Salmonella Type III Secretion-Associated Protein InvE Controls Translocation of Effector Proteins into Host Cells

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Salmonella enterica encodes a type III secretion system (TTSS) within a pathogenicity island located at centisome 63 (SPI-1) (14). This system mediates the translocation of several bacterial effector proteins that have the capacity to stimulate cellular responses leading to bacterial internalization and the reprogramming of gene expression in the infected cells. A central component of this system is a multiring structure, termed the needle complex, which spans the bacterial envelope and which likely serves as a conduit for the secreted proteins during their passage through the bacterial envelope (31). In addition, this system requires the function of a set of highly conserved inner membrane proteins and an ATPase that is presumably peripherally associated with the secretion complex and that may help to energize the system (8, 10).

Substrates destined to travel through the type III secretion pathway possess signals that allow their targeting to the secretion machinery (9, 15, 26). Two different mechanisms of substrate targeting have been described. One mechanism requires the function of a family of customized chaperones that bind discrete domains on their cognate secreted proteins and that maintain them in a conformation that is competent for secretion (47). The recently solved crystal structure of one of these chaperone–secreted-protein complexes, the Salmonella SptP-SicP complex, indicates that the secreted protein is maintained as an unfolded polypeptide that retains its secondary structure (43). The second mechanism involves information contained as an unfolded polypeptide that retains its secondary structure SicP complex, indicates that the secreted protein is maintained in a conformation that is competent for secretion (47). The recently solved crystal structure of one of these secreted proteins and that likely serves as a conduit for the secreted proteins during their passage through the bacterial envelope (31).

Addition, this system requires the function of a set of highly conserved inner membrane proteins and an ATPase that is presumably peripherally associated with the secretion complex and that may help to energize the system (8, 10).

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The mechanisms by which secreted proteins are delivered through the host cell membrane are poorly understood. Although a report has suggested that protein translocation is the result of the needle component of the needle complex “piercing” through the host cell membrane (25), this hypothesis alone cannot explain the requirement for a family of pore-forming proteins that are themselves substrates of the type III secretion machinery (21, 36, 40). In the Salmonella SPI-1 TTSS these “protein translocases” are SipB, SipC, and SipD (7). In the absence of any of these secreted proteins, protein translocation is completely inhibited although protein secretion is either unaffected (sipB or sipC mutants) or upregulated (sipD mutant) (29, 30).

Another potential component of the Salmonella SPI-1 TTSS is InvE (18). This protein is not universally conserved, and apparent homologues have only been identified in the TTSSs of Shigella spp. (1), enterohemorrhagic Escherichia coli (36), Chlamydia spp. (39), and Yersinia enterocolitica (22). We have previously shown that InvE is essential for triggering cellular responses that lead to bacterial entry (18) although it is dispensable for needle complex assembly (44). Besides these observations, nothing is known about the actual function of this protein or any of its putative homologues. In this paper, we show that InvE is required for the translocation of effector proteins into host cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. S. enterica serovar Typhimurium strains and plasmids used in this study and their sources are listed in Table 1. S. enterica serovar Typhimurium strains were grown in L broth containing 0.3 M sodium chloride at 37°C. E. coli strains were grown in L broth at 37°C. When required, antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 30 µg/ml. When appropriate, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) or 0.05% L-arabinose was added to cultures at the early log phase of growth (an optical density at 600 nm [OD600] of 0.3) to induce expression of genes carried on plasmids.

Recombinant DNA techniques. The invE mutant allele (18) was introduced into S. enterica serovar Typhimurium wild-type strain SB300 by P22HTet-mediated transduction (41) to make strain SB1204. To construct sipD with the coding sequence for the M45 epitope in the chromosome, a 1.1-kb SalI/EcoRI fragment containing the sipD-M45 allele from pSB821 (J. Urail and J. E. Galán, unpublished data) was cloned into the BamHI site of a suicide vector pSB360 (28), to yield pSB1842. This plasmid was conjugated into SB300 (wild type), SB241 (sipD) (29), SB136 (invA) (17), and SB1204 (invE) with E. coli strain SB136 (invA) as donor.

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SM10 apr to yield strains SB1213, SB1214, SB1215, and SB1216, respectively. To construct a plasmid encoding a protein consisting of a fusion between glutathione S-transferase (GST) and amino terminus of InvE, a PCR-generated DNA fragment carrying invE was cloned into the BamHI and EcoRI sites of pGEX-KG (19) yielding pSB1841. Plasmid pSB1625, which carries sicA and sipB, was constructed by removal of a SwrI fragment (which contains sipC, sipD, and sipA) from pSB511 (C. Collazo and J. E. Galán, unpublished data) and subsequent religation. Plasmid pSB1628, which carries sicA and sipC, was constructed by amplifying DNA fragments encoding sicA and sipC by PCR and subsequently cloning them into the NheI and XbaI sites of pBAD18 (20). Plasmid pSB1628, which encodes M45 epitope-tagged SicA, was constructed by amplifying a DNA fragment carrying sicA by PCR and subsequently cloning it into the EcoRI site of epitope tagging vector pSB16 (8). All plasmids used in the yeast two-hybrid analysis were made by amplifying the appropriate DNA fragments by PCR and subsequently cloning them into the bait or prey vectors (OriGene Technologies, Inc.) as appropriate.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Proteins were separated on sodium dodecyl sulfate (SDS)–12.5% or 10% polyacrylamide gels and visualized by Coomassie brilliant blue staining. For immunoblotting, the proteins on the gels were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) and treated with mouse monoclonal antibodies against InvE, SipB, SipC, SpIP, InvJ, or the M45 epitope (37). Rabbit polyclonal antibodies against outer membrane protein OmpA or cyttoplasmic protein lp-phosphogluconate dehydrogenase (generously provided by Donald Oliver, Wesley University) were used to detect the bacterial proteins that served as fractionation controls.

Analysis of proteins from culture supernatants, protein translocation, and invasion assays. Forty milliliters of L broth containing 0.3 M sodium chloride was inoculated with 0.8 ml of an overnight culture of the different S. enterica serovar Typhimurium strains. The cultures were grown with rotation at 200 rpm to an OD600 of 0.9. Proteins from whole-cell lysates and culture supernatants were prepared as described elsewhere (30). Protein translocation into infected cultured Henle-407 cells was assayed as previously described (7). Brieﬂy, semi-confluent intestinal Henle-407 cells grown in 100-mm-diameter tissue culture plates were infected with wild-type S. enterica serovar Typhimurium or its isogenic derivative strains at a multiplicity of infection of 50 for 90 min in 2.5 ml of Hanks balanced salt solution (HBSS). Infection media containing non-cell-associated bacteria were removed, and cells were washed three times with HBSS. The infection media and the cell wash solution were pooled, and bacteria were recovered by centrifugation (fraction consisting of nonadherent bacteria). Proteins from the bacterium-free supernatant were recovered by 1% trichloroacetic acid precipitation (infection medium fraction). Infected cells were incubated in 2.5 ml of Dulbecco’s modiﬁed Eagle medium containing 100 μg of gentamicin/ml for 1 h to kill extracellular bacteria followed by three washes with phosphate-buffered saline (PBS). Cells were subsequently treated with protease K (50 μg/ml) for 15 min at 37°C to remove extracellularly associated protein. The protease K treatment was terminated by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), and cells were collected by low-speed centrifugation and lysed in the presence of 0.1% Triton X-100. Triton X-100-soluble and insoluble fractions were separated by centrifugation at 15,000 × g.

Quantification of InvE molecules. Wild-type and invE S. enterica serovar Typhimurium strains were grown to an OD600 of 0.9. Proteins from whole-cell lysates of 0.3 ml of bacterial culture equivalent as well as different amounts of pure GST-InvE were separated by SDS–12.5% PAGE and transferred to polyvinylidene difluoride membranes and subjected to Western immunoblot analysis with an anti-InvE monoclonal antibody. The monoclonal antibody was

| TABLE 1. S. enterica serovar Typhimurium strains and plasmids used in this study |
|-----------------------------------|-----------------|-----------------|-----------------|
| Strain or plasmid | Relevant genotype | Reference or source |
| SB300 | Wild type | 17 |
| SB136 | invA::aphT | This study |
| SB1204 | invE::aphT | 29 |
| SB241 | sipD::aphT | 29 |
| SB174 | invE::zyIE | 45 |
| SB231 | invE::zyIE sipD::aphT | This study |
| SB319 | sicA-M45 | This study |
| SB1213 | sipD::M45 | This study |
| SB1214 | sipD::M45 | This study |
| SB1215 | invA::aphT sipD::M45 | This study |
| SB1216 | invE::aphT sipD::M45 | This study |

Plasmids

- pSB1842
- pSB823
- pSB50
- pGEX-KG
- pSB1841
- pSB511
- pSB1625
- pSB1628
- pSB1301
- pSB422
- pSB1623
- pSB680
- pEG202-NLS
- pSB1615
- pJB4-5
- pSB1843
- pSB1624
- pSB1622
- pSB1616
- pSH18-34

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<td>C. Collazo and J. E. Galán, unpublished data</td>
</tr>
<tr>
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<td>sicA sipB</td>
<td>This study</td>
</tr>
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<td>This study</td>
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a: GST, GST gene.
b: His6, coding sequence for the His6 tag.
c: B42-HA, B42 coding sequence fused to the hemagglutinin gene.
d: opr, LexA operators.
standardized by using known amounts of GST-InvE. The concentration of GST-InvE in the standard was determined by using known concentrations of bovine serum albumin (as the standard) and various amounts of GST-InvE, which were separated by SDS-PAGE and stained with Coomassie brilliant blue. The intensities of the InvE protein bands in the different bacterial fractions were measured by scanning the film and subsequently quantitating the pixels with National Institutes of Health Image. The number of InvE molecules per bacterium was determined by comparing the intensity of the signal in a lysate from a known number of bacteria with that of the purified GST-InvE protein standards. The signal for the GST moiety was subtracted from the results of the final calculation. GST pull-down assay. Forty milliliters of L broth or L broth containing 0.3 M sodium chloride was inoculated with 0.8 ml of an overnight culture of E. coli or S. enterica serovar Typhimurium strains, respectively. The cultures were grown with rotation at 200 rpm at 37 °C and harvested when they reached an OD₆₀₀ of 0.9. Bacterial cells were washed once with chilled PBS by low-speed centrifugation, the pellet was resuspended in 1.0 ml of chilled PBS containing 0.1 mg of lysosyme/ml, 1 mM PMSF, and 10 mM EDTA, and the suspension was incubated on ice for 1 h. The cells were lysed by sonication and clarified by centrifugation (three times) at 17,000 × g for 10 min. Lysates were then preabsorbed for 1 h at 4 °C with glutathione-Sepharose beads to remove nonspecific binding proteins. Preabsorbed lysates were then mixed with 30 μl of a slurry of prewashed glutathione-Sepharose beads in the presence of 0.1% NP-40, 1 mM PMSF, and equal amounts of GST-InvE or GST (purified from E. coli BL-21) in a final volume of 1 ml. The mixture was incubated for 3 h at 4 °C under gentle rocking, and proteins bound to the beads were recovered by centrifugation at 500 × g for 4 min. After beads were washed four times in 0.7 ml of PBS containing 0.1% NP-40, proteins were released from the beads by boiling in Laemmli buffer and separated by SDS-PAGE along with pre- and postincubation (unbound fraction) samples of the lysates.

Biochemical fractionations of S. enterica serovar Typhimurium cells. S. enterica serovar Typhimurium lysates were prepared as described for the GST pull-down assay. After the lysates were clarified by centrifugation, samples were passed through 0.22-μm-pore-size filter to completely remove the unlysed bacteria. The volume of the lysate was adjusted to 1.0 ml with PBS, and, after the removal of a small fraction for further analysis, the rest of the lysate was subjected to ultracentrifugation at 100,000 × g for 60 min at 4 °C. The supernatant (0.25 ml) was removed carefully to avoid contamination with the pellet fraction, and the pellet was washed again with 1.0 ml of PBS by subjecting it to ultracentrifugation at 100,000 × g for 60 min at 4 °C. The different samples (pellet, supernatant, and starting material) were subjected to SDS-PAGE and Western blot analysis.

Sucrose gradient fractionation of bacterial membranes was carried out as described previously (27) with the following modifications. S. enterica serovar Typhimurium lysates were prepared as sonication for desorption as described for the GST pull-down assay except that the bacterial pellet was resuspended in 1.0 ml of 10 mM HEPES (pH 7.4)–1 mM PMSF–5 mM EDTA–20% (wt/wt) sucrose and treated briefly with 2 μg of DNase I/ml and 10 μg of RNase A/ml in the presence of 20 mM MgSO₄. Unlysed bacteria were removed by low-speed centrifugation, and the clarified lysates were loaded onto a two-step gradient consisting of 1.6 ml of a 60% (wt/wt) sucrose cushion and 6.0 ml of 25% (wt/wt) sucrose solution in the same buffer. The membranes were pelleted on top of the 60% sucrose cushion by centrifugation at 200,000 × g at 4 °C for 4 h. Samples (1.5 ml) were collected from top to bottom and separated on an SDS–10% polyacrylamide gel.

Proteinase K susceptibility assay. The proteinase K susceptibility assay was carried out as previously described (5) with some modifications. Briefly, S. enterica serovar Typhimurium strains were grown to an OD₆₀₀ of 0.9. Cultures (1 ml) were harvested by low-speed centrifugation, and the pellets were resuspended in 0.1 ml of cold PBS. Samples were treated with proteinase K (50 or 250 μg/ml) for 30 min at 37 °C. As a control, bacterial cells were permeabilized by addition of 1% SDS (final concentration) prior to proteinase K treatment. The reactions were stopped by adding 5 mM PMSF, and the samples were analyzed by Western immunoblotting.

Yeast two-hybrid analysis. The yeast two-hybrid analysis was carried out using a DupLEX-A yeast two-hybrid system (OriGene Technologies, Inc.) according to the manufacturer’s instructions. The bait was constructed by fusing InvE to the C terminus of E. coli LexA with a simian virus 40 nuclear localization sequence in pEG202-NLS (OriGene Technologies, Inc.). The prey plasmid was constructed by fusing the InvE, SipB, SipC, or SiaC protein to the C terminus of acid blob activation domain B42 in plg4-5 (OriGene Technologies, Inc.). β-Galactosidase activity was assayed by biochemical fractionation and Western blot analysis. Consistent with its total inability to stimulate cellular responses, the invE mutant strain was unable to translocate any of the proteins assayed (SipB, SipC, and SptP) (Fig. 2, compare cytosolic fractions in lanes d). In addition, the levels of SipB and SipC in the infection medium of cells in

RESULTS

invE mutants show altered levels of type III secretion. S. enterica serovar Typhimurium strains carrying nonpolar inser- tion mutations in invE are unable to enter host cells (18). Since this entry deficiency is indistinguishable from that observed in mutant strains lacking a functional TTSS (16), we hypothesized that InvE is an essential component of the SPI-1 TTSS rather than an effector. S. enterica serovar Typhimurium invE mutants exhibit a normal needle complex, indicating that this protein is not involved in the assembly of this organelle (44). We there- fore investigated the potential role of InvE in TTSS-dependent protein secretion. We compared the total protein profile of the culture supernatant of an invE mutant strain with those of wild-type and an isogenic invA mutant strain, which is defective for type III secretion (17). The culture supernatant protein profile of the invE mutant strain showed a significant decrease in the levels of proteins with molecular weights similar to those of SipB and SipC (30) (Fig. 1A). In contrast, the levels of secreted polypeptides with mobilities identical to those of SopE (24) and SiaA (29) were slightly increased in the invE mutant strain (Fig. 1A). To more specifically test the effect of InvE on secretion through the TTSS, we examined the levels of SipB, SipC, SipD, SptP, and InvE in culture supernatant of the invE mutant strain using specific antibodies to these proteins or to an epitope tag in the case of SipD. This analysis showed that the levels of SipB, SipC, and SipD were significantly reduced in culture supernatants of the invE mutant strain (Fig. 1B and C). The reductions of SipB and SipC levels were not due to decreases in the expression of these proteins since their levels in whole-cell lysates were unaffected (Fig. 1B). Definitive conclusions regarding the relationship between the levels of SipD in culture supernatants and its total levels could not be drawn since SipD could not be detected in whole-cell lysates of any of the strains tested, even upon loading material from as much as 1 ml of bacterial culture. Additionally, the level of effector protein SptP was significantly increased in the culture supernatant of the invE mutant even though the amount of SptP in whole-cell lysates was equivalent to that of the wild type (Fig. 1D). In contrast, the levels of InvJ, a protein required for type III secretion, in both culture supernatants and whole-cell lysates of the invE mutant strain were unaffected (Fig. 1D). Therefore, these results indicate that InvE positively regulates the secretion of TTSS translocases SipB, SipC, and SipD and negatively influences the secretion of the effector proteins SiaA, SopE, and SptP.

InvE is required for TTSS-mediated protein translocation into host cells. We next examined the effect of a loss-of-func- tion mutation in invE on TTSS-mediated protein translocation into host cells. Cells were infected with wild-type S. enterica serovar Typhimurium or its isogenic derivatives carrying muta- tions in invE, invA (resulting in a secretion-incompetent mutan- t) (17), or sipD (resulting in a translocation-incompetent but secretion-competent mutant) (29), and protein transloca- tion was assayed by biochemical fractionation and Western blot analysis. Consistent with its total inability to stimulate cellular responses, the invE mutant strain was unable to translocate any of the proteins assayed (SipB, SipC, and SptP) (Fig. 2, compare cytosolic fractions in lanes d). In addition, the levels of SipB and SipC in the infection medium of cells in
fected with the invE mutant strain, which is an indication of the levels of secretion, were significantly lower than the corresponding levels for the wild type (compare lanes b in Fig. 2A). These results indicate that the secretion defect of the invE mutant strain could not be overcome by host cell contact stimulation. These results indicate that InvE is required for protein translocation into host cells, in part through the modulation of SipB, SipC, and SipD.

InvE exerts its function from within the bacterial cell. Since InvE exhibits a phenotype consistent with its involvement in protein translocation, we examined whether InvE could be recovered from culture supernatants similarly to other TTSS-associated protein translocases. InvE was not detected in culture supernatants of wild-type S. enterica serovar Typhimurium even though the assay was sensitive enough to have detected ~0.2% of the total InvE protein in the bacteria (Fig. 3A). Furthermore, proteinase K treatment of intact whole bacterial cells did not reduce the total level of InvE (Fig. 3B). InvE was degraded when cells were permeabilized prior to proteinase K treatment (Fig. 3B), suggesting that InvE is not exposed on the bacterial surface and therefore must exert its function within the bacterial cell. To further investigate this possibility, we constructed a chimeric protein consisting of GST fused to the amino terminus of InvE and examined the ability of this chimeric protein to translocate into host cells.

![FIG. 1. S. enterica serovar Typhimurium invE mutant shows altered levels of type III secretion.](image-url)
meric protein to complement an invE loss-of-function mutant. We reasoned that if InvE function requires its secretion through the TTSS, the fusion of GST at its amino terminus should interfere with its secretion and therefore with its function since TTSS secretion signals are located at the amino terminus. Introduction of a plasmid encoding a GST-InvE chimeric protein into an invE mutant strain restored the ability of this strain to enter culture cells to levels equivalent to those of the wild type (Fig. 3C). Furthermore, the chimeric protein restored the ability of the invE mutant strain to secrete proteins through the TTSS at wild-type levels (Fig. 3D). Taken together, these results indicate that InvE exerts its function from within the bacterial cell.

We then investigated the localization of InvE within the bacterial cell. We first quantified the amount of InvE in the bacterial cell. To carry out this experiment, we purified the GST-InvE protein and used it as a standard to determine the number of InvE molecules per bacterial cell (see Materials and Methods). We found that the levels of InvE are between 5,000 and 7,000 molecules per bacterial cell (Fig. 4A). We then fractionated bacterial cells and examined the fractions for the presence of InvE by Western immunoblotting analysis using a monoclonal antibody against InvE. We found that more than 40% of the total amount of InvE was present in the pellet fraction after ultracentrifugation and therefore presumably associated with the bacterial membrane (Fig. 4B). To further investigate the localization of InvE, we subjected the bacterial lysate to sucrose gradient centrifugation. InvE was broadly

![Image](http://jb.asm.org/)

**FIG. 2.** Effect of a loss-of-function mutation in invE on protein translocation into Henle-407 cells infected with wild-type or mutant strains of *S. enterica* serovar Typhimurium. Shown is detection of SipB and SipC (A) and SptP (B) in fractions of Henle-407 cells infected with wild-type and mutant strains of *S. enterica* serovar Typhimurium. Lanes a, whole-cell lysate of non-cell-associated bacteria; lanes b, bacterium-free infection medium; lanes c, Triton X-100-insoluble fraction containing internalized bacteria; lanes d, Triton X-100-soluble Henle-407 cell lysate containing translocated proteins. SipB, SipC, and SptP were detected by Western immunoblotting with monoclonal antibodies specific for these proteins. The relevant genotypes of the infecting strains are indicated at the top of the gel. The positions of relevant proteins are indicated.
to what was found for InvE, the absence of SipD results in an increase in the secretion of SipB and SipC. To gain insight into the mechanism of action of InvE, we tested whether its function was dominant over that exerted by SipD. We examined the secretion profile of a strain of \textit{S. enterica} serovar Typhimurium carrying loss-of-function mutations in both sipD and invE. As previously reported (29), the sipD mutant strain showed increased levels of secretion of several proteins, including SipB and SipC but not InvJ (Fig. 5). In contrast, the \textit{invE} sipD double mutant showed a secretion profile indistinguishable from that of the \textit{invE} single mutant, including significantly reduced levels of secretion of SipB and SipC (Fig. 5). These results indicate that \textit{invE} is epistatic over \textit{sipD} in regard to the secretion of SipB and SipC.

**InvE forms a complex with SipB, SipC, and SicA.** Since the absence of InvE drastically reduces the levels of SipB, SipC, and SipD in culture supernatants, we investigated the possibility that InvE interacts with these proteins. Whole-cell lysates of wild-type \textit{S. enterica} serovar Typhimurium were incubated with either GST-InvE or GST immobilized on glutathione-agarose beads, and the bound proteins were probed for the presence of SipB, SipC, and SipD. Both SipB and SipC, but not SipD, were readily detected in eluted proteins bound to GST-InvE but not to GST alone (Fig. 6A and B).

We then tested whether InvE could form a complex with SicA, the specific chaperone for SipB and SipC. Glutathione-agarose beads coated with GST-InvE but not GST alone were able to pull down SicA from whole-cell extracts of a strain expressing from its chromosones a functional epitope-tagged version of this chaperone (Fig. 6C). We found equivalent results in similar experiments in which plasmids encoding either GST-InvE or GST were introduced into an \textit{S. enterica} serovar Typhimurium wild-type strain and the complexed proteins were purified by affinity chromatography using glutathione-agarose beads (data not shown).

These results indicate that InvE is able to form a complex with SipB, SipC, and its cognate chaperone, SicA, but do not establish whether these interactions are direct or mediated by other TTSS-associated proteins. To address this issue, we introduced plasmids encoding SipB or SipC, either alone or in conjunction with their chaperone, SicA, into \textit{E. coli} K-12 and examined the ability of these proteins to interact with InvE in a GST pull-down assay. Beads coated with GST-InvE were able to pull down SipB or SipC when expressed in conjunction with their chaperon, SicA (Fig. 7A to D). In contrast, GST-InvE was unable to efficiently pull down SipB, SipC, or SicA when one of these proteins was expressed in \textit{E. coli} alone (Fig. 7E to G). Consistent with these results, InvE was unable to interact with SipB, SipC, or SicA in a yeast two-hybrid assay although it was able to interact with itself (Fig. 7H). These results indicate that InvE interacts with the SipB- and/or SipC-chaperone complex but does not interact with the individual components of this complex. We propose that this interaction is important for the ability of InvE to modulate SipB and SipC secretion.

**DISCUSSION**

We have shown here that the \textit{S. enterica} serovar Typhimurium protein InvE is involved in TTSS-mediated protein secretion.

![Figure 3](https://example.com/fig3.png)

**FIG. 3.** InvE exerts its function from within the bacterial cell. (A) Western blot analysis of whole-cell lysates (WC; 0.4 ml of culture equivalent) and cultured supernatants (CS; 16 ml of culture equivalent) of wild-type and mutant \textit{S. enterica} serovar Typhimurium strains. Blots were treated with a monoclonal antibody specific to InvE. Since InvE does not possess a predictable transmembrane domain, it is possible that its localization to this compartment may occur through interactions with a membrane-localized protein, presumably associated with the TTSS. Alternatively, InvE may form a supramolecular complex that can be precipitated by ultracentrifugation.

**InvE is epistatic over SipD.** In addition to InvE, \textit{Salmonella} protein SipD is known to increase the levels of secretion of a subset of type III secreted proteins (29). However, in contrast...
translocation into host cells. A strain of *S. enterica* serovar Typhimurium carrying a loss-of-function mutation in *invE* is unable to translocate effector proteins into culture cells, consistent with its inability to stimulate cellular responses. However, the *invE* mutant strain retained the ability to secrete effector proteins into culture supernatants. In fact, this mutant exhibited an increased secretion of effector proteins including SipA, SopE, and SptP. The inability of the *invE* mutant strain to translocate effector proteins into host cells is likely due, at least in part, to its deficiency in the secretion of TTSS-associated protein translocases SipB, SipC, and SipD. However, although secretion is significantly reduced, this mutant still retains the ability to secrete these proteins (data not shown). The drastic defect in cell signaling and bacterial entry exhibited by the *invE* mutant is indistinguishable from the defect of *Salmonella* mutants completely defective in type III secretion. Therefore, it is unlikely that this defect is due only to the reduction of the levels of secretion of SipB, SipC, and SipD. Perhaps the absence of InvE impairs the function of the protein translocases in some fundamental way so that even the reduced amount of secreted Sip proteins may not be functional.

**FIG. 4.** Localization of InvE in bacterial cells. (A) Determination of the number of InvE molecules per bacterial cell. The level of InvE in whole-cell lysates (WC; 0.3 ml of culture equivalent) of wild-type *S. enterica* serovar Typhimurium was determined by Western blot analysis using a monoclonal antibody specific to InvE and compared to the indicated amounts of purified GST-InvE, and the number of molecules was calculated as indicated in Materials and Methods. (B) InvE localizes to both the soluble and pelletable fractions after high-speed centrifugation. Whole-cell extracts from the indicated strains were subjected to high-speed centrifugation. Proteins in the pellet (pellet) and supernatants (sup) after this fractionation along with whole-cell lysates were analyzed by Western immunoblotting with a monoclonal antibody directed to InvE. WC, 0.4 ml of culture equivalent; Sup, 0.8 ml of culture equivalent; pellet, 4 ml of culture equivalent. (C) InvE is localized both to the bacterial membrane and cytosol. Lysates from wild-type and *invE* *S. enterica* serovar Typhimurium strains were fractionated on a sucrose gradient as indicated in Materials and Methods, and the presence of InvE in the different fractions was analyzed by Western immunoblotting with a monoclonal antibody directed to InvE. The presence in the different fractions of the membrane (Memb) and cytoplasmic proteins OmpA and 6-phosphogluconate dehydrogenase (6-PD), respectively, was determined by reprobing the blots with antibodies specific to these proteins.

**FIG. 5.** *invE* is epistatic over *sipD*. Coomassie blue staining of total protein of whole-cell lysates (WC; 0.4 ml of culture equivalent) and culture supernatants (CS; 10 ml of culture equivalent) of the indicated strains. The identities of the polypeptides in culture supernatants are indicated.
Our results indicate that InvE exerts its function from within the bacterial cell. Consistent with this hypothesis, we found that InvE is not secreted and is not exposed on the bacterial surface. Furthermore, fusion of the GST protein to the amino terminus of InvE did not impair its ability to complement a strain carrying a loss-of-function mutation. Therefore, the ability of InvE to control protein translocation is most likely not due to a direct role in protein translocation but rather to its role in regulating the function of proteins directly involved in the translocation of effectors across the host cell membrane.

Consistent with a functional interaction with the protein translocases, InvE was shown to form a complex with SipB and SipC. The ability of InvE to form a complex with these proteins was dependent on the presence of their cognate chaperone, SicA. However, InvE did not interact with SicA in the absence of the translocases, indicating that InvE apparently does not...
We have previously shown that there is a built-in hierarchy in the secretion of proteins through the Salmonella SPI-1 TTSS (8). We have specifically shown that, in the absence of InvJ secretion, no effector or translocase proteins are secreted, placing InvJ at or near the top of the secretion hierarchy (8, 32). We have also shown that some of the effector proteins (e.g., SopE and SpIP) carry out opposing functions (12, 23) and, when simultaneously delivered, they effectively cancel each other’s function (12). It is therefore likely that a hierarchy is also built into the secretion and delivery of effector proteins. Since the absence of InvE differentially affects the secretion of different proteins, it is possible that it plays a role in establishing the secretion hierarchy.

Although proteins with high degree of sequence similarity to InvE in TTSSs from Shigella spp. have been described (1), enterohemorrhagic E. coli (38), Chlamydia spp. (39), and Y. enterocolitica (22), other TTSSs do not seem to possess an obvious homologue of this protein. InvE shares very limited sequence similarity with the Yersinia YopN protein (18, 46). However, the phenotype associated with the absence of these proteins is significantly different (11, 48). Furthermore, YopN is located on the bacterial surface and is secreted via the Yersinia TTSS (5, 11), while InvE is clearly intracellularly localized. In fact, no protein with the phenotype we observed for InvE has been described so far for any other TTSS. Whether proteins in other TTSSs that may have diverged in sequence play a role equivalent to that of InvE remains to be established.

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