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The Cloned Locus of Enterocyte Effacement from Enterohemorrhagic *Escherichia coli* O157:H7 Is Unable To Confer the Attaching and Effacing Phenotype upon *E. coli* K-12

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The locus of enterocyte effacement (LEE) pathogenicity island of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 possesses the same genes in identical order and orientation as the LEE of enteropathogenic *E. coli* (EPEC) O127:H6 but is unable to form attaching and effacing (A/E) lesions or to secrete Esp proteins when it is cloned in an *E. coli* K-12 background. The A/E phenotype could not be restored by *trans* complementation with a variety of cloned EPEC LEE fragments, suggesting functional and/or regulatory differences between the LEE pathogenicity islands of EPEC O127:H6 and EHEC O157:H7.

The phenomenon known as attaching and effacing (A/E) is observed in a family of pathogens that include the prototypic A/E pathogen enteropathogenic *Escherichia coli* (EPEC) as well as enterohemorrhagic *E. coli* (EHEC), *Citrobacter rodentium*, and others (17). The A/E lesion is characterized by the loss of host cell microvilli (effacement) and intimate attachment of the bacterium to the host membrane on a pedestal of polymerized cytoskeletal elements, notably actin (4, 8, 9). Formation of the A/E lesion is accompanied by a number of signal transduction events in the host cell, including activation of protein kinase C, induction of NF-κB, and release of interleukin-8 (reviewed in references 4, 8, and 17).

All the genes necessary for A/E lesion formation by EPEC are encoded on a pathogenicity island called the locus of enterocyte effacement (LEE) (14, 15). In EPEC O127:H6, the LEE is a 35,637-bp element containing 41 predicted open reading frames (ORFs) in at least 10 operons. These genes encode a type III protein translocation complex, secreted proteins including EspA and Tir, the intestinal adhesin intimin (encoded by *eae*), and 23 ORFs of undefined function (2, 10, 12). The LEE from EHEC O157:H7 strain EDL933 has also been recently cloned and sequenced and possesses all the genes found in the EPEC LEE in the same organization (18). In general, the two LEE elements are 94% conserved at the amino acid level and differ by less than 2% in the regions encoding the protein translocation complex, although divergence of up to 34% is seen in genes encoding proteins that are believed to interact with the host (Fig. 1). The EHEC O157:H7 LEE also encodes a cryptic prophage at one end that is not found in the EPEC O127:H6 LEE. Analysis suggests that the prophage inserted into the LEE after the island was already present on the chromosome and is unlikely to encode any known virulence function (18).

The sequence divergence between the LEEs is reflected in differences in LEE-associated phenotypes. Unlike EPEC, EHEC O157:H7 does not produce a tyrosine-phosphorylated Tir (6) and requires 6 h to cause A/E lesions in an in vitro assay compared to the 3 h required for A/E formation by EPEC (11). EHEC and EPEC have different modes of pathogenesis and colonize different sites in the intestine, due at least in part to differences in intimin (17, 21). However, it is unclear to what extent the differences between EPEC and EHEC are due solely to differences in the LEE. EPEC and EHEC are known to differ at several other loci, including the large virulence-associated plasmid, pili, the Shiga toxin phage (which is absent in EPEC), and the Per regulator (absent in EHEC) (4, 5, 17). Recent work (3, 16, 20) has also demonstrated regulatory differences between EPEC and EHEC.

Isolation of the LEE in a K-12 background will assist in the examination of LEE function in isolation from other EHEC genomic factors. McDaniel and Kaper (15) demonstrated that the cloned EPEC O127:H6 LEE was able to confer the A/E phenotype upon *E. coli* K-12, including the ability to secrete EspB and translocate Tir into host cells. Therefore, the EPEC LEE is sufficient for the A/E phenotype in *E. coli* K-12. Since the LEE has been demonstrated to be necessary for A/E lesions and Esp secretion in EHEC O157:H7 strains 85-170 (3) and 86-24 (7), we hypothesized that the cloned LEE from EHEC should also be able to mediate these functions in *E. coli* K-12. By cloning the EHEC LEE into K-12 we sought to confirm this hypothesis as well as to determine the extent that the differences between EPEC and EHEC are mediated solely by variations between the two LEE elements.

Cloning and mapping the EHEC LEE. The EHEC O157:H7 LEE was cloned from strain 85-170, which is a Δ*stx* variant of strain 84-289. Both 85-170 and 84-289 secrete higher levels of Esp proteins than do EDL933 and other EHEC strains (7), and we have observed that they are also more active in the fluorescent actin stain (FAS) assay. Prior to cloning, the chromosomal locus was mapped by Southern blotting on digested 85-170 chromosomal DNA and probing with the four EPEC LEE fragments identified as LEE A through D by McDaniel et al. (14). The resulting restriction map (Fig. 1) indicated that the LEE of 85-170 was indistinguishable from that of O157:H7 strain EDL933 and similar to that of EPEC. The 85-170 LEE was cloned following the procedure of McDaniel and Kaper (15). Chromosomal DNA was partially digested with *Psp*AI, an isoschizomer of *Sma*I that generates cohesive ends, ligated into the cosmid vector pCVD551, and transfected into HB101. Colonies were probed with radiolabeled LEE probes A to D. One cosmid, pJAY1512, contained a 37-kb DNA insert that hybrid-

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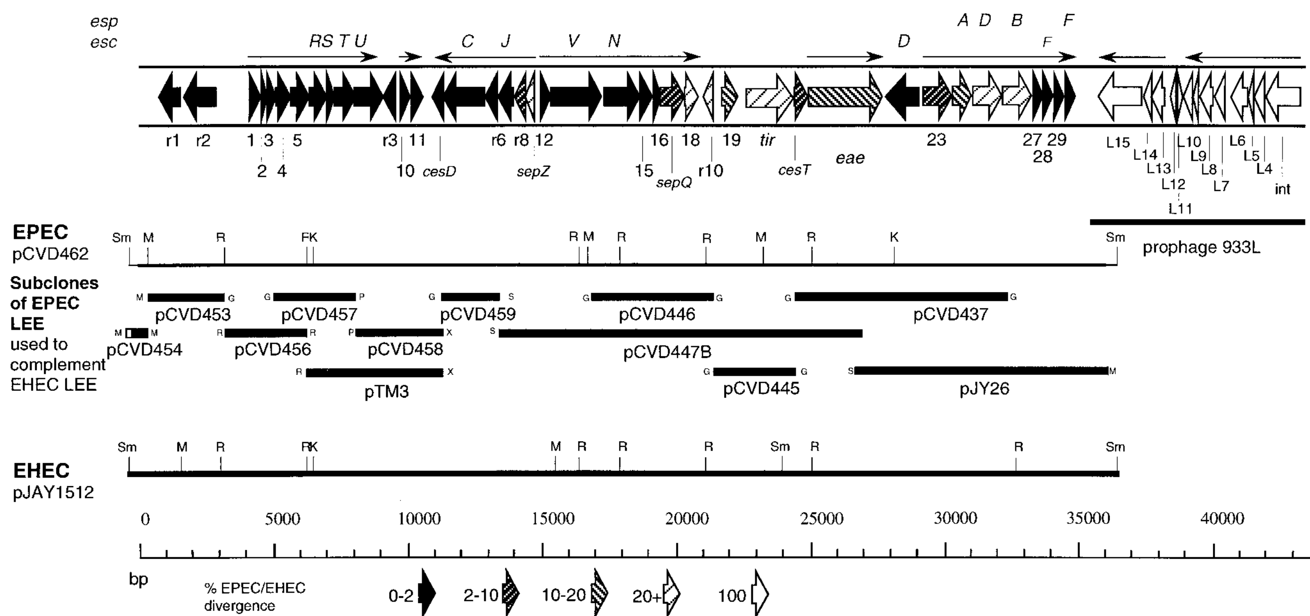

FIG. 1. LEE of EHEC O157:H7 and maps of clones of LEEs from EPEC O127:H6(pCVD462) and EHEC O157:H7(pJAY1512). The degree of genetic divergence in each ORF between the EPEC and EHEC LEEs is represented by shading. R, *Eco*RI; M, *Mlu*I; Sm, *Sma*I; K, *Kpn*I.

ized to all four EPEC LEE probes. Several partial LEE clones were also obtained. pJAY1512 was mapped with restriction enzymes and LEE probes A through D (Fig. 1). This process confirmed and extended the 85-170 LEE map obtained from chromosomal Southern blotting. We found that the cosmid clone pJAY1512 did not contain the LEE-associated prophage found at the extreme end of the EHEC LEE. In addition to pJAY1512, we also obtained the LEE clone $pLEE_{O157}$ (18) from F. Blattner. $pLEE_{O157}$ was derived from the chromosome of strain EDL933, a wild-type EHEC O157:H7 strain, by a FLP recombinase-based method (19) and has been entirely sequenced (18). It contains the LEE prophage as well as genes flanking the LEE (18).

Characterization of the cloned EHEC LEE functions. Both pJAY1512 and $pLEE_{O157}$ in their respective *E. coli* K-12 host strains (Table 1) were examined in the FAS assay for A/E lesion formation (11). The FAS assay involves incubation of bacteria on a HEp-2 cell tissue culture monolayer for 3 to 6 h during which time the bacteria form A/E lesions on the surfaces of infected cells. The addition of phalloidin-fluorescein isothiocyanate conjugate, which binds to actin, allows visualization of actin condensed below the bacteria in the A/E lesion. FAS test results were scored blindly by at least one other independent observer and were repeated at least three times. When the cloned EHEC LEE was tested in different K-12 genetic backgrounds, including HB101, W3110, and DH5α, none of the resulting strains was able to form A/E lesions (Table 1) or secrete Esp proteins (results not shown). This finding contrasts with that for the cloned EPEC LEE (Table 1).

We then examined whether the failure of HB101 (pJAY1512) to elicit A/E lesions was due to an initial failure to adhere. HB101(pJAY1512) was transformed with plasmids encoding either the afimbrial adhesin AFA-I (13) or pMAR7 (1), which encodes the bundle-forming pili of EPEC as well as other factors, notably the Per transcriptional activator of many EPEC virulence genes (5). The variants expressing AFA-I or bundle-forming pili were much more adherent to HEp-2 cells in the standard adhesion assay (1) (data not shown) but were unable to form A/E lesions.

Clearly, the cloned EHEC O157:H7 LEE is phenotypically different from the cloned EPEC O127:H6 LEE, and this difference is reflected in their sequence divergence. While many genes are highly conserved, other genes diverge quite markedly (18) (Fig. 1). These highly divergent genes may be responsible for the inability of the EHEC LEE to form A/E lesions when it is cloned into *E. coli* K-12. We hypothesized that complementation of pJAY1512 with plasmids containing regions of the EPEC LEE could restore the A/E phenotype. The ability of cloned EPEC LEE genes to complement mutated EHEC LEE genes in an EHEC background has previously been shown for *escN* (7), *ler* (3), and *eae* (21). Indeed, the cloned EPEC *eae* gene was able to restore A/E lesion formation to an EHEC Δ*eae* mutant and the resultant complemented strain colonized the intestine in a manner characteristic of EPEC rather than of EHEC (21).

To test for complementation of pJAY1512 with cloned EPEC genes, plasmids containing regions of the EPEC O127:H6 LEE were isolated to ensure that entire operons within hypervariable regions were represented (Fig. 1 and Table 1). These plasmids were transformed into HB101 (pJAY1512) and examined by the FAS assay. None of the resulting complemented strains exhibited FAS (Table 1). As a control, we transformed pJAY1512 into CVD451, an *escN* mutant of EHEC O157:H7 that is unable to form A/E lesions or to secrete Esp proteins (7). The resultant strain, CVD451 (pJAY1512), was positive in the 6-h FAS test, indicating that the EHEC LEE clone could rescue the chromosomal *escN* mutation in an EHEC background.

In summary, our data clearly suggest that the genetic differences between EPEC and EHEC LEE elements are reflected phenotypically when the LEE is cloned into an *E. coli* K-12 background. While the cloned EPEC LEE was sufficient for A/E lesion formation and Esp secretion in an *E. coli* K-12 host (15), a similarly cloned LEE from EHEC was inactive in all these assays. This result was confirmed with an independent EHEC LEE clone, $pLEE_{O157}$. This clone, which was derived from a different O157:H7 strain in a different laboratory by a different methodology, was also negative for the A/E pheno-

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TABLE 1. Strains used in this study and their activities in the FAS assay

Strain	FAS result[a] 3 h	FAS result[a] 6 h	Description
E2348/69	+++	++++	EPEC wild-type O127:H6
85-170	−	+++	Δ*stx* derivative of EHEC O157:H7 84-289 (7)
86-24	−	++	EHEC wild-type O157:H7 (7)
EDL933	−	++	EHEC wild-type O157:H7 (7)
CVD451	−	−	Strain 86-24 *escN* (7)
HB101	−	−	K-12/B F^- *mcrB mrr hsdS*20(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44* λ^-
DH5α	−	−	K-12 F^- Φ80Δ(*lacZYA-argF*)*U169 endA recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*
W3110	−	−	K-12 F^- λ^- IN(*rrnD-rrnE*)*I rph-1*
HB101(pCVD551)	−	−	Cosmid cloning vector (15)
HB101(pCVD462)	++	+++	E2348/69 LEE cloned into pCVD551 (15)
DH5α(pCVD462)	++	+++	E2348/69 LEE cloned into pCVD551 (15)
W3110(pCVD462)	++	+++	E2348/69 LEE cloned into pCVD551 (15)
HB101(pJAY1512)	−	−	Strain 85-170 LEE cloned into pCVD551
DH5α(pJAY1512)	−	−	Strain 85-170 LEE cloned into pCVD551
W3110(pJAY1512)	−	−	Strain 85-170 LEE cloned into pCVD551
XL1-Blue($pLEE_{O157}$)	−	−	EDL933 LEE cloned into pSG76-C (19)
CVD451(pJAY1512)	NT	++	Strain 86-24 *escN* complemented with 85-170 LEE clone
Complements of pJAY1512			
HB101(pJAY1512)(pMAR7)	−	−	Complemented with EPEC EAF virulence plasmid (1)
HB101(pJAY1512)(pIL14)	−	−	Complemented with AFA-I adhesin clone (13)
HB101(pJAY1512)(pCVD437)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD445)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD446)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD447B)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD453)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD454)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD457)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD458)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD456)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pCVD459)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pJY26)	−	−	Complemented with subclones of pCVD462[b]
HB101(pJAY1512)(pTM3)	−	−	Complemented with subclones of pCVD462[b]

[a] Assessment of A/E lesion formation by the FAS test: −, no lesion formation; ++ to ++++, increasing number and intensity of lesions detected. NT, not tested.
[b] Reference 14 and this paper.

type as shown by the FAS test. The inability of the EHEC LEE clone to mediate A/E lesion formation was not due to an obvious defect in the cloning of the LEE or the inability of the LEE in the native O157:H7 strain to mediate A/E lesions. The EHEC LEE diverges from its EPEC counterpart in several genes, and these genes are a likely source of phenotypic differences between both the wild-type organisms and their cloned LEEs (18). However, complementation of pJAY1512 with subclones representing the entire EPEC LEE was unable to confer A/E lesion activity. Our data also exclude the possibility that failure to form A/E lesions was due to failure to initially associate with the cell, as transformation of HB101(pJAY1512) with plasmids encoding adhesins resulted in increased adhesion but not the A/E phenotype.

These data raise the question of whether only the LEE of EPEC O127:H6 strain E2348/69 is sufficient for A/E lesion formation in an *E. coli* K-12 background and would imply that the EHEC LEE requires a factor in *trans* that is not present in *E. coli* K-12. Recent work (3, 16, 20) has shown that EPEC and EHEC have differences in regulation and also suggest that they may require different factors in *trans*, a topic which is currently being investigated in our laboratory.

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