The human diarrheal pathogens enteropathogenic Shigella flexneri (EPEC) and 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 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.

The human pathogen enterohemorrhagic Escherichia coli (EHEC) O157:H7 harbors the locus of enterohehmmagocytic lesion formation (LEE) pathogenicity island, which is necessary for A/E lesion formation in vivo (26). The LEE encodes several gene regulators, the outer membrane adhesin intimin (18), the structural components of a type III secretion system (T3SS) (17), chaperones, and translocator and several effector proteins, including Tir (19), EspG, EspF, and EspH (reviewed in reference 12), that are injected into enterocytes via the T3SS and differentially modulate cellular actin dynamics in vitro.

In addition, EPEC, EHEC, and C. rodentium use the LEE-encoded T3SS to inject a large number of effectors which are encoded by genes that are scattered around the bacterial genome, carried mainly on prophages and genomic islands (8). Among these effectors are Cif (which causes irreversible cell cycle arrest at the G2/M transition) (24), EspJ (which is involved in inhibition of receptor-mediated phagocytosis) (23), TccP/EspF1 and TccP2 (which can activate N-WASP) (1, 13, 39), Esp/LNeA (which affects exocytosis) (16, 29), and NleH of unknown function (34).

EHEC infection in humans frequently results from direct or indirect contact with ruminant feces; however, the molecular mechanisms underlying colonization of reservoir hosts are ill defined. The LEE-carried genes required for A/E lesion formation (e.g., those for Tir and intimin) play pivotal roles in colonization of such hosts by EHEC O157:H7 (4, 7, 37), and structural components of the T3SS were found by signature-tagged mutagenesis to be required for colonization of calves by EHEC O157:H7 and O26:H− (9, 36). In addition to Tir, EspK...
has been shown to influence colonization of the bovine intestine (38), while Map (9), TccP/EspF (37), and NleD (25) had no measurable effect. The roles of other TSS 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 *NleH* shares 49% sequence similarity with the *Shigella flexneri* TSS serine/threonine kinase effector protein OspG, which prevents ubiquitination and subsequent degradation of phospho-IκBα and downstream activation of the transcriptional factor NF-κB, possibly via the phosphorylation of the E3 ubiquitin ligase SCFTCP (Skp-Cullin-F box protein) complex (20). NF-κB proteins are transcriptional factors that, when activated, control the transcription of a large number of genes, many of which are involved in the immune (inflammatory) response. The similarities between NleH and OspG prompted us to investigate its role in colonization and activation of the NF-κB pathway in vivo.

**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 85-170 Δ*nleH1* Δ*nleH2* 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 nleH1 in ICC169. Primers 3 and 4 were used to amplify the chloramphenicol cassette in pKD3 for deletion of nleH1 in ICC180. For construction of the EHEC O157:H7 85-170 NaIΔ*nleH1* Δ*nleH2* 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 Δ*nleH2*, 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 nleH1, 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 nleH and 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 10⁹ CFU of wild-type 85-170 NaIΔ*nleH1* Δ*nleH2* or 85-170 NaIΔ*nleH1* Δ*nleH2* as described previously by Stevens et al. (33). The magnitude and duration of fecal excretion of the bacteria were followed daily for 14 days. Wild-type and mutant bacteria were enumerated by plating of triplicate serial dilutions of fresh feces collected by rectal palpation onto sorbitol-MacConkey agar supplemented with potassium tellurite (2.5 mg g⁻¹) (T-SMAC) and onto T-SMAC-NaI containing 25 μg ml⁻¹ kanamycin, respectively. Recovery of wild-type and mutant bacteria was confirmed by PCR from selected colonies using nleH flanking primers. The sensitivity of detection was 10⁵ CFU/g. Fecal excretion data were statistically analyzed for the effect of mutation by means of an F test, with the data taken as repeated measurements and the animal as a covariant of the data. All lambs were housed in biosecure containment level 2 accommodations. Each group was housed in a separate room with its own air supply and with a genetic separation. All lambs were dosed orally with 10⁸ CFU of either wild-type 85-170 NaI or the isogenic Δ*nleH1* Δ*nleH2* mutant separately, or with wild-type 85-170 NaI and the Δ*nleH1* Δ*nleH2* mutant together, suspended in 10 ml of 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Approximately 2 h after oral inoculation and as required thereafter for up to 28 days, rectal fecal 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 O157 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

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**TABLE 1. Strains**

| Species and strain | Characteristics* | Source or reference |
|--------------------|------------------|---------------------|
| *C. rodentium*     |                  |                     |
| ICC169             | Spontaneous NaI+ mutant of wild-type C. rodentium | 40 |
| ICC229             | ICC169 ΔnleH1 ( Kan’ ) | This study |
| ICC180             | Bioluminescent strain harboring the *Photobacterium halocDNA* operon ( Kan’ ) | 40 |
| ICC285             | ICC180 ΔnleH1 ( Cm’ ) | This study |
| E. coli 85-170     | Spontaneous Stx+ and Stx2+ EHEC O157:H7 strain | 35 |
| 85-170 NaI+        | Spontaneous NaI+ derivative of 85-170 NaI+ΔnleH1ΔnleH2 ( NaI+ Kan’ Cm’ ) | 33 |
| ICC232             | Commensal strain isolated from the cecum of a C57BL/6 mouse | This study |
| ICC299             |                  |                     |

* Kan’, Cm’, and NaI+, kanamycin, chloramphenicol, and nalidixic acid resistant, respectively.

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**TABLE 2. Plasmids**

| Plasmid | Characteristics | Source or reference |
|---------|-----------------|---------------------|
| pKD3    | oriR6 blamF; Cm’ cassette flank | 6 |
| pKD4    | oriR6 blamF; Kan’ cassette flank | 6 |
| pKD46   | ori101 repA101(ts) araBp-gam-bet-exo blam | 6 |
| pCP20   | FLP synthesis under thermal control | 3 |
| pET28-a | His6, tag expression vector | Novagen |
| pETnleH1 | Derivative of pET28-a expressing NleH1-His6 | This study |
| pET:nleH1K159A | Derivative of pET28-a expressing NleH1K159A-His6 | This study |

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**TABLE 3. Primers**

| Primer | Sequence |
|--------|----------|
| 1      | NleH1F   |
| 2      | NleH1R   |
| 3      | NleH2F   |
| 4      | NleH2R   |
| 5      | 5’-FRT   |
| 6      | 3’-FRT   |
| 7      | 5’-FRT   |
| 8      | 3’-FRT   |
antibiotic. Representative colonies were confirmed to be E. coli O157 by latex agglutination (Oxoid). For coinfection studies, the competitive index (CI) was calculated; it is 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.

**Murine models.** Specific-pathogen-free female 6- to 8-week-old mice were used in this study. Wild-type and ΔB-actin ibn65 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-B-RE-luc (Oslo) (P/N 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 (pH 7.8). The number of viable bacteria used as the inoculum was determined by retrospective plating LB agar containing the appropriate antibiotic. Representative colonies were confirmed to be E. coli O157 by latex agglutination (Oxoid). For coinfection studies, the competitive index (CI) was calculated; it is 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.

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**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.

**Immunohistochemistry.** Snap-frozen colonic tissues, embedded in OCT mounting medium (VWR BDH, Lutterworth, United Kingdom), were sectioned using a cryostat to a thickness of 5 μm. Sections were collected on polylysine 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 for 1 h. Sections were gently washed with TBS three times before addition of biotinylated anti-rat immunoglobulin G (Serotec) at a dilution of 1:200 with 4% (vol/vol) normal murine serum for blocking (Sera Laboratories International, Horsted Keynes, United Kingdom) for 30 min. After washing, a 1:200 dilution of 0.1% avidin-peroxidase (Sigma-Aldrich, Dorset, United Kingdom) was added and left for 30 min before further washing and treatment with diaminobenzamide substrate (Sigma-Aldrich) for 5 min. The reaction was stopped with excess TBS, and sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich) for 30 s, dipped in acid alcohol, and washed in tap water for 5 min. Sections were dehydrated through an ethanol gradient of 70%, 90%, and 100% solutions (2 min each), followed by clearing in xylene (VWR BDH) and mounting in DPX (VWR BDH). A control slide using no primary antibody was also made to show endogenous peroxidase-containing cells. Stained cell populations were counted in five randomly selected fields per section, and data were expressed as the number of T cells per 250 μm² of lamina propria.

**TABLE 3. Primers**

| Primer no. | Primer name | Sequence (5'→3') |
|-----------|-------------|------------------|
| 1         | NleH CRICC169 Fw | ATGGTATTACACAGCTCCCTGTAATTGGGATTTTGCTCATGGAATTCTT TTAACTCTGTTAGGCTGGAGCTGGCTTCG |
| 2         | NleH CRICC169 Rv | TAATCTCTTAATACCATGCTTATAGATCTTGTCCTCCTCCATGA TAAGCATATGATATATCTCCCTTAG |
| 3         | NleH CRICC180 Fw | ATGGTTATACAGCTCTGTAATTGGGATTTTGCTCATGGAATTCTT TTAACTCTGTTAGGCTGGAGCTGGCTTCG |
| 4         | NleH CRICC180 Rv | TAATCTCTTAATACCATGCTTATAGATCTTGTCCTCCTCCATGA TAAGCATATGATATATCTCCCTTAG |
| 5         | NleH EHEC Fw   | TGAAAGGTGGAATGATATGATATGATCTTGTCCTCCTCCATGA TAAGCATATGATATATCTCCCTTAG |
| 6         | NleH EHEC Rv   | TAATCTCTTAATACCATGCTTATAGATCTTGTCCTCCTCCATGA TAAGCATATGATATATCTCCCTTAG |
| 7         | NleH2 EHEC Fw  | ATGGTATTACACAGCTCCCTCCTCTATATAATTTGGGATTTTGCTCATGGAATTCTT TTAACTCTGTTAGGCTGGAGCTGGCTTCG |
| 8         | NleH2 EHEC Rv  | TAATCTCTTAATACCATGCTTATAGATCTTGTCCTCCTCCATGA TAAGCATATGATATATCTCCCTTAG |
| 9         | TNF-α-Fw      | ATGAGCAGACAGAAAGCATGATC |
| 10        | TNF-α-Rv      | TACAGGCTTGTGACCTGGAATT |
| 11        | B-actin-Fw    | AGAGGGAAATCGTGCGTGAC |
| 12        | B-actin-Rv    | CAATAGTGAGCTAGCCTGGCCGT |
| 13        | IFN-γ-Fw      | TGAACCGTCAACACTGCACTGTGGCCGT |
| 14        | IFN-γ-Rv      | CGACCTCCTTTCGCGCCCTCCTGGAG |

**Primer names**

- **NleH CRICC169 Fw**
- **NleH CRICC169 Rv**
- **NleH CRICC180 Fw**
- **NleH CRICC180 Rv**
- **NleH EHEC Fw**
- **NleH EHEC Rv**
- **NleH2 EHEC Fw**
- **NleH2 EHEC Rv**
- **TNF-α-Fw**
- **TNF-α-Rv**
- **B-actin-Fw**
- **B-actin-Rv**
- **IFN-γ-Fw**
- **IFN-γ-Rv**
FIG. 1. Distance tree of the NleH family. Homologues were identified using BLASTp searches of NCBI nr protein database with EPEC 2348/69 NleH1 as the query sequence. Protein sequences selected and analyzed included E. coli O111:H− NleH1 (GI:164457622), E. coli O103:H2 NleH1 (GI:16457632), E. coli O157:H7 EDL933 NleH2 (GI:15801938), C. rodentium NleH (GI:44888794), phage cdtI gp23 (GI:148609405), Y. pseudotuberculosis IP 31758 (GI:153930640), Y. frederiksenii ATCC 35641 YfleA (GI:77977208), Y. enterocolitica subsp. enterocolitica 8081 YfleA (GI:13424682), Y. enterocolitica subsp. enterocolitica 8081 YspK (GI:13442682), S. flexneri OspG (GI:13449175), and E. coli O157:H7 str EC4045 OspG (GI:168711275), as well as EPEC 2348/69 NleH2 (unpublished). The BioEdit software was used to create a ClustalW alignment. Clustal W2 (EMBL-EBI) secondary antibody was used to visualize O152-bridgedbridge, United Kingdom) secondary antibody was used to visualize O152-positive bacteria, while DNA of both bacteria and epithelial cells was counterstained with Hoechst 33342 (Sigma-Aldrich Co., United Kingdom). Sections were examined with an Axio Imager M1 microscope (Carl Zeiss MicroImaging GmbH, Germany). Images were acquired using an AxioVision (Carl Zeiss MicroImaging GmbH, Germany) 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 or gamma interferon (IFN-γ) and β-actin mRNAs were measured by semiquantitative reverse transcription-PCR (RT-PCR) using primers 9 to 14 listed in Table 3 and the one-step Reverse-iT hot-start kit (Thermo). The PCR amplification cycle was 20 s at 94°C, 30 s at 60°C, and 60 s at 72°C for 35 cycles. One microgram of RNA was used for measurement of TNF-α and IFN-γ transcript levels, and 100 ng of RNA was used for detection of β-actin mRNA. The PCR products were run on a 1% agarose gel alongside the 100-bp ladder from NEB in Tris-borate-EDTA buffer. GeneTools (Syngene) software was used to conduct densitometric analysis. All TNF-α, β-actin, and IFN-γ/β-actin 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.

RESULTS

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

EPEC O127:H6 E2348/69 and EHEC O157:H7 EDL933 and Sakai contain two NleH paralogues, which share 83 to 84% protein identity. C. rodentium contains only one nleH gene, which shares 83 and 81% amino acid sequence identity with EHEC O157:H7 NleH1 and NleH2, respectively. NleH shares 49% amino acid sequence similarity with the serine/threonine kinases OspG and YspK (Yersinia secreted protein kinase) of Yersinia enterocolitica.

We employed BLASTp searches of the NCBI protein database using EPEC E2348/69 NleH1 as the query sequence. The Clustal W2 software (EMBL-EBI) was then used to create a phylogram of the different homologues (Fig. 1). Two major clusters were formed, one containing all the NleH effectors and the other containing closer homologues of OspG, including an OspG-like effector (90.8% identity to OspG of Shigella) from...
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 both 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 were seen (Fig. 5A). However, the ΔnleH C. rodentium mutant was significantly out competed by the wild-type strain in a mixed infection (Fig. 5B).

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 1κB 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. (A) Course of fecal excretion following oral challenge of groups of six 6- to 8-week-old C57BL/6 mice with either wild-type C. rodentium ICC180 or the nleH mutant. (B) Mean CI of C. rodentium ΔnleH in mice infected with an inoculum containing a 1:2 ratio of wild-type C. rodentium and the nleH mutant. The null hypothesis that CI = 1 was tested by a two-tailed nonparametric Wilcoxon test and showed that the mean CI postchallenge is significantly less than 1 for all time points where it could be calculated.

ΔnleH, E. coli ICC299, or PBS (Fig. 7A and B). This difference was similar to the three- to fourfold-increased luminescent signal seen after mice were inoculated with TNF-α as a positive control (data not shown).

As NF-κB induces the transcription of many proinflammatory cytokines such as TNF-α and IFN-γ, we compared the levels of their transcripts in uninfected mice and mice infected with either wild-type or ΔnleH mutant C. rodentium. RNA extracted from distal colons of mice was subjected to semi-quantitative RT-PCR. Transcript levels were quantified as a
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

![Image of interaction](image-url)
the normal gut flora. 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-κB pathway. Unexpectedly, we found lower activation of NF-κB in the colon in mice infected with the C. rodentium ΔnleH mutant than in those infected with the parental wild-type strain. Consistent with these results, we found lower colonic levels of TNF-α at 14 days postchallenge in mice infected with the ΔnleH C. rodentium mutant than in those infected with the wild-type strain, while no difference was recorded for IFN-γ. These data suggest that NleH triggers local activation of NF-κB, 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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