urinary tract may be zinc-limited and that zinc acquisition may also be important for colonization of the urinary tract by other pathogens, including *P. mirabilis*. However, neither low- nor high-affinity zinc transport has been characterized in *P. mirabilis* to date; this topic will be addressed in Chapter III.

ZapA

P. mirabilis displays IgA protease activity. Of *P. mirabilis* clinical isolates that have been assessed, 100% were positive for IgA protease activity (130, 205). During initial characterization of this activity, cleavage of IgA resulted in a different pattern from

that observed with bacterial pathogens such as *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* that encode a similar activity (205). Proteases from these other species cleave the heavy chain of human IgA1 at a conserved stretch of amino acids in the hinge region; this region is absent in IgA2, which is resistant to cleavage by IgA proteases (103, 104, 158, 182). The IgA protease activity of *P. mirabilis* was correlated with production of an approximately 50-55 kDa protein (131, 132). Activity of this protein was optimal at pH 8 (131), inhibited by EDTA and other metal chelators (130-132, 205, 245), and stimulated by the addition of Mg^{2+} or Ca^{2+} ions (245). In addition, the activity of this protein (initially found to degrade IgA1) was eventually expanded to include a broad range of host proteins, including serum and secretory IgA (human and mouse), IgG, mouse IgA, secretory component, gelatin, casein, actin, β-tubulin, fibronectin, collagen, laminin, complement proteins (C1q and C3), β-insulin, and antimicrobial peptides LL-37 and human β-defensin 1 (17, 31, 130-132, 203, 208, 245). The protein displays no clear preference for a particular amino acid residue but activity is enhanced if the target protein is partially or fully denatured (31).

The protease activity was mapped to a locus termed *zapA* (245). Eventually, sequencing of the surrounding area of the genome revealed four additional genes: one upstream (*zapE*) and three downstream (*zapB*, *zapC*, and *zapD*) of *zapA*; all genes are transcribed in the same direction (239, 245). Based on homology, ZapA appears to be a zinc metalloprotease that belongs to the serralysin family of proteases (245). ZapA has several characteristics of this protein family, including putative zinc-binding residues, a calcium-binding region, and a C-terminal secretion signal (245). ZapE, located upstream of ZapA, also appears to be a metalloprotease which shares many properties with ZapA

but seems to be missing an identifiable signal sequence (239). The activity of ZapE has not been characterized to date. ZapBCD are similar to components of ABC transport systems and may function in the secretion of ZapA (239). Although this particular hypothesis has not been tested, transposon mutants with insertions in two of these genes lacked IgA protease activity (245). Interestingly, the genome sequence of *P. mirabilis* HI4320 revealed the presence of three additional copies of *zapE* directly upstream of the originally identified copy (174); it is unclear if these additional copies are present in other strains of *P. mirabilis* or what their function might be.

Historically, it has been difficult to assess the contribution of IgA proteases to virulence of bacterial pathogens since most of these enzymes have a very specific activity that is limited to human (or closely related primate) IgA molecules. It has, however, been postulated that IgA proteases are associated with virulence since non-pathogenic species appear to lack this activity (157). However, ZapA is active against mouse IgA (245). A ZapA$^-$ strain of *P. mirabilis* was generated to assess the contribution of ZapA to virulence using the mouse model of ascending UTI (239). ZapA$^-$ *P. mirabilis* lost IgA protease activity but retained normal urease production, hemolytic activity, swarmer cell differentiation, production of flagella, swarming motility, and biofilm formation (180, 239). This strain was recovered from the urine, bladders, and kidneys of experimentally infected mice in statistically significantly lower numbers than the wild-type strain (239). These results were the first demonstration that IgA proteases are, in fact, virulence factors. Interestingly, this ZapA$^-$ mutant was also attenuated in a rat model of prostatitis (180). These results suggest that the advantage offered by ZapA is not isolated to the urinary tract and may affect infection of other body sites as well. Isolates of *M. morganii*

and *P. stuartii,* two other pathogens commonly present with *P. mirabilis* during polymicrobial complicated UTI, lack IgA protease activity (206); it is interesting to consider that perhaps these species benefit *in vivo* from the ZapA activity provided by *P. mirabilis*. To that point, there is indirect evidence for the production and activity of ZapA *in vivo* during UTI in human patients (131, 208).

Type III secretion system

The genome sequence of *P. mirabilis* HI4320 encodes a seemingly intact type III secretion system (T3SS) (174). T3SSs are specialized bacterial secretion systems that secrete effector proteins directly into the cytosol of host cells; T3SSs have been identified as virulence factors in a number of pathogenic bacteria, notably *Salmonella*, *Shigella*, and some diarrheagenic *E. coli* (43). For this reason, the role of the *P. mirabilis* T3SS during experimental UTI was investigated. Twenty-four genes, including all of the components necessary for needle complex assembly and two putative effector proteins, are contained in a contiguous 22-kb region (173, 174). These sequences have a lower GC content than the surrounding HI4320 genome (30.7% compared to 38.9%), suggesting they may have been acquired by horizontal gene transfer (173). Importantly, these open reading frames all appear to be intact (with no discernible deletions or premature stop codons). To assess the potential contribution of this T3SS during infection, a mutant was constructed with an insertional mutation in a putative ATPase (*spa47*); this gene was chosen because it has been shown to be essential for the function of T3SSs in other organisms. Surprisingly, this mutant infected mice as effectively as the wild-type strain (173). Therefore, this T3SS does not appear to function or contribute to disease during experimental UTI. Pearson and Mobley postulated that perhaps the T3SS could function in the

gastrointestinal tract or other environments where *P. mirabilis* is found, but this hypothesis has not yet been experimentally tested. Alternatively, although not observed during experimental murine infection, the system could be active during human UTI.

Treatment and Prevention

More than five million patients receive urinary catheters each year (134); every single one is at risk for infection. Even patients undergoing short-term catheterization (< 7 days) have a 10-50% risk of experiencing bacteriuria; if a patient requires long-term catheterization, the percent who will experience infection approaches 100% (77, 156). Unfortunately, there are currently no effective methods for preventing these infections; at best, the onset of infection can be delayed (231). Methods for delaying the onset of complicated catheter-associated UTIs caused by *P. mirabilis*, and subsequent methods for treatment of these infections, have recently been thoroughly reviewed (90); a brief overview is presented here.

Although it seems fairly obvious, the first step in preventing catheter-associated UTIs is limiting the use of urinary catheters. These devices are overused; it has been estimated that as many as 21-38% of catheters are placed in the absence of justifiable indication (91, 159, 197). Once catheters are in place, optimal conditions for the prevention of infection include emptying drainage bags every 4-6 hours and changing the catheter every 8-10 days (161). Prompt removal of catheters once they are no longer medically necessary is crucial to infection prevention (134).

There has been much interest in the field in employing catheters made of or coated with various materials to reduce colonization of the catheter. However, to date, no single biosurface developed can effectively prevent colonization (52, 134, 221, 235).

There are extensive reports in the literature about using antibiotics and antiseptics (including silver compounds and triclosan) to coat catheters (90); however, results are conflicting, and no consensus has been reached. Results from *in vitro* experiments demonstrated that *P. mirabilis* rapidly blocked catheters coated with silver alloys and nitrofurazone (153, 155). The relatively new idea of using the catheter balloon (rather than the surface of the catheter) as a reservoir for antimicrobial compounds is promising; after seven days, catheters inflated with triclosan showed minimal encrustation and still drained freely while water-inflated controls were blocked within 24 hours (220). This treatment could potentially expand the lifespan of catheters in patients. There has also been interest in the use of probiotics to inhibit colonization by pathogenic strains. Results from both *in vitro* and *in vivo* studies suggest that colonization with nonpathogenic strains of *E. coli* can prevent the colonization of catheters by uropathogenic strains (48, 87, 186, 232-234).

Interestingly, about 90% of bacteriuria cases associated with catheterization are asymptomatic (225). Generally, treatment of asymptomatic bacteriuria with antibiotics is discouraged due to concern about the emergence of antibiotic resistance. However, there is still debate among clinicians about this matter since asymptomatic bacteriuria may progress to more a serious illness if left untreated. Regardless, once an infection is confirmed (normally through determination of bacterial cell and blood cell counts in urine), the catheter is removed (if possible) and antibiotic treatment commences (90).

If UTI results in the development of urinary stones, treatment is further complicated; *P. mirabilis* can be located in the matrix of the stone, where it is believed that bacteria are shielded from the action of antibiotics and factors of the host immune

response, including immunoglobulins (126). In addition, urinary stones are also thought to serve as a reservoir for bacteria that can rapidly colonize replacement catheters (195). Sometimes, surgery is recommended for removal of stones (90).

Due to the serious sequelae that can result from UTI with *P. mirabilis*, and the difficulties associated with treatment, the development of a prophylactic vaccine against *P. mirabilis* is an active area of research. Previous vaccine efforts are described below.

Vaccine Development against *P. mirabilis*

As with efforts for vaccination against many bacterial pathogens, immunizations began with crude preparations and moved toward more focused and pure antigens, based on what has been successful. In general, that will be the order in which previous vaccine efforts are presented here.

Infection does not significantly protect against re-infection (94). Vaccinated (or, in this case, re-infected) mice were not protected from death (94). In surviving mice, modest protection (with respect to levels of bacteria recovered from infected mice) was observed in urine and bladders (94). The protection in kidneys was statistically significant compared to control animals (94). However, kidneys were still colonized in the immunized mice; therefore, the protection observed in the kidneys, while statistically significant, may not be biologically significant. These mice generated antibody responses to a MR/P fimbriae, PMF, and flagella, as well as "numerous other, unidentified surface antigens" (94). However, there was no correlation between serum IgG and IgM levels and protection. There was a trend toward elevated serum IgA levels and protection, but this correlation was not significant, perhaps because only a small number of mice developed significant IgA levels (94). Similar to immunization with live

bacteria, immunization with heat-killed bacteria offers little protection (123). Formalin-killed bacteria, however, when administered either through the subcutaneous or intranasal routes, significantly protected mice from subsequent transurethral challenge (120).

The antibody response to outer membrane proteins (OMPs) after experimental infection was assessed. Only 15% of mice elicited specific IgM to OMPs, while 68% of mice developed OMP-specific IgG from 3-28 days post-infection (142). If antibody response analysis was delayed until 14 days post-infection, the percentage of mice that developed OMP-specific IgG rose to 87% (142). Antibodies appeared to be primarily directed towards the major OMP complex (142); this result was not surprising given that patients experiencing UTI produce antibodies against the major OMP complex of infecting bacteria (78, 163, 164, 198). Due to the production of OMP-specific antibodies during infection, an outer membrane preparation was used to immunize mice intramuscularly. Two weeks after vaccination, mice were challenged transurethrally (142). The OMP vaccine significantly protected mice from death, renal colonization, and renal damage compared to PBS controls (142). However, IgG levels did not correlate with protection. The possibility that protection observed with the OMP vaccine was due to LPS contamination of outer membrane preparations was addressed by vaccinating mice with an LPS preparation. In contrast to the OMP vaccine, immunization with LPS did not result in protection (despite the production of high levels of specific LPS antibodies) (142). Interestingly, preliminary data suggested that vaccination with OMPs enhanced clearance of three out of four heterologous strains (142). From this finding, we learned that cross-protection is possible with an OMP vaccination, but that not all

heterologous strains were affected; therefore it may be important to specifically target conserved OMP proteins.

The bulk of *P. mirabilis* vaccination studies have used vaccines targeting fimbrial proteins. Infected mice generate a strong antibody response to MR/P fimbriae following infection (20, 94). Peritoneal immunization with purified fimbrial preparations protected mice from transurethral challenge with both homologous and heterologous strains (115). An independent study determined that, although the antibody response generated by vaccination with purified fimbriae was not as robust as that generated after whole cell immunization, intranasal or transurethral immunization with purified fimbriae resulted in a significant reduction in bladder and kidney colonization in infected mice (120).

Vaccination with purified structural subunits of various fimbriae was performed; immunization with MrpA, UcaA, or PmfA offered some degree of protection (177). Mice vaccinated with MrpA via the subcutaneous route had reduced kidney colonization after transurethral challenge; bladder colonization was not affected (177). Immunization with UcaA did not protect mice from ascending UTI (177). These antigens were also assessed for protection in an intravenous (i.v.) injection model. (Although mice were infected i.v., infection was assessed by quantifying bacteria in bladders and kidneys; it should be noted that blood was never cultured, so the effect of vaccination on bacterial load in the bloodstream is unclear.) Mice immunized with either MrpA or UcaA prior to i.v. infection had reduced colonization in their bladders and kidneys (177). Immunization with PmfA did not result in protection from infection in either model (i.v. or transurethral infection), despite production of antibodies in the urine (177). Vaccination with each subunit resulted in the production of specific serum IgG response, but there was no

correlation between antibody levels and protection from infection (177). None of the tested animals, regardless of antigen or route used, developed significant serum or urine IgA production (177). These antigens (MrpA, UcaA, and PmfA) were also tested using intranasal and transurethral immunizations (201). Overall, intranasal immunization resulted in broader antibody production and led to greater protection than transurethral immunization (201). However, again, there was no significant association between protection and antibody production, in either serum or urine (201).

More recently, a new platform for the MrpA vaccine was introduced. The food-grade lactic acid bacterium *Lactococcus lactis* was used as a vehicle for antigen delivery (200). Two forms of MrpA were expressed in *L. lactis*: secreted and cell-wall-anchored (200). Following intranasal immunization with MrpA-expressing *L. lactis*, mice were infected transurethrally, and the levels of colonization of bladders and kidneys were compared to control groups receiving either PBS or *L. lactis* not expressing MrpA (200). Mice that received cell-wall-anchored MrpA had increased levels of serum IgA (which was significant when compared to PBS, but not *L. lactis*, controls). Mice that were vaccinated with *L. lactis* expressing secreted MrpA produced increased levels of serum IgG (but again, only compared to PBS, and not *L. lactis*, controls). None of the mice produced significant levels of IgA or IgG in urine following immunization. Mice that received the secreted form of MrpA were significantly protected from kidney colonization compared to mice immunized with the *L. lactis* control. However, no protection was observed in the bladders of these mice. There was no correlation between protection and antibody production. Although immunization with *L. lactis* expressing MrpA resulted in some kidney protection, vaccination did not lead to the generation of

detectable levels of mucosal antibodies (200). It is interesting to note that, in this study, intranasal immunization with MrpA-expressing *L. lactis* did not result in bladder protection (200), while earlier work by the same group showed that intranasal immunization with MrpA significantly protected mice from bladder infection (201). This difference highlights the important impact that route, platform, and adjuvant can have on the efficacy of a vaccine, even when targeting the same antigen.

All vaccines described above targeting MR/P fimbriae were aimed at the major structural subunit, MrpA. MrpH, the tip adhesin, has also been a focus of vaccine efforts. These experiments, described below, all used antigens administered via the intranasal route; the intranasal route was chosen over other tested routes (subcutaneous, transurethral, and oral) because it offered the most consistent protection in initial testing and was the only route of immunization that resulted in the production of antibodies in urine and the bladder (120). Others have also suggested that intranasal immunization induces antibodies at mucosal surfaces, including the urogenital tract (238). Mature MrpH cross-linked to the adjuvant cholera toxin, when administered intranasally, resulted in significantly lower kidney colonization following transurethral challenge, compared to control animals; although there was a trend towards lower bladder colonization, this difference was not significant (120). In response to this vaccination, there were significant increases in IgG observed in serum, bladder, and kidneys and significant increases in IgA in serum and kidneys (120). It is interesting to note that IgA was produced more highly in kidneys (which were protected) than in bladders (which were not); the authors suggested that perhaps these data may indicate a correlation between IgA production and protection (120). Next, an N-terminal truncation of MrpH

(containing the receptor-binding domain) was assessed using the same model described above for mature MrpH. This N-terminal truncation of MrpH, when chemically cross-linked to the adjuvant cholera toxin and administered intranasally, significantly protected mice from both bladder ($P = 0.002$) and kidney ($P < 0.0001$) infection compared to mice receiving control (cholera toxin only) immunization (120). Protection was also observed when MrpH was translationally fused to cholera toxin (as opposed to chemical cross-linking) (118). This translational fusion approach is interesting since it results in the production of one molecule that contains both antigen and adjuvant; bypassing the chemical cross-link step may result in a more uniform antigen preparation.

Arguably, the most successful vaccines to date have targeted two components of MR/P fimbriae: MrpA and MrpH. While there is considerable evidence that MR/P fimbriae are expressed *in vivo* (20, 121, 255) and are conserved (22, 23, 120, 188, 259), it is important to remember that this fimbria is capable of undergoing phase variation (255). This knowledge is critical when considering vaccine design, as it is easy to imagine that bacteria could potentially escape the immune response by down-regulating the targeted antigen. MR/P fimbriae contribute to infection (21, 119, 121, 125, 259); however, they are not essential for colonization. Bacteria not expressing MR/P fimbriae still bind to bladder tissue *in vivo* (93). An MrpA mutant, although recovered in significantly lower numbers than the wild-type strain, still colonized mice (21). There was not a significant difference in the level of colonization resulting from infection with an MrpH mutant and the wild-type strain (119). In addition, there was no difference between infection with the MR/P L-OFF strain (incapable of producing MR/P fimbriae) and the wild-type strain during independent challenge (121). However, MR/P L-OFF was outcompeted by the

wild-type strain during cochallenge, suggesting that MR/P fimbriae normally offer a competitive advantage during infection (121).

Targeting these antigens (MrpA or MrpH) by vaccination could introduce a new selective pressure in the urinary tract – this time, against (rather than for) expression of MR/P fimbriae. Although vaccination with either MrpA or MrpH significantly protected mice from subsequent transurethral infection, none of these vaccines resulted in sterilizing immunity; that is, there were still mice in each group that were infected (118, 120, 177, 200, 201). It would be very interesting to examine the MR/P expression profile in bacteria isolated from the non-protected mice; if these bacteria were predominately MR/P OFF, it is possible that they were able to escape the immune response directed towards MR/P fimbrial subunits induced by vaccination. The PCR-based invertible element has already been used to assess MR/P expression *in vivo* (121, 255), validating this approach. In any case, mice were not completely protected after any vaccination attempted to date; the identification of additional antigens to include in a potentially multivalent vaccine is a valid goal.

CHAPTER II. Outer Membrane Antigens of the Uropathogen *Proteus mirabilis* Recognized by the Humoral Response during Experimental Murine Urinary Tract Infection

Abstract

Proteus mirabilis, a gram-negative bacterium, is a frequent cause of complicated urinary tract infections in those with functional or anatomical abnormalities or those subject to long-term catheterization. To systematically identify surface-exposed antigens as potential vaccine candidates, proteins in the outer membrane fraction of bacteria were separated by 2D gel electrophoresis and subjected to Western blotting with sera from mice experimentally infected with *P. mirabilis*. Protein spots reactive with sera were identified by mass spectrometry, which, in conjunction with the newly completed genome sequence of *P. mirabilis* HI4320, was used to identify surface-exposed antigens. Culture conditions that may mimic *in vivo* conditions more closely than Luria broth (culture in human urine, iron-limitation, and osmotic stress) were also used. Thirty-seven antigens, to which a humoral response had been mounted, including 24 outer membrane proteins, were identified. These antigens are presumably expressed during urinary tract infection. Protein targets that are both actively required for virulence and antigenic may serve as protective antigens for vaccination; thus, five representative antigens were selected for use in virulence studies. Strains of *P. mirabilis* with mutations in three of these genes (PMI0047, *rafY*, *fadL*) were not attenuated in the murine model of urinary tract infection. Putative iron acquisition proteins PMI0842 and PMI2596, however, both contribute to fitness in the urinary tract and thus emerge as vaccine candidates.

Introduction

Proteus mirabilis, a Gram-negative bacterium, is among the most prevalent isolates from individuals suffering from complicated urinary tract infections (cUTIs) (244). cUTIs affect patients whose urinary tracts are affected by long-term catheterization or functional or anatomic abnormalities and occur via the ascending route (18). Consequences of *P. mirabilis* cUTIs include catheter encrustation, formation of urinary stones (urolithiasis), renal scarring, and progression to bacteremia (149). Catheter encrustation caused by these infections can block the flow of urine through the catheter. In addition to the possibility of causing permanent renal damage, the formation of stones, due to the action of the bacterial enzyme urease, may also make infections difficult to clear due to the presence of bacteria within the stones, where *P. mirabilis* may be shielded from the action of antibiotics (126).

Catheter-associated cUTIs are the most commonly-occurring nosocomial infection, with more than one million cases documented each year in the United States (225). Prevention of *P. mirabilis*-caused cUTIs would improve patient care and quality of life and reduce the substantial economic burden associated with their care. Since infection does not appear to fully protect against re-infection (94), a vaccine is a logical goal for several reasons. First, patients with known urinary tract abnormalities and patients at the onset of long-term catheterization could be specifically targeted for vaccination due to the high incidence of cUTIs in these populations. Second, *P. mirabilis* infections are very difficult to clear due to the presence of bacteria within the urinary stones (126). Third, based on studies of the urease and *mrpH* genes, there is evidence

that virulence factor genes are well conserved among different strains of *P. mirabilis*, which supports the notion of a cross-protective vaccine (120, 144).

Immunization with heat-killed bacterial preparations or prior infection offers little protection (94, 123). Purified fimbrial preparations, however, protect mice from subsequent transurethral challenge (115, 120). Three different structural fimbrial proteins have also been used in vaccine studies, with varying degrees of success: MrpA, UcaA, and PmfA (177). In a recent study, one of these structural proteins, MrpA, was expressed in the food-grade bacterium *Lactococcus lactis* (200). Mice were intranasally immunized with *L. lactis* expressing MrpA prior to transurethral challenge and had significantly lower bacterial colonization in the kidneys as compared to controls. An outer membrane vaccine significantly protected mice from death, renal colonization, and renal damage (142). One of the most promising vaccines to date consists of the N-terminal domain of MrpH (the tip adhesin of the MR/P fimbria) fused to domains of cholera toxin (118). A translational fusion of MrpH and the cholera toxin A2 subunit was coexpressed with the cholera toxin B subunit; the vector used for vaccine expression replaced the toxic A1 subunit of cholera toxin MrpH (76). The result of expression of these genes is the spontaneous assembly of a single chimeric protein that contains both antigen (MrpH) and adjuvant (cholera toxin), which mediates highly effective delivery to the systemic and mucosal immune systems (58). Although this vaccine was able to protect mice from infection, we have concerns about the efficacy of a vaccine solely targeting this adhesin since the *mrp* operon is capable of undergoing phase variation (255). For this reason, we believe it prudent to identify additional antigens for inclusion in a multivalent vaccine.

In this study, we used an immunoproteomic approach to identify additional

surface-exposed antigens of *P. mirabilis*. Fractions enriched for outer membrane proteins were isolated from bacterial cells and proteins were separated by two-dimensional gel electrophoresis. Immunoreactive proteins, identified by Western blot using sera from mice with experimental *P. mirabilis* UTI, were submitted for mass spectrometry analysis. Using the recently completed genome sequence (174), this study identified 37 immunoreactive antigens, including 24 outer membrane proteins. Five antigens were assessed for their role in virulence in the murine model of ascending UTI.

Materials and methods

Strain and culture conditions.

P. mirabilis HI4320 was originally cultured from the urine of a catheterized nursing home patient with bacteriuria (149). Luria broth (LB) (per liter, 10 g tryptone, 5 g yeast extract, and 0.5 g NaCl) and non-swarming agar (per liter, 10 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 15 g agar) were used to culture bacteria. Minimal medium [per liter, 200 ml 5X M9 salts (64 g $Na_2HPO_4 \cdot 7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl per liter), 2 ml 1 M $MgSO_4$, 20 ml 20% glucose, 100 μL $CaCl_2$] was inoculated with a 1:100 dilution of *P. mirabilis* cultured overnight in LB. Iron-limitation was achieved by addition of 15 μM desferoxamine (M. Pearson and H. Mobley, unpublished data) to LB cultures. Osmotic stress was induced by addition of 0.3 M NaCl to LB or minimal medium (11). Urine was collected from three healthy human donors, pooled, filter-sterilized, and stored at -20ºC until use. All cultures were incubated at 37ºC with aeration (200 rpm) unless otherwise noted.

Isolation of outer membranes.

A modification of the method of Piccini *et al*. (181) was used for isolation of outer membranes. Bacteria were harvested by centrifugation (10,000 x *g*, 15 min, 4ºC) and washed twice in 10 mM HEPES, pH 7.4. Cells were lysed by two passes through a French pressure cell (American Instrument Company, Travenol Laboratories Inc, Silver Spring, Maryland) at 20,000 psi. Intact bacteria were cleared by centrifugation (10,000 x *g*, 15 min, 4ºC). Supernatants were centrifuged to pellet membranes from the lysate (90,000 x *g*, 45 min, 4ºC). Membrane pellets were resuspended in 10 mM HEPES (pH

7.4), 10 mM $MgCl_2$, 2% Triton X-100. Following an hour-long incubation at 37ºC, Triton X-100-insoluble fractions (enriched for outer membrane proteins) were collected by centrifugation (90,000 x *g*, 45 min, 4ºC). Pellets were washed in 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2% Triton X-100. The resulting outer membrane-enriched pellets were resuspended in isoelectric focusing (IEF) solution (defined below). Protein was quantified by using 2D Quant Kit (Amersham Biosciences).

Two-dimensional gel electrophoresis.

The method of Molloy *et al.* (150) was adapted. A 17 cm pH 4-7 ReadyStrip IPG Strip (BioRad) was rehydrated for 16-24 hours with 350 µL of IEF solution (7 M urea, 2 M thiourea, 1% amidosulfobetaine-14, 40 mM tris, 2 mM tributylphosphine, and 0.5% biolytes 3-10) containing outer membrane protein. Isoelectric focusing was conducted under the following conditions in a Protean IEF Cell (BioRad): 250 V for 20 min, 10,000 V for 2.5 hours, and 10,000 V for 40,000 V-hours. Prior to the second dimension, strips were equilibrated for 20 min at room temperature by rocking in a solution of 0.15 M bisTris/0.1 M HCl, 6 M urea, 2% SDS (w/v), 20% glycerol (v/v), 5 mM TBP, and 2.5% acrylamide (w/v). Samples were run on 10% polyacrylamide gels, as described previously (150). Cathode buffer contained 0.2 M taurine, 25 mM tris, 0.1 % SDS (w/v) and anode buffer contained 0.384 M glycine, 50 mM tris, 0.1 % SDS. Gels were run at a constant current of 50 mA at room temperature until completion and then stained for 24 hours in colloidal Coomassie G-250 (151). Unstained Precision Plus Protein Standards (BioRad) were used as molecular weight standards. Proteins corresponding to immunoreactive spots on Western blots (described below) were cut from gels using a clean razor blade and submitted for mass spectrometry analysis.

Serum.

During a previous study, sera were obtained from mice 42 days after experimental urinary tract infection by *P. mirabilis* (94). In this report, we used pre-immune sera and post-rechallenge sera from those studies. Sera from 20 of the mice were individually screened for reactivity to *P. mirabilis* proteins by Western blot (details below). The five sera with the strongest reaction to *P. mirabilis* lysate were pooled in equal amounts and used in all further studies.

Western blot.

Proteins were transferred to Immobilon-P PVDF membrane (Millipore) for one hour at 400 mA at 4ºC. After transfer, membranes were blocked with 5% milk in TBS-T (0.05% Tween, 100mM tris (pH 7.5), 9% NaCl). Membranes were incubated, shaking at room temperature, with sera diluted in TBS-T. After one 15-minute wash and three five-minute washes in TBS-T, secondary antibody was applied to the membrane. Goat anti-mouse IgG conjugated to horseradish peroxidase (1:100,000 dilution in TBS-T) was applied for 45 minutes with shaking at room temperature. Precision StrepTactin-HRP Conjugate (BioRad), used for ladder detection, was added to the secondary antibody incubation. The wash procedure was repeated and detection was performed with Amersham ECL Plus Western Blotting Detection System (GE Healthcare), following the protocol recommended by the manufacturer. To screen sera from individual mice, a 1:1000 dilution was used. After sera were combined, a 1:10,000 dilution of the pooled sera were used in all further experiments.

Mass spectrometry.

Proteins were identified either by peptide mass fingerprinting (PMF) or tandem mass spectrometry (MS/MS). PMF was performed by the University of Michigan Protein Structure Facility. Samples were digested with trypsin using the Millipore Montage system, co-crystallized with alpha-cyano-hydroxy-cinnamic acid (1:1 sample:CHCA), and analyzed via MALDI-TOF MS. Protein Prospector software (http://prospector.ucsf.edu), along with the preliminary genome annotation (174), was used to identify proteins in PMF samples. MS/MS and analysis was performed by the Michigan Proteomics Consortium, as described by the protocols available on their website (http://www.proteomeconsortium.org/protocols.html). Briefly, samples were digested with trypsin and spectra were acquired by using 4700 Proteomics Analyzer (Applied Biosystems). The eight most intense peaks in each spectrum were selected for MS/MS. Ion scores are available on request.

Construction of mutants.

Insertional mutants were constructed using the TargeTron system (Sigma), as described by Pearson and Mobley (173). Briefly, genes were disrupted by insertion of an intron (containing a kanamycin resistance gene) which was targeted specifically to each gene of interest by using a set of three primers (IBS, EBS1d, and EBS2; listed in Table 1) in a mutagenic PCR. This mutated region of the intron was ligated into the vector pACD4K-C. Resultant plasmids were sequenced to confirm proper re-targeting of the intron. Correctly re-targeted plasmids were electroporated into electrocompetent *P. mirabilis* HI4320 containing the helper plasmid pAR1219 (49). Transformants were

Table 1. Primers used to generate and confirm mutations in *P. mirabilis* HI4320

Primer Name	Sequence (5' to 3')
PMI0047-IBS	AAAAAAGCTTATAATTATCCTTAGTAACCCACCCAGTGCGCCCAGATAGGGTG
PMI0047-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCACCCATATAACTTACCTTTCTTTGT
PMI0047-EBS2	TGAACGCAAGTTTCTAATTTCGATTGTTACTCGATAGAGGAAAGTGTCT
PMI0288-IBS	AAAAAAGCTTATAATTATCCTTAGCAGTCATAGTTGTGCGCCCAGATAGGGTG
PMI0288-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATAGTTTCTAACTTACCTTTCTTTGT
PMI0288-EBS2	TGAACGCAAGTTTCTAATTTCGATTACTGCTCGATAGAGGAAAGTGTCT
PMI0842-IBS	AAAAAAGCTTATAATTATCCTTACCAGCCTCTGTTGTGCGCCCAGATAGGGTG
PMI0842-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTCTGTTTCTAACTTACCTTTCTTTGT
PMI0842-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGCTGGTCGATAGAGGAAAGTGTCT
PMI1810-IBS	AAAAAAGCTTATAATTATCCTTAATAAGCTACCACGTGCGCCCAGATAGGGTG
PMI1810-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTACCACCCTAACTTACCTTTCTTTGT
PMI1810-EBS2	TGAACGCAAGTTTCTAATTTCGATTCTTATTCGATAGAGGAAAGTGTCT
PMI2596-IBS	AAAAAAGCTTATAATTATCCTTAGATGACTTGAGCGTGCGCCCAGATAGGGTG
PMI2596-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTGAGCGCTAACTTACCTTTCTTTGT
PMI2596-EBS2	TGAACGCAAGTTTCTAATTTCGATTTCATCTCGATAGAGGAAAGTGTCT
PMI0047screenFor	NNNNNNTTCGAAATGAACCATAAATATAAAC
PMI0047screenRev	NNGGTACCTTTACGAGTAATGTTGTTCG
PMI0288screenFor	NNNNNNTTCGAAATGAACATCAAACACCTTGCG
PMI0288screenRev	NNGGTAGCGAAGAAGTATTTAAAACGTGC
PMI0842screenFor	NNNNNNTTCGAAATGGTAGTGTTAAAGAAA
PMI0842screenRev	NNGGTACCGAAGTTGTAGTTTAATCCG
PMI1810screenFor	NNNNNNCATATGAACCGTAAAAACCTGTTTAC
PMI1810screenRev	NNNAGATCTGAAGCGATAGTTAAAGTTC
PMI2596screenFor	NNNNNNTTCGAAATGGAAAATAAAGGAAC
PMI2596screenRev	NNGGTACCAAAGTTAATACTTCCCTG

selected on agar containing kanamycin and screened by PCR for an insertion in the appropriate gene, using the screening primers listed in Table 1.

CBA/J mouse model of urinary tract infection.

Cochallenge and independent challenges of female CBA/J mice were carried out as previously described using a modified Hagberg protocol (75, 95). Because bacteria are introduced via a catheter and because *P. mirabilis* urease catalyzes crystal and larger stone formation, this can be classified as a model of complicated urinary tract infection. Briefly, single colonies were picked from a fresh plate and used to start overnight cultures in LB. On the day of inoculation, cultures were diluted with LB to an OD_{600} of approximately 0.2. For independent challenges, mice were inoculated transurethrally with 50 µl (containing approximately 10^7 CFU). For cochallenge experiments, the dilute wild-type and mutant cultures were pooled in a 1:1 ratio and mice were inoculated with 50 µl of this mixture (containing a total of approximately 10^7 CFU). After seven days, urine was collected, mice were euthanized, and their bladders, kidneys, and spleens were harvested asceptically and transferred to sterile tubes containing phosphate buffered saline (0.138M NaCl, 0.0027M KCl, pH 7.4). Tissues were homogenized (Omni TH Homogenizer, Omni International) and plated on agar plates using a spiral plater (Autoplate 4000, Spiral Biotech). For independent challenges, all samples were plated on LB agar. For cochallenges, all samples were plated on both LB agar plates and LB agar plates containing 25 µM kanamycin. Since the mutant strain carries a kanamycin resistance gene, the colony counts from the kanamycin plates represent mutant bacteria only; LB agar plates allow the growth of both mutant and wild-type colonies. Statistical

significance for independent and cochallenge experiments was assessed by using the Mann-Whitney test and Wilcoxin matched pairs test, respectively.

Results

Mice with experimental *P. mirabilis* urinary tract infections have varied antibody responses.

In our previous study, mice were transurethrally infected with 2 x 10^8 CFU *P. mirabilis* HI4320 and, four weeks later, treated with antibiotics to clear the infection. The same mice were challenged one week later with 2 x 10^7 CFU. Sera were collected seven days after re-infection (42 days after the initial infection) (94). In the present study, sera from 20 of these mice were individually screened for reactivity to *P. mirabilis* proteins. Proteins in a *P. mirabilis* HI4320 cell lysate were separated by SDS-PAGE, transferred to a PVDF membrane, and screened by Western blot with a 1:1000 dilution of sera from individual mice. Pre-immune sera were also screened by Western blot at a 1:1000 dilution. All post-infection sera were reactive, but showed some diversity in antigen recognition, which may reflect differences in the severity of the individual infections. Equal volumes of the five most strongly-reacting sera were pooled and used for all further Western blot analyses (Fig. 1). The five corresponding pre-immune sera were also pooled for use as a control.

Sera from infected mice recognize outer membrane antigens.

P. mirabilis was cultured in LB with aeration at 37ºC and fractions enriched for outer membranes were isolated by differential centrifugation and detergent solubilization. Proteins in the outer membrane-enriched fractions were separated by two-dimensional gel electrophoresis (Fig. 2A). Proteins were screened by Western blot using the pooled sera

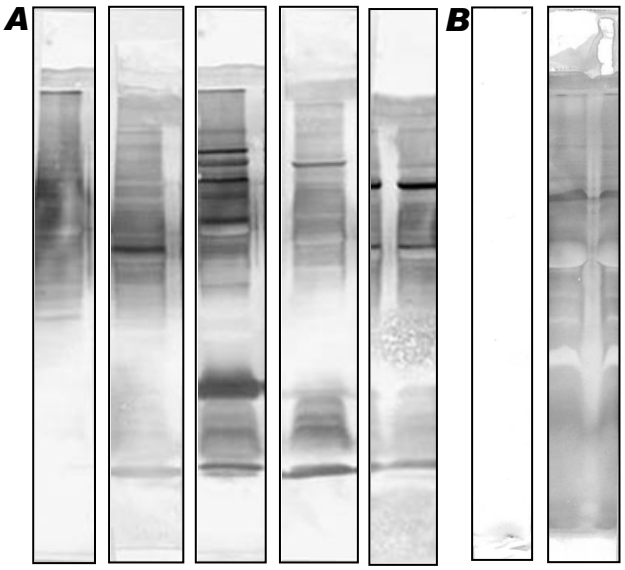


Figure 1. Western blots of *P. mirabilis* HI4320 lysate reacting with sera from mice experimentally infected with *P. mirabilis* HI4320.
(A) Western blots of *P. mirabilis* lysate probed with sera from infected mice. Each strip represents serum from one mouse. The five strips shown are the five most strongly reacting sera. (B) Pooled pre- and post-immune sera reacting with western blots of *P. mirabilis* lysate.

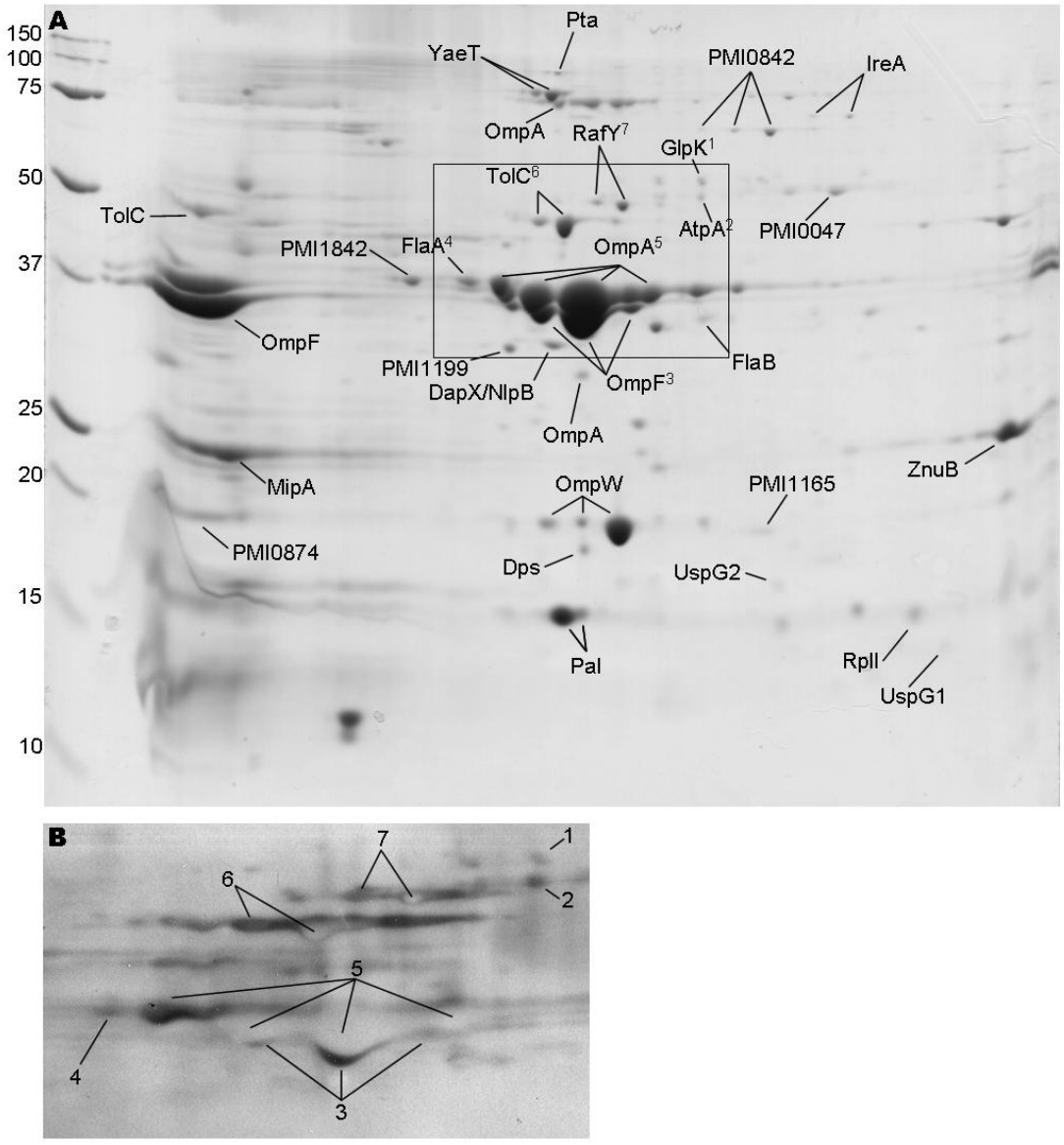


Figure 2. Sera from infected mice recognize outer membrane antigens.
(A) Coomassie-stained 2D gel loaded with 300 mg of Triton X100-insoluble outer membrane-enriched protein from *P. mirabilis* HI4320 cultured in LB. Antigenic proteins are labeled. Boxed region shows area of gel that corresponds to western blot shown in (B). pH gradient ranged from four to seven, left to right. Marker sizes are shown on the left in kilodaltons. **(B)** Western blot of a 2D gel performed with a 1:10,000 dilution of pooled sera from infected mice (shown in Figure 1B). Numbers designate proteins labeled in (A). White (negative) spots that are visible were most likely due to an overabundance of protein and high antibody concentration, as suggested by the manufacturer of the detection system used (see Materials and Methods). Proteins that reacted with sera were cut from a duplicate gel and identified by mass spectrometry.

at a dilution of 1:10,000 (Fig 2B). Antigens that reacted with sera were excised from a duplicate Coomassie-stained gel and identified by mass spectrometry.

Proteins expressed *in vivo* are not necessarily expressed in rich culture medium. To identify antigens that may not be expressed in LB, we used additional culture conditions that may more closely mimic *in vivo* conditions during urinary tract infection. For example, *P. mirabilis* was cultured in pooled human urine and also in minimal medium. Since these media are presumably more nutrient-limited than LB, bacteria may require synthesis of additional proteins for growth that may not be required during growth in LB. The production of these additional proteins increases the number of potential antigens screened. A study of the transcriptome of uropathogenic *Escherichia coli* (UPEC) during mouse infection suggested that the urinary tract is iron-limited and of high osmolarity (215). Therefore, we also isolated protein from *P. mirabilis* HI4320 cultured in media that mimic these conditions by using LB containing 15 μM desferoxamine (an iron chelator) and 0.3 M sodium chloride, respectively. The use of these culture conditions enabled the identification of nine additional antigens that were not expressed in LB, including four outer membrane proteins that appear to be related to iron acquisition (PMI0409, HmuR2, IreA, and PMI2596).

In total, 37 *P. mirabilis* immunoreactive antigens were identified. Twenty-four antigens are predicted to reside in the outer membrane (Table 2). An additional 13 proteins, not predicted to be present in the outer membrane, were also identified as immunoreactive antigens (Table 3).

Table 2. *P. mirabilis* antigens predicted to be in the outer membrane

Category	Gene Number	Protein	Culture condition				
			LB[a]	Low Iron	Urine	MM[b]	Osmotic Stress[c]
Porin	PMI0288†	RafY	**E** (*S*)[d]	**E,S**	(*S*)		(*LB*)
	PMI0765	OmpF	**E** (*S*)	**E** (*S*)	**S**	**E**	(*LB.MM*)
	PMI0785	OmpA	**E, S**	**S** (*E*)	**S**	(*E*)	**LB** (*MM*)
	PMI1017*	putative exported protein		**E**			
	PMI1350	OmpW	**E, S**		**S**		(*LB*, *MM*)
Lipoproteins	PMI0585	Pal	**E, S**		**S**		(*LB.MM*)
& Associated	PMI1563	DapX/NlpB	**E** (*S*)		(*S*)		(*LB*, *MM*)
Proteins	PMI1165	putative lipoprotein	**S** (*E*)		(*S*)		(*LB*)
	PMI1842	putative lipoprotein	**E** (*S*)		(*S*)		(*LB*, *MM*)
	PMI2277	YaeT	**E** (*S*)	**E, S**	(*S*)		**LB**
Motility	PMI1619	FlaB	**E**				(*LB*, *MM*)
	PMI1620	FlaA	**E, S**	**S** (*E*)	**S**		(*LB*, *MM*)
	PMI1651*	FlgE		**E**			
Iron	PMI0409*	TonB-dependent receptor		**E**			
Acquisition	PMI0842†	TonB-dependent receptor	**E, S**	**E** (*S*)	**S**	(*E*)	**MM** (*LB*)
	PMI1426*	HmuR2		**S** (*E*)		(*E*)	
	PMI1945	IreA	(*E*, *S*)	**E, S**	**S**	(*E*)	
	PMI2596†*	putative siderophore TonB-dependent receptor		**E, S**			
Toxins	PMI2043*	putative toxin	**S**				(*LB*)
	PMI2341	Pta	**E** (*S*)	**S**	(*S*)		(*LB*)
Other	PMI1199	putative exported protein	**S** (*E*)		(*S*)		
	PMI1810†*	FadL	**E** (*S*)		(*S*)		(*LB*, *MM*)
	PMI2349	TolC	**E** (*S*)	**E, S**	**S**	(*E*)	(*LB*, *MM*)

[a] Letters designate the growth phase of the culture from which the protein was identified: E, exponential phase; S, stationary phase

[b] Minimal medium

[c] Letters designate the medium in which the protein was identified: LB, Luria broth; MM, minimal medium

[d] Bold letters represent proteins directly identified by mass spectrometry. Italicized letters in parentheses were identified by comparison of electrophoretic mobilities with identified proteins on other gels.

† Assessed for contribution to virulence, see Table 4.

* Not on gel shown in Figure 2; identified from a subsequent gel.

Table 3. Soluble and inner membrane proteins identified as *P. mirabilis* antigens

Gene Number	Protein	Culture condition				
		Luria Broth[a]	Low Iron	Urine	MM[b]	Osmotic Stress[c]
PMI0047†	putative secreted 5'-nucleotidase	**E, S**	**E**			**LB**
PMI0103*	conserved hypothetical protein			**S**[d]		
PMI0631	Dps	**S**		(*S*)		(*LB*, *MM*)
PMI0776*	PqiA	**S**				
PMI0874	conserved hypothetical protein	**E** (*S*)		(*S*)		
PMI1150	ZnuB	**E** (*S*)		(*S*)		
PMI1449	UspG1	**S** (*E*)		(*S*)		
PMI1451	UspG2	**S** (*E*)		(*S*)		(*LB*)
PMI1488*	Hns			**S**		(*LB*)
PMI1506	MipA	**E**				
PMI1905*	conserved hypothetical protein	**E**				
PMI3062	AtpA	**E** (*S*)		(*S*)		(*LB*)
PMI3210	GlpK	(*E*, *S*)				**LB**
PMI3377	RplI	**E** (*S*)				

[a] Letters designate the growth phase of the culture from which the culture was identified: E, exponential phase; S, stationary phase

[b] Minimal medium

[c] Letters designate the medium in which the protein was identified: LB, Luria broth; MM, minimal medium

[d] Bold letters represent proteins directly identified by mass spectrometry. Italicized letters in parentheses were identified by comparison of electrophoretic mobilities with identified proteins on other gels.

† Proteins assessed for contribution to virulence.

* Not on gel shown in Figure 2; identified from a subsequent gel.

Selection of potential vaccine candidates.

For the present study, five representative antigens were chosen for analysis. We chose at least one antigen per category listed in Table 2, with the exception of lipoproteins, motility, and toxins. We did not pursue these three classes of proteins for specific reasons. Lipoproteins are generally on the inner leaflet of the outer membrane and may not, therefore, be exposed to the surface. There are two copies of the gene encoding flagellin that can recombine to form antigenically distinct flagella (25, 29, 160). Since the flagella formed by this recombination event are antigenically distinct, a vaccine employing FlaA as a protective antigen may not be able to target bacterial cells expressing recombinant flagellin. One of the toxins found in this screen, Pta (*Proteus t*oxic *a*gglutinin), recently identified as an autotransporter that is both a cytotoxin and an agglutinin (5), was assessed as a vaccine candidate in a separate study (4). We specifically chose FadL and RafY for further study because structures of similar proteins have been solved and reveal the presence of extracellular loops (61, 202, 237). If these proteins in *P. mirabilis* have similar structures, these extracellular loops could be accessible to the immune system during infection. We also chose to study two putative outer membrane iron receptors, PMI0842 and PMI2596. The urinary tract is an iron-limited environment, and another uropathogen (UPEC) upregulates a number of antigenic iron acquisition outer membrane proteins during growth in urine (13, 74, 215). Additionally, the use of iron receptors as protective antigens against *E. coli, Haemophilus influenzae,* and *Haemophilus ducreyi* infections demonstrate that iron receptors can be used successfully in vaccines (2, 193, 247). We also investigated the role of one of the antigens not predicted to be in the outer membrane, PMI0047. Although this protein is not an ideal vaccine candidate (since it is not predicted to be surface-exposed), it was

recognized by sera from infected mice. Thus, it is produced during urinary tract infection of mice and could have a role in pathogenesis.

Two outer membrane antigens involved in iron acquisition contribute to *P. mirabilis* virulence in the urinary tract.

We reasoned that if a protein target is both antigenic and actively required for colonization, it would be more likely to successfully serve as a protective antigen for vaccination. For example, if a surface protein is being used as a protective antigen, any bacterium that attempts to escape the host immune response by down-regulating this protein would be less likely to persist in the host if the protein is required for colonization. To determine contribution to virulence, we investigated the role of five representative antigens (described above) in colonization of the murine urinary tract. Isogenic insertional mutations were constructed in the genes encoding each of the potential vaccine candidates. Genes were disrupted by the insertion of an intron carrying a kanamycin resistance gene, using the method of Pearson and Mobley (173). All mutations were confirmed by PCR. Growth of each mutant was tested independently in LB; none of the mutants had *in vitro* growth rates significantly different from the wild-type strain.

Mutants were tested in the murine model of ascending urinary tract infection by co-challenge with wild-type *P. mirabilis*. Mutant and wild-type bacteria were mixed in a 1:1 ratio. This suspension was used to inoculate mice transurethrally at a total inoculum of 2×10^7 CFU per mouse. After seven days, urine was collected and mice were euthanized. Urine, bladders, kidneys, and spleens (as an indicator of systemic infection) were quantitatively cultured to determine levels of colonization by both wild-type and mutant strains. Based on data from the co-challenge experiments, PMI0047 (secreted 5'

nucleotidase), RafY, and FadL do not appear to contribute to colonization of the urinary tract (Table 4). Mutant strains, interrupted in genes encoding these proteins, colonized the urinary tract in numbers similar to the wild-type strain. Two outer membrane iron receptors do, however, contribute to colonization of the urinary tract by *P. mirabilis* (Fig. 3). PMI0842::kan was outcompeted by wild type at statistically significant levels in both the bladder and kidneys ($p < 0.05$ for both tissues, Fig. 3A). Also in cochallenge, PMI2596::kan was recovered from kidneys in significantly lower numbers than wild type ($p < 0.05$, Fig. 3C). As a control, *in vitro* cocultures were performed, in which the mutant and wild-type strains were mixed in a 1:1 ratio and used to inoculate culture medium. Neither mutant was outcompeted by the wild-type strain (Fig. 4). Thus, the mutants retained wild-type growth rates *in vitro* and likely were not outcompeted *in vivo* due to an *in vitro* growth defect.

Due to the striking phenotype of these mutants (particularly PMI0842::kan) in cochallenge, each mutant was tested for virulence in independent challenges. While cochallenges are more sensitive at detecting differences between mutant and wild-type strains because the strains are put into direct competition for niches and nutrients, they cannot conclusively reveal that a putative virulence factor is required for infection. Thus, mice were transurethrally inoculated with either the wild-type or mutant strain at a dose of 1×10^7 CFU per mouse. After seven days, urine, bladders, kidneys, and spleens were quantitatively cultured to determine the level of bacterial colonization. Independent challenge data show that PMI0842 is required for maximal colonization of the urinary tract (Fig. 3A). Although this mutant was able to colonize mice, PMI0842::kan was found in significantly lower numbers as compared to wild type in both the bladders and

Table 4. Assessment of virulence of mutants of selected antigens in cochallenge studies

Antigen	Strains	N[a]	Urine CFU/ml[b] (Ratio[c])	Bladder CFU/g (Ratio)	Kidney CFU/g (Ratio)	Spleen CFU/g (Ratio)
Secreted 5'-nucleotidase	PMI0047::kan	10	1.21×10^6 (20.07)	5.62×10^4 (0.02)	1.06×10^4 (0.41)	1.00×10^2 (1.00)
	WT		6.03×10^4	3.54×10^6	2.56×10^4	1.00×10^2
RafY	PMI0288::kan	9	6.20×10^3 (0.25)	3.73×10^4 (0.54)	1.48×10^4 (0.47)	1.00×10^2 (1.00)
	WT		2.45×10^4	6.85×10^4	3.16×10^4	1.00×10^2
TonB-dependent receptor	PMI0842::kan[d]	9	1.00×10^2 (<0.01)	1.00×10^2* (<0.01)	1.00×10^2* (<0.01)	1.00×10^2 (0.15)
	WT		8.11×10^6	1.27×10^6	3.74×10^5	6.70×10^2
FadL	PMI1810::kan	9	2.96×10^5 (0.18)	3.78×10^5 (0.11)	1.08×10^5 (0.53)	1.00×10^2(1.00)
	WT		1.61×10^6	3.53×10^6	2.03×10^5	1.00×10^2
TonB-dependent receptor	PMI2596::kan[d]	8	6.73×10^5 (0.08)	6.76×10^6 (1.19)	5.74×10^5* (0.56)	1.79×10^3 (1.25)
	WT		8.97×10^6	5.67×10^6	1.03×10^6	1.43×10^3

[a] In each experiment, 10 mice were cochallenged; any number less than 10 represents the number of live mice remaining at the end of the experiment. Urine collection from each mouse was not always possible; therefore, data for urine are based on a lower number of samples, as follows: PMI0047::kan/WT: 7 , PMI0288::kan/WT: 4, PMI0842::kan/WT: 4, PMI1810::kan/WT: 5, PMI2596::kan/WT: 5.

[b] The limit of detection (LOD) is 1.00×10^2 CFU/ml urine or /g tissue. Any sample below this limit was set to the LOD for statistical analyses. Colonization data shown are the median values for a given group of mice.

[c] Listed is the ratio of the medians of mutant versus wild type. A ratio of 1 means that wild type and mutant were present in equal levels, a ratio less than 1 represents more wild type than mutant, and a ratio greater than 1 represents more mutant than wild-type cells present in the sample.

[d] These data are shown in Figure 3.

* Statistically significant as compared to wild type colonization in the same organ during cochallenge, $p < 0.05$

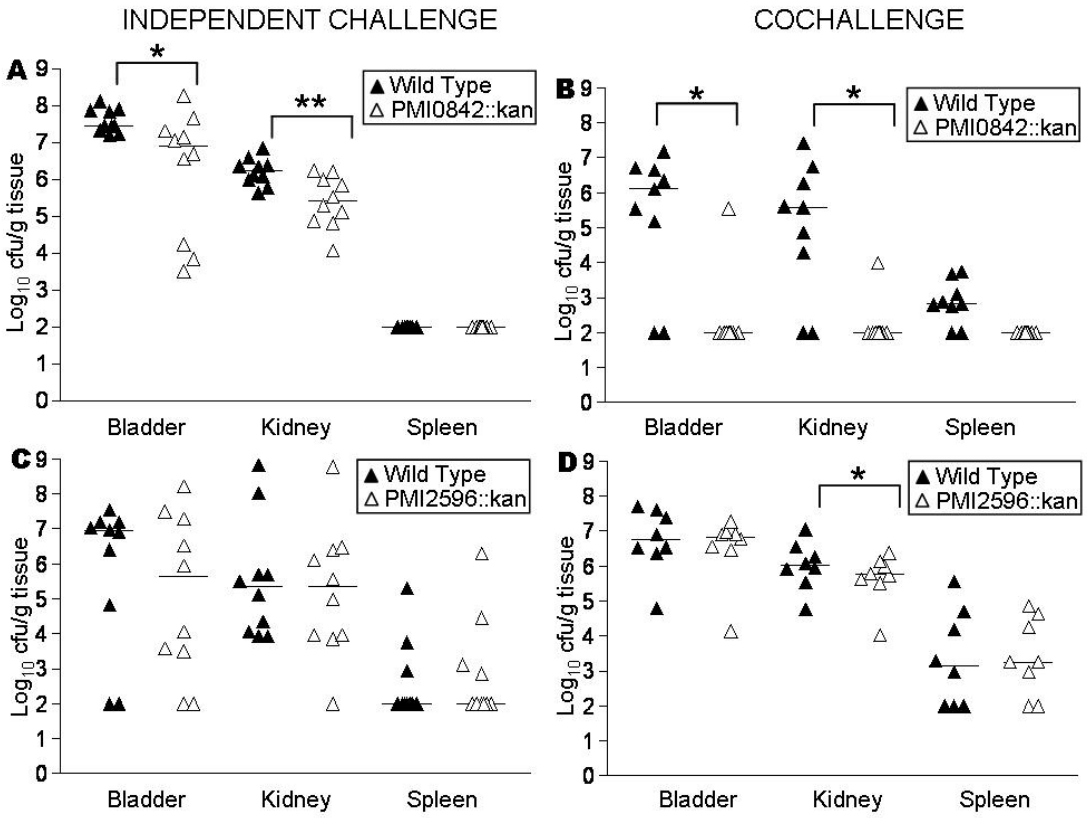


Figure 3. Putative iron acquisition outer membrane proteins contribute to *P. mirabilis* HI4320 virulence in the murine model of urinary tract infection. Each triangle represents an individual mouse. Bars represent medians. Points at 100 cfu/g of tissue represent samples at or below the limit of detection. **(A)** PMI0842::kan and wild type independent challenge. **(B)** PMI0842::kan cochallenge **(C)** PMI2596::kan and wild type independent challenge **(D)** PMI2596::kan cochallenge**.** *, $p < 0.05$; **, $p < 0.05$

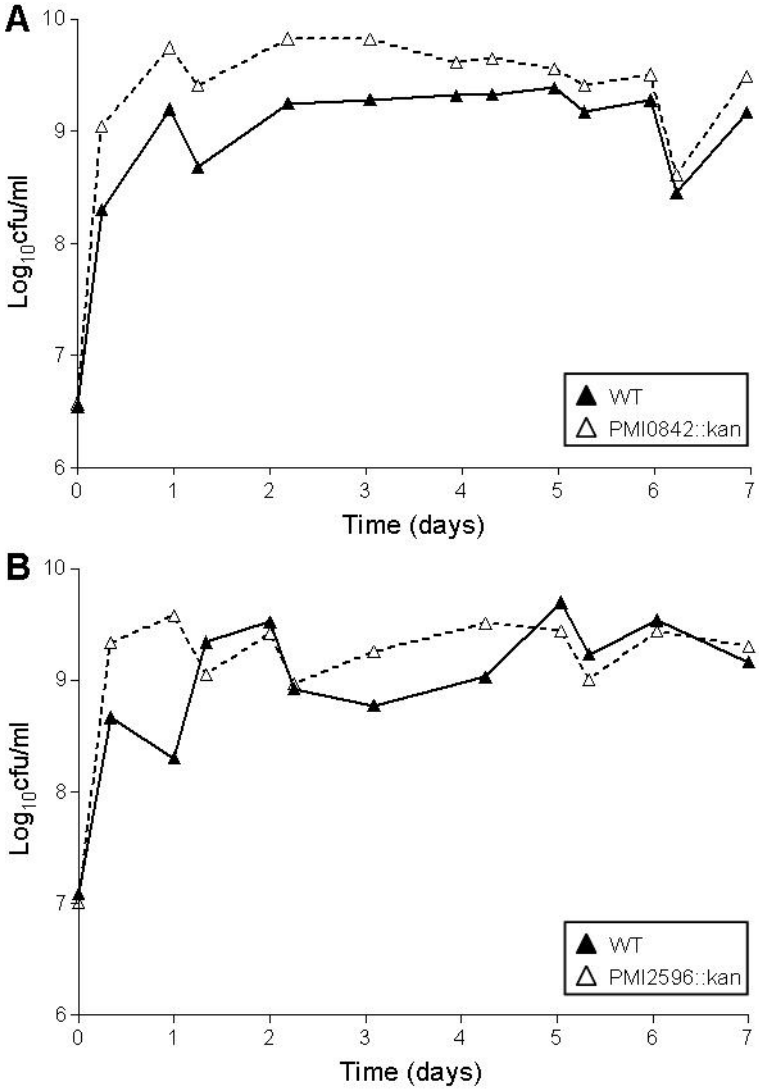


Figure 4. PMI0842::kan and PMI2596::kan are each maintained during a seven-day coculture with wild type.
Cultures were inoculated with approximately equal amounts of wild-type and mutant strains. At indicated time points, samples were taken for plating and the culture was repassaged (1:100) into fresh medium. (A) Wild type and PMI0842::kan coculture in LB. Filled symbols, wild type; open symbols, PMI0842::kan. (B) Wild type and PMI2596::kan coculture in LB. Filled symbols, wild type; open symbols, PMI2596::kan.

kidneys of infected mice ($p < 0.05$ and $p < 0.01$, respectively). In contrast, although PMI2596::kan was outcompeted in cochallenge experiments (Fig. 3D), in independent challenge experiments it colonized mice in similar numbers as the wild-type strain (Fig. 3C).

Discussion

This is the first study to identify immunoreactive outer membrane antigens of *P. mirabilis* using an immunoproteomics approach. Here, we identified 37 antigens that reacted with sera from mice experimentally infected in the urinary tract with *P. mirabilis*. Based on their annotation or homology to other proteins, 24 of these proteins are predicted to reside in the outer membrane. These proteins are attractive as potential vaccine candidates since they are expressed *in vivo*, antigenic, and exposed on the surface of the bacterium (and thus to the immune system). Five representative antigens were selected for further study: PMI0047, RafY, PMI0842, FadL, and PMI2596. Two of these proteins, both putative outer membrane iron receptors (PMI0842 and PMI2596), contribute to the fitness of *P. mirabilis* in the urinary tract. From this study, PMI0842 and PMI2596 emerge as potential vaccine candidates, since each protein both contributes to pathogenesis and is antigenic. PMI0047, RafY, and FadL do not appear to directly contribute to *P. mirabilis* HI4320 virulence in the urinary tract, as strains with insertionally interrupted copies of these genes colonized the urinary tract of mice in numbers similar to that of wild-type bacteria.

This study identified five predicted porins as immunogenic: OmpA, OmpF, OmpW, PMI1017, and RafY. OmpA, OmpF, and OmpW are major outer membrane proteins. PMI1017 appears to be a member of the OprD porin family based on homology to other family members. RafY is a predicted glycoporin, a family of proteins that typically allow passage of sugars across the bacterial outer membrane. Other well-known glycoporins include LamB (maltoporin) and ScrY (sucrose porin). In *E. coli*, RafY allows growth on raffinose but was later shown to be a general diffusion pore rather than

a carbohydrate-specific one (16). The role of RafY in *P. mirabilis* has not yet been determined. The structures of LamB from *E. coli* and ScrY from *Salmonella typhimurium* have been solved (61, 202). Both proteins contain extracellular loops which are surface-exposed. If the protein structure of *P. mirabilis* RafY is similar to other glycoporins, it may also have surface-exposed extracellular loops and therefore be an attractive candidate for inclusion in vaccine studies.

Four lipoproteins were identified: Pal, DapX, YfgL, and YeaY. Lipoproteins are typically found on the inner leaflet of the outer membrane. Two of these lipoproteins (DapX, which belongs to NlpB family, and YfgL) are involved in a complex with outer membrane protein YaeT, which was also identified as an antigen in this study. Although the complex is conserved in gram negative bacteria, DapX/NlpB and YfgL are not essential for growth of *E. coli* (36, 56, 190, 252). Peptidoglycan-associated lipoprotein (Pal) is released during gram-negative sepsis and mediates inflammation and death in mice (82). Pal is part of the Tol-Pal system, which is conserved among gram negative organisms (222). This system has been implicated in the maintenance of cell envelope integrity (128) and cell division machinery (68). Pal has been identified as an antigen in many other bacterial pathogens, including *Legionella pneumophila* and *Campylobacter jejuni* (39, 57). A homologous lipoprotein (P6) in *H. influenzae* is being assessed as a protective antigen (32, 51, 86, 108). Although we initially did not choose to investigate Pal as a vaccine candidate since we are aiming specifically for surface-exposed proteins, these findings from other bacteria suggest it may warrant further study.

Two flagellar proteins were identified: the major flagellin subunit and the flagellar hook protein (FlgE). *P. mirabilis* carries two copies of the flagellin gene, *flaA*

and *flaB* (29). Normally, *flaA* is expressed and *flaB* is silent, but the two genes can undergo recombination, resulting in the formation of antigenically distinct flagella (25, 160). Recombination of the two alleles occurs infrequently in wild-type cells (135). Such an event must have occurred under our culture conditions, since mass spectrometry data yielded results for both FlaA and FlaB proteins.

We identified five iron-related outer membrane proteins as antigenic. This is not surprising considering the important role iron acquisition plays in bacterial pathogens. Indeed, we chose to examine outer membranes from bacteria cultured under iron limitation because the urinary tract is an iron-limited environment. Iron acquisition systems are up-regulated in UPEC during infection in the murine model (215). UPEC up-regulates a number of outer membrane iron receptors when cultured in human urine and many of these were identified as antigenic (13, 74). Additionally, *P. mirabilis* iron-related outer membrane receptors have been previously identified as antigenic both in mice and in human patients (181, 210). One of these proteins, also identified as an immunoreactive antigen in the current study, was recently identified as HmuR2 (PMI1426) and characterized as a heme receptor that contributes to virulence in the urinary tract (127).

Two outer membrane antigens predicted to be iron receptors (PMI0842 and PMI2596) contribute to the fitness of *P. mirabilis* HI4320 during experimental urinary tract infection. PMI0842 was previously identified as a virulence factor by signature-tagged mutagenesis (38) and was recently shown to be up-regulated in response to iron limitation (Himpsl *et al.*, in preparation). Our co-challenge results confirm the previous finding from signature-tagged mutagenesis. Additionally, our independent challenge data

show that PMI0842 is required to achieve maximum colonization of the urinary tract. This result is surprising due to the fact that iron receptors are typically redundant in function; a defect in one system rarely results in attenuation during independent challenges since other iron uptake systems remain functional, as highlighted by Torres and colleagues (230). The role of PMI0842 in iron uptake in *P. mirabilis* remains to be determined.

This study is also the first to show a role for PMI2596 in *P. mirabilis* HI4320 virulence in the urinary tract. Although the independent challenge data show this protein is not required for virulence, cochallenge data highlight its contribution to colonization in the kidneys. PMI2596 is located roughly 2 kb upstream of the *nrp* operon, which encodes non-ribosomal peptide synthesis genes (65). NrpG was previously identified by signature-tagged mutagenesis as a virulence factor in *P. mirabilis* (38). Interestingly, a strain lacking a functional copy of NrpG was outcompeted by wild-type *P. mirabilis* HI4320 in the kidneys but not the bladders of mice in a cochallenge experiment (38), the same pattern we observed with PMI2596::kan. It is currently unknown if PMI2596 is functionally related to the *nrp* operon.

Although we were able to identify 37 antigens, we do not believe this study generated a complete list of the immunogenic outer membrane antigens expressed by *P. mirabilis in vivo*. The methods used to identify these proteins imposed some limitations. First, we were able only to identify proteins that were abundant enough to be detected by Coomassie staining. Also, since we ran the protein samples on a denaturing gel prior to screening with sera, we will have likely missed any conformational epitopes. Finally, we did not detect any fimbrial proteins under any culture condition tested. This is surprising,

especially considering the large number of fimbrial operons encoded in the genome (174). Specifically, we expected to find proteins that comprise MR/P fimbriae, since these fimbriae are expressed (although not optimally) under the conditions tested (23) and the sera used in this experiment have previously detected MR/P fimbriae (94). It is likely that fimbriae were sheared from bacterial cells during fractionation.

This study also identified 12 antigenic proteins that do not appear to be surface-exposed. These non-surface exposed antigens include two apparent DNA binding proteins, a ribosomal protein, various enzymes, and three conserved hypothetical proteins. Since the goal of this study was to identify surface-exposed antigens, these proteins were not investigated further for vaccine studies. Even though these proteins may not be ideal vaccine candidates, they were recognized by sera from infected mice, meaning they are expressed by *P. mirabilis* during urinary tract infection and could potentially play a role in pathogenesis. PMI0047 does not contribute to the fitness of *P. mirabilis* during experimental infection, since bacteria with an interrupted copy of this gene were able to colonize mice at numbers similar to the wild-type strain. Other proteins on this list, however, may play a role in pathogenesis and are currently under investigation (G. Nielubowicz and H. Mobley, unpublished data).

The sera used to identify antigens in this experiment are from our previous study in which groups of mice were either infected transurethrally with *P. mirabilis* (vaccinated with live organisms) or sham-vaccinated with PBS (94). Both groups of mice were then transurethrally challenged with 10^6 CFU of *P. mirabilis* HI4320 to determine whether having a previous infection protected mice from subsequent infection. Vaccinated mice were colonized in the urine, bladder, and kidneys, but had significantly lower

colonization levels in the kidney than the sham-vaccinated mice ($p = 0.016$). Therefore, in the study described here, we identified antigens (which we hope will be protective) by using sera from mice that were not completely protected. It may seem counterintuitive to use sera from non-protected mice to search for protective antigens, but it is important to point out that these mice had strong immunoglobulin responses to MR/P fimbriae (94). MR/P fimbriae or protein subunits (MrpH and MrpA) have been used successfully in a protective vaccine in the murine model (118, 120, 177, 200, 201). Even though these antigens can be protective and were present during infection (as evidenced by the strong immunoglobin response that was detected), their presence was not able to fully protect the mice from subsequent infection. Higher levels of antibody to specific proteins induced by subunit vaccinations may, however, be effective in protecting the urinary tract from colonization. It is important to point out that the correlates of protection in the urinary tract are not yet well understood. Multiple studies have shown that the production of serum IgG and IgM do not necessarily correlate with protection (96, 200, 201). However, data on production of local IgA and protection are not as clear. Some studies have shown no correlation (200, 201), while others have suggested one (94, 120). It is known that antigen-specific responses occur in the urinary tract and accelerate the clearance of at least one uropathogen, UPEC (227).

Future work will determine whether these proteins are conserved among *P. mirabilis* strains. We are also interested in assessing whether these antigens could provide protection against other bacteria which are commonly found in polymicrobial cUTIs, such as *Providencia* and *Morganella* species. Although these genome sequences have not been published, work is currently underway in our laboratory to determine

whether homologues of the genes encoding antigenic *P. mirabilis* proteins are present in these species (E. Flannery and H. Mobley, unpublished). We will also determine the extent of protection offered by immunization with selected newly identified antigens in the murine model of urinary tract infection. *P. mirabilis* antigenic proteins identified in this study are being overexpressed in and purified from *E. coli*. Mice will be immunized with antigen cross-linked to cholera toxin, which is known to be an effective mucosal adjuvant (58) and has previously been used with *P. mirabilis* MrpH for an effective vaccine (120). By taking this approach, we hope to develop an effective vaccine against this agent of cUTIs.

Appendix (Unpublished Data)

PMI0842, a putative TonB-dependent receptor, was identified in this study as an antigenic outer membrane protein that is required for maximal virulence during experimental murine UTI. PMI0842 met all of our criteria for an ideal vaccine candidate, and therefore was assessed as a protective antigen.

PMI0842 was cloned and purified as previously described for other antigens (4, 12, 120). Briefly, the gene encoding PMI0842 was cloned into the vector pET-30b(+) (Novagen) which resulted in the addition of a six-residue histidine tag at the C terminus. IPTG (β-D-1-thiogalactopyranoside) was used to induce expression of PMI0842 in *E. coli* BL21(DE3)pLysS cultured in Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 2.3 g/L KH_2PO_4, 12.5 g/L K_2HPO_4, 4% glycerol). Outer membranes were isolated as described in Materials and Methods, above. PMI0842 was purified from outer membrane preparations by using a Ni-NTA column (Qiagen), according to the protocol recommended by the manufacturer and as described previously for Pta (4, 5) (Fig. 5). Please note that PMI0842 was not purified to homogeneity; nonetheless, antigen preparation continued. Purified PMI0842 was conjugated to adjuvant (cholera toxin, CT) at a 10:1 ratio using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pierce), per the manufacturer's recommendations.

Mice were immunized intranasally since that route has previously been shown to be most effective for *P. mirabilis* antigens (120). Mice received a primary dose of 100 μg PMI0842 (conjugated to 10 μg of CT) on day zero; two boosters (consisting of 10 μg PMI0842 conjugated to 1 μg CT) were administered on days seven and fourteen (Fig. 6A). CT alone was administered intranasally to a group of control mice; these mice

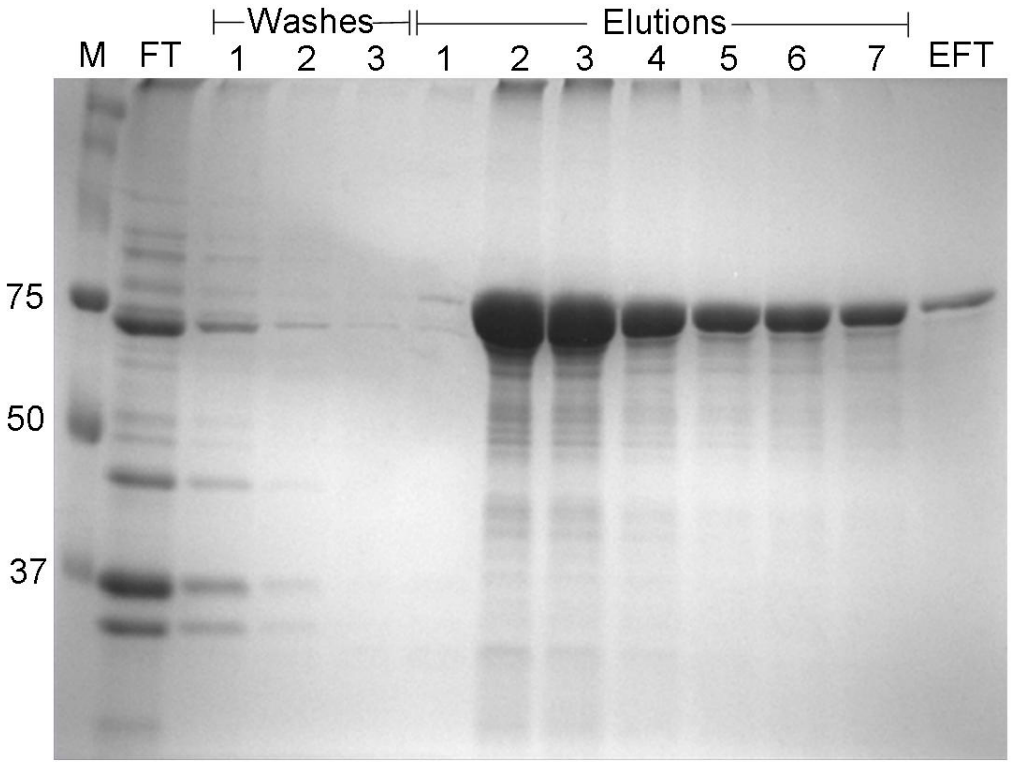


Figure 5. Sample purification of PMI0842.
M, marker (sizes of relevant bands, in kDa, are depicted to the left of the gel); FT, flow-through; EFT, elution flow-through. Proteins in elutions 2-7 were pooled and prepared for use in immunization, as described in the text.

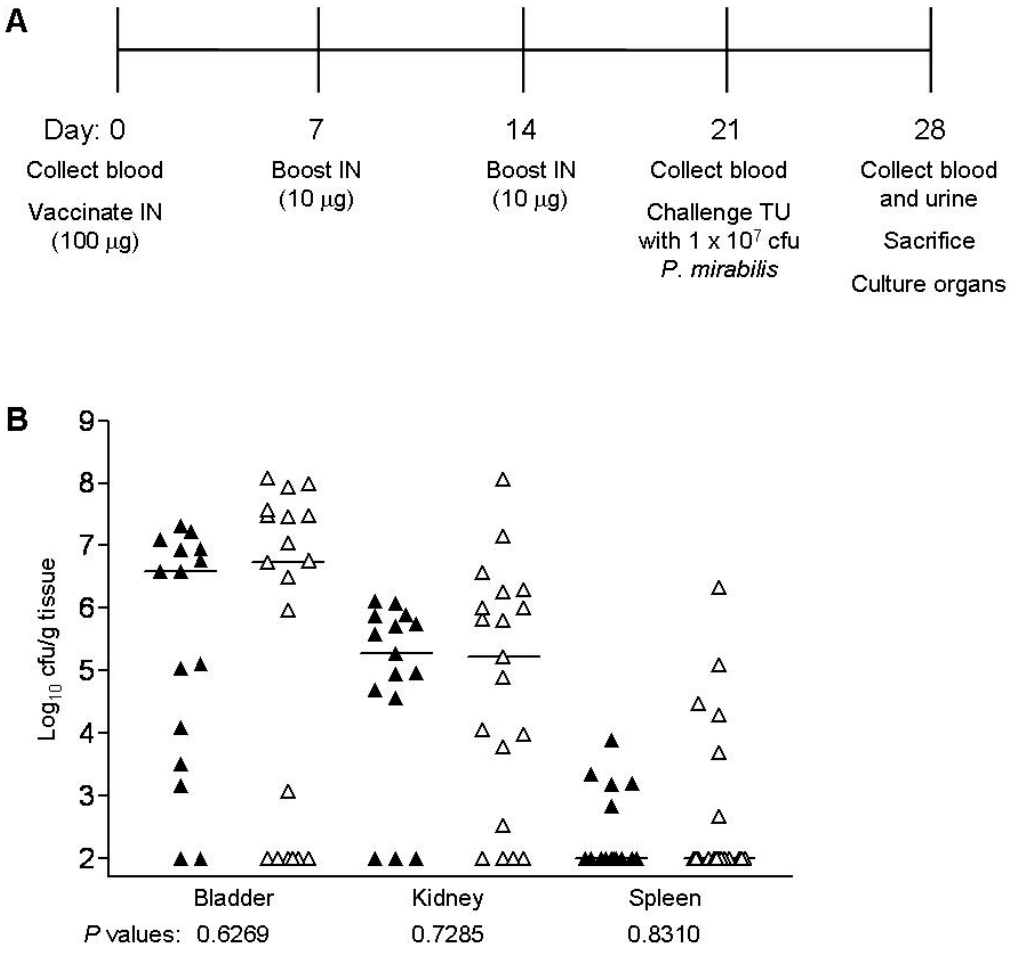


Figure 6. Intranasal vaccination with PMI0842 does not protect mice from transurethral challenge with *P. mirabilis* HI4320.
(A) Vaccination scheme. IN, intranasally; TU, transurethrally (B) Mice were immunized either with CT alone (closed symbols) or PMI0842-CT (open symbols) and then infected with *P. mirabilis* HI4320. Each symbol represents data from an individual mouse. Data shown are compiled from two independent experiments. Bars represent the median. Limit of detection is 100 CFU/gram of tissue.

received a primary dose of 10 μg CT and boosts of 1 μg CT. At the beginning of the experiment and at the time of each boost, blood was collected by an infraorbital bleed and serum was isolated to assess production of PMI0842-specific antibodies. One week following administration of the final boost, mice were infected transurethrally with approximately 1 x 10^7 CFU *P. mirabilis* HI4320. After seven days, blood and urine were collected (for antibody assessment), mice were sacrificed, and bladders, kidneys, and spleens were quantitatively cultured.

Unfortunately, mice that were immunized with PMI0842-CT were not protected from infection; levels of colonization in PMI0842-CT-vaccinated mice were not significantly different from levels of colonization in mice that received CT alone (Fig. 6B). Therefore, we conclude that PMI0842 did not serve as a protective antigen when conjugated to the adjuvant cholera toxin and administered intranasally. Although serum and urine samples were collected from mice to detect PMI0842-specific antibody production, these samples have not yet been analyzed. It is possible that vaccination did not result in the production of PMI0842-specific antibodies; this possibility could explain why no protection was observed.

The work presented in this chapter (excluding the data presented in this appendix) was published (165). The other contributing author, Sara N. Smith, assisted with animal studies; all other experiments were performed by G.R.N. Manuscript was prepared by G. R. N.

CHAPTER III. Zinc Uptake Contributes to Motility and Provides a Competitive Advantage to *Proteus mirabilis* during Experimental Urinary Tract Infection

Abstract

Proteus mirabilis, a gram negative bacterium, represents a common cause of complicated urinary tract infections in catheterized patients or those with functional or anatomical abnormalities of the urinary tract. ZnuB, the membrane component of the high affinity zinc (Zn^{2+}) transport system ZnuACB, was previously shown to be recognized by sera from infected mice. Since this system has been shown to contribute to virulence in other pathogens, its role in *P. mirabilis* was investigated by constructing a strain with an insertionally interrupted copy of *znuC*. The znuC::kan mutant was more sensitive to zinc limitation than wild type, was outcompeted by wild type in minimal medium, displayed reduced swimming and swarming motility, and produced less *flaA* transcript and flagellin protein. Production of flagellin and swarming motility were restored by complementation with *znuCB in trans*. Swarming motility was also restored by the addition of Zn^{2+} to the agar prior to inoculation; addition of Fe^{2+} to agar also partially restored swarming motility of znuC::kan but addition of Co^{2+}, Cu^{2+}, or Ni^{2+} did not. ZnuC contributes to, but is not required for, virulence in the urinary tract; znuC::kan was outcompeted by wild type during a cochallenge experiment but was able to colonize mice to levels similar to wild type during independent challenge. Since we demonstrated a role of ZnuC in zinc transport, we hypothesize that there is limited zinc present in the urinary tract and *P. mirabilis* must scavenge this ion to colonize and persist in the host.

Introduction

Proteus mirabilis, a motile gram negative bacterium, represents a common cause of complicated urinary tract infections (244). These infections typically occur in patients with functional or anatomical abnormalities of the urinary tract or patients subjected to long-term catheterization (such as those with spinal cord injuries or elderly patients residing in nursing homes). The serious sequelae (including catheter encrustation, stone formation, renal scarring, and potential for progression to bacteremia) that can result from these *P. mirabilis* infections (149, 243, 244), as well as the difficulty in treating them, have sparked active research into the mechanisms of pathogenesis (5, 38, 60, 84, 89, 216, 262) and identification of potential vaccine candidates (4, 118, 120, 123, 165, 177, 200). One of the most notable characteristics of *P. mirabilis* is swarming motility, a specialized form of flagellar-mediated motility during which bacteria differentiate into elongated hyperflagellated cells; these differentiated cells migrate together *en masse* (185). Swarming motility is clinically relevant, as *P. mirabilis* is capable of swarming across the surface of urinary catheters (219).

Our previous study, aimed at identifying antigens using sera from mice with experimental urinary tract infections (94), revealed that a protein annotated as ZnuB is an antigen expressed *in vivo* (165). ZnuB is the inner membrane component of the ZnuACB high-affinity zinc (Zn^{2+}) transport system (171). The additional components of this ABC transporter are ZnuA, a periplasmic binding protein, and ZnuC, a cytoplasmic ATPase.

Zinc is essential for life but also toxic at high concentrations; thus, its intracellular concentration must be carefully regulated (35). In bacteria, this regulation is achieved primarily by coordinated efforts to import and export zinc in environments

where the ion is limited or present in excess, respectively (79). In conditions where zinc is low, high-affinity uptake systems are employed to import zinc into the cell. In *Escherichia coli*, this process is achieved via the ZnuACB system (171). This system is synthesized from two divergently transcribed operons, *znuA* and *znuCB*. Under zinc replete conditions, the regulator Zur (zinc uptake repressor) binds as a dimer in the intergenic region between *znuA* and *znuC* and represses transcription; when zinc becomes limited, the genes are derepressed (170). Zur is exquisitely sensitive to changes in the zinc concentration in the cell; differences can be sensed in the femtomolar range (168). Under moderate conditions in which zinc is neither limited nor toxic, zinc is brought into the cell through lower-affinity transporters, namely ZupT (71), which has broad metal specificity and is expressed constitutively at low levels (70). In addition, PitA, an inorganic phosphate transporter in *E. coli*, and CitM, a citrate transporter in *Bacillus subtilis*, are capable of transporting zinc (24, 107).

Much like iron, the level of zinc available in the host is presumed to be limited (133, 254) and may actually decrease in response to bacterial infection (64). Therefore, it is not surprising that the ZnuACB system has been found to contribute to virulence in a number of pathogens, including *Campylobacter jejuni* (50), *Salmonella enterica* serovar Typhimurium (15, 40), *Haemophilus ducreyi* (117), *Brucella abortus* (105, 253), and *Pasteurella multocida* (67). Recently, ZnuACB was shown to contribute to the ability of uropathogenic *E. coli* (UPEC) to colonize the urinary tract (196), suggesting the urinary tract may be limited in zinc, as previously demonstrated for iron (13, 192, 210, 215).

Zinc uptake is uncharacterized in *P. mirabilis*. We hypothesized that the ZnuACB system functions as a zinc transport system in *P. mirabilis* and contributes to

virulence, especially considering it is expressed *in vivo* (165). In this study, we show that the presence of ZnuC allows *P. mirabilis* to grow to a higher density under zinc limitation and yields a competitive advantage during growth in minimal medium. ZnuC is required for motility; a strain with an interrupted copy of the gene swims and swarms significantly less than wild type and produces less flagellin, the major subunit of flagella. In addition, *znuA* and *znuCB* appear to be regulated by Zur. We show, for the first time, that the ability to import zinc contributes to the fitness of *P. mirabilis* during experimental urinary tract infection in the mouse model of this disease.

Materials and Methods

Strains and culture conditions.

P. mirabilis HI4320 was originally cultured from the urine of a catheterized nursing home patient with bacteriuria (149). Luria broth (LB) (per liter, 10 g tryptone, 5 g yeast extract, and 0.5 g NaCl) and non-swarming agar (per liter, 10 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 15 g agar) were used to culture bacteria. Minimal A medium was prepared as previously described (28). All cultures were incubated at 37ºC with aeration unless otherwise noted. When appropriate, kanamycin or ampicillin was added to media at a final concentration of 25 μg/ml or 100 μg/ml, respectively. Metal chelation was achieved by addition of N,N,N′,N′-Tetrakis (2-pyridylmethyl)ethylenediamine (TPEN, Sigma-Aldrich), dissolved in ethanol prior to use. Metals used to supplement media were obtained from the following sources: $FeCl_2$, Fisher Scientific; $Cu(II)SO_4 \cdot 5H_2O$, Sigma; $Co(II)Cl_2 \cdot 6H_2O$, Sigma; $NiSO_4 \cdot 6H_2O$, Sigma; $ZnSO_4 \cdot 7H_2O$, J.T. Baker. The Bioscreen C Growth Curve Analyzer (Growth Curves, USA) was used for independent growth curves.

Construction of mutants.

Insertional mutants were constructed using the TargeTron system (Sigma), as described for *P. mirabilis* by Pearson and Mobley (173). Briefly, genes were disrupted by insertion of an intron, targeted specifically to the gene of interest by using a set of three primers (IBS, EBS1d, and EBS2; listed in Table 5) in a mutagenic PCR. This mutated region of the intron was ligated into the vector pACD4K-C. Resultant plasmids were sequenced to confirm proper re-targeting of the intron. Plasmids containing a

Table 5. Primers used for mutagenesis and qRT-PCR.

Primer Name	Sequence (5' to 3')
znuC-IBS	AAAAAAGCTTATAATTATCCTTATAAACCATGTTGGTGCGCCCAGATAGGGTG
znuC-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGTTTATCGATAGAGGAAAGTGTCT
znuC-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATGTTGACTAACTTACCTTTCTTTGT
zur-IBS	AAAAAAGCTTATAATTATCCTTACGCTGCTTGCGGGTGCGCCCAGATAGGGTG
zur-EBS2	TGAACGCAAGTTTCTAATTTCGGTTCAGCGTCGATAGAGGAAAGTGTCT
zur-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTGCGGTATAACTTACCTTTCTTTGT
*znuC*ScreenFor	ATGTCTGACTTAATTCG
*znuC*ScreenRev	TCAATCATGATGACACTCC
*zur*ScreenFor	AGAGAAATTGCTCGCTCAGGCAGA
*zur*ScreenRev	AACCTTCCGGATGTGTACAGGCTT
rpoA-RT-For	ATTCTGCTTTCGTCTATGCCGGGT
rpoA-RT-Rev	GCAATAACAGGGCCAATGCCAGAT
znuA-RT-For	TTAATGGTATGGGTTGGGCCGGAT
znuA-RT-Rev	TTGTCAGTTTCCGCTAAAGCCAGC
znuC-RT-For	GCCGTATTCTTACCTTACTTGGCCCT
znuC-RT-Rev	TCTTTCCAGAAGAGGGAGCCAACA
znuB-RT-For	TGATTGAGTTGCTGTTACCGGGCT
znuB-RT-Rev	GCGCCAAACAACAAAGGAGCCTAA
flaA-RT-For	AATCGAGCGTCTGTCTTCTGGTCT
flaA-RT-Rev	GCGATAGAGATACCATCGTTCGCA
*znuCB*compFor	CTCGAGCGTAAATCCGAAGGTTTTAATGC
*znuCB*compRev	AAGCTTCTACTGCTTTGCTGGTACAAGC

correctly re-targeted intron were electroporated into electrocompetent *P. mirabilis* HI4320 containing the helper plasmid pAR1219 (49). Since the intron contains a kanamycin resistance gene, transformants were selected on agar containing kanamycin and screened by PCR for an insertion in the appropriate gene, using the screening primers listed in Table 5.

Complementation.

qRT-PCR data indicated that znuC::kan contained a polar mutation that resulted in reduced expression of *znuB*; expression of *znuB* was 50.1-fold lower in znuC::kan than in wild type. Therefore, *znuCB* were employed for complementation studies. *znuCB* were amplified from wild-type *P. mirabilis* HI4320 genomic DNA using primers listed in Table 5 (*znuCB*compFor and *znuCB*compRev) and cloned into pCR2.1-TOPO (Invitrogen). The resultant plasmid, pTOPO-*znuCB*, was transformed into electrocompetent *E. coli* TOP10 (Invitrogen); transformants were selected on agar containing kanamycin. Restriction enzymes HindIII and XhoI (New England Biolabs) were used to digest pTOPO-*znuCB* and the vector pACYC177 (New England Biolabs); the insert was ligated into the digested vector using T4 DNA ligase (Promega). The resultant plasmid, pZnuCB, was transformed into *P. mirabilis* HI4320 by electroporation to yield the complemented strain (znuC::kan + pZnuCB), which was selected for on agar containing ampicillin. pACYC177 was also transformed into znuC::kan for use as an empty vector control (strain znuC::kan + pEV).

Quantitative reverse transcriptase PCR (qRT-PCR).

Log phase culture (0.5 ml) was added to 1 ml RNA Protect solution (Qiagen). RNA was isolated using the RNeasy Mini Prep protocol (Qiagen) according to the

manufacturer's directions. Samples were treated with DNase (Turbo DNA-*free* DNase, Ambion) and cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen). Samples were analyzed by RT-PCR with primers specific to *rpoA* to confirm lack of product in negative controls with no reverse transcriptase added (-RT). qRT-PCR reactions were performed in duplicate and contained 30 ng cDNA and 12.5 µl 2× SYBR Green PCR master mix (Stratagene) per reaction. Primers were used at 100 nM (*rpoA*, *znuA*) or 200 nM (*znuC*, *znuB*, *flaA*). qRT-PCR was performed with an Mx3000P thermal cycler (Stratagene). Data were normalized to expression of *rpoA*. For experiments examining expression under conditions of zinc limitation, 35 µM TPEN (or ethanol, as a control) was added to cultures 30 minutes prior to RNA isolation.

Swimming and swarming motility.

Swimming motility was measured by stabbing the inoculum into soft agar swim plates (per liter: 10 g tryptone, 0.5 g NaCl, 2.5 g agar). Swarming motility was measured by spotting 5 µl of the inoculum onto the surface of swarming agar plates (per liter: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar). Inocula were late logarithmic phase bacterial cultures adjusted to OD_{600} = 1.0. Both swim and swarm plates were incubated at 30˚C for the times indicated. Statistical analyses were performed using two-tailed student's *t*-test with a 95% confidence interval.

Western blot.

Overnight cultures of bacteria were adjusted to an OD_{600} = 1.0. Cells were collected from 1 ml of the adjusted culture, resuspended in 100 µl of 2x Laemmli buffer (109), and boiled for 10 minutes. Proteins present in 10 µl of each sample were loaded

onto 12.5% acrylamide gels and separated by polyacrylamide gel electrophoresis. Duplicate gels were run; one gel was stained with Coomassie to confirm that protein levels were similar in each sample and the other gel was used for Western blot. Proteins were transferred to PVDF membrane (Immobilon P, Millipore) at 100V for one hour at 4˚C. The membrane was blocked overnight at 4˚C in 5% milk dissolved in TBS-T (0.05% Tween, 100mM Tris (pH 7.5), 9% NaCl). The membrane was incubated for 45 min at room temperature with anti-FlaA antibody (27), as recently described (172). Following three quick rinses with TBS-T, the membrane was subjected to one 15-minute and three five-minute washes in TBS-T. The secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (Sigma), was diluted 1:10,000 in TBS-T and applied at room temperature with shaking for 45 minutes. After the wash steps were repeated, detection was performed using Amersham ECL Plus Western Blotting Detection System (GE Healthcare) following the protocol recommended by the manufacturer.

CBA/J mouse model of urinary tract infection.

Cochallenge and independent challenges of female CBA/J mice were carried out as previously described using a modification (95) of the Hagberg protocol (75). Briefly, single colonies were picked from a fresh plate and used to start overnight cultures in LB. On the day of inoculation, cultures were diluted with fresh LB to an $OD_{600} \approx 0.2$. For independent challenges, mice were inoculated transurethrally with 50 µl containing approximately 10^7 CFU. (Actual input was 1.41×10^7 CFU per mouse for mice infected with wild type and 2.36×10^7 CFU per mouse for mice infected with znuC::kan.) For cochallenge experiments, the wild-type and mutant cultures were mixed in a 1:1 ratio and mice were transurethrally inoculated with 50 µl of the mixture containing approximately

10^7 CFU. (Actual input for the cochallenge experiment contained, per mouse, 6.15 x 10^6 CFU wild type and 1.08 x 10^7 CFU znuC::kan.) After seven days, urine was collected, mice were euthanized, and bladders and kidneys were harvested asceptically and transferred to sterile tubes containing phosphate buffered saline. Tissues were homogenized using an Omni TH Homogenizer (Omni International) and plated using a spiral plater (Autoplate 4000, Spiral Biotech). For independent challenges, all samples were plated on LB agar. For cochallenges, all samples were plated on both LB agar and LB agar containing kanamycin. Since znuC::kan harbors a kanamycin resistance gene, colony counts from kanamycin plates represent mutant bacteria only; LB agar plates allow growth of both mutant and wild-type colonies. Statistical significance was assessed by using the Mann-Whitney test and Wilcoxin matched pairs test for independent and cochallenge experiments, respectively.

Statistical analysis.

All statistical analyses were performed using GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego, California USA www.graphpad.com).

Results

ZnuC plays a role in zinc acquisition in *P. mirabilis*.

In our previous study, ZnuB was identified on an immunoblot of membrane proteins using sera from mice experimentally infected with *P. mirabilis* (165). The antisera recognized ZnuB, indicating that this protein is expressed *in vivo* and thus may represent a therapeutic target. As homologous ZnuACB uptake systems have been shown to contribute to virulence in other bacterial pathogens, its role in *P. mirabilis* was investigated.

We hypothesized that expression of a divalent cation transporter would be regulated and could be induced under zinc restriction. To test this hypothesis, bacteria were cultured in the presence and absence of the zinc chelator TPEN and expression of *znuACB* were measured by qRT-PCR (Fig. 7). All three genes were upregulated >20-fold under zinc restriction. Ethanol, used to solubilize TPEN, had no effect on induction.

To demonstrate a growth requirement for zinc, a mutation was constructed in the putative transport system using a method that has been successful in generating *P. mirabilis* mutants (173). *znuC* was inactivated by the insertion of an intron containing a kanamycin resistance gene, resulting in strain znuC::kan. Insertion of the intron within *znuC* was confirmed by PCR.

There was no difference in the growth rate of znuC::kan compared to wild type when cultured independently in LB, minimal medium, or pooled human urine (Fig. 8). However, when the two strains were cultured together, and therefore put in direct competition for resources, znuC::kan was outcompeted by the wild-type strain in minimal medium (Fig. 9A). The difference in growth was overcome by the addition of 1 mM

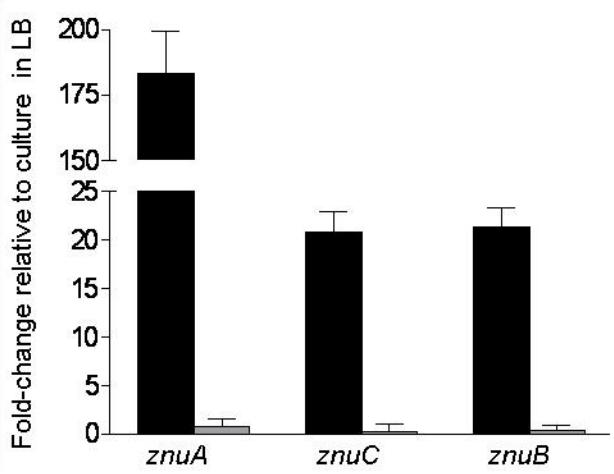
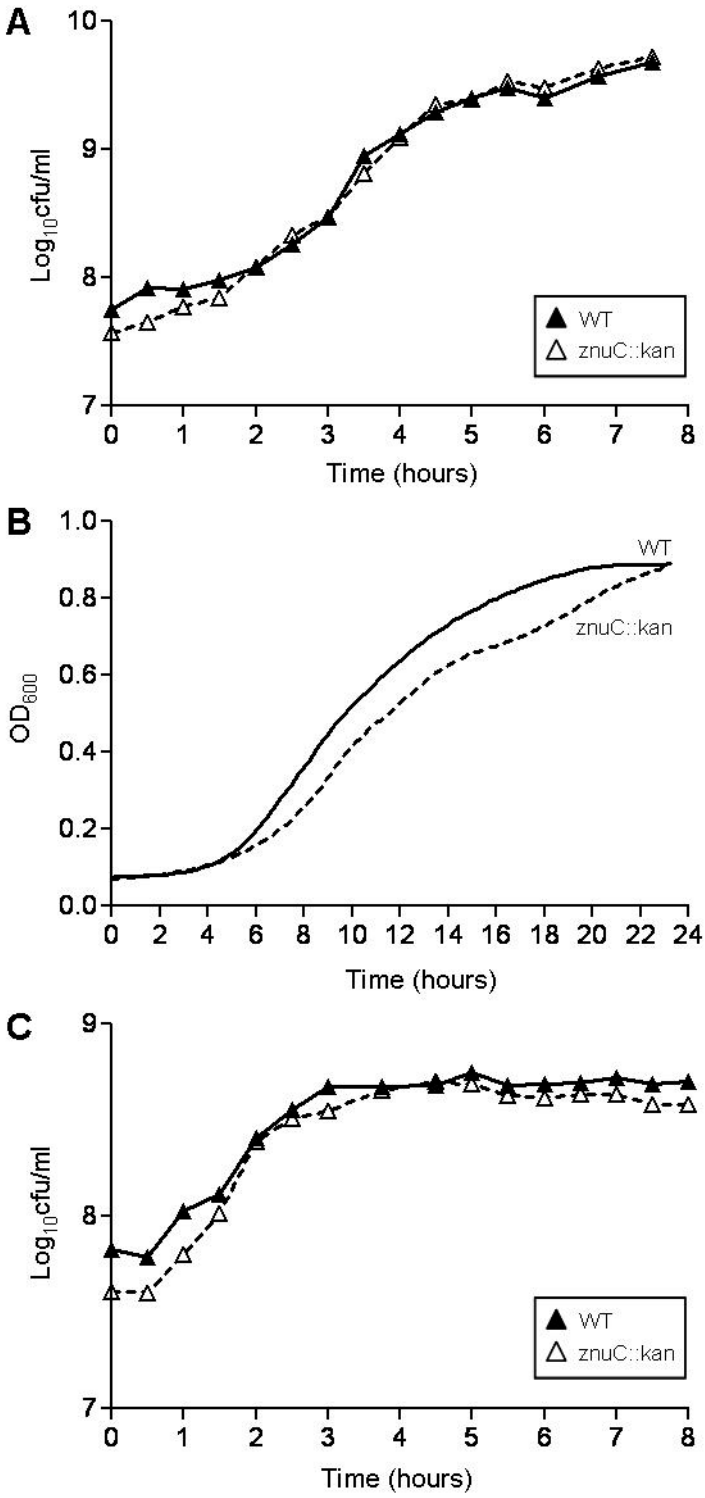


Figure 7. Expression of *znuACB* is induced by zinc limitation.
Wild type *P. mirabilis* was cultured in the absence or presence of TPEN. Gene expression was analyzed by qRT-PCR. Data were normalized to expression of *rpoA* and presented as fold-change compared to culture in LB without TPEN. Black bars, LB supplemented with 35 μM TPEN; gray bars, LB supplemented with solvent (ethanol).

Figure 8. Growth of znuC::kan compared with wild type.
(A) Wild type and znuC::kan were cultured independently in LB, and growth was monitored by plating samples at the indicated time points. Compare to OD_{600} data in Fig. 11. Filled symbols, wild type; open symbols, znuC::kan. (B) Wild type and znuC::kan were cultured independently in MM, and growth was observed by monitoring OD_{600}. Monitoring growth by OD_{600} resulted in an apparent lag in growth of znuC::kan compared with wild type; a similar pattern was observed when monitoring the growth of wild type and znuC::kan in LB, even though cfu data show no difference in growth between the two strains in LB. Solid line, wild type; hashed line, znuC::kan. (C) Wild type and znuC::kan were cultured independently in filtered, pooled human urine. Growth was monitored by plating samples at the indicated time points. Filled symbols, wild type; open symbols, znuC::kan.


A
10
9
8
7
Log10cfu/ml
0 1 2 3 4 5 6 7 8
Time (hours)
WT
znuC::kan
B
1.0
0.8
0.6
0.4
0.2
0.0
OD600
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)
WT
znuC::kan
C
9
8
7
Log10cfu/ml
0 1 2 3 4 5 6 7 8
Time (hours)
WT
znuC::kan

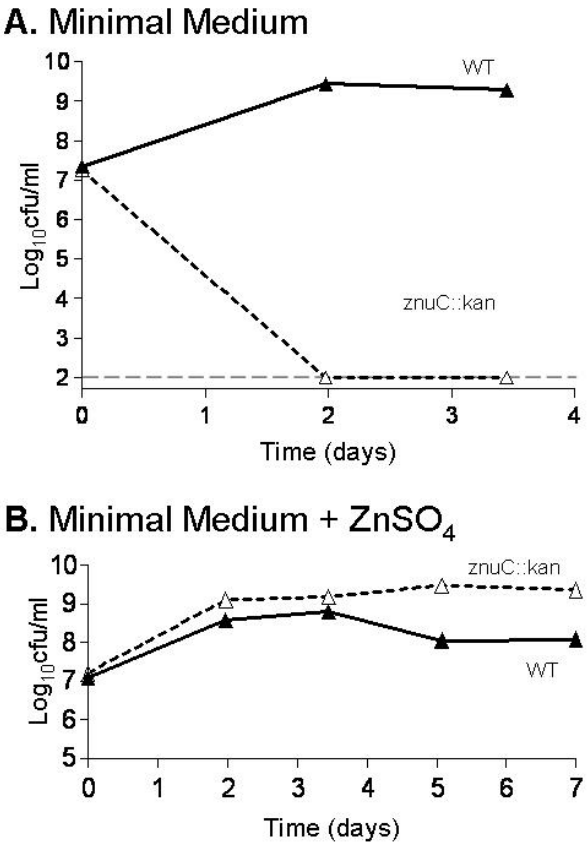


Figure 9. znuC::kan is outcompeted by wild type during coculture in minimal medium.
Cultures were inoculated with approximately equal amounts of wild type and znuC::kan. At indicated time points, samples were taken for plating and the culture was repassaged (1:100) into fresh medium. Filled symbols, wild type; open symbols, znuC::kan. (A) Minimal A Medium. Dashed gray line designates the limit of detection (100 CFU/ml). (B) Minimal A Medium supplemented with 1 mM $ZnSO_4$.

$ZnSO_4$ to the culture; under those conditions, znuC::kan was maintained in the culture, along with the wild-type strain, for the duration of the experiment (Fig. 9B). Maintenance of znuC::kan for the duration of the experiment could be achieved by the addition of as little as 5 μM $ZnSO_4$ (the lowest concentration tested) to the culture medium. The outcompetition phenotype was observed during coculture in minimal medium but not in LB (Fig. 10), suggesting the higher concentration of zinc in LB is sufficient to sustain growth of *P. mirabilis* without a functioning high-affinity uptake system, even when it is put in direct competition with the wild-type strain for this necessary nutrient. To confirm results from coculture experiments, the growth of znuC::kan was analyzed under zinc limitation achieved by the addition of TPEN to culture medium. Whereas little difference was observed in the growth rates of wild type and znuC::kan in LB, znuC::kan was more sensitive to addition of 40 μM TPEN than wild type (Fig. 11). Taken together, these data suggest that ZnuC functions in zinc acquisition in *P. mirabilis*, consistent with its function in other bacterial species.

Zur represses expression of *znuACB*.

Analogous to the *E. coli* system (170, 171), we hypothesized that *znuACB* would be repressed by the Zn-binding repressor Zur, which is distally encoded on the *P. mirabilis* chromosome (*zur*, PMI2743; *znuACB*, PMI1150-1152). To investigate the role of Zur in *P. mirabilis*, *zur* was insertionally inactivated by the method described above, resulting in the strain zur::kan. Again, this mutation was confirmed by PCR. As assessed by qRT-PCR, expression of *znuACB* was increased >10-fold in zur::kan (Fig. 12A). These results support the hypothesis that Zur acts as a repressor of *znuACB* in *P. mirabilis*.

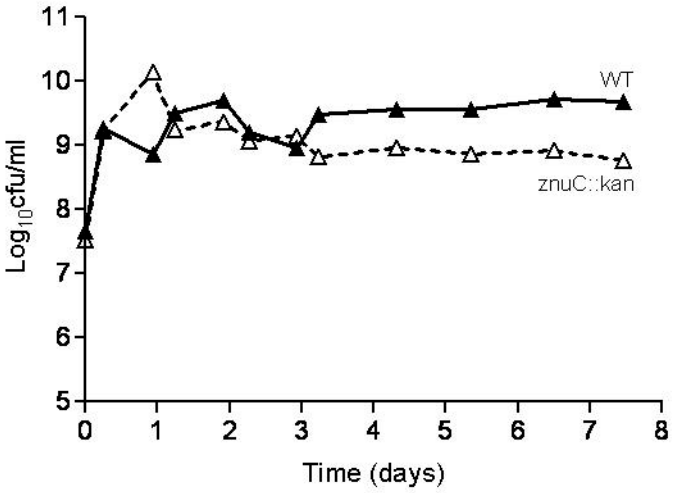


Figure 10. znuC::kan is maintained during a seven-day coculture with wild type in rich culture medium.
Culture was inoculated with approximately equal amounts of wild type and znuC::kan. At indicated time points, samples were taken for plating and the culture was repassaged (1:100) into fresh medium. Filled symbols, wild type; open symbols, znuC::kan.

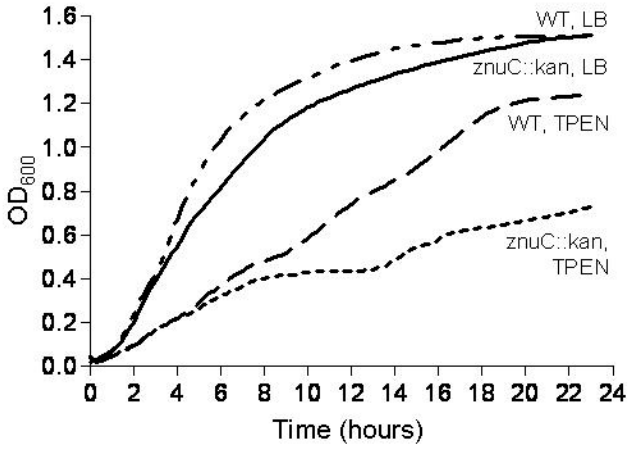
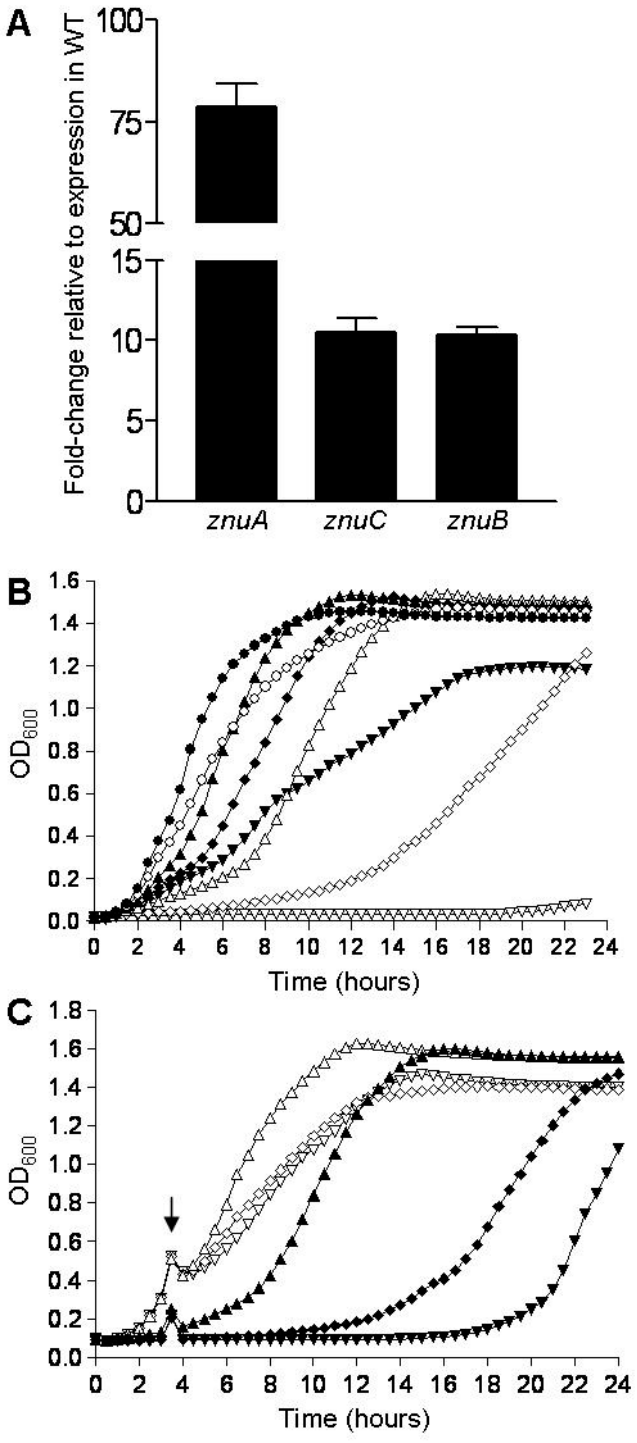


Figure 11. znuC::kan is more sensitive to TPEN than wild type.
Wild type and znuC::kan were cultured in either plain LB or LB supplemented with 40 μM TPEN. Growth was monitored by recording OD_{600} at 15 min intervals over a 24 h time period; for clarity, only 30 min time points are shown. Filled symbols, wild type; open symbols, znuC::kan; circles, growth in LB; diamonds, growth in LB supplemented with 40 μM TPEN.

Figure 12. zur::kan is more sensitive to zinc than the wild-type strain.
(A) Wild type and zur::kan were cultured in LB. Gene expression was analyzed by qRT-PCR. Data were normalized to expression of *rpoA* and presented as fold-change in zur::kan as compared to expression in wild type. (B) Wild type and zur::kan were cultured in plain LB as well as LB supplemented with 500 μM, 750 μM, or 1 mM $ZnSO_4$. Filled symbols, wild type; open symbols, zur::kan; circles, LB; triangles, LB plus 500 μM $ZnSO_4$; diamonds, LB plus 750 μM $ZnSO_4$; inverted triangles, LB plus 1 mM $ZnSO_4$. (C) zur::kan was cultured in LB supplemented with zinc either at the start of the experiment or after $OD_{600} > 0.3$. Filled symbols, cultures with zinc added at beginning; open symbols, cultures spiked with zinc during growth; triangles, LB plus 500 μM $ZnSO_4$; diamonds, LB plus 750 μM $ZnSO_4$; inverted triangles, LB plus 1 mM $ZnSO_4$. Arrow depicts when zinc was added to (open symbol) cultures.


A
Fold-change relative to expression in WT
100
75
50
15
10
5
0
znuA
znuC
znuB
B
OD600
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)
C
OD600
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)

We hypothesized that since zur::kan may overproduce the ZnuACB high-affinity uptake system, this strain may be hypersensitive to zinc toxicity. To test this hypothesis, zur::kan and the wild-type strain were cultured independently in LB supplemented with 500 μM, 750 μM, and 1 mM $ZnSO_4$. As predicted, zur::kan was more sensitive to excess zinc than the wild-type strain (Fig. 12B). Interestingly, when cultured with zinc at concentrations of 500 μM $ZnSO_4$ or above, zur::kan displayed an extended lag phase until the culture reached a specific density ($OD_{600} \approx 0.3$). Once the culture reached this density, log phase growth commenced. To rule out the possibility of a secondary or suppressor mutation leading to the ability of zur::kan to grow after extended lag phase, cultures were passaged again into fresh medium supplemented with the same concentration of $ZnSO_4$. If a suppressor mutation were responsible for the outgrowth, we would expect no extended lag phase to occur. However, an identical pattern of growth was observed (that is, an extended lag phase until OD_{600} exceeded 0.3); therefore, we concluded that zinc toxicity, rather than a suppressor mutation, was the cause of this extended lag period during growth.

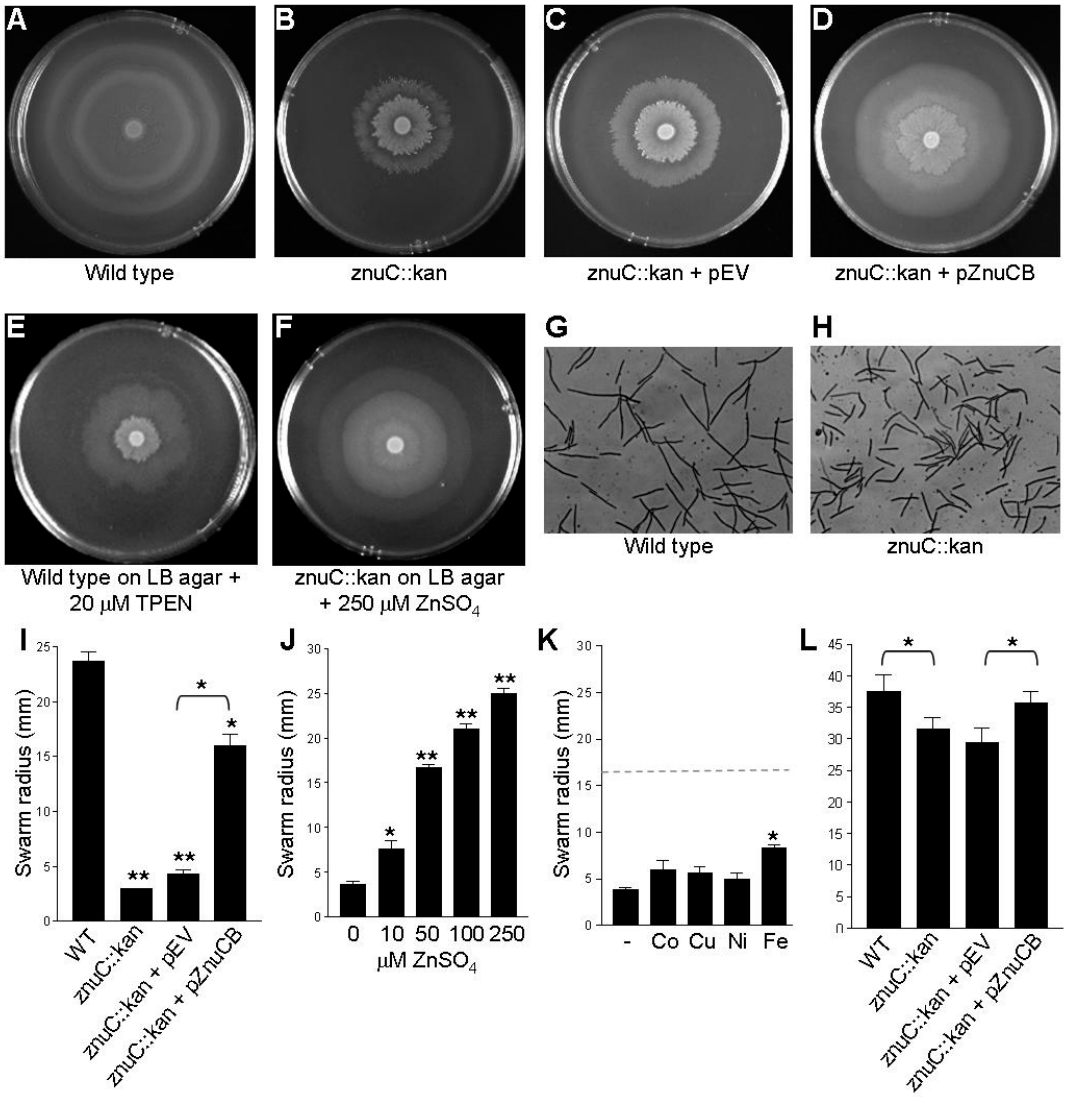
We further hypothesized that if zur::kan cells were able to divide (albeit slowly) in the presence of zinc, the culture would ultimately reach a density such that the amount of zinc present in the culture could be evenly shared by a greater number of cells and therefore become less of a burden on individual cells, at which point they would be able to overcome the concentration-dependent growth restriction. We reasoned that if the zur::kan culture was allowed to reach this critical density prior to the addition of zinc, zinc toxicity should not cause the extended lag phase observed in cultures with zinc present from the point of inoculation. To test this hypothesis, zur::kan was used to

inoculate two types of cultures: either LB supplemented with $ZnSO_4$ at the inception of the experiment or, alternately, LB supplemented with the same concentration of $ZnSO_4$ after the culture reached $OD_{600} > 0.3$. Indeed, when $ZnSO_4$ was added to cultures of zur::kan that had already passed $OD_{600} = 0.3$, the culture was able to grow at a much faster rate than its counterpart with $ZnSO_4$ added at the beginning, even though (post-$ZnSO_4$ addition to the cultures depicted by open symbols) the two cultures contained the same concentration of zinc (Fig. 12C).

Zinc uptake contributes to swarming and swimming motility.

It has been previously reported that PpaA, a P-type ATPase homologous to ZntA that functions in zinc efflux (187), plays a role in swarming in *P. mirabilis*. A strain with a transposon insertion in the gene *ppaA* showed a delayed swarming response compared with wild type (110). Since disrupted zinc efflux affects swarming motility, we hypothesized that zinc uptake would also affect motility. To test this assertion, the motility of znuC::kan was investigated. Indeed, znuC::kan displayed a modest but statistically significant reduction in swimming motility compared to the wild-type strain (P=0.025, Fig. 13L). Swarming motility was also affected; znuC::kan displayed significantly reduced swarming motility compared to the wild-type strain (P=0.0082; compare Fig. 13A and 13B, see Fig. 13I). In addition, the appearance of the swarming colony of znuC::kan was grossly different than the wild-type swarming colony. Wild-type swarming colonies displayed a smooth appearance; in contrast, znuC::kan swarming colonies had a fractured appearance, with jagged projections advancing at the swarm front rather than the smooth colony edge observed with wild type. Upon close inspection, gaps were present in the advancing colony edge containing these projections;

Figure 13. znuC::kan swarms significantly less than wild type.
(A-F) Swarm agar plates were spotted with 5 μl of log phase culture. After inoculation spot dried, plates were incubated at 30˚C. Plates shown are representative examples measured 24 hours post-inoculation. (A) Wild type (B) znuC::kan (C) znuC::kan + pEV (D) znuC::kan + pZnuCB (E) WT inoculated on LB containing 20 μM TPEN (F) znuC::kan inoculated on LB supplemented with 250 μM $ZnSO_4$ (G-H) Gram stains of wild type and znuC::kan, respectively, taken from the leading edge of a colony on swarm agar. Images shown were taken at the same magnification. (I) Swarm radii of wild type, znuC::kan, znuC::kan + pEV, and znuC::kan + pZnuCB measured 16 hours post-inoculation. Data are from three independent experiments and were analyzed by paired t-test. Asterisks above bars denote significance compared to WT. *, $P<0.05$; **, $P<0.01$ (J) Cultures of znuC::kan were spotted on swarming agar supplemented with $ZnSO_4$. Swarm radii were measured 20 hours post-inoculation. Data are from a representative experiment and were analyzed by unpaired t-test. Asterisks denote significance compared to plates with no zinc added. *, $P<0.05$; **, $P<0.001$ (K) Cultures of znuC::kan were spotted on swarming agar supplemented with 50 μM $CuSO_4$, $CoCl_2$, $NiSO_4$, or $FeCl_2$. Swarm radii were measured 20 hours post-inoculation. Dotted line represents mean swarm radius of znuC::kan spotted on agar containing 50 μM $ZnSO_4$ for comparison (refer to data shown in J). Data are from a representative experiment and were analyzed by unpaired t-test. Asterisks denote significance compared to plain swarming agar plates.*, $P<0.005$ (L) Swimming radii of wild type, znuC::kan, znuC::kan + pEV, and znuC::kan + pZnuCB measured 16 hours post-inoculation. Data are from three independent experiments and were analyzed by paired t-test. *, $P<0.05$. Radii of wild type and znuC::kan + pZnuCB were not significantly different ($P = 0.0508$).


A
B
C
D
Wild type
znuC::kan
znuC::kan + pEV
znuC::kan + pZnuCB
E
F
G
H
Wild type on LB agar +
20 μM TPEN
znuC::kan on LB agar
+ 250 μM ZnSO4
Wild type
znuC::kan
I
J
K
L
Swarm radius (mm)
WT
znuC::kan
znuC::kan + pEV
znuC::kan + pZnuCB
0 10 50 100 250
μM ZnSO4
- Co Cu Ni Fe

the swarming colony did not cover the entire agar surface in the same way that the wild-type swarming colony did. Interestingly, the wild-type strain displayed a similar fractured pattern and reduced swarming radius when inoculated onto swarming agar supplemented with TPEN (Fig. 13E). This result suggests that the defect observed in znuC::kan is a result of zinc limitation.

The swarming defects of znuC::kan were complemented by adding *znuCB* back *in trans* on a plasmid (P=0.0448 compared to an empty vector control, compare Fig. 13C and 13D; see Fig. 13I). The complemented strain also regained swimming motility (P=0.0382 compared to an empty vector control, Fig. 13L). It should be noted that only partial complementation was achieved in swarming motility; although the complemented strain swarmed less than wild type, this difference was not significant (P=0.0508, Fig. 13I). The swarming defect observed with znuC::kan could also be complemented by the addition of $ZnSO_4$ to the swarming agar prior to inoculation (compare Fig. 13B and 13F, see Fig. 13J). Under both complementation conditions, the swarming colony morphology returned to a noticeably smoother appearance. Swarming by znuC::kan could not be restored by the addition of Co^{2+}, Cu^{2+}, or Ni^{2+} to the swarm agar (Fig. 13K). When Fe^{2+} was added to the medium, znuC::kan swarmed more than on plain swarm agar, however, the radius of swarming motility did not reach the level achieved by the addition of $ZnSO_4$ and the colony retained the fractured morphology (Fig. 13K).

For swarming motility to occur, *P. mirabilis* must first differentiate into the elongated swarmer cell morphology (185). Gram staining of bacteria taken from the edge of the swarming front of both znuC::kan and the wild-type strain revealed that cells from both plates were elongated (Fig. 13G and 13H); therefore, the swarming defect of

znuC::kan does not appear to be a result of a block in the elongation process. One of the hallmark characteristics of a swarm cell is the large number of flagella synthesized by this morphotype. To determine if znuC::kan is capable of producing flagella at levels similar to wild type, flagellin transcript and protein were assessed in znuC::kan by qRT-PCR and Western blot, respectively. Transcription of *flaA*, which encodes flagellin, was reduced approximately 15-fold in znuC::kan compared to wild type (Fig. 14A), which resulted in production of lower levels of FlaA protein (Fig. 14B). FlaA protein levels were restored in the complemented strain. Transcription of *flaA* was also slightly reduced in wild type cultured in LB containing TPEN (Fig. 14A).

ZnuC contributes to, but is not required for, virulence of *P. mirabilis* in the urinary tract.

Because the ZnuACB system has been shown to contribute to virulence of several pathogens, its role in pathogenesis was investigated in the murine model of ascending urinary tract infection that has been well established for virulence factor assessment in *P. mirabilis* (5, 38, 84, 98, 256). CBA/J mice were transurethrally inoculated with approximately 1 x 10^7 CFU of either wild type or znuC::kan. After seven days, mice were sacrificed and bacteria present in urine, bladders, and kidneys were quantified. We found that znuC::kan colonized mice in numbers similar to the wild-type strain (Fig. 15A).

To assess the contribution of the ZnuACB transport system to the fitness of *P. mirabilis* in the murine urinary tract, we conducted a cochallenge experiment in which znuC::kan was put in direct competition with wild type during infection. Ten CBA/J mice were infected transurethrally with a 1:1 mixture of wild type and znuC::kan. Following a seven day infection, znuC::kan was outcompeted by wild type more than 10,000-fold in the urine (P<0.005) and was unrecoverable from the bladder

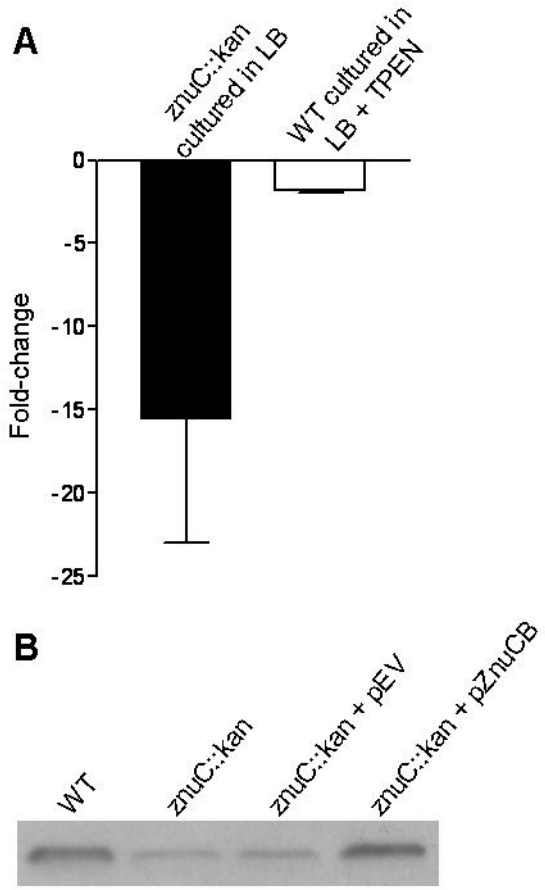


Figure 14. Flagellin transcript and protein levels are decreased in znuC::kan. (A) Analysis of *flaA* transcript by qRT-PCR. Left bar shows expression of *flaA* in znuC::kan cultured in LB relative to its expression in wild type cultured in LB. Right bar shows *flaA* expression in wild type cultured in LB supplemented with 35 μM TPEN relative to expression in wild type cultured in unsupplemented LB. (B) Western blot with anti-FlaA antibody.

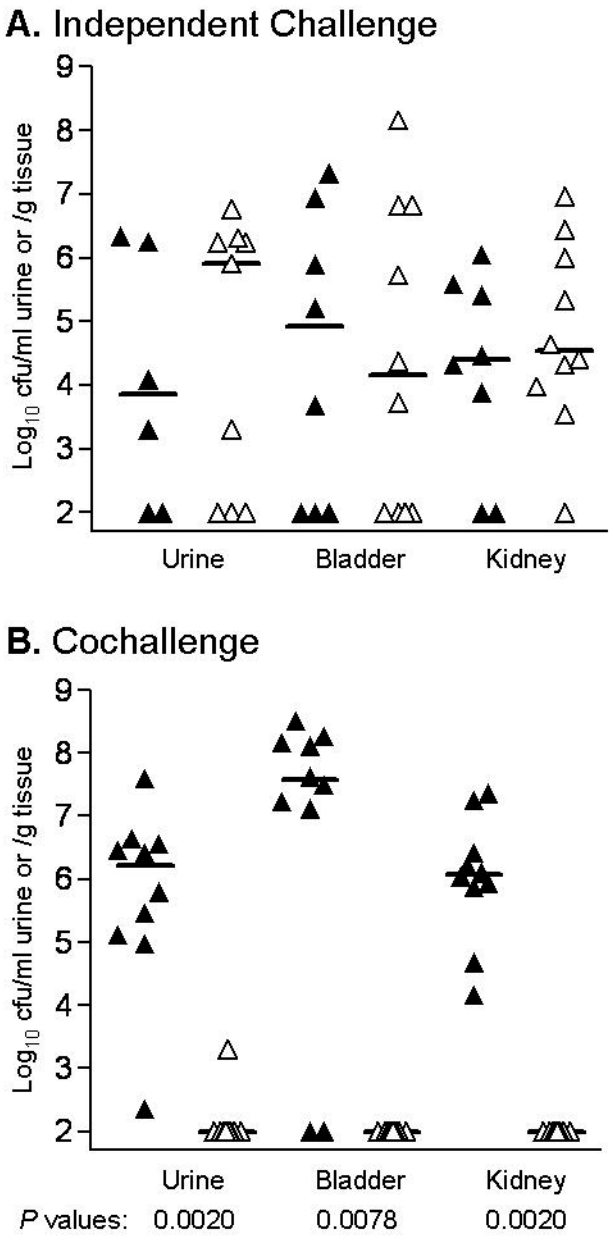


Figure 15. ZnuC contributes to fitness of *P. mirabilis* in the urinary tract, but is not required for infection.
Mice were infected transurethrally and after seven days, urine, bladders, and kidneys were quantitatively cultured. Each symbol represents data from an individual mouse – solid symbols, mice infected with wild type; open symbols, mice infected with znuC::kan. Bars represent the median. Limit of detection is 100 CFU/ml of urine or gram of tissue. (A) Independent challenge. Mice were infected with approximately 10^7 CFU of either wild type or znuC::kan. (B) Cochallenge. Mice were infected transurethrally with approximately 10^7 CFU of a 1:1 mix of wild type and znuC::kan.

and kidneys ($P<0.01$ and $P<0.005$, respectively; Fig. 15B). Taken together, these data reveal that while ZnuC is not required for *P. mirabilis* to colonize the host, it offers a competitive advantage during urinary tract infection.

Discussion

This is the first study to demonstrate the importance of zinc acquisition by *P. mirabilis* in the urinary tract during infection. We have shown that ZnuC, a component of a putative zinc ABC transporter that imports this critical ion, contributes to the fitness of *P. mirabilis* in the mouse model of ascending urinary tract infection. Based on growth differences in independent cultures supplemented with TPEN and coculture in minimal medium, ZnuC (presumably as a component of ZnuACB) appears to function as a zinc transport system in *P. mirabilis*. In addition, *znuA* and *znuCB* appear to be repressed by Zur since expression of all three genes was increased in a *zur* mutant compared with wild type. The over-expression of this high-affinity zinc uptake system rendered *P. mirabilis* hypersensitive to $ZnSO_4$, demonstrating the importance of the ability to regulate zinc homeostasis. We also discovered that zinc acquisition is required for normal swimming and swarming motility. The statistically significant reduction in swarming of znuC::kan was complemented by expressing ZnuCB *in trans* or by adding $ZnSO_4$ to swarming agar prior to inoculation, suggesting the defect resulted specifically from low levels of intracellular zinc in the mutant.

Results from the wild type and znuC::kan *in vivo* cochallenge infection suggest that there is a limited supply of zinc in the urinary tract. When znuC::kan was put in direct competition for this nutrient with wild type (which retains a functioning high affinity transport system), the mutant failed to thrive and was unrecoverable from infected animal tissue. However, during independent challenge, znuC::kan was able to utilize the zinc present and colonized to levels similar to wild type. This result suggests that in the absence of competition, znuC::kan has sufficient mechanisms for acquiring

this critical ion, perhaps through the use of lower-affinity transport systems. A similar phenotype was observed during experimental urinary tract infection with a derivative of UPEC strain CFT073 lacking a function ZnuACB transport system; the mutant was outcompeted in bladders and kidneys during cochallege (196). However, in contrast to *P. mirabilis*, UPEC CFT073 required functional ZnuACB to reach optimal infection levels in the kidney during independent challenge; although there was no difference in bladder colonization, CFT073 Δ*znuA* was recovered from infected kidneys in significantly fewer numbers than controls (196). The reason for this disparity is not immediately clear, but one possible explanation could be a difference in low affinity zinc uptake between the two species. In any case, taken together, data from two different uropathogens align well and support the hypothesis that zinc is a critical nutrient during infection of the urinary tract.

The ability of znuC::kan to colonize the urinary tract during independent challenge suggests that this mutant is capable of using other means to bring zinc into the cell. ZupT, previously thought to be a zinc-specific low affinity transporter (71), is in fact a transporter with broad metal specificity (70). However, based on the genome sequence and annotation of *P. mirabilis* HI4320 (174), this strain appears to lack a ZupT homolog. Other transporters known to import zinc include the inorganic phosphate transporter PitA (24) and the citrate transporter CitM (107). *P. mirabilis* appears to encode proteins homologous to both PitA and CitM, but the contribution of either of these proteins to zinc acquisition in this species is currently unknown.

It should be noted that we cannot rule out the possibility that the colonization defect of znuC::kan observed during cochallenge could be due, at least in part, to the

reduction in motility observed with this strain. We observed only a modest defect in swimming motility in znuC::kan, while swarming motility was greatly affected; znuC::kan produced less flagellin than the wild-type strain. Flagella-mediated motility has been implicated as a virulence factor in *P. mirabilis* (143). A FlaA$^-$ strain of *P. mirabilis* did not differentiate into swarmer cells (25), but we observed elongated forms of znuC::kan and know this strain is capable of at least this step in the differentiation process. The contribution of swarmer cells and swarming motility (as opposed to production of flagella and swimming motility) to virulence is unclear. The expression of virulence genes has been linked to swarmer cell differentiation (10). However, the presence of swarmer cells *in vivo* is debated (7, 92). In addition, some studies conclude that motile, nonswarming strains have a reduced capacity to cause infection (7) while others found that non-flagellated strains (which are therefore incapable of swarming) are still able to cause infection (116, 260).

In *P. mirabilis*, a strain with an interrupted copy of the ZntA zinc efflux protein homolog PpaA also displayed reduced swarming (110). This phenotype is interesting because we note that proteins with seemingly opposing functions (zinc uptake and efflux for ZnuC and PpaA, respectively), when interrupted, both affect swarming motility. Perhaps zinc homeostasis (as opposed to exclusively uptake or efflux) plays a role in motility. Indeed, in *E. coli*, transcription of genes involved in flagellar biosynthesis are down-regulated in response to treatment with TPEN (211) while the transcription of some motility-related genes was increased in *E. coli* treated with zinc (114).

As described above, swarming motility requires a large number of flagella per bacterium. The production of flagella occurs via a regulatory cascade mediated by the

'master regulator' FlhDC (reviewed in (42, 217)). *flhDC* comprise Class I genes of the flagellar regulon and activate expression of the Class II genes, which include proteins necessary to form the basal body and hook of the flagella as well as FliA. FliA is the transcription factor required to synthesize proteins present in Class III, including FlaA. FlhC, part of the FlhDC complex that induces the flagellar cascade, was recently determined to have a zinc-binding site (240). It is unknown what, if any, role zinc-binding has on FlhDC function. However, if FlhDC requires zinc for its function, the levels of intracellular zinc in znuC::kan may not be sufficient to fulfill the normal requirement needed for optimal FlhDC activity. We hypothesize that this potential reduction in FlhDC activity could represent one explanation for the reduced motility of znuC::kan; reduced FlhDC activity would result in reduced transcription of flagellar genes, including *flaA* (as we observed), and therefore reduced motility. This hypothesis could help explain the differences in the effect mutation of ZnuC had on swimming and swarming motility; swarming motility, which has a greater requirement for flagellin, was affected to a greater degree. Furthermore, this phenomenon may not be specific to *P. mirabilis*, since motility was also reduced in UPEC mutants lacking functional zinc uptake systems (73, 196). To elucidate the mechanism of interplay between zinc homeostasis and motility, further studies are required.

The ability of *P. mirabilis* to employ swarming motility, although described for more than a century (250), is still not well understood and remains a target of active research. Deciphering this behavior would not only benefit our understanding of a facet of the lifecycle of this pathogenic bacterium, but could potentially lead to important therapeutic targets since *P. mirabilis* may gain access to the body by swarming over the

surface of catheters. We have shown, for the first time, that the ability to acquire zinc via a high-affinity transport system is another piece of this proverbial puzzle.

Appendix (Unpublished Data)

To determine if Zur contributes to virulence, zur::kan was assessed in the murine model of ascending UTI. CBA/J mice were transurethrally inoculated with approximately 1 x 10^7 CFU of either wild type or znuC::kan. After seven days, mice were sacrificed and colonization was determined by quantifying bacteria present in urine, bladders, and kidneys. There was no difference in the level of colonization achieved by zur::kan and the wild-type strain; therefore, Zur is not required for infection of the urinary tract by *P. mirabilis* (Fig. 16A).

A cochallenge experiment in which zur::kan was put in direct competition with wild type during infection was also conducted. CBA/J mice were infected transurethrally with a 1:1 mixture of wild type and zur::kan. Following a seven day infection, zur::kan was recovered from the urine, bladders, and kidneys of infected mice at levels statistically significantly lower than the wild-type strain (Fig. 16B).

Typically, upon determination that a strain is outcompeted by wild type *in vivo*, it is assumed that the gene product is a fitness factor and offers a competitive advantage during infection. However, it is difficult to interpret the zur::kan results in that way. As an *in vitro* control for the weeklong cochallenge, a weeklong coculture experiment was performed. Overnight cultures of wild type and zur::kan were diluted to the same optical density and used to inoculate one fresh LB culture. This mixed culture was followed for seven days. Periodically, the culture was repassaged into fresh LB (typically, at the beginning and end of each day); at the time of repassage, a sample was plated to determine the levels of wild type and zur::kan present in the culture. During a weeklong

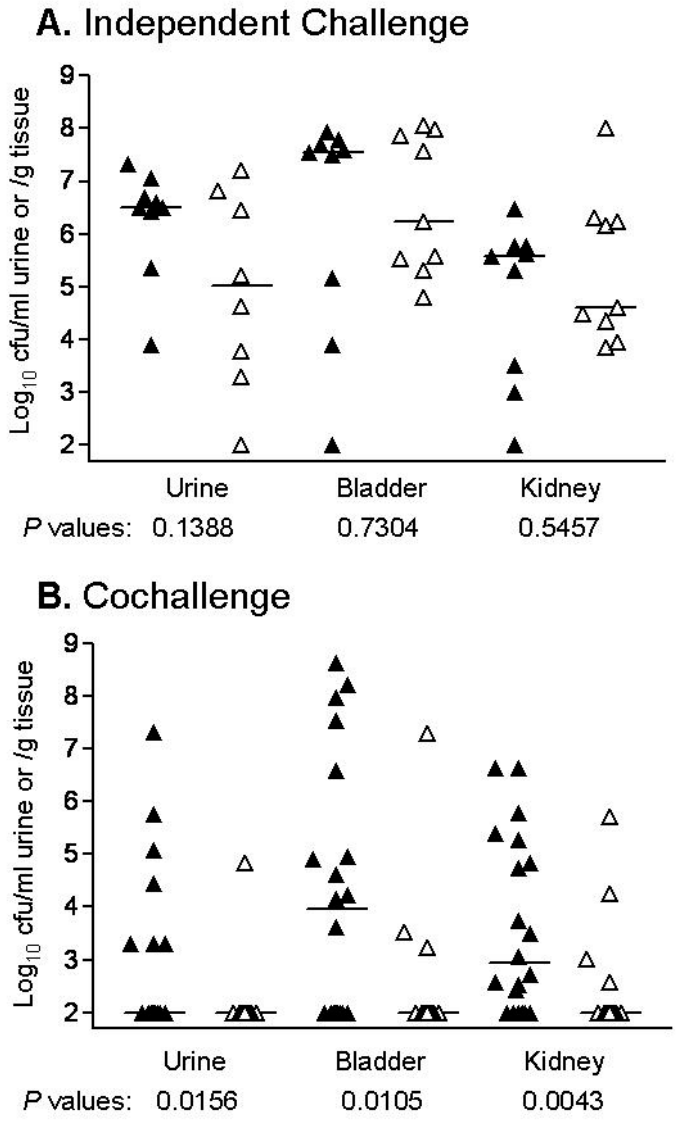


Figure 16. Zur is not required for infection.
Mice were infected transurethrally and after seven days, urine, bladders, and kidneys were quantitatively cultured. Each symbol represents data from an individual mouse – solid symbols, mice infected with wild type; open symbols, mice infected with zur::kan. Bars represent the median. Limit of detection is 100 CFU/ml of urine or gram of tissue. (A) Independent challenge. Mice were infected with approximately 10^7 CFU of either wild type or zur::kan. Actual input values (per mouse) were 2.26 x 10^7 CFU and 2.5 x 10^7 CFU for mice infected with wild type and zur::kan, respectively. (B) Cochallenge. Mice were infected transurethrally with approximately 10^7 CFU of a 1:1 mix of wild type and zur::kan. Results compiled from two independent experiments are shown. In one experiment, the actual input inoculum (1.69 x 10^7 CFU/mouse) contained 6.21 x 10^6 CFU wild type and 1.07 x 10^7 CFU zur::kan. In the second experiment, the actual input inoculum (1.53 x 10^7 CFU/mouse) contained 5.21 x 10^6 CFU wild type and 1.01 x 10^7 CFU zur::kan.

independent culture, zur::kan was maintained at high levels throughout the course of the experiment (Fig. 17A). In contrast, during coculture with wild type, zur::kan was slowly lost from the culture (Fig. 17B). Therefore, it appears that zur::kan has a slight growth defect *in vitro*. This result complicates interpretation of the cochallenge data since the outcompetition observed *in vivo* could merely be a result of the zur::kan growth defect. Additionally, interpretation of zur::kan results is difficult because, at this time, it is unclear whether Zur regulates the expression of genes other than *znuACB* in *P. mirabilis*.

The work presented in this chapter has been submitted for publication under the title "Zinc Uptake Contributes to Motility and Provides a Competitive Advantage to *Proteus mirabilis* during Experimental Urinary Tract Infection" (Nielubowicz, G. R., Sara N. Smith, and Harry L. T. Mobley); the manuscript has been reviewed and is currently under revision. Sara N. Smith assisted with animal studies; all other experiments were preformed by G. R. N. Manuscript was prepared by G. R. N.

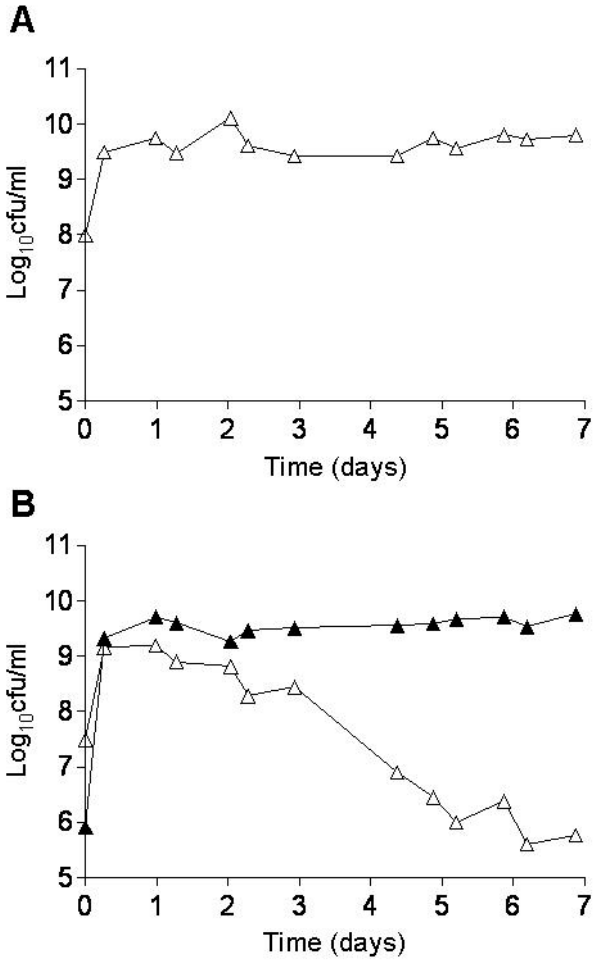


Figure 17. zur::kan is outcompeted during in vitro coculture with wild type.
Data points represent times at which the culture was repassaged 1:100 into fresh LB. (A) Weeklong zur::kan culture in LB (B) Weeklong coculture of wild type and zur::kan in LB. Closed symbols, wild type; open symbols, zur::kan.

CHAPTER IV. DISCUSSION

The main goal of this study was to identify potential antigens for inclusion in a multivalent vaccine against a major cause of complicated UTI, *P. mirabilis*. An immunoproteomic approach was used and resulted in the identification of 23 outer membrane antigens expressed by *P. mirabilis in vivo* during urinary tract infection. It also resulted in the characterization of three proteins that contribute to virulence: the TonB-dependent receptors PMI0842 and PMI2596 and the zinc transport protein ZnuC.

Previous work by others has resulted in the development of multiple vaccines that significantly reduce colonization in the mouse model. However, in each study, some mice that were vaccinated still developed an infection (*i.e.*, not all mice were protected) (118, 120, 177, 200, 201). Therefore, none of the potential vaccine antigens offer complete protection. It was our goal to identify additional antigens for inclusion in a multivalent vaccine; targeting more than one antigen simultaneously may be the most effective way to prevent infection.

Arguably, the most successful vaccines to date have targeted MR/P fimbrial proteins, namely MrpA and MrpH (118, 120, 177, 200, 201). However, as discussed in Chapter I, the *mrp* operon is capable of undergoing phase variation (255). It is currently unclear if mice susceptible to infection following vaccination with MrpA or MrpH were colonized by bacteria not expressing MR/P fimbriae. The down-regulation of the target antigen by colonizing bacteria could be one explanation for why these mice were not protected by the vaccine.

Additional vaccines have targeted UcaA and PmfA, the major structural subunits of two other fimbriae (UCA and PMF) encoded by *P. mirabilis* (177, 201). Mucosal immunization with UcaA protected mice from bladder and kidney colonization, while mucosal immunization with PmfA offered protection in the kidneys only (201). As is the case with MR/P fimbriae, PMF contribute to virulence (at least in the mouse model) (138, 261); the role of UCA during infection has not been assessed. It may be difficult to directly compare the results of these various vaccinations; experiments performed by different groups used different strains of *P. mirabilis* and different adjuvants, but it appears that intranasal immunization with MrpH reduced colonization compared to controls more effectively than intranasal immunization with MrpA (120, 201). This observation raises the question of whether the protection observed with UcaA and PmfA could be improved by targeting the tip adhesins, rather than major structural subunits, of these fimbriae. Given the number of putative fimbrial operons identified in the *P. mirabilis* genome (174), and the encouraging results achieved so far by vaccination with fimbrial proteins, it is possible that other fimbrial proteins may also serve as protective antigens. However, most of these other fimbriae have not yet been characterized experimentally; it is not known if they are expressed *in vivo* or contribute in any way to pathogenesis. Future work investigating these possibilities could also potentially contribute to vaccine design.

Interestingly, no fimbrial proteins were identified in this study (Chapter II). We found this result surprising, especially considering that MR/P fimbriae are known to elicit a strong immune response during infection (20, 94). There are a few possible reasons why no fimbrial proteins were detected: either the culture conditions used did not favor

the expression of fimbriae, or, alternately, fimbriae were sheared from the bacterial surface during sample preparation. Of course, a third possibility is that fimbriae were present, but not recognized by sera. We consider this third possibility unlikely, especially given that the sera used in our screen was previously shown to react with MR/P fimbrial proteins (94). Fimbrial preparations should be prepared and probed with the sera from infected mice to determine what, if any, fimbriae are recognized. Again, based on previous results, we predict that MR/P fimbriae will react with the sera, but it would be interesting to determine if other fimbrial proteins are also recognized.

The immunoproteomic screen detailed in Chapter II resulted in the identification of 23 outer membrane proteins that are potential vaccine candidates (165). We were encouraged that outer membrane proteins could serve as protective antigens based on previous work using outer membrane preparations for vaccination (142). Unfortunately, vaccination with PMI0842 did not result in protection from transurethral challenge (Chapter II, Appendix). However, proof of principle was offered by results of vaccination with Pta, another antigen identified in our screen. Mice that received an intranasal immunization with Pta* (an active site mutant lacking serine protease activity) had significantly lower colonization levels in their kidneys and spleens than control mice; bladder colonization was not affected by immunization with Pta* (4). Immunization with Pta* resulted in significant increases in the production of IgA in the urine and IgG in the serum directed against Pta* (as compared to pre-immunization samples) (4). A significant inverse correlation between serum IgG production and kidney colonization level was observed; that is, mice with higher levels of antibodies had reduced

colonization levels and mice that were colonized at higher levels had lower levels of antibodies (4).

Importantly, although protection was significant compared to control groups, some mice that received the Pta* vaccine were still colonized (4). These results mirror those from other *P. mirabilis* vaccine studies (118, 120, 177, 200, 201). Taken together, results from all *P. mirabilis* vaccination studies suggest that vaccination with one antigen may not be sufficient to provide complete protection. For this reason, the pursuit of a multivalent vaccine that combines multiple antigens is ongoing. The antigens identified in our immunoproteomic screen represent a pool of potential targets for inclusion in this multivalent vaccine; however, the level of protection offered by the vast majority of these candidate antigens has not yet been assessed experimentally.

The first logical step in the development of this multivalent vaccine would be to combine the two most successful antigen preparations to date: Pta* and the N-terminal region of MrpH. As discussed above, when administered individually, both antigens offered significant protection but still resulted in infection of some mice (4, 118, 120). The use of these antigens together should be fairly straightforward, for three reasons: the antigens have already been cloned into expression vectors; purification schemes have been designed and successfully implemented; an animal model (including the previously determined ideal mode of vaccine delivery) is readily available and currently in use in our laboratory. We hypothesize that mice immunized with a combination of Pta* and the N-terminal domain of MrpH will be significantly protected from transurethral infection; furthermore, we predict that mice receiving the mixed vaccine will be protected better than groups of mice only receiving one antigen.

Recent vaccine work on another uropathogen, UPEC, has also been encouraging (12); results from that study further validate that a mucosal vaccine targeting antigenic outer membrane proteins can be effective against a uropathogen. The identification of antigens used in that study resulted from a very specific approach, termed the "omics" approach, used to identify potentially protective antigens (214). These antigens all fit the criteria of *PASivE* proteins (Pathogen-specific, Antigenic, Surface-exposed, and *in vivo* Expressed) that are hypothesized to represent ideal vaccine candidates. The identification of *PASivE* proteins was made possible by the completion of many different screens, including comparative genomic hybridizations, microarray analyses, and an immunoproteomic screen (similar to the one described for *P. mirabilis* in Chapter II) (214). Target antigens were chosen because they were identified from multiple screens.

The recent improvements in resources available for *P. mirabilis* should aid in vaccine development efforts, which can be modeled after the strategy successfully employed for the UPEC vaccine. The resolution of the genome sequence of *P. mirabilis* HI4320 is a critical factor that will allow many of the screens performed with UPEC to be utilized for *P. mirabilis*. First, it allowed for design of the *P. mirabilis* microarray. This microarray has already been validated (S. Himpsl, M. Pearson, and H. L. T. Mobley, unpublished data) and is currently being used to investigate the gene expression profile of *P. mirabilis* during experimental UTI (M. Pearson and H. L. T. Mobley, unpublished data). These results will be extremely valuable for consideration during vaccine design, as it is crucial to target antigens that are expressed *in vivo*. Second, it has already afforded us the ability to easily identify outer membrane antigens by mass spectrometry.

One aspect of vaccine design that will be different for *P. mirabilis* than *E. coli* is the identification of genes common to pathogenic strains. UPEC are a specific subset of *E. coli*, but it is thought that all strains of *P. mirabilis* can cause UTI regardless of isolate origin (176, 216). In addition, many virulence genes appear to be conserved among *P. mirabilis* isolates. However, recent data suggest that there may be previously unappreciated differences between strains. For example, the *pta* gene was present in all isolates, but expression of *pta* was only detected in UTI (not fecal) isolates (4). It is currently unclear if this pattern extends to other virulence factors as well. The fact that all *P. mirabilis* isolates appear to be capable of causing UTI adds another layer of complication to vaccine design. *E. coli* and *P. mirabilis* are both found in the gut, but the UPEC vaccine targets proteins that are specifically expressed by uropathogenic, and not fecal, isolates. In this way, potential reaction of gut microbes to vaccination is hopefully minimized. However, it appears that the potential problem of targeting *P. mirabilis* in the gut cannot be handled in the same way (*i.e.*, only targeting proteins not found in commensal strains). Further work will be necessary to determine, first, how the gut microbiota (specifically *P. mirabilis*) is affected by mucosal vaccination against *P. mirabilis* and, second, how to best handle any potential negative effects that may result.

The *P. mirabilis* microarray was also recently used to identify genes shared by *P. mirabilis* and two other causative agents of complicated UTI, *M. morganii* and *P. stuartii* (60). This study employed comparative genomic hybridizations and resulted in the identification of highly conserved genes present in all three species. The identification of these highly conserved genes raises the possibility that a “complicated UTI vaccine”

could be developed that would protect against infection by all three species; however, no specific work toward this end has begun yet.

Future work that could affect vaccine design should also utilize human samples. Although the existence of an animal model has been crucial to our understanding of *P. mirabilis* pathogenesis, like all animal models, it may not exactly mirror infection dynamics in humans. For example, using sera from human patients with UTI caused by *P. mirabilis* could lead to the identification of antigens that are not expressed in mice or that are not recognized by sera from mice. Analyzing the transcriptome of *P. mirabilis* isolated from the urine of infected human patients could also lead to the identification of genes expressed during human infection that may not be expressed during *in vitro* growth or mouse infection.

Efforts to develop a vaccine against *P. mirabilis* would also benefit greatly from the identification of correlates of protection. What factors are associated with protection? Most *P. mirabilis* vaccine studies have reported no significant correlation between antibody production and protection (94, 142, 177, 200, 201); a few have suggested a correlation between the production of IgA and protection, but again, a significant correlation was not detected (94, 120). However, in contrast to all other earlier work, analysis of the recently-developed, successful Pta* vaccine data revealed a significant correlation between serum IgG levels and protection (4). It is unclear why a correlation was observed with the Pta* vaccine but not previous vaccinations with other antigens; previously used antigens have offered a similar, if not better, degree of protection from infection. It will be interesting to see if this correlation can be observed with future vaccine trials. In addition, protection from UPEC infection after vaccination has recently

been correlated with efficient antibody class switching (12). However, it is unclear if class switching correlates with protection against *P. mirabilis*.

Aside from the recognition of outer membrane proteins that can potentially serve as vaccine candidates, the immunoproteomic screen also resulted in the identification of 13 non-OMP antigens. Although these proteins are not ideal vaccine candidates, they are proteins that are expressed *in vivo*; therefore, they can offer valuable information about the state of bacteria during infection. Indeed, the identification of one of these proteins, ZnuB, led to the characterization of the zinc uptake system ZnuACB in *P. mirabilis*. ZnuACB contributes to fitness *in vivo*, a fact which was unappreciated prior to these studies. In fact, we argue that a second immunoproteomic screen could be undertaken with the sole purpose of identifying proteins from the cytosol and/or inner membrane. Again, although not suitable for vaccine development, identification of these proteins may offer some insight into, for example, bacterial metabolism during infection.

As mentioned above, the immunoproteomic screen led us to investigate ZnuACB, the zinc transport system of which ZnuB is the inner membrane component. Experiments focused on ZnuC, the cytoplasmic ATPase. We found that ZnuC is required for normal swarming motility and offers a competitive advantage. We hypothesize that it is not ZnuACB, *per se*, that are required for these functions, but perhaps other proteins that require zinc are not functional due to lower levels of intracellular zinc in znuC::kan than the wild-type strain. However, at this time, we have no experimental data to support this hypothesis. One of the methods typically used to examine differences between two strains are microarray experiments. Indeed, microarrays have been preformed analyzing *E. coli* cultured in the presence of TPEN (compared to *E. coli* cultured without TPEN)

(211). However, it is not clear that microarray experiments would answer our primary question. These experiments are designed to show differences in the levels of transcript; it is possible that zinc-binding proteins are all synthesized in the same amount, but remain inactive or are unstable when no zinc is inserted. Additionally, there are methods for differentiating between protein levels in two samples, such as the 2D-DIGE (two-dimensional fluorescence difference in gel electrophoresis) approach recently employed for a study examining expression of UPEC proteins during culture in LB or human urine (13). However, again, if target proteins are synthesized in the same amount, we would not identify them in this way. Perhaps there is a way to combine this technology with the use of a radioactive zinc isotope (^{65}Zn(II)); however, to our knowledge, no such experiments have been performed. In any case, the identification of zinc-binding proteins that are affected by decreased zinc uptake could lead to new avenues of research; the function of these proteins could contribute to the competitive advantage observed *in vivo* that is associated with the presence of a functional ZnuACB zinc transport system.

We hypothesize that multiple factors contribute to both the *in vivo* defect and the significant decrease in motility of znuC::kan. Our current working hypothesis regarding the motility defect of znuC::kan revolves around FlhDC, the master regulators of the flagellar cascade. When the structure of the FlhDC complex was solved recently, it was discovered that FlhC contained a previously unrecognized zinc-binding site (240). We hypothesize that FlhDC requires zinc and that less zinc is available in znuC::kan, leading to fewer functional copies of FlhDC. There are, of course, two major assumptions contained in this hypothesis: first, that znuC::kan contains less intracellular zinc than the wild-type strain, and, second, that FlhDC requires zinc for its function. We are currently

trying to address the first of these assumptions experimentally. It remains unknown, however, if FlhDC requires zinc to be functional; to our knowledge, this issue has not yet been addressed experimentally. The zinc ion present in the crystal structure of FlhC was ligated by four cysteine residues. Perhaps a site-directed mutagenesis approach could be used to specifically target these residues, resulting in the formation of mutant proteins unable to bind zinc. If successfully generated, these non-zinc-containing proteins could be assessed in functional assays related to motility, *i.e.* the transcription of flagellin or other motility-related genes. Again, we believe it is likely that there are multiple factors that contribute to the defect in motility of znuC::kan; the FlhDC complex is just one potential target.

We initially identified ZnuB through the immunoproteomic screen. Although we chose not to pursue ZnuB as a vaccine candidate (based on its predicted location in the cell), we learned that the ZnuACB system is important to *P. mirabilis* during UTI. This finding has led us to believe that other identified antigens may also play an important role during infection. Indeed, two putative outer membrane iron receptors, PMI0842 and PMI2596, were also found to impart a competitive advantage during infection (Chapter II) (165). The proteins we identified in the immunoproteomic screen have not all been characterized yet. Other proteins that may be interesting or contribute to pathogenesis based on their annotation include a putative RTX toxin (PMI2043) and two putative iron acquisition proteins (IreA and PMI0409).

In closing, complicated UTIs caused by *P. mirabilis* are a public health concern. Patients experiencing these infections often suffer considerable morbidity or even mortality. These infections commonly affect catheterized patients, including elderly

populations in nursing homes. As the aging population is projected to increase, it is possible an increase in catheter-associated UTIs will also occur; this possibility makes vaccine development even more pressing. The work reported here has contributed to the long-term goal of the development of a multivalent vaccine against *P. mirabilis* by identifying potential protective antigens. Indeed, one of these antigens (Pta) has already been shown to be protective in the mouse model of ascending UTI (4). The identification of proteins expressed during infection also resulted in insight into the importance of metal ion acquisition *in vivo*; the uptake of both iron and zinc are important to *P. mirabilis* during UTI.

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