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Requirement for Exported Proteins in Secretion through the Invasion-Associated Type III System of *Salmonella typhimurium*

CARMEN M. COLLAZO AND JORGE E. GALÁN*

Department of Molecular Genetics and Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-5222

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The *inv* and *spa* loci of *Salmonella typhimurium* encode a type III protein secretion system which is essential for the ability of this microorganism to gain access to cultured epithelial cells. These loci are located at centisome 63 in the *Salmonella* chromosome. We have carried out a functional analysis of several genes of these loci and have found that two exported proteins encoded in this region, InvJ and SpaO, are required for secretion through the invasion-associated type III secretion system. These findings suggest the existence of a hierarchy in the export process, since mutations in other targets of this secretory system have no effect on protein secretion. We have also shown that the *spaO*, *spaP*, *spaQ*, and *spaR* genes are required for protein secretion and for the ability of *S. typhimurium* to gain access to cultured epithelial cells. In addition, we investigated the ability of an *invJ S. typhimurium* mutant strain to present the SipB protein to the bacterial surface and demonstrated that, in contrast to Spa32, its putative *Shigella* homolog, InvJ is not involved in the surface presentation of the Sip proteins.

Infections caused by *Salmonella* spp. represent a major health problem worldwide, with an estimated 1.3 billion cases occurring every year (40). The entry of *Salmonella* spp. into cells of the intestinal epithelium is an essential step in the development of a productive infection. The internalization process is the result of an intimate interaction between the *Salmonella* organism and its host cell. As a consequence of this interaction, a host cell signaling cascade is initiated and leads to cytoskeletal rearrangements, membrane ruffling, macropinocytosis, and subsequent bacterial uptake (5).

Several bacterial genetic loci involved in the entry process have been identified, and most of them are located in the centisome 63 region of the *Salmonella* chromosome (2, 6, 9, 12, 14, 15, 17, 25–28, 30, 36, 41). It has been estimated that perhaps as much as 40 kb of a contiguous region of the *Salmonella typhimurium* chromosome, which is absent from *Escherichia coli* K-12, may encode entry determinants (35). The *inv* and *spa* loci, which are required for the ability of *Salmonella* spp. to enter host cells, are located in this region. Sequence analysis of the predicted polypeptides encoded by several genes in these loci has revealed homologies with components of protein secretion systems of other mammalian pathogens, such as *Shigella* spp., enteropathogenic *E. coli*, and *Yersinia* spp. (11). In addition, homologies have also been observed with polypeptides of secretory systems in plant-pathogenic bacteria and the flagellar export-assembly apparatus (8, 50). These systems have unique features and therefore have been classified as type III or host cell contact-dependent protein secretion systems (42). Some of these features include the absence of a typical amino-terminal signal sequence characteristic of proteins exported in a *sec*-dependent manner, the lack of processing of the amino-terminal end of the secreted polypeptides, the simultaneous export through the inner and outer membranes, and the host cell contact activation of the secretion process. The secretory machinery appears to be very complex, with several putative structural components. A number of proteins that make use of this type of system to exit the bacterial cell have been identified. These include the Yop proteins of *Yersinia* spp. (47); the Ipa (44), VirA (49), and IpgD (1) proteins of *Shigella* spp.; and the EaeB protein of enteropathogenic *E. coli* (7). Recently, we and others have identified several targets of the Inv-Spa secretory apparatus. These include the InvJ (6), SpaO (32), Sip (27, 28, 41), and SptP (29) proteins. We have shown that all but two of these targets (the SipA and SptP proteins) are required for bacterial uptake, since null mutations in these genes abolished the ability of *Salmonella* spp. to enter epithelial cells.

The *spa* locus was originally identified by Groisman and Ochman in an effort to analyze cloned DNA fragments that had been described as being specific to *Salmonella* spp. (17). It was demonstrated that two genes of this locus, *spaP* and *spaS*, play an important role in bacterial entry. However, the roles of other members of the *spa* locus in bacterial uptake have not been investigated. Furthermore, the roles of the *spa* genes in protein secretion, although postulated, have not been experimentally demonstrated. In the present study, we investigated the individual contributions of the *spa* genes to *Salmonella* entry and their involvement in protein secretion.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study and their sources are listed in Table 1. Strains were grown in L broth or on L-agar plates (31), and when required, antibiotics were added at the following concentrations: kanamycin, 50 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and streptomycin, 100 μg ml^{-1}.

Recombinant DNA techniques. All recombinant DNA techniques were performed by standard procedures (43). Isolation of plasmid DNA was performed by the method of Birnboim and Doly (4). Transformation of circular DNA into *E. coli* and *Salmonella* strains by electroporation was carried out as described elsewhere (39) with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Amplification of DNA fragments by PCR was performed with a commercial kit (GenAmp; Perkin-Elmer Cetus) according to the instructions of the manufacturer. For Southern hybridization studies, chromosomal DNA was isolated as previously described (13) and digested with *Hin*dIII. DNA fragments were separated on a 0.7% agarose gel and transferred to nylon membranes (GeneScreen Plus; Dupont, Wilmington, Del.). The membranes were then probed with an [α-^{32}P]ATP-labeled fragment generated by PCR with primers

* Corresponding author. Phone: (516) 632-8780. Fax: (516) 632-8891. Electronic mail address: galan@asterix.bio.sunysb.edu.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference or source
S. typhimurium		
SL1344	*rpsL hisG*	20
SB169	*rpsL hisG sipB*::*aphT*	28
SB241	*rpsL hisG sipD*::*aphT*	27
SB302	*rpsL hisG invJ*::*aphT*	6
SB303	*rpsL hisG spaO*::*aphT*	This study
SB304	*rpsL hisG spaP*::*aphT*	This study
SB305	*rpsL hisG spaQ*::*aphT*	This study
SB306	*rpsL hisG spaR*::*aphT*	This study
E. coli		
χ6060	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA20 galK galE recA1 rpsE argE*(Am) *rpoB thi* [F′ (*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)::Tn*5*]	Derived by R. Goldschmidt from CC118 (33)
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r) λ*pir*	34

derived from sequences upstream of the *spaO* gene (5′-GGGGTACCGTCAA ATAC-3′) and downstream of the *spaR* gene (5′-CGGGATCCGGAGTCTTC CAGC-3′).

Attachment and invasion assays. The abilities of *S. typhimurium* strains to enter into and attach to Henle-407 cells were assayed in 24-well tissue culture plates as described elsewhere (12).

Plasmid constructions. Partial restriction maps are shown in Fig. 1. Plasmid pYA2225 was constructed by cloning a 7.3-kb *Sal*I fragment from pYA2219 (12) into pUC18 (53). Digestion of pYA2225 with *Pst*I generated four DNA fragments. Two of these fragments, of approximately 1,700 and 250 bp, were subcloned into pBluescript SKII (Stratagene), to yield plasmids pSB453 and pSB455, respectively. A *Pst*I-*Sal*I fragment of approximately 760 bp was removed from pYA2225 and subcloned into pBluescript SKII, yielding plasmid pSB451. Plasmid pSB702 was generated by cloning a *Sal*I-*Bgl*II fragment of approximately 1,300 bp into the *Sal*I-*Bam*HI sites of pMob (48). These plasmids were used for the construction of the nonpolar mutations. For complementation studies of the *S. typhimurium* mutant strains, the following plasmids were generated. An 1,180-bp fragment carrying *spaO* was amplified from pYA2225 by PCR with degenerative primers derived from sequences upstream (5′-GGGGTACCGTC AAATAC-3′) and downstream (5′-CGGGATCCCAGGGCGTT-3′) of the *spaO* gene. The amplified fragment was digested with *Kpn*I and *Bam*HI and cloned into the plasmid expression vector pWKS30 (54), resulting in plasmid pSB469. An 874-bp fragment carrying *spaP* was amplified from pYA2225 by PCR with degenerative primers complementary to sequences upstream (5′-GGGGT ACCCTCGCCGAA-3′) and downstream (5′-CGGGATCCCGTCTCGTA-3′) of *spaP*. The PCR product was cloned into the *Sma*I site of the plasmid vector pLG339 (46), resulting in plasmid pSB498. An 890-bp fragment carrying the *spaQ* gene was generated by digesting pSB702 with *Hin*cII. This fragment was cloned into the *Sma*I site of pLG339 (46), yielding plasmid pSB499. A 1,014-bp fragment containing *spaR* was amplified from pSB702 by PCR with degenerative primers complementary to sequences upstream (5′-GGGGTACCTGGTAGGG TTA-3′) and downstream (5′-CGGGATCCGGAGTCTTCCAGC-3′) of *spaR*. The amplified fragment was digested with *Kpn*I and *Bam*HI and cloned into pWKS30 (54), resulting in plasmid pSB477.

Construction of nonpolar mutations. *spaO*, *spaP*, *spaQ*, and *spaR* have overlapping stop and start codons and do not show the presence of long intergenic regions, which suggests that they are part of the same transcriptional unit (17). Therefore, to examine the individual contributions of these genes in the entry process, strains carrying nonpolar mutations were constructed as follows. Mutations in *spaO* were constructed by introducing into the *Nco*I site of pSB453 a cartridge containing a modified aminoglycoside 3′-phosphotransferase gene (*aphT*) from which the transcriptional terminator had been removed (14), yielding plasmid pSB458 (Fig. 1). Insertion of this cartridge does not result in polar effects on the expression of downstream genes. A *Kpn*I-*Sac*I fragment from pSB458, carrying the mutated *spaO* gene and flanking sequences, was cloned into the *Sma*I site of pSB377, an R6K-derived replicon that cannot replicate in *S. typhimurium* in the absence of the Pir protein (26). The resulting plasmid, pSB463, was transformed into SM10λ*pir* (34), an *E. coli* strain that allows the replication and maintenance of this plasmid. pSB463 was then mobilized into *S. typhimurium* by conjugation, and transconjugants were selected on streptomycin and kanamycin plates. Streptomycin- and kanamycin-resistant and tetracycline-sensitive colonies were selected by replica plating. One transconjugant, SB303 (*spaO*::*aphT*), showed a Southern hybridization pattern consistent with the presence of the *aphT* cassette at the proper location (Fig. 2). Mutations in the *spaP*, *spaQ*, and *spaR* genes were constructed by a similar strategy. An *aphT* cassette was introduced into the *Bcl*I, *Bsp*MI, and *Cla*I sites of the *spaP*, *spaQ*, and *spaR* genes, respectively, yielding plasmids pSB459, pSB480, and pSB460, respectively. *Kpn*I-*Sac*I fragments from plasmids pSB459, pSB480, and pSB460 were cloned into the *Sma*I site of pSB377, yielding plasmids pSB464, pSB488, and pSB467, respectively. These plasmids were used to construct the *S. typhimurium* strains carrying mutations in the *spaP*, *spaQ*, and *spaR* genes as described for *spaO*. The resulting mutant strains SB304 (*spaP*::*aphT*), SB305 (*spaQ*::*aphT*), and SB306 (*spaR*::*aphT*) showed a Southern hybridization pattern consistent with the correct insertion of the *aphT* cassette (Fig. 2). These mutant strains were used in a variety of functional assays.

Analysis of proteins from culture supernatants. Two hundred fifty milliliters of L broth containing 0.3 M NaCl was inoculated with 8 ml of an overnight culture of wild-type or mutant strains of *S. typhimurium* (28). Cultures were grown with gentle aeration (180 rpm) to an optical density of 0.5 at 600 nm. Bacterial cells were removed by centrifugation at 16,000 × *g* for 20 min, and culture supernatants were harvested and filtered through a 0.45-μm-pore-size filter. Proteins were precipitated by adding trichloroacetic acid to a final concentration of 10% and incubating at 4°C for 1 h to overnight. Proteins were then recovered by centrifugation at 16,000 × *g* for 20 min. The pellets were resuspended in 5 ml of cold phosphate-buffered saline (PBS), and proteins were precipitated by adding 20 ml of cold acetone. After centrifugation at 12,000 × *g* for 20 min, the pellets were washed with 1.5 ml of cold acetone, centrifuged, dried, and resuspended in 250 ml of PBS–50 mM Tris-Cl, pH 8.0.

Western blot (immunoblot) analysis. Samples were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Specific proteins were detected by immunoblotting with the appropriate antibodies followed by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.). Blots were probed with the previously described monoclonal antibody J33.13 to InvJ (6) and with polyclonal antisera generated against the SipB and SipC proteins.

Epitope tagging of the carboxy-terminal end of SpaO. An epitope recognized by the monoclonal antibody M45 and consisting of 18 residues from the E4-6/7 protein of adenovirus (MDRSRDRLPPFETETRIL) (38) was used to tag the SpaO protein. A 920-bp DNA fragment from pYA2225 was amplified by PCR with primers complementary to sequences upstream (5′-CCCACATGTCATTG CGTGTGAGACAGATTG-3′) and downstream (5′-CCCACATGTCCCCATT ACCAGACTCGC-3′) of the *spaO* gene. The PCR fragment was digested with *Afl*III and cloned into the *Nco*I site of the tagging vector pSB504, a pBAD24 (18) derivative that carries the epitope tag, yielding plasmid pSB505. The expression of the *spaO* gene in the resulting plasmid is driven by the P_{BAD} promoter, which can be induced by the addition of arabinose.

Recovery of the tagged SpaO protein from culture supernatants. Portions (100 ml) of L broth containing 0.3 M NaCl, 100 μg of ampicillin per ml, and 0.00002% arabinose were inoculated with 2 ml of overnight cultures of different *S. typhimurium* strains carrying plasmid pSB505 grown in L broth containing ampicillin and 0.3 M NaCl. The amount of arabinose used was the minimum concentration of arabinose that allowed full complementation of a *spa* null mutation by the epitope-tagged protein and therefore more closely resembles the physiological levels of SpaO. The cultures were grown with gentle aeration (150 rpm) to an optical density of 0.40 to 0.45 at 600 nm. The proteins present in the culture supernatant were recovered as described above.

Surface presentation assay. A 96-well enzyme-linked immunosorbent assay (ELISA) plate (Dynatech Laboratories, McLean, Va.) was coated with 10 μg of *S. typhimurium* culture supernatant proteins per ml resuspended in carbonate-bicarbonate buffer (0.5 mM, pH 9.6). Coating was allowed to proceed overnight at 4°C. The following day, the wells were washed with PBS–0.05% Tween 20 (PBST). Five percent (wt/vol) bovine serum albumin in PBST was added to the plate and incubated for 1 h at 37°C. Portions (100 ml) of L broth containing 0.3 M NaCl were inoculated with 10 ml of overnight cultures of wild-type or mutant strains of *S. typhimurium*. The cultures were grown with gentle aeration (150 rpm) to an optical density of 0.8 at 600 nm. Bacteria were harvested by centrifugation and resuspended in 2 ml of PBS. Portions (50 μl) of the bacterial suspension were mixed with 50 μl of different dilutions of the monoclonal antibody 111.27.1 directed against the SipB protein. The bacterium-antibody mixture was incubated for 35 min at room temperature with gentle rocking, and

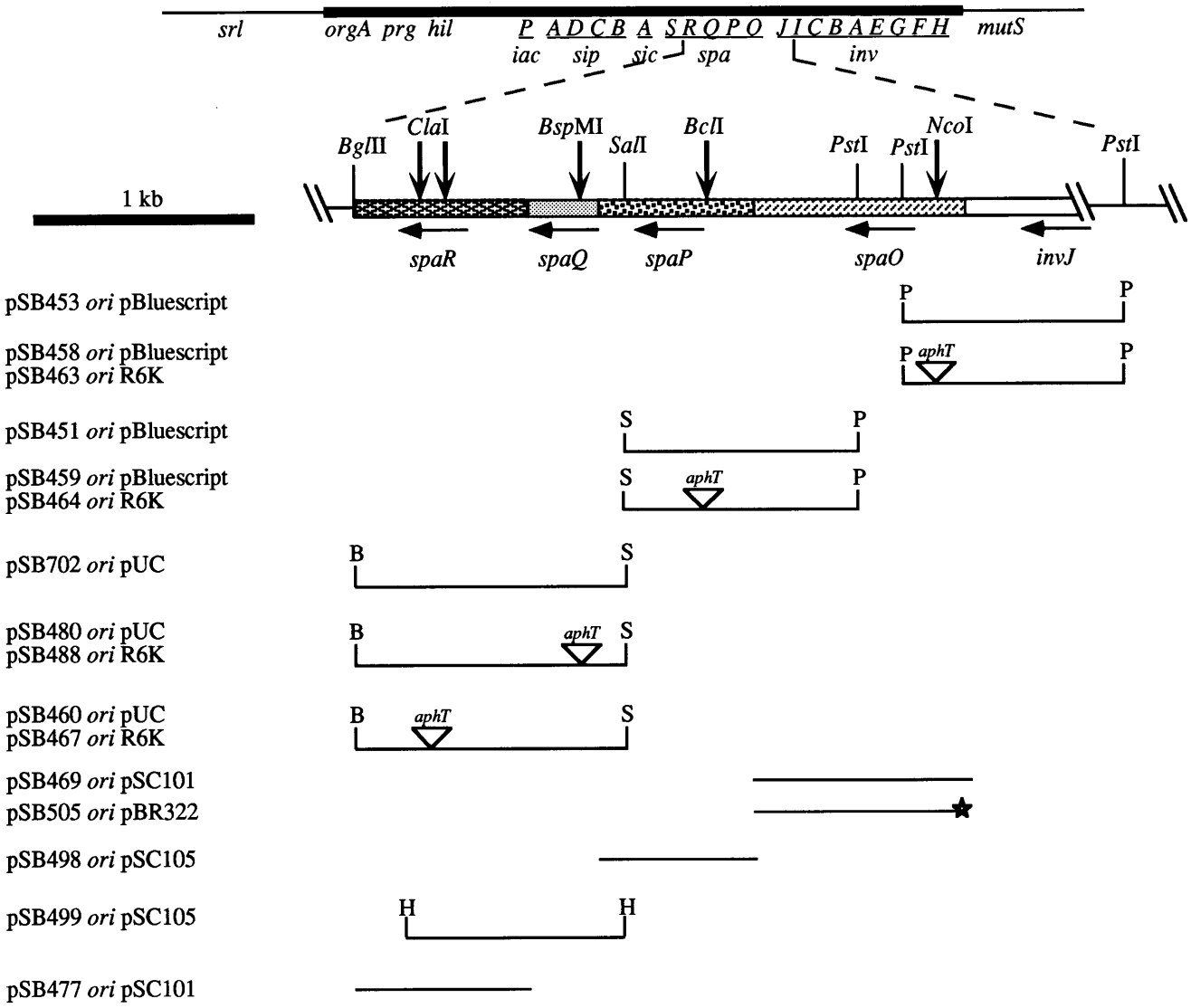

FIG. 1. Partial restriction endonuclease maps of the inserts of relevant plasmids used in this study. The position of the *inv-spa* region in relation to other invasion loci in centisome 63 of the *Salmonella* chromosome is indicated. The localization of the *spa* genes is indicated by shaded boxes, and the direction of transcription is shown by horizontal arrows. The position of the aminoglycoside 3′-phosphotransferase (*aphT*) cassette is indicated by a triangle. The cloning site of the *aphT* cartridge within each open reading frame is indicated by vertical arrows. Relevant restriction sites are indicated by capital letters: P, *Pst*I; S, *Sal*I; B, *Bgl*II; H, *Hin*cII. The position of the epitope tag recognized by the monoclonal antibody M45 is shown by a star.

bacteria were removed by centrifugation. The bacterium-free supernatant was then added to the 96-well ELISA plate and incubated for 2 h at 37°C. After 2 h, the wells were washed three times with PBST and an anti-mouse immunoglobulin G–alkaline phosphatase conjugate antiserum (Sigma ImmunoChemicals, St. Louis, Mo.) was added to the wells and subsequently incubated for 1 h at 37°C. After several washes with PBST, the substrate *p*-nitrophenyl phosphate (Sigma-Diagnostics, St. Louis, Mo.) was added and the reaction was allowed to proceed at 37°C. The A_{405} was measured with an ELISA microreader (Dynatech Laboratories). The concentrations of the antibody and bacteria were optimized by making serial dilutions until values within the linear range were obtained.

RESULTS

The *spa* genes are required for *S. typhimurium* entry into Henle-407 cells. A deletion of approximately 5.6 kbp of the *spa* region impaired the ability of *S. typhimurium* to enter cultured epithelial cells (17), suggesting that there are genetic elements in this region that are involved in the entry process. However, of the putative nine open reading frames that were described in that paper, the contributions to the invasion phenotype of only two (the *spaP* and *spaS* genes) were investigated. Previous studies in our laboratory have shown that there are three open reading frames in the centisome 63 invasion region, the *invB* (9), *sipA* (27), and *sptP* (29) genes, that when mutated do not cause a defect in the ability of the bacteria to enter cultured epithelial cells. Therefore, any inference about function based on topology cannot be conclusive. To study the potential involvement of the open reading frames encoded in the *spa* locus in *S. typhimurium* entry into cultured epithelial cells, we constructed a series of strains carrying nonpolar mutations in each of the *spa* genes. The resulting strains were tested for the ability to enter into and attach to Henle-407 cells. As shown in Fig. 3I, all strains were severely impaired in the ability to enter into cultured epithelial cells, although they were not affected in the ability to attach to the same cells (Fig. 3II). The introduction of plasmids pSB469 (*spaO*$^+$), pSB498 (*spaP*$^+$), pSB499

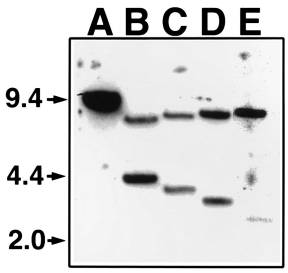

FIG. 2. Southern hybridization analysis of the *S. typhimurium spaO*, *spaP*, *spaQ*, and *spaR* mutant strains. Chromosomal DNA was isolated from the different strains and digested with *Hin*dIII, which cuts within the *aphT* cassette introduced into each open reading frame. Fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membranes as described in Materials and Methods. The membranes where hybridized to a probe, generated by PCR with primers flanking the *spaO* and *spaR* genes, that was labeled with [α-^{32}P]ATP. The lanes contain DNA isolated from the following strains: lane A, SL1344 (wild type); lane B, SB303 (*spaO*::*aphT*); lane C, SB304 (*spaP*::*aphT*); lane D, SB305 (*spaQ*::*aphT*); lane E, SB306 (*spaR*::*aphT*). The arrows and the numbers on the left indicate the positions of the λDNA/*Hin*dIII markers (in kilobase pairs).

(*spaQ*$^+$), and pSB477 (*spaR*$^+$) into the respective mutant strains restored the ability of *S. typhimurium* to enter cultured epithelial cells, which indicated that the entry defect in these mutant strains was due to the inactivation of each particular gene (Fig. 3I). These results clearly demonstrate that the *spaO*, *spaP*, *spaQ*, and *spaR* genes are essential for bacterial entry but are not required for bacterial attachment to cultured epithelial cells.

The *spa* genes are required for the secretion of the InvJ, SipB, and SipC proteins. Since the predicted polypeptides encoded by the *spa* genes are homologous to proteins involved in the secretion of virulence determinants in other microorganisms (3, 10, 16, 22–24, 37, 45, 51, 52), the roles of these genes in protein secretion were investigated. Whole-cell lysates and culture supernatants of wild-type *S. typhimurium* and *S. typhimurium* strains carrying nonpolar mutations in the *spaP*, *spaQ*, and *spaR* genes were tested for the ability to secrete InvJ, SipB, and SipC, three previously identified targets of the protein secretion apparatus encoded in the centisome 63 region of the *Salmonella* chromosome. As shown in Fig. 4A, mutations in the *S. typhimurium spaP*, *spaQ*, and *spaR* genes prevented the secretion of the InvJ, SipB, and SipC proteins to the culture supernatant. This defect in secretion was not due to decreased expression of the genes encoding the secreted targets, since similar amounts of these proteins were detected by immunoblotting in the whole-cell lysates of wild-type and the mutant strains (Fig. 4B). The ability of these mutant strains to secrete into the culture supernatant the InvJ, SipB, and SipC proteins was restored when complementing plasmids encoding wild-type copies of the different genes were introduced into the respective strains (Fig. 4A). These results establish a role for the *spa* genes in protein secretion and indicate that they are part of the type III secretory machinery encoded in centisome 63 of the *Salmonella* chromosome.

The secreted proteins InvJ and SpaO are required for protein secretion. We have previously shown that the InvJ protein is secreted to the culture supernatant in an *inv*-dependent manner (6). Similarly, Li et al. have recently reported that the SpaO protein is secreted to the culture supernatant presumably in an *inv-spa*-dependent manner, since a strain carrying a deletion of the *invCIJ* and *spaOPQRS* genes was unabled to secrete a tagged form of SpaO (32). Since *invJ* and *spaO* are immediately adjacent to genes which are part of the invasion-associated protein secretion system, we investigated their potential involvement in the secretion of known targets of this protein secretion system. Whole-cell lysates and culture supernatants of wild-type and *invJ* and *spaO* mutant strains were examined for the presence of the secreted proteins SipB and

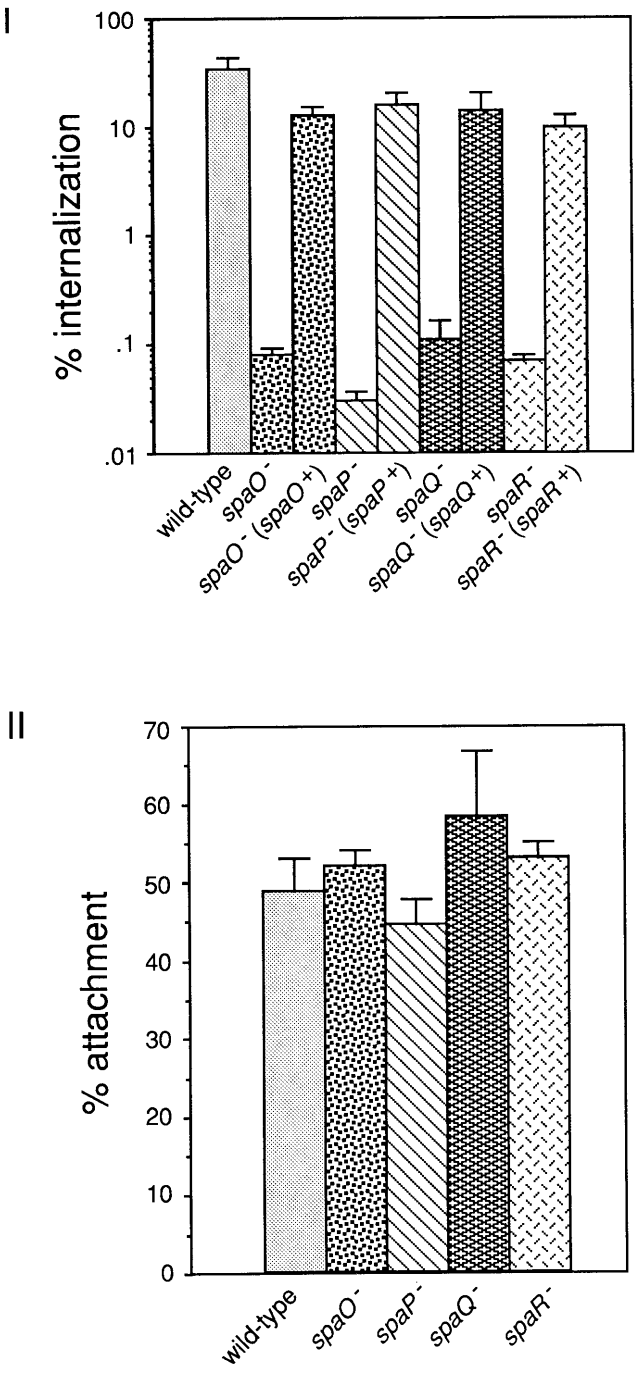

FIG. 3. Effect of *spaO*, *spaP*, *spaQ*, and *spaR* mutations on *S. typhimurium* entry into (I) and attachment to (II) cultured Henle-407 cells. Strains: wild-type, SL1344; *spaO*$^-$, SB303; *spaO*$^-$ (*spaO*$^+$), SB303(pSB469); *spaP*$^-$, SB304; *spaP*$^-$ (*spaP*$^+$), SB304(pSB498); *spaQ*$^-$, SB305; *spaQ*$^-$ (*spaQ*$^+$), SB305(pSB499); *spaR*$^-$, SB306; *spaR*$^-$ (*spaR*$^+$), SB306(pSB477). Values represent the means and standard deviations for triplicate samples and represent the percentage of the initial inoculum that survived gentamicin treatment for 2 h (12). Equivalent results were observed in several repetitions of this experiment.

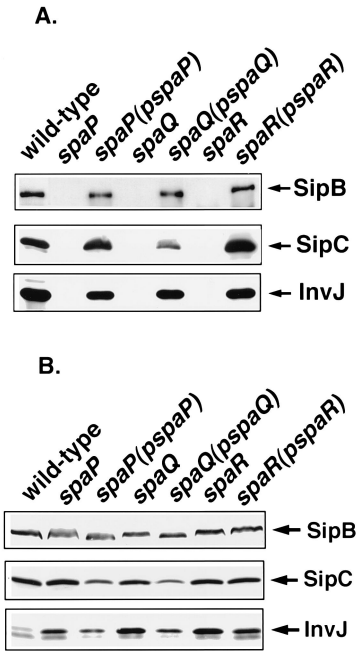

FIG. 4. Effect of *S. typhimurium spaP*, *spaQ*, and *spaR* mutations on the secretion of the SipB, SipC, and InvJ proteins. Culture supernatants (A) and whole-cell lysates (B) were prepared as described in Materials and Methods, transferred to nitrocellulose membranes, and probed with antibodies generated against the SipB, SipC, and InvJ proteins. The arrows indicate the positions of the SipB, SipC, and InvJ proteins.

SipC by Western blot analysis. Mutations in *invJ* (Fig. 5I) and *spaO* (Fig. 5II) abolished the secretion of SipB and SipC to the culture supernatant. Secretion could be restored upon complementation of the *invJ* and *spaO* mutations with plasmids pSB475 and pSB469, respectively. These results indicate that InvJ and SpaO, which are targets of the type III secretion system encoded in the *inv* and *spa* loci, are required for the secretion of the Sip proteins. We also examined the influence of InvJ and SpaO on the secretion of each other. As shown in Fig. 6, mutations in *invJ* prevented SpaO secretion, and mutations in *spaO* prevented InvJ secretion. Previously, we have shown that mutations in *sipD* result in the increased secretion of a subset of proteins that does not include InvJ (27). We therefore examined the effect of *sipD* on the secretion of SpaO. As shown in Fig. 6II, mutations in *sipD* did not result in any significant alteration in the secretion of SpaO, further supporting the notion that InvJ and SpaO belong to the same functional subgroup of secreted targets.

We have shown before that null mutations in the genes encoding the secreted proteins SipA, SipC, and SptP did not have any effect on the levels of secretion of any known target of the invasion-associated type III protein secretion system (27–29). Those observations in conjunction with the data presented here establish the existence of several functional groups of secreted proteins: one class, represented by InvJ and SpaO, is required for the secretion process; another class, represented by SipD, modulates the secretion process; and a third class, represented by SipA, SipB, SipC, and SptP, does not affect the secretion process and may have effector functions.

Effect of *invJ* on the surface presentation of SipB. It has been suggested previously that *Salmonella* InvJ and *Shigella* Spa32 may be functional homologs, since they have similar sizes and topological organizations (17). However, when these two proteins are aligned, they exhibit only 19% identity. In fact, InvJ and Spa32 are the least similar pair in the otherwise highly conserved group of homologous proteins in the type III secretion systems in *Shigella* and *Salmonella* spp. It has been postulated that Spa32 is involved in the release of the Ipa proteins into the culture supernatant, since a *spa32* mutant strain allowed the surface presentation but not the secretion of these proteins (56). These observations have led to the proposal of a two-step model for the translocation of the Ipa proteins. The first step involves the export of the proteins to the bacterial cell surface, a process mediated by several genes from the *mxi* and *spa* loci. The second step is their release to the extracellular milieu, a process mediated by Spa32. To investigate further the potential functional homology between Spa32 and InvJ, we compared the levels of the secreted protein SipB on the surfaces of wild-type *S. typhimurium* and the isogenic *invJ* and *spaP* mutant strains. SpaP is an essential component of the invasion-associated type III protein secretion apparatus. As shown in Fig. 7, SipB was not detected on the surface of either the *invJ* or *spaP* mutant strain, although it was readily detected on the surface of the wild-type strain. These results indicate that InvJ and Spa32 most likely have different functions in the secretion process. In addition, these results suggest the existence of differences in the mechanisms of secretion of *Salmonella* spp. and *Shigella* spp.

DISCUSSION

We have shown that the *spaO*, *spaP*, *spaQ*, and *spaR* genes of *S. typhimurium* play an essential role in bacterial internalization, since nonpolar mutations in each one of these genes rendered *S. typhimurium* deficient for entry into cultured epi-

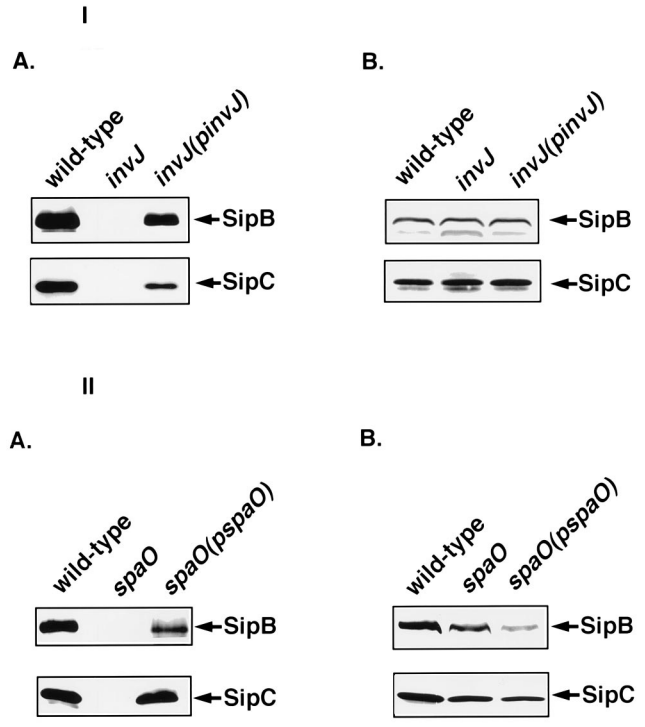

FIG. 5. Effect of *S. typhimurium invJ* (I) and *spaO* (II) mutations on the secretion of the SipB and SipC proteins. Culture supernatants (A) and whole-cell lysates (B) were prepared as described in Materials and Methods, transferred to nitrocellulose membranes, and probed with antibodies directed against SipB and SipC. The arrows indicate the positions of the SipB and SipC proteins.

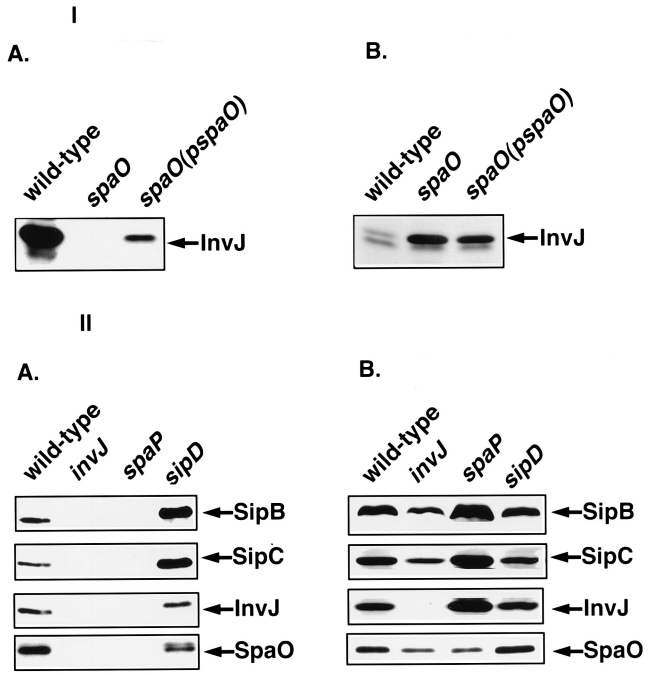

FIG. 6. (I) Effect of *S. typhimurium spaO* mutation on InvJ secretion. Culture supernatants (A) and whole-cell lysates (B) were prepared as described in Materials and Methods, transferred to nitrocellulose membranes, and probed with antibodies directed against InvJ. The arrows indicate the position of the InvJ protein. (II) Effect of mutations in the *invJ*, *spaP*, and *sipD* genes on the secretion of the SipB, SipC, InvJ, and SpaO proteins. The SpaO protein was tagged in the C terminus with an epitope consisting of 18 residues from the E4-6/7 protein of adenovirus, which is recognized by the monoclonal antibody M45. Culture supernatants (A) and whole-cell lysates (B) were prepared as described in Materials and Methods, transferred to nitrocellulose membranes, and probed with monoclonal antibody M45 directed against the epitope tag. The arrows indicate the positions of the SipB, SipC, InvJ, and SpaO proteins.

thelial cells. Furthermore, mutations in the *spa* genes prevented the secretion of several proteins known to be targets of the type III secretion apparatus encoded in the centisome 63 invasion region of the *Salmonella* chromosome. This defect was not due to reduced expression of the secreted targets, since similar amounts of these proteins were found in the whole-cell lysates of the wild-type and mutant strains. These results indicate that the *spa* gene products are part of the type III protein secretion apparatus encoded at centisome 63 of the *Salmonella* chromosome and that their role in bacterial entry is most likely due to this function. Recently, it has been reported that another putative type III secretion system required for virulence is encoded in the *Salmonella* chromosome (19). Our results, however, indicate that there is no redundancy in the function of the *spa* genes, and they confirm previous observations that suggest that there is no overlap between the type III secretion systems encoded at centisome 63 and in another region in the *Salmonella* chromosome. Alternatively, this other putative type III secretion system may not be expressed under the in vitro conditions used in these studies. A certain redundancy between these two protein secretion systems under in vivo conditions is therefore still formally possible. Indeed, mutations in genes that are components of the invasion-associated protein secretion system did not cause a virulence defect in intraperitoneally administered *Salmonella* mutant strains (12). However, mutations in components of the other type III secretion system resulted in a significant virulence defect when the microorganisms were administered intraperitoneally (19).

We have previously shown that SipD, a *Salmonella* protein that is secreted via the type III secretion apparatus encoded in the *inv* and *spa* loci, plays a role in modulating the secretion process (27). Indeed, mutations in *sipD* led to strains with enhanced secretion of a subset of secreted targets, including the Sip proteins but not InvJ. We have shown in this study that two other targets of this protein secretion apparatus, InvJ and SpaO, are also involved in the secretion process itself. Unlike mutations in *sipD*, however, mutations in either *invJ* or *spaO* completely abolished the secretion of all targets of this protein secretion system. Thus, there appears to be a hierarchy in the secretion process in which a subset of perhaps functionally related secreted proteins is modulating the export of another group of secreted targets. The specific functions of InvJ and SpaO in the secretion process, however, are not known. It is possible that secretion of InvJ and SpaO is required to render the translocon competent for engaging in the export of the other secreted proteins, including the Sip proteins. Alternatively, InvJ and/or SpaO may be the recipients of a signal(s) that could lead to the activation of the secretion process. It is not known whether the requirement for secreted proteins for protein export is a universal feature of all type III systems or whether it is a unique characteristic of *Salmonella* spp. Thus far, this requirement has not been established for other microorganisms.

It has been previously suggested that InvJ and Spa32 may be functional homologs on the basis of their similarity in size as well as in the conservation of the arrangement of the genes encoding these two proteins in relation to other genes which are highly conserved between *Shigella* and *Salmonella* spp. (17). However, the sequence similarity between InvJ and Spa32 is below statistical significance, which led us to propose

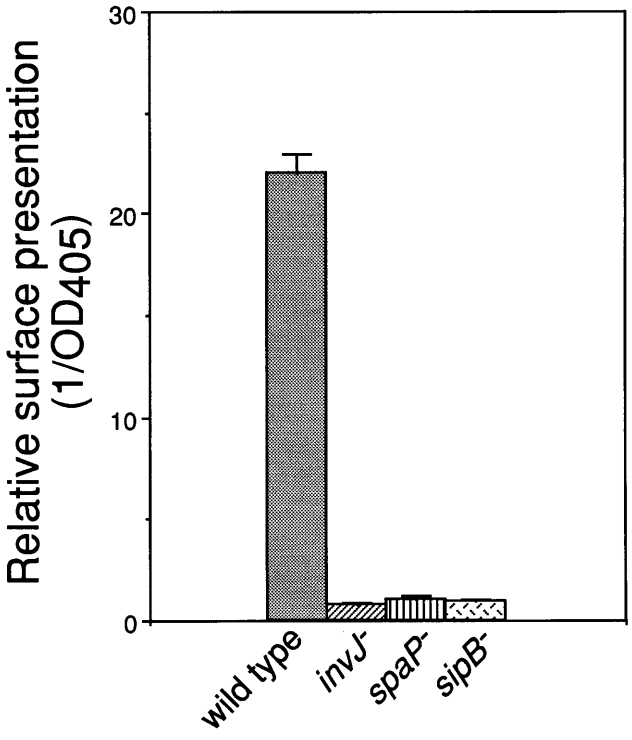

FIG. 7. Surface presentation of the SipB protein in wild-type, *spaP*::*aphT* (SB304), *invJ*::*aphT* (SB302), and *sipB*::*aphT* (SB169) strains. The presence of SipB on the surfaces of different bacterial strains was determined by competitive ELISA as described in Materials and Methods. Values represent the means and standard deviations for triplicate samples and are expressed as the inverse of the A_{405} ($1/OD_{405}$). Similar results were obtained in several repetitions of this experiment.

that it was very unlikely that these two proteins could exert similar functions (6). In this paper, we have presented evidence supporting this notion. Although not universally accepted, a two-step model has been proposed for the secretion of the Ipa proteins in *Shigella* spp. (56). The first step results in the translocation of the secreted targets to the bacterial cell surface, a process that is dependent on the *spa* and *mxi* loci. The second step results in their release into the extracellular environment, a process mediated by the Spa32 protein. Our results do not support the existence of a similar process in *Salmonella* spp. Using a quantitative competition ELISA, we failed to detect the presence of SipB and SipC (data not shown) on the surfaces of strains of *S. typhimurium* carrying mutations in several *spa* genes which encode functional homologs of the products of several *spa* genes of *Shigella* spp. More significantly, a null mutation in *invJ* also prevented the surface expression of both SipB and SipC (data not shown), which indicates that InvJ and Spa32 most likely do not have a similar function. These results also suggest the existence of differences between the mechanisms of the invasion-associated type III protein secretion systems of *Salmonella* and *Shigella* spp. These differences may be either in the actual mechanisms of protein translocation through the bacterial membranes or, most likely, in some regulatory function that determines either the timing of the secretion process or the signal responsible for the activation of the secretion apparatus itself. Indeed, these findings are not surprising, since InvJ and Spa32 are quite different proteins. The Spa32 protein is located in the outer membrane and on the bacterial cell surface and is not secreted, even when in contact with host cells (55). In contrast, InvJ is not membrane associated and it is secreted to the culture supernatant (6). In fact, the secretion of InvJ is enhanced upon contact with epithelial cells (57). In addition, Spa32 has two cysteine residues which have been postulated to be essential for its localization to the outer membrane and for its ability to release the Ipa proteins to the culture supernatant (56). InvJ, on the other hand, lacks any cysteine residues. Although there are striking similarities between the secretory machineries and the exported targets of *Salmonella* and *Shigella* spp., the pathogenesis of these two microorganisms is quite different (21, 44). Therefore, virulence determinants that are responsible for these differences may exist. These observations suggest that InvJ and Spa32 may account, at least partially, for the significant differences in the pathogenesis of these microorganisms. Perhaps these proteins play an essential role in activating the secretory system when the pathogens encounter the host microenvironment. Additional studies will be necessary to define these differences further.

In summary, we have shown that the secreted target proteins InvJ and SpaO play an essential role in the secretion process itself. In addition, we have demonstrated a role in protein secretion and bacterial entry for several genes encoded in the *spa* locus of *S. typhimurium*. Finally, our results suggest the existence of differences between the mechanisms of the related invasion-associated/Type III secretion systems of the enteric pathogens *Salmonella* and *Shigella* spp.

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