INFECTION AND IMMUNITY, Apr. 1997, p. 1327–1334 Vol. 65, No. 4
0019-9567/97/$04.00+0

Proteus mirabilis Mannose-Resistant, *Proteus*-Like Fimbriae: MrpG Is Located at the Fimbrial Tip and Is Required for Fimbrial Assembly

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Received 30 October 1996/Returned for modification 30 December 1996/Accepted 7 January 1997

The mannose-resistant, *Proteus*-like (MR/P) fimbria, responsible for mannose-resistant hemagglutination, is a virulence factor for uropathogenic *Proteus mirabilis*. Based on known fimbrial gene organization, we postulated that MrpG, a putative minor subunit of the MR/P fimbria, functions as an adhesin responsible for hemagglutination, while MrpA serves as the major structural subunit for the filamentous structure. To test this hypothesis, an *mrpG* mutant was constructed by allelic-exchange mutagenesis and verified by PCR and Southern blotting. The *mrpG* mutant was found to be negative for hemagglutination, while wild-type strain HI4320 and the complemented mutant were positive. Western blots with antiserum raised against an overexpressed MrpG′-His$_6$ fusion protein showed that MrpG was present in the fimbrial preparations of both the wild-type strain and the complemented mutant but absent in that of the *mrpG* mutant. The *mrpG* mutant was significantly less virulent in a CBA mouse model of ascending urinary tract infection. Western blots with antiserum to whole MR/P fimbriae showed that MrpA protein was also missing from the fimbrial preparation of the *mrpG* mutant. Using immunogold electron microscopy, we found that the normal MR/P-fimbrial structure was absent in the *mrpG* mutant, suggesting that MrpG is essential for initiation of normal fimbrial formation. In the wild-type strain, MrpG protein was localized to the tips of the fimbriae or at the surface of the cell when antiserum raised against overexpressed MrpG was used. Given the tip localization, MrpG may be required for initiation of assembly of MR/P fimbriae but does not appear to be the fimbrial adhesin.

Proteus mirabilis, commonly associated with complicated urinary tract infections, expresses a number of proteins that may contribute to virulence, including urease, hemolysin, flagella, and fimbriae (24). Four fimbria-like structures have been purified from *P. mirabilis* strains for which the major structural subunits have been identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subjected to N-terminal analysis (2, 3, 19, 28). The gene clusters encoding three of these fimbriae, mannose-resistant, *Proteus*-like (MR/P) fimbriae, *P. mirabilis* fimbriae, and ambient-temperature fimbriae, have been cloned and sequenced (4, 20, 21). The sequences encoding the uroepithelial cell adhesin (*ucaA*) and a *P. mirabilis* P-like pilus (*pmpA*) (6, 7) have also been reported.

Among the fimbriae of *P. mirabilis*, MR/P fimbriae are perhaps best understood. The presence of 7- to 8-nm-diameter channelled fimbriae was first observed on bacterial cells capable of mannose-resistant hemagglutination (26). Most strains express these fimbriae, which elicit an immune response during experimental infection (3, 16). Construction of an isogenic MR/P-fimbria-negative strain (mutation in *mrpA*) demonstrated a role in virulence for this surface adhesin when CBA mice were challenged transurethrally in an animal model of ascending urinary tract infection (5). Because the genetic organization of the *mrp* operon resembled that of other enterobacterial fimbrial gene clusters, including the *pap* operon, we hypothesized that *mrpG*, found near the end of the cluster, is the gene that encodes the adhesin responsible for host receptor binding.

In this study, we introduced a complementable mutation within *mrpG* into the chromosome of *P. mirabilis* HI4320. The *mrpG* mutant was characterized and used to address the hypothesis that MrpG is the receptor binding adhesin.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *P. mirabilis* HI4320 (urease positive, hemolytic, and positive for MR/P, *P. mirabilis* fimbriae, and ambient-temperature fimbriae), isolated from the urine of an elderly, long-term-catheterized woman with significant bacteriuria ($\geq 10^5$ CFU/ml) (25), has been used extensively by our group for virulence studies. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain for transformation of plasmids other than suicide vector pCVD442 (8, 23) and its derivatives. *E. coli* DH5α λpir was used for cloning with the pRK2-derived suicide vector pCVD442.

PCR. Based on the nucleotide sequence of the *mrp* gene cluster, primer 385 (5′-GCGCCATGGCTTTTGCAATACCGGACAAC-3′) and primer 386 (5′-CCAGATCTATGTCTGGTCATATTGGTCGT-3′) were designed to amplify a DNA fragment encoding the MrpG protein, beginning with the 25th amino acid residue. Primer 512 (5′-TAGCCCAAGCAAGGCAGTGT-3′) and primer 513 (5′-TGCAAAAGCCGCTGAAGTTA-3′) were used to verify the insertion of a kanamycin resistance cassette into the chromosome. Primer 527 (5′-GCAGAATTCCAATAGGAGCGTTT-3′) and primer 528 (5′-AACAAGCTTGCCGTGCAATTAT-3′) were designed to amplify a DNA fragment including the complete open reading frame of *mrpG*. All the primer sequences are from the *mrp* operon of *P. mirabilis* HI4320 (4), whose sequence has been deposited previously with GenBank under accession number Z32686.

Chromosomal-DNA preparation. Chromosomal DNA was isolated from bacterial cells by lysis with SDS and proteinase K and extraction with phenol and chloroform as described by Marmur (18).

Southern blot analysis. Chromosomal DNA was digested with *Pvu*II, electrophoresed on a 0.8% agarose gel, and transferred to a membrane (QIABRANE Nylon Plus; Qiagen Inc., Chatsworth, Calif.). Probe labeling, hybridization, and signal detection were carried out with the ECL direct nucleic acid labeling and detection system (Amersham Life Science, Amersham Place, England) according to the instructions of the manufacturer.

Western blot analysis. Protein samples were denatured in SDS-gel sample buffer, electrophoresed on an SDS–15% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Western blots were incubated with polyclonal antisera raised against either purified MR/P fimbriae or an overexpressed and gel-purified MrpG′-His$_6$ fusion protein, followed by incubation with goat anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase and developed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium)–Nitro Blue Tetrazolium as a chromogenic substrate for alkaline phosphatase (1).

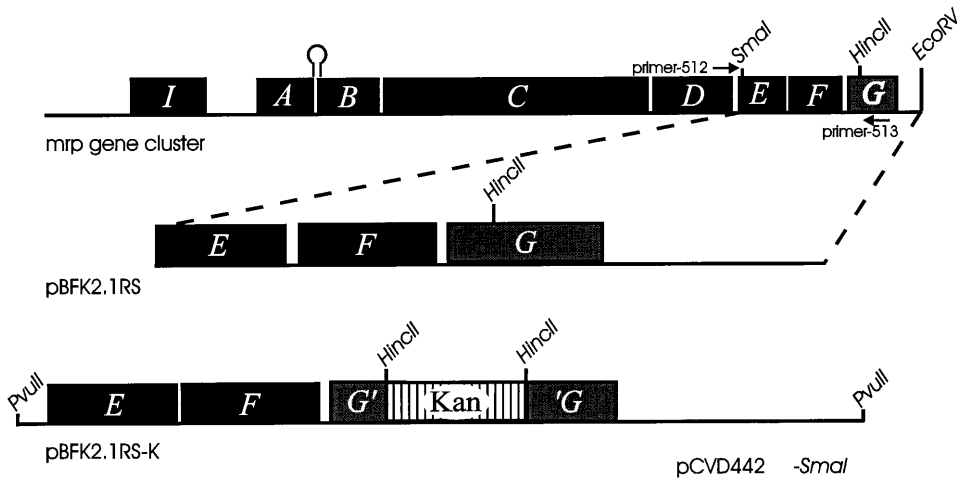


FIG. 1. Construction of an *mrpG* mutant. A 2.1-kb *Sma*I-*Eco*RV fragment from the *mrp* gene cluster was subcloned into *Sal*I-digested pBluescript for which the ends had been rendered flush and the *Hin*cII site eliminated; this plasmid was designated pBFK2.1RS. A kanamycin resistance cassette on a *Hin*cII fragment (5) was cloned into the *Hin*cII site within *mrpG*. A 3.8-kb *Pvu*II fragment containing the insertion mutation was cloned into the *Sma*I site of suicide vector pCVD442 (8). The latter construct, designated pBFK2.1RS-K, was electroporated into *P. mirabilis* HI4320; Kan^r colonies were selected and screened genotypically and phenotypically.

Hemagglutination. Bacteria were passaged statically three times for 48 h each in Luria broth at 37°C and then harvested by centrifugation (5,000 × *g*, 3 min, 4°C). Cell pellets were resuspended in phosphate-buffered saline (PBS) (containing [per liter] 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 [pH 7.2]) to approximately 10^9 CFU/ml and then mixed with an equal volume of a 3% (vol/vol) erythrocyte suspension. Hemagglutination was defined as visible clumping of erythrocytes. MR/P hemagglutination was demonstrated by the agglutination of untreated and tannic acid-treated chicken, sheep, guinea pig, horse, ox, and human erythrocytes, with chicken cells giving the strongest reaction. The reactions were not inhibited by 50 mM mannose.

Immunogold electron microscopy. *P. mirabilis* HI4320 and its derivatives were grown under conditions optimal for production of MR/P fimbriae and were passaged statically three times for 48 h each at 37°C. Immunogold labeling was performed by a modification of the method of Faulk and Taylor (9). One drop of static bacterial culture was placed on a Formvar-coated grid (Electron Microscopy Sciences, Fort Washington, Pa.) for 5 min. Excess liquid was wiped off, and the grid was air dried. PBS containing 1% bovine serum albumin (BSA) was used to block the grid. The grid was incubated for 30 min with a drop of the appropriate dilution of primary antiserum raised against either purified MR/P fimbriae or MrpG′-His_6 fusion protein. After being washed three times with PBS containing 1% BSA, the grid was incubated with a drop of a 1:25 dilution of recombinant protein G conjugated with 5-nm-diameter gold beads (AuroProbe EM protein GG5; Amersham Corp.) or goat anti-mouse IgG conjugated with 30-nm-diameter gold beads (AuroProbe EM protein GAR protein G30) for 30 min. Grids were then washed three times with PBS containing 1% BSA and three times with distilled water, negatively stained with 1% sodium phosphotungstic acid (pH 6.8), and examined by transmission electron microscopy with a JEM-1200EX II electron microscope (JEOL, Ltd., Tokyo, Japan).

Purification of MR/P fimbriae. *P. mirabilis* CFT322 was chosen for isolation of MR/P fimbriae because this strain does not produce mannose-resistant, *Klebsiella*-like hemagglutination, which may be associated with production of another fimbrial type. This selection therefore eliminated cross contamination of MR/P fimbriae with other fimbriae. Luria broth (6 liters) was inoculated with a 50-ml stationary culture of *P. mirabilis* CFT322 which had been passaged statically three times for 48 h each at 37°C, conditions shown to favor selection of fimbriated bacteria. After 48 h of static growth, cells were harvested and washed by centrifugation (4,000 × *g*, 4°C, 10 min), suspended in 250 ml of 10 mM Tris-HCl (pH 7.2), sheared by blending (5 min at setting 4 on a Waring blender 7012, model 34BL97), and centrifuged (8,000 × *g*, 4°C, 20 min). The supernatant was centrifuged (39,000 × *g*, 4°C, 30 min), and the supernatant from this spin was centrifuged again (180,000 × *g*, 20°C, 1.5 h). The pellet was resuspended in 10 mM Tris-HCl (pH 7.2) and centrifuged (117,000 × *g*, 20°C, 44 h) on a CsCl (1.3-g/ml) gradient (Beckman L8-80M ultracentrifuge; 55.2 TI rotor). Bands were extracted by needle aspiration and dialyzed against distilled water.

Overexpression of MrpG′-His_6 fusion protein. A DNA fragment encoding MrpG lacking its N-terminal 24 amino acids (signal peptide) and its C-terminal 100 amino acids was PCR amplified and cloned into the *Nco*I and *Bgl*II sites of pQE60 (QIAexpress kit; Qiagen Inc.) so that the expressed protein had a six-histidine tail at its C terminus. The construct, named pQEG1, was electroporated into *E. coli* M15, and the overexpression of the MrpG′-His_6 fusion protein was induced by 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The overexpressed protein formed inclusion bodies in *E. coli*. Cells were lysed in a French pressure cell (20,000 lb/in^2). Inclusion bodies were precipitated by centrifugation at 4,000 × *g* for 10 min, denatured in SDS-gel sample buffer, and separated on an SDS–15% polyacrylamide gel.

Antiserum preparation. Both purified MR/P fimbriae and the excised SDS-gel containing the MrpG′-His_6 fusion protein band were emulsified in Freund's complete adjuvant and injected into New Zealand White rabbits. After 4 weeks, animals were given booster injections of 100 μg of protein emulsified in Freund's incomplete adjuvant. Blood samples taken at 6 weeks were assayed for reaction with antigen by Western blotting. Sera were collected 8 weeks after the first immunization.

CBA mouse model of ascending urinary tract infection. A modification (12) of the procedure of Hagberg et al. (10) was used to assess the virulence of parent and mutant strains of *P. mirabilis* HI4320. Six- to eight-week-old female CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) were used. Prior to challenge, spontaneously voided urine was collected in a sterile petri dish; mice found to be bacteriuric were not used. Mice were challenged while anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.) by inserting a polyethylene catheter (length, 2.5 cm; outer diameter, 0.61 mm; Clay Adams, Parsippany-Troy Hills, N.J.) into the bladder through the urethra and infusing 0.05 ml of bacteria at a concentration of 2×10^8 CFU/ml for 30 s into the bladder. The urethral catheter was removed immediately after challenge, and mice were cared for by the normal routine. Mice were inspected daily for morbidity and mortality. Quantitative cultures from the urine, bladder, and kidneys were obtained as previously described (5).

RESULTS

Construction of an *mrpG* mutant. To construct a suicide vector for allelic-exchange mutagenesis of *mrpG*, a 2.1-kb *Sma*I-*Eco*RV fragment isolated from the *P. mirabilis* HI4320 *mrp* fimbrial gene cluster (which carries *mrpE*, *mrpF*, and *mrpG*) was subcloned into *Sal*I-digested pBluescript, for which the ends had been rendered blunt; this construct was designated pBFK2.1RS (Fig. 1). Into this construct, a 1.3-kb *Hin*cII kanamycin resistance cassette was inserted at the *Hin*cII site within *mrpG*. Then, the 3.8-kb *Pvu*II fragment, containing the kanamycin resistance cassette flanked by homologous sequences of 1.0 kb upstream and 1.1 kb downstream of the point of the kanamycin resistance cassette insertion, was cloned into the *Sma*I site of pCVD442 (8), a pRK2-derived suicide vector. This construct, designated pBFK2.1RS-K, was electroporated into *P. mirabilis* HI4320 (Fig. 1). Transformants were selected on Luria-Bertani agar plates containing kanamycin (50 μg/ml). All of the 34 transformants that grew on these plates were also ampicillin resistant, suggesting that they resulted from a single-crossover event and therefore represented cointegrates. The *sacB* gene on pCVD442 and the kanamycin resistance cassette inserted into *mrpG* were used to select for a double-crossover

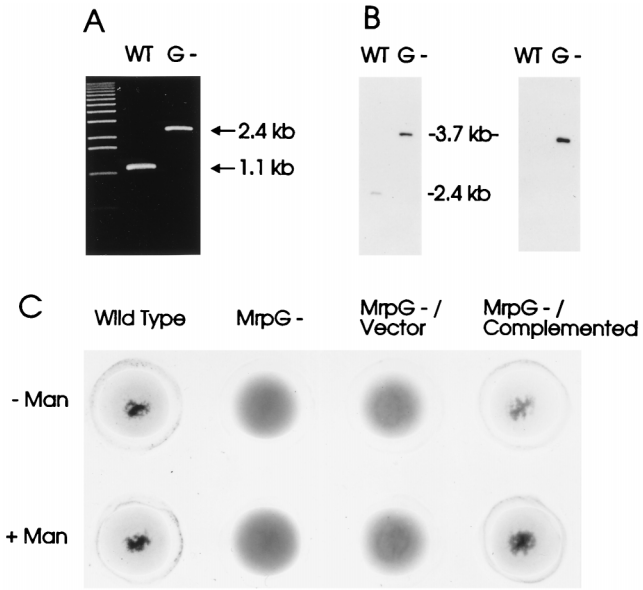


FIG. 2. Genotypic and phenotypic analysis of the *mrpG* mutant. (A) Products of PCR analysis of chromosomal preparations of the wild-type (WT) and *mrpG* mutant (G−) strains. The first lane contains the 1-kb-ladder marker. Predicted size fragments are shown and suggest insertion of a 1.3-kb kanamycin resistance cassette within *mrpG*. (B) For Southern blot analysis, chromosomal preparations of the wild-type (WT) and *mrpG* mutant (G−) strains were digested with *Pvu*II, separated on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with an *mrpG* sequence-specific probe (left panel) and an *aphA* (kanamycin resistance cassette) sequence-specific probe (right panel). Fragments of predicted sizes hybridized with the probes and suggest insertion of a 1.3-kb kanamycin resistance cassette within *mrpG*. (C) Hemagglutination assays of the wild-type strain, *mrpG* mutant strain (MrpG−), *mrpG* mutant strain containing pQE60 (MrpG−/Vector), and *mrpG* mutant strain containing pQEG2 (MrpG−/Complemented) were carried out with 3% chicken erythrocytes in the absence (−Man) and presence (+Man) of 50 mM mannose.

mutant from these cointegrates on Luria-Bertani agar plates containing 5% (wt/vol) sucrose and kanamycin (50 μg/ml). A Kan^r Amp^s colony was selected for screening as an *mrpG* mutant colony.

Genotypic analysis. PCR and Southern blotting were used to verify the integrity of the mutation in the *P. mirabilis* chromosome. One of the PCR primers, primer 512, was designed at a point just outside the upstream flanking sequence (Fig. 1) in order to confirm that the crossover took place at the correct site on the chromosome. The other primer, primer 513, was designed at a point just downstream of the *Hinc*II site within *mrpG* (Fig. 1) in order to discriminate between the mutant and the wild type by the distinctive sizes of the PCR fragments. As predicted, a 1.1-kb fragment and a 2.4-kb fragment were amplified from the chromosomal DNA samples of the wild type and the mutant, respectively (Fig. 2A), demonstrating that the mutant has a 1.3-kb insertion (the size of the kanamycin resistance cassette) within this region.

We also confirmed the integrity of the mutation by Southern blotting. Chromosomal DNA samples isolated from the wild-type and the mutant were cut with *Pvu*II and electrophoresed on an agarose gel. The blot prepared from this gel was hybridized sequentially with two probes: first with the *mrpG* probe and then with the *aphA* kanamycin resistance cassette probe (Fig. 2B). When probed with *mrpG*, a 2.4-kb fragment and a 3.7-kb fragment were labeled in the wild type and mutant, respectively, indicating that there was a 1.3-kb fragment inserted within this fragment in the mutant. When probed with the kanamycin resistance cassette, only the 3.7-kb fragment of the mutant was labeled, demonstrating that the kanamycin resistance cassette was inserted into this fragment. These results were consistent with the sizes of the predicted fragments and confirmed that the kanamycin resistance cassette insertion mutation within *mrpG* had been introduced into the chromosome of *P. mirabilis* HI4320 and that the mutant was a product of a double-crossover allelic exchange.

Phenotypic analysis. To test our hypothesis that MrpG is an adhesin, we tested the wild type and the *mrpG* mutant for the ability to hemagglutinate chicken erythrocytes in the presence of mannose (using the phenotype of MR/P-fimbriated *P. mirabilis*). We found that the isogenic *mrpG* mutant of HI4320 was negative for hemagglutination, whereas wild-type HI4320 was positive (Fig. 2C). However, after repeated passages (six to seven passages), the *mrpG* mutant did show a very weak hemagglutinating activity (data not shown).

Analysis of virulence in a mouse model of ascending urinary tract infection. To assess the contribution of the MrpG protein of the MR/P fimbria to colonization and virulence in the urinary tract, 10 CBA mice were each challenged with wild-type *P. mirabilis* HI4320 or its isogenic *mrpG* mutant. At 1 week postchallenge, the geometric mean values of urine, bladder, and kidney for wild-type and mutant strains were as follows: urine, 7.52 (wild type) versus 3.24 (mutant) $\log_{10}$ CFU/ml ($P = 0.02$); bladder, 5.61 versus 1.13 $\log_{10}$ CFU/g ($P = 0.0008$); and kidney, 4.88 versus 2.47 $\log_{10}$ CFU/g ($P = 0.0017$) (Fig. 3). The *mrpG* mutant colonized the urine, bladder, and kidneys in significantly smaller numbers than did the wild-type strain.

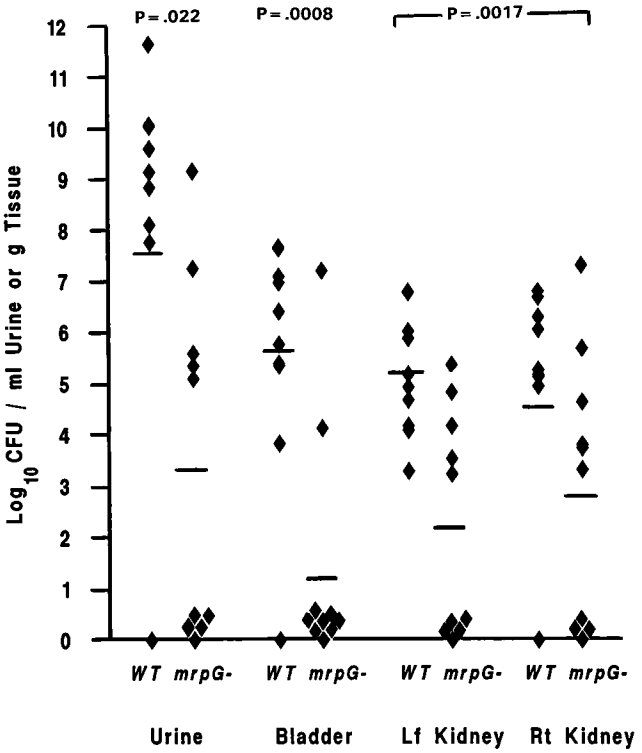


FIG. 3. CBA mouse model. Quantitative bacterial counts in urine specimens, bladders, and kidneys of mice challenged with either wild-type *P. mirabilis* HI4320 (WT) or its MrpG-negative mutant (*mrpG*−) are shown. Each diamond represents the CFU per milliliter of urine or gram of tissue from an individual mouse. Horizontal lines represent the geometric means of the colony counts. Diamonds near the *x* axis represent counts that are less than 100 CFU, the limit of detection in this assay. *P* values, shown at the top, were derived by the Mann-Whitney test. Lf, left; Rt, right.

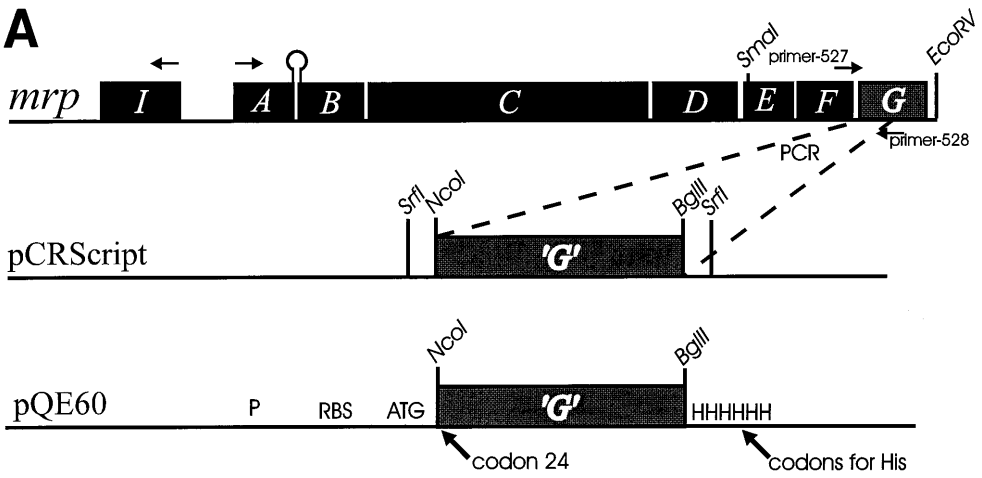
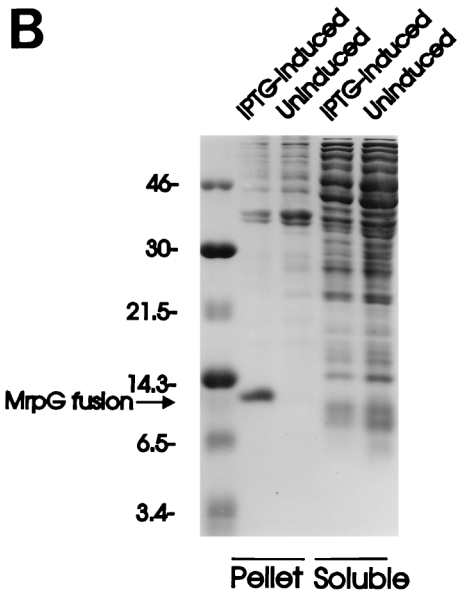


FIG. 4. Overexpression of MrpG. (A) Genetic constructs for MrpG overexpression. A 410-bp fragment carrying a truncated *mrpG* gene (*'G'*) was PCR amplified with primer 527 and primer 528 by using plasmid pBFK2.1RS (Fig. 1) and cloned into the *Srf*I site within pCRScript. A 400-bp *Nco*I-*Bgl*II fragment from this plasmid was subcloned into *Nco*I–*Bgl*II-digested pQE60, which provided the promoter (P), ribosome binding site (RBS), and start codon (ATG). (B) SDS-polyacrylamide gel electrophoresis of fractions containing the MrpG fusion protein. French press lysates of IPTG-induced or uninduced cultures were centrifuged at 4,000 × *g* for 1 h. Soluble protein was saved and insoluble protein (Pellet) was suspended in PBS. Protein was denatured in SDS-gel sample buffer, electrophoresed on an SDS–15% polyacrylamide gel, and stained with Coomassie blue. The sizes of standard protein markers run in the first lane are indicated in kilodaltons. The arrow points to the predicted electrophoretic mobility of the MrpG fusion protein.



Overexpression of MrpG′-His$_6$ fusion protein in *E. coli*. To verify the presence or absence of the fimbrial subunit, antiserum was raised against a truncated MrpG protein. MrpG was overexpressed as a fusion protein with a six-histidine tail in *E. coli* (Fig. 4A). Since the hydropathy plot of MrpG shows that it has a 24-amino-acid hydrophobic region at its N terminus, which is characteristic of a prokaryotic signal peptide, we eliminated this region in the construction of the fusion protein (see Materials and Methods). The C-terminal 100 amino acids, which contain the chaperone-binding and polymerization domains, were also not included in the fusion protein (11). The MrpG fusion protein was overexpressed in *E. coli* by induction with IPTG and electrophoresed on an SDS-polyacrylamide gel (Fig. 4B). The induced preparation had a unique band at a position of the predicted size (12 kDa) for the MrpG′-His$_6$ fusion protein derived from the insoluble protein fraction (Fig. 4). The polypeptide was not observed in the soluble protein fraction. Since the region around this band appeared to be uncontaminated by other polypeptides in both the induced and uninduced samples, we purified this fusion protein by simply excising the band from the gel and used this sample directly for rabbit immunization. Antisera against purified MrpG′-His6 fusion protein and purified whole MR/P fimbriae were collected from immunized rabbits (see Materials and Methods).

Western blot analysis. Since the amount of MrpG in whole-cell samples is too low to be detected, partially purified fimbrial samples (obtained by following the procedure for fimbrial isolation excluding the CsCl gradient centrifugation step) were used for Western blotting. Anti-MrpG antiserum showed that a specific band of about 18 kDa (mature MrpG is predicted to be 17.2 kDa) was absent from the *mrpG* mutant sample (Fig. 5A), confirming that the mutant does not express MrpG. Surprisingly, however, a Western blot analysis with anti-MR/P whole-fimbria antiserum revealed that MrpA, the major structural subunit of the fimbriae, was also absent from the *mrpG* mutant (Fig. 5B). This observation cast considerable doubt on the idea that the loss of functional MrpG alone is responsible for the loss of hemagglutination. This led us to ask whether the *mrpG* mutant was still capable of producing MR/P fimbriae.

Complementation of the *mrpG* mutant. Because *mrpG* is near the end of the *mrp* operon, we were originally not concerned that an insertional mutation would have any polar effects on upstream genes. However, following the observation that MR/P fimbriae were not produced in the *mrpG* mutant, it was necessary to demonstrate that the mutant would be complemented in trans with cloned *mrpG*.

A fragment carrying the wild-type *mrpG* gene containing its own ribosome binding site and start and stop codons was PCR amplified and cloned into the *Eco*RI and *Hin*dIII sites of pQE60; the resulting construct was designated pQEG2. *mrpG* expression was predicted to be under the control of a *lac* promoter. Because *P. mirabilis* is a *lac*-negative species, it was expected that *mrpG* would be constitutively expressed after the construct was electroporated into the mutant and that the *mrpG* mutant would be complemented.

The phenotypic characteristics of the complemented mutant were identical to those of the wild type. The mutant hemag-

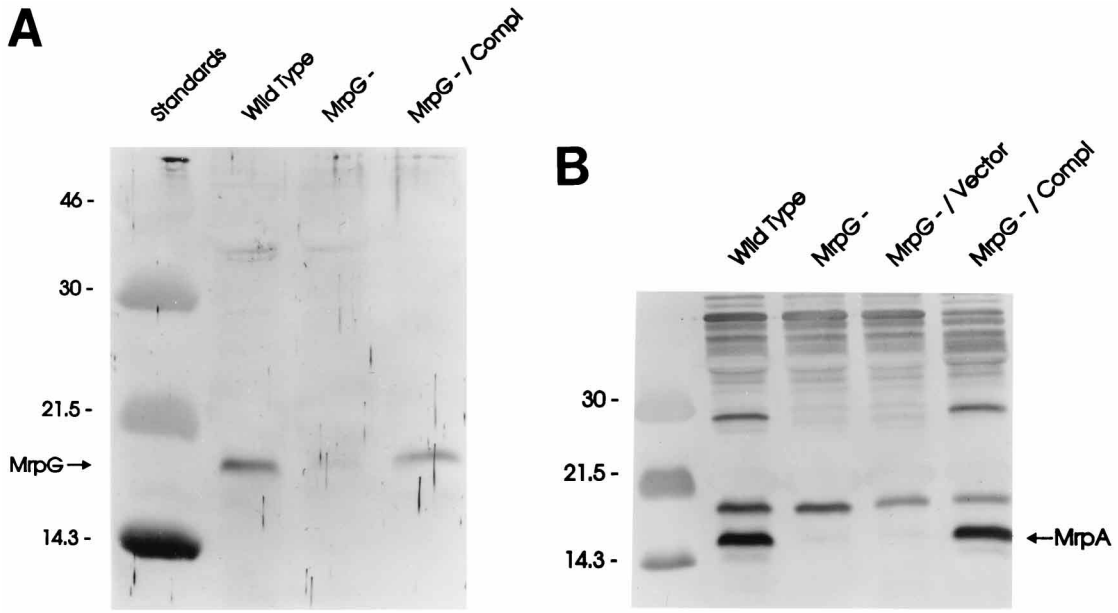


FIG. 5. Western blot analysis of partially purified fimbrial preparations. Partially purified fimbrial samples of the wild-type strain, the *mrpG* mutant strain (MrpG−), the *mrpG* mutant strain containing vector pQE60 (MrpG−/Vector), and the *mrpG* mutant strain complemented with pQEG2 (MrpG−/Compl) were electrophoresed and subjected to Western blot analysis with antiserum prepared against the MrpG fusion protein (A) or whole MR/P fimbriae (B). The sizes of standard proteins are shown on the left in kilodaltons. Immunoreactive bands predicted to represent MrpG (A) or MrpA (B) are indicated.

glutinated chicken erythrocytes in the presence and absence of 50 mM mannose (Fig. 2C), it expressed both MrpG and MrpA (Fig. 5) as identified on Western blots, and it produced normal MR/P fimbriae as observed by immunogold electron microscopy (data not shown). We therefore confirmed that the *mrpG* mutant could be complemented in *trans* with cloned *mrpG* on plasmid pQEG2.

Immunogold electron microscopy. As determined by traditional negative staining, both the wild-type strain and the *mrpG* mutant are heavily fimbriated, and MR/P fimbriae cannot be distinguished from the other fimbrial types (data not shown) when observed by transmission electron microscopy. With antiserum to MR/P whole fimbriae as the primary antibody, most of the fimbriae on the wild-type strain were labeled with gold particles (Fig. 6A). In the *mrpG* mutant, however, none of the fimbriae were gold labeled. Upon repeated static passages (six to seven passages) of the *mrpG* mutant, however, a few bacteria that had some defective filamentous structures labeled extensively with gold particles, like the MR/P fimbriae in wild-type HI4320, were identified on a grid (Fig. 6B). The morphology of these structures was clearly abnormal, and their appearance was rare. We concluded from these observations that the *mrpG* mutant was poorly capable of producing MR/P fimbriae. These extensively gold-labeled defective filamentous structures on the *mrpG* mutant observed after repeated static passages may have been the structures responsible for the weak hemagglutinating activity of these bacterial cells.

Tip localization of MrpG. With anti-MrpG antiserum as the primary antibody, MrpG was located basically at the tips of fimbriae on wild-type cells (Fig. 7). However, only a small proportion of the cells were labeled with gold particles, and most of the gold particles were attached to the tips of short, probably newly synthesized, fimbriae (Fig. 7B). In addition to the tip localization, gold beads were localized where fimbriae met between aggregating bacteria (Fig. 7).

DISCUSSION

To test the hypothesis that MrpG is expressed at the fimbrial tip and is the functional adhesin for MR/P fimbriae, we constructed an *mrpG* mutant from wild-type *P. mirabilis* HI4320. The mutation was verified at the DNA level by Southern blotting and PCR and at the protein level by Western blotting. The *mrpG* mutant was shown to have a disrupted *mrpG* gene and to not express MrpG protein. The isogenic mutant did not have the ability to hemagglutinate erythrocytes in a mannose-resistant manner, an ability characteristic of MR/P-fimbriated bacteria. The *mrpG* mutant was significantly less virulent than the parent strain in a CBA mouse model of ascending urinary tract infection. This evidence suggested that MrpG functions as an adhesin that recognizes the receptor on erythrocyte surfaces. However, a Western blot of partially purified fimbriae developed with antiserum to MR/P whole fimbriae and immunogold electron micrographs revealed that other protein components of MR/P fimbriae, including MrpA, were absent in the *mrpG* mutant, and whole MR/P-fimbrial structures were not observed. This raised the possibility that although *mrpG* is near the end of the *mrp* gene cluster, the *mrpG* mutation had an effect on expression of other genes in the operon.

To rule out any polar effects of the disruption of *mrpG* with the antibiotic resistance cassette, complementation of the mutation with cloned *mrpG* was attempted. Wild-type HI4320 and the *mrpG* mutant transformed with pQEG2, which carries *mrpG*, were positive for hemagglutination, while the *mrpG* mutant and the *mrpG* mutant carrying vector pQE60 were negative for this phenotype. Hence, despite the fact that the *mrpG* mutation was complementable with cloned *mrpG*, we were unable to directly attribute the loss of hemagglutinating activity to the disruption of the *mrpG* gene because of the effect of the mutation on the synthesis of normal MR/P fimbrial structures.

Use of the polyclonal anti-MrpG serum also did not provide

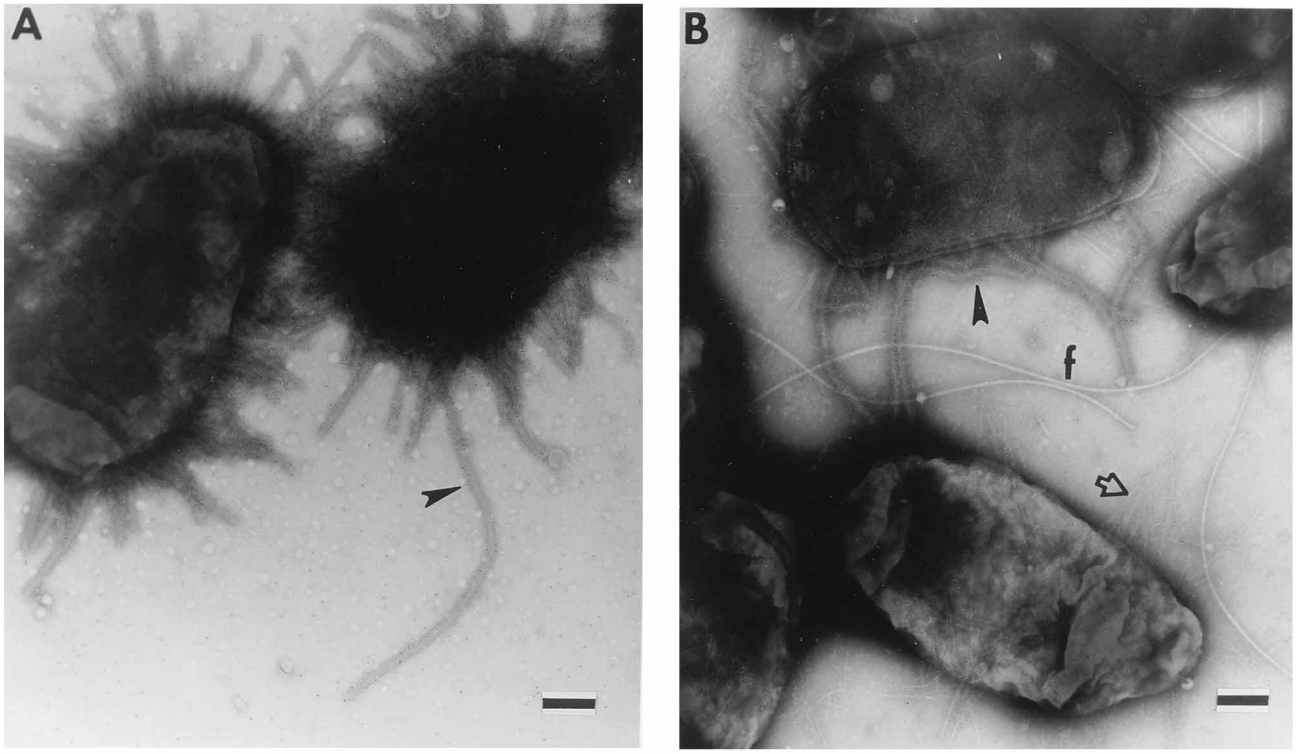


FIG. 6. Immunogold electron microscopy with anti-MR/P-fimbria serum as the primary antibody. *P. mirabilis* HI4320 (A) and its isogenic *mrpG*-negative mutant (B) were reacted first with rabbit antiserum raised against purified MR/P fimbriae and then with a secondary antibody (recombinant protein G) conjugated to 5-nm-diameter gold particles. The arrowheads point to immunoreactive protein, i.e., MR/P fimbriae (A) and defective fimbrial structures (B). The arrow points to nonreactive fimbriae (other fimbrial types in *P. mirabilis*). An "f" is placed above flagella. Bar, 200 nm.

evidence that MrpG functions as an adhesin or agglutinin. First, preincubation of strain HI4320 with antiserum to MR/P whole fimbriae inhibited hemagglutination, but preincubation of strain HI4320 with anti-MrpG antiserum did not (data not shown). In addition, antiserum to MR/P whole-fimbriae did not recognize MrpG protein clearly on a Western blot, which may mean that even though MrpG protein is not blocked with the antiserum, MR/P fimbriae lose their hemagglutinating activity. Second, strain HI4320 transformed with pQEG2 overexpressed MrpG protein and had normal MR/P-fimbrial structure but lower hemagglutinating activity than wild-type HI4320 (data not shown). Again, however, this does not prove that MrpG is not the adhesin. Antiserum to MrpG was raised against a denatured fusion protein that lacked C-terminal amino acid residues. Epitopes required for receptor binding may be conformational in nature and present only in the native protein or at the C terminus. Therefore, antibodies capable of preventing binding may not be present in the serum. On the other hand, anti-MR/P-fimbria serum was raised against native structures which would have retained such binding moieties. Antibodies elicited by these domains would be capable of inhibiting hemagglutination, which would explain the activity of the whole-fimbria antiserum.

Are the other proteins encoded by the *mrp* operon expressed in *mrpG* mutant? The successful complementation of the *mrpG* mutant with pQEG2 suggests that all the other proteins encoded by the *mrp* operon are normally expressed. Immunogold labeling of the *mrpG* mutant on the cell surface and the occasional appearance of defective filamentous structures imply that at least some of the proteins that form the MR/P-fimbrial structures are expressed and transported out of the bacterium. If so, it is clear that MrpG protein plays a critical role in fimbria formation. However, we cannot rule out the possibility that the MrpG protein contributes to a feedback mechanism; without MrpG, no high-level expression or export of the other proteins may occur.

Immunogold staining with anti-MrpG serum revealed MrpG to be at the tips of many fimbriae or at the interface of aggregating bacteria. Most fimbriae, whether colabeled or not with antiserum to whole-MRP-fimbriae, were, however, not labeled with the anti-MrpG serum. One reason that only a small proportion of the short fimbriae were gold labeled at the tips with anti-MrpG antiserum could be that the tips of longer fimbriae are more easily sheared than those of shorter fimbriae. The location of the MrpG protein at the tips of fimbriae suggests that MrpG might play a role in initiating fimbrial assembly or might simply be the first MR/P subunit to be exported out of the cell.

Although the genetic organization of the *mrp* gene cluster is similar to that of the *E. coli pap* operon (13), there appear to be distinct differences. Mutations in *papG*, encoding the digalactoside-binding adhesin, result in normal synthesis of P-fimbrial structures that lack only the tip adhesin (17). A complementable mutation of *mrpG* of *P. mirabilis*, however, prevents normal fimbrial assembly. This suggests that, unlike P fimbriae, this gene product of the *mrp* fimbrial gene cluster, which is present at the fimbrial tip, is required for normal fimbrial assembly. MrpG is not the only example of a minor pilin that is required for fimbrial biogenesis. FasG of 987P fimbriae from *E. coli*, FimD from *Bordetella pertussis*, and HifE from *Haemophilus influenzae*, minor pilins that function as adhesins, are all required for the exportation of the other pilins in their respective

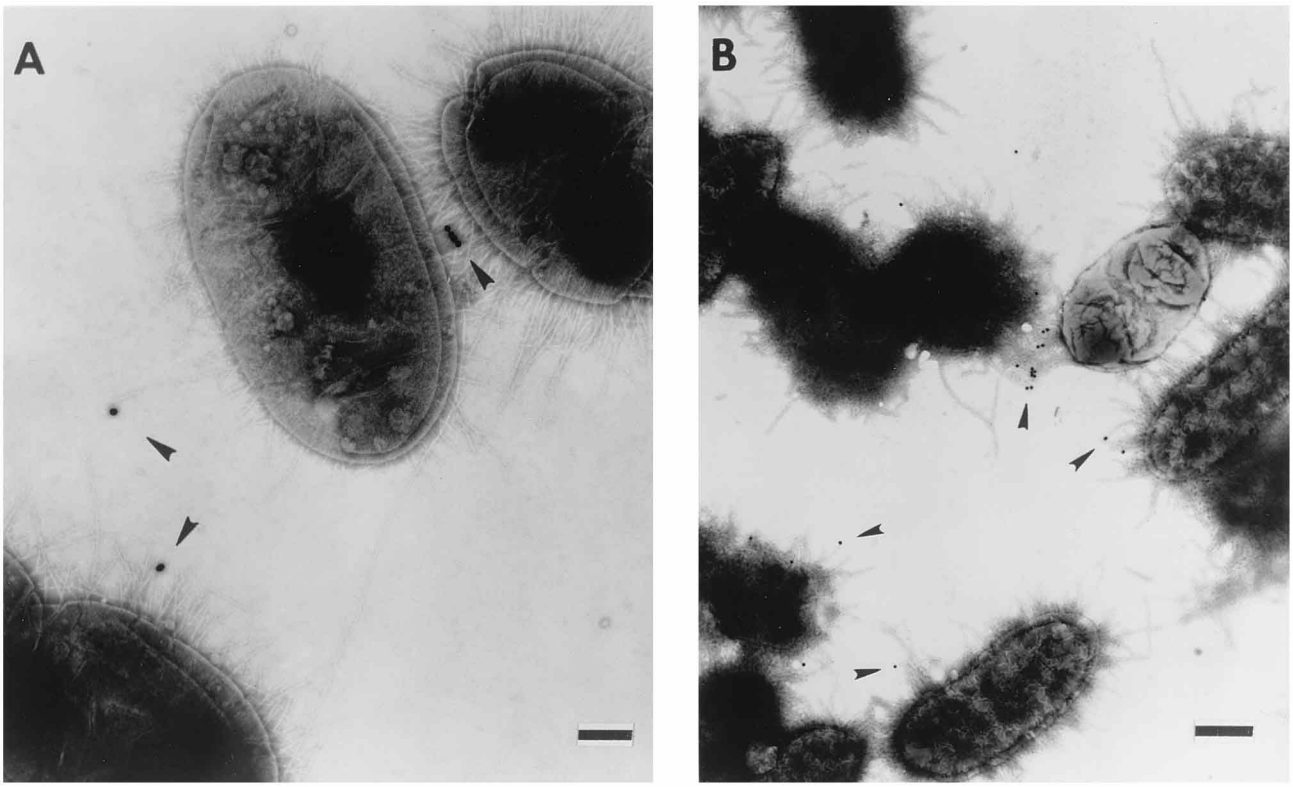


FIG. 7. Immunogold labeling of the wild-type strain with anti-MrpG serum as the primary antiserum. *P. mirabilis* HI4320 was reacted first with rabbit antiserum raised against purified MrpG fusion protein and then with a secondary antibody (goat anti-rabbit IgG) conjugated to 30-nm-diameter gold particles (A and B). For panel B, bacteria were also reacted with antiserum raised against MR/P fimbriae and then with a secondary antibody (recombinant protein G) conjugated to 5-nm-diameter gold particles. Arrowheads point to fimbrial-tip-localized 30-nm-diameter gold particles. Bars, 200 nm (A) and 500 nm (B).

systems and therefore are essential for fimbrial biogenesis (14, 22, 27). In *E. coli* type 1 fimbriae as well, minor pilins, including the adhesin FimH, have significant effects on the longitudinal morphology of the fimbriae (15).

ACKNOWLEDGMENT

This work was supported by Public Health Service grant DK47920 from the National Institutes of Health.

ADDENDUM IN PROOF

Recent studies have revealed an additional open reading frame, *mrpH*, downstream of *mrpG*, which encodes a 275-amino-acid polypeptide with significant homology to SmfG (37% identity) and PapG (22% identity). The newly acquired 3′ sequence of *mrpG* revealed that this gene encodes a homolog of SmfF and PapK. This finding is consistent with our result that MrpG is necessary for MR/P fimbrial biogenesis and is located near the fimbrial tip but does not represent the adhesin itself; a similar role was assigned for PapK in the biogenesis of P fimbriae.

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Editor: A. O'Brien