

## CesD2 of Enteropathogenic *Escherichia coli* Is a Second Chaperone for the Type III Secretion Translocator Protein EspD

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**Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* are extracellular pathogens that employ a type III secretion system to export translocator and effector proteins, proteins which facilitates colonization of the mucosal surface of the intestine via formation of attaching and effacing (A/E) lesions. The genes encoding the proteins for A/E lesion formation are located on a pathogenicity island, termed the locus of enterocyte effacement (LEE), which contains *eae* encoding intimin as well as the type III secretion system and effector genes. Many type III secreted proteins are stabilized and maintained in a secretion-competent conformation in the bacterial cytosol by specific chaperone proteins. Three type III chaperones have been described thus far within the EPEC LEE region: CesD, for the translocator proteins EspB and EspD; CesT, for the effector proteins Tir and Map; and CesF, for EspF. In this study we report the characterization of CesD2 (previously Orf27), a second LEE-encoded chaperone for EspD. We show specific CesD2-EspD protein interaction which appears to be necessary for proper EspD secretion *in vitro* and pathogenesis *in vivo* as demonstrated in the A/E-lesion-forming mouse pathogen *Citrobacter rodentium*.**

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of infant diarrhea, particularly in developing countries (42). EPEC infection is associated with formation of a typical microscopic lesion on intestinal epithelial cells, the attaching and effacing (A/E) lesion (41). A/E lesions are characterized by destruction of cellular microvilli and intimate attachment of bacteria to cup-like pedestals at the apical enterocyte cell membrane (31), triggered by activation of a number of signal transduction pathways and rearrangement of cytoskeletal proteins (46). A/E lesions are induced by other enteric pathogens, such as enterohemorrhagic *E. coli* (EHEC) (54). EHEC infection, which is frequently associated with outbreaks in the developed world, can cause severe diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (42). EPEC and EHEC are the most prominent members among the A/E-lesion-causing pathogens, which are also represented by animal pathogens, including rabbit EPEC (45) and *Citrobacter rodentium*, which causes transmissible murine colonic hyperplasia (47).

The A/E lesion phenotype is encoded by a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) region (37). The LEE consists of 41 open reading frames (11, 16), encoding a transcriptional regulator (Ler) (40); type III secretion system (TTSS) proteins (Esc and Sep) (16, 25); type III translocator (EspA, EspB, EspD) (12, 30, 34) and effector (EspF, EspG, Map, Tir) (14, 28, 29, 38, 39) proteins; an outer membrane adhesin, intimin (26); and a number of proteins of unknown function (16).

TTSSs deliver effector proteins through the bacterial inner and outer membranes, with no periplasmic intermediate, and through the plasma membrane into the eukaryotic cell cytosol (23). The structural TTSS proteins are highly conserved among pathogenic bacteria, and many show an intriguing similarity to proteins involved in flagellar biosynthesis (23). The TTSS has been purified from *Salmonella enterica* serovar Typhimurium, *Shigella flexneri* (33, 51) and most recently from EPEC (8, 49). It consists of a multiple ring structure and a needle projection. In contrast to the high degree of sequence similarity between the structural TTSS proteins, the effector, secreted, proteins show a high level of variation from one system to another. These proteins vary greatly in size, structure, and function and account for the species-specific pathogenicity phenotypes associated with different bacterial infections (23).

In EPEC there are seven LEE-encoded type III secreted proteins—EspA, EspB, EspD, EspF, EspG, Map, and Tir (12, 14, 28–30, 34, 38, 39). EspA, EspB, and EspD are translocator proteins, necessary for A/E lesion formation and signaling within host cells (19). EspA is the main or only component of a transiently expressed filamentous surface organelle (32), which is required for the translocation of other translocator and effector proteins (28, 32). In contrast, immunofluorescence assays and a calmodulin-dependent adenylate cyclase reporter system have demonstrated that EspB is translocated into the host cell, where it is distributed between the cytosol and plasma membrane compartments (61). The presence of EspB in the cytosolic fraction suggests that it may also have, in addition to its translocator activities, an effector function (52). Indeed, it has been shown that EspB causes changes in host cell morphology and reorganization of stress fibers, acting possibly as a cytoskeletal toxin (52).

The third translocator protein, EspD, is inserted into a trypt-

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TABLE 1. Bacterial strains used in this study

Strain	Description or genotype	Source or reference
<i>E. coli</i> TG1	<i>supE hsd Δ5thi Δ(lac-proAB) F'[traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15]</i>	Stratagene
<i>E. coli</i> BL21(DE3)pLysS	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3) pLysS (Cm<sup>r</sup>)</i>	Novagen
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i>	New England Biolabs
<i>E. coli</i> E2348/69	EPEC prototype strain	36
<i>C. rodentium</i>	Wild-type, nalidixic acid-resistant strain (formerly <i>C. freundii</i> biotype 4280)	47
<i>C. rodentium</i> ICC172	E2348/69 <i>ΔcesD2::aphT</i>	This study
<i>C. rodentium</i> ICC173	<i>C. rodentium cesD2::aphT</i>	This study

sin-sensitive location in the host cell plasma membrane, at sites of bacterial contact, but is not translocated into the cytoplasm (55). Based on its homology to YopB, it is believed that EspD is the main component of the TTSS translocation pore in the plasma membrane (19). Indeed, we have recently shown that EspD exhibits intermolecular interaction which involved its carboxy-terminal coiled-coil domain (7). A radical disruption of this region, although did not affect EspA filament production, caused reduced EspA filament-mediated cell attachment and EspD-mediated A/E lesion formation (7). The fact that an *espD* mutant secretes low levels of EspA and produces truncated EspA filaments (32) suggests that EspD might be a minor component of the filaments. Indeed, the current model of the EPEC TTSS predicts interaction between EspA filaments and the EspD translocation pores (19), interaction which allows protein translocation.

Many of the type III secreted proteins are dependent on specific chaperones for stabilization in the bacterial cytosol prior to secretion and prevention of premature interactions with secreted proteins and/or with parts of the secretion and translocation machinery (4, 58). In addition, chaperones have been shown to be required for the exportation of translocator and effector proteins. Three chaperones encoded within the LEE region have been described thus far. The first one described was CesD, a chaperone for EspB and EspD (56). CesD is a 17.5-kDa protein which was shown to interact specifically with EspD. In a CesD-deficient mutant, EspD secretion was abolished and the amount of EspB secreted was reduced, but little effect on secretion of EspA was observed (56). Despite

sharing features that are common to other type III secretion chaperones, CesD is distinct due to its membrane localization (56). CesT was the second reported LEE-encoded chaperone (1, 13). CesT, 15 kDa, is also a bivalent chaperone that binds to, and is required for translocation of, both Tir (1, 13) and Map (6). CesF, the most recently reported EPEC TTSS chaperone, is a 14-kDa protein which specifically interacts with EspF (15). In this study we characterize the gene product of the LEE region *orf27*, a protein with unknown function which exhibits features common to TTSS chaperones (16). We demonstrate that Orf27 is a second secretion partner for EspD and consequently have renamed it CesD2.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains used in this study are listed in Table 1. Plasmids used and constructs generated in this study are listed in Table 2. Unless otherwise stated, bacterial strains were grown in Luria-Bertani (LB) broth at 37°C. Growth media were supplemented with ampicillin (50 to 100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (30 μg/ml), or nalidixic acid (100 μg/ml) as required.

**Molecular techniques.** Cloning which required PCR amplification was performed with the proofreading DNA polymerase Deep Vent (New England Biolabs), unless stated otherwise, and all the clones were verified by sequencing. The synthetic oligonucleotides used as primers in the PCR and DNA sequencing procedures were obtained from Pharmacia (United Kingdom) and are listed in Table 3. Automated sequencing was performed at the Advanced Biotechnology Centre, Imperial College of Science, Technology, and Medicine, Faculty of Medicine. DNA analysis was performed with Gene Jockey II and programs available at <http://www.expasy.ch/>.

**Cloning, expression, and purification of histidine-tagged CesD2.** The 408-bp DNA fragment encoding *orf27* (*cesD2*) was amplified by PCR, using the primer

TABLE 2. Plasmids used in this study

Plasmid	Description or genotype	Source or reference
pET 28a	<i>P</i> <sub>T7lac</sub> N-terminal His <sub>6</sub> expression vector	Novagen
pGEM-T-Easy	ColE1-based vector for cloning of PCR products	Promega
pET3d	<i>P</i> <sub>T7</sub> expression vector	Novagen
pMal-c2X	<i>P</i> <sub>lac</sub> <i>malE</i> N-terminal fusion expression vector	New England Biolabs
pACYC184	Origin of replication from p15A; low copy number; Cm <sup>r</sup> Tc <sup>r</sup>	New England Biolabs
pICC232	pET3d-CesD2, unmodified CesD2	This study
pICC233	pMAL-c2X-CesD2; full-length CesD2 fused to MBP in N-terminal end	This study
pICC234	pET28a-CesD2; full-length CesD2 fused to His <sub>6</sub> in the N-terminal end	This study
pICC240	PCR-amplified 4.071-kbp DNA fragment containing <i>cesD2</i> ; cloned into pGEM-T-Easy	This study
pICC241	In-frame deletion subclone of pICC240; missing bp 97 to 351 of <i>cesD2</i>	This study
pICC260	<i>ΔcesD2::aphT</i> in pGEM-T-Easy	This study
pKD46	Low-copy-number plasmid encoding the phage λ Red recombinase, expressing γ, β, and exo from the arabinose-inducible <i>P</i> <sub>araB</sub>	9
pSB315	Plasmid containing cloned <i>aphT</i> kanamycin cassette	21
pICC243	PCR-amplified 1.208-kbp DNA fragment containing <i>cesD2</i> <sub>CR</sub> and cloned in pGEM-T-Easy	This study
pICC244	Insertion subclone of pICC243; <i>cesD2</i> <sub>CR::aphT</sub>	This study
pICC245	pACYC184-CesD2 <sub>CR</sub>	This study
pICC246	pACYC184-HisCesD2 <sub>EPEC</sub>	This study

TABLE 3. Oligonucleotide primers used in this study

Primer name (restriction site)	Sequence <sup>a</sup>
CesD2-F ( <i>NdeI</i> )	5' TATCATATGGTCGATACGTTTAATGATG 3'
CesD2-R ( <i>BamHI</i> )	5' TATGGATCCCTAACTATTTACGTTCAATTACGAAC 3'
LEE-4K-F	5' GGTGAAGTTACGAGTCGAACAGAG 3'
LEE-4K-R	5' GGCTATACAGGAGCGGTTTGGTCTG 3'
CesD2-INV-F ( <i>SmaI</i> )	5' CCGCCACCCGGGAAAAAAGGCCTGCCACAAAGAACTCC 3'
CesD2-INV-R ( <i>SmaI</i> )	5' AAGGGTCCCGGGCAGCAAGAAACAGAAGACGGAATTTGGTTC 3'
CesD2 <sub>CR</sub> (+1)-F ( <i>NcoI</i> )	5' TATCCATGGTCGATACGTTTAATGACG 3'
CesD2 <sub>CR</sub> (+408)-R ( <i>SalI</i> )	5' TATGTCGACCTAATTTACTTTTCATTACATACC 3'
CesD2 <sub>CR</sub> (-400)-F	5' AATCGTACATCAGGTATCGCTGATG 3'
CesD2 <sub>CR</sub> (+808)-R	5' TCTGATAAGATTTCGGGCAATACATCA 3'
CesD2 <sub>CR</sub> (-45)-F ( <i>BamHI</i> )	5' TATGGATCCATTAATCGTATGGGGCAATCGGC 3'
AphT-R	5' TCTGATGGTCGGAAGACG 3'
pACYC-F	5' TCACAGTTAAATTGCTAACGCAGTCAGGCA 3'

<sup>a</sup> Restriction sites are underlined.

pair cesD2-F (*NdeI*) and cesD2-R (*BamHI*) and EPEC genomic DNA from the prototype strain E2348/69 (36) as the DNA template. The PCR product was digested with *NdeI* and *BamHI* and cloned into *NdeI/BamHI*-digested pET28-a, generating plasmid pICC234 (Table 2). A His<sub>6</sub>-CesD2 fusion was expressed in BL21(DE3)pLysS(pICC234). An overnight culture was diluted 1:100 in 100 ml of LB broth, supplemented with kanamycin (30 µg/ml), chloramphenicol (30 µg/ml), and 0.2% glucose. The culture was grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 to 0.8, at 37°C with shaking, and was induced with the addition of 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Induction was performed for 4 h at 30°C, with shaking. Bacterial cells were pelleted by centrifugation at 8,000 × g for 20 min and resuspended in cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). Bacterial suspensions were sonicated and cell debris was removed by centrifugation at 45,000 × g for 30 min. The cell extracts were filtered through a 0.45-µm-pore-size filter device prior to the following purification procedures. His<sub>6</sub>-CesD2 was purified as recommended by the manufacturer (Novagen). Briefly, the bacterial lysate obtained as described above was loaded onto a 2.5-ml nickel-charged column, previously equilibrated with binding buffer. The column was then washed with 25 ml of binding buffer, 7.5 ml of wash buffer 1 (30 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), and 7.5 ml of wash buffer 2 (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). The bound protein was eluted with 15 ml of elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). Fractions (1 ml) were collected and monitored by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE).

**Column capture assay.** His-tag columns containing bound His<sub>6</sub>-CesD2 or His-T7 tag (negative control), were used to test the ability of recombinant CesD2 to bind the secreted forms of EspA, EspB, and EspD. The procedure was performed as described for the purification steps, involving two additional stages which consisted of loading 25 ml of culture supernatant from E2348/69, grown in Dulbecco's modified Eagle medium (DMEM), onto the columns and an extra washing step followed by elution as for the purification procedures. Fractions (1 ml) were collected, separated by SDS-12% PAGE, and analyzed by Western blotting in order to verify copurification of any of the secreted proteins with the recombinant CesD2, or the control column His<sub>6</sub>-T7.

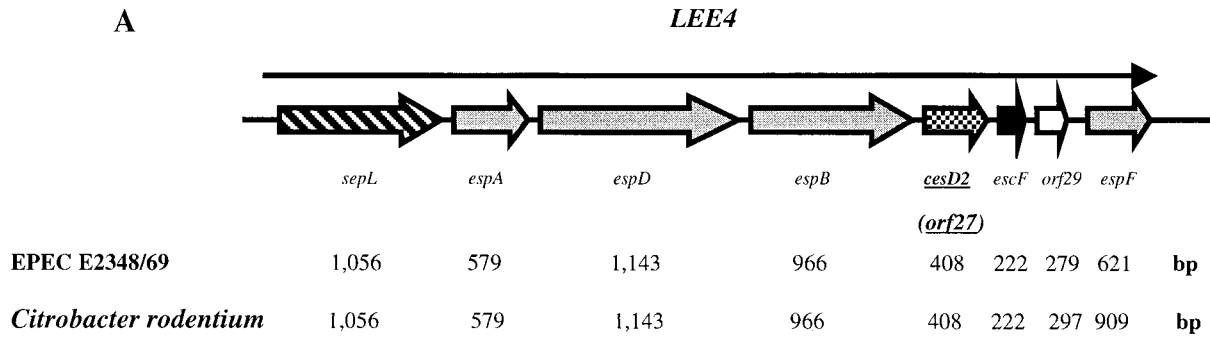
**Generation of a nonpolar cesD2 E2348/69 mutant.** PCR was performed using primers LEE-4K-F and LEE-4K-R to obtain a 4,071-bp DNA fragment of the LEE region containing *cesD2*, from strain E2348/69, which was ligated into pGEM-T-Easy vector (Promega), creating plasmid pICC240 (Table 2). Inverse PCR was used to construct an in-frame deletion within *cesD2* using primers CesD2-INV-F (*SmaI*) and CesD2-INV-R (*SmaI*) and pICC240 as the template. Digestion with *SmaI* and self-ligation excised nucleotides 97 to 351 and added a *SmaI* site to the deletion locus (Fig. 1B), creating plasmid pICC241 (Table 2). For DNA amplification, the Expand High Fidelity PCR System (Roche, Mannheim, Germany) was employed, which included a hot start of 2 min at 94°C, and the preparation of two master mixes. For a 100-µl reaction mixture, mix 1 (50 µl) contained 400 µM concentrations of the deoxynucleoside triphosphates, 600 nM concentrations of upstream and downstream primers, and 10 ng of template (genomic DNA from strain E2348/69), while mix 2 (50 µl) contained the Expand HF buffer with 15 mM MgCl<sub>2</sub> and 2.6 U of Expand High Fidelity PCR System enzyme mix. For primers LEE-4K-F and LEE-4K-R, cycling was performed as follows: 94°C for 2 min (hot start); 10 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 6 min; 20 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 6 min with 15-s increase; and a final extension at 68°C for 7 min. The same PCR cycling

conditions were used for the inverse PCR, using primers CesD2-INV-F (*SmaI*) and CesD2-INV-R (*SmaI*), except for the annealing temperature, which, in this case, was 60°C. An *aphT* cassette (21) with a *HincII* site in the correct reading frame was digested out of plasmid pSB315 and cloned into the *SmaI*-digested plasmid pICC241. The orientation of the cassette was verified by PCR, using primers CesD2-F (*NdeI*) and AphT-R, which tested one of the junction fragments. Primers LEE-4K-F and LEE-4K-R were used to amplify a fragment of approximately 4.8 kbp from plasmid pICC260, which contained the disrupted *cesD2* gene and its flanking regions. One microgram of the amplified fragment was treated with *DpnI*, purified, and transformed by electroporation into E2348/69 carrying a Red helper plasmid, pKD46 (Table 2) (9). Transformants were first selected for Km<sup>r</sup> and then tested for ampicillin sensitivity to confirm loss of the helper plasmid. The *ΔcesD2::aphT* EPEC derivative was named ICC172 (Table 1). In order to *trans*-complement ICC172, pICC234 was double digested with *XbaI/BamHI* and a 509-bp DNA fragment, which contained the entire *cesD2* and a sequence coding for an N-terminal His<sub>6</sub> tag, was ligated into the *BamHI* site of pACYC184, generating plasmid pICC246. Prior to ligation, both vector and insert were previously filled in with Klenow DNA polymerase I (New England Biolabs), according to the manufacturer's instructions. Fragment orientation was verified by PCR, using primers pACYC-F and CesD2-R (*BamHI*).

**Generation of a nonpolar cesD2 C. rodentium mutant.** PCR was used to generate a 1,208-bp fragment containing the *C. rodentium cesD2* gene (11), using primers CesD2<sub>CR</sub>(-400)-F and CesD2<sub>CR</sub>(+808)-R (Table 3) and genomic DNA from a wild-type *C. rodentium* strain (47). The amplified fragment was inserted into pGEM-T Easy vector, creating plasmid pICC243. *aphT* cassette (21) was digested out of pSB315 with *EcoRI* and ligated into *EcoRI*-cut pICC243, generating an insertion at position 76 of *cesD2* (Fig. 1C) and creating pICC244. The orientation of the cassette was verified by PCR with primers CesD2<sub>CR</sub>(+1)-F (*NcoI*) and AphT-R (Table 3). A 2,200-bp fragment containing the disrupted *cesD2* was generated by PCR using plasmid pICC244 as the template, with primers CesD2<sub>CR</sub>(-400)-F and CesD2<sub>CR</sub>(+808)-R, and treated with *DpnI*. One microgram of the PCR product was electrotransformed into the *C. rodentium* strain carrying the Red helper plasmid pKD46 (Table 2), and this was followed by screening for recombinants as described above. The generated *cesD2::aphT C. rodentium* strain was named ICC173 (Table 1). The *cesD2* (*CR27*) (11) gene was also cloned into plasmid pACYC184 (Table 2), to be used in the *trans*-complementation experiments. A fragment of 453 bp containing the full-length *cesD2* and 45 bp of upstream sequence was amplified from *C. rodentium* genomic DNA, with primers CesD2<sub>CR</sub>(-45)-F (*BamHI*) and CesD2<sub>CR</sub>(+408)-R (*SalI*) and was ligated into *BamHI/SalI*-double-digested pACYC184, generating pICC245.

**Preparation of EPEC-secreted proteins.** Analysis of culture supernatants of EPEC-secreted proteins was performed as described previously (32). Bacteria were grown in 20 ml of DMEM at 37°C with shaking to an OD<sub>600</sub> of 1.0. The bacterial cells were pelleted by centrifugation at 8,000 × g for 10 min, and supernatants were passed through filters with a pore size of 0.45 µm. Phenylmethylsulfonyl fluoride (PMSF) (50 µg/ml; Sigma), aprotinin (0.5 µg/ml; Sigma), and EDTA (0.5 mM) were added (all final concentrations), and the Esp proteins were concentrated 100-fold with centrifugal filter devices (Millipore Corporation, Bedford, Mass.). A volume of concentrated samples was loaded and separated by SDS-12% PAGE followed by Western blotting.

**Bacterial cell fractionation. (i) Periplasm extraction.** All the EPEC cultures (100 ml) were grown to an OD<sub>600</sub> of 1.0 in DMEM. The periplasmic contents



**B** **EPEC E2348/69**

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GCCCGTGATCTTACTGACCTTATTAATCGCATGGGGCAAGCGGCTCGCTTAGCTGGGTAA
A R D L T D L I N R M G Q A A R L A G *
ttgataATGGTCGATACGTTAATGATGAAGTGTTAATTACTATCTGAACAAAAGGG
M V D T F N D E V F N Y Y L E Q K G
TACACAATACAGAAGGAGTTTCTTTGGCAGTGCCTTTTTATCGGATGGCGGATTGAG
Y T I Q K E F L C G S A F F I G W R I E
ACACCTTTTTTTCATTAGCGTACAGACTGGATGAACAAGAAGTATTTGTGCTCTTTT
T P F F S L A Y R L D E Q E L I L C S F
GAAGCACGTAACCAAACAGGGCTTAACGGCCCTGTTTATCACTGACTCGTTTGCTCGAA
E A R N Q T G L N G P V L S L T R L L E
GAGTTGTATCACCACCTTTTCGGGTATTAAGAAAATCAGTCCGATGAAATCTAAGATTGGT
S L Y E E F S G I K K I S A M K S K I G
TCAGATTCAAGACGTCAAAAGCGGAAGAGTTGTTAATTACTTTCATCAGAAAGGGTCC
S D S E R Q K R E E L F N Y F I R K G A
GTGCAGCAAGAAACAGAAGCAGGAAATTTGGTTCGTAATGAACGTAATAGTTAAaaaaaa
V Q Q E T E D G I W F V M N V N S *
attattttattaactctgagggaaatttaATGAATTTATCTGAAATTACTCAACAAATG
M N L S E I T Q Q M
    
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**C** ***Citrobacter rodentium***

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CTTATTAATCGTATGGGGCAATCGGCTCGTTTAGCAGGGTAATtgaacATGGTCGATACG
L I N R M G Q S A R L A G * M V D T
TTTAATGACGAAGTGTCTAATTGCTATCTTGAACAAAAGGATATATACCAAAAAGGAA
F N D E V L N C Y L E Q K G Y I P Q K E
TTCTTTTTGGCAGTGCCTTTTTATCGGATGGCGGATTGAGACACCTTTTTTCTCATTAA
F L F G S A F F I G W R I E T P F F S L
GCGTACAGACTGGATGAACAAGAAGTATTTGTGTTCTTTGAAGCACGTAACCAACA
A Y R L D E Q E L I L C S F E A R N Q T
GGCTAAACGGCCCTGTTTATCACTGACTCGCTTGCAGAGAGTTGTACCACCATTTT
G L N G P V L S L T R L L E E L Y E E F
TCGGCTATTAAGAAAATCAGTCCATGAAATCTAAGATTGGTTTCAGTTTCAGATCGTCAA
S A I K K I S A M K S K I G S V S D R Q
AAGCGGAAGAGTTTATTAATTACTTTCATTAGAAAGGGGCGGTTTCAGCAGGAAACAGAA
K R E E L F N Y F I R K G A V Q Q E T E
AACGAAATTTGTTATGTAATGAAAGTAATAATTAGaaatatttttatttattatattat
N E I W Y V M K V N N *
gaggaaaataATGAATCTGACTCAAATTAAGATATGGGGAATATCGCTGATACG
M N L T Q I T E D M G N I A D T
    
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FIG. 1. Schematic representation of *LEE4*, DNA sequence, and deduced amino acid sequence of *cesD2* (formerly *orf27*). (A) Overview (not to scale) of the organization of the *LEE4* operon. Arrows represent each of the open reading frames, with their length indicated below. (B and C) Nucleotide and amino acid sequences of *espB* (carboxyl-terminal region), *cesD2* (*orf27*), and *escF* (amino terminal region) from EPEC E2348/69 and *C. rodentium*. Outlined amino acid sequences indicate a potential amphipathic  $\alpha$ -helix in *CesD2*. (B) The underlined DNA sequence indicates the deletion in EPEC  $\Delta$ *cesD2* and the insertion point of the *aphT* cassette. Highlighted sequences indicate the points wherein the *SmaI* sites were introduced for construction of EPEC  $\Delta$ *cesD2::aphT*. (C) Highlighted sequence indicates the *EcoRI* site where the *aphT* cassette was inserted, creating a *C. rodentium* strain that is *cesD2::aphT*.

were collected following osmotic shock as follows. Bacterial cells were pelleted by centrifugation at  $4,000 \times g$  for 20 min, at 4°C. The supernatant was removed, the pellets were washed twice and resuspended in 2.5 ml of ice-cold 20 mM Tris-HCl (pH 7.5). Two ml of ice-cold 40% sucrose and 150  $\mu$ l of 500 mM EDTA were subsequently added, followed by gentle agitation at 4°C for 20 min. Cells were once more spun down as before, the supernatant was discarded, and pellets were immediately resuspended in 1 ml of ice-cold deionized water, following gentle agitation at 4°C for 20 min. The suspension was centrifuged at  $8,000 \times g$

for 20 min at 4°C. The resulting supernatant containing periplasmic proteins was then collected (P fraction).

(ii) **Preparation of cytoplasmic and membrane fractions.** The pellet from the periplasmic extraction was then fractionated as described previously (24), with modifications. The pellet was initially resuspended in 8 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 0.5 mM PMSF, aprotinin [0.5  $\mu$ g/ml]), freeze-thawed, and passed twice through a cell disrupter (Constant Cell Disruption System). Cell envelopes and unbroken bacteria were removed by centrifuging twice ( $5,000 \times g$



for 10 min at 4°C). The supernatant, containing soluble proteins (cytosolic) and insoluble proteins (inner and outer membranes), was removed and ultracentrifuged for 1 h at 50,000 × g, at 4°C, to pellet the membranes. The supernatant containing cytoplasmic proteins (C fraction) was collected and concentrated to 0.4 ml with centrifugal filter devices (Millipore Corporation). The membrane pellet was washed once with lysis buffer and resuspended in 0.4 ml of Sarkosyl buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.5 mM PMSF, aprotinin [0.5 µg/ml], 0.5% *N*-lauroylsarcosine [Sigma]). Under this condition the inner membrane is dissolved but not the outer membrane. Following centrifugation at 50,000 × g for 1 h, at 4°C, the supernatant containing the inner membrane proteins (IM fraction) was collected. The remaining pellet was washed once with Sarkosyl buffer, under the same conditions as described above, and dissolved in 0.4 ml of 1× SDS loading buffer (OM fraction). Equivalent amount of proteins were loaded after normalization in relation to the volume of the original bacterial cultures.

Enrichment and purity of each fraction were estimated by detection of proteins known to be localized within each of the compartments. Enrichment of the cytoplasm was determined by immunoblotting using a mouse polyclonal antiserum against β-galactosidase (1:2,000; Sigma), a well-characterized cytoplasmic enzyme (35). Enrichment of the IM fraction was determined by detection of Etk (24) with a specific rabbit polyclonal antiserum (1:2,000). Maltose binding protein (antiserum diluted 1:2,000, New England Biolabs) was used as a marker for periplasmic material (27), while intimin (26), an outer membrane adhesin of EPEC (antiserum diluted 1:2,000 [2]), was used as a marker for the OM fraction.

**Immunoblot analysis.** Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (0.45-µm pore size; Bio-Rad) and immunoblotted according to the method of Towbin et al. (53). Proteins were blotted using a Bio-Rad Wet Blot apparatus, the membranes were blocked overnight in 3% bovine serum albumin (Sigma) and washed thoroughly with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Primary antisera were diluted in PBST, and the concentrations used are indicated for each experiment. Secondary antibodies, goat anti-rabbit or anti-mouse (1:5,000 dilution, Sigma), were conjugated with horseradish peroxidase. An ECL Western blotting analysis system (Amersham Life Science) was used according to the manufacturer's instructions. The immunoblots were then exposed to a high-performance chemiluminescence film, Hyperfilm ECL (Amersham Life Science), and the film developed using a Fuji X-ray film developer.

**Fluorescence actin staining (FAS).** Subconfluent cultures of HEP-2 cells on glass coverslips were placed in wells of a 24-well tissue culture plate containing 1 ml of HEPES-buffered minimal essential medium containing 2% fetal calf serum. Ten microliters of an overnight bacterial broth culture was added to each well, and the cells were incubated for 1, 3, and 6 h at 37°C; in 6-h assays fresh medium was added after 3 h. After three washes in PBS to remove nonadhering bacteria, cells were fixed in 4% buffered formalin for 30 min. Washed cells were permeabilized by treating coverslips with 0.1% Triton X-100 in PBS for 5 min. After three PBS washes, coverslips were incubated with a solution of fluorescein isothiocyanate-phalloidin (5 µg/ml; Sigma) in PBS for 20 min to stain filamentous cell actin. Bacteria were immunostained using an antibody to whole E2348/69 bacteria and a secondary Alexa-594 antibody (Molecular Probes). Coverslips were washed a further three times in PBS and mounted in Citifluor mountant (agar). Specimens were examined using a Leica TCS SP2 spectral confocal microscope.

**Challenge of mice with *C. rodentium*.** Male, specific-pathogen-free C3H/HeJ mice (4 to 5 weeks old) were purchased from Harlan Olac (Bicester, United Kingdom). All mice were housed in individual ventilated cages with free access to food and water. Bacterial inocula were prepared by culturing bacteria overnight at 37°C in LB broth containing nalidixic acid (100 µg/ml) plus chloramphenicol (30 µg/ml) (wild-type *C. rodentium* carrying pACYC184), LB broth containing kanamycin (30 µg/ml) (*C. rodentium* ICC173), or LB broth containing kanamycin plus chloramphenicol (30 µg/ml) (*C. rodentium* ICC173 carrying pICC245 or pICC246). After incubation, bacteria were harvested by centrifugation and resuspended in equal volumes of PBS. Mice were then orally inoculated with 0.2 ml of a bacterial suspension using a gavage needle (50). The viable count of each inoculum was determined by retrospective plating on LB agar containing appropriate antibiotics.

The murine infection assay was performed twice. In the first set of experiments, five mice were used per strain and two mice were used as uninfected controls. In the second set of experiments, three mice were used per group and again two mice were used as uninfected controls. There was one case of fatality among the mice infected with the wild-type strain.

**Measurement of pathogen burden.** Mice were killed 13 days postinfection, and the distal 8 cm of colon was aseptically removed and weighed, after removal of fecal pellets. Colons were then homogenized mechanically using a Seward 80

stomacher (Seward Medical, London, England), and the numbers of viable bacteria in colonic homogenates were determined by viable count on LB agar containing appropriate antibiotics as described previously (50).

**Statistical analysis.** All statistical analysis was carried out using the two-tailed Student's *t* test.

**Histological and immunofluorescence analysis.** Distal colons of noninfected mice and those of mice infected with wild-type, ICC173, or complemented strains were snap-frozen and stored at -70°C until cutting. Cryosections (6 µm thick) were cut, air dried for 1 h, fixed with acetone for 10 min, and processed for histological analysis or immunofluorescence analysis. For histological analysis cryosections were stained with hematoxylin and eosin and analyzed using a Nikon Eclipse E600 microscope.

For immunofluorescence analysis cryosections were blocked using 1% bovine serum albumin followed by the addition of a rabbit universal intimin sera (3) and were incubated for 1 h at room temperature. After extensive washing, cryosections were incubated for 45 min with goat anti-rabbit immunoglobulin G labeled with fluorescein isothiocyanate (Sigma). Host cell F-actin was stained with TRITC (tetramethyl rhodamine isothiocyanate)-conjugated phalloidin (Sigma). Specimens were mounted in Mowiol (Aldrich) and viewed through an inverted Zeiss LSM 510 Meta confocal microscope.

## RESULTS

**Binding of native secreted EspD to His<sub>6</sub>-Orf27.** Type III secretion chaperones form a heterogeneous family of proteins that have little or no similarity at the amino acid level but are generally small (14- to 19-kDa), cytoplasmic, acidic proteins with predicted carboxyl-terminal amphipathic α-helix and often adjacent to or in the proximity of the gene coding for the target protein (4, 23, 58). Although no function has been determined for *orf27* of the LEE region, its deduced gene product has all the characteristics of a putative type III secretion chaperone (16). Orf27 is encoded within the polycistronic *LEE4* operon (16, 40), which encodes the TTSS translocator (EspA, EspD, EspB, and EspF) and effector (EspF) proteins (Fig. 1A). Orf27 has a calculated molecular mass of 15.8 kDa and pI 5.3. Although it does not show primary sequence similarity to any protein currently in the databases, its carboxyl-terminal region contains a putative amphipathic α-helix, proposed to mediate interactions with target proteins (Fig. 1B). Based on these observations, we investigated whether Orf27 had the ability to directly interact with any of the translocator proteins encoded upstream on the LEE4, i.e., EspA, EspD, and EspB.

Preliminary results obtained from microarray-based transcriptional analyses in our laboratory have demonstrated that *orf27* is expressed in the early exponential growth phase (M. Batchelor, unpublished data). To examine whether the *orf27* gene produces a detectable protein product, the full-length *orf27* gene was cloned into pET3-d (pICC232) and expressed in BL21(DE3)pLysS, producing an unmodified recombinant Orf27 peptide. A protein band with an apparent molecular mass of 13 kDa, slightly smaller than that predicted from the amino acid sequence (15.8 kDa), was visualized by SDS-PAGE (data not shown). In addition, the entire Orf27 peptide was expressed as a histidine-tagged N terminus fusion protein (His<sub>6</sub>-Orf27) in *E. coli* BL21(DE3)pLysS from plasmid pET28a (pICC234). To investigate whether recombinant Orf27 binds any of the native translocator proteins from EPEC, columns containing bound His<sub>6</sub>-Orf27 or His<sub>6</sub>-T7 were overlaid with 25 ml of filtered culture supernatant from E2348/69, grown in DMEM under conditions favorable for LEE gene expression. Following elution of His<sub>6</sub>-T7 or His<sub>6</sub>-Orf27 with

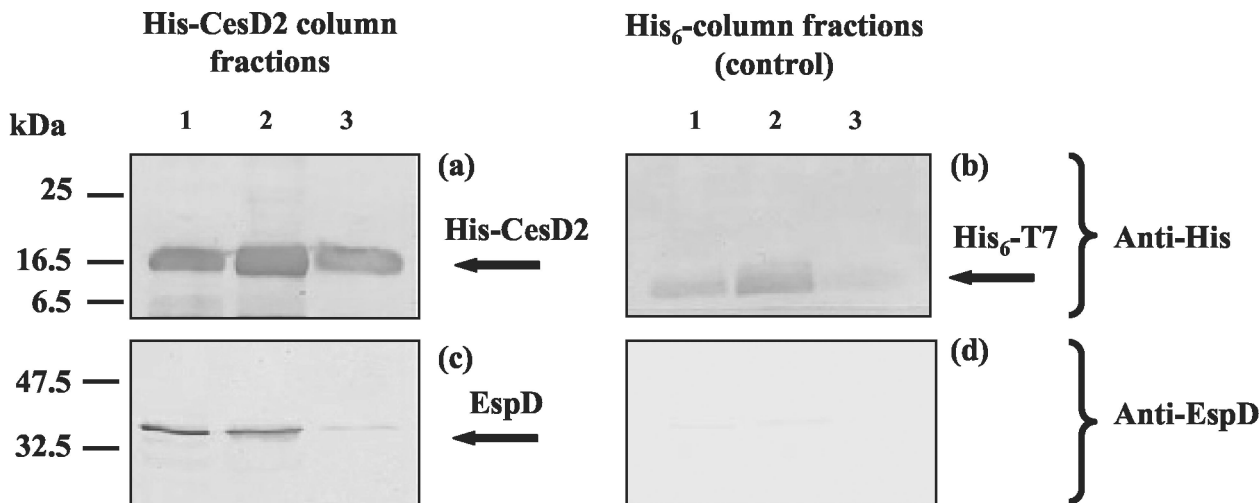


FIG. 2. Immunoblot analysis of fractions eluted from the His-CesD2 capture column. (a) Fractions 2, 3, and 4 (lanes 1, 2, and 3, respectively) probed with polyclonal anti-His sera. (c) The same fractions, probed with monoclonal anti-EspD antibody, demonstrating that the secreted form of EspD copurifies with His-CesD2. (b and d) Control, His<sub>6</sub>-T7 column fractions 2, 3, and 4, probed with anti-His polyclonal sera and monoclonal anti-EspD antibody, respectively, showing no copurification.

the imidazole buffer, 1-ml fractions were collected and probed with either His<sub>6</sub>, EspA, EspB, or EspD antisera by Western blotting. The results showed that EspD was coeluted with His<sub>6</sub>-Orf27 (Fig. 2). Neither EspA nor EspB bound to the column, as they were not detected within the eluted His<sub>6</sub>-Orf27 fractions (data not shown). The control, His<sub>6</sub>-T7 column, did not bind any of the tested proteins from the culture supernatant (Fig. 2). These results showed specific Orf27-EspD protein interaction and suggested that Orf27, like CesD, might display an EspD chaperone activity. Consequently, we renamed Orf27 CesD2.

**A nonpolar mutation in EPEC *cesD2* affects the level of intracellular and secreted EspD but not A/E lesion formation in vitro.** In order to determine the role of CesD2 during infection in vitro, we disrupted its gene in the prototype EPEC strain E2348/69 and subjected the mutant to a variety of phenotypic characterizations. For the mutagenesis, a 4-kbp DNA fragment containing *cesD2* was cloned. Inverse PCR was then performed to generate an in-frame internal deletion (Fig. 1B) in which a kanamycin resistance cassette *aphT* (21) was inserted (pICC260). The disrupted *cesD2* was amplified, and the PCR product was transformed into E2348/69 carrying plasmid pKD46 (encoding the Red recombinase, which enhances the rate of recombination of linear DNA) (9). Allelic exchange was selected, generating a  $\Delta cesD2::aphT$  EPEC, strain ICC172. The nonpolar nature of the mutation was confirmed by the detection of wild-type levels of EspF, which is encoded downstream *cesD2* (Fig. 1; data not shown), in Western blots.

Initially, the effect of the *cesD2* mutation on protein secretion was examined. Supernatants from E2348/69 and ICC172 were collected and concentrated. Proteins were separated by SDS-PAGE, electrotransferred, and probed with antisera directed against EspA, EspB, or EspD (Fig. 3). This revealed that mutation in *cesD2* resulted in a substantially reduced level of EspD in the culture supernatant, compared to that observed with the wild-type EPEC. A slight reduction in secretion of EspB and no effect on secretion of EspA were observed in the

culture supernatant of ICC172 (data not shown). Complementation of the *cesD2* mutation with pACYC-HisCesD2 (pICC246) restored secretion of EspD, but not to the wild-type levels (Fig. 3).

In order to determine if the reduced levels of secreted EspD were due to reduced stability of the protein in the absence of CesD2, we determined the levels of EspD within the intracellular compartments of wild-type, ICC172, and complemented strains. We observed that EspD could be detected predominantly in membrane fractions of both E2348/69 and ICC172 (Fig. 4B). However, and similarly to the secretion profiles, lower levels of intracellular EspD were detected in the ICC172 *cesD2* mutant strain. Interestingly, complementation of the *cesD2* mutation with pICC246 (pACYC-HisCesD2) restored the intracellular levels of EspD to nearly wild-type levels, with EspD localization predominantly in the Sarkosyl-soluble mem-

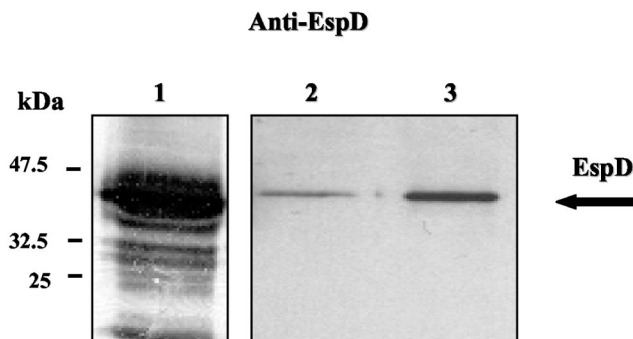


FIG. 3. Immunoblot analysis of culture supernatants showing the EspD secretion profile of EPEC strains. Secretion of EspD is greatly reduced in ICC172 (lane 2) compared with the level of secreted EspD in the supernatant of the wild-type E2348/69 strain (lane 1). Complementation of ICC172 with pICC246 resulted in an increased level of secreted EspD, although not to the wild-type level. Blots were probed with monoclonal EspD antibodies and developed by alkaline phosphates (lane 1) or chemiluminescence (lanes 2 and 3).

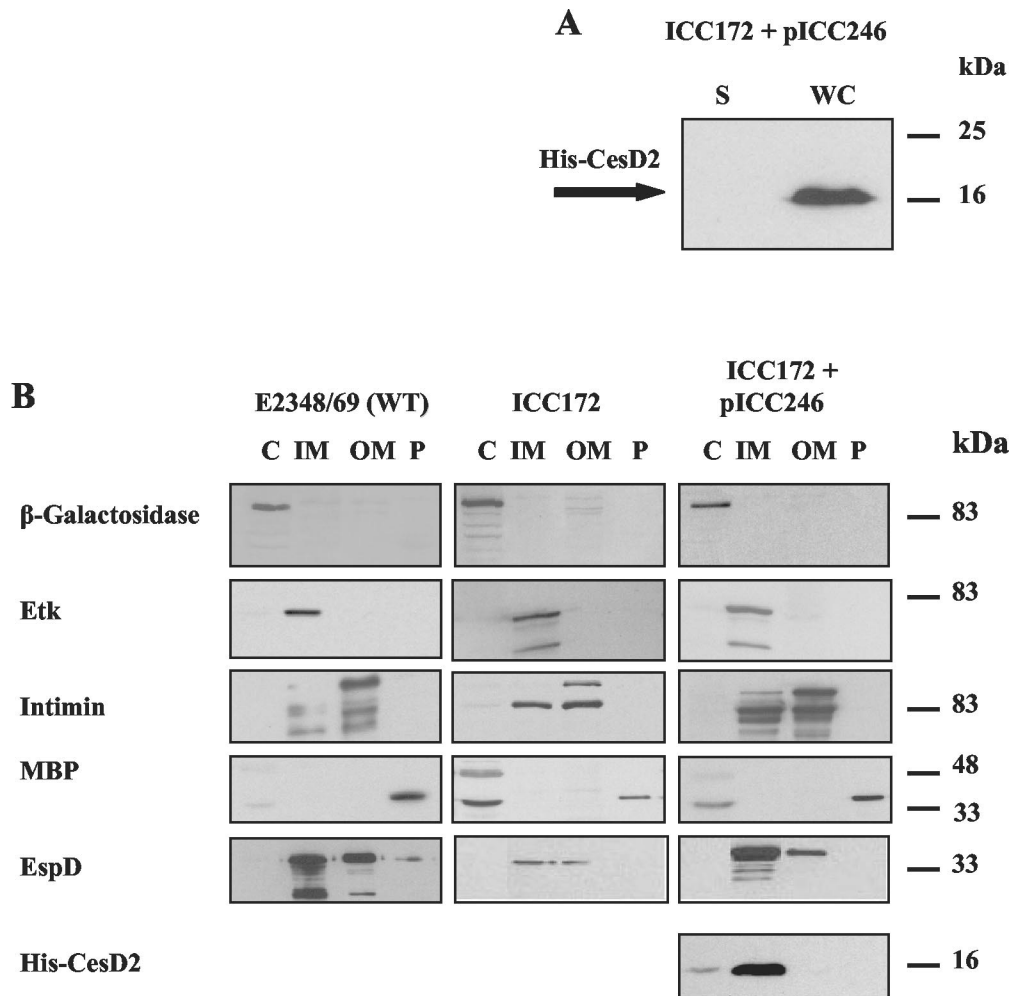


FIG. 4. Localization of CesD2 and EspD. (A) Whole ICC172(pICC246) bacterial cells (WC) and culture supernatant (S) were probed with polyclonal anti-His antiserum, demonstrating that CesD2 is intracellular. (B) EPEC strains E2348/69, ICC172, and ICC172(pICC246) were fractionated into C, IM, OM, and P fractions. Samples were analyzed by Western blotting and probed with polyclonal anti-His antiserum (ICC172-pICC246) and monoclonal anti-EspD antibody (strains E2348/69, ICC172, and ICC172-pICC246). His<sub>6</sub>-CesD2 was detected mostly in the inner membrane, with small amount detected in the cytoplasm. EspD was detected in IM and OM fractions from the three strains. Samples were also probed with anti-β-galactosidase, anti-Etk, anti-intimin, and anti-MBP to verify fraction enrichment. Anti-β-galactosidase, anti-Etk, anti-intimin, and anti-MBP reacted with bands of the size expected for β-galactosidase (116 kDa), Etk (81.2 kDa), intimin (94 kDa), and MBP (43 kDa), respectively. As predicted, β-galactosidase and Etk were detected only in C and IM fractions, respectively. Intimin, which is an outer membrane protein, was also detected in the IM fraction, probably representing the unprocessed form of the molecule. MBP was detected in the periplasmic preparation, while it was also present in some of the C fractions. The upper band that reacted with the MBP antiserum is likely to represent a cross-reactivity with another cytosolic protein. Molecular mass markers are shown at right, and fractionation markers are identified on the left side of the panel.

brane fraction, corresponding to the inner membrane cellular compartment (Fig. 4B).

To determine if the mutation in *cesD2* affected EPEC virulence properties, the FAS test, which is commonly used as an in vitro measure for A/E lesion formation, was performed. Rearrangement of filamentous actin was examined after 1, 3, and 6 h by staining infected HEP-2 cell monolayers with fluorescein-tagged phalloidin and subsequently using confocal microscopy. After 1 h the wild-type strain adhered to HEP-2 cells in small microcolonies and induced significant actin accumulation, whereas strain ICC172 showed reduced adhesion with little actin accumulation (Fig. 5). By 3 h, however, and after 6 h (data not shown) there was little difference in levels of adhesion and both strains produced discrete foci of actin accumu-

lation beneath adherent bacteria, i.e., a positive FAS reaction (Fig. 5). These results show that despite reduced secretion of EspD, CesD2 is not essential for A/E lesion formation in vitro.

**CesD2 is localized to IM and C cellular fractions.** Based on its features and the lack of any obvious signal peptide sequence, as predicted by SignalP V1.1 and iPSORT prediction (<http://hypothesiscreator.net/iPSORT/predict.cgi>), we hypothesized that CesD2, like other type III secretion chaperones, would be a cytosolic protein. In order to confirm this experimentally, we took advantage of the fact that pICC246 (pA CYC-HisCesD2), expressing a tagged CesD2, is biologically active in vivo (Fig. 4 and below). We employed a polyclonal anti-His antiserum to localize His-CesD2 in different EPEC cellular compartments.



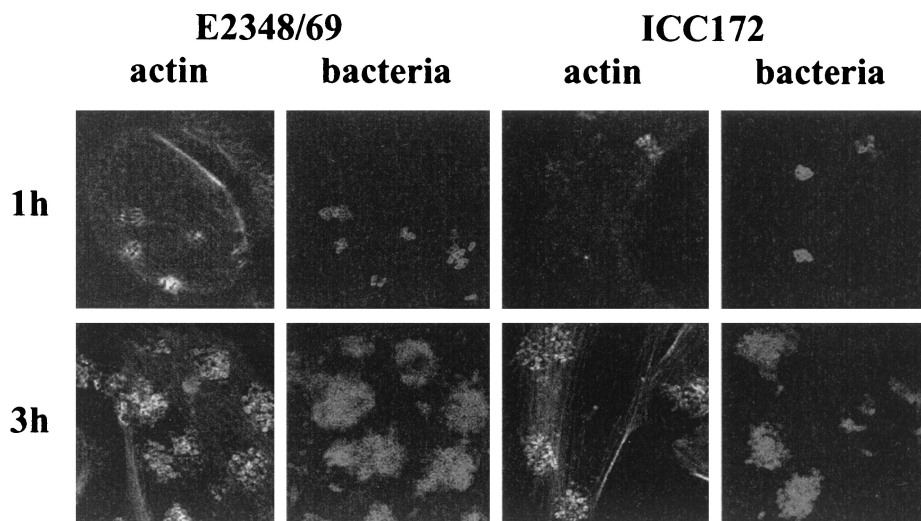


FIG. 5. FAS tests. After 1 h the wild-type strain adhered to HEp-2 cells in small microcolonies and induced disorganized actin accumulation, whereas strain ICC172 showed reduced adhesion with little actin accumulation. By 3 h however, both strains showed good adhesion and both strains produced discrete foci of actin accumulation beneath adherent bacteria, i.e., produced a positive FAS reaction.

Whole-cell lysates and concentrated culture supernatants were first examined to determine whether the His<sub>6</sub>-CesD2 was either intracellular or secreted to the external milieu. Once it was observed that CesD2 is a bacterial-associated protein (Fig. 4A), cells were fractionated into C, IM, OM, and P fractions. The fractions were assayed for enrichment by using protein markers specific for each of the tested compartments (Fig. 4).  $\beta$ -Galactosidase, the cytoplasmic marker, is an abundant *E. coli* cytosolic enzyme of 116 kDa (35). Etk, the inner membrane marker, is an 81-kDa protein tyrosine kinase associated with the inner membrane of *E. coli* (24). Maltose-binding protein (MBP), the periplasmic marker, is a 43-kDa periplasmic *E. coli* protein involved in the active transport of maltose (27). Intimin, the outer membrane marker, is a 94-kDa EPEC adhesin (26), which is also subjected to N-terminal processing during secretion and, as shown before (24), can also be detected in the inner membrane. The banding pattern of intimin is likely to represent degradation products that are commonly seen on Western blots (2). Equal amounts of subcellular fractions were separated by SDS-PAGE, electrotransferred, and probed with anti-His antiserum. The results demonstrated that CesD2 predominantly localized to the inner membrane with some present in the cytosolic compartment (Fig. 4B). These are the same cellular distributions previously reported for CesD (56). These data are consistent with the intracellular pool of EspD being membrane associated (Fig. 4B).

**The effect of *cesD2* mutagenesis on virulence in vivo.** EPEC and EHEC have a narrow host specificity range and are restricted human pathogens. Therefore, infection of mice with *C. rodentium* has been used as a surrogate model to study A/E lesion formation and colonization of A/E-lesion-causing pathogens (18, 22, 43, 47, 48). Importantly, *C. rodentium* possesses an equivalent LEE region (11), and the A/E lesion induced by *C. rodentium* is ultrastructurally identical to those formed by EHEC and EPEC in animals and humans (47).

To determine if CesD2 is required for virulence in vivo, an insertional, nonpolar mutation in *cesD2* was generated in a

wild-type *C. rodentium* strain. A 1.208-kbp PCR fragment containing *cesD2* was cloned into pGEM-T-Easy vector (pICC243), and an *aphT* cassette (21) was inserted into an *EcoRI* site within the cloned *cesD2* gene (Fig. 1C), resulting in pICC244. The disrupted *cesD2* was introduced into the chromosome of wild-type *C. rodentium* carrying pKD46, by allelic exchange, generating strain ICC173. ICC173 was complemented by either pACYC-CesD<sub>CR</sub> (pICC245) or pACYC-HisCesD<sub>EPEC</sub> (pICC246). Wild-type *C. rodentium*, ICC173 and its complemented derivatives were then used to orally infect mice. Pathogen burden was assessed 13 days postchallenge. This revealed that mice infected with the wild-type strain had ca.  $10^8$  CFU/colon (Fig. 6A) and an extensive colonic hyperplasia, as indicated by increased colon weight (Fig. 6B) and microscopic visualization of crypt length in frozen colon sections (Fig. 7). In contrast, mice infected with ICC173 had only ca.  $10^6$  CFU/colon (Fig. 6A), and no hyperplasia was observed, either by colon weight, which was similar to that in the uninfected control, or microscopic examination of crypt length (Fig. 6B and 7). Importantly, in ICC173 complemented with either the *C. rodentium* or EPEC *cesD2* genes, the virulence properties were restored to wild-type levels, showing high numbers of challenged bacteria in the colons and hyperplasia (Fig. 6 and 7).

Challenged bacteria in the frozen colon sections were also stained for intimin using immunofluorescence. Actin staining was used to counterstain the tissue. High numbers of challenged bacteria were observed along the epithelial surface of mice infected with the wild-type (Fig. 8) or complemented (not shown) strains. In contrast, and in agreement with the viable counts, a very low level of challenged bacteria, mainly in the lumen, was observed in mice infected with ICC173 (Fig. 8).

## DISCUSSION

The hallmark of EPEC infection is the A/E lesion histopathology. A/E lesion formation and intimate bacterial attach-



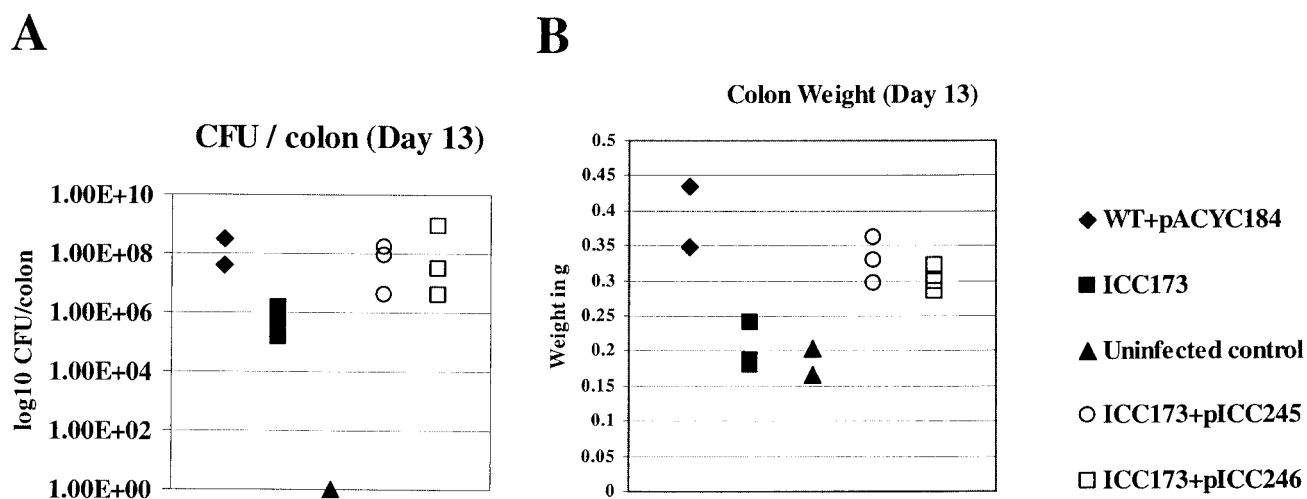


FIG. 6. Virulence of *C. rodentium* strains. (A) Data depict the number of *C. rodentium* CFU recovered from colonic tissues of individual mice, on day 13 postinfection. Mice infected with the wild-type strain had a high pathogen burden. In contrast, mice infected with the *cesD2*-mutant strain (ICC173) had lower bacterial counts, although the difference was not statistically significant ( $P > 0.05$ ). In mutant strains complemented with either pICC245 or pICC246, the CFU level was restored to wild-type level. (B) The distal 8 cm of the colons were weighed 13 days postchallenge. The weights of colons from mice infected with the wild-type (WT) strain or complemented mutant strains, carrying either pICC245 or pICC246, were equivalent and significantly greater than those of colons from mice infected with the mutant strain and uninfected controls ( $P < 0.1$ ). There was no significant difference between the colon weights of uninfected mice and mice infected with ICC173 ( $P > 0.05$ ).

ment are mediated by close interaction between the outer membrane adhesin intimin and the translocated intimin receptor, Tir, which is believed to be delivered to the host cell plasma membrane via the TTSS needle complex and through EspA filaments and an EspD-associated translocation pore

(19). Similar to translocator proteins in other TTSSs, EspD was reported to require a chaperone, CesD, for its proper secretion (56). Here we report that EspD is unique among the TTSS translocator proteins, in that it requires a second chaperone, CesD2, for stabilization and secretion.

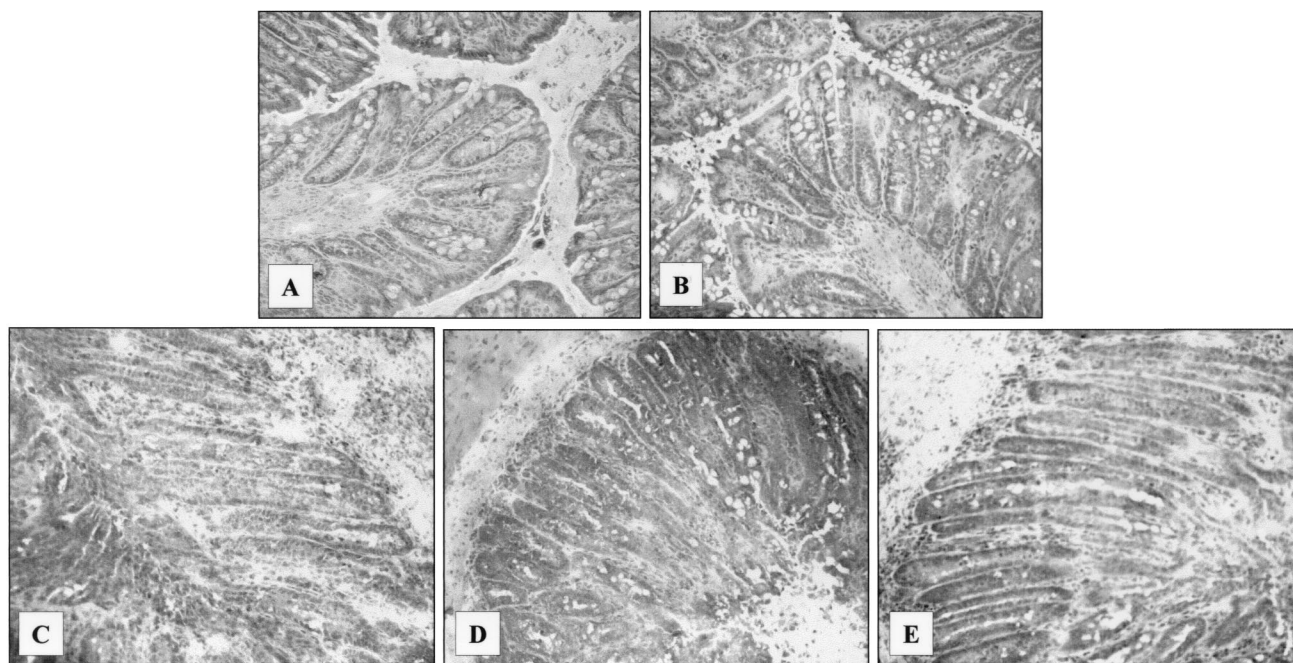


FIG. 7. Hematoxylin and eosin-stained colonic frozen sections from C3H/HeJ mice experimentally infected with *C. rodentium*. (A) Colonic sections from an uninfected mouse, showing normal colonic architecture. (B) Crypt hyperplasia and inflammation in a mouse infected with wild-type *C. rodentium*. (C) Infection with *cesD2*-minus derivative (ICC173), similarly to the uninfected control, revealed neither hyperplasia nor inflammatory response. (D and E) Complementation of ICC173 with pICC245 (D) or pICC246 (E) restored both hyperplastic and inflammatory responses. Original magnification,  $\times 200$ .

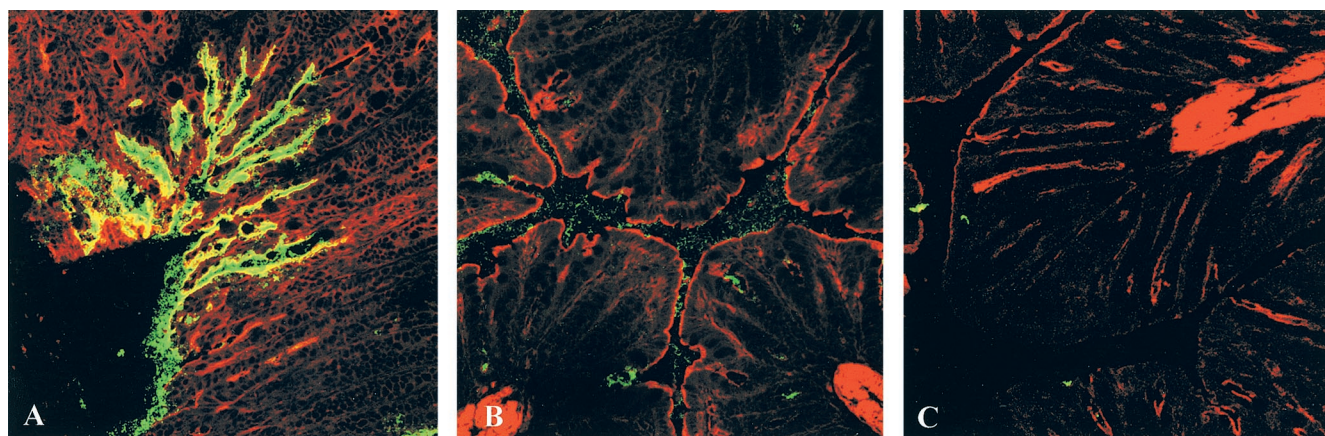


FIG. 8. Actin and intimin staining of *C. rodentium*-infected mice. Colonic sections were stained with anti-intimin antiserum to directly label adherent bacteria and counterstained with phalloidin to stain tissue actin. Large numbers of wild-type challenged bacteria can be seen in association with the surface epithelium (A), while only small numbers of ICC173, mainly luminal, were observed (B). (C) No intimin staining was seen in uninfected colon sections. Original magnification,  $\times 200$ .

TTSS chaperones form a disparate group of proteins with little or no sequence similarity, but which commonly share a predicted  $\alpha$ -helical propensity, small size (14 to 19 kDa), acidic nature (pH 4 to 7), and invariably a carboxyl-terminal amphipathic  $\alpha$ -helical segment (4, 58). The recognition that not all chaperones have single substrate specificity, but rather are able to bind and are required for the proper secretion of more than one substrate, suggests that the chaperones may not form a single homogeneous family but may rather be segregated into two subfamilies. The first is typified by the *Yersinia* SycE chaperone (57) that serves a single substrate, YopE. The archetype of the second family, the *Yersinia* SycD protein, is required for the proper secretion of two proteins, YopB and YopD (44). Interestingly, CesD2 belongs to the SycE family, since it binds only EspD, while CesD belongs to the SycD family as it has chaperone activity for both EspB and EspD (56). Importantly, CesD and CesD2 are unique not only because they chaperone and are required for the stability and secretion of a single translocator protein, EspD, but also because they are located mainly within the bacterial inner membrane.

CesD2 shows all the characteristics of a TTSS chaperone. Indeed, direct and specific EspD-CesD2 protein interaction was demonstrated using the column pull down methods. Interestingly, the phenotypes of the *cesD2* mutant (ICC172) are similar to those described previously for the *cesD* mutant (56). Specifically, mutagenesis of *cesD2*, like that of CesD (56), substantially affected the stability of EspD and consequently the level of its secretion. However, unlike the *cesD* mutant, which also affected secretion of EspB but not of EspA (56), mutation in *cesD2* had no major effect on secretion of either translocator protein EspA or EspB. However, evaluating the virulence potential of the *cesD2* mutant by the FAS test revealed that while after 1 h of infection the wild-type strain adhered to HEp-2 cells in small microcolonies and induced significant actin accumulation, ICC172 showed reduced adhesion with little actin accumulation. Nevertheless, by 3 h there was little difference in the levels of adhesion, and both strains produced a positive FAS reaction. Of note is the fact that the *cesD* mutant also required a longer incubation time to produce

a typically positive FAS test (56). These results suggest that either chaperone enables EPEC to maintain and secrete sufficient amounts of EspD for interaction with epithelial cells in vitro.

The contribution of CesD2 for pathogenesis in vivo was assessed using the *C. rodentium* mouse model. This revealed that in vivo CesD, on its own, is insufficient for colonization and disease as, in comparison with the wild-type strain, 100-fold fewer challenged bacteria were recovered following oral infection with ICC173, with no sign of colonic hyperplasia. Interestingly, the fact that a mutation of *cesD2* caused significant reduction virulence in vivo without affecting FAS and hence A/E lesion formation in vitro suggests that actin polymerization and pedestal formation might not be directly associated with colonization and virulence. The *trans* complementation of *cesD2* mutation with either EPEC or *C. rodentium cesD2* restored the virulence potential to wild-type levels. These results demonstrate that CesD2 plays an important role during infection in vivo.

Like EspD, YopN, a secreted protein in *Y. pestis*, requires two "chaperones" for its secretion, SycN and YscB (10). SycN and YscB form a complex, a heterodimer, that functions as a specific chaperone for YopN. The present study is the first report of a TTSS translocator protein (EspD) having two chaperones (CesD and CesD2).

The precise role of chaperones has not been determined. They have been described as antiassociation factors or "bodyguards," whereby chaperone binding to interactive surfaces prevents premature and nonproductive homo- or hetero-oligomeric cytosolic substrate interactions, which would otherwise target the substrate for degradation (4, 20, 44, 60). Chaperones were also implicated in the hierarchical release of effector proteins from the bacterium organizing trafficking through the secretion apparatus (5) and in control of virulence gene expression, by establishing a regulatory hierarchy of protein synthesis during infection (17, 59).

EspD, with its two putative transmembrane domains (55), is completely insoluble (7). Indeed, large EspD aggregates were seen in EPEC supernatants (7). Moreover, as EspD is essential





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