JOURNAL OF BACTERIOLOGY, Apr. 1998, p. 1793–1802 Vol. 180, No. 7
0021-9193/98/$04.00+0

Identification of a Novel *Salmonella* Invasion Locus Homologous to *Shigella ipgDE*

K. HERAN HONG AND VIRGINIA L. MILLER*

Department of Microbiology and Molecular Genetics, University of California, Los Angeles, Los Angeles, California 90095, and Departments of Pediatrics and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 19 September 1997/Accepted 16 January 1998

Genes essential for *Salmonella typhimurium* invasion have been localized to *Salmonella* pathogenicity island 1 (SPI1) on the chromosome. However, it is clear that other genes are required for the invasion process. Mutations that abolish the SPI1 invasion type III secretion system do not significantly reduce invasion into Chinese hamster ovary tissue culture cells. Two invasion defective mutants were isolated by screening 2,500 Tn*10*dTc insertion mutants of *S. typhimurium* in the tissue culture invasion assay. One of the invasion mutants, SVM167, has an insertion between centisomes 24.5 and 25.5 in an operon homologous to the *ipgDEF* operon of the *Shigella flexneri* and *Shigella sonnei* virulence plasmid. A second mutant, SVM168, has an insertion in an IS*3*-type element with homology to the *Salmonella enteritidis* IS*1351* element and *Yersinia enterocolitica* IS*1400* element from a high-pathogenicity island. Further characterization of SVM167 showed that culture supernatants from this mutant lack a previously uncharacterized protein that is also missing from culture supernatants of a SPI1 mutant, suggesting it can be secreted by the SPI1 type III secretion system. In addition, transcription of this operon, *sigDE* (*Salmonella* invasion gene), is dependent on the presence of *sirA*, an activator of *hilA* expression. HilA activates transcription of several of the SPI1 genes but does not appear to have a major role in activation of transcription from the *sigDE* promoter.

In order for salmonellae to cause disease (enteric fever or gastroenteritis) via an oral route of infection, the bacteria must penetrate the epithelial cells of the intestine (10, 33, 54, 69). Several studies have shown that salmonellae preferentially attach and enter phagocytic M cells of the Peyer's patches (33, 37, 56). However, studies have also shown that bacteria can be found in nonphagocytic enterocytes and can also cause a systemic infection when the small intestine is bypassed (10). Much research has focused on the process of invading host cells, as this ability appears to be an important aspect of pathogenesis (for reviews, see references 17, 18, and 21). Various tissue culture cell lines, including HEp-2, Henle-407, and Chinese hamster ovary (CHO), have been used as model systems to investigate this process. Upon contact with intestinal or cultured epithelial cells, salmonellae induce a dramatic ruffling of the cell surface caused by cytoskeletal rearrangements, facilitating the engulfment of bacteria (19, 20). Mutants that are not able to actively invade host cells are noticeably attenuated in intragastrically, but not intraperitoneally, infected mice (22). Invasion requires the expression of many genes, including those encoding transcriptional regulators (2, 4, 5, 38, 44, 49, 55), components of a supramolecular secretory apparatus (13, 14, 22, 23, 26, 38), and potential effector proteins secreted by this apparatus (12, 13, 32, 34, 39, 40, 73). The majority of the known invasion genes map between centisomes 62 and 64 of the *Salmonella* chromosome, a region known as *Salmonella* pathogenicity island 1 (SPI1). These genes include the *inv*, *hil*, *prg*, *org*, *spa*, and *sip*/*ssp* genes (for reviews, see references 21 and 43).

Various techniques, including complementation of a naturally occurring attenuated isolate with a genomic cosmid library (22), mutagenesis with a transposon containing a strong outward facing promoter to find hyperinvasive mutants (44), and identification of anaerobically induced genes (36), have been used to identify invasion genes. Recently, signature-tagged transposon mutagenesis was used to isolate attenuated *Salmonella typhimurium* from intraperitoneally infected mice (30). Several loci were found to affect survival in the mouse, but most notable was the characterization of *Salmonella* pathogenicity island 2 (SPI2) (31, 64). This region had also been identified in another study examining sequences of the *Salmonella* chromosome which are not found in *Escherichia coli* and appear to be necessary for macrophage survival (53). Although it appears that the main function of the SPI2-encoded proteins is survival in the host, certain mutations in this region also have a measurable effect on invasion *in vitro* (31).

Despite the numerous creative techniques used to find invasion genes, none of these strategies has saturated the *Salmonella* chromosome; each study has failed to reidentify all known invasion genes. In addition, although SPI1 mutants are significantly defective for invasion of the intestinal epithelium, they are not completely attenuated in mice, suggesting that bacteria can still reach the deeper tissues in the mouse model after oral inoculation (22). These observations strongly suggest the existence of additional, unidentified invasion genes.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. Table 1 provides descriptions of the bacterial strains, bacteriophage, and plasmids used in this study. Chemical transformation and electroporation of plasmids into bacteria were carried out as previously described (61). Plasmids that were manipulated in *E. coli* were passaged through a restriction-minus (*res*) *S. typhimurium* LT2 strain (LB5000) prior to transformation or electroporation into other *S. typhimurium* strains.

Growth conditions. *Salmonella* and *E. coli* strains were grown in Luria-Bertani (LB) Miller broth (Difco) at 37°C with aeration on a roller drum or without aeration in standing cultures, depending on the assay. Minimal medium was made with M63 salts, glucose, magnesium sulfate, and vitamin B_1 (48). Evans-Blue uranine (EBU) agar and Bochner-Maloy media were made as previously described (45). Antibiotics were used at the following final concentrations: am-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid, Campus Box 8230, St. Louis, MO 63110. Phone: (314) 747-2132. Fax: (314) 747-2135. E-mail: virginia@borcim.wustl.edu.

TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this work

Strain, plasmid, or phage	Genotype	Source or reference[a]
S. typhimurium		
14028s	Wild type	ATCC
SVM167	*sigE*::Tn*10*dTc	This work
SVM168	IS*1351*::Tn*10*dTc	This work
SVM173	*invA*::Ω*cat*	This work; J. Galan
SVM169	Same as SVM173 with a linked Tn*10*dTc insertion	This work
SVM174	*sigE*::Tn*10*dTc *invA*::Ω*cat*	This work
SVM175	IS*1351*::Tn*10*dTc *invA*::Ω*cat*	This work
EG9527	*mgtCB9232*::MudJ (Kn^r)	E. Groisman
VV302	SL1344 Δ*hilA*	4
SVM214	Δ*hilA*	4; this work
CJ010	*sirA*::Tn*10*dTc *phoN*::Tn*10*dCm	35
CS015	*phoP*::Tn*10*dCm	49
SVM226	*ssaJ*::mTn*5*	31
SVM227	*ssaT*::mTn*5*	31
SVM255	4-bp deletion in *sigD*	This work
SA536	LT2 HfrK6 (origin min 76)	K. E. Sanderson (SGSC)
SA975	LT2 HfrK13 (origin min 78)	K. E. Sanderson (SGSC)
TT10423	LT2 *proAB*47/F′ *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10*dTc	15
SB154	SL1344 *invA*::Ω*cat*	Jorge Galan
LB5000	LT2 *flaA66 metA22 trp-2 rpsL xyl-401 ilv-452 leu res mod*$^+$	B. Stocker
E. coli DH5α	F$^-$ p80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco, BRL
Plasmids		
pHG329	Ap^r; medium-copy-number cloning vector	66
pNK2881	Ap^r; P_{tac}-*tnpA ats-1 ats-2*	41
pRS415 and pRS528	*lacZYA* transcriptional reporter fusion vectors	65
pWSK29 and pWKS30	Ap^r; low-copy-number cloning vectors	70
pWSK129 and pWKS130	Kn^r; low-copy-number cloning vectors	70
pMAK705	Cm^r; temperature-sensitive origin of replication vector used for gene replacement	29
pCJ13	Ap^r; *sirA uvrC* in pWKS30	35
pGP1-2	Kn^r; λ p_L-T7 RNA polymerase	68
pVV214	Ap^r; *hilA* in pACYC177	4
pHH10	Ap^r; 4.1-kb *Eco*RI *sigDE* fragment cloned into pHG329	This work
pHH20	Ap^r; 3.2-kb *Bam*HI *sigDE* fragment cloned into pWKS30	This work
pHH21	Ap^r; 0.9-kb *Eco*RI/*Pst*I *sigDE* promoter and partial coding sequences in pRS415	This work
pHH22	Ap^r; same as pHH20 but in opposite orientation (*Bam*HI/*Eco*RI fragment)	This work
pHH25	Kn^r; 2.2-kb *sirA Pst*I fragment from pCJ13 in pWSK129	This work; 35
pHH26	Ap^r; same as pHH22 but with deleted *Pst*I site in *sigD*	This work
pHH31	Cm^r; 1.7-kb *Kpn*I fragment from pHH26 encompassing the *sigD* deletion cloned into pMAK705	This work
Bacteriophage P22HT *int*	High-frequency transducing phage	45

[a] ATCC, American Type Culture Collection; SGSC, *Salmonella* Genetics Stock Centre.

picillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 25 μg/ml; erythromycin, 150 μg/ml; and chloramphenicol, 25 μg/ml. For detection of β-galactosidase activity, solid medium was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 40 μg/ml; when necessary, isopropylthio-β-D-galactoside (IPTG) was used at 100 μM. P22HT *int* lysates were harvested and used for transductions as previously described (45).

Transposon mutagenesis. To create random Tn*10*dTc (41) insertions in the *S. typhimurium* chromosome, pNK2881 (ampicillin resistant [Ap^r]), containing a transposase gene (*tnpA*), was transformed into the wild-type parent strain 14028s. A P22 HT *int* lysate was prepared from TT10423 (15), a strain which carries a Tn*10*dTc on an F′ element. This lysate was used to transduce Tn*10*dTc into 14028s (pNK2881); transductions were performed as previously described (45) and plated on minimal (M63) salts agar supplemented with tetracycline. Transductants were pooled (ca. 1,000 per pool) and inoculated with P22 HT *int* broth (LB, E salts, bacteriophage P22HT *int*) (45) to make a lysate of these transductants. These lysates were then used to transduce the Tn*10*dTc insertions into SVM169 (*invA*::Ω*cat*). Transductants were plated on minimal medium as described above. Individual transductants were inoculated into 200 μl of LB-tetracycline-chloramphenicol-EGTA broth in each well of 96-well microtiter plates and incubated overnight without shaking at 37°C, and 10 μl of each was used for qualitative invasion assays. Before further characterization, putative mutants were plated on EBU-chloramphenicol-tetracycline agar plates to purify bacteria away from phage (45). Once free of phage, bacteria were cross-streaked against P22 HT *int* to determine if they were P22 sensitive (true transductants) or resistant (pseudolysogens). The motility phenotype was determined by stabbing bacteria into 0.25% LB-agar plates. The Tn*10*dTc insertions were separated from the *invA* mutation by transduction into the wild-type background and purified as described above.

pHH10, encoding *sigDE*, was mutagenized with Tn*Max2* as previously described (28). Plasmids with insertions in *sigD* were transformed into strain 14028s, and culture supernatant proteins were examined as described below (data not shown).

Construction of a *sigD* mutant. To construct the *sigD* mutant, SVM255, pMAK705 (chloramphenicol resistant [Cm^r]), with a temperature-sensitive origin of replication, was used as previously described (29). At 44°C, pMAK705 derivatives form cointegrates, but at 30°C, these cointegrates resolve from the chromosome as replicating plasmids. A 1.7-kb *Kpn*I fragment from pHH26 carrying a partial *sigD* fragment with a 4-bp deletion within the coding sequence was cloned into pMAK705, creating pHH31. This plasmid was electroporated into and purified from LB5000 before being electroporated into wild-type *S. typhimurium* 14028s. After several shifts in temperature between 30 and 44°C, 40 colonies were purified, cured of the plasmid, and screened in the invasion assay. Approximately 50% of the tested clones were reduced for invasion. Culture supernatants from three of the invasion mutants were analyzed and found to lack

the same protein absent from SVM167 (*sigE*::Tn*10*dTc). Southern analysis using a probe to *sigD* confirmed the exchange of the disrupted *sigD* allele with the wild-type allele on the chromosome; this was easily detected because the 4-bp deletion in *sigD* removed a *Pst*I site (data not shown).

Construction of isogenic *invA* and *hilA* mutants. SVM173 was constructed by transducing an *invA*::Ω*cat* mutation (courtesy of Jorge Galan) from strain SB154 into our parent strain, 14028s. The original *hilA* deletion mutant, VV302 (courtesy of Catherine Lee), was made in *S. typhimurium* SL1344 (4) and does not contain an associated selectable marker. To transfer the *hilA* deletion into 14028s, a P22HT *int* lysate was prepared from VV302 and used to transduce the deletion into SVM169, a 14028s derivative which contains the *invA*::Ω*cat* mutation linked to an unmapped Tn*10*dTc insertion. Transductants were plated on Bochner-Maloy medium, which selects against tetracycline resistance (Tc^r). SVM169 is Tc^r; thus, only transductants which have lost the Tn*10*dTc marker could grow. Because the wild-type *hilA*, *invA*::Ω*cat* and Tn*10*dTc were all linked, some Tc^s transductants were expected to have replaced the $hilA^+$ and *invA*::Ω*cat* with Δ*hilA* and $invA^+$ sequences. Forty-four Tc^s transductants were purified on EBU plates and confirmed to be P22 sensitive and phage free. All 44 were also Cm^s sensitive, indicating that the *invA*::Ω*cat* insertion was replaced with a wild-type *invA* copy. Four spontaneous Tc^s colonies (from a control plate which had bacteria only, no phage) had also been tested on chloramphenicol plates, and all were resistant as expected. Invasion assays in the HEp-2 cell line were done with the control strains (wild type and VV302) and 44 putative 14028sΔ*hilA* strains; all except the wild type were equally reduced for invasion (data not shown). Southern analysis (61) was performed on chromosomal DNA purified from four of the new Δ*hilA* strains, VV302, and 14028s. An *Nsi*I fragment containing *hilA* from pVV214 (4) was used as a probe to confirm the deletion in the 14028s Δ*hilA* derivatives (data not shown).

Plasmid constructions. All relevant plasmids used in this study are described in Table 1. The junctions between the transposon insertions and chromosomal sequences were cloned as follows. Chromosomal DNA preparations isolated from SVM167 and SVM168 were digested with *Eco*RI, separated on a 0.7% Tris-acetate-EDTA-agarose gel, and transferred to nitrocellulose filters (61). Because the *tetAR* genes are lethal to *E. coli* in multicopy (41), the cloning of random fragments and selection for Tc^r was not possible; *Eco*RI was chosen since it has a site within the Tc^r genes. The filters were probed with a [^{32}P]dATP-labeled *Bgl*II fragment from pNK81 encoding the *tetAR* locus (41) to determine the approximate sizes of the junction fragments that needed to be cloned. Size-fractionated fragments of chromosomal DNA isolated from the mutants were cloned into pHG329 (66), and transformants were screened by colony hybridization using the same *tetAR* probe. Once cloned, the sequences were used to probe subgenomic libraries for wild-type sequences. For SVM167, pHH10 was isolated and found to have a 4.1-kb *Eco*RI fragment carrying the wild-type *sigDE* locus.

For complementation studies, a 3.2-kb *Bam*HI fragment from pHH10 was subcloned into the low-copy-number cloning vector pWKS30 (70). For T7 expression, a 3.2-kb *Eco*RI/*Bam*HI *sigDE* fragment from pHH20 was cloned into pWSK29 (the sister plasmid to pWSK30 with the polylinker in the opposite orientation), creating pHH22. To create pHH26, a small deletion was made in *sigD* in pHH22 as follows. pHH22 was digested with *Pst*I to completion and then incubated with Deep Vent DNA polymerase (New England Biolabs), Deep Vent DNA polymerase buffer, and 200 μM deoxynucleoside triphosphates at 72°C for 20 min as described by the manufacturer. These fragments were recircularized with ligase and recovered. DNA sequence analysis showed that a 4-bp deletion was created as expected (data not shown). *sigDE* was expressed in the T7 expression system as previously described (3, 68). Sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gels were prepared as previously described (42), dried under vacuum, and exposed to X-ray film for 1 and 16 h (3).

To create the reporter plasmid pHH21, a 1-kb *Pst*I fragment from pHH10 was cloned into pHG329 to create pHH15. This fragment contained sequences starting about 408 bp upstream of the putative start codon of *sigD*. From pHH15, the *sigDE* promoter region was isolated as an 1.0-kb *Eco*RI fragment and was cloned into pRS528, a *lacZYA* transcriptional fusion reporter vector (65), to create pHH17. Although dark blue colonies on X-Gal were selected, sequence analysis revealed the *sigDE* promoter was in the wrong orientation relative to *lacZYA*, probably because of the presence of an activated or constitutively expressed divergent promoter in pHH17. This construct was used to directionally clone the *sigDE* promoter region into the sister transcriptional fusion vector, which has a polylinker in the opposite orientation of pRS528 (pRS415). The resulting plasmid with a *sigD*::*lacZYA* transcriptional fusion was named pHH21.

Tissue culture attachment and invasion assays. CHO and HEp-2 cells were maintained and passaged as recommended by the American Type Culture Collection. For tissue culture invasion (TCI) assays, 2×10^5 cells/ml were seeded in Falcon 24-well tissue culture plates to obtain about 90% confluent monolayers on the following day. For bacterial cultures, single colonies were inoculated into 2 ml of medium and grown for 18 h without shaking; 5 μl (10^7 to 10^8 CFU) was used per well of tissue culture cells. Trays were centrifuged for 10 min at 1,000 rpm in a tabletop swing-bucket centrifuge and incubated for 1 h at 37°C with 5% CO_2. For the mutants isolated in this study, centrifugation was ultimately found not to make a difference. Monolayers were then washed three times with phosphate-buffered saline (PBS) for tissue culture and then incubated for another 90 min in the presence of RPMI 1640 (Cellgro) with gentamicin at 100 μg/ml to kill extracellular bacteria. The monolayers were then washed with PBS as described previously (67), lysed with double-distilled water for 20 min, diluted, and plated on selective LB-agar plates to determine the number of intracellular bacteria. Quantitative TCI assay results are calculated as follows: percent invasion = 100 × (number of bacteria resistant to gentamicin/total number of bacteria added). Attachment assays were performed in the same manner but with five washes after the first incubation and omission of the gentamicin killing step.

Mapping and transduction analysis. The Tn*10*dTc insertion in SVM167 was mapped by using a set of MudP22 mapping strains (6, 45). SVM168 could not be mapped by the MudP22 mapping phage and was therefore mapped with a set of Hfr (high-frequency recombination) strains (*Salmonella* Genetics Stock Centre). Cultures of each Hfr strain were mixed with equal volumes of SVM168 containing *invA*::Ω*cat* (in order to select against the donor Hfr strain), diluted, and plated onto Bochner-Maloy medium (45). Two of ten Hfr strains representing regions of the chromosome not represented by the MudP22 mapping phage (SA536 and SA975, with origins of transfer at centisomes 76 and 78, respectively) were able to recombine out the Tc^r marker from SVM168.

Analysis of culture supernatants. Cultures were grown in 2 ml of LB broth with antibiotics for 18 h, without aeration, and harvested. Bacteria were pelleted at 8,000 × *g* for 15 min, and the supernatants were immediately transferred to clean tubes. The supernatants were filtered through 0.45-μm-pore-size Tuffryn (low-protein-binding) Gelman Acrodiscs, and proteins were precipitated with cold trichloroacetic acid (TCA) at a final concentration of 10%. The proteins were collected by centrifugation at 8,000 × *g* at 4°C and resuspended in 1 ml of cold acetone. These mixtures were centrifuged for 10 min at 8,000 rpm at 4°C, and pellets were resuspended in 30 μl of PBS. Sample buffer with β-mercaptoethanol (61) was added to samples, the samples were boiled for 5 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5%) (42). Proteins were visualized with silver stain (8). Western analysis using antibodies to SipA and SipB was performed with the Amersham Life Science ECL Western blotting detection system.

Enzyme assays. β-Galactosidase assays were performed and values were calculated as previously described (48).

Sequence analysis. The majority of the sequencing reactions were performed by Joan Strange (University of Montana, Missoula) and Bio S&T (Quebec, Quebec, Canada). For reactions done in the lab, Sequenase 2.0 (United States Biochemical) was used, and labeled fragments were separated in a 6% acrylamide gel (Sequagel-6; National Diagnostics). Sequence analyses (homologies, mapping, etc.) were performed with the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc.).

Nucleotide sequence accession number. The nucleotide sequence of *sigDE* has been submitted to GenBank and assigned the accession no. AF021817.

RESULTS

Identification of invasion loci. To find novel *S. typhimurium* invasion genes, two issues were addressed: (i) reidentification of known invasion genes and (ii) redundancy of *Salmonella* invasion systems (21). The ability to quickly map or clone mutations was necessary to eliminate regions that have previously been identified (i.e., SPI1). Tn*10*dTc was chosen because the Tc^r (*tetAR*) marker could be exploited by rapid MudP22 transduction mapping (6, 45). This would allow us to eliminate mutants with insertions in regions under intense study, i.e., SPI1. In addition, Tn*10*dTc does not encode a transposase and therefore would be fairly stable upon insertion into the chromosome.

The possible existence of multiple invasion systems was another issue addressed. Since SPI1 mutants were still invasive in CHO cells, albeit not at wild-type levels (data not shown), and able to cause systemic disease in vivo, it was likely that at least one other invasion system might be present in *Salmonella*; single-mutation phenotypes in one invasion system might be masked by the presence of a second invasion system. Thus, we screened for novel invasion genes in an $SPI1^-$ mutant background. Wild-type *S. typhimurium* 14028s was mutagenized with Tn*10*dTc (41); Tn*10*dTc was transduced by P22 *int* into 14028s containing pNK2881, a plasmid which provides transposase for Tn*10* in *trans* (41). Transductants were plated on minimal medium to eliminate auxotrophic mutants, thus reducing the number of mutants to be screened in the TCI assay. Twenty-five transductions were done, resulting in several hundred to more than 1,000 transductants per plate; transductants on each plate were pooled. Aliquots from these pools were

TABLE 2. Characterization of novel invasion mutants

Strain	% Invasion[a] (% of wild-type level)		Map position	Homology to region of insertion[c]
	CHO	HEp-2		
14028s (wild type)	2.2 ± 0.2 (100)	5.3 ± 0.3 (100)		
SVM167	0.2 ± 0.2 (9)	0.4 ± 0.02 (7.5)	~25.5[b]	*Shigella ipgDE(F)*
SVM168	0.5 ± 0.6 (22)	1.8 ± 0.07 (34)	~78[d]	*S. enteritidis* IS*1351* ORFB; *Y. enterocolitica* high-pathogenicity island ORFB

[a] Percentage of intracellular bacteria ± range after bacteria were allowed to invade during a 1-h incubation; duplicate assays performed. Values are representative of several assays done in this study.
[b] Map (centisome) position of the Tn*10*dTc insertions was determined by MudP22 transduction mapping (SVM167). The MudP22 element in strain TT15240 (*putA1019*::MudP) cotransduced with the Tn*10*dTc insertion in SVM167.
[c] Homologies are at the amino acid sequence level.
[d] The insertion in SVM168 was mapped by using Hfr strains with origins of transfer at 76 and 78 min of the LT2 chromosome.

used to prepare P22 *int* lysates, which were then used to transduce the Tn*10*dTc insertions into SVM169 (*invA*::Ω*cat*). Transductants were plated on minimal medium with tetracycline and chloramphenicol to select for Tn*10*dTc insertions and the *invA*::Ω*cat* mutation.

Approximately 2,500 Tc^r transductants from the 25 independent transduction pools plated on minimal medium were screened in the TCI assay for reduced invasion into CHO tissue culture cells. The Tn*10*dTc insertions from putative mutants were separated from the *invA*::Ω*cat* mutation by transducing the Tc^r marker into 14028s (wild type). Tc^r transductants were purified and assayed in both CHO and HEp-2 (human laryngeal epithelium) tissue culture cells. Mutants which appeared reduced for invasion were also tested for motility in 0.25% LB agar; several motility mutants were found and omitted from further study. Two motile, prototrophic mutants, SVM167 and SVM168 (both *invA*$^+$), were 5- to 10-fold less invasive than the wild type in both HEp-2 and CHO cell lines (Table 2). Both mutants were wild type for attachment to the tissue culture cells (data not shown). Chromosomal DNA was isolated from these mutants and analyzed by Southern hybridization using *tetAR* genes as a probe (data not shown). Based on these data, DNA fragments containing the junctions between the transposon and the chromosome were isolated. The junction sequences were cloned from each mutant and used to screen subgenomic libraries for the wild-type loci. Clones containing wild-type DNA which hybridized to junction DNA were sequenced.

SVM168 appears to have an insertion at about centisome 78 in an IS*3* element homologous to *Salmonella enteritidis* IS*1351* (A. P. Burnens, direct submission to GenBank) and *Yersinia enterocolitica* high-pathogenicity island (HPI) IS*1400* (9) elements. These insertion sequence elements contain open reading frame A (ORFA) and ORFB sequences, and SVM168 has an insertion in the ORFB homolog. The possibility that SVM168 maps to a putative third pathogenicity island, SPI3 (7), was tested. A marker in *mgtCB* of SPI3, which is located at centisome 82.5, could not be cotransduced with the mutation in SVM168, suggesting that the mutation in SVM168 was outside this region.

SVM167 was determined to have an insertion in a homolog of the *Shigella flexneri* and *Shigella sonnei* virulence plasmid encoded gene *ipgE* of the *ipgDEF* operon (1). In *Shigella*, *ipgDEF* was characterized due to its location between the *ipa* and *mxi* virulence plasmid loci (46, 62); however, this operon has no observable virulence function for *Shigella* either in in vitro invasion assays or in Serény tests in guinea pigs (1). The *Salmonella* operon contains homologs to *ipgD* and *ipgE* but not *ipgF*. Interestingly, a homolog of *ipgF*, *iagB*, has been identified downstream of *hilA* in SPI1 and in an unmapped location in *Salmonella typhi* (51). As in *Shigella*, *iagB* has no known virulence phenotype (4). Unlike the *Shigella* genes, the *Salmonella* homologs, *sigD* and *-E*, are not located between what are believed to be homologs of the *ipa* and *mxi* virulence genes at SPI1 (27); instead, *sigDE* maps between centisomes 24.5 and 25.5. The predicted amino acid sequence of *sigD* (62 kDa) is 59% similar and 41% identical to that of IpgD (60 kDa), and SigE (13 kDa) is 57% similar and 29% identical to IpgE (14 kDa) (Fig. 1). Recently, a *sigD* homolog (*sopB*) in *Salmonella dublin* was identified; however, a *sigE* homolog was not found (24). SopB did not appear to have a role in invasion in either in vitro or in vivo invasion assays in the *S. dublin* study.

Complementation of the SVM167 invasion defect by *sigDE*. The wild-type copy of *sigDE* on a 4.1-kb *Eco*RI fragment (defined by Southern analysis described above) was cloned into pHG329, a pBR322-based plasmid of medium copy number (66), creating pHH10 (Fig. 2). This plasmid was transformed into SVM167 but did not reproducibly restore invasion into tissue culture cells. It was possible that the invasion phenotype of SVM167 was affected by having the genes in a multicopy plasmid. A slightly smaller fragment containing *sigDE* was subcloned into pWKS30, a low-copy-number cloning vector (about six copies per cell) (70). The resulting plasmid, pHH20 (Fig. 2), was transformed into both the wild-type and *sigE* strains. Invasion by SVM167 harboring pHH20 increased fourfold and was similar to that of the wild type; however, 100% complementation was never observed (Table 3). This is often the case in complementation studies of invasion mutants (13, 14, 23, 26, 35). It is also possible that the insertion in *sigE* affects downstream genes. However, the nearest ORF is more than 500 bp downstream of *sigE*, and there is a putative transcriptional terminator between *sigE* and the downstream ORF.

T7 expression of *sigDE*. To identify the protein products of *sigDE*, *sigDE* was introduced into a T7 expression system (68) to specifically express *sigD* and *sigE*. The clone used to complement invasion, pHH20, contained a T7 promoter but was in the wrong orientation relative to *sigDE*. Therefore, *sigDE* from pHH20 was directionally cloned downstream of the T7 promoter in pWSK29, the sister vector to pWKS30 (70) used to construct pHH20. The resulting plasmid, pHH22, was transformed into an *E. coli* strain harboring pGP1-2, which encodes T7 polymerase under the control of a temperature-sensitive λ repressor protein (68). Induction and expression of the plasmid-encoded proteins were done in the presence of rifampin and a mixture of [^{35}S]methionine and [^{35}S]cysteine (3, 68). One of the proteins expressed, most likely SigD, migrated at about 77 kDa, as observed in the culture supernatant gels (see below), strongly suggesting that it was the same protein (Fig. 3). SigD is predicted to have a molecular size of about 62 kDa, not the observed 77 kDa. This discrepancy in protein migration

FIG. 1. Amino acid alignment of *S. typhimurium* SigD and SigE with *S. flexneri* IpgD and IpgE, respectively. SigD (62 kDa) is 59% similar and 41% identical to IpgD (60 kDa), and SigE (13 kDa) is 57% similar and 29% identical to IpgE (14 kDa). The Wisconsin sequence analysis package (Genetics Computer Group) was used to identify homologies to *sigDE*.

was apparently due to a departure from the typical bisacrylamide concentration used in our protein gels. When a standard SDS-polyacrylamide gel (30:0.8, acrylamide to bisacrylamide) (42) was used, SigD migrated more closely to the predicted size of 62 kDa (data not shown); however, in these gels, SigD was not well separated from other proteins. Fortuitously, the acrylamide gels used in this work had a 30:1.6 ratio of acrylamide to bisacrylamide, which optimally resolved SigD from other proteins.

To further demonstrate that the 77-kDa protein was SigD, a small deletion was made in *sigD*, resulting in a truncated protein, SigD′. pHH22 was digested with *Pst*I (unique in *sigD* and pHH22), treated with Deep Vent DNA polymerase (New England Biolabs) to remove the 3′ overhangs, and ligated. This plasmid, pHH26, contains a 4-bp deletion in *sigD*, resulting in a frameshift mutation. SigD′ was expressed from pHH26 (Fig. 3); however, SigD′ migrated at about 29 kDa rather than the predicted 22 kDa, consistent with the observation that the full-length SigD (62 kDa, predicted size) appears to be larger in the higher-percentage bisacrylamide gels.

SigE was also synthesized in the T7 expression system and appeared to be about 13 kDa, as predicted by sequence analysis (Fig. 3). The SigE band was more intense than SigD in these gels; SigD is predicted to have 16 cysteines and methionines, while SigE is predicted to have only 5. Nucleotide sequence analysis revealed that *sigE* has a consensus Shine-Dalgarno ribosome-binding site whereas *sigD* has no conspicuous ribosome-binding site, suggesting that SigE may be translated more efficiently than SigD.

Analysis of culture supernatants. IpgD of *Shigella* (1) and several proteins required for *Salmonella* invasion appear to be secreted (12, 13, 32, 34, 39, 40, 73), suggesting that SigD may also be secreted. Culture supernatant profiles of the *sigE* mutant and several control strains were examined. Supernatants from nonaerated (inducing conditions) (16) overnight cultures were harvested and filtered; proteins in the filtrates were TCA precipitated from wild-type, *invA* (SPI1$^-$), *sigE*, and *sigDE*$^+$ plasmid-carrying *sigE* strains. Proteins were separated by SDS-PAGE and visualized by silver staining (Fig. 4). The *sigE* mutant was missing one band at about 77 kDa. Interestingly, the *invA* mutant was also missing the 77-kDa protein in addition to the previously identified Sip/Ssp proteins, suggesting that under these conditions this 77-kDa protein is translocated by the SPI1 type III secretion system. In addition, absence of the 77-kDa protein did not significantly affect the secretion of the Sip/Ssp proteins. Presence of this protein in the supernatant was restored in the *sigE* mutant harboring pHH10 (*sigDE*$^+$) (Fig. 4, lane 5). Consistent with these results, transposon insertions in the *sigD* coding region of pHH10 eliminated this protein from the supernatant (data not shown).

Further support for the hypothesis that the 77-kDa protein is SigD came from the analysis of a *sigD* mutant. The *sigD* allele encoding the truncated SigD from pHH26 was exchanged with the wild-type allele on the chromosome. SVM255 was equally defective for invasion into both CHO and HEp-2 cells as the *sigE* mutant SVM167 (data not shown). T7 expression data (Fig. 3) suggests that SigE may be expressed in wild-type amounts in the *sigD* mutant. The *sigD* disruption

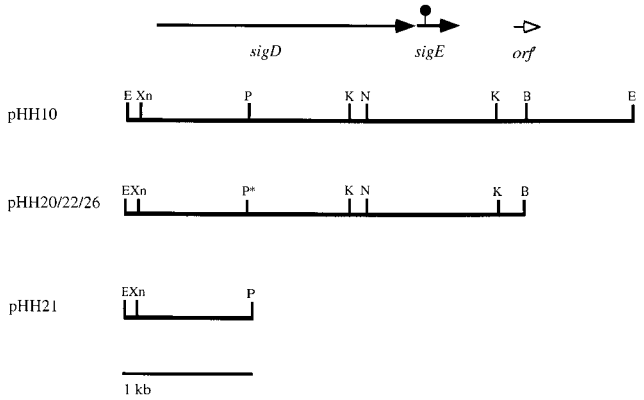

FIG. 2. Restriction map of *sigDE*$^+$ plasmids and the transcriptional reporter plasmid pHH21. pHH10 is a pHG329-based, medium-copy-number plasmid. pHH20 (pWKS30-based, low-copy-number plasmid) contains a 3.2-kb *Bam*HI fragment from pHH10. The *sigDE* fragment from pHH20 was cloned into the pWKS30 sister cloning vector, pWSK29, to obtain a clone with the insert in the opposite orientation with respect to the T7 promoter. The coding regions of *sigD* and *sigE* are indicated by the closed arrows. The original Tn*10*dTc insertion in *sigE* is represented by the black circle. An uncharacterized partial ORF (*orf'*) is indicated by the open arrowhead. pHH26 is pHH22 with a deleted *Pst*I (p*) site (see Materials and Methods). pHH21 contains an *Eco*RI/*Pst*I fragment containing about 0.9 kb of sequence from *sigDE* fused to *lacZYA*. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Xn, *Xmn*I; K, *Kpn*I; N, *Nde*I.

strain lacked the same protein from culture supernatants as SVM167 (Fig. 5). As with SVM167, transformation of SVM255 with a plasmid encoding *sigDE* restored the 77-kDa protein to culture supernatants (Fig. 5). Together, these data suggest that the 77-kDa protein missing from the supernatant of SVM167 is SigD.

The possibility that SigD is secreted by the SPI2-encoded type III secretion system was also tested (31, 53, 64). A mutation in one of the proposed SPI2 secretion apparatus genes, *ssaT*, has been shown to have reduced levels of SipC in its supernatant (31). SipC is a SPI1-secreted protein and is thought to be at least one of the effectors for invasion (12, 40). Culture supernatant proteins from the *ssaT* mutant were harvested and visualized as described above. Wild-type amounts of SigD were still present in the *ssaT* mutant (data not shown). Moreover, wild-type amounts of SipC were also present. It is notable that Hensel and coworkers also reported that in a small proportion of their experiments, SipC was still found (31). Thus, under these conditions the SPI2 type III secretion system was not required for SigD secretion.

Complementation of SVM167 with *sigE*$^+$. In the T7 expression system, SigE was produced by *E. coli* harboring pHH26 (*sigD'sigE*$^+$) in amounts similar to that produced by the pHH22 strain (*sigDE*$^+$). If SVM167 could still produce but not secrete SigD, it was possible that pHH26 could restore secretion of SigD and complement the invasion defect. To test this, pHH26 was transformed into SVM167 and 14028s, and the resulting strains were assayed for invasion in both CHO and HEp-2 cell lines. pHH26 was able to complement the *sigE* mutation in SVM167 to the same degree as pHH20 (Table 3).

To determine if pHH26 could restore the secretion of wild-type SigD (chromosomal copy of *sigD* is wild type) or secrete a truncated SigD′, proteins from culture supernatants were precipitated and examined on an SDS-polyacrylamide gel as previously described. Secretion of SigD from SVM167 carrying pHH26 was restored (Fig. 5), suggesting that SigD was still being produced but retained by the mutant. Furthermore, pHH26 did not restore secretion of SigD in the *sigD* mutant

TABLE 3. Complementation of SVM167

Strain	CHO		HEp-2	
	% Invasion[a]	% of wild-type level	% Invasion	% of wild-type level
14028s (wild type)	4.8 ± 0.3	100	5.3 ± 0.3	100
14028s (pWKS30)	2.3 ± 0.0	100	5.7 ± 0.8	100
14028s (pHH20)	2.2 ± 0.05	100	3.3 ± 0.2	100
14028s (pHH26)	4.5 ± 0.15	100	7.8 ± 0.1	100
SVM167 (*sigE*::Tn*10*dTc)	0.3 ± 0.07	6.3	0.53 ± 0.06	10
SVM167 (pWKS30)	0.2 ± 0.02	8.7	0.4 ± 0.07	7
SVM167 (pHH20)	0.8 ± 0.05	36	1.6 ± 0.3	49
SVM167 (pHH26)	1.5 ± 0.2	33	2.3 ± 0.02	35

[a] Percentage of intracellular bacteria ± range after allowing bacteria to invade during a 1-h incubation; duplicate assays were performed. Values are representative of several assays done in this study.

(Fig. 5). It is notable that in several independent assays, there appeared to be less SigD in supernatant preparations from SVM167 and SVM255 that harbored pHH26 than in samples from a strain which contained pHH10 (medium-copy-number clone of *sigDE*$^+$) or pHH20 (low-copy-number clone of *sigDE*$^+$). A novel, truncated form of SigD was never observed in these gels.

***sirA*-dependent regulation of *sigDE*.** It is clear that *sigDE* plays a role in invasion and that SigD may be secreted through the SPI1 secretion system. Many of the known invasion genes at SPI1 appear to be coordinately regulated. Therefore, the possibility that expression of *sigDE* was also coordinated with that of other invasion genes was considered. The current model of SPI1 invasion gene regulation suggests that HilA activates the expression of several invasion genes, including that of another regulator, InvF (5, 38). Transcription of *hilA* is activated by SirA (35) but repressed by PhoP, a response regulator of virulence and housekeeping genes (5, 25, 49). To determine if any of the known invasion gene regulators af-

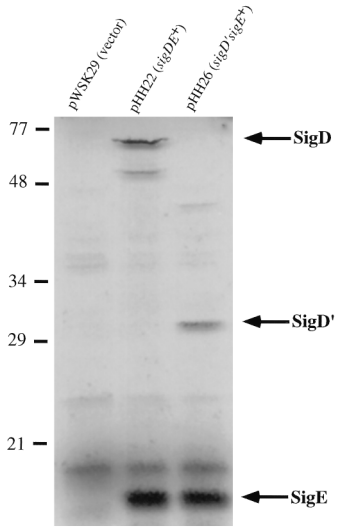

FIG. 3. Expression of *sigDE* from a T7 promoter in *E. coli*. Expression of genes from pWSK29 (vector) (lane 1), pHH22 (*sigDE*$^+$) (lane 2), and pHH26 (*Pst*I site deleted from *sigD*) (lane 3) was done in DH5α harboring pGP1-2. pGP1-2 contains a T7 RNA polymerase gene under the control of the λ p_L promoter and a gene which encodes a temperature-sensitive λ repressor protein (cI857). Proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine after the addition of rifamipin. Sizes are indicated in kilodaltons.

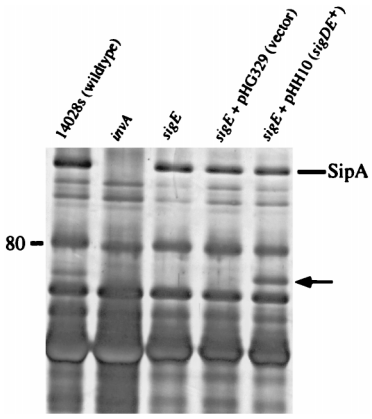

FIG. 4. Supernatant proteins from wild-type (lane 1), SVM169 (*invA* SPI1$^-$) (lane 2), SVM167 (*sigE*) (lane 3), SVM167 (pHG329) (lane 4), and SVM167 (pHH10) (lane 5) *S. typhimurium* strains. Cultures were grown without aeration overnight (18 h). Culture supernatants were TCA (10%, final concentration) precipitated and separated by SDS-PAGE (7.5% gel). Proteins were fixed and visualized silver stain. Position of the size marker is shown in kilodaltons on the left. The location of SipA was determined by Western analysis using polyclonal antibodies to SipA (data not shown). The putative location of SigD is indicated by the arrow.

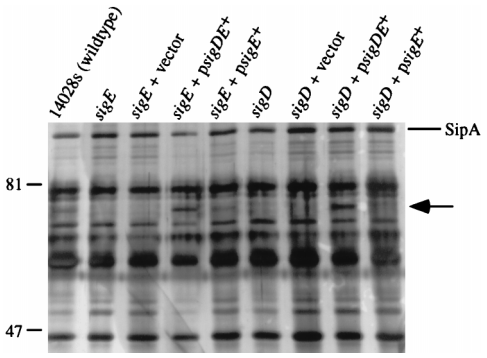

FIG. 5. Complementation of SigD secretion by SVM167 (*sigE*) and SVM255 (*sigD*). Cultures were grown and proteins were precipitated as described for Fig. 4. Shown are results for supernatant proteins from wild-type (lane 1), SVM167 (*sigE*) (lane 2), SVM167 (pWKS30) (vector) (lane 3), SVM167 (pHH20) (*sigDE*$^+$) (lane 4), SVM167 (pHH26) (*sigE*$^+$) (lane 5), SVM255 (*sigD*) (lane 6), SVM255 (pWKS30) (lane 7), SVM255 (pHH20) (lane 8), and SVM255 (pHH26) (lane 9). Positions of size standards are indicated in kilodaltons on the left. The position of SipA is indicated on the right. The putative location of SigD is indicated by the arrow.

fected expression of *sigDE*, a plasmid with a *lacZYA* fusion to *sigD*, pHH21, was made. β-Galactosidase activity was measured in isogenic *hilA* (4), *sirA* (35), and *phoP* (50) mutants containing pHH21. β-Galactosidase activity was also measured in SVM167 containing the reporter plasmid (data not shown). Overnight cultures were subcultured 1:100, and samples were assayed for β-galactosidase activity over a time course from 2 to 24 h. Expression from the *sigDE* promoter region was clearly activated in late log to early stationary phase in both anaerobic (inducing condition) (Fig. 6A) and aerobic (data not shown) cultures of all of the strains except the *sirA* mutant, suggesting that *sigDE* expression is dependent on SirA. When the *sirA* strain was transformed with a low-copy-number clone containing only *sirA* (35), activation of *lacZYA* expression was restored above wild-type levels. Curiously, in nonaerated cultures, the *hilA*, *sirA*, and *phoP* mutants reached nearly their maximum optical densities at 600 nm at about 8 h, unlike wild-type *S. typhimurium* or *E. coli* (Fig. 6B). Nevertheless, induction of β-galactosidase was consistently observed at about the same cell culture density or growth phase and at the same amounts in the wild type and the *hilA* and *phoP* mutants. However, due to the multicopy nature of the reporter plasmid used, it is conceivable that HilA and PhoP have subtle effects on regulation that were not detected under the conditions tested.

The possibility that the *Salmonella* SirA can activate transcription of the *sigD-lacZYA* construct in *E. coli* was tested. SirA, encoded on a low-copy-number plasmid, did not activate transcription of *sigDE* in *E. coli*. This result was not unexpected because *E. coli* has a SirA homolog, UvrY (96% identity to SirA), that was also unable to activate transcription (data not shown). This finding suggests that other factors required for regulation of *sigDE* might be absent in *E. coli*. However, it is not known whether the *E. coli* strain used for this experiment (DH5α) actually expresses UvrY (35, 52, 63).

DISCUSSION

Only 2 insertion mutants of 2,500 were found to retain motility and be reduced for invasion in this study. Of course, because the SPI1 invasion system had been inactivated, finding mutations in this region of the chromosome was not anticipated. In this screen, a novel invasion locus homologous to the *Shigella ipgDEF* operon was identified by transposon mutagenesis and direct screening for invasion mutants in the TCI assay. A Tn*10*dTc insertion was found in the *S. typhimurium* homolog of *ipgE* which was not well characterized in the *Shigella* study (1). Complementation for invasion of the *Salmonella* mutant was accomplished by both *sigDE*$^+$ and *sigE*$^+$ in *trans*. A mutation in *sigD* was also introduced onto the *S. typhimurium* chromosome and found to have the same phenotype as the *sigE* mutant. Failure to complement a *sigD* mutant with *sigE* alone suggests that both SigD and SigE are required. In *Shigella*, IpgD appeared to be secreted into culture supernatants, and an IpgF-PhoA fusion protein was found in the periplasmic space (1). Mutations in either of these genes did not affect invasion or virulence in the guinea pig model of infection; it was proposed that *ipgDEF* function might be observed only within the natural primate host (1). In *S. typhimurium*, SigD, like the IpgD homolog, appeared to be in culture supernatants. Unlike in *Shigella*, an *ipgF* homolog was not found in this region, although a homolog, *iagB*, has been found in SPI1 of *S. typhimurium* (4) and in an unmapped region of *S. typhi* (51). Recently, a homolog of SigD, SopB (*Salmonella* outer protein B; 98% identity) was identified in *S. dublin* by reverse genetics (24). In *S. dublin*, SopB appears to be translocated into eucaryotic cells and to promote an inflammatory response in infected calf ileal loops. Interestingly, SopB did not appear to be necessary for the invasion of *S. dublin* into HeLa cells or calf intestinal cells. It will be necessary to assess the role of *sigDE* in *S. typhimurium* pathogenesis in the mouse model of infection and to determine whether the primary function of SigD and SigE is for invasion or the stimulation of the immune response.

SigD appears to be secreted through the SPI1-encoded type III secretion apparatus and requires SigE for this process to occur. Several other proteins secreted by the SPI1-encoded apparatus that are required for *Salmonella* invasion into cultured epithelial cells have been identified. It has been proposed that these proteins act by stimulating endocytosis of the bacteria by the host cell (bacterium-mediated endocytosis or BME) (21, 57). How these proteins stimulate the host cells has

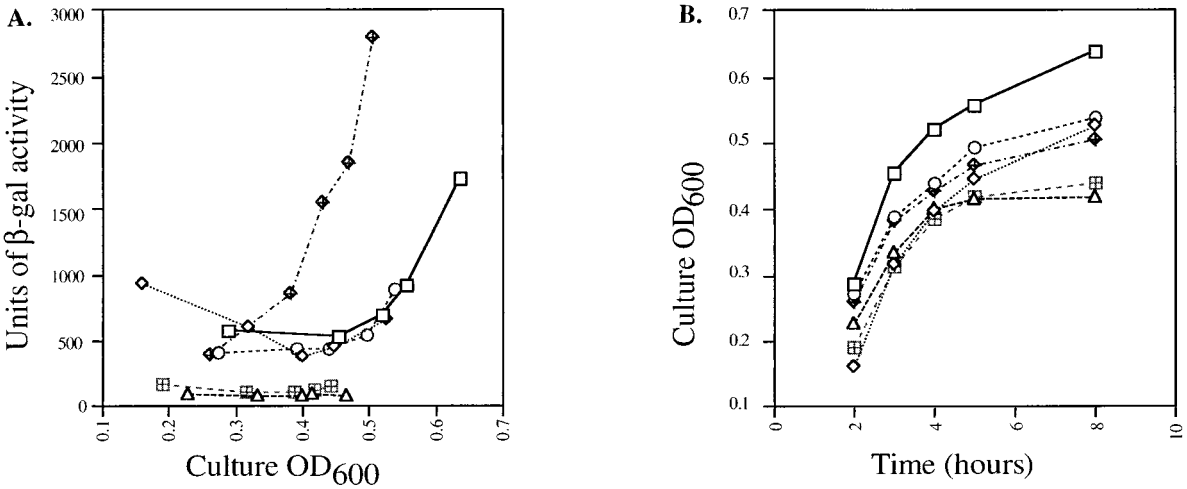

FIG. 6. Effect of regulatory mutations on *sigD-lacZYA* expression. (A) *lacZYA* expression from *sigD* in plasmid pHH21 plotted versus culture density. Expression from this plasmid was measured in strains 14028s (wild type) (squares), SVM214 (*hilA*) (diamonds), CS015 (*phoP*) (circles), CJ010 (*sirA*) (triangles), CJ010 (pWSK129) (vector) (hatched squares), and CJ010 (pHH25) (*sirA*$^+$) (hatched diamonds). β-gal, β-galactosidase. (B) Growth curves of cultures in panel A. Overnight cultures were subcultured 1:100 and grown without aeration; 1-ml samples were taken to determine cell optical density at 600 nm (OD_{600}) at time points between 2 and 24 h. The same samples were then used to measure β-galactosidase activity in these strains. The 24-h time point was omitted from these graphs since the mutant culture densities were virtually saturated by 8 h (β-galactosidase activity was also maximal for all strains at this time).

yet to be determined, but it seems that many proteins may be involved in this process.

Although SigE has not been detected in our analysis of secreted proteins, it is possible that it is secreted, perhaps in conjunction with SigD, as IpaB and IpaC are cosecreted from *Shigella* (47). Another possibility for the role of SigE is that it may target SigD to the SPI1 type III secretion apparatus. In a recent study of *Y. enterocolitica*, YopE was shown to be secreted through a single type III secretion apparatus via one of two independent signals; one involves the recognition of the first 15 amino acids of YopE by the secretion system, and the second is the YopE-SycE complex, which is proposed to be recognized by or targeted to the type III secretion machinery (11).

It is unlikely that SigE is a component of the SPI1 secretion apparatus since the absence of SigE does not affect secretion of the Sip/Ssp proteins. It is possible, however, that SigE acts as a chaperone for stability or secretion of SigD, as precedence for this has been found in other type III secretion systems in pathogenic bacteria. In one scenario, SigE may ensure the stability of the SigD polypeptide prior to secretion; a *sigE* mutation would in this case result in the rapid degradation of SigD in the cytoplasm. In *Yersinia*, SycE (YerA) appears to prevent the degradation of YopE, an antiphagocytic cytotoxin, prior to secretion (59, 60, 71). Similarly, the IpaB and IpaC invasion proteins in *Shigella* are rapidly degraded in the absence of the IpgC chaperone (47). In *Shigella* and other organisms, it has been proposed that inappropriate association of proteins such as IpaB with IpaC within the cytoplasmic compartment might result in unstable structures; these would be subject to proteolysis. IpgC, therefore, was proposed to prevent association of IpaB with IpaC prior to secretion, thereby protecting them from degradation (47). In addition, IpaB and IpaC individually could possibly misfold in the cytoplasm in the absence of IpgC to produce aberrant structures subject to proteolysis.

SigE has some similarities to these and other putative chaperones in that it is small (13 kDa), has a predicted acidic isoelectric point (pI = 3.99), and may have an amphipathic alpha helix at its C terminus (72). In addition, T7 expression of *sigDE* suggested that SigE is translated in higher amounts than SigD. It has been proposed that in *Yersinia*, SycE exists in the bacterial cytoplasm as a homodimer and binds to YopE in this form (71). Perhaps SigE also exists as a multimer and must therefore be translated in higher amounts in order to accommodate SigD molecules.

Several studies have reported that mutations in the SPI1 secretion apparatus result in the absence of up to 25 proteins in culture supernatants (34, 39, 40). Only a handful of these proteins has been characterized and found to be encoded in SPI1. An unique aspect of the *sigDE* locus is that although the secretion of SigD relies on proteins encoded in SPI1 at centisome 63, it is encoded near centisome 25. It is certainly possible, if not probable, that there are other invasion or virulence factors whose genes, like *sigDE*, map to locations outside of SPI1 and are secreted.

Another feature of *sigDE* is that its regulation is dependent on SirA, a regulator of SPI1 genes. SirA has at least two homologs, UvrY and GacA from *E. coli* and *Pseudomonas* sp., respectively (35, 52, 58, 63). Both *uvrY* and *gacA* are encoded immediately upstream of *uvrC* or its homolog and appear to be transcribed with *uvrC* as a single message in their respective organisms. UvrC is believed to have an excision repair function, and mutants have an increased sensitivity to UV light damage (52, 63). The function of UvrY in *E. coli* has yet to be determined. In *Pseudomonas*, GacA appears to be a response regulator for the LemA sensor kinase (58). Mutations in either *gacA* or *lemA* result in a decreased pathology in a plant model of infection, suggesting decreased expression of virulence factors in these strains. In *Salmonella*, a sensor kinase partner for SirA has not yet been identified. However, previous work has identified at least two different loci which complement a *sirA* mutation (35). Although SirA is believed to be a DNA binding protein based on sequence analysis, it is possible that SirA itself does not bind the *sigDE* promoter. In *Salmonella*, it appears that several regulators activate or repress expression of the SPI1-encoded genes, among them genes encoding additional regulators. It is possible that SirA may activate tran-

scription of another regulator which in turn regulates *sigDE* expression. This may indeed be the case since the *E. coli* SirA homolog (UvrY) could not activate transcription of *sigDE* in *E. coli*. This observation suggests that there is a factor in *Salmonella* but absent in *E. coli* that is necessary for *sigDE* expression; however, it is not known whether UvrY is expressed in the *E. coli* strain used in this study.

Why is the phenotype of a mutation in a gene whose product is secreted by the SPI1 secretion system more dramatic when in an SPI1$^-$ background? The original mutation was identified by screening a pool of Tn*10*dTc insertions in an SPI1$^-$ background. The reduced invasion phenotype of the double mutant (*invA sigE*) compared to *invA* alone in both CHO and HEp-2 cell lines was consistently reproducible early in this study. It is conceivable that there exist other invasion systems that require the expression of *sigD* and/or *sigE* that are independent of SPI1. SigD secretion through another secretion apparatus could be triggered in a SPI1 mutant under conditions that we have not been able to reproduce. The functions of the identified secreted proteins of *S. typhimurium* have not been characterized, but it is tempting to suggest that specific proteins may be required for invasion of particular cell types or tissues. Perhaps certain invasion proteins are produced or secreted only upon contact with a specific target cell type. It has been observed that SipC, a SPI1-secreted protein, was absent from culture supernatants of certain SPI2 mutants, suggesting that an interaction between the two type III secretion systems might exist (31). Although SigD secretion from bacteria cultured in the laboratory is not affected by a defective SPI2 apparatus, it is not inconceivable that SPI2 or yet another type III secretion apparatus may exist that secretes SigD from bacteria associated with host cells.

ACKNOWLEDGMENTS

We thank Andrew Darwin and Glenn Young for critical review of the manuscript. We also thank Jorge Galan, Eduardo Groisman, David Holden, Sidney Kushner, Catherine Lee, Samuel Miller, and Kenneth Sanderson for strains used in this work. Jorge Galan also graciously provided antibodies to SipA and InvJ. We especially thank Stanley Maloy and Valley Stewart for strains and technical advice.

This work was supported by the National Institutes of Health grant AI01230 and AI31403 to V.L.M.

REFERENCES

1. **Allaoui, A., R. Ménard, P. J. Sansonetti, and C. Parsot.** 1993. Characterization of the *Shigella flexneri ipgD* and *ipgF* genes, which are located in the proximal part of the *mxi* locus. Infect. Immun. **61:**1707–1714.
2. **Alpuche-Arande, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89:**10079–10083.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology, 2nd ed. Greene Publishing Associates and John Wiley & Sons, New York, N.Y.
4. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. *hilA* is a novel *ompR*/*toxR* family member that activates the expression of *Salmonella typhimurium* invasion genes. Mol. Microbiol. **18:**715–727.
5. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
6. **Benson, N. R., and B. S. Goldman.** 1992. Rapid mapping in *Salmonella typhimurium* with Mu*d*-P22 prophages. J. Bacteriol. **174:**1673–1681.
7. **Blanc-Potard, A., and E. A. Groisman.** 1997. The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. EMBO J. **16:**5376–5385.
8. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:**93–99.
9. **Carniel, E., I. Guilvout, and M. Prentice.** 1996. Characterization of a large chromosomal "high-pathogenicity island" in biotype 1B *Yersinia enterocolitica*. J. Bacteriol. **178:**6743–6751.
10. **Carter, P. B., and F. M. Collins.** 1974. The route of enteric infection in normal mice. J. Exp. Med. **139:**1189–1203.
11. **Cheng, L. W., D. M. Anderson, and O. Schneewind.** 1997. Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. Mol. Microbiol. **24:**757–765.
12. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of the Sip proteins into the host cell. Mol. Microbiol. **24:**747–756.
13. **Collazo, C. M., M. K. Zierler, and J. E. Galan.** 1995. Functional analysis of the *Salmonella typhimurium* invasion genes *invI* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. Mol. Microbiol. **15:**25–38.
14. **Eichelberg, K., C. C. Ginocchio, and J. E. Galan.** 1994. 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. J. Bacteriol. **176:**4501–4510.
15. **Elliot, T., and J. R. Roth.** 1988. Characterization of Tn*10*d-Cm: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213:**332–337.
16. **Ernst, R. K., D. M. Dombroski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.
17. **Falkow, S., R. R. Isberg, and D. A. Portnoy.** 1992. The interaction of bacteria with mammalian cells. Annu. Rev. Cell Biol. **8:**333–363.
18. **Finlay, B. B., and S. Falkow.** 1988. A comparison of microbial invasion strategies of *Salmonella*, *Shigella* and *Yersinia* species. Bacteria-Host Cell Interact. **64:**227–243.
19. **Finlay, B. B., S. Ruschkowski, and S. Dedhar.** 1991. Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. J. Cell Sci. **99:**283–296.
20. **Francis, C. L., T. A. Ryan, B. D. Jones, S. J. Smith, and S. Falkow.** 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. Nature **364:**639–642.
21. **Galan, J. E.** 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. **20:**263–271.
22. **Galan, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
23. **Galan, J. E., C. Ginocchio, and P. Costeas.** 1992. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174:**4338–4349.
24. **Galyov, E. E., M. W. Wood, R. Rosqvist, P. B. Mullan, P. R. Watson, S. Hedges, and T. S. Wallis.** 1997. A secreted effector protein of *Salmonella dublin* is translocated into eucaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Mol. Microbiol. **25:**903–912.
25. **Garcia-Vescovi, E., F. C. Soncini, and E. G. Groisman.** 1996. Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell **84:**165–174.
26. **Ginocchio, C., J. Pace, and J. E. Galan.** 1992. Identification and molecular characterization of a *Salmonella typhimurium* gene involved in triggering the internalization of salmonellae into cultured epithelial cells. Proc. Natl. Acad. Sci. USA **89:**5976–5980.
27. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. **12:**3779–3787.
28. **Haas, R., A. F. Kahrs, D. Facius, H. Allmeier, R. Schmitt, and T. F. Meyer.** 1993. Tn*Max*—a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. Gene **130:**23–31.
29. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. **171:**4617–4622.
30. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
31. **Hensel, M., J. E. Shea, B. Raupach, D. Monack, S. Falkow, C. Gleeson, T. Kubo, and D. W. Holden.** 1997. Functional analysis of *ssaJ* and the *ssaK*/*U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. Mol. Microbiol. **24:**155–167.
32. **Hermant, D., R. Ménard, N. Arricau, C. Parsot, and M. Y. Popoff.** 1995. Functional conservation of the *Salmonella* and *Shigella* effectors of entry into epithelial cells. Mol. Microbiol. **17:**781–789.
33. **Hohmann, A. W., G. Schmidt, and D. Rowley.** 1978. Intestinal colonization and virulence of *Salmonella* in mice. Infect. Immun. **22:**763–770.
34. **Hueck, C. J., M. J. Hantman, V. Bajaj, C. Johnston, C. A. Lee, and S. I. Miller.** 1995. *Salmonella typhimurium* secreted invasion determinants are homologous to *Shigella* Ipa proteins. Mol. Microbiol. **18:**479–490.
35. **Johnston, C., D. A. Pegues, C. J. Hueck, C. A. Lee, and S. I. Miller.** 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. **22:**715–727.
36. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
37. **Jones, B. D., N. Ghori, and S. Falkow.** 1994. *Salmonella typhimurium* initiates

murine infection by penetrating and destroying the M cells of the Peyer's patches. J. Exp. Med. **180:**15–23.
38. **Kaniga, K., J. C. Bossio, and J. E. Galan.** 1994. The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues of the AraC and PulD family of proteins. Mol. Microbiol. **13:**555–568.
39. **Kaniga, K., D. Trollinger, and J. E. Galan.** 1995. Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. J. Bacteriol. **177:**7078–7085.
40. **Kaniga, K., S. Tucker, D. Trollinger, and J. E. Galan.** 1995. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. J. Bacteriol. **177:**3965–3971.
41. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
42. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
43. **Lee, C. A.** 1997. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? Trends Microbiol. **5:**148–156.
44. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
45. **Maloy, S. R., V. J. Stewart, and R. K. Taylor.** 1996. Genetic analysis of pathogenic bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonnetti.** 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49:**164–171.
47. **Ménard, R., P. Sansonetti, C. Parsot, and T. Vasselon.** 1994. Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. Cell **79:**515–525.
48. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
50. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2490.
51. **Miras, I., D. Hermant, N. Arricau, and M. Y. Popoff.** 1995. Nucleotide sequence of *iagA* and *iagB* genes involved in invasion of HeLa cells by *Salmonella enterica* subsp. *enterica* ser. Typhi. Res. Microbiol. **146:**17–20.
52. **Moolenar, G. F., C. A. van Sluis, C. Backendorf, and P. van de Putte.** 1987. Regulation of the *Escherichia coli* excision repair gene *uvrC*. Overlap between the *uvrC* structural gene and the region coding for a 24 kD protein. Nucleic Acids Res. **15:**4273–4289.
53. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. USA **93:**7800–7804.
54. **Ozawa, A., J. Goto, Y. Ito, and H. Shibata.** 1973. Histopathological and biochemical responses of germfree and conventional mice with *Salmonella* infection, p. 325. *In* J. B. Heneghan (ed.), Germfree research, biological effect of gnotobiotic environments. Academic Press, New York, N.Y.
55. **Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller.** 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. Mol. Microbiol. **17:**169–181.
56. **Penheiter, K. L., N. Mathur, D. Giles, T. Fahlen, and B. D. Jones.** 1997. Non-invasive *Salmonella typhimurium* mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. Mol. Microbiol. **24:**697–709.
57. **Portnoy, D. A., and G. A. Smith.** 1992. Devious devices of *Salmonella*. Nature **357:**536–537.
58. **Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis.** 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. **176:**7468–7475.
59. **Rosqvist, R., A. Forsberg, and H. Wolf-Watz.** 1991. Intracellular targetting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect. Immun. **59:**4562–4569.
60. **Rosqvist, R., S. Håkansson, A. Forsberg, and H. Wolf-Watz.** 1995. Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae. EMBO J. **14:**4187–4195.
61. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
62. **Sasakawa, C., K. Kamata, T. Sakai, S. Makino, M. Yamada, N. Okada, and M. Yoshikawa.** 1988. Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. J. Bacteriol. **170:**2480–2484.
63. **Sharma, S., T. F. Stark, W. G. Beattie, and R. E. Moses.** 1986. Multiple control elements for the *uvrC* gene unit of *Escherichia coli*. Nucleic Acids Res. **14:**2301–2318.
64. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**2593–2597.
65. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
66. **Stewart, G. S. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15:**172–181.
67. **Stone, B. J., C. M. Garcia, J. L. Badger, T. Hassett, R. I. F. Smith, and V. L. Miller.** 1992. Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. J. Bacteriol. **174:**3945–3952.
68. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
69. **Takeuchi, A.** 1967. Electron microscope studies of experimental *Salmonella* infection. Am. J. Pathol. **50:**109–119.
70. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
71. **Wattiau, P., and G. R. Cornelis.** 1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. Mol. Microbiol. **8:**123–131.
72. **Wattiau, P., S. Woestyn, and G. R. Cornelis.** 1996. MicroReview: customized secretion chaperones in pathogenic bacteria. Mol. Microbiol. **20:**255–262.
73. **Wood, M. W., R. Rosqvist, P. B. Mullan, M. H. Edwards, and E. E. Galyov.** 1996. SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a *sip*-dependent mechanism and promotes bacterial entry. Mol. Microbiol. **22:**327–338.