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Pathogenic Potential of Emergent Sorbitol-Fermenting Escherichia coli O157:NM

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Non-sorbitol-fermenting (NSF) Escherichia coli O157:H7 is the primary Shiga toxin-producing E. coli (STEC) serotype associated with human infection. Since 1988, sorbitol-fermenting (SF) STEC O157:NM strains have emerged and have been associated with a higher incidence of progression to hemolytic-uremic syndrome (HUS) than NSF STEC O157:H7. This study investigated bacterial factors that may account for the increased pathogenic potential of SF STEC O157:NM. While no evidence of toxin or toxin expression differences between the two O157 groups was found, the SF STEC O157:NM strains adhered at significantly higher levels to a human colonic cell line. Under the conditions tested, curli were shown to be the main factor responsible for the increased adherence to Caco-2 cells. Notably, 52 of 66 (79%) European SF STEC O157:NM strains tested bound Congo red at 37°C and this correlated with curli expression. In a subset of strains, curli expression was due to increased expression from the csgBAC promoter that was not always a consequence of increased csgD expression. The capacity of SF STEC O157:NM strains to express curli at 37°C may have relevance to the epidemiology of human infections as curliated strains could promote higher levels of colonization and inflammation in the human intestine. In turn, this could lead to increased toxin exposure and an increased likelihood of progression to HUS.

Shiga toxin-producing Escherichia coli (STEC) strains of serogroup O157 are associated with human disease, including diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS). While non-sorbitol-fermenting (NSF) STEC O157:H7 strains are important human pathogens worldwide (37), sorbitol-fermenting (SF) STEC O157:NM strains have emerged as important pathogens in continental Europe (28). SF STEC O157:NM strains were first recognized causing HUS in children in Bavaria, Germany, in 1988 (28), and since then, these strains have been isolated from patients throughout Germany (20, 29), including a further outbreak in Bavaria that resulted in 28 cases of HUS and three fatalities (2). The first isolation of SF STEC O157:NM strains outside Germany occurred in the Czech Republic in 1995 (7). There have since been reports of this pathogen causing diarrhea or HUS in Hungary (28), Finland (30), another region of the Czech Republic (8), Austria (41), and Sweden (S. Löfdahl, personal communication). Infection with SF STEC O157:NM was not reported outside continental Europe until 2002, when it was isolated in Australia (4) and Scotland (1). It was subsequently isolated from patients in England (24) and Ireland (18). Between April and May 2006, 18 cases of SF STEC O157:NM infection were identified in Scotland, 13 of which were associated with a nursery (13). A further two cases of SF STEC O157:NM infection were identified in August 2006.

There is some evidence to suggest that SF STEC O157:NM strains are more frequently associated with HUS than NSF STEC O157:H7 strains. For example, in Germany the probability of development of HUS after infection with SF STEC O157:NM is 1:2, whereas after infection with NSF STEC O157:H7, this ratio is 1:6 (M. Bielaszewska and H. Karch, personal communication). A similar situation appears to be the case in Scotland, where 10 out of 20 cases of SF STEC O157:NM infection identified between April and August 2006 progressed to HUS. While host susceptibility between outbreaks may account for such differences, it is also possible that SF STEC O157:NM strains are more virulent than NSF STEC O157:H7 strains. Shiga toxins are responsible for the serious consequences of STEC O157 infection, including HC and HUS. Increased toxicity could be accounted for by (i) a more potent toxin variant, (ii) increased toxin expression, or (iii) colonization of the human intestine at higher levels leading to a greater exposure to toxin. Recently published work has indicated that there are no significant differences in the Stx2 proteins encoded by SF STEC O157:NM and NSF STEC O157:H7 strains (6), and a report indicates that the toxicity levels may be similar (20).

In terms of intestinal colonization, there are multiple factors that may contribute to STEC O157 adherence. Both SF and NSF STEC O157 strains possess a type III secretion system that is responsible for intimate attachment and attaching and effacing lesion formation. In contrast to NSF STEC O157:H7, SF STEC O157:NM strains contain a complete efa1 gene (26),

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⁷ Published ahead of print on 13 October 2008.
which encodes the enterohemorrhagic *E. coli* factor for adherence (Efa1) (39). Attachment may also be mediated by surface-expressed appendages such as fimbriae. SF STEC O157:NM uniquely possess the *sfp* gene cluster which encodes novel Sfp fimbriae (SF enterohemorrhagic *E. coli* O157 fimbriae, plasmid encoded) (9). At least 16 putative fimbrial operons have been identified in NSF STEC O157:H7 (22, 36, 44), including the well-characterized adhesins type 1 fimbriae and curli. While the function (25) or capacity to express many of these fimbrial adhesins appears to have been lost by NSF STEC O157:H7 strains (36), SF STEC O157:NM strains may be different.

The objective of this study was to compare both phenotypic and genotypic characteristics of SF STEC O157:NM and NSF STEC O157:H7 strains isolated from cases of infection in Scotland. In particular, the research addressed whether SF STEC O157:NM strains could be considered more virulent as a result of increased toxin expression or enhanced colonization potential.

**MATERIALS AND METHODS**

**Bacterial strains.** The STEC O157 strains used in this study are detailed in Table 1. Wild-type SF STEC O157:NM strains H8824, H8432, H8489, H8757, and H8478 were isolated during the 2006 Scottish outbreak and shared an indistinguishable pulsed-field gel electrophoresis profile. A single SF STEC O157:NM isolate (H2687) was obtained from an isolated case in Scotland (35) and was associated with bloody diarrhea. Four NSF STEC SF O157:H7 strains were included for comparison. Three strains (H77, H511, and 1477/AI) were analyzed in the phenotypic assays. A further two NSF STEC O157:NM strains (H2687 and H8824) and two NSF STEC O157:H7 isolate was the sequenced strain EDL933 (44). Two representative SF STEC O157:NM H2687 Scotland 35 from separate Scottish outbreaks (11, 42, 47), and the remaining NSF STEC O157:H7 strains (36), SF STEC O157:NM strains may be different.

**TABLE 1. STEC O157 strains used in this study**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain(s)</th>
<th>Country of origin</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF STEC O157:H7</td>
<td>EDL933, H77, H511, 1477/AI</td>
<td>United States</td>
<td>44</td>
</tr>
<tr>
<td>SF STEC O157:NM</td>
<td>H2687, H8824, H8432, H8489, H8757, H8478</td>
<td>Scotland</td>
<td>11, 42, 47</td>
</tr>
<tr>
<td></td>
<td>H2687</td>
<td>Scotland</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>H8824</td>
<td>Germany</td>
<td>2006 Scottish outbreak cluster</td>
</tr>
</tbody>
</table>

**Characterization PCRs.** Multiplex PCR for the detection of genes encoding Shiga toxin 1 (*stx*), Shiga toxin 2 (*stx*), intimin (*eae*), and enterohemolysin (*hlyA*) was performed as described previously (43). The gene encoding the flagellar subunit (*flc*) was detected and characterized using the *flc* restriction fragment length polymorphism method (16). PCR to detect the presence of the 16-bp deletion in the *fim* switch that controls type 1 fimbriae expression was performed as previously described (34).

**Nucleotide sequencing.** Nucleotide sequencing was performed using an ABI Prism BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems), and reactions were analyzed on an ABI 3730 DNA sequencer. The nucleotide sequences of *stx*2 from the six Scottish SF STEC O157:NM strains were determined following PCR amplification of *stxB*1 and *stxB*2 using a *Taq* PCR core kit (Qiagen) and primer pairs *stx2Aseq_F1* and *stx2Aseq_R2* and *stx2Bseq_F* and *stx2Bseq_R*, respectively. (Table 2 details the primer sequences and cycling conditions for the PCRs.) The amplicons were purified and sequenced directly with the PCR primers and internal *stx* primers *stx2Aseq_F2* and *stx2Aseq_R1* (Table 2). For sequence analysis of the region required for curli formation (*csgRABC* and *csgDEFG* operons and the *csgB*-to-*csgD* intergenic region), the entire region was amplified with five separate PCRs using *ip*007 high-fidelity DNA polymerase (Bio-Rad) and primer pairs curli1F and curli1R, curli2F and curli2R, curli3F and curli3R, curli4F and curli4R, and curli5F and curli5R (Table 2). PCR products were purified and ligated into the Stratagene PCR cloning vector, pSc-C, with a Stratagene blunt PCR cloning kit (Stratagene); transformed following the manufacturer’s instructions; and sequenced with primers T3 and T7.

**Vero cell cytotoxicity assay.** Bacterial cultures were cultured in minimal essential medium-HEPES, supplemented with glucose (final concentration of 0.2%) and 0.25 mM Fe(III)Cl3 at 37°C at 200 rpm for 24 h. Mitomycin C (0.5 µg/ml) was added when required to cultures at an optical density at 600 nm (OD600) of 0.3. Bacterial cultures were centrifuged (4,000 × g for 20 min), and supernatants were filter sterilized through low-protein-binding 0.22-µm filters. Culture filtrates were added to Vero cells (seeded in 96-well plates), and the cytotoxicity assay was performed at 37°C in 5% CO2 for 72 h. Following the 72-h incubation, Vero cells were washed with phosphate-buffered saline (PBS) with 0.5% formalin in PBS. Quantification of viable Vero cells was determined by staining with crystal violet solution (0.13% crystal violet, 0.065% phenol, 5% ethanol, 2% formaldehyde in PBS) for 1 h. Plates were rinsed with distilled water and dried. Crystal violet was released from the cells with 10% acetic acid. Absorbance measurements were taken at 590 nm.

**Adherence assays to Caco-2 cells.** Caco-2 cells were seeded into eight-chamber microscope slides at a density of 1 × 105 cells/well and incubated at 37°C in 5% CO2 for 48 h prior to adherence assays. Bacteria were cultured for 24 h at 37°C on CFA agar plates (1% Casamino Acids, 0.15% yeast extract, 0.005% MgSO4, 0.0005% MnCl2, 1.5% agar) and in CMDF broth [13 mM K2HPO4, 6 mM KH2PO4, 8 mM (NH4)2SO4, 2 mM sodium citrate, 0.4 mM MgSO4, 0.2% Casamino Acids, 0.2% glucose, 5 µM CaCl2, 0.01% tryptone], which have both been used previously to promote fimbrial expression (9, 15). Bacteria were harvested from CFA plates in PBS. Bacterial cultures were diluted to an OD600 of 0.5, and 300 µl was added to the Caco-2 cells. Adherence assays were performed at 37°C in 5% CO2 for 2 h. This time point was chosen because we wanted to study initial attachment and avoid the complication of adherence due to induction of type III secretion. The bacterial suspension was removed, and the cells were washed with PBS three times prior to fixation with 4% paraformaldehyde. Bacteria attached to cells were labeled by indirect immunofluorescence using rabbit anti-O157 antisera (1:100 [Mast Assure]) and Alexa Fluor 568 secondary antibody (1:1,000 [Molecular Probes]). Slides were examined by fluorescence microscopy, and the number of bacteria per field was counted for randomly selected fields in each well for at least 20 fields. Images were captured with Leica software.
Biofilm assays. Bacteria were cultured statically overnight at 28°C in LB, CFA, and CDMT broths. Cultures were diluted 1:100 into 3 ml medium, and 200 μl was added to 96-well polystyrene plates. Plates were incubated at 28°C for 24 h and 48 h. Bacterial adherence to microtiter well surfaces was determined by crystal violet staining, as described earlier, except that wet sheets were washed with PBS. Sterile medium was included as a control, and the A_{405} was subtracted from the other values.

Yeast cell agglutination. A single colony of the strain to be tested was inoculated into 5 ml LB broth and incubated statically at 37°C for 24 h. The culture was subcultured into a further 5 ml LB broth and incubated statically for another 24 h. Subculturing was repeated once more. Yeast cell agglutination was carried out by mixing 15 μl bacterial culture with an equal volume of baker's yeast (10 mg/ml) on glass slides, and the degree of clumping was assessed. Mannose inhibition of agglutination was confirmed using 3% α-D-mannose in the yeast suspension.

fim switch orientation assay. Orientation of the fim switch was carried out as described previously (23, 33, 45). Briefly, PCR was used to amplify a 603-bp region that incorporates the invertible fimC promoter element. Template DNA for the PCR was prepared by adding 50 μl of bacterial culture (following 3 days of consecutive subculture in LB broth) or a colony from cultures grown on CFA agar to 50 μl MilliQ H₂O and boiling at 100°C for 15 min. The PCR-amplified product was digested asymmetry with HinfI, and the fragments were separated on a 4% polyacrylamide Tris-borate-EDTA gel. Fragments were visualized after staining with ethidium bromide. Immunostaining of surface curli. Bacteria were cultured on CFA agar and in CFA and CDMT broths. Bacterial cultures were fixed in 4% paraformaldehyde. Curli filaments were labelled using mouse anti-SEF17 monoclonal antibody (1: 100) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin secondary antibody (1:500 [Sigma-Aldrich]). Slides were examined by fluorescence microscopy, and the images were captured with Leica software.

**TABLE 2. Primers and PCR conditions used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’→3’)</th>
<th>PCR conditions (30 cycles)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx2Aseq_F</td>
<td>TACGAGGCTCGGTTTGCGGG</td>
<td>Denaturing: 94°C, 30 s, 60°C, 45 s, 72°C, 80 s</td>
<td>1,110</td>
</tr>
<tr>
<td>stx2Aseq_R2</td>
<td>CCGATTGCGTTAACACGAGGC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>stx2Aseq_R1</td>
<td>CTCATTACGCCGAGGAGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>stx2Bseq_F</td>
<td>CCAGAATTGTGAAATCCTGCCC</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>stx2Bseq_R</td>
<td>GGGACTGTGCACTGAGTCG</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
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<tr>
<td>curli1F</td>
<td>CGCTTAAACGTTAAAGGCGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli1R</td>
<td>CCACATG TGACGTTAAGGGG</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli2F</td>
<td>TTCCTTATGGCTTACGTTGCC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli2R</td>
<td>CGGATACGCGCTTACGCGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli3F</td>
<td>CGCTGATTGAAACACGACG</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
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<tr>
<td>curli3R</td>
<td>CCGTCTGCTGATTCGTC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli4F</td>
<td>TCTCACAACCACTGGGAGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli4R</td>
<td>GCTTGCAAGAAGCACTGGAGC</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli5F</td>
<td>CGCGATGCTTTACGCGC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>curli5R</td>
<td>CTTGAGGGTTGTGTTATCC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
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<tr>
<td>csgBACprom_F</td>
<td>cggcggatccCAATTGTTTCTGTTACCGCC</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td>524</td>
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<tr>
<td>csgBACprom_R</td>
<td>cggcggatccCAATTGTTTCTGTTACCGCC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
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</tr>
<tr>
<td>stx2Aseq_R</td>
<td>aaggatccTCACCCTGGACCTGGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>stx2Aseq_R1</td>
<td>aaggatccTCACCCTGGACCTGGC</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
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</tr>
<tr>
<td>stx2Aseq_R2</td>
<td>aaggatccTCACCCTGGACCTGGC</td>
<td>Annealing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>csgBAC1</td>
<td>aaggatccTCACCCTGGACCTGGC</td>
<td>Extension: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>csgBAC2</td>
<td>aaggatccTCACCCTGGACCTGGC</td>
<td>Denaturing: 94°C, 30 s, 58°C, 45 s, 72°C, 30 s</td>
<td></td>
</tr>
</tbody>
</table>

* The lowercase portions of some sequences are nonmatching, and the underlined bases show the incorporation of restriction sites.

Inhibition adherence assays. Bacteria were cultured on CFA agar as described previously and prior to adding bacteria to Caco-2 cells, aliquots of bacterial cultures were incubated in the presence or absence of anticiurlic monoclonal antibody (1:100). Adherence to Caco-2 cells and subsequent staining, visualization, and enumeration of results were carried out as described previously.

Detection of curli expression. To screen for surface curli expression bacteria were cultured on CFA-Congo red (CR) indicator plates (0.01% CR) for 24 h at 37°C. Curliated bacteria were able to bind CR dye and stain red. The ability of selected strains to bind CR was also assessed quantitatively as described previously (17). Briefly, bacteria harvested from CFA agar plates were incubated in the presence of 100 μM CR at 37°C in an orbital shaker for 30 min. Bacterial suspensions were centrifuged (10 × g for 10 min) and the absorbance of free CR in the supernatant was measured at 490 nm.

Construction of a csgB deletion and complementation. The csg4 and csgB genes were deleted from SF O157:NM strain H8284 by allelic exchange (45, 46). Briefly, regions flanking csgB were amplified using primer pairs FlupCsgBA/FdownCsgBA and F2upCsgBA/FdownCsgBA (Table 2) and cloned into pIB307 (45, 46) to produce pTD08. A csgB deletion strain for one strain background acted as a control for background fluorescence and was subtracted from the other values.
TABLE 3. Details and accession numbers of nucleotide sequences determined in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence description</th>
<th>Sequence length (bp)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2687</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1,248</td>
<td>EU526759</td>
</tr>
<tr>
<td>H8824</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1,248</td>
<td>EU526760</td>
</tr>
<tr>
<td>H8432</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1,248</td>
<td>EU526761</td>
</tr>
<tr>
<td>H8489</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1,248</td>
<td>EU526762</td>
</tr>
<tr>
<td>H8757</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>H8478</td>
<td>stx&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1,248</td>
<td>EU526764</td>
</tr>
<tr>
<td>340/97</td>
<td>csgBA and csgDEFG operons and csgB-to-csgD intergenic region</td>
<td>4,544</td>
<td>EU554557</td>
</tr>
<tr>
<td>080/01</td>
<td>csgBA and csgDEFG operons and csgB-to-csgD intergenic region</td>
<td>4,544</td>
<td>EU554558</td>
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<tr>
<td>E06/486</td>
<td>csgBA and csgDEFG operons and csgB-to-csgD intergenic region</td>
<td>4,544</td>
<td>EU554559</td>
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<td>H8824</td>
<td>csgBA and csgDEFG operons and csgB-to-csgD intergenic region</td>
<td>4,544</td>
<td>EU554560</td>
</tr>
</tbody>
</table>

by analysis of variance in Genstat, 10th ed. Caco-2 adherence assays with the curli deletion mutant and the European SF STEC O157:NM strains and the promoter expression data were analyzed using the Student’s t test in Excel.

Nucleotide sequence accession numbers. Nucleotide sequences from this study have been deposited in the GenBank database under the accession numbers listed in Table 3.

RESULTS

Characterization of STEC O157 isolates. The six SF STEC O157:NM strains isolated from infections in Scotland (Materials and Methods) were found to possess an H7 fliC restriction fragment length polymorphism pattern. Multiplex PCR for the detection of genes encoding Shiga toxins 1 and 2, intimin, and enterohemolysin demonstrated that the SF STEC O157:NM isolates were positive for stx<sub>2</sub>, eae, and hlyA and negative for stx<sub>1</sub>. The three NSF STEC O157:H7 isolates from Scottish outbreaks were also positive for stx<sub>2</sub>, eae, and hlyA and negative for stx<sub>1</sub>. STEC O157:H7 EDL933 was confirmed to be positive for stx<sub>1</sub>, stx<sub>2</sub>, eae, and hlyA.

Sequencing of stx<sub>2</sub>. The nucleotide sequences of stx<sub>2</sub> were determined for the six Scottish SF STEC O157:NM isolates. The sequences from the five isolates obtained from the 2006 Scottish cluster (with GenBank accession numbers detailed in Table 3) were identical and the same as those published for three previously characterized SF STEC O157:NM strains (6). Their A and B subunit genes differed from the published E. coli EDL933 sequence by seven nucleotides and one nucleotide, respectively, and encode a Stx2 protein which differed from Stx2 from EDL933 by a single amino acid residue in each of the subunits. The stx<sub>2</sub> sequence from SF STEC O157:NM isolate H2687 (GenBank accession no. EU526759) differed from EDL933 by a single nucleotide in the A subunit, and the B subunits were identical. This SF STEC O157:NM isolate had a predicted protein sequence for Stx2 identical to that of EDL933.

Vero cell cytotoxicity assays. To determine if Stx2 expression levels could vary between the two STEC O157 groups, standard Vero cell cytotoxicity assays were carried out with culture filtrates of strains without and after induction with mitomycin C. A single SF STEC O157:NM isolate from the 2006 Scottish outbreak cluster (H8824) was analyzed along with SF STEC O157:NM strain H2687 isolated from a single case in 2003 and compared with NSF STEC O157:H7 strains EDL933 and 1477/AI (Fig. 1). One-way analysis of variance analysis of Vero cell survival following exposure to basal levels of toxin in culture filtrates from the four STEC O157 uninduced cultures demonstrated significant variation (P < 0.001). The source of this variation is almost entirely due to EDL933, which has a different Shiga toxin background (Stx1 and Stx2) from the other three STEC O157 isolates (Stx2 alone). Induction with mitomycin C reduced Vero cell survival for all toxin-positive strains tested, but there was no significant difference in Vero cell survival rates between the four STEC O157 strains examined following induction (P = 0.723). E. coli O157:H7 TUV93-0, a toxin-negative derivative of EDL933, was included as a negative control. Therefore, under the in vitro conditions tested there was no evidence of increased toxin expression and activity from the SF STEC O157:NM isolates in comparison with the NSF STEC O157:H7 isolates.

Adherence assays. To investigate if there are differences in the capacities of the two O157 groups to adhere to human intestinal epithelial cells, NSF STEC O157:H7 strains EDL933 and 1477/AI and SF STEC O157:NM isolates H2687 and H8824, cultured under two different conditions, were tested for their capacity to bind to Caco-2 cells. Significantly higher levels of adherence (50-fold) were observed for the SF STEC O157:NM strains compared with the NSF STEC O157:H7 strains when cultured on CFA agar, whereas little difference was observed between the two groups when cultured in CDMT broth (Fig. 2A). Images showing the adherence of STEC O157 strains when cultured on CFA agar, to Caco-2 cells are provided in Fig. 2B. It was apparent that the SF STEC O157:NM strains, but not NSF STEC O157:H7 strains, autoaggregated following CFA culture, and as a result, biofilm formation was also tested. SF STEC O157:NM isolates demonstrated increased adherence to polystyrene microtiter plates in comparison with the NSF STEC O157:H7 isolates under the different conditions tested (Fig. 2C and data not shown). It was evident that cul...
The two SF STEC O157:NM strains in CDMT broth promoted biofilm formation but did not increase adherence to Caco-2 cells. This indicates that the same combination of factors is not required for the two different adherence phenotypes.

Analysis of type 1 fimbria expression by NSF STEC O157:H7 and SF STEC O157:NM. NSF E. coli O157:H7 strains have been shown to contain a 16-bp deletion in the fim switch that controls type 1 fimbria expression (34, 45), thus preventing inversion of the fim switch to the on orientation and the expression of type 1 fimbriae. A PCR designed to detect the presence of this deletion was used to analyze the two groups of STEC O157 strains. The SF STEC O157:NM strains did not contain the deletion (Fig. 3A, lanes 5 to 10), whereas the deletion was present in the NSF STEC O157:H7 strains tested (Fig. 3A, lanes 1 to 4). When the two groups of STEC O157 strains were cultured under conditions optimal for expression promoting the two SF STEC O157:NM strains in CDMT broth promoted biofilm formation but did not increase adherence to Caco-2 cells. This indicates that the same combination of factors is not required for the two different adherence phenotypes.
of type 1 fimbriae (3-day subculture in LB broth, statically at 37°C), only the SF STEC O157:NM strains exhibited mannose-sensitive agglutination of yeast cells and had the fim switch in both the off and on orientations (Fig. 3B). In comparison, when SF STEC O157:NM strains were cultured on CFA agar, as for the adherence assays, the majority of the bacterial population had the fim switch in the off orientation (Fig. 3C, lanes 3 and 4).

Analysis of curli expression by NSF STEC O157:H7 and SF STEC O157:NM. CR binding is a well-established method to demonstrate surface curli expression by enteric bacteria (3, 21, 52). The STEC O157 strains were cultured on CFA-CR indicator plates at 37°C, and while the NSF STEC O157:H7 strains, EDL933 and 1477/Al, were negative as expected (10, 52), the SF STEC O157:NM strains, H2687 and H8824, were positive (data not shown). Additional evidence for curli expression was provided by immunostaining with an anticurli monoclonal antibody. When the bacteria were cultured on CFA agar or in CFA broth, the majority of aggregated SF STEC O157:NM bacteria exhibited fluorescent surface staining (Fig. 4A, middle panels), while the NSF STEC O157:H7 strains were negative (Fig. 4A, top panels). When the bacteria were cultured in CDMT, there was no surface staining of either STEC O157 group (data not shown). Furthermore, the SF STEC O157:NM strains demonstrated significantly higher levels of expression from the csgBAC curli promoter compared with the NSF STEC O157:H7 strains when cultured on CFA agar (Fig. 4B). Taken together, these data indicate that the SF STEC O157:NM strains tested express curli at 37°C on CFA agar.

The contribution of curli to SF STEC O157:NM adherence. To establish if sequence variation in curli

![FIG. 4. Analysis of curli expression by STEC O157 strains.](image)

A

 EDL933

 H8824

 EDL933

 B

 EDL933 1477/Al H2687 H8824

 FIG. 4. Analysis of curli expression by STEC O157 strains. (A) Curli detection by immunofluorescence microscopy. Phase-contrast and fluorescence micrographs are shown of NSF STEC O157:H7 EDL933 (top panel), SF STEC O157:NM strain H8824 (middle panel), and SF STEC O157:NM strain H8824ΔcsgB (bottom panel) stained for curli using an anticurli monoclonal antibody following culture on CFA medium at 37°C as described in Materials and Methods. (B) csgBAC curli promoter activity in STEC O157 strains. Expression from the csgBAC promoter was determined in the indicated strains using a promoter-GFP fusion as described in Materials and Methods. Shaded and unshaded bars represent values obtained following culture of the bacteria on CFA agar and in CDMT broth, respectively. Background fluorescence levels were determined for the promoterless plasmid pAJR70 and subtracted. The data are expressed as relative fluorescence units (RFU). A single asterisk indicates that, following culture on CFA agar, expression from the csgBAC promoter for SF STEC O157:NM strain H2687 was significantly higher than that for NSF STEC O157:H7 strains EDL933 and 1477/Al (P < 0.001). Double asterisks indicate that, following culture on CFA agar, expression from the csgBAC promoter for SF STEC O157:NM strain H8824 was significantly higher than that for NSF STEC O157:H7 strains EDL933 and 1477/Al (P < 0.001). Error bars indicate standard errors of the means.

Analysis of curli expression by European SF STEC O157:NM. To determine whether curli expression at 37°C is a common attribute of SF STEC O157:NM strains, a collection of 66 SF STEC O157 strains collected by Helge Karch’s group (Muenster, Germany) (9, 26, 28) were analyzed for their potential to bind CR on CFA-CR indicator plates. CR binding was confirmed for a subset of the strains by a quantitative method (Materials and Methods). A total of 52/66 (79%) were assessed as positive for CR binding. To further demonstrate the association of CR binding with curli expression and Caco-2 cell adherence, two of these CR-positive strains (E02/879 and E06/486) and two CR-negative strains (340/97 and 080/01) were analyzed by immunofluorescence using the anticurli monoclonal antibody and tested in adherence assays. Both CR-positive strains expressed curli and adhered at significantly higher levels to the Caco-2 cells compared with the CR-negative strains that did not express curli (Fig. 6A and B). Curli expression on CFA at 37°C is therefore a common property of SF STEC O157:NM strains.

Investigation of differential curli expression in SF STEC O157:NM strains. To establish if sequence variation in curli loci could account for the differences in curli expression between strains, the nucleotide sequences of the relevant regions (csgBAC and csgDEFG operons and the csgB-to-csgD inter-
levels than the O157:NM strain H8824 adhered to Caco-2 cells at significantly higher deviations. The asterisk indicates that the CR-positive SF STEC absence of this strain to Caco-2 cells was compared with its parent and not to bind CR or express curli (Fig. 4A, bottom panel). The adher-

esion mutant and wild-type strains. Strain SF STEC O157:NM H8824 back-transformed predicted means, and error bars define 95% confi-
dence interval. The graph represents back-transformed predicted means, and error bars define 95% confidence interval values. (B) Comparative binding studies of a curli deletion mutant and wild-type strains. Strain SF STEC O157:NM H8824 was deleted for csgBA (Materials and Methods) and was demonstrated not to bind CR or express curli (Fig. 4A, bottom panel). The adher-

ence of this strain to Caco-2 cells was compared with its parent and NSF STEC O157:H7 EDL933. Microscopy was used to determine the number of bacteria per field for 30 fields. Error bars define standard deviations. The asterisk indicates that the CR-positive SF STEC O157:NM strain H8824 adhered to Caco-2 cells at significantly higher levels than the ΔcsgBA derivative of this strain (P < 0.001).

**DISCUSSION**

SF STEC O157:NM strains have emerged as significant human pathogens in continental Europe, causing diarrheal disease often resulting in life-threatening HUS. Of particular concern is that 10 out of 20 Scottish patients infected with SF STEC O157:NM in 2006 progressed to HUS. This is consistent with findings in Germany, where humans infected by these strains are more likely to progress to HUS. This compares to NSF STEC O157:H7 infections that are typically associated with HUS at lower frequencies (5 to 10%) (19, 37). As clinical cases with SF STEC O157:NM are uncommon, host factors in these cases may be responsible for a bias toward HUS. These include age, clinical history, or treatment issues such as the administration of antibiotics and/or antimotility agents. However, the aim of this research was to investigate the hypothesis that bacterial factors are the cause of the increased association of SF STEC O157:NM with HUS.

Basic characterization of the Scottish SF STEC O157:NM strains showed that they possessed the genes encoding Shiga toxin 2, intimin, and hemolysin. This is in accord with all previous studies on SF STEC O157:NM strains (4, 7, 8, 14, 20, 30). It is possible that a higher HUS rate following SF STEC O157:NM infection compared to NSF STEC O157:H7 infection could be accounted for by a more active toxin or the release of more toxin during infection. In the present study, the predicted protein sequence for Stx2 produced by the SF STEC O157:NM strains was either identical to or differed by a single amino acid in the A and B subunits from Stx2 from NSF STEC O157:H7 EDL933. The levels of toxin released by strains under induced and uninduced in vitro conditions were examined, and there was no evidence of increased toxin expression from the SF STEC O157:NM isolates. Thus, toxin sequence variation and increased toxin release are unlikely to account for any differences in virulence between SF and NSF STEC O157 strains. However, it is appreciated that in vivo conditions may result in expression differences not observed in vitro.

Alternatively, SF STEC O157:NM strains may colonize the human intestine in higher numbers, resulting in increased exposure to toxin. To test this, we compared the potential of SF STEC O157:NM and NSF STEC O157:H7 strains to adhere to a human colon carcinoma cell line. Culture of the strains on CFA agar plates at 37°C resulted in up to 50 times more SF STEC O157:NM bacteria than NSF STEC O157:H7 bacteria adhering to Caco-2 cells within 2 h. While type III secretion-based intimate attachment is likely to be important for intestinal colonization (12, 49), type III secretion profiles and levels were similar for the strains analyzed in this study (data not
Consequently, initial adherence is more likely to involve surface-expressed factors such as fimbriae.

An initial observation in this study was that the SF STEC O157:NM strains tested could bind CR when cultured on CFA medium at 37°C and as described in Materials and Methods. Adherence of selected strains to Caco-2 cells was determined after 2 h of incubation at 37°C. SF STEC O157:NM strain H8824 was included as a positive control. Microscopy was used to determine the number of bacteria per field for 30 fields. CR binding which correlates with curli expression (CFA, 37°C) is indicated for the strains below the graph. Error bars define standard deviations. Single asterisks indicate that, following culture on CFA agar, the CR-positive SF STEC O157:NM strain E02/879 adhered to Caco-2 cells at significantly higher levels than the CR-negative SF STEC O157:NM strains 340/97 and 080/01 (P < 0.001). Double asterisks indicate that, following culture on CFA agar, the CR-positive SF STEC O157:NM strain E06/486 adhered to Caco-2 cells at significantly higher levels than the CR-negative SF STEC O157:NM strains 340/97 and 080/01 (P < 0.01). Curli expression and adherence among selected European SF STEC O157:NM strains. (A) Curli detection by immunofluorescence microscopy. Phase-contrast and fluorescence micrographs are shown for the indicated strains stained for curli using an anti-curli monoclonal antibody following culture on CFA medium at 37°C as described in Materials and Methods. (B) Adherence of selected strains to Caco-2 cells. The indicated strains were cultured on CFA agar, and their adherence to Caco-2 cells was determined after 2 h of incubation at 37°C. SF STEC O157:NM strain H8824 was included as a positive control. Microscopy was used to determine the number of bacteria per field for 30 fields. CR binding which correlates with curli expression (CFA, 37°C) is indicated for the strains below the graph. Error bars define standard deviations. Single asterisks indicate that, following culture on CFA agar, the CR-positive SF STEC O157:NM strain E02/879 adhered to Caco-2 cells at significantly higher levels than the CR-negative SF STEC O157:NM strains 340/97 and 080/01 (P < 0.001). Double asterisks indicate that, following culture on CFA agar, the CR-positive SF STEC O157:NM strain E06/486 adhered to Caco-2 cells at significantly higher levels than the CR-negative SF STEC O157:NM strains 340/97 and 080/01 (P < 0.01). (C) csgBAC and csgD promoter activity in selected strains. The indicated strains were transformed with plasmid-based promoter-GFP fusion constructs, and expression of csgBAC::gfp (shaded bars) and csgD::gfp (unshaded bars) was measured following the culture of transformed strains on CFA-CAM agar plates. Background fluorescence levels were determined for the promoterless plasmid pAJR70 and subtracted. The data are expressed as relative fluorescence units (RFU). Error bars indicate standard errors of the means. Single asterisks indicate that, following culture on CFA-CAM agar, expression from the csgBAC promoter for CR-positive SF STEC O157:NM strains E02/879, E06/486, and H8824 was significantly higher than that for CR-negative SF STEC O157:NM strain 340/97 (P < 0.02). Expression from the csgBAC promoter for strains E02/879 and H8824 was also significantly higher than that for strain 080/01 (P < 0.001). Double asterisks indicate that, following culture on CFA-CAM agar, expression from the csgD promoter for strain H8824 was significantly higher than that for strains 340/97, 080/01, E02/879, and E06/486 (P < 0.001).
In *E. coli*, two divergently transcribed operons, *csgBAC* and *csgDEFG*, are required for curli formation (21). The *csgBAC* operon encodes CsgA, the main subunit protein, and CsgB, the nucleator protein (3). CsgC, encoded by the third gene (*csgC*) in the *csgBAC* operon, has no reported role in curli formation (3). The *csgDEFG* operon encodes four accessory proteins required for curli assembly, where CsgD is the positive transcriptional regulator of the *csgBAC* operon (3). The curli loci from four SF STEC O157:NM strains—two curli-expressing strains and two that did not express curli under the conditions tested—were sequenced, and there were no changes detected that could account for the differences in curli expression. In particular, there were no sequence differences in the *csgB*-to-*csgD* intergenic region. This is significant as nucleotide changes in the *csgD* promoter region have been associated with variation in curli expression in *E. coli* O157:H7 (52). Transcription from the *csgBAC* promoter requires expression of the CsgD-positive regulator (21). In the present study, we have demonstrated that *csgBAC* expression was higher in strains expressing curli. In one case, this increased level coincided with a higher level of *csgD* expression, but in the other strains, levels of *csgD* expression were equivalent irrespective of curli expression phenotype. Therefore, it appears that curli expression in SF STEC O157:NM strains can be controlled either by increased *csgD* expression or by regulators acting directly on the *csgBAC* promoter with CsgD. Curli expression is controlled by osmolarity and various stress responses, in particular those that affect membrane integrity (27). Regulators controlled by these responses can act directly on the *csgBAC* promoter: for example, RcsB and CpxR, which are modified by phosphorylation. Further research will investigate differences in signaling pathways that control curli expression in SF STEC O157:NM strains.

Although previous studies have shown that in *E. coli* curli are generally expressed optimally at temperatures below 30°C (40), we have demonstrated that SF STEC O157:NM strains can express curli at 37°C. There is clear evidence that curli are an important virulence factor. For example, CR-binding variants of *E. coli* O157:H7 were more virulent in a mouse model (53) and expression of curli at 37°C has been reported for a significant number of human *E. coli* sepsis isolates (5). Curli expression may contribute to the virulence of the pathogen in several ways. First, curli can contribute to increased colonization through adherence to host cells (31, 32; this study) and extracellular matrix (3, 40) and through bacterial aggregation. Second, the interaction of curliated bacteria with certain host proteins may facilitate the spread of the bacteria through the host (3) and as bacteria expressing curli can bind to human contact-phase proteins, including fibrinogen, the clotting cascade may be inhibited (3). Third, curli can activate the innate immune system and have been shown to be a pathogen-associated molecular pattern recognized by TLR2 (51). This is in agreement with an earlier study which demonstrated that curli induce the proinflammatory cytokines tumor necrosis factor alpha, interleukin-6, and interleukin-8 (5). This final point may be critical in terms of toxin translocation during SF STEC O157:NM infections as there is evidence that toxin circulates on the surface of neutrophils (50). Therefore, the more inflammation that is induced by bacteria in the gastrointestinal tract, the more neutrophils are recruited that could traffic toxin into the bloodstream via the lymph system.

In conclusion, the emergence of SF STEC O157:NM strains is of concern since these pathogens appear to be associated with a higher incidence of HUS compared to the more common NSF STEC O157:H7 strains. We investigated potential factors which could account for increased virulence and found that, under the in vitro conditions investigated, SF STEC O157:NM can colonize a human colon cell line at higher levels than NSF STEC O157:H7. We have shown that curli are the major factor contributing to this increased adherence observed in vitro. However, the ability of SF STEC O157:NM cells to express functional type 1 fimbrae and Sfp fimbrae on their surface (38, 48; this study) suggests that these adhesins are also likely to contribute to the adherence of these pathogens in vivo. The ability of SF STEC O157:NM to express fimbral adhesins and colonize the human intestine at higher levels could have serious implications for human health where the presence of greater bacterial numbers, and perhaps more persistent colonization, in the intestine leads to higher toxin levels and associated sequelae.

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