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# 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

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**Enteropathogenic *Escherichia coli* (EPEC) secretes at least five proteins. Two of these proteins, EspA and EspB (previously called EaeB), activate signal transduction pathways in host epithelial cells. While the role of the other three proteins (39, 40, and 110 kDa) remains undetermined, secretion of all five proteins is under the control of *perA*, a known positive regulator of several EPEC virulence factors. On the basis of amino-terminal protein sequence data, we cloned and sequenced the gene which encodes the 110-kDa secreted protein and examined its possible role in EPEC signaling and interaction with epithelial cells. In accordance with the terminology used for *espA* and *espB*, we called this gene *espC*, for EPEC-secreted protein C. We found significant homology between the predicted EspC protein sequence and a family of immunoglobulin A (IgA) protease-like proteins which are widespread among pathogenic bacteria. Members of this protein family are found in avian pathogenic *Escherichia coli* (Tsh), *Haemophilus influenzae* (Hap), and *Shigella flexneri* (SepA). Although these proteins and EspC do not encode IgA protease activity, they have considerable homology with IgA protease from *Neisseria gonorrhoeae* and *H. influenzae* and appear to use a export system for secretion. We found that genes homologous to *espC* also exist in other pathogenic bacteria which cause attaching and effacing lesions, including *Hafnia alvei* biotype 19982, *Citrobacter freundii* biotype 4280, and rabbit diarrheagenic *E. coli* (RDEC-1). Although these strains secrete various proteins similar in molecular size to the proteins secreted by EPEC, we did not detect secretion of a 110-kDa protein by these strains. To examine the possible role of EspC in EPEC interactions with epithelial cells, we constructed a deletion mutant in *espC* by allelic exchange and characterized the mutant by standard tissue culture assays. We found that EspC is not necessary for mediating EPEC-induced signal transduction in HeLa epithelial cells and does not play a role in adherence or invasion of tissue culture cells.**

Secretion of proteins by gram-negative bacteria requires their transfer across both the cytosolic (inner) and outer membranes in addition to the intervening periplasmic space. To facilitate this, these organisms have developed a variety of secretion mechanisms, as exemplified by the *Escherichia coli* hemolysin (type I pathway) system and the *sec*-dependent release of pullulanase (type II pathway), which have been studied extensively (9, 36, 46–48). Recently, a type III secretion system family has been identified in many gram-negative animal pathogens such as *Yersinia* sp. (8, 41), *Shigella flexneri* (2–4, 55), and *Salmonella typhimurium* (11, 21) and in plant pathogens such as *Pseudomonas syringae*, *Pseudomonas solanacearum*, and *Xanthomonas campestris* pv. campestris (54). A distinguishing characteristic of proteins secreted by the type III system is the lack of a cleavable leader peptide and the requirement for specific chaperones. Many of the proteins released by the type III system have been shown to be virulence factors, such as the invasion plasmid antigens (Ipas) of *Shigella* spp. (25, 26, 51) and the *Yersinia* outer membrane proteins (Yops) (12).

Recently, it has been shown that enteropathogenic *E. coli* (EPEC), a human pathogen that causes neonatal diarrhea, also encodes a type III secretion system mediated by the *sep* gene products (27). This system is responsible for secretion of at least four proteins, two of which (EspA and EspB) have been characterized as proteins essential for transducing signals to host cells (15, 19, 31). EPEC induced signaling leads to inositol phosphate fluxes (20), increased intracellular calcium levels (5), host protein phosphorylation (38, 39, 49), and accumulation of actin and other cytoskeletal proteins beneath adherent bacteria (18, 35). However, strains containing mutations in tested *sep* genes, *espA*, or *espB* are deficient in activating epithelial signaling (13, 19, 30, 31).

In addition to these secreted effector molecules, an EPEC outer membrane protein, intimin, is required for intimate bacterial attachment to host cells (28, 29) and to focus the polymerization of cytoskeletal components beneath the adherent bacteria (18, 35). In the gut, EPEC attachment and EPEC-mediated cytoskeletal rearrangements lead to the disruption of the intestinal microvilli. This phenotype is known as the attaching and effacing (A/E) lesion phenotype (42).

In addition to the secretion of the four proteins mentioned above via the type III pathway, EPEC also releases a fifth protein by an alternative mechanism. Although secretion of this protein is not altered by mutations in the *sep* genes, its secretion is coregulated with *sep*-mediated protein secretion (30). Both *sep*-dependent and independent secretion pathways are affected by temperature, $CO_2$, and a positive regulator, *perA* (23, 24, 27, 30). *perA* is responsible for the regulation of several known virulence factors such as intimin and BfpA (a protein involved in the formation of microcolonies and efficient binding to host cells) (22). In this study, we examined the

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype or description | Reference or source |
|---|---|---|
| Strains | | |
| EPEC | | |
| E2368/69 | Wild type | 13 |
| JPN15 | pMAR2-cured E2368/69 | 13 |
| UMD864 | E2368/69 with *espB* deletion | 23 |
| MAS111 | E2368/69 with *espC* deletion | This study |
| MAS112 | MAS111 complemented with pMS14 | This study |
| MAS113 | MAS111 complemented with pMS15 | This study |
| MAS120 | JPN15/pCVD450 with *espC* deletion | This study |
| MAS121 | *E. coli* K-12 transformed with pMS16 | This study |
| Rabbit diarrheagenic *E. coli* RDEC-1 | Wild type | 10 |
| *Citrobacter freundii* | | |
| DBS100 (*C. freundii* biotype 4280) | EaeA$^+$ EspB$^+$ | 6 |
| DBS342 | Type strain | |
| *Hafnia alvei* | | |
| 19982 (biotype) | A/E$^+$ *eaeA*$^+$ | 1 |
| ATCC 13337 | Type strain | |
| *E. coli* | | |
| DH5α | *supE44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Bethesda Research Laboratories Life Technologies Inc. |
| K-12 | | |
| SM10λpir | Permissive host for pCVD422 | 52 |
| Plasmids | | |
| pACYC184 | Low-copy-number plasmid; Cm$^r$ Tc$^r$ | New England Biolabs |
| pBluescript SK(+) | High-copy-number phagemid; Ap$^r$ | Stratagene |
| pCVD422 | Suicide plasmid for positive selection of allelic DNA exchange | 14 |
| pMW119 | Low-copy-number plasmid, Ap$^r$ | |
| pCVD450 | pACYC184 containing the *perA* regulator | 15 |
| pMS6 | *espC* 5′ end in pbluescript; Ap$^r$ | This study |
| pMS7 | *espC* 3′ end in pbluescript; Ap$^r$ | This study |
| pMS14 | *espC* in pACYC184; Cm$^r$ | This study |
| pMS15 | Δ*espC* in pACYC184, Cm$^r$ | This study |
| pMS16 | *espC* in pMW119, Ap$^r$ | This study |

role of the 110-kDa secreted protein in EPEC signal transduction in epithelial cells. We describe the cloning of the structural gene, characterization of the 110-kDa product, and distribution of the gene and product among other A/E pathogens.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown overnight in Luria-Bertani (LB) broth or in Dulbecco's minimal Eagle medium (DMEM) at 37°C without shaking.

HeLa (ATCC CCL2), HEp-2, and CaCo-2 cells were grown at 37°C 5% $CO_2$ in minimal Eagle medium supplemented with 10% (vol/vol) fetal calf serum.

**Cloning and DNA sequence analysis of *espC*.** To clone the *espC* gene, the degenerate oligonucleotide MS2 [5′-GCCCAIACA(G)TTA(G)TCA(G,T)ATA(G)TT-3′], which was derived from N-terminal protein sequencing (30), was hybridized with *Eco*RI-*Sal*I-digested chromosomal DNA from EPEC E2368/69 under stringent hybridization conditions. This probe hybridized to a 2,000-bp *Eco*RI-*Sal*I fragment which was cloned into the vector pBluescript SK(+). The resulting plasmid was called pMS6. Since this fragment was insufficient to encode the entire 110-kDa protein, it was used as a hybridization probe to identify a 5,500-bp *Bgl*II chromosomal fragment. This fragment, which contained a 130-bp overlap with pMS1, was also cloned into the vector pBluescript SK(+) and called pMS7. To construct a clone which contained contiguous DNA from both fragments, the 2,000-bp *Eco*RI-*Sal*I fragment from pMS1 was cloned into pACYC184. The resulting clone was digested with *Sal*I-*Eag*I and recombined with the 5,500-bp *Sal*I*-*Eag*I fragment (* indicates partial digestion) of plasmid pMS7, and the larger resulting chimera was called pMS14. To express EspC under the control of the *lac* promoter in *E. coli* K-12, we amplified *espC* by PCR with the oligonucleotides MS66 (5′-AAAGGATCCCTGGTGATAAAAACATTATGTG-3′) and MS67 (5′-AAAGAATTCGCAGTATATAAACATACTCAG-3′) and cloned the resulting fragment behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter of the vector pMW119. The resulting plasmid was called pMS16 and transformed into the *E. coli* laboratory strain K-12.

The plasmids pMS1 and pMS7 were then digested with exonuclease III (Erase-a-Base; Promega) to obtain a set of nested deletions. Priming of DNA sequencing reactions utilized the M13 forward primer and the T3 primer. By using the exonuclease III deletion clones, both DNA strands were sequenced, including a 250-bp overlap between pMS1 and pMS7. The DNA sequence of *espC* was determined with the *Taq* DyeDeoxy terminator cycle sequencing kit from Applied Biosystems as described in the manufacturer's instructions. Sequence similarity searches were carried out with the BLAST program of the National Center for Biotechnology Information. The Seqapp program from Don Gilbert (Biocomputing Office, Biology Department, Indiana University, Bloomington) was used for alignments of the predicted EspC sequence.

**Construction of an *espC* deletion mutant in EPEC E2348/69.** We used plasmid pMS1, which contained the first 1,250 bp of *espC*, to create a 5′ deletion of 369 bp between bp 51 and bp 420 of the open reading frame. This was done by inverse PCR amplification with the oligonucleotides MS9 (5′-CAAAGCTTCCGGATGACGAATGGAGAT-3′) and MS10 (5′-CAAAGCTTTCAGGCCAGTTCGGATACAGC-3′). Both oligonucleotides contained a *Hin*dIII site as a 5′ extension, allowing the religation of the amplification product. The oligonucleotide MS9 also introduced a stop codon in front of the newly created *Hin*dIII site to terminate protein translation. The 1,600-bp *Sma*I-*Sal*I fragment of the deleted clone was then transferred into pCVD422 (14), a positive-selection suicide vector containing the *sacB* gene, and transformed into SM10λpir (51). Selection for homologous recombination was carried out as described before (14). One recombinant clone which was deleted in the chromosomal *espC* locus as shown by whole-cell PCR was identified. This strain failed to secrete the 110-kDa protein and was designated MAS111 and used in further investigations.

To create an in-frame deletion mutant which was missing the putative catalytic serine endopeptidase site (GDSGS), we used inverse PCR as described above. The oligonucleotides MS64 (5′-TTTCCCGGGGGTGACAATACTACAGTCGACTGG-3′) and MS65 (5′-TTTCCCGGGAGCTCCACCTATAGATGTAGTAGC-3′) were used to delete 831 bp (bp 386 to 1217) in plasmid pMS14 and also to introduce a *Sma*I site to allow religation. The resulting plasmid was called pMS15 and transformed into the *espC* deletion strain MAS111. The new strain

expressed an EspC product with an apparent molecular mass that was 30 kDa smaller than that of the wild-type protein and was designated MAS113.

**IgA protease activity.** EPEC and MAS111 were grown as overnight standing cultures at 37°C in DMEM. The next morning, the bacteria were removed by centrifugation for 5 min at 8,000 × *g*. Proteases in the supernatant were removed by cation-exchange chromatography (Bio-Rad; S Cartridge) since, otherwise, degradation of the supernatant proteins was observed during concentration. This procedure did not remove the EspC protein, as the flowthrough still contained all EPEC-secreted proteins as tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant proteins were then concentrated 500-fold by ultrafiltration (Amicon) through a 30-kDa filter membrane (Diaflo ultrafilters; model PM30). Eight microliters of a 1-mg/ml concentration of human immunoglobulin A (IgA) was added to 17 μl of concentrated supernatant (about 75 μg of EspC per ml, as estimated from SDS-PAGE), and the mixture was incubated overnight at 37°C. Alternatively, fresh culture supernatant was incubated with IgA. Five microliters of IgA protease of *Neisseria gonorrhoeae* (100 μg/ml) was used as a positive control (purified IgA protease was a gift from T. F. Meyer). Samples were analyzed by SDS-PAGE on a 10% polyacrylamide gel, and the gel was silver stained.

**Adherence and invasion assay.** Adherence and invasion assays were carried out as described by Finlay and Falkow (17) with slight modifications. Briefly, HeLa cells were seeded in 24-well tissue culture plates at a density of $10^5$ cells per well and grown overnight at 37°C under 5% $CO_2$. Ten microliters of fresh bacterial overnight cultures was added to each well, and the cells were infected for 3 h at 37°C under 5% $CO_2$. To measure invasion, bacterium-infected monolayers were washed twice with phosphate-buffered saline (PBS) and incubated with fresh tissue culture medium containing 100 μg of gentamicin per ml to kill extracellular EPEC. After 2 h, monolayers were washed with PBS and lysed by incubating with 200 μl of 1% Triton X-100 per well for 5 min. Appropriate dilutions were made in PBS and plated onto LB agar plates. To determine the number of adherent bacteria, washed monolayers were lysed immediately before the gentamicin step.

**Protein secretion assay.** Culture supernatants were obtained as described above and elsewhere (30). Proteins were then precipitated with 10% trichloroacetic acid for 1 h on ice. The proteins were collected by centrifugation (18,000 × *g*, 15 min), resuspended in 1× SDS-PAGE loading buffer, and analyzed by SDS-PAGE on a 10% polyacrylamide gel. For radioactive labeling, bacteria were grown in DMEM without methionine and cysteine at 37°C to an optical density at 600 nm of 0.2. Then, 10 μCi of [$^{35}$S]methionine and [$^{35}$S]cysteine (Tran$^{35}$S label; ICN) was added, and the bacteria were incubated for 30 min at 37°C. Secreted proteins were separated by SDS-PAGE and detected by autoradiography.

**Southern blot and colony blot hybridization.** Southern and colony blot hybridizations using stringent hybridization conditions were carried out as described before (58). Hybridization of chromosomal DNA with $^{32}$P-labeled oligonucleotide MS2 was carried out at 42°C. Following overnight incubation, the membranes were washed twice at 23°C for 30 min and once at 50°C for 10 min in washing buffer (2× SSPE, 0.05% SDS) and exposed with X-ray film overnight (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7]). Hybridizations with fluorescein-labeled DNA were carried out by using the random prime DNA labeling kit from Amersham as described in the manufacturer's recommendations.

**Immunofluorescent microscopy and Western blotting (immunoblotting).** Immunofluorescent microscopy and Western blotting were carried out as described before (49). To raise antibody against the EspC protein, EPEC supernatant proteins were concentrated by ultrafiltration, separated by 10% polyacrylamide gel electrophoresis, and transferred onto nitrocellulose. Nitrocellulose containing the 110-kDa band was then excised, fragmented by sonication, and injected into rabbits.

## RESULTS

**Identification, cloning, and sequencing of the chromosomal *espC* locus.** We previously determined the N-terminal sequence for the first 20 amino acids of the 110-kDa EPEC-secreted protein (30). On the basis of this sequence, we designed a degenerate 20-bp oligonucleotide (MS2) to hybridize with the 5′ end of the corresponding gene. MS2 was the first hybridization probe we used to detect the fragment that resulted in clone pMS1 and encoded the 5′ end of *espC*. This *espC* 5′ fragment was used in a second hybridization step to identify and clone the 3′ end of *espC* (including an overlapping region with clone pMS1) that was called pMS7. We then created a set of nested deletions in the cloned DNA regions of pMS1 and pMS7. From these deletion clones, the DNA sequence of the gene encoding the 110-kDa protein was determined. An open reading frame of 3,924 bp was identified and subcloned (pMS14). Analysis of this gene and the deduced protein sequence revealed the presence of an N-terminal extension not present on the mature secreted protein. This 53-amino-acid extension presumably encodes a signal peptide which is processed during the transport of EspC across the cytoplasmic bacterial membrane (33).

We found that EspC was also secreted by an EPEC strain (JPN15) missing the virulence plasmid, indicating that *espC* is not located on the virulence plasmid pMAR2 (known to encode *perA* and *bfpA*) but is probably on the chromosome. It has been recently reported that a larger region of the EPEC chromosome, the LEE region, is necessary for A/E lesion formation (40) and contains all known bacterial loci involved in this phenotype. We probed two cosmid clones, 5A10 and 9B3, which span this region, for hybridization with an *espC*-specific oligonucleotide (MS2). However, we were unable to detect a homologous region in either cosmid clone (data not shown). Additionally, sequence data of the LEE region (29a) does not match the 5′ end of the *espC* sequence. On the basis of the results described above, *espC* is not contained within the LEE region.

**Data bank comparison of the deduced amino acid sequence of EspC.** We next compared the EspC sequence with other known protein sequences with the Blast P and Blast X programs of the National Center for Biotechnology Information. This data bank comparison revealed a high homology of EspC throughout the entire sequence with two proteins, SepA of *Shigella flexneri* and Tsh of avian pathogenic *E. coli*, although the degree in homology was significantly higher in the carboxy-terminal portions of these proteins (Fig. 1). SepA *Shigella* mutants are attenuated for virulence in a rabbit-based ileal loop model (7), while Tsh is responsible for hemagglutination of *E. coli* at low temperatures (45). The carboxy-terminal region of EspC was homologous to SepA with 94% similarity and 89% identity over the last 470 amino acids. The homology of EspC to Tsh was somewhat lower, with 75% similarity and 57% identity over the carboxy-terminal 450 amino acids. However, EspC exhibited greater homology with Tsh than with SepA at the amino-terminal region. A second group of proteins from pathogenic bacteria showed a lesser degree of homology with EspC. This group included IgA protease of *H. influenzae* and *N. gonorrhoeae*. EspC exhibited homology to a higher degree with the amino-terminal protease domain. Twenty-four percent identity and 46% similarity also exist between the Hap protein of *H. influenzae* and EspC. Hap is responsible to some extent for mediating *H. influenzae* adherence and penetration into tissue culture cells (53). Additionally, there were similar regions within the carboxy-terminal region to the sequences of virulence proteins BrkA and pertactin of *Bordetella pertussis*. These proteins mediate serum resistance and adhesion to host cells, respectively (16, 37). C-terminal homology also exists for Hsr, a 150-kDa ring-forming surface antigen of *Helicobacter mustelae* (43).

**Test for IgA protease activity.** Because of the sequence homology between EspC and the family of IgA proteases, we subsequently investigated the possibility that EspC contained IgA protease activity or has a similar secretion pathway to that used for the export of the IgA protease family proteins. Supernatant proteins of EPEC and MAS111 were concentrated by ultrafiltration and extensively washed with PBS. Human IgA was then incubated with concentrated or unconcentrated supernatants and subsequently analyzed by SDS-PAGE under reducing conditions. In both cases, IgA was not cleaved or degraded (Fig. 2). However, purified *N. gonorrhoeae* IgA protease, which was used as a positive control, cleaved the human IgA.

```
EspC MNKIYALKYC HATGGLIAVS ELASRVMKKA AR-----GSL LALFNLSLYG   50
SepA MNKIYYLKYC HITKSLIAVS ELARRVTCKS HRRLSRRVIL TSVAALSLSS   50
Tsh  MNRIYSLRYS AVARGFIAVS EFARKCVHKS VRRLCFPVLL LIPV---LFS   50
                          N-domain             H-domain

EspC AF--LSASQA AQLNIDNVWA RDYLDLAQNK GVFKAGATNV SIQLKNGQTF  100
SepA AWPALSATVS AEIPYQI--- --FRDFAENK GQFTPGTTNI SIYDKQGNLV  100
Tsh  A-GSLAGTVN NELGYQL--- --FRDFAENK GMFRPGATNI AIYNKQGEFV  100
     C-domain

EspC -NFPNVPIPD FSPASNKGAT TSIGG----- -AYSVTATHN GTTHHAISTQ  150
SepA GKLDKAPMAD FSSATITTGS LPPGDHTLYS PQYVVTAKHV SGSDTMSFG-  150
Tsh  GTLDKAAMPD FSAVDSE--- --IGVATLIN PQYIASVKHN GGYTNVSFGD  150

EspC NWGQSSYKYI DRMTNGDFAV T--RLDKFVV ETTGVK-NSV DFSLNSHDAL  200
SepA -YAKNTYTAV GTNNNSGLDI KTRRLSKLVT EVAPAEVSDI GAVSGAYQAG  200
Tsh  --GENRYNIV DRNNAPSLDF HAPRLDKLVT EVAPTAVTAQ GAVAGAYLDK  200

EspC ERYGVEINGE KKIIGFRVGA GTTYTAQNGN TYST-GQVYN P--LLLSASM  250
SepA GRFT-EF--- -----YRLGG GMQYVKDKNG --NR-TQVYT NGGFLVGGTV  250
Tsh  ERYPV-F--- -----YRLGS GTQYIKDSNG QLTQMGGAYS ---WLTGGTV  250

EspC FQLNW---DN KRPYNNTTPF YNETTG---- ----GDSGSG FYLYDNVKKE  300
SepA SALNSYNNGQ MITAQTGDIF LIPPNGPLAN YLNMGDSGSP LFAYDSLQKK  300
Tsh  GSLSSYQNGE MISTSSGLVF DYKLNGAMPI YGEAGDSGSP LFAFDTVQNK  300

EspC WVMLGTLF-- -GIASSGADV GSILNQYDEN TVNGLKNKFT QKVQL-NNNT  350
SepA WVLIGVLS-- -SGTNYGNNW VVTTQDFLGQ QPQ---NDFD KTIAY-TSGE  350
Tsh  WVLVGVLTAG NGAGGRGNNW AVIPLDFIGQ K---FNEDND APVTFRTSEG  350

EspC MSLNSDSFTL AGNNTAVAKN NNNY------ --------KD LSFSGG-GSI  400
SepA GVLQWK-YDA ANGTGTLTQG NTTWGYAWKE RKLILNAGKN LLFTGNNGEV  400
Tsh  GALEW-SFNS STGAGALTQG TTTYAMH-GQ QGNDLNAGKN LIFQGQNGQI  400

EspC NFDNDVNIGS GGLIFDAGHH YTVTG-NNKT FKGAGLDIGD NTTVDWNVKG  450
SepA VLQNSVNQGA GYLQFAGD-- YRVSALNGQT WMGGGIITDK GTHVLWQVNG  450
Tsh  NLKDSVSQGA GSLTFRD--N YTVTTSNGST WTGAGIVVDN GVSVNWQVNG  450

EspC VVGDNLHKIG AGTLNVNVS- -QGNNLKTGD GLVVLN---- ---SANAFDN  500
SepA VAGDNLHKTG EGTLTVNGTG VNAGGLKVGD GTVILNQQAD RDGKVQAFSS  500
Tsh  VKGDNLHKIG EGTLTVQGTG INEGGLKVGD GKVVLNQQAD NKGQVQAFSS  500

EspC IYMASGHGVV KINHSAALNQ NNDYRGIFFT ENGGTLDLNG YDQSFNKIAA  550
SepA VGIASGRPTV VLSDSQQVNP DNISWGY--- -RGGRLELNG NNLTFTRLQA  550
Tsh  VNIASGRPTV VLTDERQVNP DTVSWGY--- -RGGTLDVNG NSLTFHQLKA  550

EspC TDIGALITNS AVQKAVLSVN NQSHYMYHGS VSGNTEINHQ FDTQKNNSRL  600
SepA ADYGAIITNN SEKKSTVTLD LQT--LKASD INVPVNTVSI FGGRGAPGDL  600
Tsh  ADYGAVLANN VDKRATITLD YA---LRADK VALNGWSES- --GKGTAGNL  600

EspC I-LDG----- ---------- ---------- -----NVDIT NDINIKNSQL  650
SepA Y-YDSS---T KQYFILKASS YSPFFSDLNN SSVWQNVGKD HNKAIDTVKQ  650
Tsh  YKYNNPYTNT TDYFILKQST YGYFPTDQSS NATWEFVGHS QGDAQKLVAD  650

EspC HHAGTCLHLM LFLERVG--V TCMLPGVICE KDYVS---GI QQQENSANKN  700
SepA QKIEASSQPY MYHGQLNGNM DVNIPQL-SG KDVLALDGSV NLPEGSITKK  700
Tsh  RFNTAG---Y LFHGQLKGNL NVDNRLPEGV TGALVMDGAA DIS-GTFTQE  700

EspC NNT-DYKTNN QV-------- ---------- ------SSFE QPDWENRLFK  750
SepA SGTLIFQGHP VIHA------ ---------- ---GTTTSSS QSDWETRQFT  750
Tsh  NGRLTLQGHP VIHAYNTQSV ADKLAASGDH SVLTQPTSFS QEDWENRSFT  750

EspC FKTLNLINSD FIVGRNAIVV GDISANNSTL SLSGKDTKVH IDMYDGKNIT  800
SepA LEKLKLDAAT FHLSRNGKMQ GDINATNGST VILGS-SRVF TDRSDG---T  800
Tsh  FDRLSLKNTD FGLGRNATLN TTIQADNSSV TL--GDSRVF IDKNDGQ---  800

EspC GDGFGFRQDI KDGVSVSPES SSYFGNVTLN NHSLLDIGNK FTGGIEAYDS  850
SepA GNAVSSVEGS ATATTVG-DQ SDYSGNVTLE NKSSLQIMER FTGGIEAYDS  850
Tsh  GTAFTLEEGT SVATK-DADK SVFNGTVNLD NQSVLNINDI FNGGIQANNS  850

EspC SVSVTSQNAV FDRVGSFVNS SLTLEKGAKL TAQGGIFSTG AVDVKENASL  900
SepA TVSVTSQNAV FDRVGSFVNS SLTLGKGAKL TAQSGIFSTG AVDVKENASL  900
Tsh  TVNISSDSAV LGN-STLTST ALNLNKGANA LASQSFVSDG PVNISD-AAL  900

EspC ILTGTPSAQK QEYYSPVIST TEGINL-GDK ASLSVKNMGY LSSDIHAGTT  950
SepA TLTGMPSAQK QGYYSPVIST TEGINL-EDN ASFSVKNMGY LSSDIHAGTT  950
Tsh  SLNSRPDEVS HTLL-PVYDY AGSWNLKGDD ARLNVGPYSM LSGNINVQDK  950

EspC AATINLGDGD AE---T-DSP LFSSLIRGYN AVLSGNITGE QSTVNMNNAL 1000
SepA AATINLGDSD ADAGKT-DSP LFSSLMKGYN AVLRGSITGA QSTVNMINAL 1000
Tsh  GTVTLGGEGE LSPDLTLQNQ MLYSLFNGYR NIWSGSLNAP DATVSMTDTQ 1000

EspC WYSDGNSTIG TLKSTGGRVE LGGGKD-FAT LRVKELNANN ATFLMHTNTS 1050
SepA WYSDGKSEAG ALKAKGSRIE LGDGKH-FAT LQVKELSADN TTFLMHTNNS 1050
Tsh  WSMNGNSTAG NMKLNRTIVG FNGGTSPFTT LTTDNLDAVQ SAFVMRTDLN 1050

EspC QADQLNVTNK LLGSNNTVLV DFLNKPASE- -MNVTLITAP KGSDEKTFTA 1100
SepA RADQLNVTDK LSGSNNSVLV DFLNKPASE- -MSVTLITAP KGSDEKTFTA 1100
Tsh  KADKLVINKS ATGHDNSIWV NFLKKPSNKD TLDIPLVSAP EATADNLFRA 1100

EspC GTQQIGFSNV TPVISTEKTD DATKWMLTGY QTVSDAGASK TATDFMASGY 1150
SepA GTQQIGFSNV TPVISTEKTD DATKWVLTGY QTTADAGASK AAKDFMASGY 1150
Tsh  STRVVGFSDV TPILSVRKED GKKEWVLDGY QVARNDGQGK AAATFMHISY 1150

EspC KSFLTEVNNL NKRMGDLRDT QGDAGVWARI MNGTGSADGG YSDNYTHVQI 1200
SepA KSFLTEVNNL NKRMGDLRDT QGDAGVWARI MNGTGSADGD YSDNYTHVQI 1200
Tsh  NNFITEVNNL NKRMGDLRDI NGEAGTWVRL LNGSGSADGG FTDHYTLLQM 1200

EspC GADRKHELDG VDLFTGALLT YTDSNASSHA FSGKTKSVGG GLYASALFDS 1250
SepA GVDRKHELDG VDLFTGALLT YTDSNASSHA FSGKNKSVGG GLYASALFNS 1250
Tsh  GADRKHELGS MDLFTGVMAT YTDTDASADL YSGKTKSWGG GFYASGLFRS 1250

EspC GAYFDLIGKY LHHDNQYTAS FASLGTKDYS SHSWYAGAEV GYRYHLSEES 1300
SepA GAYFDLIGKY LHHDNQHTAN FASLGTKDYS SHSWYAGAEV GYRYHLTKES 1300
Tsh  GAYFDVIAKY IHNENKYDLN FAGAGKQNFR SHSLYAGAEV GYRYHLTDTT 1300

EspC WVEPQMELVY GSVSGKSFIW EDRGMALSMK DKDYNPLIGR TGVDVGRTFS 1350
SepA WVEPQIELVY GSVSGKAFSW EARGMALSMK DKDYNPLIGR TGVDVGRAFS 1350
Tsh  FVEPQAELVW GRLQGQTFNW NDSGMDVSMR RNSVNPLVGR TGVVSGKTFS 1350

EspC GDDWKITARA GLGYQFDLLA NGETVLRDAS GEKRFEGEKD SRMLMNVGMN 1400
SepA GDDWKITARA GLGYQFDLLA NGETVLQDAS GEKRFEGEKD SRMLMTVGMN 1400
Tsh  GKDWSLTARA GLHYEFDLTD SADVHLKDAA GEHQINGRKD SRMLYGVGLN 1400

EspC AEIKDNMRFG LELEKSAFGK YNVDNAINAN FRYSF                 1435
SepA AEIKDNMRLG LELEKSAFGK YNVDNAINAN FRYVF                 1435
Tsh  ARFGDNTRLG LEVERSAFGK YNTDDAINAN IRYSF                 1435
```

FIG. 1. Amino acid sequence alignment of EspC with SepA of *Shigella flexneri* (7) and Tsh of avian pathogenic *E. coli* (44). Regions of identity are shaded. Typical signal sequence features (N, H, and C domains) for *sec*-dependent secretion are indicated below the protein sequence. The first amino acid of the mature proteins is underlined and in bold type. The catalytic site of IgA proteases (residues 285 to 290) is underlined and in bold italic type, as is the putative carboxy-terminal cleavage site of the helper domain for protein export across the outer membrane (residues 1162 to 1168).

**The putative serine endopeptidase site of EspC is not necessary for cleavage of the protease domain from the C-terminal helper domain.** The last step in secretion of IgA proteases from *N. gonorrhoeae* and *H. influenzae* into the extracellular space involves the autoproteolytic activity of the catalytic serine endopeptidase site. This event leads to the cleavage of the protease domain from the C-terminal helper domain which resides in the outer membrane (32, 33, 44). Because of the striking homology between IgA proteases and EspC, we tested whether the process of autocleavage is also necessary for secretion of EspC in EPEC. We constructed the strain MAS113, which carried a 429-bp in-frame deletion in the *espC* gene including the consensus region of the putative serine endopeptidase site (GDSDG). The mutant still secreted EspC (Fig. 3), indicating that the consensus sequence of the catalytic site is not necessary for release of EspC from the helper domain. As expected, the EspC protein secreted by the deletion strain MAS113 was 277 amino acids shorter than the wild-type protein and about 75 kDa in size. This result was also supported by the secretion of EspC into the supernatant by *E. coli* K-12. This strain was transformed with the plasmid pMS16, which contained the *espC* gene under control of the *lac* promotor (Fig. 3).

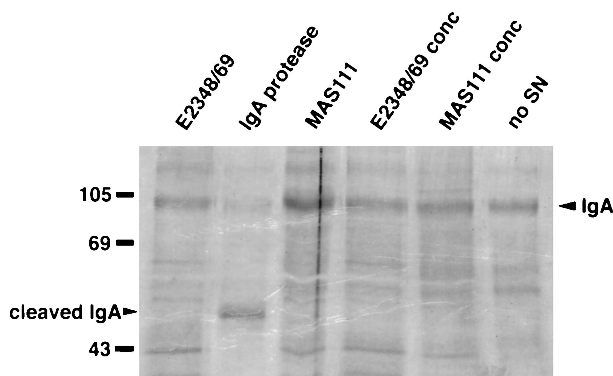


FIG. 2. Test for EspC IgA protease activity. Concentrated (conc) and unconcentrated supernatants of EPEC wild-type strain E2348/69 and MAS111 were incubated with human IgA at 37°C overnight. As a positive control, purified IgA protease of *N. gonorrhoeae* was incubated with IgA under the same conditions. IgA alone was also tested. Incubation mixtures were analyzed by SDS-PAGE on a 10% polyacrylamide gel, and proteins were stained by silver staining. Lanes are labeled to indicate IgA incubated with supernatant from the identified strains, with purified IgA protease, or with no supernatant (no SN). Molecular mass standards (in kilodaltons) are indicated on the left.

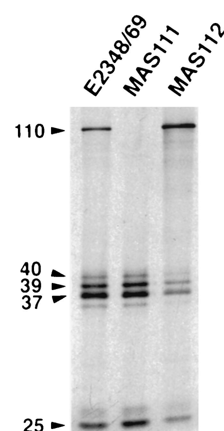


FIG. 4. Profile of secreted proteins of EPEC wild-type strain E2348/69, MAS111, and MAS112. EspC (110 kDa) is not secreted by the Δ*espC* deletion mutant MAS111. The mutant was complemented by introduction of the plasmid pMS14 (MAS112). Bacteria were grown in DMEM to an optical density at 600 nm of 0.2. Bacterial proteins were then labeled for 30 min with [$^{35}$S]cysteine and [$^{35}$S]methionine. After removal of bacteria by centrifugation, supernatant proteins were precipitated with 10% trichloroacetic acid, separated by 12% polyacrylamide gel electrophoresis, and visualized by autoradiography. Arrowheads mark the five EPEC-secreted proteins, and their apparent molecular sizes in kilodaltons are indicated on the left.

**EspC is not involved in EPEC-mediated A/E lesion formation in cultured epithelial cells.** Because of the critical role two other EPEC-secreted proteins, EspA and EspB, play in the signaling process and A/E formation, we tested whether EspC was also necessary for this activity. We constructed a 369-bp deletion mutation followed by a stop codon in the 5′ region of the *espC* gene by use of the suicide plasmid pCVD422 (14). This vector allowed positive selection for allelic exchange of an *espC* deletion with the chromosomal *espC* gene. To confirm that the *espC* deletion mutant, designated MAS111, was lacking the 110-kDa secreted protein, we examined radiolabeled secretion profiles from MAS111 by methods described elsewhere (30). As expected, we found that MAS111 was lacking a secreted protein of 110 kDa (Fig. 4). When a plasmid containing the cloned 110-kDa protein (pMS14) was transformed into MAS111, this strain, designated MAS112, now secreted a 110-kDa protein, indicating that the Δ*espC* mutation could be complemented (Fig. 4).

We then tested MAS111 and the isogenic parental EPEC strain for their ability to adhere to and invade cultured epithelial cells. We did not observe significant differences between these strains in their levels of adherence to or invasion of epithelial cell lines, including HeLa (Fig. 5), HEp-2, or polarized Caco-2 cells. To exclude the possibility that differences in adherence and invasion levels between EPEC and MAS111 were being obscured by strong adherence and invasion levels

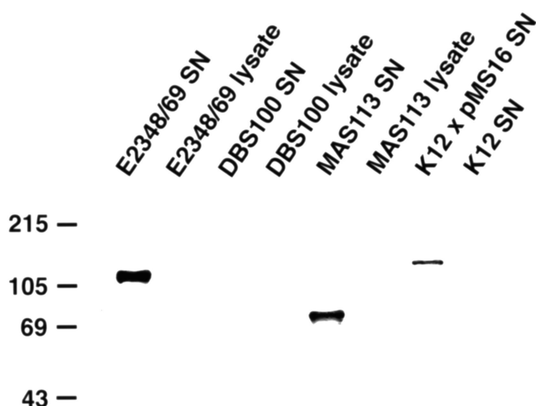


FIG. 3. Immunoblot analysis. Supernatants and whole-cell lysates of various strains (EPEC, *C. freundii* biotype 4280 (DBS100), and *E. coli* K-12) were separated by SDS-PAGE on a 10% polyacrylamide gel, electrophoretically transferred onto nitrocellulose, and probed with polyclonal anti-EspC antibody. Similar results to those obtained for DBS100 were also obtained for RDEC-1 and *Hafnia alvei* biotype 19982 (data not shown). In all three strains, neither secreted proteins nor proteins of whole-cell lysates cross-reacted with the anti-EspC antibody. The apparent molecular sizes in kilodaltons are indicated on the left.

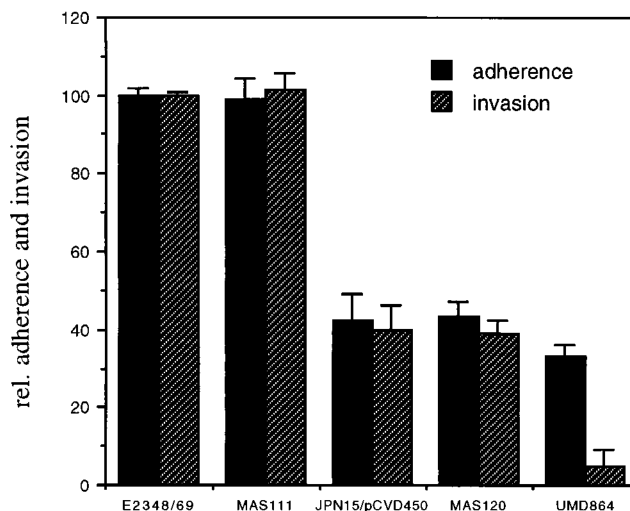


FIG. 5. Relative adherence to and invasion of various EPEC strains to HeLa cells. EPEC wild-type strain E2348/69 and JPN15/pCVD450 were compared with the corresponding strains deleted in the *espC* gene (MAS111 and MAS120). UMD864 carries a deletion in the *espB* gene. This mutant does not invade Hela cells and is also attenuated in adhesion. Adherence and invasion assays were performed in triplicate, and values are expressed relative to that of the EPEC wild-type strain. The total number of adherent and invasive EPEC bacteria are $3.5 \times 10^6$ and $4.5 \times 10^4$ bacteria per $10^6$ HeLa cells, respectively. Error bars represent standard deviations.

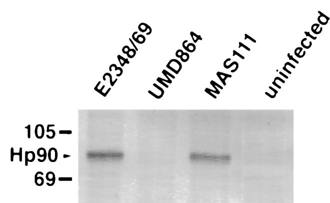


FIG. 6. Immunoblot analysis. After 3 h of infection of HeLa cells with various EPEC strains wild-type strain E2348/69, UMD864 [*espB* deletion mutant], and MAS111), Triton X-100-soluble HeLa cell membrane proteins were separated by SDS-PAGE on a 10% polyacrylamide gel, electrophoretically transferred onto nitrocellulose, and probed with anti-phosphotyrosine antibody. Lanes are labeled to indicate the infecting EPEC strains and the uninfected control. Molecular mass standards (in kilodaltons) are indicated on the left.

mediated by the bundle-forming pilus, we also constructed the same *espC* deletion in JPN15/pCVD450, which is an EPEC strain cured of the virulence plasmid and therefore lacking bundle-forming pili (22). This strain was designated MAS120. JPN15/pCVD450 contains a cloned copy of the positive regulator *perA* (23) that is necessary for transcription and expression of EPEC-secreted proteins. Because of the inability to form microcolonies, JPN15/pCVD450 adheres and invades less than the parental strain. However, no detectable difference in adherence or invasion levels between JPN15/pCVD450 and MAS120 was detected (Fig. 5).

To further test whether EspC was involved in mediating signals in EPEC-infected epithelial cells, we examined the effect of MAS111 ($\Delta$*espC*) on epithelial signaling and resulting cytoskeletal rearrangements. MAS111 was not defective for signaling or cytoskeletal rearrangements. Immunofluorescent microscopy studies of Hela cells infected with MAS111 using anti-phosphotyrosine and anti-$\alpha$-actinin antibodies demonstrated that there was still colocalization of tyrosine-phosphorylated proteins and actin condensation beneath MAS111 as has been previously reported for EPEC (18, 35, 49).

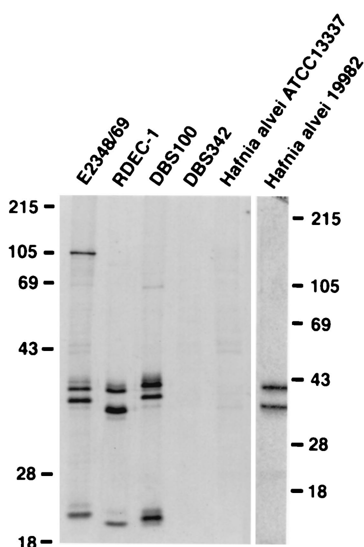


FIG. 7. Comparison of secreted protein profiles from A/E lesions causing pathogens (wild-type strain E2348/69, *C. freundii* biotype 4280 [DBS100], *Hafnia alvei* biotype 19982, and RDEC-1) and nonpathogenic strains (*C. freundii* [DBS342] and *Hafnia alvei* type strain ATCC 13337). Bacteria were grown in DMEM to an optical density at 600 nm of 0.2. Bacterial proteins were then labeled for 30 min with $^{35}$S. After removal of bacteria by centrifugation, supernatant proteins were precipitated with 10% trichloroacetic acid, separated by 12% polyacrylamide gel electrophoresis, and visualized by autoradiography. Molecular mass standards (in kilodaltons) are indicated on the left.

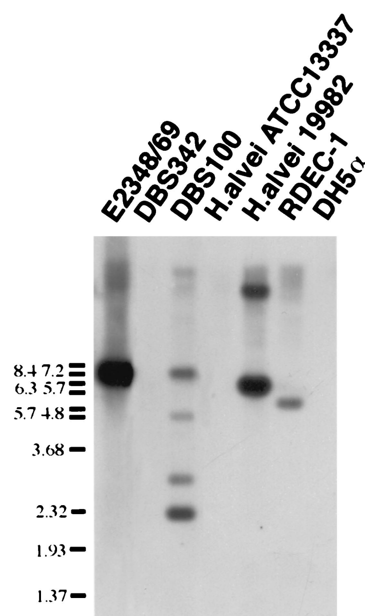


FIG. 8. High-stringency Southern hybridization analysis of chromosomal DNAs from various bacterial species with an *espC* probe. DNA of A/E lesion-causing bacteria (wild-type strain E2348/69, *C. freundii* biotype 4280 [DBS100], *Hafnia alvei* biotype 19982, and RDEC-1) and nonpathogenic bacteria (*C. freundii* [DBS342], *Hafnia alvei* type strain ATCC 13337, and *E. coli* DH5$\alpha$) were digested with *Bgl*II and hybridized with an internal 2,000 bp of *espC* DNA probe. Markers (in kilobases) are $\lambda$ DNA digested with *Bst*EII.

We have previously shown that a 90-kDa host protein, Hp90, becomes tyrosine phosphorylated following EPEC infection of epithelial cells (49). However, we also found that MAS111 still caused tyrosine phosphorylation of Hp90, as demonstrated by immune blotting HeLa membrane fractions with anti-phosphotyrosine antibodies (Fig. 6).

On the basis of all these results, we conclude that the 110-kDa protein does not play a role in EPEC-mediated epithelial cell signaling and cytoskeletal rearrangements, processes which are critical to A/E lesion formation in vitro.

**Distribution of *espC* in other A/E pathogens.** Rabbit diarrheagenic *E. coli* (RDEC-1), *Citrobacter freundii* biotype 4280 (DBS100), and *Hafnia alvei* biotype 19982 all cause A/E lesions (1, 6, 10). We examined whether these bacteria also secrete a 110-kDa protein. However, no member of this group secreted a protein of this size. Interestingly, two of the tested strains, RDEC-1 and DBS100, secreted proteins of similar sizes to the 25-, 37-, 39-, and 40-kDa proteins of EPEC, which might correspond to the four EPEC-secreted proteins exported by the type III pathway, including EspA and EspB. *Hafnia alvei* biotype 19982 secreted two proteins of 35 and 40 kDa, while secretion of a 25-kDa protein was only weakly detected. In contrast, type strains of *Hafnia alvei* (ATCC 13337) or *C. freundii* (DBS342) that do not cause A/E lesions, and are not associated with disease, do not secrete any proteins into the culture supernatant (Fig. 7).

Using Southern blotting, we also examined these various strains for the presence of a DNA sequence which hybridizes to an *espC* DNA probe, which corresponded to an internal 2,000-bp fragment of the *espC* gene. We identified homologous sequences to *espC* in RDEC-1, *C. freundii* biotype 4280 (DBS100), and *Hafnia alvei* biotype 19982, while chromosomal

DNA from nonpathogenic strains such as *C. freundii* DBS342, *Hafnia alvei* ATCC 13337, and *E. coli* DH5 did not hybridize with the *espC* probe (Fig. 8).

To examine whether some of these strains not only have homologous gene sequences but also express a protein homologous to EspC, we used polyclonal antiserum raised against the secreted form of EspC. We analyzed supernatants and whole-cell lysates of EPEC, RDEC-1, *C. freundii* biotype 4280 (DBS100), and *Hafnia alvei* biotype 19982 grown in cell culture medium by immunoblotting. EspC was detected only in EPEC supernatant (Fig. 3).

## DISCUSSION

In this study, we examined one of five proteins reported to be secreted by EPEC (30). This protein, which has also been reported to be secreted by others (27), is 110 kDa and was designated EspC for EPEC-secreted protein C. Starting with the amino-terminal protein sequence of mature secreted EspC, which was determined by protein sequencing (30), we identified a chromosomal gene that encoded an open reading frame of 1,306 amino acids. The first 53 predicted amino acids are missing from the secreted 110-kDa protein and have several characteristics found in signal peptides, which mediate *sec*-dependent export across the cytoplasmic membrane (46). These features include a polar stretch of positively charged amino acids (N domain), followed by a longer region of hydrophobic residues (H domain), and, finally, a less-hydrophobic region (C domain) that contains a signal peptidase recognition site (Fig. 1). The EspC open reading frame corresponds to a precursor molecule of about 140 kDa, approximately 30 kDa more than the mature, secreted 110-kDa form found in culture supernatant. This result suggests a possible secretion mechanism for EspC. Unlike the four other EPEC-secreted proteins (25 kDa [EspA], 37 kDa [EspB], 39 kDa, and 40 kDa), which are exported by a type III pathway using *sep*-encoded machinery (27, 30), EspC presumably uses a mechanism similar to that of IgA protease from *N. gonorrhoeae* and *H. influenzae*. As previously shown for *Neisseria* strain MS11, the IgA protease is made as a 169-kDa precursor that is transported through the cytoplasmic membrane by processing a signal sequence, using a *sec*-dependent pathway (33, 44). Transport through the outer membrane occurs via the core region of the carboxy-terminal β-domain (32). This region, whose predicted structure is a β-barrel, is 274 amino acids (31 kDa) in size and sufficient for its correct integration into and the transport of IgA protease across the outer membrane. The IgA protease domain is then cleaved off by autoproteolytic activity, while the β-core remains in the outer membrane. Although EspC is more similar to IgA proteases within the IgA protease domain, the similarity exists throughout the entire protein sequence and includes the putative motif for cleavage of the carboxy-terminal helper domain as well as the catalytic site of the IgA serine endopeptidase (Fig. 1). These motifs are also conserved in SepA, Tsh, and Hap, which are secreted proteins found in various pathogens (see below), although IgA protease activity has not been attributed to any of these proteins. We also did not detect IgA protease activity for EspC. Furthermore, the *hap* gene is a separate locus distinct from the IgA protease gene in *H. influenzae* (53). Although no other protease activity nor substrate has been found for these proteins, the proteolytic activity of the amino-terminal region may play a crucial role for the function of this protein family. We found that an *espC* mutant deleted in a region containing the catalytic serine endopeptidase domain was still able to secrete a truncated EspC product. This result does not exclude an autoproteolytic activity of the EspC protease domain but shows that autocleavage is not necessary to release EspC into the extracellular space. It appears likely that there is another substrate for the protease domain which is associated with a function of EspC and which remains to be identified. Proteases responsible for the release of EspC might be outer membrane proteins of EPEC with an intrinsic proteolytic activity such as that of OmpT. OmpT was shown to release passenger proteins fused to the helper domain of IgA protease of *N. gonorrhoeae* into the extracellular space (34). The involvement of a general outer membrane protease is also indicated by the fact that EspC is secreted from *E. coli* K-12 when expressed from a *lac* promoter. No EspC was detected in a whole-cell lysate of EPEC by immunoblotting with polyclonal antibodies against the secreted form of EspC. This means that either no EspC remains associated with the outer membrane or, less likely, that the antibody cannot recognize the membrane-bound form of EspC. There are three other secreted proteins previously described that display a high homology to the IgA protease family. Tsh was found in avian pathogenic *E. coli* and characterized as a protein which agglutinates erythrocytes at 26°C but not at 37°C (45). Hap is secreted by *H. influenzae* and restored the ability of a laboratory strain (DB117) to adhere to and penetrate HeLa cells at up to 4% of the wild-type level (53). DB117 itself was completely deficient in these properties. The most recently identified addition to this family of IgA protease-like proteins is SepA. This protein is secreted by *Shigella flexneri* into the supernatant. A deletion mutant in *sepA* exhibited attenuated virulence in the rabbit ligated ileal loop model (7), although the exact mechanisms for this attenuation have not been defined.

We also tested whether EspC was needed for mediating signal transduction and cytoskeletal rearrangements in epithelial cells. We constructed a deletion in *espC*, and tested this strain (MAS111) for various phenotypes after infecting epithelial cells. We found that MAS111 was indistinguishable from its isogenic parent for adherence, invasion, actin rearrangement, and Hp90 phosphorylation, events which are crucial for A/E lesion formation. Because of its homology to other proteins, we also excluded the possibility that EspC inhibits complement killing or causes hemagglutination (data not shown), as is the case for BrkA of *B. pertussis* and Tsh of *E. coli*, respectively (16, 45).

The comparison of the protein secretion profiles of EPEC with other organisms that cause the A/E phenotype demonstrated that neither RDEC-1, which causes an EPEC-like infection in rabbits, nor the mouse pathogen *C. freundii* biotype 4280, nor the putative human pathogen *Hafnia alvei* biotype 19982 secretes a detectable protein of this size. Polyclonal EspC antibodies did not cross-react with a homologous protein in either supernatants or whole-cell lysates of any of these three strains. This result also makes it extremely unlikely that an EspC homolog is expressed in these strains but remains associated with the outer membrane. On the basis of hybridization experiments under stringent conditions, these pathogens appear to have a gene homologous to *espC*. However, the conditions necessary to induce secretion may be different than those used in the present study or the gene may be nonfunctional. The presence of homologous sequences to *espC* in the pathogenic strains of RDEC-1, *C. freundii*, and *Hafnia alvei* yet not in nonpathogenic strains implies that EspC may have a role in the virulence of A/E-inducing pathogens. A possible function in EPEC virulence is supported by the fact that EspC is a highly immunogenic protein. Human serum collected from a volunteer 28 days after infection with EPEC strongly recognized EspC, while serum collected prior to infection did not (27).

The actual function of members of the IgA protease-like proteins in different pathogenic bacteria remains unclear. Cell culture techniques, standard assays, and homology searches imply that these proteins might help bacteria escape the immune response of the host, facilitate adherence to epithelial cells, and even participate in the process of invasion. However, these functions remain speculative. There is currently no evidence that the high level of protein sequence homology in this protein group corresponds to similar functions for its different members. Although we tested EspC for several of the reported functions of the IgA protease family, we could not confirm any of these functions for EspC. Indeed, the only common feature for these proteins might exist in their secretion mechanism.

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