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Immunochemical Characterization of P Pili from Invasive *Escherichia coli*

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P pili (or fimbriae) are present on most pyelonephritogenic *Escherichia coli* strains, and they mediate binding to erythrocytes and epithelial cells. To determine the antigenic diversity of P pili, we purified the pili from 14 bacteremic *E. coli* strains which caused mannose-resistant hemagglutination. Pilus preparations consisted of one to three bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ranged in molecular weight from 14,000 to 19,500. There was no single band common to all the strains. An enzyme-linked immunosorbent inhibition assay detected 20 ng of pilus antigen. When four different rabbit antisera were used, only two or fewer heterologous strains could inhibit the enzyme-linked immunosorbent assay. Immunoblots yielded the same results. Protein sequences of four P pili had identical N termini. These results show that despite having identical amino-terminal sequences, P pili are antigenically heterogeneous. The receptor-binding domains which are likely to be identical in all strains must be immunorecessive.

Adhesion to human cells is thought to be an important attribute of some pathogenic *Escherichia coli* strains. At least part of this binding is mediated by pili (or fimbriae). These organelles can vary considerably with respect to their chemical structure, binding specificity, and immunologic cross-reactivity.

In functional assays, *E. coli* adhesins are broadly classified into mannose sensitive (MS) and mannose resistant (MR), depending on the ability of mannose to inhibit *E. coli* binding to erythrocytes and epithelial cells. MS binding is mediated by type 1 pili, which characteristically cause agglutination of guinea pig erythrocytes (23) as well as attachment to yeast cells, buccal epithelial cells, and a mannose-containing urinary glycoprotein, the Tamm-Horsfall protein (18). The importance of these pili is uncertain, as they appear to be present on both commensal and pathogenic *E. coli* strains from a wide variety of isolation sites. MR binding is usually mediated by pili (14) (although nonpilus MR adhesion does occur [4]) and is more clearly associated with *E. coli* strains isolated from extraintestinal sites (24). MR binding to uroepithelial cells and MR hemagglutination (HA) occur frequently in pyelonephritogenic *E. coli* strains, and these are mediated in most cases by a chromosomally encoded protein termed P pilus, P fimbria, or Pap pilus (pyelonephritis-associated pilus) (14, 31). P fimbriae bind to the P blood group substance (globoside), which contains the active receptor disaccharide alpha-D-Gal-(1-4)-beta-D-Gal (9).

Many other MR adhesins have been described; K88 antigen and 987P pili bind to the gastrointestinal mucosa of piglets (7, 8, 27); K99 antigen is associated with gastroenteritis in cows and lamb (20); colonization factor antigen has a similar association in humans (5); and gonococcal pili mediate binding to erythrocytes and epithelial cells in vitro (2). The complete sequences have been determined for K88, CFA1, gonococcal, and P pili (1, 10, 11, 25).

One of the major aims of such investigations is to develop pilus vaccines which can induce antibodies that block bacterial adhesion. Vaccines containing the K88 antigen protect against gastroenteritis in piglets (22), but a gonococcal pilus vaccine for gonorrhea (J. Boslego, R. Chung, J. Sadoff, D. McChesney, M. Piziak, J. Ciak, J. Brown, W. Caldwell, D. Berliner, G. Seitter, C. Brinton, and E. Tramont, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 294, 1984) and a type 1 *E. coli* pilus vaccine for gastroenteritis (16) were found not to be protective in studies with humans.

An essential element of vaccine development is the detection of a pilus or pilus fragment which occurs uniformly among most strains, is immunogenic, and shares functional (binding) activity. Previous studies have indicated that a number of P pilus antigenic types exist (14, 21). We have attempted to clarify the antigenic diversity of P pili derived from invasive *E. coli* strains by studying their immunochemical variability by Western blotting, enzyme-linked immunosorbent assay (ELISA) inhibition, and limited protein sequencing.

MATERIALS AND METHODS

Bacteria. Thirty *E. coli* strains were obtained from patients at the Toronto General Hospital and were identified as *E. coli* by routine diagnostic tests performed in the clinical bacteriology laboratory. All bacteria had been cultured directly from the bloodstream of clinically infected patients. They were subcultured once and stored as suspensions in 5% (wt/vol) monosodium glutamate–5% (wt/vol) albumin at −80°C. For subsequent studies, bacteria were partially thawed, and a sample was removed with a sterile cotton-tipped swab which was rubbed onto agar plates. Bacteria were routinely grown on colonization factor agar (CFA) as described by Evans et al. (5).

HA. Strains were screened for HA by preparing a suspension of bacteria in phosphate-buffered saline on a glass slide. Heparinized group AB or citrated guinea pig blood was prepared as a 3% suspension, and an equal volume was added to the slide. Agglutination was viewed after the suspension was mixed for 30 s. To test for mannose inhibition, we prepared the bacterial suspension in phosphate-buffered saline containing 1% D-mannose. Quantitative HA was tested in microtiter plates as previously described (23).

Electron microscopy. Piliation was assessed after negative

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TABLE 1. Effect of culture conditions on the expression of P pili

Agar[a]	Mean titer[b]	Broth[c]	Mean titer[b]
Hektoen enteric	0.4	Brain heart infusion	1
Desoxycholate citrate	1.1	Nutrient	1.4
MacConkey	1.4	Tryptic soy	1.8
Mueller-Hinton	2.0	Colonization factor	3.4
Nutrient	2.6		
Tryptic soy	3.4		
Chocolate	4.4		
Colonization factor	4.8		
Blood	6.8		

[a] 18-h growth.
[b] Reciprocal of highest dilution causing complete agglutination.
[c] 48-h growth, static conditions.

staining with 2% phosphotungstic acid (pH 6.0) for 15 s as previously described (24).

Pilus purification. Rapid (crude) preparations of pili were prepared as follows. Overnight confluent growth from two standard-sized petri dishes containing CFA was harvested with a bent glass rod after the plate was flooded with 0.05 M Tris buffer (pH 8.5). The bacterial suspension was blended for 10 min, in a Sorvall Omni-mixer at medium speed. Bacterial cells and fragments were removed by centrifugation at 20,000 × *g* for 30 min. The supernatant was concentrated in an Amicon ultrafiltration cell fitted with an XM300 filter.

Larger-scale preparations were made by modifying the procedure of Dodd and Eisenstein (3). Overnight growth from large (150-mm-diameter) petri dishes containing CFA was harvested and blended twice. Large fragments were pelleted by centrifugation at 20,000 × *g* for 30 min, and the pili were removed by centrifugation at 200,000 × *g* for 3 h. The pellet was suspended in 5 M urea buffer for 4 h at 4°C to solubilize the flagella. After further high-speed centrifugation, the purified pili were suspended in distilled water and stored at −80°C.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in vertical slab gels with samples which had been boiled for 5 min in 2% sodium dodecyl sulfate–1% mercaptoethanol at pH 6.8. Gels contained 15% acrylamide and were run at a constant current of 25 mA for 4 to 6 h (15). Protein bands were visualized by staining gels with Coomassie brilliant blue R250.

Immunization. Female New Zealand White rabbits were injected intramuscularly with 50 μg of pilus antigen and complete Freund adjuvant. On day 14, the injection was repeated in incomplete Freund adjuvant. On day 35, an intravenous booster dose was given, and serum was collected 1 to 2 weeks later.

N-terminal amino acid sequences. Sequence analyses were carried out in a Beckman-Spinco model 890C automatic sequencer by methods previously described (24).

ELISA. The pilus antibody titers of the sera were determined by an ELISA with purified pili as the coating antigens. The ELISA was similar to that described by Voller et al. (32). *E. coli* pili were diluted to a final concentration of 25 ng/ml in the coating buffer (0.05 M bicarbonate buffer, pH 9.6). The pilus solution was adsorbed onto ELISA microtiter plates (Flow Laboratories, Inc.) by overnight incubation at 37°C. Tenfold dilutions of antisera were added, and the plates were incubated for 2 h at 37°C. The plates were washed three times with PBS-T (0.02 M phosphate buffer, 0.136 M NaCl, 0.015 M KCl, 0.05% Tween-20 [pH 7.4]), and goat antirabbit immunoglobulin G-horseradish peroxidase conjugate was then added for 1 h at 37°C. The plates were again washed three times with PBS-T, and the substrate *o*-phenylenediamine was added for 30 min at 37°C. Optimal coating of antigen and dilution of conjugate were determined by chessboard titrations with hyperimmune serum at a 1:100 dilution in PBS-T. The enzyme reaction was stopped by the addition of 2 N H_2SO_4, and the optical density was determined at a wavelength of 492 nm with an automated ELISA plate reader (Multiscan; Flow Laboratories).

The ELISA inhibition assay was performed as described above except that standardized suspensions or twofold dilutions of the potential inhibitor were added to the wells, followed by serum. Inhibitory antigens consisted of suspensions of bacteria which had been grown overnight at 37°C and which were then suspended in PBS-T to an optical density of 0.4 at 540 nm. The appropriate dilution of antiserum was selected from the linear portion of the curve derived from the ELISA described above. The dilution was usually ca. 1:100,000. The serum and the inhibitor were incubated for 2 h at 37°C, followed by three washes with PBS-T. The remainder of the test was then performed as described above for the indirect ELISA. Results were ex-

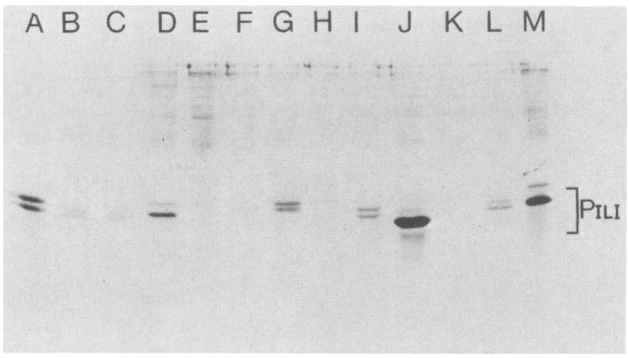

FIG. 1. SDS-PAGE of crude pilus preparations isolated by the rapid method, which minimizes pilus degradation. The lanes contain the following strains: A, 688; B, 714; C, 715; D, 799; E, 800; F, 871; G, 884; H, 917; I, 918; J, 950; K, 974; L, 993; and M, 01.

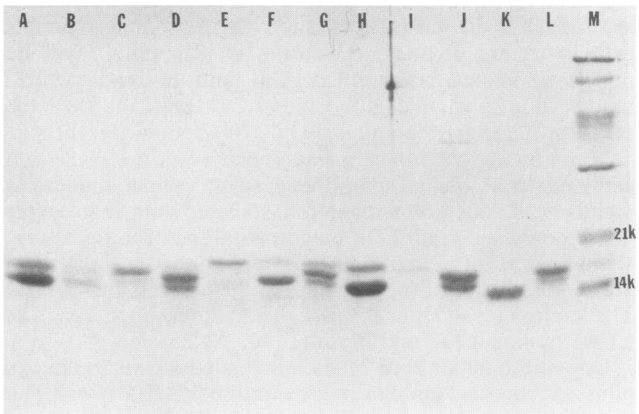

FIG. 2. SDS-PAGE of P pili purified from 12 consecutive clinical isolates. The lanes contain the following strains: A, 799; B, 800; C, 828; D, 848; E, 854; F, 871; G, 884; H, 892; I, 917; J, 918; K, 950; and L, 993. Lane M, Molecular weight markers (in thousands [k]).

pressed as the percent inhibition of control wells that received no inhibitor.

Western blotting. Bacterial antigens were separated by SDS-PAGE and then transferred from the unfixed, unstained polyacrylamide gels to nitrocellulose paper (30 V, 18 h) by the method of Towbin et al. (30). The subsequent steps were performed as described in a Bio-Rad Laboratories technical bulletin. Briefly, after all binding sites were blocked with albumin and the paper was reacted with antiserum and then with goat antirabbit immunoglobulin G-horseradish peroxidase conjugate, the transferred bands that bound antibody were visualized with 4-chloro-1-naphthol.

RESULTS

HA. After 18 h of growth on CFA, 17 of the 30 bacteremic strains caused agglutination of human erythrocytes when screened by the slide agglutination method; 1 strain caused MS HA. The 16 strains which caused MR HA were tested against erythrocytes of the rare phenotype p, which does not have the P globoside receptor. Of the 16 strains, 15 failed to agglutinate p cells, indicating that these strains probably had P pili. Only one strain (strain 884) caused MS HA of guinea pig erythrocytes.

We then studied the effects of different culture media on the expression of the MR hemagglutinin (Table 1). The highest titers after growth on agar occurred on chocolate agar, CFA, and, particularly, blood agar. Titers were lower after growth in broth, but colonization factor broth yielded the highest titers of all the liquid media.

Purification of pili. Strain 917 was initially selected because it had consistently shown the highest HA titers. Pili were initially purified after growth on blood agar, but this inevitably resulted in the copurification of hemoglobin from the agar together with the pili. All subsequent purifications were done with CFA. Some preparations of purified strain 917 pili were examined by SDS-PAGE and found to contain one major pilus subunit with a molecular weight of 17,500, but inconsistently there were one or two smaller bands with greater mobility. This was also observed for other strains. Some of the lower-molecular-weight bands were only present after prolonged incubation in urea at 37°C. Examination of the rapidly prepared pili, simply prepared by shearing and filtering, often revealed fewer bands in the area where pili would be expected on polyacrylamide gels (Fig. 1).

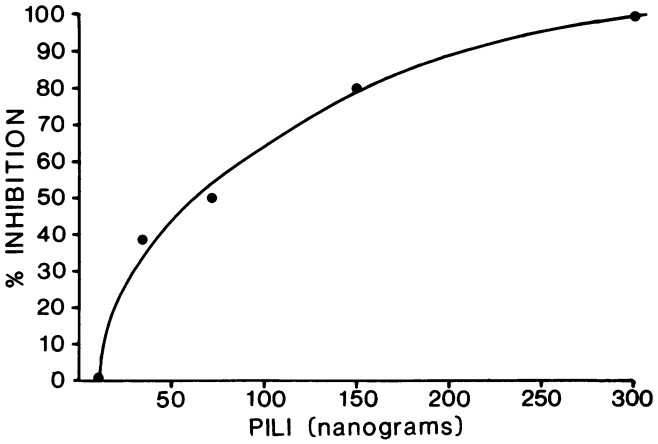

FIG. 3. Inhibition of ELISA by increasing amounts of purified pili from strain 917 and the homologous antiserum.

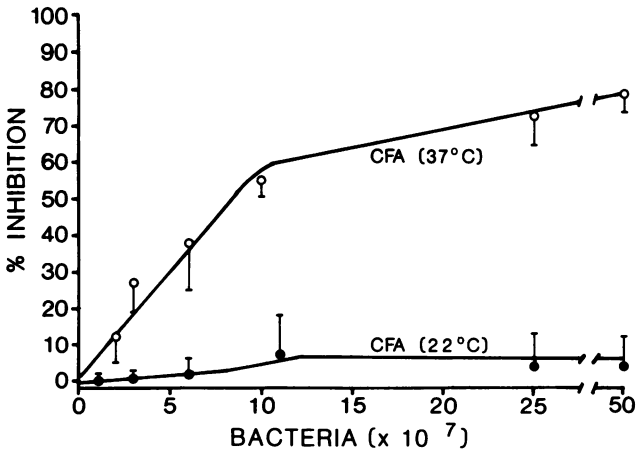

FIG. 4. ELISA inhibition. Increasing amounts of bacterial strain 917 grown on CFA at 37°C or room temperature (22°C) were used to inhibit the binding of monospecific antipilus serum to the homologous pili. Note the absence of pilus antigen on bacteria grown at room temperature.

Urea incubation, which is necessary to disaggregate flagella, was then changed to 4 h at 4°C; this was sufficient to disaggregate flagella, as judged by electron microscopy, but did not alter the appearance of the pilus bands in SDS-PAGE.

Strain 917 consisted of a single pilus subunit. Examination of SDS-PAGE samples of pili purified from all P-piliated strains revealed large interstrain differences in both the number and the position of the pilus subunits (Fig. 2). This was confirmed by the rapid preparation method, which considerably reduced the likelihood of proteolytic digestion of the pili and the formation of apparently smaller peptides (Fig. 1). The pilus subunit molecular weights ranged from 14,000 to 19,500, with one to three subunits per strain. There was no one subunit common to all the strains (Fig. 2).

ELISA. Hyperimmune antipilus rabbit serum was found to have a titer of ca. 1/500,000, whereas the titer of the preimmune serum under the same conditions was <1/64.

An ELISA inhibition assay was developed to determine the specificity of the serum and to survey the antigenic relatedness of different P-piliated strains. If purified homologous pili were used to inhibit the ELISA, ca. 20 ng of pili was detectable, and inhibition continued up to 300 ng (Fig. 3). The homologous P-piliated bacteria (5×10^8 CFU of strain 917 per ml) grown at 37°C completely inhibited the ELISA (Fig. 4). When the same strain was grown at room temperature, no pili were visible by electron microscopy or detectable by the ELISA inhibition assay (Fig. 4).

TABLE 2. Relationship between HA and inhibition of ELISA by strain 917 grown on different media

Medium	HA titer[a]	% ELISA inhibition
Blood agar	12	84
CFA (37°C)	8	83.2
MacConkey agar	0	43.6
CFA (18°C)	0	0

[a] Reciprocal of highest dilution causing complete agglutination. Initial concentration of strain 917, 10^8 CFU/ml.

TABLE 3. Pilus cross-reactivity determined by inhibition of ELISA with P-piliated bacteria and monospecific antipilus sera

Bacterial strain	Inhibition[a] with the following antisera raised against pilus filaments			
	828	854	950	917
688	−	−	−	−
799	−	−	−	−
800	−	−	−	−
828	+	−	−	+
848	−	−	−	−
854	−	+	−	−
871	−	−	−	−
884	−	−	−	−
892	−	−	−	−
917	+	−	−	+
918	−	−	−	−
950	−	−	+	−
993	−	−	−	−
7714	+	−	−	+

[a] −, Less than 35% inhibition; +, Greater than 75% inhibition.

Strain 917 was grown under different culture conditions, and its ability to inhibit the ELISA and cause HA was then determined (Table 2). The results indicated that the ELISA was much more sensitive in determining piliation than the functional assay (HA). The ELISA also confirmed the observations that culture conditions can influence the expression of the hemagglutinin.

The ELISA inhibition assay was then used to determine how many P-piliated strains could remove heterologous pilus antibodies. Fourteen strains were tested with four antisera, and only two or fewer heterologous strains could inhibit the ELISA to any significant extent (Table 3).

Western blotting. A blot of strain 917 pili with the antipilus serum indicated the presence of other bacterial components which had not been seen in SDS-PAGE, most likely indicating antibodies directed against lipopolysaccharide. This antiserum was then extensively absorbed with strain 917 grown at room temperature. The resulting antiserum contained antibodies which were only directed against the pilus subunit (Fig. 5).

The cross-reactivity of heterologous strains was then determined by Western blotting. Of the 14 strains tested, pili of only 2 or fewer heterologous strains could be detected (Fig. 5 and 6; Table 4). When a strain had more than one pilus subunit seen on the gel, only one of these reacted with the heterologous antiserum. These results confirmed those of the ELISA inhibition assay, indicating a lack of cross-reactivity between strains.

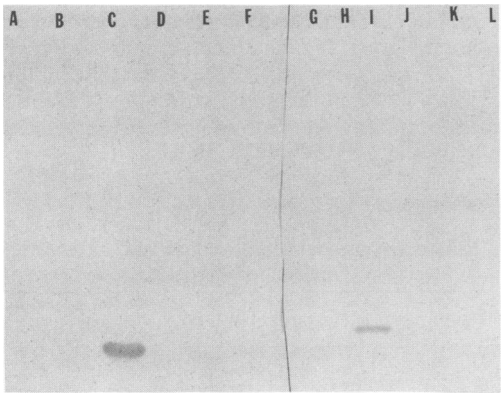

FIG. 5. Protein immunoblot of P pili from 12 different strains reacted against antiserum to strain 917 pili. Lanes are as in Fig. 2. The homologous strain (strain 917) is in lane I. Note that the only cross-reactive heterologous strain is strain 828 (lane C).

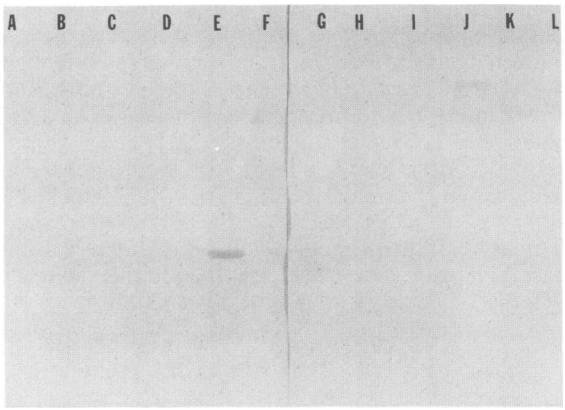

FIG. 6. Protein immunoblot of P pili from 12 different strains reacted against antiserum to strain 854 pili (lane E). Lanes are as in Fig. 2. Note that there are no cross-reactive strains.

When whole bacteria were boiled in sodium dodecyl sulfate and run in SDS-PAGE, the pilus subunit was usually not seen at all or was seen very poorly. However, if Western blotting was done with whole bacteria and antipilus serum, the pilus subunit was easily seen. This indicates the superior sensitivity of Western blotting over that of Coomassie blue or even silver staining and also indicates that crude pilus preparations and even whole bacteria can be used in Western blotting to determine pilus cross-reactivity.

Protein sequences. The N-terminal amino acid sequences were determined on purified pili from four different strains while the amino terminus of strain 950 was blocked (Fig. 7). The sequences were identical through the first 10 residues for all four strains and are identical to that of strain J96, for which the complete sequence has been previously determined (1). One strain (strain 892) had another pilus subunit which was identical to a type 1 pilus variant termed type 1c (12).

TABLE 4. Pilus cross-reactivity detected by Western blotting

Bacterial strain	Inhibition with the following antisera raised against pilus filaments			
	828	854	950	917
688	−	−	−	−
799	−	−	−	−
800	−	−	−	−
828	+	−	−	+
848	−	−	−	−
854	−	+	−	−
871	−	−	−	−
884	−	−	−	−
892	−	−	−	−
917	+	−	−	+
918	−	−	−	−
950	−	−	+	−
993	−	−	−	−
7714	ND[a]	ND	ND	+

[a] ND, Not done.

	1				5					10
7714	Ala	– Pro	– Thr	– Ile	– Pro	– Gln	– Gly	– Gln	– Gly	– Lys
917	Ala	– Pro	– Thr	– Ile	– Pro	– Gln	– Gly	– Gln	– Gly	– Lys
828	Ala	– Pro	– Thr	– Ile	– Pro	– Gln	– Gly	– Gln	– Gly	– Lys
J96	Ala	– Pro	– Thr	– Ile	– Pro	– Gln	– Gly	– Gln	– Gly	– Lys
892(A)	Ala	– Pro	– Thr	– Ile	– Pro	– Gln	– Gly	– Gln	– Gly	– Lys
892(B)	Val	– Thr	– Thr	– Val	– Asn	– Gly	– Gly	– Thr	– Val	– His
1c	Val	– Thr	– Thr	– Val	– Asn	– Gly	– Gly	– Thr	– Val	– His

FIG. 7. Amino-terminal sequences of pili from four of our strains (strains 7714, 917, 828, and 892). Sequences for strains J96 (reference 1), a P pilus type, and type 1c (type 1C in reference 12), a type 1 pilus variant, are given for comparison. (A) and (B) represent the two pilus subunits of strain 892 (see text).

DISCUSSION

The pathogenesis of urinary tract infections caused by *E. coli* strains has been linked to the ability of these strains to bind to the urinary tract epithelium (28). This is particularly true of strains associated with acute pyelonephritis. Most of these isolates also cause MR HA of human erythrocytes (24, 31). The receptor on the erythrocytes is the P blood group substance (globoseries glycolipids); the minimal receptor within these glycolipids is alpha-D-Gal-(1-4)-beta-D-Gal (9). Pili isolated from digalactoside-binding bacteria have been variously termed Pap pili (pyelonephritis-associated pili) or P fimbriae. Purified P pili bind to urinary tract epithelial cells, and homologous pilus antibody inhibits the binding (29). There is thus considerable evidence that P pili are one virulence factor in such *E. coli* strains. Local antibody from a natural infection or from immunization may render the *E. coli* strains less virulent (28). We have studied invasive *E. coli* strains cultured from the blood of patients. Because of the nature of the source of isolation, these strains were virulent. Many of them originated from urinary tract infections, but a smaller number had other portals of entry. Many of these bacteremic *E. coli* strains caused MR HA of human erythrocytes and had P pili.

All P-piliated bacteria have the same receptor specificity, so it is likely that their receptor-binding domains are very similar. Because some patients have repeated infections with strains bearing P pili, several possibilities are likely: (i) the receptor-binding portion is not immunogenic in humans, (ii) immunity is present but short-lived, (iii) there are a large number of receptor-binding domains, or (iv) a long-lived immune response to the receptor-binding domain occurs but is not involved in protection against disease. To explore some of these possibilities, we studied the immunochemistry of P pili derived from 14 different strains.

The *E. coli* strains had one to three pilus subunits each, and there was no one subunit, as determined by SDS-PAGE, which was identical in all the strains. This confirms previous studies which implied that more than one pilus type occurs in a single strain (14, 24) and even on a single bacterium (13, 19). In some cases, we found protein bands to be an artifact of the purification process. This was confirmed by more rapid purification methods. The ELISA was found to be useful for quantitating pilus antigen and for assessing the cross-reactivity of the pilus organelles. The enzyme immunoassay for pili was quite sensitive and could detect as little as 20 ng of pili. This result is comparable to that of a previously published enzyme assay for type 1 pili (3).

Very limited cross-reactivity between strains was found by either the ELISA inhibition assay or Western blotting. The agreement between both methods indicates that the antibodies raised against the pilus organelles are detectable by using the pilus monomers, as was done in Western blotting.

The amino-terminal protein sequences of the P pili were identical and are also identical to that of F12 reported by Klemm et al. (13). It is unlikely that this portion participates in binding to human cells, as another P pilus termed F9 (13) has a sequence most closely resembling those of type 1 pili. It is also clear that our antisera did not detect this amino-terminal sequence, as greater cross-reactivity would have been noted. The N-terminal sequence is also highly conserved among pili mediating MS HA (24). In addition, pili from *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella nonliquefaciens*, *Pseudomonas aeruginosa*, and *Bacteroides nodosus* have highly homologous amino-terminal sequences through residue 20 (6, 17).

It is likely then that the functional differences between pili are not to be found in the N-terminal amino acid sequence. In addition, the receptor-binding domains of the P pili must be immunologically recessive in the native state. If antibodies against P pili are protective, there would be very limited cross-reactivity, and protection would similarly be limited to the homogolous strain and a limited number of heterologous strains. Further studies are thus necessary to expose the receptor-binding domain, as has been done for gonococcal pili (26).

ACKNOWLEDGMENT

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