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Molecular and Functional Characterization of the *Salmonella* Invasion Gene *invA*: Homology of InvA to Members of a New Protein Family

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One of the earliest steps in the pathogenic cycle of the facultative intracellular pathogen *Salmonella* spp. is the invasion of the cells of the intestinal epithelium. We have previously identified a genetic locus, *inv*, that allows *Salmonella* spp. to enter cultured epithelial cells. *invA* is a member of this locus, and it is the first gene of an operon consisting of at least two additional invasion genes. We have constructed strains carrying nonpolar mutations in *invA* and examined the individual contribution of this gene to the invasion phenotype of *Salmonella typhimurium*. Nonpolar *S. typhimurium invA* mutants were deficient in invasion of cultured epithelial cells although they were fully capable of attaching to the same cells. In addition, unlike wild-type *S. typhimurium*, *invA* mutants did not alter the normal architecture of the microvilli of polarized epithelial cells nor did they cause any alterations in the distribution of actin microfilaments of infected cells. The invasion phenotype of *invA* mutants was readily rescued by wild-type *S. typhimurium* when cultured epithelial cells were simultaneously infected with both strains. On the contrary, in a similar experiment, the adherent *Escherichia coli* strain RDEC-1 was not internalized into cultured cells when coinfected with wild-type *S. typhimurium*. The *invA* locus was found to be located at about 59 min on the *Salmonella* chromosome, 7% linked to *mutS*. The nucleotide sequence of *invA* showed an open reading frame capable of encoding a polypeptide of 686 amino acids with eight possible membrane-spanning regions and a predicted molecular weight of 75,974. A protein of this size was visualized when *invA* was expressed in a bacteriophage T7 RNA polymerase-based expression system. The predicted sequence of InvA was found to be homologous to *Caulobacter crescentus* FlbF, *Yersinia* LcrD, *Shigella flexneri* VirH, and *E. coli* FlhA proteins. These proteins may form part of a family of proteins with a common function, quite possibly the translocation of specific proteins across the bacterial cell membrane.

Invasion of eukaryotic cells is an essential step in the pathogenic life cycle of a number of microbial pathogens (48). It is now clear that different organisms have evolved different strategies to enter and survive inside the eukaryotic host cells (1, 5, 9, 11, 12, 18, 20, 29, 40, 47, 54, 68, 73). Furthermore, evidence is beginning to accumulate suggesting that a given organism may utilize more than one entry pathway (9, 20, 28–30, 46, 54, 65).

Microbial invasion can be studied in vitro by using a variety of cultured cell lines, and this has allowed the identification of determinants involved in the entry process. The best-characterized invasion determinant is invasin, a *Yersinia pseudotuberculosis* protein that can direct the internalization of the normally noninvasive *Escherichia coli* K-12 (30, 32). The host cell receptors for invasin have been identified and found to be members of the β1 chain integrin family of proteins (31). Other determinants of invasion so far identified include the Ail protein of *Yersinia enterocolitica* (46), internalin of *Listeria monocytogenes* (18), penetrin of *Trypanosoma cruzi* (54), and the Ipa proteins of *Shigella* sp. (63), but no eukaryotic cell receptors for these determinants have been identified thus far.

Our laboratory is interested in understanding the molecular bases of the invasive phenotype of the facultative intracellular pathogen *Salmonella* spp. There is a large number of *Salmonella* serotypes or species that can cause a variety of diseases in different hosts. Some serotypes (i.e., *Salmonella typhi*, *Salmonella gallinarum*) are host adapted, while others (i.e., *Salmonella typhimurium*, *Salmonella enteritidis*) can cause disease in a large variety of hosts (27). Nevertheless, a common essential pathogenic feature of all of these organisms is their ability to invade the cells of the intestinal epithelium.

Transmission electron microscopic studies by Takeuchi showed the sequence of events leading to bacterial internalization in vivo (72). Upon reaching close proximity to the brush border, *S. typhimurium* caused dramatic changes, albeit transient, in the structure of the microvilli of the intestinal epithelium. These changes were localized to the point of bacterial contact. After bacterial internalization, the brush border recovered the normal appearance, and the organisms were seen in membrane-bound vesicles but never free in the cytosol of the epithelial cells. Similar changes have been observed in vitro when cultured polarized epithelial cells were used (13, 25).

A number of laboratories have identified different *Salmonella* genetic loci that are involved in conferring to these organisms the invasion phenotype (9, 15, 17, 20, 21, 25, 38, 49). Elsinghorst et al. have identified an *S. typhi* genetic locus that conferred invasive properties to the normally noninvasive *E. coli* K-12 (9). This locus was mapped at approximately 58 min on the *Salmonella* chromosome, closely linked to the *recA* and *srlC* genes. We have previously identified four *S. typhimurium* genes (*invA*, *-B*, *-C*, and

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TABLE 1. Bacterial strains used in these studies

Strain	Relevant genotype	Description, reference, or source
S. typhimurium		
SR11	Wild type	67
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
χ3642	*invA*::Tn*phoA-61*	20; derived from SR11
SB109 (SR11)	*invE*::*aph*	25
SB147 (SR11)	*invA*::*aphT*	P22HT*int* [χ3477(pSB154)] ⇒ SR1 this study
SA535 (LT2)	*serA13 rfa-3058* HfrK5	60
SB148 (LT2)	*serA13 rfa-3058* HfrK5 *invA*::*aphT*	P22HT*int* [SB147] ⇒ SA535 (this study)
E. coli		
χ6060	Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galE galK* Δ*phoA20 thi rpsE rpoB argE*(Am) *recA1*	Derived by R. Goldschmidt from CC118; reference 42
D301	RP487 *recD1903* Δ(*laclZYA-u169*)	58
RDEC-1	*gyrA*	4

-D) by virtue of their ability to complement an invasion-defective mutant of *S. typhimurium* (20). A polar mutation in *invA*, which also abolished the expression of downstream *inv* genes, severely impeded the invasive phenotype of virulent strains of *S. typhimurium* and caused a significant increase in the 50% lethal doses when these organisms were administered orally to BALB/c mice (20). Interestingly, this mutation did not affect the virulence of *S. typhimurium* when the organisms were administered intraperitoneally, suggesting that the *inv* genes are needed for the display of virulence by *S. typhimurium* in the intestinal tract, presumably to gain access to the intestinal epithelium. More recently, it was found that the *inv* locus was present and functional in most, if not all, *Salmonella* serotypes (22).

We report here the molecular and functional characterization of *invA*, the first gene in the *invABC* operon. We have determined the location of the *invA* locus in the *Salmonella* chromosome and constructed strains carrying nonpolar mutations in *invA* to assess its individual contribution to the invasive phenotype. We have also determined its nucleotide sequence and found that the predicted translated product of *invA* is homologous to *Yersinia* LcrD (56, 75), *Caulobacter crescentus* FlbF (57), *Shigella flexneri* VirH (64), and *E. coli* FlhA (44). These proteins may represent members of a new family of proteins, quite possibly with a similar function. The implications of these findings are discussed.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. The bacterial strains used in this study and their sources are listed in Table 1. The Hfr strains of *S. typhimurium* have been described elsewhere (60). Bacteriophage P22HT*int* was used for transduction of markers into *Salmonella* strains (66). The M13 derivative phage R408 (59) was used to rescue single-stranded DNA from pBluescript IISK-derived plasmids (pSK) (Stratagene, La Jolla, Calif.). Strains were grown in L broth or on L agar plates (37). When appropriate, antibiotics were added to the growth medium at the following concentrations: kanamycin, 30 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml.

Recombinant DNA, genetic techniques, and nucleotide sequencing. Recombinant DNA techniques were performed by standard procedures (41). P22HT*int* transduction was carried out as described previously (66). Conjugation of Hfr strains was carried out as described elsewhere (7). Transformation of circular DNA into *E. coli* and *Salmonella* strains by electroporation was carried out as described elsewhere (40) by using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Transformation of linear DNA was performed by using a method described elsewhere for circular DNA (39). 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 (71). Polymerase chain reaction amplification of DNA fragments was carried out by using a commercial kit (GenAmp; Perkin-Elmer Cetus, Norwalk, Conn.), following the instructions of the manufacturer. Nucleotide sequence determination was carried out by the dideoxy chain termination procedure (62) using Sequenase according to the manufacturer's instructions (U.S. Biochemical Corp., Cleveland, Ohio). Both single- and double-stranded DNA were used as templates, and both strands were sequenced. Unidirectional deletions of pSKII-derived plasmids were obtained by using the procedure described by Henikoff (26). Single-stranded DNA templates from pSKII-derived plasmids grown in the *E. coli* host χ6060 were obtained by using the helper phage RK408 (59). Sequencing of the fusion junctions of Tn*phoA* insertions was performed by using double-stranded templates and a primer derived from the *phoA* region of Tn*phoA* (5′-GCCCTGTTCTGG AAAACCGG-3′) (42). Nucleotide sequence analysis was performed by using the GCG package from the University of Wisconsin (version 7) (8).

Plasmid constructions. Plasmids carrying *invA* were derived from pYA2220 (20) (Fig. 1). Deletions for nucleotide

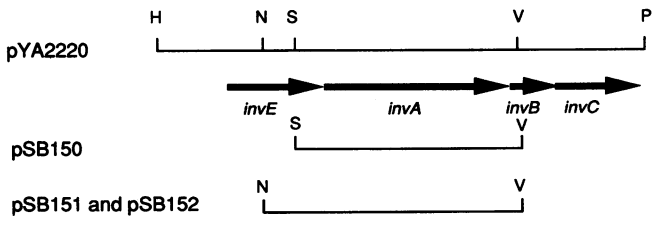

FIG. 1. Restriction endonuclease map of the *invEABC* region of *S. typhimurium*. Positions of relevant restriction endonuclease sites are shown. The location and direction of transcription of the different *inv* genes are shown by the arrows. H, *Hin*dIII; N, *Nru*I; S, *Sal*I; V, *Eco*RV; P, *Pst*I.

TABLE 2. Adherence and invasion of wild-type and *invA S. typhimurium* strains into cultured epithelial cells[a]

Strain	Relevant phenotype	Henle-407 cells		Polarized MDCK cells	
		% Adherence	% Invasion	% Adherence	% Invasion
SR11	Inv^+	39 ± 1	36 ± 2	9 ± 0.7	16 ± 1.7
$_x$3642	$InvA^-$ (polar)	22 ± 1	0.1 ± 0.01	ND[b]	ND
$_x$3642(pSB152)	$InvA^-$ ($InvA^+$)	18 ± 2	0.1 ± 0.2	ND	ND
SB147	$InvA^-$ (nonpolar)	24 ± 1	0.09 ± 0.01	11 ± 0.9	0.02 ± 0.006
SB147(pSB152)	$InvA^-$ ($InvA^+$)	23 ± 2	19 ± 1	10 ± 0.8	6 ± 0.6

[a] Values are means ± standard deviations of triplicate samples. Similar results were observed in several repetitions of this experiment.
[b] ND, not done.

sequencing were constructed in plasmid pSB002. This plasmid contains the *Hin*dIII-*Pst*I fragment of pYA2220 cloned into the *Hin*dIII and *Pst*I sites of pSKII. For expression of *invA*, plasmid pSB150 was constructed (Fig. 1). This plasmid carries a *Sal*I-*Eco*RV fragment from pYA2220 containing *invA* plus 500 bp of nucleotide sequence upstream of the beginning of the *invA* open reading frame (ORF). This fragment was cloned into the *Sal*I-*Eco*RV site of pSKII so that *invA* expression could be driven by the bacteriophage T7 promoter present in this plasmid vector. For complementation studies, plasmid pSB152 was constructed as follows. The *Nru*I-*Eco*RV fragment of pYA2220 containing the *invA* gene was cloned into the *Eco*RV site of pSKII, yielding plasmid pSB151 (Fig. 1). A *Hin*dIII-*Eco*RV fragment from pSB151 carrying the *invA* gene was then cloned into the *Hin*dIII-*Eco*RV sites of pACYC184 (3), yielding plasmid pSB152 (Fig. 1). For nucleotide sequencing, deletions of both ends of *invA* in plasmids pSB002 and pSB150 were constructed by following the protocol described by Henikoff (26). Plasmid pSB315 carrying the aminoglycoside 3′-phosphotransferase gene (*aphT*) (which encodes for kanamycin resistance) devoid of its transcription terminator was constructed as follows. A degenerate primer derived from the *aph* sequence (5′-ATACTGCAGATTAGAAAAACTCAT CG-3′) containing a newly-created *Pst*I site was used to remove the transcription terminator from the *aph* gene (53). This primer in conjunction with another primer (5′-GTTTTC CCAGTCACGAC-3′) derived from pUC4K (74) located 5′ from the beginning of the *aph* gene were used to amplify by polymerase chain reaction a 1.2-kb DNA fragment from pUC4K containing the *aph* gene without its transcription terminator. The amplified fragment was digested with *Pst*I and cloned into pSB314, yielding plasmid pSB315. Plasmid pSB314 is a derivative of pUC4K in which the *aph* gene has been removed by *Pst*I digestion and religation. The *aph* gene without its transcription terminator (*aphT*) can be retrieved from pSB315 by using *Pst*I, *Hin*cII, *Sal*I, or *Eco*RI.

Attachment and invasion assay. Attachment and invasion assays of nonpolarized Henle-407 and Madin-Darby canine kidney (MDCK) cells were performed as described elsewhere (20). For experiments requiring polarized cells, MDCK cells were grown in transparent collagen-treated Transwell filters (Costar, Cambridge, Mass.) for at least 5 days before the assay to allow for proper polarization and cell differentiation. The invasion and attachment assays were then carried out as described for nonpolarized cells.

Scanning electron microscopy. Infection of polarized confluent monolayers of MDCK cells and processing of samples for scanning electron microscopy were carried out as described elsewhere (25). Briefly, cells were infected with a multiplicity of infection of 50 for different times (10, 30, 60, and 120 min) as described above and then washed with phosphate-buffered saline (PBS) and fixed in cold (4°C) 2.5% glutaraldehyde in PBS for 1 h. Membranes were then removed from Transwell holders, washed in PBS, and treated with 2% OsO_4 in PBS for 30 min. Samples were then washed in PBS, dehydrated in a series of alcohols from 35 to 100%, critical point dried, sputter coated by gold evaporation, and examined in a JEOL scanning electron microscope.

Fluorescent microscopy. Cells grown to 60% confluency on glass coverslips were infected for 0, 15, 30, and 60 min with wild-type or *invA S. typhimurium* with a multiplicity of infection of 50. Coverslips were then washed in PBS, fixed in 3.7% formaldehyde in PBS, and treated with acetone for 5 min to permeabilize the cells. After washing with PBS, coverslips were treated with rhodamine-labelled phalloidin (Molecular Probes, Eugene, Oreg.), following the instructions of the manufacturer. Slides were then treated with rabbit-anti-*Salmonella* antiserum (group B, factors 1, 4, 5, and 12) (Difco Laboratories, Detroit, Mich.) and fluorescein isothiocyanate-labelled anti-rabbit antibody (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Coverslips were finally mounted in mounting medium (2 mg of *p*-phenylenediamine per ml in equal volumes of PBS and glycerol) and examined in a Zeiss RSIII fluorescent microscope.

Nucleotide sequence accession number. The nucleotide sequence published in this article has been assigned GenBank accession number M90846.

RESULTS

Construction of nonpolar *invA* mutants of *S. typhimurium*. *invA* is the first gene of an operon; therefore, polar mutations in this gene affect the expression of additional downstream genes also required for invasion (20). As shown in Table 2, plasmid pSB152, which carries only the *invA* gene (Fig. 1), failed to complement the invasion phenotype of *S. typhimurium* $_x$3642, which carries a polar mutation in *invA* (20). Therefore, to study the individual contribution of *invA* to the invasion phenotype, it was necessary to construct a nonpolar *invA* mutant of *S. typhimurium*. A cassette containing a modified aminoglycoside 3′-phosphotransferase (*aphT*) gene, from which the transcription terminator had been removed (see Materials and Methods), was introduced into the *Bgl*II site of plasmid pYA2222 (20), located 1,115 bp from the beginning of the *invA* ORF (see below), yielding plasmid pSB153. The inactivated *invA* gene was introduced into the chromosome of wild-type *S. typhimurium* by following the procedure described earlier (20) with some modifications. Briefly, plasmid pSB153 was linearized and transformed into the *recD* strain of *E. coli* D301 (58) carrying the plasmid pYA2217 (20). The *recD* mutation in this *E. coli* strain allows linear transformation without significantly affecting homologous recombination (58). Plasmid pYA2217 carries an insert

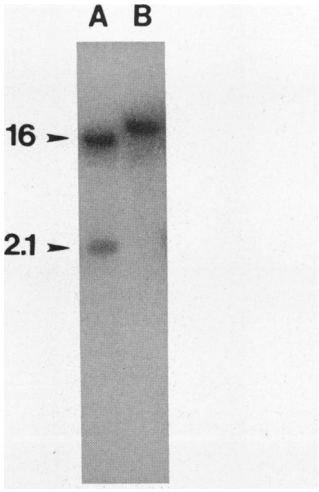

FIG. 2. Southern hybridization analysis of nonpolar *invA* *S. typhimurium* mutants. Total cell DNA was isolated from the different strains and digested with *Hin*dIII which cuts the *aphT* cassette. Fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membranes as described in Materials and Methods. Membranes were then hybridized to a [^{32}P]dATP-labeled probe containing the *invA* gene. Lanes contain DNA isolated from the following strains: A, SB147 (*invA* nonpolar mutant); B, *S. typhimurium* SR-11.

of approximately 50 kb that contains the entire *inv* region of *S. typhimurium* (20). Transformants that had undergone recombination of the *aphT* gene into the *invA* locus present in pYA2217 were identified by restriction analysis. One of the resulting plasmids, pSB154, was transformed into *S. typhimurium* $_{x}$3477. The size of pSB154 exceeds the size of a DNA fragment that can be packaged into phage P22 heads, thereby allowing the mobilization by transduction of the mutated *invA* allele into the chromosome of *S. typhimurium* without mobilization of the entire plasmid. A P22HT*int* lysate of one of the transformants was prepared and used to transduce the *invA*::*aphT* allele into wild-type *S. typhimurium* strains. Ampicillin-sensitive, kanamycin-resistant transductants were examined by Southern hybridization for the correct recombination of the mutated *invA* allele. One such transductant was *S. typhimurium* SB147 (Fig. 2).

Nonpolar *invA* mutants of *S. typhimurium* are deficient for invasion of cultured epithelial cells. *S. typhimurium* SB147 carrying a nonpolar insertion mutation in *invA* was tested for its ability to attach to and invade into polarized and nonpolarized cultured epithelial cells. As shown in Table 2, strain SB147 was significantly defective in its ability to invade cultured cells although it was not affected in its ability to attach to the same cells. Introduction of plasmid pSB152, which carries only the *invA* gene, restored the invasion phenotype. These results demonstrated that *invA* is essential for the efficient invasion by *S. typhimurium* of cultured cells. In addition, these results showed that the *aphT* promoter can successfully drive the expression of genes downstream of *invA*.

***invA* mutants of *S. typhimurium* failed to trigger the formation of membrane blebs in cultured polarized MDCK cells.** We have previously observed that shortly after coming into close contact with the surface of cultured epithelial cells, *S. typhimurium* caused dramatic changes in the architecture of the surface microvilli (25). These alterations were characterized by the formation of membrane blebs at the point of bacterial contact (25) (Fig. 3, panels 2 and 3). The blebs were most often seen associated with one or more bacteria which were located in the middle of these blebs. These changes were transient, and after 2 h, the microvilli started to regain their normal appearance. As shown in Fig. 3, the *invA* mutant of *S. typhimurium* SB147 failed to cause changes in the microvilli of polarized MDCK cells even 2 h after infection, despite the fact that numerous bacteria were observed attached to the surface of the cultured cells (Fig. 3, panel 4). Introduction of pSB152 carrying the *invA* gene into SB147 rendered this strain capable of altering the architecture of the microvilli (Fig. 3, panel 3).

***S. typhimurium invA* mutants did not alter the actin filament distribution of Henle-407 cells.** It has been shown that drugs that affect the normal structure of the cytoskeleton prevent *Salmonella* entry into cultured cells (2, 33). In addition, it has been shown that there is an accumulation of microfilaments around invading *Salmonella* spp. (14, 25). To examine the effect of *invA* mutations on the ability of *Salmonella* spp. to cause cytoskeletal rearrangements, we used rhodamine-labelled phalloidin and fluorescent microscopy. The fungal compound phalloidin has the property of binding to polymerized actin filaments (77). The *invA* mutant strain SB147 failed to cause changes in the microfilament distribution of cultured Henle-407 cells, despite the fact that a number of organisms were seen associated with the monolayer by fluorescein isothiocyanate-labelled anti-*Salmonella* antibodies (Fig. 4, panels 3 and 4). On the contrary, as reported before (14, 25), areas of condensed actin were readily observed in association with wild-type organisms (Fig. 4, panels 1 and 2).

Wild-type *S. typhimurium* can rescue the invasion phenotype of *invA* mutants. Despite their inability to invade cultured epithelial cells, *invA* mutants of *S. typhimurium* were fully capable of attaching to the same cells. We have recently reported that invasion-deficient, attachment-proficient *invE* mutants of *S. typhimurium* could be internalized into Henle-407 cells when coinfected with wild-type *S. typhimurium* (25). To test the possibility that *invA* mutants could also be rescued by wild-type *S. typhimurium*, cultured Henle-407 cells were infected with equal numbers of wild-type and *invA* mutants of *S. typhimurium*. The invasion-defective *invA* mutant SB147 was readily internalized when simultaneously infected with wild-type *S. typhimurium* (Table 3). On the contrary, as previously reported (25), *E. coli* RDEC-1 was not internalized into cultured epithelial cells when coinfected with wild-type *S. typhimurium*, despite the fact that this strain of *E. coli* readily attaches to cultured epithelial cells (data not shown) (4). The possibility of phenotypic intercomplementation between *invE* and *invA* *Salmonella* mutants was also tested. Henle-407 cells were simultaneously infected with equal numbers of the *invE* mutant SB109 (25) and the *invA* mutant SB147. As shown in Table 3, no phenotypic complementation between these mutants was observed.

Nucleotide sequence of *invA*. The nucleotide sequence of *invA* was determined on both strands as described in Materials and Methods. An ORF capable of encoding a 686-amino-acid polypeptide with a predicted molecular weight of 75,974 was identified (Fig. 5). A potential rRNA binding site (AGGAT) was found 7 nucleotides upstream from a GTG codon. No Shine-Dalgarno sequences were found near the next two ATG codons of the same ORF. The observed size of the InvA polypeptide (see below) and the size of the Tn*phoA* productive fusion *invA-61*::Tn*phoA* (20) suggest that the GTG codon is the translation initiation site. Confirmation of this hypothesis awaits until amino-terminal se-

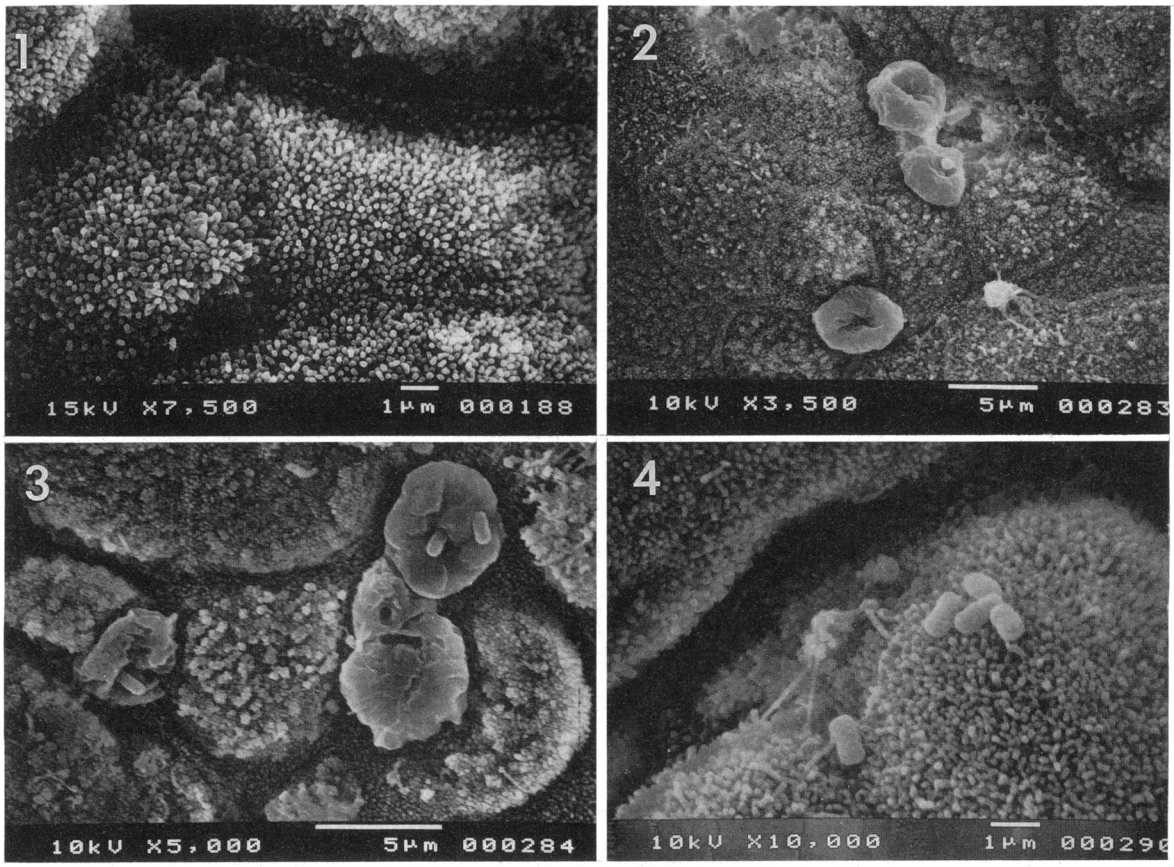

FIG. 3. Scanning electron microscopy of polarized MDCK cells infected with wild-type and nonpolar *invA* mutants of *S. typhimurium*. (1) Uninfected monolayer; (2) low magnification of a monolayer infected with wild-type *S. typhimurium* showing the formation of blebs (30 min after infection); (3) higher magnification showing the membrane blebs associated with bacteria (60 min after infection); (4) monolayer infected with the *invA* nonpolar mutant of *S. typhimurium* SB147 (2 h after infection).

quencing of InvA is performed. Putative promoter sequences were identified upstream of the predicted rRNA binding site and GTG start codon.

Hydropathy analysis of the predicted polypeptide suggests that InvA may be an integral membrane protein with eight hydrophobic stretches of at least 20 amino acids that could span a membrane. These proposed transmembrane domains are located in the amino-terminal half of the protein (Fig. 6). The carboxy-terminal half is hydrophilic, suggesting that it may be located in the periplasm or in the cytoplasm. Preliminary topological analysis indicates that the latter is more likely because in-frame fusions of the C terminus of InvA to PhoA (without its signal sequence) gave rise to a fusion protein with no significant alkaline phosphatase activity (19).

Homology of *invA* to a new family of proteins. Comparison of the InvA sequence with translated sequences in GenBank (release 69) revealed significant homology to several proteins (Fig. 6). The partially sequenced fifth ORF of the *lcrE* region of *Y. enterocolitica* (75), more recently shown to correspond to the *lcrD* gene of *Yersinia pestis* (56), showed significant homology to InvA. The LcrD protein has been reported to be a membrane-bound regulator of the *Yersinia* low-calcium response (56). The *C. crescentus* FlbF protein also showed homology to InvA (57). This protein is a cell-cycle-regulated flagellar gene located at the top of the flagellar gene hierarchy of *C. crescentus* (50). In addition, recent communication with C. Sasakawa (64) has revealed that VirH, an *S. flexneri* protein involved in the secretion of the plasmid-encoded Ipa proteins, shares homology with InvA. The *Shigella* Ipa proteins, which lack a typical signal sequence, are essential for the ability of this organism to enter cultured epithelial cells. Furthermore, communication with P. Matsumura (44) has established that InvA also shares substantial homology with the *E. coli* protein FlhA. It has been suggested that FlhA may be involved in the translocation through the membrane of a number of signalless flagellar proteins (76).

The multiple alignment of these proteins is shown in Fig. 7. Proteins can be aligned over the entire length of the sequence although homology is higher in the hydrophobic amino-terminal half. Nevertheless, clusters of residues on the carboxy-terminal half are also highly conserved and the overall secondary structures of these proteins, as determined by the method of Kyte and Doolittle (35), show significant similarity (Fig. 6).

Expression of *invA* in a bacteriophage T7 RNA polymerase expression system. We had previously shown by in vitro transcription-translation analysis of insertion and deletion mutants of plasmid pYA2219 and pYA2220 that InvA had an estimated molecular weight of 54,000. This is not consistent with the predicted size of InvA based on the nucleotide sequence. The *SalI-Eco*RV fragment of pYA2220 containing *invA* was placed behind the T7 promoter of plasmid pBluescript SKII. The resulting plasmid, pSB151, was introduced

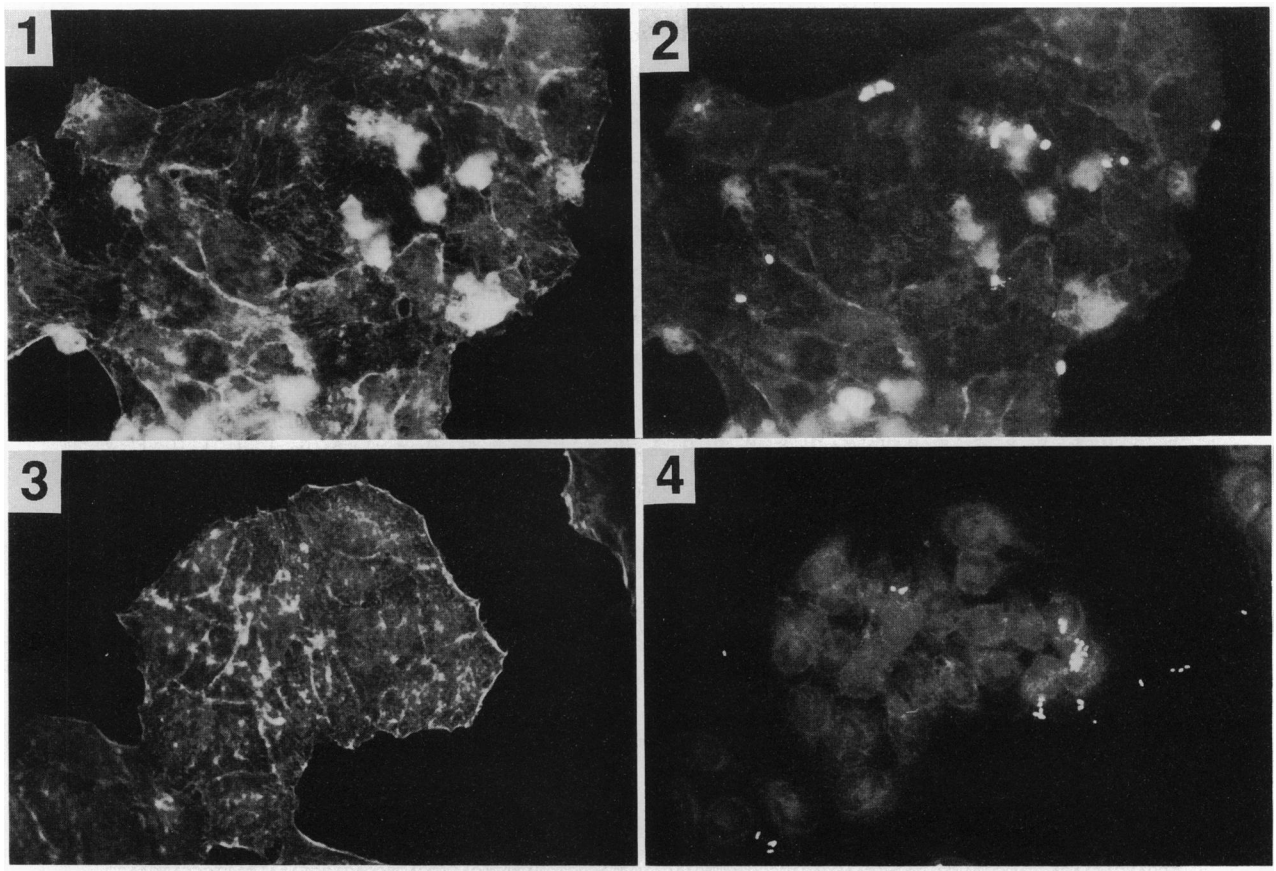

FIG. 4. Actin filament distribution in Henle-407 cells infected with wild-type and nonpolar *invA S. typhimurium* strains. Microfilaments were visualized by rhodamine-phalloidin staining (1 and 3) while bacteria were visualized by fluorescein isothiocyanate-labelled anti-*Salmonella* antibodies (2 and 4). Cells were infected with wild-type (1 and 2) and *invA* (3 and 4) *S. typhimurium* strains.

into *E. coli* BL21 (DE3), a strain that carries the T7 RNA polymerase gene under the control of p*lac* (70). Upon induction of the RNA polymerase gene, plasmid-encoded polypeptides were selectively labelled with [^{35}S]methionine as described in Materials and Methods. Cell lysates of pSB151 and vector-containing strains were then lysed and separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. As shown in Fig. 8, a polypeptide with an approximate molecular weight of 70,000 was observed in lysates of strains carrying pSB151 but was absent from strains carrying the vector alone. The misinterpretation of the previous data (20) was due to the presence of Tn*phoA*-encoded polypeptides that comigrated with proteins encoded by pYA2219 and to the presence of the recently identified gene *invE* immediately upstream of *invA* (25), which was unknown to us at that time.

TABLE 3. Invasion of *invA* mutants into cultured epithelial cells when coinfected with wild-type or *invE S. typhimurium*[a]

Strain(s) (relevant phenotype)	Henle-407 (% invasion)
SR11 (wild type)	35 ± 1
SB147 (InvA$^-$ Kan^r)	0.09 ± 0.01
SB147 (InvA$^-$ Kan^r) + SR11 (wild type)	8 ± 2[b]
SB109 (InvE$^-$) + SB147 (InvA$^-$)	0.07 ± 0.01
RDEC-1 (Nal^r) + SR11 (wild type)	0.002 ± 0.0001[c]

[a] Values are means ± standard deviations of triplicate samples. Similar results were observed in several repetitions of this experiment.

[b] Value is based on kanamycin-resistant colonies indicating invasion of *invA* mutants.

[c] Value is based on nalidixic acid-resistant colonies indicating invasion of *E. coli* RDEC-1.

Mapping of *invA-61*::Tn*phoA* within *invA*. The insertion mutant *invA-61*::Tn*phoA* was originally used to define *invA* and to construct *invA* polar mutations in wild-type *S. typhimurium* (20). Therefore, it was important to determine the localization of this insertion within the *invA* ORF. The precise location of this insertion was determined as described in Materials and Methods by sequencing the fusion junction site in pYA2224 (20), a pYA2219 derivative carrying *invA-61*::Tn*phoA*. The insertion point of *invA-61*::Tn*phoA* was located at nucleotide 82 of the *invA* ORF (Fig. 6). This location is in agreement with the size of the fusion protein previously determined in an in vitro transcription-translation system (20). In addition, the *invA-61*::Tn*phoA* insertion falls within the first putative periplasmic domain of the predicted structure of InvA, which is consistent with the observation that this fusion exhibits significant alkaline phosphatase activity (21). Tn*phoA* insertions in the equivalent site of LcrD have also been shown to yield fusions with alkaline phosphatase activity (56).

Mapping the *invA* locus in the *Salmonella* chromosome. The location of the *invA* locus in the *Salmonella* chromosome was determined by Hfr conjugation and phage P22 transduc-

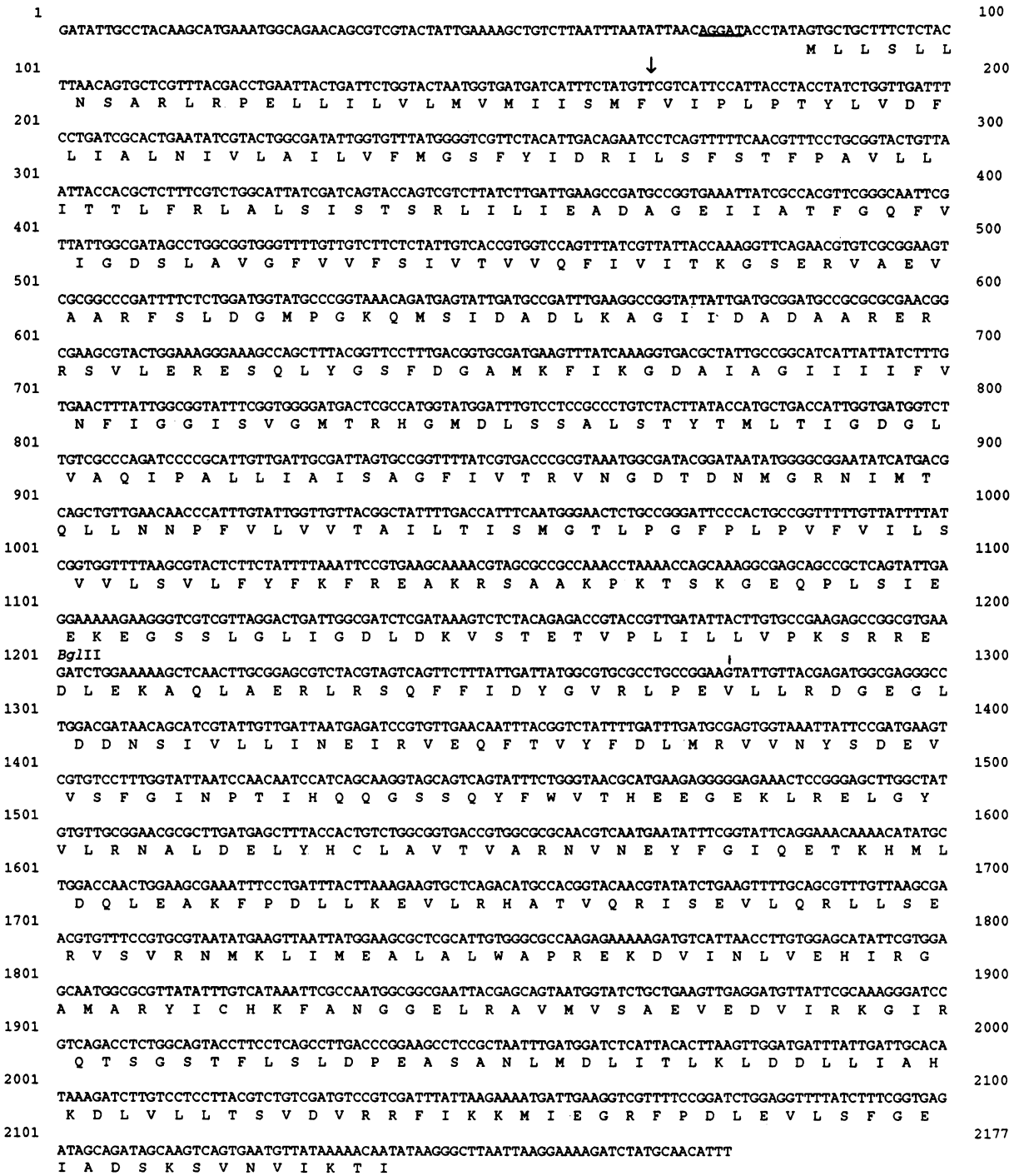

FIG. 5. Nucleotide sequence of *invA*. The sequence of the coding strand is shown with the deduced protein sequence. The end of the upstream *invE* gene is indicated. The putative Shine-Delgarno sequence is underlined. The position of the *invA-61*::Tn*phoA* insertion (20) is indicated by an arrow. The location of the *Bgl*II site where the *aphT* cassette was inserted is also shown.

tion as described in Materials and Methods. The *invA*::*aph* allele was recombined by P22HT*int* into the chromosome of several *Salmonella* Hfr strains with different origins of transfer (60). The Hfr strain SB148, with clockwise origin of transfer at 43 min, was shown to transfer the *invA*::*aph* allele at high frequency after short mating times. On the basis of this information, a series of transposon Tn*10* insertions from the Kukral collection (34) located downstream from the origin of transfer of SB148 was introduced into this strain and the frequency of cotransfer of both markers was determined (61). By using this methodology, it was possible to place the *invA* gene at approximately 58 min on the *Salmonella* chromosome. P22HT*int*-mediated transduction was then used to establish linkage to markers in this area of the

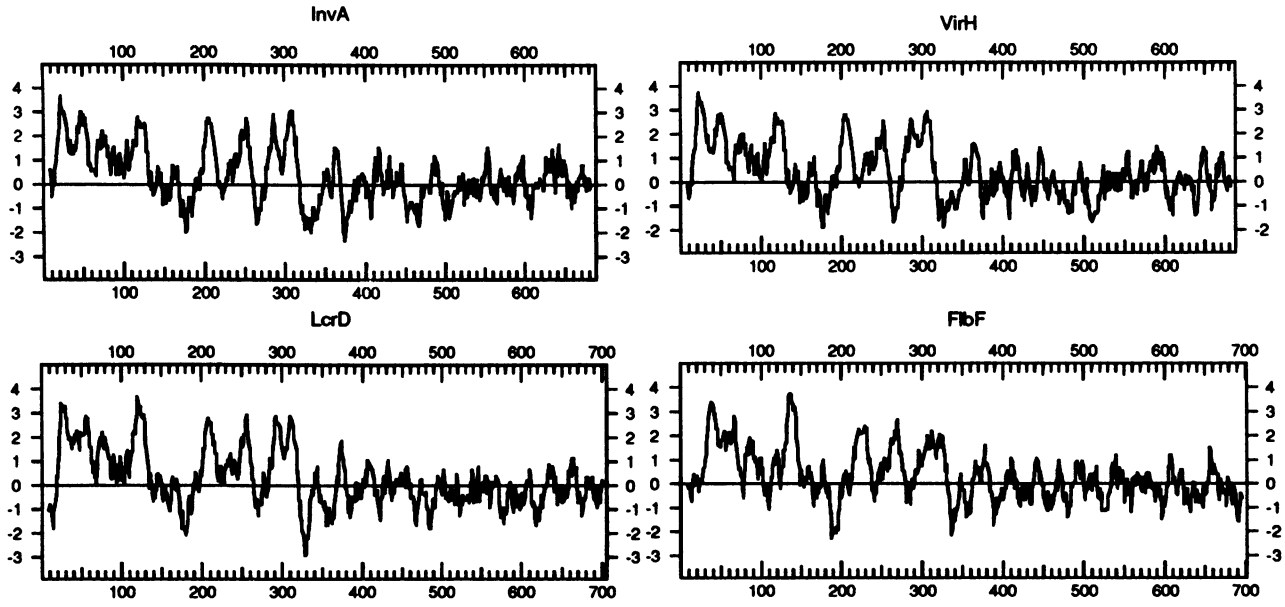

FIG. 6. Hydropathy analysis of the predicted amino acid sequences of InvA, LcrD, FlbF, and VirH. The method of Kyte and Doolittle (35) with a span of 11 residues was used to calculate the hydropathy index. Positive values indicate hydrophobic regions; negative values indicate hydrophilic regions.

Salmonella chromosome. It was found that the *invA* locus is 7% linked to *mutS*, placing *invA* at approximately 59 min on the *Salmonella* chromosome (Fig. 9) (61). An *S. typhi* invasion locus has been mapped immediately adjacent to *srlC* and *recA*, which map at about 58 min on the *Salmonella* chromosome (9). The *invA* locus was not linked by P22 transduction to either of these markers, indicating that even though *invA* is located near the *S. typhi* invasion locus, it is separate from these genes.

DISCUSSION

We have reported here the functional and molecular characterization of *invA*, an *S. typhimurium* gene that is required for the ability of this organism to invade cultured epithelial cells. *invA* is the first gene of an operon containing three or possibly more genes arranged in the same transcriptional unit (20). These genes have been shown to be present and functional in most (if not all) *Salmonella* serotypes (22). We constructed *S. typhimurium* strains carrying nonpolar mutations in *invA* by interrupting the *invA* ORF by inserting an *aphT* gene cassette in which the transcription terminator had been removed to allow transcriptional read-through of downstream genes. This allowed us to assess the individual contribution of *invA* to the invasion phenotype. Nonpolar *invA* mutants were significantly impeded in their ability to enter cultured epithelial cells and were readily complementable in *trans* by a plasmid carrying only *invA* (Table 2). These results established that *invA* is essential for the invasion phenotype of *S. typhimurium*.

The invasion process can be conceptualized as at least a two-step event. First, the organisms must come into close proximity to the target cell, and second, they must trigger intracellular signals that lead to bacterial internalization. Part of this sequence of events are the remarkable changes that occurred in the morphology of the microvilli of the intestinal epithelial cell surface upon bacterial contact. Takeuchi, in pioneering electron microscopic studies, showed that subsequent to *Salmonella* spp. coming into close proximity to the brush border, a localized degeneration of the microvilli took place (72). After internalization, the microvilli recovered the original architecture, an indication of the transient nature of these changes. We and others have observed similar changes in cultured polarized epithelial cells infected with *S. typhimurium* (13, 25). Shortly after infection, the formation of membrane blebs in close apposition to the bacteria was readily apparent. Those alterations in the architecture of the microvilli were accompanied by profound changes in the cytoskeleton of the host cell, characterized by the accumulation of polymerized actin and other cytoskeletal components at the point of bacterial entry (14, 25). In addition, we have found that intracellular free Ca^{2+} ($[Ca^{2+}]_i$) plays an important role in bacterial internalization. In work to be published elsewhere, we showed that a rapid increase in $[Ca^{2+}]_i$ levels followed shortly after the exposure of cultured epithelial cells to *S. typhimurium* (55). These changes were not simply a consequence of bacterial entry since wild-type *S. typhimurium* was able to trigger this increase in cytochalasin-treated cells (55).

We report here that despite the fact that *invA* mutants were fully capable of attaching to cultured epithelial cells, they failed to trigger changes in the morphology of the microvilli of polarized cells (Fig. 4) as well as changes in the actin filament distribution of infected epithelial cells (Fig. 5). In addition, *invA* mutants failed to cause an increase in the $[Ca^{2+}]_i$ levels of infected Henle-407 cells (55). These results suggest that *invA* may be involved in triggering the signal transduction pathway that leads to endocytosis of the microorganisms. This is consistent with the observation that the invasion phenotype of *invA* mutants can be efficiently rescued by wild-type *S. typhimurium* in simultaneous challenge of Henle-407 cells (Table 3). This rescue was specific for the *S. typhimurium invA* mutant since the adherent *E. coli* strain RDEC-1 was not internalized in a similar experiment (Table 3).

```
      1                                                                  80
InvA  ---------- --MLLSLLNS ARLRPELLIL VLMVMIISMF VIPLPTYLVD FLIALNIVLA ILVFMGSFYI DRILSFSTFP
VirH  ---------- --:IQ:F:KQ VSTK:::I:: :::::::A:L I::::::::: :::G:::::: :::::::::: E:::::::::
LcrD  ---------M NPHD:EW::R IGE:KDIMLA ::LLAVVFMM :L:::PLVL: I::AV:MTIS VVLL:IAI:: NSP:Q:SA::
FlbF  MADAAAPNAS SMPSAKS:LD GLM:G:MGLA LGV:G::VLL I::V:AP:L: V:LAISLTGS V:IL:TAIL: KKP:E:TS::
      81                                                                 160
InvA  AVLLITTLFR LALSISTSRL ILIE-----A DAGEIIATFG QFVIGDSLAV GFVVFSIVTV VQFIVITKGS ERVAEVAARF
VirH  S::::::::: :::::::::: ::VD-----: :::K::T::: :::::::::: :::::::::: :::::::::: ::::::::::
LcrD  ::::V::::: ::::V::T:M ::LQ-----: :::Q:VY::: N::VGGN:I: :I:I:L:I:I :Q:L:::::: ::::::S:::
FlbF  T:::V::::: :G:N:AST:: ::SHGQEGTG G::AV:EA:: HLMMQGNFVI :VI::I:LI: :N:M:V:::: G:I:::A:::
      161                                                                240
InvA  SLDGMPGKQM SIDADLKAGI IDADAARERR SVLERESQLY GSFDGAMKFI KGDAIAGIII IFVNFIGGIS VGMTRHGMDL
VirH  :::::::::: :::::::::: :::AG:K::: :I:::::::: :::::::::: :::::::::: ::::L::::: :::S::::S:
LcrD  :::A:::::: :::G:MR::V ::VNE::::: ATI:K:::MF ::::::M::: :::::::L:: ::::IL::VT :::T:K:LAA
FlbF  T::S:::::: A::A::ST:L :SQ:E:KI:: KE::Q::TFF :A::::S::: :::::::L:: TAINI::::I :::V:HK:PF
      241                                                                320
InvA  SSALSTYTML TIGDGLVAQI PALLIAISAG FIVTRVNG-D TDNMGRNIMT QLLNNPFVLV VTAILTISMG TLPGFPLPVF
VirH  :G::::::I: :::::::S:: :::::S:::: :::::::::- S::::::::S :IFG:::::I ::SA:ALAI: M:::::FF::
LcrD  AE::QL:SI: :V:::M:S:V :::::::T:: I:::::SSE: SSDL:SD:GK :VVAQ:KAML IGGV:LLLF: LI::::TVT:
FlbF  GD:A::::IM :I:::::S:: :::I:S:A:: MV:SKAGV:G S--ADKALT: ::AM::VG:G MVSASSGIIA LI::M:IFP:
      321                                                                400
InvA  VILSVVLSVL FYF-KFREAK RSAAKPK--- ----T:KGEQ PLSIEEKEGS SLGLIGDLDK VSTETVPLIL LVPKSRREDL
VirH  FLIA:T:TA: ::YK:VV:KE KSLSESD--- ----S:GYTG TFD:DNSHD: ::AM:EN::A ISS::::::: :FAENKINAN
LcrD  L::ALLVGCG G:MLSRKQSR NDE:NQDLQS ILTSGSGAPA ARTKAKTS:A NK:RL:EQEA FAM.::::LI D:DS:QQ:A:
FlbF  AAMALAAGA: A:KRVQDAK: PK:LD:ADLE AAAPSEPE:E :I:ASLAIDD -VKI-----E LGYGLLT::- -------N::
      401                                                                480
InvA  EKAQLAE--- RLRSQFFIDY GVRLPEVLLR DGEGLDDNSI VLLINEIRVE QFTVYFDLMR VVNYSDEVVS FGIN..PTIH
VirH  DMEG:I:--- :I:::::::: :::::TI:Y: TSNE:KVDD: ::::::V:AD S:NI:::KVC ITDENGDIDA L::P..VVST
LcrD  :ANA:NDELV :V:RALYL:L ::PF:GIH:: FN::MGEGEY IISLQ:VP:A RGELKAGYLL :RESVSQLEL L::PYEKGE:
FlbF  DGRK:TDQIR A::KTLASE: :FVM:P:RIL :NMR:ANQGY AIR:K:MEAG AGE:RLGCLM CMDPRGGQ:E L..PGEHVRE
      481                                                                560
InvA  QQGSSQYFWV THEEGEKLRE LGYVLRNALD ELYHCLAVTV ARNVNEYFGI QETKHMLDQL EAKFPDLLKE VLR-HATVQR
VirH  SYNERVIS:: DVSYT:N:TN IDAKIKS:Q: :F::Q:SQAL LN:I::I::: ::::N::::F :NRY:::::: :F:-:V:I::
LcrD  LLPDQEA::: SV:YE:R:EK SQLEFFSHSQ V:TWH:SHVL REYAEDFI:: :::RYL:E:M :GGYGE:I:: :Q:-IVPL::
FlbF  PAFGLPAT:I ADDLR:EATF R::TVVDPAT V:TTH:TEIL KE:MADLLSY A:VQKL:KE: PETQKK:VDD LIPGTV:ATT
      541                                                                640
InvA  ISEVLQRLLS ERVSVRNMKL IMEALALWAP REKDVINLVE HIRGAMARYI CHKFANGGE- LRAVMVSAEV EDVIRKGIRQ
VirH  :::::::::G :NI::::::: :::S:::::: ::::::T::: :V:ASLS::: :S:I:VS::- IKV::L:GYI ::A:::::::
LcrD  MT:I::::VG :DI:I:::RS :L::MVE:GQ K::::VQ:T: Y::SSLK::: :Y:Y::GNNI :P:YLFDQ:: :EK::S:V::
FlbF  VQR:::S::R ::::I:DLPQ :L:GVGEA:P HTAS:TQ::: QV:ARL::QL :WANRGDDGA :PIITL::DW :QAFAEALIG
      641                                                                720
InvA  TSGSTFLSLD PEASANLMDL ITLKLDDLLI AHKDLVLLTS VDVRRFIKKM IEGRFPDLEV LSFGEIADSK SVNVIKTI--
VirH  :::GS::NM: I:V:DEVMET LAHA:RE:RN :K:NF:::V: ::I:::V:RL :DN::KSIL: I:YA::DEAY TI::L:::--
LcrD  ::AGSY:A:E :AVTESLLEQ VRKTIG::SQ IQSKP::IV: M:I::YVRKL ::SEYYG:P: :SYQ:LTQQI NIQPLGR:CL
FlbF  PGDDKQ:A:P :SRLQDFIRG VRDSFERAAL AGEAP:::T: PG::PYVRSI ::RFRGQTV: M:QN::HPRA RLKTVGMV--
```

FIG. 7. Alignment of *S. typhimurium* InvA, *Y. pestis* LcrD, *C. crescentus* FlbF, and *S. flexneri* VirH. Proteins were aligned by using the program Pileup from GCG (8). A vertical dash indicates identical amino acids, two dots indicates conservative substitutions, and one dot indicates no similarity.

Nucleotide sequence analysis of *invA* showed a single ORF capable of encoding a protein of 686 amino acids with a predicted molecular weight of 75,974 (Fig. 5). A protein of this size was observed when *invA* was expressed in a bacteriophage T7 RNA polymerase expression system (Fig. 8). The predicted protein displays the characteristics of an integral membrane protein. The amino terminus is largely hydrophobic, with eight potential transmembrane domains, while the carboxy-terminal end is hydrophilic and most likely located in the cytoplasm (Fig. 6). This topology is supported by the location of the *invA-61*::Tn*phoA* productive fusion (Fig. 5) and also by the fact that a signalless PhoA fusion to the carboxy terminus yielded a hybrid protein with no significant enzymatic activity (19).

InvA shows significant similarity to a new family of bacterial proteins (Fig. 6 and 7). This family includes *C. crescentus* FlbF (57), *Yersinia* sp. LcrD (56, 75), *Shigella* VirH (64), and *E. coli* FlhA (44) proteins. The precise function of these proteins is not known, but there are well-defined phenotypes associated with mutations in these genes. Mutations in *flbF* prevent the expression of a large number of *C. crescentus* flagellar genes (50, 57). Expression of these flagellar genes is coordinately regulated in a complex hierarchy in which *flbF* is at or very near the top (51). It has been suggested that FlbF may play a role in the signal transduction across the bacterial membrane that leads to transcriptional activation of other *fla* genes (57). *lcrD* is involved in the low-Ca^{2+}-response phenotype of *Yersinia* species (56). This phenotype is characterized by the Ca^{2+}-dependent growth and expression of a number of outer membrane proteins (Yops). Mutations in *lcrD* renders *Y. pestis* Ca^{2+} independent and affects the expression of genes encoding a number of Yops, which are essential for the display of virulence and host invasion by this organism (56,

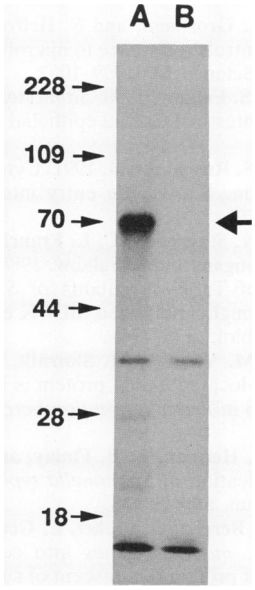

FIG. 8. Expression of *invA*. The *invA* gene was placed downstream from the bacteriophage T7 promoter of pSKII and introduced into *E. coli* BL21 (DE3) that carries a bacteriophage T7 RNA polymerase gene under the control of *plac*. After induction, whole-cell lysates were separated on an SDS-polyacrylamide gel as described in Materials and Methods. Lanes: A, pSB151 (carrying *invA*); B, pSKII (vector). The arrow on the right indicates the position of InvA, and arrows on the left indicate the positions of the molecular weight standards.

69). It has been recently hypothesized that LcrD may be involved in the sensing and/or transmembrane signalling of the environmental cues of Ca^{2+} (56). The *virH* gene of *S. flexneri* has been shown to be involved in the translocation of the Ipa proteins which are essential for the ability of this organism to penetrate intestinal epithelial cells (64). The Ipa

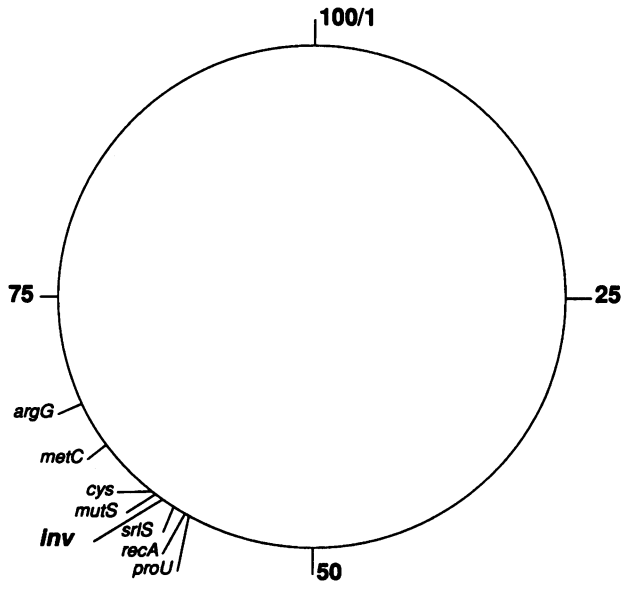

FIG. 9. Localization of the *inv* locus on the *Salmonella* chromosome.

proteins are surface located although they lack a typical signal sequence. Mutations in *virH* prevent the membrane localization of the Ipa polypeptides although they do not alter the expression levels of the *ipa* genes (64). These results indicate that VirH plays a role in the translocation apparatus of the *Shigella* invasion proteins. Similarly, mutations in *flhA* affect the translocation of a number of *E. coli* flagellar proteins which are believed to be exported by a specific mechanism, since these proteins also lack a typical signal peptide (44, 76). Therefore, it appears that the function of this new family of proteins may involve either the regulation of expression of other genes (FlbF, LcrD) or the translocation of other proteins through the membrane (FlhA and VirH). It should be noted that the latter function could also account for the phenotype observed in *flbF* and *lcrD* mutations. If, for example, FlbF or LcrD are involved in the translocation of a sensory protein(s), their mutations would be expected to have pleiotropic effects, similar to those observed in *flbF* and *lcrD* mutants (56, 57).

Currently we have no evidence for the involvement of InvA in the regulation of expression of other genes. It is known, however, that *invA* expression is regulated by changes in the level of DNA supercoiling as a consequence of a variety of stimuli such as osmolarity and oxygen tension (21). It is also known that conditions that alter the level of DNA supercoiling have a profound effect on the ability of *Salmonella* spp. to invade cultured epithelial cells (10, 21, 36). One may speculate that perhaps *invA* is involved in sensing the environmental cues that lead to changes in the level of DNA supercoiling, although we have been unable to detect any differences between the levels of DNA superhelicity of *S. typhimurium invA* mutants and those of the wild type (6).

We have reported elsewhere that InvE is homologous to the *Yersinia* YopN (LcrE) protein (25). We have not yet determined the cellular location of InvE, but it is noteworthy that YopN is found under certain growth conditions in the membrane and culture supernatants despite the fact that YopN, like InvE, lacks a typical signal sequence (16). The phenotype of *invA* mutants is almost identical to that of the *invE* mutants of *S. typhimurium*. The relationship between these two genes is not clear at the moment, but most likely both genes are part of the same entry pathway. This is suggested not only by their location immediately adjacent to each other but also by the fact that *invA* strains could not be rescued by *invE* mutants in simultaneous infection of cultured epithelial cells, despite the fact that either mutant could be rescued by wild-type *S. typhimurium* (Table 3). Therefore, it is tempting to speculate that InvA may be involved in the translocation of InvE. The possible involvement of InvA in the translocation of InvE is currently under intense investigation in our laboratory.

Besides *invA*, other *Salmonella* genes located in the same locus have been shown to be similar to other *Yersinia* virulence-plasmid genes. We have recently reported that *invE* is homologous to *lcrE* (*yopN*) (25), and we have also found that *invG*, a recently identified member of the *Salmonella inv* locus (23), is homologous to YcsA, a *Y. enterocolitica* protein involved in the secretion of Yops (45). In addition, the GC content of the *inv* genes so far sequenced is 45%, a value significantly different from the 52% reported for *Salmonella* DNA (43). This has led us to propose that perhaps *Salmonella* spp. may have acquired the *inv* genes from a different organism, quite possibly *Yersinia* spp., since its reported GC content is 46% (25, 43). In fact, phylogenetic

analysis of the InvA family of proteins strongly supports this hypothesis (24).

In summary, we have constructed nonpolar *invA* mutants of *S. typhimurium* and examined their phenotypes. We have presented evidence suggesting that *invA* may be involved in triggering the internalization of *S. typhimurium* into cultured epithelial cells. In addition, nucleotide sequence analysis has shown that InvA belongs to a new family of proteins from a wide variety of organisms. The possible functions of these proteins, derived from analysis of the mutant phenotypes, include signal transduction, transcription regulation, and protein translocation. Further studies are under way to define the precise mechanisms by which InvA exerts its function. This information will help in understanding the role of this new family of proteins.

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