INFECTION AND IMMUNITY, Nov. 1993, p. 4894–4898
0019-9567/93/114894-05$02.00/0

Vol. 61, No. 11

Virulence Determinants in Nontoxinogenic *Escherichia coli* O157 Strains That Cause Infantile Diarrhea

HERBERT SCHMIDT, HOLGER RÜSSMANN, AND HELGE KARCH*

Institut für Hygiene und Mikrobiologie der Universität Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany

Received 1 June 1993/Returned for modification 22 July 1993/Accepted 24 August 1993

Ten sorbitol-fermenting *Escherichia coli* O157 strains that cause infantile diarrhea and are positive in the fluorescence actin staining test were determined to be negative for Shiga-like toxin (SLT) genes. We amplified their complete *eae* genes, contrasting them with those of SLT-producing *E. coli* O157 by restriction fragment length polymorphism analysis and nucleotide sequence analysis of a 400-bp stretch of the 3′ end of *eae*. The data substantiated the presence of two *eae* genotypes within serogroup O157, one resembling *eae* of enteropathogenic *E. coli* (EPEC) strain E2348/69, found in nontoxinogenic *E. coli* O157 strains, and the other resembling *eae* of EHEC strain EDL 933, found in toxinogenic *E. coli* O157 strains. Another EPEC-specific virulence determinant was also shown to be large plasmids harboring EPEC adherence factor sequences. The SLT-negative *E. coli* O157 strains described here fall under the heading of EPEC, which serves as an explanation for their virulence in infants, and represent a third pathogroup within serogroup O157.

Identification of virulence genes in *Escherichia coli* strains has led to better understanding of the pathogenesis of diarrheal diseases caused by these organisms, providing a new dimension to their diagnostics. This identification has also allowed these strains associated with enteric disease to be categorized more plausibly than by conventional serotyping. Furthermore, the occurrence of distinct pathogroups could be shown within one serogroup (18). This also applies to strains of serogroup O157, which can now be broken down into three pathogroups based on the various virulence determinants expressed by them. (i) Enterohemorrhagic *E. coli* (EHEC) H7 and H^- strains, which produce Shiga-like toxin (SLTs) as obligatory virulence factors, harbor large plasmids which favor adherence to intestinal cells and possess the intimin-coding gene *eae*, which is important for attaching-and-effacing lesions. A comparison of EHEC *eae* and enteropathogenic *E. coli* (EPEC) *eae* genes showed a major divergence in the 3′ ends of the genes. EHEC O157 strains cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), resulting in very serious outbreaks of these diseases throughout the world (5, 11, 13, 17, 21, 23). (ii) Enterotoxigenic *E. coli* strains of various H-antigen types express K88 fimbriae and enterotoxins. They are associated mainly with colibacillosis in pigs (22). (iii) A hitherto uncategorized pathogroup of *E. coli* O157 strains is associated with infantile diarrhea (ID) and has no demonstrable *slt* genes but a capacity to induce a positive fluorescence actin staining (FAS) test. In this study, we characterized their *eae* genes and discovered that they closely resemble the EPEC *eae* gene. Without doubt, this pathogroup must be categorized as EPEC because of the presence of EPEC adherence factor (EAF), thus representing a third pathogroup within serogroup O157.

The sources and characteristics of the 20 FAS test-positive *E. coli* O157 strains used in this study are given in Table 1. In addition, we used EPEC O127:H6 isolate E2348/69, O157:H7 strain EDL 933, and five FAS test-negative *E. coli* O157 strains that produce H antigens 19 and 43 (1, 2) or are nonmotile. The DNA probes used to detect *slt* genes were a 282-bp polymerase chain reaction (PCR) product from the *slt*-IB subunit gene, derived from amplification of strain C600(H19J) with primers specific for *slt*-IB, and a 288-bp PCR fragment from the *slt*-IIB gene, which was derived from amplification of *E. coli* C600 (933W) with primers complementary to *slt*-IIB (6). The CVD 419 probe was a 3.4-kb *Hin*dIII fragment (15), and the EAF probe was a 1-kb *Sal*I-*Bam*HI fragment (16). The *eae* probe used here was an 863-bp DNA fragment of the conserved region seen in EPEC and EHEC *eae* genes resulting from amplification of DNA from strain E 2348/69 with primer pair SK1-SK2 (10). Probe fragments were excised from Tris acetate-EDTA-agarose gels and randomly labeled with digoxigenin as described in the Boehringer manual (Boehringer GmbH, Mannheim, Germany). For amplification of the whole EPEC *eae* gene (2,817 bp), primers LP1 (5′-ccc ggg atc cat gat tac tca tgg ttt tt-3′) and LP2 (5′-ccc gaa ttc tta ttt tac aca agt ggc-3′) were deployed, whereas primer LP3 (5′-ccc gaa ttc tta ttc tac aca aac cgc-3′) in combination with LP1 was used to amplify the entire EHEC *eae* gene (2,802 bp). The precise PCR conditions were as follows. The samples were incubated at 94°C for 45 s to denature the DNA, at 48°C for 1 min to anneal the primers, and at 72°C for 150 s to extend the annealed primers. Amplifications were performed in a total volume of 50 μl containing deoxynucleoside triphosphates at 200 μM, 50 pmol of each primer, 5 μl of 10-fold-concentrated polymerase synthesis buffer, and 2.5 U of *Taq* DNA polymerase (Amersham Laboratories, Buckinghamshire, United Kingdom). To confirm the identity of the *eae* PCR products and to distinguish between EHEC and EPEC *eae* genes, restriction enzyme (RE) analysis with *Pst*I and *Hae*III was performed. Total genomic DNA was digested with *Eco*RI and separated by agarose gel electrophoresis. Plasmids were prepared from 30-ml overnight cultures grown in Luria broth by using the Quiagen plasmid midi kit (Diagen, Düsseldorf, Germany). The FAS test was performed as described previously (10). For nucleotide sequence analysis, the *eae* genes of EPEC and EHEC strains were amplified with primer pairs LP1-LP2 and LP1-LP3, respectively, to obtain the 2.8-kb PCR products comprising the entire *eae* genes. PCR prod-

* Corresponding author.

TABLE 1. Sources and phenotypic and genotypic characteristics of FAS test-positive *E. coli* O157 strains used in this study

Strain	Serotype	Disease associated with strain (source or reference)	Sorbitol fermentation	Hybridization with gene probe			PCR result		Classification based on RFLP[a] of *eae* PCR fragments
				slt	CVD 419	EAF	LP1-LP2	LP1-LP3	
589/90	O157:H7	HC (1)	−	+	+	−	−	+	EHEC
356/89	O157:H7	HC (1)	−	+	+	−	−	+	EHEC
4220/87	O157:H7	HC (2)	−	+	+	−	−	+	EHEC
634/88	O157:H7	HC (1)	−	+	+	−	−	+	EHEC
5769/87	O157:H7	HUS (2)	−	+	+	−	−	+	EHEC
703/88	O157:H$^-$	HUS (1)	+	+	+	−	−	+	EHEC
425/88	O157:H$^-$	HUS (1)	+	+	+	−	−	+	EHEC
493/89	O157:H$^-$	HUS (10)	+	+	+	−	−	+	EHEC
817/90	O157:H$^-$	HUS (1)	+	+	+	−	−	+	EHEC
658/91	O157:H$^-$	HUS (10)	+	+	+	−	−	+	EHEC
202/88	O157:H45	ID (10)	+	−	−	+	+	−	EPEC
966/89	O157:H45	ID (10)	+	−	−	+	+	−	EPEC
904/90	O157:H45	ID (1)	+	−	−	−	+	−	EPEC
006/91	O157:H45	ID (10)	+	−	−	+	+	−	EPEC
1083/87	O157:H45	ID (1)	+	−	−	+	+	−	EPEC
159/91	O157:H45	ID (this study)	+	−	−	+	+	−	EPEC
423/92	O157:H45	ID (this study)	+	−	−	+	+	−	EPEC
1324/91	O157:H$^-$	ID (this study)	+	−	−	+	+	−	EPEC
2814/91	O157:H$^-$	ID (this study)	+	−	−	+	+	−	EPEC
3504/92	O157:H$^-$	ID (this study)	+	−	−	+	+	−	EPEC

[a] RFLP, restriction fragment length polymorphism.

ucts were separated on 0.8% agarose gels. Gel slices with the appropriate fragments were excised, and the DNA was purified with a gene clean kit (Dianova, Hamburg, Germany) as described by the manufacturer. One microgram of the double-stranded PCR product was subjected to *Taq* cycle sequencing reactions by using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Darmstadt, Germany). Briefly, 9.5 μl of a terminator premixture (containing 4 μl of reaction buffer, 1 μl of a deoxynucleoside triphosphate mixture, 1 μl of DyeDeoxy A [900 μM], 1 μl of DyeDeoxy T [450 μM], 1 μl of DyeDeoxy G [15 μM], 1 μl of DyeDeoxy C [4 μM], and 0.5 μl [4 U] of AmpliTaq DNA polymerase), template DNA (1 μg), and 3.2 pmol of primer LP3 were mixed in a 0.6-ml reaction tube that was filled with water to a final volume of 20 μl. The tube was placed in a thermal cycler preheated to 96°C and subjected to 25 cycles with the following parameters: 96°C for 15 s, 55°C for 15 s, and 60°C for 4 min. The cycle sequencing products were extracted with phenol-chloroform and ethanol precipitated. The resulting DNA pellets were dissolved in 4 μl of a mixture of formamide and 50 mM EDTA (pH 8.0) at a ratio of 5:1. Separation of sequencing products was performed on 7% denaturing polyacrylamide gels in an A737 automatic sequencer (Applied Biosystems). Sequence analyses were carried out in triplicate with the DNAsis program, version 2.0, from Pharmacia LKB.

The *E. coli* O157 strains listed in Table 1 comprise clinical isolates with and without *slt* genes that all had positive FAS tests. They were therefore subjected to PCR analysis to establish the presence of *eae*. We managed to amplify the complete *eae* gene of EPEC control strain E2348/69 by deploying primer pair LP1-LP2. Likewise, we amplified the complete *eae* gene of EHEC control strain EDL 933 with primer pair LP1-LP3. With the latter primer pair, however, PCR provided a fragment of the expected size only with all of the SLT-producing *E. coli* O157 strains, whereas the nontoxinogenic *E. coli* O157 strains surprisingly indicated a positive PCR result with primer pair LP1-LP2 (Table 1). The FAS-negative *E. coli* O157:H$^-$ H19 and H43 strains did not react at all with any of the primer pairs (data not shown). To establish differentiation within the *eae* genes of the *E. coli* O157 strains listed in Table 1, RE analysis of the PCR products was performed with *Pst*I and *Hae*III. Within the 20 strains, we observed only two restriction fragment length polymorphism profiles with each of these enzymes. All of the SLT-producing O157:H strains had *Pst*I and *Hae*III RE profiles identical to those obtained after analysis with strain EDL 933 (Fig. 1, lanes 1 and 5). The second *eae* RE profile is represented by all of the nontoxinogenic *E. coli* O157 strains and is identical to the profile of strain E2348/69 (Fig. 1, lanes 2 and 6). Two representative *Pst*I and *Hae*III RE analyses of the *eae* genes from nontoxinogenic *E. coli* O157 strains are depicted in Fig. 1, lanes 3 and 4 and 7 and 8. This direct comparison clearly reveals RE pattern identity to the *eae* gene of strain E2348/69, suggesting the presence of two *eae* genotypes among strains of *E. coli* serogroup O157. In addition to PCR and restriction fragment length polymorphism analyses, the nucleotide sequence of a 400-bp stretch of the 3′ end of the *eae* genes was determined for two nontoxinogenic *E. coli* O157 strains. The data from these analyses (Fig. 2) revealed 99.3% homology for the *eae* gene of strain 904/90 and 99.8% homology for the *eae* gene of strain 1083/87 compared with the published nucleotide sequence (9) for this specific region of the *eae* gene of strain E2348/69. In contrast to these results, homologies of only 52.8 and 53.4% for the above-described strains were revealed when they were compared with the published nucleotide sequence (23) of strain EDL 933.

To localize the *eae* genes in nontoxinogenic *E. coli* O157 strains, Southern blot hybridization of total DNA with the *eae* probe was performed. As can be seen from Fig. 3B, lanes 7 to 10, one type of hybridization signal, 7 kb long, was

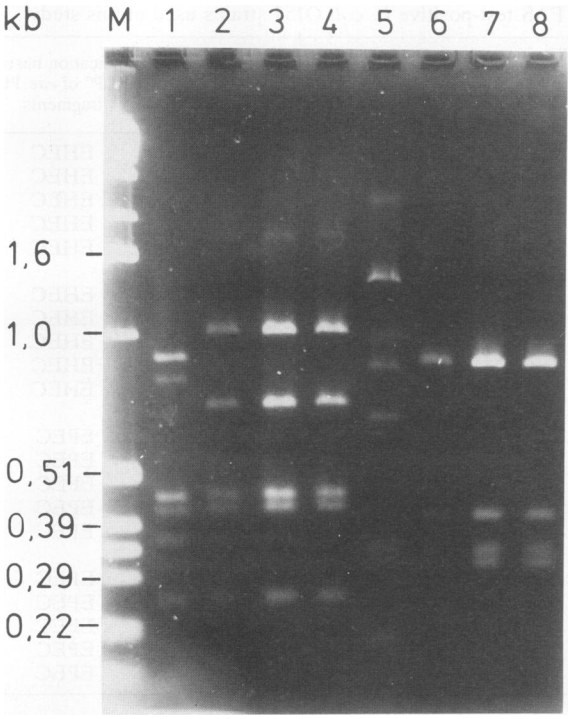

FIG. 1. RE analysis of the complete *eae* genes of EHEC strain EDL 933 with *Pst*I (lane 1) and *Hae*III (lane 5) and EPEC strain E2348/69 with the same enzymes (lanes 2 and 6, respectively). The *Pst*I pattern of *eae* of nontoxinogenic *E. coli* O157 strains 1083/87 and 3504/92 are depicted in lanes 3 and 4, and their respective *Hae*III patterns are in lanes 7 and 8. Lane M contained molecular size markers.

```
E2348/86    5' AATTGCTTCG GTGGATGCTT CTTCTGGTCA GGTCACCTTA AAAGAGAAGG
904/90          ---------- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          T--C--GA-T --C------- -A--A--GA- A-----T--G --T-GT--A-

E2348/86        GAACTACAAC TATTTCCGTT ATCTCAAGTG ATAATCAAAC TGCAACTTAT
904/90          ---------- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          -C-G-GTCGT AATTAAA-CC -CA--TG--- ----G----C A-T--G---C

E2348/86        ACTATTGCAA CACCTAATAG TCTGATTGTT CCTAATATGA GCAAGCGTGT
904/90          ---------- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          -----AAA-G ----GTCGTA -A----AAAA GTGG------ ------C-A-

E2348/86        GACCTATAAT GATGCTGTGA ATACATGTAA GAATTTTGGA GGAAAGTTGC
904/90          ---------- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          ·------GC- ------A-GT CC-TT--C-- A-----ATT- ··········

E2348/86        CGTCTTCTCA GAATGAACTG GAAAATGTCT TTAAAGCATG GGGGGCTGCA
904/90          ---------- ---------- ---------- ---------- -------C--
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          -A--CA-A-- --CG-T-T-G TC-G--A-T- A-G-CT---- ----------

E2348/86        AATAAATATG AATATTATAA GTCTAGTCAG ACTATAATTT CATGGGTACA
904/90          ---------- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          ---------A GCC------G T----TGA-C T-A----C-G -T---A-TA-

E2348/86        ACAAACAGCT CAAGATGCGA AGAGTGGTGT TGCAAGTACA TACGATTTAG
904/90          ---------- -------T-- ---------- ---------- ----------
1083/87         ---------- -------T-- ---------- ---------- ----------
EDL933          ---G---T-- AGT--GCA-C GTTC---A-- AT----C-CT --TA-CC--A

E2348/86        TTAAACAAAA CCCTCTGAAT AATATTAAGG CTAGTGAATC TAATGCTTAT 3
904/90          ------C--- ---------- ---------- ---------- ----------
1083/87         ---------- ---------- ---------- ---------- ----------
EDL933          -A-C------ ------TCC- GGGG----T- T--A-ACTC- A----T----
```

FIG. 2. Comparison of the nucleotide sequences of a 400-bp stretch from the 3′ end of the *eae* structural gene of strain E2348/68 (top line) with those of the *eae* genes of O157:H45 strain 904/90, O157:H45 strain 1083/87, and O157:H7 strain EDL 933. Dashes indicate nucleotides identical to the *eae* gene of strain E2348/69. Dots represent residues for which nucleotides were absent and were inserted with the DNAsis software for better homology.

detected. For categorization as an EPEC strain, besides *eae*, EAF sequences are mostly present (5). We therefore tested the strains listed in Table 1 by colony blot hybridization for EAF and CVD419 sequences; the latter are characteristic of EHEC. Of the 10 nontoxinogenic, FAS-positive *E. coli* O157 strains, 9 hybridized with the EAF probe but not with the CVD419 probe (Table 1). Conversely, all of the SLT-producing O157 strains reacted with the CVD419 probe but not with the EAF probe (Table 1). To locate the EAF sequences, plasmids were isolated, separated by electrophoresis, blotted onto nylon membranes, and hybridized with either the EAF or the CVD419 probe. Figure 4A, B, and C shows the resultant plasmid profiles, the hybridization pattern with the EAF probe, and the hybridization pattern with the CVD419 probe, respectively. A plasmid present in the nontoxinogenic *E. coli* O157 strains, estimated to be approximately 95 kb long hybridized with the EAF probe (Fig. 4B, lanes 1, 3, and 5), whereas hybridization signals with EAF were absent with the 90-kb plasmids (Fig. 4B, lanes 2 and 4) from the two SLT-producing *E. coli* O157 strains used as controls here. On the other hand, with the CVD419 probe, specific signals occurred with the plasmids from the SLT-producing *E. coli* O157 strains (Fig. 4C, lanes 2 and 4) but not with the plasmids from the nontoxinogenic strains (Fig. 4C, lanes 1, 3, and 5). The bands of about 50 kb that hybridized in addition to the large plasmids probably represent other conformations of the same plasmids also present in plasmid preparations of laboratory strains transformed with 90-kb plasmids (11).

It was the search for sorbitol-fermenting SLT-producing *E. coli* O157, which is an important enteric pathogen in Germany (1, 6, 10), that revealed sorbitol-fermenting *E. coli* O157 strains that were nontoxinogenic. Because these strains were obtained from infants suffering from diarrhea and we were unable to find any other pathogens in the stool samples, the question of whether these strains had any potential virulence arose. This was demonstrated by the

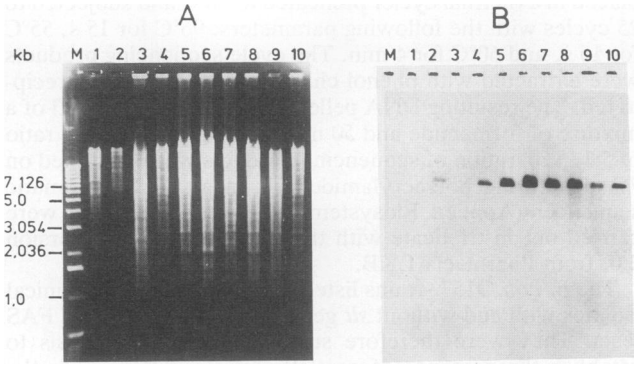

FIG. 3. Agarose gel electrophoresis (A) and Southern blot analysis (B) with the *eae* probe of *Eco*RI-digested total cellular DNA isolated from FAS-negative *E. coli* O157:H19 and H43 strains (lanes 1 and 3), from FAS-positive, SLT-producing O157:H7 strains (lanes 2, 4, 5, and 6), and from FAS-positive but *slt*-negative *E. coli* O157:H45 and O157:H$^-$ strains (lanes 7 to 10). Lanes M contained molecular size markers.

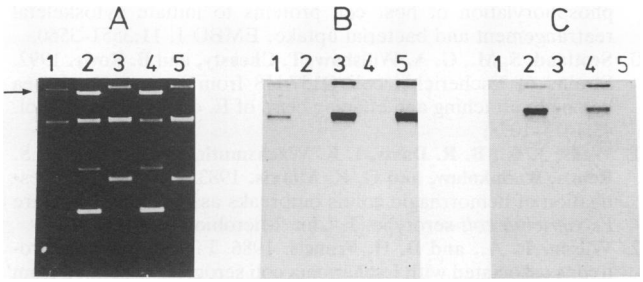

FIG. 4. Agarose gel electrophoresis of plasmids from SLT-producing *E. coli* O157:H7 strains and nontoxinogenic *E. coli* O157:H45 strains (A) and Southern blot analysis with digoxigenin-labeled EAF (B) and CVD419 (C) probes. Lanes: 1, 3, and 5, plasmids from FAS-positive, nontoxinogenic O157 strains; 2 and 4, plasmids from SLT-producing O157 strains. The arrow indicates the location of the 90-kb plasmid.

presence of *eae*, a positive FAS test, and the presence of plasmids harboring EAF sequences. In agreement with the proposal of Donnenberg and Kaper (5) to define EPEC on the basis of the above-mentioned virulence determinants and not by membership in a classical enteropathogenic serotype, we found that nontoxinogenic *E. coli* O157 strains must be categorized as EPEC. Therefore, it is unlikely that they represent isolates that lost their *slt* genes during subcultivation, a phenomenon frequently observed in non-O157 strains (12). The homology of their *eae* genes with the *eae* gene found in EPEC O127:H6 strain E2348/69 supports the concept, over and above the O antigens, that EPEC strains are clonally related organisms that share a high degree of homology in their virulence genes (5). From tissue culture assays, human volunteer studies, and epidemiological findings, there is ample evidence that EAF plasmids are vital for potentiation of diarrheal disease (4, 5, 7, 8, 14, 16). They were shown to be necessary for efficient adherence (7, 16); for enhanced production of the *eae* gene product termed intimin (8), which is involved in attaching-and-effacing lesions; and for internalization by epithelial cells (3, 4), which seems to be induced by tyrosine phosphorylation of eucaryotic proteins (19). Although the clinical significance of invasion in EPEC pathogenesis is not fully clear, in this context it will be of interest to determine whether sorbitol-fermenting *E. coli* O157 strains also possess this capacity. Scotland et al. (20) described nontoxinogenic *E. coli* O157:H8 strains that were FAS test positive. These strains hybridized with the *eae* probe but, in contrast to the strains described here, not with the EAF probe. Our finding of large plasmids in nontoxinogenic *E. coli* O157:H45 strains was also made by Wells et al. (21), who described the first outbreaks of *E. coli* O157:H7. In this context, they compared plasmids of *E. coli* O157:H7 with plasmids of *E. coli* strains of other H types, among them four *E. coli* O157:H45 isolates of which three contained a large plasmid. It is possible that their strains also harbor *eae* and EAF. The absence of the EAF plasmid in 1 of our 10 FAS test-positive *E. coli* O157 strains suggests the loss of this plasmid during subcultivation or in the host, a phenomenon already known in EPEC strains (14).

Until we were aware of EAF and *eae* sequences, we classified sorbitol-fermenting *E. coli* O157 isolates with no demonstrable SLT genes as nonpathogenic. In at least a portion of strains, this diagnosis was misleading. As a consequence, we now perform the *eae* PCR on SLT-negative *E. coli* O157 strains. Since the *eae* genes are stably expressed, in contrast to EAF, this procedure is thought to be slightly more sensitive than EAF detection. Our findings presented here should stimulate further epidemiological research on nontoxinogenic *E. coli* O157 strains to ascertain their importance in ID.

We thank Jürgen Heesemann for helpful discussions and Barbara Plaschke and Beatrix Henkel for excellent technical assistance.

This work was supported by grant Ka-717-2 from the Deutsche Forschungsgemeinschaft.

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