JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 7078–7085
0021-9193/95/$04.00+0

Vol. 177, No. 24

Identification of Two Targets of the Type III Protein Secretion System Encoded by the *inv* and *spa* Loci of *Salmonella typhimurium* That Have Homology to the *Shigella* IpaD and IpaA Proteins

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Received 13 July 1995/Accepted 3 October 1995

An important virulence factor of *Salmonella* spp. is their ability to gain access to host cells. A type III secretion system encoded in the *inv* and *spa* loci of these organisms is essential for this phenotype. We have identified two proteins, SipA and SipD, whose secretion from the bacterial cells is dependent on this system. The genes encoding these proteins are located at centisome 63 on the *S. typhimurium* chromosome, immediately downstream of the previously identified *sipB* and *sipC* genes (K. Kaniga, S. Tucker, D. Trollinger, and J. E. Galán, J. Bacteriol. 177:3965–3971, 1995). Nucleotide sequence analysis of the genes encoding these proteins indicated that SipA and SipD have significant sequence similarity to the *Shigella* IpaA and IpaD proteins. A nonpolar null mutation in *sipD* rendered *S. typhimurium* severely deficient for entry into cultured epithelial cells. In addition, this mutant strain exhibited increased secretion of a selected group of proteins whose export is controlled by the *inv*- and *spa*-encoded translocon. In contrast, a nonpolar mutation in *sipA* did not result in an invasion defect or in a significant decreased in virulence in a mouse model of infection. In addition, we have found an open reading frame immediately downstream of SipA that encodes a predicted protein with significant similarity to a family of acyl carrier proteins.

The ability of *Salmonella typhimurium* to gain access to host cells is largely encoded by a contiguous region of the chromosome located at centisome 63 (4, 9, 13, 18, 20, 22, 25, 29, 38–40, 43, 56). At least two loci of this region, *inv* (13, 18, 22, 25, 39) and *spa* (29), encode components of a *sec*-independent protein secretion system. This protein secretion apparatus, termed type III, is also present in other mammalian pathogens such as *Shigella* spp. (1, 2, 6, 7, 60, 65), *Yersinia* spp. (3, 10, 52, 53, 70), and enteropathogenic *Escherichia coli* (37) as well as in a variety of plant-pathogenic bacteria from the erwiniae and the families *Pseudomonaceae* and *Xhanthomonaceae* (19, 23, 27, 28, 35, 44, 64, 68). These secretion systems are required for the export of proteins thought to, directly or indirectly, elicit responses in infected host cells. These responses include the induction of membrane ruffling and bacterial internalization (*Salmonella* and *Shigella* spp.), cytotoxicity and other cellular responses (*Yersinia* spp.), intestinal epithelial cell damage (enteropathogenic *E. coli*), and induction of pathogenicity or local defense reactions in susceptible or resistant plant hosts (plant pathogens). Identification of proteins that exit the bacterial cells via these systems is of great interest since such proteins presumably include effectors of the various responses elicited by the corresponding pathogens. Protein targets of these type III secretion systems have been identified in *Shigella* spp. (Ipas) (reviewed in reference 59), *Yersinia* spp. (Yops) (reviewed in reference 63), enteropathogenic *E. coli* (EaeB) (17), *Pseudomonas syringae* and *Erwinia amylovora* (harpins) (33, 69), and *Pseudomonas solanacearum* (PopA1) (8). To the extent that they have been examined, they share the distinct feature that their amino termini are not cleaved upon secretion even though, at least in the case of the Yops, the secretion signal is located at the amino-terminal end (54). Interestingly, despite the extensive homology among the components of these different secretion apparatuses, the exported target proteins have very little homology at the primary amino acid sequence level. This lack of homology not only reflects the different responses that the various pathogens can elicit in their infected hosts but also suggests the existence of common structural features among the secretion signal sequences of these proteins that are not revealed by standard computer analysis.

We have previously shown that contact of *S. typhimurium* with cultured epithelial cells results in the transient assembly on the bacterial surface of appendage-like structures termed invasomes (24). The transient assembly of these structures correlated with the ability of *S. typhimurium* to enter cultured epithelial cells and was dependent on the presence of an intact *inv*-encoded type III secretion system. We have recently identified several proteins whose secretion into the culture supernatant of *S. typhimurium* is dependent on this secretion system (40) and that therefore are potential components of the invasome structures (24). We have identified the genes encoding at least three of these proteins, *invJ*, *sipB*, and *sipC*, and found that they encode polypeptides that have significant sequence similarity to the EaeB protein of enteropathogenic *E. coli* and the IpaB and IpaC proteins of *Shigella* spp., respectively (13, 40). The aforementioned homologs are themselves targets of type III secretion systems. We have also found that secretion of at least one of these proteins, InvJ, is significantly stimulated upon contact with the host cell (72). Such stimulation required live cultured cells but did not require de novo bacterial protein synthesis. In this report, we describe the identification of two additional targets of the *inv*- and *spa*-encoded translocon,

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TABLE 1. Bacterial strains used

Strain	Relevant genotype	Reference or source
S. typhimurium		
SL1344	Wild type	34
SB161	Δ*invG*	39
SB164	*invF*::*xylE*	39
SB165	*invA*::*xylE*	39
SB174	*invE*::*xylE*	39
SB233	*invJ*::*xylE*	This study
SB227	*sipC*::*xylE*	This study
SB225	*sipA*::*aphT*	This study
SB241	*sipD*::*aphT*	This study
SB230	*sipD*::*aphT invF*::*xylE*	This study
SB232	*sipD*::*aphT invA*::*xylE*	This study
SB231	*sipD*::*aphT invE*::*xylE*	This study
SB235	*sipD*::*aphT invJ*::*xylE*	This study
SB229	*sipD*::*aphT sipC*::*xylE*	This study
E. coli		
χ6060	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74* *galK*Δ*phoA20 galE recA1 rpsE* *argE*(Am) *rpoB thi* [F′ (*traD36* *proA*$^+$ *proB*$^+$ *lac*I^q *lacZ*Δ*M15*)::Tn*5*]	(26a)
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r) λ*pir*	55

termed SipD and SipA. We found that SipD and SipA are homologs of the *Shigella* IpaD and IpaA proteins, respectively. Mutations in *sipD* but not in *sipA* rendered *S. typhimurium* deficient for entry into cultured epithelial cells. In addition, mutations in *sipD* resulted in enhanced secretion of other targets of this protein secretion system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were grown in L broth or on L agar; when required, the following antibiotics were added at the concentrations indicated: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; and streptomycin, 100 μg/ml. For invasion assays, bacteria were grown under conditions that stimulate their ability to enter into cultured epithelial cells as described elsewhere (13).

Recombinant DNA, genetic techniques, and nucleotide sequencing. All recombinant DNA procedures were carried out according to standard procedures (48). P22HT*int* transduction and bacterial conjugation were carried out as described elsewhere (39). Nucleotide sequence determination was carried out by the dideoxy-chain termination procedure with Sequenase as instructed by the manufacturer (U.S. Biochemical Corp., Cleveland, Ohio).

Invasion assay. Entry of *S. typhimurium* strains into cultured Henle-407 cells was assayed in 24-well tissue culture plates as described previously (20).

Animal experiments. Peroral infections of 8- to 10-week-old female BALB/c mice with the different *S. typhimurium* strains were carried out as previously described (20).

Construction of nonpolar mutations. Strains carrying nonpolar mutations in *sipA* and *sipD* were constructed as follows (Fig. 1 and Table 1). Mutations in *sipA* were constructed by inserting into the unique *Sna*BI site of pSB417 a cassette containing a modified aminoglycoside 3′-phosphotransferase (*aphT*) gene which lacks a transcription terminator (22), yielding plasmid pSB423. A *Sal*I-*Sac*I fragment from pSB423, carrying the mutated *sipA* allele and flanking sequences, was cloned into the *Bam*HI site of the R6K-derived replicon pSB360 after filling in of the termini with the large fragment of DNA polymerase I. The mutation was then introduced into the chromosome of the wild-type *S. typhimurium* strain SL1344 by allelic exchange as described previously (39). Mutations in *sipD* were constructed by inserting an *aphT* cassette in the unique *Bam*HI site of plasmid pSB412 (40), and the mutated allele was subsequently recombined into the *S. typhimurium* chromosome as described above.

Construction of reporter-gene fusions. To monitor expression of the different genes, strains carrying operon fusions to *xylE*, a *Pseudomonas putida* Tol plasmid gene that encodes catechol 2,3-dioxygenase, were constructed. The construction of strains SB164, SB165, and SB174, carrying *xylE* fusions to *invF*, *invA*, and *invE*, respectively, has been described elsewhere (39). A *Bgl*II cassette from pSB383, which carries the coding sequence of *xylE* and its rRNA binding site but lacks its transcription terminator (39), was introduced into the *Kas*I site of pSB412 (40) and the *Nsi*I site of pSB453 (13), generating gene fusions to *sipC* and *invJ*, respectively. All gene fusions were subsequently integrated into the chromosomes of both the *S. typhimurium* wild-type strain SL1344 and the *sipD* isogenic derivative SB241 as previously described (39). Insertion of the *xylE* reporter gene does not result in polar effect on downstream genes (reference 39 and data not shown).

Catechol 2,3-dioxygenase assay. The activity of catechol 2,3-dioxygenase was measured as described previously (39).

Analysis of *S. typhimurium* culture supernatant proteins. Wild-type and mutant strains of *S. typhimurium* were grown in 25 ml of L broth containing 0.3 M NaCl to an optical density at 600 nm of 0.5. Bacterial cells were removed from cultures by centrifugation at 7,000 × *g* for 20 min and subsequent filtration through a 0.45-μm-pore-size filter. Proteins from the cell-free culture supernatants were then precipitated by addition of 10% (vol/vol) trichloroacetic acid and recovered by centrifugation at 7,000 × *g* for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and proteins were precipitated again by addition of 20 ml of cold acetone. After centrifugation at 7,000 × *g* for 20 min, the pellets were washed once with cold acetone, dried, and resuspended in 25 μl of PBS.

Western blotting (immunoblotting). Samples were separated by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose, and proteins were detected by immunoblot analysis with the appropriate antibodies followed by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, Ill.). InvJ was detected with the monoclonal antibody J33.13 previously described (13). Polyclonal antibodies to purified SipA, SipB, and SipC were raised in rabbits according to standard immunization procedures (32). The cytoplasmic protein 6-phosphogluconate dehydrogenase was detected by using a polyclonal antibody kindly provided by Donald Oliver (Weslyan University).

Computer analysis of nucleotide and protein sequences. Nucleotide and protein sequence analysis was performed with the Genetics Computer Group package (version 8) from the University of Wisconsin (16) and the MacProt software package. Comparison of translated nucleotide sequences with sequences in the available databases was carried out with the program BLAST at the server of the National Center for Biotechnology Information at the National Library of Medicine (5).

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited in GenBank under accession number U40013.

RESULTS

Identification of *sipA* and *sipD*, which encode homologs of the *Shigella* IpaD and IpaA proteins. We have previously identified in the centisome 63 region of *S. typhimurium* two genes required for bacterial entry, *sipB* and *sipC*, which encode proteins homologous to *Shigella* IpaB and IpaC (40). We have now extended the nucleotide sequence analysis of this region and identified three additional open reading frames (ORFs) en-

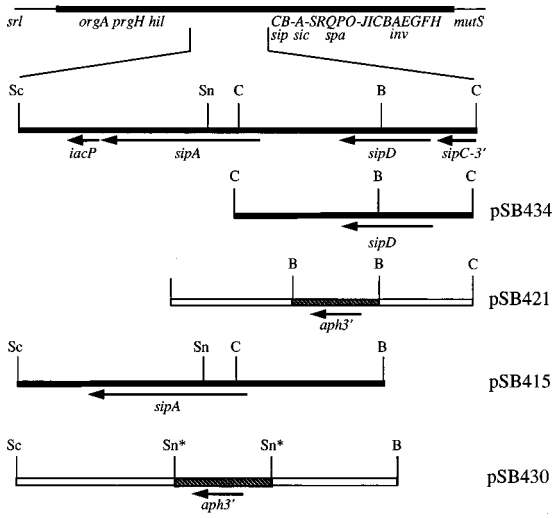

FIG. 1. Partial restriction endonuclease maps of the inserts of relevant plasmids used in this study. The position of the relevant region in relation to other invasion loci in the centisome 63 region of the *Salmonella* chromosome is indicated. The locations and directions of transcription of the different genes are shown by the arrows. C, *Cla*I; Sn, *Sna*BI; Sc, *Sac*I.

FIG. 2. Sequence alignment of *S. typhimurium* SipD and *Shigella flexneri* IpaD. Complete identity between residues is indicated by a line, and conservative substitutions are indicated by colons and periods.

coded immediately downstream of *sipC* (Fig. 1). The organization of these genes suggests that they constitute an operon, since the coding regions overlap slightly or are separated by very short intergenic regions. The first ORF encodes a polypeptide of 338 amino acids with a predicted molecular size of 37,785 Da. Sequence homology searches in the available databases revealed that this ORF encodes a polypeptide with significant similarity to the IpaD protein of *Shigella* spp. The overall sequence identity is 40% and increases to 56% when conservative substitutions are taken into consideration (Fig. 2). However, the similarity is much higher at the carboxy-terminal end, in particular over a stretch of 60 residues that exhibits 73% amino acid identity, which increases to 98% similarity when conservative substitutions are considered. We therefore have called this protein SipD (*Salmonella* invasion protein D). Secondary structure analysis of SipD with the algorithms of von Heijne (67) and Klein et al. (41, 42) did not predict the presence of a typical signal sequence or membrane-spanning domains. The second ORF encodes a polypeptide of 684 residues with a predicted molecular size of 73,776 Da which we have named SipA. Protein sequence homology analysis showed that SipA has significant sequence similarity (25% overall identity and 46% similarity) to the *Shigella* IpaA protein (Fig. 3). Although clearly significant, the overall sequence homology between these two proteins is much less than that of other Sip and Ipa proteins. The similarity is more evident at the amino termini of the proteins (29% identity and 48% similarity over the first 300 residues) but drops significantly toward the carboxy termini (only 18% identity over the last 200 residues). Secondary structure analysis did not indicate the presence of potential signal sequences or membrane-spanning regions. The third ORF encodes an 82-amino-acid polypeptide with a predicted molecular size of 9,192 Da. The predicted polypeptide has extensive sequence identity with several acyl carrier proteins (ACPs) involved in the biosynthesis of essential lipids in a variety of bacteria, yeasts, and plants (reviewed in reference 47) (Fig. 4); therefore, we have named this gene *iacP* (invasion-associated ACP). IacP also exhibits strong homology to a *Shigella dysenteriae* protein encoded by an ORF similarly located immediately downstream of IpaA (71).

The *S. typhimurium sipD* gene product is required for entry into host cells. To address the potential involvement of the Sip proteins in the *Salmonella* entry phenotype, we constructed strains carrying nonpolar mutations in both *sipA* and *sipD* and evaluated their ability to enter cultured Henle-407 cells. Mutations in *sipD* rendered *S. typhimurium* severely deficient (500-fold) for entry into cultured mammalian cells (Fig. 5). Introduction of a plasmid carrying a wild-type allele of *sipD* efficiently complemented the entry phenotype, indicating that the invasion defect on *S. typhimurium* SB241 was due only to the disruption of the *sipD* gene (Fig. 5). These results demonstrated that the *sipD* gene is essential for *S. typhimurium* entry into cultured cells. In contrast, introduction of a nonpolar mutation in *sipA* had no effect on the entry phenotype (Fig. 5), indicating that SipA is not required for bacterial entry into host cells or, alternatively, that the *sipA* phenotype cannot be revealed with the in vitro assay system used in this study. Simi-

FIG. 3. Sequence alignment of *S. typhimurium* SipA and *Shigella flexneri* IpaA. Complete identity between residues is indicated by a line, and conservative substitutions are indicated by colons and periods.

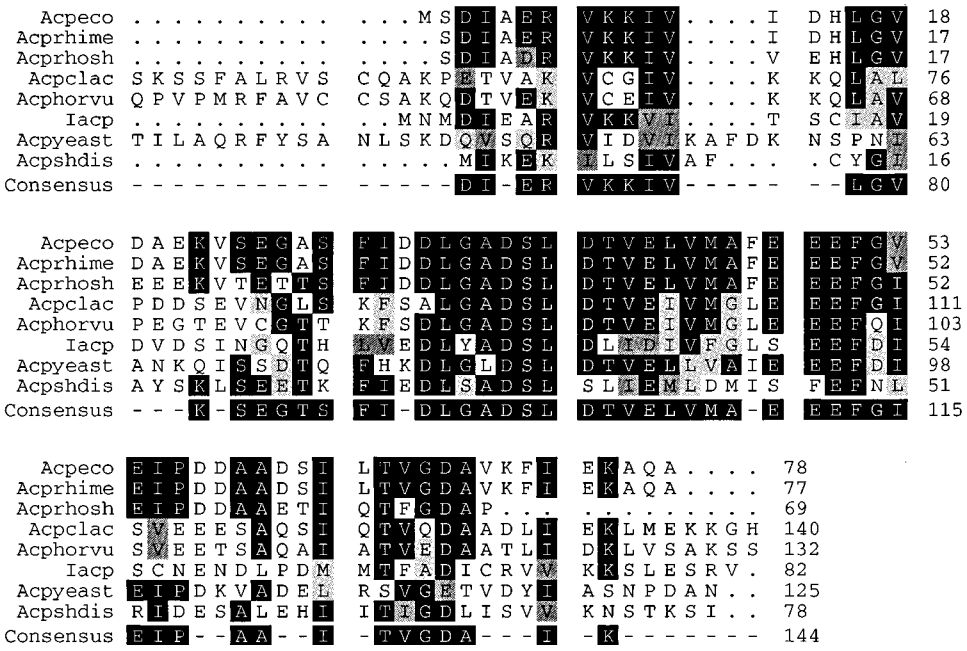

FIG. 4. Multiple sequence alignment of several ACPs that exhibit sequence similarity to *S. typhimurium* IacP. Black boxes indicate complete identity, and shaded boxes indicate conservative amino acid substitutions. Sequences were obtained from GenBank, release 90. The ACPs are from the following organisms: Acpeco, *E. coli* (58); Acprhime, *Rhizobium meliloti* (57); Acprhosh, *Rhodobacter sphaeroides* (14); Acpclac, *Cuphea lanceolata* (66); Acphorvu, Hordeum vulgare (barley) (31); Acpyeast, *Saccharomyces cerevisiae* (12); Acpshdis, *Shigella dysenteriae* (71).

larly, the *Shigella* SipA homolog, IpaA, plays no role in bacterial entry.

***S. typhimurium sipA* mutants retained wild-type virulence in a mouse model of infection.** Failure to implicate SipA in the entry phenotype prompted us to examine the phenotype of an isogenic *S. typhimurium sipA* mutant in a mouse model of *S. typhimurium* infection. As shown in Table 2, the *S. typhimurium sipA* mutant strain SB225 exhibited wild-type virulence after peroral infection of BALB/c mice. The biological significance of the slight but reproducible increase in the mean time to death of the mutant strain is unclear. These results indicate that SipA may not be essential for *S. typhimurium* virulence in this model of infection.

Localization of SipD and SipA in culture supernatants of *S. typhimurium*. We have previously identified several proteins whose secretion into the culture supernatant of *S. typhimurium* is dependent of the type III secretion system encoded in the *inv* and *spa* loci (40). Among those proteins, we have visualized polypeptides with sizes equivalent to those of SipD and SipA predicted from the nucleotide sequence. Furthermore, SipD and SipA are homologous to the Ipa proteins of *Shigella* spp., which are exported via a type III secretion system. These findings prompted us to examine the possibility that SipA and SipD were among those proteins whose secretion into the culture supernatant of invasion-competent *S. typhimurium* is dependent on the protein secretion apparatus encoded in the *inv* locus. Cultured supernatant proteins from wild-type *S. typhimurium* SL1344 and its isogenic *sipA* and *sipD* mutants were analyzed by SDS-PAGE and Coomassie blue staining. A protein of approximately 80 kDa was absent from culture supernatants obtained from the *sipA* strain SB225 (Fig. 6). Introduction of plasmid pSB415, which encodes *sipA*, into the *sipA*

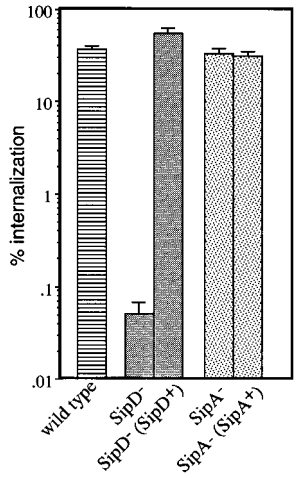

FIG. 5. Effects of *sipA* and *sipD* on *S. typhimurium* entry into cultured Henle-407 cells. Wild type, SL1344; SipD, SB241; SipA, SB225; $^{-}$, nonpolar mutation by insertion of an *aphT* cassette; $^{+}$, mutant strain carrying the complementing plasmid. Each value represents the mean ± standard deviation of triplicate samples. Similar results were obtained in several repetitions of this experiment.

TABLE 2. Mouse virulence of an *S. typhimurium sipA* mutant after peroral infection[a]

Strain	Relevant genotype	Infecting dose	Mean time to death (days) ± SD
SL1344	Wild type	3.5×10^7	6.3 ± 0.5
SB225	*sipA*	3.8×10^7	7.3 ± 0.5
SL1344	Wild type	3.5×10^6	6.6 ± 0.5
SB225	*sipA*	3.8×10^6	7.6 ± 1
SL1344	Wild type	3.5×10^5	8 ± 1
SB225	*sipA*	3.5×10^5	9.6 ± 0.5

[a] Mice (three per group) were infected perorally with the indicated doses of bacteria as indicated in Materials and Methods. There were no survivors.

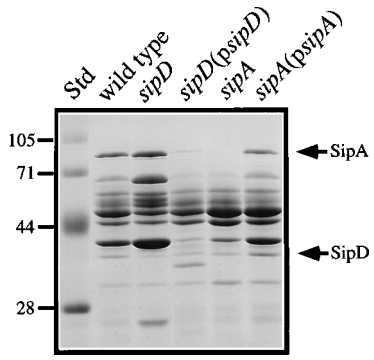

FIG. 6. Protein secretion profile of *S. typhimurium sipA* and *sipD* mutants. Culture supernatant proteins from wild-type *S. typhimurium* SL1344 and the isogenic *sipA* and *sipD* mutants SB225 and SB221 with and without the complementing plasmids were prepared as indicated in Materials and Methods, separated on an SDS–10% polyacrylamide gel, and stained with Coomassie blue. Wild type, SL1344; *sipD*, SB241; *sipD*(p*sipD*), SB241(pSB434); *sipA*, SB225; *sipA* (p*sipA*), SB225(pSB415); Std, molecular weight standards. Sizes are indicated in kilodaltons.

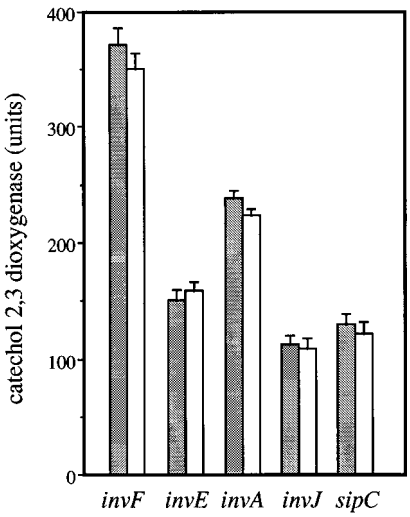

FIG. 8. Effects of *sipD* on *inv* and *sip* gene expression. Expression of the *invF*, *invE*, *invA*, *invJ*, and *sipC* genes was monitored by measuring the catechol 2,3-dioxygenase activity encoded by the reporter gene *xylE* as indicated in Materials and Methods. Open bars, $SipD^-$ background; hatched bars, $SipD^+$ background. Each value represents the mean ± standard deviation of triplicate samples. Similar results were obtained in several repetitions of this experiment.

mutant strain restored the presence of the 80-kDa protein in the culture supernatant (Fig. 6). In addition, a polyclonal antibody raised against purified SipA recognized a protein of 80 kDa in the culture supernatant of the wild type but not in that of an *invG* mutant (data not shown and Fig. 7A). These results, in conjunction with the observation that the predicted molecular size of SipA is 74 kDa, strongly suggest that this polypeptide is the *sipA* gene product and that its secretion is dependent on the *inv* locus. Attempts to determine the amino-terminal sequence of the 80-kDa protein were unsuccessful. The presence in the culture supernatant of the secreted protein InvJ, which has a molecular size identical to that of the predicted SipD protein (13, 40), prevented the unambiguous identification of SipD in the culture supernatant of wild-type *S. typhimurium*. However, the relative intensity of the 37-kDa band from culture supernatant preparations of the *sipD* mutant is clearly lower than in the wild type, despite the fact that in this mutant, the intensity of the other Sip proteins is much higher (see below). Although inconclusive, these results, in conjunction with the predicted size of SipD, suggest that the 37-kDa polypeptide band observed in wild-type *S. typhimurium* preparations contains both SipD and InvJ.

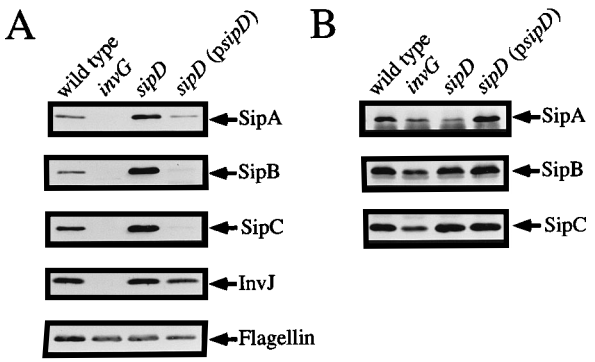

FIG. 7. Increased protein secretion of an *S. typhimurium sipD* null mutant. Culture supernatant proteins (A) and whole cell lysates (B) from wild-type *S. typhimurium* SL1344, its isogenic *invG* (SB161) and *sipD* (SB241) mutants, and the *sipD* mutant carrying the complementing plasmid pSB434 [sipD (p*sipD*)] were separated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose membranes as indicated in Materials and Methods. Blots were then probed consecutively with polyclonal antisera to SipA, SipB, and SipC, a monoclonal antibody to InvJ, and a polyclonal antibody to flagellin. Only relevant portions of the blots are shown.

***S. typhimurium sipD* mutants have increased secretion of Sip proteins.** It has been recently reported that *Shigella flexneri ipaD* mutants have enhanced secretion of the Ipa proteins (50, 51). The high degree of sequence similarity between IpaD and SipD prompted us to examine the possibility that *S. typhimurium sipD* mutants also exhibit enhanced secretion of other targets of the *inv*-encoded protein translocation system such as SipA, SipB, SipC, and InvJ. Culture supernatants from wild-type and *sipD S. typhimurium* strains were examined by SDS-PAGE and Coomassie blue staining as described in Materials and Methods. As shown in Fig. 6, culture supernatants from the *sipD* mutant contained much higher amounts of a number of proteins, including SipA, SipB, and SipC, than did those from the wild type. These results were confirmed by immunoblot analysis using polyclonal antisera to SipA, SipB, and SipC (Fig. 7A). In contrast, all samples exhibited equivalent amounts of flagellin, a protein whose export does not require the *inv*- and *spa*-encoded translocon (Fig. 7A). The increased secretion observed in the *sipD* mutant was not due to nonspecific bacterial lysis, since the very abundant cytoplasmic protein 6-phosphogluconate dehydrogenase was not detected in any of the culture supernatant preparations (data not shown). The specificity of the *sipD* phenotype is also consistent with the observation that *sipD* mutants did not exhibit increased levels of InvJ secretion, as detected by immunoblot analysis using a monoclonal antibody to this protein (Fig. 7A). This result also indicates that the effect of SipD on the secretion of other targets of the *inv*- and *spa*-encoded translocon is not global but instead is restricted to a subset of secreted proteins. We examined the possibility that the increased levels of secreted Sip proteins in the culture supernatant of the *sipD* mutant were due to an increased expression of either the genes encoding the secreted proteins themselves or the genes encoding components of the secretion apparatus. As shown in Fig. 8, the *sipD* mutation had not effect on the expression of *sipC* (40) or *invJ* (13), which encode secreted proteins, *invA* (22) and *invE* (25), which encode components of the type III secretion system, or *invF* (39),

a transcription regulator required for *Salmonella* entry. In addition, a null mutation in *sipD* did not result in increased levels of SipA, SipB, or SipC in whole cell lysate of this strain (Fig. 7B). Thus, these results indicate that the increased levels of secreted Sip proteins in the *sipD* mutant are most likely due to a direct role of SipD in modulating the secretion of a selected group of targets of the *inv*- and *spa*-encoded translocon. Interestingly, complementation of the *sipD* mutant with a plasmid-borne wild-type copy of *sipD* resulted in a strain with significantly reduced levels in the culture supernatant of the same set of secreted proteins whose secretion was enhanced by the *sipD* mutation, including SipA, SipB, and SipC (Fig. 6 and 7). Presence of a plasmid-borne *sipD*, however, did not result in decrease levels of Sip proteins in whole cell lysates (Fig. 7B). The levels of InvJ and flagellin in culture supernatants of the different strains were virtually identical (Fig. 7A), which is consistent with the notion that SipD modulates the secretion of only a selected group of proteins secreted through the *inv*- and *spa*-encoded translocon. Introduction of a plasmid-borne copy of *sipD*, however, restored the invasion phenotype of the *sipD* mutant to levels comparable to those of the wild type. These results indicate that although higher levels of *sipD* lead to lower levels of nonstimulated secretion of a subset of Sip proteins, this phenotype does not affect the ability of this strain to trigger bacterial entry into cultured epithelial cells. Presumably, overexpression of *sipD* does not affect the contact stimulated secretion of Sip proteins.

DISCUSSION

We have identified two new targets of the type III protein secretion system encoded in the *inv* and *spa* loci, which we have named SipA and SipD. The genes encoding these proteins are located immediately downstream of the previously characterized *sipB* and *sipC* genes, which encode secreted proteins required for *Salmonella* entry (40). SipA and SipD exhibited significant homology to the IpaA and IpaD proteins of *Shigella* spp. The *Shigella* Ipa proteins, which are required for these organisms to enter into host cells, are secreted via a type III secretion apparatus largely homologous to that encoded in the *inv* and *spa* loci of *Salmonella* spp. (reviewed in reference 49). The genes encoding the Ipa invasins are organized in an operon with an arrangement identical to that of the *sip* genes. The remarkable topological conservation of blocks of *Salmonella* and *Shigella* genes required for bacterial entry is also apparent in the *inv* and *spa* loci of *S. typhimurium*, which are arranged very similarly to their homologs *mxi* and *spa*. However, significant differences are observed among the relative positions of these blocks: in shigellae, the *ipa* operon is located upstream from the *mxi* locus and is transcribed in the opposite orientation (reviewed in reference 59); conversely, in salmonellae, the *sip* operon is located downstream from the *spa* locus and is transcribed in the same direction (reviewed in reference 21). Although the functional and/or evolutionary significance of the conservation of this arrangement is unknown, it is tempting to speculate that these topologically conserved blocks of genes encode proteins whose functions are closely related or that their expression needs to be coordinately regulated.

Mutations in *sipD* rendered *S. typhimurium* severely deficient for entry into cultured epithelial cells. Transcomplementation of the *sipD* mutant with a plasmid-borne copy of *sipD* completely restored the ability of this strain to enter into host cells, indicating that SipD is essential for the internalization process. What is the role of SipD in triggering bacterial entry? It has been previously shown that null mutations in the *Shigella* homolog *ipaD* as well as in *ipaB* lead to enhanced secretion through the type III export system encoded in the *mxi* and *spa* loci (50, 51). In addition, it has been shown that IpaD associates with IpaB in the bacterial envelope (51). On the basis of this observation, it has been proposed that IpaD, in association with IpaB, modulates the secretion of Ipa proteins by blocking the secretion apparatus (51). A null nonpolar mutation in *sipD* conferred a similar phenotype to *S. typhimurium*. Increased secretion was limited to a subset of proteins secreted through the *inv*- and *spa*-encoded translocon, since there was no difference in the levels of flagellin in cultured supernatants of the wild type compared with those of *sipD* mutants. Flagellin is exported through a related though completely independent secretion system (46). Furthermore, increased secretion in the *Salmonella sipD* mutant was not due to increased expression of the genes encoding either targets or components of the *inv*- and *spa*-encoded secretion apparatus. These results suggest that SipD may function similarly to the *Shigella* homolog IpaD. However, mutations in *sipD* did not affect the secretion of InvJ, indicating that the modulating effect of SipD is restricted to a subset of targets of this translocon, and therefore, it is unlikely to function as a general "plug" of this secretion system in the absence of stimulatory signals as previously proposed for *Shigella flexneri* (51). Since secretion of InvJ is stimulated upon contact with cells or serum-coated culture dishes (72), proteins other than or in addition to SipD must be involved in the control of its secretion upon receiving an export signal.

Mutations in *sipA* did not result in a measurable effect on bacterial entry into cultured epithelial cells. These results are consistent with the observation that mutations in *ipaA* did not affect the ability of shigellae to gain access to host cells (50). An *S. typhimurium sipA* mutant exhibited wild-type virulence when administered orally to BALB/c mice, indicating that SipA may not be required for *Salmonella* virulence in this model of infection. However, the remarkable conservation of SipA and its *Shigella* homolog argues in favor of a yet undiscovered important function for this protein that cannot be detected with the assays used in this study.

Immediately downstream of *sipA*, we found an ORF capable of encoding a polypeptide highly similar to a family of ACPs which we have termed IacP. This family of ACPs is necessary for the biosynthesis of essential lipids in several prokaryotic and eukaryotic organisms as well as plants (47). However, *Salmonella* spp. may have another, yet unidentified ACP molecule which may be responsible for the synthesis of essential phospholipids, and the ACP encoded by *iacP* may perform functions specific for bacterial invasion. This possibility is consistent with the observation that large deletions of the centisome 63 region of the *Salmonella* chromosome encompassing essentially all of the invasion genes resulted in strains that have normal in vitro growth properties (26). In fact, the existence of two ACPs has been documented in *Rhizobium* spp., which have one ACP involved in host-specific nodulation and another involved in the synthesis of essential lipids (15, 57, 61). The *E. coli* ACP has been also shown to play a role in the fatty acylation of the *E. coli* hemolysin (36, 62). Moreover, it is presumed that ACPs are also involved in the modification of other members of this family of membrane-targeted toxins, including leukotoxin of *Pasteurella* (45) and *Actinobacillus* (11) spp. and the adenylate cyclase-hemolysin of *Bordetella pertussis* (30). It is therefore possible that IacP is involved in the modification of some target of the type III secretion system encoded in the *inv* and *spa* loci. Given the high degree of homology between the *Shigella* and *Salmonella* proteins involved in entry as well as their remarkable topological conservation, it is likely that the *Shigella* protein encoded in the ORF located immediately downstream of *ipaA* serves a similar function.

In summary, we have identified two new protein targets of the type III secretion system encoded in the *inv* and *spa* loci. These proteins, SipA and SipD, exhibit significant sequence similarity to the *Shigella* IpaA and IpaD proteins. We have also presented evidence that SipD is essential for bacterial entry into cultured mammalian cells. Our results indicate that this function may be in part due to its role in modulating the secretion of a subset of targets of this export system.

ACKNOWLEDGMENTS

We thank Donald Oliver for providing antibodies to 6-phosphogluconate dehydrogenase and members of the Galán laboratory for critical reading of the manuscript.

This work was supported by Public Health Service grant AI30492 from the National Institutes of Health and a grant from the Pew Charitable Trust. J.E.G. is a Pew Scholar in the Biomedical Sciences and an investigator for the American Heart Association.

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