Identification of a Third EspA-binding Protein That Forms Part of the Type III Secretion System of Enterohemorrhagic Escherichia coli

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Enterohemorrhagic Escherichia coli utilizes a type III secretion system to deliver virulent effectors into cells. The secretion apparatus comprises a membrane basal body and an external needle complex of which EspA is the major component. An l0050-deletion (ΔL50) mutation was found to impair type III secretion and bacterial adherence. These phenotypes and the localization of the gene product to the inner membrane support the hypothesis that L0050, renamed EscL, forms part of the secretion apparatus. Furthermore, in ΔL50, the amount of EspA present within the cell lysate was found to have diminished, whereas the EspA co-cistron-expressed partner protein EspB remained unaffected. The decreased EspA level appeared to result from instability of the newly synthesized EspA protein in ΔL50 rather than a decrease in EspA mRNA. Using both biochemical co-purification and a bacterial two-hybrid interaction system, we were able to conclude that EscL is a third protein that, in addition to CesAB and CesA2, interacts with EspA and enhances the stability of intracellular EspA.

Enterohemorrhagic Escherichia coli (EHEC) causes hemorrhagic colitis and hemolytic uremic syndrome (1). Once EHEC gains access to the gastrointestinal tract, the bacteria adhere to the epithelial cells of the intestine, where they secrete and translocate Tir into their host cells using a type III secretion system (TTSS) (2). Tir then locates onto the host plasma membrane to serve as a receptor for the binding of the bacterial outer membrane protein intimin (2). This interaction allows a tight interaction between the bacterium and the host cell. The microvilli of the host cell are disrupted, and a pedestal-like, actin-rich structure forms beneath the bacterium (1). Other effectors secreted by the bacterium further subvert the normal cellular processes including signal transduction, cytoskeleton patterning, and mitochondria membrane potential (3). These pathological changes mark an infection presentation that is called the attaching and effacing lesion (A/E lesion) (1).

TTSS is not limited to EHEC, and it is widely found in many other Gram-negative pathogens such as Yersinia, Shigella, Salmonella, Citrobacter rodentium, and enteropathogenic E. coli (4). It has been postulated that the TTSS evolved from a bacterial flagella secretion system, as several TTSS structural proteins share similarity with various proteins from this system (4). In EHEC, the genes encoding the TTSS and their associated proteins are clustered in a 43-kilobase locus on the chromosome termed the LEE (the locus of enterocyte effacement) island (5). Similar islands with a high degree of similarity also exist in C. rodentium and enteropathogenic E. coli (6, 7). In Yersinia the TTSS components have been studied in detail (8). Based on a homology comparison, genes in the LEE found to be orthologues in Yersinia (4) are named esc (E. coli secretion) series because these parallel the Yersinia TTSS component genes, which are named ysc (Yersinia secretion) (7). However, several secretion component genes are unique to the LEE, and these genes are called sep (secretion of E. coli protein) (7).

EspA, -B, and -D are translocaters of TTSS that are required for making a 120-Å-diameter needle structure enclosing a channel of 25-Å diameter (9) for delivering the effector proteins EspF, EspG, EspH, and Map and the intimin receptor Tir into target cells (10). EspA polymerizes into the filamentous structure, and its polymerization is dependent on coiled-coil interactions between the C-terminal region of the molecule (11) in a way similar to flagella assembly (9). EspB has been reported to interact with EspA (12, 13) and EspD (14), and the complexes that form at the distal portion of the needle structure may act as a pore at the contact site with the host cell membrane and participate in the initial step of bacterial adherence (15).

The function of l0050 of the EHEC LEE has remained poorly defined. This gene has been named orf5 in the LEE of both enteropathogenic E. coli and C. rodentium (6, 7), and no counterpart with a high degree of similarity has been described in Yersinia. Recently, the gene product has been implicated as being a component of the TTSS because, during a systematic deletion of individual LEE genes in C. rodentium, the corresponding mutant failed to deliver TTS proteins, but its absence did not affect the synthesis of Tir and EspB (16). Furthermore, a low degree of similarity has been found to exist when it is compared with the YscL molecule of Yersinia (17). No additional information about this gene has been reported. Based upon these facts, we have carried out more detailed studies. We report here that l0050 affects the stability of EspA and regulates...
the level of EspA. We also provide the evidence that the product of *l0050* is inner membrane-associated and readily binds to EspA.

**EXPERIMENTAL PROCEDURES**

**Bacterial Culture**—The EHEC strain (ATCC 43888) and the cultivation conditions have been described previously (18). To prepare the TTS proteins, the bacteria were cultured at 37 °C in 5% CO₂-equilibrated M9 for 6 h.

**Plasmid Construction**—*l0050* was amplified from EHEC genomic DNA by PCR using primers L50F (ATGATTTATTTCTTAACAGACTTAA) and L50R (TCATCTCTGATATATGGCAGAAGT), and the fragment was cloned into pUC-T (MD Bio) and digested with the same enzymes, creating pEspA. The amplified DNA, thus, contained additional bases in parallel when doing PCR amplifications. The primers ESCS-R (CATTATGGATACATCAAATGCACATGCT) and ESPA-3R (CGCAGATCTTTTACACCAAGGATATTGTCATTG) were used to amplify *espA* using EHEC genomic DNA as the template. The PCR product was then treated with NcoI and BglII and cloned into pQE60 using EHEC genomic DNA as the template. The PCR product was then digested into pGEM-T (Promega) and used to generate plasmid expressing EspA. The result plasmid, pKO3-dL50, was then used to construct the *l0050* deletion mutant, and the resulting Δl50 was confirmed by PCR and sequencing (18).

**Monolayer Infection Assay**—The monolayer infection assay was carried out as described previously (22). Bacteria were stained by sequential reactions with rabbit anti-O157 antiserum (Difco) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Sigma). The cells were stained for filamentous actin with Texas Red-conjugated phalloidin (Sigma). The samples were finally examined under a fluorescence microscope (Model BX51; Olympus).

**Immunoblotting**—Immunoblotting was performed as previously described (23). All primary antibodies against the EHEC antigens were raised from mice except for anti-EspB (24) and anti-EspA (25), which were obtained by immunizing rabbits. The secondary goat antibodies (Sigma) were products conjugated with horseradish peroxidase. The blots were finally developed using the chemiluminescence reagent, and the signals were detected by exposing them to x-ray film (23).

**Fractionation of Bacterial Components**—The bacteria were fractionated into cytoplasmic, periplasmic, inner membrane, and outer membrane fractions as described previously (26). The bacteria used were overnight-cultured in Luria broth and then 1:100-inoculated into M9 medium for further cultivation at 37 °C in 5% CO₂ for 6 h.

**RNA Extraction from EHEC**—The EHEC cultures used for RNA extraction were grown at 37 °C in 5% CO₂-equilibrated M9 for 6 h. Total RNA was stabilized with RNAProtect™ Bacteria Reagent (Qiagen) and extracted with a RNaseasy™ Protect Bacteria Mini Kit (Qiagen), whereas contaminated DNA was eliminated by on-column DNase digestion. To ensure DNA was completely removed, the eluted RNA was re-treated with DNase I (New England Biolabs) (27). After digestion, DNase I was removed by phenol/chloroform extraction, and then RNA was precipitated by isopropanol precipitation. Total RNA (5 μg), quantified by NanoDrop™ (Thermo), was used to synthesize cDNA with a RevertAid™ First Strand cDNA synthesis kit (Fermentas). The obtained cDNA was annealed with primers ESPAxF (GCTCTAGAGGATACATCAAATGCGATGTTATGTTTGAGGCGC) and ESPARX (GCTCTAGACATCACTCAATTGCGATGTTATGTTTGAGGCGC) to allow PCR detection of *espB*. The primary and amounts of cDNA was amplified using primers ESPBRF (GCGCGAAAGCTACATGGATCAGTTG) and ESPBRR (TTCCCTCGTGAGAGTTGTTGCC) in a side-by-side reaction to detect *espB*. To ensure no DNA contamination, the same RNA prepared for cDNA synthesis was also incorporated in parallel when doing PCR amplifications.

**Construction of the *l0050* Deletion Mutant**—A specific gene deletion of *l0050* was created by homologous recombination as described previously (18). In brief, the 5′-flanking region of *l0050* was PCR-amplified using the primers ESCS-R (CATTA-GCCGTTTACCTTCG) and L50XR (GCTCTAGAGCACC-
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- **β-Galactosidase Assay**—The plasmids pALE and pALT were separately transformed into the parental and the ΔL50 strains, and the bacteria were then cultured accordingly. Bacterial β-galactosidase activity was measured in these cultures using o-nitrophenyl-β-galactopyranase as the substrate as described previously (28).

  - **Analysis of EspA Stability**—The method of measuring EspA stability has previously been reported (29). An EHEC strain harboring pEspA was cultured in M9 for 4.5 h, then 1 mM isopropyl 1-thio-β-D-galactopyranoside was added to induce EspA production. After 30 min of induction, 250 μg/ml chloramphenicol was added to block protein synthesis. Bacteria at different time points were sampled, and the levels of EspA in the bacterial lysate were analyzed by Western blotting with anti-His6 antibody and anti-OmpC. The level of antigen signal on the immunoblot was scanned and quantified using Labwork 4.5 software (UVP, Inc.). A stability curve of EspA was then constructed after correcting the EspA signal against the sample loading control OmpC signal.

  - **Co-purification of L0050 and EspA**—Pressure-pressed lysates derived from JM109 harboring pEspA-HisL0050 or pT5-EspA were prepared in 50 mM NaH2PO4, pH 8.0, 300 mM NaCl (containing 10 mM imidazole), cleared by centrifugation, and loaded into ProBond™ nickel-chelating resin columns (Invitrogen) that had been pre-equilibrated with the same buffer. Each column was then inverted gently at 4 °C for 1 h to allow thorough affinity binding. After a sequential washing with the same buffer containing 50 mM imidazole and 100 mM imidazole, respectively, the proteins retained on the individual columns were finally eluted by increasing the imidazole concentration up to 500 mM.

  - **Bacterial Two-hybrid Analysis**—The two-hybrid system (25) was performed by following the procedures set out in the BacterioMatch™ Two-Hybrid System manual with the reporter strain hosting the reporter genes cI at the N terminus, and a pTRG derivate was used to produce a target protein fused with an N-terminal domain of the RNA polymerase α subunit. Interaction of the bait and the target proteins in the reporter strain yields a high level of β-galactosidase expression.

**RESULTS**

- **Bacterial Attachment, the Secretion of TTSS, and the Synthesis of EspA Affected by Deletion of l0050**—To characterize the possible role of l0050 in the LEE pathogenesis, homologous recombination was used to delete most of the l0050 gene from EHEC (Fig. 1A). The resulted mutant (ΔL50) was examined in terms of the bacterial attachment to the HeLa cells. The wild-type strain gave rise to numerous bacteria adhering to the HeLa cells (Fig. 1B). Underneath the bacteria, actin filaments had condensed, as revealed by staining with Texas Red-conjugated phalloidin (Fig. 1, C and D). ΔL50, however, gave an extremely low level of bacterial attachment to the cells (Fig. 1E) and had lost the ability to induce the host cell actin rearrangement (Fig. 1, F and G). When ΔL50 was transformed with pHis-L50 to complement the lost L0050, the bacteria showed a restoration of attachment activity (Fig. 1H) and actin condensed at the sites where the bacteria had adhered (Fig. 1, I and J).

  - **EHEC attachment affected by deleting l0050**—A, schematic illustration of the gene organization of the leel operon and the relative position of the 567-bp deletion in ΔL50. B–J, monolayer infection assay. HeLa cells were infected with the parental wild-type (WT) EHEC (B–D), ΔL50 (E–G), and ΔL50/ pHis-L50 (H–J). Bacteria (EHEC) adhering to HeLa cells were indirectly stained with green fluorescein isothiocyanate (B, E, and H), whereas filamentous actin of the HeLa cells was marked with bound Texas Red-conjugated phalloidin (C, F, and I). EHEC/Actin (D, G, and J) labels the images merged from corresponding panels on the left.

  - ** Representative TTSS proteins in ΔL50**—A, Western blotting analysis of the proteins from bacterial lysates. ΔL50 was complemented by transforming with pHis-L50 which encodes L0050 tagged with His6 at the N terminus, and the proteins detected were compared with those in controls. B, analysis of the proteins from concentrated spent media. Note: Tir, EspD, EspB, and EspA are proteins secreted by TTSS, whereas CesAB is a cytoplasmic chaperone. WT, wild type.

Examination of the LEE proteins synthesized in the bacterial lysates (Fig. 2A) indicated that EspA was hardly present in ΔL50, whereas Tir, EspD, EspB, and CesAB were detected in both the wild-type strain and ΔL50 (compare lane 1 with lanes...
2 and 3). A successful complementation of L0050 with pHis-L50 (lane 4, stained with anti-His6-tag antibody) resulted in a recovered presence of EspA in ΔL50 (compare lanes 2 and 3 with lane 4). With respect to EspD and CesAB, the presence or absence of L0050 seemed to affect their levels to differing degrees; however, the effect was not as significant as the change in EspA, and the possible implications were not further explored. For the spent media, Fig. 2A clearly shows that secretions of the translocators (EspA, EspB, and EspD) and the effector (Tir) were totally lost in ΔL50 (compare lane 1 and lanes 2 and 3), and secretion was recovered by the L0050 complementation (lane 4).

Biochemical Localization of L0050 to the Inner Membrane of EHEC—Because TTSS secretion was abolished by deletion of L0050, we speculated that L0050 could be functionally associated with the type III secretion machinery, and if we could detect the presence of L0050 on the membrane, this would support the hypothesis. To test this notion, the wild-type bacteria were disrupted after activating LEE expression by cultivation in M9 (30). The disrupted bacteria were physically separated into cytoplasm, inner membrane, periplasm, and outer membrane fractions. Similar samples were also prepared from ΔL50. The proteins in each fraction were analyzed by Western blotting using specific antibodies, and the results are shown in Fig. 3. L0050 was detected in the inner membrane fraction of the wild-type strain but was not found in ΔL50. This fractionation was valid as three control proteins were detected as expected. Outer membrane protein OmpC was consistently found mainly in the outer membrane fraction. Maltose-binding protein is a periplasmic protein, and it was traced mainly to the periplasmic fraction. CesD2 is one of the chaperones for EspD and was seen in both cytoplasm and inner membrane (31). All of the above proteins except for L0050 displayed similar distributions in both the wild-type and ΔL50 strains. Conclusively, L0050 exists only in the wild-type strain and is localized to the inner membrane fraction.

Expression of EspA Enhanced by L0050—The results shown in Fig. 2A suggest that expression of authentic EspA is decreased when chromosomal I0050 is deleted. If so, it is possible that expression of EspA from an extra-chromosomal plasmid may also be affected by the presence or absence of L0050. To test this notion, plasmids expressing His6-tagged EspA and EspB molecules were separately transformed into ΔL50 and the wild-type strain, and the amounts of the His6-tagged proteins in the bacteria were then compared. Fig. 4A shows the results of Western blotting using anti-His6-tag monoclonal antibody. The fact that different samples were comparably loaded onto the gel is evidenced by the fact that similar amounts of OmpC were detected. The plasmid-encoded EspA showed at least a 10-fold difference in expression when we compared the wild-type strain to ΔL50 (Fig. 4A, lanes 3 and 4). This observation is in a great contrast with His6-tagged EspB, which showed only a small amount of variation (lanes 5 and 6) between the two strains.
The expression level of EspA from the chromosome and that from the plasmid with a T5 promoter were equally affected in ΔL50, a fact suggesting that the decrease in EspA was not due to a direct influence from the promoters, and in such a case, the mRNA level of espA would not be affected by the deletion of l0050. To confirm this notion, total RNA was isolated from ΔL50 and reverse-transcribed into cDNA. The cDNA prepared was then used to examine the level of espA mRNA present by PCR amplification (Fig. 4B). Because espA and espB are co-cistronically transcribed from the leef operon and because EspB, unlike EspA, is not affected by deletion of l0050 as seen above, espB should be able to serve as a reference control. Fig. 4C shows the side-by-side PCR results of espA and espB from the same cDNA preparation. It is obvious that, in ΔL50, the amount of espA mRNA did not significantly differ from that of espB. Therefore, the degradation of espA mRNA as a mechanism of EspA down-regulation in ΔL50 is unlikely.

Reporter Assay to Explore the Effect of L0050 on EspA—To further inspect how L0050 regulates the expression of EspA, both a translational fusion and a transcriptional fusion were examined, with β-galactosidase as the reporter. To carry out the transcriptional fusion, lacZ with its own ribosome-binding site was inserted after the stop codon of espA (Fig. 5A). In pALT, β-galactosidase and EspA are translated from the same transcript, which is driven from the same T5 promoter. If a regulator has an effect on the stability or the structure of espA mRNA, it should impact the translation of lacZ. In fact, when this plasmid was transformed into the wild-type EHEC and ΔL50, no difference in β-galactosidase activity was observed (Fig. 5C). Therefore, it was concluded again that the presence of L0050 did not affect the mRNA level of espA per se. Conversely, there was about 2.5-fold increase in β-galactosidase activity with pALE (Fig. 5C), where the bacteria carrying an authentic L0050 (in the wild-type EHEC) was compared with the bacteria without such a protein (in ΔL50). Because in pALE lacZ is translated with its sequence directly fused to the C terminus of EspA (Fig. 5B), the differences in β-galactosidase activity sought to reflect the relative abundance of EspA. The increasing β-galactosidase activity in the wild-type strain suggests that L0050 may increase the stability of EspA or enhance the synthesis of it.

Decreased EspA Stability in ΔL50—Analyzing protein stability after inhibiting de novo protein synthesis was used to clarify whether the decrease in EspA from ΔL50 was due to increased instability of this protein. With the housekeeping OmpC as a control, the amount of EspA in the bacteria was followed for a period of 16 h by Western blotting after the addition of chloramphenicol to the culture (Fig. 6B). The bacteria were harvested at different time points and disrupted by SDS sample buffer. To perform Western blotting, EspA was detected with anti-His6 antibody, whereas OmpC, a loading control, was detected by anti-OmpC. B, the stability curve of EspA was constructed by quantifying the band intensities of A followed by correction against the amount of OmpC present. WT, wild type.

Interaction between L0050 and EspA—The fact that EspA is stabilized by L0050 suggests there might be an interaction between these two LEE proteins. To substantiate this notion, EspA was engineered and co-expressed with His6-tagged L0050 from the same plasmid (pEspA-HisL0050), in which both genes in full-length were built in (Fig. 7A). By affinity purification of His6-tagged L0050 with a nickel ion column, whether EspA was co-purified with His6-tagged L0050 could be examined. Then, whether a biochemical interaction occurs between the two proteins could be deduced. Fig. 7B clearly indicates that, under such a circumstance, L0050 and EspA were both retarded on the column after buffer washing and were co-purified on a final elution. In contrast, when His6-tagged L0050 was absent in...
EspL Interacts with EspA in EHEC

The basal body of TTSS is a collection of proteins that spans the inner membrane, peptidoglycan, and the outer membrane of the bacteria. In the secretion of Yop proteins in *Yersinia* (4) or the flagella assembly in *E. coli* (32), the gene organization is somewhat suggestive of their roles as gene products within the secretion system. The expression of these genes is hierarchical; the genes for the basal body are expressed before those coding for the needle complex (4). With the LEE genes, most of them are organized into five major operons, and expression of the *lee* genes is before that of the other LEE operons (33). Within the *lee*1 (Fig. 1A), the first gene is known as a positive transcription regulator. The last four (*escR, escS, escT, escL*) of the other eight genes have been annotated by sequence comparison as the homologues of the *Yersinia* TTSS components (7, 17), and their gene products have been localized to the basal body (4). *l0050* resides immediately upstream to *escR* (Fig. 1A), and the gene product is found in the inner membrane fraction (Fig. 3). Furthermore, the *l0050* deletion mutant Δl50 has totally lost the ability to secrete proteins through the TTSS (Fig. 2B), a phenotype similar to the *orf*5 deletion mutant of *C. rodentium* (16). These properties are as typical as those observed with other mutations in TTSS apparatus components (4). Molecularly, L50 is predicted to be a cytoplasmic protein when the SOSUIGramN algorithm is used (34). Furthermore, according to PROF (35), L50 may contain a high content of α-helix (59.31%). These predications are similarly found with EscN (ref AAG58832; 38.12% in α-helix). However, EscN has been experimentally proved to be an inner membrane-associating protein (26). Intriguingly, our results in Fig. 3 have demonstrated that L50 also locates in the inner membrane fraction rather than in the predicted cytoplasm.

During a repetitive PSI-BLAST search across the bacteria protein data base, Pallen et al. (17) reported that Orf5, which is equivalent to L0050 in this study, was found to be similar to *Yersinia pestis* YscL (ref NP_395195), although the similarity is too low to be identified by a straightforward BLASTP analysis. YscL has been proposed to link YscN to the TTSS basal body (36) and has been proved to regulate YscN ATPase activity in a way similar to that of the function of FliH (37). Similarly, a FliH-like protein in *Thermotoga maritime* (ref NP_228034) was found to slightly resemble L0050 during the performance of PSI-BLAST analysis (17). When the LALIGN program (38) was used to carry out pairwise alignments among L0050, *T. maritime* FliH (Tm_FliH), and *Y. pestis* YscL (YP_YscL), the degrees of identity and similarity of any pair were relatively low as shown in Table 1. No pair has a greater identity than 25% (or 55% similarity). Such a low degree of similarity does seem not to be rare with the TTSSs as it is also seen with the YscL family (4). The similarity that L0050 shares with YscL may have some biological significance. YscL associates with YscN and YscQ and has been suggested as a regulator of the TTSS ATPase YscN (36). In *Shigella flexneri* (39), a low degree of sequence similarity between MxiN and YscL has been considered meaningful, and it was found that MxiN forms a complex with Spa33, the YscQ
EsCL Interacts with EspA in EHEC

TABLE 1
Amino acid sequence comparison between EHEC L0050 (Ec), T. maritima (Tm) FIILH, and Y. pestis (Yp) YsCL

| Protein^a | Identity^b | Similarity^b |
|-----------|-------------|--------------|
| Ec_L50 vs Tm_FIIH | 16.6 | 51.4 |
| Tm_FIIH vs Yp_YsCL | 22.0 | 54.9 |
| Ec_L50 vs Yp_YsCL | 12.9 | 49.6 |

^a Ec_L50, refAAC31529; Yp_YsCL, refNP_395195; Tm_FIIH, refNP_222803.
^b The identity and similarity were calculated by the LALIGN program (38) in a global alignment mode, with blosum62 matrix and −14/−4 for gap open/extension penalties.

homologue that participates in the ATPase complex formation. Although no association of Orf5 (L0050 in EHEC) with other LEE proteins in enteropathogenic E. coli has been reported using the yeast two-hybrid assay (40), an indirect or weak interaction of this protein with the ATPase complex within the secretion system cannot be completely excluded. Therefore, we support the proposal of Pallen et al. (17) and have named L0050 (Orf5) as EsCL.

EspA is one of the TTS substrates and easily aggregetes because it has the inherent property of forming a needle structure on the bacterial membrane surface. From the same transcript as that for EspA, two additional translocators (EspD and EspB) are translated to form the transmembrane pore that facilitates effector translocation across the host membrane (14). Secretion of the TTS translocators requires the assistance of cytoplasmic chaperones that have some common properties, namely that they have a low molecular mass (<15 kDa), have an acidic pl, and contain a C-terminal amphipathic helix (41). Up to now two chaperones (CesAB and CesA2) are reported to act with EspA (20, 29, 40), two (CesD and CesD2) with EspD (7, 31, 42), and one (CesAB) with EspB (29, 40). CesD, CesD2, CesAB, and CesA2 all have chaperone properties as described above except that CesAB has a basic pl. CesAB and CesA2 are known to chaperone EspA based on the fact that they increase the stability of EspA and show direct EspA binding activity. EspB is chaperoned by CesAB in a different way. Although EspB has been found positively bound to CesAB by yeast two-hybrid analysis (40) and the proteins are co-purified together (29), the level of EspB in the cytoplasm appears to slightly increase in the absence of CesAB (29). On the other hand, CesD binds directly to EspD and so does CesD2. Similarly, a deletion of either cesD or cesD2 affects the stability of EspD. Therefore, intracellularly, EspA and EspD appear to have some properties in common, including easy biochemical aggregation and a need for at least two binding chaperones. Neither of these properties is seen with EspB. However, hierarchically, once they are secreted, EspA must assemble first into the needle-like structure before the forming of the EspD-EspB complexes on the distal host membrane contact area. The facts that EsCL shows direct EspA binding activity and that a lack of EscL decreases the intracellular level of EspA all indicate that EspA has to be well escorted before reaching the outer membrane. Added to this possible escorting function are the facts that EscL (231 residues) is much larger than 15 kDa and that EscL may have a potential function similar to YsCL, which is unexplored as yet. Therefore, we do not favor EscL straighly as a chaperone of EspA. Taken together, our findings strongly suggest that, mechanistically, a perfect secretion of EspA involves bindings of multiple LEE components that are more than what we have previously thought.

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