Bacterial Genetic Determinants of Non-O157 STEC Outbreaks and Hemolytic-Uremic Syndrome after Infection

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Although O157:H7 Shiga toxin–producing Escherichia coli (STEC) are the predominant cause of hemolytic-uremic syndrome (HUS) in the world, non-O157:H7 serotypes are a medically important cause of HUS that are underdetected by current diagnostic approaches. Because Shiga toxin is necessary but not sufficient to cause HUS, identifying the virulence determinants that predict severe disease after non-O157 STEC infection is of paramount importance. Disease caused by O157:H7 STEC has been associated with a 26-gene pathogenicity island known as O island (OI) 122. To assess the public-health significance of this pathogenicity island, we examined the association between OI122 genes and outbreaks and HUS after non-O157 STEC infection. We found that a subset of OI122 genes is independently associated with outbreaks and HUS after infection with non-O157 STEC. The presence of multiple virulence genes in non-O157 serotypes strengthened this association, which suggests that the additive effects of a variable repertoire of virulence genes contribute to disease severity. In vivo, Citrobacter rodentium mutants lacking outbreak- and HUS-associated genes were deficient for virulence in mice; in particular, nleB mutant bacteria were unable to cause mortality in mice. The present study shows that virulence genes associated epidemiologically with outbreaks and HUS after non-O157 STEC infection are pivotal to the initiation, progression, and outcome of in vivo disease.

Hemolytic-uremic syndrome (HUS) causes acute renal failure in children and adults and follows infection with Shiga toxin–producing Escherichia coli (STEC) [1–4].

Received 9 January 2006; accepted 22 March 2006; electronically published 11 August 2006.

Potential conflicts of interest: none reported.

Financial support: Howard Hughes Medical Institute (study funding); Canadian Institutes of Health Research (study funding, including the Institute of Infection and Immunity Safe Food and Water Program, postdoctoral fellowships to M.E.W. and B.K.C., and Distinguished Investigator award to B.B.F.); Michael Smith Foundation for Health Research (postdoctoral fellowships to M.E.W., C.L., B.K.C., and N.F.B.); Honorary Killiam Postdoctoral Fellowship (to M.E.W.); Canadian Association for Gastroenterology (fellowship to C.L.); Dirección General de Asuntos del Personal Académico, Consejo Nacional de Ciencia y Tecnología (grant to J.L.P.); Howard Hughes International Research Scholar award (to J.L.P. and B.B.F.); University of British Columbia Peter Wall Distinguished Professorship (to B.B.F.).


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The Journal of Infectious Diseases 2006;194:819–27 © 2006 by the Infectious Diseases Society of America. All rights reserved. 0222-1899/2006/19406-015$15.00
The production of Shiga toxin by STEC is the primary virulence trait responsible for causing HUS. However, many non-O157:H7 strains that produce Shiga toxin do not cause HUS, which indicates that additional virulence determinants are required for disease. Work aimed at characterizing the pathogenic virulence determinants of E. coli has identified molecules—such as intimin, Tir, EspFu/TccP, and StcE—that mediate the formation of the attaching and effacing lesion and the type III secretion system, which delivers these virulence molecules into the infected host [10–14]. Additionally, a new family of STEC toxins [15] and other E. coli proteins translocated into host cells [16–19] are likely to be important in causing severe disease. Genetic typing of such determinants has revealed a variable repertoire of virulence genes among STEC strains. For instance, the virulence factor NleA/EspI, which appears to be a pivotal determinant of disease in mice [17, 18], is absent from 60% of STEC strains [20]. It is possible that the additive effect of this apparently variable repertoire of virulence determinants in a particular STEC strain governs the ability to cause severe disease.

This possibility has important consequences for the diagnosis of STEC. Although the detection of O157:H7 strains indicates a higher likelihood of developing HUS, compared with the detection of non-O157:H7 serotypes [21, 22], the clinical implications of identifying non-O157:H7 strains remain uncertain, given their variable virulence [23]. In addition, reliance on microbiological isolation only overlooks non-O157 STEC strains that might be clinically important causative agents of HUS and outbreaks. As a consequence, the contribution of non-O157 STEC to HUS and outbreaks is almost certainly underestimated. Information on the genetic determinants that contribute to the pathogenesis of non-O157 STEC is critical for the appropriate detection and management of strains associated with outbreaks and severe disease.

Outbreaks of STEC infection and HUS after STEC infection are associated with a region of the O157:H7 genome called O island (O1) 122 [24, 25]. O122 is absent from nonpathogenic E. coli strains and contains 26 genes, including homologues of putative Salmonella and Shigella virulence-associated factors. To examine the genetic determinants of non-O157:H7 STEC associated with severe disease, we analyzed the genetic content of O122 from HUS- and outbreak-associated clinical isolates of non-O157 STEC. Virulence determinants associated with HUS and outbreaks in humans were tested in an animal model of pathogenic E. coli infection for their role in disease. Pivotal, the outbreak-associated virulence determinant nleB modulated the infectious dose in mice, which suggests that it may contribute to the low infectious dose of STEC. The results revealed that bacterial genetic determinants associated with HUS and outbreaks are responsible for severe disease and the outcome of infection in vivo.

**MATERIALS AND METHODS**

**Bacterial strains.** The 72 clinical STEC isolates used in the present study are listed in table 1. All STEC strains of the same serotype were isolates from different patients, were not linked temporally, gave distinct macrorestriction enzyme–digest patterns by pulsed-field gel electrophoresis, and were known to be associated with severe disease and outbreaks in humans [25]. Nonpolar mutations in Citrobacter rodentium were generated using the lambda Red recombinase–based method described elsewhere [26].

**Investigation of strains for the presence of O1122 genes.** STEC strains were screened for the presence of the core O1122 genes Z4318, pagC, Z4322, Z4323, ent, nleB, and nleE and the lifA/efa1 open reading frames Z4332 and Z4333 (table 1). Polymerase chain reaction (PCR)–negative strains were retested by Southern hybridization using methods described elsewhere [25]. All PCR amplifications were performed in 50-μL reaction mixtures that contained 1× PCR buffer (Perkin-Elmer Applied Biosystems), 250 μmol/L dNTPs, 1 mmol/L MgCl₂, 25 pmol of each primer, and 2 U of Taq DNA polymerase (AmpliTaq; Applied Biosystems). Cycling conditions for all O1122 genes consisted of an initial denaturation step for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 1 min at the temperatures mentioned below, and elongation for 2.5 min at 72°C. There was a final elongation step for 5 min at 72°C. Southern hybridizations were performed using DIG-labeled probes generated in accordance with the manufacturer’s instructions in the PCR DIG Probe Synthesis Kit (Roche Diagnostics). Genomic DNA was extracted by using the DNeasy Tissue kit (Qiagen). Approximately 2 μg of DNA was digested with an excess of EcoRI and run on a 0.6% agarose gel. The gel was then transferred to a nylon membrane (Roche Diagnostics). Hybridizations were performed overnight at 42°C. Hybridized bands were detected by using the DIG Nucleic Acid Detection kit (Roche Diagnostics). EDL933 [24] and Sakai [27] O157:H7 strains and E. coli K-12 strain MG1655 [24] were used as controls. Oligonucleotide sequences and PCR amplification methods are listed in table 2.

**Statistical analysis.** Fisher’s exact test for 2-tailed significance was used to examine associations between HUS and outbreaks and the presence or absence of O1122 genes. The ability of O1122 genes to predict HUS- and outbreak-associated STEC strains was assessed by calculating the sensitivity, specificity, and predictive value. Sets of ≥2 groups were examined using

<table>
<thead>
<tr>
<th>Table 1. List of Escherichia coli strains used in the study.</th>
</tr>
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<tr>
<td>The table is available in its entirety in the online edition of the <em>Journal of Infectious Diseases</em>.</td>
</tr>
</tbody>
</table>
### RESULTS

**Modular OI122 in non-O157 STEC.** A survey of STEC DNA sequences (data not shown) revealed that, consistent with the results of previous work [25, 30], OI122 is incomplete in many serotypes; many non-O157 STEC strains contain a variable repertoire of virulence genes. The ability of STEC containing an incomplete OI122 to cause severe disease suggests that only a subset of genes within OI122 are virulence determinants.

Because OI122 has been associated with severe disease after infection, we examined which of the 26 genes in this island are associated with virulence in non-O157 STEC. Genetic typing of O157:H7 and non-O157 STEC strains (n = 74) (table 1) was performed for individual OI122 genes. The core region of OI122 contains many genes with hallmarks of virulence-associated genes. The association of these putative virulence genes with each other across HUS-associated non-O157 STEC strains (table 3) indicates that OI122 consists of at least 2 modules: (1) one consisting of Z4318, pagC, and Z4322 and (2) another consisting of Z4323, ent, nleB, and nleE. In non-HUS-associated STEC, the association of Z4323 with efa1/lifA is not significant (data not shown), which indicates that efa1/lifA likely makes up a third module independent of Z4323. Interestingly, the modular boundary between modules 2 and 3 contains a transposase (Z4330) in OI122 of EHEC O157:H7. It is possible that OI122 of O157:H7 strains has been generated by the acquisition of the 3 smaller genetic modules containing genes that provide a stepwise colonization advantage to STEC.

**Association of non-O157 virulence genes with HUS and outbreaks.** To determine whether the presence of the OI122 modules was associated with HUS after non-O157 STEC infection, we conducted a retrospective case-control study of 57 non-O157 STEC strains that were either associated with HUS after infection and outbreaks. The prevalence of these modules was compared in non-O157 STEC strains that were either associated or not associated with HUS. Each of the 3 modules was significantly associated with HUS (table 4), yet the non-O157 STEC–carrying module 3 was most likely to be associated with
Table 4. Non-O157 Shiga toxin–producing Escherichia coli (STEC) virulence gene modules associated with hemolytic-uremic syndrome (HUS).

<table>
<thead>
<tr>
<th>Module</th>
<th>Prevalence in non–HUS-associated STEC</th>
<th>Prevalence in HUS-associated STEC</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 (7/28)</td>
<td>59 (17/29)</td>
<td>4.3 (1.4–13.2)</td>
<td>.0156</td>
</tr>
<tr>
<td>2</td>
<td>21 (6/28)</td>
<td>55 (16/29)</td>
<td>4.5 (1.4–14.4)</td>
<td>.014</td>
</tr>
<tr>
<td>3</td>
<td>14 (4/28)</td>
<td>69 (20/29)</td>
<td>13.3 (3.6–49.9)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>2 + 3</td>
<td>7 (2/28)</td>
<td>55 (16/29)</td>
<td>16 (3.2–80.4)</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

NOTE. Data are % (no./total no.). Examination of the prevalence of virulence gene modules in HUS and non-HUS associated non-O157 STEC indicated that O island 122 modules were associated with HUS after non-O157 STEC infection. CI, confidence interval; NS, not significant; OR, odds ratio.

Table 5. Non-O157 Shiga toxin–producing Escherichia coli (STEC) virulence gene modules associated with outbreaks.

<table>
<thead>
<tr>
<th>Module</th>
<th>Prevalence in non–outbreak-associated STEC</th>
<th>Prevalence in outbreak-associated STEC</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 (18/42)</td>
<td>40 (6/15)</td>
<td>0.9 (0.3–3.0)</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>24 (10/42)</td>
<td>80 (12/15)</td>
<td>12.8 (3.0–54.6)</td>
<td>.0004</td>
</tr>
<tr>
<td>3</td>
<td>21 (9/42)</td>
<td>100 (15/15)</td>
<td>109.3 (6.0–2002)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>2 + 3</td>
<td>14 (6/42)</td>
<td>80 (12/15)</td>
<td>24 (5.2–111.1)</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

NOTE. Data are % (no./total no.), unless otherwise indicated. Examination of the prevalence of virulence gene modules in outbreak- and non–outbreak-associated non-O157 STEC indicates O island 122 modules 2 and 3 associate with non-O157 STEC outbreaks. CI, confidence interval; NS, not significant; OR, odds ratio.

HUS. The additional presence of module 2 strengthened the association of module 3 with HUS. This observation was consistent with the cumulative effects of virulence-associated genes contributing to non-O157 STEC disease.

Examination of the prevalence of OI122 modules in outbreak- and non–outbreak-associated STEC strains indicated that only modules 2 and 3 were significantly associated with non-O157 outbreaks (table 5), with module 3 being more strongly associated. The additional presence of module 2 did not strengthen the association of module 3 with non-O157 STEC outbreaks.

We then examined the prevalence of individual OI122 genes in non-O157 STEC strains associated or not associated with HUS. Table 6 shows that pagC, Z4322, ent, nleB, nleE, and efa1/lifA were more prevalent in non-O157 STEC associated with HUS after infection, which suggests these genes are involved in the pathogenesis of non-O157 STEC. Examination of the prevalence of OI122 genes in non-O157 STEC strains associated with outbreaks indicated that Z4318, pagC, and Z4322 were not associated with non-O157 outbreaks (table 7). However, the genes Z4323, ent, nleB, nleE, and efa1/lifA were each more prevalent in outbreak-associated non-O157 STEC, which implicates these genes in the pathogenesis non-O157 STEC.

We noted that the combined presence of pagC, nleB, and efa1/lifA in a single non-O157 strain increased the strength of association with HUS (odds ratio [OR], 13 [95% confidence interval [CI], 2.63–64.19]). These data are consistent with the notion that additive effects of virulence-associated genes are important in the ability of STEC to cause disease. Additionally, non-O157:H7 strains that were positive for both nleB and efa1/lifA were strongly associated with outbreaks (OR, 252.3 [95% CI, 13.22–4814]). It is important to note that all of the O157:H7 STEC strains examined (n = 14) contained all of the virulence determinants (pagC, Z4322, ent, nleB, nleE, and efa1/lifA) associated with outbreaks and human disease (table 1), which suggests that these OI122 genes also contribute to the pathogenesis of O157:H7 infection.

pagC, ent, nleB, and efa1/lifA in colonization and in vivo disease. The above results associated 6 virulence genes of non-O157:H7 STEC (pagC, Z4322, ent, nleB, nleE, and efa1/lifA) with HUS or outbreaks after infection. To assess the role of these genes in vivo, we constructed nonpolar deletion mutants in each gene in C. rodentium. C. rodentium causes attaching and effacing lesions in mice that are indistinguishable from enterohemorrhagic E. coli (EHEC)–induced lesions in humans and contains both the locus of enterocyte effacement pathogenicity island and the identified putative OI122 virulence determinants. At day 7 after infection, colonization of mice by ent and nleB mutant bacteria was significantly lower than that...
by wild-type bacteria (figure 1A). Partial complementation of colonization in mice was achieved for nleB mutant bacteria by providing nleB on a pACYC184-based plasmid (data not shown). efa1/lifA mutant bacteria were also defective for colonization at day 7 (data not shown), which is consistent with recent data [31]. pagC mutant bacteria were not attenuated for colonization (figure 1A), which suggests that pagC is dispensable for in vivo colonization at this time point.

To examine the ability of the mutant strains to cause disease in vivo, we measured crypt height as a measure of epithelial-cell hyperplasia [19] in the descending colon (figure 1B). Relative to mice infected with wild-type C. rodentium, the crypt heights in colons of mice infected with pagC, ent, or nleB mutant bacteria was significantly smaller, which indicates that pagC, ent, and nleB mutant bacteria are defective for the induction of hyperplasia.

pagC, ent, nleB, and efa1/lifA in virulence. To further examine the relative virulence properties of HUS-associated gene mutants in vivo, we performed competitive index experiments [32] (figure 1C). Mice were infected with a mixed inoculum of wild-type bacteria and isogenic OI122 mutants, and the output levels of each bacterial strain were examined 7 days after infection. In mixed infections with wild-type bacteria, pagC mutant bacteria were significantly attenuated. As was expected, ent and nleB mutant bacteria were attenuated, relative to wild-type bacteria (figure 1C). efa1/lifA mutant bacteria were also attenuated ($P < .001$; data not shown).

Necessity of nleB and ent for pathologic changes in vivo. To assess the role of the identified virulence predictors in disease, histopathologic changes were compared at day 12 after infection with mutant bacterial strains. Mice infected with wild-type bacteria exhibited significant histopathologic changes, relative to uninfected mice (figure 1D and 1E). Mice infected with pagC mutant bacteria displayed histopathologic changes not significantly different from those induced by wild-type bacteria (figure 1D), which suggests that pagC is not required for histopathologic changes in vivo.

### Table 6. Prevalence of O island 122 virulence determinants in non-O157 Shiga toxin–producing Escherichia coli (STEC) strains associated with hemolytic-uremic syndrome (HUS) after infection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prevalence in non–HUS-associated STEC</th>
<th>Prevalence in HUS-associated STEC</th>
<th>OR (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z4318</td>
<td>68 (19/28)</td>
<td>86 (25/29)</td>
<td>3.0 (0.8–11.1)</td>
<td>NS</td>
</tr>
<tr>
<td>pagC</td>
<td>32 (9/28)</td>
<td>76 (22/29)</td>
<td>6.6 (2.1–21.2)</td>
<td>.0014</td>
</tr>
<tr>
<td>Z4322</td>
<td>36 (10/28)</td>
<td>66 (19/29)</td>
<td>3.4 (1.2–10.2)</td>
<td>.0348</td>
</tr>
<tr>
<td>Z4323</td>
<td>29 (8/28)</td>
<td>55 (16/29)</td>
<td>3.1 (1.0–9.2)</td>
<td>NS</td>
</tr>
<tr>
<td>ent</td>
<td>32 (9/28)</td>
<td>69 (20/29)</td>
<td>4.7 (1.5–14.3)</td>
<td>.0081</td>
</tr>
<tr>
<td>nleB</td>
<td>32 (9/28)</td>
<td>69 (20/29)</td>
<td>4.7 (1.5–14.3)</td>
<td>.0081</td>
</tr>
<tr>
<td>nleE</td>
<td>32 (9/28)</td>
<td>66 (19/29)</td>
<td>4.0 (1.3–12.1)</td>
<td>.0173</td>
</tr>
<tr>
<td>efa1/lifA</td>
<td>14 (4/28)</td>
<td>69 (20/29)</td>
<td>13.3 (3.6–49.9)</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

**NOTE.** Data are % (no./total no.), unless otherwise indicated. CI, confidence interval; NS, not significant; OR, odds ratio.

### Table 7. Prevalence of O island 122 virulence determinants in non-O157 Shiga toxin–producing Escherichia coli (STEC) strains associated with outbreaks.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prevalence in non–outbreak-associated STEC</th>
<th>Prevalence in outbreak-associated STEC</th>
<th>OR (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z4318</td>
<td>76 (32/42)</td>
<td>80 (12/15)</td>
<td>1.3 (0.3–5.3)</td>
<td>NS</td>
</tr>
<tr>
<td>pagC</td>
<td>52 (22/42)</td>
<td>60 (9/15)</td>
<td>1.3 (0.4–4.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Z4322</td>
<td>50 (21/42)</td>
<td>53 (8/15)</td>
<td>1.1 (0.4–3.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Z4323</td>
<td>29 (12/42)</td>
<td>80 (12/15)</td>
<td>10 (2.4–41.9)</td>
<td>.0008</td>
</tr>
<tr>
<td>ent</td>
<td>33 (14/42)</td>
<td>100 (15/15)</td>
<td>60.9 (3.4–1093)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>nleB</td>
<td>33 (14/42)</td>
<td>100 (15/15)</td>
<td>60.9 (3.4–1093)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>nleE</td>
<td>33 (14/42)</td>
<td>93 (14/15)</td>
<td>28 (3.3–235.2)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>efa1/lifA</td>
<td>21 (9/42)</td>
<td>100 (15/15)</td>
<td>109.3 (6.0–2002)</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

**NOTE.** Data are % (no./total no.), unless otherwise indicated. CI, confidence interval; NS, not significant; OR, odds ratio.
Figure 1. Virulence determinants O island (OI) 122 genes pagC, ent, and nleB. A, Attenuation of ent and nleB mutant bacteria, relative to wild-type (wt) bacteria. Living bacteria were quantified in colon homogenates collected from infected C57BL/6 mice at day 7 after infection. nleB and ent mutant bacteria were attenuated for colonization relative to wild-type bacteria (denoted by asterisk; \( P < 0.001 \) and \( P < 0.01 \), respectively, 1-way analysis of variance [ANOVA] with Newman-Keuls multiple comparison test). Mean ± SE no. of colony-forming units per colon are shown. B, Crypt heights (mean ± SE) of C57BL/6 mice infected with OI122 mutants at day 12 after infection. pagC, ent, and nleB mutant bacteria were attenuated for virulence (\( P < 0.01 \), \( P < 0.01 \), and \( P < 0.001 \), respectively, 1-way ANOVA with Newman-Keuls multiple comparison test). C, Competitive indices of wt bacteria, relative to isogenic OI122 mutants in the mouse colon. pagC, ent, and nleB mutant bacteria were attenuated, relative to wt bacteria, in mixed infections (\( P < 0.001 \), \( P < 0.0001 \), and \( P < 0.0001 \), respectively, 1-sample t test). Dotted line, mean competitive index of chromosomally marked wt versus wt C. rodentium (wt C. rodentium vs. chromosomally marked wt C. rodentium gave a competitive index not significantly different from 1). Competitive index experiments were performed in C57BL/6 mice, and bacterial loads were examined on day 7 after infection. Each data point represents 1 mouse, and horizontal bars indicate means (mice with competitive index values < 0.01 were included for statistical analysis but not graphed). D, Attenuation of ent and nleB mutant bacteria for the ability to induce pathologic changes at day 12 after infection. Microscopic examination of colons 12 days after infection showed quantitative pathologic changes to be most severe in the colons of mice infected with wt bacteria and to be completely attenuated in mice infected with nleB mutant bacteria. Inflammation in the colons of mice infected with ent mutant bacteria was statistically intermediate between mice infected with wt bacteria and uninfected mice. P values were determined using 1-way ANOVA with Dunn’s multiple comparison test comparison. Representative colons of infected mice are shown in panel E. Magnification, ×100. LP, lamina propria; PMN, polymorphonuclear cells.

topathologic change. Mice infected with ent mutant bacteria exhibited histopathologic changes that were statistically intermediate, relative to changes induced during infection with wild-type bacteria and changes in uninfected mice, which indicates that ent mutant bacteria induce an intermediate level of virulence. Mice infected with nleB mutant bacteria exhibited few histopathologic changes, which suggests that nleB is pivotal to pathogenesis in vivo (figure 1D). The level of histopathologic change induced by nleB mutant bacteria resembled that of uninfected mice, despite their being colonized with \( 5 \times 10^7 \) bacteria (data not shown).

nleB and the determination of the outcome of infection in vivo. To assess the role of HUS-associated genes in causing mortality in mice susceptible to C. rodentium infection, C3H/HeJ mice were infected with HUS-associated gene mutants, and survival of the infected mice was examined. Examination of survival times indicated that mice infected with ent mutant bacteria survived significantly longer than mice infected with wild-type bacteria (figure 2A). Interestingly, nleB mutant bacteria were unable to cause mortality in C3H/HeJ mice, which indicates that this gene plays a central role in disease. To determine whether nleB mutant bacteria are able to induce mor-
infection, relative to mice infected with wild-type (wt) bacteria. Weight loss during infection with nleB mutant bacteria peaked day 11 after infection and returned to preinfection levels by day 20 after infection during nleB mutant infection, compared with mice infected with wt bacteria, which had extensive weight loss before they died.

**Modulation of the infective dose by nleB.** One of the characteristic features of O157:H7 STEC is a very low infective dose (estimated to be 100–200 bacteria) [33]. Because nleB is associated with severe disease after non-O157 STEC infection and non-O157 outbreaks and it was present in all O157:H7 STEC strains in that study, it is possible that nleB modulates the infectivity of pathogenic E. coli. To investigate this possibility in vivo, mice were infected with serial dilutions of wild-type or nleB mutant bacteria, and the infective dose was calculated. The ID_{50} of wild-type bacteria was 5.25 \times 10^{7} bacteria, whereas that of nleB mutant bacteria was 1.50 \times 10^{5} bacteria. nleB-positive bacteria were 285.7 times more infective than bacteria lacking nleB.

**DISCUSSION**

The clinical importance of non-O157 STEC may have been undervalued, because of the variable virulence of this diverse set of strains. The reliance largely on only MacConkey sorbitol agar for clinical diagnosis of O157:H7 STEC infection has further compounded this underestimation, because, without EIA for Shiga toxin or molecular typing, non-O157 STEC strains remain unidentified [23]. The importance of non-O157 STEC as a cause of HUS in regions such as Europe and Australia indicates that a subset of non-O157 STEC strains is likely as virulent as O157:H7; their clinical identification should therefore be of paramount importance. It is clear that both microbiologic and molecular diagnostic approaches should be conducted in parallel [22, 34, 35]. However, the implications of finding non-O157 STEC infection vary across different patient populations and regions, which suggests that a third diagnostic approach, such as rapid molecular typing, be used to identify pathogenic non-O157 STEC. The non-O157 disease-associated molecules that contribute to in vivo disease that we have identified may prove to be suitable targets for molecular typing in the identification of pathogenic non-O157 STEC.

The association of O1122 with severe disease that has been observed is likely due to the additive effect of many (if not all) O1122 genes associated with HUS (table 6). The present results indicate that non-O157 STEC strains contain a variable repertoire of O157:H7 virulence determinants, the additive effect of which (in concert with the rest of the genome and, pivotally, Shiga toxin) allows disease-associated STEC strains to cross a virulence threshold beyond which they are sufficiently pathogenic to humans to cause HUS after infection. The nature of such a virulence threshold remains to be determined, but it may involve the colonization of an infected host to a particular level in the face of immune assault. As a consequence, various strategies may be used by STEC to reach this threshold, including an array of adhesins and toxins [36]. Of the genes associated with HUS after non-O157 STEC infection, the present results implicate pagC, ent, nleB, and efa1/lifA as contributing substantially to reaching and/or crossing this virulence threshold. That nleB changes the outcome of lethal infection in vivo suggests that this molecule plays a major role in disease.

It is interesting that wild-type C. rodentium has a 285-fold
lower infectious dose in mice than nleB mutant bacteria. Given the strong association between nleB and non-O157 STEC outbreaks [OR, 60.9 [95% CI, 3.4–1993]], it is possible that nleB may modulate the infectious dose of non-O157 STEC to humans. One of the characteristic features of O157:H7 STEC is a very low infectious dose (estimated to be 100–200 bacteria) [33], which is thought to contribute to its ability to cause outbreaks [37, 38]. Because all O157:H7 strains examined in the present work and elsewhere [39] have been positive for nleB, it is possible that NleB is one of the many molecules that contribute to the low infectious dose of O157:H7 STEC and, therefore, to the epidemicity of O157:H7.

Genomic and proteomic approaches to examine virulence determinants of other STEC strains will likely reveal diversity in virulence determinants, including those absent from O157:H7 STEC. Already, important work has identified a new family of toxins, the subAB toxins, that are pivotal in O113:H21 STEC disease but are absent from O157:H7 [15]. This suggests that there are many different pathways toward virulence sufficient to cross the virulence threshold. Interestingly, the only HUS-associated strains in the present study that lacked ent, nleB, and efa1/lifA were 4 O113:H21 and 4 O91:H21 intimin-negative strains (serotypes known to contain subAB) [15]. This suggests that one pathway to sufficient virulence for severe disease involves the acquisition of type III secretion and the substrates secreted into the host (including nleB) and that another involves the acquisition of the subAB toxin genes. The present results also have important implications for the potential emergence of highly virulent O157:H7 strains through the acquisition of additional virulence genes (such as the subAB toxins).

Recent work examining the diversity of O157:H7 strains has indicated that, like non-O157:H7 strains, this serotype contains a variable repertoire of virulence genes [39, 40]. For instance, although the 15 O157:H7 strains examined in the present study contained a complete O1122, 2 O157:H7 strains have recently been identified that appear to lack the efa1/lifA open reading frame Z4332 [39]. Given this work and that of other researchers [31], these 2 O157:H7 strains would be predicted to be less virulent than their isogenic efa1/lifA-positive counterparts. This notion is supported by the observed variability in virulence of different O157:H7 strains in animal models [41–43].

Given the variability of O157:H7 virulence gene content that is starting to emerge [39], it remains possible that, like non-O157 STEC strains, some O157:H7 strains may associate more strongly with HUS and outbreaks than do others. Work toward the determination of this association has demonstrated the existence of 2 distinct O157:H7 lineages based on genomic diversity, with human and bovine isolates nonrandomly distributed throughout these lineages [44]. As a consequence, it is possible that there is a subset of O157 STEC strains circulating in cattle that is of lesserened pathogenic potential for humans: these strains would be predicted to lack virulence determinants contributing to human disease. Therefore, molecular typing of both non-O157 and O157:H7 STEC would seem to be warranted.

The association of nleB and efa1/lifA with both non-O157 STEC outbreaks and HUS after non-O157 STEC infection indicates that these molecules may be suitable for clinical typing of STEC to identify pathogenic strains as an adjunct to sorbitol MacConkey agar and EIA tests. Because NleB is a type III secreted molecule and requires this secretion system to function, the detection of nleB implies the presence of this secretion system and, consequently, intimin (an adhesin central to the pathogenesis of EHEC that is commonly used as a marker for the type III secretion system). Consistent with this, the co-occurrence of intimin (eae) and nleB (OR, 5073 [95% CI, 97.78–263,200]) in non-O157 strains suggests that molecular typing for nleB may be a viable alternative to typing for intimin. Although 100% of STEC strains positive for nleB are positive for intimin, it is possible that intimin-positive (and, therefore, type III secretion competent) STEC exist that lack nleB. Our work using nleB mutant bacteria (which are type III secretion competent) suggests that such STEC strains may be attenuated, because nleB mutant bacteria are defective for colonization and the ability to cause disease in vivo. Similarly, bacteria lacking nleA are unable to cause mortality in mice [17, 18], which again suggests that it is the concerted action of virulence factors, including type III effectors, that confer pathogenicity on STEC strains. Therefore, typing for type III secretion substrates pivotal to disease, rather than the presence of the system itself, may prove to be a valuable and simple approach to molecular typing for the appropriate clinical management of pathogenic STEC disease.

Acknowledgments

We thank the Finlay Laboratory and 2 anonymous reviewers, for helpful comments on the manuscript; and Michael Lowden, for technical advice.

References


