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InvB Is Required for Type III-Dependent Secretion of SopA in *Salmonella enterica* Serovar Typhimurium

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The *Salmonella* effector protein SopA is translocated into host cells via the SPI-1 type III secretion system (TTSS) and contributes to enteric disease. We found that the chaperone InvB binds to SopA and slightly stabilizes it in the bacterial cytosol and that it is required for its transport via the SPI-1 TTSS.

Type III secretion systems (TTSS) are found in many pathogenic gram-negative bacteria and mediate the injection of an array of effector proteins into the host cell cytoplasm. Once injected, the effector proteins modulate host cell signaling cascades for the benefit of the pathogen. The secretion mechanism of these effector proteins and their secretion signals are still poorly understood. It has been shown that secretion and translocation of many effector proteins require a cognate chaperone (9, 17). These chaperones usually bind to the N-terminal region and exert various functions on their cognate effector protein, i.e., cytosolic stability (10, 20, 21), transcriptional regulation (4, 5, 19), prevention of premature interactions (7, 12, 13), maintenance of the effector in a secretion-competent state (18, 21), and recognition by the TTSS (1). Type III secretion (TTS) chaperones do not exhibit sequence similarities but share some common features. They are generally small, acidic proteins with an amphipathic C-terminal α-helix and are often encoded next to or in close vicinity to the effector protein (9, 17). In contrast, the chaperone Spa15 of *Shigella* spp. is encoded within an operon encoding essential components of the TTS apparatus and binds to not just one but several effector proteins which do not show sequence similarities (16). Due to these special features, Spa15 is thought to represent a new class of TTS chaperone (16, 17).

The *Salmonella* pathogenicity island 1 (SPI-1) of *Salmonella enterica* serovar Typhimurium encodes the protein InvB, which is homologous to Spa15 of *Shigella* spp. InvB is a chaperone for the SPI-1-encoded effector SipA/SspA (2). Recently members of our group have shown that InvB also binds to SopE and SopE2, two effector proteins encoded outside of SPI-1 but secreted in a SPI-1-dependent manner (7a). Secretion and translocation of SipA, SopE, and SopE2 depend on InvB. Based on this observation, we hypothesized that InvB might be required for secretion of additional effector proteins of serovar Typhimurium.

To address this question, we expressed a glutathione *S*-transferase (GST)-InvB fusion protein (pM672) (7a) in the mutant strain M574 (*invB*::*aphT* Δ*sopE* Δ*sopE2* Δ*sopB* Δ*sipA*) (7a), which lacks all known InvB binding effector proteins and the chromosomally encoded *invB*. This strain also lacks the effector protein gene *sopB*. However, SopB/SigD is transported via its own cognate chaperone, PipC/SigE (6). Therefore, the *sopB* mutation was not expected to affect any InvB-effector protein interactions. M574 (pM672) was grown overnight in Luria broth containing 0.3 M NaCl, diluted 1:20 into fresh medium, and grown for another 4 h at 37°C (referred to as SPI-1 inducing conditions). Cells were lysed in a French pressure cell, and GST-InvB and bound proteins were purified on glutathione-Sepharose beads from the cleared cell lysate. Aliquots from every step of the purification procedure were analyzed on a Coomassie brilliant blue-stained SDS gel. A polypeptide with an apparent molecular weight of 80 kDa was copurified with GST-InvB (Fig. 1). The band was excised from the gel, trypsin digested, and eluted as described recently (7a). The protein was identified by matrix-assisted laser desorption ionization-mass spectrometry fingerprint analysis as SopA (12 matching peptides, 21% covered sequence), a known effector protein, which is encoded outside of SPI-1 but translocated in a SPI-1-dependent manner (22). Although the biochemical activity of SopA is still unknown, it was shown to play a role in bovine enterocolitis models (22, 23).

The binding of SopA to InvB was verified by a coimmunoprecipitation experiment. For this purpose, a suicide vector (pM261) encoding a C-terminally M45-tagged version of *sopA* was integrated into the chromosome of SL1344 to generate M612, which expresses $sopA_{M45}$ under its native promoter (Table 1).

M612 was grown under SPI-1 inducing conditions and lysed in a French pressure cell. $SopA_{M45}$ binding proteins were precipitated with a mouse monoclonal anti-M45 antibody from cleared bacterial lysates as described previously (7a). Aliquots from every step of the precipitation procedure were analyzed by Western blotting using a polyclonal anti-InvB antiserum (7a) and a mouse monoclonal anti-M45 antibody (14). InvB was coimmunoprecipitated with $SopA_{M45}$ from M612 but not from the control lysate of an isogenic strain (M712) lacking amino acids 2 to 782 of *sopA* (Fig. 2, lane f). This result supports the notion that InvB binds (directly or indirectly) to SopA.

InvB has been described as a chaperone necessary for secretion of the effector proteins SipA (2), SopE, and SopE2 (7a). This suggested that InvB might also be required for secretion of SopA. To explore this hypothesis, we have con-

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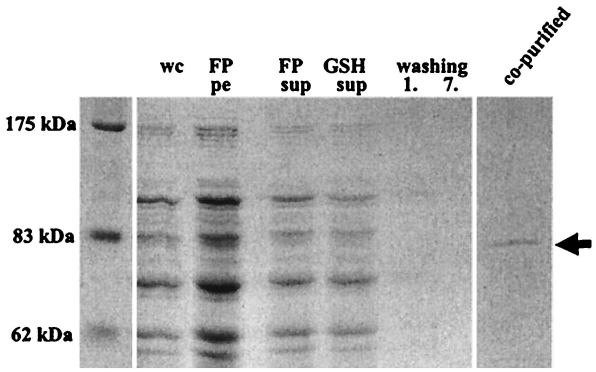

FIG. 1. Pull-down assay to isolate InvB binding proteins. GST-InvB (34 kDa) and bound proteins were purified from cleared cell lysate by incubation with glutathione (GSH)-Sepharose beads. Bound proteins larger than the GST-InvB fusion protein were analyzed by SDS-PAGE and Coomassie brilliant blue staining. wc, whole culture before harvesting of the cells; FP pe, resuspended pelleted cell debris after lysis using a French pressure cell; FP sup, cleared French pressure cell lysate; GSH sup, cleared cell lysate after binding of GST-InvB and its associated proteins; washing, supernatant after the first and seventh wash of the GSH-Sepharose beads; co-purified, GSH-Sepharose beads.

structed the isogenic serovar Typhimurium ATCC 14028 strains M619 (wild type) (2), M618 (Δ*invB*) (2), and M623 (*invC*::*aphT*), which all harbor an M45 epitope-tagged *sopA* gene in the chromosome (Table 1). The strains were grown under SPI-1 inducing conditions, and $SopA_{M45}$ secretion was analyzed by Western blotting as described elsewhere (7a).

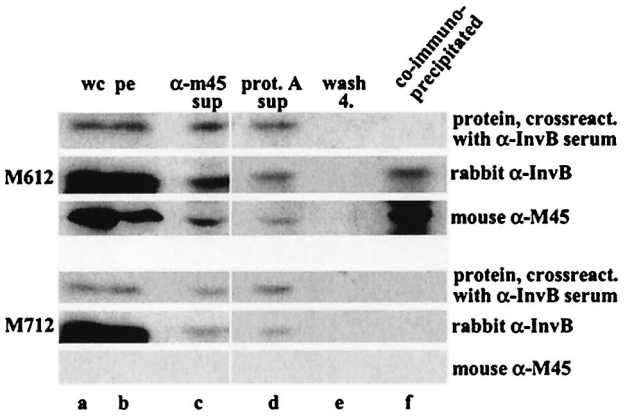

FIG. 2. InvB is coimmunoprecipitated with $SopA_{M45}$. A lysate of the $sopA_{M45}$-expressing strain M612 (top panel) was incubated with a monoclonal mouse anti-M45 (α-M45) antibody and protein A-Sepharose beads. Samples from the precipitation procedure were analyzed by Western blotting using a polyclonal rabbit anti-InvB (α-InvB) antiserum (recognizes InvB [15 kDa] and another unidentified 18-kDa *Salmonella* protein) and a mouse anti-M45 antibody (recognizes $SopA_{M45}$). To demonstrate specificity, a coimmunoprecipitation experiment was performed with M712 (bottom panel) (Table 1). The 18-kDa protein cross-reacting with the anti-InvB antiserum was not coprecipitated in either strain, which confirmed the specificity of the coimmunoprecipitation experiment. Lane a, whole culture before harvesting the cells (wc); lane b, resuspended bacterial pellet (pe); lane c, cleared cell lysate after incubation with anti-M45 antibody and removal of nonspecific aggregates by centrifugation (α-M45 sup); lane d, supernatant after incubation with protein A-Sepharose beads (prot. A sup); lane e, supernatant after the fourth wash of the protein A-Sepharose beads (wash 4); lane f, proteins bound to the protein A-Sepharose beads.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype and relevant markers[a]	Reference or source
Salmonella Typhimurium SL1344 strains		
SL1344	Wild type	Hoiseth and Stocker (11)
M574	*invB*::*aphT* Δ*sopE* Δ*sopE2* Δ*sopB* Δ*sipA*	Ehrbar et al. (7a)
M612	*sopA*::pM261	This study
M712	Δ*sopE* Δ*sopE2* Δ*sopB* Δ*sipA* Δ*sopA*	This study
Salmonella Typhimurium ATCC14028 strains		
CS401	*phoN2 zxx*::*6251* Tn*10d-Cm*, Str^r	Bronstein et al. (2)
PB502	CS401, Δ*invB*	Bronstein et al. (2)
M618	PB502, *sopA*::pM261	This study
M619	CS401, *sopA*::pM261	This study
M623	CS401, *sopA*::pM261 *invC*::*aphT*	This study
M629	CS401, *sopA*::pM261 *spaO*::*aphT*	This study
M630	PB502, *sopA*::pM261 *spaO*::*aphT*	This study
M635	CS401, *sopA*::pM265	This study
M636	PB502, *sopA*::pM265	This study
M637	CS401, *sopA*::pM265 *spaO*::*aphT*	This study
M638	PB502, *sopA*::pM265 *spaO*::*aphT*	This study
M639	CS401, *sopA*::pM265 *invC*::*aphT*	This study
Plasmids		
pM250	pBAD24 derivative, arabinose-inducible expression of *invB* (induced with 0.001% arabinose)	Ehrbar et al. (7a)
pM261	pSB377 derivative, encodes the C-terminal 287 aa of *sopA* fused to the M45 epitope	This study
pM265	pSB377 derivative, suicide vector to construct a chromosomal $sopA_{M45}$ *lacZ* transcriptional fusion	This study

[a] aa, amino acids.

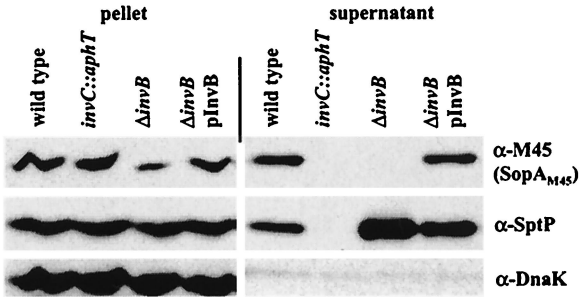

FIG. 3. *invB*-dependent expression and secretion of SopA$_{M45}$. Pelleted bacteria corresponding to 300 μl of culture and proteins recovered from 2 ml of culture supernatant of strains M619 (*sopA$_{M45}$*), M623 (*sopA$_{M45}$ invC*::*aphT*), M618 (*sopA$_{M45}$* Δ*invB*), and M618/pM250 (*sopA$_{M45}$* Δ*invB* pInvB) were analyzed by Western blotting using an anti-M45 (α-M45) antibody. The blot was reprobed with a polyclonal anti-SptP (α-SptP) antiserum to verify that a deletion of *invB* had no general effect on the TTSS and an anti-DnaK (α-DnaK) antiserum to confirm that the same amounts were loaded onto each lane.

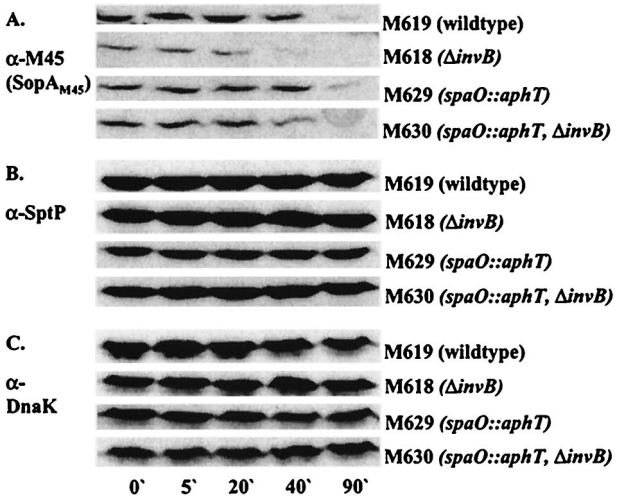

FIG. 4. Effect of an *invB* deletion on the stability of SopA$_{M45}$. Amounts of cytosolic SopA$_{M45}$ at different time points after addition of spectinomycin were analyzed by Western blotting using a mouse anti-M45 (α-M45) and polyclonal rabbit anti-SptP (α-SptP) to verify that a deletion of *invB* had no general effect on the stability of effectors. To ensure equal loading of the lanes, the blot was reprobed using an anti-DnaK (α-DnaK) antibody.

SopA$_{M45}$ was secreted from the wild-type strain M619 but not from the secretion-deficient strain M623 (*invC*::*aphT*), lacking the ATPase InvC (8), and the Δ*invB* strain M618 (Fig. 3, upper panel). The latter secretion defect could be complemented using the *invB* expression vector pM250, which expresses *invB* under control of an arabinose-inducible promoter (7a). Reprobing with a rabbit polyclonal antiserum raised against amino acids 49 to 543 of the SPI-1 effector protein SptP verified that the *invB* deletion had no general effect on the TTSS (Fig. 3, middle panel).

The cytoplasmic SopA$_{M45}$ pool was slightly lower in the Δ*invB* strain M618 than in the wild-type strain, M619 (Fig. 3). This indicated that InvB might play a role in stabilization or expression of SopA. To examine the cytoplasmic stability of SopA$_{M45}$, one has to consider that InvB might have two functions: stabilization of cytoplasmic SopA and transport of SopA via the SPI-1 TTSS. If significant amounts of SopA protein become transported to the outside during the course of the assay, this fraction might become protected from degradation by *Salmonella* proteases. To exclude this, we have analyzed the role of *invB* in stabilization of cytoplasmic SopA$_{M45}$ in secretion-deficient strains. The *invB* open reading frame is overlapping with the *invC* open reading frame. Therefore, it was not possible to combine the Δ*invB* and *invC*::*aphT* alleles (Fig. 3; Table 1) by P22 transduction. For this reason we constructed the secretion-deficient *spaO*::*aphT* strain (M629), lacking an essential subunit of the export apparatus (3) encoded 2.7 kb downstream of *invB*, and the Δ*invB spaO*::*aphT* double mutant (M630).

We then analyzed the cytosolic stability of SopA$_{M45}$ in the Δ*invB* strain M618 (*sopA$_{M45}$* Δ*invB*; does not secrete SopA$_{M45}$ [Fig. 3]), the secretion-deficient mutant M629 (*sopA$_{M45}$ spaO*::*aphT*), and the double mutant M630 (*sopA$_{M45}$ spaO*::*aphT* Δ*invB*) (Table 1). As a control we also examined the cytosolic stability of SopA$_{M45}$ in the wild-type strain M619 (*sopA$_{M45}$*). M619, M618, M629, and M630 were grown under SPI-1 inducing conditions, and protein biosynthesis was inhibited by addition of spectinomycin (final concentration, 200 μg/ml). Aliquots were removed 0, 5, 20, 40, and 90 min after spectinomycin addition. Western blot analysis of bacterial pellets revealed that SopA$_{M45}$ degradation was slightly accelerated in the absence of InvB (Fig. 4A, compare M629 and M630 or M618 and M629). In the wild-type strain background (M619), the amount of bacterium-associated SopA$_{M45}$ was slightly higher than in the secretion-deficient strain M629 at the beginning of the experiment (0′ to 20′) but decreased faster (Fig. 4A). As discussed above, this is probably due to cumulative effects of secretion of SopA$_{M45}$ from M619 into the culture supernatant and degradation. For this reason we could not base any conclusions about the role of InvB in SopA stabilization on this strain.

The stability of the effector protein SptP (cognate chaperone is SicP) was not affected by the *invB* mutation (Fig. 4). Altogether, these data suggested that InvB has a slight effect on stabilization of SopA in the bacterial cytosol.

To verify that the *invB* expression level is not altered in the secretion-deficient mutants, we performed a Western blot analysis using the strains M619 (*sopA$_{M45}$*), M618 (*sopA$_{M45}$* Δ*invB*), M629 (*sopA$_{M45}$ spaO*::*aphT*), M630 (*sopA$_{M45}$ spaO*::*aphT* Δ*invB*), and M623 (*sopA$_{M45}$ invC*::*aphT)* (Table 1). This analysis confirmed that the amount of cytosolic InvB is not altered in the secretion-deficient mutants M629 (*sopA$_{M45}$ spaO*::*aphT*) and M623 (*sopA$_{M45}$ invC*::*aphT*) and that InvB is absent from the *invB* deletion strains M618 (*sopA$_{M45}$* Δ*invB*) and (*sopA$_{M45}$ spaO*::*aphT* Δ*invB*) (Fig. 5).

To analyze whether *invB* reduces transcription of *sopA*, we constructed *lacZ* transcriptional reporter strains. The *lacZ* cassette of pSB1040 (kindly provided by D. Zhou and J. E. Galán) was cloned downstream of the *sopA$_{M45}$* stop codon into pM261 (see above) to create the suicide vector pM265 (Table 1). pM265 was integrated into the chromosome of CS401, yielding M635 (*sopA$_{M45}$ lacZ*). The control strains M636 (Δ*invB*), M637 (*spaO*::*aphT*), M638 (Δ*invB spaO*::*aphT*), and M639 (*invC*::*aphT*) were constructed by P22 transduction of the *sopA*::pM265 allele (Table 1).

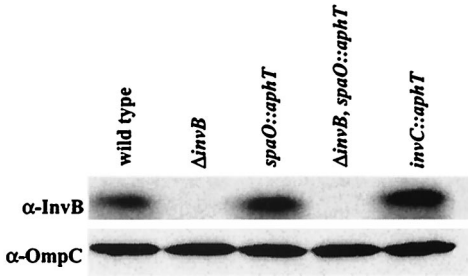

FIG. 5. Western blot analysis of *invB* expression level. Bacteria were grown under SPI-1 inducing conditions. Bacteria recovered from 200 μl of culture of strains M619 ($sopA_{M45}$), M618 ($sopA_{M45}$, Δ*invB*), M629 ($sopA_{M45}$ *spaO*::*aphT*), M630 ($sopA_{M45}$ Δ*invB spaO*::*aphT*), and M623 ($sopA_{M45}$ *invC*::*aphT*) were analyzed by Western blotting using an anti-InvB antiserum (α-InvB). The blot was reprobed with a monoclonal anti-OmpC antibody (α-OmpC) to verify that equivalent amounts of lysate were loaded onto each lane.

Thus, we could use β-galactosidase assays to study *sopA* promoter activity. The β-galactosidase activity was determined in at least eight independent experiments, and statistical analysis was performed using the exact Mann-Whitney U test. We found that *sopA* transcription was in the same order of magnitude for all strains, analyzed (Fig. 6). Disruption of *invB* did not decrease β-galactosidase activity. Rather, β-galactosidase activity was slightly but significantly increased in M636 (Δ*invB)* ($P < 0.001$), M639 (*invC*::*aphT)* ($P = 0.001$), and M638 (Δ*invB spaO*::*aphT)* ($P < 0.001$) (Fig. 6). Therefore, the decreased $SopA_{M45}$ protein levels in the cytoplasm of an *invB* mutant (Fig. 3) are attributable to a slightly decreased protein stability but not to transcriptional down regulation. However, the reasons for the slight augmentation of transcription in M636, M638, and M639 remain to be analyzed.

In summary, the copurification and coimmunoprecipitation experiments demonstrate that InvB binds directly or indirectly to SopA. In the absence of InvB, SopA is not secreted and its intracellular stability is decreased. Although SipA, SopE/SopE2, and SopA do not share sequence similarities, they all require InvB for their transport via the SPI-1 TTSS (2, 7a; also this study).

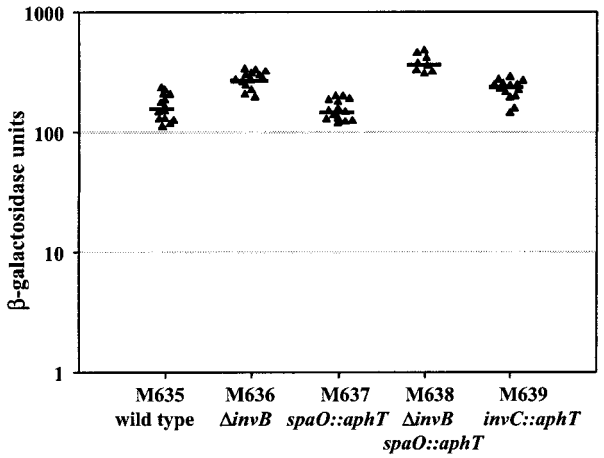

FIG. 6. Effect of an *invB* deletion on transcription of $sopA_{M45}$. Transcription of $sopA_{M45}$ was measured using transcriptional *lacZ* reporter constructs in standard β-galactosidase activity assays. β-Galactosidase activities were determined in at least eight independent experiments. Bars indicate the median.

TTS chaperones have been divided into three classes: class I, chaperones which associate with effector proteins; class II, chaperones which associate with translocators; and class III, chaperones of the flagellar system (17). Due to their unique features, InvB and its homologs Spa15 (*Shigella* spp.), YsaK (*Yersinia* spp.), and InvB (*Sodalis* spp.) are thought to represent a new family of TTS chaperones. Therefore, they have been assigned to the new subclass IB, which represents chaperones that bind several different effectors (17). This classification was based on experimental evidence from *Shigella flexneri* (16). Interestingly, Page and Parsot have hypothesized that InvB, like Spa15, might also associate with different unrelated proteins (15). This was confirmed by our findings that InvB is a chaperone not only for SipA (2) but also for SopE, SopE2 (7a), and SopA (this work).

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