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In Vivo Expression and Variation of *Escherichia coli* Type 1 and P Pili in the Urine of Adults with Acute Urinary Tract Infections

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In vivo expression of pili by *Escherichia coli* in the urine of 41 adults with lower urinary tract infections was analyzed by immunostaining with polyclonal antiserum to type 1 and P pili. Type 1 pili were detected in 31 of 41 urine specimens, while P pili were detected in 6 of 18 specimens. The piliation status of bacterial populations in urine was heterogeneous, varying from predominantly piliated to a mixture of piliated and nonpiliated cells. Bacteria frequently adhered to exfoliated uroepithelial cells and leukocytes in urine. Expression of pili in vivo did not always correlate with the hemagglutination phenotype after growth in vitro. Strains isolated from different sites in the urogenital tract of two individuals showed phenotypic variation in the state of piliation. The results demonstrate that *E. coli* type 1 and P pili are expressed and are subject to variation in vivo during acute urinary tract infections in adults.

Pilus-mediated adherence of *Escherichia coli* to uroepithelial cells is an important event in the pathogenesis of urinary tract infections (35). Type 1 pili, which mediate mannose-sensitive agglutination (MSHA) of guinea pig erythrocytes, and P pili, which mediate mannose-resistant agglutination of human erythrocytes, are frequently produced in vitro by *E. coli* isolated from patients with urinary tract infection (5, 8, 20, 22, 30, 41). Although in vitro adherence studies (15, 27, 36) and animal models (14, 19, 21, 29, 37) offer indirect evidence for the role of type 1 and P pili in urinary tract infections, direct evidence to indicate that these structures are present in vivo is limited and partially contradictory. Ljungh and Wadstrom (23) demonstrated by electron microscopy that pili were present on *E. coli* in the urine of 31 of 37 patients, but the specific pilus type was not determined. Conversely, Ofek et al. (28) showed no mannose-sensitive adhesins in 22 of 24 urine isolates from patients with indwelling catheters, and Harber et al. (16) found that organisms in 19 of 20 samples from patients with acute urinary tract infections were devoid of pili and were nonadherent until subcultured in broth. Assessment of pilus production by clinical *E. coli* isolates is further complicated by the fact that environmental growth conditions can produce rapid changes in pilus expression in vitro (8, 13, 20), with cells switching between piliated and nonpiliated phases (1, 10).

Pere et al. recently used indirect immunofluorescence to test the in vivo expression of pili on *E. coli* in the urine of 20 children with pyelonephritis or cystitis; P pili were present in 17 specimens, while type 1 pili were detected in 9 (33). In this study, we used specific polyclonal antibodies in an immunomicroscopy assay to demonstrate the presence of type 1 and P pili on single bacterial cells in the urine of adult patients with lower urinary tract infections. The variable expression of piliation by bacterial populations in urine and the observation that bacterial populations from different sites within the urinary tract differed with respect to expression of type 1 pili suggest that phase variation of *E. coli* pili may occur in vivo.

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MATERIALS AND METHODS

Patient population. Midstream or catheterized urine specimens were collected from consecutive patients with *E. coli* cystitis in a urology clinic. The group consisted of 9 men and 32 women, ages 24 to 90. A total of 79% of the patients were symptomatic (frequency of urination, dysuria, pyuria without fever), and the rest had asymptomatic bacteriuria. None of the patients had clinical evidence of pyelonephritis, were receiving antimicrobial agents, or had indwelling catheters or urinary conduits. A total of 85% of the patients had a history of previous urinary tract infections, and 12% had urinary tract abnormalities. In two female patients, cystoscopy and ureteral catheter localization studies (39) were performed, and bacteria were obtained simultaneously from the upper urinary tract as well as from the voided urine, catheterized bladder urine, bladder lavage, and vaginal mucosa.

Specimens. Urine specimens were Gram stained to identify gram-negative rods and were examined with a hemacytometer to determine the number of epithelial cells and leukocytes. All specimens contained $>10^3$ bacteria per ml, and 73% had $>10^5$ bacteria per ml. Isolated bacteria were stored on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) slants and passaged five consecutive days on blood agar plates (Gibco Laboratories, Grand Island, N.Y.) and in brain heart infusion broth.

Indirect immunomicroscopy assays. Polyclonal antibodies to purified preparations of *E. coli* type 1 and P pili were produced as previously described by Hultgren et al. (19). Before using these antibodies in the assay, we confirmed the specificity of the anti-type 1 and anti-P pilus antibodies by showing that each antibody inhibited the hemagglutination (HA) of 10 type 1 and 4 P-piliated *E. coli* clinical strains, respectively (20).

Fresh urine specimens were pelleted by centrifugation and suspended in 1 ml of phosphate-buffered saline (pH 7.2), layered on a 20 to 35 to 50% discontinuous Percoll (Pharmacia Diagnostics, Piscataway, N.J.) gradient, and centrifuged at 10,000 × *g* for 10 min. Discrete bands of bacteria, leukocytes, and epithelial cells were washed in phosphate-

buffered saline, pelleted onto slides by using a Shandon cytocentrifuge (Sewickley, Pa.), and fixed in 95% alcohol.

Slides from all 41 specimens were stained for type 1 pili by the peroxidase-antiperoxidase method of Sternberger et al. (40) or by fluorescein isothiocyanate-labeled swine anti-rabbit antibody (Accurate Chemical, Westbury, N.Y.). Slides of the last 18 consecutive specimens were also stained for the presence of P pili by using tetramethyl rhodamine B isothiocyanate-labeled swine anti-rabbit antibody (Accurate Chemical). Slides were also stained by a modified Papanicolaou method (18) to evaluate leukocyte and epithelial-cell populations.

One slide of each isolate was prepared with normal rabbit serum rather than polyclonal antibody to ensure that nonspecific staining was not occurring. A type 1-piliated strain, 149, and a P-piliated strain, 31, were used as positive controls; nonpiliated variants of the same strains were used as negative controls (20).

A total of 20 or more oil immersion fields with at least 50 bacteria each were observed per slide. When epithelial cells were present, 10 or more cells per field were observed. Individual bacteria were described as adherent if they were attached to epithelial cells. If they were not attached to epithelial cells, they were called free. Normarski differential interference contrast microscopy (7) was used to confirm that adherent bacteria were not intracellular.

HA. Mannose-sensitive and mannose-resistant HA titers of broth- and agar-grown isolates were determined as previously described (20).

Analysis of strains from multiple sites in same patient. Chromosomal DNA digests were analyzed from *E. coli* collected from the vaginal mucosa, voided urine, catheterized urine, bladder wash, and kidney urine of patient 26. The DNA was isolated (34), digested with *Hin*dIII, and run on a 0.4% agarose gel (25).

Hemolysin was assayed by noting the zone of hemolysis on blood agar plates.

Serum sensitivity was determined as described by Finn et al. (11) in a final concentration of 33% normal human serum.

RESULTS

Detection of piliated bacteria in urine. To detect pili on fresh clinical isolates, bacteria were separated directly from urine specimens in Percoll gradients and probed with pilus-specific antibodies by using immunofluorescent and immunoperoxidase staining. Bacteria with type 1 pili were identified in 31 (76%) of the 41 specimens.

Of the 41 specimens, 31 contained epithelial cells. Epithelial cells with attached bacteria (adherent bacteria) were identified in 27 of the 31 specimens. The bacteria adherent to epithelial cells possessed type 1 pili in 21 (78%) of 27 of these specimens (Fig. 1). The bacterial population was either uniformly piliated (48% of specimens) or a mixture of piliated and non-type 1-piliated cells (Fig. 2). Of the 27 specimens, 6 (22%) had adherent bacteria which did not possess type 1 pili, and in these specimens, the free bacteria (not adherent to epithelial cells) also lacked type 1 pili. Of the 31 specimens with epithelial cells, 4 had no bacteria adherent to the epithelial cells; in 3 of these specimens, the unattached bacteria possessed type 1 pili.

Free bacteria were present in 40 of the 41 specimens. The free bacteria were type 1 piliated in 58% (23 of 40) of these specimens (Fig. 1). In most (71%) of the specimens, the free-bacterium population was a mixture of type 1-piliated and non-type 1-piliated bacteria, but in 29%, all the unattached bacteria stained positively for type 1 pili.

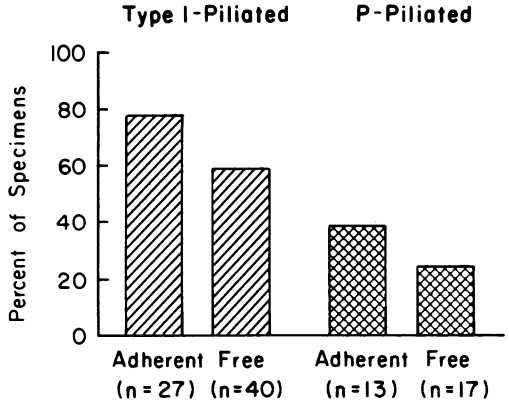

FIG. 1. Prevalence of type 1- and P-piliated bacteria in urine specimens containing bacteria that were free or adherent to uroepithelial cells.

P pili were present in 6 (33%) of 18 specimens examined. A total of 13 of the 18 specimens contained epithelial cells, and adherent bacteria were present in all 13. Adherent bacteria possessed P pili in 38% (5 of 13) of these specimens (Fig. 1), and the bacterial population was usually a heterogeneous mixture of P and non-P-piliated cells.

Free bacteria were present in 17 of the 18 specimens tested for P pili. The free bacteria were P piliated in four (24%) of these specimens (Fig. 1). In one specimen, the free population was uniformly P piliated (Fig. 3), while in the others, a heterogeneous population was seen.

The bacteria in one of the specimens containing a strain which expressed both type 1 and P pili simultaneously was examined in more detail. Of 300 individual bacteria examined, 179 (60%) were nonpiliated, 85 (28%) showed type 1 pili, 17 (6%) showed P pili, and 19 (6%) showed both type 1 and P pili. Of 13 specimens with adherent *E. coli*, 3 failed to show the presence of either type 1 or P pili in vivo. There was no statistically significant correlation among adherent or nonadherent bacteria and the expression of either type 1 or P pili.

A total of 35 specimens contained leukocytes, and 28 (80%) had *E. coli* attached to the leukocyte surface. Type 1 pili were detected in 23 (82%) of the specimens, usually as a mixed population of piliated and non-type 1-piliated cells. Bacteria with P pili were detected in 5 (45%) of the 11 specimens tested.

Few clinical correlates were found with regard to the type of pili expressed by the isolates. Patients with symptomatic urinary tract infection more frequently exhibited *E. coli* with type 1 pili (22 [58%] of 38) rather than P pili (4 [31%] of 13). The prevalence of pili types expressed on *E. coli* did not correlate with the sex of the patient, pyuria, or urinary tract pathology.

Comparison of phenotypes in urine and after growth in vitro. The expression of pili on bacteria in urine was compared with the HA reactions of isolates passaged five consecutive times in static broth or on agar. Of the 41 isolates, 30 (73%) showed MSHA of guinea pig erythrocytes. Of the 31 strains that had type 1 pili in urine, 25 (81%) also showed MSHA after in vitro passage (Table 1). A total of 11 strains showed a disparity between the phenotypes in urine and the HA reaction after in vitro growth. The sensitivity of the immunoassay was compared for bacteria in urine and after passage on five consecutive days in broth. All five strains that were immunonegative in urine and HA positive in vitro

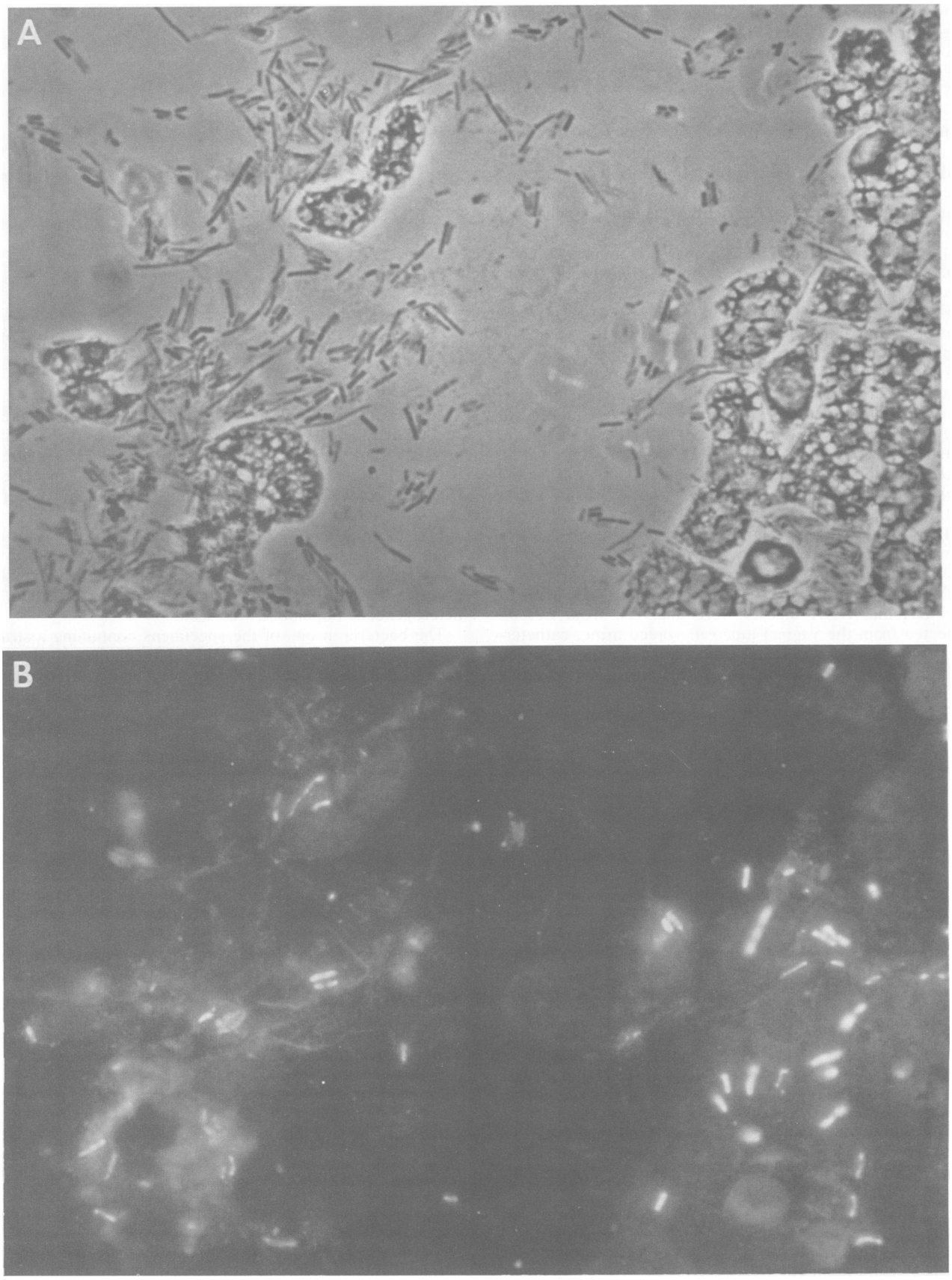

FIG. 2. Phase-contrast micrograph (A) and immunofluorescence staining (with antiserum to type 1 pili of strain 149 and with fluorescein isothiocyanate-conjugated second antibody) (B) of *E. coli* in the urine of a patient with acute urinary tract infection. A mixture of piliated and nonpiliated bacteria adherent to epithelial cells exfoliated in the urine is shown.

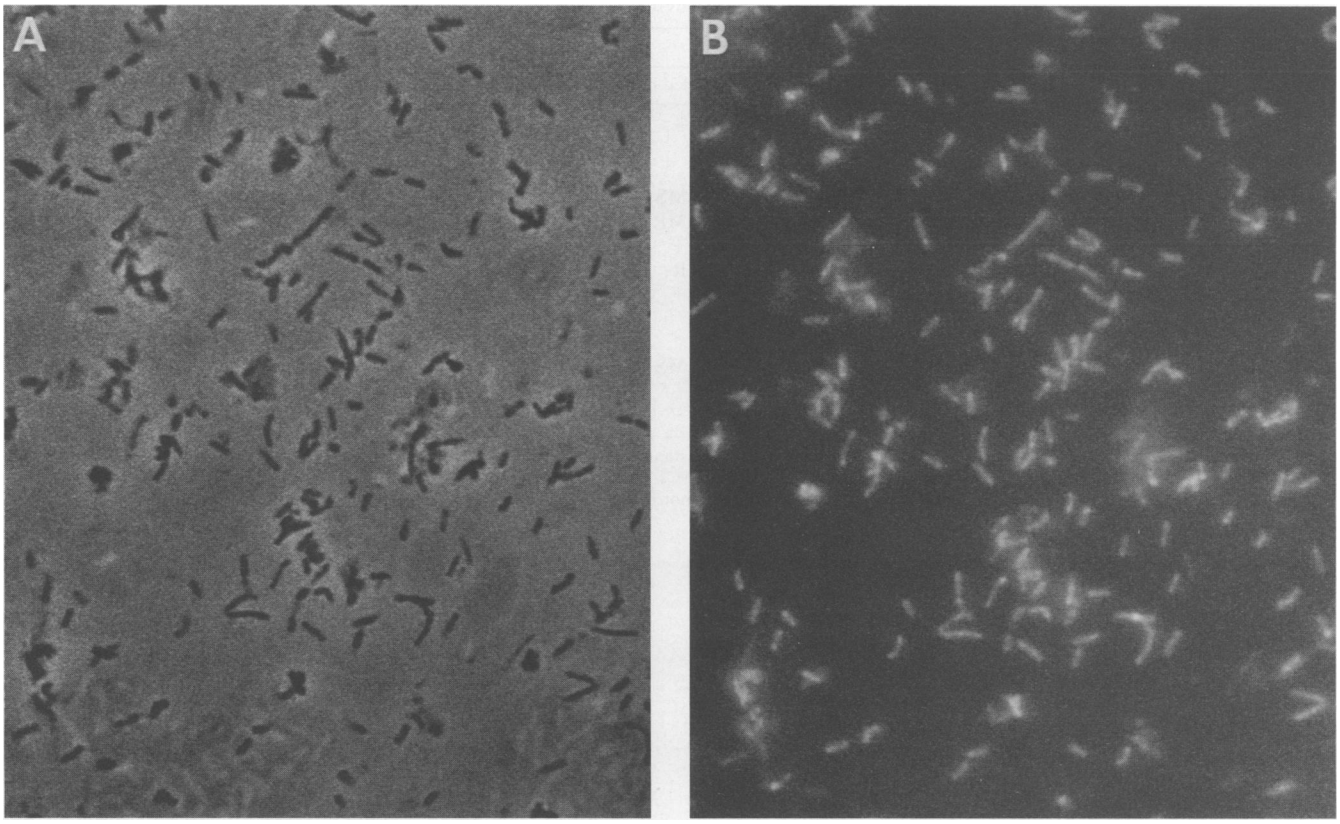

FIG. 3. Phase-contrast micrograph (A) and immunofluorescence staining (with antiserum to P pili of strain 31 and with tetramethyl rhodamine B isothiocyanate-conjugated second antibody) (B) of free *E. coli* in the urine of a patient with acute urinary tract infection. Most of the bacteria are P piliated.

were immunopositive in vitro. Of the five tested strains that were immunopositive in urine and HA negative in vitro, four were immunonegative and one was immunopositive in vitro.

Mannose-resistant agglutination of human erythrocytes after passage of strains in vitro was evident with 11 (27%) of the 41 isolates. The concordance of P pilus phenotypes in urine and in vitro was examined for 18 isolates (Table 1). Of the seven strains that showed a disparity between in vivo staining and the in vitro HA reaction, six were tested after passage on five consecutive days on agar. One of three strains that were immunonegative in urine and HA positive in vitro was immunopositive in vitro. All three tested strains that were immunopositive in urine and HA negative in vitro were immunonegative in vitro.

Expression of type 1 pili by isolates from multiple sites in the same patient. When characteristics of *E. coli* isolated from different sites in individual patients were examined, a disparity in type 1 phenotype between sites and between characteristics in urine and in vitro was observed (Table 2). For example, *E. coli* in the voided bladder urine of patient 26 had type 1 pili, whereas *E. coli* isolated from other sites did not show type 1 pili. No urine isolates showed P pili. All the strains, however, demonstrated MSHA of guinea pig erythrocytes after growth in broth and mannose-resistant agglutination of human erythrocytes after growth on agar. Serum sensitivity and production of hemolysin were the same for isolates from the different sites. Patient 22 showed a disparity between type 1 phenotypes in voided and catheter or bladder wash specimens and the phenotypes of bacteria in voided urine and after growth in vitro.

To demonstrate that organisms isolated from different sites were the same strain, we compared the restriction enzyme cleavage patterns of chromosomal DNA of each isolate from patient 26. Digestion of DNA from each isolate from the patient resulted in identical patterns of restriction fragments, although the digestion pattern of an isolate from another patient was different (Fig. 4).

TABLE 1. Comparison of type 1 and P pilus phenotypes in urine and after growth in vitro

Pilus expression in urine[a]	No. of in vitro specimens with pili			
	Type 1[b]		P[c]	
	Positive	Negative	Positive	Negative
Positive	25	6	2	4
Negative	5	5	3	9

[a] Indirect immunoperoxidase and immunofluorescence assays.
[b] MSHA of guinea pig erythrocytes.
[c] Mannose-resistant agglutination of human P but not p erythrocytes.

DISCUSSION

Our results show that *E. coli* expresses pili in urine, and these results are consistent with the hypothesis that pili are virulence factors in adults with acute urinary tract infection. Of fresh urine isolates stained by either an immunoperoxidase-antiperoxidase or a fluorescence technique, 76% exhibited type 1 pili and 33% produced P pili. Previous

TABLE 2. Characteristics of *E. coli* isolated from different sites in individual patients

Patient no.	Characteristic tested	Response of specimens from[a]:				
		Vagina	Voided urine	Catheter urine	Bladder wash	Kidney urine
26	Type 1 pili in specimen[b]	−	+	−	−	−
	In vitro					
	HA					
	Broth grown	MS	MS	MS	MS	MS
	Agar grown	MR	MR	MR	MR	MR
	Hemolysin	+	+	+	+	+
	Serum sensitivity	R	R	R	R	R
22	Type 1 pili in specimen	ND	+	−	−	ND
	In vitro					
	HA					
	Broth grown	ND	MS	MS	MS	ND
	Agar grown	ND	−	−	−	ND
	Hemolysin	ND	+	+	+	ND

[a] Abbreviations: MS, MSHA of guinea pig erythrocytes; MR, mannose-resistant agglutination of human P but not p erythrocytes; R, survives in the presence of 33% normal human serum; ND, not determined. +, Positive; −, negative.
[b] Indirect immunoperoxidase and immunofluorescence assays. P pili were not present.

studies reported that over 90% of freshly isolated *E. coli* were nonadherent or nonpiliated or both but that after subculture in vitro, the bacteria frequently became adhesive and produced pili (16, 19). The difference between our observation that *E. coli* expresses pili in urine and the lack of piliation in these previous studies may be due to the duration of infection, the patient population investigated, or the sensitivity of the methods used to detect pili. A decrease in the degree of piliation has been observed during the course of experimental pyelonephritis or peritonitis in mice (2). On the other hand, Pere et al. (33) found that P and type 1 pili were expressed in vivo and were subject to phase variation during acute urinary tract infection in pediatric patients.

As did Pere et al. (33), we identified type 1 and P pili with polyclonal antibodies in an immunomicroscopy assay rather than with the electron microscopy and agglutination assays used by Harber et al. (16) and Ofek et al. (28). Many of the piliated bacteria in our study were adherent to epithelial cells and could be nonreactive in an agglutination assay. Furthermore, only a few bacteria were piliated in some specimens, and these might not have given a positive phenotype in an agglutination assay.

E. coli populations in urine were heterogeneous with regard to piliation. If one assumes that the infecting bacteria are a single strain of *E. coli*, the results of the present study raise the possibility that piliated urinary isolates exhibit phase variation in vivo in the urinary tract. Animal models of urinary tract infection have shown that phase variation of piliation occurs in vivo and may be an important virulence factor. Hultgren et al. (19) showed that *E. coli* which adhered to the murine bladder urothelium expressed type 1 pili, whereas nonadherent bacteria in the urine frequently were nonpiliated.

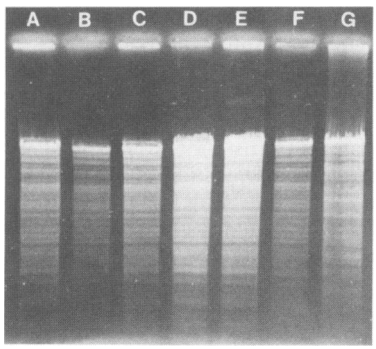

FIG. 4. Ethidium bromide-stained agarose gel (0.4%) of chromosomal DNA digested with *Hin*dIII. DNA was extracted from *E. coli* isolated from voided urine (lane A), vaginal mucosa (lane B), voided urine (lane C), catheter urine (lane D), bladder lavage (lane E), kidney urine specimens of a single patient (lane F), or another clinical isolate (strain 33) (lane G).

Additional evidence suggestive of phase variation in human urine was obtained from the ureteral catheter localization studies. *E. coli* isolated from different sites of the urinary tract in two patients with bacterial persistence associated with upper-tract stones (Table 2) showed different states of piliation, even though biotyping, in vitro HA, serum sensitivity, production of hemolysin, and DNA profiles were identical. The voided urine contained bacteria and uroepithelial cells from the bladder mucosa. Type 1 pili were identified only in isolates from the voided urine, whereas upper-tract isolates, obtained by ureteral catheter or by bladder catheterization immediately after voiding, were nonpiliated. In a murine model of ascending urinary tract infection, type 1-piliated *E. coli* were inoculated intravesically, but the bacteria recovered from the kidneys were frequently nonpiliated, suggesting that pilus loss may be advantageous to the survival of *E. coli* in the upper tract (37).

Type 1 and P pili have been identified on the same isolate in vitro (30). Our detailed examination of a single strain with both type 1 and P pili supports the observation of Nowicki et al. (26) that less than 9% of piliated cells have more than one pilus type and the report of Gander and Thomas (12) that 7% of the cells in a laboratory strain of *E. coli* expressed both type 1 and P pili simultaneously in vitro.

Adherent bacteria were frequently found on exfoliated epithelial cells in urine specimens. Since exfoliated epithelial cells are thought to represent the surface of the urothelium, these bacteria may be representative of the population bound to the mucosal lining of the bladder. Of 13 adherent isolates, 3 exhibited neither type 1 nor P pili, suggesting that adherence may be mediated by other types of pili or by factors other than pili in these instances.

E. coli was bound frequently to leukocytes. Bacterial adherence to leukocytes was associated with type 1 pili in 23 of 28 specimens and with P pili in 5 of 11 specimens. Our

results are consistent with a role for type 1 pili in mediating binding of *E. coli* to leukocytes in vitro (3, 4, 24, 38).

The in vitro HA titer did not always reflect the state of piliation in vivo. Discrepancies between in vivo and in vitro expression of pili have been reported previously (16, 23) and may be due to decreased sensitivity or specificity of the antibody probe used to detect pili in vivo. This could be due in part to interference from antibody binding by components of urine that remain bound to bacteria despite gradient purification. P pili are known to occur in many serovariants (31), and although type 1 pili are considered serologically conserved (9, 32), they have recently been shown to be serologically discrete (20, 33). However, six of the eight strains that were immunonegative with regard to the presence of type 1 or P pili in vivo and HA positive in vitro were immunopositive in vitro. Therefore, serovariants could have accounted for only two false-negative results. Buchanan et al. (6) observed MSHA of only 34 of 60 isolates which displayed DNA homology to the type 1 pilus gene cluster. Similarly, Hull et al. (17) observed that strains which reacted positively with P pilus probes did not always express P pili and mannose-resistant agglutination. Lastly, growth conditions in vivo or in vitro may limit expression of pili.

Expression of type 1 and P pili in vivo in strains of *E. coli* in urine of patients with cystitis is consistent with the role of pili in the pathogenesis of lower urinary tract infection. The observation that *E. coli* existed simultaneously in both piliated and nonpiliated states in vivo and exhibited phenotype variation at different sites within the urinary tract suggests that phase variation of pili occurs in vivo. Discordance between the in vitro HA titer and state of piliation in vivo emphasizes that passage of strains in vitro may obscure important differences present in vivo which modify the virulence of uropathogenic *E. coli*.

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