

Chromosomal Regions Specific to Pathogenic Isolates of *Escherichia coli* Have a Phylogenetically Clustered Distribution

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We studied the ancestry of virulence-associated genes in *Escherichia coli* by examining chromosomal regions specific to pathogenic isolates. The four virulence determinants examined were the alpha-hemolysin (*hly*) loci *hlyI* and *hlyII*, the type II capsule gene cluster *kps*, and the P (*pap*) and S (*sfa*) fimbria gene clusters. All four loci were shown previously to be associated with pathogenicity islands of uropathogenic *E. coli* isolates. The *hly*, *kps*, *sfa*, and *pap* regions each have an unexpected clustered distribution among the *E. coli* collection of reference (ECOR) strains, but all these regions were absent from a collection of diarrheagenic *E. coli* isolates. Strains in the ECOR subgroup B2 typically had a combination of at least three of the four loci, and all strains in subgroup D had a copy of the *kps* and *pap* clusters. In contrast, only four strains in subgroup A had either *hly*, *kps*, *sfa*, or *pap*, and no subgroup A strains had all four together. Strains of subgroup B1 were devoid of all four virulence regions, with the exception of one isolate that had a copy of the *sfa* gene cluster. This phylogenetic distribution of strain-specific sequences corresponds to the ECOR groups with the largest genome size, namely, B2 and D. We propose that the pathogenicity islands are ancestral to subgroups B2 and D and were acquired after speciation, with subsequent horizontal transfer into some group A, B1, and E lineages. These results suggest that the *hly*, *kps*, *sfa*, and *pap* pathogenicity determinants may play a role in the evolution of enteric bacteria quite apart from, and perhaps with precedence over, their ability to cause disease.

Escherichia coli is a genetically diverse species, the majority of isolates of which are commensal organisms of the intestinal tract. However, some isolates are opportunistic pathogens causing intestinal and extraintestinal infections in a range of hosts. For example, the enteropathogenic *E. coli* (EPEC) is the leading cause of severe infantile diarrhea in the developing world, and enterohemorrhagic *E. coli* (EHEC) O157:H7 has recently emerged as the cause of bloody diarrhea and hemolytic-uremic syndrome in major food-borne outbreaks in the United States, Europe, and Asia (33, 34).

Pathogenicity islands (PAIs) encompass large segments of sometimes unstable chromosomal DNA (5 to 200 kb) containing virulence gene clusters (11, 14, 19) that are often flanked by insertion sequence elements or tRNA genes (2, 6, 14). Five such islands have previously been identified in pathogenic *E. coli* isolates (10, 14). PAIs I, II, IV, and V carry a number of virulence gene clusters, among them the P (*pap*) and P-related (*prf*) fimbria gene clusters and two alpha-hemolysin loci, *hlyI* and *hlyII* (2, 3, 16, 21). The locus *LEE* (locus of enterocyte effacement) was identified in an EPEC strain (23); *LEE* carries a different set of virulence genes than those found in PAI I, II, IV, or V but is inserted in the same chromosomal site as PAI I, at 82 min at the selenocysteine-specific tRNA (2, 23).

We examined the occurrence, phylogenetic distribution, and genetic diversity of the four virulence determinants *hly*, *kps*, *pap*, and *sfa* among *E. coli* natural isolates. The purpose was to test the conventional view that chromosomal virulence determinants are temporary insertions into the bacterial chromosome which come and go as the lineage undergoes expansion

and diversification (2, 3, 11, 14, 19). The *hly* locus contains the gene for alpha-hemolysin production, whereas the *kps* gene cluster contains genes for type II capsule (4, 8, 21, 26). The *pap* and *sfa* gene clusters encode P and S fimbriae that are associated mostly with uropathogenic *E. coli* strains (3, 9, 12, 13, 17). The distribution of the four virulence determinants was confined predominately to two lineages of the *E. coli* reference collection (ECOR) strains, subgroups B2 and D, with sporadic occurrences in subgroups A, B1, and E.

Does the observed phylogenetic clustering we have found reflect common descent of the pathogenicity determinants in the subgroup B2 and D lineages? If it does, then this would imply that these particular pathogenicity determinants have a long-term persistence in the genomes of these particular bacterial lineages. Did horizontal transfer of PAIs play an important role during the evolution of *E. coli*, and what role, if any, do PAIs play in genome size evolution? To address these issues we also analyzed nucleotide sequence variation at the three loci *hlyA*, *kpsD*, and *papH* to determine levels of genetic diversity compared with the housekeeping gene *mdh* for malate dehydrogenase. These data were analyzed for evidence of horizontal transfer among ECOR subgroups and long-term persistence within subgroups.

MATERIALS AND METHODS

Bacterial strains. Two *E. coli* reference collections of natural isolates were examined: the ECOR collection and the diarrheagenic *E. coli* (DEC) collection (25, 34). The ECOR collection consists of 72 strains, 62 of which were recovered from healthy humans and animals, and 10 from women with urinary tract infections. The ECOR collection encompasses much of the total variation found within this species, and the major lineages are divided into five groups: A, B1, B2, D, and E. The DEC collection is made up of 15 strains recovered from patients infected with an organism from one of three enteric disease categories: EPEC, EHEC, and enterotoxigenic *E. coli* (ETEC). Genomic DNA was extracted with the G-Nome DNA isolation kit from Bio 101 (Vista, Calif.).

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TABLE 1. Forward and reverse primers used in construction of probes

Gene	Forward and reverse primer	PCR product size (bp)	Probe	Reference
<i>sfaA1</i>	5' CGG TGT GCG TAG TTC AAT 3'			32
<i>sfaA2</i>	5' CAC CCG CAT GGA TAA AAA 3'	641	sfa1	32
<i>sfaG1</i>	5' CAT TAA CTC CCG AAA CTT 3'			29
<i>sfaH2</i>	5' CAT TAC CGC CAC AAC TGC 3'	1,862	sfa2	29
<i>papA1</i>	5' TTA AAG GTA ATC GGG TCA T 3'			22
<i>papA2</i>	5' GGA ATC AGA GAA AAG GTT 3'	856	pap1	22
<i>papA1</i>	5' TTA AAG GTA ATC GGG TCA T 3'			22
<i>papC2</i>	5' CGC TTC AGG TCA ACA GAG G 3'	3,512	pap2	22
<i>papH1</i>	5' AAT ACT GGG GAG AAG AGC AC 3'			22
<i>papF2</i>	5' ATT ACG AAA GGG CAC TGA AG 3'	6,086	pap3	22
<i>kpsE1</i>	5' AAA AGA CCC GTG TAG AAG C 3'			26
<i>kpsC2</i>	5' CAA AAG GTC AGA GCC AAG T 3'	3,852	kps1	26
<i>kpsD2</i>	5' CGTTACGGG AAT GCT GCT T 3'			26
<i>kpsM1</i>	5' GTC CAG AAA GTC ACC GTA GA 3'			X53819 ^a
<i>kpsT2</i>	5' CAA TCG CCA CAT CAC AAA AC 3'	1,335	kps2	X53819 ^a
<i>hly1</i>	5' TCA GTC CTC TTT CTT TCC T 3'			U12572 ^a
<i>hly2</i>	5' CTC TGG CAA CGG TCT CTC C 3'	2,899	hly1	U12572 ^a
<i>hlyA1</i>	5' GAC AAA GCA CGA AAG ATG 3'			8
<i>hlyA2</i>	5' CAA CTG CAA TAA AGA AGC 3'	2,930	hly2	8

^a GenBank accession number.

PCR and DNA probe construction. Nine sets of primers for amplification of sequences of the *hly*, *kps*, *pap*, and *sfa* gene clusters were used to obtain probes to screen strains for the presence of these virulence determinants (Table 1). In the 7.9-kb *sfa* operon there are nine genes, and two probes were designed for this

region: *sfa1* from the 5' end of the gene cluster and *sfa2* from the 3' region (Table 1 and Fig. 1A). We applied long-range PCR (7) to obtain three probes, *pap1*, *pap2*, and *pap3*, from the 9-kb *pap* gene cluster (Table 1 and Fig. 1B). Probe *kps1* represents *kps* region 1, and probe *kps2* represents *kps* region 3

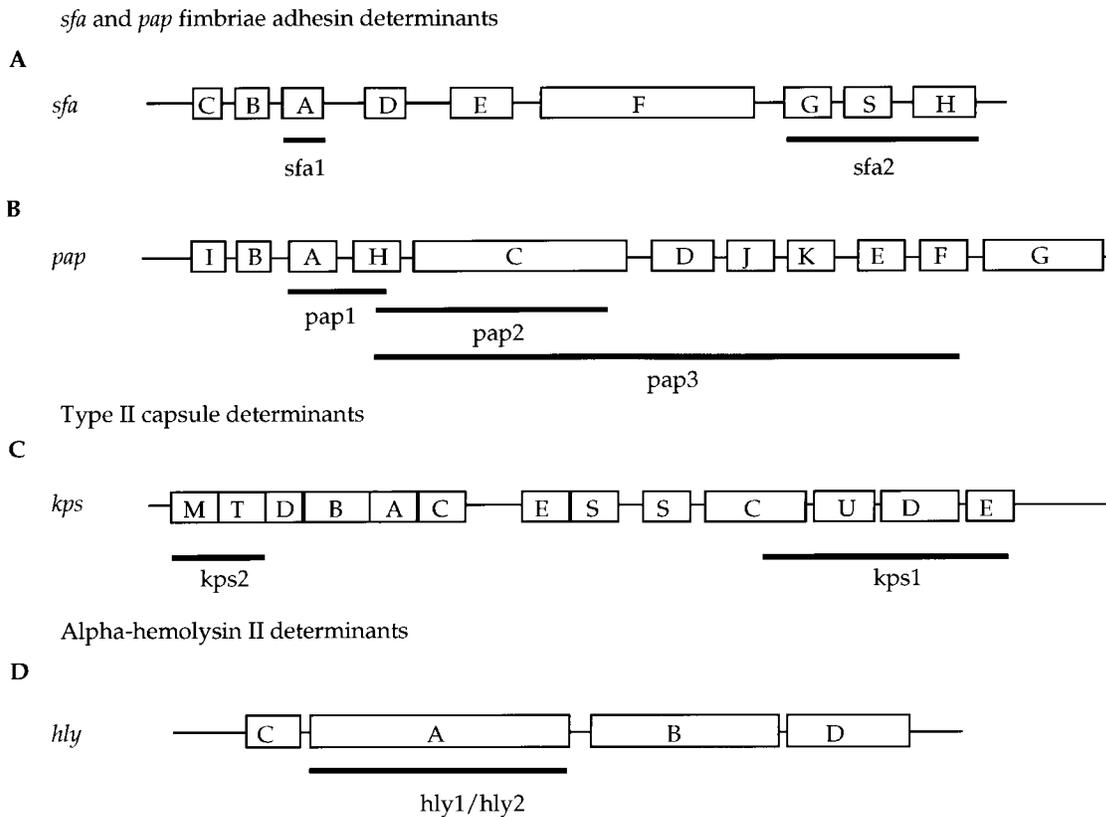


FIG. 1. Gene organization of *kps*, *sfa*, *pap*, and *hly* gene clusters. (A and B) Organization of *pap* and *sfa* gene clusters. Boxes indicate genes, and uppercase letters indicate gene designation(s). (C) The *kps* genes in region 1 (five genes [*kpsSCUDE*]) and region 3 (two genes [*kpsMT*]) are conserved among *E. coli* isolates that synthesize serologically distinct capsules. The number of genes in the central region 2 (*kpsDBACES*) reflects the size and complexity of the polysaccharide repeating unit. (D) The alpha-hemolysin determinant from *E. coli* J96 is represented; it consists of four genes. Black bars below gene clusters indicate the position of DNA fragments used as probes.

(Table 1 and Fig. 1C). Two alpha-hemolysin gene probes, *hly1* and *hly2*, were made from a hemolysin gene cluster in an EHEC strain and a uropathogenic *E. coli* strain, J96, respectively (Table 1 and Fig. 1D). Following amplification, the PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Inc., Chatsworth, Calif.). All probes were prepared with DNA from ECOR 52 as a template. The probes were labeled with fluorescein-conjugated nucleotides according to the manufacturer's instructions (Amersham, Arlington Heights, Ill.).

DNA hybridization. Bacterial genomic DNA was digested with *EcoRI*, and the digests were separated by electrophoresis in 0.6% agarose gels in 1× Tris-borate-EDTA. DNA fragments were transferred by alkaline blotting to Hybond-N+ membranes (Amersham). Membranes were prehybridized for 30 min at 65°C in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 5% dextran sulfate. Hybridizations to labeled probes were carried out overnight at 65°C. Hybridized fragments were detected with the enhanced chemiluminescence system (Amersham).

Nucleotide sequencing. PCR products from three genes, *hlyA*, *kpsD*, and *papH*, were sequenced with an Applied Biosystems model 373A automated DNA sequencing system with a DyeDeoxy terminator cycle sequencing kit. For all strains, both strands were sequenced. From eight ECOR isolates, representing three ECOR subgroups, A, B2, and D, 570 bp of the *hlyA* gene was sequenced. A 729-bp region of the *kpsD* gene was sequenced from each of nine ECOR strains from subgroups A, B2, and D. A 462-bp portion of the *papH* gene was sequenced from six ECOR isolates.

Statistical analysis. To assay possible intragenic recombination events the Stephens test was used (31). The Stephens test identifies nonrandom clustering of polymorphic sites that may reflect the results of recombination events. The Stephens method examines the distribution of polymorphic sites relative to the phylogenetic partitions they support. Polymorphic sites supporting a particular phylogenetic partition are expected to be randomly distributed along the sequence if there is no recombination. Nucleotide sequences were analyzed by use of the computer program MEGA (20). Phylogenetic trees were constructed from synonymous site variation by the neighbor-joining method (28).

Nucleotide sequence accession numbers. The nucleotide sequences of the genes described in this paper have been deposited in the GenBank database under the accession no. AF037572 to AF037588.

RESULTS

Distribution of virulence determinants associated with PAIs. We determined the distribution of four virulence-associated regions in the 72 isolates of the ECOR reference collection and the 15 isolates of the DEC reference collection. The loci assayed were the two adhesin gene clusters *sfa* and *pap*; the *kps* capsule operon; and the alpha-hemolysin loci *hlyI* and *hlyII*. The distribution of the four virulence determinants was confined predominately to two lineages of the ECOR strains, subgroup B2 and D (Fig. 2).

The *sfa* locus was found almost exclusively in ECOR group B2 strains (10 of 15) and one isolate of subgroup B1 (ECOR 58); moreover, this was the only one of the assigned regions detected in subgroup B1. The *pap* gene cluster was detected in all subgroup D strains and most subgroup B2 strains (11 of 15), as well as in three group A and two group E isolates. In two subgroup B2 strains (ECOR 55 and ECOR 64), one subgroup D isolate (ECOR 35), and one subgroup E isolate (ECOR 37), the majority of the *pap* cluster was apparently deleted, as only the *pap1* probe gave a positive hybridization signal. Four strains had multiple copies of *sfa* (ECOR 52, ECOR 54, ECOR 63, and ECOR 60) and *papA* (ECOR 11, ECOR 24, ECOR 49, and ECOR 50).

The *kps* operon was present in all subgroup D isolates, the majority of B2 isolates (12 of 15), and four isolates of group A (Fig. 2). The *hly1* probe from an EHEC *hly* sequence did not hybridize with any of the ECOR strains; however, the *hly2* probe from the uropathogenic *E. coli* strain J96 hybridized with ECOR subgroup B2 strains (9 of 16) and one strain each of subgroups A, D, and E.

Nucleotide sequence variation. The single genes *hlyA* (*hlyII*), *kpsD* (*kps*), and *papH* (*pap*) from each of the three virulence gene clusters were sequenced from representative isolates of the ECOR collection.

Within the *hlyA* alleles from nine *E. coli* strains, there were only six polymorphic sites in the 570-bp segment sequenced.

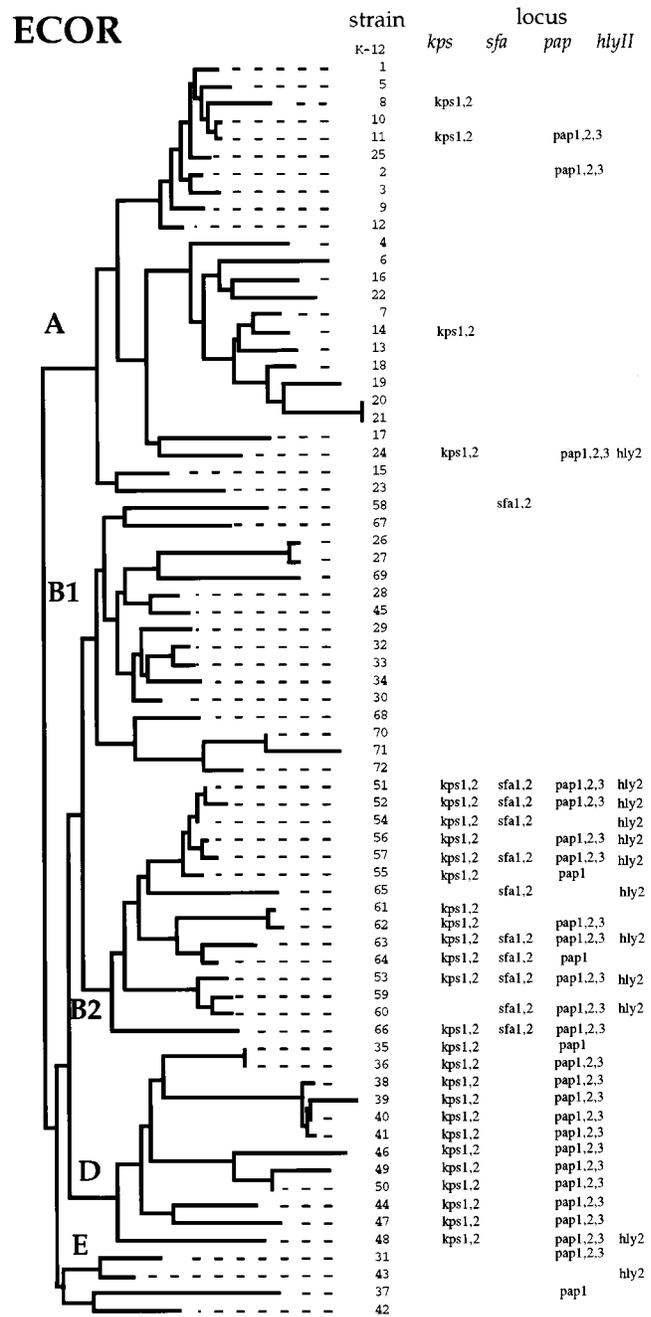


FIG. 2. Distribution of the *kps*, *sfa*, *pap*, and *hly* complexes among the ECOR collection of natural isolates. The tree is based on electrophoretic variation among 38 enzymes (15). Only for the *pap* operon did the three probes used give differing results.

ECOR 24 and ECOR 43 had virtually identical sequences and are distinct at five of the six polymorphic sites detected in the other ECOR strains examined (Fig. 3). The *hlyA* locus has a GC content of 40%, which is atypical for genes of *E. coli*, and a codon adaptation index (CAI) of 0.228.

At the *papH* locus there were 22 polymorphic sites, which resulted in nine amino acid replacements, six of which were found in ECOR 49 (Fig. 4). The *papH* locus had a typical *E. coli* GC content of 50%.

There were 48 polymorphic sites in the 729-bp region of

<i>kpsD</i>		1	111	111	111	112	222	233	333	333	444	444	444	444	456		
		45	567	992	223	355	677	790	378	923	467	999	001	122	222	345	749
ECOR8		GG	GCC	TCA	GAC	GCC	CGG	GAT	ACT	CAG	GCG	GTC	CTC	ATG	CCC	CGA	ATG
ECOR11	T	G.	G. . .	.T.	A.T.	C. A	
ECOR14		T. .	T. T	GAG	A. A	A. TCC	G. C	.T. .	A.T.AG	C. .	
ECOR38	T	G.A.	G. . .	.T.	C. .	
ECOR46		.A	T.T.T.	C. .	
ECOR48		.A	. . T	G.	TAC	. . .	G. . .	.TA	ATA	.CG	TCT	GCA	T. T	A. G	C. .	
ECOR49		. .	.AT	G. .	AGA	GT. .	.T.	A.T.	C. A	
ECOR52	T	G.A.	G. . .	TT.AG	C. .	
ECOR53	T	T. . .	G. . .	.T.	C. .	
			SA	N	Q	S	A		Y	V	V				A		
			IT	S	K	I	S		F	I	I				T		

<i>papH</i>		12	222	234	4				
		122	223	335	899	940	247	851	2
		103	454	783	113	927	896	842	3
ECOR48		GTC	ACT	GGA	GTC	TGT	ACC	TCT	G
ECOR52	
ECOR53	
ECOR49		ACT	GTC	TAG	TAA	C. C	.TA	CTC	.
ECOR50	C	T. . .	.A	.C	TT. .	C. C	.
ECOR46	AC	G. . .	.C	T
		RVP	LF	G	H	S	A		
		QAL	FL	Y	R	T	T		
			L	C					

<i>hlyA</i>		12	333
		48	132
		923	388
ECOR24		CGG	TAT
ECOR43		.A	. . .
ECOR51		TAA	CG.
ECOR52		.AA	CGC
ECOR54		.AA	CG.
ECOR60		.AA	CG.
ECOR63		.AA	CGC
ECOR48		.AA	CG.
		A	AN
		T	TS

FIG. 3. Distribution of polymorphic nucleotide and amino acid sites along the *kpsD*, *papH*, and *hlyA* genes among ECOR isolates. Vertical numbers indicate the nucleotide position along the gene. Dots indicate nucleotide identity.

kpsD sequenced in nine ECOR isolates. ECOR 14 and ECOR 48 were the most divergent in nucleotide sequence and accounted for much of the variation among the strains (Fig. 4). The GC content of *kpsD* is 52%, and this coding region has a CAI of 0.331 (30).

Rates of synonymous and nonsynonymous substitution. For *hlyA* we estimated the numbers of synonymous (silent) substitutions per 100 synonymous sites (k_S) and nonsynonymous (replacement) substitutions per 100 nonsynonymous sites (k_N) (24). There were reduced levels of synonymous and nonsynonymous site variation at the *hlyA* locus relative to the gene *mdh*, coding for malate dehydrogenase; however, the k_N/k_S ratio was high because three of six polymorphic sites encoded amino acid replacements. The k_N/k_S ratio indicates the relative degree of functional constraints experienced by an evolving protein. Examination of the genes in Table 2 indicates that both *mdh* and *kpsD* have an increased level of functional constraint relative to *hlyA* and *papH*. At the *kps* and *papH* loci, the levels of variation at synonymous sites were similar to that of the housekeeping gene *mdh* (Table 2). However, at both of these loci there were elevated levels of nonsynonymous site variation; in the case of *papH*, most of the variation was accounted for by polymorphism contributed by the ECOR 49 *papH* sequence (Fig. 2).

Distribution of polymorphic nucleotide sites. To test for nonrandom clustering of polymorphic nucleotide sites, a pat-

tern that may be indicative of intragenic recombination, we used the Stephens test (31). Among the six *papH* sequences, there were 22 polymorphic sites in the 462 bp sequenced, and the Stephens test identified two statistically significant partitions. The first identified a 177-bp segment of invariant sites ($P < 0.006$) in ECOR 49, and the second identified a 156-bp region of invariant sites shared between ECOR 49 and ECOR 50.

For the 48 polymorphic sites among the nine *kpsD* sequences (shown in Fig. 3), six partitions were found for which there were statistically significant P values for either clustered polymorphic sites or segments composed of invariant sites. The first partition grouped ECOR 8 and ECOR 46 *kpsD* sequences and was based on only three polymorphic sites (sites 2, 9, and 25) and a 159-bp segment of consecutive nonpolymorphic sites ($P < 0.006$). The second partition identified a cluster of nine polymorphic sites ($P < 0.002$) in ECOR 14, eight of which were found at the 5' end of the *kpsD* gene. The third partition involved only three clustered sites in ECOR 8 with a 324-bp segment of consecutive nonpolymorphic sites ($P < 0.006$). The fourth partition identified 15 clustered polymorphic sites ($P < 0.000$), the majority of which were in the 3' end of the *kps* gene and separated ECOR 48 from the rest. Two sites grouped strains ECOR 14 and ECOR 49, and the probability of two polymorphic sites as close as or closer than 2 bp is 0.009. The final statistically significant *kps* partition identified grouped *kps*

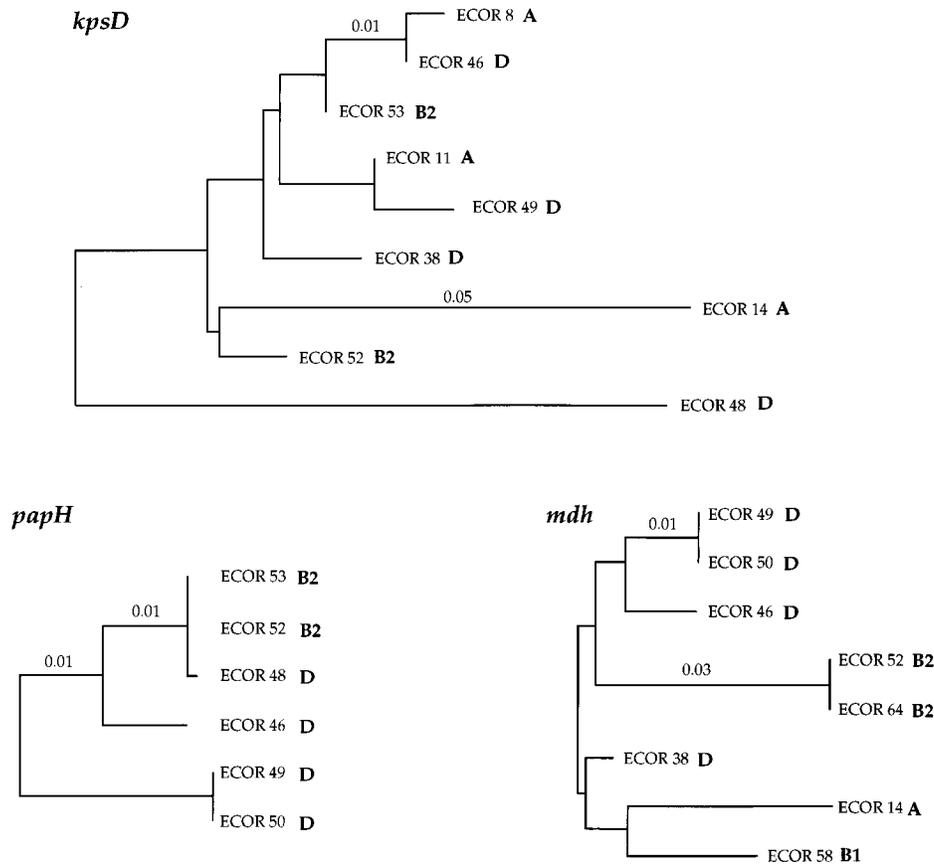


FIG. 4. Evolutionary relationships among three chromosomal loci. Pairwise genetic distances were estimated from the numbers of substitutions (18, 24). Letters indicate subgroups of the ECOR strains, and numbers indicate genetic distance.

sequences from ECOR 11 and ECOR 49 and is based on a run of 275 bp of consecutive nonpolymorphic sites ($P < 0.006$).

Evolutionary trees for the *kpsD* and *papH* sequences. For purposes of comparison, the housekeeping gene *mdh* from eight ECOR strains (5) analyzed at the *kpsD* and *papH* loci was used to construct an *mdh* gene tree (Fig. 4). Previous analysis has shown that the *mdh* gene tree is congruent with phylogenetic relationships based on multilocus enzyme electrophoresis and DNA hybridization analysis (5), thus providing a reliable measure of overall chromosomal relationships. No phylogenetic tree was constructed for *hlyA*, as there was not sufficient nucleotide sequence polymorphism to determine relationships among strains. Among all trees there are three isolates in common: ECOR 46, ECOR 49, and ECOR 52. Relationships among the ECOR strains were not congruent among the *kpsD*, *papH*, and *mdh* trees, particularly at the *kps* locus, at which phylogenetic comparisons with the *mdh* gene tree indicate several possible horizontal transfer events. The long branch

length found in the ECOR 14 and ECOR 48 *kpsD* sequences can be accounted for by possible intragenic recombination events from an unknown source, identified by the Stephens test, involving the 5' region in ECOR 14 and the 3' region in ECOR 48.

DISCUSSION

Pathogenic isolates of *E. coli* are known to carry large chromosomal regions required for virulence, which are termed PAIs. Natural isolates are polymorphic for the presence and copy number of these DNA sequences. For example, the pathogenicity islands PAI I and PAI II of uropathogenic strains (2, 3), which are 70 and 190 kb in length, as well as the class II capsule synthesis operon (*kps*), are absent from *E. coli* K-12 (4). The prevailing view of *E. coli* as a relatively benign organism obtaining novel sequences from an outside source and thereby becoming pathogenic or switching disease syn-

TABLE 2. Nucleotide sequence polymorphism among three virulence genes and one housekeeping gene from natural isolates of *E. coli*

Gene	No. of isolates	Length (bp)	No. of polymorphic:		$k_S (10^2)$	$k_N (10^2)$	k_N/k_S ratio	CAI (30)	GC content
			Nucleotides	Amino acids					
<i>hlyA</i>	8	570	6	3	0.89 ± 0.53	0.27 ± 0.16	0.33	0.23	40
<i>kpsD</i>	8	729	48	10	5.84 ± 0.99	0.52 ± 0.16	0.09	0.33	52
<i>papH</i>	6	462	22	9	5.29 ± 1.54	1.07 ± 0.35	0.21	0.24	50
<i>mdh</i>	8	864	20	2	3.10 ± 0.80	0.14 ± 0.10	0.05	0.58	52

TABLE 3. Sources of *E. coli* reference collection strains^a

Host	Strains having indicated virulence-associated region			
	<i>hlyII</i>	<i>kps</i>	<i>pap</i>	<i>sfa</i>
Human	24, 43, 48, 51, 54, 56, 60, 63	8, 11, 14, 24, 35, 36, 38, 39, 40, 41, 47, 48, 49, 50, 51, 53, 55, 56, 61, 62, 63, 64	2, 11, 24, 35, 36, 38, 39, 40, 41, 48, 49, 50, 51, 53, 54, 55, 56, 59, 60, 62, 63, 64	51, 53, 54, 60, 63, 64
Other primates	52, 57	46, 52, 57, 66	37, 46, 52, 57	52, 57, 65, 66
Nonprimate		44, 47	31, 44	58

^a Numbers are ECOR strain numbers (25).

drome or host has been supported by many authors (2, 3, 11, 14, 19). For example, Whittam and colleagues have proposed that the O157:H7 clone emerged when an EPEC O55:H7-like progenitor was lysogenized by a bacteriophage containing Shiga-like toxin genes (34).

Recently, Pupo and colleagues (27) have described the relationship between pathogenic and nonpathogenic isolates of *E. coli*. The pathogenic isolates included EPEC, EHEC, ETEC, enteroinvasive *E. coli*, and urinary tract infection strains, and the nonpathogenic isolates were represented by the ECOR strains. The authors concluded that pathogenic strains arising from *E. coli* do not have a single evolutionary origin within *E. coli* but have arisen many times, with the exception of *Shigella* and the EHEC clones. The *Shigella* isolates examined were all closely related to each other, clustering within ECOR subgroup A strains, confirming earlier conclusions that *Shigella* species are a clonal lineage of *E. coli* (35). Further, the results verified earlier findings of Whittam et al. (34) that strains causing the same disease do not form a monophyletic group. Wieler et al. (36) recently examined the insertion sites of the *LEE* locus in DEC strains and showed that the *LEE* insertion site differs in relation to the clonal lineage of the strains, which they conclude is due to multiple insertions during the evolution of these pathogens.

As shown by the phylogenetic distribution in Fig. 2, the majority of ECOR B2 and D isolates contain at least two of the four regions examined. Most isolates of subgroup B2 and all isolates of subgroup D have a copy of *kps* and *pap*; in addition, subgroup B2 strains have a copy of *sfa*. Among the other ECOR lineages there is only a sporadic occurrence of these sequences. In particular, among subgroup A strains (which is the largest set of lineages represented in the ECOR collection [25 of 72]), only five strains show hybridization to the probes for *hly*, *kps*, and *pap*, and only two strains (ECOR 11 and ECOR 24) contain sequences with homology to both the *kps* and *pap* regions.

The sporadic occurrence of *hly*, *kps*, *pap*, and *sfa* among ECOR group A isolates could have resulted from either widespread loss from strains within this group, which is unlikely, or transfer from perhaps a group B2 or D strain. The *hlyA* locus has been detected on an *E. coli* plasmid (8), which suggests a mechanism of horizontal transfer. Given the low GC content of the *hlyA* gene, 40% (compared to an average of 52% for most *E. coli* genes), and the limited nucleotide sequence polymorphism, this region may have been recently acquired from an unknown source. Alternatively, the highly conserved nucleotide sequence at *hlyA* may be due to selective constraints at the protein level. These conflicting views may be addressed with additional nucleotide sequence information from this gene cluster. Among the *kpsD* sequences examined, those of subgroup A strains ECOR 8 and ECOR 11 showed identity with those of subgroup B2, which is most easily explained by

recent horizontal transfer between these subgroups. Marklund and coworkers (22) have proposed that *E. coli* acquired the *pap* locus after the speciation of *E. coli* and suggest that the different *pap* genes could have been acquired by horizontal gene transfer. Moreover, they proposed that the recent genetic exchanges involving the entire pilin gene clusters have occurred in response to selection pressures exerted by the host. Among the *papH* sequences studied, only that of ECOR 49 is radically different, and all of the amino acid substitutions are encoded in the first hundred bases of this gene; based on additional nucleotide sequence analysis, it appears that this diversity resulted from horizontal transfer at the *papA* locus, 63 bp upstream of *papH*, and that the variation in ECOR 49 is a result of hitchhiking (5a).

The stratification in the distribution of these virulence regions may be a consequence of the fact that the majority of the isolates of groups B2 and D were recovered from primates. For example, among isolates with sequences that show hybridization to the *kps* probe, all but two were isolated from primates; likewise all *pap*- and *sfa*-positive strains, with one exception, are all from primates. In contrast, ECOR isolates of subgroup B1 were predominantly isolated from nonprimate hosts (Table 3).

Natural isolates of *E. coli* may vary in genome size by up to 1 Mb (1, 25a). The mechanism(s) of chromosome size variation in natural isolates of *E. coli* remains largely unknown. It may be relevant to these observations that isolates belonging to ECOR subgroup A have a significantly smaller genome size than those of either subgroup B2 or D, which have the largest genome size among natural isolates of *E. coli* (1). Comparisons of genome size with the presence of regions of interest in ECOR strains indicate that ECOR 14, which has a putative PAI present, has a genome size of 5,000 kb and differs in size by more than 300 kb from a closely related lineage, ECOR 13, where we found none of the four regions present. Isolates of group B2 and D (ECOR 51, 62, 63, 38, and 40) for which genome size is available range in size from 4,952 to 5,302 kb, and all isolates have two or more of the regions under study associated with them.

To determine the relationship between uropathogenic *E. coli* and DEC, isolates from the DEC collection were examined (34). The DEC collection comprises 15 clones representing *E. coli* isolates recovered from patients with enteric disease in one of three disease categories, EPEC, EHEC, and ETEC. Among the 15 DEC isolates examined, none had sequences which hybridized with the *hlyII*, *pap*, *sfa*, and *kps* probes. This result confirms earlier findings that DEC strains require a very different set of virulence determinants (the *LEE* locus) than those of uropathogenic *E. coli* isolates. The result also suggests that isolates may not typically carry PAIs for multiple disease categories. Further, our data support the view of long-term persistence of PAIs within *E. coli* lineages and indicate that whereas some pathogenicity determinants may be genetically

labile, others are maintained for long periods of evolutionary time. Currently we are investigating whether *hly*, *kps*, *pap*, and *sfa* are confined to a single PAI. Future studies will include analysis of the insertion sites of putative PAIs in subgroup B2 and D strains.

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