

## Regulation of *igaA* and the Rcs System by the MviA Response Regulator in *Salmonella enterica*<sup>∇</sup>

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**IgaA is a membrane protein that prevents overactivation of the Rcs regulatory system in enteric bacteria. Here we provide evidence that *igaA* is the first gene in a  $\sigma^{70}$ -dependent operon of *Salmonella enterica* serovar Typhimurium that also includes *yrfG*, *yrfH*, and *yrfI*. We also show that the Lon protease and the MviA response regulator participate in regulation of the *igaA* operon. Our results indicate that MviA regulates *igaA* transcription in an RpoS-dependent manner, but the results also suggest that MviA may regulate RcsB activation in an RpoS- and IgaA-independent manner.**

In *Salmonella enterica* serovar Typhimurium, the *igaA* gene is located at centisome 75, between genes *yrfE* and *yrfG* (60). *igaA* encodes an inner membrane protein of 710 amino acids which is predicted to contain five transmembrane domains (28). *igaA* is homologous to *yrfF* in *Escherichia coli* and *umoB* in *Proteus mirabilis* (29) and was identified by analysis of a mutant with an increased growth rate in fibroblasts (16). This mutant harbored a substitution mutation, and the resulting allele was designated *igaA1* (16). Mutants in the same gene (also named *mucM*) were independently isolated on the basis of their resistance to amdinocillin (23). *igaA1* and other *igaA* point mutants are mucoid (due to upregulation of the *wca* genes), show a defect in motility (due to repression of the *flhDC* operon), and are attenuated for virulence (16). The attenuation is not explained by the motility defect but is partially explained by the overproduction of colanic acid capsule (28, 40). Different mucoid *igaA* point mutants display similar phenotypes but in different degrees that correlate with the degree of activation of the Rcs system (28). *igaA* is an essential gene (15). The phenotypes of viable (leaky) *igaA* point mutants and the lethality of null alleles are suppressed by mutations in *rscC*, *rscD* (previously named *yoyN*), or *rscB* (15, 23, 24, 28, 58), suggesting that loss of IgaA results in overactivation of the Rcs system.

Rcs is a phosphorelay initially characterized in *E. coli* as a regulator of colanic acid capsule synthesis (12, 43, 73). RcsC is a hybrid histidine kinase, RcsD is an intermediate phosphotransmitter (19, 78), and RcsB is the transcriptional regulator of the system. Other components of the system are RcsA, a second transcriptional activator (74), and RcsF (41), an outer membrane protein that lies upstream of RcsC in the signaling pathway RcsF → RcsC → RcsD → RcsB (54, 55). In addition, recent evidence suggests that RcsB can directly receive signals by accepting the phosphoryl group from acetyl phosphate (37).

The Rcs system participates in many processes in addition

to the production of colanic acid capsule. The list includes synthesis of other exopolysaccharides (8, 66), synthesis of flagella (15, 36), swarming (4, 19, 44, 47), cell division (17), resistance to cell envelope stress (22, 50), and biofilm formation (35). Rcs also plays a role in the control of *Salmonella* virulence. *rscC* mutants display modest attenuation in mice (27), and mutations in either *igaA* (28) or *rscC* (40, 63) that hyperactivate the system show a much more pronounced effect, with extremely low competitive indices (CIs). This effect is partially suppressed by mutations that prevent colanic acid capsule synthesis (40, 63), suggesting that overproduction of capsule is one of the causes of attenuation in these mutants. However, additional loci involved in *Salmonella* virulence are also regulated by Rcs, including genes in *Salmonella* pathogenicity island 1 (SPI-1) (63, 84), SPI-2 (84), and SPI-4 (39). IgaA seems to prevent activation of the Rcs system during early stages of infection (28). However, this system also activates virulence genes required at later stages of infection (27, 31, 84).

The Rcs system can be transiently or moderately activated by certain environmental signals, including osmotic upshift (72) and a combination of low temperature and glucose or zinc (45). In addition, mutations in some genes or overexpression of others can lead to permanent activation of the Rcs signal transduction pathway. As mentioned above, this is the case for viable *igaA* alleles in *S. enterica* serovar Typhimurium (28). Such mutants have been successfully exploited for the study of the *Salmonella* Rcs regulon (28, 39, 84). The *igaA*, *rscB*, *rscD*, and *rscC* loci are present exclusively in enterobacteria and, interestingly, no bacterium whose genome has been sequenced has been found to contain only *igaA* or only the *rsc* genes. Such a coincidence is reminiscent of related functions (28) and suggests that the study of *igaA* is necessary for a full understanding of the Rcs system.

This work was undertaken to characterize the *igaA* transcriptional unit and to identify regulators of the expression of this gene. We show that *igaA* is the first gene in an operon driven by a  $\sigma^{70}$ -dependent promoter. We also show that MviA, a response regulator that participates in the degradation of RpoS, modulates the expression of *igaA* and hence the activation of the Rcs system.

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TABLE 1. Bacterial strains<sup>a</sup>

Strain	Description	Source or reference
<i>E. coli</i> strains		
DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	46
UQ285	<i>lacZ4 rpoD285(ts) argG75</i>	48
<i>S. enterica</i> strains		
14028	Wild type	ATCC
55130	<i>pho-24</i> (PhoP constitutive)	E. A. Groisman
LT2	Wild type	ATCC
SV4582	$\phi$ ( <i>igaA-lacZY</i> ) Km <sup>r</sup> $\Delta$ <i>rscC::MudQ</i>	Laboratory stock
SV5590	$\phi$ ( <i>igaA-lacZ</i> ) <sub>lyb</sub> Km <sup>r</sup> $\Delta$ <i>rscB</i>	This work
SV5663	<i>lon::Tn10dTc</i>	This work
SV5664	<i>mviA::Tn10dTc</i>	This work
SV4003	<i>rpoS::Ap<sup>r</sup></i>	Laboratory stock
SV5614	LT2 <i>hns</i>	Laboratory stock
SV4917	<i>flgK::MudJ</i>	Laboratory stock
SV4610	<i>flhC5213::MudA</i>	Laboratory stock
SV5737	$\Phi$ ( <i>rscB-lacZY</i> ) Km <sup>r</sup>	This work
SV5738	<i>rscB::3xFlag</i> Km <sup>r</sup>	28

<sup>a</sup> Derivatives of some of these strains were used as indicated in the text.

## MATERIALS AND METHODS

**Bacterial strains, bacteriophage, and strain construction.** *E. coli* and *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. Unless otherwise indicated, *Salmonella* strains are derived from the mouse-virulent strain ATCC 14028. Transductional crosses using phage P22 HT 105/1 *int201* (71) were used for strain construction (56). To obtain phage-free isolates, transductants were purified by streaking on green plates (18). Phage sensitivity was tested by cross-streaking with the clear plaque mutant P22 H5.

**Media and chemicals.** The standard culture medium for *S. enterica* was Luria-Bertani (LB) broth. Solid LB contained agar at a 1.5% final concentration. Antibiotics were used at the following concentrations: kanamycin (Km), 50  $\mu$ g ml<sup>-1</sup>; chloramphenicol (Cm), 20  $\mu$ g ml<sup>-1</sup>; ampicillin (Ap), 100  $\mu$ g ml<sup>-1</sup>. For some experiments 40  $\mu$ g ml<sup>-1</sup> 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside and 0.2% glucose or arabinose were added to LB. Motility assays were carried out in LB prepared without yeast extract (42). Motility agar contained agar at a 0.25% final concentration.

**DNA amplification by PCR.** Amplification reactions were carried out in a GeneAmp PCR System 2400 (Perkin-Elmer Cetus). The final volume of reaction mixtures was 50 to 100  $\mu$ l, and the final concentration of MgCl<sub>2</sub> was 1 mM. Reagents were used at the following concentrations: deoxynucleoside triphosphates, 200  $\mu$ M; primers, 1  $\mu$ M; *Taq* polymerase (Expand High Fidelity PCR system; Roche Diagnostics SL), 1 unit per reaction mixture. The thermal program included the following steps: (i) initial denaturation, 2 min at 94°C; (ii) 25 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 to 3 min); (iii) final incubation at 72°C for 7 min, to complete extension.

**Construction of plasmid pIZ1586.** DNA from strain 14028 was used as a template for PCR amplification with primers *igaA*Pro5' and *igaA*Pro3' (Table 2). The amplified fragment was purified, digested with BamHI and BglIII, and cloned on BglIII-digested pIC552. The resulting plasmid (pIZ1586) carries a *igaA::lac* transcriptional fusion.

**Construction of chromosomal *lacZ* fusions.** Disruption and replacement of *igaA* with a Cm resistance gene were performed as described previously (26). Briefly, the Cm resistance gene from plasmid pKD13 was PCR amplified with primers *igaA*-P1 and *igaA*-P4. The sequences of the primers used are shown in Table 2. The PCR product was used to transform the wild-type strain carrying the Red recombinase expression plasmid pKD46. The antibiotic resistance cassette introduced by the gene-targeting procedure was eliminated by recombination using the FLP helper plasmid pCP20 (26). The FRT site generated by excision of the antibiotic resistance cassette was used to integrate plasmid pCE40 to generate a translational *lac* fusion (30). The method for the construction of a transcriptional *rscB-lacZ* fusion was similar except that the Km resistance gene from pKD4 was amplified with primers *rscB*-P1 and *rscB*-P4 and integration of pCE36 was used to generate the fusion (30).

TABLE 2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5'-3')
A.....	GCGTGTATACCGATGCCAAC
A'.....	CGCTGCTGGTGTGTTTATG
F.....	CAGCTTTCATAGTATTCCTGG
G.....	GTTTCCGGTACCAGCTTTTG
H.....	GTAAGGGTTGCGTTCAGCTC
I.....	GAGCTGTACCGTAATGTCAC
<i>igaA</i> Pro5'.....	GTCAGAATCCAGCGTACCTGCGGCAATGG
<i>igaA</i> Pro3'.....	CGTAGGATCCAGAGACAGGCCAGCAAAGC
Tn10d5'.....	CTTTCTAAGGCAGACCAACC
Tn10d3'.....	CACGCACAACAGATTTTACG
<i>igaA</i> -P1.....	TCAGATGAGATTTTCCGGAGAGACGGTTAA CCTGGGATGTGTAGGCTGGAGCTGCTTC
<i>igaA</i> -P4.....	CGTTTTTGTACCGTGGATGATAGGCGGCGC GATCCTGCTGATTCCGGGGATCCGTC GACC
<i>rscB</i> -P1.....	ATTATGCGGATGACCACCGATGTACTGT TCGGTATTCGTGTAGGCTGGAGCTGCTTC
<i>rscB</i> -P2.....	AGAGAGATAGTTGAGCAGCGGATATCATT CTCTACGCCCATATGAATATCTCTCTTAG

**RNA extraction.** Two protocols were used, one for primer extension and a different one for reverse transcriptase PCR (RT-PCR) experiments. In the first protocol, RNA preparations were obtained by guanidinium isothiocyanate lysis and phenol-chloroform extraction (20). Saturated cultures were immersed in liquid N<sub>2</sub>, and 1.4 ml of lysis solution (5 M guanidinium isothiocyanate, 50 mM Tris [pH 7.5], 10 mM EDTA, 8% [vol/vol]  $\beta$ -mercaptoethanol) was added. Each mixture was incubated at 60°C for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and 0.28 ml of chloroform was added. After gentle shaking and incubation for 10 min at room temperature, the samples were centrifuged at 9,000 rpm for 10 min and 0.66 ml of isopropanol was added to the supernatant. The samples were incubated at -20°C for 15 min and centrifuged again at 9,000 rpm for 15 min. The pellets were then rinsed with 70% ethanol and dried. After resuspension in 75  $\mu$ l of water (with 0.1% [vol/vol] diethyl pyrocarbonate), the samples were subjected to standard treatments with DNase and proteinase K, followed by extraction with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol (70). The aqueous phase was precipitated with a 1/10 volume of 3 M sodium acetate, pH 4.8, and 2.5 volumes of absolute ethanol. The samples were then kept at -80°C for at least 30 min, centrifuged, and washed with 70% ethanol. Finally, the precipitates were dried and resuspended in 20 to 40  $\mu$ l of diethyl pyrocarbonate-water. In the second protocol, to prepare cells for RNA extraction, 5 ml of fresh LB was inoculated 1:100 from an overnight culture and grown under antibiotic selection with shaking. Three biological replicates were performed for each strain, and RNA was extracted at an optical density at 600 nm of ~3 (stationary phase). RNA extractions were performed as described by Mangan et al. (57). Briefly, 20% (vol/vol) ice-cold RNA stabilization solution (5% [vol/vol] phenol-95% ethanol) was added with mixing and the cultures were immediately incubated on ice for 30 min. The cultures were pelleted by centrifugation (3,100  $\times$  g for 30 min at 4°C) and pellets were stored at -80°C until required. RNA was extracted using a Promega SV total RNA purification kit. The RNA concentration was determined by the absorbance at 260 nm, and their quality was assessed on an Agilent 2100 Bioanalyzer.

**Primer extension.** The oligonucleotide *igaA*Pro3' (Table 2), complementary to an internal region of the *igaA* gene, was end labeled with [ $\gamma$ -<sup>32</sup>P]ATP and annealed to 25  $\mu$ g of total RNA prepared from *S. enterica* strains. For annealing, 10<sup>5</sup> cpm of oligonucleotide was used. The end-labeled primer was extended with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) under conditions described previously (59). The products of reverse transcription were analyzed in urea-polyacrylamide gels. For autoradiography, the gels were exposed to Kodak Biomax MR film.

**RT-PCR.** Three micrograms of total RNA were treated with 1 U of Turbo DNA-free (Ambion, Inc., Applied Biosystems, Foster City, CA). The RNA was reverse transcribed into cDNA by using MultiScribe reverse transcriptase with the random hexamers included in the kit. Then, 2  $\mu$ l of each retrotranscription reaction mixture was subjected to PCR by using *Taq* DNA polymerase (Promega). Positive controls were performed with genomic DNA, and negative controls were performed with RNA that had been treated with TURBO DNA-free,

and 5 U of RNase H from *E. coli* (Epicentre Biotechnologies, Madison, WI) was reverse transcribed into cDNA and subjected to PCR.

**Mutagenesis with Tn10dTc.** Strain ATCC 14028 was mutagenized with Tn10dTc (80). Pools of 4,500 colonies, each carrying an independent Tn10dTc insert, were then prepared and lysed with phage P22 HT. The lysates were used to transduce strains SV4582 and SV5590, selecting tetracycline (Tc)-resistant transductants on LB or minimal medium E plates supplemented with X-Gal, Tc, and Km.

**Cloning and molecular characterization of Tn10dTc inserts.** Genomic DNA from each Tn10dTc-carrying isolate was digested with BamHI or XhoI and ligated with T4 DNA ligase to BamHI- or XhoI-digested pBlueScript II (SK). The ligations were transformed into *E. coli* DH5 $\alpha$  cells, and Tn10dTc-containing transformants were selected on LB plates supplemented with Tc. The DNA sequences of the fusion junctions and the flanking DNA were obtained by sequencing with an automated DNA sequencer (Sistemas Genómicos SL, Valencia, Spain) using the primers Tn10d5' and Tn10d3'.

**DNA sequencing.** Sequencing reactions were carried out with the Sequenase 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, OH). The manufacturer's instructions were followed. Additionally, 1  $\mu$ l of unlabeled dATP (10  $\mu$ M) was added to the reaction mixtures. Sequencing gels were prepared in Tris-borate-EDTA and contained 6% acrylamide and 500 g/liter urea. The gels were run in a Paker Face SE1500 sequencer (Hoefer Scientific Instruments, San Francisco, CA), dried in a Slab gel dryer (model SE1160; Hoefer Scientific Instruments), and developed by exposure to X-ray film.

**Sequence analysis.** Sequence analysis was performed with molecular biology algorithms from the National Center for Biotechnology Information at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) and the European Bioinformatics Institute at [www.ebi.ac.uk](http://www.ebi.ac.uk).

**$\beta$ -Galactosidase assays.** Levels of  $\beta$ -galactosidase activity were assayed for exponential- and stationary-phase cultures in LB medium or in minimal medium as described previously (62), using the CHCl<sub>3</sub>-sodium dodecyl sulfate (SDS) permeabilization procedure.

**Motility assays.** Liquid cultures were prepared in motility medium and incubated at 37°C with shaking. At the stage of mid-exponential growth, 5  $\mu$ l of the culture was spotted in the center of a motility agar plate. The plate was incubated at 37°C. The diameter of the bacterial growth halo was measured every hour.

**Mouse mixed infections and determination of CIs.** Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were subjected to mixed infections. Groups of three to four animals were inoculated with a 1:1 ratio of two strains. Bacteria were grown overnight at 37°C in LB with shaking, diluted into fresh medium (1:100), and grown until an optical density at 600 nm of 0.3 to 0.6. Intraperitoneal inoculation was performed with 0.2 ml of saline containing 10<sup>5</sup> CFU. Bacteria were recovered from spleens 48 h after inoculation and CFU were enumerated on LB and on selective medium. A CI for each mutant was calculated as the ratio between the mutant and the wild-type strain in the output (bacteria recovered from the host after infection) divided by their ratio in the input (initial inoculum) (9, 38, 79).

**Statistical analysis.** Each reported CI or  $\beta$ -galactosidase activity value is the mean of at least three independent experiments  $\pm$  the standard error. Student's *t* test was used to analyze results. *P* values of 0.05 or less are considered significant.

**Western blotting and antibodies.** *Salmonella* strains were grown overnight in LB. The bacteria were then pelleted by centrifugation, resuspended in NP-40 buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin), and submitted to sonication and centrifugation. The soluble proteins contained in the supernatants were quantified and aliquots of each lysate were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels and electrophoretically transferred to nitrocellulose filters for immunoblot analysis using anti-Flag M2 monoclonal antibodies (1:5,000; Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibodies (Bio-Rad) were used as secondary antibodies. For stability tests, bacterial cultures were treated with chloramphenicol at 100  $\mu$ g/ml and aliquots were removed at timed intervals for lysis and Western blotting.

## RESULTS

**Characterization of the *igaA* transcriptional unit.** The *igaA* gene is annotated as *yrfF* in the *Salmonella enterica* serovar Typhimurium LT2 complete sequence (60). This open reading frame (ORF) is located at centisome 75 flanked by *yrfE*, which is divergently transcribed, and *yrfG* (Fig. 1A). Analysis of this

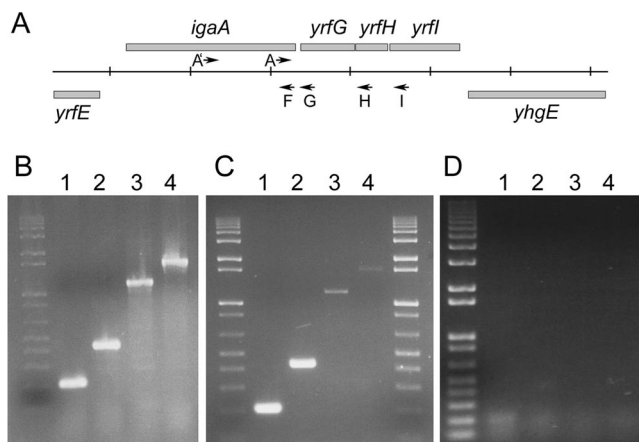


FIG. 1. Transcriptional organization of the *igaA* region. (A) Organization of the region containing the *igaA* gene in the *Salmonella* chromosome. Vertical lines are separated by 1 kb. The *igaA* (*yrfF*) ORF starts at position 3650187 and the *yrfI* ORF finishes at position 3654367 in the *Salmonella enterica* serovar Typhimurium LT2 complete sequence. The arrows indicate the positions and orientations of the primers A', A, F, G, H, and I that were used for RT-PCR. (B) Agarose gel of the products obtained when the PCR was performed with genomic DNA. Lanes: 1, primers A and F; 2, primers A and G; 3, primers A and H; 4, primers A and I. (C) Agarose gel of the RT-PCR products obtained with the same primers as for panel B. The molecular weight marker is the 1-kb ladder (Life Technologies). The expected sizes of the products are 202 bp, 422 bp, 1,155 bp, and 1,621 bp, respectively. (D) RT-PCR results for a sample of the RNA used for panel C, previously treated with RNase H; no products were observed.

region with the OperonDB algorithm (<http://www.cbcb.umd.edu/cgi-bin/operons/operons.cgi>) (32) suggests that *igaA* could be the first gene in an operon that includes *yrfG*, *yrfH*, and *yrfI*. This prediction is based on codirectional transcription, distance between genes, and conservation of adjacency in other genomes. The analysis indicates that these four genes are consecutive on the same DNA strand in 15 other genomes, from bacterial species included in the genera *Salmonella*, *Escherichia*, *Shigella*, *Yersinia*, and *Photobacterium*, and gives an 86% confidence to the conclusion that they belong to the same transcriptional unit. To get experimental support for this hypothesis, RT-PCR was performed by using oligonucleotide pairs designed to amplify regions that included a portion of *igaA* and downstream DNA sequences of various lengths. The efficacy of the primer pairs was first confirmed by carrying out PCR on genomic DNA (Fig. 1B). RT amplification products of the expected sizes (202 bp, 422 bp, 1,155 bp, and 1,621 bp) were observed with the four primer pairs used (Fig. 1C). A possible artifact due to DNA contamination was discarded by performing RT-PCR on an RNA sample previously treated with RNase H (Fig. 1D). In addition these results were confirmed by carrying out RT-PCRs using a different direct primer (A') and the same reverse primers (G, H, and I). Amplification of products of the expected sizes (1,051 bp, 1,271 bp, 2,004 bp, and 2,470 bp) was obtained (not shown). In summary, these experiments provide evidence that *igaA*, *yrfG*, *yrfH*, and *yrfI* are transcriptionally linked.

**$\sigma^{70}$  dependence of the *igaA* operon transcription.** The next step in the characterization of the *igaA* transcriptional unit was the identification of the transcriptional start site. The inter-

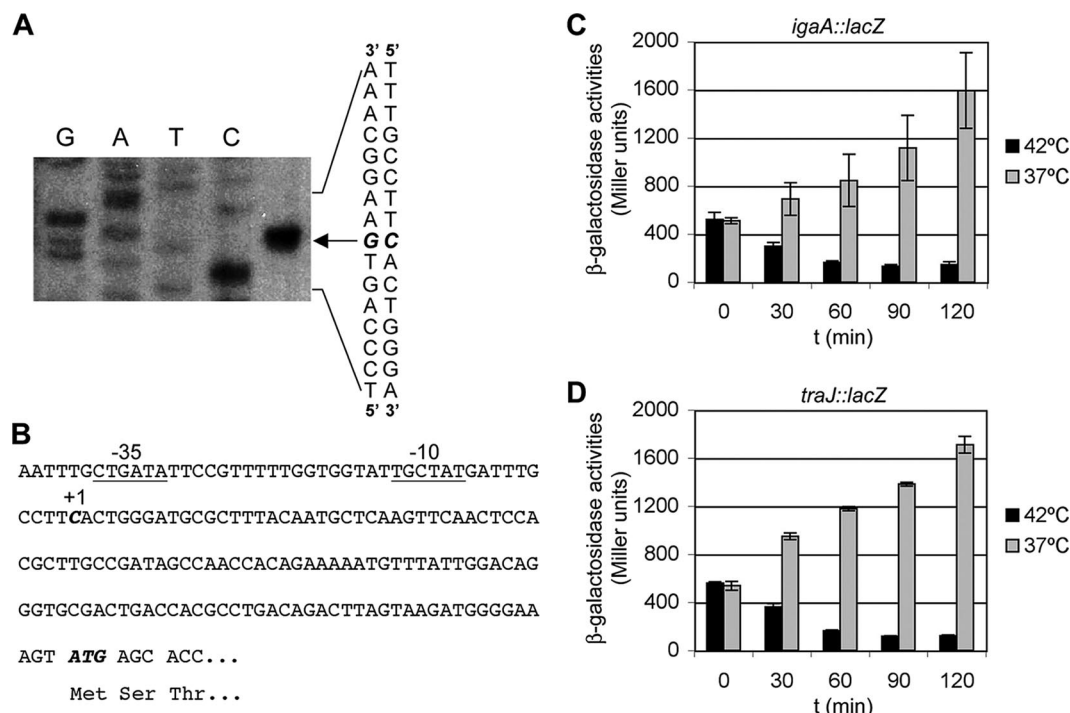


FIG. 2.  $\sigma^{70}$ -dependent transcription of *igaA*. (A) Primer extension was performed on strain 14028/pIZ1586. Lanes G, A, T, and C are the sequencing ladder. The base in italics is the transcriptional start site. (B) The sequence surrounding the transcriptional start site (in italics) and the translation initiator codon (in italics) are shown. Putative  $\sigma^{70}$  promoter consensus sequences are underlined. (C) Exponential-phase cultures of *E. coli* strain UQ285 (*rpoD* thermo-sensitive)/pIZ1586 (*igaA::lac* transcriptional fusion) were either maintained at 37°C (permissive temperature) or shifted to 42°C (restrictive temperature), and  $\beta$ -galactosidase activities were measured at the indicated times (in minutes). (D) Same experiment as in panel C but for a *traJ::lac* transcriptional fusion (plasmid pIZ898).

genic region between *yrfE* and *igaA*, together with the first part of the coding region of *igaA*, was cloned in the plasmid pIC552 to generate a transcriptional fusion with the *lacZ* gene. This construct, pIZ1586, was introduced in strain 14028. Preliminary  $\beta$ -galactosidase activity measurements confirmed that the cloned region was enough to ensure expression. RNA isolated from the strain carrying the plasmid was used in primer extension experiments with the oligonucleotide *igaA*Pro3'. Primer extension results are shown in Fig. 2A. Transcription starts at a C located 125 bases upstream of the initiator AUG codon. Visual inspection reveals the presence of  $-10$  and  $-35$  regions related to the consensus sequences of  $\sigma^{70}$ -dependent promoters (Fig. 2B).

To test whether  $\sigma^{70}$  was necessary for *igaA* transcription, we studied the effect of a thermo-sensitive *rpoD* mutation on the expression of an *igaA::lac* fusion. For this purpose plasmid pIZ1586 was electroporated into the *E. coli* strain UQ285, which carries a thermo-sensitive *rpoD* allele. Exponential-phase cultures grown in LB with Ap at 37°C (permissive temperature) were split in two; one-half was maintained at the permissive temperature and the other was shifted to 42°C (restrictive temperature). At different time points aliquots were extracted and  $\beta$ -galactosidase activities were measured. A *traJ::lac* fusion in plasmid pIZ898 (14, 81) was also used as a control for a  $\sigma^{70}$ -dependent gene. As shown in Fig. 2C and D, activities decreased over time at the restrictive temperature. Two hours after the shift, the activity of the *igaA::lac* fusion was 15-fold higher at 37°C than at 42°C. These results provide

evidence that transcription of the *igaAyrfGHI* operon requires the *rpoD*-encoded  $\sigma^{70}$  factor.

**Genetic screens for identification of genes regulating the *igaA* operon.** In order to identify genes that regulate *igaA* expression, we devised two genetic screens, one based on a chromosomal *igaA::lac* fusion and the other based on a translational fusion. Both fusions produce null alleles of the *igaA* gene, which are expected to be lethal. To avoid lethality, the fusions were obtained in an *RcsB*<sup>-</sup> background. Mutagenesis was carried out on the wild-type strain with the defective transposon Tn10dTc. Pools were prepared with resulting Tc-resistant colonies, each pool containing 4,500 independent insertions. These pools were used as donors in P22-mediated transduction experiments with the *rpsB* strains carrying the *igaA::lac* fusions as recipients. Transductants were selected in LB or minimal medium supplemented with Tc (the transposable element confers resistance to this antibiotic), Km (a resistance gene is associated with the *lac* fusions), and X-Gal (a chromogenic indicator for the activity of the fusions). With these screens we expected to detect insertions in genes for *igaA* activators (white colonies in indicator plates) and in genes for *igaA* repressors (darker colonies). Candidates were submitted to reconstruction crosses, in which the Tn10dTc element was transduced to the original strain, in order to ascertain that the observed phenotypes were associated with the insertions.  $\beta$ -Galactosidase activities of the *igaA::lac* fusions were measured from liquid cultures of every candidate. A total of 45,000 independent insertions were analyzed, both in LB and in min-

TABLE 3. *igaA* regulators found in screens with *lac* fusions

Screen	Gene interrupted	Activity of transcriptional <i>igaA::lacZ</i> fusion (Miller units)				References
		MM <sup>a</sup>		LB <sup>b</sup>		
		WT	Mutant	WT	Mutant	
Transcriptional fusion in minimal medium	<i>lon</i>	157.5 ± 8.3	82.0 ± 5.9	65.1 ± 1.7	50.5 ± 0.2	13, 21, 87
Translational fusion in LB	<i>mviA</i>	71.3 ± 12.8	37.4 ± 3.9	51.3 ± 2.7	26.7 ± 1.4	6, 75

<sup>a</sup> Activities were measured from cultures in minimal medium (MM).

<sup>b</sup> Activities were measured from cultures in LB medium. Data are the means ± standard deviations from three measures. WT, wild type.

imal medium, and two of them significantly altered the expression of the fusions. To identify the loci where the Tn10dTc had inserted, chromosomal fragments containing the defective transposon were cloned in pBluescript SK (II). DNA sequencing was performed using primers Tn10d5' and Tn10d3' (Table 2). The genes identified in these screens were *lon* and *mviA*. Both genes appear to regulate the expression of the transcriptional (Table 3) and the translational (not shown) *igaA::lac* fusions. Lon protease could have an indirect effect on *igaA* expression, perhaps through the degradation of a transcriptional regulator. On the other hand, MviA was of interest as it is a response regulator from a two-component system involved in the control of virulence in *Salmonella* (5, 7, 76). For this reason, we decided to further characterize the effect of MviA on *igaA* expression.

**Regulation of *igaA* by MviA is mediated by RpoS.** A null mutation in *mviA* (for mouse virulence gene A [7]) leads to avirulence in mice in almost all the *S. enterica* serovar Typhimurium strains examined (77). The exception is LT2, a strain that was originally described as virulent for mice in 1948 (53). Avirulent LT2 derivatives become virulent by inactivating *mviA* (7). In all strains except LT2, avirulence caused by *mviA* mutations is correlated with a growth defect reflected in the small size (Scm<sup>+</sup> phenotype) of the colonies formed on agar plates. The *E. coli* *mviA* homolog is known as *rssB* (64) or *sprE* (69). The N-terminal region of MviA/RssB is very similar to CheY and other response regulators, including an aspartate at position 58 that is predicted to be phosphorylated, causing MviA activation. The way this phosphorylation is controlled is not clear, but involvement of acetyl phosphate (10, 25) and the histidine kinase ArcB (61) have been suggested. The C-terminal region of MviA has no similarity with other known proteins and apparently does not contain a DNA binding domain. In contrast, MviA is involved in posttranslational regulation of the  $\sigma$  factor RpoS. *mviA* mutants show increased levels of RpoS that are at least partially responsible for their phenotypes (3, 64, 69). In this context, the function of MviA is in binding RpoS and acting as an adaptor for recognition and degradation by the ClpXP protease (49, 89).

To test if the increased levels of RpoS were responsible for the repression of *igaA* transcription in the *mviA* mutant, we measured expression of the *igaA::lacZ* transcriptional fusion in different genetic backgrounds combining *mviA* and *rpoS* null mutations. As shown in Fig. 3, a mutation in *rpoS* led to a small but significant increase in the activity of the fusion and suppressed the effect of the *mviA* mutation. These results are consistent with the idea that *igaA* regulation by MviA is mediated by RpoS.

**Other genes involved in posttranslational control of RpoS also regulate *igaA*.** In *E. coli*, the nucleoid-associated protein H-NS affects both RpoS mRNA translation and RpoS turnover in logarithmic growth: a 10-fold increase in the RpoS synthesis rate, as well as a 10-fold increase in RpoS stability, is observed in *hns* mutants (2, 86). An *hns* mutant of *S. enterica* serovar Typhimurium is also defective in RpoS turnover (25). In *E. coli* the effect on stability is mediated by RssB (MviA). An unidentified antiadaptor, negatively regulated by H-NS, may prevent the action of RssB on RpoS (11, 88). According to our hypothesis, a similar relationship between MviA and H-NS in *Salmonella* would lead to a decrease in the expression of *igaA* in an *hns* mutant.

In *Salmonella*, but not in *E. coli*, the response regulator PhoP controls RpoS stability through the transcriptional activation of *iraP*, whose product is an antiadaptor that prevents degradation of RpoS through a direct interaction with MviA (11, 83). Thus, one would predict a negative effect of PhoP on the expression of *igaA*.

Bearing these precedents in mind we tested the effect of *hns* and *phoP/phoQ* mutations on *igaA::lacZ*. Mutations in *Salmonella hns* are lethal unless accompanied by compensatory mutations in other regulatory loci, like *rpoS* (67). For this reason, experiments involving *hns* mutations were carried out in an LT2 background, in which lethality is prevented due to a leaky, *rpoS* mutation (52). For the PhoPQ two-component system, a mutation that confers constitutive activation of the system (*pho-24*) was used. Both an *hns* null mutation and the *pho-24*

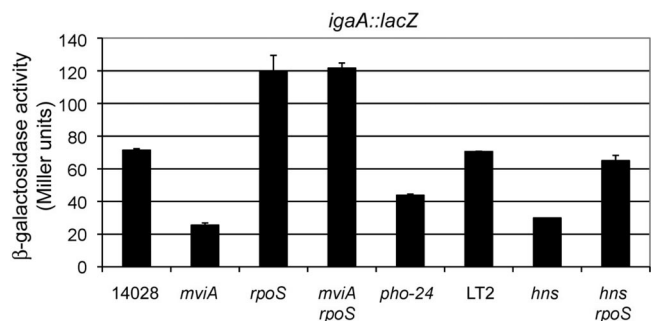


FIG. 3. *igaA* transcription is regulated by MviA, RpoS, PhoP, and H-NS. Expression levels of *igaA* were monitored with a *lacZ* transcriptional fusion in the indicated genetic backgrounds. *hns* and *hns rpoS* strains should be compared to LT2, and the rest should be compared to 14028. The data represent the means and standard deviations from at least two experiments. According to the Student *t* test differences between the single mutant backgrounds and the corresponding wild-type backgrounds were statistically significant.

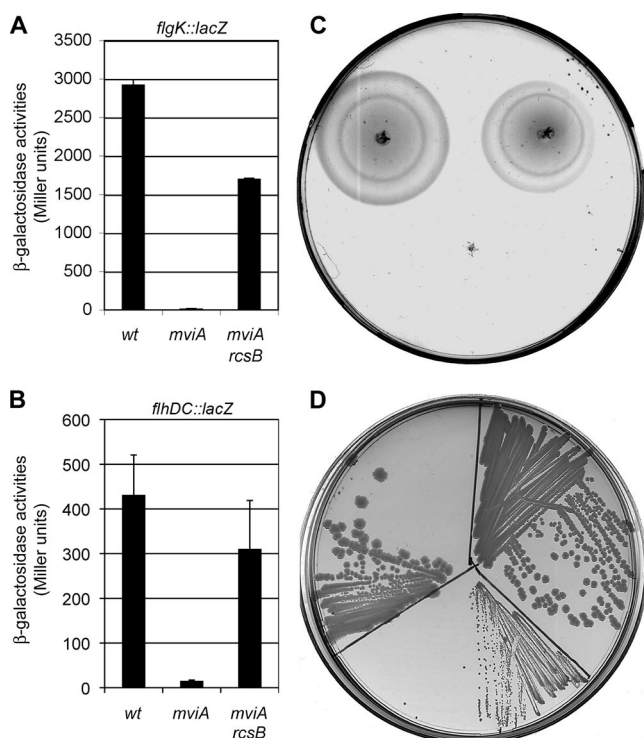


FIG. 4. MviA regulates the Rcs system. (A) Expression of an *flgK::lacZ* transcriptional fusion in wild-type, *mviA*, or *mviA rcsB* backgrounds. The data shown are means and standard deviations from three experiments. (B) Expression of a *flhDC::lacZ* transcriptional fusion in wild-type, *mviA*, or *mviA rcsB* strains. The data shown are means and standard deviations from three experiments. (C) Motility of the wild-type strain (upper left), the *mviA* mutant (center), and the *mviA rcsB* double mutant (upper right). Bacteria were spotted onto motility agar and incubated for 6 h at 37°C. (D) Colony morphology of the wild-type strain (upper left), the *mviA* mutant (center), and the *mviA rcsB* double mutant (upper right). The  $Scm^+$  phenotype of the *mviA* mutant is suppressed by an *rscB* mutation.

mutation caused decreases in *igaA* expression that were statistically significant ( $P < 0.001$ ) and that agreed with our predictions (Fig. 3).

**Some of the phenotypes of the *mviA* mutant are mediated by RcsB.** The fact that *igaA* is regulated by MviA, together with the role of *igaA* as an inhibitor of the activity of the Rcs system, suggests that some of the effects of the *mviA* mutation could be due to activation of the Rcs system. This system regulates flagellum synthesis through repression of the *flhDC* operon (16). To test the effect of an *mviA* mutation on flagellar genes we used a *flgK::lacZ* transcriptional fusion. This gene was completely repressed in an *mviA* background (Fig. 4A), a result that was in agreement with the idea that this mutation activates the Rcs system. Partial suppression of this effect by an *rscB* mutation gave support to this view (Fig. 4A). To establish the generality of these findings we studied the effects of the *mviA* mutation on the master flagellar operon *flhDC* and we got similar results: a *flhDC::lacZ* transcriptional fusion was repressed in the *mviA* mutant and this repression was suppressed by an *rscB* mutation (Fig. 4B). These results were confirmed by comparing the wild-type and the mutant strains on motility agar plates. Figure 4C shows that the *mviA* mutant is nonmo-

tile and that a mutation in *rscB* partially suppresses this phenotype. The fact that this new phenotype (lack of motility) of the *mviA* mutant is mediated by RcsB suggests that other phenotypes of this mutant could also be mediated by this regulatory system. The most conspicuous phenotype of the *mviA* mutant is the small colony morphology ( $Scm^+$ ). Comparison of the growth on LB agar of the wild type and the *mviA* and *mviA rcsB* mutants suggests that, in fact, the activity of the Rcs system is involved in this phenotype (Fig. 4D).

One of the most interesting phenotypes of *Salmonella mviA* mutants is avirulence in mice (77). The prevalent view is that this virulence defect is caused by increased levels of RpoS (77, 85). However, this hypothesis has not been directly tested because *Salmonella rpoS* null mutants are also avirulent (33). Since decreased levels of IgaA lead to activation of RcsB (28) and overactivation of Rcs causes attenuation (28, 40), a possibility is that avirulence of the *mviA* mutant is, at least partially, mediated by the Rcs system. A prediction of this hypothesis is that a null mutation in *rscB* would suppress avirulence of the *mviA* mutant. To test this possibility, competitive indexes in mice were calculated both for the *mviA* single mutant and the *mviA rcsB* double mutant after mixed infections with the wild type by the intraperitoneal route. The results (means of three experiments  $\pm$  standard deviations) were as follows: whereas the single mutant had a CI of  $0.0006 \pm 0.0002$ , the CI for the double mutant was  $0.12 \pm 0.06$ . This indicates that the double mutant is much more virulent than the single mutant but less virulent than the wild type and suggests partial suppression of the avirulence of the *mviA* mutant by the *rscB* null mutation, which is in agreement with our hypothesis.

**MviA also regulates RcsB in an RpoS- and IgaA-independent fashion.** The simplest interpretation of the data presented here is that the *mviA* mutation activates RcsB through a decrease in the levels of IgaA, which is in turn due to an increased RpoS level. In this context we would expect that the phenotypes of the *mviA* mutant that are suppressed by null *rscB* mutations were also suppressed by null *rpoS* mutations. In support of this idea, the  $Scm^+$  phenotype, which is suppressed by the *rscB* mutation, is also suppressed by an *rpoS* mutation (76) (data not shown). On the other hand this mutation is not able to suppress attenuation of the *mviA* mutant, since the *rpoS* mutant is itself avirulent, as mentioned above. We decided to examine the effects on flagellum synthesis and, surprisingly, we found that the *rpoS* mutation does not suppress the effect of the *mviA* mutation on the expression of *flgK* (Fig. 5). In addition, the *mviA rpoS* double mutant was nonmotile (Fig. 5). These results suggest that MviA may control the activity of RcsB through an RpoS- and IgaA-independent pathway. To further explore this possibility, we took advantage of a mutation in *rscC*, the gene that mediates the effects of IgaA on the Rcs system. This mutation was unable to suppress the effects of the *mviA* mutation on *flgK* expression, motility (Fig. 5), or small colony morphology (not shown). In addition, the introduction of plasmid pNG1166, a pBAD18 derivative that carries the *igaA* gene under the control of the  $P_{BAD}$  promoter, does not alter the phenotypes of the *mviA* mutant (Fig. 5 and data not shown).

**MviA controls levels of RcsB.** A tentative explanation for the results in the preceding section is that MviA, in addition to controlling the IgaA-RcsC-RcsD-RcsB pathway through

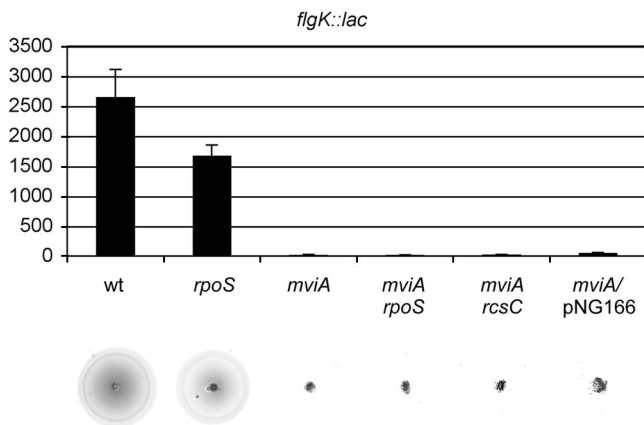


FIG. 5. MviA regulation of *flgK* transcription is RcsB dependent but RpoS, RcsC, and IgaA independent.  $\beta$ -Galactosidase activities (in Miller units) of the *flgK::lac* fusion were measured in the indicated backgrounds using stationary-phase cultures in LB medium. The data are the means  $\pm$  standard deviations of two independent experiments. The results of a motility assay for each strain are also shown.

RpoS, might regulate RcsB expression at the transcriptional or posttranscriptional level. To test the effect of *mviA* on *rcsB* transcription, we used an *rcsB::lacZ* transcriptional fusion. Expression of this fusion was only slightly altered in the *mviA* background (Fig. 6A). To test whether posttranscriptional regulation might occur, we used a strain carrying a 3xFlag tag at the 3' end of the *rcsB* gene. Immunoblot analysis revealed an increased level of RcsB in an *mviA* background (Fig. 6B). Although an *rpoS* mutation had a slight effect on *rcsB* transcription (Fig. 6A) it did not suppress the effect of *mviA* on the RcsB level (Fig. 6B). These results suggest that defects in MviA could alter either the translational efficiency or *rcsB* mRNA or proteolytic turnover of the RcsB protein. To ascertain between these alternatives, we tested the effect of an *mviA* mutation on RcsB turnover. Cultures of *mviA*<sup>+</sup> and *mviA* strains were treated with chloramphenicol to prevent further synthesis of RcsB, and samples for Western blot analysis were collected at defined time intervals. As seen in Fig. 6C, although the levels of RcsB were increased in the *mviA* strain, the stability of the protein did not appear to be altered. These results suggest that, aside from influencing activation of the Rcs system through RpoS and IgaA, MviA may also control the level of RcsB protein in an RpoS-independent manner.

## DISCUSSION

As an essential gene involved in the control of virulence in *Salmonella*, *igaA* is an attractive subject of research. However, most of the interest of the study of *igaA* has resulted from its use as a tool for the study of the Rcs system. In fact, the use of different *igaA* mutant alleles has uncovered new aspects of the Rcs system in *Salmonella*, including its role in the regulation of virulence (28). In this work, we decided to focus on *igaA* itself, and we have been able to show that (i) *igaA* is the first gene in an operon that includes four genes, (ii) its promoter is  $\sigma^{70}$  dependent, and (iii) its transcription is regulated by Lon and MviA. In addition we have unveiled relationships between the Rcs system, RpoS, and MviA.

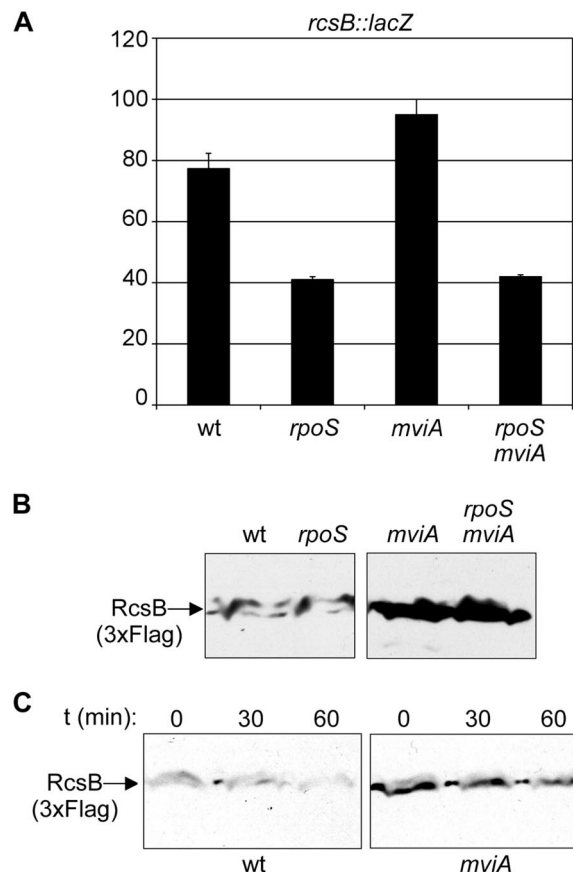


FIG. 6. MviA regulation of RcsB. (A)  $\beta$ -Galactosidase activities (in Miller units) of an *rcsB::lac* fusion in wild-type (wt), *rpoS*, *mviA*, and *mviA rpoS* backgrounds. The data are the means  $\pm$  standard deviations of two independent experiments. (B) Immunoblot analysis of the levels of RcsB-3xFlag in the wild-type, *rpoS*, *mviA*, and *mviA rpoS* backgrounds. Strains were cultured in LB medium. Protein extracts were resolved by 10% SDS-PAGE. The same amount of protein was loaded in each lane. Immunoblotting was performed with a monoclonal anti-Flag antibody. (C) Stability of RcsB-3xFlag in wild-type and *mviA* strains. The level of RcsB-3xFlag was analyzed by immunoblotting 0, 30, and 60 min after the addition of chloramphenicol to cultures in LB.

None of the phenotypes of *igaA* mutants seems to be due to polar effects on downstream genes: first, most of the mutants used are nonpolar, point mutants (15, 16, 28, 84); second, a polar insertion in the second gene of the operon, *yrfG*, is not lethal and does not produce any of the phenotypes associated with *igaA* mutations (15); third, a plasmid-borne *igaA*<sup>+</sup> allele is able to complement an *igaA* null mutation (15). There are no experimental data about the function of *yrfG*, *yrfH*, or *yrfI*, although sequence similarities suggest that they may encode a hydrolase and two heat shock proteins (Hsp15 and Hsp30), respectively.

Analysis of six viable (leaky) *igaA* mutants revealed that none of the corresponding mutations compromised the level of the protein in exponential growth phase. However, three mutations mapping in the putative cytoplasmic domains caused a significant IgaA decrease in stationary phase, and this loss of protein correlated with activation of the Rcs phosphorelay (28). These previous results support the idea that the control of *igaA* expression could be a way to regulate the activation of the

Rcs system. The results presented here show that, in fact, Lon and MviA regulate *igaA* at the transcriptional level.

Although early studies in *E. coli* identified Lon as a DNA binding protein, its ability for sequence-specific binding remains unclear. In contrast it has been clearly established that Lon is an ATP-dependent protease highly conserved in archaea, eubacteria, and eukaryotic mitochondria and peroxisomes (reviewed in reference 51). Therefore, our results are more likely explained by an indirect effect of Lon. As a heat shock protein, one of the main functions of Lon is to act as a quality control protease. However it is also important for the degradation of short-lived regulatory proteins. A possibility is that Lon may degrade an unidentified repressor of *igaA*. Interestingly, Lon is known to degrade RcsA (82), a coactivator necessary for the transcription of a subset of the Rcs-regulated genes. *igaA* is not itself regulated by the Rcs system, since an *rscB* mutation does not modify the expression of the *igaA::lacZ* transcriptional fusion (data not shown). Our results suggest that Lon could be negatively controlling the Rcs system in two ways: directly lowering the levels of RcsA and indirectly increasing the levels of IgaA.

Although MviA is a response regulator for a two-component regulatory system, it does not appear to contain a DNA binding domain. This suggests that its effect on the *igaA* transcription may be indirect. Our results show that this effect is mediated by RpoS. This is in agreement with the known role of MviA in the control of RpoS stability. In addition, we have shown that a mutation in RpoS is able not only to suppress the effect of an *mviA* mutation but also to increase the levels of *igaA* transcription. At least two hypotheses can explain the negative action of RpoS on a  $\sigma^{70}$ -dependent operon: (i) RpoS might control transcription of an *igaA* repressor; (ii) RpoS might compete with  $\sigma^{70}$  for core RNA polymerase. Sigma factor competition is not a new idea (90), and it has been proposed as a general mechanism to explain RpoS-dependent repression of  $\sigma^{70}$ -dependent genes (34, 68).

MviA acts as an adaptor for the protease ClpXP. The action of MviA is controlled by other proteins, called antiadaptors (11). One such antiadaptors is negatively regulated by H-NS (88) and another is positively regulated by the PhoPQ two-component system (83). Our results showing that both a null mutation in *hns* and a "constitutive" mutation in *phoP* result in a decrease in *igaA* transcription are consistent with the previously described relationships between these regulatory proteins and give additional support to the finding that *igaA* is regulated by MviA and RpoS.

The only known function of IgaA is repression of the Rcs system. Conditions like the *mviA* mutation that decrease the levels of IgaA are expected to result in activation of the Rcs system. Several results in this paper are consistent with this prediction: the phenotypes of avirulence, lack of motility, lack of expression of flagellar genes, and small colony type are suppressed, at least partially, by an *rscB* null mutation. Based on the results presented above, we propose a model in which RpoS has a negative effect on *igaA* transcription by competing with  $\sigma^{70}$ . Through degradation of RpoS, MviA positively controls the expression of *igaA*. H-NS and PhoP would fit in this model because they control the expression of antiadaptors that antagonize the action of MviA. Recent microarray data show that the Rcs system normally functions as a positive regulator

of SPI-2 and other genes important for the growth of *Salmonella* in macrophages, although when highly activated the system completely represses the SPI-1/SPI-2 virulence, flagellar, and fimbrial biogenesis pathways (84). It is tempting to speculate that activation of *phoP* in the intracellular environment would modulate degradation of RpoS, leading to repression of *igaA* and activation of the Rcs system, giving physiological significance to our results.

Although most of our data are consistent with the model proposed above, some results are puzzling: the repression of flagellum genes in the *mviA* mutant is suppressed by an *rscB* mutation but not by an *rpoS* mutation, by an *rscC* mutation, or by overexpression of *igaA*. These results do not fit the model but can be explained if we assume that MviA can also repress RcsB activity in an RpoS-, IgaA-, and RcsC-independent way. The idea that MviA does not act exclusively through RpoS is consistent with previous data from *Erwinia carotovora*. The ortholog of *mviA* in this plant pathogen is *expM*. An *expM* mutant exhibits reduced virulence in tobacco and is affected in production and secretion of extracellular enzymes. Suppression of both phenotypes by an *rpoS* mutation can be tested in this pathogen, since *rpoS* mutants of *Erwinia carotovora* are not avirulent (65). The conclusion is that overproduction of RpoS is an important factor to explain the phenotypes of the *expM* mutant. However an *expM rpoS* double mutant exhibits decreased virulence and secretion of extracellular enzymes in comparison with an *rpoS* single mutant, suggesting that ExpM in addition to RpoS acts on other targets (1). According to the results presented here, RcsB could be one such target in *Salmonella*. It is interesting that some of the phenotypes of the *mviA* mutant could be the result of the simultaneous effect on different targets. For instance, the *Scm*<sup>+</sup> phenotype is suppressed by both an *rpoS* mutation and an *rscB* mutation (but not an *rscC* mutation), indicating that an increase in RpoS level and RcsB overactivation are simultaneously necessary to produce this phenotype.

There are at least three hypotheses to explain this effect of MviA on RcsB: (i) the *mviA* mutation alters RcsB activation through cross-phosphorylation, perhaps using another histidine kinase or acetyl phosphate; (ii) MviA represses *rscB* transcription; (iii) MviA alters the levels of the RcsB protein influencing mRNA translation efficiency or protein stability. Hypotheses ii and iii are based on the fact that overexpression of *rscB* mimics overactivation of the Rcs system (39).

The differences detected in the immunoblot analysis shown in Fig. 6B cannot be explained by the slight differences observed in *rscB* transcription (Fig. 6A) and support RpoS-independent, posttranscriptional control of *rscB* by MviA. Data presented in Fig. 6C are more consistent with an effect in translation efficiency, since the stability of the RcsB protein does not appear to be altered in an *mviA* background.

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