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# A Third Secreted Protein That Is Encoded by the Enteropathogenic *Escherichia coli* Pathogenicity Island Is Required for Transduction of Signals and for Attaching and Effacing Activities in Host Cells

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**Enteropathogenic *Escherichia coli* strains are able to signal host cells, cause dramatic cytoskeletal rearrangements, and adhere intimately to the cell surface in a process known as the attaching and effacing effect. A pathogenicity island of 35 kb known as the locus of enterocyte effacement (LEE) is necessary and sufficient for this effect. The LEE encodes an outer membrane adhesin called intimin, a type III secretion apparatus, and the EspA and EspB secreted proteins. The DNA sequence of the region between *espA* and *espB* revealed a new gene, *espD*. The product of *espD* was demonstrated by using a T7 expression system. We constructed a nonpolar mutation in *espD* and found that the mutant is incapable of the signal transduction events that lead to activation of the putative intimin receptor in host cells and that the mutant fails to induce the attaching and effacing effect. These phenotypes were restored to the mutant by complementation with a plasmid containing the cloned *espD* locus. We demonstrated by immunoblotting and microsequencing that the EspD protein is secreted via the type III apparatus. Thus, we describe a novel locus encoding a secreted protein that is required for attaching and effacing activity.**

The ability of a number of pathogenic bacteria to secrete virulence proteins that lack signal sequences has recently been the subject of considerable attention. These type III secretion systems are found in enteric pathogens of humans such as members of the genera *Shigellae*, *Yersiniae*, and *Salmonellae* as well as in *Pseudomonas aeruginosa* and plant pathogens (33, 49). Type III secretion systems export factors that play important roles in pathogenesis include the Ipa proteins (1, 3), the Yop proteins (36), and the Sip (also known as Ssp) proteins (17, 21, 22). The secretion of these virulence factors is enhanced by environmental conditions and by contact with host cells or host proteins (34, 40, 48, 50). The YopE, YopH, and YpkA proteins are translocated directly from the bacteria into the cytosol of the host cell upon contact (16, 40, 42, 43). The secretion systems, as well as the secreted proteins, are encoded in these bacteria by blocks of genes on plasmids or pathogenicity islands. It is believed that the acquisition of these systems represented a major advance in the virulence of the species that possess them (15).

Enteropathogenic *Escherichia coli* (EPEC), a principal cause of diarrhea among infants in developing countries (7), possesses a type III secretion apparatus encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE) (18, 31). The LEE is necessary and sufficient for the attaching and effacing effect, a dramatic rearrangement of host cytoskeletal proteins induced by EPEC (32). The attaching and effacing lesion is characterized by loss of microvilli and the intimate attachment of the bacteria upon cup-like pedestals composed of host cytoskeletal proteins including actin, α-actinin, talin, ezrin, and myosin light chain (13, 25, 28). Other pathogens capable of producing attaching and effacing lesions, including enterohemorrhagic *E. coli*, *Citrobacter rodentium*, and certain strains of *Hafnia alvei*, also possess LEE sequences (31).

Recent advances have begun to clarify the molecular events that lead to attaching and effacing lesions. Following contact with cells, EPEC induces the phosphorylation of a 90-kDa host membrane protein (Hp90) (38) that is activated to become capable of binding intimin (39), an outer membrane protein adhesin encoded by the *eae* gene within the LEE (20). This tyrosine kinase signal transduction is dependent upon the *sep* genes that encode the EPEC type III secretion apparatus (18) and upon the *espA* and *espB* (formerly known as *eaeB*) genes that encode proteins secreted via the Sep apparatus (23, 24). Mutations in *sepA*, *sepB*, *espA*, and *espB* all result in the inability to induce the tyrosine phosphorylation of Hp90, the inability to alter the host cell cytoskeleton, and the inability to cause attaching and effacing lesions (14, 18, 24, 38).

The *sep* genes are located upstream of *eae* and the *esp* genes are downstream of *eae* within the LEE. The *espA* and *espB* genes are separated by approximately 1 kb. The purpose of this study was to determine whether the region between *espA* and *espB* encodes functions required for attaching and effacing by EPEC and to characterize the product(s) encoded by this region.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, tissue culture, and media.** The strains and plasmids used in this study are described in Table 1. Bacteria were stored in 50% Luria-Bertani (LB) broth–50% (vol/vol) glycerol at −70°C and grown on LB agar plates or LB broth with chloramphenicol (20 μg/ml), ampicillin (200 μg/ml), nalidixic acid (50 μg/ml), or kanamycin (50 μg/ml) added as needed to maintain plasmids. HEp-2 cells (ATCC CCL23) were grown at 37°C in an atmosphere of 95% air–5% $CO_2$ in Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

**DNA manipulation.** Routine DNA manipulation and cloning techniques were performed as described in general references (41). Oligonucleotides were con-

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TABLE 1. Strains and plasmids used in the study

| Strain or plasmid | Genotype or characteristics | Reference(s) or source |
|---|---|---|
| Strains | | |
| E2348/69 | Prototypic O127:H6 EPEC strain; nalidixic acid resistant | 27 |
| 27-3-2(1) | E2348/69 *sepA*::Tn*phoA*; previously known as *cfm* | 8, 31, 38 |
| UMD870 | E2348/69 *espD1*::*aphA-3* | This study |
| UMD872 | E2348/69 Δ*espA1*::*aphA-3* | 4 |
| UMD864 | E2348/69 Δ*espB1* | 11 |
| CVD206 | E2348/69 Δ*eae8* | 9 |
| DH5α | *supE44* Δ*lacU169* (φ80 *lacZ*Δ*M*φ *15*) *hsdR17 recA1 endA*φ *1 gyrA*φ *96 thi-1 relA*φ *1* | 41 |
| DH5αλ*pir* | DH5α (λ*pir*) | 35 |
| BL21(DE3) | *hsdS gal* (λcI*ts857 ind1 sam7 nin5 lacUV 5*-T7 gene 1) | 46 |
| Plasmids | | |
| pCRscript | High-copy-number vector for cloning PCR products | Stratagene |
| pUC18K | *aphA-3* gene cassette cloned in pUC18 | 35 |
| pCVD442 | π-dependent, *sacB*-containing positive-selection suicide vector | 9 |
| pJY26 | *Sal*I-*Mlu*I fragment from within *eaeA* to beyond K-12 junction cloned in pACYC184 derivative | 11 |
| pMSD2 | pJY26 deleted of 2.3- and 2.7-kb *Bgl*II fragments, resulting in a disruption of the open reading frame downstream of *espA* and loss of the rest of the LEE | 11 |
| pLCL115 | PCR product of reaction using Donne-18 and Donne-73 as primers and pJY26 as template, cloned into pCRscript | This study |
| pLCL116 | *aphA-3* cassette cloned into *Eco*47III site of pLCL115 | This study |
| pLCL118 | *Sma*I-*Sac*I fragment of pLCL116 cloned into pCVD442 | This study |
| pLCL123 | *Sal*I-*Nru*I fragment of pJY26 cloned into pACYC184 | This study |

structed at the Biopolymer Laboratory of the University of Maryland at Baltimore. PCR was performed by using Deep Vent polymerase (New England Biolabs, Beverly, Mass.) on 50-μl samples in a minicycler (MJ Research, Watertown, Mass.). Plasmids were introduced into wild-type EPEC strains by triparental conjugation (5) or by electroporation in 0.1-cm cuvettes with an *E. coli* pulser (Bio-Rad Laboratories, Hercules, Calif.) set at 1.8 kV.

The double-stranded sequence of the region cloned in plasmid pLCL115 was determined on both strands by using the dideoxy-chain termination method on double-stranded plasmid templates (4). A Sequenase version 2.0 kit (Amersham, Naperville, Ill.) was used for manual sequencing, and a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) was used for automated sequencing. Automated sequencing was performed by the Biopolymer Laboratory at the University of Maryland at Baltimore, using a model 373A sequencer (Applied Biosystems).

**Construction of a nonpolar mutation.** We used PCR with primers Donne-18 (5′-CAG AGC GGC TGT CGC A-3′) and Donne-73 (5′-CGA GAT CTC AGT TGC TAG TGC G-3′) to generate a fragment of 2,036 bp extending from within *espA* to within *espB*. Oligonucleotide Donne-18 represents nucleotides 414 to 399 of the published *espB* sequence (accession no. Z1555), and Donne-73 represents nucleotides 68 to 81 of the published *espA* sequence (accession no. Z54352). The PCR consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The amplification product was cloned into pCRscript (Stratagene, La Jolla, Calif.) to create pLCL115 (Fig. 1). The 850-bp *aphA-3* kanamycin resistance cassette flanked by *Sma*I sites (35) was inserted into the *Eco*47III site of pLCL115 to create pLCL116. The *Sma*I-*Sac*I fragment containing the insertion was cloned into positive-selection suicide vector pCVD442 in *E. coli* DH5αλ*pir* for introduction into wild-type EPEC strain E2348/69 by triparental conjugation. A mutant that had undergone allelic exchange to replace the wild-type locus with the locus containing the *aphA-3* insertion was selected on modified LB-kanamycin plates containing 5% sucrose and confirmed by Southern blotting as previously described (11).

**Protein expression.** Radiolabeled proteins were expressed in *E. coli* BL21(DE3) under the control of the T7 promoter, using a previously described modification (45) of the original method (47).

**Tissue culture assays for EPEC phenotypes.** The fluorescence actin staining test, using fluorescein isothiocyanate-phalloidin to detect filamentous actin as a surrogate for attaching and effacing activity (25), was performed in chamber slides as previously described (10). Proteins containing phosphotyrosine were detected in host cells by fluorescence microscopy (38) and by Western blotting (37) as previously described.

**Detection of secreted proteins.** Bacterial strains were grown in 100 ml of Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, Md.) to an $A_{600}$ of 1.0. Secreted proteins were concentrated 100-fold by using an Omega-cell disposable stirred-cell filtration device (Filtron Technology Corp., Northborough, Mass.), and whole-cell extracts were concentrated 10-fold as described previously (19). One-fortieth volume of the concentrated samples was separated by electrophoresis through an aged sodium dodecyl sulfate (SDS)–15% polyacrylamide gel (26). Separated proteins were electrotransferred to polyvinylidene difluoride (Immobilon-P; Millipore, Bedford, Mass.) membranes in 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer as previously described (29). Membranes were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.5% Tween 20 for 1 h, followed by incubation for 1 h in 0.5% BSA in phosphate-buffered saline–0.05% Tween 20 either with antiserum raised against all EPEC-secreted proteins (18) at a dilution of 1:1,000 or with EspB-specific antiserum (37) at a dilution of 1:5,000. Horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody was used at a dilution of 1:45,000 in 0.5% BSA–0.05% Tween 20 for 1 h. The immunoblots were developed by enhanced chemiluminescence (Amersham).

**Microsequencing of EspD.** One hundred microliters of a 100-fold-concentrated secreted protein sample from *espB* mutant UMD864 was separated by electrophoresis through an aged Laemmli SDS–15% polyacrylamide gel. Separated proteins were electrotransferred to a Trans-Blot membrane (Bio-Rad) in CAPS buffer as previously described (29). Transferred proteins were visualized by staining the membrane with 0.1% Coomassie blue R-250 dissolved in 40% methanol and 0.5% aldehyde-free acetic acid. The band of interest was excised, and the first six amino-terminal amino acids were determined by using a Procise 494 protein and peptide sequencer (Applied Biosystems) at the Michigan State University Macromolecular Structure Facility.

**Nucleotide sequence accession number.** The nucleotide sequence of the *espD* gene was deposited in the EMBL database under accession no. Y09228.

## RESULTS

**The *espD* gene encodes a protein of approximately 40 kDa.** We determined the nucleotide sequence of the region between *espA* and *espB* and detected a single open reading frame (ORF) of 1,143 bp beginning 12 nucleotides after the stop codon of *espA* and ending 20 nucleotides before the start codon of *espB*. A possible ribosome binding site (GGAGA) was noted six nucleotides prior to the putative start codon. The ORF is predicted to encode a protein of 381 amino acids, with a molecular mass of 39,492 Da and an isoelectric point of 4.98. No export signal sequence was detected, and no similarities were found in a search of the available databases by using BLASTP (2). The protein encoded by the ORF was detected by using a T7 expression system and found to migrate upon SDS-polyacrylamide gel electrophoresis (PAGE) with a relative mobility of 42,000 (Fig. 2). Given the proximity of the gene to *espA* and *espB* and further data described below, we designated this locus *espD*.

**The *espD* gene is required for attaching and effacing activity.** To determine the role of the protein encoded by *espD* in EPEC

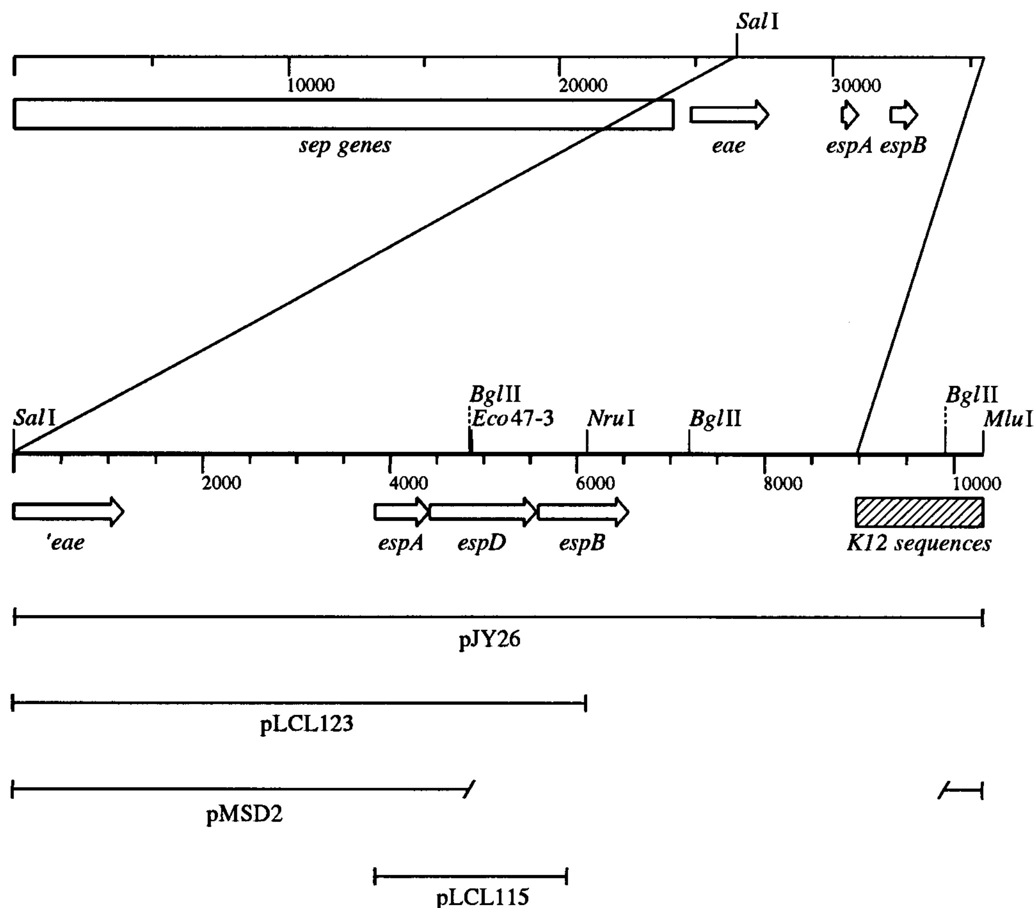


FIG. 1. Map of the LEE, with emphasis on the *esp* region. The upper map shows the relative locations of the *sep* genes encoding a type III secretion system, the *eae* gene encoding intimin, and the *esp* genes within the LEE. The unique *Sal*I restriction site is shown for reference. The bottom map shows an expanded view of the region cloned in pJY26, which extends from within the *eae* gene to beyond the junction between the LEE and ubiquitous *E. coli* sequences (represented by the hatched bar). The lines below the map depict plasmids used in this study. Selected restriction endonuclease sites important for plasmid constructions are shown. Arrows depict genes. Numbers on the scales represent base pairs.

pathogenesis, we interrupted this locus with a nonpolar mutation as described in Materials and Methods. This method disrupts the targeted locus with a promoterless kanamycin resistance gene while retaining the reading frame of the 3′ end of the target cistron and therefore allows unaffected transcription and translation of downstream genes (35). The orientation and reading frame of the insertion were confirmed by DNA sequencing. The mutated *espD* allele was cloned in positive-selection suicide vector pCVD442 and introduced into wild-type EPEC strain E2348/69 by allelic exchange. The resulting mutant, UMD870, was verified to be resistant to sucrose and kanamycin and sensitive to ampicillin. Southern analysis using the 2.3-kb *Bgl*II fragment surrounding the *Eco*47III site of the insertion as a probe confirmed the construction (data not shown).

Since *espD* is located between *espA* and *espB*, we examined whether *espD* encodes a product involved in the EPEC attaching and effacing effect. In contrast to wild-type EPEC strain E2348/69, the *espD* mutant UMD870 was unable to induce accumulations of actin or of tyrosine-phosphorylated proteins beneath adherent bacteria as assessed by fluorescence microscopy (data not shown). To test the integrity of the mutation by complementation, we chose plasmid pLCL123, which contains a fragment that extends from within *eae* through the first half

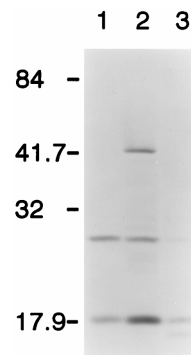


FIG. 2. Expression of the product of *espD* by using a T7 RNA polymerase system. [$^{35}$S]methionine-labeled proteins from *E. coli* BL21(DE3) transformed with pCRscript vector (lane 1), pLCL115 containing *espD* (lane 2), or pLCL116 containing *espD::aphA-3* (lane 3) were separated by SDS-PAGE and subjected to autoradiography. Proteins were labeled following induction of T7 RNA polymerase with isopropyl-β-D-thiogalactopyranoside and inhibition of host DNA polymerase with rifampin. The relative mobilities of molecular weight markers (in thousands) are indicated on the left.

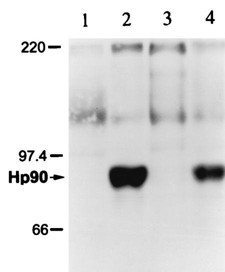


FIG. 3. Role of *espD* in tyrosine phosphorylation of HEp-2 cell proteins. HEp-2 cells were incubated for 6 h in the absence of bacteria (lane 1) or after infection with wild-type EPEC strain E2348/69 (lane 2), *espD* mutant UMD870 (lane 3), or UMD870 complemented with plasmid pLCL123 containing the cloned *espD* gene (lane 4). Triton X-100-soluble tyrosine-phosphorylated proteins were extracted, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by enhanced chemiluminescence after reaction with a monoclonal antibody against phosphotyrosine. The relative mobilities of molecular weight markers (in thousands) are indicated on the left. The position of Hp90 is indicated by the arrow.

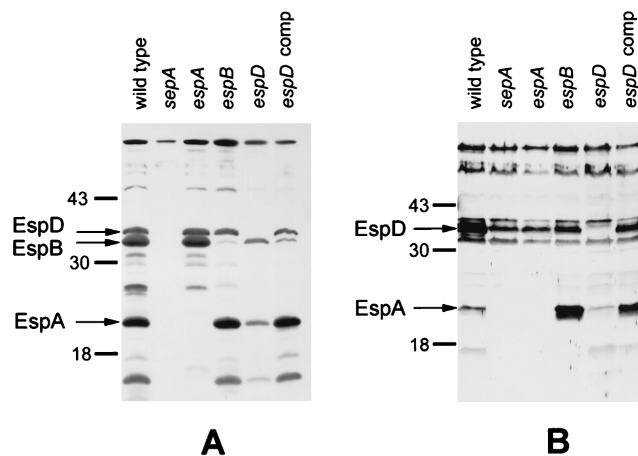


FIG. 4. Effects of mutations on secretion of Esp proteins. Culture supernatants (A) and cell pellets (B) from wild-type EPEC strain E2348/69, *sepA* mutant 27-3-2(1), *espA* mutant UMD872, *espB* mutant UMD864, *espD* mutant UMD870, and UMD870 complemented with pLCL123 containing the cloned *espD* gene were applied to an SDS–15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Secreted proteins were detected by using a rabbit polyclonal antiserum raised against proteins secreted by the wild-type strain followed by enhanced chemiluminescence reagents. The positions of Esp proteins are indicated by arrows, and the relative mobilities of molecular weight markers (in thousands) are indicated on the left.

of *espB*, so that we could express *espD* from its native promoter. When pLCL123 was introduced into UMD870, both the ability to induce actin accumulation and accumulation of tyrosine-phosphorylated proteins were restored. The ability of pLCL123, which lacks an intact *espB* gene, to complement the mutant confirms that the *espD* mutation is not polar on *espB*. In contrast, pMSD2, a construction similar to pLCL123 but ending within *espD*, was unable to complement the mutant. This result confirms that the mutation in *espD*, rather than a spurious mutation upstream, is responsible for the phenotype of the mutant. In addition, tyrosine-phosphorylated Hp90 was detected by Western blotting in cells infected with the wild-type strain and mutant UMD870 complemented with pLCL123 but not in uninfected cells or cells infected with mutant UMD870 (Fig. 3). Therefore, we conclude that *espD* is required for transduction of signals by EPEC to epithelial cells that result in the attaching and effacing effect.

**EspD is secreted via the Sep type III secretion apparatus.** The effects of the *espD* mutation on EPEC interactions with host cells are very similar to those of *sep*, *espA*, and *espB* mutations. Furthermore, *espD* is located between *espA* and *espB* in the LEE. These observations raise the possibility that, like EspA and EspB, EspD is exported via the Sep type III secretion apparatus. Therefore, we performed immunoblotting of secreted proteins and whole-cell lysates, using an antiserum raised against all of the EPEC-secreted proteins (Fig. 4A). We observed that a single band with a relative mobility of approximately 40,000 was present in supernatant samples from wild-type EPEC and *espA* and *espB* mutants but absent from the supernatants of the *espD* mutant and the *sepA* mutant. This band was restored by complementation of the *espD* mutant with a plasmid containing the *espD* gene. The 40,000-$M_r$ band was also present in the whole-cell lysates of all of the strains except the *espD* mutant and restored to the *espD* mutant by complementation (Fig. 4B). Thus, secretion of this protein is dependent on the Sep type III secretion apparatus. The size of this protein suggests that it may be the product of the *espD* gene. However, these results do not exclude the possibility that another protein that has a relative mobility similar to that of EspD is dependent on EspD for secretion. To determine whether the 40,000-$M_r$ secreted protein is EspD, we separated secreted proteins from the *espB* mutant by SDS-PAGE, transferred the proteins to a nylon membrane, and excised the 40,000-$M_r$ band after visualization by Coomassie blue staining. We chose the *espB* mutant to avoid the EspB protein, which migrates slightly faster than the band of interest. Limited amino-terminal sequencing revealed the amino acids methionine, leucine, asparagine, valine, asparagine, and asparagine, in perfect accord with the first six residues predicted by the *espD* sequence. Thus, the product of the *espD* gene is secreted by EPEC via the Sep secretion apparatus without removal of an amino-terminal signal sequence.

**Secretion of EspA and EspB by the *espD* mutant.** To determine whether the mutation in *espD* affects secretion of EspA and EspB, we examined these proteins by immunoblotting using an antiserum specific for EspB as well as the antiserum that recognizes all of the secreted proteins. We detected EspB, as well as bands that represent EspB degradation products, in the culture supernatant of the *espD* mutant and in the supernatant of wild-type EPEC and the *espA* mutant (Fig. 5A). The level of EspB appeared to be somewhat reduced in the supernatant obtained from culture of the *espD* mutant in comparison to that from wild type. However, complementation failed to restore the level of EspB to that seen in wild type. In contrast, no EspB was detected in the supernatant from cultures of the *espB* mutant or a *sepA* mutant. EspB was present in the whole-cell lysates of all strains tested except that of the *espB* mutant, although the pellet of the *sepA* mutant, and perhaps that of the *espD* mutant, contained less EspB than did the pellet of wild type (Fig. 5B). Similarly, in an assay using an antiserum against all EPEC-secreted proteins, the EspA protein was detected in culture supernatants of all strains tested except those of the *espA* mutant and the *sepA* mutant (Fig. 4A). Again, the level of the EspA protein appeared to be somewhat reduced in the supernatant from the *espD* mutant in comparison to that of wild type. As expected, EspA was present in the whole-cell lysates of all strains tested except that of the *espA* mutant but appeared to be reduced in the lysates of the *sepA* and *espD* mutants (Fig. 4B).

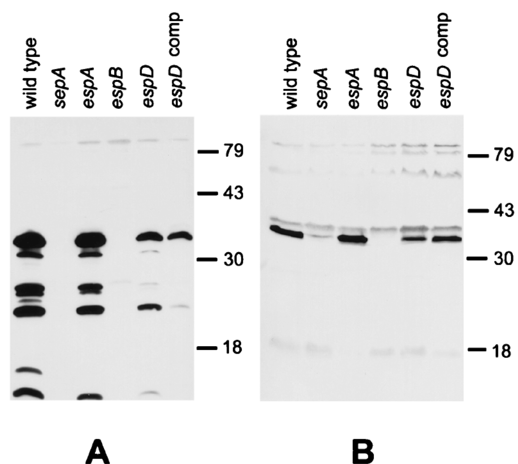


FIG. 5. Effects of mutations on secretion of EspB. Culture supernatants (A) and cell pellets (B) from strains indicated in Fig. 4 were applied to an SDS–15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. EspB was detected by using a rabbit polyclonal antiserum raised against an EspB peptide followed by enhanced chemiluminescence reagents. The relative mobilities of molecular weight markers (in thousands) are indicated on the right.

## DISCUSSION

We describe a new gene, designated *espD*, for EPEC-secreted (or signaling) protein D, that is located within the LEE between *espA* and *espB* and encodes a protein of 39,492 Da. To investigate the role of *espD* in the interactions between EPEC and host epithelial cells, we constructed a nonpolar mutation in this gene and found that the mutant is deficient in the ability to cause attaching and effacing lesions and in the ability to induce the tyrosine phosphorylation of Hp90, the putative receptor for intimin (39). Both phenotypes are restored to the mutant by introduction of a plasmid containing the cloned *espD* gene. We found that a protein with a relative mobility consistent with EspD is absent from the culture supernatants of the *espD* mutant and demonstrated that this protein is EspD. Lastly, we provide evidence that secretion of EspD is dependent on the Sep type III secretion apparatus.

The sequence of the *espD* gene sheds little light on the function of its product. EspD has no compelling similarity with other proteins in the sequence databases. There are no recognizable sequence motifs within the protein. Hydrophobicity plots do not reveal potential transmembrane segments. EspD lacks even the relative abundance of serine and threonine residues, which were noted near the amino termini of EspA and EspB but are of uncertain significance. The EspD protein was not previously detected in supernatants of EPEC (23) or enterohemorrhagic *E. coli* (12), perhaps because of its mobility close to EspB. Thus *espD* represents a novel locus potentially involved in virulence.

EspD is the sixth protein to be identified in supernatants of EPEC grown in tissue culture medium. Three of these proteins do not appear to be involved in the attaching and effacing effect. EspC, the largest secreted protein, is related to a number of high-molecular-weight proteins that encode their autologous, secretion system-independent export. A mutation in the *espC* gene has no effect on EPEC attaching and effacing activity (44). One of the proteins secreted by EPEC has an N-terminal amino acid sequence nearly identical to that of glyceraldehyde 3-phosphate dehydrogenase, while another has no significant sequence homologs (23). Since the LEE is both necessary and sufficient for attaching and effacing activity, and since the genes for these two proteins are not encoded on the LEE (30), it seems unlikely that these two proteins are required for the attaching and effacing effect. The other two proteins identified in EPEC culture supernatants, EspA and EspB, are required for attaching and effacing activity.

We found EspD in the culture supernatant of wild-type EPEC but not in the supernatant of a *sepA* mutant, indicating that EspD is secreted via the EPEC type III secretion apparatus. We have noted similar results with a *sepB* mutant (data not shown). The ability to visualize EspD by Coomassie blue staining against a background of very few other proteins indicates that the appearance of EspD in the media is not due to cell lysis. Furthermore, the amino-terminal sequence of the protein confirms that EspD is not processed by cleavage of a signal sequence, a feature consistent with type III secretion.

The *espD* gene is located between the *espA* and the *espB* genes of EPEC in an arrangement that suggests that they may be part of a single transcriptional unit. However, we previously suggested that the *espB* gene might have its own promoter (11). Direct experimentation is required to resolve this question. The *espADB* arrangement is strikingly similar to that of the *ipa* genes of *Shigella* species and the *sip* genes of *Salmonella* species, which are also exported via type III secretion systems and which also mediate critical interactions with host cells.

The phenotypes of *espA*, *espB*, and *espD* mutants are very similar. All three genes encode proteins that are secreted via the Sep system. All three mutants fail to signal host cells to phosphorylate Hp90, fail to induce cytoskeletal alterations, and fail to bind intimately to host cells. Thus, there is little to distinguish the effects of these proteins. One clue to possible divergent functions of these proteins is the effect of each on the secretion of the other Esp proteins. It has previously been noted that mutation of *espA* appears to result in increased EspB secretion (24). In contrast, we suggest that mutation of *espD* may result in decreased secretion of EspB and EspA. However, the failure of complementation of the *espD* gene to restore the levels of EspB to those of wild type indicates that this effect is likely to be sensitive to *espD* gene copy number. The potential effect of EspD on secretion of EspA and EspB is reminiscent of the effect of InvJ and SpaO of *Salmonella typhimurium* on Sip secretion (6). More quantitative assays of Esp secretion will be required to determine whether *espD* mutants indeed secrete less EspA and EspB. If decreased secretion is confirmed, further experimentation will be required to determine whether this effect is due to changes in expression, stability or export.

One may wonder whether the requirement of EspD for attaching and effacing activity is due to a direct effect of EspD on host cells or whether this requirement is mediated simply by the effect of EspD on secretion of EspA and EspB, which may be more directly involved. We propose that EspD is also directly involved in attaching and effacing lesion formation. We previously described a mutant that is unable to secrete detectable amounts of EspB but is still able to form attaching and effacing lesions (37). We postulated that very little EspB secretion is required for the attaching and effacing effect. Since the *espD* mutant is able to secrete detectable amounts of EspB, it follows that any effect of the mutation on EspB secretion is not sufficient to explain the inability of the mutant to mediate the attaching and effacing effect.

We have little information on the precise role of the Esp proteins in signal transduction leading to attaching and effacing activity. By analogy with the Yop system, it may be that secretion of these proteins into the medium is not relevant to pathogenesis. Rather, the true role of the Sep apparatus could be to translocate some or all of the Esp proteins to the host cell

cytoplasm. Experiments to address this possibility are ongoing in our laboratory.

## ACKNOWLEDGMENTS

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