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**Mutation of toxB and a Truncated Version of the efa-1 Gene in *Escherichia coli* O157:H7 Influences the Expression and Secretion of Locus of Enterocyte Effacement-Encoded Proteins but not Intestinal Colonization in Calves or Sheep**

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Enterohemorrhagic *Escherichia coli* (EHEC) strains comprise a broad group of bacteria, some of which cause attaching and effacing (AE) lesions and enteritis in humans and animals. Non-O157:H7 EHEC strains contain the gene efa-1 (referred to in previous publications as efa1), which influences adherence to cultured epithelial cells. An almost identical gene in enteropathogenic *E. coli* (lifA) mediates the inhibition of lymphocyte proliferation and proinflammatory cytokine synthesis. We have shown previously that significantly lower numbers of EHEC O5 and O111 efa-1 mutants are shed in feces following experimental infection in calves and that these mutants exhibit reduced adherence to intestinal epithelia compared with isogenic wild-type strains. *E. coli* O157:H7 strains lack efa-1 but encode a homolog on the pO157 plasmid (toxBH7095) and contain a truncated version of the efa-1 gene (efa-1’/z4332 in O island 122 of the EDL933 chromosome). Here we report that *E. coli* O157:H7 toxB and efa-1’ single and double mutants exhibit reduced adherence to cultured epithelial cells and show reduced expression and secretion of proteins encoded by the locus of enterocyte effacement (LEE), which plays a key role in the host-cell interactions of EHEC. The activity of LEE1, LEE4, and LEE5 promoters was not significantly altered in *E. coli* O157:H7 strains harboring toxB or efa-1’ mutations, indicating that the effect on the expression of LEE-encoded secreted proteins occurs at a posttranscriptional level. Despite affecting type III secretion, mutation of toxB and *efa-1’* did not significantly affect the course of fecal shedding of *E. coli* O157:H7 following experimental inoculation of 10- to 14-day-old calves or 6-week-old sheep. Mutation of *tir* caused a significant reduction in fecal shedding of *E. coli* O157:H7 in calves, indicating that the formation of AE lesions is important for colonization of the bovine intestine.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are zoonotic enteric pathogens of worldwide importance (39). In humans, infection by some EHEC serotypes may cause diarrhea, which may be complicated by hemorrhagic colitis and severe systemic sequelae, including hemolytic-uremic syndrome (45). EHEC strains are closely related to enteropathogenic *E. coli* (EPEC) strains, which are a leading cause of infantile diarrhea in developing countries, and have many of the EPEC genes implicated in virulence (13, 51).

Ruminants are an important reservoir of EHEC (15, 44), and direct or indirect contact with ruminant feces is the leading antecedent to EHEC infection in humans (17, 30, 43). Natural and experimental infections of calves or sheep with EHEC result in efficient colonization of the intestinal tract, and large numbers of bacteria are shed in the feces for several weeks (3, 5–9, 18, 59, 67, 68). Strategies to reduce the prevalence of EHEC in ruminants offer the potential to lower the incidence of human infections (58). However, little is currently known about the mechanisms underlying intestinal colonization of cattle and sheep by EHEC.

EHEC strains are defined by their ability to produce one or more Shiga toxins and to induce characteristic attaching and effacing (AE) lesions on intestinal epithelia, in which the bacteria adhere intimately to the apical surface of enterocytes on raised actin-rich pedestals and microvilli are locally destroyed. This histopathology is determined by the chromosomal locus of enterocyte effacement (LEE), which encodes a type III protein secretion system (13). One of the LEE-encoded secreted proteins (Tir) is translocated into the host cell plasma membrane, where it acts as a receptor for the bacterial outer membrane protein intimin (10). intimin is an important colonization factor for *E. coli* O157:H7 in neonatal calves (7), lambs (67), and adult cattle and sheep (5). intimin can also bind to β1-integrins (14) and cell surface-localized nucleolin (57); however, the importance of such interactions and of type III secretion in colonization of the ruminant intestine is unclear. Immunization of calves with *E. coli* O157:H7 type III
secreted proteins reduces fecal shedding of the bacteria following experimental infection; however, the contribution of individual secreted proteins in colonization and immunity in ruminants requires detailed investigation (50).

Recently, it was reported that the product of the efa-1 gene (EHEC factor for adherence; referred to in previous publications as efa1) influences intestinal colonization in calves by non-O157:H7 EHEC (59). Efa1 was first identified as a factor influencing adhesion of a clinical O111:H7 EHEC strain to cultured epithelial cells (42). EHEC O5 and O111 strains harboring mutations in the efa-1 gene were shed in the feces in significantly lower numbers than the corresponding parent strains following oral inoculation of conventional calves. Furthermore, an EHEC O111:H7 efa-1 mutant was impaired in the ability to adhere to the colonic epithelium in calves, as determined by confocal microscopy and direct recovery of bacteria from the mucosa (59).

The mechanism by which Efa1 influences intestinal colonization remains obscure. Mutation of efa-1 in EHEC O5 and O111 reduces the expression and secretion of LEE-encoded effector proteins, suggesting that it may indirectly influence colonization (59). The EHEC O111:H7 Efa1 protein is 97.4% identical at the amino acid level to EPEC lymphostatin (LiFa), which confers an ability to inhibit lymphocyte proliferation of human peripheral blood lymphocytes and the mitogen-activated synthesis of proinflammatory cytokines (25). LiFa also inhibits the proliferation of human and murine intraepithelial lymphocytes, indicating that LiFa and Efa1 may influence intestinal colonization by modulating mucosal immunity in the gut (21, 25–27, 32).

The efa-1/lifA gene is present in all non-O157:H7 EHEC serotypes tested so far and in related entero pathogens, such as Citrobacter rodentium and rabbit EPEC (REPEC) (25, 42, 60). Sorbitol-fermenting E. coli O157:H7 strains also contain efa-1; however, the intact efa-1 gene is absent from the E. coli O157:H7 strains that have been sequenced (19, 22, 47). The efa-1 gene in REPEC was recently shown to mediate bacterial adherence to cultured epithelial cells, apparently without affecting the secretion of LEE-encoded proteins, implying that it may act as an adenin in its own right (2). While E. coli O157:H7 lacks the full-length efa-1 gene, a truncated version of efa-1 (efa-1’) is present in the chromosome (z4332 and z4333 in O island 122 of the EDL933 genome). An E. coli O157:H7 mutant carrying a transposon insertion upstream of efa-1’ (G2B12) showed reduced adherence to human colon carcinoma cells (62; I. Tatsuno, personal communication), indicating that the truncated Efa1 protein may have some of the properties of full-length Efa1. Recent surveys of the prevalence of O island 122 revealed that it is frequently associated with EHEC isolates from serious and/or epidemic disease, and the authors speculated that the truncated efa-1 gene may contribute to virulence (24, 38).

In addition to the product of the truncated efa-1 gene, E. coli O157:H7 also encodes a large predicted protein (ToxB/L7095) that exhibits 28% amino acid identity to Efa1 on the pO157 large plasmid (4, 31). E. coli O157:H7 strains containing derivatives of pO157 that lack toxB exhibit reduced adherence to cultured epithelial cells, possibly as a result of reduced expression and secretion of LEE-encoded type III secreted proteins (63). The toxB gene has also been suggested to be a functional homolog of EPEC lymphostatin, since an E. coli O157:H7 strain with pO157 deleted lacked the ability to inhibit interleukin-2 and interleukin-4 synthesis in mitogen-activated human peripheral blood lymphocytes (25). However, pO157 encodes other secreted cytotoxins, including enterohemolysin (EhxA), a serine protease (EspP), and a metalloprotease (SteC), and it is unclear if ToxB alone mediates the inhibitory effect.

In order to clarify the role of the E. coli O157:H7 toxB and truncated efa-1 genes, we investigated the effect of single and combined defined mutations on the transcription and translation of LEE genes and intestinal colonization in calves and sheep.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. E. coli O157:H7 strain 85-170 is a spontaneous stxl- and stx2-negative derivative of strain 84-289 (65). 85-170 Nafl is a spontaneous nalidixic acid-resistant derivative of 85-170 that was selected by plating ca. 10^6 CFU of 85-170 on Luria-Bertani (LB) agar containing 25 μg of nalidixic acid per ml, followed by overnight incubation at 37°C. 85-170 Nafl exhibits normal growth and adhesion characteristics in vitro. Plasmids pCVD442 (11) and pDM4 (37) are positive-selection suicide vectors containing a pr dependent R6K origin of replication, the mob region from plasmid RP4, and the Bacillus subtilis sacB genes and confers resistance to ampicillin and chloramphenicol, respectively. pCVD442, pDM4, and derivatives of these plasmids were maintained in E. coli DH5α apr (49) and in E. coli S17-1pir for conjugation (56). Plasmids pAR71 and pAR74 have been described previously (52) and contain fusions of the LEE1 and LEE4 promoters, respectively, to a promoterless gene encoding an extended-half-life green fluorescent protein (eGFP). Plasmid pAR70 (52) has single BamHI and KpnI sites 5′ of a promoterless copy of the gene encoding eGFP and was used to create an LEE5 reporter construct. Bacteria were isolated on LB agar and were cultivated in LB broth with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and nalidixic acid, 25 μg/ml. For oral inoculation studies bacteria were amplified in brain heart infusion broth for 18 h at 37°C, and the absorbance at 600 nm was adjusted to within 0.05 U.

Cell lines. HeLa cells (ATCC CCL2) were cultivated in RPMI 1640 buffered with 2 g of sodium bicarbonate per liter and supplemented with 10% (vol/vol) fetal calf serum (PAA Laboratories GmbH, Haidmannweg, Austria) and 0.3 g of l-glutamine per liter. For adhesion assays and fluorescent actin staining, cells were seeded at a concentration of 2 x 10^5 cells/35-mm dish on glass coverslips and grown for 18 h at 37°C in a humidified 5% CO2 atmosphere.

Routine DNA manipulation. Standard procedures were used for DNA extraction, plasmid isolation, cloning, PCR, and verification of mutations by Southern hybridization (54).

Construction of defined E. coli O157:H7 mutants. A deletion of the entire toxB gene was created by overlapping PCR followed by allelic exchange with the positive-selection suicide vector pCVD442. Sequences flanking the E. coli O157:H7 toxB gene were separately amplified by PCR with Vent proofreading DNA polymerase (New England Biolabs, Beverly, Mass.) by using the primer pairs tox1 (5′ ATATATGTGCACCCGCACAATATTGCCG 3′)-tox2 (5′ GGA AATCAGCGCTACATATCTATACCTC 3′) and tox3 (5′ GGTAATTACGCTCACTACATATCTATAACCCTC 3′) and tox3 (5′ GGTTATAGATA TGTCGAGCTGAATTTCTCG 3′)-tox4 (5′ ATATATGTGCACCCGCACAATATTGCCG 3′) (based on the pO157 sequence; accession number AF074613). The primary PCR products were gel purified and combined in an overlapping PCR (20) by using the flanking primers tox1 and tox4. The secondary PCR product was then cloned into pCVD442 via Sall sites incorporated into the primers. The resulting plasmid, pCVDAm01, was introduced into 85-170 Nafl by conjugation from E. coli S17-1pir, and merodiploids were isolated on LB agar containing ampicillin and nalidixic acid. Double recombinants were selected by growing merodiploids to the late logarithmetic phase in LB medium lacking ampicillin and plating onto LB agar (without NaCl) containing 6% (wt/vol) sucrose at 30°C. Sucoce-resistant colonies were screened for deletions by colony PCR, and recombinants were verified by Southern hybridization. The deletion resulted in juxtaposition of the predicted start and stop codons of toxB.

To create a deletion of the E. coli O157:H7 truncated efa-1 gene, sequences flanking the gene were separately amplified by PCR with Vent DNA polymerase by using the primer pairs efa10 (5′ ATATATGTGCACCCGCACAATATTGCCG 3′)-efa11 (5′ GGGAATATGTCGACCCGCACAATATTGCCG 3′) and efa12 (5′ GGGAATATGTCGACCCGCACAATATTGCCG 3′)-efa13 (5′ ATATAGGCTTCTTATGTTCCGGTAAAGC 3′) (based on the E. coli
contiguous 3
Efa1
deletion by colony PCR and verification containing chloramphenicol and nalidixic acid. Double recombinants were selected essentially as described above, and they were screened for the efa-1 deletion by colony PCR and verified by Southern hybridization. The deletion removed the open reading frame encoding the N-terminal 433 amino acids of Efa1 and 911 bp starting at the start codon and N-terminal 97-170 Nalr amino acids of a contiguous 3′ open reading frame encoding a predicted 275-amino-acid protein that was 100% identical to amino acids 435 to 710 of Efa1.

A 3′ deletion mutant of strain 85-170 Nalr was used as a control in calf infection studies and was constructed as follows. Sequences flanking the 3′ gene were separately amplified by using the primer pairs tir3 (5′-ATATATGAGCTC TAGCATATCGAGGG-3′) and tir4 (5′-GAAACAGTGGATCAGGATATCAAT AGGCC-3′)-tir3 (5′-ATATATGAGCTCGGATAACCTTGTCAGG-3′). The primary PCR products were gel purified and combined in an overlapping PCR by using the flanking primers tir3 and tir4. The secondary PCR product was then cloned into pDM4 via SacI sites incorporated into the primers, and the resulting plasmid, pDM4tkr, was introduced into 85-170 Nalr by conjugation from E. coli S17-1pir, and merodiploids were isolated on LB agar containing chloramphenicol and nalidixic acid. Double recombinants were selected essentially as described above, and they were screened for the efa-1 deletion by colony PCR and verified by Southern hybridization. The deletion removed the open reading frame encoding the N-terminal 433 amino acids of Efa1 and 911 bp starting at the start codon and N-terminal 97-170 Nalr amino acids of a contiguous 3′ open reading frame encoding a predicted 275-amino-acid protein that was 100% identical to amino acids 435 to 710 of Efa1.

Adhesion and fluorescent actin staining assays. Adhesion of EHEC strains to HeLa cells was quantified by visual observation essentially as described previously (16, 63). Semiconfluent HeLa cells grown on 22-mm glass coverslips were overlaid with serum-free RPMI 1640 buffered with 2 g of sodium bicarbonate per liter and were incubated at a multiplicity of infection of approximately 50:1 with fresh stationary-phase LB medium cultures of 85-170 Nalr wild-type and mutant strains adjusted to the same optical density. Cells were incubated for 3 h at 37°C in a humidified 5% CO2 atmosphere, washed three times with 2 ml of phosphate-buffered saline (PBS), and then incubated in fresh medium for an additional 3 h. Cells were washed five times with 2 ml of PBS, fixed, and stained with Hemacolor rapid staining solutions (Merck, Darmstadt, Germany), and multiple images were captured at a magnification of ×400 by using a Leica DMLS microscope with a Polaroid digital microscope camera. For each independent assay 20 randomly selected fields containing 50 or more cells were examined, and the mean number of microcolonies (MC) (comprising eight or more bacteria) per field was determined. Assays were performed three times, and the microscopic observations were made by a researcher unfamiliar with the identities of the strains tested. The results are given below as the mean number of MC per cell (± standard deviation) from three independent experiments.

Fluorescent actin staining for the detection of filamentous actin under sites of bacterial adhesion to HeLa cells was performed as described previously (28), except that bacteria were incubated on the cells for a total of 8 h.

Detection of EspD by Western blotting. To measure expression and secretion of the LEE-encoded type III secreted protein EspD, bacteria were grown to the stationary phase in double-distilled water (lower limit of detection, 100 CFU/g). The primary antibody used in this study. The calves were housed in a high-containment accommodation in tanks on tenderfoot mats and were fed milk replacer twice daily, and they had free access to water. Prior to infection the calves were confirmed to be culture negative for EHEC and Salmonella by direct plating of rectal swabs on sorbitol MacConkey agar containing 2.5 µg of potassium tellurite (T-SMAC) per ml and brilliant green agar (Oxoid, Basingstoke, United Kingdom), respectively. While it is acknowledged that T-SMAC may not detect all EHEC, this medium is highly selective for serogroups O157 and O26, which are by far the most prevalent serogroups in cattle in the United Kingdom at present. Presumptive EHEC colonies were screened for stx1 and stx2 genes by PCR using the primers pairs stx1F (5′-ATATATCCTCTTGTTTGATCT-3′) and stx2F (5′-GAACGCCC ACTGAGCATC-3′) and stx1R (5′-GGGACTGCTGAACACTGCCC-3′) and stx2R (5′-TGCAGATCTGCAAGTCGAT-3′). Animals excreting stx-positive E. coli were excluded from the study. All calves were obtained from the same farm and received colostrum from their dams for the first 24 to 48 h. After this, no further colostrum was given. Total serum immunoglobulin levels were measured when the calves were 1 or 2 days old as a measure of colostrum intake by the zinc sulfate turbidity assay. Only calves with a zinc sulfate turbidity value of more than 10 were used.

Calves were orally challenged with ca. 1 × 1010 CFU of EHEC in 20 ml of antacid (5% [wt/vol] MgO, 5% [wt/vol] Mg trisilicate, and 5% [wt/vol] NaHCO3 in double-distilled water) just before the morning feeding. Mutants 85-170 Nalr ΔσToxB, 85-170 Nalr ΔσToxF, and 85-170 Nalr ΔσToxB ΔσToxF were each administered to two calves. The parent strain, 85-170 Nalr, was administered to a total of five calves. An 85-170 Nalr Δair mutant was administered to four calves. During the next 7 days, the calves were observed for clinical signs, and fecal samples were taken twice daily by rectal palpation. The numbers of viable EHEC per gram of feces were determined by plating triplicate 10-fold serial dilutions onto sorbitol MacConkey agar containing 20 µg of sodium tellurite per ml and 2.5 µg of potassium tellurite per ml (lower limit of detection, 100 CFU/g).

Oral inoculation of sheep. Eighteen 6-week-old cross-bred lambs were randomly divided into two equal groups and supplied with food and water ad libitum, and they were confirmed to be free of EHEC by enrichment culturing as described previously (67). Nine lambs were dosed orally with 1 × 109 CFU of 85-170 Nalr resuspended in 10 ml of PBS, and a separate group of nine lambs was given a matching dose of 85-170 Nalr ΔσToxB ΔσToxF-1. Approximately 24 h postadministration and at regular intervals of detection, 100 CFU of bacteria were captured at a magnification of ×400 by using a Leica DMLS microscope with a Polaroid digital microscope camera. For each independent assay 20 randomly selected fields containing 50 or more cells were examined, and the mean number of MC per cell (± standard deviation) from three independent experiments.
acid per ml. One lamb from each group was sacrificed on days 3, 8 and 18 postinfection for bacteriological and histological analyses of tissues.

All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the local ethical review committee. The fecal shedding data were statistically analyzed for the effect of mutation by using an F test, with the data taken as repeated measurements (Proc Mixed Statistical Analysis System, 1995; SAS Institute, Cary, N.C.). P values of <0.05 were considered significant.

RESULTS

Mutation of the E. coli O157: H7 toxB and truncated efa-1 genes influences bacterial adherence to cultured HeLa cells but not filamentous actin nucleation. Quantitative adherence assays were performed by using HeLa cells, the 85-170 Nal' ΔtoxB and Δefa-1' single and double mutants, and the parent strain. Mutation of the E. coli O157: H7 toxB and efa-1' genes did not alter the in vitro growth rates of the mutants compared to those of the parent strain (data not shown). The numbers of MC comprising eight or more bacteria per HeLa cell in three independent experiments were as follows: for 85-170 Nal', 0.0784 ± 0.0162 MC/cell; for 85-170 Nal' ΔtoxB, 0.0192 ± 0.0228 MC/cell; for 85-170 Nal' Δefa-1', 0.0216 ± 0.0250 MC/cell; and for 85-170 Nal' ΔtoxB Δefa-1', 0.0066 ± 0.0078 MC/cell (means ± standard deviations). Thus, mutation of toxB or efa-1' alone caused a ca. fourfold reduction in MC formation (for toxB, P = 0.006; for efa-1', P = 0.007). These phenotypes are comparable to those reported by Tatsuno et al. (62, 63). A double mutant lacking both toxB and efa-1' exhibited a ca. 12-fold reduction in MC formation compared to the wild-type strain (P = 0.002). Despite forming fewer MC, the mutant strains still nucleated filamentous actin under the sites of attachment to HeLa cells (Fig. 1), indicating that LEE-mediated rearrangement of cytoskeletal actin could still occur.

We are aware that unintended secondary mutations can result from allelic exchange when positive-selection suicide vectors are used (23), and therefore we attempted to transcomplement the defects in MC formation by cloning and expression of the toxB and efa-1' genes. Several unsuccessful attempts were made to subclone toxB on a ca. 10-kb AatII fragment from pO157 into the low-copy-number vector pACYC184. We obtained the mini-pO157 derivative containing toxB described by Tatsuno et al. (63) (pIC37, kindly supplied by C. Sasakawa, University of Tokyo). However, this plasmid could not be stably maintained in 85-170 Nal' ΔtoxB. When kanamycin-resistant bacteria containing pIC37 were obtained, they exhibited an extremely low growth rate in vitro (data not shown), which prohibited the development of MC in the adherence assay.

Badea et al. recently reported that they were unable to clone the N-terminal 376 amino acids of REPEC efa-1 (2) presumable because of toxicity. We were successful in cloning the truncated efa-1' gene from E. coli O157:H7 (z4332) together with the contiguous open reading frame z4333 in pET30-Ek/Lic (Novagen, Madison, Wis.). However, induction of expression of efa-1' caused a dramatic decrease in the growth rate, making quantification of MC formation unreliable (data not shown).

Mutation of the E. coli O157:H7 toxB and truncated efa-1 genes reduces expression and secretion of LEE4-encoded proteins. Expression and secretion of the LEE4-encoded type III secreted protein EspD by the E. coli O157:H7 wild-type strain and ΔtoxB, Δefa-1', and ΔtoxB Δefa-1' mutant strains were analyzed by Western blotting by using specific antiserum (Fig. 2). The densities of secreted EspD based on three independent experiments were as follows: for 85-170 Nal', 1,664 ± 108; for 85-170 Nal' ΔtoxB, 792 ± 123; for 85-170 Nal' Δefa-1', 1,638 ± 196; and for 85-170 Nal' ΔtoxB Δefa-1', 201 ± 87 (means ± standard deviations). Thus, mutation of toxB caused a signifi-

![FIG. 1. Nucleation of filamentous actin under sites of adherence of the 85-170 Nal' wild-type strain and ΔtoxB and Δefa-1' single and double mutant strains to cultured HeLa cells. Cells were examined by fluorescent actin staining by using Oregon Green 514-phalloidin (green) and a confocal laser scanning microscope. Bacteria were detected with rabbit anti-O157 lipopolysaccharide typing serum and anti-rabbit Ig-Alexa 568 (red). (A) 85-170 Nal'; (B) 85-170 Nal' ΔtoxB; (C) 85-170 Nal' Δefa-1'; (D) 85-170 Nal' ΔtoxB Δefa-1'. Representative images are shown. Magnification, ×630.](Image 355x151 to 505x225)

![FIG. 2. Effects of toxB and efa-1' mutations on expression and secretion of the LEE4-encoded proteins. Whole-cell and secreted protein fractions of 85-170 Nal', 85-170 Nal' ΔtoxB, 85-170 Nal' Δefa-1', and 85-170 Nal' ΔtoxB Δefa-1' (ca. 25 μg of protein) were resolved by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed by using a mouse monoclonal antibody specific for EspD. Equivalent loading of proteins was confirmed by Coomassie blue staining of gels (data not shown). WT, wild type.](Image 57x604 to 546x723)
An apparent reduction in the secretion of EspD (the value was 2.1-fold lower than the wild-type value; \( P = 0.001 \)). While mutation of \( \text{efa-1'} \) alone had little effect (\( P = 0.82 \)), a cumulative reduction in EspD expression occurred when both \( \text{toxB} \) and \( \text{efa-1'} \) were deleted (the value was ca. 8.2-fold lower than the wild-type and single \( \text{efa-1'} \) mutant values; \( P < 0.001 \)). Similar reductions in the expression of EspD were detected following analysis of whole-cell protein from the wild-type and mutant strains (Fig. 2).

We also analyzed the expression of EspA filaments by the wild type and the \( \Delta \text{toxB \Delta efa-1'} \) mutant strain by indirect immunofluorescence. It was observed previously that EspA filaments are not produced during growth in M9 minimal medium but are induced following subculture in MEM-HEPES (52). Upon induction, not all bacteria in the population express EspA filaments at a given time, and the highest proportion of bacteria with EspA filaments is detected in the late exponential phase (52). We determined the proportion of wild-type and mutant bacteria elaborating EspA filaments at \( A_{600} \) values of 0.4, 0.8, and 1.0. Mutation of \( \text{toxB} \) and \( \text{efa-1'} \) singly or in combination resulted in a reduction in the proportion of bacteria elaborating EspA filaments (Fig. 3); however, the difference was significant only for samples collected at an \( A_{600} \) of 0.4 (for 85-170 Na\(^+\) \( \Delta \text{toxB} \), \( P = 0.004 \); for 85-170 Na\(^+\) \( \Delta \text{efa-1'} \), \( P = 0.013 \); for 85-170 Na\(^+\) \( \Delta \text{toxB \Delta efa-1'} \), \( P = 0.008 \)). During the late logarithmic phase (\( A_{600} \) 0.8 and 1.0), the extent of EspA filamentation observed with the mutant strains was consistent with the levels of EspD detected by Western blotting (Fig. 2). Since the production of EspD filaments by EHEC is dependent on EspD (29), it is not possible to state whether the observed reduction in EspA filament production in the mutant strains was the result of lower EspD production (Fig. 2), lower EspA production, or a combination of the two.

Since production of LEE4-encoded proteins is growth phase dependent (52, 53), it was not possible to test-complement the defects in EspD or EspA production owing to the inhibitory effect of plasmids containing \( \text{toxB} \) or \( \text{efa-1'} \) on bacterial growth (data not shown).

**FIG. 3. Effect of mutation of \( \text{toxB} \) and \( \text{efa-1'} \) on the formation of EspA filaments.** EspA filaments were visualized by indirect immunofluorescence following subculture of bacteria grown in M9 minimal medium into MEM-HEPES. Samples were prepared at \( A_{600} \) values of 0.4, 0.8, and 1.0. The proportions of bacteria expressing EspA filaments were determined as described in Materials and Methods, and the data are the means \( \pm \) standard deviations for three independent experiments. WT, wild type.

**DISCUSSION**

It has been reported previously that mutation of the \( \text{efa-1} \) gene of EHEC serotypes O5 and O111 results in a significant reduction in fecal shedding of the organisms (\( P < 0.05 \) at 7 days postinoculation) (52). *E. coli* O157:H7 strains lack the full-length \( \text{efa-1} \) gene; however, a truncated version of \( \text{efa-1} \) is
present in the chromosome (19, 47), and a homolog of efa-1 (toxB/l7095) is present in the large plasmid (4, 31). To clarify the role of these genes, we constructed single and double ΔtoxB and Δefa-1’ mutants of E. coli O157:H7 and assessed the phenotypes of the mutants in vitro and in calves and sheep.

Consistent with the observations of Tatsuno et al. (63), we found that deletion of the E. coli O157:H7 toxB gene led to a reduction in the expression and secretion of LEE4-encoded type III secreted proteins and impaired MC formation on cultured epithelial cells. Furthermore, we found for the first time that a defined mutation of the truncated version of efa-1 alone caused a reduction in MC formation on HeLa cells. This is consistent with the finding that a transposon insertion upstream of efa-1 reduced adherence to Caco-2 cells to 25 to 50% of the wild-type adherence level (62; I. Tatsuno, personal communication). The efa-1’ single mutant expressed and secreted wild-type levels of EspD, and the production of EspA filaments was not reduced significantly during the phase of growth when such filaments are expressed maximally. This is in accordance with the finding that the G2-B12 efa-1’ mutant

FIG. 4. Effect of toxB and efa-1’ mutations on the activity of LEE1, LEE4, and LEE5 promoters. Plasmids pAJR71 (lee1::gfp), pAJR74 (lee4::gfp), and pAJR75 (lee5::gfp) were separately transformed into 85-170 Nal’, 85-170 Nal’ ΔtoxB, 85-170 Nal’ Δefa-1’, and 85-170 Nal’ ΔtoxB Δefa-1’ mutants, and the bacteria were grown in MEM-HEPES. At stages in the growth cycle the absorbance of the cultures was recorded, and the fluorescence due to accumulation of eGFP was quantified. The data are the means for two independent measurements. wt, wild type.

FIG. 5. Mutation of toxB and efa-1’ does not significantly affect the course of fecal shedding of E. coli O157:H7 in calves. The data are fecal shedding data for duplicate calves inoculated with the 85-170 Nal’ ΔtoxB, 85-170 Nal’ Δefa-1’, and 85-170 Nal’ ΔtoxB Δefa-1’ mutants. Data from five calves infected with 85-170 Nal’ and four calves infected with 85-170 Nal’ ΔtoxB are shown for comparison. The bacterial recovery from the feces was measured twice each day. The data are daily means ± standard errors of the means. wt, wild type.

FIG. 6. Mutation of both toxB and efa-1’ does not significantly affect the course of fecal shedding of E. coli O157:H7 in 6-week-old sheep. Animals were inoculated with 85-170 Nal’ or 85-170 Nal’ ΔtoxB Δefa-1’ (nine animals per group), and fecal samples were collected at intervals up to 24 days. One lamb from each group was sacrificed on days 3, 8, and 18 postinfection, and postmortem examinations were performed. The data are means ± standard deviations for different times.
identified by Tatsuno et al. produced wild-type levels of EspA as assessed by immunoblotting (62). In contrast, deletion of efa-1' in combination with toxB led to marked reductions in the expression and secretion of EspD and EspA filament production and a further reduction in the ability of the bacteria to form MC on cultured cells, implying that there is a synergistic effect. In all cases the reduction in EspD and EspA expression and secretion was not sufficient to prevent the nucleation of filamentous actin under the sites of E. coli O157:H7 attachment to cultured cells (62, 63; this study). Some bacteria harboring the ΔtoxB Δefa-1' double mutation still assembled EspA filaments on their surfaces, as detected by indirect immunofluorescence. Since actin condensation under adherent bacteria is dependent on the delivery of LEE-encoded type III secreted effectors, our data indicate that sufficient quantities of LEE4-encoded proteins are still produced and translocated into epithelial cells so that the bacteria can elicit cytoskeletal rearrangements.

We attempted to trans-complement the defects in MC formation and the expression and secretion of EspD and EspA in the mutant strains by cloning and expressing the toxB and efa-1' genes. Several unsuccessful attempts were made to subclone toxB from pO157, and it is likely that this gene is unstable and/or toxic, as has been reported for the homologous 9.5-kb efa-1 gene (22, 25, 42). A mini-pO157 derivative containing toxB described by Tatsuno et al. (63) could not be stably maintained in 85-170 NaCl ΔtoxB, most likely as a result of incompatibility with the endogenous pO157ΔtoxB plasmid. We were able to clone the truncated efa-1 gene from E. coli O157:H7; however, induction of gene expression restricted the growth rate, probably as a consequence of toxicity, as reported elsewhere (2).

Mutation of toxB caused a defect in the expression and secretion of LEE-encoded type III secreted effectors whose magnitude was similar to that seen in EHEC O5 and O111 efa-1 mutants (59). Type III secretion of EspD was impaired by ca. eightfold in an E. coli O157:H7 mutant harboring deletions of both toxB and efa-1'. Despite this, no significant reduction in fecal shedding of E. coli O157:H7 was seen in calves or sheep infected with the mutant strains. Our data are consistent with the observation that an E. coli O157:H7 strain with the pO157 plasmid which encodes toxB deleted colonizes gnotobiotic piglets and induces enteritis at least as well as the wild type (65). We cannot preclude the possibility that there were differences in the immune status of the animals used in the present study or the possibility that various levels of passively acquired EHEC-specific maternal antibody masked subtle effects of the mutations.

LEE-encoded type III secreted proteins are major virulence factors for several AE pathogens in their target animal species, including EPEC EspB in humans (61), REPEC EspA, EspB, and Tir in rabbits (1, 33), and Citrobacter rodentium EspB in mice (41). Data supporting a role for LEE-encoded type III secreted proteins in the colonization of ruminants by E. coli O157:H7 were recently provided by the finding that immunization of calves with E. coli O157:H7 type III secreted proteins reduces fecal shedding of the bacteria upon subsequent challenge (50). Considerable natural variation in the level of secretion of the LEE-encoded Tir and EspD proteins has been observed among EHEC O157:H7 strains isolated from cattle (35); however, it is not known if this correlates with the ability of the strains to colonize the intestines efficiently. While the E. coli O157:H7 ΔtoxB Δefa-1' double mutant was impaired in the ability to express and secrete LEE4-encoded proteins, the amount of EspD secreted by this mutant was comparable to the amounts secreted by naturally occurring E. coli O157:H7 strains isolated from cattle (35; Roe and Gally, unpublished observations).

When the ability of E. coli O157:H7 to form AE lesions was abolished through deletion of the tir gene, we observed a significant reduction in fecal shedding of the bacteria following oral inoculation of calves. This indicates that differences in the ability of EHEC strains to colonize the bovine intestine can be detected with our oral challenge model and that the model does not merely measure shedding of the inoculum. Furthermore, it shows for the first time that the translocated intimin receptor is required for intestinal colonization in calves. This implies that the role of intimin in intestinal colonization of calves and sheep may be explained, at least in part, by its ability to bind to its translocated receptor instead of secondary receptors, such as β1-integrins (14) and/or cell surface-localized nucleolin (57). There is strong evidence for the existence of host cell intimin receptors, and the role of such molecules in the carriage and tissue tropism of EHEC merits close investigation.

It is unclear why mutation of efa-1' in EHEC O5 and O111 impairs intestinal colonization in calves whereas mutation of toxB in EHEC O157:H7 does not, even though type III secretion is similarly affected in the two backgrounds. It is possible that different EHEC serotypes rely to different extents on the LEE-encoded type III secretion system for intestinal colonization. There is increasing evidence that different EHEC serovars colonize the intestines of calves by distinct mechanisms. We and other workers have shown that EHEC serotypes O5, O26, and O111 adhere extensively to the intestinal epithelium in the bovine cecum, colon, and rectum and can readily be seen forming AE lesions (46, 59). In contrast, E. coli O157:H7 is rarely detected in association with the intestinal epithelium at these sites following oral inoculation of calves even though high numbers of organisms may be shed (6, 8, 68). E. coli O157:H7 may exhibit a specific tropism for lymphoid follicle-dense mucosa at the terminal rectum in weaned calves and adult cattle (40); however, preliminary data from our laboratories have indicated that non-O157 EHEC serotypes do not share a tropism for this site (S. W. Naylor, A. J. Roe, and D. L. Gally, unpublished observations). It is not known if E. coli O157:H7 exhibits a specific tropism for the terminal rectum in animals that are the ages we used. Indeed, the preweaned calves and 6-week-old lambs inoculated in this study had not started to ruminate, and it is not clear if lymphoid follicle-dense epithelium has even matured in the terminal rectum at this age. The role of specific E. coli O157:H7 genes in colonization of the terminal rectum and other sites in preweaned, weaned, and adult ruminants awaits detailed investigation.

It is possible that Efa1 influences intestinal colonization of calves by EHEC O5 and O111 by acting independently of the LEE. Given that Efa1 is 97.4% identical to EPEC lymphostatin, this molecule may facilitate colonization by suppressing the activation of gastrointestinal lymphocytes. However, in a recent study it was not possible to detect lymphostatin-like effects
on bovine intraepithelial lymphocytes exposed to EHEC serotype O103:H2 in situ in a ligated ileal loop model of infection (36). It is also possible that Efa1 acts as an adhesin per se or influences adhesion indirectly by some other mechanism. Indeed, Badea et al. reported that Efa1 mediates adherence of REPEC to cultured epithelial cells, apparently without affecting the expression and secretion of LEE-encoded proteins (2). Furthermore, Efa1-specific antibody inhibited the adherence of REPEC to cultured cells (2). It may be that ToxB and the truncated version of Efa1 do not influence intestinal colonization by E. coli O157:H7 because they do not share the activities of Efa1.

Our data and those of Tatsuno et al. (63) suggest that Efa1 and ToxB influence the expression of LEE-encoded type III secreted proteins at a posttranscriptional level. Almost all LEE-positive strains tested to date contain either Efa1 or ToxB (25, 42, 60), and indeed in EHEC O26 strain 413/89-1 and REPEC strains RDEC-1 and 83/89, the LEE and efa-1 are adjacent on the chromosome (64, 69) (EHEC O26 LEE accession number AJ277443). This close genetic linkage occurs in many other EPEC and EHEC strains (38) and may imply that Efa1 is required for complete activity of the LEE-encoded type III secretion system. Furthermore, O island 122, which contains truncated efa-1’, is also significantly associated with LEE-positive EHEC strains that cause serious and/or epidemic disease (24, 38). No enzymatic activities have yet been ascribed to the EHEC Efa1, Efa1’, and ToxB proteins. Efa1 and ToxB are highly homologous to large clostridial toxins at the N terminus and share a DXD glycosyltransferase motif (4, 25, 31, 42), and both contain a cysteine protease motif that is present in numerous other secreted bacterial virulence factors (55). The 433-amino-acid truncated Efa1 protein lacks both these motifs, and their relevance to the activities of Efa1 and ToxB remains to be tested.

The mechanism by which Efa1 and ToxB influence the translation of LEE4 transcripts is unclear. For some type III secretion systems in gram-negative bacteria, it has been suggested that the secretion signal for translocated effector proteins is present in the mRNA and that translation and secretion of the proteins may be coupled at the needle complex (48). If such a situation applied to the EHEC LEE-encoded type III secretion apparatus, then it is conceivable that toxB and efa-1’ mutations may influence expression and secretion of LEE4-encoded proteins by interfering with the proper insertion and assembly of the type III secretion apparatus in the membrane and/or the targeting of LEE transcripts.

Interestingly, LifA does not appear to be required for efficient production and secretion of LEE-encoded effector proteins in human or REPEC strains (2, 59). It is noteworthy that the cloned EPEC LEE can confer AE activity upon E. coli K-12 (34), whereas the cloned LEE from E. coli O157:H7 cannot (12), suggesting that there are differences in the control of type III secretion between the strains. The involvement of Efa1, Efa1’, and ToxB in this process is the subject of ongoing studies in our laboratory.

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