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Construction and Expression of Recombinant Plasmids Encoding Type 1 or D-Mannose-Resistant Pili from a Urinary Tract Infection *Escherichia coli* Isolate

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Isolates of *Escherichia coli* from human urinary tract infections frequently express adherence properties found less often among normal intestinal isolates. These properties include adherence to human uroepithelial cells and primary monkey kidney cells, as well as D-mannose-resistant hemagglutination of human erythrocytes, and they are mediated by a pilus type different from type 1. The genes encoding this pilus type (pyelonephritis-associated pili, *pap*) and those encoding type 1 pili have been cloned from a urinary tract infection isolate of *E. coli* and transferred to an *E. coli* K-12 derivative. The recombinant plasmids were found to express functional pili and to endow the new host with all of the adherence properties of the urinary tract infection isolate. Both pilus types were found to be genetically distinct, and unlike the adherence genes from bovine, porcine, and human diarrheal isolates, both were found to be chromosomally encoded.

Escherichia coli is the microorganism most commonly isolated from human primary urinary tract infections (UTI). *E. coli* strains isolated from UTI are usually of an O serotype represented among the fecal flora of the infected individual (11) but often possess properties that distinguish them from the predominant stool strains. One such property is the ability to adhere to various mammalian cell types. *E. coli* strains isolated from patients with upper UTI have been shown to adhere more frequently to human uroepithelial cells than those strains isolated from asymptomatic lower-tract infections or from normal stools (21). This adherence is not inhibited by D-mannose (22). Similar studies have shown that virulence of uropathogenic *E. coli* is related to D-mannose-resistant (MR) adherence to human periurethral cells (8) and primary rhesus monkey kidney cells (C. Claussen, personal communication) and to MR hemagglutination (MRHA) of human erythrocytes (13, 14).

MR adherence has been found to be associated with the presence of pili on the bacterial cell surface. When viewed in an electron microscope, UTI isolates expressing strong MR adherence are also heavily piliated, and pili purified from such strains exhibit MR adherence to human erythrocytes and urinary tract epithelial cells (8, 10, 22). Expression of D-mannose-sensitive (MS) adherence to epithelial cells mediated by type 1 pili has also been implicated as a property associated with UTI virulence among strains of *E. coli* (15, 18, 24) and *Klebsiella pneumoniae* (6). In addition, type 1 pili have been shown to adhere to African green monkey kidney cells (Vero) (17). However, others have been unable to demonstrate tissue-specific adherence of type 1 pili to human urinary tract epithelial cells (16).

One difficulty inherent in studying virulence-associated properties of pathogenic bacteria is the current lack of genetically defined strains, i.e., bacterial isolates which differ in a single property. For the current study we used in vitro recombinant deoxyribonucleic acid (DNA) techniques to prepare chromosomally isogenic derivatives of *E. coli* K-12 which express either the MR or MS adherence properties of a human uropathogenic clinical *E. coli* isolate. The adherence characteristics of the recombinant strains have been examined, and evidence is presented to show that the *E. coli* strains which carry the recombinant molecules have acquired the adherence properties of the UTI isolate. Strains having cloned determinants encoding specific adherence possess demonstrable pili which mediate MRHA and those with the

cloned determinants for common pili possess pili which mediate only MS hemagglutination (MSHA).

Evidence is also presented to show that the two adherence properties are genetically distinct and that neither property is plasmid encoded in the donor UTI strain.

MATERIALS AND METHODS

Bacterial strains and growth media. P678-54 (F⁻ *thr-1 leu-6 thi-1 lacY1 malA1 xyl-7 ara-13 mtl-2 tonA2 gal-6* λ− *rpsL minA minB*) is a nonpiliated minicell-producing *E. coli* K-12 derivative; PK243(HB101, pHC79) was obtained from John Collins. The clinical isolate used in this study, *E. coli* J96 (O4,K6), was obtained from a human pyelonephritis infection. The MR adherence factor of *E. coli* J96 is antigenically cross-reactive with the MR pili from *E. coli* 3048 described by Korhonen et al. (10). In addition to its adherence properties, *E. coli* J96 is hemolytic, colicin V positive, and motile.

Unless otherwise specified, bacteria were grown in L broth (12) without glucose (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, pH 7) or on L-agar plates solidified with 1.5% Difco agar.

Enzymes. Enzymes used in this study were obtained from the following sources: restriction endonucleases *Eco*RI, *Hin*dIII, and *Bam*HI, Bethesda Research Laboratories; restriction endonuclease *Sau*3A, New England Biolabs; lysozyme, Worthington Diagnostics; proteinase K, Beckman Instruments, Inc.; phenylmethylsulfonyl fluoride, Calbiochem. T4 ligase was a generous gift from David Gelfand, Cetus Corp.

Buffers and reagents. TE buffer is 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8. BSG contains per liter: 8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of anhydrous Na_2HPO_4, and 1% gelatin, pH 7 (4). Conditions used for digestion of DNA with the various endonucleases were those recommended by the manufacturer. Ligations were performed in buffer containing 20 mM Tris, pH 7.5; 10 mM $MgCl_2$; 50 mM NaCl; 10 μM dithiothreitol; and 40 μM adenosine 5′-triphosphate. The running and gel buffers for agarose gel electrophoresis contain per liter: 10.8 g of Tris base; 0.93 g of disodium EDTA; and 5.5 g of boric acid, pH 8 to 8.5. Before loading on a gel, DNA samples were mixed with 1/4 volume of stop mix (20% Ficol 400, 7% sodium dodecyl sulfate).

Preparation of plasmid DNA. Plasmid DNA was purified by the method described by So et al. (19).

Preparation of whole-cell DNA. High-molecular-weight chromosomal DNA was prepared as follows. An exponentially growing 100-ml broth culture was harvested by centrifugation, washed once in TE buffer, and suspended in 2 ml of 25% sucrose in 50 mM Tris–1 mM EDTA (pH 8) on ice. Lysozyme was added (0.05 ml at 40 mg/ml), followed in 5 min by the addition of 0.01 ml of proteinase K at 20 mg/ml. After the proteinase K was thoroughly mixed in, 0.4 ml of EDTA (0.5 M) was added and mixed in gently. Cells were lysed by the addition of 0.25 ml of Sarkosyl (10%) and allowed to rest on ice until lysis was complete. The tubes were then covered with aluminum foil and incubated overnight at 50°C. A 35-ml amount of CsCl solution (69.6 g of CsCl in 55.2 ml of TE buffer containing 50 μg of the proteinase K inhibiter phenylmethylsulfonyl fluoride per ml) was added, and the lysate was centrifuged at 35,000 rpm for 40 h in a Beckman 60 Ti rotor. The diffuse DNA band was collected from the side through a 12-gauge needle and dialyzed against endonuclease *Sau*3A reaction buffer lacking $MgCl_2$ (50 mM NaCl, 6 mM Tris-hydrochloride, pH 7.5).

Recombinant DNA techniques. The recombinant DNA procedures used were a modification of the cosmid-cloning method described by Collins (3). High-molecular-weight DNA was digested at room temperature with *Sau*3A to ensure that cleavage did not go to completion. Portions of the digest (200 to 300 μl at 0.1 mg/ml) were layered on a 5 to 20% NaCl gradient and centrifuged at 35,000 rpm for 4.5 h in a Beckman SW41 rotor. Fractions (0.5 ml) were collected, and 7-μl samples of each, along with appropriate linear molecular weight standards, were electrophoresed overnight at 1 V/cm through a 0.35% horizontal agarose gel. Fractions containing fragments in the range of 21 to 30 megadaltons were precipitated at −20°C with 3 volumes of 95% ethanol. DNA from a single fraction (usually 2 to 3 μg) was collected by centrifugation, dried, and suspended in 10 μl of ligation buffer. *Bam*HI-cut pHC79 DNA (350 ng) was added, and the fragments were allowed to ligate overnight on ice. Recombinant molecules were then packaged in vitro into λ particles by the method described by Sternberg et al. (20).

Hemagglutination assay. Cells from citrated whole blood were washed twice with BSG and resuspended as a 3% solution in BSG. Bacteria were grown on L-agar plates (or L agar plus 100 μg of ampicillin per ml for cosmid-containing derivatives) overnight at 37°C and then suspended gently in BSG. Twofold serial dilutions of bacteria were also made in BSG. Erythrocytes (0.25 ml) and bacteria (0.25 ml) were mixed in a test tube and incubated at 4°C. Hemagglutination was read after 60 min. When used, D-mannose was added at a 50 mM final concentration.

Electron microscopy. Bacterial suspensions in buffered saline were fixed in glutaraldehyde and placed on a Parlodion-carbon-coated copper grid which was washed three times with buffered saline, dried, and shadowed in an evaporator with platinum-palladium at a 10° angle. Preparations were examined in a JOEL 200B electron microscope.

RESULTS

Characteristics of *E. coli* J96. Epidemiological studies conducted by Minshew et al. (unpublished data) have shown a high incidence of MRHA associated with *E. coli* from symptomatic upper UTI. For this study, an *E. coli* upper UTI isolate (J96) which expresses both MRHA and MSHA was chosen. In the experiments described below, an ultraviolet light-induced lactose-negative derivative of J96, called SH1, was used. Additional properties of J96 are described above.

Isolation of recombinant plasmids expressing MRHA or MSHA. Bacteriophage lambda transducing particles carrying recombinant cosmid molecules with portions of the SH1 genome were prepared as described above and used to transduce a nonpiliated *E. coli* K-12 strain, P678-54. Approximately 1% of the ampicillin-resistant transductants had also inherited the potential for MRHA of human erythrocytes or MSHA. Plasmid DNA from several of the ampicillin-resistant MRHA$^+$ transductants and MSHA$^+$ transductants and from P678-54 was extracted and visualized after electrophoresis through a 0.7% agarose gel. P678-54 was confirmed as plasmid-free, whereas each of the ampicillin-resistant isolates contained a single plasmid. Although the plasmid sizes varied among the independent isolates, all were within the expected 25- to 34-megadalton range. One recombinant of each type, HU807 (MRHA$^+$) and HU808 (MSHA$^+$), was chosen for further study.

Adherence properties. To compare the adherence properties of the recombinant derivatives with those of the nonpiliated parent and with those of the original clinical isolate, SH1, each strain was grown overnight on L-agar plates and tested for its capacity to hemagglutinate guinea pig or human erythrocytes (Table 1). HU807 exhibited MRHA of only human erythrocytes. HU808 exhibited MSHA of both human and guinea pig erythrocytes. The clinical isolate SH1 was found to exhibit both hemagglutination patterns, whereas the parent strain P678-54 could not hemagglutinate any erythrocytes. Adherence of each strain to primary rhesus monkey kidney cells was also measured with similar results. Both of the recombinant strains adhered to monkey kidney cells, but adherence of HU808 was inhibited by D-mannose.

Electron microscopy. Since it has frequently been shown that MR adherence is associated with the presence of pilus-like structures on the bacterial cell surface, the cosmid-containing derivatives were examined by electron microscopy to determine the extent of their piliation (Fig. 1). These results show that, unlike the cosmid-free strain, both HU807 and HU808 are heavily piliated. These and other electron microscopic studies with negatively stained preparations of piliated cells suggest that the two pilus types are morphologically similar.

Restriction endonuclease analysis of recombinant plasmids. MR adherence properties exhibited by enterotoxigenic *E. coli* have been shown in several instances to be plasmid encoded, whereas the gene for type 1 pilus expression resides on the chromosome of *E. coli* K-12 (1, 23). The following work was done to determine the location of genes expressing MRHA and MSHA in the clinical isolate.

SH1 was known to possess a single plasmid which encodes colicin V expression. Plasmid DNA was purified from SH1 and digested to completion with either *Eco*RI or *Hin*dIII. The restriction fragment pattern was compared with that of similarly treated cosmid DNA after electrophoresis through a 0.7% agarose gel (Fig. 2). If either of the recombinant cosmids contained DNA cloned from the plasmid in SH1, the restriction endonuclease digest pattern should represent a subset of the digest pattern of the ColV plasmid, with the exception of two vector-clone junction fragments. The results show that this is not the case, suggesting that neither of the pilus types is plasmid encoded. Moreover, we have isolated a number of plasmid-free UTI strains which possess both MR and MS hemagglutinating properties.

DISCUSSION

This study describes the preparation and characterization of genetically defined bacterial derivatives expressing single adherence properties associated with *E. coli* strains isolated from UTI. Plasmids carrying genes for MR or MS pili were prepared in vitro and transferred to a common nonpiliated *E. coli* K-12 host. Derivatives carrying pRHU807 (MR) were found to hemagglutinate human, but not guinea pig, erythro-

TABLE 1. *Hemagglutination properties of various E. coli derivatives*

Strain	Undiluted titer	Hemagglutination endpoint[b]			
		Human RBC[a]	Human RBC + D-mannose	Guinea pig RBC	Guinea pig RBC + D-mannose
SH1	4×10^9	1:8	1:8	1:2	0[c]
HU807	4×10^9	1:32	1:32	0	0
HU808	3×10^9	1:4	0	1:16	0
P678-54	9×10^9	0	0	0	0

[a] RBC, Erythrocytes.
[b] Last twofold dilution of bacteria resulting in unambiguous hemagglutination.
[c] No hemagglutination with undiluted bacteria.

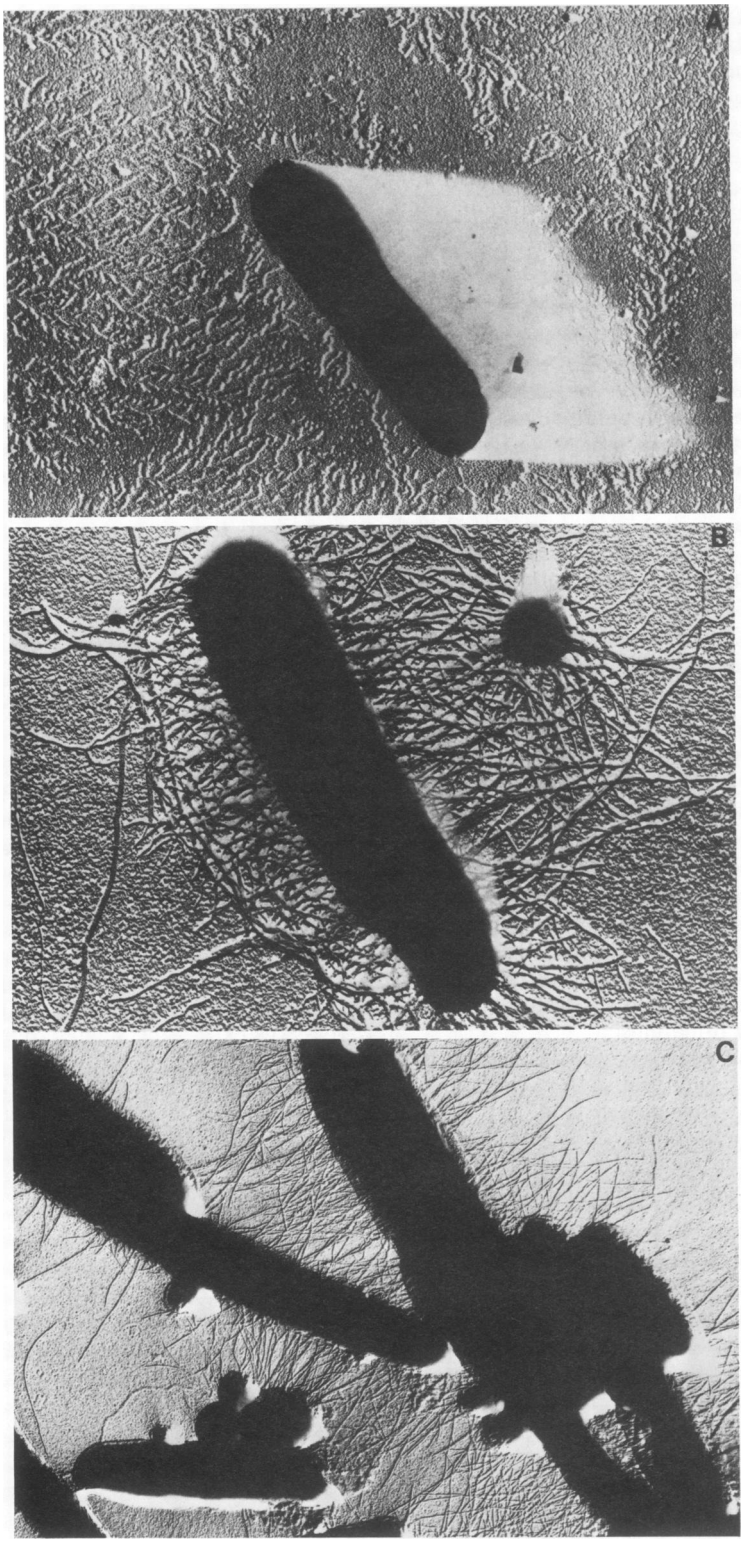

FIG. 1. *Electron micrographs of shadow-cast E. coli derivatives. (A) P678-54; (B) HU807; (C) HU808.*

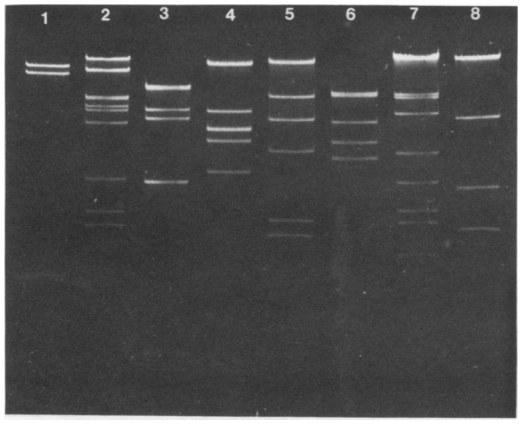

FIG. 2. *Restriction endonuclease digests of plasmid DNA. Recombinant plasmid DNA from HU807 and HU808 and ColV DNA was purified from J96 by dye-buoyant density centrifugation and digested with EcoRI or HindIII, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The lanes from the left are digestions of: (1) HU808 with EcoRI; (2) ColV with EcoRI; (3) HU807 with EcoRI; (4) bacteriophage λ DNA with EcoRI; (5) bacteriophage λ DNA with HindIII; (6) HU808 with HindIII; (7) ColV with HindIII; and (8) HU807 with HindIII.*

cytes and to adhere in the presence of D-mannose to primary rhesus monkey kidney cells. The *E. coli* K-12 with pRHU808 (MS) was found to hemagglutinate guinea pig erythrocytes and, to a lesser degree, human erythrocytes and to adhere to primary monkey kidney cells. Each of these properties was inhibited by the presence of D-mannose. Several conclusions can be drawn from these data: (i) P678-54 does not adhere to any of the cell types tested; (ii) HU807 has acquired adherence properties different from those associated with type 1 pili; (iii) HU808 expresses adherence properties consistent with the presence of type 1 pili; (iv) under the growth conditions used, SH1 expresses both type 1 and MR adherence factors.

E. coli MR adhesins associated with porcine (K88) (7), bovine (K99) (2), and human (colonization factor antigen) (5) enterotoxigenic strains are generally thought of as being plasmid encoded. We compared the restriction endonuclease digests of the 60- to 70-megadalton plasmid from *E. coli* J96 with digests of the recombinant plasmids and were able to show that the cloned sequences are not represented on the J96 plasmid. We believe that both the MR and MS adherence factors are encoded by chromosomal genes. Data from preliminary genetic mapping experiments also suggest a chromosomal position, and experiments are currently underway to more precisely locate the genes for MR and MS pili in *E. coli* J96. In addition, we have shown that the endonuclease digest patterns of pRHU808 and pRHU807 are different and that the *Eco*RI fragment encoding MR adherence is not represented in the pRHU808 digest (unpublished data). One can therefore conclude that these two pilus types are genetically and functionally distinct and do not represent a modification(s) of a single gene product.

In evaluating the contribution of individual virulence-associated factors to the overall virulence of a bacterial pathogen, one must consider the characteristics of the particular factor and how it interacts with other properties of the strain. The recombinant derivatives described here allow us to determine the virulence characteristics of two UTI-associated adherence factors in a defined genetic background. Moreover, generation in vitro of plasmids possessing specific virulence factors will allow us to move combinations of properties to an avirulent stool or laboratory *E. coli* isolate. In this manner, one might ultimately reconstruct piecemeal a fully virulent strain and thus partially fulfill a kind of Koch's postulates (9) at a molecular level.

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