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Role of the *Escherichia coli* O157:H7 O Side Chain in Adherence and Analysis of an *rfb* Locus

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Shiga-toxigenic *Escherichia coli* strains belonging to serotype O157 are important human pathogens, but the genetic basis of expression of the O157 antigen and the role played by the lipopolysaccharide O side chain in the adherence of this organism to epithelial cells are not understood. We performed Tn*phoA* mutagenesis on *E. coli* O157:H7 strain 86-24 to identify a mutant (strain F12) deficient in O-antigen expression. Nucleotide sequence analysis demonstrated that the transposon inserted within an open reading frame with significant homology to *rfbE* of *Vibrio cholerae* O1 (U. H. Stroeher, L. E. Karageorgos, R. Morona, and P. A. Manning, Proc. Natl. Acad. Sci. USA 89:2566–2570, 1992), which is postulated to encode perosamine synthetase. This open reading frame was designated *rfbE*$_{EcO157:H7}$. The guanine-plus-cytosine fraction (0.35) suggests that *rfbE*$_{EcO157:H7}$ may have originated in a species other than *E. coli*. *rfbE*$_{EcO157:H7}$ is conserved in nontoxigenic *E. coli* O157 strains expressing a variety of other flagellar antigens but is not found in *E. coli* O55:H7 strains, which are more closely related to *E. coli* O157:H7. Strain F12 was significantly more adherent to HeLa cells in a quantitative adherence assay than was its *E. coli* O157:H7 parent, but they did not differ in other phenotypes. Restoration of the expression of the O side chain by complementation of the Tn*phoA* mutation in strain F12 by a plasmid expressing intact *rfbE*$_{EcO157:H7}$ reduced the adherence of the hyperadherent strain F12. We conclude that *rfbE*$_{EcO157:H7}$ is necessary for the expression of the O157 antigen, that acquisition of *E. coli rfb* genes occurred independently in *E. coli* O157:H7 and unrelated O157 strains, and that the O side chain of *E. coli* O157:H7 lipopolysaccharide interferes with the adherence of *E. coli* O157:H7 to epithelial cells.

Shiga-toxigenic *Escherichia coli* strains expressing lipopolysaccharide (LPS) O-antigen 157 are important human enteric pathogens. Many *E. coli* strains produce Shiga toxins, but Shiga-toxigenic *E. coli* strains possessing this particular O antigen are the pathogens most frequently isolated from humans in North America and are the predominant cause of hemolytic uremic syndrome (34).

Sorbitol-nonfermenting *E. coli* O157:H7 and sorbitol-fermenting *E. coli* O157:NM (15) are clonally related on the basis of having similar or identical isoenzyme forms detected by multilocus enzyme electrophoresis (42, 43). *E. coli* O55:H7 is the serotype most closely related to *E. coli* O157:H7 and *E. coli* O157:NM by this analysis; 6-phosphogluconate dehydrogenase (EC 1.1.1.44), encoded by the *gnd* locus which is adjacent to the *rfb* region in *E. coli* (2), is the only differentiating electromorph among 20 enzymes studied. In contrast, *E. coli* O157 strains expressing flagellar antigens other than H7 are distantly related to *E. coli* O157:H7 (42, 43); *E. coli* O157 with non-H7 flagellar antigens display multiple polymorphisms for each of these 20 different enzymes and do not express Stx1, Stx2, or intimin.

We performed Tn*phoA* mutagenesis of *E. coli* O157:H7 to identify a mutant deficient in expression of the O157 antigen for the purposes of (i) identifying *rfb* loci of this serotype, (ii) determining if these sequences are conserved in *E. coli* O157 expressing different flagellar antigens and in *E. coli* O55:H7, and (iii) assessing the role played by the O side chain of *E. coli* O157:H7 in the adherence of this organism to epithelial cells in vitro.

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(These data were presented in part at the 95th General Meeting of the American Society for Microbiology [4a].)

MATERIALS AND METHODS

Bacterial strains used. *E. coli* O157:H7 strain 86-24 was used as the prototype for these studies (14, 35). A spontaneously occurring nalidixic acid-resistant mutant of this organism (designated *E. coli* O157:H7 86-24^{nalR}) was isolated from Luria-Bertani (LB) (29) agar plate containing nalidixic acid (20 mg/liter), onto which the centrifuged bacterial contents of 1 ml of overnight LB broth culture of *E. coli* O157:H7 86-24 were spread.

E. coli O157 strains were studied to determine the conservation of the *E. coli* O157:H7 *rfb* locus. Eighteen *E. coli* O157:H7 strains were analyzed, including 17 strains from a previous study (35) and the outbreak strain from the 1993 multistate *E. coli* O157:H7 epidemic (3). Five strains of *E. coli* O55:H7 and four strains of *E. coli* O157:H43 were obtained from Thomas Whittam. Seven strains of *E. coli* O157 expressing H antigens 3 (n = 1), 12 (n = 1), 16 (n = 3), 38 (n = 1), and 45 (n = 1) were kindly provided by Peter Feng. A sorbitol-fermenting, Stx2-producing *E. coli* O157:NM strain from a German patient with hemolytic uremic syndrome was contributed by Lothar Beutin. Except for this last strain, none of the non-H7 *E. coli* O157 strains studied produced Stx1 or Stx2. DNAs from non-O157 Shiga-toxigenic *E. coli* strains isolated from humans (7) were also probed.

E. coli SM10 (λ-pir) transformed with pRT733 (36), a suicide vector into which Tn*phoA* has been cloned, was used for conjugative introduction of Tn*phoA* into *E. coli* O157:H7 86-24^{nalR}. *E. coli* B171 (26), a nontoxigenic enteropathogenic strain of serotype O111:NM, *E. coli* O26:H11, which produces Stx1 but not Stx2 (35), *E. coli* O55:H7, and *E. coli* HB101 (29) were used to study the expression of a cloned *E. coli* O157:H7 *rfb* locus in various *E. coli* strains.

Tn*phoA* mutagenesis. *E. coli* SM10 (λ-pir) containing pRT733 was mated with *E. coli* O157:H7 86-24^{nalR}. Sixty separate matings produced kanamycin- and nalidixic acid-resistant transconjugants, of which approximately 0.5% expressed alkaline phosphatase (PhoA), as indicated by the presence of blue colonies when plated on agar containing phosphate (to suppress endogenous PhoA activity) and 5-bromo-4-chloro-3-indolylphosphate (X-P), a chromogenic substrate of PhoA. A total of 3,000 transconjugants (approximately 50 from each mating) were frozen in LB broth–15% glycerol at −70°C until analysis. To determine if the O157 antigen was retained by these mutants, transconjugants were thawed, grown on LB agar containing nalidixic acid (20 mg/liter) and kanamycin (37.5 mg/liter), and tested individually for expression of this antigen by using the Oxoid (Unipath) latex particle agglutination test.

DNA cloning and sequencing strategy. The region of the Tn*phoA* insertion in the mutant of interest was first mapped by hybridization to identify restriction sites in the DNA flanking the transposon. Size-selected genomic DNA fragments from *E. coli* O157:H7 strain F12 were ligated into the corresponding sites in plasmid Bluescript SK$^+$ (pSK+) (Stratagene) to clone the DNA flanking the Tn*phoA* insertion. A 6.6-bp fragment of DNA extending from the *Bam*HI site of Tn*phoA* to the *Eco*RV site in DNA adjacent to the IS*50*L flank of Tn*phoA* was ligated into corresponding sites in pSK+. Similarly, a 7.3-kb fragment of DNA extending from *Cla*I sites in Tn*phoA* and in DNA adjacent to the IS*50*R region was ligated into the *Cla*I site of pSK+. These fragments were selected by plating transformed *E. coli* HB101 on LB agar containing kanamycin.

Initial sequence from the cloned DNA adjacent to the IS*50*L region of Tn*phoA* was obtained by using a primer (5′GTAAAACGACGGCCAGT3′) complementary to vector sequences adjacent to the cloning site, single-stranded DNA template created with the VCS-M13 helper phage (Stratagene), and the dideoxy-chain termination procedure (30) with Sequenase (U.S. Biochemicals). All oligonucleotides were purchased from GenSet. The initial sequence from the DNA adjacent to the right flank of Tn*phoA* was obtained by using Sequenase, double-strand sequencing techniques suggested by the manufacturer, and primer oligonucleotides complementary to IS50R (5′CGGCCGCACGATGAAGAGC3′) and M13 reverse primer (5′GGAAACAGCTATGACCATG3′) sequences of pSK+.

After sequence analysis of the cloned DNA originating from *E. coli* O157:H7, we performed PCR with 5′GGGGATCCTAATCTTCTGGCATGATTGATTG GC3′ and 5′GGGAATTCTTTACAATTCCACCGCCCCACTCG3′ (incorporating *Bam*HI and *Eco*RI sites, respectively, for cloning purposes) as primers, which were derived from the cloned DNA furthest from Tn*phoA* in the regions adjacent to the left and right flanks, respectively. This amplified PCR product was cloned into the respective sites on pSK+ and sequenced by double-strand sequencing techniques with the *Taq* DyeDeoxy cycle-sequencing kit (Applied Biosystems) under the conditions recommended by the manufacturer, with appropriate intervening primers and an Applied Biosystems model 373A DNA sequencing unit (Molecular Pharmacology Unit, University of Washington School of Medicine). Resulting sequences were confirmed by double-strand sequencing of the cloned DNA flanking the Tn*phoA* insertion in strain F12 and compared with databases reached through the National Center for Biotechnology Information Geninfo BLAST network server (12).

The gene into which Tn*phoA* inserted was amplified from parental *E. coli* O157:H7 by PCR with oligonucleotides 5′GGGGATCCAAAAGGGTTAACT GTTATGTTGTACTGC3′ and 5′GGGAATTCTTTACAATTCCACCGCCCC ACTCG3′ (incorporating *Bam*HI and *Eco*RI sites, respectively, for ligation purposes) as primers and cloned into pSK+. The resulting construct was designated pF12.

LPS analysis. Bacterial LPS from equivalent numbers of organisms was extracted with hot phenol (16), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide), and visualized by silver staining (37). Electrophoretically separated LPS was transferred to Immobilon-P (Millipore), which was then preadsorbed in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (Sigma), and probed with polyclonal rabbit antibody to the O157 antigen (a gift of Joy Wells) diluted 1:100 in PBS–3% BSA. Immunoreactive LPS was identified by incubation of the membrane with goat anti-rabbit antibody coupled to horseradish peroxidase (Sigma) and the enhanced chemiluminescence substrate (Amersham) followed by autoradiography.

Outer membrane protein analysis. Bacterial outer membrane proteins were isolated by the method of Achtman et al. (1) after overnight growth under the same conditions as used for bacteria in the adherence assays. A 20-μg sample of protein from each strain was analyzed in separate lanes of SDS-PAGE (12% polyacrylamide) gels. The resulting separated proteins were visualized by Coomassie blue staining.

Southern hybridization. DNA was extracted from mutant and wild-type *E. coli* (28), digested, electrophoretically separated in 0.7% agarose gels in 0.5× Tris-borate-EDTA, transferred to a Nytran membrane (Schleicher & Schuell), and probed. Membranes were prehybridized in 5× SSC (1× SSC is 0.015 M sodium citrate and 0.15 M NaCl)–1 mM EDTA–0.1% 5× Denhardt's solution–100 mg of sonicated and heat denatured calf thymus DNA per ml before the addition of probe. All probes were labelled with the Megaprime DNA-labelling system (Amersham) and [α-^{32}P]dCTP (New England Nuclear Research Products). The probes used were a 0.7-kb *Sma*I-*Pst*I fragment from the Tn*5* central region of Tn*phoA* and a 1.3-kb fragment corresponding to the complete gene into which Tn*phoA* inserted in the mutant of interest (produced as a PCR-derived clone [described above]).

Phenotypic analysis of the mutant of interest. The mutant which no longer expressed the O157 antigen was tested for phenotypes associated with the parental *E. coli* O157:H7 strain, including the production of a cytotoxin for Vero cells (11), expression of the H7 antigen (kindly performed by Jay Lewis, Washington State Department of Health), and inability to ferment sorbitol when plated on sorbitol-MacConkey agar (Prepared Media Laboratory) (41) or to produce β-glucuronidase from 4-methylumbelliferyl-β-D-glucuronide (Sigma), a fluorogenic substrate of this enzyme (27).

Adherence assay. HeLa cells grown to confluence at 37°C in 5% CO_2 in minimal essential medium containing 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) were trypsinized, diluted, added to glass chamber slides, and reincubated. The adherence assay was performed 2 days later, when the cells were approximately 80% confluent.

To assay bacterial adherence, the cells in each chamber were washed with sterile PBS and covered with 0.6 ml of incubation medium (minimal essential medium, 5% fetal calf serum, 2 mM L-glutamine, nonessential amino acids, 0.5% D-mannose). A 20-μl portion of bacteria which had been grown overnight with agitation in LB broth at 37°C was added to the HeLa cells. Bacterial strains which had not been transformed with pSK+ were incubated at 37°C in 5% CO_2 for 2 h. The chambers were then washed three times with sterile PBS, covered with 0.6 ml of incubation medium, and incubated again for 2 h (toxic effects from strain F12 were noted with longer incubation periods). Bacterial strains which had been transformed with pSK+ were incubated with cells in the presence of ampicillin (100 mg/liter), and the incubation periods were 3 and 3 h (strain F12 transformed with pSK+ was not as cytocidal as strain F12, permitting the longer incubation periods). Nonadherent bacteria were removed by 10 washes of the chambers with PBS from a plastic wash bottle at the end of the assays.

Washed cells and adherent bacteria were fixed by the addition of 100% methanol to each chamber for 5 min, stained with Giemsa stain for 60 min, mounted, and examined for adherence. Positive and negative controls were locally adherent enteropathogenic *E. coli* B171, and nonadherent laboratory strain *E. coli* ORN172, an *E. coli* K-12 derivative from which cryptic type 1 pilus genes had been deleted (44), respectively. Five fields were examined in each chamber by an observer unaware of the identity of the organisms which had been added to each chamber, and the HeLa cells and adherent clusters (five or more bacilli per cluster) were enumerated. The values from at least eight experiments were used to determine the adherence ratio, which is expressed as the mean number of clusters (± standard deviation) per cell. The significance of differences between means was determined by the two-tailed *t* test.

RESULTS

Identification of a Tn*phoA* mutant of *E. coli* O157:H7 deficient in the expression of the O157 LPS antigen. A total of 3,000 Tn*phoA* mutants of *E. coli* O157:H7 were tested individually for the loss of the O157 antigen. A single mutant, designated F12, which expressed PhoA at a low level (manifested by a faint blue color when the mutant was plated on agar containing X-P), failed to react in the O157 latex particle agglutination test. Southern blot analysis demonstrated that strain F12 had sustained a single Tn*phoA* insertion in a 20-kb chromosomal *Mlu*I fragment (data not shown). Strain F12 neither fermented sorbitol after overnight incubation on sorbitol MacConkey agar nor produced β-glucuronidase but was motile, expressed the H7 antigen, and elaborated a cytotoxin for Vero cells; these phenotypes are identical to those of *E. coli* O157:H7 strain 86-24 and *E. coli* O157:H7 strain 86-24$^{\mathrm{nalR}}$, from which F12 was derived.

Cloning and sequencing of the DNA adjacent to the Tn*phoA* insertion in strain F12. (1.7 and 0.6 kb) of DNA flanking the IS*50*L and IS*50*R regions, respectively, of the Tn*phoA* insertion in strain F12 were sequenced. Tn*phoA* had inserted into an open reading frame (ORF) beginning with an ATG codon 6 bp 3′ to a Shine-Dalgarno sequence, extending to a stop codon (TAG) 1,095 nucleotides downstream (Fig. 1). This ORF encodes a putative polypeptide of 364 amino acids with a predicted molecular mass of 41,552 Da. Analysis of the deduced amino acid sequence (39) failed to demonstrate a probable cleavage site for a signal peptide. The Tn*phoA* insertion site in strain F12 is noted by an arrow in Fig. 1.

The protein encoded by the ORF into which Tn*phoA* inserted has extensive homology to the probable perosamine synthetase encoded by *rfbE* of *Vibrio cholerae* O1 (33) (Fig. 2). Like *V. cholerae*, the LPS of *E. coli* O157:H7 contains perosamine (22). Because perosamine, an unusual component of bacterial LPS, is found in the side chains of both organisms and because of the extensive homology between RfbE of *V. cholerae* O1 and the protein encoded by the ORF into which Tn*phoA* inserted in strain F12, we have designated this *E. coli* O157:H7 ORF *rfbE*$_{\mathrm{EcO157:H7}}$. Of the 364 amino acids in RfbE$_{\mathrm{EcO157:H7}}$, 197 (54.1%) can be exactly aligned with the probable perosamine synthetase of *V. cholerae* O1: an addi-

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GGGTTAACTGTTATGTTGTACTGCTTCATTTTTATATATATTGTAAATTACTTTATATGGTATAAATGTAGTTTTAAAAAACATATCGATAGACAGTTAA

ATATAAGAGGATGAAAATGAAATATATACCAGTTTACCAACCGTCATTGACAGGAAAAGAAAAAGAATATGTAAATGAATGTCTGGACTCAACGTGGATT
                 M  K  Y  I  P  V  Y  Q  P  S  L  T  G  K  E  K  E  Y  V  N  E  C  L  D  S  T  W  I

TCATCAAAAGGAAACTATATTCAGAAGTTTGAAAATAAATTTGCGGAACAAAACCATGTGCAATATGCAACTACTGTAAGTAATGGAACGGTTGCTCTTC
S  S  K  G  N  Y  I  Q  K  F  E  N  K  F  A  E  Q  N  H  V  Q  Y  A  T  T  V  S  N  G  T  V  A  L  H

ATTTAGCTTTGTTAGCGTTAGGTATATCGGAAGGAGATGAAGTTATTGTTCCAACACTGACATATATAGCATCAGTTAATGCTATAAAATACACAGGAGC
 L  A  L  L  A  L  G  I  S  E  G  D  E  V  I  V  P  T  L  T  Y  I  A  S  V  N  A  I  K  Y  T  G  A

CACCCCCATTTTCGTTGATTCAGATAATGAAACTTGGCAAATGTCTGTTAGTGACATAGAACAAAAAATCACTAATAAAACTAAAGCTATTATGTGTGTC
 T  P  I  F  V  D  S  D  N  E  T  W  Q  M  S  V  S  D  I  E  Q  K  I  T  N  K  T  K  A  I  M  C  V

CATTTATACGGACATCCATGTGATATGGAACAAATTGTAGAACTGGCCAAAAGTAGAAATTTGTTTGTAATTGAAGATTGCGCTGAAGCCTTTGGTTCTA
H  L  Y  G  H  P  C  D  M  E  Q  I  V  E  L  A  K  S  R  N  L  F  V  I  E  D  C  A  E  A  F  G  S  K

AATATAAAGGTAAATATGTGGGAACATTTGGAGATATTTCTACTTTTAGCTTTTTTGGAAATAAAACTATTACTACAGGTGAAGGTGGAATGGTTGTCAC
 Y  K  G  K  Y  V  G  T  F  G  D  I  S  T  F  S  F  F  G  N  K  T  I  T  T  G  E  G  G  M  V  V  T
                                                         ↓
GAATGACAAAACACTTTATGACCGTTGTTTACATTTTAAAGGCCAAGGATTAGCTGTACATAGGCAATATTGGCATGACGTTATAGGCTACAATTATAGG
 N  D  K  T  L  Y  D  R  C  L  H  F  K  G  Q  G  L  A  V  H  R  Q  Y  W  H  D  V  I  G  Y  N  Y  R

ATGACAAATATCTGCGCTGCTATAGGATTAGCCCAGTTAGAACAAGCTGATGATTTTATATCACGAAAACGTGAAATTGCTGATATTTATAAAAAAAATA
M  T  N  I  C  A  A  I  G  L  A  Q  L  E  Q  A  D  D  F  I  S  R  K  R  E  I  A  D  I  Y  K  K  N  I

TCAACAGTCTTGTACAAGTCCACAAGGAAAGTAAAGATGTTTTTCACACTTATTGGATGGTCTCAATTCTAACTAGGACCGCAGAGGAAAGAGAGGAATT
 N  S  L  V  Q  V  H  K  E  S  K  D  V  F  H  T  Y  W  M  V  S  I  L  T  R  T  A  E  E  R  E  E  L

AAGGAATCACCTTGCAGATAAACTCATCGAAACAAGGCCAGTTTTTTACCCTGTCCACACGATGCCAATGTACTCGGAAAAATATCAAAAGCACCCTATA
 R  N  H  L  A  D  K  L  I  E  T  R  P  V  F  Y  P  V  H  T  M  P  M  Y  S  E  K  Y  Q  K  H  P  I

GCTGAGGATCTTGGTTGGCGTGGAATTAATTTACCTAGTTTCCCCAGCCTATCGAATGAGCAAGTTATTTATATTTGTGAATCTATTAACGAATTTTATA
A  E  D  L  G  W  R  G  I  N  L  P  S  F  P  S  L  S  N  E  Q  V  I  Y  I  C  E  S  I  N  E  F  Y  S

GTGATAAATAGCCTAAAATATTGTAAAGGTCATTCATGAAAATTGCGTTGAATTCAGATGGATTTTACGAGTGGGGCGGTGGAATTG
  D  K
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FIG. 1. Complete nucleotide sequence of inserted DNA in pF12, with deduced amino acid sequences. The Shine-Dalgarno sequence is underlined. The arrow indicates the site of the Tn*phoA* insertion in strain F12.

tional 135 (37%) of the amino acids of $\mathrm{RfbE}_{\mathrm{EcO157:H7}}$ can be conservatively matched to residues in this *V. cholerae* O1 enzyme. There is 62% homology at the nucleotide level between *rfbE*$_{\mathrm{EcO157:H7}}$ and *rfbE* of *V. cholerae* O1 (results not shown).

Complementation of the Tn*phoA* insertion in *rfbE*$_{\mathrm{EcO157:H7}}$ of strain F12 with *rfbE*$_{\mathrm{EcO157:H7}}$. A DNA fragment encoding the *E. coli* O157:H7 *rfbE* locus was amplified via PCR with primers derived from sequences flanking this gene, and inserted into pSK$^+$. The resulting construct, pF12, when introduced into strain F12, complemented the Tn*phoA* mutation, as evidenced by restored reactivity in the latex particle agglutination test.

LPS analysis of *E. coli* O157:H7 strain 86-24 and derivatives. The LPS profiles of *E. coli* O157:H7 and derivatives are presented in Fig. 3A. Tn*phoA* mutant strain F12, which does not react in the latex particle agglutination test, does not express detectable O side chain on immunoblot analysis, although the core LPS is still expressed in strain F12. LPS from only the strains reactive in the O157 latex particle agglutination test (*E. coli* O157:H7 strain 86-24, *E. coli* O157:H7 strain 86-24$^{\mathrm{nalR}}$, and strain F12 transformed with pF12) display the high-molecular-weight ladder representing O-polysaccharide side chain. When immobilized LPS was probed with antibodies to the O157 antigen, LPS from strain F12 demonstrated no reactive antigens in the core region of the gels (Fig. 3B).

LPS purified from *E. coli* O26:H11, *E. coli* O55:H7, and *E. coli* O111:NM (strain B171) transformed with pF12 failed to express O side chains recognized by antibody to the O157

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RfbE_O157 1 MKYIPVYQPSLTGKEKEYVNECLDSTWISSKGNYIQKFENKFAEQNHVQY 50
               ||||:|||.|.|:.|:|:|:||.|:||:|.||::||..|||   .|..
RfbE_VC   1 ..MIPVYEPSLDGNERKYLNDCIDSGWVSSRGKYIDRFETEFAEFLKVKH 48

         51 ATTVSNGTVALHLALLALGISEGDEVIVPTLTYIASVNAIKYTGATPIFV 100
            |||||||||||||: ||||.:||||||||:||:||||.|   .|| |:|.
         49 ATTVSNGTVALHLAMSALGITQGDEVIVPTFTYVASVNTIVQCGALPVFA 98

        101 DSDNETWQMSVSDIEQKITNKTKAIMCVHLYGHPCDMEQIVELAKSRNLF 150
            : :.|.:|:||.|:..||..||||:|.||:||::||:: : :|...:.|:
         99 EIEGESLQVSVEDVKRKINKKTKAVMAVHIYGQACDIQSLRDLCDEHGLY 148

        151 VIEDCAEAFGSKYKGKYVGTFGDISTFSFFGNKTITTGEGGMVVTNDKTL 200
            :||||||||:|.   .|| ||||||:||||||||||||.||||||||.|...:
        149 LIEDCAEAIGTAVNGKKVGTFGDVSTFSFFGNKTITSGEGGMVVSNSDII 198

        201 YDRCLHFKGQGLAVHRQYWHDVIGYNYRMTNICAAIGLAQLEQADDFISR 250
            .|:||::|.||:.. :.||||:::|||||||:|||||:||||..|.:|.
        199 IDKCLRLKNQGVVAGKRYWHDLVAYNYRMTNLCAAIGVAQLERVDKIIKA 248

        251 KREIADIYKKNINSL.VQVHKESKDVFHTYWMVSILTRTAEE..REELRN 297
            ||:||:||:.::.:| :||||||.:.||.||:.||:    . |   |::|..
        249 KRDIAEIYRSELAGLPMQVHKESNGTFHSYWLTSIILDQEFEVHRDGLMT 298

        298 HLADKLIETRPVFYPVHTMPMYSEKYQK..HPIAEDLGWRGINLPSFPSL 345
             |.:. ||.||.|||.||:|||..   :|    |:.:..: ||||||||:|:|
        299 FLENNDIESRPFFYPAHTLPMYEHLAEKTAFPLSNSYSHRGINLPSWPGL 348

        346 SNEQVIYICESINEFYSDK 364
            :::||  ||::|.:::.
        349 CDDQVKEICNCIKNYFNCI 367
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FIG. 2. Homology between $\mathrm{RfbE}_{\mathrm{EcO157:H7}}$ and the probable perosamine synthetase of *V. cholerae* O1. Amino acids are aligned by using the Genetics Computer Group sequence analysis software package (10). Exact matches between aligned amino acids are denoted by vertical lines. Double and single dots represent amino acid pairs with a comparison value of ≥0.50 and 0.10 to 0.50, respectively (13). Gaps introduced in the sequence to optimize alignment are indicated by dots between adjacent residues.

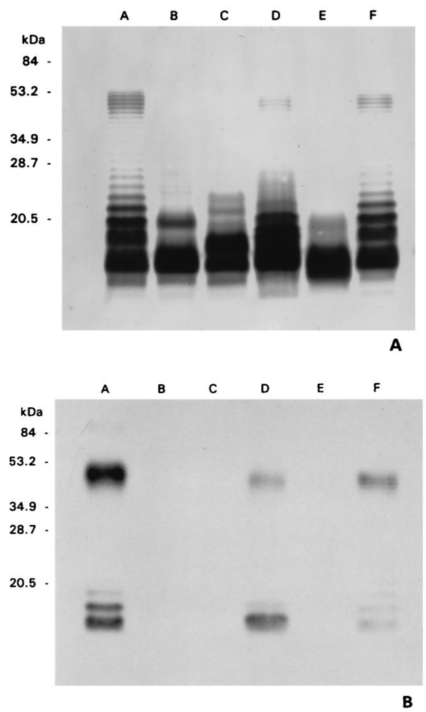

FIG. 3. LPS analysis of *E. coli* O157:H7 and derivatives. LPS from *E. coli* O157:H7 strain 86-24^{nalR} (lane A), Tn*phoA* mutant F12 (lane B), strain F12 transformed with pSK+ (lane C), strain F12 transformed with pF12 (lane D), *E. coli* HB101 transformed with pSK+ (lane E), and *E. coli* O157:H7 strain 86-24 (lane F) were separated electrophoretically, visualized with silver stain (A) and transferred to Immobilon-P and probed with antibody to the O157 antigen (B).

antigen when analyzed by immunoblotting of electrophoresed, immobilized antigen (data not shown).

Conservation of the *E. coli* O157:H7 *rfbE*$_{EcO157:H7}$ locus. We used as a probe a fragment corresponding to *rfbE*$_{EcO157:H7}$ to determine the conservation of this gene in *E. coli* O157:H7 and other *E. coli* strains. Homology was detected on a 6.5-kb *Eco*RI fragment of chromosomal DNA in each of 18 *E. coli* O157:H7 strains tested (data not shown); in each of 11 *E. coli* strains of serogroup O157 expressing H antigens 3, 12, 16, 38, 43, and 45; and in a nonmotile, sorbitol-fermenting, Stx2-producing *E. coli* O157 strain (Fig. 4). One of the *E. coli* O157:H16 strains contained a region of homology to *rfbE*$_{EcO157:H7}$ in a slightly smaller *Eco*RI fragment (Fig. 4, lane L). Homology to *rfbE*$_{EcO157:H7}$ was not detected in five strains of *E. coli* O55:H7 (Fig. 4) or in five strains of Stx-producing *E. coli*, isolated from Seattle children, which expressed a variety of O and H antigens, including *E. coli* O26 (data not shown).

Adherence properties of *E. coli* O157:H7 and O-antigen-deficient strain F12. Table 1 demonstrates the quantitative adherence characteristics of *E. coli* O157:H7 compared with those of its O-antigen-deficient mutant. *E. coli* O157:H7 strain 86-24^{nalR} adheres sparsely to epithelial cells; strain F12 adheres approximately sevenfold better (Fig. 5). The hyperadherence of strain F12 was significantly diminished by the introduction of pF12, which partially restores the O157 side chain antigen (Table 1; Fig. 5). *E. coli* ORN172 was completely nonadherent in these assays.

OMP analysis of *E. coli* O157:H7 86-24^{nalR} and F12. To determine if the hyperadherence of strain F12 could be attributed to the increased expression of a surface protein, the OMP profiles of strain F12 and *E. coli* O157:H7 86-24^{nalR}, with and

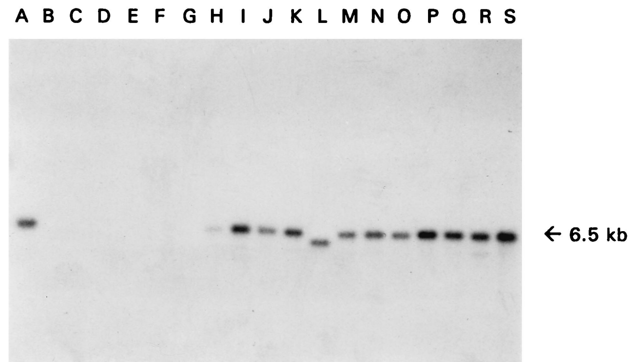

FIG. 4. Conservation of *rfbE*$_{EcO157:H7}$ in strains of *E. coli* O157 but not in strains of *E. coli* O55:H7. Shown are total DNAs from *E. coli* O157:H7 strain 86-24 (lane A), *E. coli* HB101 (lane B), *E. coli* O55:H7 strains (lanes C to G), nontoxigenic *E. coli* O157 strains expressing H antigens 43 (lanes H to K), 16 (lanes L to N), 45 (lane O), 3 (lane P), 38 (lane Q), and 12 (lane R), and toxigenic, sorbitol-fermenting, *E. coli* O157:NM (lane S), digested with *Eco*RI, electrophoresed, transferred to Nytran, and probed with *rfbE*$_{EcO157:H7}$.

without transformation with pSK+, were compared. There were no prominent bands in the F12 strains compared with the parent strains (Fig. 6).

DISCUSSION

rfbE$_{EcO157:H7}$ is necessary for expression of the O157 antigen of *E. coli* O157:H7. Complementation of the Tn*phoA* mutation in strain F12 with *rfbE*$_{EcO157:H7}$ restored O157 antigenicity. Therefore, loss of expression of this antigen was not caused by a polar effect on a gene downstream of *rfbE*$_{EcO157:H7}$. RfbE$_{EcO157:H7}$ is similar to RfbE of *V. cholerae* O1, a probable perosamine synthetase (33). The *E. coli* O157:H7 O polysaccharide consists of [→3-α-D-GalNAc*p*-(1→2)α-D-PerNAc*p*-(1→3)-α-L-Fuc*p*-(1→4)-β-D-Glc*p*-(1→]$_n$ repeats (22). Although the function of RfbE$_{EcO157:H7}$ is unknown, its homology to RfbE of *V. cholerae* and the presence of perosamine in the LPS of both organisms suggest that RfbE$_{EcO157:H7}$ is responsible for perosamine synthesis. Humans immunized with killed *V. cholerae* O1 develop antibodies which detect the *E. coli* O157 antigen (8). Perhaps the perosamine moiety contributes a cross-reactive epitope between the LPS of these organisms.

E. coli O157:H7 and sorbitol-fermenting, toxigenic *E. coli* O157:NM are more closely related to *E. coli* O55:H7 than to non-H7 *E. coli* O157 strains (43), differing only in the 6-phosphogluconate dehydrogenase electromorph (43). 6-Phosphogluconate dehydrogenase polymorphism results from intra-

TABLE 1. Adherence of *E. coli* O157:H7 86-24^{nalR} and derivatives to HeLa cells[a]

Strain	No. of clusters/cell (mean ± s.d.)	P^b
O157:H7 86-24^{nalR}	0.04 ± 0.05	
F12	0.27 ± 0.30	<0.05
F12(pSK+)	0.17 ± 0.13	
F12(pF12)	0.06 ± 0.04	<0.05

[a] Adherence is expressed as the number of clusters of bacteria (containing five or more organisms) adherent to HeLa cells. Five fields were examined for each chamber, and the clusters and cells visualized were enumerated. Values represent examination of at least eight chambers.

[b] *P* values represent comparison of the adherence of strain F12 with that of its parent, *E. coli* O157:H7 86-24^{nalR}, and of strain F12 transformed with pSK+ with that of strain F12 transformed with pF12.

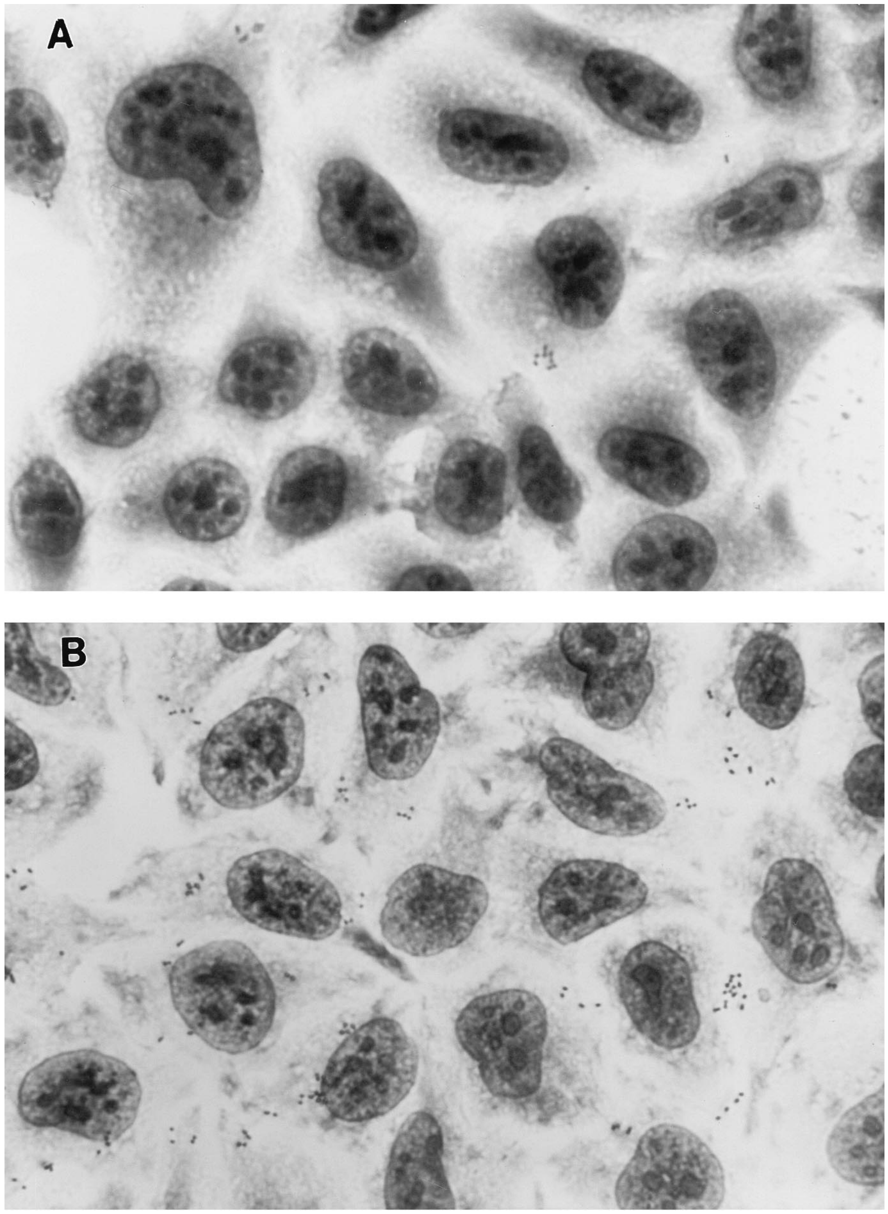

FIG. 5. Adherence of *E. coli* O157:H7 strains 86-24^{nalR} (A) and F12 (B) to HeLa cells.

genic and extragenic recombination in *gnd* (5, 20, 21). Whittam proposed that recombination involving the *rfb* clusters and *gnd* loci differentiated the O side chains of *E. coli* O157:H7 from *E. coli* O55:H7 (42). The absence of $rfbE_{EcO157:H7}$ from *E. coli* O55:H7 is consistent with this hypothesis. The guanine-plus-cytosine fraction of $rfbE_{EcO157:H7}$ (0.35) also suggests that this gene might not have originated in *E. coli*. Low G+C fractions have been identified in *rfb* loci of *E. coli* O7 (18), *E. coli* O101 (9), *E. coli* K-12 (31), and *Salmonella enterica* strains (24).

stx1 and *stx2* are contained on bacteriophages (32), which were presumably acquired by *E. coli* O157:H7 via transduction from an unidentified donor species. Intimin, which mediates the ability of *E. coli* O157:H7 to induce actin aggregation, is encoded by $eaeA_{EcO157:H7}$ (15). $eaeA_{EcO157:H7}$ is in a large segment of DNA, designated *lee* (locus of enterocyte effacement) (19). *lee* (G+C content, 0.39) is integrated at the same site in the *E. coli* O157:H7 chromosome as are large fragments

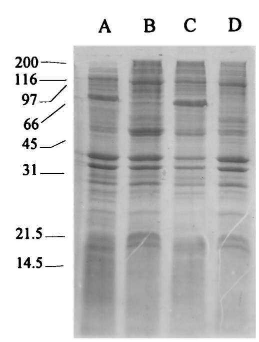

FIG. 6. OMP profiles of *E. coli* O157:H7 strains 86-24^{nalR} (lanes A and C) and F12 (lanes B and D), without (lanes A and B) and with (lanes C and D) pSK+.

which include genes encoding intimin and uropathogenic virulence factors in enteropathogenic (19) and uropathogenic (6) *E. coli* strains, respectively. Presumably, these virulence-associated segments, like $rfbE_{EcO157:H7}$, were acquired laterally from another organism.

Preliminary analysis of DNA adjacent to $rfbE_{EcO157:H7}$ demonstrates an upstream ORF with significant homology to *orf7.4* of *Yersinia pseudotuberculosis* (reference 17 and unpublished results). *orf7.4* is postulated to encode a transmembrane protein which translocates O side chains to the bacterial periphery, and it has recently been designated *rfbX* (25). The sequenced portion of this gene in *E. coli* O157:H7, which we have tentatively designated $rfbX_{EcO157:H7}$, also has a low G+C content (0.31); $rfbX_{EcO157:H7}$, like $rfbE_{EcO157:H7}$, occurs in *E. coli* strains expressing the O157 antigen but not in *E. coli* O55:H7 strains (data not shown).

Our data also suggest that the *E. coli* O157:H7 O side chain diminishes the adherence of *E. coli* O157:H7 to cultured epithelial cells. The outer membrane protein profile of strain F12 does not contain bands that are absent from strain *E. coli* O157:H7 strain 86-24^{nalR}, so it is unlikely that a protein adhesin was hyperexpressed because of Tn*phoA* insertion in $rfbE_{EcO157:H7}$. Ail-dependent invasion of Chinese hamster ovary cells by *Y. enterocolitica* is enhanced in strains deficient in the expression of O:3 side chains. Monoclonal antibodies detect Ail in larger quantities on the surface of the hyperinvasive O:3$^-$ mutants, implying that LPS side chains of *Y. enterocolitica* mask Ail (23) and possibly other virulence determinants. *Shigella flexneri* strains expressing recombinant *inv* penetrate eukaryotic cells only after the *S. flexneri* strains are mutated so that O side chains are not expressed (40). Expression of *E. coli* and *S. typhimurium* O antigens reduces the ability of antibodies to bind to porin surface epitopes (4). Additionally, expression of the O antigen of LPS reduces the ability of monoclonal antibodies to bind to porin surface epitopes in *E. coli* and *S. typhimurium* (4) and to PhoE of various members of the family *Enterobacteriaceae* (38). These observations suggest that the O side chains of bacterial LPS physically hinder contact between outer membrane virulence factors and eukaryotic cells, possibly modulating the effects of virulence traits by bacterial pathogens. The hyperadherence of the O157$^-$ mutant in vitro raises the possibility that variable expression of LPS in vivo influences adherence of *E. coli* O157:H7 to enterocytes.

In summary, the homology of $rfbE_{EcO157:H7}$ to corresponding genes in *E. coli* O157 of differing H antigens, but not to those in the more closely related *E. coli* O55:H7, and its low G+C content support the concept that O-antigen diversity results from the horizontal acquisition of genetic material, possibly from an interspecific source, rather than from point mutations. Furthermore, our data suggest that the O157 side chain inhibits the adherence of *E. coli* O157:H7 to epithelial cells. The in vivo relevance of this observation warrants further study.

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ADDENDUM IN PROOF

Since submission of the manuscript, another communication has reported that an O-side-chain-deficient mutant of *E. coli* O157:H7 is hyperadherent (F. Cockerill, G. Beebakhee, R. Soni, and P. Sherman, Infect. Immun. **64:**3196–3200, 1996).

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