Two Enteropathogenic Escherichia coli Type III Secreted Proteins, EspA and EspB, Are Virulence Factors

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Summary

Enteropathogenic Escherichia coli (EPEC) belongs to a family of related bacterial pathogens, including enterohemorrhagic Escherichia coli (EHEC) O157:H7 and other human and animal diarrheagenic pathogens that form attaching and effacing (A/E) lesions on host epithelial surfaces. Bacterial secreted Esp proteins and a type III secretion system are conserved among these pathogens and trigger host cell signal transduction pathways and cytoskeletal rearrangements, and mediate intimate bacterial adherence to epithelial cell surfaces in vitro. However, their role in pathogenesis is still unclear. To investigate the role of Esp proteins in disease, mutations in espA and espB were constructed in rabbit EPEC serotype O103 and infection characteristics were compared to that of the wild-type strain using histology, scanning and transmission electron microscopy, and confocal laser scanning microscopy in a weaned rabbit infection model. The virulence of EspA and EspB mutant strains was severely attenuated. Additionally, neither mutant strain formed A/E lesions, nor did either one cause cytoskeletal actin rearrangements beneath the attached bacteria in the rabbit intestine. Collectively, this study shows for the first time that the type III secreted proteins EspA and EspB are needed to form A/E lesions in vivo and are indeed virulence factors. It also confirms the role of A/E lesions in disease processes.

Key words: enteropathogenic Escherichia coli • attaching and effacing lesion • Esp proteins • type III secretion system • cytoskeletal rearrangement

A family of enteropathogenic Escherichia coli (EPEC) causes diarrhea in humans and animals (1), yet the mechanism by which they cause disease in vivo has not been defined. These pathogens cause a characteristic histopathological lesion termed an attaching/effacing (A/E) lesion (2), although the contribution of this lesion to disease has not been defined. Enterohemorrhagic Escherichia coli (EHEC) O157:H7 also causes this lesion (3–5). Several genes are involved in formation of the A/E lesion in tissue culture cells, including eae (which encodes intimin, an adhesin) and espB (which encodes a secreted protein), and these genes are conserved in EPEC, EHEC, and rabbit EPEC strains (6, 7).

The A/E lesion is defined by the intimate attachment between bacteria and the epithelial surface, and the effacement (disappearance) of host cell microvilli. Alteration of cytoskeletal components beneath adherent bacteria results in formation of a pedestal-like structure that can extend to a pseudopod. In vitro (tissue culture), the A/E lesion is mediated by bacteria-host cell interactions, including triggering of host signal transduction pathways (8–10). Initial localized adherence to epithelial cells is mediated by a plasmid-encoding, bundle-forming pilus (BFP) (11–15), followed by the insertion of translocated intimin receptor (Tir), a bacterial protein, into the host plasma membrane (10). Tir (10), formerly Hp90 (16), is delivered to tissue culture cells, and requires the secreted proteins EspA and EspB for its translocation. Tir has at least three possible functions that have been identified (10). Tir is translocated into the epithelial cell membrane and serves as a cell surface...

Abbreviations used in this paper: A/E, attaching and effacing; CLSM, confocal laser scanning microscopy; CPE, cytopathic effect; EHEC, enterohemorrhagic Escherichia coli; EPEC, enteropathogenic Escherichia coli; LB, Luria-Bertani; LEE, locus of enterocyte effacement; REPEC, rabbit EPEC; SEM, scanning electron microscopy; STX, Shiga toxin; TEM, transmission electron microscopy; Tir, translocated intimin receptor; TxA, Texas red.

A. Abe and U. Heczko contributed equally to this paper.
receptor for intimin. The second function of TIR is to nucleate actin after intimin binding. The third function is to transmit additional signals to host cells once TIR–intimin interaction occurs. These events trigger tyrosine phosphorylation of phospholipase Cγ (17) and other host proteins, and the resulting signals follow TIR phosphorylation and other early signaling events.

EPEC A/E lesions require products of a 35-kb locus termed the LEE (locus of enterocyte effacement; 18) that includes the esc cluster, tir, eae, espA, espD, and espB (formerly eaeB). The esc cluster encodes a type III secretion system responsible for secretion of Esp proteins (19), tir and eae encode Tir (10) and intimin (20), which are required for intimate attachment in vitro. At least three secreted proteins, EspA (21), EspB (22) and EspD (23), encoded by espA, espB, and espD, are secreted via the type III secretion pathway. These secreted proteins are essential for triggering of host signal transduction pathways in tissue culture cells. However, it is not known if these processes, and thus these secreted proteins, are needed for virulence in vivo.

To investigate the role of EspA and EspB in EPEC pathogenesis, we constructed mutant strains in E. coli serotype O103. In Europe, serotype O103 is the most common rabbit EPEC (R EPEC) strain, causing heavy diarrhea in weaned rabbits, and its rhamnose fermentation negative phenotype is linked to high virulence (24–26). E. coli O103 also causes histopathological features similar to that caused by EPEC in the intestine, including A/E lesions on the intestinal mucosa (27, 28). Recently, the E. coli O103 LEE region has been studied (29). However, the role of the type III secreted proteins EspA and EspB in virulence is still unclear, as is the role of the A/E lesion in disease for this family of pathogens. Here we report characterization of disease in rabbits infected with strains that are defective for EspA and EspB using a rabbit infection model, and show that these two secreted proteins and the resulting A/E lesions formed are essential for pathogenesis.

Materials and Methods

Bacterial Strains, Plasmids, Cell Culture and Media. E. coli serotype O103 85/150 strain causes heavy diarrhea in weaned rabbits and was provided by Dr. Johan E. Peeters (National Institute of Veterinary Research, Brussels, Belgium). The phenotypes and genotypes of this strain and other bacteria used in this study are listed in Table 1. Plasmids pAA23Δ and pAAxBΔ (7) are derivatives of a positive suicide vector pCVD442 that contains sacB, bla, and a pir-dependent R6K replicon (30). HeLa cells were maintained and assayed in MEM containing 10% (vol/vol) fetal calf sera at 37°C in a 5% CO2 incubator. For infection, R EPEC and mutant strains were grown in Luria-Bertani (LB) broth overnight at 37°C without shaking.

Construction of Nonpolar Stop Codon Mutations in R EPEC O103 espA and espB. Plasmid pAA23Δ and pAAxBΔ are positive suicide vectors, and were designed for construction of nonpolar mutations in espA and espB, respectively (7). Plasmid pAA23Δ contains RDEC-1 espA including a stop codon and a BglII site 235 bp downstream from the espA start codon. This plasmid was introduced into E. coli SM10pir (31) and was transconjugated into R EPEC O103 as described elsewhere (30). Plasmid pAAxBΔ (7) contained a stop codon in addition to a 250-bp deletion, starting 154 bp downstream of the espB start codon. This plasmid was transformed into E. coli SM10pir and transconjugated into R EPEC O103. Both mutant strains were selected by resistance to sucrose and nalidixic acid and sensitivity to ampicillin.

Preparation of Secreted Proteins. Bacterial overnight cultures were diluted 1:100 into DMEM and incubated for 6.5 h in a 5% CO2 incubator. Bacteria were removed by centrifugation (18,000 g for 10 min) and the supernatant was precipitated by addition of 10% TCA, and incubated on ice for 1 h. After centrifugation, the pellets were resuspended in Laemmli sample buffer and analyzed by 12% SDS-PAGE (32).

Immunofluorescence Microscopy. HeLa cells (104) were inoculated and grown overnight on coverslips, then infected with R EPEC O103 or its mutant strains for 3 h. Cells were washed three times with PBS and fixed with 3% PFA in PBS (pH 7.2) for 30 min at room temperature, then washed twice with PBS. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS in the presence of phalloidin–Texas Red (Molecular Probes, Eugene, OR) to stain filamentous actin, and antiphospho-tyrosine antibodies, clone 4G10 (Upstate Biotechnology, Lake Placid, N. Y.). FITC-conjugated anti-mouse IgG and IgM

| Strain | Relevant characteristics | Comments | Reference |
|--------|--------------------------|----------|-----------|
| 85/150 | Rabbit EPEC              | Prototypic O103:H2 strain, rhamnose negative | 24 |
| RDEC-1 | Rabbit EPEC              | Prototypic O155:H2 strain | 43 |
| E2348/69 | Human EPEC             | Prototypic O127:H6 EPEC strain | 44 |
| R EPEC O103 | Rabbit EPEC       | Nalidixic acid resistant strain derived from 85/150 | This study |
| AAF004 | R EPEC O103 (EspA- )   | Nonpolar mutation in R EPEC O103 espA | This study |
| AAF005 | R EPEC O103 (EspB- )   | Nonpolar mutation in R EPEC O103 espB | This study |
| SM 10xpir | Thr leu tonA lac' supE | Donor strain for constructing R EPEC | 31 |
|        | resA::R P4-2-T c::M u K m | Mutant strains |           |

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and examined with a TEM (EM 10A/B; Carl Zeiss Inc., Thorn-
sections (90 nm) were stained with uranyl acetate and lead citrate
were further dehydrated in graded ethanols and propylene oxide,
gold. The resulting sections were examined with an SEM (Ste-
critical point-dried using liquid carbon dioxide and coated with
taraldehyde, then rinsed in 0.1 M cacodylate buffer. After postfix-
tion, tissues were washed in PBS and prefixed in 2.5% glu-
tate, then dehydrated in graded ethanols. SEM sections were
visualized and photographed using a light microscope
sucrose in PBS overnight at 4
bacteria inside the orogastric tubes were flushed with an addi-
tional 2 ml PBS wash.

Tissue Preparation and Histological Procedure. After 1-4 d infec-
tion, tissues from ileum, Peyer's patches, cecum, and proximal colon were excised immediately after sacrifice by intravenous injec-
tion of ketamine and overdosing of sodium pentobarbital in
accordance to the guidelines of the Canadian Council of Animal Care and the University of British Columbia. For light micro-
copy, intestinal tissues were fixed in 10% neutral buffered forma-
alin and processed for paraffin embedding. Serial 4-μm-thick sec-
tions were cut onto glass slides and stained with hematoxylin and
evsn, and examined and photographed using a light microscope
(AH-2; Olympus America Inc., Melville, NY). For scanning
electron microscopy (SEM) and transmission electron microscopy
(TEM), tissues were washed in PBS and prefixed in 2.5% gluta-
araldehyde, then dehydrated in graded ethanols. SEM sections were
further dehydrated in graded ethanols and propylene oxide,
then embedded in Spurr's resin. After polymerization, ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate and examined with a TEM (EM 10A/B; Carl Zeiss Inc., Thorn-
wood, NY).

Preparation of Thick Cryosection of Intestinal Tissues. Intestinal tis-
sues were fixed with 4% PFA in PBS (pH 7.2) for 2 h at room
temperature, washed twice with PBS, then incubated in 20% sucrose in PBS overnight at 4°C for cryoprotection. Tissue sam-
ple were embedded in OCT Tissue-Tek (Miles Laboratories Inc.,
Elkhart, IN), then frozen in cold 3-methyl-butane (−50 to
−60°C). The frozen tissues were cut at 20 μm sections on a cryo-
stat, then kept in PBS at 4°C.

Immunohistochemistry of Thick Cryosections. Sections were incu-
bated in 10% normal goat serum in PBS for 10 min, then perme-
abilized with 100 μl permeabilization buffer (0.2% saponin, 10%
normal goat serum in PBS) in the presence of mouse antisera
against R EPEC O103 (1:200) overnight at 4°C. Sections were
washed three times in PBS, then incubated in 0.2% saponin and
10% normal goat serum in PBS in the presence of phalloidin-
TXR (1:400; Molecular Probes) and FITC-conjugated anti-
mouse IgG and IgM (ICN Pharmaceuticals Inc.) as the secondary antibody for anti-R EPEC O103 antibody. Tissue samples were
washed twice in PBS, then mounted on coverslips and visualized
as described elsewhere (34). In brief, stained sections were visual-
ized by a confocal laser scanning microscope (MRC-600; Bio-
Rad Labs., Hercules, CA) under control of COMOS software
(Bio-Rad Labs.) The resulting scanned images were analyzed by
a public domain image processing and analysis program, NIH Im-
age (National Institutes of Health, Bethesda, MD), and processed
as described elsewhere (33). In brief, stained sections were visual-
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The resulting scanned images were analyzed by a public domain image processing and analysis program, NIH Image (National Institutes of Health, Bethesda, MD), and processed
as described elsewhere (33).

Results

Construction of N onpol M utations in espA and espB. To con-
struct mutations in espA and espB, a nalidixic acid resist-
tant strain of E . coli serotype O103 85/150 (R EPEC O103) was
established, then nonpolar mutations were constructed by
inserting a stop codon into these genes as described in M aterials and Methods. To confirm mutations in espA and espB, chromosomal DNA was prepared from each mutant strain and PCR
analysis was performed with two sets of primers encompassing espA or espB. The resulting PCR products were digested with BglII to confirm the presence of this engineered restriction site (data not shown).

The mutant strains containing the stop codon in espA and espB
were designated as AAF004 (EspA−) and AAF005 (EspB−), re-
spectively.

Protein Secretion Profile of R EPEC O103 and Its M utant
Strains in Tissue Cult ure M edia. We have already shown
that another rabbit EPEC strain, R DEC-1, secretes EspA
and EspB into culture media (7). The secreted protein pro-
file of R EPEC O103 was quite similar to that of R DEC-1,
and EspA and EspB in both rabbit EPEC strains migrated
comparatively faster compared to those from the human EPEC strain (Fig. 1 A). Interestingly, EspC (110 kD) in both rabbit EPEC strains was not detectable using Coomassie blue staining. How-
ever, this protein is not involved in EPEC triggering of host signal transduction pathways and intimate adherence
in vitro (35). In contrast, AAF004 (EspA−) and AAF005 (EspB−)
lacked bands corresponding to EspA and EspB. Although we constructed both mutant strains by inser-
ting a stop codon that should not affect downstream gene expression, the amounts of other secreted proteins were less compared to the wild-type O103 strain (Fig. 1 B).

It is possible that lack of an Esp protein might affect the sec-
cretion of other Esp proteins by altering transcriptional, postranslational events, and/or Esp-Esp protein interaction.
However, these effects might be common features of type III secretion in EPEC, because the reduced amount of
secreted proteins were also found in the EspB (36) and
EspD (23) mutant strains in EPEC O127. The reduced amount of other secreted proteins in EspA and EspB
mutant strains might contribute to changes in the virulence phenotypes.

Figure 1. Secreted protein profiles of R EPEC O103, R DEC-1,
EPEC (A), and R EPEC O103 mutant strains (B). Bacteria were grown in DMEM and the secreted proteins were precipitated by addition of 10% TCA, and then analyzed by 12%
SDS-PAGE stained with Coomassie blue. Strains R EPEC O103, O103;
AAF004 (EspA−), ΔespA; AAF005 (EspB−), ΔespB.
EspA and EspB Proteins Are Involved in Cytoskeletal Rearrangements in HeLa Cells.

EPEC EspA and EspB are involved in accumulation of cytoskeletal actin and tyrosine-phosphorylated proteins beneath attached bacteria in tissue culture cells (21, 37). To confirm the role of REPEC O103 EspA and EspB in triggering these events in HeLa cells, cytoskeletal actin and tyrosine-phosphorylated proteins were stained with phalloidin-TxR and FITC-labeled antiphosphotyrosine (αPY) antibodies for detection of colocalization in tissue culture cells by immunofluorescence microscopy. Strains: REPEC O103, O103; AAF004 (EspA⁻), ΔespA; AAF005 (EspB⁻), ΔespB.

Virulence of EspA⁻ and EspB⁻ Strains in Rabbits. To determine if EspA and EspB are involved in disease, rabbits were infected with REPEC O103 and the mutant strains via an orogastric route, and the virulence of these mutant strains was compared to that of the wild-type strain. Rabbits (n = 5) infected with REPEC O103 (7 × 10⁸) suffered weight loss and one rabbit died with watery diarrhea 7 d after infection. Rabbits that survived (n = 4) showed significant weight loss compared to rabbits given PBS (n = 3; Fig. 3 A). In contrast, rabbits given AAF004 (EspA⁻, n = 5) and AAF005 (EspB⁻, n = 4) did not show any diarrheagenic symptoms or weight loss (Fig. 3, B and C). These results clearly show that EspA and EspB are involved in these disease processes.

Histological Analysis of Intestinal Tissues from Rabbits Infected with REPEC O103 and Mutant Strains. After 72 h of infection, ileal sections were obtained from rabbits infected with REPEC O103, AAF004 (EspA⁻), and AAF005 (EspB⁻), and stained with hematoxylin and eosin. Only animals infected with REPEC O103 showed histological evidence of blunting of intestinal villi, necrosis of mucosal epithelial cells and sloughing, and increased inflammation consisting of a mixture of polymorphonuclear leukocytes, lymphocytes, and plasma cells (Fig. 4 A). In contrast, animals infected with AAF004 (EspA⁻) and AAF005 (EspB⁻) did not have altered villous architecture, nor increase inflammatory cell infiltrates in the lamina propria or muscularis layers (Fig. 4, B and C), much like the uninfected controls (Fig. 4 D). Furthermore, increased injury and inflammation were also observed in Peyer’s patches when rabbits were infected with REPEC O103, but not with EspA and EspB mutant strains (data not shown). These results indicate that EspA and EspB are involved in inflammation and disruption of the mucosal epithelial surface.
Adeherene of REPEC O 103 and M utant Strains to Intestinal Villi. To analyze how EspA and EspB contribute to adherence to the mucosal surface, rabbits were killed 1-4 d after infection, and intestinal sections, including ileum, Peyer’s patches, cecum, and proximal colon, were visualized by SEM. REPEC O 103 was detected in all intestinal tissues (summarized in Table 2). In the ileum, bacteria were detected 24 h after infection, and showed diffuse adherence 48-72 h after infection (Fig. 5A). The bacteria were sometimes aggregated and clumped on top of each other, and embedded into the ileal surface (Fig. 5B). At higher magnifications, cup-like structures were also observed on the epithelial surface, presumably caused by detached bacteria (Fig. 5C). In Peyer’s patches, REPEC O 103 appeared to adhere preferentially to the domed villi (follicle associated epithelium) 48 h after infection, but bacteria were also observed in normal villi of Peyer’s patches 72 h after infection (data not shown). In the proximal colon, REPEC O 103 showed a similar adherence pattern, but bacteria appear to bind to mucus rather than the epithelial surface. Although we could detect bacteria in the cecum, bacterial adherence was less than in other intestinal sections.

Although the adherence of AAF004 (EspA⁻) and AAF005 (EspB⁻) was somewhat delayed compared to that of wild-type, both strains adhered to the ileum (Fig. 5D and E). Peyer’s patches, and proximal colon 72-96 h after infection (summarized in Table 2). However, we could not detect embedded bacteria as shown in Fig. 5 B, nor formation of cup-like structures on epithelial surfaces as in Fig. 5C. These results suggest that formation of embedded and cup-like structures require EspA and EspB, although the initial (nonintimate) adherence of both AAF004 (EspA⁻) and AAF005 (EspB⁻) is similar to that of the parental strain.

EspA and EspB Are Required for A/E Lesions In Vivo. To examine bacterial adherence in more detail, rabbit intestinal samples were analyzed using TEM. The results showed that REPEC O 103 caused typical A/E lesions in vivo and intimately associated with the ileal surface (Fig. 6A). Elongated and swollen microvilli and microbodies were

Table 2. Phenotypes of REPEC O103 and Mutant Strains

| Strain             | Adherence* | A/E lesions and cup-like structures | Adherence* | A/E lesions and cup-like structures |
|--------------------|------------|------------------------------------|------------|------------------------------------|
| IL     | PP     | CE     | PC     | IL     | PP     | CE     | PC     |
| REPEC O103        | ++      | +++    | +      | +++    | +      | -      | +      |
| AAF004 (EspA⁻)    | ++      | +      | +      | +++    | +      | -      | +      |
| AAF005 (EspB⁻)    | ++      | +      | +      | +++    | +      | -      | +      |

*Degree of adherent efficiency was assigned as follows: +, few adherent bacteria; ++, diffuse adherence; ++++, intimate adherence.

Tissue samples: IL, ileum; PP, Peyer’s patches; CE, cecum; PC, proximal colon.
observed closely associated with adherent bacteria. The bacteria rested on cups or pedestals, sometimes oriented perpendicularly or invaginated into host epithelial membranes. The glycocalyx and microvilli were completely disrupted beneath attached bacteria. Indicative of progressive intracellular damage, the enterocytes showed a pale vacuolated watery cytoplasm and disappearance or dysmorphism of mitochondria, ribosomes, and endoplasmic reticulum, as well as pale nuclei, with the enterocytes often being extruded into the lumen. These typical A/E lesions were found in the ileum, Peyer’s patches, and proximal colon, but not in the cecum (summarized in Table 2). In contrast, when rabbits were infected with AAF004 (EspA<sup>−</sup>) and AAF005 (EspB<sup>−</sup>), no A/E lesions were detected in ileal tissue (Fig. 6, B and C) or other intestinal sections examined. The microvilli beneath these bacteria still exhibited its normal brush border, and no sign of intracellular damage could be found. These results suggest that EspA and EspB are needed to trigger A/E lesions and cell damage in vivo.

We have already shown that EspA and EspB were needed for efficient delivery of Tir, which was required for intimate adherence to HeLa cells (10). In this study, both mutant strains did not adhere intimately and did not make A/E lesions, presumably due to the inability to deliver Tir to the host membrane. However, these strains still adhered at a distance above the ileal surface. An adhesive factor, AF/R2, was previously identified in REPEC O103 and is needed for nonintimate adherence (38). Therefore, the initial (nonintimate) adherence at a distance from ileal surfaces (Fig. 6, B and C) is probably mediated by AF/R2 or another adhesin. Indeed, adherent patterns in EspA and EspB mutant strains were quite similar to that of the EPEC EspB mutant strain in Hep-2 cells (22), although EPEC initial adherence to epithelial cells is mediated by bundle-forming pilus.

EspA and EspB Are Required for Cytoskeletal Rearrangements and Diarrhea. The role of cytoskeletal actin rearrangements mediated by EPEC infection in vivo is still unclear. To determine if cytoskeletal rearrangements also occur in vivo and are similar to those seen with tissue culture cells, cytoskeletal actin of epithelial surfaces were analyzed by confocal laser scanning microscopy (CLSM) using anti-O103 antibody and phalloidin-TxR, which specifically stains filamentous actin. We selected sections from ileum and Peyer’s patches at 72 h after infection for examination. REPEC O103 adhered well to Peyer’s patches (Fig. 7) confirming the SEM and TEM results. When tissue was stained with phalloidin-TxR, we found that the cytoskeletal actin was highly reorganized and formed cup-like structures beneath attached bacteria (Fig. 7). Bacterial colonization occurred in the ileum and Peyer’s patches when rabbits were infected with AAF004 (EspA<sup>−</sup>) and AAF005 (EspB<sup>−</sup>). However, both mutant strains never triggered cytoskeletal rearrangements beneath the attached bacteria (Fig. 7). These results indicate that EspA and EspB are involved in triggering of cytoskeletal actin rearrangement and cup-like structures in vivo, processes that are needed for disease.

### Discussion

We show here that formation of A/E lesions mediated by the type III secreted proteins, EspA and EspB, is in-
involved in virulence using histology, SEM, and TEM. Furthermore, thick tissue sections prepared from infected intestinal organs were analyzed by CLSM, revealing for the first time that the A/E lesion is formed by extensive cytoskeletal actin rearrangement in vivo.

Due to host tropism of human EPEC and their limitation to human in vivo studies, we used a natural rabbit infection model, EPEC serotype O103, to study disease events. Human EPEC serotype O127 normally infects only young children and does not infect animals. We have pre-

Figure 7. Confocal laser scanning micrographs of Peyer's patches infected with REPEC O103, AAF004, and AAF005. Peyer's patches were taken 72 h after infection and cryosections (20-μm-thick) were stained with phalloidin-TxR (red for overlay) and anti-O103 antibody (green for overlay). REPEC O103 adhered and colonized to the Peyer's patches, cytoskeletal actin beneath the attached bacteria was rearranged, and cup-like structures were also observed. In contrast, a small number of EspA and EspB mutant strains were observed in villi crypts, but no actin rearrangements occurred. Strains: REPEC O103, O103; AAF004 (EspAΔ), ΔEspA; AAF005 (EspBΔ), ΔEspB; PBS inoculation, Control.
vously shown that maximal expression of EPEC Esp proteins occurs at 37°C, and their expression is reduced at 39°C, which corresponds to rabbit body temperature (7). The adaptation to human body temperature is one possible explanation of the narrow host range of EPEC. Although an EPEC infection study was carried out using human adult volunteers (39), this does not reflect a natural infection as adults were used and a high infection inoculum was required. In contrast, the advantages of using R EPEC O 103 are that this pathogen naturally infects weaned rabbits, forms the A/E lesion, and has a conserved LEE locus similar to EPEC O 127. Thus the natural rabbit infection model using REPEC O 103 is a useful model of human EPEC infections.

REPEC O 103 secreted at least three proteins, EspA, EspB, and EspD, and its secretion profile was similar to that of another rabbit EPEC strain, R DEC-1 serotype O 15. Indeed, DNA sequencing alignment analysis showed that the open reading frame encoding intimin (eae, 2820 bp) in REPEC O 103 84/110/1 strain (24) is very similar to that of R DEC-1 (99.8% identity) with two nucleotide changes REPEC O103 84/110/1 strain (24) is very similar to that of another rabbit EPEC strain, RDEC-1 serotype O15. Indeed, DNA sequencing alignment analysis showed that the open reading frame encoding intimin (eae, 2820 bp) in REPEC O103 84/110/1 strain (24) is very similar to that of R DEC-1 (99.8% identity) with two nucleotide changes.

These similarities indicate that other genes located in the REPEC LEE locus are highly conserved between serotype O 103 and 015. We have previously established RDEC-1 espA and espB mutant strains using suicide vectors pAA23 and pAABxbD, which were originally constructed using R DEC-1 espA and espB (7). As expected, both vectors also functioned in EPEC O 103 due to their sequence similarity. However, we were unable to produce any disease in rabbits infected with high doses of R DEC-1, and thus we switched to infection studies using REPEC O 103 and its espA and espB nonpolar mutant strains.

REPEC O 103 causes actin nucleation beneath the attached bacteria and forms horseshoe-like structures on HeLa cells, and this accumulation requires EspA and EspB. In contrast to our results, De Rycke et al. (29) have reported that REPEC O 103 B10 strain does not accumulate actin beneath adherent bacteria 4 h after infection. Instead of actin accumulation, B10 induces a cytopathic effect (CPE) by causing vinculin accumulation, and the accumulation of vinculin forms a rod-shaped pattern 48 h after infection. Although human EPEC E2348/69 serotype O 127 does not show a CPE, this CPE in B10 strains is dependent on the type III secretion system (29). De Rycke et al. suggested that the lack of EPEC-like actin accumulation is due to the diffuse adherence mediated by an AF/R 2 (38, 40). However, REPEC O 103 used in this study showed the same diffuse adherence pattern, and the secretion profiles of REPEC O 103 and B10 appear similar. These results indicate that O 103 strains might differ in causing cytoskeletal rearrangements and that the REPEC O 103 strain used in this study may be more similar to the human EPEC strain.

Studies with adult human volunteers indicate that EPEC intimin mutant strains are decreased for virulence (39). Diarrhea still developed in 4 out of 11 volunteers who ingested the intimin mutant strain, indicating that other virulence factors are also involved in EPEC pathogenesis. In this study, the virulence of EspA and EspB mutants in REPEC O 103 was greatly decreased, and growth curves from rabbits infected with both mutant strains were similar to a control group. Furthermore, the infection of REPEC O 103, but not EspA and EspB mutant strains, showed histopathological features including blunting of villi, necrosis, and inflammation (Fig. 4). These results clearly indicate that both EspA and EspB proteins are virulence factors that are needed for these histopathological effects.

Using SEM, the initial adherence of both mutant strains was quite similar to that of the wild-type strains (Fig. 5), indicating that other adhesins such as AF/R2 fimbriae may contribute to initial levels of adherence. In contrast, only the wild-type strain showed formation of embedded (Fig. 5B) and cup-like (Fig. 5C) structures on the epithelial surface. These adherent characteristics were further analyzed by TEM. We could detect formation of the A/E lesion and this formation only occurred in rabbits infected with the wild-type, but not EspA and EspB mutant strains. Furthermore, pedestal-like structures (using TEM) were only seen in the wild-type strain. These results suggest that formation of the A/E lesion followed by pedestal-like structures required EspA and EspB. This is the first study suggesting that EspA and EspB are needed for formation of A/E lesions and pedestal-like structures in an in vivo natural infection model and that these processes are needed for disease.

CLSM studies showed that extensive cytoskeletal rearrangements beneath the attached bacteria only occurred when rabbits were infected with wild-type, but not EspA and EspB mutant strains, indicating that EspA and EspB trigger cytoskeletal rearrangements in vivo (Fig. 7) similar to that seen in vitro. Immunostained thick sections with phalloidin-TxR revealed that heavy cytoskeletal rearrangements were composed of filamentous actin and were observed as cup-like structures beneath attached bacteria. Collectively, these results suggest that EspA and EspB are required for cytoskeletal actin rearrangements, and A/E lesions followed by pedestal-like structures are formed by actin reorganization. Therefore, continued cytoskeletal rearrangements might contribute to disease in infected animals, probably by disrupting the brush border.

We confirmed that REPEC O 103 does not produce Shiga toxin (STX) I and II using an enzyme-linked assay (data not shown), since some REPEC O 103 strains produce STX I or II (27, 41, 42) and this might facilitate their virulence. Thus although R DEC O 103 does not encode STX and appears to have no invasive ability, this pathogen still disrupted the epithelial surface, and this was correlated to A/E lesion production.

In conclusion, we showed that EspA and EspB proteins are required for virulence and to trigger formation of the A/E lesion in vivo, which contains cytoskeletal actin rearrangements. The role of the type III secretion system and its secreted components have been extensively studied in...
vitro using cultured epithelial cells. We have now extended those observations to a natural infection model and confirmed that these events do indeed occur in vivo, and, more importantly, are critical for disease.

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References

1. Donnenberg, M.S., and J.B. Kaper. 1992. Enteropathogenic Esherichia coli. Inf. Immun. 60:3953–3961.
2. Moon, H.W., S.C. Whipp, R.A. Argenzio, M.M. Levine, and R.A. Giannella. 1983. Attaching and effacing activities of rabbit and human enteropathogenic Esherichia coli in pig and rabbit intestines. Inf. Immun. 41:1340–1351.
3. Francis, D.H., J.E. Collins, and J.R. Duimstra. 1986. Infection of gnotobiotic pigs with an Esherichia coli O157:H7 strain associated with an outbreak of hemorrhagic colitis. Inf. Immun. 51:953–956.
4. Tzipori, S., K.I. Wachsmuth, J. Smithers, and C. Jackson. 1988. Studies in gnotobiotic piglets on non-O157:H7 Esherichia coli serotypes isolated from patients with hemorrhagic colitis. Gastroenterology. 94:590–597.
5. Tzipori, S., H. Karch, K.I. Wachsmuth, R.M. Robins-Browne, A.D. O'Brien, H. Lior, M.L. Cohen, J. Smithers, and M.M. Levine. 1987. Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic Esherichia coli O157:H7 in gnotobiotic piglets. Inf. Immun. 55:3117–3125.
6. Agin, T.S., J.R. Cantey, E.C. Boedeker, and M.K. Wolf. 1996. Characterization of the eae gene from rabbit enteropathogenic Esherichia coli strain R DEC-1 and comparison to other eae genes from bacteria that cause attaching-effacing lesions. FEMS Microbiol. Lett. 144:249–258.
7. Abe, A., B. Kenny, M. Stein, and B.B. Finlay. 1997. Characterization of two virulence proteins secreted by rabbit enteropathogenic Esherichia coli, EspA and EspB, whose maximal expression is sensitive to host body temperature. Inf. Immun. 65:3547–3555.
8. Finlay, B.B., I. Rosenshine, M.S. Donnenberg, and J.B. Kaper. 1992. Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic Esherichia coli adherence to HeLa cells. Inf. Immun. 60:2541–2543.
9. Rosenshine, I., M.S. Donnenberg, J.B. Kaper, and B.B. Finlay. 1992. Signal transduction between enteropathogenic Esherichia coli (EPEC) and epithelial cells; EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO (Eur. Mol. Biol. Organ.) J. 11:3551–3560.
10. Kenny, B., R. Devinney, M. Stein, D.J. Renscheid, E.A. Frey, and B.B. Finlay. 1997. Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell. 91:511–520.
11. Vuopio-Varkila, J., and G.K. Schoolnik. 1991. Localized adherence by enteropathogenic Esherichia coli is an inducible phenotype associated with the expression of new outer membrane proteins. J. Exp. Med. 174:1167–1177.
12. Donnenberg, M.S., J.A. Giron, J.P. Nataro, and J.B. Kaper. 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic Esherichia coli associated with localized adherence. Mol. M.icrobiol. 6:3427–3437.
13. Giron, J.A., A.S. Ho, and G.K. Schoolnik. 1993. Characterization of fimbriae produced by enteropathogenic Esherichia coli. J. Bacteriol. 175:7391–7403.
14. Sohel, I., J.L. Puente, W.J. Murray, J. Vuopio-Varkila, and G.K. Schoolnik. 1993. Cloning and characterization of the bundle-forming pilin gene of enteropathogenic Esherichia coli and its distribution in Salmonella serotypes. Mol. M.icrobiol. 7:563–575.
15. Stone, K.D., H.Z. Zhang, L.K. Carlson, and M.S. Donnenberg. 1996. A cluster of fourteen genes from enteropathogenic Esherichia coli is sufficient for the biogenesis of a type IV pilus. Mol. Microbiol. 20:325–337.
16. Rosenshine, I., S. Ruschkowski, M. Stein, D.J. Renscheid, S.D. Mills, and B.B. Finlay. 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO (Eur. Mol. Biol. Organ.) J. 15:2613–2624.
17. Kenny, B., and B.B. Finlay. 1997. Intimin-dependent binding of enteropathogenic Esherichia coli to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase c-γ1. Inf. Immun. 65:2528–2536.
18. McDaniel, T.K., K.G. Jarvis, M.S. Donnenberg, and J.B. Kaper. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl.

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20. Jerse, A.E., J.A. Giron, A.E. Jerse, T.K. McDaniel, M.S. Donnenberg, and J.B. Kaper. 1990. A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA. 87:7839–7843.

21. Kenny, B., L.C. Lai, B.B. Finlay, and M.S. Donnenberg. 1996. EspA, a protein secreted by enteropathogenic Escherichia coli, is required to induce signals in epithelial cells. Mol. Microbiol. 20:313–323.

22. Donnenberg, M.S., J. Yu, and J.B. Kaper. 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic Escherichia coli to epithelial cells. J. Bacteriol. 175:4670–4680.

23. Lai, L.C., A. Wainwright, K.D. Stone, and M.S. Donnenberg. 1997. A third secreted protein encoded by the enteropathogenic Escherichia coli pathogenicity island is required for transduction of signals and for attaching and effacing activities in host cells. Infect. Immun. 65:2211–2217.

24. Peeters, J.E., R. Geeroms, and F. Orskov. 1988. Biotype, serotype, and pathogenicity of attaching and effacing enteropathogenic Escherichia coli strains isolated from diarrheic commercial rabbits. Infect. Immun. 56:1442–1448.

25. Camguilhem, R., and A. Milon. 1989. Biotypes and O serogroups of Escherichia coli involved in intestinal infections of weaned rabbits: clues to diagnosis of pathogenic strains. J. Clin. Microbiol. 27:743–747.

26. Milon, A., J. Esslinger, and R. Camguilhem. 1990. Adhesion of Escherichia coli strains isolated from diarrheic weaned rabbits to intestinal villi and HeLa cells. Infect. Immun. 58:2690–2695.

27. Hall, G.A., C.R. Dorn, N. Chanter, S.M. Scotland, H.R. Smith, and B. Rowe. 1990. Attaching and effacing lesions in vivo and adhesion to tissue culture cells of Vero-cytotoxin-producing Escherichia coli belonging to serogroups O5 and O103. J. Gen. Microbiol. 136:797–796.

28. Liccis, D., A. Reynaud, M. Federighi, B. Gaillard-Martine, J.F. Guillot, and B. Joly. 1991. Scanning and transmission electron microscopic study of adherence of Escherichia coli O103 enteropathogenic and/or enterohemorrhagie strain GV in enteric infection in rabbits. Infect. Immun. 59:3796–3800.

29. De Rycke, J., E. Comtet, C. Chalareng, M. Boury, C. Tasca, and A. Milon. 1997. Enteropathogenic Escherichia coli O103 from rabbit cecal actin stress fibers and focal adhesions in HeLa epithelial cells, cytopathic effects that are linked to an analog of the locus of enterocyte effacement. Infect. Immun. 65:2555–2563.

30. Donnenberg, M.S., and J.B. Kaper. 1991. Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect. Immun. 59:4310–4317.

31. Simon, R., U. Priever, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology. 1:784–791.

32. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

33. Finlay, B.B., S. R uschikowski, and S. Dedhar. 1991. Cytoskeletal rearrangements accompanying Salmonella entry into epithelial cells. J. Cell. Sci. 99:283–296.

34. Richter-Dahlfors, A., A. Buchan, and B.B. Finlay. 1997. Characterization of EspC, a 110-kilodalton protein secreted by enteropathogenic Escherichia coli which is homologous to members of the immunoglobulin a protease-like family of secreted proteins. J. Bacteriol. 179:6546–6554.

35. Kenny, B., and B.B. Finlay. 1993. Protein secretion by enteropathogenic Escherichia coli is essential for transducing signals to epithelial cells. Proc. Natl. Acad. Sci. USA. 92:7991–7995.

36. Fiederling, F., M. Boury, C. Petit, and A. Milon. 1997. Adhesive factor/rabbit 2, a new fimbrial adhesin and a virulence factor from Escherichia coli O103, a serogroup enteropathogenic for rabbits. Infect. Immun. 65:847–851.

37. Donnenberg, M.S., C.O. Tacket, S.P. James, G. Losonsky, J.P. Nataro, S.S. Waserman, J.B. Kaper, and M.M. Levine. 1993. Role of the eaeA gene in experimental enteropathogenic Escherichia coli infection. J. Clin. Invest. 92:1412–1417.

38. Pillien, F., C. Chalareng, M. Boury, C. Tasca, J. De Rycke, and A. Milon. 1996. Role of adhesive factor/rabbit 2 in experimental enteropathogenic Escherichia coli O103 diarrhea of weaned rabbit. Vet. Microbiol. 50:105–115.

39. Ritchie, M., S. Partington, J. Jessop, and M.T. Kelly. 1992. Comparison of a direct fecal Shiga-like toxin assay and sorbitol-MacConkey agar culture for laboratory diagnosis of enterohemorrhagic Escherichia coli infection. J. Clin. Microbiol. 30:461–464.

40. Tarr, P.I., L.S. Fouser, A.E. Stapleton, R.A. Wilson, H.H. Kim, J. Vary, Jr., and C.R. Clausen. 1996. Hemolytic-uremic syndrome in a six-year-old girl after a urinary tract infection with Shiga-toxin-producing Escherichia coli O103:H2. N. Engl. J. Med. 335:635–638.

41. Cantey, J.R., and R.K. Blake. 1977. Diarrhea due to Escherichia coli in the rabbit: a novel mechanism. J. Infect. Dis. 135:454–462.

42. Levine, M.M., E.J. Bergquist, D.R. Nalin, D.H. Waterman, R.B. Hornick, C.R. Young, and S. Sotman. 1978. Escherichia coli strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet. 1:1119–1122.