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# Essential Role of the Type III Secretion System Effector NleB in Colonization of Mice by *Citrobacter rodentium*

Michelle Kelly,[1] Emily Hart,[2] Rosanna Mundy,[3] Olivier Marchès,[3] Siouxsie Wiles,[3] Luminita Badea,[1] Shelley Luck,[1] Marija Tauschek,[2] Gad Frankel,[3] Roy M. Robins-Browne,[2] and Elizabeth L. Hartland[1,3]*

*Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Victoria 3800, Australia[1]; Australian Bacterial Pathogenesis Program, Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia[2]; and Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, United Kingdom[3]*

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**Attaching and effacing (A/E) pathogens are a significant cause of gastrointestinal illness in humans and animals. All A/E pathogens carry a large pathogenicity island, termed the locus for enterocyte effacement (LEE), which encodes a type III secretion system that translocates several effector proteins into host cells. To identify novel virulence determinants in A/E pathogens, we performed a signature-tagged mutagenesis screen in C57BL/6 mice by using the mouse A/E pathogen *Citrobacter rodentium*. Five hundred seventy-six derivatives of *C. rodentium* were tested in pools of 12 mutants. One attenuated mutant carried a transposon insertion in *nleB*, which encodes a putative effector of the LEE-encoded type III secretion system (T3SS). *nleB* is present in a genomic pathogenicity island that also encodes another putative effector, NleE, immediately downstream. Using translational fusions with β-lactamase (TEM-1), we showed that both NleB and NleE were translocated into host cells by the LEE-encoded T3SS of enteropathogenic *Escherichia coli*. In addition, deletion of the gene encoding NleB in *C. rodentium* resulted in reduced colonization of mice in single infections and reduced colonic hyperplasia. In contrast, the deletion of other non-LEE-encoded effector genes in *C. rodentium*, *nleC*, *nleD*, or *nleE*, had no effect on host colonization or disease. These results suggest that *nleB* encodes an important virulence determinant of A/E pathogens.**

The attaching and effacing (A/E) pathogen *Citrobacter rodentium* has emerged as a valuable tool for the study of infections caused by the diarrheagenic human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). As well as causing diarrhea (N. Simpson et al., submitted for publication), *C. rodentium* is the causative agent of murine transmissible colonic hyperplasia (2, 33). A/E pathogens share a distinctive mechanism of intestinal colonization that leads to the formation of a characteristic histopathological lesion termed the A/E lesion. A/E lesions are characterized by localized damage to intestinal microvilli and the rearrangement of host cytoskeletal proteins beneath bacteria intimately attached to the host cell surface (16, 17). In vivo, *C. rodentium* induces A/E lesions, which are morphologically indistinguishable from those caused by EPEC and EHEC (33). *C. rodentium* shares a number of essential virulence determinants with EPEC and EHEC, most notably the locus of enterocyte effacement (LEE), which encodes regulators, the adhesin intimin, a type III secretion system (T3SS), chaperones, and translocator and effector proteins (7, 10, 15). LEE is essential for A/E lesion formation and is well conserved among A/E pathogens (21, 36).

The translocated effector proteins encoded within LEE include Tir, Map, EspF to EspH, and EspZ, of which only Tir plays a direct role in A/E lesion formation (14, 22, 23, 27, 37). Several recent studies have revealed that many effectors of A/E pathogens are encoded by genes carried on prophages and small pathogenicity islands which are nevertheless secreted and translocated into host cells by the LEE-encoded T3SS. These effectors include the Golgi-associated NleA/EspI, which is essential for full virulence of *C. rodentium* (19, 29); EspJ, which may play a minor role in intestinal colonization (6); and EspG2, which, similarly to EspG, triggers the dissociation of microtubules beneath adherent bacteria (34). Other recent effectors show some strain and/or pathogen specificity, including the cycle inhibiting factor Cif, which induces host cell cycle arrest and reorganization of the actin cytoskeleton (4), and EspFU, or TccP (Tir-cytoskeleton coupling protein), which functions as an adapter protein of bacterial origin necessary for Tir-dependent recruitment and activation of N-WASP at the site of EHEC O157:H7 cell attachment (3, 18).

Several other non-LEE-encoded secreted proteins, NleB to NleH, have been identified for *C. rodentium* and have been shown to be secreted by the LEE-encoded T3SS (11). Of these, NleC and NleD have also been shown to be translocated into host cells (26). Although the gene encoding NleD was identified during a signature-tagged mutagenesis (STM) screen of EHEC O157:H7 mutants in cattle, further studies were unable to show any significant contribution of *nleD* to colonization and/or virulence of EHEC O157:H7 in cattle or lambs (12, 26). Presently, the contribution of *nleB* to *nleH* to the virulence of *C. rodentium* is unknown.

Previously, we performed an STM screen in C3H/HeJ mice to identify mutants of *C. rodentium* that were attenuated for virulence (30). As well as several LEE-encoded virulence de-

* Corresponding author. Mailing address: Department of Microbiology, Monash University, Victoria 3800, Australia. Phone: (61) 3 9905 4323. Fax: (61) 3 9905 4811. E-mail: Liz.Hartland@med.monash.edu.au.

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristic(s)[a] | Source/reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| EPEC E2348/69 | Wild-type EPEC O127:H6 | 25 |
| ICC192 | E2348/69 *escN*::Kan | 18 |
| *C. rodentium* | | |
| ICC169 | Spontaneous nalidixic acid-resistant derivative of wild type *C. rodentium* biotype 4280 (Nal$^r$) | 30 |
| CRΔ*nleB* | *C. rodentium nleB*::Kan (Nal$^r$ Kan$^r$) | This study |
| CRΔ*nleE* | *C. rodentium nleE*::Kan (Nal$^r$ Kan$^r$) | This study |
| ICC197 | *C. rodentium nleC*::Kan (Nal$^r$ Kan$^r$) | This study |
| ICC213 | *C. rodentium nleD*::Kan, *nleD2*::Cm (Nal$^r$ Kan$^r$ Cm$^r$) | This study |
| Plasmids | | |
| pCX340 | Cloning vector used to construct TEM-1 β-lactamase fusions, Tet$^r$ | 4 |
| pACYC184 | Medium-copy-number cloning vector (Cm$^r$ Tet$^r$) | New England Biolabs |
| pICC309 | Derivative of pCX340 encoding a NleD–TEM-1 fusion | 26 |
| pCX:*nleB* | Derivative of pCX340 encoding a NleB–TEM-1 fusion | This study |
| pCX:*nleE* | Derivative of pCX340 encoding a NleE–TEM-1 fusion | This study |
| pACYC:*nleB* | Derivative of pACYC184 encoding *nleB* (Tet$^r$) | This study |

[a] Kan, kanamycin; Nal, nalidixic acid; Cm, chloramphenicol; Tet, tetracycline.

terminants, the screen identified a novel type IV pilus operon, *cfc*, and the type III effector *espI* as essential for colonization of the host and disease (29). In this study, we continued screening the STM library constructed previously in *C. rodentium* by using the more resistant mouse strain C57BL/6 as a host. From this screen, we identified a number of previously characterized virulence determinants of A/E pathogens as well as the non-LEE effector gene *nleB*. Here we show that NleB is a translocated effector protein of EPEC and that *nleB* is required for colonization of the mouse intestine by *C. rodentium* and the development of disease.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *C. rodentium* strains were grown in Luria-Bertani (LB) broth or in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine and incubated aerobically with shaking at 37°C overnight. HeLa cells were passaged at 37°C in the presence of 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. Where necessary, the following antibiotics were added to growth media: kanamycin, 100 μg/ml; nalidixic acid, 50 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 25 μg/ml.

**Cloning of *nleB* and *nleE* into pCX340 and TEM-1 translocation assays.** A 989-bp fragment and a 674-bp fragment containing the *nleB* and *nleE* genes, respectively, were amplified by PCR using primers nleBF and nleBR (*nleB*) and nleF and nleR (*nleE*) and genomic DNA from EPEC E2348/69 as a template (Table 2). The PCR products were digested with KpnI/EcoRI and NdeI/EcoRI for *nleB* and *nleE*, respectively, and ligated into pCX340, generating plasmids pCX:*nleB* and pCX:*nleE* encoding NleB–TEM-1 and NleE–TEM-1 fusions. Secretion of the TEM-1 fusions was checked by immunoblotting using anti-TEM-1 antibodies. Translocation assays were performed as described previously, and images were captured using a Nikon Digital DXM1200 camera (26).

**Construction of nonpolar *nleB*, *nleC*, *nleD*, and *nleE* mutations in *C. rodentium*.** Nonpolar mutations in genes encoding the type III effectors *nleB*, *nleC*, *nleD*, and *nleE* were constructed in *C. rodentium* by use of the λ Red recombination system (9). The target genes were deleted and replaced by either a kanamycin resistance cassette or a chloramphenicol resistance cassette. All mutations were obtained using either pKD3 (chloramphenicol resistance cassette) or pKD4 (kanamycin resistance cassette) as a template (9) with the following primer pairs: nleB855 and nleB856 (*nleB*), nleE872 and nleE873 (*nleE*), NleCCitrFRTfor and NleCCitrFRTrev (*nleC*), NleD1CitrFRTfor and NleD1CitrFRTrevr (*nleD*), and NleD2CitrFRTfor and NleCCitrFRTrev (*nleD2*) (Table 2). The PCR products were electroporated into wild-type *C. rodentium* carrying the Red system expression plasmid pKD46, and mutants were selected for on LB plates supplemented with kanamycin. All mutations were confirmed by PCR using primers flanking the targeted region and primers within the kanamycin resistance gene.

***trans*-complementation of the *C. rodentium nleB* mutant.** A *trans*-complementing vector encoding *nleB* was constructed in pACYC184, which has been used previously for in vivo studies with *C. rodentium* (29, 30). *nleB* was amplified by PCR to include a putative upstream promoter sequence, using the primers nlebFP954 and nleBFR955 (Table 2). The resulting product was cloned into the EcoRI site of pACYC184, reading in the same direction as the chloramphenicol resistance gene.

**Screening of a *C. rodentium* STM library in C57BL/6 mice.** Construction of the STM library has been described previously (30). In this study, pools of 12 mutants were used to infect groups of 4- to 5-week-old male C57BL/6 mice by oral gavage. Two mice per group were inoculated with 200 μl phosphate-buffered saline (PBS) containing approximately $2 \times 10^9$ CFU of an overnight culture of each mutant. Each mutant was grown individually in LB broth at 37°C overnight with shaking, and the optical density at 600 nm was measured to check for growth defects. The viable count for the inoculum strain was determined retrospectively by serial dilution and plating onto LB agar containing the appropriate antibiotic. The remaining inoculum was used to harvest DNA for the amplification of input pool DNA. Stool samples were taken from the mice at day 5 after inoculation, and the mice were killed and the colons removed at day 7 after inoculation. Stools and colon contents were homogenized in PBS and plated onto LB agar containing appropriate antibiotics. More than 10,000 colonies were used to isolate DNA at each time point, and these DNA samples were then combined. The oligonucleotide signature tags from the input and output pools were amplified by PCR using the primers P2 and STM-Rev2 (Table 2) and spotted in triplicate onto nylon membranes. Input and output pools were then probed simultaneously with a digoxigenin-labeled, 40-bp oligonucleotide primer homologous to one of the 12 signature tag sequences. Twelve different hybridizations were performed to detect all 12 tags. Signature tags that were present in the input pool but absent or reduced in the output pool indicated putative attenuated mutants.

**Mixed-strain infections of mice and determination of the in vivo CI.** To determine the in vivo competitive index (CI) for mutant derivatives of *C. rodentium* identified by STM, overnight cultures of bacterial strains for testing were grown as described above. Approximately $2 \times 10^9$ CFU of the mutant was combined 50:50 with wild-type *C. rodentium* in 200 μl PBS and used to inoculate 4- to 5-week-old male C57BL/6 mice by oral gavage. At day 7 postinoculation, mice were killed, colons were dissected, and colon scrapings were homogenized as described above. To determine the proportion of wild-type bacteria to mutant bacteria, dilutions of the inoculum and the recovered bacteria were plated onto

TABLE 2. Oligonucleotide primers used in this study

| Primer | Sequence (5′–3′) |
|---|---|
| P2 | TACCTACAACCTCAAGCT |
| STM-Rev2 | CAAACCGTTATTCATTCG |
| nleBF | GGGGTACCATGTTATCTTCATTAAATG |
| nleBR | CGAATTCTCCCATGAACTGCAGGTATAC |
| nleEF | GGAATTCCATATGATTAATCCTGTTACTAAT |
| nleER | CGAATTCTCCTCAATTTTAGAAAGTTT |
| nleB855 | GTTCTTCAATTTAATTTCAGAGGAGAGACCGCTTTATCAGATAGTGCTCCTCTCCAGACTGTGTAGGCTGGAGCTGCTTC |
| nleB856 | CGCTTGAGTCCCTTTCCAACACCATCATAATATGGATGCGCATCAACTTTACTCTTCATACATATGAATATCCTCCTTAG |
| nleE872 | CCCATCACAAATACTCAGAACTTACTTTCTATAAATACTAAGCATGCTGAATATGTGGTGGTGTAGGCTGGAGCTGCTTC |
| nleE873 | GGCTGACCTTCTCGCCTCAATTTGAGCAAGTTTATTATTCATATTTTTCATATAACTATCCATATGAATATCCTCCTTAG |
| NleCCitrFRTfor | CATAGTTTATTCTGTTGTAATAAATGATTAGCATGGTATTAGGTATCAACATGTGTGTAGGCTGGAGCTGCTTCG |
| NleCCitrFRTrev | TAGCCTTGTGTCGCTGCAAAGACGCGTCAGCGCTGATTGTGTTTGTCCACCCATATGAATATCCTCCTTAG |
| NleD1CitrFRTfor | GCTTATCCTCAGGAGGTCTTAATGCGCCCTACATCCCTTAACCTGACATTTGTGTAGGCTGGAGCTGCTTCG |
| NleD1CitrFRTrev | CCTGAGTCACTGTATTGTCATCATGAATGAGAGATGAATCGTGCGGGTAGCATATGAATATCCTCCTTAG |
| NleD2CitrFRTfor | ACCTTCGTTACCTCTACCCTCATCTTCAAATTCAATTTCAGCCACAGACAGCCTACCTGTGACGGAAGATCA |
| NleD2CitrFRTrev | AATGTTCTGCGGGGCATCCCAATCTCTTCACGAAATTTATTTTCTGAAAGTCATTTAAATGGCGCGCCTTAC |
| nleBFP954 | GAGGAATTCCGTCAGCAATGTTCTGAATC |
| nleBFR955 | GAGGAATTCCATCTACATCTCCATAACG |

LB agar containing nalidixic acid only and onto LB agar containing nalidixic acid and kanamycin. The ability of each mutant to compete with the wild-type strain was analyzed for at least three animals, and the CI was calculated as the proportion of mutant to wild-type bacteria recovered from animals divided by the proportion of mutant to wild-type bacteria in the inoculum (30). Mutants with a CI of less than 0.5 were considered to be attenuated.

**Single-strain infections of mice.** Overnight cultures of *C. rodentium* derivatives were resuspended in PBS for inoculation as described above. At least four male 4- to 5-week-old C57BL/6 mice per strain were inoculated by oral gavage with approximately $2 \times 10^9$ CFU in 200 μl PBS. The viable count of the inoculum was determined by retrospective serial dilution and plating on LB agar containing the appropriate antibiotic. Stool samples were recovered aseptically at various time points after inoculation, and the number of viable bacteria per gram of stool was determined by plating onto antibiotic selective media. The limit of detection was 10 CFU $g^{-1}$ feces.

**Assay for colonic hyperplasia.** Overnight cultures of *C. rodentium* derivatives were resuspended in PBS for inoculation as described above. Male 8- to 10-week-old C3H/HeJ mice were inoculated by oral gavage with approximately $2 \times 10^8$ CFU in 200 μl PBS. The viable count of the inoculum was again determined by retrospective serial dilution and plating on LB agar containing the appropriate antibiotic. At selected time points postinfection, mice were killed and the distal section of colon from the cecum to the rectum was aseptically removed and weighed after the removal of fecal pellets and cecal contents. Organs were homogenized mechanically in 5 ml of sterile PBS by use of a Seward 80 Stomacher, and the number of viable bacteria per gram of organ homogenate was determined by plating onto LB agar containing the appropriate antibiotics.

## RESULTS

**Identification of attenuated mutants of *C. rodentium* by STM.** STM has been used extensively to identify novel virulence determinants in a range of bacterial pathogens, including A/E pathogens (1, 5, 8, 12, 13, 30, 35, 39). Previously, we used STM to identify genes required for the survival of *C. rodentium* in C3H/HeJ mice (30). In this study, we continued screening the *C. rodentium* library constructed previously by performing mixed infections in C57BL/6 mice. Mutants already tested in C3H/HeJ mice were not repeated in this STM screen. Five hundred seventy-six mutants were tested for their ability to colonize 4- to 5-week-old male C57BL/6 mice. Upon analysis by dot blot hybridization, the transposon insertion sites of mutants that went missing from output pools after 5 to 7 days were cloned and sequenced. We identified two insertions into known LEE-encoded virulence determinants, EscN and CesT, as well as a putative regulator and genes encoding proteins involved in cysteine synthesis and sugar transport and two outer membrane proteins (Table 3). In addition, this screen identified three known virulence determinants of *C. rodentium*: *cfcF*, which is essential for type IV pilus biogenesis; the adhesin/toxin *efa1*(*lifA*); and *nleA*(*espI*), which encodes a T3SS effector protein. Mutants with insertions into genes that had not been characterized before were retested in 50:50 competition experiments with wild-type *C. rodentium*. Some mutants with insertions into known virulence determinants were included as controls (Table 3). Mutants with a competitive index of less than 0.5 were considered highly attenuated. One novel determinant identified by this screen was *nleB*, which encodes a putative effector protein of the LEE-encoded T3SS. NleB is encoded by Z4328, located within O island 122 in EHEC O157:H7 strain EDL933, and Z4329 (encoding the T3SS protein NleE), which lies directly downstream. *nleB* and *nleE* are also located together in EHEC O157:H7 Sakai, *C. rodentium*, EPEC E2348/69, and rabbit-specific enteropathogenic *E. coli* (REPEC) strain 83/39 (Fig. 1). In the case of REPEC, both genes are located adjacent to the core LEE region (36).

**Secretion and translocation of NleB and NleE into host cells.** NleB is known to be secreted by the LEE-encoded T3SS

TABLE 3. Transposon insertion sites of *C. rodentium* mutants attenuated for colonization

| Strain(s) and characterization | Disrupted gene or homologue | Function | In vivo CI |
|---|---|---|---|
| LEE | | | |
| 33A5 | *escN* | T3SS | 0.00017 |
| 9A2, 40E6 | *cesT* | T3SS chaperone | 0.00007, ND[a] |
| Non-LEE | | | |
| 30E1 | *nleB* | Type III effector | 0.0515 |
| 34F2 | *nleA* | Type III effector | ND |
| 21E1, 33E1, 35F5 | *efa1*(*lifA*) | Adhesin/toxin | ND |
| 42E1, 47E6 | *cfcF* | Type IV pilus | ND |
| Regulator | | | |
| 1A2 | *fapR* (*regA*) | AraC-like regulator | 0.0067 |
| Metabolism | | | |
| 9F2 | *cys* operon | Cysteine synthesis | 0.0113 |
| 47F2 | *cysA* | Sulfate permease | 0.197 |
| 35F2 | Z4304 | Methylgalactoside transport protein | 0.000031 |
| 32F5 | Z0002 | Aspartokinase I | 0.00086 |
| 17E3 | *ydaM* | GGDEF domain protein | 0.43 |
| Other | | | |
| 20E3 | *ytfM* | Putative exported protein (*S. enterica*) | 0.0032 |
| 15E1 | STM4242 | Putative exported protein (*S. enterica*) | 0.0042 |
| 35H2 | *fliR*-like | Lateral flagellar export and assembly (Flag-2 locus) | 0.022 |

[a] ND, not determined.

of *C. rodentium* (11). To determine if NleB is also secreted by the LEE-encoded T3SS of EPEC and translocated into host cells, we generated translational fusions of NleB to TEM-1 β-lactamase by using the vector pCX340 (4). In addition, we generated a TEM-1 fusion with NleE to determine if this protein was also a translocated effector of EPEC. The TEM-1 fusions were introduced into EPEC E2348/69 and the *escN* T3SS mutant ICC192 (18). Production and export of the TEM-1 fusions were detected by Western blotting with anti-bodies to TEM-1, confirming that the secretion of both NleB and NleE was LEE dependent (data not shown). The presence of TEM-1 in infected eukaryotic cells can be measured directly by the addition of the fluorescent substrate CCF2-AM (4). To determine if the NleB– and NleE–TEM-1 fusions were translocated into host cells by the LEE-encoded T3SS, EPEC E2348/69 and ICC192 expressing the NleB– and NleE–TEM-1 fusions were used to infect HeLa cell monolayers loaded with CCF2-AM, as described previously (26). TEM-1 translocation was analyzed by fluorescence microscopy of infected cells, and EPEC E2348/69 and ICC192 expressing NleD–TEM-1 were used as positive and negative controls, respectively. HeLa cells infected with EPEC E2348/69 carrying pCX:*nleB* or EPEC E2348/69 carrying pCX:*nleE* exhibited blue fluorescence, whereas HeLa cells infected with ICC192 carrying pCX:*nleB* or ICC192 carrying pCX:*nleE* exhibited green fluorescence (Fig. 2). This indicated that both NleB and NleE were translocated into host cells in a LEE-dependent manner and therefore constituted novel effectors of the LEE-encoded T3SS.

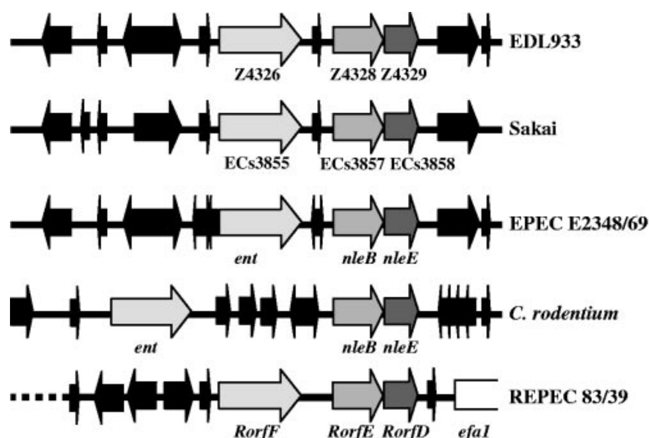


FIG. 1. Diagram showing the genetic arrangement of the locus containing *nleB* and *nleE* from several A/E pathogens, including EHEC O157:H7 strains EDL933 (GenBank accession no. AE005174) and Sakai (GenBank accession no. BA000007), EPEC E2348/69 (www.sanger.ac.uk/Projects/Escherichia_Shigella), *C. rodentium* (www.sanger.ac.uk/Projects/C_rodentium), and REPEC O15:H$^{-}$ strain 83/39 (GenBank accession no. AF453441). Open reading frames and their direction of transcription are indicated by arrows. Protein homologs are indicated by shading in gray.

**Colonization of C57BL/6 mice by *nleB* and *nleE* mutants of *C. rodentium*.** Loss of the *nleB* transposon mutant 30E1 from output pools during STM suggested that this derivative of *C. rodentium* was defective for colonization when in competition with the wild-type strain. To investigate further the role of *nleB* in colonization of mice by *C. rodentium*, we tested the ability of the *nleB* deletion mutant of *C. rodentium* to colonize 4- to 5-week-old male C57BL/6 mice in mixed and single infections. In addition, we tested a deletion mutant of *nleE* for attenuation in mixed and single infections. In a mixed infection with wild-type *C. rodentium*, the CI of the *nleB* deletion mutant was 0.069, similar to that of the STM mutant 30E1 (Table 3). In contrast, the CI of the *nleE* deletion mutant was 0.21, suggest-

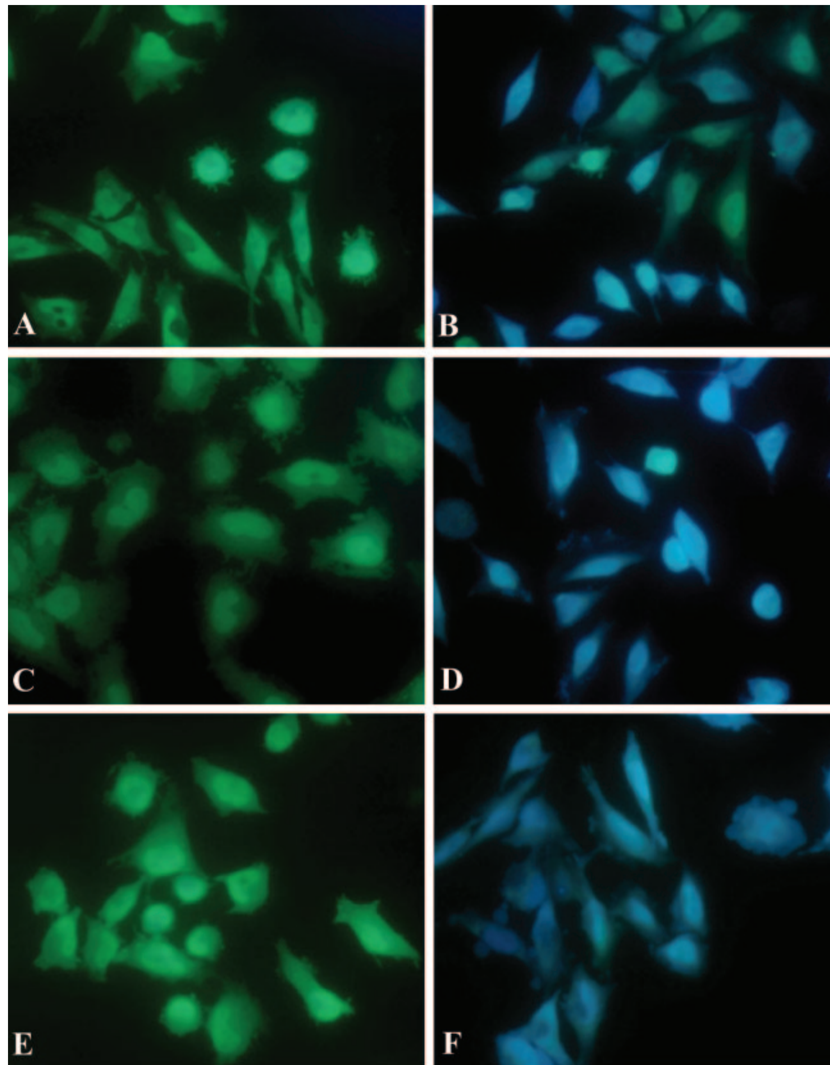


FIG. 2. Translocation of NleB and NleE into eukaryotic cells. Immunofluorescence microscopy of HeLa cells loaded with CCF2/AM and infected with (A) ICC192 (pCX:*nleB*), (B) EPEC E2348/69 (pCX:*nleB*), (C) ICC192 (pCX:*nleE*), (D) E2348/69 (pCX:*nleE*), (E) ICC192 (pICC309), or (F) EPEC E2348/69 (pICC309).

ing that this strain was not as attenuated as the *nleB* mutant but that it was still outcompeted by wild-type *C. rodentium* in vivo.

In single infections, stool samples from groups of mice inoculated with wild-type *C. rodentium* and the *nleB* and *nleE* deletion mutants were collected at selected time points and the ability of the wild-type and mutant strains to establish an infection and colonize the mice was monitored by performing viable counts on recovered stools. Stool counts showed that there was no significant difference in the abilities of the *nleE* mutant and wild-type *C. rodentium* to colonize mice, with the strains reaching a maximum mean CFU $g^{-1}$ feces of $2.7 \times 10^7$ and $3.8 \times 10^7$, respectively (Fig. 3). In contrast, the *nleB* mutant reached a maximum mean CFU $g^{-1}$ feces of only $6.7 \times 10^5$, with three mice in the group maintaining very low numbers of the *nleB* mutant ($<10^3$ CFU $g^{-1}$) throughout the experiment (Fig. 3). Differences in the levels of colonization between wild-type *C. rodentium* and the *nleB* deletion mutant were significant on days 3, 5, 7, 9, and 13 postinoculation ($P < 0.05$ by unpaired, two-tailed *t* test). These results strongly suggested that when tested in a single infection, *nleB* was essential for full colonization of mice by *C. rodentium*, whereas *nleE* did not make a significant contribution to colonization. The data also indicated that attenuation of the *nleB* mutant was not due to an effect on the expression of *nleE* directly downstream.

***trans*-complementation of the *C. rodentium nleB* mutant.** To confirm that attenuation of the *nleB* deletion mutant was not due to an unforeseen secondary mutation, we complemented the *nleB* mutant strain with pACYC:*nleB* and tested the ability of the complemented strain to infect mice. As a control, pA CYC184 was introduced into wild-type *C. rodentium* and into the *nleB* mutant. The results showed that carriage of pACYC: *nleB* was able to restore the colonization defect of the mutant to wild-type levels (Fig. 4). Whereas there was no significant difference in the abilities of wild-type *C. rodentium* (pACYC184) and CRΔ*nleB* (pACYC:*nleB*) to colonize mice, levels of colonization by CRΔ*nleB* (pACYC184) were significantly reduced at days 2, 4,

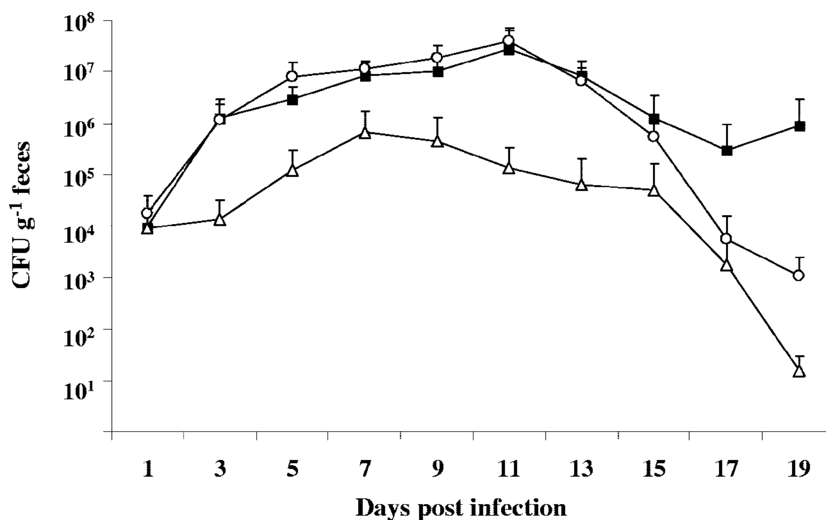


FIG. 3. Colonization of C57BL/6 mice with derivatives of *C. rodentium*. Results are expressed as the mean $\log_{10}$ CFU $g^{-1}$ feces for at least four mice, measured at selected time points postinoculation. Mice were infected with approximately $2 \times 10^9$ CFU of wild-type *C. rodentium* (■), CRΔ*nleB* (△), and CRΔ*nleE* (○). Error bars indicate standard deviations.

9, and 12 postinfection compared to levels of colonization by CRΔ*nleB* (pACYC:*nleB*) ($P < 0.05$ by unpaired, two-tailed *t* test). This confirmed that *nleB* was required for full colonization of mice by *C. rodentium*.

**Induction of colonic hyperplasia in C3H/HeJ mice.** We also tested the ability of the *nleB* and *nleE* mutants of *C. rodentium* to colonize and induce colonic hyperplasia in the more sensitive C3H/HeJ mouse strain. Male 8- to 10-week-old C3H/HeJ mice were killed 10 days postinoculation, and the region from the cecum to rectum was removed for weighing, as an indication of colonic hyperplasia. The results showed that there was no significant difference in the average colon weights of mice infected with the wild-type and *nleE* mutant strains (0.423 ± 0.045 g and 0.420 ± 0.033 g, respectively) (Fig. 5A). In contrast, mice infected with the *nleB* mutant showed significantly reduced levels of colonic hyperplasia (0.327 ± 0.032 g) compared with mice infected with either the wild type or the *nleE* mutant ($P = 0.005$ and $P = 0.002$, respectively, by unpaired, two-tailed *t* test) (Fig. 5A). In addition, the *nleB* mutant was recovered in significantly lower numbers from the colons of

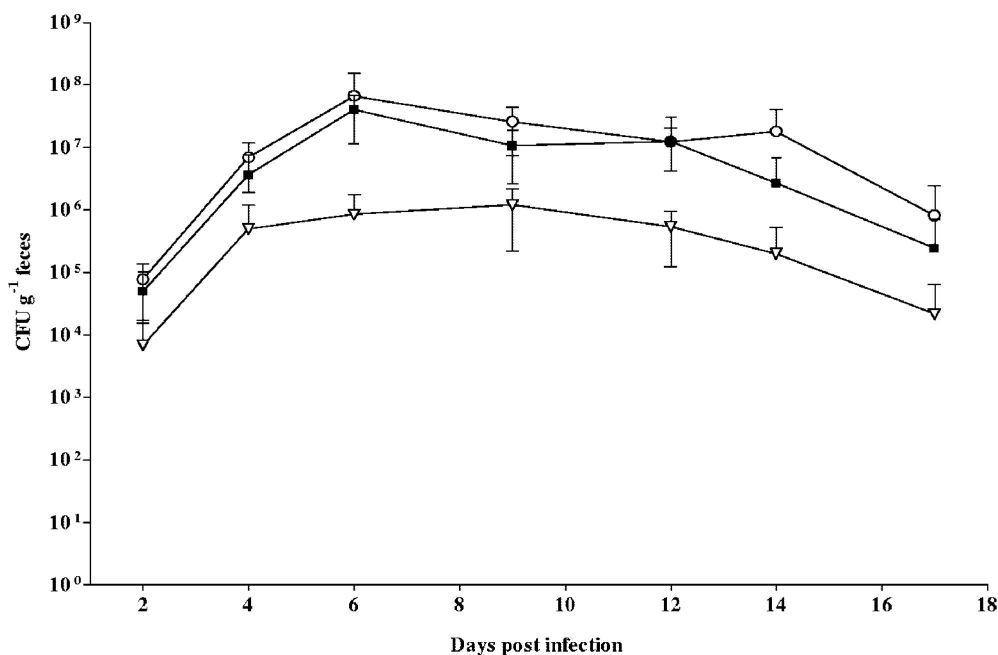


FIG. 4. Colonization of C57BL/6 mice with derivatives of *C. rodentium*. Results are expressed as the mean $\log_{10}$ CFU $g^{-1}$ feces for at least four mice, measured at selected time points postinoculation. Mice were infected with approximately $2 \times 10^9$ CFU of wild-type *C. rodentium* (pACYC184) (■), CRΔ*nleB* (pACYC184) (▽), and CRΔ*nleB* (pACYC:*nleB*) (○). Error bars indicate standard deviations.

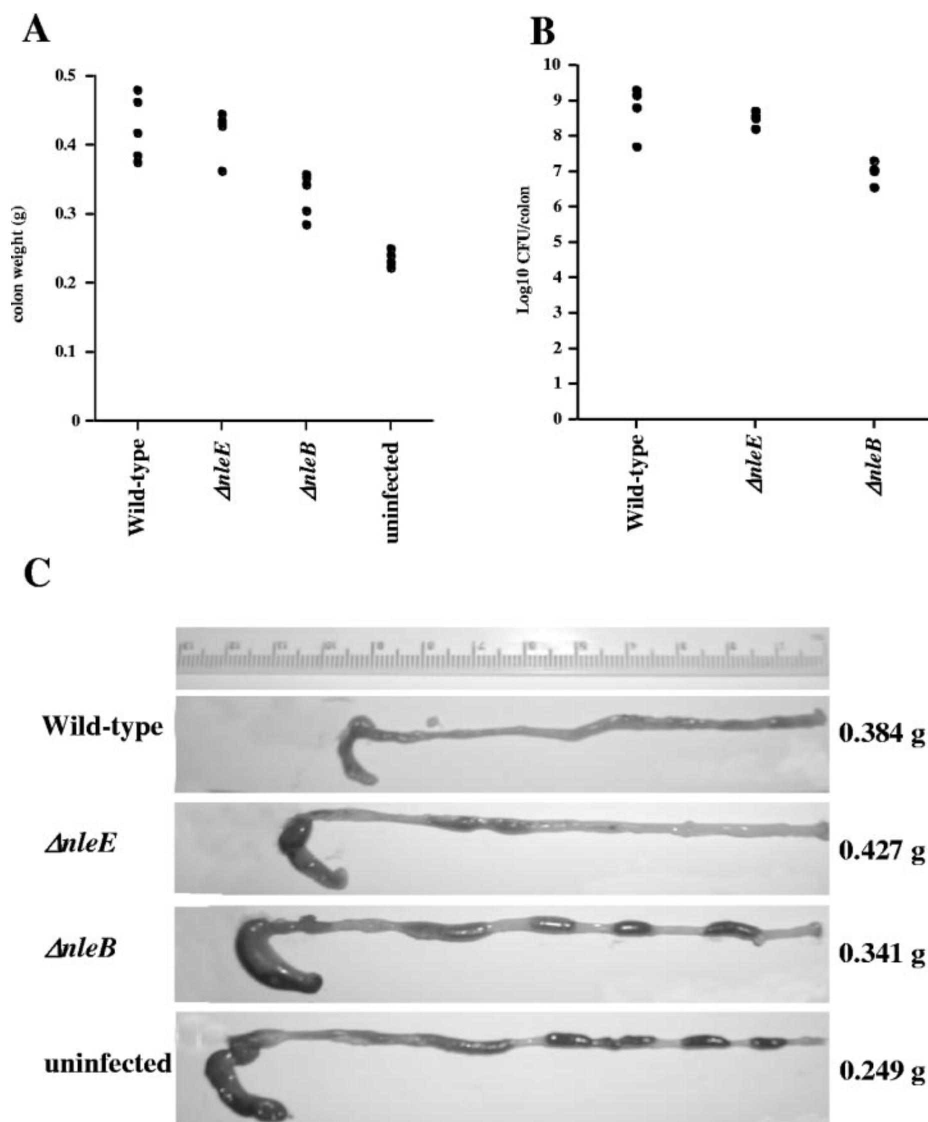


FIG. 5. Induction of colonic hyperplasia in C3H/HeJ mice by derivatives of *C. rodentium*. (A) Weights of colons dissected from C3H/HeJ mice on day 10 postinoculation. Stools were removed from the dissected colon, and remaining tissue was weighed. Data are shown as individual weights for each animal. (B) Viable counts of *C. rodentium* recovered from the colons of individual C3H/HeJ mice. (C) Photographs of the dissected ceca and colons from representative mice killed at day 10 postinoculation. Total colon weight from the cecum to rectum is indicated next to each picture.

C3H/HeJ mice (mean $\log_{10}$ CFU per colon, 6.97 ± 0.27) than from the colons of mice infected with either wild-type *C. rodentium* (mean $\log_{10}$ CFU per colon, 8.84 ± 0.67; $P = 0.0004$ by unpaired, two-tailed *t* test) or the *nleE* mutant (mean $\log_{10}$ CFU per colon, 8.5 ± 0.20; $P = 0.000008$ by unpaired, two-tailed *t* test) (Fig. 5B). Macroscopic examination of the intestine showed that wild-type-infected and *nleE* mutant-infected colons were shorter and thicker than colons dissected from uninfected mice or those infected with the *nleB* mutant (Fig. 5C). Therefore, whereas *nleE* did not appear to make a major contribution to colonization of the host or disease in the *C. rodentium*/mouse model of infection, *nleB* was essential for both colonization and the development of colonic hyperplasia.

**Colonization of C57BL/6 mice by *nleC* and *nleD nleD2* mutants of *C. rodentium*.** The genome of *C. rodentium* is unique among A/E pathogens, as although it harbors only a single copy of *nleC* (rod314d12-ORF7 [where ORF7 is open reading frame 7]) it possesses two copies of *nleD* (rod368e02-ORF41 and rod76e07-ORF103). To extend our study of the role of T3SS effector proteins in the pathogenesis of infections with *C. rodentium*, we assessed the ability of an *nleC* single deletion mutant (ICC197) and an *nleD nleD2* double deletion mutant (ICC213) to infect 5- to 6-week-old C57BL/6 mice. The ability of the wild-type and mutant strains to establish an infection and colonize the mice was monitored by performing viable counts on bacteria recovered from stools at selected time points postinoculation. There was no significant difference in levels of colonization between the mutant strains and wild-type *C. rodentium* at any time point (Fig. 6). There was also no significant difference in the induced levels of colonic hyperplasia

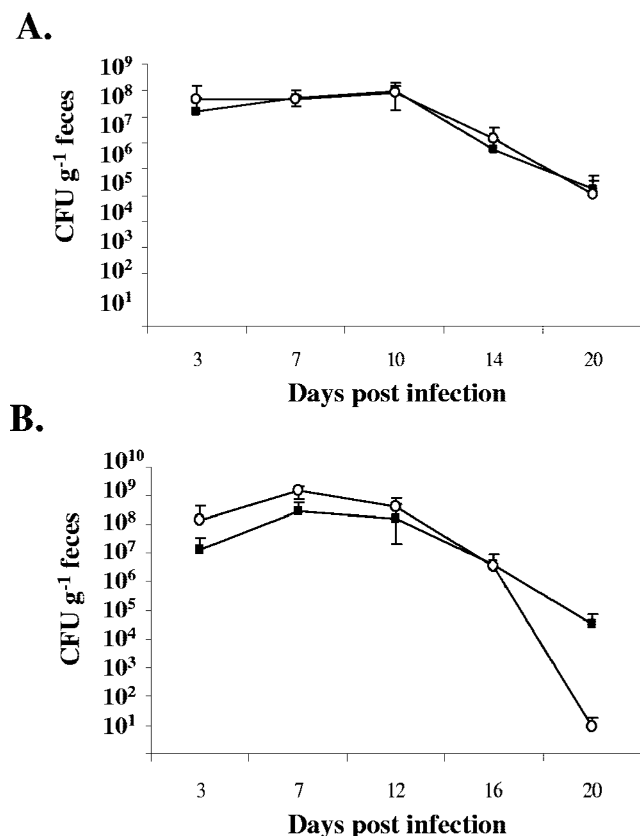


FIG. 6. Colonization of C57BL/6 mice with derivatives of *C. rodentium*. Results are expressed as the mean $\log_{10}$ CFU $g^{-1}$ feces for at least four mice, measured at different time points postinoculation. Mice were infected with approximately $2 \times 10^9$ CFU of (A) wild-type *C. rodentium* (■) or ICC197 (○) or (B) wild-type *C. rodentium* (■) or ICC213 (○). Error bars indicate standard deviations.

between the wild-type and mutant strains, as indicated by increased colon weight (data not shown). Therefore, neither *nleC* nor *nleD* and *nleD2* appeared to make an important contribution to colonization of the host or disease in the *C. rodentium*/mouse model of infection.

## DISCUSSION

The identification of genes important for the survival of bacteria in vivo has been greatly facilitated by the invention of STM (20). STM permits the rapid identification of genes essential for virulence and survival in vivo by screening mixed pools of transposon mutants in an animal model of infection (35). The infection of mice with *C. rodentium* serves as a valuable small-animal model for the study of host colonization by A/E pathogens. Recently, we performed an STM screen with *C. rodentium* mutants in the highly susceptible C3H/HeJ mouse strain (30). This initial study identified the type IV pilus gene cluster *cfc* and the prophage-carried T3SS effector protein EspI, as well as eight mutants with insertions into the LEE region (29, 30). Of the 576 mutants tested, 14 colonization-defective mutants were found, resulting in a "hit rate" of 2.4%. To identify further determinants required for survival in vivo, we continued screening the *C. rodentium* STM library in the more resistant C57BL/6 mouse strain. The susceptibility of mice to infection with *C. rodentium* shows significant host variation (38). C3H/HeJ mice, which lack an innate immune response to lipopolysaccharide, are colonized more rapidly and to higher bacterial numbers, suffering more extensive colonic hyperplasia and higher mortality rates, than mouse strains such as NIH Swiss and C57BL/6, which are more resistant to *C. rodentium* infection and disease (38). In more-resistant mouse strains, the CI for attenuated mutants is lower than that observed with C3H/HeJ mice (19). Given that STM relies on a competitive infection, it may be possible to uncover additional factors necessary for full virulence in a resistant background. In this study, we screened 576 mutants of *C. rodentium* in C57BL/6 mice and identified 19 attenuated strains, representing a "hit rate" of 3.3%. Several insertions occurred in previously identified virulence genes, including *cfcF*, *efa1*, and *espI*, and three insertions occurred within the LEE region (24, 29–31).

Novel genes identified here by STM included a putative regulator with similarity to the AraC-like regulator PerA from EPEC, which is a transcriptional activator of the *bfp* operon and LEE (28). This gene has been named *regA* for regulation factor A. Two insertions occurred within genes encoding putative outer membrane proteins with similarity to hypothetical proteins from *Salmonella enterica*, and other insertions were found to occur in genes required for cysteine synthesis and sugar transport, suggesting that these processes are important for the survival of *C. rodentium* in vivo and perhaps for A/E pathogens in general. One insertion was found to occur in the *C. rodentium* Flag-2 locus, which contains genes associated with lateral flagellum assembly (32).

Of particular interest was the identification of the gene encoding a putative translocated effector of A/E pathogens, NleB (11). In EHEC O157:H7 strain EDL933, *nleB* is present in O island 122 and is located directly upstream from a gene encoding another putative T3SS effector, NleE. In *C. rodentium*, EPEC, and REPEC, *nleB* and *nleE* are also located together, and, interestingly, in REPEC these genes are found to be closely associated with the LEE region (36). This suggests that *nleB* and *nleE* may have entered A/E pathogens with the LEE region but that in EPEC and EHEC, genomic rearrangements have led to the relocation of these genes to different positions in the genome.

The secretion of NleB and NleE by the LEE-encoded T3SS of *C. rodentium* has been demonstrated previously (11). In this study, we confirmed that NleB and NleE were secreted by LEE in EPEC and demonstrated that NleB and NleE are also translocated into host cells in a LEE-dependent manner. To determine the contribution of *nleB* and *nleE* to virulence, we constructed *nleB* and *nleE* defined mutants of *C. rodentium* and tested the mutants' ability to colonize C57BL/6 mice in competition with wild-type *C. rodentium* and in single infections. Like the *nleB* transposon mutant 30E1, the *nleB* deletion mutant was outcompeted by wild-type *C. rodentium* in a mixed infection. In single infections, *nleB* was also essential for colonization and virulence. In contrast, the *nleE* deletion mutant showed no difference from wild-type *C. rodentium* in its ability to colonize mice and induce disease.

To extend our study of the role of non-LEE-encoded type III effectors in *C. rodentium* colonization and virulence, we constructed deletion mutants of other effector genes *nleC*,

*nleD*, and *nleD2*. *nleD* was identified recently during an STM screen of EHEC O157:H7 mutants in calves (12). Due to the presence of a second copy of *nleD* in the *C. rodentium* genome, *nleD2*, we generated a double deletion mutant, *nleD nleD2*. The results showed that none of these genes (*nleC*, *nleD*, or *nleD2*) played a role in *C. rodentium* infection of mice. This finding parallels the results of our previous work which found that *nleC* and *nleD* were not required for colonization of lambs and calves by EHEC O157:H7 (26). At this stage, the role of NleC and NleD in the pathogenesis of infections with AEEC is unclear.

In this study, we identified *nleB* by STM and found that NleB is an essential virulence determinant of the A/E pathogen *C. rodentium*. The identification of a growing number of T3SS effectors that are carried on prophages and small pathogenicity islands of A/E pathogens with no obvious phenotype in vitro emphasizes the importance of A/E models of infection to elucidate the role of these new effectors in pathogenesis. Although the *C. rodentium*/mouse model of infection is an invaluable small-animal model in which to test the role of new effector proteins in disease, mice do not serve as a definitive representative host for all A/E pathogens. The testing of effector mutants in other A/E pathogens and infection models, such as rabbits and calves, will help to identify essential virulence factors shared by all A/E pathogens and those which may be host specific.

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