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Identification of Two Different Hemolysin Determinants in Uropathogenic *Proteus* Isolates

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DNA sequences similar to those of the *Escherichia coli* hemolysin genes were detected among uropathogenic isolates of *Proteus vulgaris* and *Morganella morganii* by using the Southern blotting technique and *hly* gene-specific DNA probe. Immunoblotting revealed that among the hemolytic *P. vulgaris* and *M. morganii* isolates there was expressed a polypeptide species similar in molecular size (110 kilodaltons) and antigenicity to *Escherichia coli* HlyA. A plasmid-mediated *P. vulgaris* hemolysin determinant identified by Southern blotting analysis was molecularly cloned, and the recombinant plasmid (pWPV100) was characterized by restriction endonuclease fragment mapping. A second recombinant library of genomic DNA prepared from a hemolytic, urinary tract isolate of *Proteus mirabilis* was constructed in *E. coli*. A 5.5-kilobase *XhoI* fragment encoding an extracellular hemolytic activity was molecularly cloned (pWPM100), and this plasmid was subjected to transposon-mediated mutagenesis with Tn*phoA*. The *P. mirabilis* hemolytic phenotype was determined to be encoded by a polypeptide species (HpmA) with an estimated molecular size of 140 kilodaltons based on minicell polypeptide analysis of pWPM100 and its mutant derivatives. Southern blotting analysis with a *HpmA*-specific DNA probe revealed that this novel determinant is commonly found in both *Proteus* species but is not present in hemolytic isolates of *M. morganii*, *E. coli*, *Citrobacter freundii*, and *Serratia marcescens*.

Escherichia coli and *Proteus* species stand out as two major causes of opportunistic and nosocomial infections in humans (18, 29, 36). In particular, these two organisms represent the two most common gram-negative bacteria that cause urinary tract and surgical wound infections (29). The identification and characterization of virulence determinants involved in the pathogenesis of extraintestinal *E. coli* diseases is the subject of intensive research in many laboratories. The study of the pathogenesis of *Proteus* diseases is meager by comparison. Urease activity has been suggested to be an important factor in pyelonephritis caused by *Proteus* species (19, 22). Recently, Wray and co-workers (35) identified a *Proteus mirabilis* adhesin that was responsible for adherence to urinary epithelial cells. Peerbooms et al. (25) correlated hemolytic activity with Vero cell invasiveness by isolates of *P. mirabilis*. The *P. mirabilis* hemolytic activity remains uncharacterized and has not been demonstrated to be directly involved in cellular invasion.

E. coli hemolysin is a protein cytotoxin capable of disrupting respiratory function and killing isolated rat renal tubular cells (16). There are numerous reports that the hemolysin can interfere with important functions of human neutrophils (3, 10, 31). These data reinforce the long-standing epidemiologically based view that the hemolysin plays a significant role in several extraintestinal human diseases caused by *E. coli* (2, 6, 9). Although hemolysin is a factor that adds to the virulence of *E. coli* in rodent models of peritonitis (12, 32) and pyelonephritis (30), the specific contribution that it makes to the pathogenesis of extraintestinal *E. coli* diseases remains to be demonstrated conclusively.

The analysis of the DNA sequence of a chromosomal hemolysin of an *E. coli* urinary tract isolate lead to the conjecture that hemolysin is a determinant which, in an evolutionary sense, only recently came to reside within the *E. coli* gene pool (8). This was based on the observation that the guanine and cytosine content of the 8.2-kilobase (kb) *hly* region was only 39% compared with the *E. coli* genomic content of approximately 50 to 52% (8). In addition, the codon usage pattern was atypical for *E. coli*, with an abundance of codons requiring rare iso-accepting species of tRNAs (8). This evidence supports previous DNA hybridization results that indicated that there is limited dissemination of *hly* sequences among *E. coli* (12, 34). Recently, C. Strathdee and R. Lo (personal communication) discovered that the *Pasteurella haemolytica* leukotoxin shared extensive amino acid sequence homology with the *E. coli* hemolysin structural gene (*hlyA*). Evidence is presented here which shows that an *E. coli hlyA*-like gene product can be found in *Proteus vulgaris* as well as *Morganella morganii*. In addition, a second novel extracellular hemolysin common to uropathogenic *Proteus* isolates is identified.

MATERIALS AND METHODS

Bacteria and bacteriophage strains. The clinical isolates used in this study were taken from collections of the Wisconsin State Health Laboratory, the University of Wisconsin Medical School teaching laboratory, and the University of Wisconsin Hospital Clinical Microbiology Laboratory. Some of the isolates of *M. morganii* were provided by D. J. Flourney, University of Oklahoma Health Science Center (Norman, Okla.). The origin of recombinant *E. coli* WAF270 and the clinical isolate J96 have been described elsewhere (33). *E. coli* CC118 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74* *phoA*Δ*20* *galE* *galK* *thi* *rpsE* *rpoB* *argE*(Am) *recA1*] and bacteriophage lambda::Tn*phoA-1* were provided by Colin Manoil and Jonathan Beckwith (21). *E. coli* DH1 [F^- *recA1* *endA1* *gyrA96* *thi-1* *hsdR17* (r^- m^+) *supE44*] and P678-54 (*minA* *minB*) were acquired from Duard Walker, University of Wisconsin, and Steve Moseley, University of Washington, Seattle, respectively.

Media, reagents, and chemicals. L-broth and L-agar were prepared by the method described by Maniatis et al. (20).

Antibiotics, 5-bromo-4-chloro-3-indolyl phosphate, salts, and buffers were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases and other DNA-modifying enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.) and Boerhinger Mannheim Biochemicals (Indianapolis, Ind.). Nitrocellulose was acquired from Schleicher & Schuell, Inc. (Keene, N.H.). Defibrinated sheep blood was provided courtesy of Sarah Hurley and the University of Wisconsin Department of Veterinary Sciences. Reagents for agarose and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were bought from Bio-Rad Laboratories (Richmond, Calif.) and Bethesda Research Laboratories (Gaithersburg, Md.).

Immunoblotting. The growth of L-broth cultures was monitored by measuring the optical density at a wavelength of 600 nm. At various intervals, 25 μl of culture was removed and added to an equal amount of 2× crack buffer (0.13 M Tris [pH 6.8], 4% SDS, 20% glycerol, 0.002 bromphenol blue, 2% 2-mercaptoethanol). An 8-μl sample of this mixture was subjected to SDS-PAGE. Prestained molecular weight markers (Sigma) were included to monitor the progress of polypeptide separation. The polypeptides present in the gel were then transferred electrophoretically to nitrocellulose filters, and immunoblotting was performed by the method described by Blake et al. (1). Hyperimmune anti-HlyA serum was prepared by immunization of rabbits with electrophoretically purified HlyA. The antiserum was diluted 1,000-fold in saline and added to the blots. This was incubated overnight, the blots were washed, and the presence of bound rabbit antibody was detected by the addition of goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma) and colorimetric development of bound alkaline phosphatase.

Southern blotting analysis. A total of 2 ml of a late-log-phase L-broth culture was subjected to a Triton X-100 method of rapid plasmid isolation, and the DNA was fractionated by agarose gel electrophoresis as described previously (34). The DNA was mildly depurinated by acid treatment and then transferred to sheets of nitrocellulose by Southern blotting (28). A pSF4000 7.0-kb *Ava*I fragment internally spanning all four *E. coli hly* cistrons (8) was isolated by electroelution and radiolabeled by nick translation (26) with a kit and directions given by the supplier (Bethesda Research Laboratories). Both [α-^{32}P]ATP and [α-^{32}P]CTP (New England Nuclear Corp., Boston, Mass.) were used to radiolabel the DNA probes. The blots were hybridized for 20 h at 37°C in 35% formamide in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Following hybridization the blots were washed in 0.1× SSC at 60°C. The washed blots were dried and then subjected to autoradiography.

Construction of pWPV100 and pWPM100. Cesium chloride gradient purification of genomic DNA from *P. vulgaris* 414-3 and *P. mirabilis* 477-12 was performed by the method described by Hull et al. (14). These DNAs were digested with a restriction endonuclease and mixed with appropriately digested pUC19 vector DNA, and ligation was achieved by using T4 DNA ligase under conditions recommended by the supplier (New England BioLabs). The DNA mixture was transformed into *E. coli* DH1, and selection of transformants was made on ampicillin-containing (100 μg/ml) sheep erythrocyte agar plates (34). Hemolytic recombinant clones were reisolated under selective conditions, and the recombinant plasmid DNA was isolated by the method described by Ish-Horowicz and Burke (15). The plasmid DNA was subjected to restriction endonuclease digestion with fragment maps constructed by pairwise digestion with various species of enzymes and analysis of the resulting fragment patterns by agarose gel electrophoresis.

Tn*phoA*-mediated mutagenesis of pWPM100. Bacteriophage lambda::Tn*phoA* at a multiplicity of infection of 0.1 was used to infect CC118 isolates transformed with pWPM100. Selection of transductants was made by plating the infected cells on L-agar containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 30°C. The colonies were pooled by scraping them from the plate, and plasmid DNA was prepared from these cells (15). The plasmid DNA was used to transform CC118, with selection being made on ampicillin-kanamycin sheep erythrocyte agar plates incubated at 37°C. Both hemolytic and nonhemolytic transformants were observed and reisolated for further analysis. The pWPM100::Tn*phoA* derivatives were subjected to restriction endonuclease fragment analysis to determine the location of the Tn*phoA* inserts.

Minicell analysis of plasmid-encoded polypeptides. Several mutant derivatives as well as pWPM100 were transformed into the minicell-producing *E. coli* P678-54. Minicells were purified and polypeptides were labeled with [^{35}S]methionine by methods described previously (8). The labeled plasmid-encoded polypeptides were subjected to SDS-PAGE. Size estimates and analysis of mutant results were performed by examination of flourograms of those gels.

Extracellular hemolysin assay. A modified protocol described by Welch and Falkow (33) was used in which late-log-phase L-broth culture supernatants were filtered (pore size, 0.2 μm; Gelman Sciences, Inc., Ann Arbor, Mich.). A sample of 200 μl of the filtered material was mixed with 800 μl of lysis assay buffer (with or without 10 mM $CaCl_2$) containing a suspension of washed sheep erythrocytes at a final concentration of 1%. The supernatant-erythrocyte mixture was incubated for 1 h at 37°C, the mixture was then centrifuged for 30 s in a microfuge, and the optical density of the supernatant was measured at 540 nm (3). The extracellular hemolysin activity (optical density at 540 nm per hour) of *P. mirabilis* 477-12 culture supernatants was arbitrarily set at 100 hemolytic units.

RESULTS

Nine isolates of *P. mirabilis*, three isolates of *P. vulgaris* and seven isolates of *M. morganii* were initially examined for the presence of a polypeptide species similar in size and immunoreactivity to *E. coli* HlyA by immunoblotting. The blots were done in duplicate so that the immunoreactivity of proteins to the hyperimmune sera could be compared with that of the preimmune sera. *E. coli* HlyA is maximally expressed at the late-log phase (7). In this study culture material from each strain was examined at three different intervals representing mid-log, late-log, and early-stationary phases. One *P. vulgaris* isolate and six *M. morganii* isolates produced a polypeptide species that comigrated with the 110-kilodalton (kDa) *E. coli* HlyA. This protein species was maximally produced in the late-log-phase cultures (data not shown). Shown in Fig. 1 is an example of these results with late-log-phase culture material. In each case it is apparent that there was some background reactivity; however, a comparison of the polypeptide pattern for the preimmune sera with that of the immune sera indicates antigenic identity to *E. coli* HlyA. It was observed consistently that greater reactivity occurred among several of the *Morganella* isolates than with *P. vulgaris* 414-3. This was reflected also in the

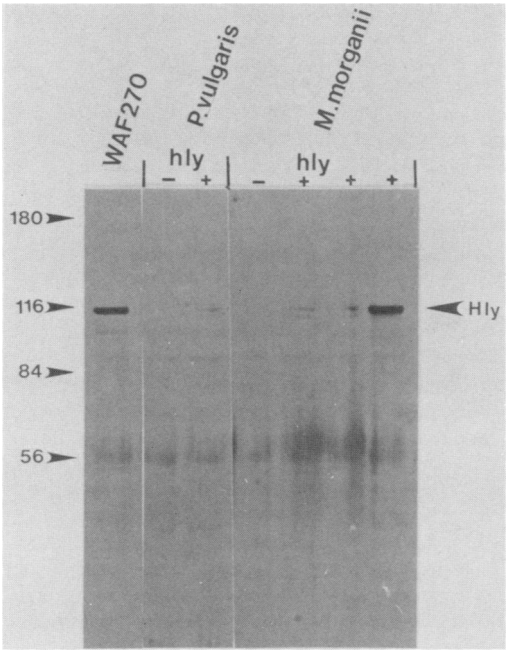

FIG. 1. Immunoblotting analysis. Shown is an immunoblot of total cell protein preparations of a recombinant, hemolytic *E. coli* strain (WAF270), two *P. vulgaris* isolates (414-5, 414-3), and four M. morganii isolates (W17, W1, W5, W6), from left to right, respectively. The hemolytic phenotype for each strain is denoted by plus and minus signs at the top of the figure. The presence of a polypeptide species among the hemolytic isolates that is similar in molecular size (indicated to the left of the gels, in kilodaltons) and antigenicity to *E. coli* HlyA is indicated by the arrowhead to the right of the figure.

difference in the hemolytic phenotype exhibited by these isolates on sheep erythrocyte agar. After overnight incubation, a zone of typical beta-hemolysis surrounding colonies of hemolytic *E. coli* was evident only for the *Morganella* strains. The *P. vulgaris* 414-3 isolate displayed hemolysis after 2 or 3 days of incubation. It was necessary to sweep away the cells to observe the hemolysis underneath the *Proteus* colonies.

The examination of the *Proteus* and *Morganella* isolates for DNA sequences similar to an *E. coli hly* probe sequence by Southern blotting revealed clear hybridization signals for the hemolytic *Morganella* isolates but marginal darkening of the X-ray film for any of the *Proteus* isolates. The hybridization signals for the six *Morganella* strains occurred in the gel region enriched for the linear chromosomal DNA that was present in the rapid plasmid preparations (Fig. 2). A clear hybridization signal with this particular probe and target DNA preparation was apparent only in one *P. vulgaris* isolate. This coincided with a 17-kb plasmid found in *P. vulgaris* 414-3.

Recombinant DNA libraries of genomic DNA from the hemolytic isolates *P. vulgaris* 414-3 and *P. mirabilis* 477-12 were constructed by using the simple shotgun methodology of insertion of DNA fragments into the vector pUC19. In the case of *P. vulgaris* 414-3, hemolytic clones were acquired with *Sal*I-digested genomic DNA but not with *Xho*I nor *Xba*I. The very small zone of hemolysis surrounding the *Sal*I-derived recombinant colonies was apparent only after 48 h of incubation at 37°C. Six hemolytic colonies were reisolated, and their plasmid DNAs were subjected to restriction endonuclease fragment mapping. All six clones harbored an identical plasmid that was called pWPV100. Its restriction endonuclease fragment map is depicted in Fig. 3. Based on the map of pWPV100, it is apparent that this plasmid is the result of the cloning of a partial *Sal*I digest fragment of the original 17-kb plasmid that was present in *P. vulgaris* 414-3. To help localize the pWPV100 *E. coli hly*-like determinant, a *Pst*I deletion derivative of pWPV100 was constructed in vitro by religation of the largest pWPV100 *Pst*I fragment (Fig. 3). The hemolytic phenotype that the pWPV100 *Pst*I deletion conferred was a distinct zone of beta-hemolysis around the colony which was evident after overnight incubation.

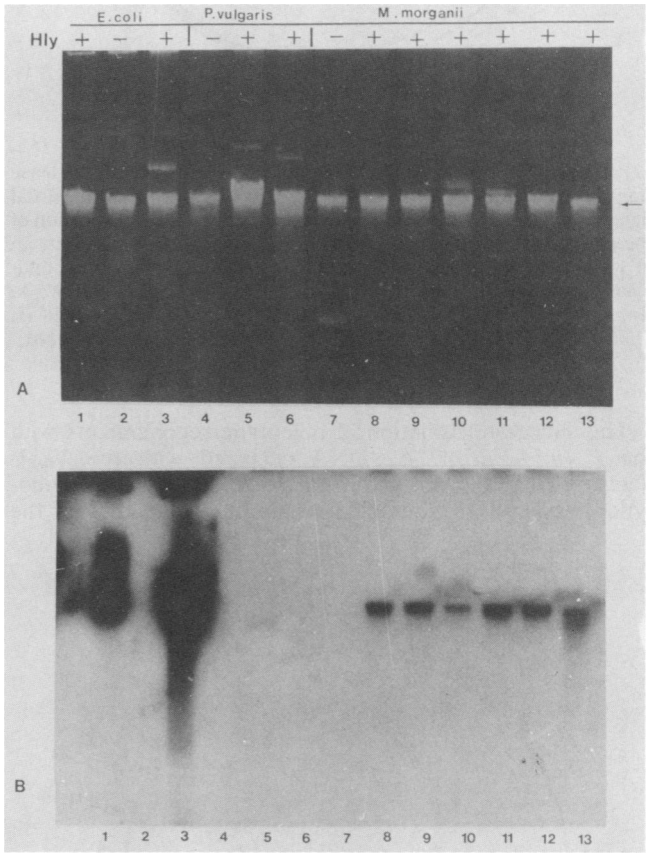

FIG. 2. Southern blotting analysis. (A) Photograph of an ethidium bromide-stained agarose gel containing DNA preparations of clinical isolates of *E. coli*, *P. vulgaris*, and *M. morganii*. The rapid plasmid-chromosomal preparation technique results in substantial contamination of linear chromosomal fragments, indicated by the arrow. Lanes 1 and 3 contain *E. coli* strains known to harbor *hly* DNA sequences (J96 and WAF270, respectively), whereas lane 2 is a nonhemolytic control strain (J198). Lanes 4 to 6 harbor DNA preparations from three clinical isolates of *P. vulgaris* (414-5, 414-3, and 478-2, respectively). The hemolytic phenotype for these strains is indicated by a plus or minus sign above the lane. Lanes 7 to 13 contain DNA from one nonhemolytic (W17) and six hemolytic isolates of *M. morganii* (W1 to W6), respectively. (B) Photograph of an autoradiogram of *E. coli hly*-specific hybridization signals present on a nitrocellulose blot of the gel depicted in panel A. The positive *E. coli* controls show strong hybridization signals, a faint signal is evident in the hemolytic *P. vulgaris* 414-3 isolate (lane 5) in the region of a 17-kb plasmid species migrating just in front of the chromosomal fragments, and a moderate signal can be seen in all six hemolytic *M. morganii* isolates in the chromosomal region.

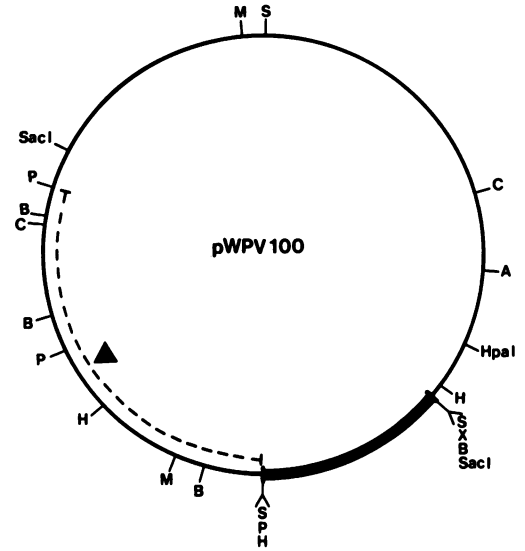

FIG. 3. Map of pWPV100. Shown is the restriction endonuclease fragment map for pWPV100 which consists of a 17-kb *Sal*I partial digest fragment inserted into the vector pUC19 (thickened portion of the circle). The inner arc labeled with a solid triangle represents an in vitro-constructed deletion of pWPV100. The restriction endonuclease digestion sites for Fig. 3 and 4 are abbreviated as follow: A, *Apa*I; Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

The successful isolation of hemolytic recombinants with the *P. mirabilis* 477-12 DNA occurred with the *Xho*I-digested DNA but not with *Sal*I-treated DNA. Four hemolytic colonies were identified within 18 h of plating of the

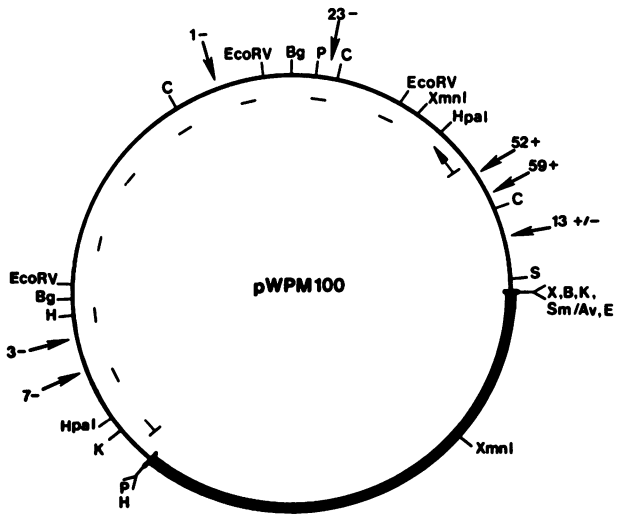

FIG. 4. Map of pWPM100. pWPM100 represents the insertion of a 5.5-kb *Xho*I fragment into the *Sal*I site of pUC19 (thickened portion of the circle). The dashed inner arc shows where the HpmA polypeptide is encoded. The location of Tn*phoA* insertions into pWPM100 are denoted by numbered arrows. If an insertion resulted in the loss of the hemolytic phenotype, the numbered arrow has a negative sign beside it. The pWPM100::Tn*phoA* insertion 13 derivative encodes a reduced hemolytic phenotype which is depicted as a plus or minus sign. The abbreviations used in this figure are identical to those given in the legend to Fig. 3.

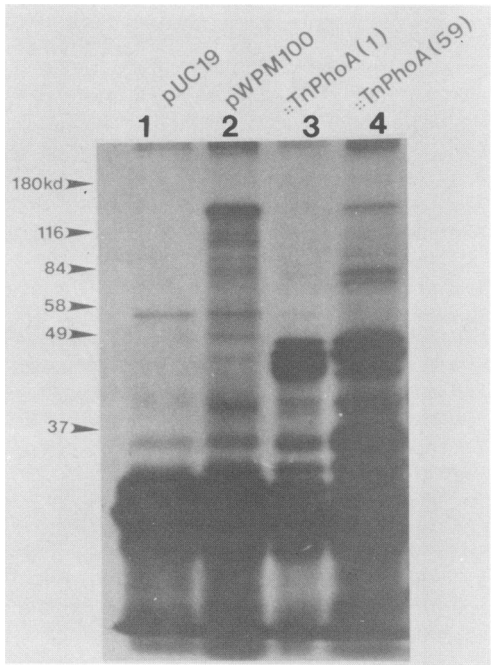

FIG. 5. Polypeptides encoded by different plasmids in minicells. Fluorogram of SDS-10% PAGE containing [^{35}S]methionine-labeled polypeptides encoded by different recombinant plasmid derivatives present in purified minicells. The plasmids employed are listed across the top of the figure. The 140-kDa HpmA polypeptide is evident in pWPM100 and the hemolytic pWPM100::Tn*phoA* insertion 59 derivative but is missing in the nonhemolytic Tn*phoA* insertion 1 mutant.

transformants. Restriction endonuclease fragment mapping of the plasmids harbored by these four clones revealed that they were identical. The physical map of one of these plasmids (pWPM100) is shown in Fig. 4. In this particular case, the hemolytic phenotype was encoded by a 5.5-kb *Xho*I fragment. Based on the DNA sequence and mutant analysis of the *E. coli hly*, just over 7.3 kb of DNA is required to encode the hemolytic phenotype (8). The apparent smaller *P. mirabilis* hemolysin determinant prompted an investigation to localize better the hemolysin gene(s) present on pWPM100. This was accomplished by use of the recombinant transposon Tn*phoA* and by minicell polypeptide analysis. Tn*phoA* acts as an insertional mutagen and also has the capability of creating gene fusions between a target gene and the transposon-encoded, leader peptideless alkaline phosphatase gene that is present in the terminus of the left repeat element (21). Sixty-four pWPM100::Tn*phoA* derivatives were isolated, and the location of the Tn*phoA* insertions was determined by restriction endonuclease fragment analysis. The numbered arrows shown in Fig. 4 represent the location of seven well-characterized, independent Tn*phoA* insertions. There was approximately a 1-kb region of the 5.5-kb *Xho*I insert of pWPM100 where Tn*phoA* insertions did not result in the loss of the hemolytic phenotype (e.g., pWPM100::Tn*phoA* insertion 59). The four insertions shown in Fig. 4 (counterclockwise, insertions 7, 3, 1, and 23) that inactivate the hemolytic phenotype also yielded active alkaline phosphatase gene fusions by virtue of the fact that they conferred a blue colony phenotype on L-agar plates containing the alkaline phosphatase indicator substrate 5-bromo-4-chloro-3-indolyl phosphate. The orientation of the Tn*phoA* element in each instance indicates that the fusions were to a

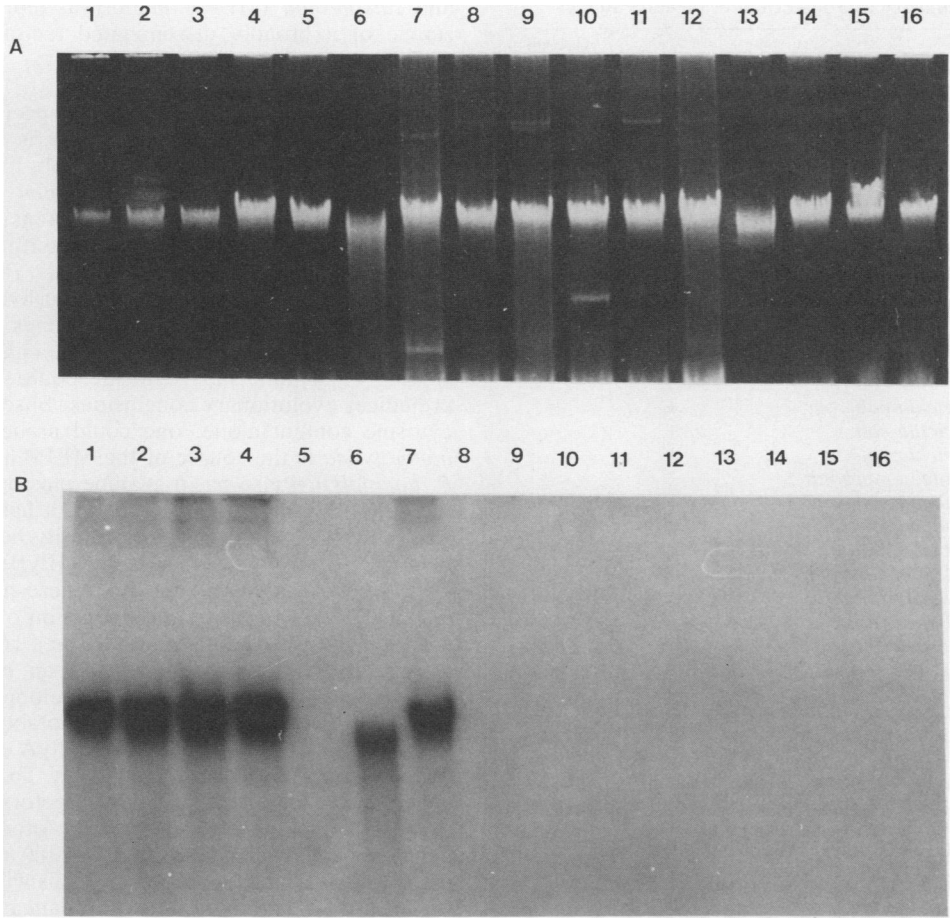

FIG. 6. Southern blotting analysis. (A) Photograph of an ethidium bromide-stained agarose gel containing rapid plasmid-chromosomal DNA preparations of clinical isolates of *Proteus*, *E. coli*, and *Morganella*. The numbers above the gel correspond to the isolates listed in Table 1. (B) Photograph of an autoradiogram of the 1.7-kb pWPM100-*Eco*RV probe-specific hybridization signals present on a nitrocellulose blot of the gel shown in panel A.

gene(s) encoded in the counterclockwise direction on the pWPM100 map.

The use of *E. coli* minicells harboring pWPM100 and several of its Tn*phoA* insertion derivatives permitted the determination that a single polypeptide (HpmA [hemolysin of *Proteus mirabilis*]) of 140 kDa was responsible for the hemolytic phenotype. The HpmA polypeptide is lost in the pWPM100::Tn*phoA* insertion 1 derivative (Fig. 5, lane 3) but is present in pWPM100::Tn*phoA* (insertion 59; Fig. 5, lane 4). Therefore, based on the length of DNA that is necessary to encode a polypeptide of that size and the orientation direction determined by the active alkaline phosphatase fusions, HpmA is encoded in the counterclockwise direction in the region shown as a dashed arc on the inside of the pWPM100 map (Fig. 4). The smaller molecular weight polypeptide species that are encoded by pWPM100 (Fig. 5, lane 2) but that are absent in the pUC19 background (Fig. 5, lane 1) are most likely proteolytic products of HpmA. This breakdown phenomenon is common to secreted polypeptides examined in a minicell background (7, 13).

Hemolytic activity was detected in the cell-free, late-log-phase culture supernatants of *P. mirabilis* 477-12. The hemolytic activity occurred in lysis assay buffer with or without 10 mM $CaCl_2$. The culture supernatants of the *E. coli* recombinant strain WPM100 possessed 10-fold greater hemolytic activity units relative to *P. mirabilis* 477-12. The cloned WPM100 hemolytic activity was also $CaCl_2$ independent. A negative control of *E. coli* DH1 had no detectable extracellular hemolytic activity. A Coomassie blue-stained polypeptide of 140 kDa was detected by SDS-PAGE of trichloroacetic acid-precipitable material from 1 ml of WPM100 culture supernatants (data not shown).

The discovery of the novel *P. mirabilis* extracellular hemolysin prompted a survey of its dissemination among other *Proteus* isolates as well as members of the family *Enterobacteriaceae* and other hemolytic, gram-negative bacilli. A 1.7-kb pWPM100-*Eco*RV fragment representing an internal encoding portion of HpmA was used as a probe in Southern blots containing DNA preparations similar to those employed in Fig. 2. An example of these results is shown in Fig. 6. There were DNA sequences similar to the *Eco*RV probe in all four of the hemolytic *P. mirabilis* isolates and two of the three hemolytic *P. vulgaris* isolates tested. *P. vulgaris* 414-3, which harbored the *E. coli hly*-like determinant, did not have HpmA-like gene sequences. In fact, other than *Proteus* strains, no isolate tested gave a positive hybridization signal. The results of this survey are summarized in Table 1.

TABLE 1. Summary of Southern hybridization studies

Strain[a]	Organism	Hemolytic phenotype	Hybridization to pWPM100-*Eco*RV 1.7-kb probe
1. 477-12	*Proteus mirabilis*	+	+
2. 468-1	*Proteus mirabilis*	+	+
3. 471-20	*Proteus mirabilis*	+	+
4. 434-21	*Proteus mirabilis*	+	+
5. 414-3	*Proteus vulgaris*	+	−
6. 478-2	*Proteus vulgaris*	+	+
7. WPV-1	*Proteus vulgaris*	+	+
8. J96	*Escherichia coli*	+	−
9. J198	*Escherichia coli*	−	−
10. 3629	*Escherichia coli*	+	−
11. 4432	*Escherichia coli*	+	−
12. 4476	*Escherichia coli*	+	−
13. JR-1	*Escherichia coli*	+	−
14. W17	*Morganella morganii*	−	−
15. W1	*Morganella morganii*	+	−
16. W5	*Morganella morganii*	+	−
17. W6	*Morganella morganii*	+	−
18. WPS-1	*Providencia stuartii*	−	−
19. WPR-2	*Providencia rettgeri*	±[b]	−
20. WPR-4	*Providencia rettgeri*	−	−
21. WSM-1	*Serratia marcescens*	+	−
22. WSM-2	*Serratia marcescens*	+	−
23. WCF-1	*Citrobacter freundii*	+	−
24. WCF-2	*Citrobacter freundii*	+	−
25. WPH-1	*Pasteurella haemolytic*[a]	+	−
26. WPH-2	*Pasteurella haemolytic*[a]	+	−
27. WAC-1	*Acinetobacter calcoaceticus*	+	−

[a] Numbers (1 to 17) correspond to the numbers above the lanes in Fig. 6.
[b] ±, Hemolysis was barely apparent on sheep erythrocyte agar plates after 48 h of incubation at 37°C.

DISCUSSION

Results of this study indicate that *E. coli* hemolysinlike determinants are present in at least two other genera of the family *Enterobacteriaceace*. It is not particularly surprising that this hemolysin can be found among different bacterial genera. Emody et al. (5) have suggested previously that the *M. morganii* and *E. coli* hemolysins were similar in virulence properties based on the use of different mouse models of extraintestinal infections. De la Cruz and co-workers (4) provided evidence that the *E. coli* hemolysin is, or had been, part of a transposable element because the hemolysin determinant could be localized to *E. coli* plasmids of different incompatibility groups. Direct evidence that the *E. coli* hemolysin behaves as a transposable element has not been documented. However, additional suggestive evidence of a hemolysin-transposon association is presented here, in which it has been shown that the *E. coli hly*-like sequences can be found on a plasmid in *P. vulgaris* 414-3 and in the chromosome of *M. morganii*. Previous DNA hybridization studies by a colony blotting method did not reveal a similarity between a *E. coli hly*-specific probe and DNAs among a broad survey of hemolytic bacteria, including members of the genera *Vibrios*, *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Streptococcus*, *Staphylococcus*, and *Clostridia* (unpublished data). The possible divergence of related DNA sequences to a degree that compromises their detection by DNA-DNA hybridization techniques makes such analyses equivocal. Yet with the results described here and the recent finding of a *E. coli* hemolysinlike determinant in *Pastuerella haemolytica*, it is clear that the *Morganella*, *E. coli*, *Proteus*, and *Pastuerella* (MEPP) hemolysin represents a new instance of a broadly disseminated factor involved in the pathogenesis of bacterial diseases.

The apparent divergence of the MEPP hemolysin sequence between *E. coli* and *Proteus* species is curious. It is debatable whether *Proteus* species is the evolutionary source of the *E. coli* hemolysin simply because the *E. coli* hemolysin determinant and the *Proteus* genome share a similar 39% guanine plus cytosine content (8). It is difficult to explain how the *E. coli* hemolysin determinant continued to diverge in DNA sequence from the *Proteus* hemolysin determinant, as witnessed by the faint hybridization signals seen in Fig. 2, yet the *E. coli hly* genes maintain a codon usage pattern that is atypical for *E. coli* (8). Perhaps the sharing of guanine plus cytosine content in this instance prejudices evolutionary conclusions. Based on guanine plus cytosine content alone, one could argue that *Pastuerella haemolytica* is the source of the MEPP hemolysin because *P. haemolytica* also has a guanine plus cytosine content in the 40 to 43% range (23). Recently, it has been discovered that there is 60% amino acid similarity between the *E. coli* hemolysin transport gene product (HlyB) and the multiple drug resistance determinant of Chinese hamster ovary cell lines (11). This suggests that deduction of the origin of the MEPP hemolysin determinant will be a complex issue.

The observation that there was an increased zone of beta-hemolysis surrounding the colonies harboring the pWPV100-*Pst*I deletion plasmid is notable. It suggests that there may be a negative effector of HlyA expression present on the original pWPV100 recombinant. To date, physical and genetic localization of negative effectors of MEPP HlyA expression has not been described. Alternatively, the *Pst*I deletion may have brought the pWPV100 *hly* genes under the control of a more active promoter. This will be the subject of further investigations because the data presented here are insufficient to permit a distinction between the two possibilities.

The evidence that the *P. mirabilis* 477-12 hemolysin has a novel determinant that is unrelated to that of the MEPP hemolysin is severalfold. First, there was no detectable hybridization of the 1.7-kb pWPM100-*Eco*RV probe to the DNA isolated from the source of the MEPP hemolysin in *P. vulgaris* 414-3. Second, the extracellular hemolytic activity encoded by the pWPM100 recombinant was on a 5.5-kb DNA fragment that was approximately 1.5 kb too small to encode MEPP *hly* genes. Tn*phoA*-mediated mutagenesis of pWPM100 indicates that the hemolytic phenotype is encoded in an even smaller (4.5-kb) region. The extracellular activity encoded by pWPM100 was found to be Ca^{2+} independent, in contrast to the Ca^{2+}-dependent MEPP hemolysin (27). Lastly, the analysis of pWPM100-encoded polypeptides in isolated minicells indicated that a single large-molecular-weight polypeptide (HpmA) of 140 kDa was responsible for the hemolytic character. Presently, it is assumed that the reduction in the hemolytic zone surrounding the colonies harboring pWPM100::Tn*phoA* insertion 13 is the result of Tn*phoA* insertion into the promoter area for *hpmA* or that a second gene is present in that area but one that is not absolutely required to encode the hemolytic phenotype.

A polypeptide similar in molecular weight to HpmA was found in the WPM100 culture supernatants. Definitive identification of this as HpmA and characterization of potential HpmA processing is dependent on DNA and amino-terminal protein sequence analysis. Such work is under way. The fact that active alkaline phosphatase gene fusions with *hpmA*

were isolated suggests that HpmA contains secretory signals, but precise cellular localization of potential HpmA precursors and the pathway for HpmA secretion await further analysis. HpmA is unlike the MEPP hemolysin in that there do not appear to be several closely linked genes involved in its activation and transport.

Unlike the case for the MEPP hemolysin, the HpmA determinant does not appear to be broadly disseminated among gram-negative bacilli. In the limited survey reported here, the gene sequences for this hemolysin were found in four of four hemolytic *P. mirabilis* isolates and two of three hemolytic *P. vulgaris* isolates. It could not be detected by DNA hybridization in any other hemolytic member of the family *Enterobacteriaceae*, *Pastuerella haemolytica*, or *Acinetobacter calcoaceticus*. It will be interesting to examine the epidemiology of the HpmA determinant among a large collection of *Proteus* isolates that are differentiated on the basis of their clinical source (blood versus urine versus feces). This may help establish a role for this hemolysin in the pathogenesis of diseases caused by *Proteus* species.

It is striking that the MEPP and HpmA hemolysins are present in the microorganism which, next to *E. coli*, is the most prevalent cause of urinary tract infection (18, 29, 36). In terms of what roles the MEPP hemolysin plays for *Proteus* in urinary tract infections, it is likely that the continued study of the MEPP hemolysin among uropathogenic *E. coli* will help illuminate its part in the pathogenesis of *Proteus* disease. On the other hand, the discovery of the novel HpmA hemolysin opens new areas of investigation. It will be of interest to see if there is HpmA-mediated cytolytic and cytotoxic activity against renal tubular cells and neutrophils. The suggestion that *Proteus* hemolytic activity is associated with invasiveness (25) also points the way toward some exciting avenues of investigation. Lastly, mouse models of urinary tract infection for *Proteus* are available (24). It will be very informative to use these models with isogenic *Proteus* strains that have various combinations of the MEPP and HpmA hemolysins.

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ADDENDUM

Recently, evidence for the presence of the *E. coli* hemolysinlike determinant in *Proteus* species and *M. morganii* appeared elsewhere (17).

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