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Hybridization of Strains of *Escherichia coli* O157 with Probes Derived from the *eaeA* Gene of Enteropathogenic *E. coli* and the *eaeA* Homolog from a Vero Cytotoxin-Producing Strain of *E. coli* O157

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A total of 375 *Escherichia coli* O157 strains were tested by colony hybridization with the *eae* probe from the central portion of the *eaeA* gene of the classical enteropathogenic *E. coli* strain E2348/69. They were also tested with a probe, *eae*O157, from the C-terminal end of the *eae* gene homolog from a Vero cytotoxin (VT)-producing strain of *E. coli* (VTEC) of serotype O157:H7. Both probes hybridized with all 246 O157:H7 or H$^-$ VTEC strains tested. The majority were from human infections, and the remainder were from cattle. A further 10 strains (H7 or H$^-$) hybridized with both *eae* and *eae*O157 sequences but not with VT probes. They resembled O157 VTEC and were probably naturally occurring derivatives that had lost VT genes. The remaining 119 strains of O157 were from human, animal, and food sources and belonged to 16 H types other than H7 or were H$^-$. They were VT negative and differed in their properties from O157 VTEC: generally they fermented sorbitol in 1 day, produced β-glucuronidase, and could not be phage typed by the scheme for O157 VTEC. The *eae* probe but not the *eae*O157 sequence hybridized with 18 H8 or H39 strains, predominantly from human diarrhea. The remaining 101 VT-negative strains hybridized with neither probe. However, 16 strains of O157:H45 hybridized with a probe for diffusely adherent *E. coli* and attached to HEp-2 cells in a diffuse pattern. Serogroup O157 comprises strains with heterogeneous properties. The *eae*O157 probe is a valuable addition to the VT probes used to differentiate O157 strains.

Strains of *Escherichia coli* that produce Vero cytotoxin (VT) are associated with disease in humans and animals. Vero cytotoxin-producing *E. coli* (VTEC) has been isolated from human cases of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. VTEC strains belonging to serogroup O157 (O157 VTEC) are most commonly associated with human disease and have the flagellar (H) antigen 7 or are nonmotile (H$^-$) (17). However, VTEC strains of many other serotypes from human, animal, and food sources have been reported (17). VTEC strains produce one or both of the two principal VTs. VT1 is almost identical to Shiga toxin produced by *Shigella dysenteriae* type 1, whereas VT2 is antigenically distinct (28). Strains of O157 VTEC isolated in Britain commonly produce VT2 only, strains elaborating both toxins are found less frequently, and VT1 producers are very rare (34).

Strains of O157 VTEC colonize the intestines of experimentally infected gnotobiotic piglets (36), where they produce attaching and effacing (AE) lesions that are similar microscopically to those caused by some strains of enteropathogenic *E. coli* (EPEC) (19). Formation of AE lesions is a major factor in the pathogenesis of EPEC and has been observed in animal models and human biopsy material (20, 23). The process is characterized by effacement of microvilli at the site of initial localized adherence, followed by intimate attachment involving cytoskeletal changes including accumulation of filamentous actin (9). Production of AE lesions in vitro by using HEp-2 cells forms the basis of the fluorescence actin-staining (FAS) test (19). Formation of AE lesions by EPEC is associated with a chromosomally located gene, *eaeA*, from which a gene probe has been derived (9, 14).

Jerse et al. (14) showed that 29 of 30 VTEC strains belonging to serotype O157:H7 or O26:H11 hybridized with the *eae* probe. In a study of other VTEC serogroups, however, a much smaller proportion of strains (35%) was *eae* positive (40). An *eaeA* gene homolog from O157 VTEC has been cloned and sequenced independently by two groups (5, 44). The gene from the O157 strain was 97% homologous to EPEC *eaeA* over the first 2,200 bp but only 59% homologous over the last 800 bp at the C-terminal end of the sequence. The region corresponding to the *eae* probe was in the central part of the gene within the highly conserved region.

Pathogenic O157 strains that do not produce VT were first described for cases of porcine colibacillosis (24). These organisms were enterotoxigenic and sometimes produced the K88 adhesin. Scotland et al. (31) described another group of VT-negative O157:H45 strains, associated with diarrhea and extraintestinal infections in humans. Strains of serotype O157:H8 have been isolated from cases of diarrhea and possess properties associated with virulence (29). Studies in this and other laboratories have identified several different VT-negative O157 strains from raw beef products, healthy dairy cattle, and beef cattle at slaughter (7, 8, 39, 41). However, the status of such strains as human pathogens is unknown.

We have investigated the distribution of genes associated with the AE property within the O157 serogroup by screening VT-positive and VT-negative strains from human, animal, and food sources for hybridization with the *eae* probe. A probe from the 3′ end of the *eaeA* gene homolog of an O157 VTEC strain has been used to determine whether *eae* probe-positive

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TABLE 1. Strains of *E. coli* O157 that hybridized with the *eae* gene probe but did not produce VT

Strain	H type	Source[a]	Phage type	Utilization of:		Hybridization with DNA probe	
				Sorbitol	MUG	*eae*	*eae*O157
E67085	7	Human D	14	−	−	+	+
E67152	7	Human ?	14	−	−	+	+
E81186	7	Human D	54	−	−	+	+
E81226	7	Bovine	32	−	−	+	+
E83742	7	Bovine	32	−	−	+	+
E85629	7	Human BD	4	−	−	+	+
E81616	NM[b]	Human D	4	−	−	+	+
E83192	NM	Human D	4	−	−	+	+
E81192	NM	Human D	RDNC[c]	+	+	+	+
E78860	NM	Human D	RDNC	+	+	+	+
E34573[d]	8	Human	NT[e]	+	+	+	−
E39080	39	Human D	NT	+	+	+	−
E75218	39	Human D	NT	+	+	+	−
E27023	39	Human	NT	+	+	+	−

[a] D, diarrhea; BD, bloody diarrhea; ?, diarrhea status not known. The bovine strain E81226 was from a healthy animal (8), and E83742 was from a scouring calf (33). Strain E27023 was isolated from urine.
[b] NM, nonmotile (H).
[c] RDNC, Reacts but does not conform. The strain produced a pattern of lysis with the typing phages that did not conform to the pattern of any type recognized at present.
[d] Fourteen other strains of O157:H8 gave identical results. At least 12 of these were from cases of diarrhea.
[e] NT, nontypeable.

strains of serogroup O157 differ from each other within the variable region of the *eaeA* gene.

MATERIALS AND METHODS

Bacterial strains. A total of 375 strains of serogroup O157 comprising 246 VTEC strains and 129 strains that did not produce VT was examined. The VTEC strains possessed H-antigen 7 or were nonmotile (H^-). They included 138 strains from human cases of diarrhea, bloody diarrhea, or hemolytic uremic syndrome, predominantly from the United Kingdom. Five sorbitol-fermenting strains from Germany were provided by S. Aleksic. Some of these strains have been described elsewhere (3, 11, 15, 34). The 64 O157 VTEC strains from cattle were from diseased animals (33) and healthy animals (7, 8; also unpublished data). A panel of 44 reference (type) strains from the phage typing scheme for O157 VTEC was also tested (1, 11, 18). These were provided by H. Lior (Laboratory Centre for Disease Control, Ottawa, Canada).

The 129 VT-negative strains are listed in Tables 1 and 2. They were mainly from the United Kingdom. However, strains E81192 and E81186 were from Austria (32) and E85629 was from Iceland (Table 1); two strains of serotype O157:H10 were isolated in Mexico and provided by A. Cravioto (Table 2). Some of the O157:H8 and O157:H45 strains of human origin have been reported previously (29, 31). VT-negative strains of O157 from cattle included those from an abattoir (7, 8; also unpublished data) and from dairy cattle (39). Some additional porcine strains (31) and isolates from raw beef (41) and water were also examined.

Positive control strains of VTEC for DNA probe tests were E32511 (O157:H^- $VT2^+$ eae^+) and H19 (O26:H11 $VT1^+$ eae^+) (28). The EPEC strain E2347/69 (O127:H6 VT^- eae^+) was a reference strain used in hybridizations with the *eae* and *eae*O157 probes (14, 30). Strains E66438 (O75:H^-) and E60725 (O92:H33) were the respective positive controls in tests with the probes for diffusely adherent (6) and aggregatively adherent (4) *E. coli*.

TABLE 2. Strains of *E. coli* O157 that did not hybridize with either the *eae* or the *eae*O157 probe[a]

H antigen	Source	No. tested	Utilization of:		Hybridization with DA probe	Other properties
			Sorbitol	MUG		
2	Bovine	1	+	+	−	
	Human D	1	+	+	−	
3	Human D	1	+	+	−	
	Human D	1	−	−	−	
10	Human D	2	+	+	−	
11	Human	1	+	+	−	
12	Bovine	9	+	+	−	
	Human D	2	+	+	−	
18	Food	3	+	+	−	
19	Bovine	7	+	+	−	
	Human D	1	+	+	−	
	Porcine	1	+	+	−	LT[b]
30	Human ?D	1	+	+	−	
32	Bovine	1	+	+	−	
34	Food	1	+	+	−	
38	Bovine	7	+	+	−	
42	Bovine	5	+	+	−	
	Food	3	+	+	−	
	Water	1	+	+	−	
43	Bovine	1	+	+	−	
	Porcine	2	+	+	−	LT
45	Bovine	1	+	+	−	
	Human[c]	17	+	+	−, +[d]	
NM	Bovine	15	−, +[e]	+	−	
	Human[f]	12	+	+	−, +[g]	
	Human D	1	+	+	+	PT21[h]
	Human D	2	+	+	−	PT31
	Porcine	1	+	+	−	LT

[a] The strains did not hybridize with the VT probes and unless otherwise stated did not give patterns of lysis with the typing phages that corresponded to patterns of recognized phage types.
[b] LT, heat-labile enterotoxin production.
[c] Eleven strains were fecal isolates from patients with diarrhea, two cultures were from the feces of patients not known to have diarrhea, and four cultures were from extraintestinal sources.
[d] A negative result was obtained for 1 strain; positive results were obtained for 16 strains.
[e] Negative results were obtained for 13 strains; positive results were obtained for 2 strains.
[f] Ten strains were isolated from feces: eight from cases of diarrhea, one from a case of hemolytic uremic syndrome, and one from a patient without diarrhea. Two strains were from extraintestinal sources.
[g] Negative results were obtained for seven strains; positive results were obtained for five strains.
[h] PT, phage type.

Characterization of strains. The strains had been identified biochemically and serotyped with antisera to O antigens 1 to 173 and H antigens 1 to 55 (13). Fermentation of sorbitol within 24 h was tested in Andrade peptone water containing

0.5% sorbitol. Production of β-glucuronidase was assessed by hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG) (27). All strains were tested by the phage typing scheme for *E. coli* O157 VTEC (1, 11, 18).

Detection of *eae* gene sequences. The *eae* gene probe of Jerse et al. (14) comprised a 1-kb *Kpn*I-*Sal*I fragment from the central one-third of the *eae* gene of the EPEC strain E2348/69. A second probe, *eae*O157, was based on the nucleotide sequence of the 3′ end of the *eae* gene homolog of *E. coli* O157 strain CL8 (5). Oligonucleotide primer sequences flanked a 410-bp region at the 3′ end of the gene where homology with the EPEC *eae* gene was low. The upstream primer was 5′GCGACGATAACACTAACTTCC (bases 2366 to 2386), and the downstream primer was 5′GGACATAGCATCAG CATAATAGG (bases 2776 to 2754). An internal oligonucleotide probe, 5′ACTGAAAGCAAGCGGTGGTG (2560 to 2579), was used to verify the identity of the 410-bp fragment.

The *eae*O157 probe was produced by amplification of the 410-bp sequence by PCR, using as a template 2 μl of a log-phase culture of strain CL8. The amplification reaction mixture contained 67 mM Tris-HCl (pH 8.3), 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Tween 20, 1 μM each primer, 200 μM deoxynucleoside triphosphates, and 1 U of *Taq* polymerase. The fragment was purified from a 2% agarose gel by using Qiaex resin (Qiagen) and was labelled with deoxyadenosine 5′-[α-^{35}S]thiotriphosphate by the random primer method. Alternatively, the probe was labelled by PCR with a mixture of digoxigenin-11–dUTP (70 μM) and dTTP (130 μM) before gel purification. The *eae*O157 probe hybridized with O157 VTEC strain E32511 but not with EPEC strain E2347/69, whereas the *eae* probe of Jerse et al. (14) hybridized with both strains.

Detection of other gene sequences. The strains were tested with digoxigenin-labelled VT1 and VT2 probes (35, 42, 43). To detect sequences other than *eae* that may be associated with adhesion, the VT-negative O157 strains were tested with radioactively labelled probes for *E. coli* showing diffuse or aggregative patterns of adherence to HEp-2 cells. The diffuse adherence (DA) probe was a 370-bp *Pst*I fragment (6); the probe for aggregative adhesion was a 1-kb *Eco*RI-*Pst*I fragment (4).

DNA hybridization. The strains were prepared for colony hybridization as described previously (43). Hybridization with digoxigenin-labelled probes was by the method of Thomas et al. (35) except that for the *eae*O157 probe, the final stringency wash was at 68°C for two periods of 30 min each in 0.1× SSC containing 0.1% sodium dodecyl sulfate (1× SSC contained 0.015 M trisodium citrate and 0.15 M sodium chloride, pH 7.0). Radioactively labelled probes were used under the stringent conditions of hybridization and washing described by Maniatis et al. (22).

Adhesion to HEp-2 tissue culture cells. A proportion of strains that hybridized with the DA probe were tested for adherence to HEp-2 cells in culture (30). The FAS test (19) was performed on strains of H types 8 and 39.

RESULTS

Hybridization with the *eae* and *eae*O157 probes subdivided the *E. coli* O157 strains, and results shown in Tables 1 and 2 indicate how this division related to other properties of the organisms.

Strains positive with the *eae* gene probe. All 246 *E. coli* O157:H7 or O157:H$^-$ strains that hybridized with one or more of the VT probes hybridized with the *eae* probe that detects the central portion of the gene homologous to the *eae* gene of EPEC. These O157 VTEC strains were also positive with the *eae*O157 probe directed against the C-terminal end of an *eae* gene homolog from an O157 VTEC strain. Strains belonging to a total of 27 phage types were screened, including at least 10 representatives of the most common types from the United Kingdom and 44 O157 VTEC reference strains. O157 VTEC strains that gave patterns of lysis with the typing phages that did not correspond to patterns for presently recognized types were also tested. Nine O157 VTEC strains were atypical in that they produced urease, and five strains from Germany differed from the majority of O157 VTEC strains, since they fermented sorbitol and utilized MUG (3, 15). However, all these strains possessed the *eae* sequences of O157 VTEC.

Ten O157 strains that did not hybridize with VT sequences were detected with the *eae* and *eae*O157 probes (Table 1). They included isolates from human diarrhea and two strains from cattle feces or rectal swabs. Eight strains had all the characteristics of O157 VTEC except the presence of VT genes. Thus, they were of H type 7 or nonmotile, did not utilize sorbitol or MUG, and could be typed by the phage typing scheme for O157 VTEC. Two strains (E81192 and E78860) hybridized with both *eae* and *eae*O157 probes but fermented sorbitol and utilized MUG. Strain E81192 was isolated in Austria (32) and gave a lysis pattern with the typing phages that was identical to that of sorbitol-positive O157 VTEC strains isolated in Germany (see above). It may thus represent a VT-negative variant of an O157 VTEC strain.

VT-negative strains of O157:H8 and O157:H39 hybridized with the *eae* probe but not with *eae*O157 (Table 1). These serotypes have never been associated with VT production, but the majority of the strains were from cases of diarrhea. Strains of both serotypes showed localized adhesion to HEp-2 cells and were FAS positive. The adhesion of a strain of O157:H8 is shown in Fig. 1A.

Strains negative with the *eae* gene probe. Neither the *eae* nor the *eae*O157 probe hybridized with 101 serogroup O157 strains that were VT negative and belonged to 14 H types other than 7, 8, or 39 or were nonmotile (Table 2). The sources of the strains were diverse. The largest group of strains from humans were of serotype O157:H45 or O157:H$^-$, predominantly from cases of diarrhea but including some isolates from extraintestinal sites. Some strains, such as those of H types 12, 38, and 43, were isolated almost exclusively from animal feces or rectal swabs. Strains from raw beef products belonged to H types 18, 34, and 42.

Consistent with their lack of VT and *eae* genes, the strains generally lacked other properties associated with O157 VTEC. Most isolates fermented sorbitol and were MUG positive. However, a strain of H type 3 from a case of bloody diarrhea failed to utilize sorbitol or MUG, and 13 nonmotile strains from healthy cattle at slaughter were also sorbitol negative but hydrolyzed MUG. The isolates were from a single abattoir (7, 8) and probably represent a single strain. With the exception of three nonmotile strains, the organisms listed in Table 2 did not give recognized patterns of lysis with the typing phages for O157 VTEC.

Some strains possessed other virulence properties or putative virulence markers that further differentiate them from O157 VTEC (Table 2). Three porcine isolates were reported to produce heat-labile enterotoxin (31). The majority of the O157:H45 strains (16 of 17) hybridized with a probe that detects diffusely adherent *E. coli*. All DA probe-positive strains that were tested for adhesion attached in large numbers over the surface of HEp-2 cells (Fig. 1B). Six of the 15 strains of serotype O157:H$^-$ also hybridized with the DA probe (Table 2). They were all of human origin, five from cases of diarrhea

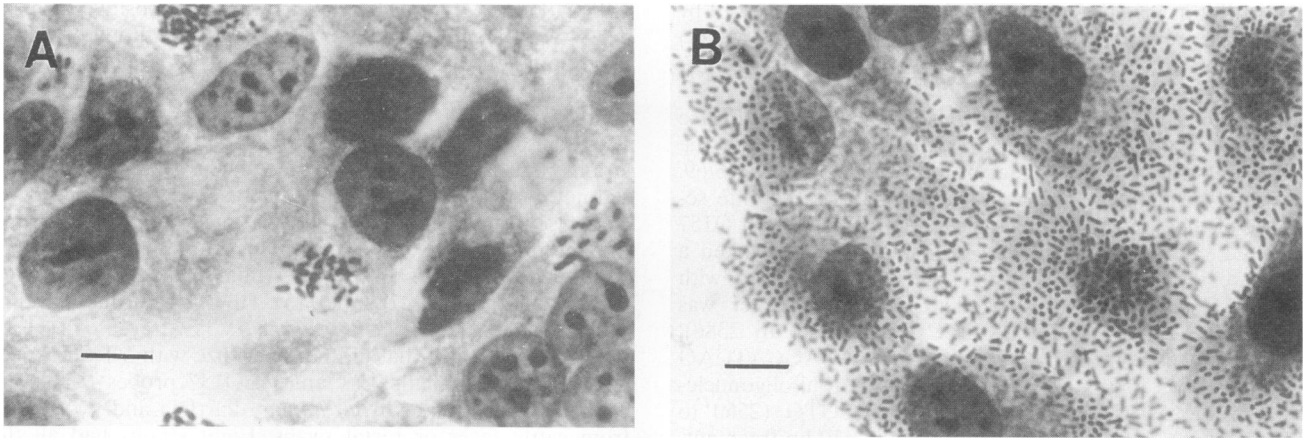

FIG. 1. Adhesion of *E. coli* E72509 serotype O157:H8 (A) and *E. coli* E20579 serotype O157:H45 (B) to HEp-2 cells in a 6-h test. Bar = 10 μm.

and one from urine. All the strains gave a pattern of adherence similar to that shown in Fig. 1B. None of the strains hybridized with a probe for enteroaggregative *E. coli.*

DISCUSSION

The *eaeA* genes of EPEC and O157 VTEC are homologous to the invasin gene of *Yersinia* species (5, 14, 44) and to a gene in a strain of *Citrobacter freundii* associated with AE lesions in mouse colons (25). Homology is greatest in the central part of the gene that, in *Yersinia* species, may be necessary for the stable location of the gene product in the outer membrane (9). There is most divergence at the C-terminal end; this may be related to antigenic variation and receptor binding. Some strains of *Hafnia alvei* from children with diarrhea hybridize with the *eae* probe, produce AE lesions in rabbit intestines, and are FAS positive (2). There may be a family of *eae*-like genes in different bacterial species with analogous roles as virulence determinants. Despite variation in their DNA sequences, the *eaeA* gene products of EPEC and O157 VTEC are functionally interchangeable since the genes complement each other in vivo (10) and in vitro (21).

Results of hybridization with the *eae* and *eae*O157 probes extend previous studies of a limited number of O157 VTEC strains (12) and show that the central and C-terminal regions of the *eaeA* gene are conserved irrespective of the sources, phage types, or unusual biochemical properties of the strains. In the study by Gannon et al. (12), one pair of primers from the 3′ region of the *eaeA* gene of an O157 VTEC strain was relatively specific when used in PCR. There was amplification of a 1,087-bp fragment in O157:H7 or H^- strains but also in one VTEC strain of serogroup O145 and one VT-negative *E. coli* O55 strain. The *eae*O157 probe hybridized with two VTEC strains of serotype O145:H^- and all O55:H7 strains tested, including one that produced VT (unpublished data). Multilocus enzyme electrophoresis demonstrated that the VT-producing clone of O157:H7 was most closely related to a clone of *E. coli* O55:H7 associated with infantile diarrhea (38), and it was predicted that the sequences of the *eae* genes in the two organisms would be very similar.

Ten O157 strains, of the more than 2,000 *E. coli* O157 strains in our collection, carried the *eae* gene of O157 VTEC but were VT negative. They were predominantly from cases of diarrhea. The VT genes are carried on bacteriophages in some O157 VTEC strains and other VTEC strains (17, 42), and the property of toxin production can be unstable on cultivation (16). VT-negative O157 strains that hybridized with both *eae* and *eae*O157 gene probes may be rare, naturally occurring derivatives of VT-producing organisms. Both VT-positive and VT-negative O157 strains of phage type 32 were recovered from cattle in the same studies (7, 8, 33). A derivative of an O157 VTEC strain that no longer produced VT still caused diarrhea in a piglet model (37). Thus, such derivatives may retain their diarrheagenic potential for humans.

Although strains of serotypes O157:H8 and O157:H39 hybridized with the *eae* probe, the C-terminal end of the gene differed from that in O157 VTEC. Strains of O157:H8 have been shown to be FAS positive (29), and this property is shared by the H39 strains. However, AE lesions in the intestines of experimental animals have not yet been demonstrated. Other probes based on the variable region of the *eaeA* gene might establish the relatedness of the gene in O157:H8 and H39 strains to the gene in classical EPEC strains such as E2348/69. The properties of strains of O157:H8 indicate that they may cause human diarrhea (29), but too few O157:H39 strains have been isolated for their importance to be assessed.

VT-negative O157 strains that did not hybridize with the *eae* probes belonged to 14 different H types. Those of H type 45 were most commonly isolated from humans, and the majority of them carried genes encoding DA to HEp-2 cells. More than 40% of the nonmotile strains from humans also possessed this putative virulence factor, and some could derive from strains of H type 45, although we have no evidence that DA probe-positive strains can be grouped on the basis of any other property. The DA probe-positive strains differ from a group of VT-negative O157:H45 and H^- strains from Germany that were FAS positive but possessed the *eae* gene of EPEC rather than that of O157 VTEC and carried the EPEC adherence factor plasmid EAF (26). O157 strains with a variety of H antigens including 12, 19, 38, and 42 and isolated from foods, cattle, carcasses, and drain swabs have been reported (8, 39, 41; also unpublished data). The organisms are negative in current tests for putative virulence factors, and their pathogenic potential for humans is unknown.

Hybridization with a probe such as *eae*O157 is valuable in the differentiation of O157 strains. Probing of colonies from food or fecal specimens with VT and *eae*O157 sequences in combination targets O157 VTEC, whereas techniques based on immunological detection of the O157 antigen identify all strains of this serogroup and some cross-reacting organisms

(41). Although it appears that the C-terminal regions of the *eae* gene may be very similar in VTEC strains of serogroups O157, O55, and O145, VT-producing strains of the latter two groups are rare. A probe such as *eae*O157 would be of practical value in discriminating O157 VTEC, provided that the identity of VT-positive isolates were confirmed serologically.

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