Supporting Information

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SI Text

SI Results

ppGpp-Mediated Induction of SPI-1 and SPI-2 Regulons. The role of the stringent response in the HilD-dependent activation of *ssrAB* was addressed by constructing an *S. typhimurium* Δ*relA* Δ*spoT* double mutant and by analyzing the expression of the *cat* transcriptional fusions to *sirA, hilD, hilA, invF, ssrAB*, and *ssaG* in this background. Expression levels of both SPI-1 and SPI-2 genes were highly reduced in the mutant growing in LB (Fig. S3) as well as in minimal media (data not shown), except for the *sirA-cat* fusion, which was not significantly reduced in the Δ*relA* Δ*spoT* double mutant. Together with the results shown in Figs. 1 and 2, our data indicate that ppGpp plays a fundamental role in the HilD-mediated activation of both SPI-1 and SPI-2 regulons as well as in the HilD-independent induction of the SPI-2 genes observed in minimal media, and thus in *Salmonella* virulence as previously reported (1–3). However, the stringent response molecule ppGpp does not seem to be involved in the mechanism controlling the transition from SPI-1 to SPI-2 expression in LB.

HilD and OmpR Regulate the Transcriptional Activation of the *ssrAB* Operon. The genes encoding the two-component system SsrA/B are organized in tandem with an intergenic region of only 30 bp between *ssrA* and *ssrB* (Fig. S5*A*). Despite this organization, it has been proposed that transcription of *ssrA* and *ssrB* is uncoupled, beginning 175 and 167 bp upstream from their respective start codons, and directly regulated by OmpR and PhoP (4–7). To define whether HilD regulates SsrA and SsrB production at the transcriptional level, two *ssrAB* transcriptional fusions to the *cat* reporter gene were constructed. The *ssrAB-cat* fusion contains the *ssrA* and *ssrB* promoters and the *ssrB-cat* fusion contains only the putative *ssrB* promoter plus 796 bp upstream of its reported transcriptional start site (5) (Fig. S5*A*). The expression of these two fusions was analyzed in WT *S. typhimurium* and in its Δ*hilD* and Δ*ompR* derivatives grown to the stationary phase in LB. In agreement with the results shown in Figs. 2*C* and 3, the expression of the *ssrAB-cat* fusion was drastically reduced in the Δ*hilD* and Δ*ompR* mutants carrying plasmid pMPM-T3 (vector), but it was restored in the presence of plasmid pT3-HilD1 in the Δ*hilD* or pT3-OmpR1 in the Δ*ompR* (Fig. S5*B*). In contrast, expression of the *ssrAB-cat* fusion was not restored in the Δ*ompR* strain carrying pT3-HilD1 or in the Δ*hilD* strain carrying pT3-OmpR1 (Fig. S5*B*), which indicates that both HilD and OmpR are required for the expression of *ssrAB*. Surprisingly, the *ssrB-cat* fusion showed only low background levels of activity, comparable to the WT strain and all mutants tested (Fig. S5*B*), even in the presence of plasmids pT3-HilD1 or pT3-OmpR1, indicating that the sequence contained in this fusion lacks a functional promoter for *ssrB* under the conditions tested.

To clarify whether in fact the *ssrAB* genes are transcribed as an operon, we followed two strategies. First, RT-PCR analysis was performed using total RNA purified from WT *S. typhimurium* SL1344 grown in LB medium, three different pairs of primers that amplify internal regions of *ssrA* or *ssrB* used as positive controls, and a region spanning *ssrA* to *ssrB*. RT-PCR products of the expected size were obtained with the three pairs of primers in the presence of reverse transcriptase but not in its absence (Fig. S5*C*), indicating that the *ssrA* and *ssrB* genes are cotranscribed.

To confirm our results further, we constructed a strain in which the promoter region of *ssrA* was deleted from the chromosome. This was produced by replacing the intergenic region between *spiC* and *ssrA* with a kanamycin (kan) resistance marker, generating mutant strain Δ*PssrA*::kan (JPTM18). In this mutant, expression of *ssrAB* is driven by read-through transcription coming from the kan cassette. This cassette was then excised from strain Δ*PssrA*::kan, generating strain Δ*PssrA* (JPTM19), which consequently lacks the entire *ssrA* promoter region. We then assessed levels of SsrB in these strains grown to the stationary phase in LB or in N-minimal medium. Under both growth conditions, expression of SsrB was detected in the WT and Δ*PssrA*::kan strains but not in Δ*PssrA* (Fig. S5*D*). Together, these results indicate that HilD acts in coordination with OmpR to induce the transcription of the *ssrAB* operon from a promoter located upstream of *ssrA*.

SI Materials and Methods

Construction of Mutant Strains. Non–polar gene-deletion mutants, the strain lacking the *ssrA* promoter, and strains carrying a chromosomal FLAG-tagged *hilA* or *ssrA* gene were generated by the λ Red recombinase system, as described previously (8, 9), using gene-specific primer pairs, as shown in Table S2. The FRT-flanked kan cassette was excised from strains JPTM8, JPTM18, and JPTM20 after transformation with pCP20, as described previously (8), generating strains JPTM9, JPTM19, and JPTM21, respectively. The Δ*relA* Δ*spoT*::*kan* double mutant (JPTM22) was generated by introducing the Δ*spoT*::*kan* deletion in the Δ*relA* mutant (JPTM21). The Δ*ompR*::*kan*, Δ*hilA*::*kan*, Δ*hilC*::*kan*, and Δ*hilD*::*kan* alleles from strains JPTM3, VV341, JPTM6, and JPTM5, respectively, were transferred to strain JPTM9 by P22 transduction, generating strains JPTM10, JPTM11, JPTM12, and JPTM13, respectively. All strains were verified by PCR amplification and sequencing.

Construction of Plasmids. Primers used for PCR amplification are listed in Table S2. Transcriptional fusions *sirA-cat*, *hilD-cat*, *hilA-cat*, *invF-cat*, *sopB-cat*, *ssaG-cat*, *pipB-cat*, *ssrAB-cat*, *ssrB-cat*, and *hns-cat* were generated by cloning a PCR product containing the corresponding regulatory region into pKK232–8, a plasmid carrying a promoterless *cat* gene (Amersham Pharmacia LKB Biotechnology). PCR products were digested with different restriction enzymes as indicated in Table S2 and ligated to pKK232–8 digested with the same enzymes, except for HpaI ends, which were ligated to SmaI-generated ends. The resulting constructs, psirA-cat1, philD-cat1, philA-cat1, pinvF-cat1, psopB-cat1, pssaG-cat1, ppipB-cat1, pssrAB-cat1, pssrB-cat2, and phns-cat1 are described in Table S1.

Primers hilDpFSal and hilDBHR were used to amplify the *S. typhimurium* SL1344 *hilD* structural gene. The resulting PCR product was digested with SalI and BamHI and ligated into pMPM-T3 (10), digested with the same restriction enzymes, generating plasmid pT3-HilD1. This plasmid contains the promoterless *hilD* gene, including its putative ribosome-binding site, which is expressed from the *lac* promoter contained in the vector. Primers HilDexF and HilDexR were also used to amplify *hilD*, but the resulting PCR product was used to construct a plasmid encoding an MBP-tagged HilD protein to facilitate its purification by digesting it with BamHI and PstI and ligating it into pMAL-c2X digested with the same restriction enzymes, generating plasmid pMAL-HilD1. The *in vivo* functionality of the MBP-HilD protein was confirmed by its ability to complement the Δ*hilD* mutant (data not shown). pT3-OmpR1 (Table S1) was

generated by subcloning the BamHI-HindIII DNA fragment from pFM2000 (11), carrying *ompR*, into pMPM-T3 digested with the same enzymes.

Primers hns-Nco and hns-22R were used to amplify the WT *hns* structural gene from *E. coli* CU241 or the Q92am *hns* mutant allele from *E. coli* HM60 (12). The resulting PCR products were digested with NcoI and HindIII and ligated into pMPM-T6Ω (10) digested with the same restriction enzymes, generating plasmids pT6-HNS-WT and pT6-HNS-Q92am, respectively (Table S1). The resulting plasmids express, under the control of the arabinose-inducible *araBAD* promoter, WT H-NS and H-NS^{Q92am}, an H-NS with a C-terminal truncation at position 92, which acts as a dominant negative form. All constructs were confirmed by DNA sequencing.

Western Blotting. Whole-cell extracts were prepared from samples taken from cultures grown in LB or N-minimal media. Ten micrograms of total protein was subjected to SDS/PAGE analysis and transferred to 0.45-μm pore size nitrocellulose membranes (Bio-Rad) using a semidry transfer apparatus (Bio-Rad). Immunoblots were performed using polyclonal anti-SsrB or anti-OmpR antibodies (kindly provided by L.J. Kenney) and anti-FLAG M2 (Sigma) or anti-DnaK (StressGen) monoclonal antibodies at 1:10,000, 1:2,000, 1:1,000, and 1:20,000 dilutions, respectively. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Pierce), diluted at 1:20,000, was used as a secondary antibody. Detection was with the Western lightning chemiluminescence reagent plus (PerkinElmer) and Kodak X-Omat film.

Expression and Purification of MBP-HilD and H-NS-His$_6$. *E. coli* BL21/pLys containing pMAL-HilD1 was grown in 100 ml of LB media with 0.2% glucose at 37°C in a shaken water bath. At an optical density of 0.6, expression of MBP-HilD was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside and the cells were allowed to grow for another 4 h at 30°C. Bacterial cells were then collected by centrifugation at 4°C. The pellet was washed once with ice-cold column buffer (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol) and 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 an amylose column equilibrated with column buffer; the column was then washed with 20 volumes of buffer. MBP-HilD was eluted with column buffer containing 10 mM maltose (Bioxon). Fractions were analyzed by SDS/PAGE, and those containing the purified protein were loaded into a Slide-A-Lyzer 7K cassette (Pierce) and dialyzed at 4°C in a buffer containing 20 mM Tris-HCl (pH 8), 40 mM KCl, 1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol. Protein concentration was determined by the Bradford procedure. Aliquots of the purified protein were stored at −70°C.

The H-NS-His$_6$ protein was purified from cultures of *E. coli* BL21/pLys containing pT6-HNS-His$_6$ (13) by using a HiTrap Ni^{2+}-chelating column as previously described (13).

RT-PCR Assays. Total RNA was purified from the *S. typhimurium* WT strain grown in LB medium at 37°C for 8 h using the Qiagen RNeasy mini kit. One microgram of purified RNA was subjected to DNase I (Invitrogen) treatment according to the manufacturer's instructions. Then, 0.01 μg of the DNA-free RNA sample was used as a template in a reverse transcription reaction, followed by PCR amplification of the generated cDNA, by using the SuperScript III One-Step RT-PCR System with Platinum TaqDNA Polymerase (Invitrogen). The PCR amplification step was performed using the following pairs of primers, each at a final concentration of 0.5 μM: pair a, SBF-PE22/SBR-PE11, spanning an *ssrB* region of 295 bp; pair b, SsrAF-BD9/SsrBR-S4C, spanning an *ssrA* region of 260 bp; and pair c, SsrAF-BD9/SsrBRSal, spanning an *ssrA*-to-*ssrB* region of 630 bp. RT-PCR cycles were as follows: one cycle of 30 min at 53°C for the cDNA synthesis; one cycle of 2 min at 94°C for denaturation; 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 68°C for PCR amplification; and a final elongation step of 3 min at 94°C. Negative controls were performed by substituting the SuperScript III RT/Platinum Taq mix for Platinum TaqDNA polymerase in the reactions. Controls for the expected size of the PCR products were performed by using chromosomal DNA from *S. typhimurium* as the template instead of the DNA-free RNA and substituting the SuperScript III RT/Platinum Taq mix for Platinum TaqDNA polymerase in the reactions. The PCR products were analyzed by 1% agarose gel electrophoresis.

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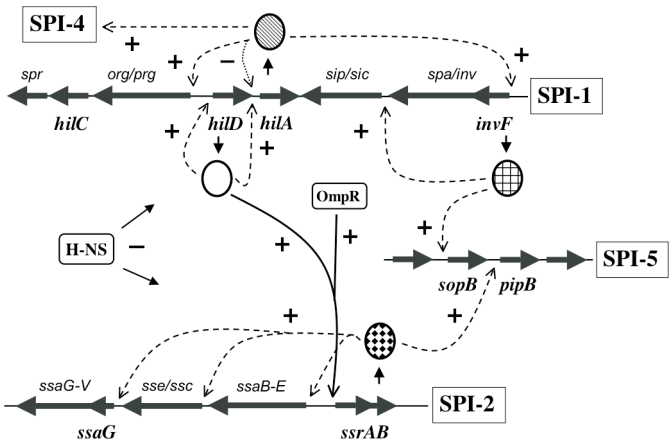

Fig. S1. Schematic representation of the HilD-dependent transcriptional cross-talk between SPI-1 and SPI-2. A regulatory cascade integrated by the SPI-1–encoded regulators HilD and InvF, which are members of the AraC family of transcriptional regulators, and HilA, which belongs to the OmpR/ToxR family of transcriptional regulators (14–17), controls the expression of type III secretion system components and effectors encoded within and outside SPI-1 under conditions resembling the intestinal environment (e.g., late log/early stationary phase in LB medium). HilD as well as HilC and RtsA induces expression of HilA, which, in turn, activates the expression of *invF* and SPI-1 genes encoding type III secretion system components, whereas InvF activates the expression of effector genes both within and outside SPI-1. In addition, several other global transcriptional regulators positively or negatively control expression of *hilA* or *hilD* (14–17). Expression of genes encoding SPI-2 type III secretion system components and effector proteins is regulated by the SPI-2–encoded two-component system SsrA/B (18–20). Expression of the *ssrAB* genes is positively regulated by the two-component systems OmpR/EnvZ and PhoP/PhoQ and the MarR-like regulator SlyA (4–7, 16, 21) in response to the intracellular environment (e.g., minimal medium) (22–24). In addition, the SPI-1–encoded regulator HilD helps to set the activation of the SPI-2 regulon, in a nonoverlapping manner, at nonclassic SPI-1– or SPI-2–inducing conditions (e.g., late stationary phase in LB medium), which probably resemble SPI-1 and SPI-2 expression during the intestinal phase of the infection but not necessarily at the same niche. SPI-1 inactivation could be mediated by a *hilA*-specific negative regulatory mechanism that turns SPI-1 activation off even in the presence of HilD, probably redirecting it to overcome the H-NS–mediated repression exerted on *ssrAB*, and thus of SPI-2, in a niche- or environment-specific manner. Current knowledge suggests that this could be achieved by, for example, HilA, which has been shown to autoregulate its own expression negatively (25) or by HilE (26).

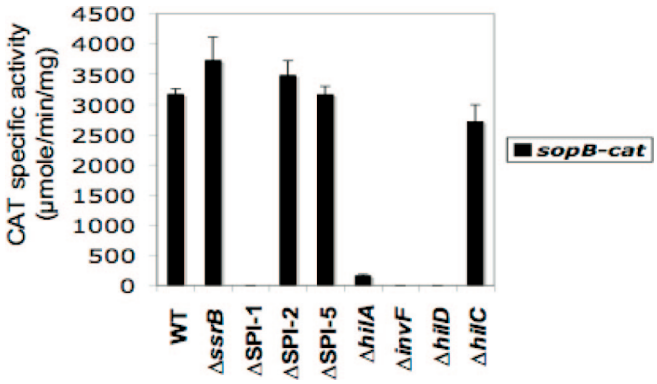

Fig. S2. HilD, HilA, and InvF are required for expression of the SPI-1 regulon. Expression of the *sopB-cat* transcriptional fusion was analyzed in WT *S. typhimurium* and its Δ*ssrB*, ΔSPI-1, ΔSPI-2, ΔSPI-5, Δ*hilA*, Δ*invF*, Δ*hilD*, and Δ*hilC* derivatives containing plasmid psopB-cat1. CAT activity was determined from samples taken from LB cultures after 5 h. The data are the average of three different experiments done in duplicate.

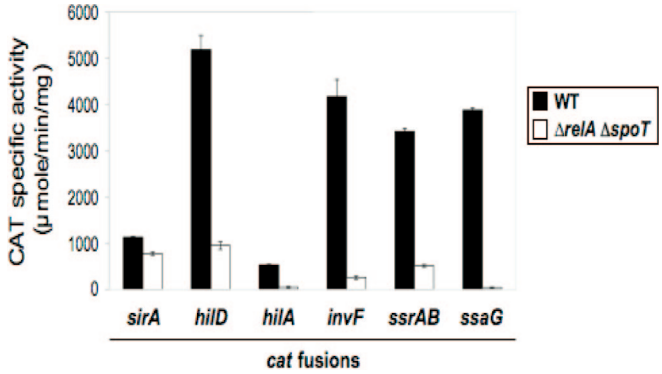

Fig. S3. The effector molecule for the stringent response ppGpp is required for the expression of the SPI-1 and SPI-2 regulons. Expression of the *sirA-cat*, *hilD-cat*, *hilA-cat*, *invF-cat*, *ssrAB-cat*, and *ssaG-cat* transcriptional fusions was analyzed in WT *S. typhimurium* and its Δ*relA* Δ*spoT*::*kan* derivative mutant. CAT activity was determined from samples taken from LB cultures after 5 or 9 h. The data are the average of three different experiments done in duplicate.

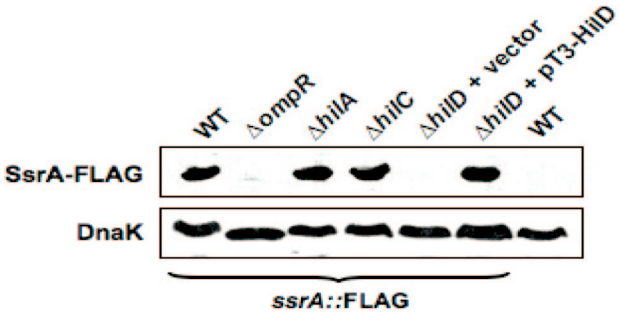

Fig. S4. HilD and OmpR are required for the expression of *ssrA* in *Salmonella* grown to the stationary phase in LB. SsrA levels in WT *S. typhimurium* and in its Δ*ompR*, Δ*hilA*, Δ*hilC*, and Δ*hilD* derivatives, carrying a chromosomal FLAG-tagged *ssrA* gene, were analyzed by immunoblotting using a monoclonal anti-FLAG antibody and whole-cell lysates prepared from samples of bacterial cultures grown in LB for 12 h. As a control for protein loading, the expression of DnaK was determined using a monoclonal antibody. pT3-HilD, used to complement the expression of SsrA-FLAG in the Δ*hilD* mutant, expresses HilD from the vector *lac* promoter.

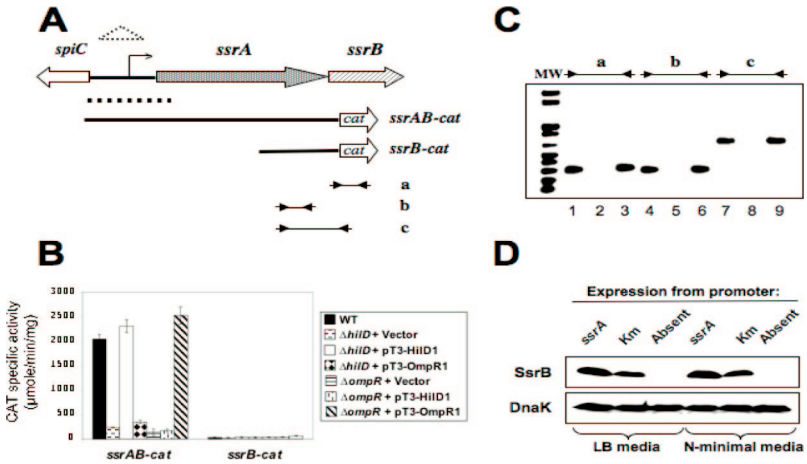

Fig. S5. HilD and OmpR regulate the expression of the *ssrAB* operon promoter at the transcriptional level. (*A*) Schematic representation of the *ssrAB* operon and the *cat* transcriptional fusions used to monitor its expression. A bent arrow indicates the previously reported transcriptional start site (+1) for *ssrA* (5). A dotted triangle represents the deleted promoter region in strains JPTM18 (Δ*PssrA*::kan) and JPTM19 (Δ*PssrA*). A dashed line and the three lines between the arrows located below the *ssrAB* scheme indicate the *ssrAB* DNA fragment used for the EMSAs and the regions amplified in the RT-PCR reactions, respectively. (*B*) Expression of the *ssrAB-cat* and *ssrB-cat* transcriptional fusions was determined from 12-h LB culture samples of WT *S. typhimurium* and its Δ*hilD* and Δ*ompR* derivatives carrying plasmids pssrAB-cat1 or pssrB-cat2 and containing or not containing pMPM-T3 (vector), pT3-HilD1, or pT3-OmpR1. The data are the average of three different experiments done in duplicate. (*C*) RT-PCR assay. Total RNA was isolated from the WT *S. typhimurium* SL1344 grown in LB at 37°C for 8 h and was then subjected to an RT reaction and PCR amplification of the fragments indicated in *A* and shown above the gel. RT-PCR reactions for each region were performed with a reverse transcriptase/TaqDNA polymerase mix (lanes 1, 4, and 7) or only with TaqDNA polymerase (lanes 2, 5, and 8). PCR reactions using SL1344 chromosomal DNA as a template and the same set of primers were performed as controls (lanes 3, 6, and 9). PCR products were analyzed in a 1% agarose gel. (*D*) SsrB levels in WT *S. typhimurium* and its Δ*PssrA*::kan (JPTM18) and Δ*PssrA* (JPTM19) derivatives. SsrB was detected by immunoblotting using whole-cell lysates prepared from samples of bacterial cultures grown for 12 h in LB media or for 16 h in N-minimal media and a polyclonal anti-SsrB antibody.

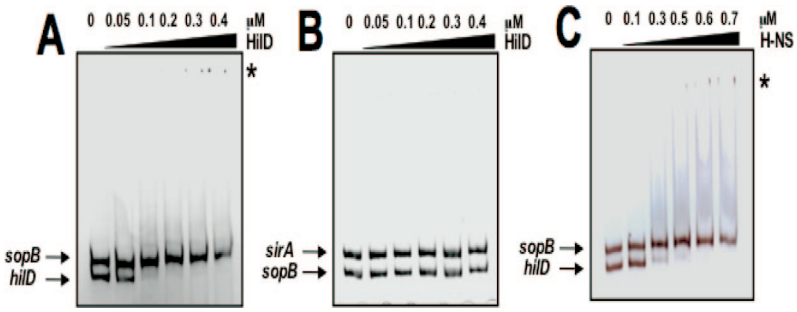

Fig. S6. EMSAs show HilD and H-NS binding to the *hilD* regulatory region. Fragments encompassing the regulatory regions of *hilD* (−364 to +88), *sirA* (−563 to +98), and *sopB* (−400 to +128), with respect to the corresponding transcriptional start sites, were incubated with increasing concentrations of purified MBP-HilD (0, 0.05, 0.1, 0.2, 0.3, and 0.4 μM) or H-NS-His$_6$ (0, 0.1, 0.3, 0.5, 0.6, and 0.7 μM). The DNA-protein complexes were resolved in 6% polyacrylamide nondenaturing gels in 0.5× Tris-borate-EDTA buffer at room temperature and stained with ethidium bromide. The *hilD* fragment was used as a positive control for the binding activity of the purified MBP-HilD and H-NS-His$_6$ proteins, and the *sirA* and *sopB* fragments were used as negative controls. DNA-protein complexes are indicated by an asterisk.

Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Ref. or source
Strains		
S. enterica serovar Typhimurium SL1344	WT, Sm^R	27
ΔSPI-1	Δ*SPI-1*::*kan*	24
ΔSPI-2	Δ*SPI-2*::*kan*	28
ΔSPI-5	Δ*SPI-5*::*kan*	This study
MJW112	Δ*ssrB*::*kan*	M. Worley and F. Heffron
VV341	Δ*hilA*::*kan-339*	29
JPTM3	Δ*ompR*::*kan*	This study
JPTM4	Δ*invF*::*kan*	This study
JPTM5	Δ*hilD*::*kan*	This study
JPTM6	Δ*hilC*::*kan*	This study
JPTM7	*hilA*::*3*×*FLAG-kan*	This study
JPTM8	*ssrA*::*3*×*FLAG-kan*	This study
JPTM9	*ssrA*::*3*×*FLAG*	This study
JPTM10	*ssrA*::*3*×*FLAG* Δ*ompR*::*kan*	This study
JPTM11	*ssrA*::*3*×*FLAG* Δ*hilA*::*kan*	This study
JPTM12	*ssrA*::*3*×*FLAG* Δ*hilC*::*kan*	This study
JPTM13	*ssrA*::*3*×*FLAG* Δ*hilD*::*kan*	This study
JPTM14	Δ*phoP*::*kan*	This study
JPTM15	Δ*rpoS*::*kan*	This study
JPTM16	Δ*sdiA*::*kan*	This study
JPTM17	Δ*luxS*::*kan*	This study
JPTM18	Δ*PssrA*::kan	This study
JPTM19	Δ*PssrA*	This study
JPTM20	Δ*relA*::*kan*	This study
JPTM21	Δ*relA*	This study
JPTM22	Δ*relA* Δ*spoT*::*kan*	This study
E. coli BL21/pLys	Strain for expression of recombinant proteins	Invitrogen
E. coli MC4100	Cloning strain, K12 derivative	Laboratory collection
Plasmids		
pKK232-8	pBR322 derivative containing a promoterless chloramphenicol acetyltransferase (*cat*) gene, Ap^R	30
pinvF-cat1	pKK232-8 derivative containing a *invF-cat* transcriptional fusion from nucleotides −306 to +213	This study*
psopB-cat1	pKK232-8 derivative containing a *sopB-cat* transcriptional fusion from nucleotides −400 to +128	This study*
pssaG-cat1	pKK232-8 derivative containing a *ssaG-cat* transcriptional fusion from nucleotides −232 to +361	This study*
ppipB-cat1	pKK232-8 derivative containing a *pipB-cat* transcriptional fusion from nucleotides −737 to +70	This study*
pssrAB-cat1	pKK232-8 derivative containing a *ssrAB-cat* transcriptional fusion from nucleotides −303 to +3054	This study*
pssrB-cat2	pKK232-8 derivative containing a *ssrB-cat* transcriptional fusion from nucleotides +1966 to +3054	This study*
psirA-cat1	pKK232--8 derivative containing a *sirA-cat* transcriptional fusion from nucleotides −563 to +98	This study*
philD-cat1	pKK232-8 derivative containing a *hilD-cat* transcriptional fusion from nucleotides −364 to +88	This study*
philA-cat1	pKK232-8 derivative containing a *hilA-cat* transcriptional fusion from nucleotides −410 to +446	This study*
phns-cat1	pKK232-8 derivative containing a *hns-cat* transcriptional fusion from nucleotides −967 to +73	This study*
pMPM-T3	p15A derivative low-copy-number cloning vector, *lac* promoter, Tc^R	10
pT3-HilD1	pMPM-T3 derivative expressing HilD from the *lac* promoter	This study
pT3-OmpR1	pMPM-T3 derivative expressing OmpR	This study
pMPM-T6Ω	p15A derivative cloning vector containing an arabinose-inducible promoter, Tc^R	10
pT6-HNS-WT	pMPM-T6Ω derivative expressing WT H-NS from an arabinose-inducible promoter	This study
pT6-HNS-His_6	pMPM-T6Ω derivative expressing H-NS-His_6 under the arabinose-inducible promoter	13

Strain or plasmid	Genotype or description	Ref. or source
pT6-HNS-Q92am	pMPM-T6Ω derivative expressing dominant negative C-terminal truncated H-NS at position 92 (H-NS^{Q92am}) from an arabinose-inducible promoter	This study
pKD46	Red recombinase system under an arabinose-inducible promoter	8
pKD4	Template plasmid containing the kan cassette for λ Red recombination	8
pCP20	Plasmid expression FLP recombinase from a temperature-inducible promoter	8
pSUB11	Template plasmid for FLAG epitope tagging	9
pMAL-c2X	Vector for constructing MBP fusions	New England Biolabs
pMAL-HilD1	pMAL-c2X derivative expressing MBP-HilD	This study

*The coordinates for the *cat* fusions are indicated with respect to the transcriptional start site reported for each gene.

Table S2. Primers used in this study

Primer	Sequence* (5′-3′)	Target gene	RS†
For *cat* transcriptional fusions			
pKK-invF-F (fw)	CGCGGATCCAAGAATGAGGCGCCATGT	*invF*	BamHI
pKK-invF-R (rv)	CCCAAGCTTACAACGGCCTGCTCGCAA		HindIII
SigDBHIF (fw)	TCCCGACAGGATCCTTTTACCC	*sopB*	BamHI
SigDH3R (rv)	CGTTGTATAAGCTTTTTTGTAG		HindIII
pKK-ssaH-F (fw)	CGCGGATCCTGGTAGTTTGGGACTACA	*ssaG*	BamHI
pKK-ssaH-R (rv)	CCCAAGCTTATAACCGTTAGCGCTGGT		HindIII
pipBBHIF (fw)	TATTTTAATGGATCCGATCATAC	*pipB*	BamHI
pipBH3R (rv)	CAGGTAAAAAGCTTGACAAACC		HindIII
ssaBFHpaI (fw)	GGCTGTTAACTTCGGCCCTGATATCCTG	*ssrAB*	HpaI
ssrBRSalI (rv)	ATGGTCGACAATTTTAAAATGAGGCCAG		SalI
ssrAFHpa (fw)	TTTGTTAACAACAATAATTATTTGGCTG	*ssrB*	SmaI
sirAFBamHI (fw)	GCCGGATCCATCGCCTGCAGCATCAGC	*sirA*	BamHI
sirARHindIII (rv)	AGCAAGCTTCACCGACAACTTTAATGC		HindIII
hilDBamHI (fw)	AATGGATCCTCACAGCCGTTCAGTGAG	*hilD*	BamHI
hilDRHindIII (rv)	CTGAAGGTTATCTGCGGCAGGACGC		HindIII
hilAIFBamHI (fw)	ATCGGATCCCTCTGAGAACTATTTGC	*hilA*	BamHI
hilA2RHindIII (rv)	GACAAGCTTTTCTGAGCGTAGCAGGG		HindIII
hns-bIF (fw)	TGTCGGATCCTGCCAGTAAGTA	*hns*	BamHI
hns-H3R (rv)	CACGAAGCTTACGGATGTTG		HindIII
For gene deletions			
SPI5-KOF (fw)	AGAATATTTCCCGAAAAAACGGACTACGCGA GTCTTTAGTTTCTTTTCGTTTCCCGATGTAGG CTGGAGCTGCTTCG	SPI-5	
SPI5-KOR (rv)	AAAGGATGCCGGCAAAGAATATTGCTGGCTC GATCACTTTCTCGGTTTCCAATCCAGGGTCAT ATGAATATCCTCCTTAG		
SompRH1P1 (fw)	TTGGGAGTACAGACAATGCAAGAGAATTATAA GATTCTGGTGTGTAGGCTGGAGCTGCTTCG		*ompR*
SompRH2P2 (rv)	AAAGACGTAGCCCAGGCCCCATACGGTCTGA ATATAACGCGGCATATGAATATCCTCCTTAG		
SinvFH1P1 (fw)	TCCGCGCAAAAGCTGCATATGTCATTTTCTG AAAGCCGACACTGTAGGCTGGAGCTGCTTCG	*invF*	
SinvFH2P2 (rv)	CAATTGAATAATATTTGATAATTTCCGCGGCG AAACGCCGATCATATGAATATCCTCCTTAGTTC		
ShilDH1P1 (fw)	CATCAACAAAGGGATAATATGGAAAATGTAAC CTTTGTAAGTTGTAGGCTGGAGCTGCTTCG	*hilD*	
ShilDH2P2 (rv)	TGACAGATACAAAAAATGTTAATGGAACGCCA TTTTTATGAACATATGAATATCCTCCTTAG		
ShilCH1P1 (fw)	TAGCACACAGGATAAAATATGGTATTGCCTTC AATGAATAAATGTAGGCTGGAGCTGCTTCG	*hilC*	
ShilCH2P2 (rv)	CGTTTAAAATAATTTACAAAATCAATGGTTCAT TGTTCGCATCATATGAATATCCTCCTTAG		
SphoPH1P1 (fw)	TAACACAAGGGAGAAGAGATGATGCGCGTAC TGGTTGTAGAGTGTAGGCTGGAGCTGCTTCG	*phoP*	
SphoPH2P2 (rv)	TCCGCGTACGGTGGTAATGACATCGTGCGGA TACTGGGCCTGCATATGAATATCCTCCTTAG		
SrpoSH1P1 (fw)	CACGGGTAGGAGCCACCTTATGAGTCAGAATAC GCTGAAAGTTCATTGTAGGCTGGAGCTGCTTCG	*rpoS*	
SrpoSH2P2 (rv)	TTTTTTTGACAAGGGTACTTACTCGCGGAACAG CGCTTCGATCATATGAATATCCTCCTTAG		
SsdiAH1P1 (fw)	TCAAAGGCGTGACCATAAAATATGCAGGAAAA TGATTTCTTCACCTGTAGGCTGGAGCTGCTTCG	*sdiA*	
SsdiAH2P2(rv)	CGAAGCATCGTCAGCACGTCATATCAGACCTG TCGCCGCAGCGTACATATGAATATCCTCCTTAG		
SluxSH1P1 (fw)	AAAATTATCGGAGGTGACTAAATGCCATTATTA GATAGCTTCGCATGTAGGCTGGAGCTGCTTCG	*luxS*	
SluxSH2p2 (rv)	TGGAACCGCTTACAAATAAGACTAAATATGCAG TTCCTGCAGTTTCATATGAATATCCTCCTTAG		
RelAH1P1 (fw)	AGAAGTGCACATATTAATAAAGCTGGTGAATTT GATCCGAAGTGTAGGCTGGAGCTGCTTCG	*relA*	
RelAH2P2 (rv)	ATTACTGTCTGGGGTTTACCCCCCGTGCAGTC GCCGTGCATCCATATGAATATCCTCCTTAG		

Primer	Sequence* (5′-3′)	Target gene	RS†
SpoTH1P1 (fw)	GAAAGCCTGAATCAACTGATTCAAACCTACCTG CCGGAAGACTGTAGGCTGGAGCTGCTTCG	*spoT*	
SpoTH2P2 (rv)	GGTGACTTTAATGACGTCTGGCATCACGCGGA TTTTGCGCATCATATGAATATCCTCCTTAG		
For deletion of the *ssrA* promoter			
SSP33FP (fw)	TAAAACTGCCAGCATGAATCCCTCCTCAGA CATAAATGGGTGTAGGCTGGAGCTGCTTCG	*ssrA*	
SSP44RP (rv)	CGTATTCTTGAGATTGAGCAAATTCATAATG CTTCCCTCCCATATGAATATCCTCCTTAGTTC		
For gene FLAG-tagging			
hilAflagF (fw)	AAAAGATGGAAACAGGATCCCCGCTTGATT AAATTACGGGACTACAAAGACCATGACGGT	*hilA*	
hilAflagR (rv)	CGATGATAAAAAAATAATGCATATCTCCTCT CTCAGATTCATATGAATATCCTCCTTAGTTC		
SssrAFTAG1 (fw)	CGCCAGAAAATACGTATGATCTTCAAAAACTA CACCATTACTGACTACAAAGACCATGACGG	*ssrA*	
SssrARTAG2 (rv)	ATATTCTTTCATTTTGCTGCCCTCGCGAAAAT TAAGATAATACATATGAATATCCTCCTTAG		
For gene cloning			
hilDpFSal (fw)	TTT*GTCGAC*AGGATACCAGTAAGGAACAT	*hilD*	SalI
hilDBHR (rv)	GTATAAGCTG*GGGA*TCCGGACAGAAC		BamHI
HilDexF (fw)	GAT*GGATCC*GAAAATGTAACCTTTGTAAGTAATAG	*hilD*	BamHI
HilDexR (rv)	TCCC*TG*CAGAACAATGATATTGAATAGC		PstI
hns-Nco (fw)	CTACCATG*GG*CGAAGCACTTA	*hns*	NcoI
hns-22R (rv)	GCAATCTACAA*GCTT*TTATTGCTTGATC		
For EMSAs			
ssaBFBglII (fw)	GGCTA*AGATC*TTCGGCCCTGATATCCTG	*ssrA*	BglII
ssrBRS6E (rv)	TTG*GTC*GACCGACAGATAGATGCCGG		SalI
hilA1FBamHI (fw)	ATC*GGA*TCCCTCTGAGAACTATTTGC	*hilA*	BamHI
hilA2RHindIII (rv)	GAC*AAG*CTTTTCTGAGCGTAGCAGGG		HindIII
hilDFBamHI (fw)	AATGGA*TC*CTCACAGCCGTTCAGTGAG	*hilD*	BamHI
hilDRHindIII (rv)	CTG*AAGC*TTATCTGCGGCAGGACGC		HindIII
sirAFBamHI (fw)	GCC*GGATC*CATCGCCTGCAGCATCAGC	*sirA*	BamHI
sirARHindIII (rv)	AGCA*AGC*TTCACCGACAACTTTAATGC		HindIII
sigDBHIF (fw)	Described above for *cat* transcriptional fusions	*sopB*	
sigDH3R (rv)			
For RT-PCR analysis			
SBF-PE22 (fw)	GATCTTATTAGTAGACGATCATG	*ssrB*	
SBR-PE11 (rv)	CATAGCCATTAGCACCTGCGGC		
SsrAF-BD9 (fw)	CGATTATGGCATGATGAGCC	*ssrA*	
SsrBR-S4C (rv)	ATTAATGACCATATCATCTGTCGCCAG		
SsrBRSal (rv)	Described above for *cat* transcriptional fusions	*ssrB*	

fw, forward; rv, reverse.
The sequence corresponding to the template plasmid pKD4 or pSUB11 (SI Table 2) is underlined.
*Italic letters indicate base changes in the primer sequence with respect to the WT sequence, designed to introduce restriction enzyme sites.
†Restriction enzymes used to digest the PCR products.