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# InvF Is Required for Expression of Genes Encoding Proteins Secreted by the SPI1 Type III Secretion Apparatus in *Salmonella typhimurium*

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**The expression of genes encoding proteins secreted by the SPI1 (*Salmonella* pathogenicity island) type III secretion apparatus is known to require the transcriptional activators SirA and HilA. However, neither SirA nor HilA is believed to directly activate the promoters of these genes. *invF*, the first gene of the *inv-spa* gene cluster, is predicted to encode an AraC-type transcriptional activator and is required for invasion into cultured epithelial cells. However, the genes which are regulated by InvF have not been identified. In this work, an in-frame deletion in *invF* was constructed and tested for the expression of Φ(*sigD-lacZYA*), *sipC*::Tn*5lacZY*, and a plasmid-encoded Φ(*sicA-lacZYA*). SigD (*Salmonella* invasion gene) is a secreted protein required for the efficient invasion of *Salmonella typhimurium* into cultured eucaryotic cells. *sicA* (*Salmonella* invasion chaperone) is the first gene of a putative operon encoding the Sip/Ssp (*Salmonella* invasion/*Salmonella* secreted proteins) invasion proteins secreted by the SPI1 type III export apparatus. *invF* was required for the expression of the *sigD*, *sicA*, and *sipC* fusions. This is the first demonstration that there is a functional promoter in the intergenic sequence between *spaS* and *sicA*. In addition, several proteins were either absent from or found in reduced amounts in the culture supernatants of the *invF* mutant. Therefore, *invF* is required for the optimal expression of several genes encoding SPI1-secreted proteins. Genetic evidence is also presented suggesting there is HilA-dependent readthrough transcription from the *invF* promoter at least through *sipC*.**

The expression of genes required for the invasion of eucaryotic cells is stimulated by environmental cues including osmolarity (12), pH (4), and growth state and oxygen tension (10, 27, 38). Several regulators of SPI1 (*Salmonella* pathogenicity island 1) gene expression have been identified, but it is not known how they recognize environmental signals which affect gene expression. A *pho-24* mutant produces constitutively active PhoP, a response regulator which directly or indirectly represses the expression of SPI1 genes (4, 6, 15, 21). One of these genes, *hilA*, encodes an activator of SPI1 gene expression (3, 28). HilA is believed to directly activate expression from the *invF* and *prgH* promoters (3), although this has not been established biochemically. *invF* is predicted to encode an AraC-type transcriptional activator (for a review, see reference 13), but it is not known which genes it activates (23). *invF* is not autoregulated, nor does it activate the expression of *invH*, a gene which encodes an outer membrane lipoprotein component of the SPI1 type III secretion apparatus and which is divergently transcribed from *invF* (2, 8, 23). Nonetheless, *invF* is required for efficient invasion of cultured epithelial cells (23), suggesting that it is important for the expression of other genes required for invasion. This hypothesis was tested by examining the effect of an *invF* mutation on the expression of genes known to be required for invasion.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are described in Table 1. Electroporation of plasmids into bacteria was carried out as previously described (36). Plasmids that were manipulated in *Escherichia coli* were passaged through a restriction-minus (*hsd*) *Salmonella typhimurium* LT2 strain (LB5000) (37) prior to electroporation into *S. typhimurium* SL1344 (19). P22 HT *int* lysates were harvested and used for transductions as previously described (30).

pDL7-2 (generously provided by Catherine Lee) is a pLAFR2-based clone (11) containing the *inv-spa* and *sicAsipB* genes (32). Two subclones were made by digesting pDL7-2 with *Eco*RI and cloning an ~8-kb fragment containing *invH-FGEABC′* and a 6.7-kb fragment containing the *invIJ spaOPQRS sicA* sequence into pWKS130 (40), resulting in pHD7 and pHD8, respectively. pHD7 was digested with *Pst*I to subclone a 1.7-kb fragment containing *invF*, creating pHD9-1. This fragment includes about 250 bp of sequence upstream of the *invF* start codon and is transcribed in the same direction as the *lacZ* promoter in pWKS130. The same *Pst*I fragment was cloned into the medium-copy-number vector pHG329 (39), forming pHD10-1.

To construct pHD14, a 2.5-kb *Sal*I-*Bam*HI fragment containing *spaQRS* was cloned from pHD8 into pMAK705 (16). A unique *Bgl*II site was used to clone a ~2-kb streptomycin-spectinomycin resistance cassette from pSmUC into *spaS*, resulting in pHD14.

To construct the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *sigDE* clone pHH37, a 3.2-kb *Eco*RI-*Bam*HI fragment from pHH20 (20) was cloned into pVLT33 (9). Induction of *sigDE* expression was done by the addition of IPTG to a final concentration of 100 μM in overnight cultures.

pHD3 (*invF-lacZYA*) and pHD11 (*sicA-lacZYA*) were made by PCR amplification (*Pfu* polymerase; Stratagene) of putative promoter sequences and cloning into pRW50 (29). For pHD3, an *invH* primer with an *Eco*RI linker (*invH*-1 [5′-GGAATTCCGGCGCCATGTTTTTACACAACCGTCAGAAC-3′]) and an *invF* primer with a *Bam*HI linker (*invF*-1 [5′-CGGGATCCCGGCAGCTTTTGCGCGGAACACGTCTGTATAAACC-3′]) (Gibco BRL) were used to amplify ~460 bp between *invF* and *invH* from pDL7-2. The resulting fragment was cloned into the *Bam*HI and *Eco*RI sites of pRW50. For pHD11, the primers *spaS-Eco*RI-3 (5′-GGAATTCCGCGGAGAAGGTTGGCGTACCTG-3′) and *sicA-Bam*HI-1 (5′-CGGGATCCCGGCGTGGCGCCTTCACTAACGGCATCC-3′) were used to amplify the intergenic sequence (137 bp) between *spaS* and *sicA* along with 192 bp of the 3′ end of *spaS* and 76 bp of *sicA*. This amplified product was cloned into pRW50 as described above, and both strands were sequenced with the same primers used for amplification to confirm the sequence.

To construct the *sigD* chromosomal *lacZYA* reporter, plasmid pFUSE was used (5). pFUSE contains an R6K origin of replication and cannot replicate in SL1344. A 1-kb *Xba*I fragment from pHH15 (20) containing 0.4 kb of sequence upstream of *sigD* and 0.6 kb of *sigD* coding sequence was cloned into the unique

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TABLE 1. Bacterial strains and plasmids used in this work

| Strain | Genotype | Reference or source |
|---|---|---|
| ***S. typhimurium*** | | |
| SL1344 | Wild type | 19 |
| SVM473 | $Cm^r$ Φ(*sigD-lacZYA*)/*sigDE*$^+$ | This work |
| SB154 | $Cm^r$ $Str^R$ *invA*::Ω*cat* | J. Galán |
| SVM514 | $Str^r$ $Sp^r$ *spaS*::ΩStr/Sp | This work |
| SVM579 | Δ*invF* (in-frame deletion of 465 bp) | This work |
| VV302 | Δ*hilA-523* | 3 |
| BJ68 | $Tc^r$; *sipC*::Tn*5lacZY* | 34 |
| SVM725 | $Tc^r$ *sipC*::Tn*5lacZY* Δ*invF* | This work |
| SVM733 | $Str^r$ $Sp^r$ *spaS*::ΩStr/Sp Δ*invF* | This work |
| SVM754 | $Str^r$ $Sp^r$ $Tc^r$ Δ*invF spaS*::ΩStr/Sp *sipC*::Tn*5lacZY* | This work |
| LB5000 | LT2, *flaA66 metA22 trp-2 rpsL xyl-401 ilv-452 leu hsd mod*$^+$ | 37 |
| **Plasmids** | | |
| pHG329 | $Ap^r$ medium-copy-number cloning vector | 39 |
| pWKS130 | $Kn^r$, low-copy-number cloning vector | 40 |
| pVV214 | $Ap^r$, *hilA* in pACYC177 | 3 |
| p*hilA* | $Kn^r$, 2.2-kb *Nsi*I fragment from pVV214 cloned into the *Pst*I site of pWSK130 | This work |
| pHH21 | $Ap^r$, *sigD-lacZYA*, 0.9-kb *Eco*RI/*Pst*I *sigDE* promoter and partial coding sequences in pRS415 | 20 |
| pVLT33 | $Kn^r$, low-copy-vector for IPTG-inducible expression downstream of $P_{tac}$ | 9 |
| pHH37 | $Kn^r$, 3.2-kb *Eco*RI-*Bam*HI *sigDE* fragment in pVLT33 | This work |
| pRW50 | $Tc^r$, low-copy-number transcriptional reporter fusion vector | 29 |
| pHD3 | $Tc^r$, Φ(*invF-lacZYA*) in pRW50 | This work |
| pHD11 | $Tc^r$, Φ(*sicA-lacZYA*) in pRW50 | This work |
| pFUSE | $Cm^r$, suicide vector for integration of *lacZYA* transcriptional reporters onto the chromosome | 5 |
| pHD5 | $Cm^r$, 1-kb sequence including *sigDE* promoter in pFUSE | This work |
| pDL7-2 | $Tc^r$, cosmid clone containing *inv-spa* and *sicAB* genes | 32 |
| pHD7 | $Kn^r$, ~8-kb *Eco*RI fragment from pDL7-2 containing *invHFGEABC′* in pWKS130 | This work |
| pHD8 | $Kn^r$, ~6.7-kb *Eco*RI fragment from pDL7-2 containing *invC′IJ spaOPQRS sicA* sequence in pWSK130 | This work |
| pHD9-1 | $Kn^r$, 1.7-kb *Pst*I fragment from pHD7 containing *invF* in pWKS130 | This work |
| pHD10-1 | $Ap^r$, 1.7-kb *Pst*I fragment from pHD7 containing *invF* in pHG329 | This work |
| pHD17 | $Cm^r$, 1.7-kb *Hin*dIII *invF* fragment from pHD10-1 cloned into pACYC184 | This work |
| pHD13 | $Ap^r$, same as pHD10-1 but with 465-bp deletion in *invF* | This work |
| pMAK705 | $Cm^r$, temperature-sensitive origin of replication vector used for gene replacement | 16 |
| pHD15 | $Cm^r$, ~1.25-kb *Pst*I fragment from pHD13 cloned into pMAK705 | This work |

*Xba*I site of pFUSE. Ligations were transformed into *E. coli* S17-1λ*pir*, and clones were screened by restriction digestion with *Pst*I and *Eco*RI for inserts in the correct orientation relative to *lacZYA*. One clone was selected and named pHD5. pHD5 was transferred by conjugation into SVM252 (14028s *sirA*::Tn*10*dTc) (20, 22), where it integrated into *sigD*, leaving an intact *sigDE*$^+$ copy in addition to a *sigD-lacZYA* operon fusion. The *sirA*::Tn*10*dTc strain was used as a recipient in order to provide a selectable marker (tetracycline resistance) for *S. typhimurium*. Correct integration of pHD5 was confirmed in several exconjugants by transduction linkage analysis using SVM167 (*sigE*::Tn*10*dTc) (86% linkage). The Φ(*sigD-lacZYA*) fusion was then transduced into the appropriate strains.

**Growth conditions.** *S. typhimurium* and *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco) at 37°C with aeration on a roller drum or without aeration in standing cultures, as indicated. Antibiotics were used at the following final concentrations: chloramphenicol, 25 μg/ml; kanamycin, 100 μg/ml; streptomycin, 100 μg/ml; spectinomycin, 100 μg/ml; and tetracycline, 15 or 30 μg/ml for single-copy or multicopy tetracycline resistance, respectively. For the detection of β-galactosidase activity, solid medium (LB agar) was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 40 μg/ml. IPTG was used at a final concentration of 100 μM.

**Tissue culture invasion assays.** HEp-2 cells were maintained and passaged as recommended by the American Type Culture Collection. For invasion assays, $2 \times 10^5$ cells/ml were seeded into Falcon 24-well tissue culture plates (Becton Dickinson, Lincoln Park, N.J.) to obtain about 90% confluent monolayers on the following day. For bacterial cultures, single colonies were inoculated into 2 ml of LB broth and grown for 18 h without shaking at 37°C. Aliquots of 5 μl ($10^7$ to $10^8$ CFU) were used per well of tissue culture cells. The invasion assay was performed as previously described (20).

**Construction of *spaS* disruption mutants.** To construct the *spaS*::ΩStr/Sp mutant SMV514, a 2.5-kb *Sal*I-*Bam*HI fragment containing *spaQRS* was cloned into pMAK705, creating pHD12. A *Bam*HI streptomycin-spectinomycin resistance cassette from pSmUC was cloned into the unique *Bgl*II site within the 5′ region of *spaS*. This plasmid, pHD14, was used to exchange the disrupted *spaS* allele with the wild-type allele on the chromosome as previously described (16).

To confirm the disruptions on the chromosome, Southern analysis was performed (36). Labeling of a DNA probe and detection of disrupted sequences were done with enhanced chemiluminescence (ECL). Southern blotting detection reagents (Amersham Pharmacia Biotech). To confirm the *spaS* disruption, chromosomal DNA was digested with *Bam*HI and probed with a *spaS* PCR-amplified product. As predicted, the *spaS* probe hybridized to a 7-kb fragment of SL1344 chromosomal DNA and a 9-kb fragment in the *spaS*-disrupted strains (data not shown). One *spaS*::ΩStr/Sp mutant was chosen and called SVM514.

**Construction of the *invF* in-frame deletion mutant.** To make an in-frame deletion in *invF*, pHD10-1 was digested with *Cla*I and *Sac*II, the 5′ and 3′ single-stranded overhangs were removed with mung bean nuclease (New England Biolabs), and the blunt ends were ligated with T4 DNA ligase (New England Biolabs). Mung bean nuclease can sometimes remove double-stranded DNA in addition to single-stranded DNA; therefore, several clones were sequenced to identify clones with an in-frame deletion in *invF*. One clone, pHD13, contained an in frame deletion of 465 bp. A 1.25-kb *Pst*I fragment from pHD13 was subcloned into pMAK705, producing pHD15, which was then used to exchange the deletion onto the chromosome as previously described (16).

To screen for the *invF* deletion on the wild-type chromosome, bacteria with resolved plasmids were pooled in phosphate-buffered saline, subcultured into LB broth, and infected with P22 HT *int* containing pHH21 (*sigD-lacZYA* reporter plasmid, medium copy number) (20). If *invF* were required for the expression of *sigD* as predicted, the expression of Φ(*sigD-lacZYA*) would be reduced in an Δ*invF* strain. Transductants were grown on MacConkey lactose agar supplemented with ampicillin. Four of several hundred transductants that formed less red or white colonies were purified and subsequently cured of the reporter plasmid by growing bacteria in LB broth for 5 days, subculturing 1:500 on each day. Individual colonies were screened for ampicillin sensitivity on LB agar containing ampicillin. One mutant was chosen and called SVM579. The *invF* mutation in this strain was complemented by *invF* in a low-copy-number vector

(pHD9-1), suggesting that the phenotype of this mutant (see Results) was due to the deletion in *invF* and not another mutation elsewhere on the chromosome. The same technique was used to introduce the *invF* deletion onto the chromosome of BJ68 (*sipC*::Tn*5lacZY*) (34), creating SVM725. The Δ*invF* resulted in reduced *lacZY* expression from the *sipC*::Tn*5lacZY* fusion when tested on LB agar plates supplemented with X-Gal.

To confirm the presence of the deletion on the chromosome and the absence of any gross chromosomal rearrangements, Southern analysis was performed (36). Chromosomal DNA was digested with *Pst*I and probed with the 1.7-kb *Pst*I fragment from pHD10-1. The probe hybridized to a 1.7-kb *Pst*I fragment of chromosomal DNA from SL1344 and a 1.2- to 1.3-kb fragment of SVM579 and SVM725 as predicted (data not shown).

To construct the Δ*invF spaS*::ΩStr/Sp *sipC*::Tn*5lacZY* triple mutant, SVM754, the *spaS*::ΩStr/Sp mutation in SVM514 was transduced into SVM579 (Δ*invF*). Nine purified transductants were screened by Southern hybridization to confirm that the *invF* deletion had not been lost upon transduction due to its *spaS*-linked location on the chromosome (data not shown). One mutant, SVM733 (Δ*invF spaS*::ΩStr/Sp), was used to make a P22 HT *int* lysate. The *spaS*::ΩStr/Sp mutation from SVM733 was transduced into SVM725 (Δ*invF sipC*::Tn*5lacZY*). Tetracycline-, spectinomycin-, and streptomycin-resistant transductants were purified and checked for P22 sensitivity.

**Analysis of culture supernatants.** Cultures were grown in 5 to 10 ml of LB broth with antibiotics for 18 h without aeration, and equivalent units of optical density at 600 nm were harvested as previously described (20). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26) on 7.5% gels for silver stain analysis (7) and 5% gels for immunoblot (Western) analysis (ECL Western blotting detection system; Amersham Pharmacia Biotech). It is notable that in these gels (30% acrylamide to 1.6% bisacrylamide), some proteins migrated more slowly than through gels poured with 0.8% acrylamide (data not shown). For immunoblots, proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore).

**Antibodies.** To make antibodies to SigD, the first 575 bp of *sigD* were cloned into the expression vector pMAL-c1 (New England Biolabs). Expression and affinity purification of the maltose binding protein (MBP)-SigD′ fusion protein expressed from this plasmid were performed as described by the manufacturer. Protein eluted from the amylose column was separated on a 7.5% SDS-polyacrylamide gel, and the fusion protein was excised from the gel. Lyophilized gel slices containing MBP-SigD′ were used to raise rabbit antibodies to SigD (Covance Research Services, Denver, Pa.). The anti-SigD antibodies were preadsorbed with an acetone powder of *E. coli* DH5α expressing MBP (17) to reduce cross-reactivity of the antibody to proteins other than SigD. The preadsorbed antiserum was used at a 1:1,000 dilution. Anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma (St. Louis, Mo.).

**Enzyme assays.** β-Galactosidase assays were performed and values were calculated as previously described (31). Cultures were grown in 5 ml of LB with appropriate antibiotics for 18 h at 37°C in 13- by 100-mm screw-capped tubes. Samples of 1.5 ml were harvested, washed once in 0.88% NaCl, and resuspended in 1 ml of working buffer; 100 μl of each cell suspension was used per assay.

**Sequence analysis.** Sequencing was performed by using the BigDye Terminator Cycle Sequencing Ready Reaction system (PE Applied Biosystems, Foster City, Calif.). Reactions were analyzed by the Washington University Nucleic Acid Chemistry Laboratory (St. Louis, Mo.). Sequence analyses (homologies, mapping, etc.) were performed using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc.).

## RESULTS

**InvF is essential for the expression of effector genes.** *invF* is the first open reading frame of a large gene cluster encoding components of the SPI1 type III secretion apparatus (23). It has been proposed that InvF activates the expression of genes required for invasion (23), but this has not been tested. Therefore, a strain with an in-frame deletion of 465 bp in *invF*, SVM579, was constructed and tested for expression of the Φ(*sigD-lacZYA*) (chromosomal), *sipC*::Tn*5lacZY*, and Φ(*sicA-lacZYA*) (episomal) reporters. *sigD*, which is unlinked to SPI1, encodes a protein secreted by the SPI1 type III secretion apparatus and is required for efficient invasion into cultured epithelial cells (20). The SigD homologue in *S. dublin*, SopB (*Salmonella* outer protein), has been implicated as a factor important for causing enteritis in a calf model of infection (14). Contrary to our previous report (20), *sigD* expression was found to be *hilA* dependent (1). Therefore, strain VV302 (Δ*hilA-523*) was resent to us and retested for the expression of Φ(*sigD-lacZYA*) in pHH21. In addition to the episomal *sigD-lacZYA* fusion, a chromosomal reporter fusion, Φ(*sigD-lacZYA*)/*sigDE*$^+$, was constructed and tested in the wild-type and VV302 strains. The expression of both Φ(*sigD-lacZYA*) fusions was significantly reduced in the *hilA* background (data not shown). Thus, although the *hilA* deletion in VV302 in our previous work was confirmed by Southern analysis and by tissue culture invasion assays (20), it appeared that the original strain of VV302 we received had acquired a suppressing mutation which allowed for *hilA*-independent expression of Φ(*sigD-lacZYA*).

TABLE 2. Complementation of the Δ*invF* mutant for regulation of gene expression[a]

| Strain | β-Galactosidase activity[b] (U) | | |
|---|---|---|---|
| | pWKS130 | pHD9 | p*hilA* |
| SVM473 [Φ(*sigD-lacZYA*)/*sigDE*$^+$] | 74 | 580 | 1,847 |
| SVM579 [Δ*invF* Φ(*sigD-lacZYA*)/*sigDE*$^+$] | 3 | 642 | 5 |
| SL1344/pHD11 [Φ(*sicA-lacZYA*)] | 114 | 986 | 3,612 |
| SVM579 (Δ*invF*)/pHD11 [Φ(*sicA-lacZYA*)] | 2 | 1,095 | 5 |
| BJ68 (*sipC*::Tn*5lacZY*) | 962 | 4,473 | 6,277 |
| SVM725 (BJ68Δ*invF*) | 196 | 4,268 | 1,291 |
| SVM754 (BJ68Δ*invF spaS*::ΩStr/Sp) | 9 | 4,430 | 16 |

[a] See Table 1 for descriptions of strains and plasmids.
[b] Average of duplicate β-galactosidase assays performed on duplicate cultures, representative of several independent assays. The standard deviation of β-galactosidase activity was less than 15% between cultures except for BJ68/pWKS130, in which case it was 24%.

*sicA* is predicted to encode a chaperone for one or more of the secreted proteins encoded in SPI1 (*sip*/*ssps*) and is believed to be cotranscribed with these genes (18, 21, 24, 25). The expression of Φ(*sigD-lacZYA*) and Φ(*sicA-lacZYA*) in SVM579 was found to be much lower than in the wild-type strain SL1344 (Table 2). The regulation defect due to the *invF* deletion was complemented by pHD9-1 (*invF*$^+$), demonstrating that the regulation phenotype was due to Δ*invF*. Expression of the Φ(*sicA-lacZYA*) and Φ(*sigD-lacZYA*) reporters was not complemented by p*hilA* (*hilA*$^+$) (Table 2), suggesting that the regulatory effect of InvF on the *sigD* and the *sicA* promoters is downstream of HilA.

The expression of a *sipC*::Tn*5lacZY* chromosomal reporter fusion from strain BJ68 (34) was also tested in the Δ*invF* background. The expression of *sipC*::Tn*5lacZY* was significantly reduced in the Δ*invF* mutant (Table 2). This regulatory defect could be complemented by pHD9-1 (*invF*$^+$). Interestingly, *hilA* provided in multicopy could also increase the expression of *sipC*::Tn*5lacZY*, suggesting that *sipC* expression could be activated from either a HilA- or an InvF-dependent promoter (Table 2). The HilA-dependent expression of *sipC*::Tn*5lacZY* in the Δ*invF* mutant could be virtually eliminated by a polar disruption in *spaS*, a gene upstream of *sicA* and *sipC*. In contrast, the InvF-dependent expression of *sipC*::Tn*5lacZY* was not affected by the polar disruption in *spaS*. These results suggest the production of a readthrough transcript beginning upstream of *spaS* that is HilA dependent and a second transcript beginning downstream of *spaS* (probably the *sicA* promoter) that is InvF dependent (Table 2).

Expression from the *invF* promoter was measured from pHD3 (*invF-lacZYA*) in SVM579 to determine if *invF* was autoregulated. As previously reported for a different *invF* mutant (23), the in-frame deletion in *invF* did not significantly affect expression of the *invF* promoter (64 (wild type) versus 53 [Δ*invF*] U of β-galactosidase activity).

**InvF is not sufficient to activate the expression of Φ(*sicA-lacZYA*) or Φ(*sigD-lacZYA*) in *E. coli*.** Previous work demonstrated that an Φ(*invF-lacZYA*) reporter (pVV562) could be

TABLE 3. Expression of Φ(*invF-lacZYA*), Φ(*sigD-lacZYA*), and Φ(*sicA-lacZYA*) in *E. coli*[a]

| Reporter genotype (name) | β-Galactosidase activity[b] (U) | | |
|---|---|---|---|
| | *hilA*$^+$ | *invF*$^+$ | *hilA*$^+$ *invF*$^+$ |
| Φ(*invF-lacZYA*) (pHD3)[c] | 2,798 | ND | ND |
| Φ(*sigD-lacZYA*) (pHH21)[d] | 84 | 89 | 81 |
| Φ(*sicA-lacZYA*) (pHD11)[d] | <1 | <1 | <1 |

[a] See Table 1 for descriptions of plasmids.
[b] Average of duplicate β-galactosidase assays performed per culture, representative of several assays done on different days. The standard deviation of β-galactosidase activity was less than 10% between assays. ND, not determined.
[c] The control strain, DH5α containing pHD3 and the cloning vector pWSK130, produced 2 U of β-galactosidase activity. *hilA* was provided by p*hilA*.
[d] The amount of β-galactosidase activity produced by strains containing the cloning vector pWSK130 was comparable to the values obtained with *hilA* provided in *trans*. Due to plasmid incompatibility constraints, different *hilA* and *invF* constructs were used. For the pHH21 assays, *hilA* was provided by p*hilA* and *invF* was provided by pHD17 (Table 1). For the pHD11 assays, *hilA* was provided by pVV214 and *invF* was provided by pHD9-1.

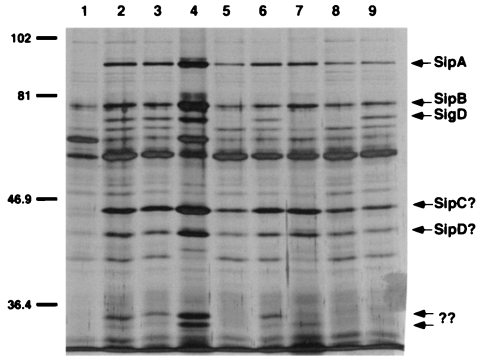


FIG. 1. Supernatant proteins from wild-type and Δ*invF* strains. Lane 1, supernatant proteins from the secretion defective *spaS* mutant SVM514; lanes 2 to 7, *S. typhimurium* SL1344 containing pWSK130, pHD9 (*invF*$^+$), and p*hilA* (lanes 2 to 4) and the *invF* mutant SVM579 containing the same plasmids in the same order (lanes 5 to 7); lanes 8 and 9, supernatant proteins from SVM579 strains containing the vector pVLT33 (lane 8) and the IPTG-inducible *sigDE* clone pHH37 (lane 9). Positions of molecular weight standards are indicated in kilodaltons on the left; previously identified secreted proteins are indicated on the right. Question marks denote proteins that have not been confirmed by immunoblot analysis. Proteins were prepared and analyzed as described in Materials and Methods.

activated by *hilA* (in pVV214) expressed in *E. coli* (3). To determine if HilA and/or InvF directly activates *sigD* or *sicA* expression, the effect of providing *hilA* or *invF* in *trans* on the expression of Φ(*sigD-lacZYA*) in pHH21 (20) and of Φ(*sicA-lacZYA*) in pHD11 was tested in *E. coli*. Although p*hilA* and pVV214 (data not shown) were able to activate Φ(*invF-lacZYA*) (pHD3) in *E. coli*, neither pVV214 nor p*hilA* could activate Φ(*sicA-lacZYA*) or Φ(*sigD-lacZYA*), respectively, in *E. coli* (Table 3). Expression of these fusions was also tested in the presence of *invF*. Like *hilA*, *invF*, with or without *hilA*, was unable to activate the expression of either fusion in *E. coli* (Table 3). Therefore, *invF* may require additional factors or signals that are absent from *E. coli* for the activation of the *sigD* and the *sicA* promoters.

**InvF is required for efficient invasion into cultured epithelial cells.** Invasion into cultured epithelial cells was also quantitated for the SVM579 mutant and wild-type *S. typhimurium*. Invasion of HEp-2 cells by the *invF* deletion strain was reduced significantly (Table 4), but not to the extent previously reported (23). It is possible that the *invF* mutation tested by Kaniga et al. (23) was having a polar effect on the expression of the *inv* genes and thus had a stronger phenotype. Moreover, invasion by the Δ*invF* mutant was not as reduced as invasion by a secretion-defective *invA* (SB154) or *spaS* (SVM514) mutant (Table 4). Invasion by SVM579 into HEp-2 cells was fully complemented to wild-type levels by pHD9-1. Interestingly, invasion was partially restored by p*hilA* (Table 4).

**Analysis of secreted proteins from the *invF* mutant.** Proteins that are secreted by the SPI1 type III machinery are believed to be the effectors which stimulate the uptake of bacteria by eucaryotic host cells (18, 20, 21, 24, 25). Because the expression of genes encoding several of these proteins was reduced in the *invF* mutant, the secreted protein profile of SVM579 was analyzed by SDS-PAGE and silver staining. Compared to the wild-type strain, several proteins were missing from the culture supernatants of the *invF* mutant, including SigD (confirmed by immunoblotting with anti-SigD antibodies [data not shown]) and a smaller protein migrating at about 36 kDa (Fig. 1, lanes 2 and 5) (it is notable that most of the known proteins, including SipA, SipB, and SigD, did not migrate according to their predicted molecular weights in these gels [see Materials and Methods]). Unexpectedly, Sip/SspA was clearly observed in culture supernatants of the *invF* mutant, although in slightly lower amounts, compared to the wild-type supernatant proteins. Sip/SspA and the other SPI1 secreted proteins were clearly absent from the supernatants of the *spaS* mutant SVM514 (Fig. 1, lane 1). pHD9-1 restored the secretion of SigD and the ~36-kDa protein into culture supernatants of the *invF* mutant and increased Sip/Ssp secretion to wild-type levels (Fig. 1, lane 6).

Unlike pHD9-1, p*hilA* did not restore SigD or the ~36-kDa protein into the culture supernatants of the Δ*invF* mutant. However, p*hilA* did increase the amounts of several other proteins including the Sip/Ssps to culture supernatants of the Δ*invF* mutant (Fig. 1, lane 7). Because p*hilA* could increase the expression of *sipC*::Tn*5lacZY* in the Δ*invF* mutant, it is not surprising that the Sip/Ssp proteins in culture supernatants are increased accordingly (Fig. 1, lane 7). This result may explain why p*hilA* partially restores invasion of an *invF* mutant into tissue culture cells.

To determine if InvF was required for the secretion of SigD in addition to the regulation of *sigDE* expression, an inducible

TABLE 4. Complementation of the Δ*invF* mutant for invasion of HEp-2 cells

| Strain | Mean % invasion ± SD[a] (% of wild-type level) | | | |
|---|---|---|---|---|
| | No plasmid | pWKS130 (vector) | pHD9 (*invF*$^+$) | p*hilA* |
| SL1344 | 12.4 ± 0.3 (100) | 7.7 ± 1.4 (100) | 7.1 ± 1.3 (100) | 8.0 ± 2.0 (100) |
| SVM579 (Δ*invF*) | 0.6 ± 0.1 (5) | 0.6 ± 0.1 (7) | 6.8 ± 1.3 (96) | 3.2 ± 0.3 (40) |
| SB154 (*invA*::Ω*cat*) | 0.02 ± 0.006 (0.1) | ND | ND | ND |
| SVM514 (*spaS*::ΩStr/Sp) | 0.02 ± 0.001 (0.1) | ND | ND | ND |

[a] After bacteria were allowed to invade during a 1-h incubation. Values are representative of several assays done in duplicate. ND, not determined.

clone of *sigDE*, pHH37 ($P_{tac}$-*sigDE*), was transformed into SVM579. If *invF* were required for secretion, SigD would not appear in culture supernatants even after the induction of *sigDE* expression with IPTG. The secretion of SigD was clearly restored to the supernatants of the *invF* mutant containing pHH37, demonstrating that InvF is not required for the secretion of proteins by the SPI1 type III secretion system (Fig. 1, lane 9).

## DISCUSSION

*invF* was identified by sequence analysis of the SPI1 region and was predicted to encode an AraC-type transcriptional activator (23). However, InvF-dependent genes were not identified. In this study, *invF* was shown to be required for the expression of Φ(*sigD-lacZYA*) and Φ(*sicA-lacZYA*) fusions. An in-frame *invF* deletion mutant was fully complemented for the expression of these reporters by *invF* cloned into a low-copy-number vector but was not complemented by *hilA*, a central activator of SPI1 gene expression (3). In contrast, although a *sipC*::Tn*5lacZY* reporter also required *invF* for optimal expression, *hilA* provided in multicopy was also able to increase *sipC* expression in the Δ*invF* mutant.

Several of the genes encoding secreted proteins, specifically the *sip*/*ssp* genes, are immediately downstream of the *inv-spa* gene cluster (18, 21, 24, 25). Because the *sip*/*ssp* locus is only 137 bp downstream of the *spa* genes, it was possible that *sip*/*ssp* expression could be activated from the *invF* promoter ~12 kb upstream of *sicA*; no transcriptional terminator is evident in this intergenic region. This observation is supported by the analysis of secreted proteins from the *invF* mutant which revealed the presence, arguably in lesser amounts, of several of the Sip/Ssp proteins. Moreover, these proteins could be restored to wild-type levels in the *invF* mutant by providing *hilA* on a low-copy-number plasmid. *hilA* could also partially complement the invasion defect of the *invF* mutant. Most importantly, the expression of *sipC*::Tn*5lacZY* could be activated in an *invF* mutant with the addition of *hilA* in multicopy, and this expression could be eliminated by a polar disruption in *spaS*. These results suggest that readthrough expression of the *sip*/*ssp* genes could be activated from the *invF* promoter by HilA.

To determine if a promoter was present immediately upstream of the *sip*/*ssp* gene cluster, an episomal, Φ(*sicA-lacZYA*) reporter fusion in a low-copy-number vector was made. Expression of this fusion was dramatically reduced in the *invF* mutant. The regulation defect was complemented by *invF* but not *hilA*. This result demonstrates for the first time the presence of an InvF-dependent promoter immediately upstream of *sicA*. From these results taken together with the invasion assays, the β-galactosidase assays, and the SDS-PAGE data, it appears that expression of the *sip*/*ssp* genes can be driven in part from the HilA-dependent *invF* promoter in addition to an InvF-dependent promoter immediately upstream of *sicA* (Fig. 2).

The expression of a Φ(*sigD-lacZYA*) fusion in the *invF* deletion mutant was also dramatically reduced. SigD was absent from culture supernatants of the Δ*invF* mutant and restored by pHD9-1 (*invF*$^+$). In contrast to the Sip/Ssp proteins, SigD was not restored to culture supernatants when p*hilA* was placed in the *invF* mutant. In addition to SigD, a ~36-kDa protein was absent from *invF* mutant culture supernatants. This protein was also restored by the presence of *invF* but not *hilA* on a low-copy-number plasmid. Therefore, unlike the *sip*/*ssp* genes, *sigD* (and possibly the ~36-kDa protein) is not likely to be directly dependent on *hilA* for expression. It is notable that unlike the case for *hilA*, providing *invF* in multicopy does not result in the hypersecretion of proteins found in the culture supernatants of the wild-type strain (Fig. 1, lane 3 versus lane 4). Although InvF activates the expression of genes encoding secreted proteins, it probably does not increase the transcription of the apparatus genes required for the secretion of these proteins. This may explain why the hypersecretion of proteins does not occur despite the hyperexpression of the effector genes observed when *invF* is present in multicopy.

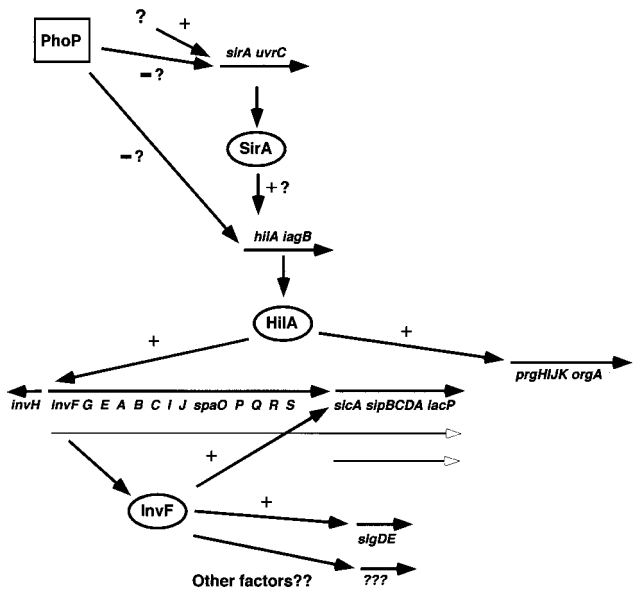


FIG. 2. Model for the regulation of invasion/virulence gene expression in *S. typhimurium*. The direction of transcription for each gene cluster is indicated by closed arrows; open arrows represent putative transcripts of the *inv-spa* and *sip*/*ssp* genes. Question marks indicate either unidentified regulatory factors or unclear relationships between the designated regulator and the noted promoter.

It is clear that the regulation of SPI1 gene expression is complicated and multifactorial (Fig. 2). *hilA* expression is dependent, directly or indirectly, on SirA, a protein which is known to be conserved in several of the *Enterobacteriaceae* (22, 33, 35). HilA, a member of the OmpR/ToxR family of regulators, in turn activates the expression of genes encoding the type III secretion apparatus (3). This effect is predicted to be direct because *hilA* expressed in *E. coli* can activate the expression of either Φ(*invF-lacZYA*) or Φ(*prgH-lacZYA*) (3). This work provides the first demonstration that the AraC-type transcriptional activator InvF is required for the expression of genes encoding proteins secreted by the type III secretion system. Further analysis will be necessary to determine if InvF itself binds to sequences upstream of *sicA* and *sigD* or if it activates the expression of another gene required for their expression. Because *invF* and *hilA* cannot activate the expression of Φ(*sicA-lacZYA*) or Φ(*sigD-lacZYA*) in *E. coli*, it seems unlikely that InvF alone is sufficient for activation. Perhaps InvF, like AraC, requires a cofactor (in the case of AraC, arabinose) (13) which induces a conformational change in InvF allowing it to bind to the appropriate promoters. Future studies will elucidate if and how InvF directly interacts with the promoters of genes encoding secreted effectors.

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