INFECTION AND IMMUNITY, May 1989, p. 1604–1611
0019-9567/89/051604-08$02.00/0

Vol. 57, No. 5

Distribution of the P-Associated-Pilus (*pap*) Region among *Escherichia coli* from Natural Sources: Evidence for Horizontal Gene Transfer

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Received 29 August 1988/Accepted 4 February 1989

Variation in chromosomal DNA in *Escherichia coli* was studied with probes specific for the *P*-associated-pilus (*pap*) region. The presence of DNA homologous to *pap* was determined by dot blots. Variation in the number of copies of *pap* and in the organization of internal and flanking sequences was determined by Southern blot hybridization. The 229 strains studied were also classified by O:K:H serotyping and multilocus enzyme electrophoresis. There was considerable heterogeneity in the presence of *pap* and distribution of *pap*-homologous DNA in these *E. coli* strains from natural sources. In general, there was less variation in *pap* among strains of the same specific O:K:H serotype and enzyme electrophoretic type than among random isolates. There were, however, *E. coli* strains identified as members of the same clone by O:K:H serotyping and enzyme electrophoresis that were *pap* positive and *pap* negative or had different Southern blot patterns for the *pap* probes (*pap* type). There were also isolates of the same *pap* type that differed in two of three O:K:H serotype antigens and the majority of enzymes that determined their enzyme electrophoretic type. These latter two observations were interpreted as evidence for the horizontal (infectious) transfer of the *pap*-homologous sequences among clones of *E. coli*.

Populations of *Escherichia coli* appear to exist as arrays of lineages among which there is little exchange of chromosomal genes. This was first inferred from the nonrandom coappearance of serotype and biotype markers among strains isolated from different sources (37, 46, 51). Additional evidence for the temporal and geographic stability of *E. coli* genotypes has come from investigations using multilocus enzyme electrophoresis (7, 8, 43, 44), and studies of outer membrane protein variation (1). While the initial evidence for the genetic isolation of bacterial lineages came from studies of *E. coli*, similar observations have been made for other bacteria: *Haemophilus* spp. (33, 41), *Legionella* spp. (45), and meningococci (6). This idea of the structure of bacterial populations as collections of genetically stable and separate lineages has been called the clone concept (37).

The evidence upon which the clone concept is founded is largely phenotypic, based on gene products rather than genes themselves (for an exception, see reference 31). While the implication is that the entire chromosomes of bacteria maintain clonal identity, this has not been demonstrated. It is conceivable that although most of the chromosomes of bacteria identified as members of the same clone are identical or nearly so, portions may have different ancestries and be derived, by horizontal (infectious) transfer, from other clones of the same or different species. Horizontal transfer between clones is certainly the case for plasmids (5, 12, 36).

The *pap* gene cluster of *E. coli* (15, 35) codes for the pili and adherence proteins that are required for the attachment of bacteria to carbohydrate receptors in mucosal surfaces (19, 22, 23). These receptors are antigens of the P blood group system (29), and attachment is associated with the ability of a bacterium to cause symptomatic kidney infections (pyelonephritis)—hence, the name *pap*, P-associated-pili. Attachment is a virulence determinant (11, 49, 50). By enabling bacteria to invade and maintain populations in the urinary tract better than cells without those genes (13), the Pap phenotype augments the range of habitats available to *E. coli* (niche expansion). It is a character that would be favored in a manner analogous to antibiotic and heavy metal resistance, characters that are commonly encoded for by genes borne on plasmids.

In this investigation, probes specific for the *pap* gene cluster were used to examine variability in this chromosomal DNA in *E. coli* isolated from different kinds of urinary tract infections. The results suggest that the *pap* sequence has been horizontally transferred between genetically distinct clones of this bacterial species.

MATERIALS AND METHODS

Bacteria. A total of 229 *E. coli* isolates from patients with urinary tract infections were examined. Of these strains, 194 came from girls aged 3 months to 15 years living in Göteborg, Sweden. They included 68 isolates taken from patients with the first known episode of acute pyelonephritis and 71 from patients with acute cystitis (18). A sample of 55 isolates from children with asymptomatic bacteriuria were obtained during a school screening program (26). The remaining 35 *E. coli* strains were from a single clinic in Houston, Tex. These were derived from patients with symptomatic urinary tract infections (either cystitis or pyelonephritis). The Swedish strains were chosen by clinical criteria and independently of their *pap* genotypes or phenotypes. The strains in the Houston collection were selected because they were positive for the *pap* region and had restriction fragment length classes which, on first consideration, appeared to be present in the Swedish samples.

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The Göteborg strains were obtained between 1968 and 1973 and were stored in deep agar stab culture. The Houston strains were isolated after 1980 and were stored at either −20 or −70°C in 30% glycerol–1% peptone.

Serotyping. The lipopolysaccharide O, the capsular polysaccharide K, and the flagellar H antigens were determined as described in detail by Ørskov and Ørskov (38). Briefly, the O antigen was determined by testing agglutination of boiled cultures against 165 O antisera distributed in pools, followed by examination against single unabsorbed and absorbed antisera. Strains agglutinating in saline were designated as spontaneously agglutinating or OR (rough). Strains that did not react with available antisera were designated O-nontypable (ON). The presence of a K antigen was assayed for by the ability of a bacterial extract to precipitate with Cetavlon in an electrophoretic test. When the test was positive, the serotype of the K antigen was determined according to the outcome of a countercurrent immunoelectrophoresis test involving the 72 known K antisera. When no reaction was seen, the K antigen was designated KN. The H antigen was determined by agglutination tests with the 53 known H antisera. When no reaction was seen, the H antigen was designated HN. Each isolate was assigned a serotype based on the combination of O:K:H antigens or the reactions to the tests for those antigens.

Multilocus enzyme electrophoresis. The procedures for antigen preparation and starch gel electrophoresis have been described in detail previously (7). Briefly, an extract of soluble cytoplasmic material was prepared from broth cultures of each strain. The suspension was electrophoresed in starch gels with cooling. Each gel was sliced longitudinally to permit parallel staining with several substrates. The position of each allozyme was recorded.

The enzymes detected by staining for were malate dehydrogenase, 6-phosphogluconate dehydrogenase, β-galactosidase, adenylate kinase, phenylalanyl-leucine peptidase, leucyl-leucyl-glycine peptidase, isocitrate dehydrogenase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, and glutamic oxaloacetic transaminase. The majority of these are coded for by single well-characterized loci (8, 44). The multilocus enzyme electrophoresis type (ET) of each isolate was defined by the combination of alleles at 13 loci. Allozymes not expressed by individual strains were designated null alleles (8).

***pap* region analysis.** DNA probes specific for the *pap* sequence were derived from the recombinant plasmid pRHU845. Two probes containing DNA sequences within the functional limits of the *pap* operon were constructed: (i) a *Hin*dIII fragment containing the structural gene for pili as well as for transport and anchoring at the bacterial surface and (ii) a *Sma*I fragment containing the structural genes for the minor proteins involved in adhesion (17). Plasmid DNA was isolated as described by So et al. (47). The probes were radiolabeled with [α-^{32}P]deoxynucleotides by using Klenow fragment and random oligonucleotide primers (oligonucleotide labeling kit; Pharmacia, Uppsala, Sweden).

Whole-cell DNA from each *E. coli* strain was isolated as follows. Brain heart infusion broth cultures (30 ml) were inoculated and grown overnight with shaking at 37°C. Bacteria were collected, washed once with TE buffer (10 mM Tris, 1 mM ethylenediamine tetraacetic acid, pH 8), and resuspended in 3 ml of TE buffer. Lysozyme (0.3 ml of 10 mg/ml in 0.25 M Tris, pH 8) and EDTA (0.15 ml of 0.5 M, pH 8) were added, and the tubes were incubated on ice for 10 min. Proteinase K (1 μl of 10 mg/ml in 10 mM $CaCl_2$) was added, and lysis was initiated with the addition of 0.4 ml of 10% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.). The lysates were then incubated overnight at 50°C and stored at 4°C until used.

For the preparation of dot blots, whole-cell DNA (5 μl) was mixed with 20 μl of 0.5 NaOH and incubated at 37°C for 15 min. After neutralization with 20 μl of 1 M Tris (pH 7), portions containing 10 to 20 ng of DNA were spotted onto a sheet of nitrocellulose. The filters were then soaked for 10 min on a double thickness of paper (3MM; Whatman, Inc., Clifton, N.J.) saturated with 1 M Tris–1.5 M NaCl (pH 7.0), dried, and baked for 2 h in a vacuum at 80°C.

For Southern analysis, whole-cell DNA was further purified on a cesium chloride-ethidium bromide gradient. One part of this DNA was cut with the restriction endonuclease *Eco*RI, and the other was cut with *Hin*dIII, under conditions specified by the supplier. The endonuclease digests were separated according to size on 0.35% agarose (*Eco*RI digests) or 0.7% agarose (*Hin*dIII digests), at 35 V for 18 h. The DNA was transferred to nylon filters according to the method of Southern (17, 28).

The filters were prehybridized for 4 to 18 h at 68°C in a solution containing 2× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate, and 0.5% skim milk. Hybridization with heat-denatured, labeled probe DNA was carried out in the same solution at 68°C for 18 h by using probes with activity of at least 10 cpm per filter. Heat-denatured salmon sperm or calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was added to the incubation mixture for a final concentration of 100 μg/ml. Filters were rinsed briefly in 2× SSC–0.1% sodium dodecyl sulfate at room temperature, washed at 68°C for 1 h in the same solution, rinsed in 0.1× SSC at room temperature, and air dried. The filters were then exposed to Kodak X-Omat AR film for 24 h at −70°C.

The hybridization with the dot blot probes was described as follows: 0, no reaction (blank); 100, a strong (intense black) hybridization reaction; and 200, a positive but weak (grey) hybridization reaction. Furthermore, each dot-blot-positive strain subjected to Southern blot analysis was assigned a *pap* type based on the combined sizes of *Eco*RI fragments hybridizing with the *Hin*dIII and *Sma*I probes and those of the *Hin*dIII fragments hybridizing with the *Hin*dIII probe.

Statistical analysis. The (i) O:K:H serotype, (ii) the relative electrophoretic mobilities of 13 enzymes, and (iii) either the *pap* type or the dot blot reaction was recorded for each strain in the collection. The data were analyzed using a FORTRAN 77 computer program, CLAN (Clone Analysis Package), written by Neal Bogdanovich (copies of this MSDOS program are available from Bruce Levin). The CLAN program assigned a number to each unique O:K:H serotype, ET, and Southern blot pattern, i.e., *pap* type or dot blot reaction.

The mean allozyme difference among pairs, *D* (7), was used as the primary measure of enzyme variation within serotypes and *pap* types. *VD* is the variance of *D*; Hi*D* and Lo*D* describe the range of the *D* values. A "bootstrapping" procedure was used to determine whether there is less enzyme variation within serotypes and *pap* types than expected by chance alone (statistical significance). To generate the test distribution, the *D* values among ETs were calculated for different numbers of isolates (for 5, 10, 15, up to 50) selected at random from the collection at large. The probability distribution of *D* values used for this statistical analysis was generated by 500 independent samplings.

TABLE 1. Enzyme and *pap* type variation within O:K:H serotypes represented by more than one isolate[a]

Serotype[b] O:K:H	ET	Isoenzyme pattern														*pap* type	Restriction fragment length (kb)[c]			No. of strains
																	*Eco*RI-*Hin*dIII	*Eco*RI-*Sma*I A	*Hin*dIII-*Hin*dIII	
1 1 7	16	F	B	A2	B	F	A	M	S	C	M	B	B	B	8	100	100		3
	16	F	B	A2	B	F	A	M	S	C	M	B	B	B	11	25	25	4	1
	3	F	B	A	B	F	A	M	S	C	M	B	B	B	31	19.5	19.5	4	1
	136	F	B	A2	B	F1	A	M	S	C	M	B	B	B	5	22	22	4	1
	92	F	B	A	B	F	A	M	S	C	M	B		B	8	100	100		1
1 1 N	139	S	C	A2	A		A	M	S	C	M	B		B	7	0	0		1
	129	S	C	A2	A	M	A	M	S	C	S	B	B	B	30	200	200		1
	129	S	C	A2	A	M	A	M	S	C	S	B	B	B	64	22	3.5, 2.5		1
	56	S	C	A2	A	M	A		S1	C	S			B	22	19.5	19.5	8	1
2 1 4	11	F	B	C	B	F	A	M	S	B	M	B		B	8	100	100		2
	26	F	B	C	B	F	A	M	S	B	M	B	B	B	19	16	16	4	1
	26	F	B	C	B	F	A	M	S	B	M	B	B	B	8	100	100		3
	26	F	B	C	B	F	A	M	S	B	M	B	B	B	11	25	25	4	1
2 1 7	13	F	B	C	B	F	A	M	S	C	M	B	B	B	11	25	25	4	3
	3	F	B	A	B	F	A	M	S	C	M	B	B	B	11	25	25	4	1
	34	F	B	A	B	F1	A	M	S	C	M	B	B	B	11	25	25	4	1
2 2 1	72	F	B	A	B	F	A		S	C	M	B	A	B	7	0	0		1
	38	F	B		B	F	A		S	C	M	B	A	B	25	22	22	8	1
	44	F	B	A	B	F	A	M	S	C	M	B	A	B	25	22	22	8	1
2 5 4	106	M	B	C	A	M	A	M	S1	A	S	B	B	B	8	100	100		1
	114	M	B	C	A	M	A	M	S	A	S	B	B	B	14	200	100		1
	14	M	B	C	A	M	B	M	S1	C	S	B	B	B	12	19.5	19.5, 13.5	10	1
4 3 5	117	F	B	A	A	F1	A	M	S	B	M	B	B	B	8	100	100		1
	93	F	B	A	A	F1	A	M	S	C	M	B	B	B	22	19.5	19.5	8	1
4 12 5	9	F	B	A	A	F	A	M	S	C	M	B	B	B	8	100	100		1
	3	F	B	A	B	F	A	M	S	C	M	B	B	B	3	25, 22, 19.5	25, 22, 19.5	4	1
4 12 N	3	F	B	A	B	F	A	M	S	C	M	B	B	B	3	25, 22, 19.5	25, 22, 19.5	4	2
4 12 5	9	F	B	A	A	F	A	M	S	C	M	B	B	B	3	25, 22, 19.5	25, 22, 19.5	4	6
	28	F	B	A	A	F1			S	C	M	B	B	B	8	100	100		1
4 N 5	9	F	B	A	A	F	A	M	S	C	M	B	B	B	3	25, 22, 19.5	25, 22, 19.5	4	1
	20	F	B	A	A	F	A	S	S	C	M	B	B	B	8	100	100		1
	42	F	B	A	A	F		M	S	C	M	B	B	B	28	15.5	15.5	4	1
6 2 1	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	7	0	0		1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	13	16	6.5	8	1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	49	>25, 22	>25, 22	4	8
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	30	200	200		1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	31	19.5	19.5	4	1
	90	F	B	A		F2	A	M	S	C	M	B	B	B	46	>25	>25	4	1
	7	F	B	A	B	F2	A	M	S	D	F	B	B	B	8	100	100		1
6 2 31	44	F	B	A	B	F	A	M	S	C	M	B	A	B	48	15	15	12	2
	92	F	B	A	B	F	A	M	S	C	M	B		B	48	15	15	12	1
	3	F	B	A	B	F	A	M	S	C	M	B	B	B	7	0	0		1
6 5 1	110	M	B	A	B	F2	A	M	S	B	M	B	B	B	8	100	100		1
	110	M	B	A	B	F2	A	M	S	B	M	B	B	B	60	25, 22	25, 22	4	1
6 N 7	138	M	B	A	B	F1	A	M	S	C	M	B	B	B	48	15.5	15.5	12	1
	25	M	B	A	B	F	A	M	S	C	M	B	B	B	8	100	100		1
6 13 1	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	8	100	100		1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	29	22, 19.5	22, 19.5	8, 4	1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	22	19.5	19.5	8	1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	20	22	2.5	10, 8	1
6 13 N	100	F	B	A	B	F2	A	M	S	B	M	B	B	B	8	100	100		1
	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	8	100	100		1
6 14 N	111	F	B	A	B	M	A	M	S	B	M	B	C	B	8	100	100		1
	89	F	B	A	B	M	A	M	S	C	M	B	B	B	11	25	25	4	1
	88	F	C	A	B	M	A	M	S	C	M	B	B	B	45	22, 10	4	12	1
	89	F	B	A	B	M	A	M	S	C	M	B	B	B	45	22, 10	4	12	2
	89	F	B	A	B	M	A	M	S	C	M	B	B	B	11	25	25	4	1
7 1 N	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	8	100	100		4
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	28	15.5	15.5	4	1
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	46	16	22	8	1
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	4	10	17.5, 3	4	1
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	5	22	22, 3	4	1
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	24	10	25, 22, 6.5	4	1
	125	S	B	A2	B	M	A	M	S	C		B	B	B	8	100	100		1
	125	S	B	A2	B	M	A	M	S	C		B	B	B	62	10	20, 10	4	1

Continued on following page

TABLE 1—*Continued*

Serotype[b] O:K:H	ET	Isoenzyme pattern													*pap* type	Restriction fragment length (kb)[c]			
																*Eco*RI-*Hin*dIII	*Eco*RI-*Sma*I A	*Hin*dIII-*Hin*dIII	No. of strains
8 25 9	109	F	A	A2	A	S	A	M	F	C	M	B	N	B	70	200	0		1
	31	F	A	A2	A	S	A	M	F	C	M	B		B	70	200	0		1
16 1 6	137	F	B	A	B	F	A	M	S	C	M	C	B	B	63	15.5	3.5		1
	1	F	B	A	B	F	B	M	S	C	M	C	B	B	1	22, 16	22, 16, 4	8, 4	1
	1	F	B	A	B	F	B	M	S	C	M	C	B	B	8	100	100		1
16 1 N	124	F	B	A2	B	F1	A	M	S	C	M	C	B	B	58	16, 15	15, 3.5	10, 8	1
	27	F	B	A	B	F1	A	M	S	C	M	C	B	B	20	22	2.5	10, 8	1
	27	F	B	A	B	F1	A	M	S	C	M	C	B	B	8	100	100		2
	27	F	B	A	B	F1	A	M	S	C	M	C	B	B	59	16, 15	10, 4	10, 8	2
18 1 7	22	F	B	B	B	F1	A	M	S	B	M	B	B	B	7	0	0		2
	70	M	B	B	B	F	A	M	S	C	M	B	B	B	7	0	0		1
18 5 N	36	F	B	B	B	F2	A		S	B	S	B	B	B	23	10	7.5, 3	10	1
	102	F	B	B	B	F1	A	M	S	B	S	B	B	B	8	>25	11	8	2
	18	F	B	B	B	F1	A	M	S	A	S	B	B	B	8	>25	11	8	1
	71	F	B	B	B	F1	A	M	S	D	S	B	B	B	39	25	9	10	1
25 5 1	115	F	B	A	B	F	A	M	S	B	M	B	B	B	14	22	22	4	1
	29	F2	B	A	B	F1	A	M	S	D	M	B	B	B	11	25	25	4	1
75 N 5	99	M	C	A	B	F1	A	M	S	A	S	B	B	B	30	200	200		1
	99	M	C	A	B	F1	A	M	S	A	S	B	B	B	17	200	0		1
75 5 N	99	M	C	A	B	F1	A	M	S	A	S	B	B	B	30	200	200		1
	99	M	C	A	B	F1	A	M	S	A	S	B	B	B	17	200	0		1
	53	F	B	A	B	F	A	M	S	B	S	B		B	35	22	2.5	10	2
	43	F	B	A	B	F	A	M	S	C	S	B	B	B	20	22	2.5	10, 8	1
	43	F	B	A	B	F	A	M	S	C	S	B	B	B	43	22	2.5	8	1
	95	F	B	A	B	F	A	M	S	C	S	B	B		13	16	6.5	8	1
	21	F	C	A	B	F	A	M	S	C	S	B	A	B	17	200	0		1
	30	F	B	C	B	S	A	FY	M	C	M	B		B	17	200	0		1
	45	F	B	A	B	F	A		S	C	S	B	B	B	30	200	200		1
83 N 31	112	M	B	A	A	F1	A	M	S	B	M	B		B	30	200	200		1
	101	M	B	A	A	F1	A	M	S	B	M	B	B	B	40	22	22	4	1
R 5 N	8	F	B	A1	B	F	B		S	D	S	B	B	B	8	100	100		1
	62	F	B	A	B	F1	A	M	M		S	B	B	B	38	25	9	10	1
	34	F	B	A	B	F1	A	M	S	C	M	B	B	B	40	22	22	4	1
R 2 1	5	F	B	A	B	F2	A	M	S	C	M	B	B	B	7	0	0		1
	15	F	B	A	B	F1	A	M	S	D	M	B	B	B	13	16	6.5	8	1
	17	F	B	A	B	F1			S	C	M	B	B	B	36	200	100		1
	59	F	B	A	B	F2	A		S	C	M	B		B	25	22	22	8	1
R N N	52	F	B	A2	A	M	A	M	S	C	S	B	B	B	34	0	100		1
	81	F	C	A	A	M	B	FY	M	C	M	B	A	B	36	200	100		1
	49	S	B	A	B	M	A		S	C	S	B		B	33	16	25, 2.5	4	1
R 1 N	68	S	C	A2	A	M	A		S	C	S	B	B	B	7	0	0		1
	66	S	B	A2	A	M	A	M	S	C	S	B	B	B	30	200	200		1
	4	S	B	A2	B	M	A	M	S	C	S	B	B	B	39	10	2.5	10	1
	80	F	B	A	B	F	A		S	C	M	D	B	B	44	16, 15	16, 3.5	10, 8	1
R 5 1	34	F	B	A	B	F1	A	M	S	C	M	B	B	B	11	25	25	4	1
	65	F	B	A	B	F1	A	M	S	A	S	B	B	B	31	19.5	19.5	4	1
	64	F	B	A	B	F2	A		S		M	B			40	22	22	4	1
R N 2	75	M	C	A	A	M	B	FY	M	C	M	B	A	B	7	0	0		1
	83	F	C	A	A	M	A		M	C	M	B	A	B	7	0	0		1

[a] The complete set of primary data is available from the corresponding author.
[b] N, Nontypable; R, rough.
[c] kb, Kilobase.

RESULTS

Overall distribution of serotypes, ETs, and *pap* types. Of the 229 *E. coli* strains in the collection, 90 had complete three-antigen serotypes, resulting in 31 specific serotypes; 60 had a complete serotype for O and K antigens but were nonmotile (HN); and 79 had incomplete serotypes, i.e. were rough or otherwise nontypable for one or two antigens. While ETs were obtained for all strains in the collection, a number of enzymes were not active, i.e., null alleles. Including these null alleles as a separate class, among the 229 isolates there was a total of 133 distinct ETs for the 13 enzymes considered. A total of 191 strains were positive (100 or 200) for at least one of the *pap* probes. Among these, 176 were positive for both probes, 15 hybridized weakly with only the *Hin*dIII probe, and 3 hybridized only with the *Sma*I probe. Southern blot hybridization data were obtained for 111 of these strains, resulting in 49 distinct *pap* types. The asymptomatic bacteriuria strains in the Swedish sample included relatively

TABLE 2. Examples of *pap* type variation within ET[a]

ET	*pap* type	Restriction fragment length (kb)[b]			No. of strains
		*Eco*RI-*Hin*dIII	*Eco*RI-*Sma*I	*Hin*dIII-*Hin*dIII	
3	3	25, 22, 19.5	25, 22, 19.5	4	1
	31	19.5	19.5	4	1
	11	25	25	4	1
	7	0	0		2
5	13	16	6.5	8	10
	5	22	22	4	1
	31	19.5	19.5	4	1
	22	19.5	19.5	8	1
	20	22	2.5	10, 8	1
	7	0	0		6
9	3	25, 22, 19.5	25, 22, 19.5	4	8
13	11	25	25	4	3

[a] The remaining ETs with *pap* variation were ET 1, 4, 11, 16, 18, 22, 26, 27, 34, 43, 44, 53, 89, 92, 99, 101, 102, 106, 110, 112, 117, 125, and 129 (Table 1).
[b] kb, Kilobases.

fewer *pap*-positive isolates (65%) than the pyelonephritis and cystitis isolates (88 and 86%) ($P < 0.05$ for the contingency χ^2).

***pap* variation among serotypes.** The distribution of ETs and *pap* types for complete O:K:H serotypes represented by at least two isolates is shown in Table 1. Most isolates with the same complete O:K:H serotype had identical ETs, and there was significantly less enzyme variation among isolates of the same three-antigen serotype than among randomly chosen strains.

The variation in *pap* DNA within and between clones was analyzed at two levels: the presence of *pap* DNA detected by dot blots and the organization of the DNA in *pap*-positive isolates as detected by Southern blot analysis. The serotypes O2:K2:H1, O6:K2:H1, and O6:K2:H31 included *pap*-positive strains which hybridized with both the *Hin*dIII and *Sma*I probes as well as *pap*-negative strains which failed to hybridize with either probe after repeated dot blot analyses. For the latter two, the *pap*-positive and *pap*-negative isolates had the same ET as well as the same complete serotype.

Among the *pap*-positive isolates, the variation in the Southern blot pattern had two components, the number of copies of *pap* ranged from one to three and the lengths of the restriction fragments hybridizing with either probe varied. In most strains which contained more than one *pap*-homologous restriction fragment, the two probes reacted with two fragments of the same size. There were, however, exceptions in which the *Hin*dIII probe hybridized with one *Eco*RI fragment, while the *Sma*I probe detected two fragments, and vice versa. Such variation was seen for O1:K1:HN, O2:K5:H4, O7:K1:HN, O16:K1:H6, and O18:K5:HN. Some isolates sharing both O:K:H serotype and ET varied in both the number and the size of *pap*-homologous restriction fragments, e.g., O6:K13:H1 and ET5, and O6:K2:H1 and ET5. In general, isolates of the same ET and complete O:K:H serotype contained the same number of fragments.

***pap* variation among ETs.** As can be seen from Table 2, some ETs represented by multiple isolates were homogeneous for *pap* type, e.g., ET 9 and ET 13. However, the majority of the ETs represented by five or more isolates included strains with very different restriction fragment length classes for the *pap* probes. Furthermore, ETs 3 and 5 included isolates that were both *pap* positive and *pap* negative.

Serotype variation within *pap* types. The majority of strains of a particular *pap* type had either the same three-antigen O:K:H serotype or serotypes that differed by one antigen that for some reason was not completely typable, e.g., OR, ON, KN, or HN. There were, however, a number of exceptions which are listed in Table 3. *pap* types 11, 22, and 40 were represented by isolates that differed in all three antigens.

TABLE 3. Examples of serotype variation within *pap* type[a]

pap type	Restriction fragment length (kb)[b]				No. of strains
	*Eco*RI-*Hin*dIII	*Eco*RI-*Sma*I	*Hin*dIII-*Hin*dIII	Serotype[c] O : K : H	
11	25	25	4	1 1 7	1
				2 1 4	1
				2 1 7	3
				2 1 N	1
				6 14 N	2
				25 5 1	1
				R 5 1	1
				157 52 45	1
22	19.5	19.5	8	1 1 N	1
				4 3 5	1
				6 13 1	1
				75 95 N	1
31	19.5	19.5	4	1 1 7	2
				6 2 1	1
				75 95.5 N	1
				R 5 1	1
				R R 5	2
				117 53 N	2
40	22	22	4	1 1 7	1
				R 5 1	1
				R 5 N	1
				25 5 1	1
				83 N 31	1

[a] Serotype variation also occurred among *pap* types 3, 5, 13, 20, 25, 28, 35, 38, 45, 46, 48, and 59 (Table 1).
[b] kb, Kilobases.
[c] N, Nontypable; R, rough.

ET variation within *pap* types. In general, there was less allozyme variation among isolates of the same *pap* type than among randomly chosen isolates. Isolates of the same *pap* type which differed, mostly showed small differences, e.g., null alleles or one or two allozymes (Table 4). There were, however, exceptions. *Pap* types 22, 28, 40, 46 included isolates that differed by four or more allozymes, and there were isolates with identical restriction patterns, e.g., *pap* type 31, which differed by 12 of the 13 enzymes scored.

DISCUSSION

Variation in chromosomal DNA among *E. coli* strains from natural sources was studied with probes specific for the *P*-associated pilus (*pap*) region. The presence of DNA homologous to *pap*, variation in the number of copies, and sizes of these sequences and those flanking it were determined by dot and Southern blot hybridization. The results of this survey reveal considerable heterogeneity in *pap* also among strains identified as members of the same clone by O:K:H serotyping and multilocus enzyme electrophoresis. We interpret these observations as an illustration of the limitations of the clone concept and as evidence for the horizontal transfer of chromosomal genes in natural populations of *E. coli*.

The three procedures used to identify clones in this investigation are fundamentally different. Serotyping distin-

TABLE 4. Pairwise difference in ETs among isolates of the same *pap* type

pap type	Restriction fragment length (kb)[a]			No. of strains	No. of ETs	*D*[b]	*VD*[c]	Hi*D*[d]	Lo*D*[d]
	*Eco*RI-*Hin*dIII	*Eco*RI-*Sma*I	*Hin*dIII-*Hin*dIII						
3	25, 22, 19.5	25, 22, 19.5	4	11	3	0.6[e]	0.7	3	0
11	25	25	4	13	8	2.1[e]	1.8	5	0
13	16	6.5	8	12	3	0.8[e]	1.6	4	0
20	22	2.5	10, 8	3	3	2.3	0.3	3	2
22	19.5	19.5	8	4	4	6	11.6	10	2
25	22	22	8	4	4	2.8[e]	0.5	4	2
28	15.5	15.5	4	2	2	6		6	6
31	19.5	19.5	4	7	6	4.6	16.5	12	0
35	22	2.5	10	2	1	0.0		0	0
38	25	9	10	2	2	3.0		3	3
40	22	22	4	5	5	3.9	3.4	7	1
45	22, 10	4	12	5	2	0.4[e]	0.3	1	0
46	>25	>25	4	3	3	6.7	0.3	7	6
48	15	15	12	4	2	0.7	0.3	1	0
54	15.5	15.5	12	2	2	2.0		2	2
59	16, 15	10, 4	10, 8	2	1	0.0		0	0

[a] kb, Kilobases.
[b] *D* is the mean number of allozyme differences among all pairs of isolates of the noted *pap* type.
[c] *VD* is the variance in the number of allozyme differences among all pairs of isolates of the noted *pap* type.
[d] Hi*D* and Lo*D* are, respectively, the maximum and minimum number of allozyme differences among pairs of isolates of the noted *pap* type.
[e] Significant difference, i.e., the probability of a higher value of *D* by chance alone is greater than 0.95. The test was employed only when the number of isolates in a group exceeds 5.

guishes variation in surface antigens that are products of multiple chromosomal genes, all of which have not been identified and mapped (40). Multilocus enzyme electrophoresis detects variation in enzymes, the majority of which are products of single chromosomal genes that have been mapped in *E. coli* K-12 (6–8, 43). The dot and Southern blot hybridization procedures identify variation in the structure and organization of the *E. coli* chromosome that may or may not be reflected as differences in phenotype. Although whole-cell DNA was used for these analyses, we interpret the *pap* data as evidence for chromosomal rather than plasmid variation. A separate analysis of plasmid DNA from 60 isolates revealed no homology with *pap* probes (S. Hull and R. Hull, unpublished data).

The variation in *pap* type reported here reflects strain differences in the DNA sequences flanking the *pap* operon and in the *pap* operon itself. The *pap* probes used for the dot and Southern blot analyses hybridize with a region that has been cloned and partially sequenced (2, 15, 27). It was previously determined that there are no *Eco*RI sites within the functional limits of the *pap* operon for *E. coli* SH1, from which the probes were derived (17). Thus, the Southern blot hybridization analyses with the *Eco*RI digests detect primarily variation in the distribution of the DNA sequences flanking the *pap* operon. However, some of the natural isolates could have *Eco*RI sites within their *pap* operons, either within or between the regions recognized by the two probes, and if so, they would have two or more fragments that hybridize with the probes. The restriction endonuclease *Hin*dIII recognizes and cuts two sites within the *pap* operon of SH1. Therefore, hybridization with the *Hin*dIII probe would detect primarily variation in the distribution of DNA sequences within the *pap* operon.

Since the sensitivity of the *pap* probes used here was close to 100% for strains binding to galactoseα1→4galactoseβ-containing receptors (16) we consider the lack of hybridization (*pap* negative) most likely due to the absence of the *pap* region than to extensive sequence divergence in this region. However, we cannot exclude the possibility that the latter mechanism contributes to some of the negative hybridization reactions. In fact, sequence divergence seems to be a reasonable explanation for the cases in which hybridization obtained for only one of the two probes used for the dot blot survey.

In the main, the results presented here are consistent with the clone concept (37). The majority of isolates from Houston and Göteborg with a given *pap* type also had the same, or nearly the same, ET and O:K:H serotype. Most isolates of a specific O:K:H type and ET had the same *pap* type. There were, however, exceptions. First, strains of the same ET and specific O:K:H serotype were both positive and negative for *pap*. Second, strains with the same clonal markers had different *pap* restriction fragment patterns. Third, isolates with the same *pap* type differed by O:K:H serotype and ET. We do not consider these exceptions to be fundamental violations of the clone concept or to call into question the utility of typing procedures for identifying pathological strains of *E. coli* in epidemiological studies. These results suggest only that O:K:H serotype and ET data may not be sufficient to infer identity for other properties and that it would be useful to also employ typing procedures that directly detect the relevant virulence factors.

The three clone identification characters used here are likely to differ in the extent to which they are subject to selection and in the nature of that selection. Multilocus enzyme electrophoresis detects different forms of the same functional enzyme and, almost exclusively, metabolic enzymes that are operating internally. It has been argued that this type of enzyme variation is undetectable to selection, i.e., selectively neutral (21, 34, 43). The surface antigens that determine serotypes, including fimbrial antigens (20), are recognized by the mammalian immune system. A variety of observations suggests that surface antigens used for serotyping are under selection (40; S. Hansson, D. A. Caugant, U. Jodal, and C. Svanborg Edén, Br. Med. J., in press). The *pap* gene products function both as antigens and as adhesins (17). Their antigenicity is determined mainly by the *pap* A gene product (39), the major fimbrial protein. The receptor-

binding function is determined by the tip adhesin (14, 25, 32). Bacteria may retain the binding phenotype while the antigenic structure of the fimbriae varies. As a consequence, selection for *pap* can operate in a number of ways. Frequency-dependent selection would favor novel antigenic types, in analogy with the O, K, and H antigens and antigens of parasites (4, 9). The adherence phenotype could facilitate its maintenance in traditional habitats as well as colonization and persistence in new habitats (niche expansion). Galactoseα1→4galactoseβ-containing receptors are present in the human large intestine (10, 53) and may enhance the persistence at that site. These receptors are also present in the urinary tract, and the capacity to bind to them enhances persistence of *pap*-positive *E. coli* strains in kidneys and bladders (13). As long as the intensity of selection favoring changes in *pap* phenotype is substantial, these changes would occur, even if the rate at which *pap* variation is generated is low. The processes postulated to account for the clonal structure of bacterial populations, random extinction of lineages (30) and periodic selection (24), do not rule out the substitution of specific genes that are favored by selection.

The primary evidence for the horizontal transfer of the *pap* chromosomal region is the finding of the same *pap* type in isolates with O:K:H serotypes that differ by two and three antigens and with ETs that differ by large numbers of allozymes. In addition, there was extensive heterogeneity in the size and number of copies of *pap* among naturally occurring isolates. This suggests that *pap* or the DNA sequences flanking it are evolutionary labile; they change at a substantial rate. The occurrence of the same *pap* type in genetically distant clones suggests either that the DNA sequence was constrained by the same selection pressures in these different clones or that the region of the chromosome was recently obtained from a common source (horizontal transfer).

The second line of evidence for the horizontal transfer of *pap* is variation in the existence, number of copies, and sizes of *pap* hybridizing fragments among isolates of the same ET and complete O:K:H serotype. This suggests that the *pap* region is transposable; it can be completely excised from the *E. coli* chromosome and can move as a unit to different sites on that chromosome or onto plasmid or bacteriophage vectors. While not essential, transposition makes horizontal transfer of a chromosome region more likely than if homologous, "legitimate" recombination were required for gene insertion or exchange. There is evidence that other virulence determinants are transposable (48, 52) or associated with extinct transposons (3, 42). In the case of *pap*, neither of these lines of evidence is available.

The horizontal transfer of *pap* has to be considered a hypothesis. It is, however, a hypothesis that makes a number of predictions. (i) The *pap* region is (or recently had been) transposable. (ii) The *Sma*I and *Hin*dIII cuts of the *E. coli* chromosome that hybridize with the *pap* probes are within the presumptive *pap* transposon. (iii) There is identity or near identity in *pap* nucleotide sequences of *E. coli* of the *pap* types but with different ETs and serotypes. (iv) There are conjugative plasmids or temperate phage vectors which still carry the *pap* genes. Finally, if horizontal transfer of chromosomal genes involved with niche expansion is a general phenomenon among the bacteria, their chromosomes would, to some extent, be chimeras containing segments of different ancestry. DNA sequence data for different regions of chromosomes of bacteria from natural sources should reveal whether this is so.

ACKNOWLEDGMENTS

We gratefully thank Anneli Lundell and Susanne Schmeisser for excellent technical assistance. We wish to express our gratitude to those who support and maintain BITnet, the computer communication network, which greatly facilitated this international collaboration.

This research was supported by grants from the Swedish Medical Research Council (K.P. and C.S.E.) and the Volkswagen Foundation (I.Ø. and F.Ø.) and by Public Health Service grants AI18462, AI121009 (S.I.H. and R.A.H.), and GM33782 (B.R.L.).

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