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Molecular Subtyping and Genetic Analysis of the Enterohemolysin Gene (*ehxA*) from Shiga Toxin-Producing *Escherichia coli* and Atypical Enteropathogenic *E. coli*[▽]

Adrian L. Cookson,[1]* Jenny Bennett,[2] Fiona Thomson-Carter,[2] and Graeme T. Attwood[1]

Food, Metabolism and Microbiology Section, Food and Health Group, AgResearch, Grasslands Research Centre, Tennent Drive, Palmerston North,[1] and Enteric Reference Laboratory, ESR Kenepuru Science Centre, Porirua,[2] New Zealand

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Analyses of the distribution of virulence factors among different *Escherichia coli* pathotypes, including Shiga toxin-producing *E. coli* (STEC), may provide some insight into the mechanisms by which different *E. coli* strains cause disease and the evolution of distinct *E. coli* types. The aim of this study was to examine the DNA sequence of the gene for enterohemolysin, a plasmid-encoded toxin that readily causes the hemolysis of washed sheep erythrocytes, and to assess the distribution of enterohemolysin subtypes among *E. coli* isolates from various human and animal sources. The 2,997-bp *ehxA* gene was amplified from 227 (63.8%) of 356 *stx*- and/or *eae*-positive *E. coli* strains isolated from cattle and sheep and from 24 (96.0%) of 25 STEC strains isolated from humans with diarrheal disease. By using PCR and restriction fragment length polymorphism (RFLP) analysis of *ehxA*, six distinct PCR-RFLP types (A to F) were observed, with strains of subtypes A and C constituting 91.6% of all the *ehxA*-positive strains. Subtype A was associated mainly with ovine strains with *stx* only ($P < 0.001$), and subtype C was associated with bovine *eae*-positive strains ($P < 0.001$). Eleven *ehxA* alleles were fully sequenced, and the phylogenetic analysis indicated the presence of three closely related (>95.0%) *ehxA* sequence groups, one including *eae*-positive strains (subtypes B, C, E, and F) and the other two including mainly *eae*-negative STEC strains (subtypes A and D). In addition to being widespread among STEC strains, *stx*-negative, *eae*-positive strains (atypical enteropathogenic *E. coli* strains) isolated from cattle and sheep have similar *ehxA* subtypes and hemolytic activities.

Diarrheagenic *Escherichia coli* pathotypes, such as Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC), are conveniently subdivided according to the expression of pathogenic determinants that are implicated in animal and human disease (26). The acquisition of such virulence factors often reflects the extensive horizontal transfer of genetic material, such as the insertion of mobile elements, and can provide some insight into the evolution of separate *E. coli* pathotypes (8). However, the convenient subdivision of *E. coli* strains into specific groups often fails to address fundamental intrapathotype variation and interpathotype similarities. For example, all STEC strains exhibit a marked cytotoxic effect on human vascular endothelial cells mediated by the Shiga toxins encoded by stx_1 and/or stx_2 (27). Some STEC strains also express a number of proteins, including intimin (encoded by the *eae* allele), that coordinate the formation of attaching and effacing (A/E) lesions on gastrointestinal epithelial cells (19), but other STEC strains are *eae* negative and do not produce such proteins. Similarly, all EPEC strains are *eae* positive and typically possess a cluster of plasmid-borne genes (*bfp*) that encode bundle-forming pili that stabilize microcolony formation (26). However, atypical EPEC strains, commonly isolated from ruminants, are *bfp* negative (26, 41). Therefore, the structural analysis of virulence determinants within and between different *E. coli* diarrheagenic pathotypes should provide valuable clues on the evolutionary relationship of these pathotypes and on possible mechanisms by which pathogenesis occurs. One such virulence factor is the plasmid-encoded enterohemolysin of STEC that readily causes the hemolysis of washed sheep erythrocytes (4, 5, 34, 39) by serotypes including O157:H7 (5, 32) and O111:H$^-$ (33). Indeed, serum samples from patients with hemolytic-uremic syndrome (HUS) react specifically to the hemolysin from O157:H7 strains (32).

The main genetic determinants for the production of the O157:H7 STEC enterohemolysin (4) are associated with pO157 (34). This large plasmid of approximately 94 kb contains the *ehx* locus, which has the gene order *ehxCABD* and is >60% homologous to the alpha-hemolysin gene of *E. coli* (35). Like alpha-hemolysin, enterohemolysin is a pore-forming RTX (*r*epeats in *t*o*x*in) cytolysin that is active on sheep erythrocytes and certain bovine lymphoma cell lines (2, 32, 34, 35, 44). Overnight incubation on washed sheep blood agar is required for enterohemolysin expression, in contrast to the rapid (4-h) hemolysis associated with alpha-hemolysin (4). Enterohemolysin is also likely to be synthesized as an inactive protoxin requiring the acylation activity of EhxC protein and secretion, mediated by EhxB and EhxD, by a specific membrane translocator system (38, 44).

The precise role of enterohemolysin in STEC disease remains to be fully established, but enterohemolysin from a STEC O128:H12 isolate has been observed to induce increased levels of the proinflammatory cytokine interleukin-1β from human monocytes during in vitro studies (39). Other studies

* Corresponding author. Mailing address: Food, Metabolism and Microbiology Section, Food and Health Group, AgResearch, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand. Phone: 64 6 351 8229. Fax: 64 6 351 8003. E-mail: adrian.cookson@agresearch.co.nz.

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using bacterial strains from which pO157 has been removed have failed to provide definitive proof of the importance of the large plasmid or enterohemolysin in STEC pathogenesis (18, 43); however, *ehxA* is found in many STEC serotypes, such as O157:H7, O26:H11, O103:H2, O111:H$^-$, O113:H21, O5:H$^-$, and O84:H2/H$^-$, that are commonly associated with diarrheal disease and HUS, and thus, *ehxA* has been commonly used as a possible marker for STEC (4, 13, 24, 31, 32, 33, 45, 46). Several *ehxA*-positive serotypes, including O157:H$^-$ (16) and O111:H$^-$ (33), do not exhibit the enterohemolytic phenotype on washed sheep blood agar, indicating that the precise conditions for the regulation and optimum expression of enterohemolysin may remain to be determined.

More recently, *stx*-negative *E. coli* strains that possess the A/E-lesion determinant have been increasingly recognized as having the same enterohemolytic phenotype on washed sheep erythrocytes as STEC (1, 10, 21). The link between atypical EPEC strains that are *bfp* negative and human diarrheal disease has yet to be readily established (29, 37, 41, 42). However, some *stx*-negative strains of STEC-associated serotypes O157:H7 and O157:H$^-$, commonly associated with human clinical disease (diarrhea and HUS), have also occasionally been isolated in clinical cases (36). Furthermore, atypical EPEC strains (serotypes O26:H11, O69:H32, O76:H$^-$, O84:H38, O115:H$^-$, O123:H11, O123:H$^-$, O145:H$^-$, O149:H$^-$, O149:H7, and O168:H8) lacking bundle-forming pili have been isolated from cattle and sheep on a number of occasions (1, 10, 21), but no information is available on the relationship between the *ehxA* gene from these atypical EPEC strains and that for the enterohemolysin more commonly associated with STEC. Therefore, the aim of this work was to identify and characterize the genetic and phenotypic diversity of the enterohemolysin from *stx*- and/or *eae*-positive *E. coli* strains from various sources (animal and human) to better understand the possible evolutionary relationship among bacteria from different *E. coli* diarrheagenic pathotypes. In addition, this study describes a rapid technique for the typing of *ehxA* genes from *E. coli* and the identification of three new *ehxA* sequences that are similar to those from STEC O157:H7.

MATERIALS AND METHODS

Bacterial strains and culture conditions for enterohemolysin expression. Most of the bacterial strains used in this study have been described elsewhere (3, 11, 12, 13). Of the strains examined for *ehxA*, 215 were positive for *stx* only (99 for stx_1 only, 33 for stx_1 and stx_2, and 83 for stx_2 only) and 141 were *eae* positive (115 were positive for *eae* only, 25 were stx_1 and *eae* positive, and 1 was stx_2 and *eae* positive). These strains were isolated from rectoanal mucosal swabs taken from healthy cattle and sheep on the lower North Island, New Zealand, over a period of 3 months. The *E. coli* strains ($n = 25$; 1 positive for stx_1 only, 2 stx_1 and stx_2 positive, 6 positive for stx_2 only, 9 stx_1 and *eae* positive, 5 stx_2 and *eae* positive, and 2 *eae* positive) from human patients with diarrheal disease were obtained from the Enteric Reference Laboratory, ESR Ltd. Luria-Bertani broth or agar was used routinely for the growth of bacteria. For the detection of enterohemolytic activity, strains were inoculated from a well-spaced single colony onto washed sheep blood agar plates and the plates were incubated at 37°C for 18 h, followed by 6 h at room temperature. Defibrinated sheep blood was washed three times in phosphate-buffered saline (pH 7.4) at 950 × *g* and added (5%, vol/vol) to Luria-Bertani agar cooled to 50°C (9).

PCR-RFLP subtyping. All bovine and ovine bacterial strains that were *stx* and *eae* positive ($n = 38$), *stx* positive only ($n = 115$), or *eae* positive only ($n = 115$) were chosen for further study to detect the presence of *ehxA* by PCR amplification of the complete 2,997-bp *ehxA* gene with oligonucleotide primers (Invitrogen, Auckland, New Zealand) *ehx*ARFLP F (5′ ATGACAGTAAATAAAATA AAGAAC 3′) and *ehx*ARFLP R (5′ TCAGACAGTTGTCGTTAAAGTTG 3′), corresponding to positions 1 to 24 (*ehx*ARFLP F) and 2975 to 2997 (*ehx*ARFLP R), and the Px2 system (Thermo Hybaid, Ashford, United Kingdom). Amplification was performed with a DNA template prepared from heat-treated bacterial cells as described previously (11). The PCR mixture included the DNA template and 2.5 pM (each) primers, and the volume was made up to 20 μl by using the *Taq* polymerase-deoxynucleoside triphosphate supermix (Invitrogen, Auckland, New Zealand). For PCR amplification of the *ehxA* gene, an initial denaturing cycle of 95°C for 5 min was followed by 30 cycles of 95°C for 45 s (denaturing), 52°C for 45 s (annealing), and 72°C for 2 min, with a final extension step at 72°C for 5 min. Initially, 5 μl of each PCR mixture was used to detect the presence of the 2,997-bp *ehxA* amplicon by agarose gel electrophoresis (Innovative Sciences Ltd., Dunedin, New Zealand). Approximately 0.5 μl of 10× gel loading buffer (Invitrogen, Auckland, New Zealand) was added to the sample before it was electrophoresed across a 2.5% (wt/vol) agarose (Boehringer Mannheim, Germany) gel in Tris-acetate-EDTA buffer. Each gel was electrophoresed at 180 V (approximately 15 V cm^{-1}) for 55 min. Approximately 8 μl of a 10,000× SYBR safe DNA stain (Molecular Probes, Auckland, New Zealand) solution was added to each 100-ml molten agarose (2.5%, wt/vol)–Tris-acetate-EDTA buffer gel mix immediately prior to pouring in order to visualize DNA on a transilluminator. For those strains that were *ehxA* positive, approximately 1.1 μl of 10× restriction enzyme buffer and 5 U of TaqI restriction endonuclease (Invitrogen, Auckland, New Zealand) were added to 9 μl of each PCR mixture and mixed gently. Digestion mixtures were incubated for 90 min at 65°C and then subjected to DNA electrophoresis and visualization as described previously. Restriction fragment length polymorphism (RFLP) electrophoretic gel images were stored as TIFF files by using a Gel Logic 200 imaging system (Kodak; Biolab, Auckland, New Zealand) to compare the images with those generated in silico. Virtual gels of GenBank *ehxA* gene sequences AF043471 (O8:H19), AY258503 (O113:H21), and AB032930 (O128:H12), corresponding to subtype A; X79839 (O157:H7), corresponding to subtype B; and X94129 (O111:H$^-$), corresponding to subtype C, were constructed using Vector NTI Advance (version 9.1.0; Invitrogen Corporation). The TaqI sites from each *ehxA* sequence were identified, and the apparent electrophoretic mobility of the TaqI fragments was assessed using the Vector NTI Advance program.

Colony blot hybridization. Bacteria were streaked out onto gridded Hybond N+ nylon membranes (GE Healthcare Life Sciences, Auckland, New Zealand) immobilized on MacConkey agar plates and grown for 16 h at 37°C. The membrane was removed from the agar plate, and bacteria were lysed using sodium dodecyl sulfate (10%, wt/vol). Bacterial DNA was denatured, and the membrane was neutralized according to the manufacturer's instructions. Excess bacterial cell debris was removed by washing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and DNA was fixed to the membrane by baking at 80°C for 2 h. A 534-bp fragment of *ehxA* was amplified using hlyAF and hlyAR primers, corresponding to positions 70 to 90 and 581 to 602 of the *ehxA* sequence, as described previously (11) and labeled using the AlkPhos direct labeling and detection system according to the instructions of the manufacturer (GE Healthcare Life Sciences). Hybridization reactions were performed overnight at 50°C. Membranes were washed at 50°C, and *ehxA*-positive bacteria were visualized using CDP-Star (GE Healthcare Life Sciences). *E. coli* O157:H7 NCTC12900 and *E. coli* DH5α were used as positive and negative controls, respectively.

DNA sequencing. The complete *ehxA* gene sequence was obtained from 11 *ehxA*-positive strains (2 strains each of *ehxA* subtypes A, B, C, D, and F and 1 strain of *ehxA* subtype E) displaying a wide variety of enterohemolytic characteristics. Initially, the *ehxA* gene was amplified using the *ehx*ARFLP F and *ehx*ARFLP R primers as described previously. The PCR product was purified using the QIAGEN PCR purification kit and sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Auckland, New Zealand). Initial sequencing was performed with the *ehx*ARFLP F and *ehx*ARFLP R primers and then proceeded with internally designed oligonucleotides for the sequencing of the whole *ehxA* gene fragment. Capillary separation of sequencing reaction mixtures was performed on an ABI 3730 machine (Applied Biosystems) at the Allan Wilson Centre Genome Service Facility (Massey University, New Zealand).

Serological analysis. The evaluation of O (O1 to O181)- and H (H1 to H56, excluding H13, H22, and H50)-antigens from selected strains was carried out according to standard World Health Organization methods using antisera raised for all known O and H groups (Statens Serum Institute, Copenhagen, Denmark). O-antigens were identified using overnight broth cultures steamed for 1 h. H-antigens were identified from strains after repeated passage through semisolid agar medium followed by treatment with 0.05% (vol/vol) formalin. Strains that did not agglutinate any of the specified O or H antisera were designated O

TABLE 1. Distribution of *ehxA*-positive STEC and EPEC strains and associated *ehxA* PCR-RFLP subtypes

PCR-RFLP subtype	No. (%) of strains	No. (%) of strains from:		
		Cattle	Sheep	Humans
A	115 (45.8)	4	103	8
B	5 (2.0)	1	0	4
C	115 (45.8)	74	29	12
D	3 (1.2)	3	0	0
E	2 (0.8)	2	0	0
F	11 (4.4)	9	2	0
ehxA positive	251 (65.9)	93 (66.4)	134 (62.0)	24 (96.0)
ehxA negative	130 (34.1)	47 (33.6)	82 (38.0)	1 (4.0)

nontypeable (ONT) and H nontypeable (HNT), respectively, and strains that were considered nonmotile were designated H^-.

Phylogenetic analysis. DNA sequences were edited with Contig Express (Vector NTI Advance, version 9.1.0; Invitrogen Corporation). Phylogenetic trees were compiled with MEGA version 3.1 (22; http://www.megasoftware.net/) using the neighbor-joining method of Saitou and Nei (30). ClustalW included in the DNASTAR (University of Wisconsin) software was used to calculate genetic distances (40).

Statistical analysis. Comparisons of the distribution of *ehxA* PCR-RFLP subtypes with that of (i) *E. coli* pathotypes and (ii) strains from cattle or sheep was performed using χ^2 analysis (GenStat 7.0; VSN International, Hemel Hempstead, United Kingdom) where a P value of <0.05 was considered significant.

Nucleotide sequence accession numbers. The complete *ehxA* sequences of strains O90:H8 AGR047 (subtype C), O131:H25 AGR053 (subtype C), O153:H^- AGR119 (subtype F), O98:H^- AGR151 (subtype A), O101:H^- AGR158 (subtype E), O121:H19 AGR270 (subtype B), O91:H^- AGR340 (subtype A), O5:H^- AGR374 (subtype F), ONT:H^- AGR670 (subtype D), ONT:H^- AGR674 (subtype D), and O157:H7 ER03/4238 (subtype B) were submitted to GenBank and have been assigned the accession numbers EF204919 to EF204929, respectively.

RESULTS

Identification of novel *ehxA* subtypes by PCR-RFLP analysis. *E. coli* strains that were *stx* and/or *eae* positive were examined by PCR to detect the presence of the full-length 2,997-bp *ehxA* gene. Overall, 227 (63.8%) of the *stx*- or *eae*-positive strains from animals were *ehxA* positive, of which 134 (59.0%) were sheep strains and 93 (41.0%) were cattle strains (Table 1). Twenty-four (96.0%) of the 25 human *E. coli* isolates examined were *ehxA* positive. Of the 134 *ehxA*-positive sheep *E. coli* strains that were *stx* or *eae* positive, 103 (76.9%) were *stx* positive only, 4 (3.0%) were *stx* and *eae* positive, and 27 (20.1%) were *eae* positive only. Of the 93 *ehxA*-positive cattle *E. coli* strains that were *stx* or *eae* positive, 3 (3.2%) were *stx* positive only, 21 (22.6%) were *stx* and *eae* positive, and 69 (74.2%) were *eae* positive only. After an initial PCR screening for the presence of *ehxA*, the remainder of the *ehxA* PCR product was cut with TaqI and electrophoresed on a 2.5% agarose gel to determine the *ehxA* PCR-RFLP type (Fig. 1). Six distinct *ehxA* subtypes could be differentiated after digestion with TaqI and agarose gel electrophoresis (Fig. 1). *ehxA* subtypes A and E had the most similar PCR-RFLP profiles but could be readily distinguished when broad-toothed gel combs were used to give high-definition electrophoresis profiles. The predicted lengths of TaqI restriction fragments from GenBank *ehxA* sequences AF043471 (O8:H19), AY258503 (O113:H21), AB032930 (O128:H12), X79839 (O157:H7), and X94129 (O111:H^-) corresponded to the lengths of fragments of subtype A, B, or C. Subtypes D, E, and F appeared to be novel.

Four further *E. coli* strains (one positive for stx_2 only and three positive for *eae* only) were identified as *ehxA* positive by colony blot analysis using a 534-bp *ehxA* fragment, but the full-length *ehxA* gene could not be amplified using the PCR primers or the conditions outlined above, indicating that there may be other *ehxA* subtypes or that the *ehxA* gene in these strains was not complete.

***ehxA* PCR-RFLP subtyping of *E. coli* strains from human patients with diarrheal disease and ruminants.** In this study, two PCR-RFLP types (A and C) represented the majority (230 of 251; 91.6%) of the strains that were identified as *ehxA* positive (Table 1). The remaining PCR-RFLP types (B, D, E, and F) were noted less frequently. Only 4 (8.3%) of 48

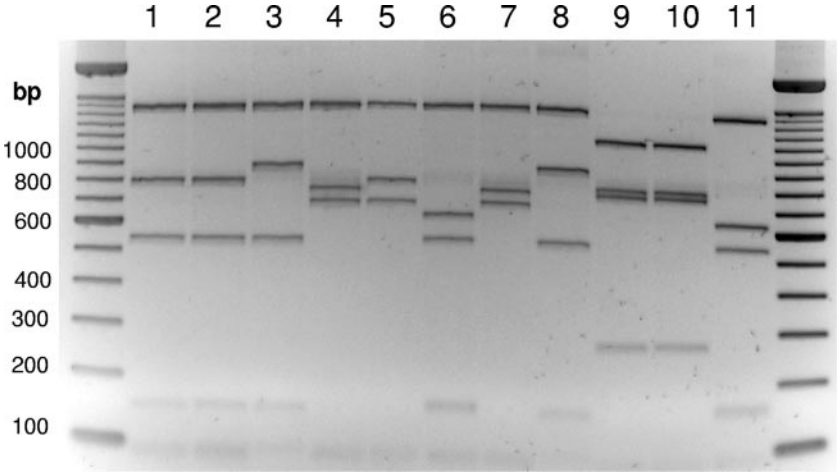

FIG. 1. RFLP profiles of *ehxA* PCR amplicons after TaqI digestion. PCR products were subjected to agarose (2.5%, wt/vol) gel electrophoresis for the analysis of *exhA* PCR-RFLP profiles. Lanes: 1, O90:H8 AGR047 (subtype C); 2, O131:H25 AGR053 (subtype C); 3, O153:H^- AGR119 (subtype F); 4, O98:H^- AGR151 (subtype A); 5, O101:H^- AGR158 (subtype E); 6, O121:H19 AGR270 (subtype B); 7, O91:H^- AGR340 (subtype A); 8, O5:H^- AGR374 (subtype F); 9, ONT:H^- AGR670 (subtype D); 10, ONT:H^- AGR674 (subtype D); and 11, O157:H7 ER03/4238 (subtype B).

TABLE 2. Virulence factors associated with *ehxA* PCR-RFLP subtypes of *E. coli* strains

PCR-RFLP subtype	No. (%) of strains with virulence determinant(s):		
	stx only	*stx* and *eae*	*eae* only
A	114	0	1
B	0	4	1
C	0	31	84
D	3	0	0
E	0	0	2
F	0	3	8
ehxA positive	117 (51.8)	38 (100)	96 (82.1)
ehxA negative	109 (48.2)	0 (0)	21 (17.9)

stx-positive, *eae*-negative strains from cattle were *ehxA* positive, compared to 103 (61.7%) of 167 *stx*-positive, *eae*-negative sheep strains. Of the *eae*-positive strains, 81.3% of cattle strains (74 of 91) and 59.2% of sheep strains (29 of 49) were *ehxA* positive. Of the strains positive for *stx* only, 51.8% (117 of 226) were *ehxA* positive, of which 114 (99.0%) were *ehxA* subtype A ($P < 0.001$) (Table 2). All the *stx*- and *eae*-positive STEC strains ($n = 38$) were *ehxA* positive and were subtype C, except for four O157:H7 isolates that were subtype B and three O5:H$^-$ isolates that were subtype F. Most (96 of 217; 82.1%) *eae*-positive, *stx*-negative atypical EPEC strains were *ehxA* positive, and 84 (87.5%) of 96 had *ehxA* subtype C ($P < 0.001$) (Table 2). However, four additional subtypes (A, B, E, and F) were less frequently associated with the *eae*-positive, *stx*-negative strains. Further studies may indicate whether these data represent a general trend or whether *E. coli* populations isolated from geographically distinct areas or from different animal cohorts have different ratios of *ehxA* subtypes.

Hemolytic activity on sheep blood agar plates. Enterohemolytic activity is exhibited as a narrow zone or a very narrow zone of hemolysis on washed sheep red blood cells (31). In the present study, hemolytic activity on washed sheep blood agar characteristically required overnight incubation but varied by strain, from barely discernible zones of hemolysis more suggestive of enterohemolytic activity as previously described (4, 31, 32, 34) (Fig. 2a) to large (≥3-mm) zones of visible clearing of the agar surrounding individual bacterial colonies (Fig. 2c). The degree of hemolysis varied between and within *ehxA* subtypes, but under the incubation conditions and with the solid growth media described above, the *eae*-positive, *stx*-negative subtype F strains generally displayed the most extensive clearing of the washed sheep blood agar, similar to alpha-hemolysis (Fig. 2c). The hemolytic activity associated with the *ehxA* subtype F O5:H$^-$ STEC strains, however, was more typically characteristic of enterohemolytic activity, with small zones of turbidity (Fig. 2h). Whether this variable hemolytic activity is attributable to altered enterohemolysin expression or secretion is unknown. However, a variation in the hemolytic activities of *ehxA*-positive STEC strains has been noted previously (9, 39). Generally, cattle strains were more likely than sheep strains to be hemolytic (Table 3). Under the incubation conditions tested, strains of the less frequently identified *ehxA* PCR-RFLP subtypes B, E, and F were hemolytic on the blood agar plates. Most bacterial strains (93.0%) that were *ehxA* subtype C were hemolytic. However, approximately 40% of the subtype A and all ($n = 3$) of the subtype D strains did not display the hemolytic phenotype (Table 3).

Serotyping of *ehxA*-positive and -negative strains. Of the 132 strains for which serotyping was attempted, including the 38 *eae*-positive STEC strains, 40 were O-antigen nontypeable (Table 4). The most common O serogroup was O84 ($n = 16$), of which 11 strains were serotype O84:H$^-$ and 5 were O84:H2. Some serotypes corresponded to different *ehxA* subtypes, with single O153:H$^-$ strains being of subtype C and F and O91:H$^-$ strains being of subtype A or C or being *ehxA* negative. Both *eae*-positive STEC strains and *stx*-negative, *eae*-positive strains of serotypes O145:H$^-$ and O26:H11 had identical *ehxA* subtypes (Table 4). Whether the *stx* prophage had been lost during storage is unknown, but all strains were subcultured from the original −85°C freezer stocks to eliminate the likelihood of the loss of *stx* during successive subcultures.

Comparative sequence analysis and phylogenetic profiling. The *ehxA* gene from strains that possessed representative enterohemolysin PCR-RFLP subtypes was fully sequenced and aligned along with five other enterohemolysin gene sequences available from GenBank. Virtual gel profiles generated from in silico TaqI digestion of *ehxA* sequence subtypes from GenBank and *ehxA* sequence subtypes from this work matched exactly with TaqI profiles generated using the PCR-RFLP method. A phylogenetic tree was generated using the neighbor-joining method with MEGA (version 3.1) (Fig. 3). Based on the *ehxA* sequence analysis and alignment, subtypes B, C, and F were closely related and were associated with *eae*-positive strains exclusively. Subtypes A and E formed a distinct group; all strains with these subtypes were *stx* positive and *eae* negative, except two subtype E strains that were *stx* negative and *eae* positive. The *ehxA* sequences from the two subtype D strains (*stx* positive and *eae* negative) were the most distantly related and were well separated from those of the other strains representative of the other five *ehxA* PCR-RFLP subtypes (Fig. 3). These data were reflected in the percentages of sequence similarity calculated using a sequence identity matrix for the 16 *ehxA* genes (Table 5). The *ehxA* sequences of AGR670 and AGR674 were identical (100.0%), and there was 95.6 to 96.6% identity to sequences associated with non-subtype D *ehxA* types. All other *ehxA* ($n = 14$) sequences had ≥98.1% sequence similarity. Two *ehxA* subtype A sequences, those of AGR340 (O91:H$^-$) and O128:H12, were identical (100.0%). An analysis of the amino acid sequences of the protoxins of AGR670 and AGR674 indicated the deletion of a codon encoding one of four concurrent glycine residues in the 11th RTX toxin repeat associated with enterohemolysin. All other enterohemolysin sequences had the predicted 13 tandem arrays of the 9-amino-acid repeat consensus sequence GGXGX DX[L/I/V/W/Y/F]X (where X is any amino acid) associated with RTX proteins and spanning amino acid residues 706 to 832 at the C terminus (17, 23, 32). The lysine residues at positions 550 and 675 required for the activation of the protoxin through fatty acylation of the enterohemolysin were also present in all deduced amino acid sequences (38), as were histidine 841 (14) and aspartate 845 (15), required for pH- and Ca^{2+}-dependent activity, respectively.

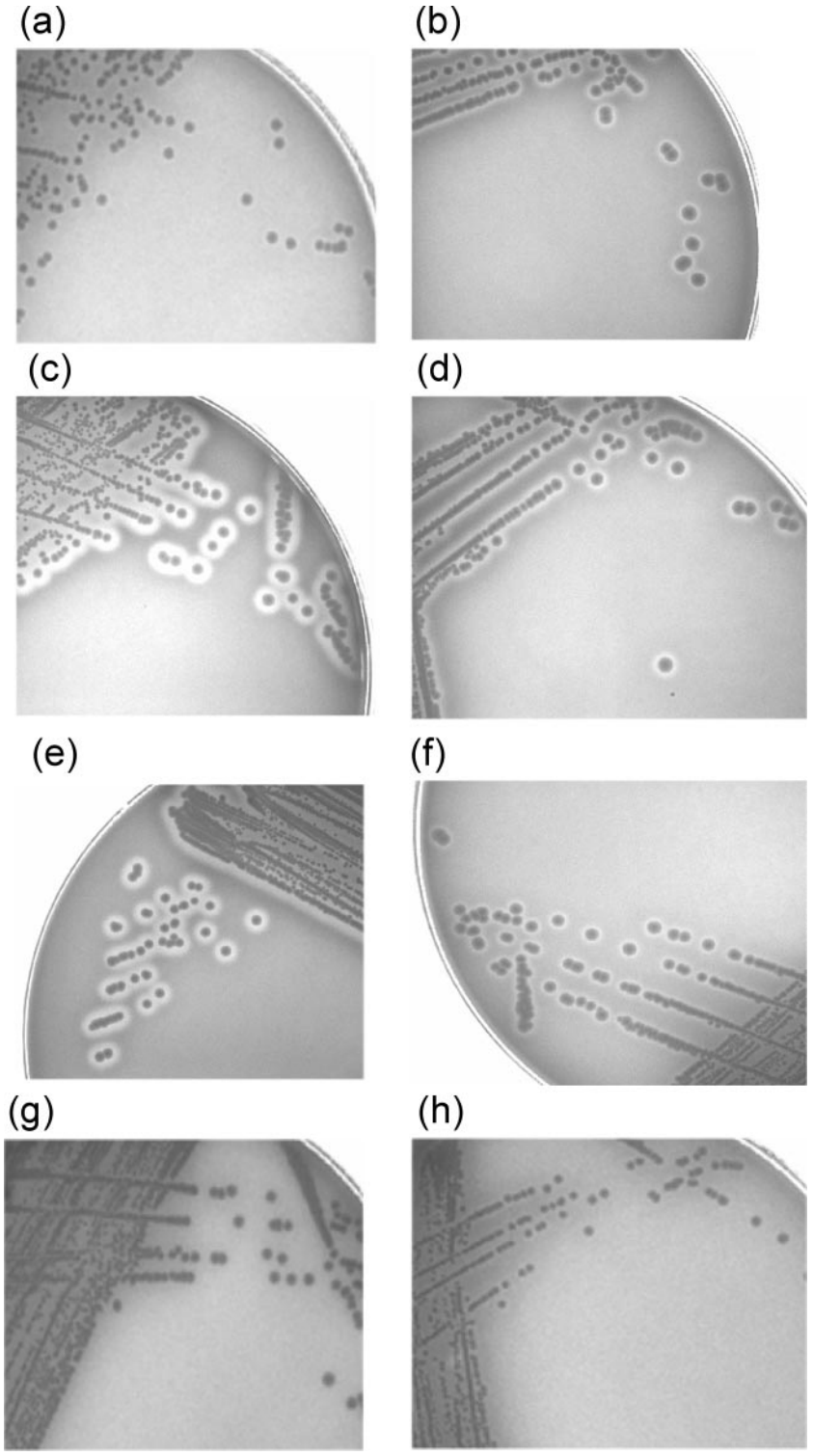

FIG. 2. Enterohemolytic phenotypes and hemolytic scores of ruminant *E. coli* strains after growth (18 h at 37°C and 6 h at room temperature) on washed sheep blood agar plates. (a) O157:H7 ER03/4238 (subtype B); (b) O131:H25 AGR053 (subtype C); (c) O153:H$^-$ AGR119 (subtype F); (d) O98:H$^-$ AGR151 (subtype A); (e) O101:H$^-$ AGR158 (subtype E); (f) O121:H19 AGR270 (subtype B); (g) ONT:H$^-$ AGR674 (subtype D); (h) O5:H$^-$ AGR374 (subtype F).

TABLE 3. Hemolytic phenotypes of *ehxA*-positive *E. coli* strains after growth (18 h at 37°C and 6 h at room temperature) on washed sheep blood agar

PCR-RFLP subtype	No. of indicated strains from:					
	Cattle		Sheep		Humans	
	Hemolytic	Nonhemolytic	Hemolytic	Nonhemolytic	Hemolytic	Nonhemolytic
A	4	0	58	46	8	0
B	1	0	0	0	4	0
C	66	7	29	1	12	0
D	0	3	0	0	0	0
E	2	0	0	0	0	0
F	9	0	2	0	0	0

DISCUSSION

As yet, the role of enterohemolysin as an *E. coli* virulence factor has not been fully elucidated. It is likely that the enterohemolysin is expressed during human infection and subsequent disease, as patients suffering from O157-associated HUS produce serum antibodies specific to the enterohemolysin from STEC O157 in almost all cases (32). Any possible role for enterohemolysin in bacterial colonization and pathogenesis in ruminants remains to be established, and although receptors for stx_1 on bovine intestinal epithelia have been noted previously (20), STEC strains do not normally cause disease in cattle. To our knowledge, *E. coli* strains that are *ehxA* and *bfp* positive have not been recognized. Therefore, by virtue of being *ehxA* positive, *eae* positive, and *stx* negative, atypical EPEC strains from cattle and sheep that lack the bundle-forming pilus may have a closer evolutionary relationship with STEC strains than with other EPEC strains, despite the absence of *stx* genes (1, 10, 21). However, the expression of enterohemolysin by these strains or STEC in the animal host has not been established. Atypical EPEC strains that lack the *bfp* locus are becoming more apparent, especially in ruminants (41). However, additional studies are required to identify the role of enterohemolysin in pathogenesis and to determine whether the formation of A/E lesions without concomitant bundle-forming pilus expression (as in typical EPEC strains) or the expression of *stx* (as in STEC strains) is sufficient for diarrheal disease to occur.

With in vitro culture on washed sheep blood agar, a variety of enterohemolysin activity levels were noted in this study (Fig. 2). It has also been suggested previously that the variation in levels of enterohemolysin secretion and, therefore, of visible hemolysis may be a characteristic of the double or single methionine residue in the N-terminal region of EhxB (39). However, this possibility has not been substantiated, and additional work is required to establish a correlation between the number of methionine residues and enterohemolytic activity. Previous studies have noted the discrepancy between the presence of the *ehxA* gene and the enterohemolytic phenotype. None of nine STEC O157:H$^-$ strains from Finland were hemolytic on enterohemolysin agar (16), and 2 of 22 STEC O111:H$^-$ strains displayed the nonhemolytic phenotype despite being *ehxA* positive (33). The enterohemolysins of the two PCR-RFLP subtype D strains that were sequenced had a deletion in tandem repeat 11 that may have been a cause of the nonhemolytic phenotype. However, for other apparently nonhemolytic strains, different culture conditions may be required for the effective expression of enterohemolysin.

Recent work indicates that genes found on pO157 regulate some chromosomal genes involved in colonization by STEC O157 strains and the persistence of these strains in cattle (25); however, there was no discernible activity associated with enterohemolysin. Analyses of the pO157 plasmid and other large plasmids from STEC strains suggest that many virulence factors are on potentially mobile elements and that their acquisition and evolution may be in part a reflection of the association of specific STEC serotypes with pathogenicity (8). The genetic diversity of *ehxA* and enterohemolysin expression as deter-

TABLE 4. Serotypes of STEC and *eae*-positive *E. coli* strains[a]

PCR-RFLP subtype	Serotype(s) with virulence determinant(s):		
	stx only	*stx* and *eae*	*eae* only
A	O75:H8, O91:H21, O91:H$^-$, O113:H21, O128:H$^-$, O128:H2, O130:H11, ONT:H7, ONT:H8, ONT:H10, ONT:H$^-$, OR:H2, OR:H$^-$		O98:H$^-$
B		O157:H7	O121:H19
C		O26:H11, **O26:H$^-$**, O84:H2, O84:H$^-$, **O84:H$^-$**, **O145:H$^-$**, ONT:H11, ONT:H$^-$, ONT:HNT, OR:H$^-$, **OR:H11**, OR:H21	O26:H$^-$, O26:H11, O70:H11, O76:H$^-$, O90:H8, O91:H$^-$, O103:H25, O103:HR, O108:H25, O115:H$^-$, O129:H$^-$, O131:H25, O136:H$^-$, O145:H46, **O145:H$^-$**, O150:H$^-$, O153:H$^-$, O172:H$^-$, O177:H11, O180:H$^-$, OR:H11, **ONT:H?**, ONT:H8, ONT:H25, ONT:H$^-$
D	ONT:H$^-$		
E			O101:H$^-$
F		O5:H$^-$	O103:H$^-$, O153:H$^-$
None	O9:H51, O65:H$^-$, O69:H6, O91:H$^-$, **O91:H$^-$**, O149:H8, O150:H8, O174:H8, ONT:H6, ONT:H8, ONT:H10, ONT:H14		O37:H$^-$, O49:H10, O109:H$^-$, O158:H11, ONT:H10, ONT:H$^-$, ONT:H$^-$

[a] Serotypes listed in bold correspond to strains obtained from human patients with diarrheal disease and identified in this study. Underlined designations represent serotypes of strains from which the full-length *ehxA* PCR product was not obtained but which hybridized with the 534-bp *ehxA* probe during colony blotting.

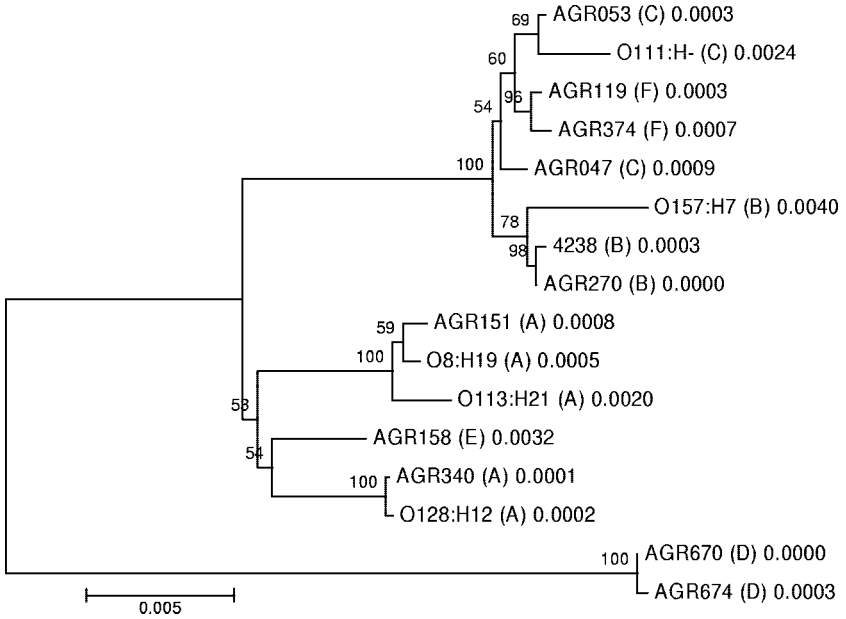

FIG. 3. Phylogenetic positions of *E. coli ehxA* sequences from this study and the following *ehxA* sequences available in GenBank, based on neighbor joining: AF043471 (O8:H19), AY258503 (O113:H21), and AB032930 (O128:H12), corresponding to subtype A; X79839 (O157:H7), corresponding to subtype B; and X94129 (O111:H$^-$), corresponding to subtype C. Bootstrap values (expressed as percentages of 1,000 replications) of ≥50% are shown at branch points. The number after the *ehxA* PCR-RFLP subtype corresponds to the branch length and indicates the genetic distance between the two *ehxA* sequences that the branch connects. Bar, 0.5% sequence dissimilarity.

mined in the present study may give some insight into the potential virulence of *E. coli* strains isolated from ruminants.

This is the first study to use a simple and rapid method, such as PCR-RFLP analysis, for the comprehensive subtyping of *ehxA* genes and the comparison of *exhA* genes from atypical EPEC strains with the *ehxA* alleles from STEC strains in order to assess the genetic relatedness of the corresponding enterohemolysins. The *ehxA* gene was more commonly associated with those strains that were *eae* positive (86.2%) than with those that were *stx* positive only (51.8%). Previously, *ehxA* has been used as a marker for identifying specific *eae*- and *ehxA*-positive STEC serotypes such as O26, O103, O111, O145, and O157, which are more commonly associated with outbreaks of serious human clinical disease, including bloody diarrhea and HUS, than other serotypes (3, 5, 13, 31, 32, 33, 45, 46). Indeed, all the *eae*-positive STEC strains examined in this study (n = 38) were also *ehxA* positive. STEC strains that are *eae* negative are only rarely isolated as a cause of diarrheal disease (45). However, STEC O113:H21 strains, which are often *eae* negative and exhibit *ehxA* subtype A, are a well-recognized serotype associated with severe diarrhea and HUS (3, 28) and provide an example of a particularly virulent serotype.

The association of *ehxA* with *eae*-positive, *stx*-negative ruminant *E. coli* strains has only recently been noted (1, 10, 21). The

TABLE 5. Percentages of identity of enterohemolysin gene sequence pairs as determined using ClustalW alignment

Sequence from strain	% Identity to sequence from strain:															
	4238	AGR047	AGR053	AGR119	AGR151	AGR158	AGR270	AGR340	AGR374	AGR670	AGR674	O111	O113	O128	O157	O8
4238	100.0	99.7	99.6	99.7	98.4	98.6	100.0	98.5	99.6	96.1	96.1	99.4	98.3	98.5	99.5	98.4
AGR047		100.0	99.8	99.8	98.4	98.7	99.8	98.6	99.8	96.1	96.1	99.5	98.3	98.5	99.3	98.4
AGR053			100.0	99.8	98.3	98.6	99.7	98.5	99.8	96.0	96.0	99.7	98.2	98.4	99.2	98.3
AGR119				100.0	98.4	98.6	99.7	98.5	99.9	96.1	96.0	99.6	98.3	98.5	99.3	98.4
AGR151					100.0	99.0	98.4	99.1	98.3	96.5	96.5	98.1	99.6	99.0	98.0	99.9
AGR158						100.0	98.6	99.3	98.6	96.6	96.5	98.4	98.9	99.3	98.2	99.0
AGR270							100.0	98.5	99.7	96.1	96.1	99.4	98.3	98.5	99.6	98.4
AGR340								100.0	98.5	96.5	96.5	98.3	99.0	100.0	98.1	99.1
AGR374									100.0	96.0	96.0	99.5	98.2	98.4	99.2	98.3
AGR670										100.0	100.0	95.8	96.5	96.5	95.7	96.5
AGR674											100.0	95.8	96.4	96.5	95.6	96.5
O111												100.0	98.1	98.3	99.3	98.2
O113													100.0	99.0	97.9	99.8
O128														100.0	98.1	99.1
O157															100.0	98.0
O8																100.0

bacterial strains in this study were isolated from apparently healthy animals, and therefore, the role of *eae*- and *ehxA*-positive strains in ruminant diarrheal disease remains equivocal. Only bacterial strains that were *stx* and/or *eae* positive were assessed for the presence of *ehxA* in this study. Other studies have found that *ehxA* may commonly be associated with *E. coli* strains lacking the *stx* and *eae* virulence factors (6, 21). Strains positive for *ehxA* only (*stx* negative and *eae* negative) were isolated from 6 (3.1%) of 191 bovine fecal samples derived from animals with gastrointestinal infections in Australia (21). A total of 338 strains that were isolated from effluent from municipal wastewater treatment were also found to be *ehxA* positive. However, none were *stx* positive and only two were *eae* positive (6). These data indicate that the *ehx* locus may be commonly associated with environmental *E. coli* strains that lack well-recognized virulence factors such as *stx* and *eae*, in addition to strains from cattle and sheep, and that enterohemolytic functionality may not necessarily be directly involved with virulence per se but may have a role in survival and persistence outside of the gastrointestinal tract under conditions in which trace elements such as iron are required for maintenance.

By using a single restriction endonuclease, six distinct *ehxA* PCR-RFLP subtypes could be distinguished in this study. The only previous study investigating the sequence diversity and evolution of the *ehxA* gene separated STEC strains from diverse geographical areas into two major groups that corresponded to strains that were *ehxA* and *eae* positive and strains that were *ehxA* positive and *eae* negative by PCR analysis (7). Although *eae*-positive, *stx*-negative strains were not included in the study by Boerlin et al. (7), STEC strains were divided into two groups comprising *eae*-positive and *eae*-negative STEC strains upon analysis of the various *ehxA* alleles identified. The same division of STEC strains according to *ehxA* sequence similarity was also found in this present study, but all the *eae*-positive, *stx*-negative strains were grouped with the *eae*-positive STEC strains. This study has identified an additional subtype (D) not noted in previous work that has a distinct PCR-RFLP profile type and a DNA sequence that represents a new *ehxA* group associated with *stx*-positive, *eae*-negative strains from cattle. In addition, the *eae*-positive PCR-RFLP subtype E strains clustered with the *eae*-negative PCR-RFLP subtype A strains, indicating that there may be some intermediate *ehxA* subtypes that do not fit the previous *ehxA* grouping model. A further example of a strain that did not conform to the previous *ehxA* grouping scheme was a single O98:H$^-$ (AGR151) strain that was *eae* positive but had an *ehxA* PCR-RFLP profile that corresponded to the subtype A profile more usually associated with the *eae*-negative, *stx*-positive strains. The *ehxA* gene from this strain was fully sequenced and, upon phylogenetic analysis, grouped with the cluster comprising mostly *eae*-negative, *stx*-positive strains (Fig. 3). For the most part, RFLP analysis of the complete *ehxA* sequence is able to distinguish STEC strains that are positive for *stx* only from *E. coli* strains that are positive for *eae* only or *eae*-positive STEC strains. In addition to the analysis of *ehxA*, further sequence analysis of the large plasmid of STEC has confirmed the clear division of this pathotype into two groups (7), but the analysis of the same sequences of the large plasmids of *stx*-negative, *eae*-positive strains is required to determine whether these strains too are more closely related to *eae*-positive STEC. Furthermore, the *ehxA* sequence analysis of the *stx*-negative, *eae*-negative strains isolated from wastewater (6) is warranted to assess the relationship of these strains to those *stx*-positive and/or *eae*-positive strains examined in this work.

The predominance of subtypes A and C and the apparent scarcity of subtypes D and E requires confirmation from further *ehxA* subtyping studies. Indeed, two of the three subtype D strains were isolated from the same dairy cow, and the remaining strain was isolated from the same cohort of dairy cattle on the same day, indicating that this *ehxA* subtype may be rare. The two subtype E strains were isolated from cattle from two separate sites. Subtype F, although uncommon, may be fairly widespread in cattle and sheep as it was isolated from animals on seven separate sampling occasions and from all four separate locations where sampling took place.

No O157 strains were identified among this collection of bacteria isolated from cattle and sheep (11); however, STEC O157:H7 and O157:H$^-$ strains have been identified previously using this TaqI PCR-RFLP method as having a specific subtype found exclusively in STEC serogroup O157 strains (7), and this subtype corresponds to subtype B from the present study. Therefore, the *ehxA* PCR-RFLP subtypes of four New Zealand O157:H7 human strains were included in this study to give a characteristic profile for this serotype (Fig. 1). The *ehxA* gene of one of the New Zealand O157:H7 strains (ER03/4238) was also sequenced to establish the identity of its sequence to others available in the GenBank database. Of note, a single strain (O121:H19) gave a TaqI *ehxA* PCR-RFLP profile that was indistinguishable from that of the *ehxA* gene of O157. When sequenced, the *ehxA* gene from this *stx*-negative, *eae*-positive subtype B strain grouped closely with the sequence from the New Zealand O157:H7 ER03/4238 strain. These data indicate that the use of *ehxA* subtype A, for the identification of *eae*-negative STEC strains that are less frequently associated with disease than other STEC strains, and the use of subtype B, for the identification of the serotypes O157:H7 and O157:H$^-$ that are commonly associated with large outbreaks of diarrheal disease and HUS, cannot be exhaustively relied upon for distinguishing *eae*-negative from *eae*-positive strains.

It should be highlighted that the use of descriptive terms for diarrheagenic *E. coli* pathotypes, such as EPEC and STEC, must be viewed with caution as there have been several instances in which analyses of virulence factors, such as *ehxA*, shared by STEC and *stx*-negative *E. coli* strains have indicated that there may be some shared evolutionary relationships. In contrast, within EPEC and STEC groups, there are other virulence factors, such as *bfp*, *eae*, and *ehxA*, that clearly separate bacteria of each pathotype into different groups.

In summary, this exhaustive study has shown a strong link not only between the presence of the *ehxA* and *eae* alleles in STEC strains from cattle and sheep but also between *ehxA* and *eae*-positive atypical EPEC strains isolated from cattle and sheep. There is no apparent difference in *ehxA* genes from ruminant *E. coli* strains and those isolated from humans that may indicate specific roles for enterohemolysin in the separate hosts. Whether the reduced frequency of *ehxA* in *stx*-positive, *eae*-negative STEC strains may be in part a reflection of the apparently reduced virulence of these strains and their association with hemorrhagic colitis and HUS in humans is un-

known, but several important roles of the enterohemolysin in pathogenesis have been identified only in humans. However, these roles and an immune response to enterohemolysin from atypical EPEC strains and *eae*-negative STEC strains such as those of O113:H21 in the context of human infection remain to be established. Generally, the *ehxA* gene is highly conserved (>95%) among a large number of atypical EPEC and STEC strains, but as demonstrated by the data from this study, certain distinguishing features of different *ehxA* subtypes are readily established by PCR-RFLP methods that give some possible insight into the pathogenicity and associated virulence factors of these subtypes. It is likely that the horizontal transfer of the *ehx* locus to *E. coli* may have occurred at least three times, (i) to *eae*-positive *E. coli* strains (including atypical EPEC and *eae*-positive STEC strains), (ii) to *eae*-negative STEC strains, and (iii) to *ehxA* subtype D *eae*-negative STEC strains, but selection pressures ensure that the locus remains highly conserved. Recent data also indicate that *ehxA* is present in *stx*-negative, *eae*-negative *E. coli* strains (6, 21), but the *ehxA* subtypes of these strains remain to be established. Further studies are also required to establish the role of the enterohemolysin in the animal host and to determine the mechanisms by which enterohemolysin expression may be regulated.

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