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RESEARCH ARTICLE

SlyA and HilD Counteract H-NS-Mediated Repression on the *ssrAB* Virulence Operon of *Salmonella enterica* Serovar Typhimurium and Thus Promote Its Activation by OmpR

María M. Banda,[a] Crispín Zavala-Alvarado,[a] Deyanira Pérez-Morales,[b] Víctor H. Bustamante[a]

[a]Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México
[b]CONACYT, Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México

ABSTRACT H-NS-mediated repression of acquired genes and the subsequent adaptation of regulatory mechanisms that counteract this repression have played a central role in the *Salmonella* pathogenicity evolution. The *Salmonella* pathogenicity island 2 (SPI-2) is an acquired chromosomal region containing genes necessary for *Salmonella enterica* to colonize and replicate in different niches of hosts. The *ssrAB* operon, located in SPI-2, encodes the two-component system SsrA-SsrB, which positively controls the expression of the SPI-2 genes but also other many genes located outside SPI-2. Several regulators have been involved in the expression of *ssrAB*, such as the ancestral regulators SlyA and OmpR, and the acquired regulator HilD. In this study, we show how SlyA, HilD, and OmpR coordinate to induce the expression of *ssrAB* under different growth conditions. We found that when *Salmonella enterica* serovar Typhimurium is grown in nutrient-rich lysogeny broth (LB), SlyA and HilD additively counteract H-NS-mediated repression on *ssrAB*, whereas in N-minimal medium (N-MM), SlyA antagonizes H-NS-mediated repression on *ssrAB* independently of HilD. Interestingly, our results indicate that OmpR is required for the expression of *ssrAB* independently of the growth conditions, even in the absence of repression by H-NS. Therefore, our data support two mechanisms adapted for the expression of *ssrAB* under different growth conditions. One involves the additive action of SlyA and HilD, whereas the other involves SlyA, but not HilD, to counteract H-NS-mediated repression on *ssrAB*, thus favoring in both cases the activation of *ssrAB* by OmpR.

IMPORTANCE The global regulator H-NS represses the expression of acquired genes and thus avoids possible detrimental effects on bacterial fitness. Regulatory mechanisms are adapted to induce expression of the acquired genes in particular niches to obtain a benefit from the information encoded in the foreign DNA, as for pathogenesis. Here, we show two mechanisms that were integrated for the expression of virulence genes in *Salmonella* Typhimurium. One involves the additive action of the regulators SlyA and HilD, whereas the other involves SlyA, but not HilD, to counteract H-NS-mediated repression on the *ssrAB* operon, thus favoring its activation by the OmpR regulator. To our knowledge, this is the first report involving the coordinated action of two regulators to counteract H-NS-mediated repression.

KEYWORDS H-NS, HilD, OmpR, SPI, *Salmonella*, SlyA, transcriptional regulation, *ssrAB*

Salmonella enterica serovar Typhimurium, a facultative intracellular pathogen, generally causes mild self-limiting gastroenteritis in humans and several animals, but it can also produce severe systemic infections in different hosts, including humans (1, 2). Thus, *S.* Typhimurium has been extensively used as a model for studying the molecular mechanisms governing *S. enterica* virulence (1, 3, 4).

Around one-quarter of the *S.* Typhimurium genome was shaped by the gain of DNA

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Address correspondence to Víctor H. Bustamante, victor@ibt.unam.mx.

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through several horizontal gene transfer (HGT) events that occurred at different evolutionary times (5). The *Salmonella* pathogenicity island 2 (SPI-2) is an acquired chromosomal region containing 44 genes which encode a type III secretion system (T3SS-2), their chaperones and effector proteins, and the two-component system SsrA-SsrB (4). The SPI-2 genes are required for survival and replication of *S.* Typhimurium inside host cells, such as macrophages, which leads to the systemic disease (1, 4). Additionally, the SPI-2 genes contribute to the induction of the intestinal inflammatory response (6–8). Consistently, the SPI-2 genes are expressed when *S.* Typhimurium is within host cells and in the intestinal lumen (9–15). *In vitro*, the expression of SPI-2 is induced when *S.* Typhimurium is grown in minimal medium containing low concentrations of phosphate, calcium, and magnesium (11, 16, 17), as well as during the late-stationary phase of growth in nutrient-rich medium (18, 19), conditions that somehow resemble the intracellular and the intestinal lumen environments, respectively.

The *ssrA* and *ssrB* genes, located in SPI-2, code for the two-component system SsrA-SsrB, with SsrA being the sensor kinase and SsrB the response regulator, which directly induces the expression of the SPI-2 genes and many other virulence genes located outside SPI-2 (4, 20–22). The *ssrA* and *ssrB* genes are transcribed as an operon (18); also, transcription of *ssrB* independent of *ssrA* has been reported (17, 23), which seems to be dependent on the growth conditions tested. To simplify, these genes are referred to here as the *ssrAB* operon. Multiple regulators have been involved in the expression of *ssrAB*, including SlyA, HilD, OmpR, PhoP, and SsrB, which act positively and directly on this operon (4, 23–29). SlyA, OmpR, and PhoP are transcriptional regulators that control the expression of a large number of genes, encoding distinct cellular functions in different bacteria; SlyA is a member of the MarR family of transcriptional factors, whereas OmpR and PhoP are the response regulators of the two-component systems EnvZ-OmpR and PhoQ-PhoP, respectively (24, 27, 30–40). HilD is an AraC-like transcriptional regulator, present only in *Salmonella* spp. and encoded in the *Salmonella* pathogenicity island 1 (SPI-1), which controls the expression of the SPI-1 genes and many other virulence genes (4, 18, 19, 41–51). On the other hand, the expression of *ssrAB* is also controlled by negative regulators, such as the nucleoid-associated protein H-NS (18, 19, 52–54), which acts as a global transcriptional factor in many bacteria (55, 56). H-NS is considered a genome sentinel that has played an important role during the evolution of *Salmonella* pathogenicity by preventing uncontrolled expression of acquired DNA that could be deleterious to bacterial fitness (52, 53, 56). Different regulatory proteins have been adapted to antagonize H-NS-mediated repression in specific promoters, which allows the expression of acquired genes only under those conditions where the encoded information is beneficial for bacteria, as in particular niches during infection of a host (57, 58). For instance, HilD induces the expression of *ssrAB* by directly displacing H-NS-mediated repression on the promoter upstream of *ssrA* under *in vitro* growth conditions that resemble the intestinal environment (18, 19).

In this work, we determine how the ancestral regulators SlyA and OmpR and the acquired regulator HilD induce the expression of the *S.* Typhimurium *ssrAB* operon under different growth conditions. During growth in nutrient-rich lysogeny broth (LB), SlyA and HilD additively counteract H-NS-mediated repression on *ssrAB*, whereas during growth in N-minimal medium (N-MM), SlyA antagonizes the H-NS-mediated repression on *ssrAB* independently of HilD. In both cases, the expression of *ssrAB* also requires the action of OmpR, even in the absence of the repression by H-NS.

RESULTS

SlyA is required for the expression of *ssrAB* under both rich and minimal growth conditions. The *ssrAB* operon, and thus the SPI-2 genes, are expressed when *S.* Typhimurium is grown in nutrient-rich media, such as LB, as well as in minimal media like N-MM (18, 19), which mimic different niches that *S.* Typhimurium finds in its hosts. Several studies have shown that SlyA is required for the expression of *ssrAB* in minimal medium (42, 59). Additionally, some reports indicate that overexpression of SlyA

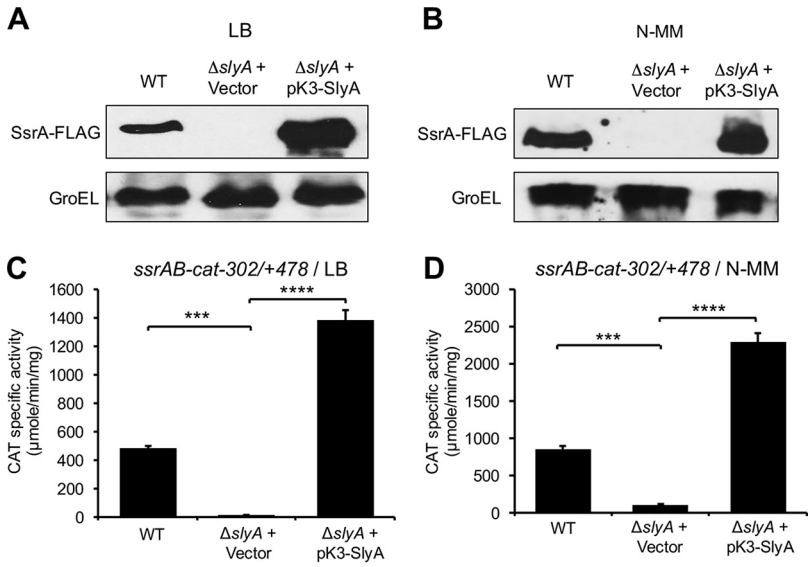

FIG 1 SlyA is required for expression of the *ssrAB* operon in LB and N-MM. (A and B) Expression of SsrA-FLAG in WT *S.* Typhimurium and its Δ*slyA* derivative mutant containing the pMPM-K3 vector or the pK3-SlyA plasmid that expresses SlyA from a constitutive promoter was analyzed by Western blotting using monoclonal anti-FLAG antibodies. Whole-cell lysates were prepared from samples of bacterial cultures grown for 9 h in LB (A) or for 16 h in N-MM at pH 7.4 (B) at 37°C. As a control, the expression of GroEL was also determined using polyclonal anti-GroEL antibodies. (C and D) Expression of the *ssrAB-cat−302/+478* transcriptional fusion carried by the pssrAB-cat-302/+478 plasmid was tested in WT *S.* Typhimurium and its isogenic Δ*slyA* mutant containing the pMPM-K3 vector or the pK3-SlyA plasmid. CAT-specific activity was determined from samples of bacterial cultures grown for 9 h in LB (C) or for 16 h in N-MM at pH 7.4 (D) at 37°C. Data represent the mean with standard deviation from the results from three independent experiments performed in duplicate. Statistically different values are indicated (***, $P < 0.001$; ****, $P < 0.0001$).

induces the expression of *ssrAB* in LB (25, 28). To confirm whether physiological levels of SlyA regulate the expression of *ssrAB* in LB, we examined the chromosomal expression of the C-terminal 3×FLAG-tagged SsrA protein (SsrA-FLAG) in the wild-type (WT) *S.* Typhimurium strain SL1344 and its isogenic Δ*slyA* mutant, grown in LB at 37°C. As a control, the expression of SsrA-FLAG was also analyzed in these same strains grown in N-MM. In both LB and N-MM, SsrA-FLAG was detected in the WT strain but not in the Δ*slyA* mutant; as expected, the pK3-SlyA plasmid expressing SlyA, but not the control vector pMPM-K3, restored the expression of SsrA-FLAG in the Δ*slyA* mutant at levels even higher than those reached in the WT strain (Fig. 1A and B). To confirm these results, similar assays were performed by analyzing the expression of the *ssrAB-cat−302/+478* transcriptional fusion (*ssrAB* sequence from −302 to +478 and the *cat* reporter gene) that we have successfully used before (19), which carries the promoter upstream of *ssrA*. In agreement with our results obtained by assessing SsrA-FLAG, the activity of the *ssrAB-cat−302/+478* fusion was drastically reduced in the Δ*slyA* mutant with respect to the WT strain, and it was induced in the presence of the pK3-SlyA plasmid (Fig. 1C and D). Taken together, these results show that SlyA is required for the expression of *ssrAB* under different growth conditions.

SlyA counteracts repression exerted by H-NS on *ssrAB* during growth in LB. Previous studies indicate that SlyA induces gene expression mainly by counteracting the repression exerted by H-NS on target promoters (33, 39). Furthermore, we have shown that H-NS directly represses the expression of *ssrAB* when *S.* Typhimurium is grown in LB (18, 19). Therefore, in order to determine if SlyA counteracts H-NS-mediated repression on *ssrAB*, during growth in LB, we tested whether SlyA is required for the expression of *ssrAB* in the absence of H-NS activity. The *S.* Typhimurium Δ*hns* mutant shows severe growth defects (53); however, activity of WT H-NS can be inactivated by overexpressing the H-NS^{G113D} mutant, which does not have DNA binding activity but still forms heterodimers with WT H-NS monomers (60) and thus

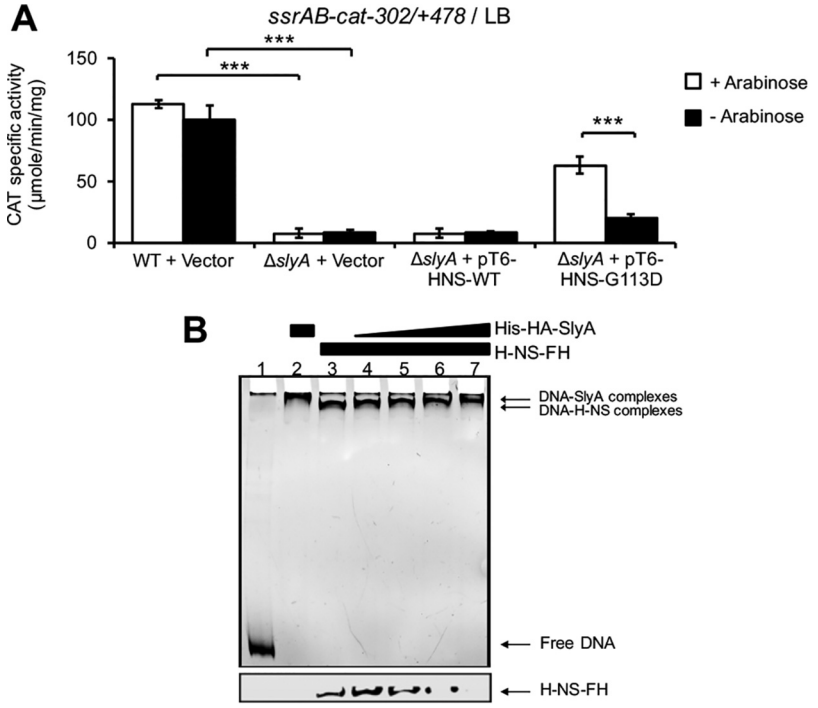

FIG 2 SlyA directly displaces H-NS-mediated repression on *ssrAB* during growth in LB. (A) Expression of the *ssrAB-cat−302/+478* transcriptional fusion carried by the pssrAB-cat-302/+478 plasmid was tested in WT *S.* Typhimurium and its isogenic Δ*slyA* mutant containing or not containing the pMPM-T6Ω vector or the pT6-HNS-WT or pT6-HNS-G113D plasmid, which expresses WT H-NS or the dominant negative H-NS^{G113D} mutant, respectively, from an arabinose-inducible promoter. CAT-specific activity was determined from samples of bacterial cultures grown for 9 h in LB at 37°C. L-Arabinose (0.1%) was added (+) or not added (−) to the medium for inducing the expression of WT H-NS and H-NS^{G113D} from pT6-HNS-WT and pT6-HNS-G113D, respectively. Data represent the mean with standard deviation of the results from three independent experiments performed in duplicate. Statistically different values are indicated (***, $P < 0.001$). (B) Competitive nonradioactive EMSAs between H-NS and SlyA on the −302/+478 region of *ssrAB*. Purified H-NS−FH protein was added at 0.5 μM (lanes 3 to 7), and purified His-HA-SlyA protein was added at 1, 2, 2.5, and 3 μM (lanes 4 to 7, respectively). No proteins were added in lane 1, and His-HA-SlyA was added at 3 μM in lane 2. The DNA-protein complexes were resolved in a nondenaturing 6% polyacrylamide gel. Top, protein-DNA complexes stained with ethidium bromide; bottom, immunoblot detection of H-NS−FH from the DNA-protein complexes. Similar results were obtained from three different experiments.

acts as a dominant negative mutant (61). In this way, the expression of the *ssrAB-cat−302/+478* fusion was assessed in the WT *S.* Typhimurium strain and its derivative Δ*slyA* mutant containing the empty vector pMPM-T6Ω, or the pT6-HNS-WT or pT6-HNS-G113D plasmids, which express WT H-NS and the H-NS^{G113D} mutant, respectively, from an arabinose-inducible promoter (44, 61). The strains were grown in LB at 37°C in the presence or absence of 0.1% arabinose to induce or not induce the expression of the H-NS proteins. As shown in Fig. 2A, overexpression of H-NS^{G113D}, but not WT H-NS, induced the activity of the *ssrAB-cat−302/+478* fusion in the Δ*slyA* mutant strain, indicating that the overproduction of the dominant negative H-NS mutant partially suppresses the need of SlyA for the expression of *ssrAB*. In similar experiments, we previously showed that overproduction of the H-NS^{Q92am} dominant negative mutant also suppresses the need of HilD for the expression of *ssrAB* (19).

To investigate if SlyA counteracts directly the repression of H-NS on *ssrAB*, we performed competitive electrophoretic mobility shift assays (EMSAs) to examine the effect of SlyA on H-NS bound to the region −302/+478 of *ssrAB*. A DNA fragment spanning this region was first incubated with a constant concentration of purified H-NS-FLAG-His (H-NS−FH) protein, and then increasing amounts of purified His-HA-SlyA protein were added. The DNA-H-NS and DNA-SlyA complexes were detected by staining the DNA fragments. Furthermore, the presence of H-NS−FH or His-HA-SlyA on

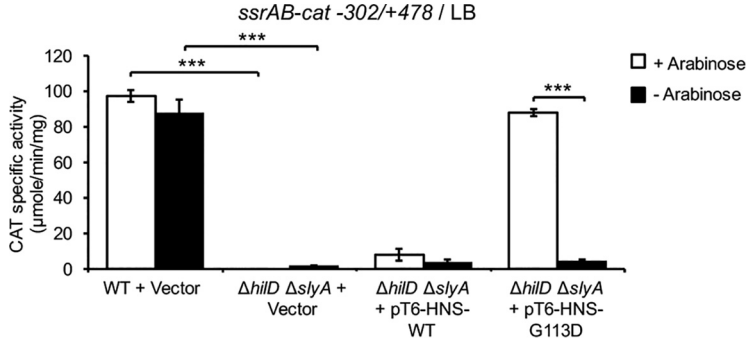

FIG 3 HilD and SlyA are not required for expression of *ssrAB* during growth in LB when H-NS is inactivated. Expression of the *ssrAB-cat−302/+478* transcriptional fusion carried by the pssrAB-cat-302/+478 plasmid was determined in the WT *S.* Typhimurium strain and its derivative Δ*hilD* Δ*slyA* mutant containing the pMPM-T6Ω vector, as well as in the Δ*hilD* Δ*slyA* mutant containing the pT6-HNS-WT or pT6-HNS-G113D plasmid, which expresses WT H-NS or the H-NS^{G113D} dominant negative mutant, respectively, from an arabinose-inducible promoter. CAT-specific activity was determined from samples of bacterial cultures grown for 9 h in LB at 37°C. Expression of WT H-NS or H-NS^{G113D} from plasmids pT6-HNS-WT and pT6-HNS-G113D, respectively, was induced by adding 0.1% L-arabinose to the medium (+ arabinose). Data represent the mean with standard deviation of the results from three independent experiments done in duplicate. Statistically different values are indicated (***, $P < 0.001$).

these complexes was monitored by immunoblotting with anti-FLAG and anti-HA antibodies, respectively. As shown in Fig. 2B, increasing amounts of His-HA-SlyA shifted the DNA-H-NS−FH complex to a slower-migrating complex, similar to that formed by only His-HA-SlyA; additionally, the immunoblots indicated that increasing amounts of His-HA-SlyA correlate with decreasing amounts of H-NS−FH bound to the DNA fragments. We were unable to detect His-HA-SlyA bound to the DNA fragments, probably due to a low sensitivity of the anti-HA antibodies used (data not shown).

In all, these results show that SlyA counteracts H-NS-mediated repression on *ssrAB* by directly displacing H-NS from the region −302/+478.

SlyA and HilD additively antagonize repression of H-NS on *ssrAB* during growth in LB. In previous studies, we demonstrated that HilD also induces the expression of *ssrAB* during the growth of *S.* Typhimurium in LB by antagonizing H-NS-mediated repression (18, 19). To confirm that both SlyA and HilD act as antirepressors of H-NS to induce the expression of *ssrAB*, we analyzed if the inactivation of H-NS leads to the expression of *ssrAB* independently of both SlyA and HilD. For this, we determined the expression of the *ssrAB-cat−302/+478* fusion in the WT *S.* Typhimurium strain and its derivative Δ*hilD* Δ*slyA* double mutant containing the empty vector pMPM-T6Ω or the plasmids pT6-HNS-WT or pT6-HNS-G113D in cultures grown in LB at 37°C. As could be expected, the activity of the *ssrAB-cat−302/+478* fusion was drastically reduced in the Δ*hilD* Δ*slyA* mutant; in addition, overexpression of the H-NS^{G113D} dominant negative mutant, but not WT H-NS, restored the activity of the *ssrAB-cat−302/+478* fusion in this strain (Fig. 3). These results demonstrate that when the activity of H-NS is inactivated, the expression of *ssrAB* becomes independent of both SlyA and HilD.

We next analyzed the expression of *ssrAB* in the absence of each SlyA or HilD, or both, when the repression of *ssrAB* by H-NS is blocked or not blocked. For this, the activities of the *ssrAB-cat−302/+478* fusion and its derivative *ssrAB-cat−302/+10* fusion were determined in the WT *S.* Typhimurium strain and its isogenic Δ*hilD*, Δ*slyA,* and Δ*hilD* Δ*slyA* mutants grown in LB. The *ssrAB-cat−302/+10* fusion lacks H-NS-binding sites required for the repression by H-NS; repression of *ssrAB* by H-NS involves the H-NS-binding sites located on the promoter as well as those located on the translational start codon of *ssrA* (Fig. 4) (19). Since OmpR is required for the expression of *ssrAB* in LB, even in the absence of the H-NS activity (18), a Δ*ompR* mutant was assessed as control in these assays.

The activity of the *ssrAB-cat−302/+478* fusion (repressed by H-NS) was reduced around 4-fold in the Δ*hilD* and Δ*slyA* mutants, with respect to the WT strain; interest-

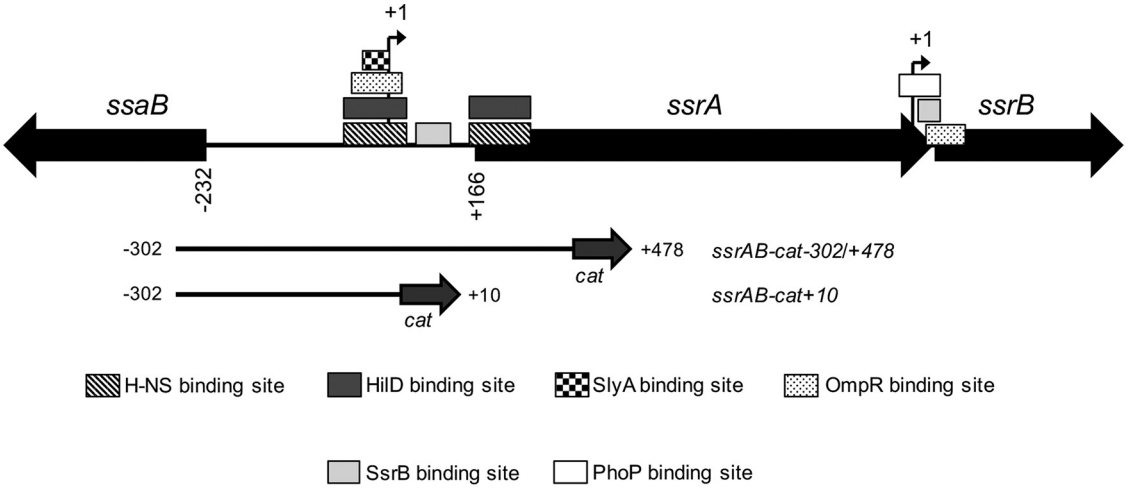

FIG 4 Schematic representation of *ssrAB* and the *ssrAB-cat* transcriptional fusions analyzed. The transcriptional start sites (tss) (+1) upstream *ssrA* and *ssrB* are indicated by a bent arrow. HilD-, OmpR-, SlyA-, SsrB-, PhoP- and H-NS-binding sites on *ssrAB*, which have been previously reported (19, 23–26, 28), are indicated. The DNA fragments carried by the *ssrAB-cat−302/+478* and *ssrAB-cat+10* transcriptional fusions are shown. All positions indicated are relative to the tss upsteam of *ssrA*.

ingly, the Δ*hilD* Δ*slyA* double mutant showed a higher reduction (40-fold) in this activity (Fig. 5A) compared with the Δ*hilD* or Δ*slyA* single mutants, supporting the idea that SlyA and HilD have an additive effect on *ssrAB*. On the other hand, the activities of the *ssrAB-cat−302/+10* fusion (not repressed by H-NS) were similar in the WT strain and its isogenic Δ*hilD*, Δ*slyA,* and Δ*hilD* Δ*slyA* mutants (Fig. 5B), whereas the activities of

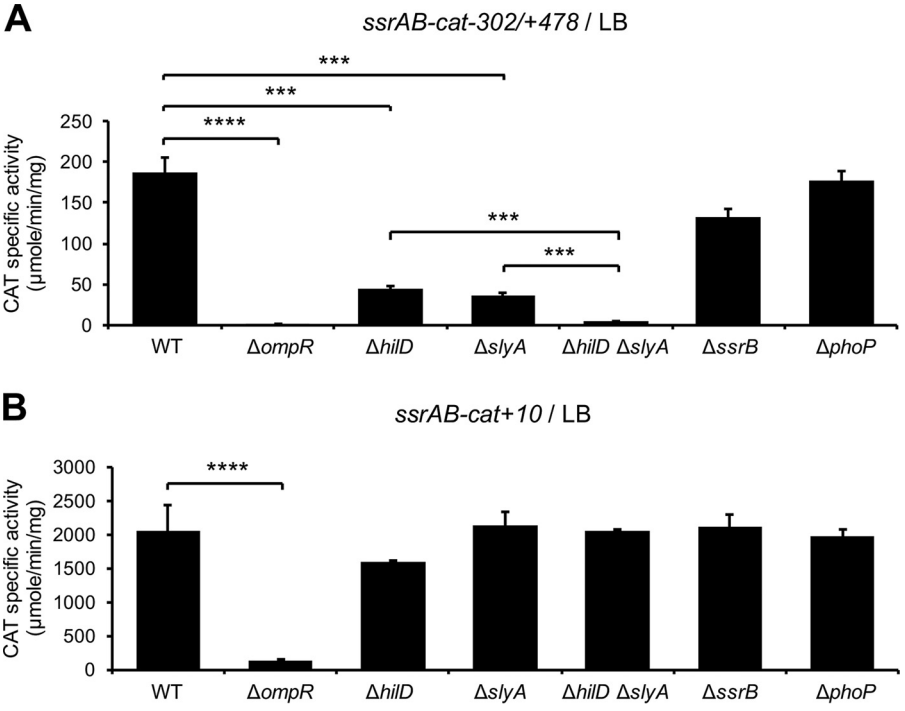

FIG 5 HilD and SlyA act additively as anti-H-NS factors, whereas OmpR acts independently of H-NS, to induce expression of *ssrAB* during growth in LB. Expression of the *ssrAB-cat−302/+478* (A) and *ssrAB-cat+10* (B) transcriptional fusions carried by the pssrAB-cat-302/+478 and pssrAB-cat+10 plasmids, respectively, was tested in WT *S.* Typhimurium strain and its isogenic Δ*ompR*, Δ*hilD*, Δ*slyA*, Δ*hilD* Δ*slyA,* Δ*ssrB,* and Δ*phoP* mutants. CAT-specific activity was determined from samples of bacterial cultures grown for 9 h in LB at 37°C. Data represent the mean with standard deviation of the results from three independent experiments performed in duplicate. Statistically different values are indicated (***, $P < 0.001$; ****, $P < 0.0001$).

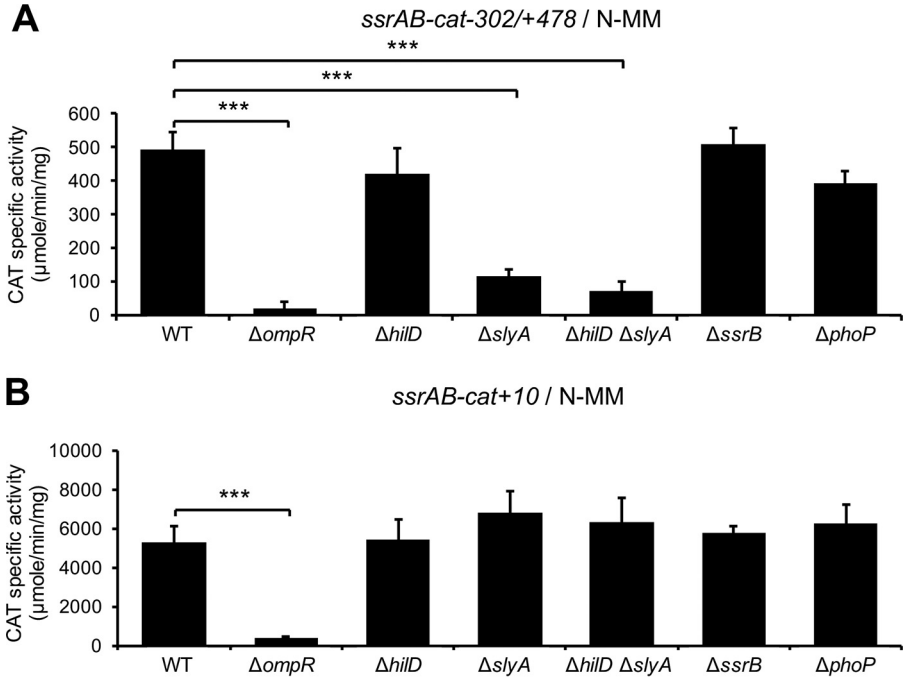

FIG 6 SlyA, but not HilD, acts as an anti-H-NS factor, whereas OmpR acts independently of H-NS, to induce expression of *ssrAB* during growth in N-MM. Expression of the *ssrAB-cat−302/+478* (A) and *ssrAB-cat+10* (B) transcriptional fusions carried by the pssrAB-cat-302/+478 and pssrAB-cat+10 plasmids, respectively, was determined in the WT *S.* Typhimurium strain and its isogenic Δ*ompR*, Δ*hilD*, Δ*slyA*, Δ*hilD* Δ*slyA,* Δ*ssrB,* and Δ*phoP* mutants. The CAT-specific activity was determined from samples of bacterial cultures grown for 6 h in N-MM at pH 5.8 and 37°C. Data represent the mean with standard deviation of the results from three independent experiments performed in duplicate. Statistically different values are indicated (***, $P < 0.001$).

the two fusions analyzed were severely reduced in the Δ*ompR* mutant (Fig. 5). These results confirm that SlyA and HilD act on *ssrAB* by counteracting H-NS-mediated repression and further support the idea that OmpR acts on *ssrAB* as a classical activator.

Collectively, these results indicate that SlyA and HilD act additively to counteract H-NS-mediated repression on *ssrAB* during the growth of *S.* Typhimurium in LB, which would favor the activation of *ssrAB* by OmpR.

SlyA, but not HilD or OmpR, counteracts H-NS-mediated repression on *ssrAB* during growth in N-MM. In order to investigate if SlyA also antagonizes repression of *ssrAB* by H-NS in N-MM, a growth condition where HilD is not involved in the expression of *ssrAB* (18, 19), the activities of the *ssrAB-cat−302/+478* and *ssrAB-cat−302/+10* fusions were tested in the WT *S.* Typhimurium strain and the Δ*slyA* mutant, grown in N-MM at pH 5.8 and 37°C, growth conditions that somehow resemble the intracellular environment where *S. enterica* survives (11). As controls, the Δ*hilD* and Δ*hilD* Δ*slyA* mutants were also assessed in these assays. The activity of the *ssrAB-cat−302/+478* fusion (repressed by H-NS), but not that of the *ssrAB-cat−302/+10* fusion (not repressed by H-NS), was similarly decreased in the Δ*slyA* and Δ*hilD* Δ*slyA* mutants compared with the WT strain (Fig. 6). As expected, the activities of these fusions were not affected in the Δ*hilD* mutant (Fig. 6). Nearly the same results were obtained using N-MM at pH 7.4 (data not shown).

These results show that SlyA, but not HilD, counteracts the repression of *ssrAB* by H-NS during growth in N-MM.

On the other hand, OmpR is also required for the expression of *ssrAB* in N-MM (18); however, it is unknown whether, under these growth conditions, OmpR is still needed for the expression of *ssrAB* in the absence of repression by H-NS. Therefore, to determine this, we tested the expression of the *ssrAB-cat−302/+478* and *ssrAB-*

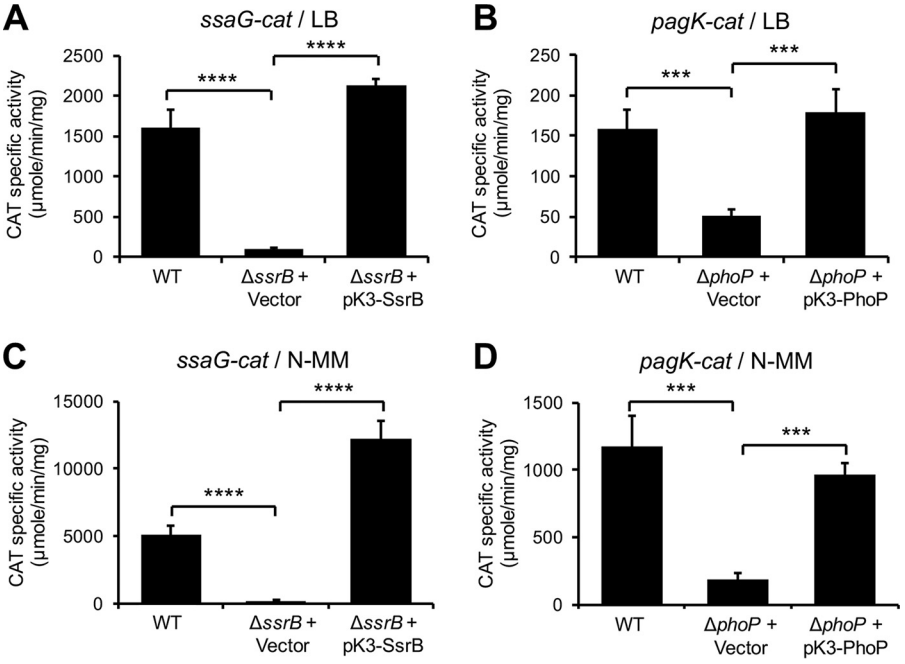

FIG 7 Positive controls for *cat* reporter assays in the Δ*ssrB* and Δ*phoP* mutants. (A and C) Expression of the *ssaG-cat* transcriptional fusion carried by the pssaG-cat plasmid was determined in the WT *S.* Typhimurium strain and its isogenic Δ*ssrB* mutant containing the pMPM-K3 vector or the pK3-SsrB plasmid that expresses SsrB from a constitutive promoter. (B and D) Expression of the *pagK-cat* transcriptional fusion carried by the ppagK-cat plasmid was determined in the WT *S.* Typhimurium strain and its isogenic Δ*phoP* mutant containing the pMPM-K3 vector or the pK3-PhoP plasmid that expresses PhoP from a constitutive promoter. The CAT-specific activity was determined from samples of bacterial cultures grown for 9 h in LB (A and B) or 6 h in N-MM (C and D) at pH 5.8 and 37°C. Data represent the mean with standard deviation of the results from three independent experiments performed in duplicate. Statistically different values are indicated (***, $P < 0.001$; ****, $P < 0.0001$).

cat−302/+10 fusions in the Δ*ompR* mutant grown in N-MM at pH 5.8. As shown in Fig. 6, the activities of both fusions were drastically reduced in the Δ*ompR* mutant with respect to the WT strain. Similar results were obtained using N-MM at pH 7.4 (data not shown).

These results indicate that OmpR is required for the expression of *ssrAB* during growth in N-MM, independently of the repression by H-NS.

PhoP and SsrB are not involved in the transcription of *ssrAB* coordinated by SlyA, HilD, and OmpR. PhoP and SsrB seem to have no major effect on the promoter located upstream of *ssrA* but are required for the transcription of an additional promoter on *ssrAB*, located upstream of *ssrB* (23, 26), which is not contained in the *ssrAB* transcriptional fusions used in our study (Fig. 4). To discard a possible role of these regulators on the transcription of *ssrAB* mediated by SlyA, HilD, and OmpR, under the conditions tested in this work, where the *ssrA* and *ssrB* genes are transcribed as an operon (18), the activities of the *ssrAB-cat−302/+478* and *ssrAB-cat−302/+10* fusions were now analyzed in the Δ*ssrB* and Δ*phoP* mutants grown in LB or N-MM at pH 5.8. As shown in Fig. 5 and 6, the expression of these fusions was not affected in the Δ*ssrB* and Δ*phoP* mutants with respect to the WT strain. As controls for these assays, the expression of the *ssaG-cat* and *pagK-cat* transcriptional fusions, which is dependent on SsrB (62) and PhoP (44), respectively, was also analyzed in the respective Δ*ssrB* or Δ*phoP* mutant. As expected, the expression of *ssaG-cat* and *pagK-cat* decreased specifically by the absence of SsrB or PhoP, respectively, in both LB and N-MM (Fig. 7).

Together, these results indicate that PhoP and SsrB are not involved in the coordinated regulation of *ssrAB* revealed in this study, mediated by SlyA, HilD, and OmpR.

DISCUSSION

Evolution of *Salmonella* pathogenicity has involved the adaptation of regulatory mechanisms for a tight control of the expression of virulence genes acquired through horizontal transfer. Different studies have shown that the nucleoid-associated protein H-NS plays a major role in these mechanisms by acting as a global transcriptional repressor (4, 52, 53, 55, 57, 63). For instance, H-NS represses the expression of SPI-1 and SPI-2, two chromosomal regions acquired in different evolutionary times which contain genes with essential roles for the *Salmonella* pathogenicity (1, 52, 53, 64, 65). Therefore, the expression of most acquired genes requires transcriptional regulators that antagonize the repression by H-NS.

SlyA, HilD, and OmpR have been involved in the expression of the *ssrAB* regulatory operon located in SPI-2, HilD by antagonizing H-NS-mediated repression (18, 19) and SlyA and OmpR by until-now unknown mechanisms, although it has been reported that the control of *ssrAB* expression by OmpR requires relaxation of DNA supercoiling (66). In this study, we show how these three regulators cooperate to induce the expression of *ssrAB* in LB and N-MM, two *in vitro* growth conditions that somehow mimic different niches that *S. enterica* colonizes in hosts, the intestinal and intracellular environments, respectively.

Previous studies have demonstrated that SlyA induces the expression of *ssrAB* and several other genes during growth in minimal medium (27, 42, 59). We found that SlyA induces the expression of *ssrAB* during growth in LB, which is consistent with previous reports indicating that the overexpression of SlyA induces the expression of *ssrAB* in LB (25, 28), as well as with results from a previous transcriptomic analysis supporting the idea that SlyA positively regulates *ssrAB* and several other genes during growth in LB (36). These data strongly suggest that SlyA is present and active under different growth conditions. Accordingly, the *slyA* gene is expressed during growth in rich and minimal media (17, 36, 67, 68). Furthermore, SlyA is required for the expression of the *grhD1* virulence gene in both LB and N-MM (44). Our results show that SlyA induces the expression of *ssrAB* during growth in LB by counteracting H-NS-mediated repression. Previously, we reported that HilD also induces the expression of *ssrAB* in LB by counteracting H-NS-mediated repression (19). *In vitro*, both SlyA and HilD can independently displace H-NS from the promoter of *ssrAB*. However, our results indicate that the additive action of SlyA and HilD is required to antagonize the repression of *ssrAB* by H-NS during growth in LB. To our knowledge, this is the first report indicating a concerted action of SlyA and HilD to induce gene expression, and it is the first showing an additive action of two different regulators to antagonize H-NS-mediated repression.

Our results indicate that SlyA also induces the expression of *ssrAB* during growth in N-MM by counteracting H-NS-mediated repression. Interestingly, HilD is not required for the expression of *ssrAB* in N-MM (18, 19). It is tempting to speculate that another transcriptional factor replaces the action of HilD during growth in N-MM. Alternatively, a different H-NS repressor complex on *ssrAB* could be formed during growth in N-MM, probably caused by local changes in DNA structure, which could be displaced by only SlyA.

Our data show that even when the repression by H-NS is displaced, the expression of *ssrAB* requires OmpR. Several studies support the idea that OmpR mainly acts as a classical transcriptional activator on *ssrAB* and many other genes (18, 23–25, 66); classical activators favor RNA polymerase binding on promoters (69). Therefore, the expression of *ssrAB* mediated by SyA, HilD, and OmpR would involve two steps, as follows: the relief of H-NS-mediated repression by SlyA and HilD or only SlyA and the recruitment of the RNA polymerase by OmpR (Fig. 8). Similarly, expression of the *ugtL* and *pagC* genes of *S. enterica* first requires the action of SlyA to counteract H-NS-mediated repression and then the action of the PhoP classical activator that recruits the RNA polymerase on the promoters of these genes (38). The results from our study illustrate the integration of ancestral (H-NS, SlyA, and OmpR) and previously acquired

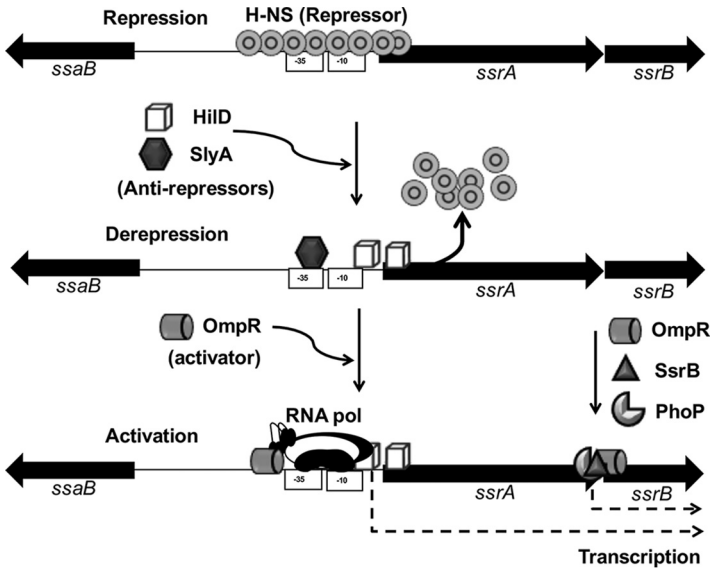

FIG 8 Model for the regulation of *ssrAB* by SlyA, HilD, OmpR, and H-NS. H-NS binds a region spanning the promoter upstream of *ssrA* and thus constitutively represses the expression of *ssrAB*, probably by blocking the access of both OmpR and the RNA polymerase. Under inducing conditions, SlyA and HilD (as during growth in LB) or only SlyA (as during growth in N-MM) displace the H-NS complex bound to the promoter upstream of *ssrA*. This allows binding of OmpR that recruits the RNA polymerase on this promoter, which finally induces the transcription of the *ssrAB* operon. The previously reported transcription of *ssrB*, from an additional promoter upstream of this gene, which involves the OmpR, PhoP, and SsrB regulators (23, 25, 26), is also indicated.

(HilD) regulators into mechanisms that control the expression of newly acquired virulence genes, such as those from SPI-2.

Important to note, under growth conditions other than those assessed in this study, there is transcription of *ssrB* independent of *ssrA*, which is directly mediated by OmpR, PhoP, and SsrB (23, 25, 26) (Fig. 4 and 8). Even more, in the absence of SsrA, unphosphorylated SsrB induces the expression of genes required for biofilm formation (70). We found that PhoP and SsrB do not play an evident role in transcription from the promoter upstream of *ssrA*, which is consistent with findings from previous reports (23, 26); however, PhoP controls the expression of *ssrAB* at the posttranscriptional level (26). Thus, our results, together with those from previous studies, show the high complexity of the mechanisms governing the expression of *ssrAB*, which favor the expression of the SPI-2 virulence genes in different *in vivo* niches.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 1. Bacterial cultures for chloramphenicol acetyltransferase (CAT) and Western blot assays were grown in LB or N-MM containing low Mg^{2+} (10 μM) at pH 5.8 or pH 7.4, as described previously (18, 19). Culture samples were taken after 9 h of growth in LB and 6 or 16 h of growth in N-MM at pH 5.8 or pH 7.4, respectively. When appropriate, antibiotics were used at the following final concentrations: ampicillin, 200 μg/ml; streptomycin, 100 μg/ml; tetracycline, 12 μg/ml; and kanamycin, 20 μg/ml.

Construction of plasmids. The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. The pK3-SlyA plasmid was constructed by amplification of *slyA* from chromosomal DNA of *S.* Typhimurium SL1344, using the primers SlyA-RV11 and SlyA-FW22. This PCR product was digested with BamHI and HindIII restriction enzymes, purified, and then cloned into the same restriction sites of the pMPM-K3 vector (71). In *Salmonella* spp., the pK3-SlyA plasmid constitutively expresses SlyA under the control of a *lac* promoter (P*lac*), since both *Salmonella* spp. and the pMPM-K3 vector lack the gene encoding LacI, the repressor of P*lac*. The pQE30-His-HA-SlyA plasmid was generated by amplifying *slyA* from chromosomal DNA of *S.* Typhimurium, with primers SlyA/HA/His-F and SlyA/HA/His-R. This PCR product was digested with the BamHI and HindIII restriction enzymes, purified, and cloned into the same restriction sites of the vector pQE30. The pQE30-His-HA-SlyA plasmid expresses SlyA fused to the hemagglutinin (HA) epitope and 6×His (His-HA-SlyA) from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter.

Construction of deletion mutant strains and strains expressing FLAG-tagged proteins. The bacterial strains used in this work are listed in Table 1. Nonpolar deletion of the *slyA* gene in the *S.*

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
S. Typhimurium		
SL1344	Wild type; *xyl hisG rpsL* Sm^r	74
JPTM3	Δ*ompR*::*kan*	18
JPTM5	Δ*hilD*::*kan*	18
JPTM8	*ssrA*::*3*×*FLAG-kan*	18
JPTM25	Δ*hilD*	75
JPTM28	Δ*ompR*	75
DTM99	Δ*ssrB*	62
DTM104	Δ*phoP*	44
DTM115	Δ*slyA*::*kan*	This study
DTM116	Δ*slyA*	This study
DTM117	Δ*hilD* Δ*slyA*::*kan*	This study
DTM118	Δ*hilD* Δ*slyA*	This study
DTM119	Δ*slyA ssrA*::*3*×*FLAG-kan*	This study
DTM120	Δ*slyA ssrA*::*3*×*FLAG*	This study
E. coli		
M15	Strain for expression of recombinant proteins	Qiagen
DH10β	Laboratory strain	Invitrogen
Plasmids		
pKK232-8	pBR322 derivative containing a promoterless chloramphenicol acetyltransferase (*cat*) gene, Ap^r	76
pssrAB-cat-302 + 478	pKK232-8 derivative containing a *ssrAB-cat* transcriptional fusion from nucleotides −302 to +478	19
pssrAB-cat-302 + 10	pKK232-8 derivative containing a *ssrAB-cat* transcriptional fusion from nucleotides −302 to +10	19
pssaG-cat	pKK232-8 derivative containing a *ssaG-cat* transcriptional fusion from nucleotides −303 to +361	18
ppagK-cat	pKK232-8 derivative containing a *pagK-cat* transcriptional fusion from nucleotides −880 to +251	44
pMPM-K3	p15A derivative low-copy-number cloning vector, *lac* promoter, Kan^r	71
pK3-SlyA	pMPM-K3 derivative expressing SlyA from the *lac* promoter	This study
pK3-SsrB	pMPM-K3 derivative expressing SsrB from the *lac* promoter	62
pK3-PhoP	pMPM-K3 derivative expressing PhoP from the *lac* promoter	44
pMPM- T6Ω	p15A derivative low-copy-number cloning vector, arabinose-inducible promoter, Tc^r	71
pT6-HNS-WT	pMPM-T6Ω derivative expressing promoter WT H-NS from the arabinose-inducible	18
pT6-HNS-G113D	pMPM-T6Ω derivative expressing H-NS^{G113D} from the arabinose-inducible promoter	44
pQE30	Vector for expression of recombinant proteins, *lac* promoter, Ap^r	Qiagen
pQE30-His-HA-SlyA	pQE30 derivative expressing His-HA-SlyA from the *lac* promoter, Ap^r	This study
pBAD-H-NS-FH	pBADMycHisC derivative expressing H-NS−FH from an *ara* promoter, Ap^r	44
pKD46	pINT-ts derivative expressing red recombinase under the control of an arabinose-inducible promoter, Ap^r	72
pKD4	pANTsγ derivative template plasmid containing the kanamycin cassette for λRed recombination, Ap^r	72
pCP20	Plasmid expressing FLP recombinase from a temperature-inducible promoter, Ap^r	72

[a]The coordinates for the *cat* fusions are indicated with respect to the transcriptional start site of *ssrA*, *ssaG*, or *pagK*. Sm^r, streptomycin resistance; Ap^r, ampicillin resistance; Kan^r, kanamycin resistance; Tc^r, tetracycline resistance.

Typhimurium SL1344 strain was performed with the λ Red recombinase system, as reported previously (72), using the respective primers described in Table 2, thus generating the strain DTM115. P22 transduction was used to transfer the Δ*slyA*::*km* allele from strain DTM115 into strain JPTM25, generating strain DTM117, as well as to transfer the *ssrA*::*3*×*FLAG-kan* allele from strain JPTM8 into strain DTM116,

TABLE 2 Primers used in this work

Primer by use	Sequence (5′–3′)[a]	Target gene	RE[b]
Gene cloning			
SlyA-RV11	ACGGGATCCTCGGCAGGTCAGCGTGTCG	*slyA*	BamHI
SlyA-FW22	TAAAAGCTTAGCAAGCTAATTATAAGGAG	*slyA*	HindIII
SlyA-HA-His-F	GATGGATCCTCTATCCGTATGATGTTCCTG ATTATGCTAGCCAAATTCGAATCGCCACTA GGTTC	*slyA*	BamHI
SlyA-HA-His-R	CTAAAGCTTTGTCGTGCTCGCCAGCAACG	*slyA*	HindIII
EMSAs			
SsaBF (fw)	GGCTAAGATCTTCGGCCCTGATATCCTG	*ssrAB*	
SsrBRS6E (rv)	TTGGTCGACCGACAGATAGATGCCGG	*ssrAB*	
Gene deletions			
slyA-H1P1	GCTAATTATAAGGAGATGAAATTGGAATC GCCACTAGGT*TGTAGGCTGGAGCTGCTT* CG	*slyA*	
slyA-H2P2	GTATGCCCCTGCACCTCAATCGTGAGAG TGCAATTCCAT*CATATGAATATCCTCCTT AG*	*slyA*	

[a]Underlined letters indicate the respective restriction enzyme site in the primer. The sequences corresponding to the template plasmid pKD4 (Table 1) are in italics.
[b]RE, restriction enzyme for which a site was generated in the primer.

generating strain DTM119. The kanamycin resistance cassette was excised from strains DTM115, DTM117, and DTM119 by using the pCP20 plasmid expressing the FLP recombinase, as described previously (72), generating strains DTM116, DTM118, and DTM120, respectively. All mutant strains were verified by PCR amplification and sequencing.

Chloramphenicol acetyltransferase assays. The chloramphenicol acetyltransferase (CAT) assay and protein determinations to calculate CAT specific activities were performed as described previously (73).

Statistical analysis. Data from CAT assays were analyzed with Prism 5.0 software version 5.04 (GraphPad, Inc., San Diego, CA) using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test. A P value of <0.05 was considered significant.

Expression and purification of His-HA-SlyA. *Escherichia coli* M15(pREP4) containing pQE30-His-HA-SlyA was grown in 200 ml of LB at 37°C in a shaken water bath. At an optical density at 600 nm of 0.6, the expression of His-HA-SlyA was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were allowed to grow for an additional 4 h at 30°C. Bacterial cells were then collected by centrifugation at 4°C. The pellet was washed once with ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 8]) and then resuspended in 10 ml of the same buffer. The bacterial suspension was sonicated for 8 min, combining 9.9-s pulses with 9.9-s resting cycles, in a Soniprep 150 sonicator (Sonics and Materials, Inc.). Bacterial debris was separated by centrifugation at 4°C, and the soluble extract was loaded into a Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA)–agarose affinity column equilibrated with lysis buffer; the column was then washed with 20 volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole [pH 8]). His-HA-SlyA was eluted with washing buffer containing 250 mM imidazole. Fractions were analyzed by SDS-PAGE, and those containing the purified protein were loaded into a Slide-A-Lyzer G2 dialysis cassette (Thermo) and dialyzed at 4°C in a buffer containing 20 mM Tris (pH 8), 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), and 20% (vol/vol) glycerol. Protein concentration was determined by the Bradford procedure. Aliquots of the purified protein were stored at −70°C.

Expression and purification of H-NS−FH. The His-tagged fusion protein H-NS−FH was expressed in *E. coli* BL21(DE3) containing the pBAD-H-NS-FH plasmid and purified by using a Ni^{2+}-NTA–agarose affinity column, as described previously (18).

Competitive electrophoretic mobility shift assays. The DNA fragment containing the regulatory region of *ssrAB* was amplified by PCR using the SsaBF/SsrBRS6E primer pair and chromosomal DNA of *S.* Typhimurium SL1344 as the template. PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research). Binding reactions were performed by mixing ≈100 ng of the PCR product first with 0.5 μM H-NS–FH for 15 min and then incubated with increasing concentrations of His-HA-SlyA for an additional 40 min in binding buffer containing 10 mM Tris (pH 9), 50 mM KCl, and 0.1% of Triton X-100 in a total volume of 20 μl. Protein-DNA binding reactions were electrophoretically separated in 6% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 4°C. The DNA fragments were stained with ethidium bromide and visualized with an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

Western blotting. H-NS–FH–DNA complexes from EMSAs were transferred to 0.45-μm-pore-size nitrocellulose membranes (Bio-Rad) using a semidry transfer apparatus (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated with anti-FLAG M2 (Sigma) monoclonal antibodies at a dilution of 1:3,000. Horseradish peroxidase-conjugated anti-mouse antibody (Pierce), at a dilution of 1:10,000, was used as the secondary antibody. Bands on the blotted membranes were developed by incubation with the Western Lightning chemiluminescence reagent plus (PerkinElmer) and exposed to Kodak X-Omat films.

SsrA-FLAG and GroEL were detected from whole-cell extracts as described above, using anti-FLAG M2 monoclonal antibody (Sigma) or anti-GroEL polyclonal antibody (StressGen) at 1:2,000 and 1:100,000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Pierce), at a dilution of 1:10,000, was used as a secondary antibody.

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REFERENCES

1. Haraga A, Ohlson MB, Miller SI. 2008. Salmonellae interplay with host cells. Nat Rev Microbiol 6:53–66. https://doi.org/10.1038/nrmicro1788.
2. Haselbeck AH, Panzner U, Im J, Baker S, Meyer CG, Marks F. 2017. Current perspectives on invasive nontyphoidal *Salmonella* disease. Curr Opin Infect Dis 30:498–503. https://doi.org/10.1097/QCO.0000000000000398.
3. Ohl ME, Miller SI. 2001. *Salmonella*: a model for bacterial pathogenesis.

Annu Rev Med 52:259–274. https://doi.org/10.1146/annurev.med.52.1.259.
4. Fàbrega A, Vila J. 2013. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev 26:308–341. https://doi.org/10.1128/CMR.00066-12.
5. Porwollik S, McClelland M. 2003. Lateral gene transfer in *Salmonella*. Microbes Infect 5:977–989. https://doi.org/10.1016/S1286-4579(03)00186-2.
6. Bispham J, Tripathi BN, Watson PR, Wallis TS. 2001. *Salmonella* pathogenicity island 2 influences both systemic salmonellosis and *Salmonella*-induced enteritis in calves. Infect Immun 69:367–377. https://doi.org/10.1128/IAI.69.1.367-377.2001.
7. Coburn B, Li Y, Owen D, Vallance BA, Finlay BB. 2005. *Salmonella enterica* serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. Infect Immun 73:3219–3227. https://doi.org/10.1128/IAI.73.6.3219-3227.2005.
8. Coombes BK, Coburn BA, Potter AA, Gomis S, Mirakhur K, Li Y, Finlay BB. 2005. Analysis of the contribution of *Salmonella* pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileal loop model and a murine model of infectious enterocolitis. Infect Immun 73:7161–7169. https://doi.org/10.1128/IAI.73.11.7161-7169.2005.
9. Knodler LA, Vallance BA, Celli J, Winfree S, Hansen B, Montero M, Steele-Mortimer O. 2010. Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. Proc Natl Acad Sci U S A 107:17733–17738. https://doi.org/10.1073/pnas.1006098107.
10. Cirillo DM, Valdivia RH, Monack DM, Falkow S. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol Microbiol 30:175–188. https://doi.org/10.1046/j.1365-2958.1998.01048.x.
11. Deiwick J, Nikolaus T, Erdogan S, Hensel M. 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Mol Microbiol 31:1759–1773. https://doi.org/10.1046/j.1365-2958.1999.01312.x.
12. Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JCD. 2002. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol Microbiol 47:103–118. https://doi.org/10.1046/j.1365-2958.2003.03313.x.
13. Srikumar S, Kröger C, Hébrard M, Colgan A, Owen SV, Sivasankaran SK, Cameron ADS, Hokamp K, Hinton JCD. 2015. RNA-seq brings new insights to the intra-macrophage transcriptome of *Salmonella* Typhimurium. PLoS Pathog 11:e1005262. https://doi.org/10.1371/journal.ppat.1005262.
14. Westermann AJ, Förstner KU, Amman F, Barquist L, Chao Y, Schulte LN, Müller L, Reinhardt R, Stadler PF, Vogel J. 2016. Dual RNA-seq unveils noncoding RNA functions in host–pathogen interactions. Nature 529:496–501. https://doi.org/10.1038/nature16547.
15. Brown NF, Vallance BA, Coombes BK, Valdez Y, Coburn BA, Finlay BB. 2005. *Salmonella* pathogenicity island 2 is expressed prior to penetrating the intestine. PLoS Pathog 1:e32. https://doi.org/10.1371/journal.ppat.0010032.
16. Miao EA, Miller SI. 2000. A conserved amino acid sequence directing intracellular type III secretion by *Salmonella* Typhimurium. Proc Natl Acad Sci U S A 97:7539–7544. https://doi.org/10.1073/pnas.97.13.7539.
17. Kröger C, Colgan A, Srikumar S, Händler K, Sivasankaran SK, Hammarlöf DL, Canals R, Grissom JE, Conway T, Hokamp K, Hinton JCD. 2013. An infection-relevant transcriptomic compendium for *Salmonella enterica* serovar Typhimurium. Cell Host Microbe 14:683–695. https://doi.org/10.1016/j.chom.2013.11.010.
18. Bustamante VH, Martínez LC, Santana FJ, Knodler LA, Steele-Mortimer O, Puente JL. 2008. HilD-mediated transcriptional cross-talk between SPI-1 and SPI-2. Proc Natl Acad Sci U S A 105:14591–14596. https://doi.org/10.1073/pnas.0801205105.
19. Martínez LC, Banda MM, Fernández-Mora M, Santana FJ, Bustamante VH. 2014. HilD induces expression of *Salmonella* pathogenicity island 2 genes by displacing the global negative regulator H-NS from *ssrAB*. J Bacteriol 196:3746–3755. https://doi.org/10.1128/JB.01799-14.
20. Walthers D, Carroll RK, Navarre WW, Libby SJ, Fang FC, Kenney LJ. 2007. The response regulator SsrB activates expression of diverse *Salmonella* pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. Mol Microbiol 65:477–493. https://doi.org/10.1111/j.1365-2958.2007.05800.x.
21. Walthers D, Li Y, Liu Y, Anand G, Yan J, Kenney LJ. 2011. *Salmonella enterica* response regulator SsrB relieves H-NS silencing by displacing H-NS bound in polymerization mode and directly activates transcription. J Biol Chem 286:1895–1902. https://doi.org/10.1074/jbc.M110.164962.
22. Tomljenovic-Berube AM, Mulder DT, Whiteside MD, Brinkman FSL, Coombes BK. 2010. Identification of the regulatory logic controlling *Salmonella* pathoadaptation by the SsrA-SsrB two-component system. PLoS Genet 6:e1000875. https://doi.org/10.1371/journal.pgen.1000875.
23. Feng X, Oropeza R, Kenney LJ. 2003. Dual regulation by phospho-OmpR of *ssrA/B* gene expression in *Salmonella* pathogenicity island 2. Mol Microbiol 48:1131–1143. https://doi.org/10.1046/j.1365-2958.2003.03502.x.
24. Lee AK, Detweiler CS, Falkow S. 2000. OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. J Bacteriol 182:771–781. https://doi.org/10.1128/JB.182.3.771-781.2000.
25. Feng X, Walthers D, Oropeza R, Kenney LJ. 2004. The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. Mol Microbiol 54:823–835. https://doi.org/10.1111/j.1365-2958.2004.04317.x.
26. Bijlsma JJE, Groisman EA. 2005. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. Mol Microbiol 57:85–96. https://doi.org/10.1111/j.1365-2958.2005.04668.x.
27. Linehan SA, Rytkönen A, Yu X-J, Liu M, Holden DW. 2005. SlyA regulates function of *Salmonella* pathogenicity island 2 (SPI-2) and expression of SPI-2-associated genes. Infect Immun 73:4354–4362. https://doi.org/10.1128/IAI.73.7.4354-4362.2005.
28. Okada N, Oi Y, Takeda-Shitaka M, Kanou K, Umeyama H, Haneda T, Miki T, Hosoya S, Danbara H. 2007. Identification of amino acid residues of *Salmonella* SlyA that are critical for transcriptional regulation. Microbiology 153:548–560. https://doi.org/10.1099/mic.0.29259-0.
29. Fass E, Groisman EA. 2009. Control of *Salmonella* pathogenicity island-2 gene expression. Curr Opin Microbiol 12:199–204. https://doi.org/10.1016/j.mib.2009.01.004.
30. Miller SI, Kukral AM, Mekalanos JJ. 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella* Typhimurium virulence. Proc Natl Acad Sci U S A 86:5054–5058. https://doi.org/10.1073/pnas.86.13.5054.
31. Groisman EA, Chiao E, Lipps CJ, Heffron F. 1989. *Salmonella* Typhimurium *phoP* virulence gene is a transcriptional regulator. Proc Natl Acad Sci U S A 86:7077–7081. https://doi.org/10.1073/pnas.86.18.7077.
32. Groisman EA. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183:1835–1842. https://doi.org/10.1128/JB.183.6.1835-1842.2001.
33. Ellison DW, Miller VL. 2006. Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol 9:153–159. https://doi.org/10.1016/j.mib.2006.02.003.
34. McVicker G, Sun L, Sohanpal BK, Gashi K, Williamson RA, Plumbridge J, Blomfield IC. 2011. SlyA protein activates *fimB* gene expression and type 1 fimbriation in *Escherichia coli* K-12. J Biol Chem 286:32026–32035. https://doi.org/10.1074/jbc.M111.266619.
35. Wyborn NR, Stapleton MR, Norte VA, Roberts RE, Grafton J, Green J. 2004. Regulation of *Escherichia coli* hemolysin E expression by H-NS and *Salmonella* SlyA. J Bacteriol 186:1620–1628. https://doi.org/10.1128/JB.186.6.1620-1628.2004.
36. Navarre WW, Halsey TA, Walthers D, Frye J, McClelland M, Potter JL, Kenney LJ, Gunn JS, Fang FC, Libby SJ. 2005. Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Mol Microbiol 56:492–508. https://doi.org/10.1111/j.1365-2958.2005.04553.x.
37. Lithgow JK, Haider F, Roberts IS, Green J. 2007. Alternate SlyA and H-NS nucleoprotein complexes control *hlyE* expression in *Escherichia coli* K-12. Mol Microbiol 66:685–698. https://doi.org/10.1111/j.1365-2958.2007.05950.x.
38. Perez JC, Latifi T, Groisman EA. 2008. Overcoming H-NS-mediated transcriptional silencing of horizontally acquired genes by the PhoP and SlyA proteins in *Salmonella enterica*. J Biol Chem 283:10773–10783. https://doi.org/10.1074/jbc.M709843200.
39. Dorman CJ, Dorman MJ. 2017. Control of virulence gene transcription by indirect readout in *Vibrio cholerae* and *Salmonella enterica* serovar Typhimurium. Environ Microbiol 19:3834–3845. https://doi.org/10.1111/1462-2920.13838.
40. Shimada T, Takada H, Yamamoto K, Ishihama A. 2015. Expanded roles of two-component response regulator OmpR in *Escherichia coli*: genomic SELEX search for novel regulation targets. Genes Cells 20:915–931. https://doi.org/10.1111/gtc.12282.
41. Cordero-Alba M, Ramos-Morales F. 2014. Patterns of expression and translocation of the ubiquitin ligase SlrP in *Salmonella enterica* serovar Typhimurium. J Bacteriol 196:3912–3922. https://doi.org/10.1128/JB.02158-14.
42. Colgan AM, Kröger C, Diard M, Hardt W-D, Puente JL, Sivasankaran SK, Hokamp K, Hinton JCD. 2016. The impact of 18 ancestral and

horizontally-acquired regulatory proteins upon the transcriptome and sRNA landscape of *Salmonella enterica* serovar Typhimurium. PLoS Genet 12:e1006258. https://doi.org/10.1371/journal.pgen.1006258.
43. Smith C, Stringer AM, Mao C, Palumbo MJ, Wade JT. 2016. Mapping the regulatory network for *Salmonella enterica* serovar Typhimurium invasion. mBio 7:e01024-16. https://doi.org/10.1128/mBio.01024-16.
44. Banda MM, López C, Manzo R, Rico-Pérez G, García P, Rosales-Reyes R, De la Cruz MA, Soncini FC, García-del Portillo F, Bustamante VH. 2018. HilD and PhoP independently regulate the expression of *grhD1*, a novel gene required for *Salmonella* Typhimurium invasion of host cells. Sci Rep 8:4841. https://doi.org/10.1038/s41598-018-23068-0.
45. Golubeva YA, Sadik AY, Ellermeier JR, Slauch JM. 2012. Integrating global regulatory input into the *Salmonella* pathogenicity island 1 type III secretion system. Genetics 190:79–90. https://doi.org/10.1534/genetics.111.132779.
46. Ellermeier CD, Ellermeier JR, Slauch JM. 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator *hilA* in *Salmonella enterica* serovar Typhimurium. Mol Microbiol 57:691–705. https://doi.org/10.1111/j.1365-2958.2005.04737.x.
47. Saini S, Ellermeier JR, Slauch JM, Rao CV. 2010. The role of coupled positive feedback in the expression of the SPI1 type three secretion system in *Salmonella*. PLoS Pathog 6:e1001025. https://doi.org/10.1371/journal.ppat.1001025.
48. Petrone BL, Stringer AM, Wade JT. 2014. Identification of HilD-regulated genes in *Salmonella enterica* serovar Typhimurium. J Bacteriol 196:1094–1101. https://doi.org/10.1128/JB.01449-13.
49. Main-Hester KL, Colpitts KM, Thomas GA, Fang FC, Libby SJ. 2008. Coordinate regulation of *Salmonella* pathogenicity island 1 (SPI1) and SPI4 in *Salmonella enterica* serovar Typhimurium. Infect Immun 76:1024–1035. https://doi.org/10.1128/IAI.01224-07.
50. Martínez-Flores I, Pérez-Morales D, Sánchez-Pérez M, Paredes CC, Collado-Vides J, Salgado H, Bustamante VH. 2016. *In silico* clustering of *Salmonella* global gene expression data reveals novel genes co-regulated with the SPI-1 virulence genes through HilD. Sci Rep 6:37858. https://doi.org/10.1038/srep37858.
51. Singer HM, Kühne C, Deditius JA, Hughes KT, Erhardt M. 2014. The *Salmonella* Spi1 virulence regulatory protein HilD directly activates transcription of the flagellar master operon *flhDC*. J Bacteriol 196:1448–1457. https://doi.org/10.1128/JB.01438-13.
52. Lucchini S, Rowley G, Goldberg MD, Hurd D, Harrison M, Hinton JCD. 2006. H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS Pathog 2:e81. https://doi.org/10.1371/journal.ppat.0020081.
53. Navarre WW, Porwollik S, Wang Y, McClelland M, Rosen H, Libby SJ, Fang FC. 2006. Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. Science 313:236–238. https://doi.org/10.1126/science.1128794.
54. Duong N, Osborne S, Bustamante VH, Tomljenovic AM, Puente JL, Coombes BK. 2007. Thermosensing coordinates a cis-regulatory module for transcriptional activation of the intracellular virulence system in *Salmonella enterica* serovar Typhimurium. J Biol Chem 282:34077–34084. https://doi.org/10.1074/jbc.M707352200.
55. Dorman CJ. 2004. H-NS: a universal regulator for a dynamic genome. Nat Rev Microbiol 2:391–400. https://doi.org/10.1038/nrmicro883.
56. Dorman CJ. 2007. H-NS, the genome sentinel. Nat Rev Microbiol 5:157–161. https://doi.org/10.1038/nrmicro1598.
57. Navarre WW, McClelland M, Libby SJ, Fang FC. 2007. Silencing of xenogeneic DNA by H-NS-facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. Genes Dev 21:1456–1471. https://doi.org/10.1101/gad.1543107.
58. Stoebel DM, Free A, Dorman CJ. 2008. Anti-silencing: overcoming H-NS-mediated repression of transcription in Gram-negative enteric bacteria. Microbiology 154:2533–2545. https://doi.org/10.1099/mic.0.2008/020693-0.
59. Yoon H, McDermott JE, Porwollik S, McClelland M, Heffron F. 2009. Coordinated regulation of virulence during systemic infection of *Salmonella enterica* serovar Typhimurium. PLoS Pathog 5:e1000306. https://doi.org/10.1371/journal.ppat.1000306.
60. Ueguchi C, Suzuki T, Yoshida T, Tanaka K, Mizuno T. 1996. Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. J Mol Biol 263:149–162. https://doi.org/10.1006/jmbi.1996.0566.
61. De la Cruz MÁ, Fernández-Mora M, Guadarrama C, Flores-Valdez MA, Bustamante VH, Vázquez A, Calva E. 2007. LeuO antagonizes H-NS and StpA-dependent repression in *Salmonella enterica ompS1*. Mol Microbiol 66:727–743. https://doi.org/10.1111/j.1365-2958.2007.05958.x.
62. Pérez-Morales D, Banda MM, Chau NYE, Salgado H, Martínez-Flores I, Ibarra JA, Ilyas B, Coombes BK, Bustamante VH. 2017. The transcriptional regulator SsrB is involved in a molecular switch controlling virulence lifestyles of *Salmonella*. PLoS Pathog 13:e1006497. https://doi.org/10.1371/journal.ppat.1006497.
63. Schmidt H, Hensel M. 2004. Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev 17:14–56. https://doi.org/10.1128/CMR.17.1.14-56.2004.
64. Baños RC, Vivero A, Aznar S, García J, Pons M, Madrid C, Juárez A. 2009. Differential regulation of horizontally acquired and core genome genes by the bacterial modulator H-NS. PLoS Genet 5:e1000513. https://doi.org/10.1371/journal.pgen.1000513.
65. Hansen-Wester I, Hensel M. 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect 3:549–559. https://doi.org/10.1016/S1286-4579(01)01411-3.
66. Cameron ADS, Dorman CJ. 2012. A fundamental regulatory mechanism operating through OmpR and DNA topology controls expression of *Salmonella* pathogenicity islands SPI-1 and SPI-2. PLoS Genet 8:e1002615. https://doi.org/10.1371/journal.pgen.1002615.
67. Norte VA, Stapleton MR, Green J. 2003. PhoP-responsive expression of the *Salmonella enterica* serovar Typhimurium *slyA* gene. J Bacteriol 185:3508–3514. https://doi.org/10.1128/JB.185.12.3508-3514.2003.
68. Mouslim C, Hughes KT. 2014. The effect of cell growth phase on the regulatory cross-talk between flagellar and Spi1 virulence gene expression. PLoS Pathog 10:e1003987. https://doi.org/10.1371/journal.ppat.1003987.
69. Browning DF, Busby SJW. 2016. Local and global regulation of transcription initiation in bacteria. Nat Rev Microbiol 14:638–650. https://doi.org/10.1038/nrmicro.2016.103.
70. Desai SK, Winardhi RS, Periasamy S, Dykas MM, Jie Y, Kenney LJ. 2016. The horizontally-acquired response regulator SsrB drives a *Salmonella* lifestyle switch by relieving biofilm silencing. Elife 5:e10747. https://doi.org/10.7554/eLife.10747.
71. Mayer MP. 1995. A new set of useful cloning and expression vectors derived from pBlueScript. Gene 163:41–46. https://doi.org/10.1016/0378-1119(95)00389-N.
72. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.120163297.
73. Puente JL, Bieber D, Ramer SW, Murray W, Schoolnik GK. 1996. The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. Mol Microbiol 20:87–100. https://doi.org/10.1111/j.1365-2958.1996.tb02491.x.
74. Hoiseth SK, Stocker BAD. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 291:238–239. https://doi.org/10.1038/291238a0.
75. Martínez LC, Yakhnin H, Camacho MI, Georgellis D, Babitzke P, Puente JL, Bustamante VH. 2011. Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the *Salmonella* SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol 80:1637–1656. https://doi.org/10.1111/j.1365-2958.2011.07674.x.
76. Brosius J. 1984. Plasmid vectors for the selection of promoters. Gene 27:151–160. https://doi.org/10.1016/0378-1119(84)90136-7.