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Secretion of Extracellular Proteins by Enterohemorrhagic *Escherichia coli* via a Putative Type III Secretion System

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Enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) infections result in attaching and effacing lesions on intestinal epithelial cells. Secretion of extracellular proteins via a type III secretion apparatus is necessary for the formation of attaching and effacing lesions by EPEC. We now show that EHEC also secretes polypeptides via a putative type III secretion system. The secreted EHEC proteins are recognized by rabbit antiserum raised against the proteins secreted from EPEC and by human serum from a patient infected with an EHEC O157:H7 strain.

Enterohemorrhagic *Escherichia coli* (EHEC) is the most common cause of hemorrhagic colitis, a bloody diarrhea which can lead to the life-threatening hemolytic-uremic syndrome (HUS) (3, 8, 21). EHEC produces Shiga-like toxins I and II (recently renamed Stx1 and Stx2) and variants which are potent cytotoxins closely related to Shiga toxin of *Shigella dysenteriae* type I (26). Infections caused by EHEC and another diarrheagenic pathogen, enteropathogenic *E. coli* (EPEC), result in a histopathology called attaching and effacing (AE) (20, 28). AE lesions are characterized by effacement of intestinal microvilli, followed by intimate association of bacteria to host cells and accumulation of polymerized actin and other cytoskeletal components beneath the adherent bacteria (15, 20). For EPEC, specific signal transduction events have been associated with AE lesions; these include tyrosine phosphorylation of a 90-kDa host cell protein (Hp90) (22), fluxes in inositol phosphate levels (6), increased intracellular Ca^{2+} levels (1), and phosphorylation of myosin light chain (18). In a recent study EHEC infections were also found to stimulate fluxes in inositol phosphates and to increase intracellular Ca^{2+} levels, but they did not cause tyrosine phosphorylation of host epithelial cell proteins (10).

Another feature common to both EPEC and EHEC is the presence of a large chromosomal locus called the LEE (locus for enterocyte effacement), which encodes all of the known virulence factors necessary for AE lesions (19). One gene within the LEE, *eaeA* (*E. coli* attaching and effacing), encodes an outer membrane protein called intimin, which is required but not sufficient for AE-lesion formation (2, 12, 30). Two other genes within the EPEC LEE, *espA* (encoding a 25-kDa polypeptide) (14) and *espB*, formerly called *eaeB* (encoding a 37-kDa polypeptide) (4, 13), are necessary for epithelial cell signal transduction events during AE-lesion formation (5). The *espA* and *espB* gene products are secreted into the culture supernatant during in vitro growth and are presumed to interact with host cells during natural infections (11, 13, 14). A transport apparatus that secretes the Esp proteins and several other polypeptides is also encoded by genes in the EPEC LEE (11). These genes, called *sepA* through *sepI* (secretion of *E. coli* proteins) in EPEC, are similar to the genes in *Shigella*, *Salmonella*, and *Yersinia* spp. encoding type III secretion system proteins, which are responsible for secretion of virulence factors in these pathogens (9, 29). An EPEC *sepB* mutant is defective in type III protein secretion, tyrosine phosphorylation of Hp90, and formation of AE lesions in tissue culture cells (11).

The presence of regions homologous to the EPEC *sep* genes in the EHEC LEE region prompted us to test EHEC strains for secretion of extracellular proteins. We examined O157:H7 and O26:H11 strains, since these are the most common serotypes associated with hemorrhagic colitis and HUS in the United States (8, 17) (Table 1). In our previous study, culture supernatants of EPEC were analyzed for secreted proteins after a laborious ammonium sulfate precipitation procedure which gave variable results (11). A modification of this method was employed as follows. Bacteria were grown as previously described in 100 ml of Eagle's minimal essential medium at 37°C with shaking to an optical density at 600 nm of 1.0. Bacteria were pelleted by centrifugation (10,000 × *g*, 10 min), and phenylmethylsulfonyl fluoride (50 μg/ml; Sigma), aprotinin (0.5 μg/ml; Sigma), and EDTA (0.5 μM; Sigma) were added to the supernatants, which were then passed through 0.45-μm-pore-size filters and concentrated to 1.0 ml in Omegacell disposable stirred-cell filtration devices (Filtron Technology Corp., Northborough, Mass.) (11). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Western blotting (immunoblotting) with rabbit antiserum (diluted 1:1,000) raised against EPEC-secreted proteins or with human serum (diluted 1:10,000) from an individual who developed HUS subsequent to an O157:H7 EHEC infection (a gift from Phillip Tarr). Polyclonal rabbit antiserum was raised as follows. Four polypeptides (37, 28, 25, and 23 kDa) were excised from SDS-polyacrylamide gels, and the gel slices were lyophilized, macerated, and suspended in phosphate-buffered saline. A New Zealand White rabbit was immunized subcutaneously with the antigen mixture in an equal volume of complete Freund's adjuvant. Two booster injections in incomplete Freund's adjuvant were given (11).

To test for the presence of a type III secretion apparatus in EHEC, a *sepB* insertion mutation was constructed in strain 86-24 by using a cloned *sepB* gene and methods previously used

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TABLE 1. Strains used in this study[a]

Strain	Serotype	Stx1	Stx2	Origin or description (reference)
86-24	O157:H7	−	+	Hemorrhagic colitis outbreak, Walla Walla, Wash. (7)
84-289	O157:H7	+	+	Food handler in a Canadian nursing home (27)
85-170	O157:H7	−	−	Spontaneous Stx-negative derivative of 84-289 (27)
EDL933	O157:H7	+	+	Hemorrhagic colitis outbreak, Michigan (21)
NF4	O157:H7	−	+	Canadian isolate from a patient with HUS
6549	O26:H11	−	+	German isolate from a child with bloody diarrhea
83-574	O26:H11	+	−	Canadian isolate from a patient with HUS
E2348/69	O127:H6	−	−	Wild-type EPEC strain (16)
CVD452	O127:H6	−	−	*sepB* mutant of EPEC E2348/69 (11)
CVD451	O157:H7	−	+	*sepB* mutant of EHEC 86-24
CVD451(pCVD446)	O157:H7	−	+	CVD451 plus plasmid pCVD446; 4.5-kb *Bgl*II subclone from the EPEC LEE region containing the *sepB* gene (11)
CVD451(pKJ2)	O157:H7	−	+	CVD451 plus plasmid pKJ2; 1.3-kb subclone from pCVD446 (11)
HS-4	O9:H14	−	−	U.S. isolate from a healthy adult (16)

[a] Stx, Shiga toxin.

to mutate the EPEC *sepB* gene (11). As can be seen in Fig. 1, the EHEC *sepB* mutant, CVD451 (lane 4), does not secrete the 37- or 24-kDa polypeptide, which is analogous to results obtained when the EPEC *sepB* gene is mutated (11) (Fig. 1, lane 2; proteins below 40 kDa are no longer secreted). Protein secretion was restored to the *sepB* mutant, CVD451, by adding plasmid pCVD446, which contains an intact EPEC *sepB* gene (Fig. 2, lane 3). A plasmid containing a smaller insert, pKJ2, was unable to complement the mutation in CVD451 (Fig. 2, lane 4). Previous attempts to restore protein secretion to an EPEC *sepB* mutant by using pKJ2 were also unsuccessful (11). It has now been determined that, contrary to an initial report, pKJ2 does not contain the complete *sepB* gene because of a sequencing error, which explains the inability of this plasmid to restore protein secretion (unpublished data).

Six additional EHEC strains were tested for the presence of secreted proteins. Figure 3 shows an SDS-PAGE gel (Fig. 3A) and an identical Western immunoblot (Fig. 3B) of the secreted proteins from five O157:H7 strains (lanes 1 through 5) and two O26:H11 strains (lanes 6 and 7). Culture supernatants from EPEC strain E2348/69 (lane 8) and *E. coli* HS-4 (lane 9), an avirulent fecal isolate, are shown for comparison. All of the EHEC strains secrete 100- to 110-, 37-, and 24-kDa polypeptides, with the exception of 6549, which does not secrete the 100- to 110-kDa protein. The 100- to 110-, 37-, and 24-kDa polypeptides are recognized by antiserum raised against EPEC-secreted proteins (Fig. 3B), suggesting the presence of cross-reacting epitopes on EHEC- and EPEC-secreted proteins.

Under the conditions used here the EHEC strains secrete a 24-kDa polypeptide (Fig. 3A, lanes 1 through 7), while EPEC strain E2348/69 (Fig. 3A, lane 8) secretes EspA, which has a molecular mass of 25 kDa (14). All of these polypeptides are recognized by the antiserum raised against EPEC-secreted proteins, again suggesting the presence of cross-reacting epi-

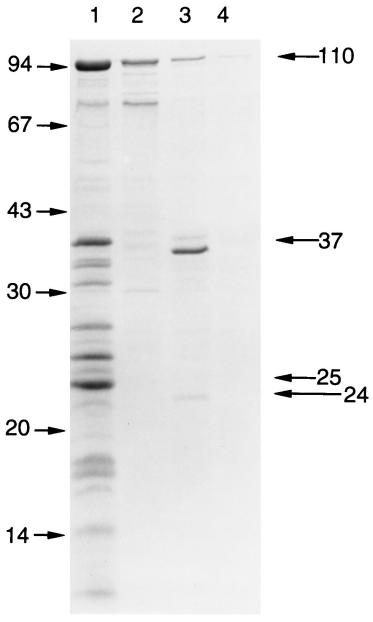

FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel of culture supernatants from EPEC E2348/69 (lane 1); the EPEC *sepB* mutant, CVD452 (lane 2); EHEC 86-24 (lane 3); and the EHEC *sepB* mutant, CVD451 (lane 4). Molecular mass markers and molecular masses of secreted proteins, both in kilodaltons, are shown on the left and right, respectively.

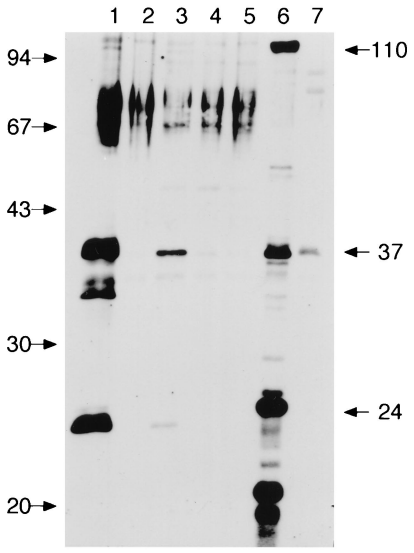

FIG. 2. Immunoblot of culture supernatants from EHEC 86-24 (lane 1), CVD451 (lane 2), CVD451(pCVD446) (lane 3), CVD451(pKJ2) (lane 4), CVD451(pBluescript) (lane 5), EPEC E2348/69 (lane 6), and *E. coli* HS-4 (lane 7), probed with rabbit antiserum against EPEC-secreted proteins. Molecular mass markers and molecular masses of secreted proteins, both in kilodaltons, are shown on the left and right, respectively.

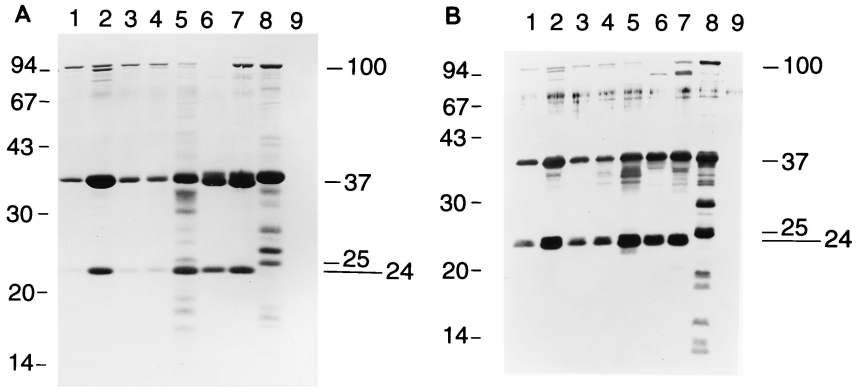

FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel (A) and identical immunoblot probed with rabbit antiserum against EPEC-secreted proteins (B) of culture supernatants from EHEC 84-289 (lanes 1), 85-170 (lanes 2), 86-24 (lanes 3), EDL933 (lanes 4), NF4 (lanes 5), 6549 (lanes 6), and 83-574 (lanes 7); EPEC E2348/69 (lanes 8), and *E. coli* HS-4 (lane 9). Molecular mass markers and molecular masses of secreted proteins, both in kilodaltons, are shown on the left and right, respectively.

topes on EPEC- and EHEC-secreted polypeptides (Fig. 3B, lanes 1 through 8). It is presumed that the 24-kDa protein secreted by EHEC is analogous to EspA of EPEC, and this possibility is being investigated.

O157:H7 strains 84-289, 85-170, 86-24, EDL933, and NF4 (Fig. 3A, lanes 1 through 5) secrete two polypeptides in the 100- to 110-kDa region. We previously determined that the secretion of a 110-kDa polypeptide in EPEC strain E2348/69 was not affected by a mutation in the type III secretion apparatus (11). We found similar results with the EHEC *sepB* mutant, CVD451 (Fig. 1), which still secretes the 110-kDa protein. Stein et al. have shown that the 110-kDa polypeptide of EPEC has homology with the immunoglobulin A protease-*Haemophilus influenzae* family of adhesins and that mutation of the gene encoding this protein has no effect on AE-lesion formation (25). It is not clear what the role of this polypeptide in EPEC pathogenesis is and whether one of the high-molecular-mass proteins secreted by EHEC is homologous to this polypeptide. It is likely that one of the EHEC proteins in this region is the plasmid-encoded hemolysin known to occur in O157:H7 strains (23). Supporting this possibility is the fact that all of the strains used in this study hybridize with the DNA probe specific for this hemolysin (17) with the exception of strain 6549, which does not secrete either of the high-molecular-mass proteins (Fig. 3A and data not shown).

We determined the N-terminal sequence of a 37-kDa polypeptide from EHEC strain 84-289 supernatants and found that the first four amino acids (Met-Asn-Thr-Ile) are identical to those of EspB of EPEC, which also has a molecular mass of 37 kDa (4, 13) (only the first four residues were determined). Figure 3A shows that all of the EHEC strains examined here secrete polypeptides in the 37-kDa range, suggesting that they all produce EspB. Furthermore, antiserum specific for the EspB polypeptide reacts with a 37- but not the 24-kDa polypeptide in supernatants from all of the strains tested in this study (data not shown). The two O26:H11 strains examined secrete two polypeptides in the 37-kDa region (Fig. 3A, lanes 6 and 7). The identity of the lower-molecular-mass protein is not known, but it does not react with antiserum specific for the EPEC EspB protein (data not shown) or the antiserum raised against the EPEC-secreted proteins (Fig. 3B).

The varied protein secretion profiles among EHEC strains and EPEC could be due to membrane blebbing as opposed to actual protein secretion. To test this possibility, a centrifugation step (124,000 $\times$ *g* for 1.5 h) was added to the protein purification method described above after passage of the supernatants through a 0.45-μm-pore-size filter. Figure 4 shows that the secretion profiles of strains 86-24, 6549, and E2348/69 are unaffected by this high-speed centrifugation. These data and the fact that a mutation in a type III secretion gene (*sepB*) in strain 86-24 eliminates the secretion of the 37- and 24-kDa polypeptides constitute good evidence that these polypeptides are actually secreted rather than being part of bacterial membrane blebs.

Immunoblot analysis was performed to determine whether the secreted polypeptides would be recognized by human serum collected from a patient who developed HUS as a result of an O157:H7 EHEC infection. Figure 5 shows that the EHEC-secreted proteins are recognized by human serum and that the recognition pattern is similar to that seen with rabbit antiserum against the EPEC-secreted proteins (Fig. 3B). All of the strains secrete polypeptides of 37 and 24 kDa that are recognized by the serum from an HUS patient (Fig. 5) but not by serum from uninfected children (data not shown). The presence of cross-reacting epitopes on the proteins secreted by EHEC and EPEC is shown again in Fig. 5, for which human serum was used to detect the EPEC polypeptides. These data strongly suggest that the proteins secreted by EHEC are produced in vivo and play a role in human infections.

Since the identification of EHEC in 1982, this pathogen has

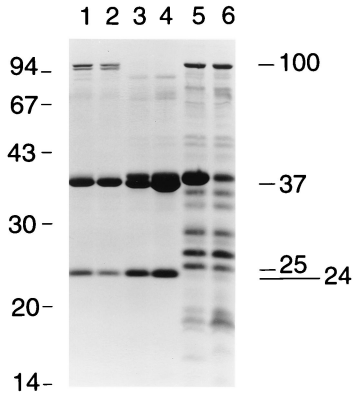

FIG. 4. Coomassie blue-stained SDS-polyacrylamide gel of culture supernatants from EHEC 86-24 (lanes 1 and 2) and 6549 (lanes 3 and 4) and EPEC E2348/69 (lanes 5 and 6). Culture supernatants in lanes 1, 3, and 5 were concentrated as described in the text, and those in lanes 2, 4, and 6 were subjected to a high-speed centrifugation step (124,000 $\times$ *g*) to eliminate membrane blebs. Molecular mass markers and molecular masses of secreted proteins, both in kilodaltons, are shown on the left and right, respectively.

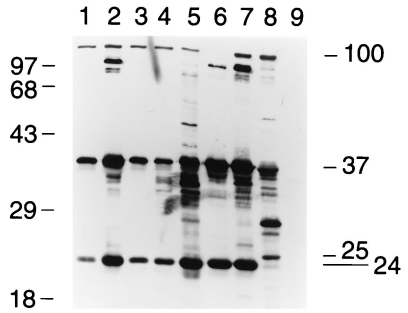

FIG. 5. Immunoblot of culture supernatants from EHEC 84-289 (lane 1), 85-170 (lane 2), 86-24 (lane 3), EDL933 (lane 4), NF4 (lane 5), 6549 (lane 6), and 83-574 (lane 7), EPEC E2348/69 (lane 8); and *E. coli* HS-4 (lane 9) probed with serum from a patient who developed HUS. Molecular mass markers and molecular masses of secreted proteins, both in kilodaltons, are shown on the left and right, respectively.

engendered great public interest and intense study because of their frequent association with food-borne outbreaks of diarrhea, which can result in hemorrhagic colitis and/or the life-threatening HUS (3, 21). Characteristics that have been implicated as virulence factors include production of one or more Shiga toxins, possession of a large (60-MDa) virulence plasmid, and formation of AE lesions (17). A limited number of serotypes of *E. coli*, including O157:H7 and O26:H11, with some or all of the above-mentioned characteristics have frequently been associated with cases and outbreaks of hemorrhagic colitis and HUS (8, 17, 24). As with EPEC, the presence of a type III secretion apparatus component, *sepB*, was observed in O157:H7 EHEC. In EPEC, the type III secretion components and the secreted polypeptides are necessary for AE-lesion formation, which is critical for EPEC pathogenesis. The presence of secreted proteins in each of the EHEC strains studied here suggests the existence of previously unidentified virulence factors in these pathogens. The fact that one of the secreted polypeptides has amino acid sequence identity with EspB of EPEC strongly suggests that the proteins secreted by EHEC contribute to the pathogenic mechanisms involved in AE lesions. Future investigations will demonstrate the role that the secreted polypeptides play in EHEC infections.

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