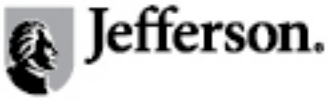

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Expression of Enteropathogenic *Escherichia coli* Map Is Significantly Different than That of Other Type III Secreted Effectors *In Vivo*

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The enteropathogenic *Escherichia coli* (EPEC) locus of enterocyte effacement (LEE)-encoded effectors EspF and Map are multifunctional and have an impact on the tight junction barrier while the non-LEE-encoded proteins NleH1 and NleH2 possess significant anti-inflammatory activity. In order to address the temporal expression of these important genes *in vivo*, their promoters were cloned upstream of the *luxCDABE* operon, and luciferase expression was measured in EPEC-infected mice by bioluminescence using an *in vivo* imaging system (IVIS). Bioluminescent images of living mice, of excised whole intestines, and of whole intestines longitudinally opened and washed were assessed. The majority of bioluminescent bacteria localized in the cecum by 3 h postinfection, indicating that the cecum is not only a major colonization site of EPEC but also a site of EPEC effector gene expression in mice. *espF*, *nleH1*, and *nleH2* were abundantly expressed over the course of infection. In contrast, *map* expression was suppressed at 2 days postinfection, and at 4 days postinfection it was totally abolished. After 2 to 4 days postinfection, when *map* is suppressed, EPEC colonization is significantly reduced, indicating that *map* may be one of the factors required to maintain EPEC colonization. This was confirmed in a competitive colonization study and in two models of chronic infection, repeated exposure to ketamine and *Citrobacter rodentium* infection. Our data suggest that *map* expression contributes to the maintenance of EPEC colonization.

Enteropathogenic *Escherichia coli* (EPEC) is a human pathogen that causes infantile diarrhea in developing countries (1–7). EPEC adheres to host intestinal epithelium and induces attaching and effacing (A/E) lesions, which are characterized by intimate attachment of the organisms to host epithelial cells and effacement of the surrounding enterocyte microvilli (8). A pathogenicity island known as the locus of enterocyte effacement (LEE) encodes the proteins that promote the attachment and effacement phenotype, including the type III secretion system (T3SS) and T3SS effector proteins (9, 10). The T3SS translocates bacterial effector proteins into host cells, thus mediating host cell responses and alterations. Effector proteins secreted through the T3SS are comprised of not only LEE-encoded proteins but also non-LEE-encoded proteins. The temporal hierarchy of translocated effectors through the T3SS has been defined using *in vitro* models. Tir is the first effector to be translocated from EPEC into cultured cells, followed by EspZ, EspF, EspH, EspG, and Map (11). Therefore, it is likely that the expression of effector proteins *in vivo* also varies in accordance with time and localization of infection. A recent report described the expression of virulence genes in *Citrobacter rodentium*, a natural mouse pathogen, by studying the expression of Ler, a regulator for most LEE genes (12). This study showed that virulence genes were expressed and required for pathogen growth during early infection but that they were downregulated in the late phase of infection (12). It is possible that expression of LEE effector genes in EPEC may have a similar pattern; however, this has not been addressed *in vivo*.

Understanding the expression patterns of different EPEC effector proteins aids in interpreting their contribution to EPEC pathogenesis *in vivo*. Previously, we reported the use of a bacterial luciferase expression plasmid, pCM17 (13), to study the colonization pattern of EPEC in a murine model (14). The plasmid pCM17 contains a constitutive promoter of an outer membrane gene, *ompC*, upstream of the *luxCDABE* operon isolated from *Photorhabdus luminescens*. It can be employed to study gene expression patterns of different EPEC virulence effector proteins by introducing the promoter of the virulence gene of interest upstream of the luciferase operon, instead of the *ompC* promoter, into EPEC and studying its expression in mice.

EPEC produces at least 26 virulence effector proteins, including LEE and non-LEE proteins (15). EspF and Map (mitochondrial associated protein) are among the most extensively studied LEE effector proteins and have multiple functions. EspF induces mitochondrial lysis (16, 17), disrupts tight junctions (16, 18), promotes degradation of antiapoptotic proteins (19), inhibits function of Na^+/H^+ exchanger isoform 3 (NHE3) (20) and the sodium glucose transporter SGLT-1 (21), and inhibits phagocytosis (22). Map induces transient filopodia at bacterial attachment sites (23, 24), contributes to tight junction disruption (18), and triggers mitochondrial dysfunction (25). *espF* is located in the polycistronic LEE4 operon. *map* is located in a monocistronic operon in LEE. Two homologous non-LEE-encoded proteins, NleH1 and NleH2 (NleH1/2), that contain antiapoptotic and anti-inflamma-

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tory properties were also investigated. NleH1/2 block apoptosis via inhibition of Bax-1 inhibitor and caspase-3 activation (26, 27). They also dampen host cell inflammation via inhibition of the NF-κB signaling pathway (28, 29). We previously reported that NleH1/2 inhibit NF-κB through a mechanism involving attenuation of IκBα ubiquitination (28). NleH1, but not NleH2, was reported to prevent ribosomal protein S3 (RPS3) association with NF-κB in the nucleus by inhibiting IKKβ phosphorylation of RPS3 (29).

In this study, the temporal expression profiles of *espF*, *map*, *nleH1*, and *nleH2* were assessed in a C57BL/6J mouse model of EPEC infection using an *in vivo* imaging system (IVIS) to determine luciferase expression, as previously described (14). Mice were infected by gastric gavage with EPEC strains carrying the luciferase reporter driven by different native EPEC effector gene promoters (*LEE4*, *map*, *nleH1*, and *nleH2*). Gene expression was assessed at various times postinfection by an IVIS in live animals and in excised intestine. *map* expression was found to be differentially regulated compared to other EPEC effectors. Moreover, the role of *map* and EPEC colonization was assessed in a competitive index (CI) study. The correlation between *map* expression and EPEC colonization was also assessed in mice repeatedly exposed to ketamine to prolong colonization and in a natural *C. rodentium* infection. Our data indicate that *map* may contribute to EPEC colonization.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material.

Strain construction. Promoter regions were predicted using BPROM bacterial promoter prediction software (30). Primers are listed in Table S2 in the supplemental material.

Construction of an *luxCDABE* reporter with multiple cloning sites (MCS), pCM18. Because pCM10 (13) has only two unique restriction sites, EcoRI and BamHI, an 88-nucleotide DNA fragment carrying multiple unique restriction sites was generated in between these EcoRI and BamHI sites, upstream of *luxCDABE*. This fragment was generated from two complementary primers, 18P1 with overhanging EcoRI and BamHI sites (5′-AATTCATACGATCGATACCTGCAGGATAGGTACCTAAGG CCTATTACCCGGGTAGCACTTTAATTAAGTTAACTTAGGCCGGC CTATG; restriction sites are underlined) and 18P2 with overhanging BamHI and EcoRI sites (5′-GATCCATAGGCCGGCCTAAGTTAACTT AATTAAAGTGCTACCCGGGTAATAGGCCTTAGGTACCTATCCTG CAGGTATCGATCGTATG; restriction sites are underlined). 18P1 and 18P2 were hybridized by incubation at 65°C for 10 min and then ligated into pCM10 at EcoRI and BamHI sites.

p*fliC*, p*LEE4*, p*nleH1*, p*nleH2*, and p*map* were constructed by PCR amplification of the respective promoter regions and then subcloning them into pCM10 or pCM18 to fuse with *luxCDABE*.

Mouse infection. Six-week-old male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in a specific-pathogen-free facility at the University of Illinois at Chicago for 5 to 7 days with free access to food and water. Mice were infected with bacteria by oral gavage as previously described (14). Mice were pretreated with 5 g/liter streptomycin sulfate in drinking water for 24 h to reduce the normal intestinal flora and then switched to regular drinking water for 24 h prior to infection. To prepare the inocula, bacteria were grown overnight (O/N) at 37°C in LB broth with appropriate antibiotics (50 μg/ml kanamycin) and then inoculated at 1:33 into serum-free and antibiotic-free Dulbecco's modified Eagle medium (DMEM)/F12 medium for 3 h at 37°C. Bacteria was centrifuged at 3,700 rpm for 30 min, and then cell pellets were washed with sterile phosphate-buffered saline (PBS) once; the bacterial concentration was adjusted to $\sim 2 \times 10^7$ CFU/200 μl in PBS for mouse gastric gavage. This inoculation was 10 times lower than a typical EPEC inoculation in order not to maximize the luciferase measurement by the IVIS machine at the peak time point (around 3 h postinfection). Bacteria were administered by gastric gavage using a 22-gauge, ball-tipped feeding needle.

In the competitive colonization studies, a Δ*map* (Amp^r Kn^r) strain, SE1174, from the SE882 background (31), provided by James Kaper, University of Maryland, and a wild-type (WT) EPEC strain carrying plasmid pACYC184 (Cm^r) were grown in serum-free and antibiotic-free DMEM/F12 medium for 3 h, and equal amounts of bacteria (10^8 CFU of each strain in 100 μl of PBS) were mixed and delivered to mice by gastric gavage. The competitive index was calculated as described previously in *Salmonella enterica* serovar Typhimurium infection studies (32). The competitive index (CI) of the mutant was calculated as the output ratio (mutant CFU count/WT CFU count) divided by the input (inoculum) ratio (mutant CFU count/WT CFU count). Animal protocols were approved by IACUC at the University of Illinois at Chicago and Loyola University Chicago.

BLI *in vivo* and *ex vivo*. Mice were assessed for bioluminescence imaging (BLI) by an IVIS Spectrum instrument (Xenogen Corporation, Hopkinton, MA, USA) at 30 min, 3 h, 6 h, 24 h, 48 h, and 4 days postinfection. For *in vivo* BLI, mice were anesthetized either with 2.5% isoflurane and then maintained at 1% or with an intraperitoneal (i.p.) injection of a ketamine (100 mg/kg)-xylazine (5 mg/kg) mixture and maintained using 1% isoflurane. To remove the black fur which interferes with the bioluminescence signal, Nair Hair Remover (Church and Dwight Co., Princeton, NJ) was used on the day of infection. For *ex vivo* BLI, mice were euthanized at different times postinfection, and the entire intestine was immediately excised, positioned on a petri dish, and then imaged by an IVIS. To detect adherent bacteria, the small intestine, cecum, and colon were opened longitudinally and washed extensively with sterile PBS before imaging with an IVIS. Quantification of bioluminescence was conducted using Living Image software, version 3.1.

Bacterium enumeration from fecal pellets. Freshly collected stool from infected mice was weighed, homogenized, and serially diluted in PBS and then plated on agar plates supplemented with appropriate antibiotics. EPEC was plated on sorbitol MacConkey agar plates supplemented with 100 μg/ml nalidixic acid, and *Citrobacter rodentium*/p*map* was plated on LB plates supplemented with 50 μg/ml kanamycin.

***In vitro* bioluminescence imaging.** Bacteria were grown O/N in LB medium in the presence of appropriate antibiotics and then inoculated at 1:500 into different types of media: LB medium, LB medium with 18.4 mM NH_4Cl, DMEM, DMEM with 18.4 mM NH_4Cl, PBS, and PBS with 18.4 mM NH_4Cl. At 1 h postinfection, triplicate samples of 100 μl of each culture were pipetted into 96-well dark-walled, clear-bottom plates (Corning Incorporated, Corning, NY). BLI was measured using an IVIS.

Statistical analysis. All data are reported as means ± standard errors of the means (SEM). Data comparisons were made using a nonparametric Kruskal-Wallis test with Dunn's multiple-comparison test or with Student's *t* test. Differences were considered significant when the *P* value was ≤0.05. Data were graphed using GraphPad Prism software, version 5 (La Jolla, CA).

RESULTS

Expression of EPEC effector proteins in mice within the first 24 h postinfection. To compare the expression profiles of the EPEC effector proteins Map, EspF, NleH1, and NleH2 in a murine model of EPEC infection, EPEC was transformed with p*map*, p*LEE4*, p*nleH1*, and p*nleH2*, respectively. *espF* is expressed under the regulation of *LEE4* promoter; therefore, p*LEE4* was used to study the expression of *espF* and other proteins in the LEE4 operon (SepL, EspA, EspD, CesD2, and EscF). The regulation of expression of flagellin (FliC) was previously reported in uropathogenic *Escherichia coli* (33). Therefore, a luciferase reporter driven by the EPEC *fliC* promoter, p*fliC*, served as a positive control in these experiments. Promoter regions were predicted using BPROM bacterial promoter prediction

software (30). Primers were designed so that each subcloned promoter covered a region of 700 to 1,000 kb.

Constructed promoters were tested first *in vitro* to determine whether they could be regulated by an exogenous stimulus. It has been reported that EPEC secretion is reduced in the presence of 18.4 mM NH_4Cl (34). Strains carrying different EPEC effector protein promoters were inoculated into PBS or DMEM, and then the luciferase signal was measured by an IVIS (see Fig. S1A and B in the supplemental material) in the presence and absence of NH_4Cl. The promoterless pCM10 plasmid carrying *luxCDABE* was used as a negative control. pCM17 (the plasmid carrying an outer membrane OmpC promoter) and p*fliC* served as positive controls. Luciferase expression of all tested promoters was significantly reduced 10 to 25% in the presence of NH_4Cl at 1 h postinoculation, in both PBS and DMEM, confirming that the expression of these constructs was regulated. In addition, gene expression was 20 times higher in DMEM than in PBS, confirming known regulatory patterns for the genes.

To test the expression of these genes *in vivo*, 7-week-old C57BL/6J mice were infected with EPEC carrying p*fliC*, p*LEE4*, p*map*, p*nleH1*, or p*nleH2* as described in the Materials and Methods section. Mice were anesthetized with isoflurane, and BLI imaging was performed at 30 min, 3 h, 6 h, and 24 h postinfection and quantified (Fig. 1A and B). After imaging, the mice were sacrificed, and the intestine was immediately excised and imaged by an IVIS in order to better localize the site of expression (Fig. 1C and D). The gene expression profiles of effector proteins in bacteria adherent to the intestine were also examined by opening the intestine longitudinally and washing it extensively with sterile PBS before *in vivo* imaging was performed (Fig. 1E and F). For all genes examined, the expression patterns were similar within the first 24 h postinfection, with peak expression occurring at 3 h. In addition, by 3 h postinfection, the majority of bioluminescent bacteria occupied the cecum (Fig. 1C); this pattern persisted at 24 h postinfection, suggesting that the cecum is the main site for EPEC colonization and for expression of EPEC effector proteins in mice. No significant difference was observed between the expression levels of *fliC*, *LEE4*, *nleH1*, *nleH2*, and *map* within the first 24 h postinfection (Fig. 1B, D, and F).

Map expression is totally abolished by 4 days postinfection. The initiation of EPEC infection requires the establishment of bacterial adherence and creation of a niche. We were therefore interested in assessing the level of effector expression at later time points postinfection. Again, imaging and bioluminescence were examined and quantified at 2 and 4 days postinfection. Interestingly, at 2 days postinfection, *map* expression was suppressed, while the other effector promoters remained active (Fig. 2A and B). By 4 days postinfection, other effector promoters continued to produce strong luciferase signals, while the *map* signal was undetectable (Fig. 2D). Colonization levels of all strains were comparable at both 2 and 4 days postinfection (Fig. 2C and E), indicating that the suppression of *map* promoter signal was not due to loss in bacterial colonization.

We previously demonstrated the pCM17 plasmid to be 100% stable in EPEC when the bacteria are grown in LB medium for at least 7 days without antibiotic selection (14). To further confirm that the loss of the *map* signal was not due to plasmid instability, we similarly tested the stability of the p*map* plasmid in nonselective LB medium. After each consecutive day of growth, cultures were plated on LB plates without antibiotics, and the percentage of luminescent colonies was determined. We confirmed that p*map* was stable in 100% of colonies at least until day 4, and it remained highly stable in 96% of colonies by day 5 (see Fig. S2 in the supplemental material). This confirms that the loss of map expression was not due to the instability of the plasmid.

In many cases, gene transcription is regulated by at least one transcriptional regulator that binds to the regulatory region. In this study, the promoters were constructed to include possible regulator regions upstream of the core promoters. Although a *map* regulatory region has not been reported, it is likely that one exists. To determine if the upstream region of the *map* promoter is crucial for its expression, this region was truncated to 125 nucleotides and cloned into the luciferase reporter plasmid designated p*map*T. The construct p*map*T was transformed into EPEC and assessed for *map* expression in infected mice (Fig. 2F). The EPEC/p*map*T colonization level was comparable to that of EPEC/p*map* (data not shown). As anticipated, luciferase expression driven by the truncated *map* promoter was significantly lower than that under the control of the full-length promoter (Fig. 2G). No signal was observed in mice, even at 3 h postinfection, which is the normal time point for peak expression of effector proteins, suggesting that the upstream region of the *map* promoter is required for its expression.

Map expression is restored in a model of prolonged infection. Ketamine, an anesthetic drug used in animals, is known to prolong the colonization of bacteria (14, 35). Specifically, EPEC colonization in mice is prolonged for at least 30 days postinfection, instead of the typical 7 to 10 days, when ketamine is received daily (14). We examined the pattern of *map* expression in ketamine-treated mice in comparison to that in untreated mice. For these experiments, the same mice were repeatedly assessed for luciferase expression at different time points, i.e., 3 h, 6 h, 24 h, 2 days, and 4 days postinfection (Fig. 3A), and anesthetized intraperitoneally (i.p.) with a ketamine (100 mg/kg)-xylazine (5 mg/kg) mixture prior to each image acquisition. Similar to the results in untreated mice, *map* expression was absent at 2 days postinfection in ketamine-treated mice while the other effectors were abundantly expressed (Fig. 3A). In contrast, at 4 days postinfection, animals that received repeated ketamine injections expressed *map* at levels comparable to those of the other effectors (Fig. 3A). In addition, EPEC/p*map* colonization remained stable over these 4 days (Fig. 3B).

In order to confirm that the elevated expression of *map* at day 4 was due to the effect of repeated exposure to ketamine, we examined *map* expression at 2 and 4 days postinfection in mice sedated with ketamine-xylazine only immediately prior to IVIS imaging. Only 1 out of 4 mice expressed *map* at 2 days postinfection while no mice showed a luciferase signal at 4 days postinfection (Fig. 3C and D), supporting the hypothesis that the elevated expression of *map* at 4 days postinfection in animals receiving repeated exposure to ketamine was, in fact, due to the effect of ketamine and correlated with enhanced colonization. These data also support the idea that the p*map* plasmid is stable in the *in vivo* model. With repeated ketamine administration, bacterial colonization remained at similar levels at days 2 and 4 postinfection (Fig. 3B), whereas in the absence of ketamine, colonization levels dropped more than 1 log at 4 days compared to the level at 2 days postinfection (Fig. 3E). These data suggest that Map expression contributes to the extended colonization associated with repeated ketamine administration.

Map is required for competitive colonization. Map has been reported to promote colonization in mixed-infection studies with *Citrobacter rodentium* (36, 37) but has not been tested with EPEC. To investigate the requirement of Map in competitive coloniza-

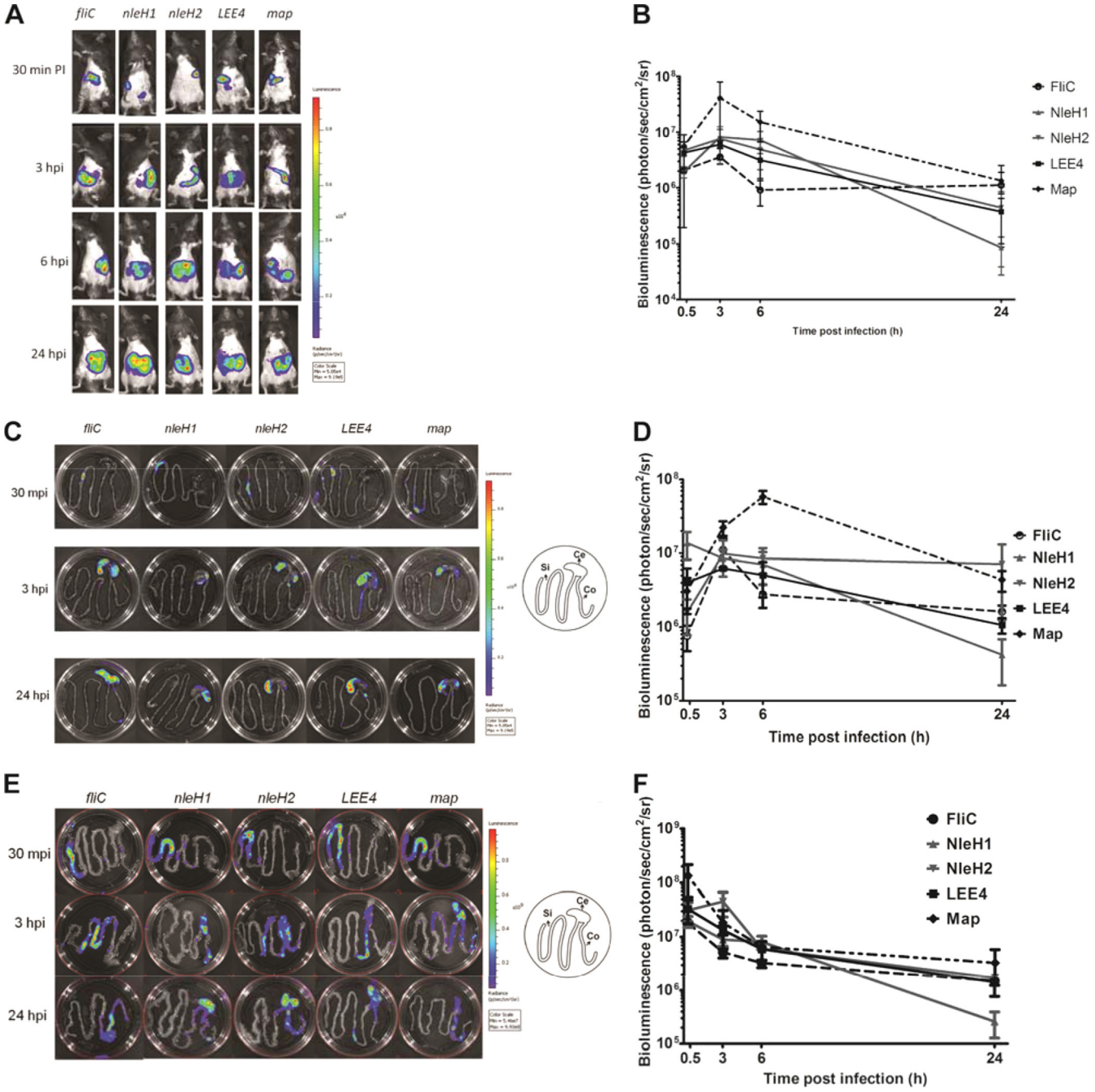

FIG 1 Bioluminescent imaging of EPEC expressing p*fliC*, p*nleH1*, p*nleH2*, p*LEE4*, and p*map in vivo*. (A) Representative bioluminescent imaging of infected mice at 30 min, 3 h, 6 h, and 24 h postinfection. Mice were anesthetized with 2.5% isoflurane, maintained at 1%, and then imaged by an IVIS. (B) Quantification of bioluminescence in the images in panel A. (C) Representative bioluminescent imaging shows colonization of EPEC expressing p*fliC*, p*nleH1*, p*nleH2*, p*LEE4*, and p*map* in excised intestines. (D) Quantification of bioluminescence in the images in panel C. (E) Representative bioluminescent imaging of excised, longitudinally opened and washed intestines showing adherent bacteria. (F) Quantification of bioluminescence in the images in panel E. In panels C and E, the entire intestine was positioned on a petri dish with arrangement of small intestine, cecum, and colon from left to right, and then imaged by IVIS. Si, small intestine; Ce, cecum; Co, colon. Signal strength is as indicated by the colored bars on the figure. In panels B, D, and F, values are means; error bars indicate SEM ($n = 3$). p.i. postinfection; hpi, hours postinfection; mpi, minutes postinfection.

tion, a mixture of approximately 50% WT EPEC (carrying a low-copy-number Cm^r plasmid) and 50% EPEC Δ*map* (Kn^r Amp^r) was given to mice. Bacterial input levels were confirmed by growth on selective LB plates with the specified antibiotics as described in Materials and Methods. Stools were collected for colonization from day 1 until day 5 postinfection. Calculation of the competitive index (CI) was as described in the Materials and Methods section. A CI of 1 indicates that the test strain was able to compete at the same level as the WT strain, while a CI of less than 1 indicates that the test strain was out-competed by the WT. The EPEC Δ*map*

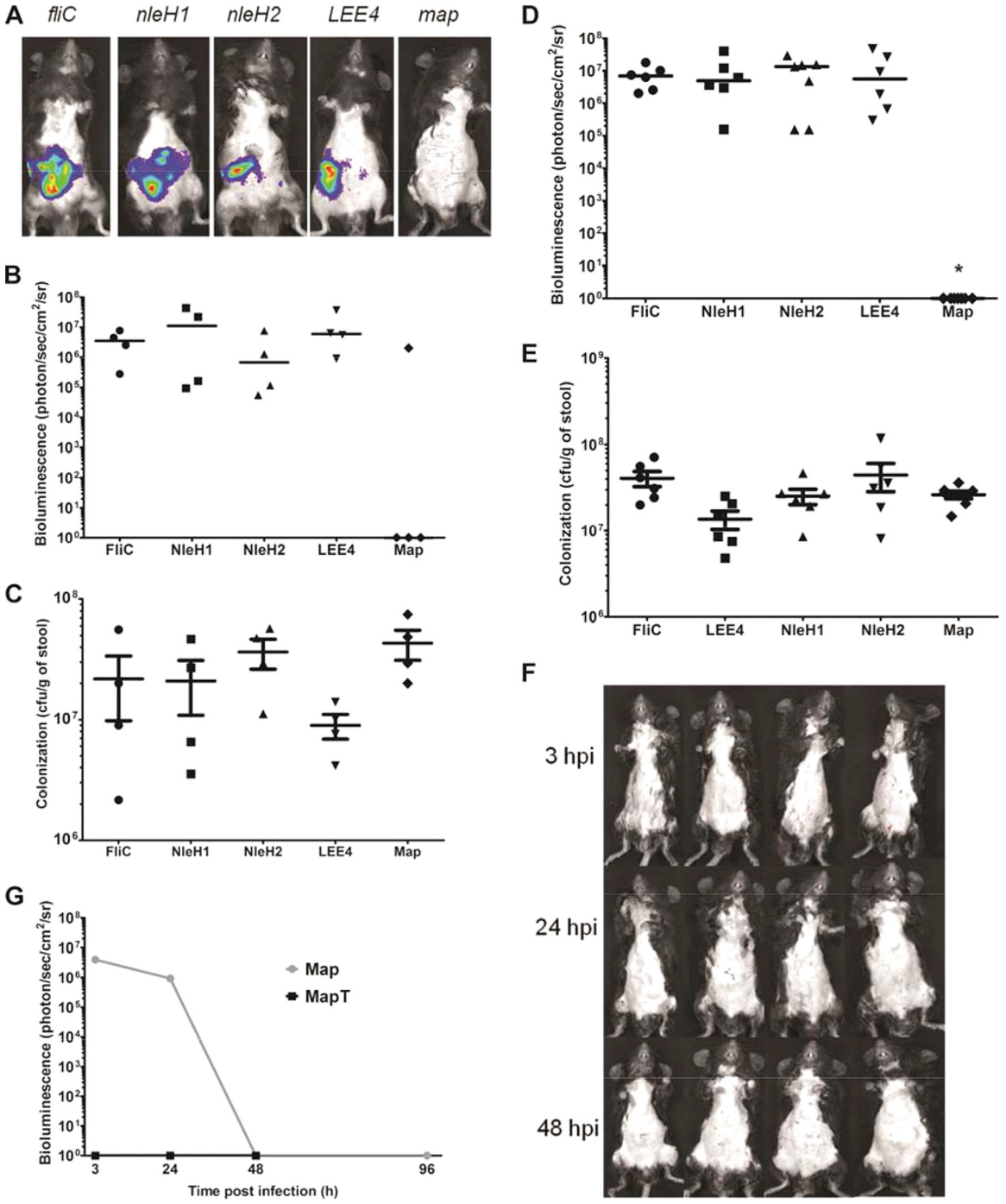

FIG 2 *map* expression at days 2 and 4 postinfection. Mice were anesthetized with 2.5% isoflurane, maintained at 1%, and then imaged by an IVIS. (A) Representative bioluminescent imaging of EPEC expressing p*fliC*, p*nleH1*, p*nleH2*, p*LEE4*, and p*map* at day 2 postinfection ($n = 4$). (B) Quantification of bioluminescence in the images in panel A. (C) Colonization of EPEC at day 2 postinfection. (D) Quantification of bioluminescence at day 4 postinfection ($n =$ 6 to 7). *, $P < 0.05$ for *map* expression compared with either *fliC*, *nleH1*, *nleH2*, or *espF* under the control of *LEE4* promoter. (E) Colonization at day 4 postinfection. (F) Bioluminescent imaging of EPEC/p*mapT* at 3 h, 24 h, and 48 h postinfection ($n = 4$; all mice are shown). (G) Quantification of p*map* and p*mapT* signals ($n = 4$). *, $P \leq 0.05$. Horizontal bars (B to E), medians; error bars (C and D), SEM.

strain has a CI of less than 0.2 from day 1 to day 5 postinfection (Fig. 4), indicating that the expression of *map* provides an advantage for EPEC colonization.

EPEC Map expression in *Citrobacter rodentium*. As opposed to the 7- to 10-day colonization period of EPEC infection in the murine model with streptomycin pretreatment, the related mouse pathogen *Citrobacter rodentium* colonizes mice naturally for 17 to 21 days. EPEC Map and *C. rodentium* Map are 80% identical (NCBI protein-protein BLAST), while their possible regulatory and promoter regions share no significant similarity. Therefore,

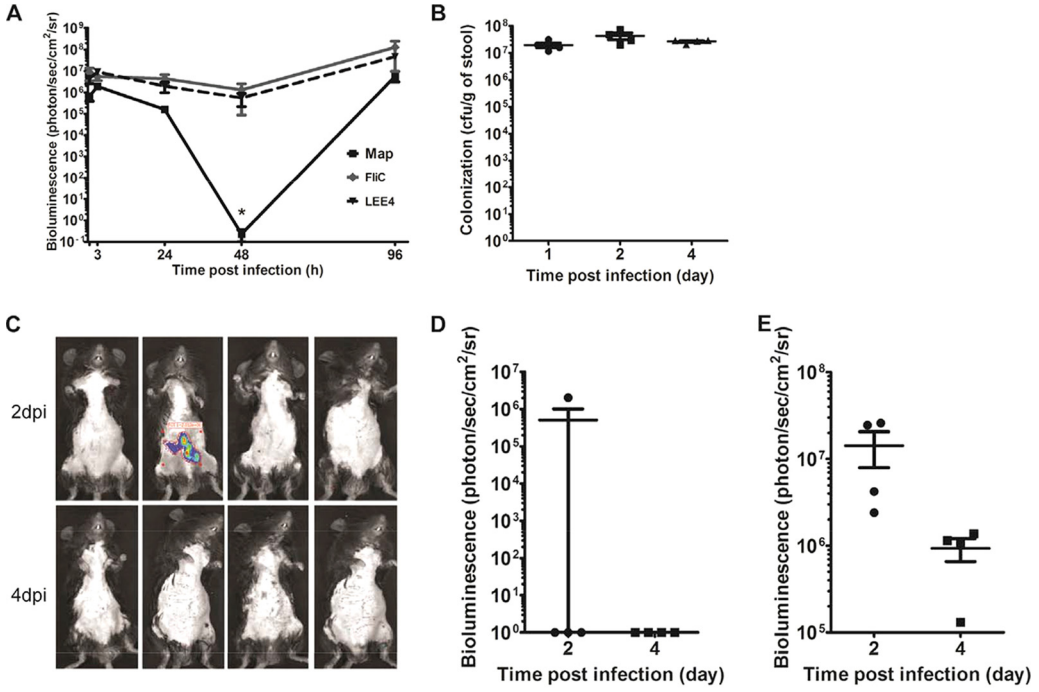

FIG 3 *map* expression in mice receiving repeated exposure to ketamine and in untreated mice. (A) Mice infected with EPEC expressing p*map*, p*fliC*, and p*LEE4* were anesthetized with an i.p. injection of a mixture of ketamine (100 mg/kg)-xylazine (5 mg/kg), and sedation was maintained using 1% isoflurane. Bioluminescence was imaged and quantified at 30 min, 3 h, 6 h, 24 h, 48 h, and 4 days postinfection ($n = 4$ to 8). Error bars, SEM. *, $P \leq 0.05$ for expression of *map* compared with either *fliC* or *espF* under the control of the *LEE4* promoter. (B) Colonization of EPEC/p*map* from mice repeatedly exposed to ketamine. (C) Bioluminescent imaging of EPEC/p*map* *in vivo* at days 2 and 4 postinfection in untreated mice. Mice were anesthetized only once with ketamine-xylazine immediately before *in vitro* imaging. All mice are shown. (D) Quantification of bioluminescence in the images in panel C. (E) Colonization of EPEC/p*map* in untreated mice at 2 days and 4 days postinfection. Stools were collected at days 2 and 4 postinfection. Horizontal bars (B, D, and E), means; error bars (D and E), SEM ($n = 4$). *, $P \leq 0.05$.

we used *C. rodentium* as the infecting agent to examine the level of EPEC *map* expression in a natural model of prolonged colonization. Mice were infected with *C. rodentium* carrying the construct p*map*, and *map* expression was determined by IVIS. Interestingly, EPEC *map* expression was maintained throughout the course of infection in *C. rodentium* (Fig. 5A), in contrast to its limited expression in EPEC. Interestingly, the maintenance of expression of *map* correlates with the prolonged colonization of *C. rodentium* (Fig. 5B). The differential expression of the EPEC *map* promoter in EPEC versus *C. rodentium* indicates a divergent regulatory mechanism of this gene in the two species.

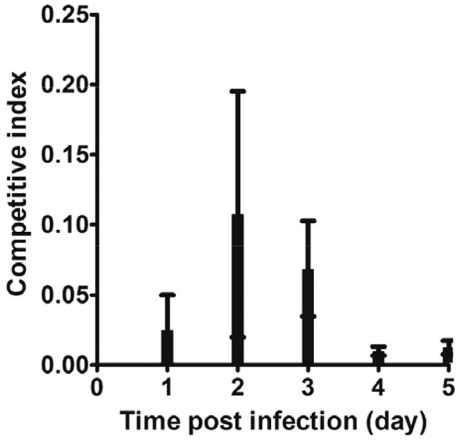

FIG 4 Competitive index (CI) of EPEC Δ*map* strain in competitive colonization studies with WT EPEC. Mice were infected with a mixture of equal amounts of WT EPEC and a Δ*map* strain. Colonization was determined by growth of each strain on LB plates containing its corresponding selection antibiotic. CI was calculated as described in Materials and Methods. Error bars, SEM ($n = 8$).

DISCUSSION

This paper reports for the first time the investigation of expression patterns of different EPEC effector proteins *in vivo*. We observed that all of the studied effector genes, *map*, *espF*, *nleH1*, and *nleH2*, have their highest expression in the cecum. This is consistent with our previous report that the cecum was the main colonization site for EPEC in mice (14). In a *C. rodentium* mouse model, the colon and the cecum were reported to be the major expression sites for LEE-encoded proteins during the early phase of infection (12).

Moreover, all studied effector genes were abundantly expressed within the first 24 h postinfection, with peak expression occurring at 3 h postinfection. However, at 48 h postinfection, while *nleH1*, *nleH2*, and *espF* were still robustly expressed, *map* expression was significantly reduced, and it was totally suppressed by 4 days postinfection. The fact that *map* is suppressed at day 4 while EPEC colonizes the gut for only 7 to 10 days suggests that *map*, together with other factors, may play a role in maintaining EPEC colonization. In fact, there is a sharp drop in colonization at day 4 postinfection, which correlates with the suppression of *map* expression (Fig. 3D). A role for *map* in EPEC colonization was confirmed in competition assays with WT EPEC and a Δ*map* strain, which revealed that colonization by an EPEC strain lacking *map* was significantly impaired in mice over the 5-day period

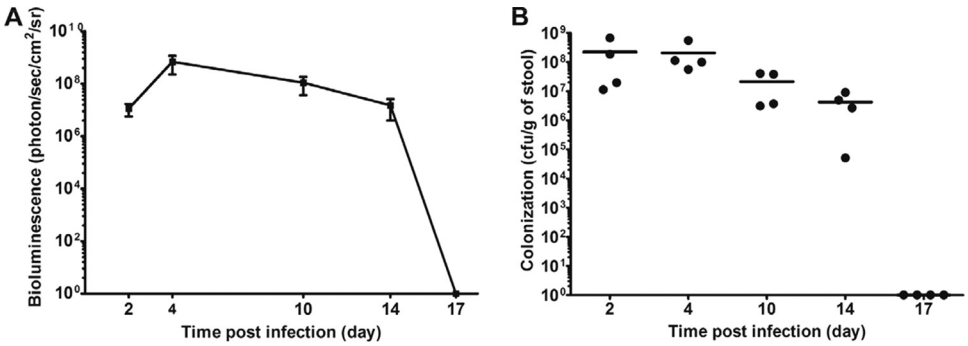

FIG 5 Expression and colonization of p*map* in *C. rodentium*. (A) Quantitation of bioluminescent imaging of *C. rodentium* expressing p*map in vivo* at 2, 4, 10, 14, and 17 days postinfection. (B) Colonization of p*map* in *C. rodentium*. Horizontal bars, mean values; error bars, SEM ($n = 4$).

tested (Fig. 4). Of greater interest perhaps is the demonstration that in two models of prolonged colonization, one induced with repeated ketamine exposure and the other through natural infection by *C. rodentium*, *map* expression is maintained at the same level as other effectors except for a transient dip on day 2 postinfection in ketamine-treated mice.

The role of *map* in colonization has also been studied in other A/E pathogens, enterohemorrhagic *E. coli* (EHEC) (38), and *C. rodentium* (36, 37, 39). In an EHEC-infected infant rabbit model, EHEC with a deletion of *map* showed a significant reduction in colonization in small intestine (38). In *C. rodentium*-infected mice, although Map deletion did not have an effect on colonization (39), a *C. rodentium* Δ*map* strain was impaired in competitive assays with the WT (36, 37). Together with our results, these findings suggest that *map* contributes to the colonization of A/E pathogens although perhaps at a subtle level.

In addition to Map, intimin (40) and many other effectors, such as Tir (41), EspF (38), EspH (38), and EspG (38), promote colonization of A/E pathogens. Tir is required for *ex vivo* colonization of both EPEC and EHEC in human intestinal mucosa in *in vitro* organ culture experiments (41). However, intimin but not Tir is required for colonization of *C. rodentium* in C57BL/6 mice pretreated with streptomycin (40). EspF, EspH, and EspG are all required for EHEC colonization in infant rabbits (38). However, EspG does not promote colonization of *C. rodentium* in mice (36). Together, it is possible that Map may act in harmony with others factors like intimin, Tir, and other effectors to maintain EPEC colonization.

Map has multiple known functions, including filopodium formation at bacterial attachment sites, disruption of tight junctions, and mitochondrial dysfunction, which may be important for the earlier phases of infection. Of these known Map activities, transient filopodium formation is most associated with attachment and perhaps plays a role in initial colonization. Attachment is associated with actin pedestal formation mediated by Tir-intimin complex (34). Map is translocated into host cells after Tir (11) and induces filopodium formation caused by actin polymerization at the site of bacterial infection (37, 38). Although Map signaling is inhibitory to Tir-intimin-triggered pedestal formation, it may still play an important role in optimizing the process of pedestal formation (23). It is unclear why filopodia are induced transiently at early phases of infection (23, 24), and we can only speculate as to the cause. One possibility is that filopodium formation may assist in localizing cellular components required for pedestal formation to regions surrounding the bacterial attachment site. It is therefore possible that transient, but not sustained, filopodium formation assists in pedestal establishment and bacterial attachment.

Finally, our findings suggest that there are specific regulatory events that control the expression of EPEC effector proteins, such as Map. Further investigation of the mechanisms involved in Map's contributions to colonization and other functions is important in order to gain a better understanding of Map's role in pathogenesis of A/E organisms. Moreover, elucidation of the expression profiles of other effectors such as EspG, Tir, intimin, EspH, NleC, NleD, and others would provide deeper insight into the pathogenesis of these bacteria.

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