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Molecular Epidemiology of Adhesin and Hemolysin Virulence Factors among Uropathogenic *Escherichia coli*

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The *pap*, *prs*, *pil*, and *hly* operons of the pyelonephritic *Escherichia coli* isolate J96 code for the expression of P, F, and type 1 adhesins and the production of hemolysin, respectively; the *afal* operon of the pyelonephritic *E. coli* KS52 encodes an X adhesin. Using different segments of these operons as probes, colony hybridizations were performed on 97 *E. coli* urinary tract and 40 fecal clinical isolates to determine (i) the presence in the infecting bacteria of nucleotide sequences related to virulence operons, and (ii) the phenotypic properties associated with such sequences. Coexpression of P and F adhesins encoded by *pap*-related sequences was detected more frequently among isolates from patients with pyelonephritis (32 of 49, 65%) than among those with cystitis (11 of 48, 23%; $P < 0.0001$) or from fecal specimens (6 of 40, 15%; $P < 0.0001$). Therefore, the expression of both adhesins appears to be critical in the colonization of the upper urinary tract. In contrast, *afal*-related sequences were detected significantly more frequently among isolates from patients with cystitis, suggesting that this class of X adhesin may have a role in lower urinary tract infections. Urinary tract isolates differed from fecal isolates by a low incidence of type 1 adhesin expression among *pil* probe-positive isolates. *hly*-related sequences were only detected in *pap* probe-positive isolates. The frequency of hemolysin production among *pap* probe-positive isolates was not associated with a particular pattern of infection. The distribution of these virulence factors was similar in the presence or absence of reflux, indicating that structural abnormalities of the urinary tract did not facilitate colonization by adhesin-negative isolates.

Bacterial infections of the urinary tract encompass a wide spectrum of clinical syndromes ranging from asymptomatic bacteriuria to symptomatic cystitis and pyelonephritis. A single bacterial species, *Escherichia coli*, causes more than 80% of these infections, and neither anatomical differences nor defects in host defense mechanisms provide an adequate explanation for the different clinical patterns that have been observed (31). *E. coli* isolated from infected urinary tracts often express specific properties that are not prevalent among strains from the commensal fecal flora (33). These properties include the expression of adhesins mediating attachment to specific receptors on uroepithelial cells, production of hemolysin, serum resistance, release of aerobactin, and the presence of particular surface antigens.

The ability to adhere to uroepithelial cells is considered to be a critical virulence factor which allows particular fecal clones of *E. coli* to colonize the urinary tract. Several multicistronic operons encoding adhesins of uropathogenic *E. coli* have been analyzed by DNA cloning techniques. Some adhesin operons encode for the production of fimbrial appendages (pili), while others appear to encode afimbrial structures (13, 19).

pap (pyelonephritis-associated pili) and *prs* (pap-related sequence) are two structurally and functionally related operons cloned from the chromosomal DNA of the pyelonephritic *E. coli* isolate J96 (13, 22, 23). The *pap* operon encodes a fimbrial adhesin that specifically recognizes the globoseries of glycolipids present on human erythrocytes and uroepithelial cells bearing the P blood group antigen (16, 20, 22, 23). The minimal binding receptor of this adhesin is the disaccharide α-D-galactosyl-(1→4)-β-D-galactopyranose (Gal-Gal). The *prs* operon encodes an adhesin that preferentially binds to the Forssman antigen, a major constituent on sheep erythrocyte membranes that has also been isolated from the human renal pelvis (22, 23). The binding epitope for this adhesin was identified as the galactose-*N*-acetyl-α(1→3) galactose-*N*-acetyl moiety.

The *pap* operon encodes an adhesin specific for the P blood group antigen and is called the P adhesin; by analogy, we refer to the adhesin encoded by the *prs* operon and specific for the Forssman antigen as the F adhesin.

The role of the P adhesin as a virulence factor in urinary tract infections is well documented. In numerous epidemiologic surveys, the majority of pyelonephritic *E. coli* isolates express a P adhesin, whereas this phenotype is found less frequently among isolates from patients with cystitis and fecal isolates (for a review, see reference 33). In a mouse infection model, *E. coli* expressing a P adhesin showed an increased ability to colonize the upper urinary tract (27). A soluble receptor analog for the adhesin was protective against ascending urinary tract infections in the same model (34). Thus, the P adhesin promotes ascending infection of the urinary tract, presumably by allowing bacteria to evade the normal cleansing action of urine flow in the ureters.

As distinguished from other adhesins, type 1 adhesins bind to α-D-mannose, and thereby agglutinate guinea pig erythrocytes (29). Nucleotide sequences related to the *pil* operon, which encodes a type 1 adhesin in *E. coli* J96, have been

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detected in the majority of *E. coli* isolates from extraintestinal infections (4).

Adhesins recognizing the receptors that are present on human erythrocytes lacking the P antigen are referred to as X adhesins. This phenotype defines a heterogeneous group of adhesins, and several genetically unrelated operons encoding X-type adhesins have been cloned (17). Among these, the *afaI* operon encodes an afimbrial adhesin that mediates binding to human uroepithelial cells, although the specific receptor remains undefined (18, 19). The incidence of the different X adhesins among uropathogenic *E. coli* isolates has not been studied extensively (17).

Epidemiologic surveys of *E. coli* virulence factors have typically been based on phenotypic assays. Such assays require the expression of virulence factors under laboratory conditions that likely differs from expression at the site of infection. Recently, the use of DNA hybridization has led to genotypic assays for virulence factors that do not rely on gene expression (5, 17, 28). We used both of these approaches to survey 137 *E. coli*, including 97 isolates from children with urinary tract infections and 40 fecal isolates. For each isolate we determined the genotype and the phenotype with respect to hemolysin production and the expression of P, F, type 1, and X adhesins. Results of this study demonstrated that (i) genotypic versus phenotypic assays can reveal significantly different distributions of virulence factors, and (ii) *E. coli* from different patient populations can be differentiated not only by the frequency of nucleotide sequences related to virulence operons but also by the frequency of the phenotypes associated with such sequences. In the accompanying report (3), we describe a further analysis by Southern blot hybridization of 30 of the 137 isolates initially characterized in this report.

MATERIALS AND METHODS

Patient population. Pediatric primary-care patients between the ages of 2 weeks and 13 years with bacteriologically proven, symptomatic urinary tract infections treated at Cleveland Metropolitan General Hospital (Cleveland, Ohio) were eligible for entry into this study, as described previously (15). Patients with asymptomatic bacteriuria and myelodysplasia, children attending the pediatric urologic clinic, and children who had previously had either cystograms or intravenous pyelograms were excluded from this study. After urine was cultured, the history of the patient was recorded and a physical examination was performed. Additional investigations, which were carried out within 5 days of presentation, included erythrocyte sedimentation rate, quantitative determination of the serum C-reactive protein, and D-deamino arginine vasopressin concentrating ability. At 4 to 6 weeks following the initiation of antibiotic therapy, a renal and pelvic ultrasound and a voiding cystogram were performed. If either test result was abnormal, an intravenous pyelogram was then done. Patients were categorized as having acute pyelonephritis if at least two of the following four criteria were positive: (i) rectal temperature of >38°C or oral or axillary temperature of >37°C; (ii) erythrocyte sedimentation rate of >25 mm/h; (iii) serum C-reactive protein of >1.0 μg/ml, and (iv) D-deaminoarginine vasopressin test of renal concentrating ability abnormal for the age of the patient. The last test was not performed on children who were under 2 years of age because of concern for excessive fluid load if the parents forced fluids. Children with less than two positive tests were classified as having cystitis.

Forty additional *E. coli* fecal isolates were obtained from 40 consecutive stool specimens submitted to the Microbiology Laboratory of Boston City Hospital (Boston, Mass.).

Cultures of specimens from patients. Bacteria were initially isolated from urine or stool specimens by culturing the specimens on bimedia dip slides consisting of eosin-methylene blue and tryptic soy agar (Culturia; Clinical Convenience Products, Madison, Wis.). Biotypes were determined with the API 20E system (Analytab Products, Plainview, N.Y.), and a subculture was kept frozen at −80°C in 22% glycerol until it was analyzed. One isolate per patient was studied. All cultures were prepared at 37°C on Luria agar or Luria broth with aeration unless otherwise specified.

Agglutination assays. Hemagglutination was performed by using human erythrocytes P1 MM (Ortho Diagnostics, Inc., Raritan, N.J.), human erythrocytes pp NN (generously provided by Gamma Biologicals Inc., Houston, Tex.), human P1 NN erythrocytes (obtained in our laboratory), sheep erythrocytes (Organon Teknika Corp., Chester, Pa.), and guinea pig erythrocytes (obtained from Hartley strain guinea pigs). Erythrocytes were washed and suspended at 3% (vol/vol) in Alsever solution. Adhesin phenotypes were identified by using 10 μl of a suspension of bacteria grown on agar (10^{10} CFU/ml in 0.1 M phosphate-buffered saline; pH 7.2) and mixed with 10 μl of erythrocytes. Inhibition of agglutination by D-mannose was assessed by using bacteria that were suspended in phosphate-buffered saline with 4% (wt/vol) D-mannose. Bacterial agglutination by latex particles coated with synthetic Gal-Gal (Chembiomed Ltd., Edmonton, Alberta, Canada) was also determined. Agglutinations were performed at 4°C on an orbital shaker (60 rpm). Assays were considered positive only if agglutination was present after 10 min. All reagents and suspensions were kept at 4°C. Isolates were considered to express a P adhesin if hemagglutination was positive with P1 MM erythrocytes, positive with P1 NN erythrocytes, and negative or comparatively weaker and slower with pp NN erythrocytes. Isolates were considered to express an F adhesin if hemagglutination was positive with sheep erythrocytes and to express an X adhesin if hemagglutination was positive with pp NN human erythrocytes. The type 1 adhesin was considered to be present if hemagglutination was positive with guinea pig erythrocytes. D-Mannose always inhibited hemagglutination of guinea pig erythrocytes, but it never inhibited hemagglutination of human or sheep erythrocytes. Type pp MM erythrocytes were unavailable, and therefore, assays for adhesins specific for the M antigen (30) could not be performed in isolates expressing the P adhesin or other X adhesins.

Hemolysin production assay. The production of hemolysin was tested on Columbia agar supplemented with 4% sheep erythrocytes. Colonies surrounded by a clear halo after overnight culture at 37°C were defined as hemolysin positive.

Plasmids. The *pap*, *prs*, *hly*, and *pil* operons were isolated by cloning them from chromosomal DNA of the pyelonephritic isolate *E. coli* J96 (13, 22, 35). The *afaI* operon was cloned from chromosomal DNA of the pyelonephritic isolate KS52 (19). The properties and sources of five hybrid plasmids carrying these operons are given in Table 1. Using the laboratory strain *E. coli* C1a (32), which had none of these virulence factors, we constructed derivatives harboring each of these plasmids for use throughout this study as control strains expressing a single defined virulence factor.

DNA hybridization. Nucleotide sequences related to *E. coli* virulence operons were detected by colony hybridization. Plasmid DNA was purified by two consecutive band-

TABLE 1. Plasmids used in this study and their origins

Plasmid	Relevant characteristics	Chromosome origin	Reference(s)
pRHU845	*pap* operon, P-pilus adhesin	J96	26
pPAP601	*prs* operon, F-pilus adhesin	J96	22
pSH2	*pil* operon, type-1-pilus adhesin	J96	11, 25
pIL14	*afaI* operon, afimbrial X adhesin	KS52	18, 19
pSF4000	*hly* operon, hemolysin	J96	9, 35

ings in cesium chloride-ethidium bromide gradients (24). The DNA restriction fragments used to generate the probes (Fig. 1) were separated by electrophoresis in horizontal slab gels (25 by 16 by 0.7 cm) containing 0.8% agarose (type VII; Sigma Chemical Co., St. Louis, Mo.). DNA from a slice of the gel containing the relevant restriction fragment was radiolabeled in the agarose by random oligo priming with [α-^{32}P]dCTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) (7, 8). Bacteria were inoculated with a 25-well replicator on nitrocellulose filters (BA-85; Schleicher & Schuell, Inc., Keene, N.H.) and lysed in situ (24). DNA hybridizations were performed at 42°C in the presence of 50% formamide (24). All isolates were tested twice.

Statistical analyses. Statistical analyses were performed on a microcomputer (Macintosh SE; Apple Computer Co, Cupertino, Calif.) by using StatView 512+ software (Brain Power, Inc, Calabasas, Calif.) to perform Student *t* tests and chi-square tests with continuity correction; $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical characteristics of the patients. Among 117 episodes of urinary tract infections in children, 6 were caused by coagulase-negative staphylococci, 6 were caused by *Klebsiella* spp., and 1 was caused by each of *Pseudomonas* sp. and *Enterobacter* sp. Of the 103 patients infected with *E. coli*, complete clinical and radiologic data were available for 97 of them (Table 2). A total of 9 of these patients were under the age of 1 year, 61 were between the ages of 1 and 5 years, and 27 were between the ages of 5 and 12 years. A total of 4 of the 9 children under the age of 1 year were male, and 6 of the remaining 88 children were male. Of the 97 children for whom data were available, 17 (18%) had a history consistent with recurrent urinary tract infection, while the remaining 80 were experiencing their first urinary tract infection. Treatable urologic abnormalities were identified in 17 patients: 16 children had vesicoureteral reflux (grade I in 4 patients, grade II in 9 patients, and grade III in 3 patients, 1 of whom also had a ureterocele); and 1 child had obstruction at the ureteropelvic junction and ipsilateral hydronephrosis. Among these 17 children, 3 were male and 14 were between the ages of 1 and 5 years. One had a previous episode of urinary tract infection.

Of the 97 episodes of urinary tract infection, 49 (51%) were diagnosed as pyelonephritis and 48 (49%) were diagnosed as cystitis (Table 2). Among the patients with pyelonephritis, 8 were male (including all 4 males under 1 year of age), 7 had experienced previous urinary tract infections, and 12 had treatable urologic abnormalities (including all those with grade III reflux or structural deformities). The patients with pyelonephritis were significantly younger than those with cystitis (2.87 ± 0.36 versus 4.90 ± 0.42 [mean + standard error; $P < 0.001$ by the Student *t* test]). This was true even when patients who were males, who had prior infection, or who had treatable abnormalities were excluded.

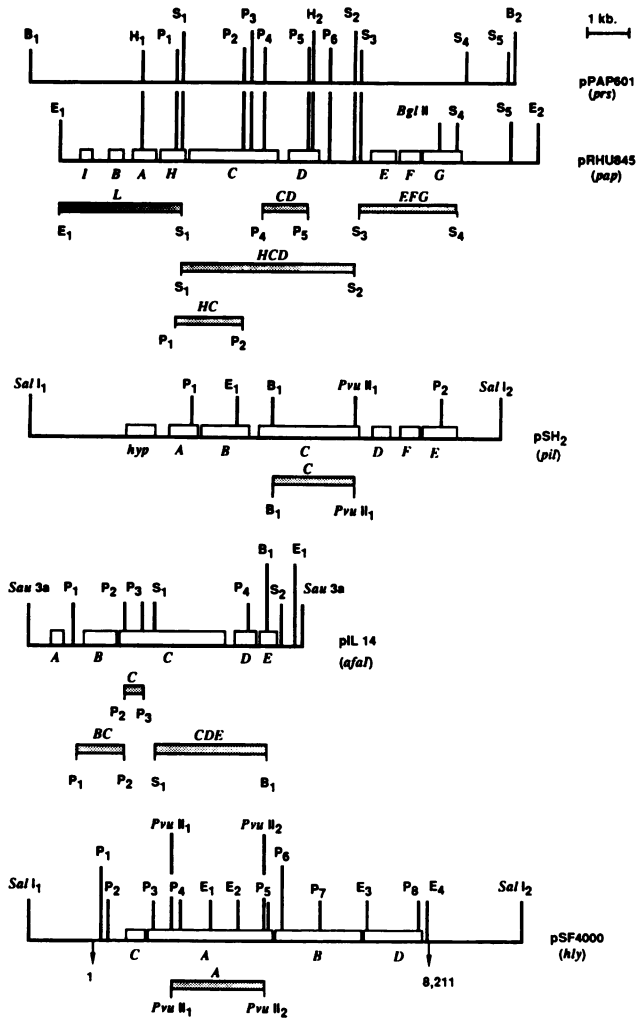

FIG. 1. Positions of the DNA restriction fragments used to generate the probes in the physical and genetic maps of the virulence operons. The inserts found in the recombinant plasmids listed in Table 1 are represented by horizontal lines. Open boxes represent the open reading frames in the inserts. Shaded boxes below the inserts indicate the positions of restriction fragments that were used as probes. The probes are named according to the open reading frames that they span, except for the *papL* probe (left-hand end of the *pap* operon). Restriction sites abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I. For pSF4000, the map displays only the 8,211-base-pair portion of the insert for which the sequence has been determined. The positions of the first and last base pairs according to previously published sequence numbering (9) are indicated by arrows. kb, Kilobases. The subscript numbers following restriction enzyme designations refer to consecutive cutting sites.

Phenotypic properties of the isolates. Of 58 clinical isolates expressing a P adhesin, all but two agglutinated latex beads coated with Gal-Gal, the minimal receptor of the P-specific adhesin encoded by the *pap* operon of *E. coli* J96. None of the other isolates agglutinated the Gal-Gal-coated latex beads.

Expression of a P adhesin among isolates from patients with pyelonephritis (referred to hereafter as pyelonephritis isolates) (36 of 49, 73%) was significantly more frequent than

TABLE 2. Clinical characteristics of the 97 children with pyelonephritis and cystitis caused by *E. coli*

Disease (no. of patients)	No. (%) of females	Age (yr [mean ± SE])	No. (%) with history of prior urinary tract infection	No. (%) with treatable urologic abnormalities
Pylonephritis (49)	41 (84)	2.87 ± 0.36	7 (14)	12 (24)
Cystitis (48)	46 (96)	4.90 ± 0.42	10 (22)	5 (10)
P	NS[a]	<0.001[b]	NS	NS

[a] NS, Not significant by chi-square test with continuity correction.
[b] *P* value determined by Student *t* test.

among isolates from patients with cystitis (referred to hereafter as cystitis isolates) (15 of 48, 31%; $P = 0.0001$) or isolates from fecal specimens (referred to hereafter as fecal isolates) (7 of 40, 18%; $P = 0.0001$; Fig. 2). Expression of an F adhesin among pyelonephritis isolates (32 of 49, 65%) was significantly more frequent than that of cystitis (13 of 48, 27%; $P = 0.0004$) or fecal (15 of 40, 38%; $P = 0.02$) isolates. For both the P and F adhesins, the differences observed between cystitis and fecal isolates were not statistically significant.

Expression of type 1 adhesin was detected with similar frequencies in pyelonephritis (10 of 49, 20%) and cystitis (12 of 48, 25%) isolates. This phenotype was more frequent in fecal isolates (23 of 40, 58%) than in pyelonephritis or cystitis isolates ($P < 0.005$ for each).

The incidence of X-adhesin expression was similar in pyelonephritis (24 of 49, 49%) and cystitis (23 of 48, 48%) isolates. This phenotype was less frequent in fecal isolates (4 of 40, 10%) than in pyelonephritis or cystitis isolates ($P < 0.0005$ for each).

Hemolysin production was found in 20 (41%) of 49 pyelonephritis, 9 (19%) of 48 cystitis, and 12 (30%) of 40 fecal isolates. The only statistically significant difference was between pyelonephritis and cystitis isolates ($P = 0.03$).

Distribution of nucleotide sequences related to the *pap* operon. The 137 isolates were studied by colony hybridization by using five different *pap* probes (Fig. 3). Four of the five probes (*papHC*, *papCD*, *papHCD*, and *papEFG*) were within the *pap* operon (Fig. 1), while the fifth probe (*papL*) included most of the *papA* gene plus the regulatory region of the *pap* operon and a chromosomal sequence of *E. coli* J96 external to the *pap* operon. Three probes within the central portion of the operon, *papHC*, *papCD*, and *papHCD*, detected the same 76 isolates. The *papEFG* probe detected these 76 isolates plus 6 additional isolates, thereby indicating the presence of genetic elements structurally related to only part of the *pap* operon. The *papL* probe gave hybridization signals which varied in intensity over a wide spectrum. This was in contrast to the other probes used in this study, which gave distinct positive and negative classes of hybridization. Less than 10% of the 137 isolates appeared to completely lack nucleotide sequences homologous to the *papL* probe. None of these isolates was positive when the other *pap* probes were used.

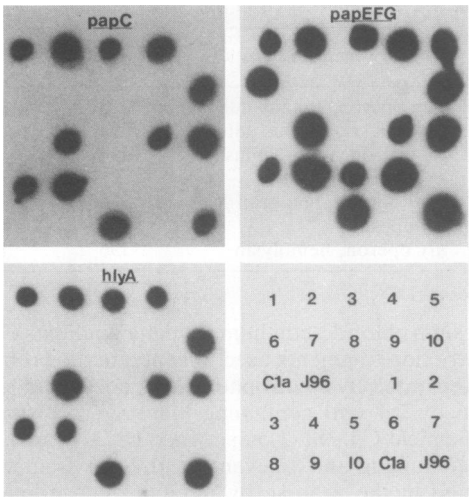

FIG. 3. Analysis of DNA by colony hybridization. Twelve strains were inoculated in duplicate with a replicator on a nitrocellulose membrane according to the positions shown at the bottom right. Bacteria were lysed in situ, and the DNA transferred to the membrane was tested for hybridization with the probe listed above each autoradiogram.

Adhesin expression in isolates detected by the *pap* probes. The *papL*, *papHC*, *papCD*, *papHCD*, and *papEFG* probes detected all 9 isolates expressing only a P adhesin, all 11 isolates expressing only an F adhesin, and all 49 isolates

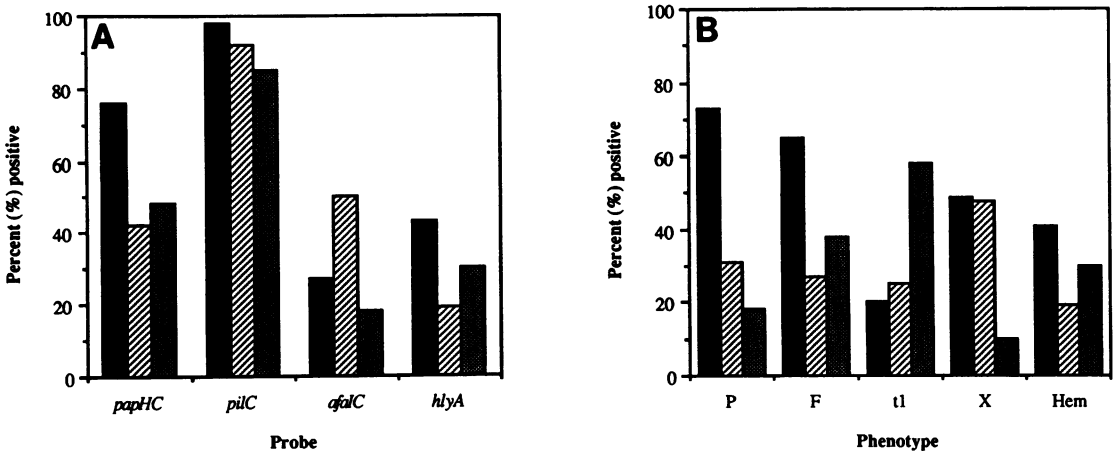

FIG. 2. Histogram of the distribution of virulence factors in the three types of isolates. (A) Distribution of nucleotide sequences related to the probes. Solid bars, pyelonephritis isolates ($n = 49$); striped bars, cystitis isolates ($n = 48$); shaded bars, fecal isolates ($n = 40$). (B) Distribution of virulence *factor* expression. Abbreviations: P, P adhesin; F, F adhesin; t1, type 1 adhesin; X, X adhesin; Hem, hemolysin.

TABLE 3. Correlations between the presence of virulence operon-related nucleotide sequences and expression of virulence factors

Probe (total no. of isolates detected by probe) and phenotype	% of strains detected by probe among strains expressing phenotype (no. probe positive/ no. phenotype positive)
papHC (76)	
P adhesin only	100 (9/9)
F adhesin only	100 (11/11)
Both P and F adhesins	100 (49/49)
Neither P nor F adhesins	11 (7/61)
pilC (126)	
Type 1 adhesin	100 (45/45)
Negative	88 (81/92)
afaIC (44)	
X adhesin	63 (32/51)
Negative	14 (12/86)
hlyA (42)	
Hemolysin	100 (41/41)
Negative	1 (1/96)

expressing both a P and an F adhesin (Table 3). A single hybridization class defined by positive hybridization with each of these five probes was therefore found for isolates expressing one or both binding specificities encoded by the *E. coli* J96 *pap* and *prs* operons. In addition, 13 isolates that expressed neither a P nor an F adhesin possessed genetic information related to the *pap* operon. Seven of these isolates were detected by the *papL*, *papHC*, *papCD*, *papHCD*, and *papEFG* probes; and the six remaining isolates were detected only by the *papL* and *papEFG* probes.

Strains detected by the *papL*, *papHC*, *papCD*, *papHCD*, and *papEFG* probes occurred more frequently among pyelonephritis isolates (37 of 49, 76%) than among cystitis (20 of 48, 42%; $P = 0.002$) or fecal (19 of 40, 48%; $P = 0.01$) isolates (Fig. 2). The differences observed between cystitis and fecal isolates were not statistically different.

The incidence of *pap* probe-positive isolates expressing both a P and an F adhesin was significantly higher in pyelonephritis isolates (32 of 49, 65%) than in cystitis isolates (11 of 48, 23%; $P < 0.0001$) or in fecal isolates (6 of 40, 15%; $P < 0.0001$; Fig. 4). The properties of the cystitis and fecal isolates were similar. Strains expressing only a P adhesin occurred rarely among the pyelonephritis (4 of 49, 8%), cystitis (4 of 48, 8%), and fecal (1 of 40, 3%) isolates. No pyelonephritis isolates and only 2 (4%) of the 48 cystitis isolates expressed an F adhesin only. This phenotype occurred more frequently in fecal isolates (9 of 40, 23%). Isolates detected by the five *pap* probes and expressing neither a P nor an F adhesin occurred infrequently among the pyelonephritis (1 of 49, 3%), cystitis (3 of 48, 6%), and fecal (3 of 40, 8%) isolates. Taken together these results indicate that adhesin expression varies among *pap* probe-positive isolates in the three groups of isolates. Expression of a P and an F adhesin was found in the majority of *pap*-positive pyelonephritis (32 of 37, 86%) and cystitis (11 of 20, 55%) isolates. In contrast, the most frequent pattern of adhesin expression among the fecal isolates detected by the *pap* probes was the expression of an F adhesin only (9 of 19, 47%).

Type 1 fimbrial adhesin. The *pilC* probe that was internal to the *pilC* open reading frame (Fig. 1) detected 126 (92%) of the 137 *E. coli* isolates. Only a minority 45 (34%) of these 126

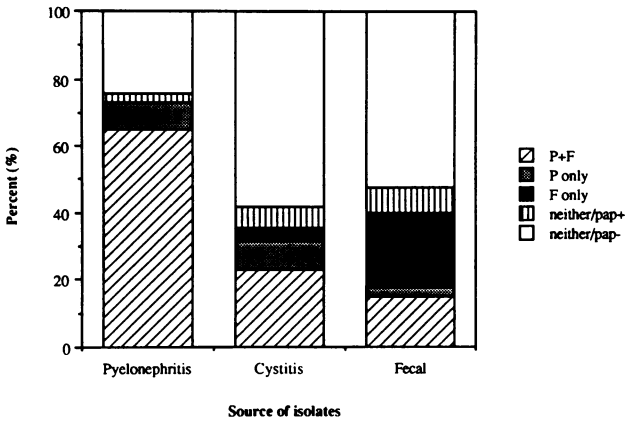

FIG. 4. Histogram of P- and F-adhesin expression among the three types of *E. coli* isolates. *pap*$^+$, Positive hybridization with the *papHC* probe; *pap*$^-$, absence of hybridization with the *papHC* probe.

isolates expressed a type 1 adhesin. The 11 isolates which were not detected by the *pilC* probe did not express a type 1 adhesin (Table 3). We tested 18 clinical isolates for the effects of serial subculturing at 42°C on the expression of type 1 pilus adhesin. Stationary-phase cultures were diluted 500-fold each day in tryptic soy broth, and expression of type 1 adhesin was tested after the fifth subculture. Five isolates that were not detected by the *pilC* probe remained negative for type 1 adhesin expression. In contrast, of 13 isolates detected by the *pilC* probe, 7 were converted to type 1 adhesin expression by serial passages, while 5 remained negative. The subculture procedure did not affect the phenotypes of the J96 or C1a controls.

The incidence of *pil*-related sequences in the pyelonephritis (48 of 49, 98%), cystitis (44 of 48, 92%), and fecal (34 of 40, 85%) isolates was similar ($P > 0.05$ for each type of isolate; Fig. 2). Thus, the higher incidence of type 1 adhesin expression in fecal isolates (see above) was not due to a higher incidence of *pil*-related sequences, but to a higher incidence of type 1 adhesin expression among *pil*-related sequences from fecal isolates.

Expression of X adhesins and distribution of nucleotide sequences related to the *afaI* operon. The distribution of nucleotide sequences structurally related to the *afaI* operon encoding the afimbrial adhesin was studied by colony hybridization by using three different internal fragments of the operon as probes (Fig. 1). The *afaIC* probe was internal to the *afaIC* open reading frame. The three probes gave identical results and detected 44 (32%) of the 137 isolates. Among these 44 isolates, 32 expressed an X adhesin (Table 3). Five serial passages in liquid medium, as described above, did not convert the 12 remaining isolates to X-adhesin expression. Nineteen isolates expressed an X adhesin and were not detected by the *afaI* probes, indicating the presence of operons that were genetically distinct from *afaI* operons that encode X adhesins.

Nucleotide sequences related to the *afaI* operon were detected more frequently in cystitis (24 of 48, 50%) than in pyelonephritis (13 of 49, 27%; $P = 0.03$) or fecal (7 of 40, 18%; $P = 0.003$; Fig. 2) isolates. The properties of pyelonephritis and fecal isolates were similar. Among isolates detected by the *afaI* probes, expression of an X adhesin was detected in 11 (85%) of 13 pyelonephritis, 18 (75%) of 24 cystitis, and 3 (43%) of 7 fecal isolates. Thus, the uneven distribution of X adhesin expression in the three groups of

TABLE 4. Hybridization classes defined by a positive or negative hybridization with the *papHC*, *papEFG*, *hlyA*, *afaIC*, and *pilC* probes

Probe	No. of isolates with homology to the following probes:			
	afaIC$^+$ *pilC*$^+$	*afaIC*$^+$ *pilC*$^-$	*afaIC*$^-$ *pilC*$^+$	*afaIC*$^-$ *pilC*$^-$
papHC$^+$ *papEFG*$^+$ *hlyA*$^{+a}$	9	0	31	2
papHC$^+$ *papEFG*$^+$ *hlyA*$^-$	9	0	24	1
papHC$^-$ *papEFG*$^+$ *hlyA*$^-$	4	2	0	0
papHC$^-$ *papEFG*$^-$ *hlyA*$^-$	17	3	32	3

[a] Homology to the *hlyA* probe was only found in isolates detected by the *papHC* and *papEFG* probes.

TABLE 5. Properties of strains isolated from patients with and without reflux

Adhesin or probe	No. (%) of strains expressing virulence factors or detected by probes in patients with or without reflux[a]:			
	Pyelonephritis and cystitis isolates (n = 97)		Pyelonephritis isolates only (n = 49)	
	+ (n = 17)	− (n = 80)	+ (n = 12)	− (n = 37)
P adhesin	10 (59)	41 (51)	9 (75)	27 (73)
F adhesin	7 (41)	38 (48)	6 (50)	26 (70)
Type 1 adhesin	5 (29)	17 (21)	3 (25)	7 (19)
X adhesin	11 (65)	36 (45)	9 (75)	15 (41)
Hemolysin	5 (29)	24 (30)	5 (42)	15 (41)
papHC probe	11 (65)	46 (58)	10 (83)	27 (73)
afaIC probe	5 (29)	25 (31)	4 (33)	9 (24)

[a] Patients with (+) and without (−) reflux are indicated.

isolates (Fig. 2) did not result from differences in adhesin expression among strains detected by the *afaIC* probe. Among strains expressing an X adhesin, nucleotide sequences related to the *afaI* operon were detected in 11 (46%) of 24 pyelonephritis, 18 (78%) of 23 cystitis, and 3 (75%) of 4 fecal isolates. Thus, the high incidence of X-adhesin expression among urinary tract isolates (see above; Fig. 2) correlates with a high incidence of *afaI*-related sequences in cystitis isolates, but in pyelonephritis isolates the majority of X adhesins appeared to be encoded by operons distinct from *afaI*.

Hemolysin. The distribution of nucleotide sequences that were structurally related to the *hly* operon encoding the production of hemolysin in *E. coli* J96 was studied by colony hybridization by using the *hlyA* probe that was internal to the *hlyA* cistron (Fig. 1). The probe detected all 41 isolates that lysed sheep erythrocytes in blood agar plates (Table 3). The DNA of one isolate hybridized with the probe but did not produce a hemolysin by this assay. Ninety-five isolates were negative both for sheep erythrocyte lysis and for the colony hybridization assay.

Association of virulence factors. Theoretically, five probes can define 32 (2^5) hybridization classes based on combinations of positive and negative hybridization signals. When we used the *papHC*, *papEFG*, *pilC*, *afaIC*, and *hlyA* probes, only 12 of the 32 possible hybridization classes were found to exist among the 137 isolates (Table 4). This was predominantly due to the fact that all 42 isolates detected by the *hlyA* probe were also detected by the *papHC* and *papEFG* probes. Hemolysin expression was detected in 28 (57%) of the 49 isolates that expressed both a P and an F adhesin, in 2 (22%) of the 9 isolates that expressed only a P adhesin, in 10 (91%) of the 11 isolates that expressed only an F adhesin, and in 1 (1%) of 68 isolates that expressed neither a P nor an F adhesin. No association was detected between *pap*-related sequences and *afaI*- or *pil*-related sequences. In fact, the incidence of *afaIC*-related sequences was slightly lower among isolates detected by the *papHC* probe (18 of 76, 24%) than among *papHC* probe-negative isolates (24 of 61, 39%).

Twenty different phenotypic classes were defined based on the assays for P, F, type 1, and X adhesins and hemolysin (data not shown). Forty-eight different classes of isolates were defined on the basis of both the phenotypic and colony hybridization assays. Thus, isolates belonging to the same hybridization class may express different phenotypes, and isolates expressing the same phenotype may belong to different hybridization classes. Therefore, expression of each adhesin does not appear to depend on the presence or expression of other virulence operons.

Virulence factors of isolates from patients with reflux. The properties of clinical isolates from patients with reflux were very similar to those found in patients without reflux (Table 5). This was also true when the properties of pyelonephritis isolates from patients with and without reflux were compared. Thus, the distribution of P adhesins as well as the other virulence factors did not appear to correlate with the presence of reflux. There was no association between the virulence factors and patient age, sex, or history of prior infection (data not shown).

Physical characterization of adhesins and adhesin-mediated agglutination. Using high-resolution transmission electron microscopy, we examined 30 of the clinical isolates for which the phenotypes and genotypes were determined as described above. Although the degree of piliation varied, the pilus morphology associated with expression of P, F, and type 1 pili was indistinguishable (Fig. 5). All isolates expressing X-type adhesins were surrounded by an amorphous mesh of fine fiber appendages; these fibers were often found to be released into the surrounding media (Fig. 5). Transmission electron microscopy was also used to analyze both the agglutination of erythrocytes and Gal-Gal latex beads as mediated by clinical isolates expressing a P adhesin. In both cases, tight binding complexes of piliated bacteria and the receptor-carrying substrate were resolved (Fig. 6).

DISCUSSION

Current hypotheses for the pathogenesis of *E. coli*-mediated urinary tract infections postulate that bacterial adhesins act as essential virulence factors by facilitating colonization of the uroepithelium. Molecular epidemiologic analyses with either phenotypic or genotypic assays have sought to correlate adhesins and particular patterns of infection (17, 28, 33). Each of these approaches has intrinsic limitations. Phenotyping may be spurious because of variations in gene expression, uncertainties as to appropriate receptor-based assays, and the likelihood that most clinical isolates can express several different classes of adhesins. On the other hand, colony hybridization studies do not discriminate homologous operons encoding different binding specificities and, as discussed in the accompanying paper (3), may be further confounded by other alterations at the nucleotide level.

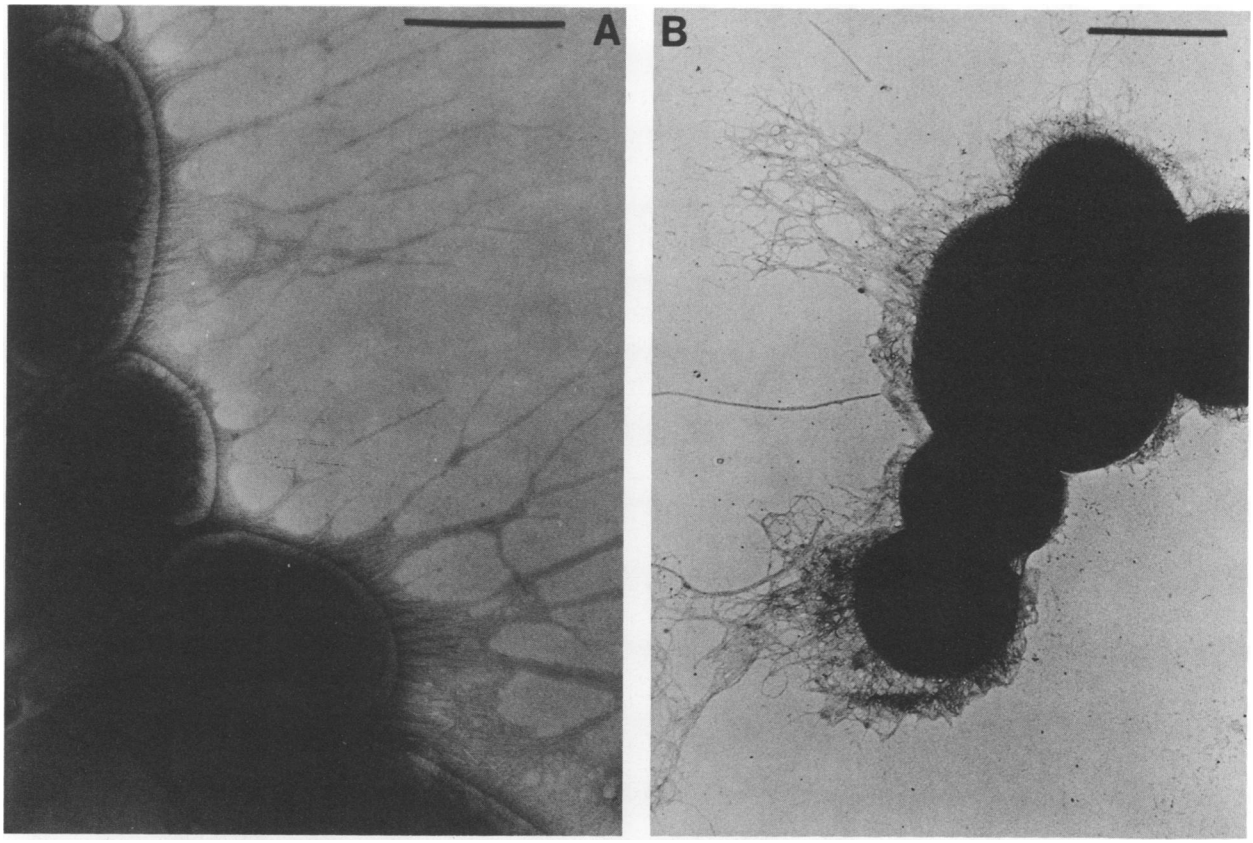

FIG. 5. Transmission electron micrographs of *E. coli* appendage adhesins. (A) Bacteria grown from a clinical isolate expressing only a P and an F adhesin. (B) Bacteria grown from a clinical isolate expressing only an X adhesin. Electron microscopy was performed with a transmission electron microscope (JEOL JEM 100C) at 80 kV and a nominal magnification of ×10,000. Copper 400-mesh grids coated with a film of 1% Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) and a thin layer of carbon were used for all samples. A 20-μl sample suspension was placed on a grid, and excess sample was removed by rinsing the grid with several drops of 2% ammonium acetate–3 mM $MgCl_2$, (pH 7.0) buffer. One drop of a negative staining solution of 1% phosphotungstic acid was added to the grid, and excess fluid was removed immediately by blotting. Either polystyrene beads of known unit size or P2 phage tails of known length (10) were sprayed on the back of all grids for magnification calibrations. Bars, 0.5-μm.

Although a combination of both approaches has been used in recent studies (4, 5, 17, 28), the study described here involved a substantially larger number of isolates than has been analyzed previously, more definitive phenotypic assays, and probing with DNA fragments from several virulence factor operons as well as multiple suboperon fragments. This allowed us to establish genetically defined correlations between the properties of infecting bacteria and different types of urinary tract infections, taking into account the significance of particular associations of bacterial phenotypes and genotypes.

Molecular epidemiology of *pap*-related sequences. Recent studies by Lund et al. (22, 23) with the pyelonephritic isolate *E. coli* J96 demonstrated that the *pap* (P adhesin) and *prs* (F adhesin) operons are closely related. In our study all 69 clinical isolates expressing *pap*-related adhesins belonged to a single class defined by a positive hybridization with the *papL*, *papHC*, *papCD*, *papHCD*, and *papEFG* probes (Fig. 3 and Table 3). Thus, among the 137 clinical isolates thus characterized, both P and F adhesins appear to be encoded by genetic elements related to the entire *pap* operon. These findings also indicate that the striking similarities between the P- and F-adhesin operons of *E. coli* J96 as observed by Lund et al. (22, 23) are widespread among clinical isolates. The implications of this observation are relevant to all molecular epidemiologic studies involving adhesins; i.e., alterations that are undetectable by extensive probing with suboperon DNA segments may dramatically alter the receptor-binding specificities of a typical adhesin operon. Thus, physical characterization of virulence operon fine structure is beyond the resolution of colony hybridization. Studies of this nature based on Southern blotting are described in the accompanying paper (3). The results provide relevant new data with respect to copy number, deletions, duplications, and chromosomal locales of *pap*-related sequences that have been found among clinical isolates expressing distinct adhesin phenotypes.

Based on three agglutination assays, five phenotypes were associated with the presence of *pap*-related nucleotide sequences, as follows. (i) Seven isolates mediated P-adhesin-specific hemagglutination of human erythrocytes and the agglutination of Gal-Gal-coated beads. This agglutination profile is indistinguishable from that observed for the P adhesin encoded by the *pap* operon in J96 (23). (ii) Eleven isolates hemagglutinated sheep erythrocytes. In *E. coli* J96 hemagglutination of sheep erythrocytes is caused by the expression of the F adhesin, which is encoded by the *prs* operon and recognizes the Forssman antigen (23). Hemagglutination of sheep erythrocytes in clinical isolates likely is caused by the presence of an F adhesin recognizing the

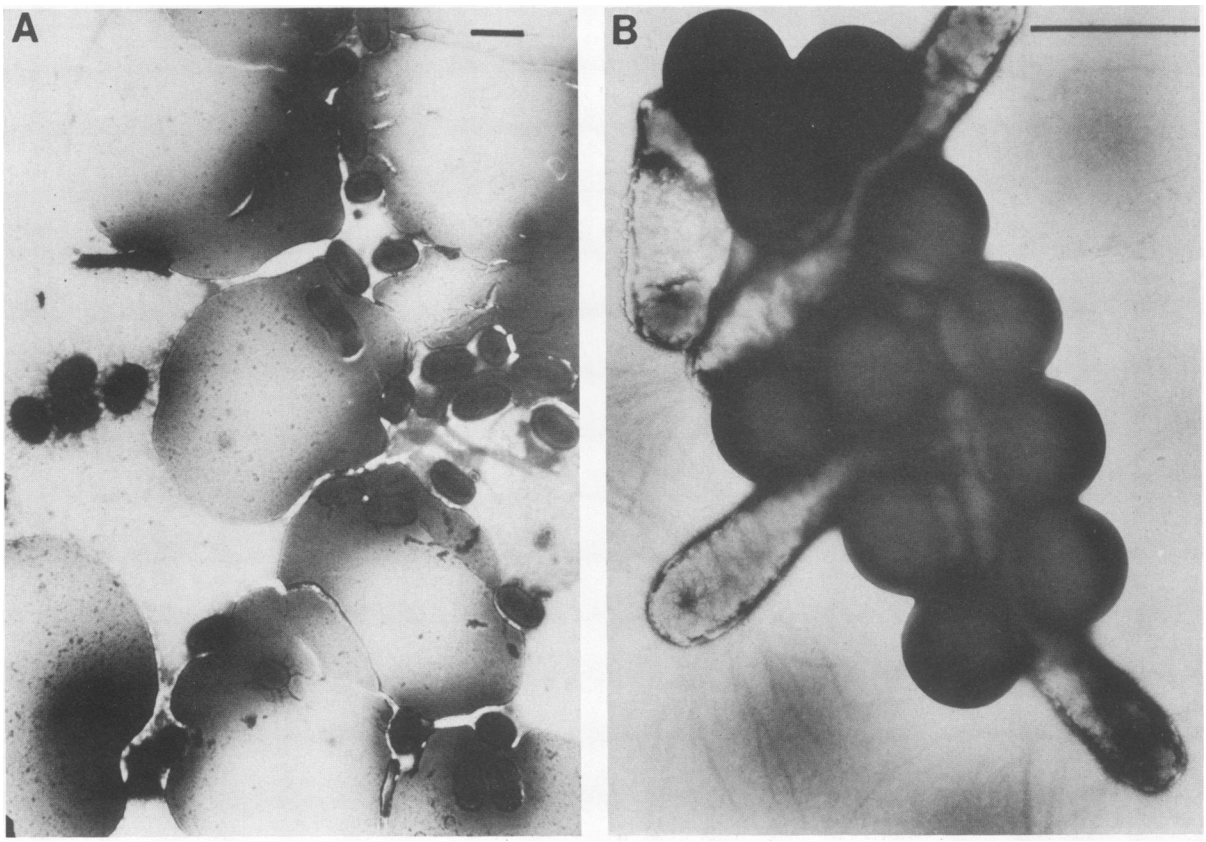

FIG. 6. Trasmission electron micrographs of agglutination reaction of bacteria from a clinical isolate expressing P and F adhesins only with human P1 NN erythrocytes (A) and, Gal-Gal-coated latex beads (B). Sample preparation and electron microscopy were as described in the legend to Fig. 5. Bars, 1 μm.

Forssman antigen, but we could not assay for receptor specificity because neither sheep erythrocytes lacking the Forssman antigen nor latex beads coated with the binding epitope were available. (iii) Forty-nine isolates mediated P-adhesin-specific hemagglutination of human erythrocytes, hemagglutination of sheep erythrocytes, and agglutination of Gal-Gal-coated latex beads. In J96 this agglutination profile is caused by the presence of two structurally related operons, *pap* and *prs*, encoding two adhesins with distinct binding specificities (23). In clinical isolates this phenotype may correspond to the coexpression of two distinct adhesins as found in *E. coli* J96, but alternatively, a single adhesin may recognize the Gal-Gal moiety of the P antigen and a receptor on sheep erythrocytes. It should be noted that the Forssman antigen contains an internal Gal-Gal disaccharide. However, as expressed at the surface of sheep erythrocytes, this epitope is not recognized by the P adhesin of J96 (23). (iv) Two isolates mediated P-adhesin-specific hemagglutination of human erythrocytes and hemagglutination of sheep erythrocytes but no agglutination of Gal-Gal-coated beads. This rare agglutination profile may be caused by the expression of an F adhesin plus a P adhesin that does not recognize the Gal-Gal minimal receptor of the J96 P adhesin when it is present on latex beads. (v) Seven isolates were negative for all three agglutination assays. Nucleotide sequences related to the *pap* operon were detected in 13 clinical isolates that expressed neither P nor F adhesins. Seven of these isolates were detected by all the probes, while DNA of six isolates hybridized with the *papL* and *papEFG* probes but proved negative for the *papHC*, *papCD*, and *papHCD* probes. Thus, genetic elements related to a part and, apparently, to the entire *pap* operon were detected in isolates that expressed neither a P nor an F adhesin.

Clinical correlates of P and F adhesins. The detailed clinical investigations and radiological studies performed provide a basis to correlate different types of urinary tract infections with the virulence factors found among the infecting bacteria (Fig. 2). For comparison with uropathogens, we also analyzed *E. coli* fecal isolates.

As expected, results of this study confirmed the previously observed prevalence of P-adhesin expression in pyelonephritis isolates (for a review, see reference 33), and is therefore in agreement with the model that the P adhesin confers a selective advantage to *E. coli* in colonizing the upper urinary tract. The use of additional phenotypic assays for non-P adhesins allowed us to identify an unforeseen novel association between pyelonephritis and the coexpression of both the P and the F adhesins encoded by *pap*-related sequences. The expression of these two adhesins among pyelonephritis isolates (32 of 49, 65%) was significantly higher than that among cystitis isolates (11 of 48, 23%; $P < 0.0001$) or fecal isolates (6 of 40, 15%; $P < 0.0001$). In contrast, the properties of the cystitis and fecal isolates proved to be similar. The same statistically significant differences or similarities were observed when the distributions of P adhesin, F adhesin, or *pap*-related sequences among the three groups of isolates were considered independently (Fig. 2). Furthermore, the frequencies of the different adhesion phenotypes associated with *pap*-related sequences were found to vary among urinary tract and fecal isolates (Fig. 4).

Coexpression of a P and an F adhesin was detected in the majority of *pap* probe-positive pyelonephritis (32 of 37, 86%) and cystitis (11 of 20, 55%) isolates. In contrast, expression of an F adhesin only was the most frequent phenotype among *pap* probe-positive fecal isolates (9 of 19, 47%), while this phenotype was not detected in pyelonephritis isolates and was rare in cystitis probe-positive isolates (2 of 20, 10%).

The results suggest that distinct *pap*-related adhesins either alone or in combination may play different roles in virulence. Expression of the F adhesin alone appears to confer no selective advantage in the colonization of the upper urinary tract, since this phenotype was not detected in pyelonephritis isolates but was common in fecal *pap* probe-positive isolates. Since expression of only a P adhesin was rare in the three groups of isolates, the association of P and F adhesins may reveal either that P and F adhesins in consort are critical for virulence or that the F adhesin is a neutral passenger of a selection process that is focused on the P adhesin alone.

Molecular epidemiology of *pil*-related sequences. The *pilC* probe that was used for colony hybridization analyses was also derived from the pyelonephritogenic isolate *E. coli* J96 (Table 1 and Fig. 1). A total of 126 (92%) of the 137 clinical isolates studied displayed homology with the *pilC* probe (Table 3). However, only 45 of these 126 isolates initially expressed a type 1 adhesin based on phenotypic assay results. For 7 of the 13 isolates tested, we were able to obtain expression of the type 1 adhesin phenotype after serial subculturing. Thus, it is likely that most of the 126 *pilC* probe-positive isolates maintained the capacity to express a type 1 adhesin. These observations could be accounted for by phase variation, which, in *E. coli* CSH50, has been shown to result from the inversion of a DNA fragment controlling the transcription of the type 1 adhesin operon (1). Phase variation of type 1 adhesin expression occurs both in vivo, as demonstrated for experimental peritonitis in mice (2), and under laboratory conditions (14).

Clinical correlates of type 1 adhesins. Expression of the type 1 adhesin among *pilC* probe-positive isolates was detected more frequently among fecal isolates (23 of 34, 68%) than among pyelonephritis (10 of 48, 21%; $P < 0.0001$) or cystitis (12 of 44, 27%; $P = 0.0009$) isolates. These results suggest that the lower incidence of type 1 adhesin expression among urinary tract isolates is caused by the regulation of functional *pil*-related operons and may indicate that infective conditions in the urinary tract select against the expression of *pil*-related sequences. However, phase variation of type 1 adhesin expression under laboratory conditions as described above precludes the establishment of a strong conclusion.

Molecular epidemiology of *afaI*-related sequences. Among the adhesins surveyed in this study, the broad class of X adhesins is the least well characterized at the molecular level. This may account for the observed deviation between genotypic and phenotypic characterizations. In this survey the three *afaI* internal probes (Fig. 1) detected the same group of 44 isolates. The observation that 12 of these clinical isolates were phenotypically negative for X-adhesin expression (Table 3) could be explained by regulation or by partial deletions leading to the absence of the X-adhesin phenotype, as proposed recently based on Southern blot analysis of *afaI*-related sequences (17). Likewise, there may be multiple classes of cross-hybridizing *afaI*-related adhesin operons encoding different receptor specificities, as was found for the *pap* operon.

Analysis of *afaI* epidemiology is further complicated by the observation that 12 of the 93 *afaI* probe-negative isolates expressed an X adhesin. This suggests that these isolates have as yet unresolved adhesins that can bind to the pp, NN erythrocytes that were used to detect the expression of X adhesins.

Clinical correlates of X adhesins. X-adhesin expression proved significantly higher in pyelonephritis (24 of 49, 49%) and cystitis (23 of 48, 46%) isolates than in fecal isolates (4 of 40, 10%; $P = 0.0005$; Fig. 2). In contrast, *afaI* sequences were found among cystitis isolates (24 of 48, 50%) significantly more frequently than among pyelonephritis (13 of 49, 27%; $P = 0.03$) or fecal (7 of 40, 18%; $P = 0.003$) isolates. The frequency of X-adhesin expression among *afaI* probe-positive strains was similar in the three groups of isolates.

The simplest extrapolation of these results is that X adhesins encoded by *afaI*-related sequences contribute to the virulence of *E. coli*-mediated cystitis. Because the *afaI*- and *pap*-related sequences were not associated (Table 4), our results suggest that *afaI*-related adhesins facilitate lower urinary tract colonization, while *pap*-related adhesins facilitate upper urinary tract colonization.

Molecular epidemiology of the *hly*-related sequences. The distribution of nucleotide sequences structurally related to the *hly* operon encoding the hemolysin of pyelonephritogenic *E. coli* J96 was surveyed with the *hlyA* probe, which is internal to the *hlyA* cistron (Fig. 1). All 41 clinical isolates that lysed sheep erythrocytes in blood agar plates were detected by the *hlyA* probe (Table 3). Only a single probe-positive isolate displayed a negative phenotype. The remaining 95 isolates were negative with respect to both the hybridization and phenotypic assays. These results indicate that (i) hemolysin is encoded by a single class of genes homologous to the *hly* operon, and (ii) with one exception, *hly*-related sequences encode a functional hemolysin and are expressed.

It should be noted that all 42 clinical isolates detected by the *hlyA* probe also hybridized with the full set of *pap* probes (Table 4). Results of the study of Hull et al. (12) indicate that while the *pap* and *prs* operons are located far apart in *E. coli* J96, the *hly* operon is closely linked to the *prs* operon in this strain. This genetic linkage may be responsible for the codissemination found in clinical isolates.

Clinical correlates of hemolysin. Expression of hemolysin was similar in pyelonephritis (20 of 49, 41%), cystitis (9 of 48, 19%) and fecal (12 of 40, 30%) isolates (Fig. 2). Only the difference in hemolysin expression between pyelonephritis and cystitis isolates was significant ($P = 0.03$). Hemolysin expression was only detected in *pap* probe-positive isolates (see above). The distribution of hemolytic strains among the *pap* probe-positive pyelonephritis, cystitis, and fecal isolates was similar. These observations suggest that hemolysin confers no particular selective advantage in the colonization of the urinary tract.

Adhesins and reflux. Among the whole collection of 97 isolates from patients with urinary tract pathogens and the subset of 49 isolates from patients with pyelonephritis, the distribution of virulence factors was similar among organisms infecting hosts with or without radiologically documented structural abnormalities (Table 5). In particular, the 12 isolates from patients with both structural abnormalities and pyelonephritis expressed P, F, and X adhesins at rates comparable to those of the other pyelonephritis isolates, suggesting that even in the presence of reflux most infections were caused by strains expressing pathogenic adhesins.

There is some conflict among the data that have been published on this issue, probably relating to differences in the patient populations that have been studied. Lomberg et

al. (21) have reported that only 25% of the isolates from patients with pyelonephritis and reflux expressed P adhesins, compared with 68% of the isolates from patients without reflux. This patient population was notably different from ours in that all patients had recurrent pyelonephritis (≥two episodes) and the overall mean age was 12 years. In subsequent studies by the same investigators (21), patients who went on to develop scarring were significantly less likely to be infected with *E. coli* expressing P adhesins than were patients with recurrent pyelonephritis who did not develop scarring (H. Lomberg, M. Hellström, U. Jodal, and C. Svanborg-Edén, Proceedings of the IVth International Symposium on Pyelonephritis, p. 116, 1986). Reflux was, as might be expected, strongly associated with scarring; but independent of scarring, reflux did not correlate with the presence or absence of P adhesins. The specific host factors related to the development of scarring and the mechanism by which these factors might relate to bacterial adhesins remain undefined.

Mårlid and co-workers have recently studied patients during their first episode of pyelonephritis, similar to the majority of our patients, and found little influence of reflux on the prevalence of P-adhesin expression (S. Mårlid, M. Hellström, U. Jodal, B. Kaijser, I. Orskov, F. Orskov, and C. Svanborg-Edén, Proceedings of the IVth International Symposium on Pyelonephritis, p. 58, 1986). Elo et al. (6) have studied the infecting organisms from 74 children with pyelonephritis, 57% of whom had a recurrent infection and 41% of whom had reflux. Among these isolates, 77% expressed P adhesins, and this percentage did not vary with the presence of the degree of reflux. Overall, the results of several studies, including this one, indicate that the substantial majority of pyelonephritogenic *E. coli* express P adhesins. This appears to be true even in patients with reflux, at least for their initial episode of infection. In contrast, the role of adhesins in the pathogenesis of recurrent urinary tract infections remains to be elucidated, particularly for that subgroup of patients with reflux who proceed to develop renal scarring.

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