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RtsA and RtsB Coordinately Regulate Expression of the Invasion and Flagellar Genes in *Salmonella enterica* Serovar Typhimurium

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***Salmonella enterica* serovar Typhimurium encounters numerous host environments and defense mechanisms during the infection process. The bacterium responds by tightly regulating the expression of virulence genes. We identified two regulatory proteins, termed RtsA and RtsB, which are encoded in an operon located on an island integrated at $tRNA^{PheU}$ in *S. enterica* serovar Typhimurium. RtsA belongs to the AraC/XylS family of regulators, and RtsB is a helix-turn-helix DNA binding protein. In a random screen, we identified five RtsA-regulated fusions, all belonging to the *Salmonella* pathogenicity island 1 (SPI1) regulon, which encodes a type III secretion system (TTSS) required for invasion of epithelial cells. We show that RtsA increases expression of the invasion genes by inducing *hilA* expression. RtsA also induces expression of *hilD*, *hilC*, and the *invF* operon. However, induction of *hilA* is independent of HilC and HilD and is mediated by direct binding of RtsA to the *hilA* promoter. The phenotype of an *rtsA* null mutation is similar to the phenotype of a *hilC* mutation, both of which decrease expression of SPI1 genes approximately twofold. We also show that RtsA can induce expression of a SPI1 TTSS effector, *slrP*, independent of any SPI1 regulatory protein. RtsB represses expression of the flagellar genes by binding to the *flhDC* promoter region. Repression of the positive activators *flhDC* decreases expression of the entire flagellar regulon. We propose that RtsA and RtsB coordinate induction of invasion and repression of motility in the small intestine.**

Salmonella serovars cause a range of human diseases from self-limiting gastroenteritis to life-threatening systemic infections (62). The disease process is initiated by bacterial interaction with and invasion of the intestinal epithelium. Analyses of the early steps in colonization and invasion by *Salmonella enterica* serovar Typhimurium with the mouse model of infection and in vitro tissue culture models have significantly increased our understanding of these events (20). Serovar Typhimurium invades intestinal epithelial cells by using a type III secretion system (TTSS) encoded by *Salmonella* pathogenicity island 1 (SPI1). The SPI1 TTSS forms a needle-like structure that injects effector proteins directly into the cytosol of host cells (45, 46, 79). The various effector proteins are implicated in a number of physiological responses, including actin rearrangement that promotes invasion (89), fluid accumulation and transepithelial migration of polymorphonuclear leukocytes (83), and necrosis of Peyer's patch macrophages (12, 63).

The expression of the SPI1 TTSS is controlled in response to a specific combination of environmental signals that presumably act as a cue that the bacteria are in the appropriate anatomic location (7, 71, 75). In the laboratory, the system is active when cells are grown under SPI1-inducing conditions, i.e., high osmolarity and low oxygen. Regulation is mediated primarily via control of the level of HilA, a member of the OmpR/ToxR family of transcriptional regulators encoded on SPI1 (6, 49, 54). HilA directly activates expression of the *prg*/*org* and *inv*/*spa* operons in SPI1 by binding just upstream of the −35 sequences of P_{prgH} and P_{invF} (54). Activation of P_{invF} increases production of InvF, a member of the AraC family of transcriptional regulators (44). InvF then induces expression of effector proteins encoded both within and outside SPI1, including the products of *sicA* (SPI1), *sopE* (SopEϕ in strain SL1344), and *sopB* (*sigD*) (SPI5) (54). Activation of these promoters by InvF requires SicA, a TTSS chaperone (19, 21, 24), which has been suggested to stabilize a complex between InvF, RNA polymerase, and DNA (22).

Two SPI1-encoded proteins, HilC (SirC or SprA) and HilD, both members of the AraC/XylS family of transcriptional regulators, induce expression of *hilA* (26, 67, 71). Loss of HilD decreases expression of *hilA* ~10-fold under SPI1-inducing conditions, whereas a mutation of *hilC* reduces expression of *hilA* ~2-fold (26, 67, 71). HilC and HilD bind to the *hilA* promoter region, and it is believed that this binding induces the expression of *hilA* (64, 71, 72). Recent data suggest that HilD acts as a direct activator of *hilA* (11) as opposed to a derepressor as previously inferred (71, 72). HilC and HilD also induce expression of the *inv*/*spa* operon independent of HilA (2, 26, 67). This induction is due to activation of a second promoter located 5′ to the known P_{invF} (2).

Genetic studies have identified a number of regulatory proteins encoded outside SPI1 that control expression of *hilA*. These include the two-component regulatory systems PhoPQ (9, 66), PhoBR (56), OmpR/EnvZ (55), and SirA/BarA (1, 43, 67). With the exception of OmpR/EnvZ, which alters *hilA* expression via *hilC* (55), it is not known whether control of *hilA* is direct or indirect. Other proteins reported to affect *hilA* expression include Hha (29), Lon (81), Fis (85), integration host factor (IHF) (28), FadD (56), FliZ (25, 42, 56), and HilE (28). With the exceptions of Hha, which binds to the *hilA* promoter region (29), and HilE, which interacts with HilD (8), it remains mechanistically unclear how these proteins control

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expression of *hilA*. The *csrAB* genes encode a protein-RNA pair that act as both positive and negative regulators of *hilA* expression (3, 4, 69). A *csrA* null mutation decreased the steady-state levels of *hilC* and *hilD* mRNAs (3), suggesting that the control of *hilA* is via these two regulators.

Serovar Typhimurium produces peritrichous flagella required for motility. There are more than 40 genes involved in flagellar biosynthesis. These genes are controlled by a regulatory cascade, which is initiated by the production of FlhDC. These regulatory proteins induce expression of the class 2 flagellar genes, including *fliA*, which encodes an alternative sigma factor required for transcription of the class 3 flagellar genes. This cascade serves to control the timing of gene expression to coincide with assembly of the flagellar apparatus (16, 57). Serovar Typhimurium can produce two immunologically distinct flagellin proteins, FliC or FljB. The production of these proteins is phase variable and mediated by DNA inversion (57).

A number of regulators affect expression of the flagellar regulon. The two-component regulators SirA/BarA act to repress expression of most flagellar operons, including *flhDC* (35). Interestingly, this repression was observed only during growth in motility agar (35). It remains unclear whether the repressing effects of SirA are via direct interaction with the promoter region of *flhDC* or whether this repression is mediated through some other regulator. It has been shown in *Escherichia coli* that CsrA binds the *flhDC* transcript, protecting it from degradation (84). Other regulators implicated in control of *flhDC* include catabolite gene activator protein (48, 74), LrhA (51) and HNS, via HdfR (47).

Here we describe two regulatory proteins, one of which induces expression of the SPI1 TTSS (RtsA) whereas the other represses expression of flagellar genes (RtsB). We show that RtsA is capable of directly binding to the promoter region of *hilA*, suggesting that RtsA can directly induce expression of *hilA*. We also show that RtsB can directly bind just downstream of the *flhDC* promoter, suggesting that RtsB directly represses expression of *flhDC* and thus of the entire flagellar regulon.

MATERIALS AND METHODS

Media, reagents, and enzymatic assays. Luria-Bertani medium (LB) was used in all experiments for growth of bacteria, and SOC was used for the recovery of transformants (58). MacConkey medium was prepared according to the manufacturer's directions and supplemented with 1% lactose and the appropriate antibiotics (Difco). Motility agar contained 0.3% Bacto agar, 1% tryptone, and 0.5% NaCl supplemented with either 0.2% glucose or 0.2% L-arabinose. Bacterial strains were routinely grown at 37°C, except for strains containing the temperature-sensitive plasmid pCP20 or pKD46, which were grown at 30°C. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. The β-galactosidase chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 80 μg/ml. Enzymes were purchased from Invitrogen or New England Biolabs and were used according to the manufacturer's recommendations. Primers were purchased from IDT Inc. β-Galactosidase assays were performed with a microtiter plate assay as previously described (76) on strains grown under the indicated conditions. β-Galactosidase activity units are defined as (micromoles of *o*-nitrophenol [ONP] formed minute^{-1}) × 10^3/(optical density at 600 nm [OD_{600}] × milliliters of cell suspension) and are reported as means ± standard deviations, where *n* is ≥2. Cultures requiring SPI1-inducing conditions were grown statically in LB with 1% NaCl. Cultures requiring SPI1-repressing conditions were grown in LB without NaCl and were highly aerated.

Strain and plasmid construction. Bacterial strains and plasmids are described in Table 1. All *S. enterica* serovar Typhimurium strains created for this study are isogenic derivatives of strain 14028 (American Type Culture Collection) and were constructed by using P22 HT105/1 *int*-201 (P22)-mediated transduction (58). Random MudJ transcriptional fusions to the *lac* operon were created by using strain TT10286 and transitory *cis* complementation as previously described (40). Sequencing of the RtsAB-regulated MudJ fusions was performed by utilizing the semi-random-primed sequencing method previously described (17, 39). The Pi-dependent plasmids used in this study were maintained in DH5αλpir. All plasmids were passaged through a restriction-negative, modification-positive Pi$^+$ serovar Typhimurium strain (JS198) prior to transformation into derivatives of strain 14028.

Standard recombinant DNA methods were used for the construction of plasmids (70). The *rtsA*, *rtsB*, and *rtsAB* genes were cloned by using the Gateway PCR method (Invitrogen) (38). Primers flanked with the λ*attB* sites were used to PCR amplify *rtsA* (RtsA1, GGGGACAAGTTTGTACAAAAAAGCAGGCTTACACGCACATTTAATAAAAGG; RtsA2, GGGGACCACTTTGTACAAGAAAGCTGGGTCAGTATTAACATATTGATACG), *rtsB* (RtsB1, GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTCCTCTCGTCATCAATATG; RtsB2, GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATTACGTAATATCGACTG), and *rtsAB* (RtsA1and RtsB2). The PCR products were gel purified and cloned into plasmid pDONR201 (38) with a mixture of λInt and IHF, which catalyzes a site-specific recombination between the λ*attB*-flanked PCR products and the λ*attP* sites located on the plasmid. This resulted in plasmids that contained the gene of interest flanked by λ*attL* sites (38). The inserts were then sequenced to ensure that they did not contain mutations. To create plasmids in which expression of these genes was L-arabinose inducible, we cloned a λ*attR*-flanked *ccdB*$^+$ *cat* cassette into a blunt-ended *Eco*RI site of pBAD30, creating pCE46 (36, 38). λInt, λXis, and IHF were used to recombine the λ*attL*-flanked *rtsA*, *rtsB*, and *rtsAB* fragments into pCE46 to create the plasmids pRtsA, pRtsB, and pRtsAB (38). To create c-Myc epitope-tagged versions of RtsA and RtsB, we utilized a two-step overlapping PCR method in conjunction with Gateway cloning. The Myc tag was located in the 5′ primer. The primers were used to PCR amplify *rtsA* (myc-RtsA, GATAGAACCATGGAACAAAAATTAATTTCTGAAGAAGATTTACTAAAAGTATTTAATCCC; RtsA2) or *rtsB* (myc-RtsB, GATAGAACCATGGAACAAAAATTAATTTCTGAAGAAGATTTACAGTATAAGAACAAAGCA; RtsB2). A primer with homology to the Myc tag (AttBmyc, GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGAAGATAGAACCATGGAAC) was then used to add the AttB1 sequences to the 5′ end of *rtsA* or *rtsB* in separate PCR amplifications. The resulting PCR products were cloned by using a single-step reaction into pCE46 according to the directions of the manufacturer (Invitrogen).

Construction of chromosomal deletion-insertions and *lac* fusions. Deletions of the *hilA*, *hilC*, *hilD*, *invF*, *hilC-D*, *hilC-A*, and *slrP* genes and concomitant insertion of a chloramphenicol resistance cassette were carried out by lambda Red-mediated recombination (23, 88) as described previously (27). PCR products containing the antibiotic resistance cassette flanked by 30 bp of homology to the gene of interest in the 5′ and 3′ ends were transformed into a serovar Typhimurium strain containing pKD46, which is temperature sensitive and carries the λ *red*, *gam*, and *bet* genes under L-arabinose control (23). The endpoints of each deletion are indicated in Table 1. The *tetRA* genes from Tn*10* were inserted upstream of *rtsAB* by using this system. The *tetRA* cassette was amplified by using rtsA-tet1 and rtsA-tet2, which have 15 nucleotides of homology to *tetRA* on the 3′ end and 30 nucleotides of homology to the DNA 10 bp upstream of the *rtsA* open reading frame (ORF). In all cases, the appropriate insertion of the antibiotic resistance marker was checked by P22 linkage to known markers and/or by PCR analysis. In each case, the constructs resulting from this procedure were moved into a clean wild-type background (14028) by P22 transduction. In-frame deletions were created or antibiotic resistance cassettes were removed by using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (15). Mutations constructed with the pKD3 template plasmid (23) were converted to transcriptional *lac*$^+$ fusions by using an FLP/FRT-mediated site-specific recombination method as previously described (27).

Analysis of *Salmonella* secreted proteins. Overnight cultures of the indicated strains were diluted 1/20 into 10 ml of LB–ampicillin–0.2% L-arabinose and grown with shaking at 225 rpm on a platform shaker for 4 h at 37°C. Ten milliliters of culture supernatant was centrifuged two times at 5,000 × *g*. The culture supernatant was then filter sterilized by using a 0.2-μm-diameter syringe filter. Proteins were precipitated with ice-cold trichloroacetic acid at a final concentration of 10% after incubation on ice for 30 min. The proteins were pelleted by centrifugation at 15,000 × *g* for 30 min at 4°C. The supernatant was then removed, and the pellets were washed with 5 ml of cold acetone. The samples were then centrifuged for another 20 min at 15,000 × *g*. The supernatant was removed, and the pellets were allowed to air dry. The pellets were then resuspended in 50 μl of 50 mM Tris (pH 8), and 20 μl of 4× sodium dodecyl

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype[a] or relevant characteristics	Deletion or cloned end points[b]	Source or reference[c]
Strains			
14028	Wild-type serovar Typhimurium		ATCC[d]
JS135	*zii-8104*::Tn*10*dTc		78
JS247	Φ(*tetA*$^+$-*rtsA*$^+$*B*$^+$)3	4561769–4561768	
JS248	Δ*rtsA5*	4561755–4560884	
JS249	Δ*rtsB6*	4560868–4560602	
JS250	Δ*rtsAB7*	4561769–4560602	
JS251	Δ*hilA112*::Cm	3019885–3021480	
JS252	Δ*hilC113*::Cm	3012135–3012976	
JS253	Δ*hilD114*::Cm	3017865–3018730	
JS254	Δ*invF100*::Cm	3043931–3043290	
JS255	Δ*hilC-A2914*::Cm	3012135–3021480	
JS256	Δ*hilC-D2915*::Cm	3012135–3018730	
JS257	Δ*slrP100*::Cm	866973–869216	
JS258	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 P_{hilD}*111*::MudJ		
JS259	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 *invC101*::MudJ		
JS260	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 *sopA100*::MudJ		
JS261	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 *sopB101*::MudJ		
JS262	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 *icgA1*::MudJ		
JS263	Φ(*tetRA-rtsA*$^+$*B*$^+$)3 *yhgF*:231:MudJ		
JS264	*zii-8104*::Tn*10*dTc P_{hilD}*111*::MudJ		
JS265	*zii-8104*::Tn*10*dTc *invC101*::MudJ		
JS266	*zii-8104*::Tn*10*dTc *sopA100*::MudJ		
JS267	*zii-8104*::Tn*10*dTc *sopB101*::MudJ		
JS268	*zii-8104*::Tn10dTc *icgA1*::MudJ		
JS269	*zii-8104*::Tn10dTc *yhgF231*::MudJ		
JS270	Δ*rtsAB7* Δ*hilA112*::Cm		
JS271	Δ*rtsAB7 invC101*::MudJ		
JS272	Δ*rtsAB7* Δ*hiD114*::Cm		
JS273	Δ*rtsAB7* Φ(*hilD-lac*$^+$)*114*		
JS274	Δ*rtsAB7* Φ(*hilC-lac*$^+$)*113*		
JS275	Δ*rtsAB7* Φ(*hilA-lac*$^+$)*112*		
JS276	Δ*rtsAB7* Φ(*invF-lac*$^+$)*100*		
JS277	Δ*rtsAB7 flhC5456*::MudJ		
JS278	Δ*rtsAB7 fliC5050*::MudJ		
JS279	Φ(*hilA-lac*$^+$)*112*		
JS280	Δ*rtsA5* Φ(*hilA-lac*$^+$)*112*		
JS281	Δ*rtsB6* Φ(*hilA-lac*$^+$)*112*		
JS282	Φ(*invF-lac*$^+$)*100*		
JS283	Δ*rtsA5* Φ(*invF-lac*$^+$)*100*		
JS284	Δ*rtsB6* Φ(*invF-lac*$^+$)*100*		
JS285	*sopA100*::MudJ		
JS286	Δ*rtsA5 sopA100*::MudJ		
JS287	Δ*rtsB6 sopA100*::MudJ		
JS288	Δ*rtsAB7 sopA100*::MudJ		
JS289	*sopB101*::MudJ		
JS290	Δ*rtsA5 sopB101*::MudJ		
JS291	Δ*rtsB6 sopB101*::MudJ		
JS292	Δ*rtsAB7 sopB101*::MudJ		
JS293	*invC101*::MudJ		
JS294	Δ*rtsA5 invC101*::MudJ		
JS295	Δ*rtsB6 invC101*::MudJ		
JS296	*icgA1*::MudJ		
JS297	Δ*rtsA5 icgA1*::MudJ		
JS298	Δ*rtsB6 icgA1*::MudJ		
JS299	Δ*rtsAB7 icgA1*::MudJ		
JS300	Δ*rtsAB7* Δ*hilC113*::Cm Φ(*hilA-lac*$^+$)*112*		
JS301	Δ*rtsAB7* Δ*hiD114*::Cm Φ(*hilA-lac*$^+$)*112*		
JS302	Δ*rtsAB7* Δ*hilC-D2915*::Cm Φ(*hilA-lac*$^+$)*112*		
JS303	Δ*rtsAB7* Δ*hilA112*::Cm Φ(*invF-lac*$^+$)*100*		
JS304	Δ*rtsAB7* Δ*hilC-D2915*::Cm Φ(*invF-lac*$^+$)*100*		
JS305	Δ*rtsAB7* Δ*hilC-A2914*::Cm Φ(*invF-lac*$^+$)*100*		
JS306	Δ*rtsAB7 sopA100*::MudJ		
JS307	Δ*rtsAB7* Δ*hilA112*::Cm *sopA100*::MudJ		
JS308	Δ*rtsAB7* Δ*invF100*::Cm *sopA100*::MudJ		
JS309	Δ*rtsAB7 sopB101*::MudJ		
JS310	Δ*rtsAB7* Δ*hilA112*::Cm *sopB101*::MudJ		
JS311	Δ*rtsAB7* Δ*invF100*::Cm *sopB101*::MudJ		

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Genotype[a] or relevant characteristics	Deletion or cloned end points[b]	Source or reference[c]
JS312	Δ*rtsAB7 invC101*::MudJ		
JS313	Δ*rtsAB7* Δ*hilA112*::Cm *invC101*::MudJ		
JS314	Δ*rtsAB7* Δ*invF100*::Cm *invC101*::MudJ		
JS315	Δ*rtsAB7 icgA1*::MudJ		
JS316	Δ*rtsAB7* Δ*hilA112*::Cm *icgA1*::MudJ		
JS317	Δ*rtsAB7* Δ*invF100*::Cm *icgA1*::MudJ		
JS318	Δ*rtsAB7* Φ(*slrP-lac*$^+$)*100*		
JS319	Δ*rtsAB7* Δ*hilC-D2915*::Cm Φ(*slrP-lac*$^+$)*100*		
JS320	Δ*rtsAB7* Δ*invF100*::Cm Φ(*slrP-lac*$^+$)*100*		
JS321	Δ*rtsAB7* Δ*hilA112*::Cm Φ(*slrP-lac*$^+$)*100*		
JS322	Δ*rtsA5* Δ*hilC-D2915*::Cm Φ(*hilA-lac*$^+$)*112*		
JS323	Δ*rtsA5* Δ*hilC-D2915*::Cm Φ(*slrP-lac*$^+$)*100*		
JS324	Φ(*rtsA-lac*$^+$)*5*		
JS325	Φ(*rtsB-lac*$^+$)*6*		
TT10286	LT2 *hisD9953*::MudJ *his-9944*::MudI		40
TH1077	LT2 *fliC5050*::MudJ		34
TH4054	LT2 *flhC5456*::MudJ		18
DH5αλpir	*E. coli* K-12 *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lac-argF*)*U169 deoR* ϕ80 Δ(*lac*)M15 λ*pir*$^+$		Lab stock
MG1655	*E. coli* K-12 F$^-$ λ$^-$ *ilvG rfb-50 rph-1*		Lab stock
Plasmids			
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *oriTS*		23
pCP20	*bla cat* cI857 λP_R *flp* pSC101 *oriTS*		15
pKD3	*bla* FRT *cat* FRT PS1 PS2 *oriR6K*		23
pKD13	*bla* FRT *ahp* FRT PS1 PS4 *oriR6K* (for creating in-frame deletions)		23
pCE36	*ahp* FRT *lacZY*$^+$ t_{his} *oriR6K*		27
pCE37	*ahp* FRT *lacZY*$^+$ t_{his} *oriR6K*		27
pBAD30	*bla araC* P_{BAD} pACYC184 *ori*		36
pCE46	*bla araC* λ*AttL1 ccdB*$^+$ *cat* λ*AttL2* pACYC184 *ori*		
pRtsA	*bla* P_{BAD} λ*AttB1 rtsA*$^+$ λ*AttB2* pACYC184 *ori*	4561766–4560885	
pRtsB	*bla* P_{BAD} λ*AttB1 rtsB*$^+$ λ*AttB2* pACYC184 *ori*	4560890–4560595	
pRtsAB	*bla* P_{BAD} λ*AttB1 rtsA*$^+$*B*$^+$ λ*AttB2* pACYC184 *ori*	4561766–4560595	
pCE81	*bla* P_{BAD} λ*AttB1 myc-rtsA* λ*AttB2* pACYC184 *ori*	4561737–4560885	
pCE82	*bla* P_{BAD} λ*AttB1 myc-rtsB* λ*AttB2* pACYC184 *ori*	4560850–4560595	
pLS118	*bla* P_{BAD} *hilD-myc*-His pACYC184 *ori*		72
pLS119	*bla* P_{BAD} *hilC-myc*-His pACYC184 *ori*		72

[a] Unless otherwise noted, all strains are isogenic derivatives of 14028.
[b] Numbers indicate the base pairs (inclusive) that are deleted (for strains) or cloned (for plasmids), as defined in the *S. enterica* serovar Typhimurium LT2 genome sequence in the National Center for Biotechnology Information database.
[c] This study, unless otherwise noted.
[d] ATCC, American Type Culture Collection.

sulfate (SDS) loading buffer (5) was added. The samples (15 μl total) were then separated by SDS–12.5% polyacrylamide gel electrophoresis (PAGE) (5). The proteins were stained with GelCode Blue according to the directions of the manufacturer (Pierce).

Gel shift assays. Whole-cell extracts for gel shift assays were prepared by subculturing overnight cultures 1/100 in LB and growing them to an OD_{600} of 0.5, at which time 0.2% L-arabinose was added and cultures were grown for an additional 3 h at 37°C. Cultures were then centrifuged, and the pellets were resuspended in 10 mM Tris-Cl (pH 8)–50 mM KCl–10% glycerol–1 mM dithiothreitol–0.5 mM EDTA. Samples were lysed by passage through a French press. Lysates were clarified by centrifugation at 16,000 × *g* for 30 min at 4°C. The protein concentration in each sample was determined by using a bicinchoninic acid assay (Pierce). Binding reaction mixtures contained approximately 0.1 ng of ^{32}P-labeled DNA, 50 μg of herring sperm DNA per ml, 10 mM Tris-Cl (pH 8), 50 mM KCl, 100 μg of bovine serum albumin per ml, 10% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA, and the appropriate concentration of whole-cell extract in a final volume of 20 μl. To determine if RtsA or RtsB directly bound to the DNA, 100 ng of anti-Myc antibody (Invitrogen) were added to the appropriate reaction mixtures. Binding reaction mixtures were incubated for 20 min at room temperature and then subjected to electrophoresis on a 5% native polyacrylamide gel in 0.5× Tris-borate-EDTA at room temperature (5). Gels were dried on filter paper in a vacuum drier and visualized with a Fuji phosphorimager (FLA-3000) and Image Reader software.

RESULTS

Identification of *rtsAB*. The completed *S. enterica* serovar Typhimurium LT2 genome reveals a large number of putative genes with no known function. We identified two regulatory genes that are present in most *Salmonella* serovars but absent from *E. coli* K-12 (10, 59, 65). In serovar Typhimurium, these regulators are located on a recently described 15-kb island inserted near the $tRNA^{PheU}$ gene (37). The $tRNA^{PheU}$ island in serovar Typhimurium contains one previously identified gene, *phoN*, which encodes a nonspecific acid phosphatase. It also contains several unknown ORFs, including an operon that encodes a homolog of dimethyl sulfoxide reductase, an operon encoding a putative acetyltransferase and a transcriptional regulator belonging to the CopG family, an IS*200* element, and a regulatory operon encoding two putative transcriptional regulators that we have named *rtsAB* (for regulator of TSS; STM4315 and STM4314, respectively). Sequence analysis of the regulatory operon revealed that RtsA belongs to the AraC/

XylS family of transcriptional regulators and is homologous to both HilC (37% identical and 55% similar overall) and HilD (34% identical and 55% similar overall). RtsB is a small protein that contains a putative helix-turn-helix DNA binding motif.

Identification of RtsAB-regulated genes. To identify RtsAB-regulated genes, we created a strain in which expression of *rtsAB* was conditional. The strain JS247 contains the *tetRA* genes from Tn*10* integrated 10 bp upstream of the *rtsA* ORF by the λ Red recombinase method (23, 88). This construct uncoupled expression of *rtsAB* from the putative *rtsA* promoter and placed expression of *rtsAB* under the control of the *tetA* promoter (68). In the absence of tetracycline, TetR represses expression of *tetA* and thus *rtsAB*. In the presence of tetracycline, expression of both *tetA* and *rtsAB* is induced 5.5-fold (data not shown).

We generated 50,000 independent MudJ transcriptional *lac* fusions in the Φ(*tetA*$^+$-*rtsA*$^+$*B*$^+$)*3* strain. The resulting colonies were replica plated onto LB–X-Gal and LB–X-Gal–tetracycline or on MacConkey-lactose and MacConkey-lactose-tetracycline. Using this method, we identified 39 *lac* fusions with altered levels of expression in the presence of tetracycline. Two classes of false positives were anticipated in this screen. First, the *lac*$^+$ fusion could be controlled directly by the *tetRA* promoters. These fusions would be linked to the Φ(*tetA*$^+$-*rtsA*$^+$*B*$^+$)*2* construct by P22 transduction. None of the MudJ fusions fell into this class. Second, the expression of the fusion could be altered by the presence of tetracycline independent of the effect of tetracycline on the expression of *rtsAB*. To eliminate these fusions, we transduced each MudJ into a strain containing *zii-8104*::Tn*10*dTc, which does not affect the expression of *rtsAB* (78), and screened for those fusions in which expression was not altered by tetracycline. Six fusions had increased expression in the Φ(*tetA*$^+$-*rtsA*$^+$*B*$^+$)3 background but were unaffected by tetracycline in the *zii-8104*::Tn*10*dTc strain. These MudJ fusions were considered candidates for RtsA-or RtsB-regulated genes and were studied further.

Sequence analysis of potential RtsAB-regulated genes. To identify the genes regulated by either RtsA or RtsB, the insertion site of each MudJ was sequenced. Sequence analysis revealed that two of the fusions were to genes located on SPI1. One fusion appeared to be to the *hilD* promoter, although the fusion joint was located 39 bp upstream of the *hilD* translational start site. The other fusion was to *invC*, which is directly induced by binding of HilA to the *invF* promoter. We also isolated fusions to *sopA* and *sopB* (*sigD*), which encode TTS effectors not encoded on SPI1. It is known that InvF and SicA act in concert to activate expression of *sopB* (21). The regulation of *sopA* remains unclear. The fifth gene identified was STM4261, a large gene located on SPI4, which we have termed *icgA* (for invasion-coregulated gene A) because it was previously shown to be regulated by SirA in a HilA-dependent manner (1). Based on sequence analysis, *icgA* appears to lie in an operon with genes that contain homology to an ABC-like transport apparatus. IcgA itself shows homology to both putative RTX pore-forming toxins and putative adhesins. This suggests that IcgA may be a type 1 secreted toxin or adhesin. We also isolated a fusion to *yhgF*, a gene with no known function. Sequence analysis of YhgF showed that it contains a putative S1 RNA binding domain and similarity to RNase R (13, 14). YhgF also contains similarity to the Tex protein of *Bordetella pertussis*, which has been implicated in control of toxin expression (30).

Requirements for induction of the RtsAB-regulated genes. Our screen identified six genes that were induced by increased production of RtsAB. We wanted to determine if the RtsA and RtsB regulators could act independently of one another and, if so, which of the regulators was required for induction. Therefore, we constructed strains that had the chromosomal *rtsAB* genes deleted and contained plasmids in which the expression of *rtsA*, *rtsB*, or both was arabinose inducible (36). We transduced each of the fusions into the four plasmid-bearing strains (carrying pBAD30, pRtsA, pRtsB, and pRtsAB) and measured the β-galactosidase activity of each strain after growth for 3 h in L-arabinose. RtsA alone was required for increased expression of *hilD*, *sopA*, *sopB*, *invC*, and *icgA*. Indeed, the *sopA*, *sopB*, and *invC* fusions were induced 50- to 80-fold by RtsA, while expression of *hilD* was induced ~5- to 7-fold. Induction of these genes was independent of RtsB (data not shown). RtsB induced expression of *yhgF* approximately sevenfold, while RtsA had no effect (data not shown). Simultaneous production of RtsA and RtsB induced expression of all genes to the same levels observed with the single regulator (data not shown). Thus, RtsA and RtsB act independently to control expression of these genes.

RtsA induces secretion of SPI1 TTS effectors in a HilA-dependent manner. Our screen for RtsA-regulated genes identified five genes known to be part of the SPI1 regulon. To determine if RtsA increased expression of the entire SPI1 TTSS, we assayed the secretion of SPI1 TTS effectors into culture supernatants. Plasmid-bearing strains were grown under SPI1-repressing conditions with 0.2% L-arabinose. The resulting cell-free supernatant was trichloroacetic acid precipitated, and the proteins were separated by SDS-PAGE. As shown in Fig. 1, the strain containing the pBAD vector alone secreted few proteins into the culture supernatant, with the flagellin filament FliC being the predominant protein. The pRtsB strain resembled the vector control except that the band corresponding to the flagellin subunit FliC was decreased in this background. This phenotype was repeatable and is addressed below. In contrast, a strain expressing RtsA had increased amounts of SPI1 TTS effector proteins in the culture supernatant. The strain containing pRtsAB had both increased SPI1 effector proteins and decreased flagellin protein FliC in the culture supernatant. In order to ensure that the proteins found in the culture supernatants were dependent upon the presence of the SPI1 TTS apparatus, we assayed protein secretion in strains containing pRtsA and *hilA*, *hilD*, or *invC* mutations. Mutation of either *hilA* or *invC* blocked secretion of the SPI1 TTS proteins. In contrast, a *hilD* mutation did not appear to decrease RtsA-dependent induction of the SPI1 TTSS (Fig. 1). Thus, production of RtsA induces expression of the entire SPI1 TTSS. This induction is dependent upon the master regulator HilA but is independent of HilD.

RtsA induces expression of *hilA*, *invF*, *hilC*, and *hilD*. To understand the mechanism of RtsA induction of the invasion genes, we constructed *lac* fusions to the major SPI1 regulatory genes by using an FLP-mediated method that we recently described (27). These included fusions to *hilA*, *hilC*, and *invF* and a new fusion to *hilD*, in which the fusion joint is downstream of

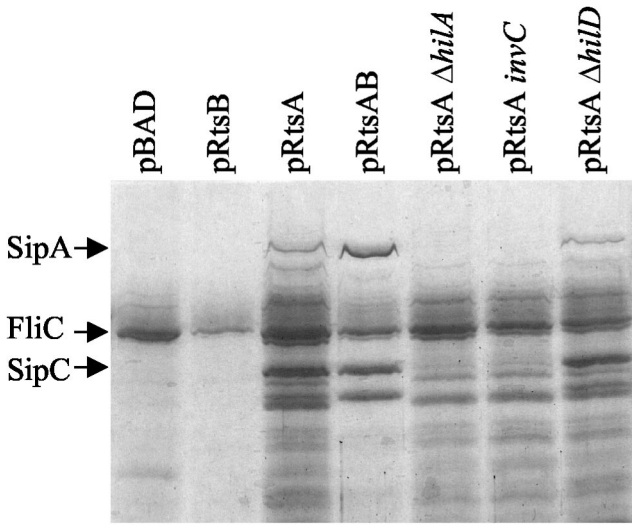

FIG. 1. Effect of RtsA and RtsB on secretion of proteins into culture supernatants. The strains are Δ*rtsAB7* and contain the plasmid and mutations specified. Overnight cultures were subcultured into LB (without salt)–ampicillin–0.2% L-arabinose and grown with aeration to an OD_{600} of ~0.8. Culture supernatants were prepared as described in Materials and Methods. An equivalent amount of sample from the supernatant of each strain was separated by SDS–12.5% PAGE. The gel was stained for total protein with GelCode Blue. Proteins of greater than ~25 kDa are shown. The strains used were plasmid-containing derivatives of JS250, JS270, JS271, and JS272.

the translational start site. These fusions were introduced into strains containing Δ*rtsAB* and either pBAD, pRtsA, pRtsB, or pRtsAB. As shown in Fig. 2A, the expression of *hilD* and *hilC* was induced approximately five- to sevenfold by production of RtsA but not RtsB. Thus, RtsA can induce expression of *hilC* and *hilD*, both of which encode positive regulators of *hilA*. Figure 2A also shows that the *hilA* and *invF* fusions are induced approximately 40- to 60-fold by RtsA but not RtsB. Thus, RtsA can induce expression of both *hilA* and *invF*, and this induction is approximately 10-fold higher than RtsA induction of *hilC* and *hilD*.

A functional chromosomal copy of *rtsA* is required for maximal expression of the invasion genes. Production of RtsA under normally SPI1-repressing conditions results in the induction of several genes, which belong to the SPI1 TTSS regulon. In order to determine if RtsA is required for expression of the SPI1 genes under SPI1-inducing conditions, we constructed strains with in-frame deletions in *rtsA*, *rtsB*, or both. We then introduced *hilA-lac*, *invF-lac*, *sopA100*::MudJ, *sopB101*::MudJ, *invC101*::MudJ, and *icgA1*::MudJ fusions into wild-type and mutant strains and assayed the β-galactosidase activities of the resulting strains. Figure 3 shows that deletion of *rtsA* or *rtsAB*, but not *rtsB* alone, decreased expression of each of the fusions approximately 1.5- to 2-fold. This is similar to the effect seen in a *hilC* mutant (reference 55 and data not shown). This suggests that RtsA is normally required for maximal expression of the SPI1 TTSS. Under these laboratory conditions, deletion of *rtsA* confers a modest decrease in expression of the SPI1 regulon. Therefore, we chose to perform subsequent genetic analysis by ectopic production of RtsA and/or RtsB.

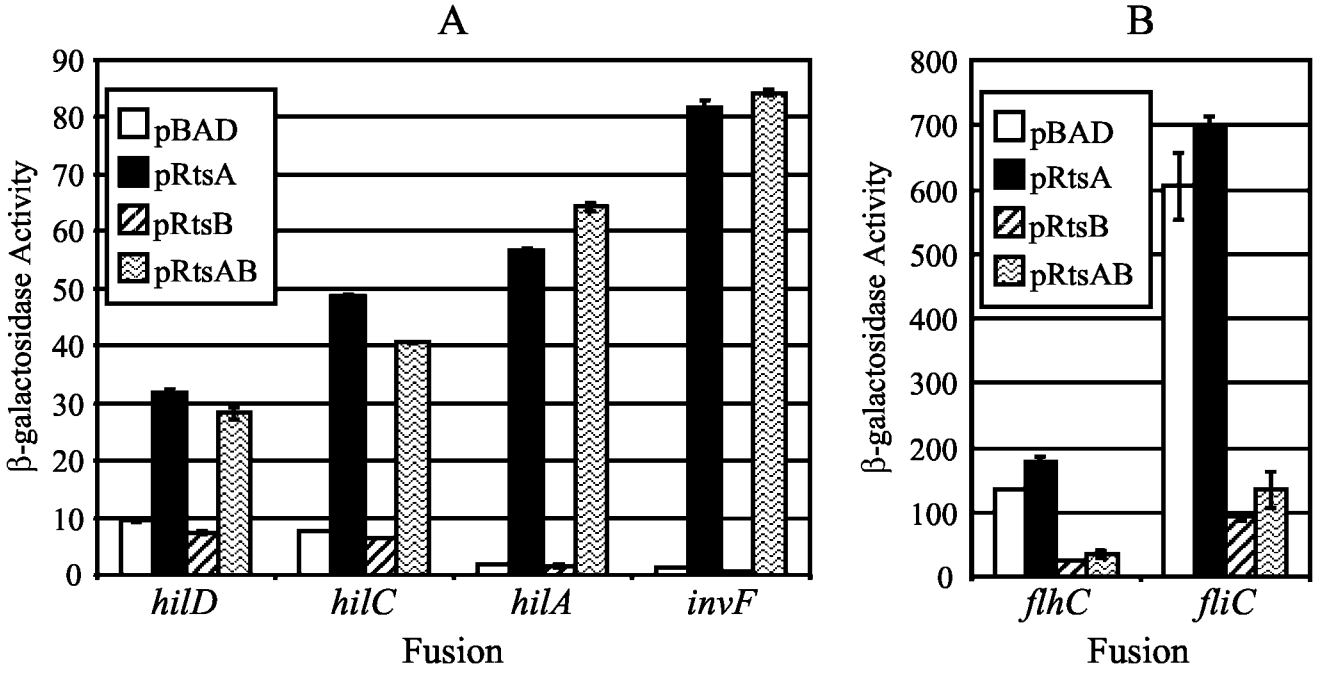

FIG. 2. Effect of RtsA, RtsB, or RtsAB on the expression of the SPI1 regulatory genes (A) and the flagellar genes (B). The strains are Δ*rtsAB7* and contain pBAD30, pRtsA, pRtsB, or pRtsAB and a *lacZ* transcriptional fusion to the specified gene. Overnight cultures were subcultured into LB (without salt)–ampicillin–0.2% L-arabinose and grown to an OD_{600} of ~0.6. β-Galactosidase activity units are defined as (micromoles of ONP formed $minute^{-1}$) × 10^3/(OD_{600} × milliliters of cell suspension) and are reported as means ± standard deviations, where $n = 4$. The strains used were plasmid-containing derivatives of JS273 through JS278.

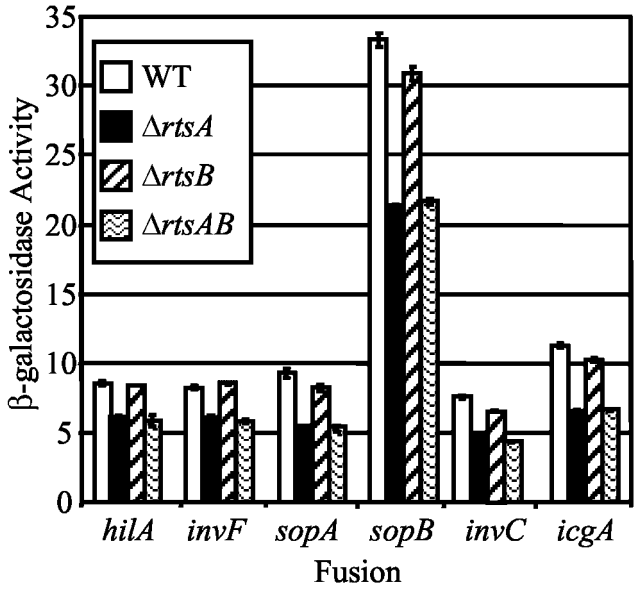

FIG. 3. Effect of Δ*rtsA5*, Δ*rtsB6*, or Δ*rtsAB7* mutations on the expression of RtsA-regulated genes. Stationary-phase cultures were subcultured 1/100 into LB–1% NaCl and grown statically overnight at 37°C, at which point β-galactosidase activities were determined. β-Galactosidase activity units are defined as (micromoles of ONP formed minute^{-1}) × 10^3/(OD_{600} × milliliters of cell suspension) and are reported as means ± standard deviations, where $n = 4$. The strains used were plasmid-containing derivatives of JS271, JS275, JS276, and JS279 through JS299. WT, wild type.

RtsA induction of *hilA* does not require HilC or HilD. It is possible that RtsA increases expression of *hilC* and *hilD*, whose products then act to induce expression of *hilA* (26, 67, 71). A second possibility is that RtsA induces expression of *hilA* independent of HilC and HilD. To distinguish between these models, we constructed strains that had *hilC*, *hilD*, or both genes deleted. As shown in Fig. 4A, production of RtsA results in increased expression of *hilA* in the absence of either *hilC* or *hilD*. Deletion of both regulators decreased the absolute level of expression from the *hilA-lac* fusion but did not alter the fold induction of *hilA* by RtsA, which remained approximately 50- to 60-fold. This suggests that increased expression of *hilA* by RtsA is independent of the increase in *hilC* and *hilD* expression. This is consistent with the observation that RtsA-dependent secretion of TSS effector proteins was independent of HilD (Fig. 1).

RtsA directly binds the *hilA* promoter region. Does RtsA increase expression of *hilA* by directly interacting with the *hilA* promoter region or by altering the expression of another regulator of *hilA*? RtsA belongs to the AraC/XylS family of transcriptional regulators and contains similarity to HilC and HilD. To determine if RtsA binds to the *hilA* promoter region, we first constructed an antibody epitope-tagged version of RtsA, Myc-RtsA. This fusion protein is functional as evidenced by its ability to activate expression of a *hilA-lac* fusion (data not shown). We performed gel shift assays with the minimal DNA required for HilC and HilD activation of the *hilA* promoter (64, 71, 72). This region of DNA contains both HilC and HilD binding sites (64, 72). As shown in Fig. 5, increasing protein concentrations of whole-cell extracts from an *E. coli* strain

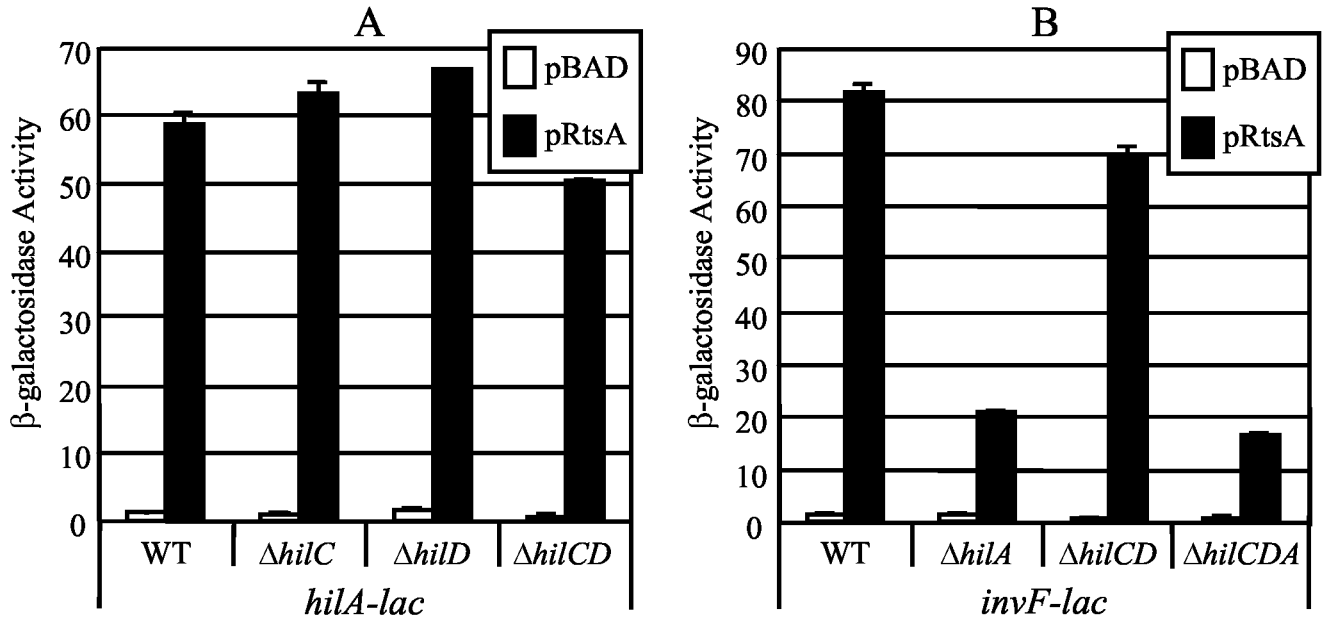

FIG. 4. Effect of mutations in SPI1 regulatory genes on RtsA-induced expression of *hilA-lac*$^+$ (A) and *invF-lac*$^+$ (B) fusions. The *hilA-lac*$^+$ or *invF-lac*$^+$ transcriptional fusion strains are Δ*rtsAB*; contain pBAD30, pRtsA, pRtsB, or pRtsAB; and have the indicated regulatory gene(s) deleted. The *hilC*, *hilD*, and *hilA* mutations are simple deletion-insertions of a chloramphenicol cassette. The Δ*hilC-D* mutation deletes *hilC* to *hilD*, including the *prgHIJK* operon, and the Δ*hilC-A* deletion removes *hilC*, *prgHIJK*, *hilD*, and *hilA*. Overnight cultures were subcultured into LB (without salt)–ampicillin–0.2% L-arabinose and grown to an OD_{600} of ~0.6 before assay of β-galactosidase. β-Galactosidase activity units are defined as (micromoles of ONP formed minute^{-1}) × 10^3/(OD_{600} × milliliters of cell suspension) and are reported as means ± standard deviations, where $n = 2$. The strains used were plasmid-containing derivatives of JS275, JS276, and JS300 through JS305. WT, wild type.

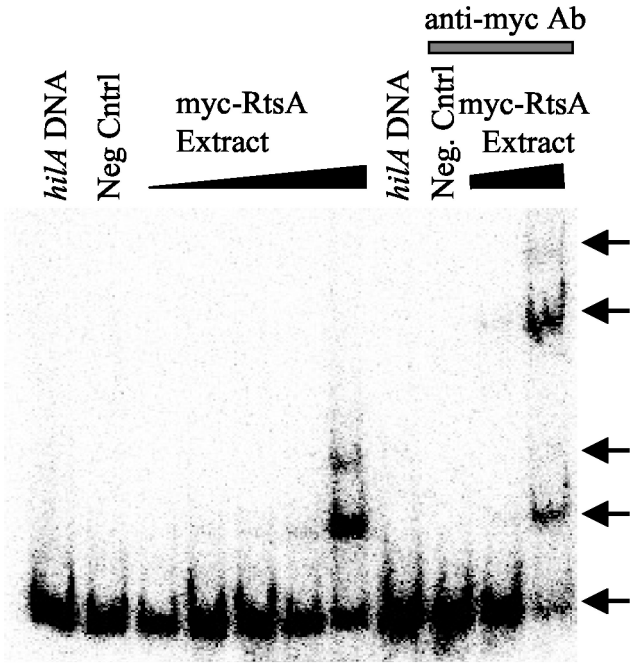

FIG. 5. Gel shift analysis of RtsA binding to the *hilA* promoter region. Binding reaction mixtures contained ~0.1 ng of ^{32}P-labeled *hilA* promoter region DNA corresponding to −189 to +19 bp from the start site of transcription. Increasing amounts of the Myc-RtsA whole-cell extract from 2 to 1,000 ng were included in the indicated reaction mixtures. The *hilA* DNA lane has no extract. The negative control (Neg Cntrl) lane contains 1,000 ng of whole-cell extract of the vector control strain. One hundred nanograms of anti-Myc antibody (Ab) was added to the indicated reaction mixtures. The strain used was MG1655 with either pBAD or pCE81.

(which lacks *hilC*, *hilD*, and *rtsA*) producing a Myc-RtsA fusion protein resulted in the altered migration of *hilA* DNA through the gel matrix. There was also a supershift, suggesting the presence of multiple RtsA binding sites similar to those seen with HilC binding to the *hilA* promoter region (64, 72). The addition of the anti-Myc antibody to the binding reactions containing Myc-RtsA causes a further retardation of the *hilA* DNA, showing that the shift is mediated directly by RtsA. Production of wild-type RtsA also shifts the *hilA* promoter region (data not shown). Thus, RtsA binds to the *hilA* promoter, suggesting that it directly activates expression.

RtsA can induce expression of *invF* independently of HilA, HilC, and HilD. RtsA induces expression of the *invF-invC* operon. Transcription of *invF* is known to be regulated by HilA, HilC, and HilD. To determine if RtsA-mediated induction was dependent on these regulators, we constructed *invF-lac* fusion strains with pBAD or pRtsA and either *hilA*, *hilCD*, or *hilCDA* deletions. Figure 4B shows that loss of HilC and HilD caused only a slight decrease in *invF* transcription. A deletion of *hilA* or *hilCDA* decreased but did not abolish RtsA induction of the *invF-lac*$^+$ fusion. This suggests that RtsA can induce expression of *invF* independent of HilA, similar to what has been observed for both HilC and HilD (2, 26, 67). Thus, RtsA-dependent induction of *invF* is apparently a combination of direct and indirect effects.

HilA and/or InvF is required for maximal RtsA induction of the MudJ fusions. RtsA induces expression of *hilA* and *invF*, whose products can directly or indirectly regulate expression of several genes encoded both within and outside SPI1. This information prompted us to test whether HilA and InvF were required for RtsA induction of the fusions identified in our screen. The MudJ fusions were transduced into strains containing pBAD or pRtsA and deletions of *hilA* or *invF*. As shown in Fig. 6, RtsA-dependent induction of *sopA* and *sopB* was significantly decreased, but not abolished, in the *hilA* deletion strain, whereas a deletion of *invF* completely blocked transcription of these fusions. This suggests that HilA regulates expression of these genes indirectly by activating expression of InvF, consistent with previous studies (2, 26, 67). Because RtsA can induce *invF* independently of HilA, some induction is still apparent in the *hilA* deletion strain.

Expression of the *invC* fusion was analogous to that observed in the *invF* fusion described above; these genes are in an operon. Indeed, there was an apparent decrease in *invC* expression observed in the *invF* deletion, but we believe that this is due to polarity of the *invF* deletion construct on the *invC* fusion; InvF is not known to autoregulate (19, 44). Interestingly, RtsA induction of *icgA* (located on SPI4) was completely abolished by a mutation in *hilA* but was unaffected by the *invF* mutation (Fig. 6). This suggests that HilA regulates expression of *icgA* in an InvF-independent manner, but it is not known whether this regulation is direct or indirect.

RtsA induces expression of *slrP* independently of HilA and InvF. We wanted to determine whether RtsA could induce expression of other SPI1 effectors. Expression of *slrP* is induced in a HilA-independent manner (61). It is also known that SlrP is secreted primarily by the SPI1 TTSS and is required for colonization of Peyer's patches in the mouse small intestine (61, 82). Figure 7A shows that when RtsA is produced from the arabinose promoter under SPI1-repressing conditions, expression of *slrP* is induced 20- to 30-fold compared to the vector-only control. However, in contrast to other effectors examined, RtsA induction of *slrP* is independent of HilA, InvF, and HilD or HilC (Fig. 7A). This suggests that RtsA may induce expression of a subset of SPI1 effector proteins that are not directly controlled by HilA or InvF.

RtsA is a better inducer of *slrP* expression than HilC or HilD. Our data suggest that HilC, HilD, and RtsA function in similar manners by binding to the same fragment of *hilA* to induce its expression. Therefore, we wanted to determine if HilC or HilD could also induce expression of *slrP*. To test this, we constructed strains containing Δ*rtsA* and Δ*hilC-D*::Cm mutations, either a *hilA-lac* or a *slrP-lac* fusion and pBAD, pRtsA, pLS118 (HilD), or pLS119 (HilC). Figure 7B shows that expression of *hilA* was induced ~40-fold by RtsA, ~20-fold by HilD, and ~120-fold by HilC. These levels are consistent with previous data (72). Interestingly, RtsA induces expression of *slrP* 45-fold, while HilD and HilC are able to induce expression of *slrP* only four- and fivefold, respectively (Fig. 7B). Simplistically this suggests that RtsA acts as a better inducer of *slrP* expression than does either HilC or HilD, at least compared to their ability to induce *hilA* expression.

Expression of *rtsA* is induced under SPI1-inducing conditions. It is clear that production of RtsA induces expression of the SPI1 invasion genes. We wanted to determine whether *rtsA*

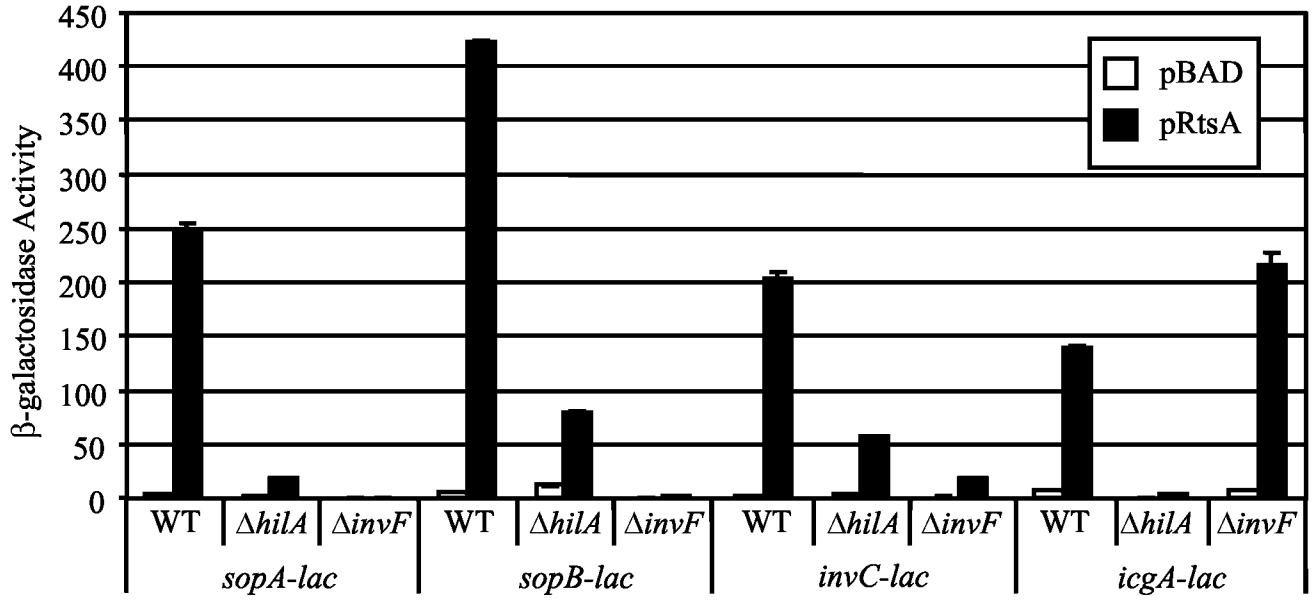

FIG. 6. Effect of *invF* and *hilA* mutations on RtsA induction of *sopA*, *sopB*, *invC*, and *icgA* fusions. The strains are Δ*rtsAB7*; contain pBAD30, pRtsA, pRtsB, or pRtsAB; and are Δ*hilA*::Cm or Δ*invF*::Cm. The stains contain a *lacZ* transcriptional fusion to the gene specified. Overnight cultures were subcultured into LB (without salt)–ampicillin–0.2% L-arabinose and grown to an OD_{600} of ~0.6 before assay of β-galactosidase. β-Galactosidase activity units are defined as (micromoles of ONP formed minute^{-1}) × 10^3/(OD_{600} × milliliters of cell suspension) and are reported as means ± standard deviations, where $n = 2$. The strains used were plasmid-containing derivatives of JS306 through JS317. WT, wild type.

and *rtsB* were induced under the same conditions as the SPI1 invasion genes. We constructed *rtsA-lac* and *rtsB-lac* fusions in an otherwise wild-type background (JS324 and JS325, respectively) and then assayed the β-galactosidase activity in these strains after growth under SPI1-repressing and SPI1-inducing conditions. The β-galactosidase activity produced by the *rtsA-lac* fusion under SPI1-repressing conditions was 1.39 ± 0.08 U. Under SPI1-inducing conditions, the β-galactosidase activity

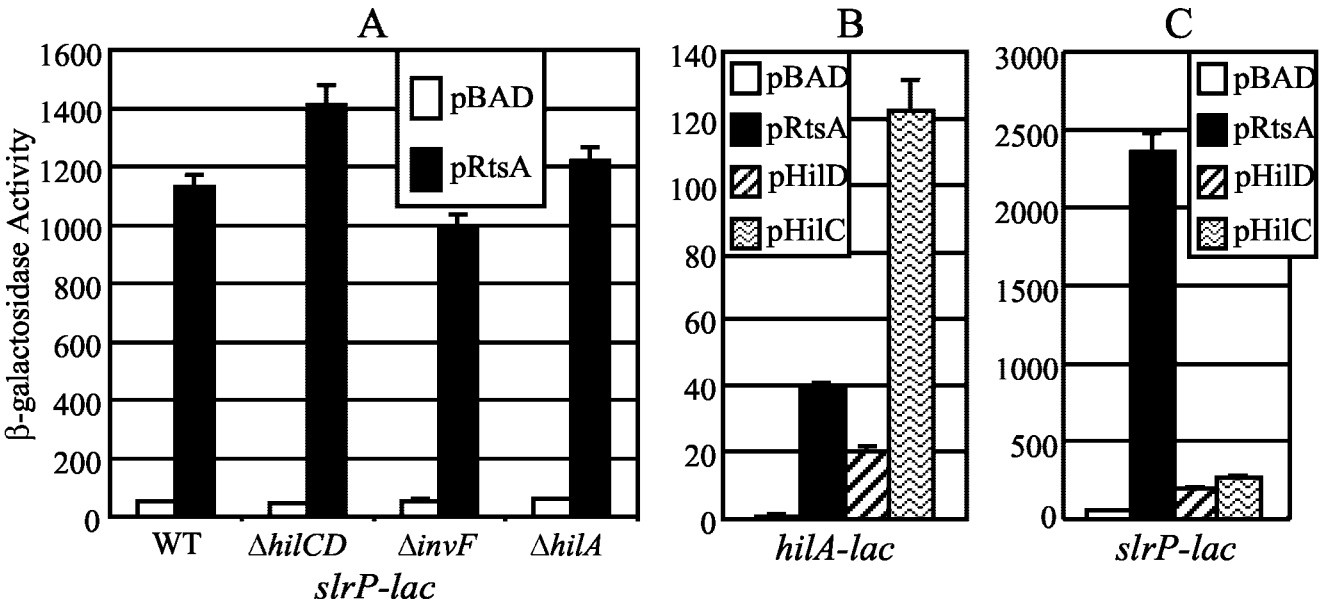

FIG. 7. Effect of *invF*, *hilA*, and *hilC-D* mutations on RtsA induction of *slrP* (A) and ability of HilC, HilD, and RtsA to induce expression of *hilA* (B) and *slrP* (C). (A) The strains are Δ*rtsAB7* and contain pBAD30 or pRtsA; Δ*hilC-D*::Cm, Δ*hilA*::Cm, or Δ*invF*::Cm; and an *slrP-lac* fusion. (B and C) The strains contain a *lac* transcriptional fusion with *hilA* and *slrP*, respectively, and the chromosomal Δ*rtsA* Δ*hilC-D*::Cm mutations along with the plasmid specified. pHilD is pLS118, and pHilC is pLS119 (72). Overnight cultures were subcultured into LB (without salt)–ampicillin–0.2% L-arabinose and grown to an OD_{600} of ~0.6 before assay of β-galactosidase. β-Galactosidase activity units are defined as (micromoles of ONP formed minute^{-1}) × 10^3/(OD_{600} × milliliters of cell suspension) and are reported as means ± standard deviations, where $n = 4$. The strains used were plasmid-containing derivatives of JS318 through JS323. WT, wild type.

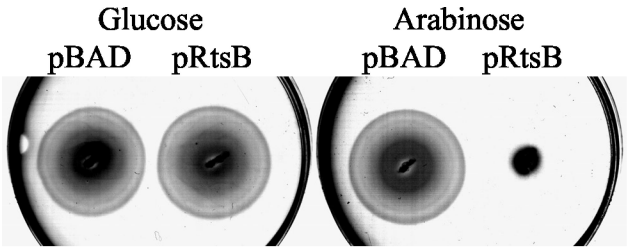

FIG. 8. Effect of RtsB production on motility. The strains are Δ*rtsAB7* (JS250) and contain pBAD30 or pRtsB. Motility assays were performed in plates with 0.3% agar supplemented with either 0.2% glucose or 0.2% L-arabinose.

increased to 21.65 ± 2.48 U. The β-galactosidase activity produced from the *rtsB-lac* fusion under SPI1-repressing conditions was 1.79 ± 0.44 U, versus 12.22 ± 0.86 U under SPI1-inducing conditions. Thus, the expression of *rtsA* and *rtsB* is significantly increased under SPI1-inducing conditions. Given that the *rtsAB* genes are separated by 11 bp and appear to be coordinately regulated, this suggests that they form an operon.

RtsB represses expression of the flagellin subunit gene *fliC* and regulatory genes *flhDC*. It appeared from our analysis of secreted proteins that strains producing RtsB had reduced levels of the flagellin subunit FliC/FljB in the culture supernatant (Fig. 1). To characterize this further, we tested the effect of RtsB production on motility by inoculating motility agar containing 0.2% glucose or 0.2% L-arabinose with strains containing either pRtsB or the pBAD vector alone. Both strains were motile in the presence of glucose. However, when grown in the presence of 0.2% L-arabinose, the strain containing pRtsB exhibited a significant motility defect compared to the control strain (Fig. 8).

To analyze the effect of RtsB on flagellar gene expression, we introduced a *fliC5050*::MudJ or *flhC5456*::MudJ fusion into strains containing pBAD, pRtsA, pRtsB, and pRtsAB. We then assayed the β-galactosidase activity of the resulting strains after growth for 2.5 h in the presence of 0.2% L-arabinose. As shown in Fig. 2B, increased production of RtsB, but not RtsA, decreased expression of the *fliC5050*::MudJ and *flhC5456*::MudJ fusions approximately 5.5-fold. Thus, the decreased FliC/FljB observed in the culture supernatant is due to transcriptional repression of the flagellin genes by RtsB. Moreover, RtsB apparently acts as a repressor of flagellar expression by repressing expression of the master regulators of the flagellar operon, *flhDC*. We also examined the effect of loss of *rtsA*, *rtsB*, and *rtsAB* deletion mutations on both *fliC-lac* and *flhC-lac* expression; there was no significant decrease under the conditions tested (data not shown).

RtsB directly binds to the *flhDC* promoter region. We wanted to determine whether repression of the flagellar master regulators was due to the direct binding of RtsB to the promoter region of *flhDC*. We performed gel shift assays with the *flhDC* promoter region and whole-cell extracts of *E. coli* strains (which otherwise lack *rtsB*) producing RtsB or Myc-RtsB. The Myc-RtsB construct represses expression of the *flhDC-lac* fusion (data not shown). Using whole-cell extracts of strains producing RtsB, we narrowed the region of the promoter that could be gel shifted to a 110-bp region corresponding to −4 to +106 relative to the start site of transcription (data not shown).

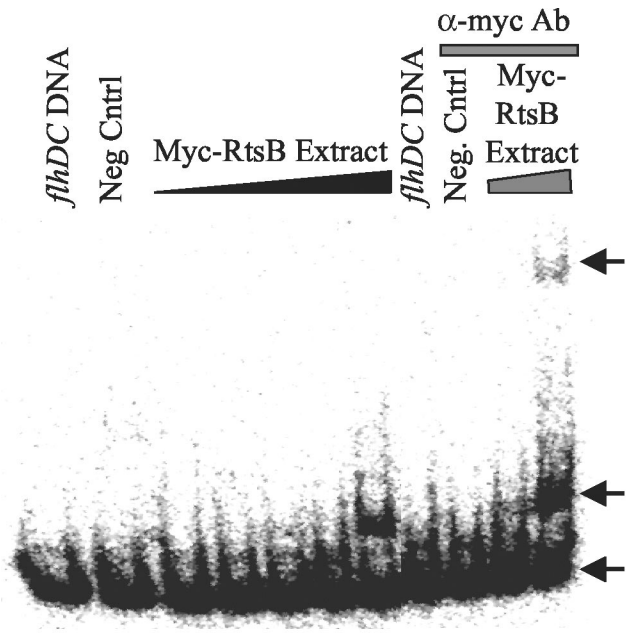

FIG. 9. Gel shift analysis of RtsB binding to the *flhDC* promoter region. Binding reaction mixtures contained ~0.1 ng of ^{32}P-labeled *flhDC* promoter region DNA corresponding to −4 to +106 bp from the start site of transcription. Increasing amounts of the Myc-RtsB whole-cell extract from 2 to 1,000 ng were included in the indicated reaction mixtures. The *flhDC* DNA lane has no extract. The negative control (Neg Cntrl) lane contains 1,000 ng of whole-cell extract of the vector control strain. One hundred nanograms of anti-Myc antibody (Ab) was added to the indicated reaction mixtures. The strain used was MG1655 with either pBAD or pCE82.

Figure 9 shows that increasing concentrations of whole-cell extract from strains producing Myc-RtsB caused a significant shift in the migration of this *flhDC* DNA. The addition of anti-Myc antibody to the binding reactions caused a further shift in the migration of the *flhDC* DNA, showing that the gel shift is due to direct binding of RtsB. This suggests that RtsB decreases motility and represses expression of flagellar genes by acting as a repressor of *flhDC*.

DISCUSSION

The ability of *S. enterica* serovar Typhimurium to sense its location within the host and respond by inducing and repressing expression of the appropriate virulence factors is critical to its survival. We have identified a regulatory operon on a *Salmonella*-specific island near tRNA^{PheU} that encodes RtsA and RtsB. Sequence analysis suggests that RtsA is a member of the AraC/XylS family of transcriptional regulators and is related to HilC and HilD. This homology is most significant in the carboxy-terminal third of the proteins, which contains the putative DNA binding domain (RtsA versus HilC, 56% identity and 76% similarity; RtsA versus HilD, 60% identity and 75% similarity; HilC versus HilD, 58% identity and 72% similarity). However, the N-terminal domains, which presumably act to sense some environmental parameter, also show limited homology (RtsA versus HilC, 25% identity and 42% similarity; RtsA versus HilD, 21% identity and 46% similarity; HilC versus HilD, 24% identity and 50% similarity).

RtsA, HilC, and HilD apparently function in very similar fashion. All three proteins bind to approximately the same region of the *hilA* promoter. The results of our gel shift assays suggest that RtsA binds within the region from −189 to +19 from the start site of transcription. Much like HilC, RtsA appears to bind to at least two sites within this region as evidenced by multiple bands in the gel shift (64). HilC and HilD are known to independently bind to two regions of the *hilA* promoter, from approximately −231 to −179 and from −101 to −49, although they differ in their sequence requirements for recognition of the binding sites (64, 72). RtsA also induces expression of both *hilC* and *hilD*. HilC and HilD have been shown to bind both the *hilC* and *hilD* promoters, and either protein can activate expression of the *hilC* promoter in vitro (64). RtsA can induce expression of *invF* in a HilA-independent fashion. Both HilC and HilD also directly activate *invF*, and it has been suggested that they activate a promoter located upstream of the HilA-dependent promoter P_{invF} (2). The homology in the DNA binding domains of these proteins and the fact that they apparently activate the same set of promoters suggest that they may function by binding to similar DNA sites to directly activate transcription.

Although these three proteins are similar, they can each act independently. RtsA induces expression of *hilA* in the absence of both HilC and HilD. It has also been shown that both HilD and HilC can independently induce expression of a *hilA-lac*$^+$ fusion in *E. coli* (72). RtsA is absent in this system. Why does serovar Typhimurium maintain three regulators of *hilA* that apparently perform the same function? We have three working hypotheses, which are not mutually exclusive: (i) RtsA, HilC, and HilD are active under different conditions or induce expression of *hilA* in response to different environmental cues. (ii) RtsA, HilC, and HilD differentially regulate expression of other genes independent of their effects on *hilA* expression. Indeed, it appears that RtsA, HilC and HilD are all capable of inducing expression of *hilA*, *hilC*, *hilD*, and *invF* but that RtsA is preferentially capable of inducing expression of *slrP*. This strongly supports a role for RtsA in expression of the SPI1 TTSS independent of its effects on *hilA*. (iii) RtsA, HilC, and HilD are required for signal amplification such that once one of these genes is turned on, expression of the others is also induced, resulting in greater induction of *hilA*. Regulation of *hilA* expression by RtsA, HilC, and HilD is an example of a feedforward loop. This type of regulatory cascade is common in yeast and presumably allows for increased sensitivity and/or tight temporal regulation (50). We believe that the inductions of RtsA, HilC, and HilD are self-reinforcing events leading to rapid and fully induced expression of *hilA*. Thus, this genetic switch is poised such that expression of the SPI1 TTSS is either essentially on or off.

The SPI4-carried gene *icgA* appears to encode a type 1 exported RTX pore-forming toxin or adhesin. SirA had previously been shown to induce expression of several SPI4 genes in a HilA-dependent manner, but the requirement for InvF in this induction was not determined (1). The previously identified fusions were within *icgA*, although at that time this region was incorrectly annotated (86). Here we show that RtsA induces expression of *icgA* in a HilA-dependent but InvF-independent manner. This suggests that HilA may directly regulate the expression of genes located outside SPI1 independently of InvF. It is not known what role IcgA plays during a serovar Typhimurium infection, but it is intriguing that a putative toxin or adhesin is coordinately regulated with the SPI1 invasion genes.

RtsB transcriptionally represses the flagellar operon by binding to and repressing expression of the *flhDC* promoter. Sequence analysis suggests that RtsB is a small protein (~10 kDa), which contains a helix-turn-helix DNA binding motif that most closely resembles those in the LuxR/GerE family. However, RtsB does not appear to contain any distinct regulatory domain. RtsB binds directly to the *flhDC* promoter region within a 110-bp region from −4 to +106 from the start site of transcription (87). Ectopic production of RtsB in *E. coli* also blocks motility (data not shown), suggesting that RtsB can repress the *E. coli flhDC* operon. The *E. coli* and serovar Typhimurium *flhDC* promoters have the most significant homology immediately downstream of the −10 region (77, 87). Thus, RtsB binding may include the major transcriptional start site, and this binding could sterically hinder RNA polymerase, thus explaining how such a seemingly simple protein could act as a regulator. Regulation could be mediated by controlling the level of RtsB rather than by some conformational change in the protein in response to a particular signal. RtsB also induces expression of at least one other gene, *yhgF*. However, it is not clear whether RtsB-mediated induction is direct or indirect.

Although flagella are not essential for virulence of serovar Typhimurium in BALB/c mice (52), they affect interaction with the host at multiple levels (33, 41, 53, 73). Several studies have identified the class 3 flagellar protein FliZ as an activator of *hilA* expression, seemingly antagonistic to the role of RtsB (25, 42, 56). There are conflicting data about the role of the flagella and flagellar regulators and expression of *hilA*. A *fliZ* mutation decreases expression of *hilA* 2-fold (25, 42, 56), while a *sirA* mutation decreases expression of *hilA* 10-fold and induces expression of the flagella 100-fold (35). This suggests that the relationship between expression of the flagella and the SPI1 TTSS is complex and will require further study. The FliC/FljB flagellin proteins interact with Toll-like receptor 5 on the basolateral surface of epithelial cells to activate the NF-κB pathway in epithelial cells (31). This results in secretion of interleukin-8 and production of human β-defensin 2 (32, 80). FliC has also been shown to be a major $CD4^+$ T-cell epitope in mice (60). However, strains that produce only FljB are attenuated during systemic infection in a mouse model (41).

Why does the cell want to upregulate expression of the SPI1 invasion genes while repressing expression of the flagellar genes? There are several possibilities. First, when serovar Typhimurium is invading epithelial cells, motility may be detrimental. The bacteria do not want to swim away from a host cell primed for invasion. Second, the flagellum is immunogenic and elicits a number of host responses (32, 60, 80). Therefore, when serovar Typhimurium is invading host tissue, it may be advantageous to stop producing the appendage. A problem with both of these arguments is that transcriptional repression of the flagellar genes would not be expected to immediately block motility or the immunological effects of preexisting flagella. Third, simultaneous production of SPI1 and the flagella could result in interference between the two TTSSs. Interference could manifest itself as misappropriate secretion of effectors or flagellin or more indirectly as increased periplasmic stress.

A number of genes have been shown to control expression of

hilA. However, in many cases the mechanism of *hilA* regulation is unknown. Future experiments must address the regulation of *rtsAB* in order to place RtsA, RtsB, and the other regulators into regulatory pathways that control invasion and flagellar gene regulation. Of the known regulators of the SPI1 TTSS and flagellar genes, SirA is of particular interest, as it is known to induce expression of *hilA* while repressing expression of *flhDC* (35). At this time it is unclear how SirA regulates these two different TTSSs. RtsA and RtsB are induced by SPI1-inducing conditions. Thus, these environmental parameters are sensed upstream of RtsA in the regulatory scheme. To ultimately understand how RtsA affects expression of *hilA* and the SPI1 invasion genes during an infection, the regulation of *rtsAB* must be studied in detail.

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