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Comparative Analysis of Locus of Enterocyte Effacement Pathogenicity Islands of Atypical Enteropathogenic *Escherichia coli*

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The pathogenicity of enteropathogenic *Escherichia coli* (EPEC) is linked to the locus of enterocyte effacement, or LEE, encoding a type III secretion system (T3SS) that directly transfers bacterial effector proteins into eukaryotic cells. Atypical diffusely adhering EPEC (DA-EPEC) strains that harbor homologues of the LEE but lack the EPEC adherence factor plasmid have been increasingly associated with outbreaks of diarrhea. In this study, we have completely sequenced and functionally characterized LEE pathogenicity islands derived from the clinical DA-EPEC isolates 3431 (O8:H$^-$) and 0181 (O119:H9:K61). LEE_{3431} and LEE_{0181} exhibit genetic organization analogous to that of the prototype $LEE_{E2348/69}$. Genes constituting the T3SS apparatus are highly conserved. However, LEE-encoded effector proteins exhibit major differences. Transfer and functional expression of LEE_{0181} in an *E. coli* XL1 blue MR background demonstrated that LEE_{0181} contains all the information for signal transduction and pedestal formation.

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of persistent diarrhea among infants, primarily in developing countries (7, 8, 23). Characteristic features of EPEC are the presence of the EPEC adherence factor (EAF) plasmid encoding bundle-forming pili (BFP), the lack of the known enterotoxins (heat-stable enterotoxin, heat-labile enterotoxin, and Stx), and the induction of attaching and effacing (A/E) lesions. A/E lesions are characterized by the destruction of brush border microvilli, the rearrangement of host cytoskeletal proteins, and the apical formation of an actin-raised platform ("pedestal") directly underneath the adherent bacteria. All factors responsible for the A/E phenotype are encoded by a chromosomal pathogenicity island, the locus of enterocyte effacement, or LEE (20, 21). LEE homologues have been characterized in other pathogenic *E. coli* strains, such as the EHEC strain EDL933 and the RDEC-1 strain, and also in *Citrobacter rodentium*. The LEE is a 35.6-kb cluster of genes containing five polycistronic operons (LEE1 to LEE5) that encode a type III secretion system (T3SS) which transfers effector proteins into the target host cells (18). These proteins are responsible for the ensuing pathology.

In recent years, atypical EPEC strains that harbor homologues of the LEE but lack the EAF plasmid and thus BFP as well as the regulator Per (plasmid-encoded regulator) have been identified (3, 19, 29). Due to the lack of BFP, these strains exhibit a diffuse adherence pattern and therefore have been described as diffusely adhering EPEC (DA-EPEC) (3). As epidemiological studies show that these DA-EPEC strains are increasingly associated with outbreaks of diarrhea (5, 15, 30), they have been recognized as emerging human and animal pathogens (6, 7, 25, 29). Nonetheless, LEE pathogenicity islands derived from the emerging DA-EPEC strains have not been investigated yet. In this study, we have characterized and comparatively analyzed the LEEs derived from the clinical DA-EPEC strains 3431 (O8:H$^-$) and 0181 (O119:H9:K61) (courtesy of L. R. Trabulsi, Saõ Paulo, Brazil).

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To characterize and compare the LEEs, cosmid libraries of the DA-EPEC strains 0181 and 3431 employing the SuperCos1 cosmid vector (Stratagene) were generated. LEE-harboring cosmid clones were identified by colony hybridization. Cosmid clones used for sequencing encompassed the LEEs of the two DA-EPEC strains as depicted in Fig. 1. In addition, the sequence between the *eae* and *espD* genes of LEE_{3431} was obtained using a 3-kb PCR fragment generated with the primer pair JG31-JG32 (Fig. 1; Table 1). The 3′ sequence of LEE_{3431} from the *espD* gene to the 3′ end had been determined previously (3).

G+C contents, insertion sites, and flanking regions of the LEEs of the DA-EPEC strains 0181 and 3431. Pathogenicity islands are large clusters of functionally cooperative virulence genes that are horizontally transferred between different species, resulting in significant changes in fitness and also in pathogenicity ("quantum leaps in evolution") (16). The G+C contents of the atypical LEEs were determined to be 38.3% (LEE_{0181}) and 39.5% (LEE_{3431}). These are considerably lower than that of the *E. coli* chromosome but similar to those of LEEs of other *Enterobacteriaceae*, such as the EPEC strain E2348/69 (38.4%), the enterohemorrhagic *E. coli* (EHEC) strain EDL933 (39.6%), *Citrobacter rodentium* (38.1%), and the RDEC-1 strain (41.3%) (10, 12, 31).

LEE_{0181} is inserted into the *selC* tRNA gene of the *E. coli* K-12 genome (Fig. 2), as has been reported for $LEE_{E2348/69}$ and the LEE of the EHEC strain EDL933 as well as those of several EPEC 1 and EHEC 1 serovars (12, 20). The 5′- and 3′-flanking regions of LEE_{0181} are identical to the flanking

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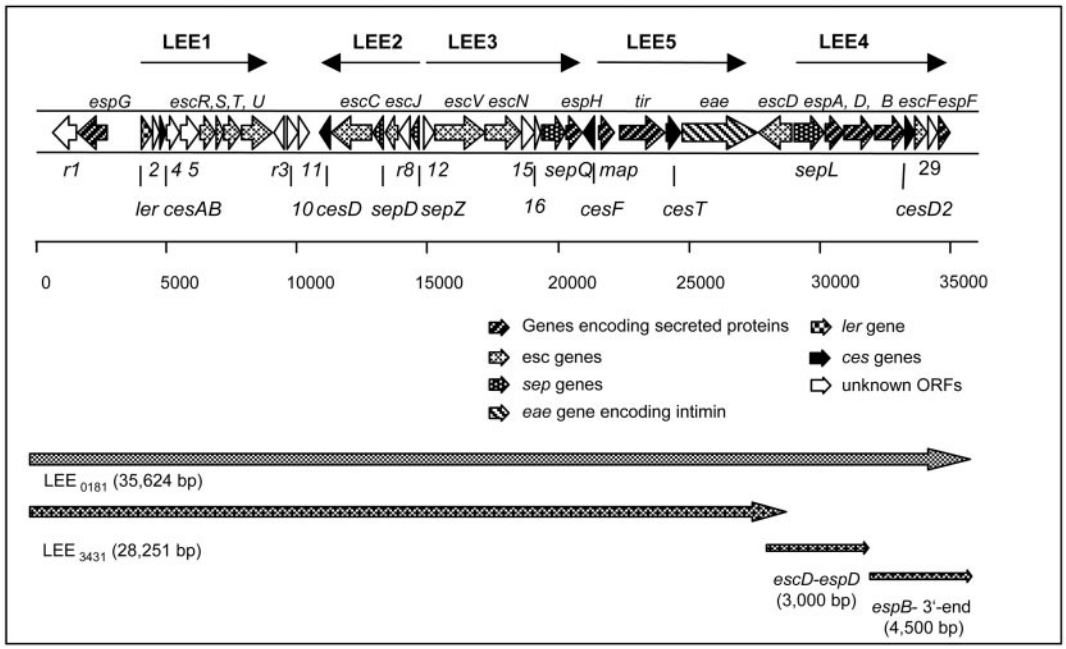

FIG. 1. Organization of the prototype $LEE_{E2348/69}$ and analogous segments of atypical LEEs covered by cosmid clones. The cosmid clone LEE_{0181} covers the complete LEE of the DA-EPEC strain 0181, and the cosmid LEE_{3431} encompasses 28,251 bp of the LEE of the DA-EPEC strain 3431. The sequence between the *eae* and the *espD* genes of LEE_{3431} was obtained using a 3-kb PCR fragment; the 3′ sequence of LEE_{3431} from the *espD* gene to the 3′ end had been determined previously.

regions of $LEE_{E2348/69}$. The 5′-flanking region of about 700 bp begins in the *yicK* gene of the *E. coli* K-12 genome, which as a result of the integration is largely truncated (250 instead of 1,185 bp). The 3′-flanking region of about 790 bp is followed by an insertion element, which is identical to the IS*600* flanking the 3′ end of $LEE_{E2348/69}$, as well as by the *selC* tRNA gene of the *E. coli* K-12 genome (12).

LEE_{3431} is inserted next to the *pheU* tRNA gene of the *E.*

TABLE 1. Oligonucleotides used as primer pairs and probes

Primer (reference)	Primer sequence (5′ to 3′)	Product (no. of bp)	T_A[a] (°C)
EspB3431(+) (16a)	TCC CCC GGG ATG AAT ACT ATT GAT AAT AAT	*espB* (924)	56
EspB3431(−) (16a)	TCC CCC GGG TTA CCC GGC TAA GCG ACC CGA		
EspD3431(+) (16a)	AAT CTG TTC ACG CTA GGC GGA TCC GCG ATG CTT AAT	*espD* (1200)	60
EspD3431(−) (16a)	TAA ACC AAT TCC CCC GGG GGA TTA AAT TCG ACC ACT		
K260 (20)	GAG CGA ATA TTC CGA TAT CTG GTT	orf394/*selC* (527)	60
K261 (20)	CCT GCA AAT AAA CAC GGG GCA T		
JG31	CGTCTGGGCGGGAGATGTTGATACCATCTT	Strain 3431 *eaeA-espD* (3 kb)	64
JG32	CATCTGAAGTAGCCGAAGCAGCATTAGCCC		
K295 (20)	CGC CGA TTT TTC TTA GCC CA	orf394/LEE (405)	60
K296 (20)	CAT CTC GAA ACA AAC TGG TC		
C6 (2a)	GAT ATA TAA GGG ATT AGA AGG GG	LEE/*selC* (381)	50
K260 (20)	GAG CGA ATA TTC CGA TAT CTG GTT		
C14 (2a)	CGA ACT GTT AAC CAC ACT G	LEE/*pheU* (2,006)	53
C23 (2a)	CCT ATG AGC AAT CGA AGA AAG G		
Ler R1 (22a)	GTT AAA TAT TTT TCA GCG GTA	*ler* (390)	52
Ler F1 (22a)	CAT GCG GAG ATT ATT TAT TAT		
SK1	CCC GAA TTC GGC ACA AGC ATA AGC	*eaeA* (863)	52
SK2	CCC GGA TCC GTC TCG CCA GTA TTC G		
Tir 19R (22a)	CGG AAT TCT TAA ACG AAA CGT ACT GGT	*tir* (1650)	58
Tir 19F (22a)	AAG GAT CCA TGC CTA TTG GTA ACC TTG G		

[a] T_A, annealing temperature.

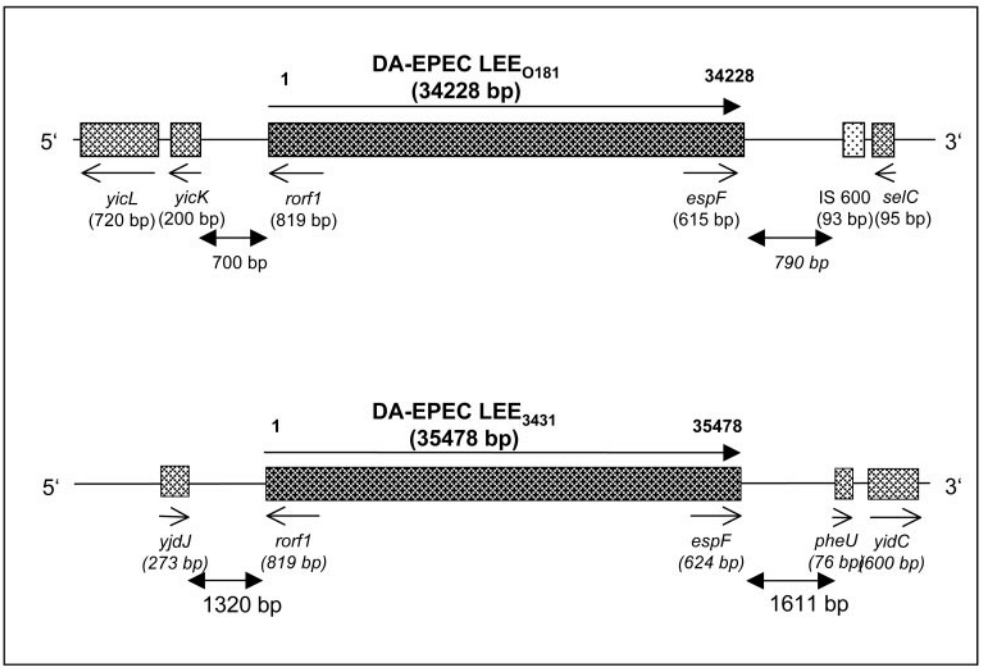

FIG. 2. Schematic overview of the chromosomal junctions of the LEEs of the DA-EPEC strains 0181 and 3431.

coli genome (Fig. 2). At the 5′ end, LEE_{3431} has been incorporated in the *yjdK* gene, resulting in the deletion of the gene. The 3′ end of LEE_{3431} is followed by a 240-bp fragment, which is identical to the 3′-flanking region of $LEE_{E2348/69}$. Downstream (1,260 bp) is a 134-bp repeat region, which has also been found at the 3′ junction of the LEE of the Shiga toxin-producing *E. coli* strain 413/89-1 (O26:H$^-$) (27). Directly adjacent to the 3′ end of LEE_{3431}, the *pheU* gene followed by the *yjdC* gene has been identified, indicating a deletion of the intervening 10-kb chromosomal DNA encompassing the *lysU*, *ydlL*, *cadA*, and *cadC* genes.

Sequence characteristic of the cloned LEE. The nucleotide sequence of the LEE of the DA-EPEC strain 0181 (35,624 bp) covers the core region of the LEE, defined as the region encompassing the first (rorf1) and the last (*espF*) genes of the LEE as well as 675 and 797 bp of flanking sequences at the 5′ and 3′ ends. The nucleotide sequence of the LEE of the DA-EPEC strain 3431 (38,235 bp) contains the core region of the LEE as well as 777 and 1,904 bp of noncoding sequences at the 5′- and 3′-flanking regions, respectively.

LEE pathogenicity islands are highly conserved in terms of size and organization of genes. Each LEE harbors 41 open reading frames (ORFs) organized into five polycistronic operons (LEE1, LEE2, LEE3, LEE5, and LEE4) that exhibit identical orders and orientations. It is noteworthy that all of the identified ORFs in LEE_{0181} and LEE_{3431} correspond to genes located on the LEEs of the prototype EPEC strain E2348/69, the EHEC strain EDL933, *Citrobacter rodentium*, and the RDEC-1 strain, indicating that these LEEs may have descended from a common ancestor. The increased size of LEE_{3431} (35,478 bp) compared to $LEE_{E2348/69}$ (34,066 bp) is due to the insertion of a 1,431-bp Tn*5* transposon into the *ler* gene. The functional significance of the *ler*-mediated regulation is currently under examination in our laboratory. With 34,228 bp, LEE_{0181} is only slightly (162 bp) larger than $LEE_{E2348/69}$. Comparisons of sequences of specific operons indicated a closer relationship of LEE_{0181} with $LEE_{E2348/69}$ than with LEE_{3431} (Table 2).

Genes involved in the T3SS. Sequence analysis clearly demonstrated that the proteins constituting the T3SS apparatus (Esc and Sep proteins) are highly conserved. The Sep (secretion of *E. coli* proteins) and Esc (*E. coli* secretion) proteins of the DA-EPEC strain 0181 and the EPEC strain E2348/69 are nearly identical (>96.9% identity). The corresponding proteins of the DA-EPEC strain 3431 and those of the EPEC strain E2348/69 and the DA-EPEC strain 0181 share lower identities of 87.9 to 97.9% (Table 2). This remarkable conservation of Esc and Sep proteins is paralleled in other A/E pathogens, such as the EHEC strain EDL933, the RDEC-1 strain, and *Citrobacter rodentium* and—once again—emphasizes the stringent requirements for the T3SS (10, 12, 31).

In contrast, the SepZ protein turned out to be almost hypervariable among T3SS proteins and to represent one of the most divergent proteins of the LEE (Table 2) (10, 12, 31). Interestingly, the SepZ proteins of the DA-EPEC strain 0181 and the EPEC strain E2348/69 are almost identical (96.9% identity). In contrast, the SepZ proteins of the DA-EPEC strain 3431 and the EPEC strain E2348/69 as well as that of the DA-EPEC strain 0181 exhibit very low identity of only 59.2%. These differences point to a possible role for SepZ in the specificity of the T3SS.

LEE-encoded effector proteins. In contrast to the proteins involved in the T3SS, the LEE-encoded secreted effector proteins exhibit differences which are larger than would have been expected for clonal divergence among *E. coli* strains (Table 2) (12, 31). These variations may reflect greater evolutionary pressure on the secreted effector proteins both from the host immune system and from differences among hosts. We found a

TABLE 2. Comparison of ORFs of the LEEs identified in DA-EPEC strains 0181 and 3431 with the corresponding ORFs of the prototype EPEC strain E2348/69

ORF	No. of aa corresponding to ORF of LEE from:			Gene designation	Function of protein	% Similarity of protein sequences from strains:		
	Prototype EPEC strain E2348/69	DA-EPEC strain 3431	DA-EPEC strain 0181			3431 and E2348/69	0181 and E2348/69	3431 and 0181
rorf1	272	272	272		Unknown	81.2	98.9	81.6
rorf2	398	387	398	*espG*	Secreted protein	69.5	99.7	69.5
orf1	129	86	129	*ler*	LEE regulator	93.0	99.2	93.0
orf2	72	72	72	*cesAB*	Chaperone for EspA and EspB	93.1	98.6	91.7
orf3	107	107	107		Type III secretion?			
orf4	199	199	199		Type III secretion?	93.5	100	93.5
orf5	231	231	231		Type III secretion?	79.7	98.7	79.7
orf6	217	217	217	*escR*	Type III secretion	95.9	99.5	96.3
orf7	89	89	89	*escS*	Type III secretion	95.5	100	95.5
orf8	258	258	258	*escT*	Type III secretion	93.8	99.6	93.4
orf9	345	345	345	*escU*	Type III secretion	95.1	100	95.1
rorf3	152	154	152		Unknown		99.3	
orf10	123	112	123		Unknown	85.7	99.2	85.7
orf11	137	137	137		Unknown	94.2	100	94.2
rorf4	151	151	151	*cesD*	Chaperone for EspD	96.7	100	96.7
rorf5	512	512	512	*escC*	Type III secretion	97.5	99.6	97.9
rorf6	151	151	151	*sepD*	Type III secretion	95.4	99.3	96.0
rorf7	190	190	190	*escJ*	Type III secretion	97.9	99.5	97.4
rorf8	142				Type III secretion?			
rorf9	98	99	98	*sepZ*	Type III secretion	59.2	96.9	59.2
orf12	117				Type III secretion?			
orf13	675	675	675	*escV*	Type III secretion	96	99.7	96.1
orf14	446	446	446	*escN*	Type III secretion	95.7	99.6	96.0
orf15	125	125	125		Type III secretion?	93.6	100	93.6
orf16	138	138	138		Type III secretion?	89.1	97.1	90.6
orf17	305	305	305	*sepQ*	Type III secretion	87.9	100	87.9
orf18	176		177	*espH*	Type III secretion			
rorf10	120	120	120	*cesF*	Chaperone for EspF	78.3	98.3	77.5
orf19	203	203	203	*map*	Secreted protein	74.9	88.7	76.4
orf20	550	549	550	*tir*	Intimin receptor	56.5	95.8	56.8
orf21	156	156	156	*cesT*	Chaperone for Tir	96.8	100	96.8
orf22	939	937	948	*eae*	Intimin	79.5	83.2	79.8
rorf11	406	406	406	*escD*	Type III secretion	92.9	97.8	94.1
orf23	351	351	351	*sepL*	Type III secretion	94.6	97.7	94.3
orf24	192	192	190	*espA*	Secreted protein	81.2	74.2	73.7
orf25	380	380	379	*espD*	Secreted protein	75.8	85.5	71.1
orf26	321	307	321	*espB*	Secreted protein	61.9	100	61.9
orf27	135	135	135	*cesD2*	Chaperone for EspD	92.6	100	92.6
orf28	73	73	73	*escF*	Type III secretion	97.3	100	97.3
orf29	92	92	92		Unknown			
orf30	206	207	204	*espF*	Secreted protein	75.7	85.8	73.0

considerably large variance among the Esp's (*E. coli* secreted proteins), namely, EspA, EspB, EspD, and EspF (Table 2).

The sequence analysis of the EspA proteins of the two DA-EPEC strains as well as that of the EPEC prototype strain E2348/69 revealed identities of 73.7 to 81.4% (Table 2). EspB together with EspD forms a translocation pore in the target cell membrane and has been described as a cytosolic effector protein tampering with cellular signaling processes (17, 28). The LEE_{3431}-encoded EspB is 14 amino acids (aa) shorter (307 aa) than the E2348/69 counterpart and shares only 61.9% identity with that counterpart. In contrast, $EspB_{0181}$ is completely identical to $EspB_{E2348/69}$. $EspD_{3431}$ is the same size as and shares 75.8% identity with $EspD_{E2348/69}$, whereas $EspD_{0181}$ is 1 aa shorter than $EspD_{2348/69}$ and shares 85.5% identity with that protein. These findings reflect a relatively close relationship of LEE_{0181} with $LEE_{2348/69}$ that is also corroborated by the shared inability of the Esp's of E2348/69 and 0181 to induce hemolysis. In contrast, the Esp proteins of the DA-EPEC strain 3431 are sufficient for erythrocyte lysis (17).

The impairment of gastrointestinal barrier functions is regarded as an important step in EPEC pathogenesis. In this process, the T3SS-secreted effector protein EspF has been suggested to play a major role (22). EspF exhibits differences in amino acid sequences and also in protein sizes in different A/E pathogens. EspF contains 301 aa residues in *Citrobacter rodentium*, 248 aa in the EHEC strain EDL933, 206 aa in the EPEC strain E2348/69, 204 aa in the DA-EPEC strain 0181, 207 aa in the DA-EPEC strain 3431, and only 160 aa in the RDEC-1 strain (10, 12, 31). These size differences in EspF are largely due to the number of proline-rich repeats, and it has been speculated that these repeats may be involved in host specificity (10) (Fig. 3). While RDEC-1 has two repeats, the EPEC and DA-EPEC strains have three, the EHEC strain EDL933 carries four, and *Citrobacter rodentium* harbors five repeats.

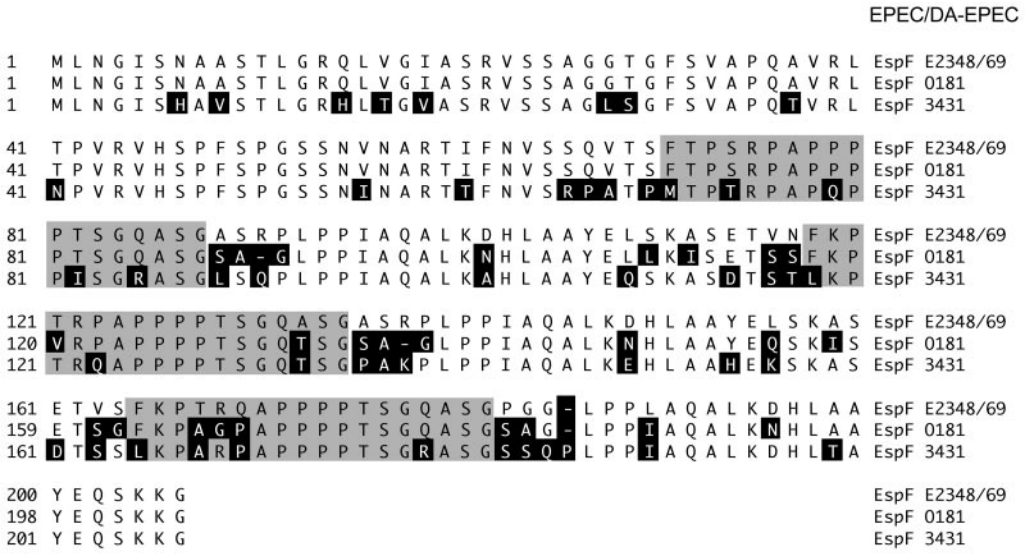

FIG. 3. Alignments of the EspF protein of the EPEC protoype strain E2348/69 with those of the DA-EPEC strains 0181 and 3431. Residues differing from those of $EspF_{E2348/69}$ are boxed in black. The three proline-rich repeats in EspF are shaded in grey.

The $EspF_{0181}$ protein is again more closely related to $EspF_{E2348/69}$ (85.8% identity) than to the EspF protein from DA-EPEC strain 3431 (73% identity) (Fig. 3).

Additional highly variable genes include the *eae* gene encoding the outer membrane adhesin intimin and the *tir* gene encoding the intimin receptor Tir (translocated intimin receptor). The interaction of intimin and Tir mediates the intimate attachment between bacterium and host cell and plays an important role in EPEC pathogenesis (11, 23). The intimin proteins of the three strains compared in this study differ both in their sequences and their lengths. While intimin of the DA-EPEC strain 0181 comprises 948 aa, the corresponding proteins of the DA-EPEC strain 3431 and the EPEC strain E2348/69 have 937 and 939 aa. Moreover, $intimin_{E2348/69}$ is 79.5 and 83.2% identical to $intimin_{3431}$ and $intimin_{0181}$, which are only 79.8% identical to each other (Table 2). Differences in intimin sequences are thought to reflect the evolutionary lineages of the various LEEs (2, 26) and have been employed to classify A/E *E. coli* isolates. Several genetically and serologically distinct intimin types (intimin α to intimin λ) have been identified. The comparison of the intimin sequences of the DA-EPEC strains showed that the intimin of the DA-EPEC strain 3431 is 99.8% identical to intimin φ2 and that the intimin of strain 0181 is identical to intimin ε2. The intimin of the EPEC prototype strain E2348/69 shows an identity of 94.4% to intimin α2. Based on the intimin sequences, the relationship among the intimins derived from the DA-EPEC strains and the prototype EPEC strain E2348/69 can be displayed as a phylogenetic tree (Fig. 4) that again reflects the relatively close relationship of LEE_{0181} to $LEE_{E2348/69}$.

The genes encoding the chaperones involved in the stabilization and secretion of the effector proteins EspB, EspD, Tir, and Map as well as EspF, CesD, CesD2, CesT, and CesF (9, 14, 24) turned out to be highly conserved. These proteins are unique to the LEE and have not been found in other T3SSs. The high level of sequence identity between chaperones of different strains supports a prominent role in the maintenance of effector functions. Interestingly, whereas the Tir effector proteins differ in the three EPEC strains, the corresponding chaperone CesT, which binds to the N-terminal region of Tir (1), is highly conserved among the three LEEs examined in this study (Table 2). The Tir of LEE_{0181} is 95.8% identical to the Tir of $LEE_{E2348/69}$. In contrast, the Tir of LEE_{3431} shows an identity of 56.5% to the Tir of $LEE_{E2348/69}$.

Functional analysis of the cloned LEE_{0181}. The recombinant *E. coli* JG-LEE_{0181}/XL1 blue MR strain harboring the complete LEE_{0181} adhered to host cells and, furthermore, induced the formation of pedestal-like structures underneath adherent bacteria in HeLa cells (Fig. 5). In addition, the recombinant strain secreted EspB and induced a fluorescence actin staining-positive reaction as well as the accumulation of tyrosine-phosphorylated proteins (presumably Tir) at the site of infection (data not shown). These results indicate that the LEE of DA-EPEC also encodes all information necessary for bacterial adherence to the host cell and the activation of signal transduction pathways leading to A/E lesions and pedestal formation.

The lack of the EAF plasmid and thus of the BFP reduces the ability of DA-EPEC to form biofilm-embedded microcolonies or "bioclips" on the host cell surface and consequently impairs quorum sensing, inducing the activation of LEE and

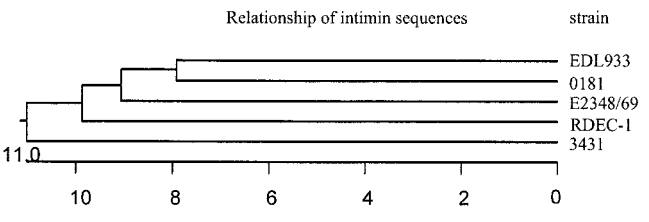

FIG. 4. Phylogenetic tree of intimin proteins of DA-EPEC strains, the prototype EPEC strain E2348/69, the EHEC strain EDL933, and the rabbit EPEC strain RDEC-1 as a visualization of sequence alignments. The tree was generated using DNASTAR MegAlign software.

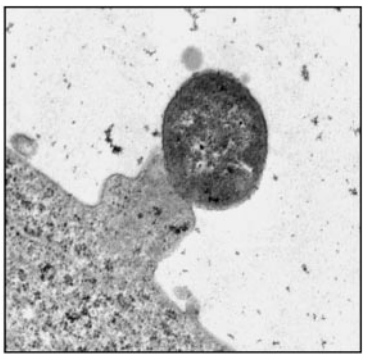

FIG. 5. Transmission electron microscopy of a HeLa cell infected with JG-LEE$_{0181}$/XL1 blue MR (magnification, ×19,000). The recombinant strain is able to adhere to HeLa cells and induces the formation of host cell protrusions (pedestals) underneath adherent bacteria.

the following signal transduction. Besides the F1845 fimbriae (4), which have been identified in strain 0181 (3), little is known about possible adhesins expressed by DA-EPEC. In addition, Per, which is largely regulated by the LEE-encoded Ler, has been shown to be responsible for maximal expression of LEE-encoded proteins (13). Therefore, due to the lack of *per*, regulation of LEE expression in DA-EPEC strains lacking the EAF may be quite different. In further studies it will be interesting to see whether an alternative regulatory system may be involved in DA-EPEC strains.

Nucleotide sequence accession numbers. Sequences of the LEEs of DA-EPEC strains 0181 and 3431 have been deposited in GenBank under accession numbers AJ633129 and AJ633130, respectively.

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