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Molecular and Functional Characterization of the *Salmonella typhimurium* Invasion Genes *invB* and *invC*: Homology of InvC to the F_0F_1 ATPase Family of Proteins

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Entry into intestinal epithelial cells is an essential step in the pathogenesis of *Salmonella* infections. Our laboratory has previously identified a genetic locus, *inv*, that is necessary for efficient entry of *Salmonella typhimurium* into cultured epithelial cells. We have carried out a molecular and functional analysis of *invB* and *invC*, two members of this locus. The nucleotide sequence of these genes indicated that *invB* and *invC* encode polypeptides with molecular masses of 15 and 47 kDa, respectively. Polypeptides with the predicted sizes were observed when these genes were expressed under the control of a T7 promoter. Strains carrying nonpolar mutations in these genes were constructed, and their phenotypes were examined in a variety of assays. A mutation in *invC* rendered *S. typhimurium* defective in their ability to enter cultured epithelial cells, while mutations in *invB* did not. Comparison of the predicted sequences of InvB and InvC with translated sequences in GenBank revealed that these polypeptides are similar to the *Shigella* spp. proteins Spa15 and Spa47, which are involved in the surface presentation of the invasion protein antigens (Ipa) of these organisms. In addition, InvC showed significant similarity to a protein family which shares sequence homology with the catalytic β subunit of the F_0F_1 ATPase from a number of microorganisms. Consistent with this finding, purified preparations of InvC showed significant ATPase activity. Site-directed mutagenesis of a residue essential for the catalytical function of this family of proteins resulted in a protein devoid of ATPase activity and unable to complement an *invC* mutant of *S. typhimurium*. These results suggest that InvC may energize the protein export apparatus encoded in the *inv* locus which is required for the surface presentation of determinants needed for the entry of *Salmonella* species into mammalian cells. The role of InvB in this process remains uncertain.

Salmonella spp. have the ability to enter mammalian cells. This process is essential for the pathogenicity of these organisms, because it may allow them to reach deeper tissues or gain access to a more permissive environment. The internalization event is the outcome of an intimate interaction between the bacterium and the host in which biochemical signals are presumably exchanged. As a consequence of this biochemical dialogue or cross-talk, novel structures are assembled on the *Salmonella* surface, and, subsequently, a signaling cascade is triggered in the host cell (25). This cascade involves tyrosine phosphorylation of host proteins, phospholipase activity, calcium mobilization, and cytoskeletal rearrangements, resulting in membrane ruffling and, ultimately, the internalization of the infecting organisms (22, 44).

A number of *Salmonella* genetic loci that allow these organisms to enter cultured mammalian cells have been identified (4, 15, 17, 19, 20, 24, 35, 39, 53). We have previously isolated a genetic locus, *inv*, on the *Salmonella* chromosome that is essential for these organisms to efficiently enter into cultured epithelial cells (19). This locus was originally identified by its ability to complement a noninvasive strain of *Salmonella typhimurium*. Mutations in *inv* rendered *S. typhimurium* deficient for entry into cultured epithelial cells and also increased the 50% lethal dose when the organisms were administered orally into BALB/c mice (19). *invB* and *invC* were originally identified as members of the *invABC* operon; however, their molecular characterization has not been previously reported. In this paper, we present a molecular and functional analysis of these genes as well as an examination of their individual contributions to the ability of *S. typhimurium* to enter cultured epithelial cells.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study, including their sources, are listed in Table 1. Bacterial strains were grown in L broth or on L agar plates (36), and, when appropriate, antibiotics were added to the growth medium at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml.

Recombinant DNA, genetic techniques, and nucleotide sequencing. Recombinant DNA techniques were carried out by standard protocols (41). P22HT*int* transduction was carried out as described previously (50). Transformation of circular and linear DNA into *Escherichia coli* and *Salmonella* strains was carried out as described elsewhere (40). Expression and [^{35}S]methionine labelling of plasmid-encoded polypeptides in a bacteriophage T7 RNA polymerase expression system were carried out as described by Tabor and Richardson (55). Amplification of DNA fragments by the PCR was performed using a commercial kit (GenAmp; Perkin-Elmer Cetus, Norwalk, Conn.), according to the instructions of the manufacturer. DNA sequencing of both strands was carried out by the dideoxy chain termination method (47) using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions. Unidirectional deletions of appropriate plasmids for nucleotide sequence determinations were con-

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
S. typhimurium		
SL1344	Wild type	30
SB178	*invB*::*aphT*	This study
SB566	*invC*::*aphT*	This study
χ3477	*hsdL6* Δ(*galE-uvrB*)*-1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	Derived from AS68 of T. Palva by S. Tinge and R. Curtiss (unpublished data); Δ(*galE-uvrB*) obtained from SL5400 from B. D. L. Stocker
E. coli		
χ2991	Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galE galK* Δ*phoA20 thi rpsE rpoB argE*(Am) *recA1*	Derived by R. Goldschmidt from CC118 (42)
BL21 (DE3)	F$^-$ *ompT hsdS*	54
D301	RP487 *recD1903* Δ(*lacIZYA-u169*)	46
SM10λ pir	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu λ *pir* R6K	51

structed according to the method described by Henikoff (29). Nucleotide sequence analysis was performed using the Genetics Computer Group package from the University of Wisconsin (version 7) (12).

Plasmid constructions. Plasmids carrying *invB* and *invC* were derived from pYA2220 (19) (Fig. 1). The *Eco*RV-*Pst*I fragment of pYA2220 was cloned into the *Hin*cII and *Pst*I sites of pBluescript SKII (Stratagene), yielding plasmid pSB553. This plasmid was used as a substrate for the exonuclease III deletions constructed for sequence determinations. Plasmid pSB555 was constructed by cloning the *Dra*I-*Pst*I fragment of pYA2220 into the *Eco*RV and *Pst*I sites of pBluescript SKII so that the expression of both, *invB* and *invC*, was placed under the control of a T7 promoter. To express *invB* under the control of a bacteriophage T7 promoter, plasmid pSB560 was constructed by cloning a 959-bp *Bam*HI fragment from pSB555 into the *Bam*HI site of pBluescript SKII. Plasmid pSB561 was constructed by cloning a 959-bp *Bam*HI fragment from pSB560 into the *Bam*HI site of pUC18 (57).

For complementation studies of *S. typhimurium* mutants, the *invB* and *invC* genes were cloned into the plasmid vector pACYC184 (9) so that the expression of these genes was placed under the control of the *tet* promoter present in this vector. A 959-bp *Bam*HI fragment from pSB561, containing *invB*, was cloned into the vector pACYC184 that had been digested with *Hin*dIII and *Bam*HI, yielding plasmid pSB577. A 2,212-bp *Pvu*II fragment of pSB553 containing *invC* was cloned into the *Eco*RV and *Nru*I sites of pACYC184, yielding plasmid pSB558.

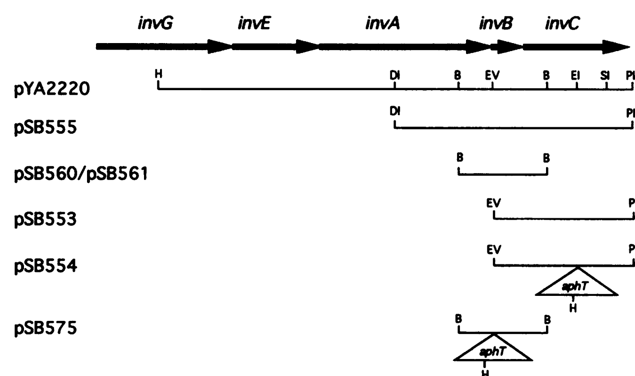

FIG. 1. Partial restriction endonuclease maps of the inserts of relevant plasmids utilized in this study. The positions of relevant restriction endonuclease sites are shown. The locations and directions of transcription of the different *inv* genes are shown by the arrows. H, *Hin*dIII; DI, *Dra*I; B, *Bam*HI; EV, *Eco*RV; EI, *Eco*RI; SI, *Sal*I; PI, *Pst*I.

Site-directed mutagenesis. Site-directed mutagenesis of InvC was carried out using the Alter Sites system of Promega (Madison, Wis.). A *Xba*I-*Xba*I fragment from pSB573 containing *invC* was cloned into the *Xba*I site of the vector pALTER (Promega), yielding plasmid pSB586. Site-directed mutagenesis was conducted with the mutagenic primer 5′-CATGGTCT CACCGCATC-3′ following the instructions of the manufacturer. The mutagenic primer was designed to change a lysine at position 165 to glutamic acid by changing the codon AAG to GAG. The mutation generated a diagnostic *Bsa*I site that was used to screen for the presence of the mutation. For complementation studies with the mutated allele of *invC*, plasmid pSB589 was constructed by exchanging a *Bam*HI fragment containing the mutated codon for the equivalent fragment of pSB553 which contains the wild-type codon.

High level of expression and purification of InvC and the site-directed mutant K165E. Fusion proteins between InvC or its site-directed mutant K165E and the glutathione *S*-transferase (GST) (52) were constructed for overexpression and purification of these proteins. PCR was used to create a *Xba*I restriction site at nucleotide 23 of the InvC coding sequence. A degenerative primer (5′-GCTCTAGAATATCTGGCCTAC CCAC-3′) complementary to the 5′ end of *invC* and the universal reverse primer (5′-AACAGCTATGACCATG-3′) complementary to the cloning vector were used to amplify a fragment of pSB553 which contains *invC*. The resulting amplified fragment was digested with *Xba*I and cloned into the *Xba*I site of the cloning expression vector pGEX-2T (27), yielding pSB573. For overexpression of the K165E mutant, the *Xba*I fragment of pSB587 containing the mutated codon was exchanged for the equivalent fragment of pSB573, yielding plasmid pSB588. Overexpression and purification of GST and the fused protein were carried out as described elsewhere (27), except that bacteria were grown and induced at 30°C. When required, thrombin cleavage of the fused protein was carried out as described elsewhere (27). The yields of the GST-InvC and GST-K165EInvC fusion proteins were equivalent.

ATPase activity measurements. A modification of the malachite green ATPase assay (38) was used to measure ATP hydrolysis. A typical GST-InvC ATPase reaction mixture (900 μl) contained 15 μl (9 μg) of purified GST-InvC or GST-K165EInvC proteins in dialysis buffer, 90 μl of 10× reaction buffer [500 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-

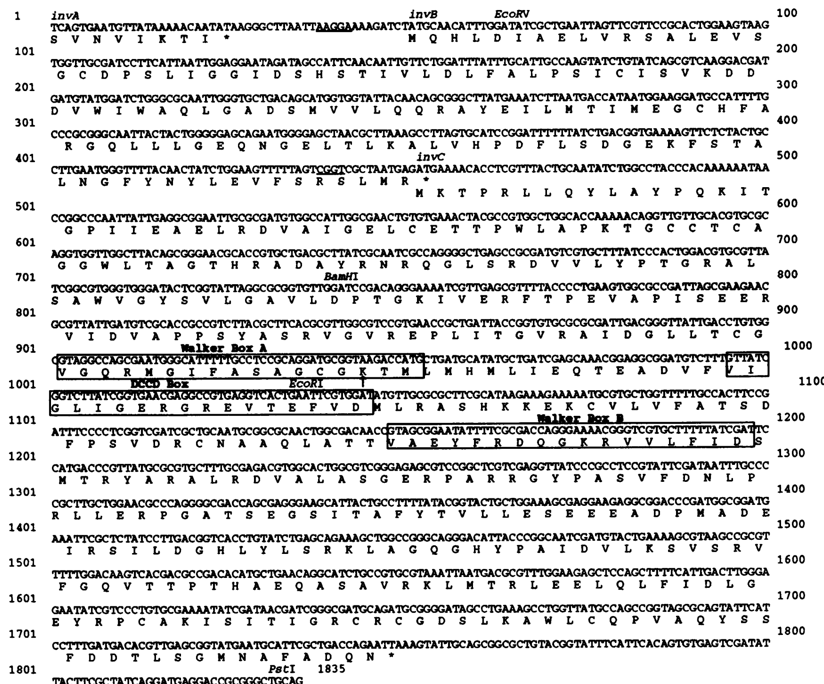

FIG. 2. Nucleotide sequences of *invB* and *invC*. The sequence of the coding strand is shown with the deduced protein sequence. The end of the upstream *invA* gene is indicated. The putative Shine-Dalgarno sequences upstream of the predicted ATG start codons are underlined. The positions of relevant restriction sites, the Walker boxes A and B (59), and the DCCD-binding box (62) are indicated. The K residue subjected to site-directed mutagenesis is indicated by a vertical arrow.

ethanesulfonic acid), 300 mM KCl, 300 mM NH_4Cl, 10 mM dithiothreitol, 50 mM $Mg(acetate)_2$], 90 μl of bovine serum albumin (5 mg/ml), 36 μl of freshly prepared ATP (0.1 M; pH 7.0; titrated with 1 M KOH), and 669 μl of H_2O. The components were mixed together on ice, and the samples were incubated at 37°C. At various time points (1, 5, 10, 15, 20, 25, and 30 min), 100 μl of the reaction mixture was withdrawn and the reaction was stopped by adding 800 μl of freshly prepared malachite green-ammonium molybdate reagent. This reagent contained 3 volumes of 0.045% malachite green hydrochloride (Sigma), 1 volume of ammonium molybdate (4.2% in 4 N HCl), and 1/50 volume of 1% Triton X-100. After 1 min at room temperature, 100 μl of 34% citric acid was added to stop the color development. The samples were kept at room temperature and measured photometrically within the next 2 h at a fixed wavelength of 660 nm. To quantitate the amounts of enzymatically released P_i, the samples (in triplicates) were compared with a standard curve which was prepared with dilutions of a standard solution (1 mM KH_2PO_4 in 0.01 N H_2SO_4) over a range of 1 to 30 nM phosphate.

***S. typhimurium* adherence and invasion assay.** *S. typhimurium* attachment to and entry into Henle-407 cells were assayed as described elsewhere (21).

Fluorescence microscopy. Staining of *S. typhimurium*-infected Henle-407 cells with rhodamine-labelled phalloidin was carried out as described elsewhere (21).

Nucleotide sequence accession number. The nucleotide sequence published in this paper has been assigned GenBank accession number U08279.

RESULTS

Molecular characterization of the *invB* and *invC* genes. The entire nucleotide sequences of both strands of the *Eco*RV-*Pst*I fragment immediately downstream of *invA* were determined, and two open reading frames were identified (Fig. 2). The first open reading frame, *invB*, starts 22 bp downstream of *invA* and is capable of encoding a 134-amino-acid polypeptide with a predicted molecular weight of 14,852. A good rRNA consensus-binding site (AGGAA) is present at the appropriate distance from the putative start codon. The second open reading frame, *invC*, starts with an ATG codon that overlaps the termination codon of *invB* and is capable of encoding a 432-amino-acid polypeptide with a predicted molecular weight of 47,393. A weak rRNA consensus-binding site (CGGT) was identified upstream of the putative ATG start codon. No apparent transcription termination signals were identified upstream or downstream of *invB*, confirming previous observations that these genes are in the same transcriptional unit as *invA* (19).

DNA fragments carrying either or both of these genes were cloned behind the T7 promoter present in the vector pBluescript SKII and plasmid-encoded polypeptides examined as described in Materials and Methods. Cell lysates of *E. coli* BL21 (DE3) carrying plasmid pSB560, which encodes *invB*, or plasmid pSB553, which encodes *invC*, showed polypeptides with molecular masses of 15,000 and 47,000 Da, respectively, which were absent from lysates of cells carrying the plasmid vector alone (Fig. 3). The sizes of the expressed polypeptides are in complete agreement with the predicted sizes of the encoded polypeptides. A polypeptide with a molecular mass of 31,000 Da corresponding to the product of the *aphT* gene was observed in lysates of *E. coli* carrying plasmid pSB554. This plasmid contains an insertion of a cassette carrying the *aphT* gene in the *Eco*RI site of the *invC* open reading frame (Fig. 2). Polypeptides with molecular masses of 14,000 and 47,000 Da corresponding to InvB and InvC, respectively, were also observed in lysates of *E. coli* BL21 (DE3) carrying pSB555 (Fig.

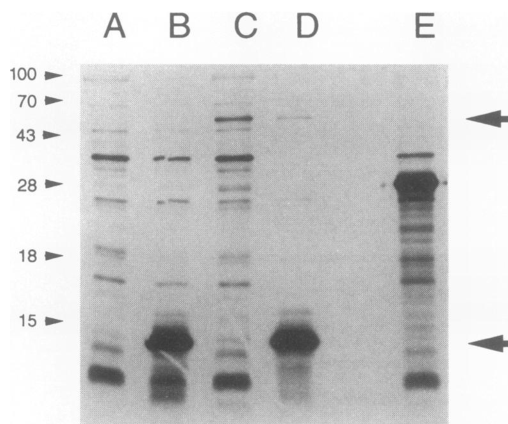

FIG. 3. Expression of *invB* and *invC*. The expression of *invB* and *invC* was placed under the control of the bacteriophage T7 promoter present in pBluescript SKII and introduced into *E. coli* BL21 (DE3) that carries a bacteriophage T7 RNA polymerase gene under the control of p*lac*. After induction, whole-cell lysates were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as described in Materials and Methods. Lanes: A, pBluescript SKII; B, pSB560 (*invB*); C, pSB553 (*invC*); D, pSB555 (*invB* and *invC*); E, pSB554 (*invC*::*aphT*). Arrows on the right indicate the positions of InvB (bottom) and InvC (top), and numbers on the left indicate the positions of the molecular weight standards.

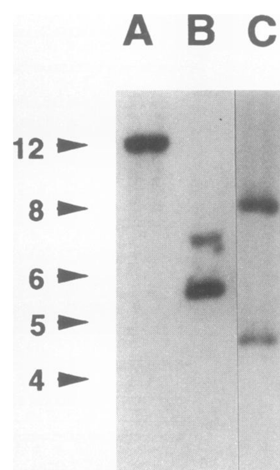

FIG. 4. Southern hybridization analysis of *invB* and *invC S. typhimurium* mutants. Total-cell DNA was isolated from the different strains and digested with *Hin*dIII, which cuts the *aphT* cassette. Fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membranes as described in Materials and Methods. Membranes were then hybridized to a [^{32}P]dATP-labeled probe containing the *Dra*I-*Pst*I fragment of pSB555 which contains the *invB* and *invC* genes. Lanes contain DNAs isolated from the following strains: A, SL1344 (wild type); B, SB566 (*invC*::*aphT*); C, SB178 (*invB*::*aphT*).

3). This plasmid carries a *Dra*I-*Pst*I fragment encoding both *invB* and *invC*. Interestingly, *invB* was expressed at significantly higher levels than *invC*, most likely reflecting differences in the translation efficiencies of these genes. The poor translation of *invC* is consistent with the presence of an rRNA-binding site (CGGT) that is significantly deviated from the canonical consensus sequence.

Construction of nonpolar mutations in *invB* and *invC*. *invB* and *invC* are part of a larger transcriptional unit. Therefore, in order to examine their individual contributions to *S. typhimurium* internalization, nonpolar mutations in these genes were constructed by two strategies. Mutations in *invB* were constructed by inserting into the *Eco*RV site of pSB561 a cassette containing a modified aminoglycoside 3′-phosphotransferase (*aphT*) gene from which the transcription terminator had been removed (4), yielding plasmid pSB575. A *Bam*HI fragment from pSB575 carrying the mutated *invB* gene and flanking sequences was cloned into pKNG121 (33), an R6K-derived replicon that cannot replicate in *S. typhimurium* in the absence of the λ Pir protein, yielding pSB576. This plasmid was then mobilized into *S. typhimurium* by conjugation. Transconjugants were selected by the sucrose selection method as described elsewhere (33). One transconjugant, strain SB178, showed a Southern hybridization pattern consistent with the presence of the *aphT* cassette in the proper location (Fig. 4). This mutant strain was used in a variety of functional assays. Mutations in *invC* were constructed as follows. The *aphT* cassette was cloned into the *Eco*RI site of pSB553, yielding plasmid pSB554. A *Pvu*II fragment of this plasmid containing the mutated allele of *invC* was cloned into the *Eco*RV and *Nru*I sites of the plasmid vector pACYC184, yielding plasmid pSB559. Subsequent introduction of the mutated allele of *invC* into the *S. typhimurium* chromosome was carried out by a combination of linear transformation and P22 transduction as described elsewhere (21), yielding strain SB566. Southern hybridization analysis confirmed the correct position of the insertion mutation (Fig. 4).

Effect of nonpolar mutations in *invB* and *invC* on *S. typhimurium* entry into cultured epithelial cells. Strains SB178 and SB566, carrying nonpolar mutations in *invB* and *invC*, respectively, were tested for their ability to attach to and enter into cultured Henle-407 cells. As shown in Table 2, a nonpolar mutation in *invC* severely impeded the ability of *S. typhimurium* to enter into cultured cells, although this mutation did not affect the ability of this organism to attach to these cells. This is consistent with the finding that this mutant strain did not cause cytoskeletal rearrangements in cultured Henle-407 cells (data not shown), an indication of their failure to trigger the signal transduction pathway that leads to bacterial uptake (44). Introduction of the plasmid pSB558, which carries a wild-type copy of *invC* under the control of the *tet* promoter, into strain SB566 restored the ability of this strain to enter into cultured epithelial cells. These results indicate that *invC* is required for the entry phenotype (Table 2). In contrast, introduction of a nonpolar mutation in *invB* did not significantly affect the ability of *S. typhimurium* to attach to or enter into cultured epithelial cells (Table 2), indicating that, under the assay conditions used, this gene does not contribute to the entry phenotype.

Sequence homologies of InvB and InvC. The predicted

TABLE 2. Adherence and invasion of wild-type and *invB* and *invC* mutants of *S. typhimurium* into cultured Henle-407 cells[a]

Strain	Relevant phenotype	% Adherence	% Invasion
SL1344	Wild type	78 ± 4	58 ± 2
SB178	InvB$^-$	49 ± 3	43 ± 3
SB566	InvC$^-$	23 ± 1	0.11 ± 0.01
SB566(pSB558)	InvC$^-$ (InvC$^+$)	ND	5.13 ± 0.9

[a] Values are means ± standard deviations of triplicate samples and are the percentages of the initial inoculum that survived gentamicin treatment for 2 h as described in Materials and Methods. Similar results were observed in several repetitions of this experiment. ND, not done.

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                    .         .         .         .         .
InvB     1 MQHLDIAELVRSALEVSGCDPSLIGGIDSHSTIVLDLFALPSICISVKDD 50
           | ::::.:|||..| . ||.||:|.::||||.|.:.| .:|.| |.: ::
Spa15    1 MSNINLVQLVRDSLFTIGCPPSIITDLDSHSAITISLDSMPAINIALVNE 50
                    .         .         .         .         .
InvB    51 DVWIWAQLGADSMVVLQQRAYEILMTIMEGCHFARGQLLLGEQNGE.LTL 99
           :| :||.::|.| | || .||:||  :: .  :. .:|:  ...:| | |
Spa15   51 QVMLWANFDAPSDVKLQSSAYNILNLMLMNFSYSINELVELHRSDEYLQL 100
                    .         .         .
InvB   100 KALVHPDFLSDGEKFSTALNGFYNYLEVFSRSL 132
           :.:::..|:: ||  |.. |::||. :|::.  |
Spa15  101 RVVIKDDYVHDGIVFAEILHEFYQRMEILNGVL 133
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FIG. 5. Sequence alignment of *S. typhimurium* InvB and *S. flexneri* Spa15. The alignments were constructed using the program Best Fit from the Genetics Computer Group package from the University of Wisconsin (12). Identical residues are indicated with vertical lines, and conserved substitutions are indicated by periods or colons.

sequences of InvB and InvC were compared with translated sequences in GenBank (release 78). InvB showed significant similarity to Spa15, a *Shigella* spp. protein involved in the surface presentation of the invasion protein antigens (Ipa) of these organisms (Fig. 5) (56). In addition, InvB showed weak similarity to FliH from enteric bacteria (58) and *Bacillus subtilis* (1) (previously known as Orf3 of the *fla* operon of this organism) (Table 3). These proteins are involved in the assembly of flagella in these organisms. InvC showed significant similarity to a number of proteins related to the catalytic subunits of the F_0F_1 ATPases (Fig. 6). Members of this family include the Spa47 protein from *Shigella* spp., involved in the surface presentation of the Ipa proteins (56); YscN from *Yersinia* spp., involved in the secretions of the Yops (60); FliI proteins of enteric bacteria and *B. subtilis* (previously known as the Orf4 of the *fla* operon), involved in flagellar assembly (1, 58); and HrpB6 (16) of several gram-negative bacterial plant pathogens involved in the secretion of harpin, a protein responsible for the induction of the hypersensitive response of plants to these plant pathogens (28). Interestingly, the genetic organization of *invB* and *invC* and their homologs in several microorganisms is the same, suggesting evolutionary conservation and, perhaps, a functional relationship between these two proteins.

Nucleotide binding motifs in InvC. Sequence comparison of proteins that are known to bind ATP have determined that a large proportion of these proteins share sequence motifs (59). The functional significance of these motifs has been confirmed by mutagenesis as well as by crystallographic datum analysis (18). The best conserved of these motifs is a glycine-rich region which forms a phosphate-binding, flexible loop between a beta strand and an alpha helix. This sequence motif is generally referred to as the Walker box A. An additional conserved sequence among nucleotide binding proteins is the Walker box B, which consists of a hydrophobic β-sheet region with a conserved aspartic acid residue. InvC has regions of significant homology to both Walker boxes (Fig. 6). In addition, it presents similarity to a region around a conserved glutamic acid residue that has been shown, in other ATP-binding proteins, to react with dicyclohexylcarbodiimide (DCCD) (62). This compound is known to inhibit the function of a number of ATPases, presumably because it modifies the active sites of these enzymes.

Complementation by a site-directed mutant of InvC. Several members of the F_0F_1 ATPase family of proteins have been subjected to site-directed mutational analysis (13, 45). In all cases, a mutation that changes a Lys in the Walker motif A has resulted in loss of function, indicating that this residue is critical for the catalytic function of these proteins. We therefore carried out a site-directed mutagenesis at the corresponding position in InvC by changing Lys-165 to Glu. This substitution was chosen because Glu has side chains with approximately the same size but different charges. A similar mutation resulted in loss of function of the highly related member of this family, FliI (13). Plasmid pSB589, encoding K165EInvC, was introduced into the *S. typhimurium* strain SB566, and the resulting strain was tested for its ability to enter into cultured epithelial cells. As shown in Table 4, pSB589 failed to complement the entry phenotype of the *invC* strain of *S. typhimurium*. Introduction of the same plasmid into the wild-type strain of *S. typhimurium* SL1344 did not have any effect on the ability of this strain to enter into cultured epithelial cells, indicating that the site-directed mutant of InvC is not transdominant.

ATP hydrolysis by InvC. The observation that InvC shares significant sequence homology with the F_0F_1 ATPase family of proteins prompted us to investigate its ability to hydrolyze ATP. InvC was purified to homogeneity using the GST expression purification system as described in Materials and Methods (Fig. 7). The purified fusion protein was then tested for its ability to hydrolyze ATP as described in Materials and Methods. As shown in Fig. 8, purified GST-InvC protein fusion showed an ATPase activity of ~250 pmol of phosphate per min per μg of protein. Similar results were obtained with the purified InvC protein after cleavage with thrombin (data not shown). This activity is comparable to those of other ATPases, including SecA, a component of the main secretory apparatus of gram-positive and gram-negative bacteria (37). On the contrary, purified GST-K165EInvC fusion protein did not show any significant ATPase activity. These results are consistent with the inability of this protein to complement an *invC* mutant of *S. typhimurium* and indicate that ATP hydrolysis is required for InvC function.

DISCUSSION

The ability of *Salmonella* spp. to enter into mammalian cells is an essential pathogenic feature of these organisms. We have previously identified a genetic locus, *inv*, which is required for *Salmonella* spp. to enter into cultured epithelial cells. In this paper, we report the molecular and functional characterization

TABLE 3. Proteins with sequence homology to InvB and InvC

Protein (organism)	Protein compared	% Identity	% Similarity	Proposed function
FliH (*E. coli* and *Salmonella* spp.)	InvB	22	43	Flagellar assembly
FliH (*B. subtilis*)	InvB	23	54	Flagellar assembly
Spa15 (*Shigella* spp.)	InvB	33	59	Ipa secretion
FliI (*E. coli* and *Salmonella* spp.)	InvC	38	60	Flagellar assembly
FliI (*B. subtilis*)	InvC	37	59	Flagellar assembly
Spa47 (*Shigella* spp.)	InvC	51	68	Ipa secretion
YscN (*Yersinia* spp.)	InvC	45	65	Yops secretion
HrpB6 (*Xanthomonas* spp.)	InvC	42	51	Harpin secretion
β subunit of F_0F_1 ATPase	InvC	29	52	Proton translocation
α subunit of F_0F_1 ATPase	InvC	24	51	Proton translocation

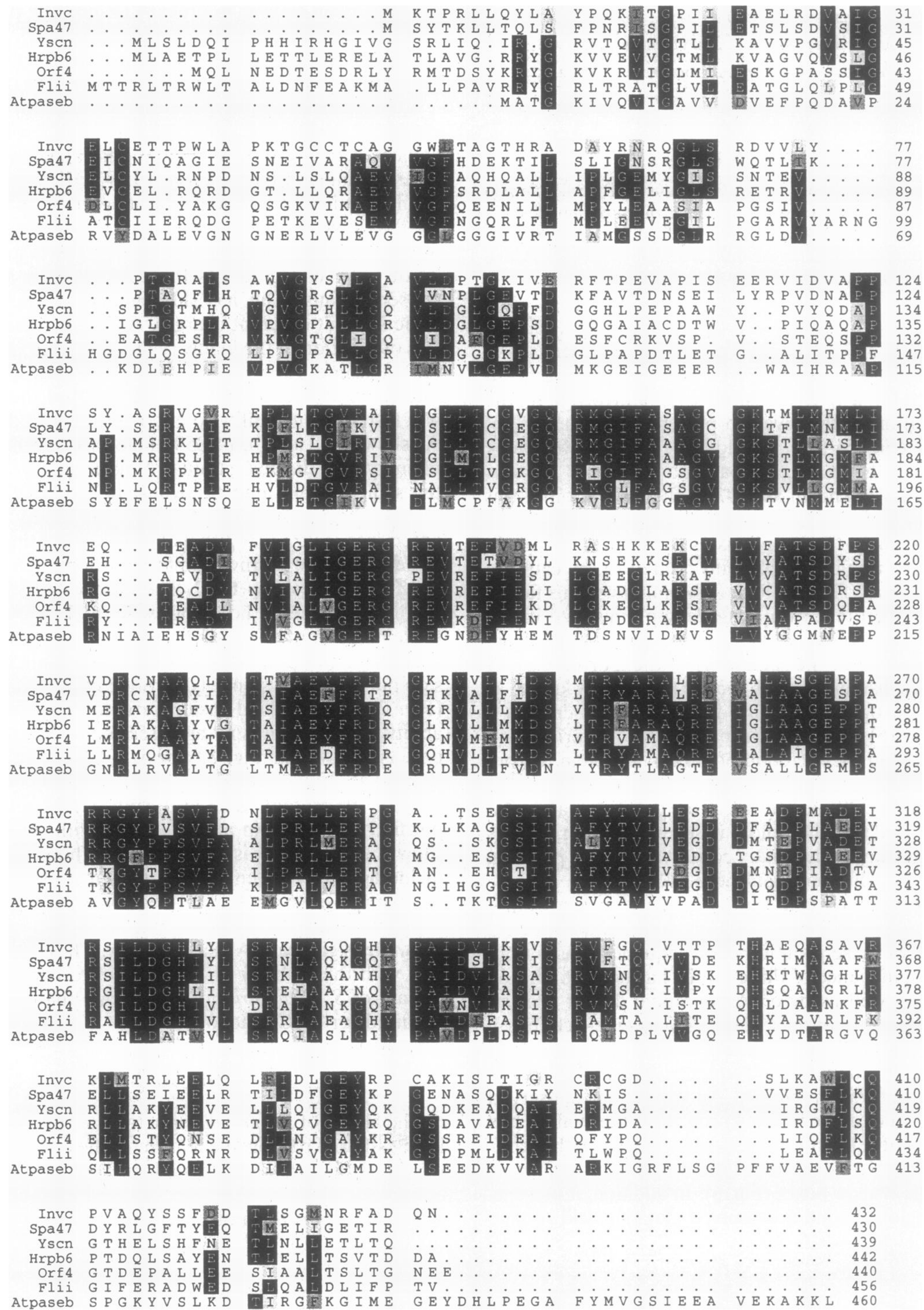

FIG. 6. Multiple sequence alignment of the InvC homologous proteins. The alignments were constructed using the program Pileup from the Genetics Computer Group package from the University of Wisconsin (12). The output of this program was further processed for display using the program Pretty Box of the same package.

of two members of this locus, *invB* and *invC*. We determined their nucleotide sequences and examined their gene products. *invB* and *invC* encode 134- and 432-amino-acid polypeptides, respectively, with predicted molecular weights of 14,852 and 47,393. Polypeptides with similar sizes were visualized when these genes were expressed in a T7 RNA polymerase expression system. The nucleotide sequences suggested that these genes are in the same transcriptional unit as *invA* and downstream genes. Therefore, to investigate the individual contributions of *invB* and *invC* to the entry phenotype, we con-

TABLE 4. Ability of K165EInvC to complement the entry phenotype of the *S. typhimurium invC* mutant[a]

Strain	Relevant phenotype	Plasmid-encoded protein	% Invasion
SL1344	Wild type		88 ± 4
SB566	$InvC^-$		0.10 ± 0.03
SB566(pSB558)	$InvC^-$ ($InvC^+$)	InvC	30 ± 2.1
SB566(pSB553)	$InvC^-$ ($InvC^+$)	InvC	21 ± 1.6
SB566(pSB589)	InvC ($InvC^-$)	K165EInvC	0.08 ± 0.04
SL1344(pSB553)	Wild type ($InvC^+$)	InvC	46 ± 3.4
SL1344(pSB589)	Wild type ($InvC^-$)	K165EInvC	38 ± 1.6

[a] Values are means ± standard deviations of triplicate samples and are the percentages of the initial inoculum that survived gentamicin treatment for 2 h as described in Materials and Methods. Similar results were observed in several repetitions of this experiment.

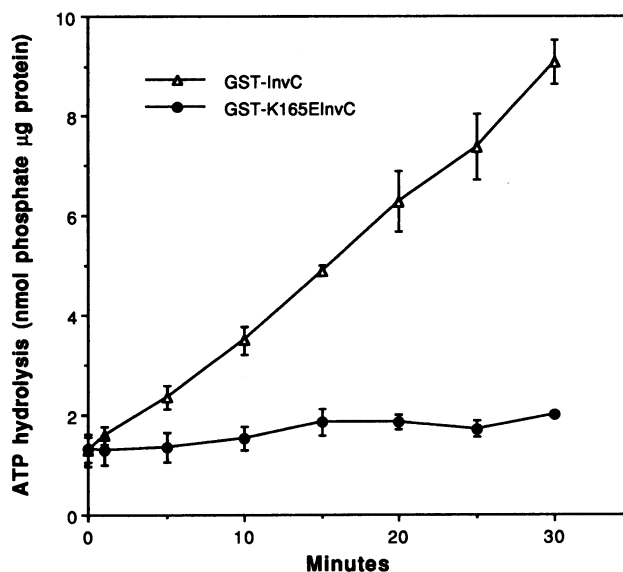

FIG. 8. ATP hydrolysis by purified InvC and K165EInvC. Hydrolysis of ATP was measured by the malachite green method as described in Materials and Methods. △, purified GST-InvC; ●, purified GST-K165EInvC.

structed nonpolar mutations in each one of these genes by inserting an *aphT* cassette from which the transcription terminator had been removed. Insertion of this cassette allows transcription of downstream genes from the *aphT* promoter. This approach has been successfully used to construct nonpolar mutations in *invE* and *invA* (21, 24).

S. typhimurium SB566 carrying a mutation in *invC* was defective for entry into but not for attachment to cultured Henle-407 cells. This observation was consistent with the fact that this mutant strain was unable to induce cytoskeletal rearrangements in infected cells, an indication of its inability to trigger the host-cell signalling pathways that lead to bacterial uptake (44). The phenotype of *S. typhimurium* SB566 was complemented in *trans* by a plasmid carrying only *invC*, indicating that the defect in entry observed in this strain was solely due to the mutated *invC*. Comparison of the translated sequence of *invC* with translated sequences in GenBank revealed that this protein is homologous to members of what is becoming a new family of ATPases (13). Members of this family include proteins required for the export of flagellar proteins in enteric bacteria (58) and *B. subtilis* (FliI) (1) and proteins involved in conferring pathogenic properties to animal (*Shigella* spp. [Spa47] and *Yersinia* spp. [YscN]) (56, 60) and plant (*Xanthomonas campestris* [HrpB6]) (16) pathogens. This protein family is related to the catalytic β subunit of the bacterial F_0F_1 proton-translocating ATPase and to equivalent subunits of vacuolar and archaebacterial ATPases (11, 31). The F_0F_1 ATPase is a multisubunit complex found in a large variety of prokaryotic and eukaryotic cells (8). The enzyme plays an important role in energy transduction in the final step of oxidative phosphorylation. By using the electrochemical potential over the membrane built up by the proton gradient, the F_0F_1 ATP synthase catalyzes the formation of ATP from ADP and P_i. The complex is composed of an integral membrane proton channel (F_0) and a membrane-associated catalytic subunit (F_1). Solubilized F_1 exhibits ATP-hydrolyzing activity, which represents the reverse of its normal reaction (14). The two major subunits of the F_1 component, α and β, can both bind adenine nucleotides and are homologous to each other (31). However, affinity labeling and chemical modifications showed that only the β subunit contributes to the catalytic function (61, 63). As with other members of this protein family (58), no other similarities between the members of the *inv* locus and the other subunits of the F_0F_1 ATPase have been identified.

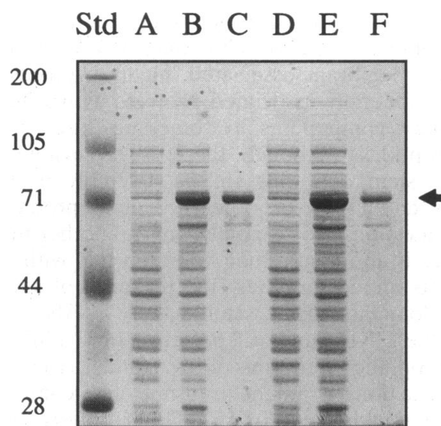

FIG. 7. SDS-polyacrylamide gel electrophoresis of purified InvC and the site-directed mutant K165EInvC. Protein fusions between InvC or the site-directed mutant K165EInvC and the GST were constructed as described in Materials and Methods to facilitate their expression and purification. Following induction, cells were disrupted with a French press, and the cell lysates were run through a glutathione column as indicated in Materials and Methods. Lanes contain samples from the following preparations: A, whole-cell lysate of the *E. coli* strain χ2991 carrying pSB573 which encodes GST-InvC before induction with isopropyl-β-D-thiogalactopyranoside (IPTG); B, same as A, after IPTG induction; C, purified GST-InvC; D, whole-cell lysate of the *E. coli* strain χ2991 carrying plasmid pSB588, which encodes GST-K165EInvC before induction with IPTG; E, same as D after IPTG induction; F, purified K165EInvC. Arrow to the right indicates the position of the GST-InvC or GST-K165EInvC fusions, and Std lane shows the molecular weight standards.

InvB was found to be homologous to the Spa15 protein of *Shigella* spp. This protein is part of a large operon that has been implicated in the translocation and surface expression of the *Shigella* Ipa proteins (56). In addition, InvB showed similarity, although weak, to FliH, a protein involved in flagellar assembly of enteric bacteria and *B. subtilis* (1, 58). Interestingly, the proteins encoded by genes located immedi-

ately upstream and downstream of FliH in these organisms, FlhA and FliI (58), are highly homologous to InvA and InvC, suggesting that the similarity between InvB and FliH may be evolutionarily or perhaps functionally significant. However, *S. typhimurium* SB178, carrying a mutation in *invB*, was not affected in its ability to enter into or attach to cultured Henle-407 cells, suggesting that this gene may not be required for *S. typhimurium* entry into these cells or that it encodes a redundant function. These results are consistent with the observations of Sasakawa et al. (49), who found that mutations in *spa15*, which encodes a protein homologous to InvB, did not affect the ability of *Shigella flexneri* to enter into cultured epithelial cells. Alternatively, the phenotype of the *invB* mutant may not be apparent in the assay system used.

In addition to *Salmonella* spp., other members of the family *Enterobacteriaceae*, such as enteroinvasive *E. coli*, *Yersinia* spp., and *Shigella* spp., are capable of entering mammalian cells. Interestingly, it is becoming evident that the molecular bases of the interaction of these microorganisms with host cells may share more features than originally suspected. Of particular interest is the remarkable similarity between *Salmonella* spp. and *Shigella* spp. (and presumably enteroinvasive *E. coli*) genes required for entry into mammalian cells. We have previously reported the characterization of four members of the *S. typhimurium inv* locus, *invA* (21), *invE* (24), *invF*, and *invG* (32). The predicted sequences of InvA, InvE, InvF, and InvG share extensive homology with the MxiA, MxiC, MxiE, and MxiD protein sequences of *Shigella* spp., respectively, which are required for the surface presentation of the Ipa proteins and are located in the virulence-associated plasmids present in these organisms (5, 48). We have not extended our molecular analysis of this *Salmonella* locus and have found that the similarities expand through a rather large region of the *Shigella* virulence plasmid (10, 32). This region encompasses at least 12 genes of the *mxi* and *spa* loci, arranged in the same order and presumably with the same transcriptional organization as the *Salmonella inv* locus (2, 3, 5, 49, 56). Similar results have been recently reported by Groissman and Ochman (26). This functional similarity is further strengthened by the fact that mutations in some of these genes (e.g., *invA*) can be complemented by the cognate *Shigella* genes (e.g., *mxiA*) (23). It appears, then, that *Salmonella* spp. and *Shigella* spp. share a similar translocation system that has been adapted to assemble a supramolecular structure (25) or to export proteins required for the interaction of these organisms with mammalian cells (2, 3, 5, 49, 56). Since the interactions of these organisms with their hosts appear to be significantly different, it is likely, then, that each secretory system has been tailored to perform specific functions in these two different pathogens. Alternatively, *Shigella* spp. may also assemble a surface organelle similar to that of *Salmonella* spp., and the differences between these two organisms may reside on the effector molecules.

Consistent with the sequence homologies that suggest that InvC may function as an ATPase, we showed that this protein, purified under nondenaturing conditions, has ATP-hydrolyzing activity. Under the assay conditions utilized, InvC had an ATPase activity of ~250 pmol of phosphate per min per μg of protein. This activity is equivalent to that observed in other ATPases, including SecA, a component of the main secretory pathway of gram-positive and gram-negative bacteria (43). It is possible that under in vivo conditions, this activity is significantly higher. For example, InvC may be part of a multisubunit complex, and the presence of the other components may enhance its activity. This has been shown to be the case for the β subunit of the F_0F_1 ATPase complex whose ATP-hydrolyzing activity is significantly enhanced in the presence of the other components (11). Alternatively, consistent with the protein-translocating function proposed for the homologs of InvC, the ATP-hydrolyzing activity may be enhanced in the presence of the translocation target(s). This has been observed for SecA, a member of the general secretory pathway whose ATPase activity increases dramatically in the presence of translocation-competent preproteins and membranes (38). Dreyfus et al. failed to demonstrate ATPase activity in purified FliI, a protein homologous to InvC involved in flagellar assembly in enteric bacteria, despite the fact that FliI is capable of binding ATP (13). As they suggested, this may have been due to the purification protocol, which included guanidinium hydrochloride and might have yielded denatured protein devoid of enzymatic activity.

Consistent with the hypothesis that ATP hydrolysis is required for InvC function, a site-directed mutant of InvC in which a Lys residue at position 165 was changed to a Glu, failed not only to hydrolyze ATP but also to complement an *invC* mutation of *S. typhimurium*. The Lys-165 residue resides in the Walker box A and has been shown to be critical for the catalytic activities of several members of this family of ATPases (45).

What is the functional significance of the ATPase activity of InvC? The similarity with proteins thought to be involved in organelle assembly and/or protein secretion in other bacterial systems strongly suggests a similar function for InvC in *S. typhimurium*. This is consistent with the observation that *invC* mutants failed to assemble the appendages observed on the surface of *S. typhimurium* upon contact with cultured epithelial cells (25). ATP hydrolysis has been shown to be a requirement for the translocation of proteins across the membrane through the general secretory pathway (37) or through other binding-protein-dependent transport systems (6). InvC may therefore couple ATP hydrolysis to the transport across the membranes of proteins required for bacterial entry into host cells. A number of the ATP-binding, protein-dependent transport systems have significant structural homology (7). They are usually composed of membrane-associated, highly hydrophobic proteins and peripherally associated proteins with ATP-binding cassettes. These components are sometimes fused in multidomain polypeptides. Although there is no evidence of an equivalent system operating in the assembly of the entry apparatus, examination of other *inv* gene products raises interesting questions. For example, InvA, another member of the *inv* locus, is an integral membrane protein with structural features in its amino terminus (the presence of seven transmembrane domains) that resemble those of the membrane domain of the HlyB family of proteins. InvC might interact with the hydrophilic C terminus of InvA, and, as a consequence of this interaction, it might transmit any conformational changes that could result from ATP hydrolysis, aiding the putative protein transport across the membrane. At this point, we have no evidence of physical interaction between InvA and InvC to support this hypothesis. Elucidating the function of InvC and other proteins encoded in the *inv* locus will help us to understand not only the way *Salmonella* spp. interact with host cells but also the interactions of other plant and mammalian pathogens with their hosts, since it is clear that this signal sequence-independent protein secretion system is widespread among other pathogens.

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