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A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens

(bacterial pathogenesis/epithelial cells/attaching and effacing lesions)

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ABSTRACT **Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* O157:H7 are intestinal pathogens that profoundly damage the microvilli and subapical cytoskeleton of epithelial cells. Here we report finding in EPEC a 35-kbp locus containing several regions implicated in formation of these lesions. DNA probes throughout this locus hybridize to *E. coli* O157:H7 and other pathogens of three genera that cause similar lesions but do not hybridize to avirulent members of the same species. The EPEC locus and a different virulence locus of uropathogenic *E. coli* insert into the *E. coli* chromosome at the identical site and share highly similar sequences near the point of insertion.**

Enteropathogenic *Escherichia coli* (EPEC) are a long-recognized and leading cause of severe infantile diarrhea in the developing world (1). Enterohemorrhagic *E. coli* (EHEC) O157:H7 have recently emerged as the cause of bloody diarrhea and hemolytic uremic syndrome in large food-borne outbreaks in the western United States and elsewhere (1–3). Although the two organisms cause different diseases, they share a trait that distinguishes them from other pathogenic *E. coli* and from normal flora *E. coli*—the ability to elicit a histopathologic effect on intestinal epithelial cells termed the attaching and effacing (AE) lesion (1, 4). These lesions are marked by degenerated microvilli and "pedestals" of densely clustered cytoskeletal proteins that protrude from the apical membrane and intimately cup individual bacteria (4, 5). Coincident with AE lesion formation the bacteria induce a host signal-transduction pathway (6–8). Signal transduction is linked to AE lesion formation, as mutations that interfere with signaling also interfere with AE (7, 8).

The gene first associated with AE lesion formation, EPEC's *eaeA* (*E. coli* attaching and effacing), encodes an outer-membrane adhesin that is necessary for AE lesion formation but is not necessary for adherence *per se* (9–12). Homologs of *eaeA* have been cloned from other AE-forming diarrheagenic bacteria, including EHEC, RDEC-1 (an *E. coli* pathogen of rabbits), strains of *Hafnia alvei* isolated from children with diarrhea, and *Citrobacter freundii* biotype 4280, the cause of transmissible colonic hyperplasia in mice (11, 13, 14). On the EPEC chromosome 4.4 kb downstream of *eaeA* is the *eaeB* gene, which is necessary (like *eaeA*) for AE lesion formation and (unlike *eaeA*) for inducing signal transduction (8, 15). Additional genes are apparently necessary for AE lesion formation, as three AE-negative, signal transduction-negative transposon Tn*phoA* mutants have previously been generated whose transposon insertions lie outside of the *eae* gene cluster (refs. 8 and 16; I. Rosenshine and B. Finlay, personal communication). One of these, mutant 30-5-1(3), contains a single transposon. The remaining two, class four mutants (*cfm*), each contain two Tn*phoA* insertions, making it difficult to tell which insertions are responsible for the mutant phenotype (16).

In this study, we report the localization of these Tn*phoA* insertions and the discovery of a large (≈35 kb) region of DNA that encodes all necessary determinants for the AE phenotype thus far identified. This region is conserved among other species that produce the AE lesion and is inserted into the *E. coli* chromosome at the identical site where a block of virulence genes of uropathogenic *E. coli* is located.

MATERIALS AND METHODS

Bacterial Strains. Normal flora *E. coli* include strain HS4 (O9:H14), isolated from a healthy American adult male, and nine strains collected from healthy American adult females. Pathogenic bacteria came from the University of Maryland Center for Vaccine Development strain collection. EPEC were defined by serogroup and AE ability (17, 18). Other *E. coli* pathogens were identified by using DNA probes specific for the various classes of pathogenic *E. coli* (17).

Pulsed-Field Gel Electrophoresis and Southern Blotting. Chromosomal DNA was extracted in agarose plugs and electrophoretically separated as described (19). For Southern blotting DNA was transferred to a GeneScreen II nylon membrane (DuPont), hybridized, and washed according to manufacturer's instructions. The most stringent wash step was 65°C, 2× standard saline citrate/0.1% SDS.

Cosmid Screening and Restriction Mapping. In previous work *Mlu* I clones p27B, p27W, and p30 containing Tn*phoA* insertions from mutants 27-3-2(1) and 30-5-1(3) had been cloned and mapped for several restriction enzyme sites (16). Also, prior work had produced a restriction map spanning from the *Bgl* II fragment downstream of *eaeB* to the *Sal* I site upstream of *sepA* (refs. 9 and 13; J. Yu and J.B.K., unpublished data). The order of the Tn*phoA* insertions established by pulsed-field gel electrophoresis (Fig. 1) enabled us to align these previously unrelated maps to create the integrated map shown in Fig. 2. To extend the map rightward, we screened a pHC79 cosmid bank of EPEC E2348/69 genomic DNA with a radiolabeled 2.4-kb *Eco*RI/*Mlu* I fragment from p27W. This probe hybridized to clones 2G11 and 2F1 shown in Fig. 2. To extend the map leftward, the 2.8-kb *Eco*RI/*Mlu* I fragment from the left end of p30 was used as a probe against the same library; this hybridized to three clones including 9B3 shown in Fig. 2. This clone linked the *Mlu* I fragment of p30 to that of p27B. Colony hybridizations were done as in ref. 22, except that hybridizations were done at 65°C without formamide. The most stringent washes were at 65°C in 2× standard saline citrate/0.1% SDS.

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Abbreviations: EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *E. coli*; AE, attaching and effacing; LEE, locus of enterocyte effacement; pai, pathogenicity island.

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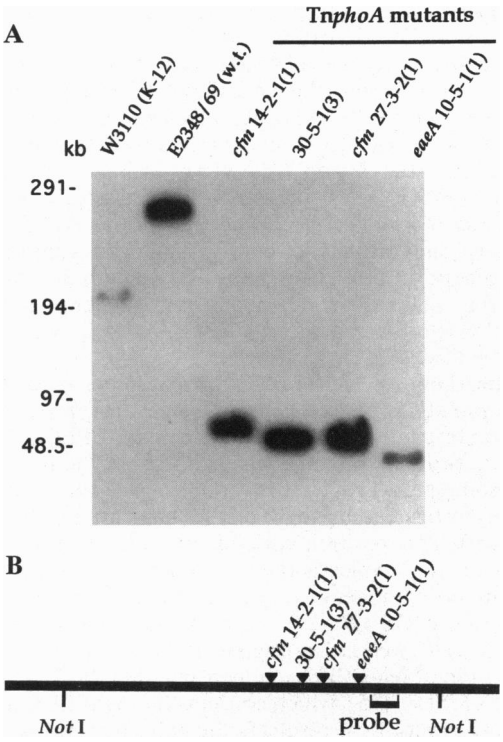

FIG. 1. EPEC Tn*phoA* mutants contain transposon insertions in a single large locus. (*A*) DNA from wild-type EPEC E2348/69 and four Tn*phoA* mutants with diminished or absent AE phenotypes were digested with *Not* I, separated by pulsed-field gel electrophoreses, blotted to a nylon membrane, and then hybridized to the ^{32}P-labeled 2.5-kb insert of plasmid pMSD 1, which lies ≈700 bp downstream of *eaeB* (see Fig. 2). The probe hybridizes to a 275-kb fragment in the wild type, which is disrupted by a transposon in each mutant. Mutant bands are smaller because of the presence of *Not* I sites in Tn*phoA* (20, 21). (*B*) Map of the 275-kb wild-type *Not* I fragment. Arrowheads indicate insertion points of Tn*phoA* in the mutants. Map is not to scale.

Genomic Mapping. A 9.8-kb *Eco*RI/*Sal* I fragment from cosmid 2F1 lying to the right of LEE and a 2.4-kb *Eco*RV fragment from p27W lying to the left of LEE (Fig. 2) were radiolabeled and used to probe a nylon membrane containing an ordered library of the *E. coli* strain W3110 chromosome (ref. 23; Takara Biochemical, Otsu, Shiga, Japan). The p27W probe hybridized to clones 569 and 570 at 82 min on the chromosome, whereas the 2F1 probe hybridized to clones 570 and 571 at 82 min.

DNA Sequencing. The right LEE junction was sequenced from pMSD 1 (Fig. 2 and ref. 16) by the Ready Reactions Dye-Deoxy Terminator cycle sequencing kit (Applied Biosystems) and run on an Applied Biosystems model 373A automated sequencer. Sequencing primers were as follows: K260, 5′-GAGCGAATATTCCGATATCTGGTT; K261, 5′-CCTGCAAATAAACACGGCGCAT; and K-255, 5′-GGTTGAGTCGATTGATCTCTGG. The left LEE junction was sequenced by the Sanger dideoxynucleotide chain-termination method (Sequenase kit: United States Biochemical) from pCVD 454, an ≈850-bp *Mlu* I fragment containing the left LEE junction cloned into pSPORT 1 (BRL; see Fig. 2). The clone was initially sequenced with universal vector primers (T7 and SP6) and then with insert-specific primers based on the generated sequence. Insert-specific primer sequences were as follows: K295, 5′-CGCCGATTTTTCTTAGCCCA; K296, 5′-CATTCTGAAACAAACTGCTC; and K300, 5′-CTAAATGACCAAATGACCAA.

PCR. Single bacterial colonies were picked from agar plates and added to 50-μl reaction mixtures containing 20 mM Tris (pH 8.4), 500 mM KCl, 1 unit of *Taq* DNA polymerase (BRL), 1.5 mM $MgCl_2$, 200 mM each of dNTP, and 20 μM each of primers K255, K260, and K261 (see above for primer sequences). Reactions were done in an MJ Research (Cambridge, MA) minicycler: 5 min at 94°C, followed by 30 cycles of 2 min at 92°C, 2 min at 50°C, and 3 min at 72°C, followed by 5 min at 72°C.

RESULTS

Mapping Transposon Insertions. Because each of the *cfm* mutants contains two Tn*phoA* insertions, our initial aim was to determine whether they contain disruptions in a common locus, which might identify which insertion in each strain is responsible for the mutant phenotype. We ran pulsed-field electrophoretic gels of *Not* I-digested chromosomal DNA from each *cfm* mutant, as well as the parental strain, EPEC

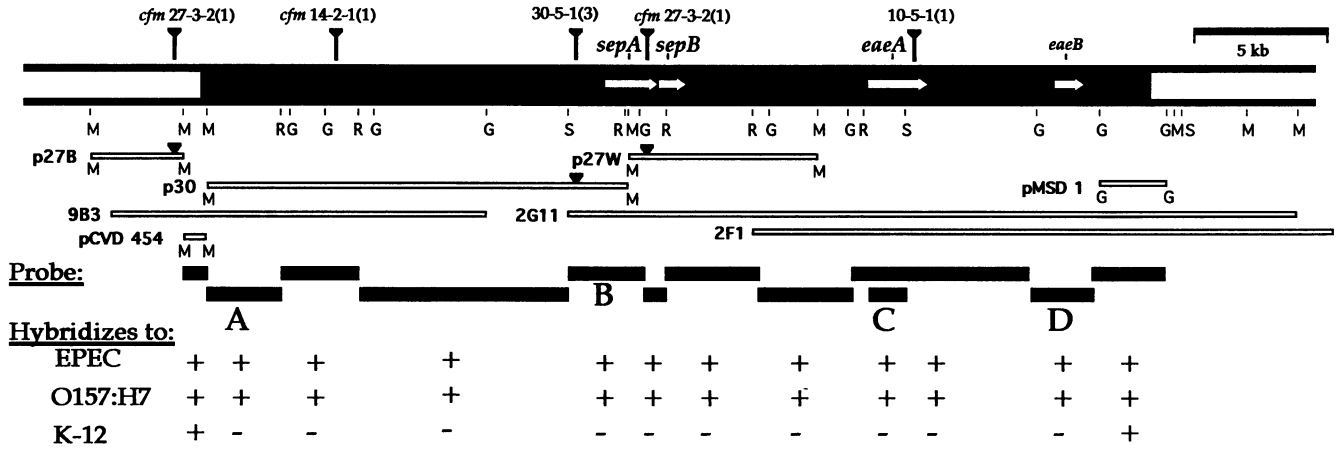

FIG. 2. Map of the EPEC locus of enterocyte effacement (LEE). Black portions of map, LEE DNA; white portions, DNA present in K-12 and EPEC; arrows, genes; arrowheads, Tn*phoA* insertion points. Note that *cfm* mutant 27-3-2(1) contains two transposon insertions in the region: one within the LEE's *sepA* gene and one within ubiquitous *E. coli* sequence 2.3 kb to the left of LEE. Plasmid and cosmid inserts used to map LEE are depicted as open lines and aligned with their corresponding position of the map. Probes used in hybridization experiments are similarly aligned and shown as black bars. Results of hybridization of these probes to EPEC (strain E2348/69), EHEC (EDL-933), and K-12 *E. coli* (DH5-α) colony blots are indicated beneath each probe. The large stretch of DNA that is present in EPEC but absent in K-12 defines LEE. The exact boundaries of LEE were determined by comparing the LEE sequence of LEE junctions to the published corresponding K-12 sequence (Fig. 3). Restriction sites: G, *Bgl* II; M, *Mlu* I; R, *Eco*RI; S, *Sal* I. This restriction map combines new data with previous work (refs. 9 and 15; J. Yu and J.B.K., unpublished data).

E2348/69. The IS50 repeats that flank Tn*phoA* (20) contain recognition sites for *Not* I (21), so any band containing the transposon should be cleaved by this enzyme. Compared with the parent, each mutant was missing a band of ≈275 kb (data not shown). Mutant 30-5-1(3) and an *eaeA*::Tn*phoA* mutant (16) also had a disruption in this band: thus, the *cfm* mutants and one other AE-deficient mutant have transposons in a locus linked to *eae*. To localize the Tn*phoA* insertions with respect to each other, radiolabeled DNA fragments near the sites of insertion were used to probe Southern blots of *Not* I-digested DNA from EPEC E2348/69 and each mutant. The results shown in Fig. 1 and those obtained from other probes (data not shown) indicate that all mutants contain transposon insertions within a region of <50 kb.

LEE: A 35-kb Locus of AE Enterobacteria. The *eaeA* locus is absent from normal flora *E. coli* (9). Because *cfm* transposons insert near *eaeA*, we hypothesized that all of these loci were part of a single large locus, present in EPEC but absent from avirulent *E. coli*. To test this possibility, we first generated a restriction map (Fig. 2) that links the *cfm* insertions to the *eae* locus and each other. Eleven adjacent restriction fragments from the region were used to probe colony blots of EPEC strain E2348/69, EHEC strain EDL-933, and *E. coli* K-12 (Fig. 2). Nine of these probes, spanning 33.2 kb of sequence, hybridized to EPEC and EHEC but did not hybridize to K-12 (Fig. 2). Thus, a large locus absent from avirulent *E. coli* is present in both EPEC and EHEC. Because this locus contains multiple regions implicated in AE lesion formation, we designate it LEE, for locus of enterocyte effacement.

We next determined whether the entire LEE is present in AE bacteria other than *E. coli* E2348/69 and EDL-933. We used four restriction fragments distributed throughout the LEE (probes A, B, C, and D in Fig. 2) to probe colony blots of 27 AE strains including a variety of EPEC and EHEC, RDEC-1, *H. alvei* isolated from children with diarrhea, and *C. freundii* biotype 4280. Previous work had shown that all of these strains cause AE lesions in cultured human cells and contain the *eaeA* gene (refs. 18, 24, and 25; A. Jerse and J.B.K., unpublished results). We used the same probes to test 79 non-AE bacteria, including normal flora *E. coli*, non-AE *E. coli* belonging to EPEC serogroups, pathogenic *E. coli* other than EPEC and EHEC, non-AE *C. freundii* and *H. alvei*, and a variety of non-AE pathogens comprising six enteric genera. In a total of 106 isolates tested, the four LEE probes were either all present or all absent in a given strain and were 100% sensitive and 98.7% specific for strains with the AE phenotype (Table 1).

Table 1. Results of hybridizing LEE probes A, B, C, and D to bacterial colony blots

Strain type	No. strains hybridizing/no. tested with probe			
	A	B	C	D
AE				
E. coli				
EPEC*	19/19	19/19	19/19	19/19
EHEC†	3/3	3/3	3/3	3/3
RDEC-1	1/1	1/1	1/1	1/1
C. freundii	1/1	1/1	1/1	1/1
H. alvei	3/3	3/3	3/3	3/3
Total	27/27	27/27	27/27	27/27
Non-AE				
E. coli				
EPEC serogroups‡	1/5	1/5	1/5	1/5
Misc. pathogens§	0/18	0/18	0/18	0/18
Normal flora	0/10	0/10	0/10	0/10
K-12	0/1	0/1	0/1	0/1
C. freundii	0/2	0/2	0/2	0/2
H. alvei	0/1	0/1	0/1	0/1
Other pathogens¶	0/42	0/42	0/42	0/42
Total	1/79	1/79	1/79	1/79

Colony blots of overnight-grown bacterial colonies were incubated as in ref. 22 with radiolabeled LEE probes A, B, C, and D (see Fig. 2).
*Includes O55, O111, O119, O125, O126, O127, O128, and O142 serogroups.
†Includes O26:H11 and O157:H7 serotypes.
‡Includes O86, O111, O114, O126, and O158 serogroups. The strain that hybridized with the LEE probes (an O111 strain) does not adhere to cultured cells (18), which probably accounts for its inability to cause AE lesions.
§Includes 5 enteroinvasive, 7 enterotoxigenic, 1 enteroaggregative, 3 uropathogenic, and 2 diffusely adherent *E. coli* strains.
¶Includes 3 *Klebsiella pneumoniae*, 3 *Salmonella typhi*, 1 *Shigella boydii*, 3 *Shigella dysenteriae*, 19 *Shigella flexneri*, 8 *Shigella sonnei*, 2 *Yersinia enterocolitica*, and 2 *Vibrio cholerae* strains.

Localization of LEE in EPEC Chromosome. To determine the location of LEE in the EPEC chromosome, we hybridized restriction fragments lying just outside of the EPEC E2348/69 LEE to an ordered (Kohara) phage library of the K-12 *E. coli* chromosome (23). Probes from both sides of LEE hybridized to clones corresponding to 82 min on the chromosome (data not shown). This position contains the *selC* (selenocysteine) tRNA locus (26), which is the site of insertion of a virulence region in uropathogenic *E. coli* strain 536 and of *E. coli* retronphage φR73 (26, 27). To establish whether LEE also inserts into *selC*, we used an oligonucleotide complementary to the 5′ region of *selC* (28) to sequence within the 2.7-kb *Bgl* II insert of pMSD 1 (15), which contains the right LEE junction (Fig. 2). Comparing the results to the published corresponding K-12 sequence (28) revealed that LEE disrupts the EPEC genome at a position 16 bp downstream of *selC* (Fig. 3*A*). This exact position is disrupted in uropathogenic *E. coli* strain 536 by pai I, a 78-kb virulence locus containing hemolysin genes (30, 31). The sequences just inside the right LEE junction and the corresponding portion of pai I are 71% identical with a 1-bp gap over 93 bp of DNA—i.e., over the entire length of the published pai I junctional sequence (Fig. 3*B*, ref. 31). LEE and pai I are clearly different loci, differing greatly in size and encoding different functions. However, the strikingly similar sequences inside of their junctions imply evolutionary relatedness between the two elements.

The left junction of LEE was sequenced from the 850-bp *Mlu* I insert of pCVD 454 (Fig. 2). The sequence (Fig. 3*A*) reveals that this region of the EPEC chromosome differs from that of K-12 not only by the insertion of LEE but also by the deletion of two regions (Fig. 3*C*). The deletions remove 1570 bp of total sequence including the putative promoter and 949 bp of o394, an open reading frame of unknown function (28). A PCR-based assay designed to distinguish an intact *selC* region from one containing the LEE insertion and o394 deletions found in E2348/69 showed that the E2348/69 structure is conserved in O157:H7 strain EDL-933 and a variety of EPEC isolates (Fig. 3*D*).

DISCUSSION

In this paper we have shown that a large chromosomal locus called LEE encodes all known determinants of an essential feature of the pathogenesis of EPEC diarrhea, the AE phenotype. We have further shown that this region is conserved among all intestinal pathogens that produce the AE lesion including EPEC, EHEC, RDEC-1, *H. alvei*, and *C. freundii* biotype 4280. We and others have previously shown that EPEC, EHEC, and *C. freundii* 4280 strains mutated in the LEE-encoded *eaeA* gene are deficient in AE and that EPEC and *C. freundii eaeA* mutants have attenuated virulence (14, 29). Mutation of the EPEC *eaeB* locus eliminates AE lesion formation and the associated signal transduction (8). We (K.G.J., T.K.M., and J.B.K., unpublished data) have recently found that the region of LEE which is disrupted in *cfm* mutant 27-3-2(1) contains genes similar to those encoding pathways

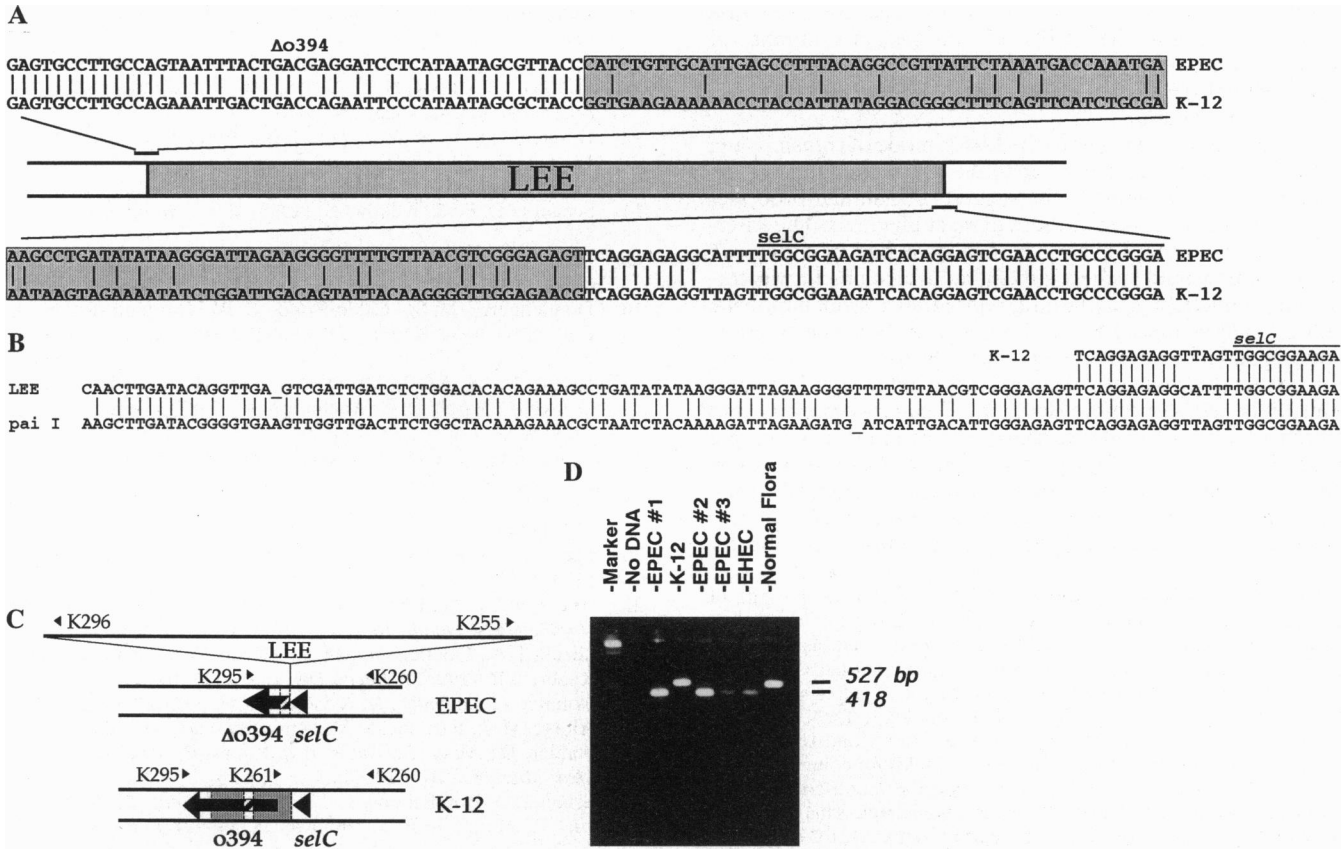

FIG. 3. Structure of the EPEC LEE and flanking sequences. (*A*) Nucleotide sequences of LEE junctions aligned with corresponding K-12 sequences. Sequences project from a schematic representation of LEE and surrounding portions of the EPEC chromosome. LEE sequences are shaded; ubiquitous *E. coli* sequences are plain. The left LEE junction truncates open reading frame o394, whereas the right junction diverges from the K-12 sequence 16 bp downstream from the *selC* tRNA gene. (*B*) Alignment of the right LEE junction and corresponding sequences of pathogenicity island I (pai I) of uropathogenic *E. coli* strain 536 (29). (*C*) Schematic comparison of the EPEC and K-12 chromosomes in the region surrounding LEE. LEE is shown as a large insertion in the EPEC chromosome. Shaded sequences are those present in K-12 but absent from EPEC. A 61-bp internal fragment of o394 present in both EPEC and K-12 is shown as a striped box. PCR primers described in *D* are indicated by arrowheads. Diagram is not to scale. (*D*) Three-primer PCR to distinguish an intact *selC* o394 locus from one disrupted by LEE. Primers K255, K260, and K261 shown in *C* were used in a single reaction to amplify DNA from various *E. coli* strains. Primers K255 and K260 are predicted to produce a 418-bp amplicon in strains containing a LEE-disrupted locus (e.g., EPEC E2348/69) but not to produce one in strains lacking LEE (e.g., K-12). Primers K261 and K260 should produce the 527-bp amplicon in strains with a K-12-type genome; strains with an EPEC E2348/69-type genome (in which the target of primer K261 is deleted) will produce no product with these primers. EPEC isolates representing three serotypes and one EHEC strain produced the predicted 418-bp LEE product, whereas K-12 and normal flora *E. coli* produced the 527-bp product, indicating an intact *selC* locus. A PCR using primers K295 and K296 confirmed that the EHEC and three EPEC strains contained the E2348/69-type structure (data not shown). Strains are as follows: EPEC #1, E2348/69 (O127:H6); K-12, W3110; EPEC #2, O55:H6; EPEC #3, O55:H7; EHEC, EDL933 (O157:H7); Normal Flora, HS4.

for secretion of virulence factors in a variety of pathogenic bacteria (32). Mutation of one of these genes, *sepB* (secretion of EPEC proteins; Fig. 2), eliminates the ability of EPEC E2348/69 to induce AE lesions and to secrete at least five proteins (K.G.J., T.K.M., and J.B.K., unpublished data). Independent work by Kenny and Finlay (B. Kenny and B. Finlay, personal communication) has shown that the N-terminal sequence of one of the secreted proteins is identical to that predicted by the *eaeB* gene sequence. These results suggest that LEE encodes factors that directly participate in AE lesion formation as well as factors necessary for their secretion.

These results are of particular interest given recent developments in the epidemiology of EHEC O157:H7. *E. coli* O157:H7 first emerged as an important pathogen in the United States in 1982 and is now the second or third most commonly isolated pathogen from stool (1–3). In 1993, an outbreak of O157:H7 infections in Washington, Idaho, California, and Nevada resulted in >500 culture-confirmed cases of diarrhea or hemolytic uremic syndrome (2). Whittam *et al.* (33) have proposed that the O157:H7 clone emerged when an EPEC O55:H7-like progenitor already capable of producing the AE lesion was lysogenized by a bacteriophage containing Shiga-like toxin genes.

The present findings associate the AE phenotype with a large genetic locus found in pathogens of humans, mice, and rabbits. The LEE's conservation as an apparently intact unit among AE members of *C. freundii, E. coli*, and the divergent *H. alvei* (34) is consistent with a cluster of virulence genes that spread horizontally among unrelated bacteria during recent evolution. A continuing effort to sequence the entire LEE in our laboratories has revealed no sequences that would offer clues as to the putative vehicle that delivered LEE to EPEC. No flanking repeat or transposon-, phage-, or plasmid-like elements have been found in >17 kb of sequence now generated (ref. 9; K.G.J., T.K.M., Y. K. Kang, and J.B.K., unpublished data). However, the nucleotide composition of these 17 kb (39% G + C) differs greatly from the 51.4% value found in the *E. coli* genome sequenced to date,‖ further supporting

‖Calculated from the 919,888 bp generated by *E. coli* genome sequencing projects and submitted to GenBank as of release 82 (April 15, 1994). Acquisition numbers of these sequences are as follows: D10483, D26526, L10328, L19201, M87049, U00006, and U00039.

recent acquisition from a foreign source. Because a deletion apparently exists at the LEE's left junction in a number of other EPEC strains (Fig. 3), it is possible that clues about the nature of the LEE's mode of insertion in these strains may have been lost in evolution.

If little evidence exists as to the LEE's mode of transmission, the similarity of its right junction to the corresponding junction of pai I of uropathogenic *E. coli* suggests that similar processes carried out the insertion of these distinct elements. The insertion of LEE and pai I at the identical base pair of the *E. coli* chromosome would suggest either that each element was carried on a vehicle (e.g., a plasmid) containing a stretch of DNA with enough similarity to the interrupted sequence to guide homologous recombination or that a site-specific recombination system inserted both elements. Seen in this light, one may think of the *selC* region as a slot in the *E. coli* chromosome into which cartridges of virulence factors can be inserted in discrete units. The sequences stably transform the organism into a pathogenic clone, whose associated disease depends on the program of the cartridge inserted. Our results imply a mechanism for the past (and perhaps future) creation of pathogens.

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