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Role of NleH, a Type III Secreted Effector from Attaching and Effacing Pathogens, in Colonization of the Bovine, Ovine, and Murine Gut

Cordula Hemrajani,1 Olivier Marches,1 Siouxsie Wiles,1 Francis Girard,1 Alison Dennis,2 Francis Dživa,3 Angus Best,4 Alan D. Phillips,5 Cedric N. Berger,1 Aurelie Mousnier,1 Valerie F. Crepin,2 Laurens Kruidenier,6 Martin J. Woodward,4 Mark P. Stevens,3 Roberto M. La Ragione,4 Thomas T. MacDonald,2 and Gad Frankel1,*

Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, United Kingdom; Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London E1 2AT, United Kingdom; Division of Microbiology, Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN, United Kingdom; Department of Food & Environmental Safety, Veterinary Laboratories Agency (Weybridge), Surrey KT15 3NB, United Kingdom; Centre for Paediatric Gastroenterology, Royal Free Hospital, London, United Kingdom; and Immuno-Inflammation CEDD, GlaxoSmithKline Medicines Research Centre, Stevenage, Hertfordshire SG1 2NY, United Kingdom

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The human pathogen enterohemorrhagic Escherichia coli (EHEC) O157:H7 colonizes human and animal gut via formation of attaching and effacing lesions. EHEC strains use a type III secretion system to translocate a battery of effector proteins into the mammalian host cell, which subvert diverse signal transduction pathways implicated in actin dynamics, phagocytosis, and innate immunity. The genomes of sequenced EHEC O157:H7 strains contain two copies of the effector protein gene nleH, which share 49% sequence similarity with the gene for the Shigella effector OspG, recently implicated in inhibition of migration of the transcriptional regulator NF-κB to the nucleus. In this study we investigated the role of NleH during EHEC O157:H7 infection of calves and lambs. We found that while EHEC ΔnleH colonized the bovine gut more efficiently than the wild-type strain, in lambs the wild-type strain exhibited a competitive advantage over the mutant during mixed infection. Using the mouse pathogen Citrobacter rodentium, which shares many virulence factors with EHEC O157:H7, including NleH, we observed that the wild-type strain exhibited a competitive advantage over the mutant during mixed infection. We found no measurable differences in T-cell infiltration or hyperplasia in colons of mice inoculated with the wild-type or the nleH mutant strain. Using NF-κB reporter mice carrying a transgene containing a luciferase reporter driven by three NF-κB response elements, we found that NleH causes an increase in NF-κB activity in the colonic mucosa. Consistent with this, we found that the nleH mutant triggered a significantly lower tumor necrosis factor alpha response than the wild-type strain.
has been shown to influence colonization of the bovine intestine (38), while Map (9), TccP/EspFU (37), and NleD (25) had no measurable effect. The roles of other T3SS effectors in colonization of ruminants are not known.

* C. rodentium* is a natural mouse pathogen that, while causing colonic hyperplasia, shares many virulence factors with EPEC and EHEC (reviewed in reference 28). Following inoculation via the oral route, bacteria colonize the colon, typically peaking at day 9 before clearance at around day 17 (27). Recently we refined the *C. rodentium* mouse model by developing noninvasive real-time bioluminescence imaging (BLI) to monitor infection dynamics and tissue tropism in vivo (41). Using this method, we have shown that *C. rodentium* first targets the murine cecal patch and rectum before the infection spreads to the large intestine.

The genome sequences of EHEC O157:H7 strains EDL933 and Sakai, EPEC strain E2348/69, and *C. rodentium* strain ICC168 revealed that EHEC and EPEC contain two *nleH* alleles (34; A. Iguchi et al., unpublished data), while *C. rodentium* harbors only one *nleH* gene. NicH shares 49% sequence similarity with the *Shigella flexneri* T3SS serine/threonine kinase effector protein OspG, which prevents ubiquitination and subsequent degradation of phospho-IκBα by FRT sites

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and primers.** The bacterial strains, plasmids, and primers used in this study are described in Tables 1, 2, and 3, respectively. Bacteria were grown at 37°C in Luria-Bertani (LB) broth or agar supplemented with ampicillin (100 μg ml⁻¹), chloramphenicol (25 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and nalidixic acid (NaI) (15 to 25 μg ml⁻¹) as appropriate. *C. rodentium* ICC169 *ΔnleH*, luminescent *C. rodentium* ICC180 *ΔnleH*, and EHEC O157:H7

### TABLE 1. Strains

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. rodentium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC169</td>
<td>Spontaneous Nal⁺ mutant of wild-type</td>
<td>40</td>
</tr>
<tr>
<td>ICC229</td>
<td><strong>ICC169 ΔnleH1 (Kan')</strong></td>
<td>This study</td>
</tr>
<tr>
<td>ICC180</td>
<td>Bioluminescent strain harboring the <em>Photorhabdus luminescens</em> operon (Kan')</td>
<td>40</td>
</tr>
<tr>
<td>ICC285</td>
<td><strong>ICC180 ΔnleH1 (Cm')</strong></td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85-170</td>
<td>Spontaneous Stxl⁻ and Stx2⁺ EHEC O157:H7 strain</td>
<td>35</td>
</tr>
<tr>
<td>85-170 Nan⁻ ICC232</td>
<td><strong>85-170 Nan⁺ ΔnleH1ΔnleH2 (Nan⁺ Kan⁺ Cm⁻)</strong></td>
<td>This study</td>
</tr>
<tr>
<td>ICC299</td>
<td>Commensal strain isolated from the cecum of a C57BL/6 mouse</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Kan', Cm', and Nan⁺, kanamycin, chloramphenicol, and nalidixic acid resistant, respectively.

**TABLE 2. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD3</td>
<td>orfR blaM; Cm' cassette flanked by FRT sites</td>
<td>6</td>
</tr>
<tr>
<td>pKD4</td>
<td>orfR blaM; Kan' cassette flanked by FRT sites</td>
<td>6</td>
</tr>
<tr>
<td>pKD46</td>
<td>orf101 repAI (18S) araB-gampet-eso blaM</td>
<td>6</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP synthesis under control</td>
<td>3</td>
</tr>
<tr>
<td>pET28-a</td>
<td>His6 tag expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET::nicH</td>
<td>Derivative of pET28-a expressing NicH1-His6</td>
<td>This study</td>
</tr>
<tr>
<td>pET::nicH1K159A</td>
<td>Derivative of pET28-a expressing NicH1K159A-His6</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Note:** The bacterial strains, plasmids, and primers used in this study are described in Tables 1, 2, and 3, respectively. Bacteria were grown at 37°C in Luria-Bertani (LB) broth or agar supplemented with ampicillin (100 μg ml⁻¹), chloramphenicol (25 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and nalidixic acid (NaI) (15 to 25 μg ml⁻¹) as appropriate. *C. rodentium* ICC169 *ΔnleH*, luminescent *C. rodentium* ICC180 *ΔnleH*, and EHEC O157:H7

85-170 Nan⁺ *ΔnleH1ΔnleH2* mutant strains (strains ICC229, ICC285, and ICC232, respectively) were generated using the one-step PCR λ Red-mediated mutation protocol (6) (Table 1). Primers 1 and 2 (Table 3) were used to amplify the kanamycin cassette in pKD4 for deletion of *nleH* in ICC169. Primers 3 and 4 were used to amplify the chloramphenicol cassette in pKD4 for deletion of *nleH* in ICC180. For construction of the EHEC O157:H7 85-170 Nan⁺ *ΔnleH1ΔnleH2* mutant, primers 5 and 6 for *nleH1* and 7 and 8 for *nleH2* were used for amplification of the kanamycin cassette from pKD4. Prior to deletion of *nleH1* from 85-170 *ΔnleH2*, the kanamycin cassette was deleted as described previously (6) using the pCP20 vector (3). The PCR product of the resistance cassette, flanked by approximately 50 bp of *nleH*, was digested with DpnI and the cassette electroporated into the recipient strains carrying pKD46, encoding the λ Red recombinase. Mutants were selected on selective LB plates with kanamycin or chloramphenicol. Recombinant clones were cured of pKD46 and the mutation confirmed by PCR using primers flanking *nleH1* and *nleH2* primers within the antibiotic resistance gene. Growth curves have confirmed that the mutant and wild-type strains have identical growth rates in LB and minimal media. Mutations of the *nleH1* and *nleH2* genes created using the same method in EPEC strains were successfully complemented in trans during in vitro studies.

**Oral inoculation of calves.** All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the local Ethical Review Committee. Groups of four 12-day-old Friesian bull calves were separately inoculated with approximately 10¹⁰ CFU of wild-type 85-170 Nan⁺ or 85-170 Nan⁺ *ΔnleH1 ΔnleH2* mutant, primes 5 and 6 for *nleH1* and 7 and 8 for *nleH2* were used for amplification of the kanamycin cassette from pKD4. Prior to deletion of *nleH1* from 85-170 *ΔnleH2*, the kanamycin cassette was deleted as described previously (6) using the pCP20 vector (3). The PCR product of the resistance cassette, flanked by approximately 50 bp of *nleH*, was digested with DpnI and the cassette electroporated into the recipient strains carrying pKD46, encoding the λ Red recombinase. Mutants were selected on selective LB plates with kanamycin or chloramphenicol. Recombinant clones were cured of pKD46 and the mutation confirmed by PCR using primers flanking *nleH1* and *nleH2* primers within the antibiotic resistance gene. Growth curves have confirmed that the mutant and wild-type strains have identical growth rates in LB and minimal media. Mutations of the *nleH1* and *nleH2* genes created using the same method in EPEC strains were successfully complemented in trans during in vitro studies.

**Oral inoculation of lambs.** Fifteen 6-week-old crossbred lambs were randomly divided into three equal groups, supplied with food and water ad libitum, and confirmed to be free of *E. coli* O157:H7 by enrichment and O157 immunomagnetic separation. All lambs were housed in biosecure containment level 2 accommodations. Each group was housed in a separate room with its own air handling. The animals were visited by experienced staff, who changed protective clothing between groups. Five lambs were each dosed orally with 10⁹ CFU of either wild-type 85-170 Nan⁺ or the isogenic *ΔnleH1 ΔnleH2* mutant separately, or with wild-type 85-170 Nan⁺ and the *ΔnleH1 ΔnleH2* mutant together, suspended in 10 ml of 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Approximately 24 h after oral inoculation and as required thereafter for up to 28 days, fecal rectal samples from each lamb were collected for direct plating onto SMAC supplemented with either 15 μg of NaI/ml or 25 μg of kanamycin/ml. Samples that were 85-170 negative on direct plating were enriched in buffered peptone water for 6 h at 37°C and then plated onto SMAC plates supplemented with the appropriate...
antibiotic. Representative colonies were confirmed to be E. coli O157 by latex agglutination (Oxoid). For co-infection studies, the competitive index (CI) was calculated; it is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the host after infection) divided by their ratio within the input (initial inoculum). For this experiment, the input ratio was 1:1. The null hypothesis that CI = 1 was tested by a two-sided t-test using the GraphPad InStat software (GraphPad Software Inc., San Diego, CA).

**Murine models.** Specific-pathogen-free female 6- to 8-week-old mice were used in this study. Wild-type inbred C57BL/6 and DBA-1 mice were purchased from Harlan UK Ltd. (Bicester, United Kingdom), while the transgenic light-producing animal model DBA-1 NF-κB-luc (Oslo) (PN 119335) was purchased from Xenogen-Caliper Corp. (Alameda, CA). The transgenic light-producing animal model NF-κB-RE-luc (Oslo)-Xen, commonly called NF-κB-RE-luc (Oslo), carries a transgene containing three NF-κB responsive element (RE) sites from the immunoglobulin κ light-chain promoter and modified firefly luciferase cDNA. All animals were housed in individually HEPA-filtered cages with sterile bedding and free access to sterilized food and water. Independent experiments were performed at least twice (but only once for histology) using at least four mice per group.

**Oral infection of mice, harvesting and collection of tissues, and bacterial stool counts.** Mice were orally inoculated using a gavage needle with 200 μl of overnight LB-grown bacterial suspension in PBS (∼5 × 10^9 CFU). The number of viable bacteria used as the inoculum was determined by retrospective plating onto LB agar containing antibiotics. Stool samples were recovered aseptically at various time points after inoculation and the number of viable bacteria per gram of stool determined after homogenization at 0.1 g/ml in PBS and plating onto LB agar containing the appropriate antibiotics. At selected time intervals post-infection, blood was collected by cardiac puncture and mice were sacrificed by cervical dislocation. Sections of distal colon were collected and snap frozen in liquid nitrogen before storage at −70°C prior to analysis. For coinfection studies, the competitive index (CI) was calculated; it is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the host after infection) divided by their ratio within the input (initial inoculum). For this experiment, the input ratio was 1:1. The null hypothesis that CI = 1 was tested by a two-sided t-test using the GraphPad InStat software (GraphPad Software Inc., San Diego, CA).

**Histopathology.** Segments of the cecum and the terminal colon of each mouse were collected at 9 and 14 days postinoculation, rinsed of their content, and fixed in 10% buffered formalin for microscopic examination. Formalin-fixed tissues were then processed, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) according to standard techniques. Sections were examined by light microscopy for the presence of intimately adhering bacteria on intestinal cells, as previously described (15). Crypt length was also evaluated, and the lengths of at least four well-oriented crypts were measured for each section. A nonparametric analysis of variance (ANOVA) with a posteriori comparisons was performed using commercially available GraphPad InStat v3.06 software (GraphPad Software Inc., San Diego, CA). P values of ≤0.05 were considered significant.

**Immunohistochemistry.** Snap-frozen colonial tissues, embedded in OCT mounting medium (VWR BDH, Lutterworth, United Kingdom), were sectioned using a cryostat to a thickness of 5 μm. Sections were mounted on poly-L-lysine slides (VWR BDH) and air dried overnight before fixing in acetone at room temperature for 20 min. After air drying for 1 h, sections were rehydrated in Tris-buffered saline (TBS) for 5 min and then incubated with antibodies against CD3, CD4, and CD8 (Serotec, Oxford, United Kingdom) at a dilution of 1:50 to 1:100 in PBS for 5 min and then incubated with antibodies against CD3, CD4, and CD8 (Serotec, Oxford, United Kingdom) at a dilution of 1:50 to 1:100 in PBS (pH 7.8) was administered by intraperitoneal injection 5 min prior to imaging. As a positive control for luciferase gene expression from the NF-κB-RE-luc (Oslo) mice, tumor necrosis factor alpha (TNF-α) (2 μg per mouse) was administered by intraperitoneal injection.

**TABLE 3. Primers**

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NeH CRICC169 Fw</td>
<td>ATGTTATCACCAGCTCCTGTAAATTGGAATGTTATCGAGATCTTT</td>
</tr>
<tr>
<td>2</td>
<td>NeH CRICC169 Rv</td>
<td>TAACGGCTTGATGAGCTGCGATCTTGCAGGAAGC</td>
</tr>
<tr>
<td>3</td>
<td>NeH CRICC180 Fw</td>
<td>ATGTTATCACCACCGTCCTGTAAATTGGGATGTTATCGAGATCTTT</td>
</tr>
<tr>
<td>4</td>
<td>NeH CRICC180 Rv</td>
<td>TAACGGCTTGATGAGCTGCGATCTTGCAGGAAGC</td>
</tr>
<tr>
<td>5</td>
<td>NeH1 EHEC Fw</td>
<td>AGAGGGTAATTGATTTAGCGCCATATCTGTGAATATTGGGGA</td>
</tr>
<tr>
<td>6</td>
<td>NeH1 EHEC Rv</td>
<td>TGTGCGTGATGGCTGAACTGCTTGCG</td>
</tr>
<tr>
<td>7</td>
<td>NeH2 EHEC Fw</td>
<td>ATGTTATCGCCCTCTTCATATAATTGGGATGTTATCGAGATCTTT</td>
</tr>
<tr>
<td>8</td>
<td>NeH2 EHEC Rv</td>
<td>TAACGGCTTGATGAGCTGCGATCTTGCAGGAAGC</td>
</tr>
<tr>
<td>9</td>
<td>TNF-α-Fw</td>
<td>ATGAGGCACAGAAGACGTATGC</td>
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<tr>
<td>10</td>
<td>TNF-α-Rv</td>
<td>TACAGGCTTGATGAGCTGCGATCTTGCAGGAAGC</td>
</tr>
<tr>
<td>11</td>
<td>B-actin-Fw</td>
<td>AGAGGGAAATCTGCTGACGTAC</td>
</tr>
<tr>
<td>12</td>
<td>B-actin-Rv</td>
<td>CAAATGCTGACCTGCTGGAGC</td>
</tr>
<tr>
<td>13</td>
<td>IFN-γ-Fw</td>
<td>TGAACCCCTCATACACTGCTATGCTTGCAGGAAGC</td>
</tr>
<tr>
<td>14</td>
<td>IFN-γ-Rv</td>
<td>CGACTCCCTTTCGCCCTTCGAG</td>
</tr>
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</table>
**RESULTS**

The NleH T3SS effector homologues. NleH belongs to a family of T3SS effectors found in diverse enteric pathogens.

**EPEC O127:H6**

**EHEC O157:H7 EDL933**

**Y. pseudotuberculosis**

**S. flexneri**

**Y. enterocolitica**

**EHEC O157:H7**

**E. coli**

**C. rodentium**

**Shigella**

**Yersinia**

**SEM.** Intestinal segments were fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy (SEM) as previously described (14). SEM samples were examined blindly at 25 kV using a JEOI JSM-5300 SEM [JEOL (UK) Ltd., Herts, United Kingdom].

**IFA.** An indirect immunofluorescence assay (IFA) was used for the detection of C. rodentium (serotype O152) in formalin-fixed, paraffin-embedded sections as previously described (14). Tetramethyl rhodamine isothiocyanate-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) secondary antibody was used to visualize O152-positive bacteria, while DNA of both bacteria and epithelial cells was counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). Sections were examined with an Axio Imager M1 microscope (Carl Zeiss MicroImaging GmbH, Germany). Images were acquired using an AxioCam MRm monochrome camera and computer processed using AxioVision (Carl Zeiss MicroImaging GmbH, Germany) and Adobe Photoshop 5.0 and Adobe Illustrator 8.0 software (Adobe Systems Incorporated, CA).

**RNA extraction and quantitative RT-PCR.** Total RNA was isolated from frozen colonic tissue using the RNeasy Plus minikit (Qiagen). All tissues used were harvested from mice 14 days after oral gavage. Total RNA was measured using a Nanodrop. TNF-α and IFN-γ primers were previously described (14). The PCR products were run on a 1% agarose gel alongside the 100-bp ladder from NEB in Tris-borate-EDTA buffer. GeneTools (Syngene) was used to conduct densitometric analysis. All TNF-α mRNA ratios were compared between mice infected with wild-type and nleH mutant bacteria. Statistical analyses were conducted with the one-way ANOVA Bonferroni multiple-comparison test using the GraphPad InStat software (GraphPad Software Inc., San Diego, CA), as all groups displayed normal distributions.
EHEC O157:H7 strain EC4045. To determine the role of NleH proteins in vivo, we generated EHEC O157:H7 (strain 85-170) ΔnleH1 ΔnleH2 and C. rodentium (ICC169 and bioluminescent strain ICC180) ΔnleH mutants.

The EHEC O157:H7 ΔnleH1 ΔnleH2 double mutant is shed in greater numbers than the parental strain from orally challenged calves. To assess the role of NleH in intestinal colonization of calves by EHEC O157:H7, wild-type and mutant bacteria were separately inoculated into four 12-day-old Friesian bull calves. Both wild-type and mutant strains colonized the calves efficiently, and the mutant was excreted at lower levels than the wild type during the first 4 days. From day 7 the mutant was shed at higher levels than the wild-type strain, and the difference became statistically significant (P < 0.05) from day 10 onwards (Fig. 2). The course of the fecal excretion of the wild-type strain was consistent with previously observed patterns (10, 33, 37).

**Contribution of NleH1 and NleH2 to colonization of conventional 6-week-old-lambs.** A 6-week-old lamb model was next used to compare the persistence of an E. coli O157:H7 ΔnleH1 ΔnleH2 double mutant and the isogenic wild-type strain. The ability of the mutant to establish itself and persist in lambs was investigated by monitoring the viable counts recovered in fecal pellets collected per animal. When given as a single inoculum, the wild-type E. coli O157:H7 isolate produced the classical shedding pattern in lambs, as described previously (5, 42), persisting in relatively high numbers during the early stages of infection and then declining by day 11 postinoculation (Fig. 3A). The ΔnleH1 ΔnleH2 mutant demonstrated a similar shedding pattern, except that positive fecal samples were noted only until day 12 postinoculation (Fig. 3A). When both isolates were administered in the same inoculum, the wild type persisted for 4 days longer than the ΔnleH1 ΔnleH2 mutant (data not shown). The mean CI was signifi-
cantly less than 1 for all time points where it could be calculated except day 1 (Fig. 3B), demonstrating that the wild-type strain outcompeted the ΔnleH1 ΔnleH2 mutant in the ovine model.

**Contribution of NleH to colonization in the C. rodentium murine model.** We followed the anatomical localization and pathogenic burden using BLI and viable counts in stools of mice infected with either wild-type or ΔnleH C. rodentium. Using BLI, we observed that while the wild-type and mutant strains colonized the cecal patch and rectum by day 3 to 4 postinfection. After adaptation to the in vivo environment (day 6 to 7), the entire distal colon was then heavily infected. Clearance began by day 10, at which point light intensity decreased. By day 14 the gastrointestinal tract had mostly cleared the infection (Fig. 4). Viable counts of bacteria in stool mirrored the results obtained by BLI; no significant differences in viable bacterial counts of the wild-type and mutant strains (data not shown). Immunohistochemistry, performed to investigate the influx of CD3+ CD4+, and CD8+ T cell subsets into the colonic lamina propria at 14 days postinoculation, revealed that although the ΔnleH mutant-infected animals showed slightly fewer CD3- and CD4-positive T cells in the lamina propria, no significant differences were observed between animals infected with the mutant or wild-type C. rodentium strains (data not shown). Immunofluorescent staining with anti-O152 and SEM analysis at day 9 or day 14 postinoculation did not reveal any differences between the wild-type and ΔnleH C. rodentium strains (Fig. 5).

**NleH does not affect A/E lesion formation and T-cell infiltration in the C. rodentium murine model.** The hallmarks of C. rodentium infection include induction of extensive colonic hyperplasia and influx of T cells into the colonic lamina propria. Histological examination and measurement of crypt length did not reveal any differences between the parent and mutant strains (data not shown). Immunohistochemistry, performed to investigate the influx of CD3+, CD4+, and CD8+ T cell subsets into the colonic lamina propria at 14 days postinoculation, revealed that although the ΔnleH mutant-infected animals showed slightly fewer CD3- and CD4-positive T cells in the lamina propria, no significant differences were observed between animals infected with the mutant or wild-type C. rodentium strains (data not shown). Immunofluorescent staining with anti-O152 and SEM analysis at day 9 or day 14 postinoculation did not reveal any differences between the wild-type and ΔnleH C. rodentium strains (Fig. 6).

**NleH influences NF-κB levels and expression of TNF-α in vivo.** A recent study has shown that OspG inhibits degradation of 1xB and hence activation of NF-κB and that a Shigella ospG mutant induces a stronger inflammatory response than the wild-type strain after inoculation of rabbit ileal loops (20). In order to determine if NleH influences activation of NF-κB in vivo, we infected NF-κB-RE-luc reporter mice with C. rodentium wild-type strain ICC169 or ICC169 ΔnleH; mice inoculated with the commensal E. coli strain ICC299 (Table 1) or PBS were used as controls. The number of nleH mutant bacteria shed from this mouse strain was similar to that of the wild type (data not shown). No significant differences in whole-body (including chest, neck, abdomen, and rectum) luminescence counts were recorded by live imaging, at any time point, between PBS-gavaged animals and those gavaged with wild-type C. rodentium, the nleH mutant, or the commensal E. coli. On day 14 postchallenge, we recorded luminescence counts in different organs. While no significant difference in the luminescence counts was seen in the mesenteric lymph nodes, spleens, and ceca of the different mouse groups (Table 4), there was a significant (P = 0.0024) 2.5-fold increase in the signal from the colon in mice infected with the wild-type C. rodentium compared to those inoculated with C. rodentium

![Fig. 5](image-url)
ratio of TNF-α or IFN-γ to β-actin. This revealed that the levels of TNF-α mRNA were significantly higher in tissues extracted from infected mice than in those from uninfected control mice. Mice infected with the wild-type C. rodentium had significantly higher TNF-α transcript levels than those infected with the ΔnleH mutant (Fig. 8). The levels of IFN-γ transcript were comparable in all mouse groups (data not shown).

DISCUSSION

Many gram-negative bacterial pathogens use T3SS effectors to modulate host cell signaling pathways. Effector proteins can trigger local (e.g., alteration in the cytoskeleton) or systemic (e.g., immune response) changes. Colonization of the mucosal surface requires temporal modulation of the host immune status. While downregulation of innate immunity might aid the pathogen to launch an infection and to reach a critical mass, activation of the immune system might assist the pathogen in its competition with the resident gut microflora. It is therefore not surprising that pathogenic bacteria are equipped with virulence factors which have antagonistic immune modulation activities. Indeed, in a recent report Lupp et al. demonstrated that C. rodentium population expansion in vivo is mediated by an inflammatory response that disrupts the endogenous intestinal microbiota (22). In contrast, using polarized culture models, Ruchaud-Sparagano et al. have shown that EPEC inactivates innate immune responses in vitro (32).

In this study we investigated the role of NleH in colonization, competitiveness, and activation of the NF-κB pathway in vivo. EPEC O127:H6 E2348/69 and EHEC O157:H7 EDL933 and Sakai contain two, almost identical, copies of nleH, while C. rodentium harbors only a single nleH gene. The functional consequences of this gene duplication are not known. Interestingly, a recent shotgun sequencing of a number of EHEC O157:H7 isolates has shown that they contain, in addition to nleH, a gene whose product shares 90.8% sequence identity with OspG (NCBI BLASTp).

Investigation of the contribution of NleH toward colonization and competitiveness of EHEC O157:H7 in bovine and ovine hosts, which are important animal reservoirs of the pathogen, showed that the EHEC O157 ΔnleH1 ΔnleH2 double mutant was shed in greater numbers than the parental strain from orally challenged calves, significantly from day 10 postinoculation. The precise effect of deleting nleH on factors or regulatory mechanisms involved in EHEC O157:H7 colonization in bovine intestines remains to be investigated. In single-infection studies with lambs, there was no statistical difference in shedding after oral inoculation with the same strains. However, CIs measured following oral inoculation of lambs with a mixture of wild-type EHEC O157:H7 and the ΔnleH1 ΔnleH2 double mutant revealed that the mutant was significantly out-competed. The reasons for the different phenotypes observed in the bovine and ovine models are currently not known. However, these results show that effector proteins might have dissimilar or even opposite functions in different hosts.

In order to determine the contribution of NleH to infection with C. rodentium, we mutated nleH in the wild-type ICC169 and luminescent ICC180 strains. Deletion of nleH from C. rodentium had no significant effect on in vivo tissue tropism, bacterial burden, colonic hyperplasia, or infiltration of CD3+, CD4+, and CD8+ cells to the lamina propria. A subtle phenotype was recently reported for C. rodentium ΔnleH in single infection, as expansion of the mutant population in vivo lagged behind that of the wild-type population during early stages (6 days postchallenge), although at later stages (10 days) the mutant and wild type colonized at comparable levels (11). The reasons for the different results might be due to the mouse status (i.e., composition of the normal gut flora), preparation of the inocula, or bacterial strains. Importantly, we found that in a mixed infection the nleH mutant was significantly out-competed by the wild-type strain, suggesting that expression of NleH increases the bacterial fitness in vivo. This could explain the need for conservation and multiplicity of the gene in EHEC and EPEC strains.

As we did not observe any difference in colonization and clearance dynamics, hyperplasia, and T-cell infiltration between the wild-type and ΔnleH mutant strains, NleH appears to have a local rather than systemic role, possibly in displacing

![FIG. 6. Interaction of wild-type C. rodentium strain ICC180 and its ΔnleH mutant with the colonic epithelium in vivo. (A) At day 9 postchallenge, typical foci of intimately adherent bacteria (arrows) were observed on H&E-stained sections, accompanied by a highly disorganized epithelium. IFA staining revealed these bacteria to be of the O152 serotype (arrowheads), corresponding to C. rodentium serotype. These foci of intimately adherent bacteria were confirmed to be A/E lesions by SEM. Neither O152-positive bacteria nor A/E lesions were observed on sections or samples derived from uninfected mice. (B) No adherent bacteria were observed in the colons of mice infected with ICC180 and the ICC180 ΔnleH mutant at day 14 postchallenge, although crypt hyperplasia was still present. For the IFA panel: Hoechst 33342 (blue, false color), DNA; tetramethyl rhodamine isothiocyanate (intense blue, false color), O152-positive bacteria. Representative micrographs are shown. Bar, 20 μm (H&E and IFA) or 100 μm (B).](image-url)
Considering that both NleH (C. Hemrajani, unpublished data) and OspG (20) are protein kinases and share a high level of sequence identity, we investigated, using reporter mice, if NleH plays a role in activation of the NF-$\kappa$B pathway. Unexpectedly, we found lower activation of NF-$\kappa$B in the colon in mice infected with the \textit{C. rodentium} \textit{nleH} mutant than in those infected with the parental wild-type strain. Consistent with these results, we found lower colonic levels of TNF-$\alpha$ at 14 days postchallenge in mice infected with the \textit{C. rodentium} \textit{nleH} mutant than in those infected with the wild-type strain, while no difference was recorded for IFN-$\gamma$.

These data suggest that NleH triggers local activation of NF-$\kappa$B, which in turn leads to a differential increase in the levels of proinflammatory cytokines. Collectively these results demonstrate that the role of NleH during infection is host specific. NleH, one of the core and conserved T3SS effectors in A/E pathogens, is likely to work with other effectors in optimizing the level of local gut inflammatory responses and the relationship with the endogenous gut flora for the benefit of the pathogen.

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REFERENCES

ROLE OF NleH IN EHEC AND C. RODENTIUM INFECTION


