

Frequency Among *Enterobacteriaceae* of the DNA Sequences Encoding Type 1 Pili

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Type 1 pili, characterized by mannose-inhibitable agglutination of fowl or guinea pig erythrocytes, have been found throughout the family *Enterobacteriaceae*. A radiolabeled probe was prepared from a restriction endonuclease-digested fragment of the *Escherichia coli* pil operon and used to detect homologous DNA sequences in 236 bacteria representing 11 genera of *Enterobacteriaceae*. Only isolates identified as *E. coli* or *Shigella* spp. exhibited homology. In contrast, mannose-sensitive hemagglutination was observed in nine genera. Probe DNA did not hybridize to plasmid DNA, indicating a chromosomal location for the pil operon. Analysis of restriction nuclease-digested whole-cell DNA from 60 *E. coli* and two *Shigella* sp. isolates indicated that internal sequences were conserved in most strains, but that changes in flanking sequences in the chromosome were common.

Type 1, or common, pili are short, rigid, hair-like fibers found on the surface of bacteria of the family *Enterobacteriaceae* (3, 4, 7, 8). They are composed of 17,000-dalton protein subunits, called pilin, polymerized into a pilus 7 nm in diameter and 0.5 to 2.0 μ m in length (3, 19). They are distinguished from other types of pili by their ability to agglutinate fowl or guinea pig erythrocytes and by the inhibition of this hemagglutination by α -D-mannose and other mannosides (22). A functional role for type 1 pili has not been determined, although several hypotheses have been suggested, including roles in colonization of the animal bowel and in more efficient acquisition of oxygen and nutrients (4, 6, 8, 23, 29). Ørskov et al. (26) have observed that type 1-piliated *Escherichia coli* adhere strongly to human urinary mucus and suggested that type 1-piliated bacteria may be captured by free mucus in the bladder and removed from the body upon micturition. However, results of studies with animal models have indicated that type 1 pili have a positive role in colonization of the lower urinary tract in mice by *E. coli* and in rats by *Klebsiella pneumoniae* (9, 11, 14).

Type 1 pili have been purified and partially characterized from only three species: *E. coli*, *Salmonella typhimurium*, and *K. pneumoniae* (10, 16, 19). The physical properties of the different pili were reported to be similar, but differences were observed in the molecular weight and the amino acid composition of the pilin subunits.

DNA hybridization is a rapid and sensitive method for determining relatedness among gene sequences (2). This report describes the use of this technique to probe for DNA sequences homologous to the structural gene for pilin cloned from a urinary isolate of *E. coli* (12) among more than 200 isolates representing 11 genera of *Enterobacteriaceae*. In addition, all isolates were tested for mannose-sensitive agglutination of guinea pig erythrocytes. Although mannose-sensitive hemagglutination (MSHA) was observed in nine genera, only isolates identified as *E. coli* or *Shigella* spp.

were found to contain DNA sequences homologous to the pilin structural gene from *E. coli*.

MATERIALS AND METHODS

Bacteria and plasmid. Bacteria from human cases of extra-intestinal infections and representing the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, and *Serratia* were obtained from the Ben Taub County Hospital, Houston, Tex., or from the U.S. Army Institute for Surgical Research, Fort Sam Houston, San Antonio, Tex. Bacteria of the genera *Salmonella*, *Shigella*, and *Yersinia* were obtained from the Centers for Disease Control, Atlanta, Ga. *Salmonella typhimurium* LT2 was provided by S. Moseley, University of Washington, Seattle. *E. coli* SH1 is the pyelonephritis isolate from which the DNA sequence encoding MSHA was cloned (12). The isolates were stored at -20°C in 30% glycerol-1% peptone.

The recombinant plasmid pSH2 has been described previously (24).

Isolation of whole-cell DNA from *Enterobacteriaceae*. Whole-cell DNA was isolated by using lysozyme, Sarkosyl, and proteinase K as described by Hull et al. (13).

Dot blot hybridization. Samples of whole-cell DNA were denatured in NaOH, neutralized, and spotted onto nitrocellulose paper as described by Hull et al. (13). Probe DNA was radiolabeled with α - ^{32}P -deoxynucleotides by nick translation (17). Hybridization of probe DNA to blots was carried out as previously described (13).

Plasmid DNA isolation. Plasmid DNA was isolated as described by So et al. (30).

Southern blot analysis of restricted DNA. The method of Southern (31) was used for the transfer of endonuclease-digested DNA to nitrocellulose. Hybridization conditions were as described for dot blot analysis.

Hemagglutination assay. Clinical isolates were subcultured from glycerol-peptone media to MacConkey agar and incubated at 37°C overnight. Isolated colonies were used to inoculate 2-ml LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) cultures which were incubated at 48 h at 37°C under static conditions to enhance piliation. The hemagglutination assay was performed with fresh guinea pig erythrocytes in

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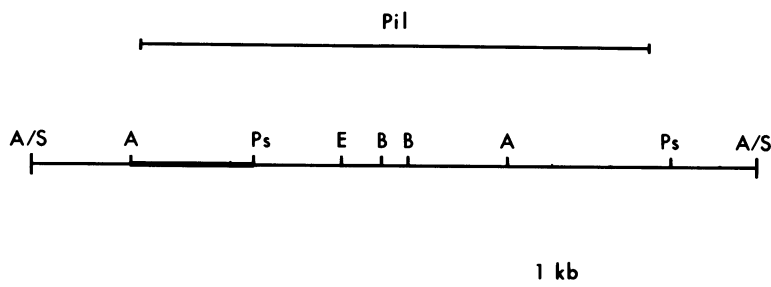


FIG. 1. Restriction endonuclease map of the *Sall* fragment encoding type 1 pili. The heavy line indicates the probe used in this study. Restriction site abbreviations: A, *AccI*; B, *BamHI*; E, *EcoRI*; Ps, *PstI*; S, *Sall*.

phosphate-buffered saline or phosphate-buffered saline with 1% D-mannose, as described by Minshew et al. (21).

Rapid plasmid extraction from *Enterobacteriaceae*. Plasmid DNA was extracted from clinical strains by the alkaline lysis procedure described by Portnoy et al. (27). One-third of the final sample was electrophoresed through a 0.7% agarose gel in Tris-borate buffer, stained with ethidium bromide, and photographed as described previously (27).

RESULTS

Selection of probe. The *Sall* fragment encoding the DNA sequence necessary for MSHA from a pyelonephritis isolate of *E. coli* is presented in Fig. 1. The *AccI-PstI* fragment (heavy line in Fig. 1) on the left end of the operon was chosen as a probe for homologous sequences in other members of *Enterobacteriaceae*. This fragment includes the entire structural gene for pilin and an additional 23,000-dalton peptide which appears to have a regulatory role in pilin synthesis (24).

Hybridization of probe DNA with dot blots. α -³²P-radiolabeled probe DNA was hybridized to nitrocellulose filters onto which samples of undigested whole-cell DNA from 236 isolates of *Enterobacteriaceae* had been spotted (Table 1). This procedure was found to be superior to the colony hybridization method, as many clinical strains were poorly lysed in situ on nitrocellulose. Only bacteria identified as *E. coli* and *Shigella* spp. exhibited homology with the probe (Fig. 2). Only nine *E. coli* strains failed to hybridize.

Hemagglutination. All 236 isolates were grown in LB without aeration for 48 h at 37°C to enhance type 1 piliation. MSHA was observed in a total of 76 bacterial isolates,

representing nine different genera (Table 1). Only *Shigella* spp. and *Yersinia* spp. failed to exhibit MSHA under the conditions used. None of the nine *E. coli* isolates which failed to hybridize was MSHA positive.

Analysis of homologous sequences among restriction nuclease-digested DNA. Cesium-chloride-purified whole-cell DNA from all 60 dot blot-positive *E. coli* isolates, 2 *Shigella* isolates, (*Shigella boydii* and *Shigella flexneri*), and 69 *Enterobacteriaceae* isolates from seven other genera were digested to completion with *AccI* and with *HindIII* in separate reactions, electrophoresed through 0.35% agarose, and transferred to nitrocellulose by the method of Southern (31). No discrepancies were seen in hybridization results between the dot blots and the Southern blots. Again, only *E. coli*- and *Shigella* spp.-digested DNA hybridized with probe DNA (Fig. 3 and 4).

AccI has a site within the *pil* operon of *E. coli* SH1 and sites near both ends (Fig. 1); it can therefore be used to determine sequence conservation within the operon. The

TABLE 1. Comparison of hemagglutination reactions and hybridization of *pil* probe to undigested DNA

Genus	Total no. of isolates tested	No. of MSHA-positive isolates	No. of isolates showing DNA homology
<i>Escherichia</i>	69	34	60
<i>Shigella</i>	6	0	6
<i>Salmonella</i>	7	3	0
<i>Citrobacter</i>	10	3	0
<i>Klebsiella</i>	48	3	0
<i>Enterobacter</i>	48	14	0
<i>Serratia</i>	17	10	0
<i>Proteus</i>	18	4	0
<i>Morganella</i>	5	1	0
<i>Providencia</i>	5	1	0
<i>Yersinia</i>	3	0	0

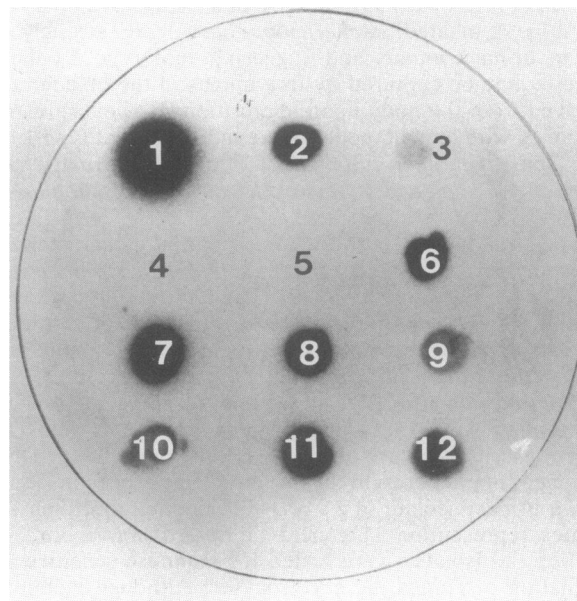


FIG. 2. Autoradiograph of radiolabeled probe DNA hybridization to undigested bacterial DNA spotted onto nitrocellulose. Spot 1, Purified plasmid pSH2 DNA; spot 2, *E. coli* K-12; spot 3, *E. coli* O157:H7; spot 4, *Salmonella typhimurium* LT2; spot 5, *S. brazil*; spot 6, *Shigella dysenteriae*; spot 7, *Shigella flexneri*; spot 8, *Shigella boydii*; spot 9, *Shigella flexneri*; spot 10, *Shigella boydii*; spot 11, *E. coli* H10407; spot 12, *Shigella boydii*. Spots 4 and 5 are negative; all others are positive.

results (Fig. 3; Table 2) indicate that 54 of 62 strains, including one *Shigella* sp., were identical with *E. coli* SH1 in the size of the *AccI* restriction fragment which hybridized with the probe. All 34 MSHA-positive *E. coli* fell into this category. Fragments of other sizes were detected in eight isolates; two fragments were detected in four of these eight. The biotypes, antibiograms, and plasmid profiles of the *E. coli* isolates were rechecked to ensure that they were, indeed, unique isolates.

In contrast, greater diversity was observed when the probe was hybridized to *HindIII*-digested whole-cell DNA (Table 3). *HindIII* has no sites within the *Sall* fragment (Fig. 1); its use, therefore, may detect changes in sequences flanking the operon. Only 39 of 62 strains contained a hybridizing sequence identical in size to that in SH1, but not all of the MSHA-positive strains fell into this category. Almost half (15 of 34) of the MSHA-positive strains fell into five different categories, including one in which two small fragments hybridized.

Location of homologous sequences in clinical isolates. Plasmids were prepared from all 60 dot blot-positive *E. coli* isolates by the rapid alkaline lysis method of Portnoy et al. (27). Radiolabeled probe DNA was hybridized to nitrocellulose to which electrophoresed crude plasmid extracts had been transferred. The probe never hybridized with plasmid bands but only to the band containing linear fragments of chromosomal DNA (data not shown). Thus, it appears that the location of the *pil* operon is always chromosomal.

DISCUSSION

Our results indicate that, although functionally equivalent, the DNA sequences encoding type 1 pili in the family

TABLE 2. Sizes of *AccI* fragments hybridizing with *pil* probe DNA and hemagglutination reactions of 60 *E. coli* and 2 *Shigella* sp. isolates

Group	No. of isolates tested	Size (kb) of <i>AccI</i> fragment(s) hybridizing with <i>pil</i> probe	No. of MSHA-positive isolates
1 ^a	54 ^b	6.0	34
2	1	7.0	0
3	1 ^c	7.5	0
4	1	8.0	0
5	2	9.0, 8.0 ^d	0
6	1	5.0	0
7	2	5.0, 1.0 ^d	0

^a Mobility of hybridizing fragment appeared identical to that of SH1.

^b Includes one MSHA-negative isolate of *Shigella boydii*.

^c *Shigella flexneri*.

^d Two different fragments exhibited homology.

Enterobacteriaceae have undergone considerable divergence from any common ancestral gene. Radiolabeled probe DNA encoding the structural gene for type 1 pilin from *E. coli* hybridized only to those bacteria identified as *E. coli* or *Shigella* spp. The conditions used here, incubation at 37°C in 50% formamide-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), mimic hybridization conditions at a temperature of approximately 25°C below the thermal denaturation point of *E. coli* (1, 18). Thus, only sequences exhibiting at least 75% homology with the *E. coli* sequence form stable duplexes. The results presented here are in excellent agreement with those obtained by Brenner and Falkow (2), who found that 71 to 85% of *E. coli* DNAs formed stable heteroduplexes with *Shigella* DNA under

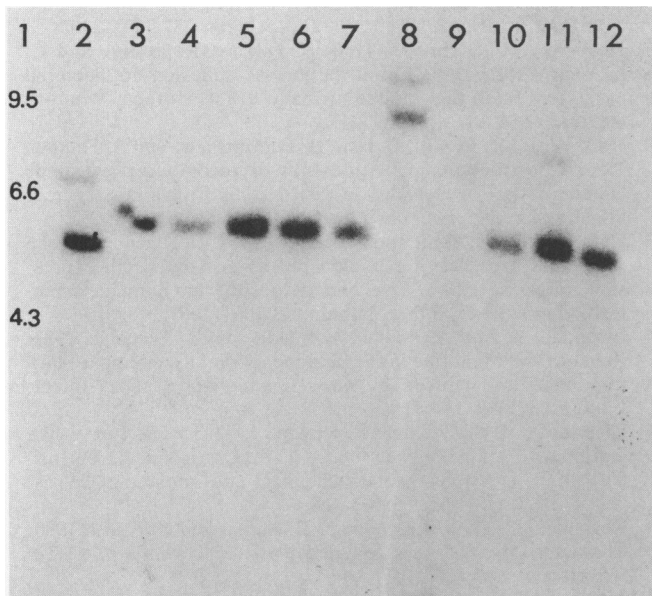


FIG. 3. Hybridization of radiolabeled probe DNA to *AccI*-digested samples of DNA from clinical *E. coli* DNA sources: lane 1, phage lambda (digested with *EcoRI*); lane 7, *E. coli* SH1 control; lanes 2 through 6 and 8 through 12, *E. coli* strains isolated from extraintestinal infections. Fragment sizes are given in kilobases. Lanes 3 through 6 and 10 through 12 belong to the group identical to SH1 (Table 2). Lane 2 contains the single representative of group 6, and lane 8 contains the single representative of group 4 (the light upper band in lane 8 was interpreted as a partial digest).

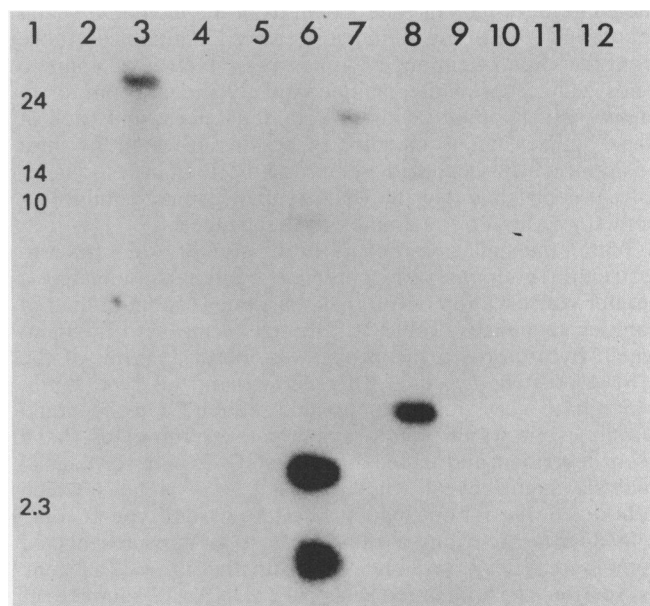


FIG. 4. Hybridization of radiolabeled probe DNA to *HindIII*-digested samples of DNA from clinical *Enterobacteriaceae*. DNA sources: lane 1, phage lambda (digested with *BglII*); lane 2, *Enterobacter* sp.; lane 3, *E. coli* (Table 3, group 3); lane 4, *Enterobacter* sp.; lane 5, *Klebsiella* sp.; lane 6, *E. coli* (Table 3, group 7); lane 7, *E. coli* SH1 control; lane 8, *E. coli* (Table 3, group 4); lane 9, *Klebsiella* sp.; lane 10, *Klebsiella* sp.; lane 11, *Enterobacter* sp.; lane 12, *Klebsiella* sp. Fragment sizes are given in kilobases.

TABLE 3. Sizes of *Hind*III fragments hybridizing with *pil* probe DNA and hemagglutination reactions of 60 *E. coli* and 2 *Shigella* sp. isolates

Group	No. of isolates tested	Size (kb) of <i>Hind</i> III fragment(s) hybridizing with <i>pil</i> probe DNA	No. of MSHA-positive
1 ^a	39 ^b	24	19
2	1 ^c	18	0
3	5	26	1
4	6	5	5
5	1	1	1
6	1	3	1
7	7	3, 1 ^d	7
8	2	24, 3 ^d	0

^a Mobility of hybridizing fragment appeared identical to that of SH1.

^b Includes one MSHA-negative isolate of *Shigella flexneri*.

^c *Shigella boydii*.

^d Two different fragments exhibited homology.

stringent conditions, whereas DNAs from all other *Enterobacteriaceae* isolates tested exhibited 50% or less reaction with *E. coli* DNA. In contrast, certain gene sequences, for example, those encoding 23S rRNA, tRNAs, cystathionase (*metC*), and thymidylate synthetase (*thyA*), appear to have been more highly conserved within the family (2, 20, 28).

Duguid and Campbell (5) found antigenic differences among type 1 pili of various *Enterobacteriaceae* isolates. Antisera were prepared against whole, piliated bacteria, absorbed with homologous, nonpiliated bacteria, and used in agglutination reactions against bacteria of seven genera. Their results support those reported here except that antisera prepared against *E. coli* or *Shigella flexneri* also agglutinated *K. aerogenes*.

Fader et al. (10) sequenced the first 25 amino acids from the amino terminus of type 1 pilin from *K. pneumoniae* and found that 79% of the amino acids were identical with those from the amino terminus of *E. coli* pilin. However, 4 amino acids, valine, threonine, alanine, and glycine, account for 16 of the first 24 amino acids in both sequences, and each of these amino acids is specified by codons in which the third position can be occupied by any of the four bases. Therefore, it is possible that the DNA sequences may exhibit less homology than do the amino acid sequences.

Within the species *E. coli*, hybridization of probe DNA to restriction endonuclease-generated fragments indicated a greater conservation of internal sequences (Table 2) than of flanking sequences (Table 3). The size of the *AccI* fragment which hybridized to the probe was identical in all of the MSHA-positive *E. coli*. Antigenic variants of type 1 pili, which have very similar amino acid sequences at the amino terminus but which fail to agglutinate erythrocytes, have been described and called type 1C (15, 25). A serological analysis might reveal whether the *E. coli* isolates which hybridized but did not exhibit MSHA possess type 1C pili.

In addition to being a sensitive test for the presence of homologous DNA sequences even in the absence of gene expression, hybridization of probe DNA to samples of restriction nuclease-digested DNA separated by size by agarose electrophoresis is capable of detecting differences among strains not readily apparent by other techniques. For example, in this study, 34 of 60 *E. coli* isolates were identical when screened for MSHA yet fell into six different categories when samples of their DNA were digested by a restriction endonuclease with sites external to the *pil* operon (Table 3).

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