Development of an Intranasal Vaccine To Prevent Urinary Tract Infection by *Proteus mirabilis*

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*Proteus mirabilis* commonly infects the complicated urinary tract and is associated with urolithiasis. Stone formation is caused by bacterial urease, which hydrolyzes urea to ammonia, causing local pH to rise, and leads to the subsequent precipitation of magnesium ammonium phosphate (struvite) and calcium phosphate (apatite) crystals. To prevent these infections, we vaccinated CBA mice with formalin-killed bacteria or purified mannose-resistant, *Proteus*-like (MR/P) fimbriae, a surface antigen expressed by *P. mirabilis* during experimental urinary tract infection, via four routes of immunization: subcutaneous, intranasal, transurethral, and oral. We assessed the efficacy of vaccination using the CBA mouse model of ascending urinary tract infection. Subcutaneous or intranasal immunization with formalin-killed bacteria and intranasal or transurethral immunization with purified MR/P fimbriae significantly protected CBA mice from ascending urinary tract infection. Subcutaneous or intranasal immunization with formalin-killed bacteria and intranasal or transurethral immunization with purified MR/P fimbriae significantly protected CBA mice from ascending urinary tract infection by *P. mirabilis* (*P* < 0.05). To investigate the potential of MrpH, the MR/P fimbrial tip adhesin, as a vaccine, the mature MrpH peptide (residues 23 to 275, excluding the signal peptide), and the N-terminal receptor-binding domain of MrpH (residues 23 to 157) were overexpressed as C-terminal fusions to maltose-binding protein (MBP) and purified on amylose resins. Intranasal immunization of CBA mice with MBP-MrpH (residues 23 to 157) conferred effective protection against urinary tract infection by *P. mirabilis* (*P* < 0.002).

The urinary tract is a complicated epithelium-lined tube with an opening to the body surface. It is susceptible to infections by exogenous organisms that can colonize the peri-urethral area, enter the bladder via the urethra, and ascend the ureters to the kidneys. In some cases the organisms can enter the bloodstream and cause sepsis. Most urinary tract infections (UTIs) occur in otherwise healthy women with normal urinary tracts, but a significant proportion of UTIs develop in those with complicated urinary tracts, including those that are catheterized or otherwise instrumented or obstructed due to structural abnormalities that prevent the normal flow of urine (42).

*Proteus mirabilis* infects a high proportion of patients with complicated urinary tract infections (29, 44). Importantly, once in the urinary tract, the bacterium appears to have a predilection for the kidney (10). Furthermore, this bacterium causes not only cystitis and acute pyelonephritis (9, 11, 13, 38, 41) but also urethral stones (14), further complicating already abnormal urinary tracts.

Stone formation is caused by the expression of bacterial urease, which hydrolyzes urea to ammonia, causing local pH to rise, and leads to the subsequent precipitation of magnesium ammonium phosphate (struvite) and calcium phosphate (apatite) crystals (14, 31, 32). The stones resulting from aggregation of such crystals begin forming at the surface of the epithelium (25), which complicates infection for three reasons. First, the

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immunoglobulin response was mounted against MR/P fimbiae as assessed by Western blot (4, 17). In addition, when urine and bladder samples were assessed by PCR, we found that the \textit{mep} invertible element, which carries the promoter and controls transcription of \textit{mep} genes, is mostly (>90%) in the on position during infection (23, 45). This indicates that most of the bacterial population is synthesizing MR/P fimbiae during infection. Competition experiments between the phase-locked mutants (the locked-on and the locked-off mutants) and the wild-type strain revealed that MR/P fimbia is an important bladder colonization factor (23).

To prevent the serious consequences of infection with \textit{P. mirabilis}, we have conducted studies with a goal of developing a vaccine that protects against infection by this species and thus the development of urolithiasis. We believe that several criteria make the development of a vaccine against \textit{P. mirabilis} UTI both useful and feasible. First, a well-defined population that would benefit from immunization can be easily identified. Three categories of patients comprise this population: (i) those with known anatomically or functionally abnormal urinary tracts, including neurogenic bladders and urinary diversions; (ii) those early in the course of long-term catheterization (urethral, suprapubic, intermittent, and condom); and (iii) possibly, women with apparently normal urinary tracts but who are experiencing recurrent \textit{Escherichia coli} UTIs (before they develop \textit{P. mirabilis} infection). Although Proteus is only a small percentage, the denominator is so large that most patients with struvite stones and recurrent urinary infection are women who do not have abnormal or instrumented urinary tracts (12). This intimates that \textit{P. mirabilis}, through the process of stone formation, can convert an uncomplicated UTI into a complicated one.

The second reason for preventing \textit{P. mirabilis} infections is that they are often difficult to clear, as the bacteria reside within the urease-induced crystals, constituting a persistent reservoir for recurrent infection. Third, \textit{P. mirabilis} infections can be particularly serious because the combination of stones and infection may result in renal damage, including acute pyelonephritis, bacteremia, and chronic pyelonephritis (38, 43). Fourth, \textit{P. mirabilis} appears to have a large number of conserved surface antigens that do not differ significantly from strain to strain, regardless of whether the bacteria are from feces, urine of asymptomatic individuals, or urine of patients with catheter-associated bacteriuria or acute pyelonephritis (38, 43). Fifth, \textit{P. mirabilis} is present in the fecal flora of <5% of individuals (40), meaning that a vaccine that clears the organism from the stool would have little effect on the normal fecal flora.

The concept of an adhesin-based vaccine has earned credence from a report of a successful FinH (the adhesin of type 1 fimbiae) vaccine that has been shown to prevent experimental UTI by a uropathogenic strain of \textit{E. coli} (20). The FinH adhesin was held in the native conformation by its chaperone FinC and used for systemic vaccination. Significant protection from transurethral (TU) challenge was reported (20).

In this report, we describe the use of a fimbrial component, MrpH, as a vaccine to prevent infection by \textit{P. mirabilis} in a CBA mouse model of ascending UTI to test the efficacy of vaccination. We assessed protection after infection with live bacteria (17) and after vaccination with formalin-killed bacte-
PCR products were electrophoresed on a 0.8% agarose gel, and DNA bands when comparing percentages of mice protected.

To identify bacterial antigen preparations that were capable of protecting mice from infection with *P. mirabilis* by vaccination, we began our protection studies using crude preparations and then moved to more refined approaches using specific antigens. To test the efficacy of the whole-cell preparation, we immunized groups of 10 CBA mice on days 0, 7, and 14 with formalin-killed whole cells of *P. mirabilis* at a dose of 10⁵ cells per mouse. Four routes of immunization were tested: s.c., i.n., TU, and OR. For s.c. immunization, the formalin-killed whole-cell preparation was emulsified in Freund's complete adjuvant for the primary injection on day 0 and in Freund's incomplete adjuvant for the two boosters on days 7 and 14. Groups of CBA mice were immunized s.c., i.n., TU, or OR with formalin-killed whole cells of *P. mirabilis* on days 0, 7, and 14 (see Materials and Methods for details). On day 21, immunized and naive mice were challenged TU with 5 × 10⁷ CFU of *P. mirabilis* HI4320. Seven days after challenge, bacteria in bladder and kidneys of surviving mice were quantitatively cultured.

For immunization with whole killed *P. mirabilis*, significant decreases in colonization were achieved by s.c. (bladder, *P* = 0.005; kidney, *P* = 0.003) and i.n. (bladder, *P* = 0.004; kidney, *P* = 0.01) immunization (Table 1). After s.c. and i.n. immunization, 60 and 67% of mice, respectively, had detectable numbers (limit of detection = 10⁵ CFU/g) of bacteria in their bladder and kidneys 1 week after TU challenge; this compared to only 10% of unimmunized mice that were protected (*P* < 0.05 using Fisher's exact test).

<table>
<thead>
<tr>
<th>Route</th>
<th>Bladder</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c.</td>
<td>0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>i.n.</td>
<td>0.004</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Mucosal immunization via the TU and the OR routes failed to induce significant protection of mice against UTI by *P. mirabilis*.

### Antibody response to vaccination with formalin-killed *P. mirabilis*

Groups of mice that displayed statistically significant levels of protection against homologous challenge (i.e., those vaccinated by s.c. and i.n. routes) mounted high titers in serum of IgG to whole killed bacteria (Table 1). However, mice vaccinated by the s.c. route did not produce detectable serum IgA or IgG and IgA in urine, bladder, vaginal wash, or bile. On the other hand, vaccination by the i.n. route elicited a broad antibody response yielding high titers of IgG and IgA in serum and detectable levels in urine, bladder, vaginal wash, and bile. Indeed, the i.n. route was the only group with immunoglobulin in the urine and bladder directed against *P. mirabilis*.

### RESULTS

Protection against homologous challenge. Immunity to re-infection with a homologous strain of *P. mirabilis* was previously assessed following experimental infection (17). CBA mice were inoculated TU with 5 × 10⁷ CFU of *P. mirabilis* HI4320. After 4 weeks, this group of mice and a control group were treated with ampicillin for 3 days followed by a 4-day washout period. Both groups were then challenged with a *Nal*³ mutant of the homologous strain. One week later, animals were sacrificed, and urine, bladder, and kidneys were quantitatively cultured. Only modest protection of the kidneys but not the bladder was noted. Thus, UTI with *P. mirabilis* does not protect against homologous urinary challenge; this is consistent with the clinical occurrence of recurrent infections with *P. mirabilis*.

High serum antibody titers to specific antigens including MR/P fimbrine and flagella, however, were observed (4).
Vaccine to prevent Proteus mirabilis UTI

TABLE 1. Immunization with whole killed P. mirabilis

<table>
<thead>
<tr>
<th>Group</th>
<th>% Protected</th>
<th>Log_{10} CFU/g of bladder</th>
<th>Log_{10} CFU/g of kidney</th>
<th>Mean Antibody titer on day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>P</td>
</tr>
<tr>
<td>Naive</td>
<td>10 (1/10)</td>
<td>6.59</td>
<td>7.05</td>
<td>NA</td>
</tr>
<tr>
<td>s.c.</td>
<td>60 (6/10)^b</td>
<td>3.44</td>
<td>2.00</td>
<td>0.005</td>
</tr>
<tr>
<td>i.n.</td>
<td>67 (6/9)^b</td>
<td>3.51</td>
<td>2.00</td>
<td>0.004</td>
</tr>
<tr>
<td>TU</td>
<td>33 (3/9)</td>
<td>4.65</td>
<td>5.97</td>
<td>0.04</td>
</tr>
<tr>
<td>OR</td>
<td>29 (2/7)</td>
<td>5.09</td>
<td>6.30</td>
<td>0.28</td>
</tr>
</tbody>
</table>

The percentage of mice with undetectable numbers (<10^2 CFU/g tissue) of bacteria in their bladder and kidneys (the number of mice with undetectable level of colonization in their bladder and kidneys/number of mice survived 7 days after transurethral challenge). The total number of mice for each group is 10.

The percentage of mice protected from infection is significantly higher in the vaccinated mice than in the naive mice (P < 0.05 using one-tailed Fisher’s exact test).

P values are derived from one-tailed Mann-Whitney test comparing the colonization level of the vaccinated mice with that of the naive mice.

NA, not applicable.

Titers measured just prior to transurethral challenge; values expressed as the reciprocal of the highest detectable dilution.

Samples from individual mice were assayed by ELISA independently.

The percentage of mice with undetectable numbers (P < 0.005 using Fisher’s exact test).

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Samples from individual mice were assayed by ELISA independently.

The percentage of mice with undetectable numbers (P < 0.005 using one-tailed Fisher’s exact test).

For MR/P fimbriae, we previously noted that infected mice mounted a strong serum antibody response against MR/P fimbriae, indicating that these cell surface structures were expressed in vivo (4). In vivo expression of MR/P fimbriae was further confirmed by the invertible element assay, a PCR-based assay that measures phase variation of MR/P fimbria, which indicated that in the heavily colonized mouse bladder, >90% of bacteria expressed MR/P fimbriae (23). We hypothesized that proteinaceous structures expressed on the surface of the bacterium could serve as effective antigens for vaccination. To test the efficacy of purified antigen preparations, MR/P fimbriae were sheared from the bacterial surface and then purified by differential centrifugation. Groups of CBA mice were immunized via i.n., TU, s.c., or OR routes on days 0, 7, and 14 at a dose of 100 to 200 μg of MR/P fimbria per mouse (see Materials and Methods for details). On day 21, immunized and naive mice were challenged TU with 5 × 10^7 CFU of P. mirabilis HH4320. Seven days after challenge, bacteria in bladder and kidneys of surviving mice were quantitatively cultured.

For MR/P fimbriae immunization, a significant reduction in colonization was observed with i.n. (bladder, P = 0.005; kidneys, P = 0.0004) and TU (bladder, P = 0.009; kidneys, P = 0.0005) immunization (Table 2). After i.n. and TU immunization, 60 and 63% of mice, respectively, had undetectable numbers (<10^2 CFU/g) of bacteria in their bladder and kidneys 1 week after TU challenge; this compared to only 10% of the naive mice that were protected (P < 0.05 using Fisher’s exact test).

Antibody response to vaccination with MR/P fimbriae. In groups of mice protected from homologous challenge by i.n. and TU vaccination with MR/P fimbriae, the antibody response (Table 2) was not as vigorous as in mice vaccinated with whole killed cells (Table 1). Again the i.n.-vaccinated mice displayed the most broad response with detectable immunoglobulin measured in serum, urine, vaginal wash, and bile. Mice vaccinated by the TU route had detectable IgG only in the serum with no detectable response in any other compartment. The highest single titer was IgG in serum (1:40,960) in mice vaccinated by the s.c. route; high levels of immunoglobulin in serum, however, did not correlate with protection.

Selection of i.n. route for subsequent studies. i.n. immunization with formalin-killed bacteria or MR/P fimbriae offered the most consistent protection. Furthermore, i.n. was the only route that provided protection in the absence of detectable IgM response.

TABLE 2. Immunization with purified MR/P fimbriae

<table>
<thead>
<tr>
<th>Group</th>
<th>% Protected^a</th>
<th>Log_{10} CFU/g of bladder</th>
<th>Log_{10} CFU/g of kidney</th>
<th>Mean Antibody titer on day 21^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>P</td>
</tr>
<tr>
<td>Naive</td>
<td>10 (1/10)</td>
<td>6.09</td>
<td>7.01</td>
<td>NA</td>
</tr>
<tr>
<td>s.c.</td>
<td>25 (2/8)</td>
<td>4.67</td>
<td>5.43</td>
<td>0.09</td>
</tr>
<tr>
<td>i.n.</td>
<td>60 (6/10)^b</td>
<td>3.24</td>
<td>2.00</td>
<td>0.005</td>
</tr>
<tr>
<td>TU</td>
<td>63 (5/8)^b</td>
<td>3.11</td>
<td>2.00</td>
<td>0.60</td>
</tr>
<tr>
<td>OR</td>
<td>33 (3/9)</td>
<td>5.17</td>
<td>6.33</td>
<td>0.13</td>
</tr>
</tbody>
</table>

^a The percentage of mice with undetectable numbers (<10^2 CFU/g tissue) of bacteria in their bladder and kidneys (the number of mice with undetectable level of colonization in their bladder and kidneys/number of mice survived 7 days after transurethral challenge).

^b The percentage of mice protected from infection is significantly higher in the vaccinated mice than in the naive mice (P < 0.05 using one-tailed Fisher’s exact test).

P values are derived from one-tailed Mann-Whitney test comparing the colonization level of the vaccinated mice with that of the naive mice.

NA, not applicable.

Titers measured just prior to transurethral challenge; values expressed as the reciprocal of the highest detectable dilution.

Samples from individual mice were assayed by ELISA independently.

Comparisons were made by Fisher’s exact test.
route that elicited vaginal antibody production, which may be protective because the vagina may serve as a source of UTI organisms capable of causing UTI. Thus, the i.n. route of immunization was chosen for subsequent studies.

**i.n. immunization with MrpH, the adhesin of MR/P fimbriae.** We have previously provided evidence that MrpH is the fimbrial tip adhesin component of MR/P fimbriae (22). These observations include that (i) MrpH shares 30% amino acid sequence identity with PapG, the Gal(1-4)Gal-binding adhesin of *E. coli* P fimbriae; (ii) amino acid residue substitution of the N-terminal cysteine residues (C66S and C128S) of MrpH abolished receptor-binding activity (hemagglutinating ability) of MrpH but allowed normal fimbrial assembly; (iii) immunogold electron microscopy of *P. mirabilis* strain HI4320 revealed that MrpH was located at the tip of MR/P fimbriae, consistent with its role in receptor binding; and (iv) when aligned with other MR/P pilins (MrpA, -B, -E, -F, and -G), MrpH contains extra N-terminal amino acid sequence of 121 amino acid residues putatively associated with receptor binding (22).

MrpH, the MR/P fimbrial tip adhesin, represents a minor protein component in the MR/P fimbrial preparation (Fig. 1). Given the functional importance of MrpH, we hypothesized that increased specific immune response to MrpH would result in more effective protection of mice from urinary infection by *P. mirabilis*. To examine the effect of using the fimbrial tip adhesin as antigen, an MBP fusion of mature MrpH (amino acid residues 23 to 275) was constructed, overexpressed, and purified on an amylose column (Fig. 2, lanes 2 and 3). Purified MBP-MrpH (residues 23 to 275) was covalently conjugated to cholera toxin using SPDP and then used for i.n. immunization of mice on days 0, 7, and 14 at a dose of 10^7 CFU of *P. mirabilis* strain HI4320 (lane 2) and its isogenic MR/P pilin-negative mutant (mutation in *mrpH*) (lane 4). The molecular masses of the bands shared by the biotinylated protein markers and the Rainbow protein markers are indicated on the right in kilodaltons. Western blot analysis using antibodies against MrpH indicated that the fimbrial tip adhesin (+) is a minor component in the MR/P fimbrial preparation, which consists of mostly the major structural subunit MrpA (#).

As shown in Fig. 3, the immunized mice were colonized by significantly fewer bacteria in kidneys (median log_{10} CFU/g of tissue: 5.76 for the naive mice versus 2.00 for the immunized mice; *P* = 0.0001) and they also tended to be colonized by fewer bacteria in the bladder (median log_{10} CFU/g of tissue: 7.49 for the naive mice versus 5.45 for the immunized mice; *P* = 0.056). 36% (4 of 11) of the immunized mice had undetectable levels of colonization (<10^2 CFU/g of tissue) in bladder and kidneys, compared to 0% (0 of 10) of the naive mice (*P* = 0.055).

**Antibody response to i.n. vaccination with MrpH.** Highly significant IgG responses to MrpH vaccination were observed in the serum, bladder, and kidney and clearly measurable responses were quantified in urine and vaginal washes (Table 3). The latter responses rose in response to vaccination and primary and secondary boost. Significant IgA responses were also noted in serum, vaginal wash, and kidneys; the response in the bladder and urine was weak. This less robust response in the bladder correlated with less protection compared to the protection in the kidneys.

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i.n. immunization with MrpH truncate. The amino-terminal receptor-binding domain (amino acid residues 23 to 157) of MrpH was also tested as a vaccine candidate for the following reasons. First, the advantage of using adhesin polypeptide as vaccine is that the stimulated immune response may specifically block bacterial adherence. Secondly, the carboxyl-terminal domain of MrpH shares strong sequence homology with other MR/P pilins (22), the building blocks of MR/P fimbriae, which require the periplasmic chaperone MrpD for proper folding. In the absence of MrpD, the carboxyl-terminal pilin domain of MrpH is not properly folded and could interfere with the folding of the receptor-binding domain. In our study, the fusion protein MBP-MrpH (residues 23 to 275) was >90% soluble when overexpressed in *E. coli*, but often precipitated out of solution at high concentrations, which complicated vaccine preparation. We reasoned that removing the carboxyl-terminal domain of MrpH would solve this problem.

The truncate fusion, MBP-MrpH (residues 23 to 157) was constructed, overexpressed, and purified on an amylose column (Fig. 2, lanes 4 and 5). As we expected, the truncate MrpH fusion protein did not precipitate out of solution at high concentrations (5 mg/ml). i.n. immunization with MBP-MrpH (residues 23 to 157) was carried out according to the same procedure described above. Twelve naive mice and 12 immunized mice were used in this experiment; 1 of the 12 naive mice died before the day of challenge. We observed that, compared to the naive mice, mice immunized with the truncate form of MrpH were colonized by significantly fewer bacteria in the bladder (median log_{10} CFU/g of tissue: 7.21 for the naive mice versus 2.00 for the immunized mice; *P = 0.002*) and kidneys (median log_{10} CFU/g of tissue: 5.45 for the naive mice versus 2.00 for the immunized mice; *P < 0.0001*) (Fig. 4). The percentage of mice with undetectable levels of colonization in bladder and kidneys (≤2 log_{10} CFU/g of tissue) was significantly higher for the immunized mice than the naive mice (1 of 11 [9%] for the naive mice versus 9 of 12 [75%] for the immunized mice; *P = 0.002*).

**Conservation of mrpH and MrpH among strains.** For an MrpH vaccine to be effective against challenge with heterologous strains, MrpH must be conserved among diverse strains. Genetic variation of MR/P fimbriae was examined among different *P. mirabilis* strains, including 10 pyelonephritis isolates, 10 catheter-associated isolates, and 10 fecal isolates. Western blot analysis using antibodies against MrpA, the major pilin of MR/P fimbriae, indicated that Mrp/P fimbriae were expressed at

![Figure 3](http://iai.asm.org/)

**Table 3.** Antibody response in mice to i.n. vaccination with MBP-MrpH

<table>
<thead>
<tr>
<th>Mouse sample</th>
<th>Time of sampling</th>
<th>Antibody response</th>
<th>Naive</th>
<th>MrpH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG^c</td>
<td>IgA^c</td>
<td>IgG^c</td>
</tr>
<tr>
<td>Serum (n = 4)</td>
<td>Post-primary immunization</td>
<td>5 ± 10</td>
<td>0</td>
<td>&gt;20,480^b</td>
</tr>
<tr>
<td></td>
<td>Post-first booster</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Post-second booster (prechallenge)</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Urine (pool of 10)</td>
<td>Post-primary immunization</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Post-first booster</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Post-second booster (prechallenge)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal wash (pool of 10)</td>
<td>Post-primary immunization</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Post-first booster</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>Post-second booster (prechallenge)</td>
<td>0</td>
<td>0</td>
<td>640</td>
</tr>
<tr>
<td>Bladder (n = 4)</td>
<td></td>
<td>2,240 ± 2,123^b</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Kidney (n = 4)</td>
<td></td>
<td>7,040 ± 3,840^b</td>
<td>7,020</td>
<td>3,840</td>
</tr>
</tbody>
</table>

^a* P = 0 reading at 1:20 dilution.

^b *P < 0.001 compared to naive control.

^c Some data are given as mean ± standard deviation.

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**FIG. 3.** *P. mirabilis* colonization in bladder and kidneys of the naive mice (N) and the mice immunized with MBP-MrpH (residues 23 to 275) (H). Each diamond represents log_{10} CFU per gram of tissue from an individual mouse. Samples with undetectable colonization were given a value of 2 log_{10} CFU/g of tissue (gray horizontal line). The black bars represent the median log_{10} CFU per gram of tissue. One-tailed *P* values were determined by Mann-Whitney test comparing the colonization levels in bladder and kidneys of the naive mice with those of the immunized mice.
various levels in all 30 isolates (data not shown). Dot blot analysis using an mrpH probe revealed that all of the 30 isolates examined were mrpH positive (data not shown). Subsequently, 10 of the 30 isolates (four pyelonephritis, three catheter-associated, and three fecal strains) and the vaccine strain P. mirabilis HI4320 were subjected to the Dienes test, a mutual inhibition test for identifying unique strains with a discriminatory power of 0.98 (Fig. 5) (37). The mrpH genes from these 10 isolates were PCR amplified and sequenced. As shown in Table 4, among the 10 isolates, four belong to the same Dienes type (B), three each belong to a different Dienes type (C, D, and E), and the other three failed to swarm and thus were typed. Isolates belonging to the same Dienes type share 100% identity in the sequence of mrpH, but isolates that share 100% identity in the sequence of mrpH do not necessarily belong to the same Dienes type (Table 4). Overall, the mrpH gene sequences from the 10 isolates representing five different Dienes types share 99.4 to 100% identity with the mrpH gene sequence from P. mirabilis strain HI4320. Most significantly, none of these nucleotide changes in the mrpH gene resulted in any amino acid substitutions in the MrpH protein. These results demonstrated that MrpH is conserved among various P. mirabilis strains.

DISCUSSION

A previous study by our group showed that experimental UTIs by P. mirabilis yield little protection to mice from reinfection by a homologous strain (17). In this study, we investigated the efficacy of vaccination via four different routes (s.c., i.n., TU, and OR) with a formalin-killed whole-cell preparation, a purified MR/P fimbrial preparation, and recombinant MBP fusions of MrpH, the tip adhesin of MR/P fimbria. Our results indicate that i.n. vaccination with recombinant MBP fusion of truncate MrpH (MBP-MrpH [residues 23 to 157]) is most effective in preventing UTI with P. mirabilis in mice.

Infection of the urinary tract by P. mirabilis is governed by the general principles of bacterial pathogenesis. Proteins produced by this species during infection may contribute to (i) colonization of the host, (ii) evasion of host defense, and (iii) damage of host tissue. Colonization requires movement of P. mirabilis to its niche, attachment to specific receptors, and survival in the urinary tract. At least four fimbriae have been identified in this species, including MR/P fimbriae, PMF, ATF, and the nonagglutinating fimbriae (1, 3, 4, 26, 33, 34). MR/P fimbriae and PMF appear to contribute to colonization by P. mirabilis in the CBA mouse model of ascending UTI. When separate groups of mice were TU inoculated with the parent strain and an MR/P-deficient isogenic mutant (constructed in our laboratory), the mutant was recovered in lower numbers than the parent strain from the bladder and kidneys of mice 1 week after experimental inoculation of the bladders (5). In similar experiments, the PMF fimbrial mutant was found to be present at a significantly lower concentration in the bladder only (27). An isogenic ATF-negative mutant of P. mirabilis was shown not to be attenuated in its ability to colonize mouse urinary tract (46). The contribution of nonagglutinating fimbria has not yet been tested but is of doubtful significance because of its lack of identifiable adherence property.

Observations from our laboratory and those of other investigators suggest that P. mirabilis vaccination may confer pro-
Protection against infection with heterologous strains. At least four antigen preparations have been tested previously. Decades ago, Pazin and Braude immunized rats parenterally with purified flagella (35). They demonstrated that antisera immobilized bacteria by binding to flagella and prevented the organisms from spreading from one kidney to the other kidney (i.e., down one ureter, into the bladder, and back up the other ureter). Moayeri et al. have shown that immunization with an outer membrane protein preparation protects BALB/c mice from homologous intravesicular challenge (28). Legagni-Fajardo et al. demonstrated that parenteral immunization with purified fimbrial preparation protects mice from TU challenge with homologous and heterologous strains (21). In an important follow-up to this study, Pellegrino and colleagues (36) purified recombinant fimbrial antigens of *P. mirabilis* including MrpA, PmIa, and UcaA and used these for vaccination and protection from hematogenous and TU challenge. They found that MrpA protected s.c. immunized mice in both models, demonstrating that, indeed, the MR/P fimbria is an important vaccine target. We had previously noted a strong serum immunoglobulin response to MR/P fimbriae in mice following experimental UTI, suggesting that this antigen is expressed in vivo and is immunogenic (4). In this report, we demonstrated that i.n. immunization with the adhesin molecule of the MR/P fimbria, MrpH, or with its N-terminal receptor-binding domain alone protected mice from experimental UTIs.

We tested four different immunization routes with the formalin-killed whole-cell preparation and purified MR/P fimbrial preparation. The immunization routes significantly affect the efficacy of the vaccine. The killed whole-cell vaccine is effective via s.c. or i.n. routes, whereas the MR/P fimbria vaccine is effective via i.n. or TU routes. Overall, the i.n. route of vaccination elicited the broadest antibody response, elevating titers in serum, urine, bladder, vaginal wash, and bile (Tables 1 and 2). Systemic immunization via the s.c. route induced the strongest serum IgG response, but it did not necessarily result in the most effective protection from infection. This is consistent with our previous finding that there is no significant correlation between serum IgG level and protection from infection (17).

Our data indicated that both the killed whole-cell vaccine and the MR/P fimbria vaccine were effective protecting mice from *P. mirabilis* infection, up to 67 and 63% protection, respectively. The recombinant MBP-MrpH (residues 23 to 157) vaccine resulted in a higher percentage of protection, 75%. However, the most significant advantage of the recombinant MBP-MrpH formulation over the first two is its reduced side effects. Compared to the naive mice, higher mortalities were observed in mice immunized with the killed whole-cell vaccine and the MR/P fimbria vaccine (Table 1 and 2). Such increases in mortality may due to possible side effects of vaccination; a few immunized mice suffered weight losses (data not shown).

The protection of mice by the truncate MrpH vaccine from *P. mirabilis* UTI is comparable to the protection by the FimH vaccine from *E. coli* UTI (20). For comparison to this study, if one uses a mean bladder weight of 0.5 g to convert the data of Langermann et al. (20), then protection of the bladder from colonization following challenge of vaccinated animals was nearly identical for *E. coli* and *P. mirabilis*. For the kidney, the *P. mirabilis* vaccine appeared to offer better protection (1.5 logs). This is especially important since *P. mirabilis* localizes to the kidney and can cause serious histological damage to these organs, even after a single infection. There are a few other differences as well. The protection from *E. coli* UTI was achieved by systemic immunization with the FimH vaccine. In our study, however, systemic immunization via the s.c. route with the MrpH vaccine, despite inducing a strong serum IgG response, yielded no protection from *P. mirabilis* UTI (data not shown). The FimH adhesin was coexpressed with and then copurified with the FimC chaperone based on the affinity binding of FimH to mannos. In our study, we tried coexpressing the MrpH adhesin with the MrpD chaperone, but >90% of overexpressed protein was composed of the MrpD-MrpD dimer. However, the lack of information about the receptor of MrpH made it difficult to separate the MrpH-MrpD dimer from the MrpD-MrpD dimer efficiently. We also tried overexpressing the mature MrpH polypeptide as a six-histidine-tagged recombinant protein, which resulted in formation of inclusion bodies. The addition of the ~40-kDa MBP fusion to the N terminus of the mature MrpH polypeptide appeared to keep the recombinant protein in a soluble form. Thus, the MBP fusions of the MrpH adhesin were chosen for our vaccine study.

In our study, systemic immunization via the s.c. route did not...
yield protection from *P. mirabilis* infection (data not shown). Pellegrino et al. reported that s.c. immunization of mice with MrpA (the major structural subunit of the MR/P fimbriae) afforded great protection from intravenous challenge of *P. mirabilis* and a much smaller degree of protection (limited to kidneys only) from TU challenge of *P. mirabilis* (36). These results further suggest that systemic immunization yields better protection from systemic infections than from mucosal infections. Pellegrino et al. also found that there is no significant association between the degree of infection and IgG response in serum (36). In this study, we chose the i.n. route of mucosal immunization to test the efficacy of the MrpH vaccine because (i) it yielded consistent protection from *P. mirabilis* infection when the formalin-killed whole-cell preparation and purified MR/P fimbriae preparation were tested as vaccine candidates, (ii) it induced broader antibody responses, and finally (iii) the route of vaccination is simple. Even though TU immunization with the formalin-killed whole-cell preparation and purified MR/P fimbriae preparation did not yield consistent protection, it cannot be ruled out that TU immunization with MrpH may be protective.

For the vaccine to protect against heterologous challenge, it would be desirable for MrpH to be conserved among *P. mirabilis* strains. We have good evidence that virulence genes are conserved in *P. mirabilis*. For example, when the urease gene cluster was examined, we probed *Hind*III restriction digests of chromosomal preparations of 100 strains using the entire urease gene cluster (~8 kb) as a probe. All 100 strains showed identical Southern blots without exception (30). To demonstrate that *mrpH* is conserved among strains, a fragment carrying the open reading frame was PCR-amplified from 10 *P. mirabilis* isolates representing five distinct Dienes types (8): four pyelonephritis isolates, three catheter-associated isolates, and three fecal isolates. Sequencing of these PCR-amplified fragments revealed that the nucleotide sequences of the *mrpH* genes are 99.4 to 100% identical and the predicted amino acid sequences of the MrpH proteins are 100% identical. Given the conservation of MrpH among various *P. mirabilis* strains, an MrpH-based vaccine would likely protect against heterologous infections.

The truncate MrpH vaccine protected 75% of mice from *P. mirabilis* UTI. To further improve the efficacy, other adhesin molecules may be added to the vaccine formulation. As discussed above, *P. mirabilis* produces at least four different types of fimbriae. Recent studies in our laboratory indicated that increased MR/P fimbrial expression in vivo might be a result of the outgrowth of (as a result of selection for) MR/P fimbral bacteria (unpublished data). It is possible that immune responses to MrpH may allow an outgrowth of (i.e., selection of) bacteria expressing a different type of fimbiae in vaccinated mice. In this case, addition of other adhesin molecules in the vaccine formula may improve its efficacy.

In summary, UTI is the most frequently diagnosed kidney and urological disorder. While *E. coli* is the most common isolate in normal individuals, those with structural abnormalities of the urinary tract or catheterized individuals are very frequently infected with *P. mirabilis*. Because we are now beginning to understand the molecular mechanisms by which *P. mirabilis* establishes infection, evades the host defense, and damages host tissue; because we can identify a large and well-defined group of patients who are affected by this species; and because there is evidence that vaccination would prevent infections and thus urolithiasis, we believe that the development of a vaccine that could be used to prevent these often devastating infections in a high-risk group of individuals is useful.

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REFERENCES


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