JOURNAL OF BACTERIOLOGY, Mar. 2009, p. 1855–1867 Vol. 191, No. 6
0021-9193/09/$08.00+0 doi:10.1128/JB.01604-08

Genome Expression Analyses Revealing the Modulation of the *Salmonella* Rcs Regulon by the Attenuator IgaA[▽]†

Javier F. Mariscotti and Francisco García-del Portillo*

Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CSIC), Darwin 3, 28049 Madrid, Spain

Received 12 November 2008/Accepted 29 December 2008

Intracellular growth attenuator A (IgaA) was identified as a *Salmonella enterica* regulator limiting bacterial growth inside fibroblasts. Genetic evidence further linked IgaA to repression of the RcsCDB regulatory system, which responds to envelope stress. How IgaA attenuates this system is unknown. Here, we present genome expression profiling data of *S. enterica* serovar Typhimurium *igaA* mutants grown at high osmolarity and displaying exacerbated Rcs responses. Transcriptome data revealed that IgaA attenuates gene expression changes requiring phosphorylated RcsB (RcsB∼P) activity. Some RcsB-regulated genes, *yciGFE* and STM1862 (*pagO*)-STM1863-STM1864, were equally expressed in wild-type and *igaA* strains, suggesting a maximal expression at low levels of RcsB∼P. Other genes, such as *metB*, *ypeC*, *ygaC*, *glnK*, *glnP*, *napA*, *glpA*, and *nirB*, were shown for the first time and by independent methods to be regulated by the RcsCDB system. Interestingly, IgaA-deficient strains with reduced RcsC or RcsD levels exhibited different Rcs responses and distinct virulence properties. *spv* virulence genes were differentially expressed in most of the analyzed strains. *spvA* expression required RcsB and IgaA but, unexpectedly, was also impaired upon stimulation of the RcsC→RcsD→RcsB phosphorelay. Overproduction of either RcsB$^+$ or a nonphosphorylatable RcsB(D56Q) variant in strains displaying low *spvA* expression unveiled that both dephosphorylated RcsB and RcsB∼P are required for optimal *spvA* expression. Taken together, our data support a model with IgaA attenuating the RcsCDB system by favoring the switch of RcsB∼P to the dephosphorylated state. This role of IgaA in constantly fine-tuning the RcsB∼P/RcsB ratio may ensure the proper expression of important virulence factors, such as the Spv proteins.

Bacteria use two-component signaling systems to sense, respond to, and adapt to changes in the environment (38, 57). These systems participate in processes such as chemotaxis, motility, biofilm formation, adaptation to stress, and virulence (1, 5, 24). A sensor histidine kinase and a cytosolic response regulator are essential elements of these systems (38, 43). In response to the external stimulus, the histidine kinase autophosphorylates a conserved histidine residue to further transfer the phosphoryl group to a conserved aspartate residue of the response regulator. Once phosphorylated, the response regulator binds target genes to modulate their expression levels. Transfer of the phosphate group also occurs between modules of the same protein or intermediate proteins that act as phospho-transmitters (43). A widely studied two-component signaling system is the RcsCDB signaling system, first identified in *Escherichia coli* as a regulator of the production of the colanic acid capsule (53). This system can be activated by diverse envelope-related stresses (13, 14, 19, 20, 35, 36, 47, 49, 52, 61) or the overproduction of proteins such as DjlA, LolA, and OmpG (11, 37).

The RcsCDB system operates with two membrane proteins, the hybrid sensor RcsC and the phospho-transmitter RcsD, together with the cytosolic response regulator RcsB (34, 39). Most genes subjected to regulation by the RcsCDB system encode envelope proteins and factors related to the synthesis of diverse exopolysaccharides (20, 22, 23, 27, 30, 35). It has been proposed that, upon sensing envelope alterations, bacteria activate the RcsCDB system to remodel the envelope composition accordingly. The stimulus sensed by the system is transmitted via an RcsC→RcsD→RcsB phosphorelay (11). Phosphorylated RcsB (RcsB∼P) modulates target gene expression by interacting with DNA through its C-terminal DNA-binding domain. RcsA is another protein playing a coregulatory role in a subset of the RcsB-regulated genes, such as those involved in the production of the colanic acid capsule (39). The outer membrane protein RcsF also modulates the RcsCDB system by activating RcsC via a yet-unknown mechanism (8, 40). RcsF-independent signaling requiring RcsC has also been shown previously (8). Another important regulatory mechanism is the phosphorylation of RcsB mediated by acetylphosphate under conditions in which RcsC functions mainly as phosphatase (26).

In our previous studies, we obtained genetic evidence linking an inner membrane protein of *Salmonella enterica* serovar Typhimurium, named intracellular growth attenuator A (IgaA), to repression of the RcsCDB system (6). Mutations affecting IgaA stability result in strong RcsCDB responses, manifested by the inhibition of flagellum production and the overproduction of capsule material and cell division proteins (6, 42). Activation of the RcsCDB system in *E. coli* has been correlated in most cases to envelope stress (34, 39). However, mutations in IgaA causing partial loss of function (*igaA1* allele) do not alter envelope integrity in serovar Typhimurium (17, 42). Thus,

* Corresponding author. Mailing address: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CSIC), Darwin 3, 28049 Madrid, Spain. Phone: (34) 91 585 4923. Fax: (34) (91) 585 4506. E-mail: fgportillo@cnb.csic.es.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 5 January 2009.

activation of the RcsCDB system does not necessarily impair envelope homeostasis, and some remodeling may be tolerable without compromising basic envelope functions. On the other hand, IgaA is an essential protein in serovar Typhimurium but dispensable in mutants lacking any of the three main components of the RcsCDB system (6, 15, 42). Spontaneous mutations consisting of deletions leading to a diminished production of RcsC or RcsD are also known to suppress IgaA essentiality (42). Important traits of serovar Typhimurium, such as its virulence potential, depend on the fine-tuning of the activity of the RcsCDB system (17, 28, 45). IgaA and the RcsCDB system are present in the same genera of enteric bacteria (17, 20, 34), and this concurrence strongly supports the functional link existing between IgaA and the RcsCDB system.

Microarray analyses performed with *E. coli* have been instrumental in defining the composition of the Rcs regulon under diverse environmental conditions (23, 30, 46). Although common genes were found in these studies, there were also substantial differences, probably due to the distinct growth conditions and the diverse mutations used. Thus, 46 genes with altered expression were identified when *rcsB*$^+$ and Δ*rcsB* strains were compared (46); 32 genes were identified when *rcsC*$^+$ and Δ*rcsC* strains grown at low temperatures, in glucose, and with high zinc concentrations were compared (30); and 149 genes were identified when profiles of *rcsC*$^+$ and Δ*rcsC* bacteria overexpressing DjlA were compared (23). The Rcs regulon of *Yersinia pseudotuberculosis* has been estimated to be formed by at least 136 genes, with 60% of the genes encoding functions related to envelope homeostasis (31). In serovar Typhimurium, 26 RcsB-dependent but RcsA-independent genes were reported upon exposure of bacteria to sublethal concentrations of antimicrobial peptides (20). Recent studies performed with serovar Typhimurium expressing IgaA(T191P), a variant previously shown by us to be unable to repress the RcsCDB system (17), revealed that one-fifth of the genes analyzed displayed expression changes higher than twofold (55).

In this work, we analyzed the serovar Typhimurium Rcs regulon in bacteria exposed to high osmolarity, a condition known to stimulate the RcsCDB system (61). We also investigated the Rcs regulon in strains producing an IgaA(R188H) variant (R188H is the first mutation characterized for this attenuator to cause overactivation of the RcsCDB system [6, 7, 17, 27, 54]) and in recently described IgaA-deficient strains having reduced RcsC or RcsD levels (42). The study has provided new insights into the mode of attenuation exerted by IgaA on the RcsCDB system and has led to the first evidence supporting a role for dephosphorylated RcsB as a transcriptional regulator.

TABLE 1. *S. enterica* serovar Typhimurium strains and plasmids used in this study

Strain	Relevant genotype	Reference or source
Strains		
SL1344	*hisG46 rpsL*, virulent parental isolate	33
SV4450	*igaA1*	6
MD0862	*igaA1 rcsB70*::Tn*10d* Cm	42
SV4406	*rcsB70*::Tn*10d* Cm	6
SV4343	*igaA1 zhf-6311*::Tn*10d* Tet *rcsA51*::Mu*d*Q	6
SV4379	*rcsA51*::Mu*d*P	6
SV4380	*rcsC52*::Mu*d*Q	6
SV4404	*igaA1 zhf-6311*::Tn*10d* Tet *rcsC52*::Mu*d*Q	6
MD2023	Δ*rcsD69*	This work
MD2026	*igaA1 zhf-6311*::Tn*10d* Tet Δ*rcsD69*	This work
MD0835-J1	*igaA2*::KIXX Δ(*apbE′-rcsC′*)	42
MD0842-J4	*igaA2*::KIXX *rcsC67′*	42
MD0855-J10	*igaA2*::KIXX Δ(*ompC′-micF*)	42
MD0890-J4	*igaA3*::Cam^r *rcsC67′*	This work
MD1313-J10	*igaA3*::Cam^r Δ(*ompC′-micF*)	This work
SV4407	*wcaH21*::Mu*d*J	6
MD0099	*igaA1 wcaH21*::Mu*d*J	6
MD0870	*igaA1 rcsB70*::Tn*10d* Cm *wcaH21*::Mu*d*J	This work
MD0895-J4	*igaA3*::Cam^r *rcsC67′ wcaH-21*::Mu*d*J	42
MD1326-J10	*igaA3*::Cam^r Δ(*ompC′-micF*) *wcaH21*::Mu*d*J	42
SV4312	*spvA103*::Mu*d*J	7
MD1368	*igaA1 spvA103*::Mu*d*J	This work
MD1369	*igaA1 rcsB70*::Tn*10d* Cm *spvA103*::Mu*d*J	This work
MD1370	*rcsB70*::Tn*10d* Cm *spvA103*::Mu*d*J	This work
MD1371-J4	*igaA3*::Cam^r *rcsC67′ spvA103*::Mu*d*J	This work
MD1372-J10	*igaA3*::Cam^r Δ(*ompC′-micF*) *spvA103*::Mu*d*J	This work
MD1396	*igaA1 zhf-6311*::Tn*10d* Tet *rcsA51*::Mu*d*Q *spvA103*::Mu*d*J	This work
MD1397	*rcsA51*::Mu*d*Q *spvA103*::Mu*d*J	This work
MD2007	*rcsC52*::Mu*d*Q *spvA103*::Mu*d*J	This work
MD2008	*igaA1 zhf-6311*::Tn*10d* Tet *rcsC52*::Mu*d*Q *spvA103*::Mu*d*J	This work
MD2027	Δ*rcsD69 spvA103*::Mu*d*J	This work
MD2028	*igaA1 zhf-6311*::Tn*10d* Tet Δ*rcsD69 spvA103*::Mu*d*J	This work
MD2053	*igaA1 rcsB70*::Tn10*d* Cm/pIZ1589	This work
MD2054	*igaA1 rcsB70*::Tn10*d* Cm/pJM0029	This work
MD2055	*spvA103*::Mu*d*J/pIZ1589	This work
MD2056	*igaA1 spvA103*::Mu*d*J/pIZ1589	This work
MD2057	*igaA1 rcsB70*::Tn10*d* Cm *spvA103*::Mu*d*J/pIZ1589	This work
MD2058	*rcsB70*::Tn10*d* Cm *spvA103*::Mu*d*J/pIZ1589	This work
MD2059	*spvA103*::Mu*d*J/pJM0029	This work
MD2060	*igaA1 spvA103*::Mu*d*J/pJM0029	This work
MD2061	*igaA1 rcsB70*::Tn10*d* Cm *spvA103*::Mu*d*J/pJM0029	This work
MD2062	*rcsB70*::Tn10*d* Cm *spvA103*::Mu*d*J/pJM0029	This work
Plasmids		
pIZ1589	pBAD18 *rcsB*$^+$	27
pJM0029	pBAD18 *rcsB*(*D56Q*)	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Salmonella enterica* serovar Typhimurium strains and plasmids used in this work are listed in Table 1. All strains are isogenic derivatives of the mouse-virulent strain SL1344 (33). Bacteria were grown at 37°C in Luria-Bertani (LB) broth, on LB agar plates, or in minimal intracellular-salt medium (ISM) as indicated previously (6, 58). ISM, which is of high osmolarity (403 mosmol), provides an optimal stimulus for induction of the RcsCDB system in strains with defects in IgaA (see below). ISM has also been shown to be suitable to assess expression of the *spv* virulence genes in serovar Typhimurium (58). Unless otherwise indicated, glycerol (38 mM) was used as the carbon source in the ISM. When appropriate, kanamycin (30 μg ml^{-1}), tetracycline (10 μg ml^{-1}), ampicillin (100 μg ml^{-1}), or chloramphenicol (10 μg ml^{-1}) was used.

Generation of a nonphosphorylatable RcsB(D56Q) variant. A nonphosphorylatable RcsB(D56Q) variant was constructed using as the template the plasmid pIZ1589, a gift of J. Casadesús (Universidad de Sevilla, Spain). pIZ1589 expresses the *rcsB*$^+$ wild-type allele under the control of the arabinose-inducible P_{BAD} promoter (27). Mutagenesis of the phosphorylatable aspartate residue at position 56 of RcsB was designed to replace codon 56, GAC, with codon CAG, specific for glutamine. The mutagenesis was performed by overlap extension PCR as described previously (32) using the following oligonucleotides: rcsB5′ (5′-AGC GGA ATT CAG GAG GAA TAC ATG AAC AAT ATG AAC G-3′), rcsB3′D56Q (5′-CAT GGA GAG CTG AGT GAT CAA C-3′), rcsB5′D56Q (5′-GTT GAT CAC TCA GCT CTC CAT G-3′), and rcsB3′ (5′-GTG AAA

GCT TGT CGA CAA GCG ATT TAT TCT TTG TCT G-3′). The underlined oligonucleotide sequences are the sites changed in the mutagenesis procedure.

Construction of a Δ*rcsD* nonpolar deletion mutant. To avoid any polar effect on *rcsB* transcription, the deletion of *rcsD* was designed from 155 nucleotides upstream of the start codon (*micF-rcsD* intergenic region) to position 2,523 of the 2,670-nucleotide open reading frame of *rcsD*. The deletion was created by following the one-step inactivation procedure described by Datsenko and Wanner and using plasmid pKD3 as the template (16). The oligonucleotides used for this procedure were rcsD-P1 (5′-AGC AAA TAA TTT CTT GAT ATT TAG TGC TAA ACA TTT ATA AGT AGT CTT TAT GTG TAG GCT GGA GCT GCT TC-3′) and rcsD-P2 (5′-TAA CTG CTT GCC GGG TAC CAG ATT AAG CAT GGC AAA CAC CCC TTT CAG GCG CAT ATG AAT ATC CTC CTT AGT-3′). Underlined in these sequences are the regions that anneal to the flanking regions of the *cat* cassette (Cm^r) present in plasmid pKD3. This allelic exchange generated the strain MD2022 (Δ*rcsD69*::*cat*). The *cat* cassette was further eliminated with plasmid pCP20, expressing the FLP recombinase, as described previously (16), and resulting in strain MD2023 (Δ*rcsD69*). Control experiments showed that *rcsB* transcription is not affected in strain MD2023 (see Fig. S1 in the supplemental material). Oligonucleotides specific for *rcsD*, *rcsB*, and *rcsC* used in these reverse transcription-PCR (RT-PCR) assays are listed in Table S1 in the supplemental material. The loss of the wild-type $rcsD^+$ allele was confirmed with oligonucleotides yojN-4D and yojN-2R (see Table S1 in the supplemental material).

Phage transductions. The transductional crosses were made using the P22 HT 105/1 *int201* phage (51), as described previously (41). Phage-free transductants were screened on green plates as described previously (10) and tested for sensitivity to the clear-plaque mutant P22 H5.

Microarray analyses. A 70-mer oligonucleotide microarray was constructed using the genome sequence of *S. enterica* serovar Typhimurium strain SL1344, made available by the Wellcome Trust Sanger Institute (see the supplemental material). Hybridization of nucleic acids was conducted using cDNA as the "expression sample" of the respective strain mixed with genomic DNA (gDNA) as the internal reference. As previously reported (21), this method allows transcriptome comparisons among different strains. RNA was purified in six independent experiments from bacteria grown in 10 ml of ISM-glycerol medium at exponential phase (optical density at 600 nm [OD_{600}], ~0.2) by using the SV total RNA isolation system kit (catalog no. Z3100; Promega). These RNAs were combined in pools of three samples and used in two independent hybridizations. Prior to this manipulation, a 1:5 volume of a 95% ethanol-5% phenol solution was added to the bacterial culture (final concentration, 19% ethanol-1% phenol). As described previously, this step ensures the full integrity and stability of the RNA (21). The quality of the RNA was tested using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). RT of RNA was performed with SuperScript III reverse transcriptase (catalog no. 18080-044; Invitrogen) according to the instructions of the manufacturer. cDNA was purified with the QIAquick‰ PCR purification kit (catalog no. 28106; Qiagen). Reference gDNA was purified from bacteria grown in 6 ml of LB broth to stationary phase. Upon centrifugation (10,000 × *g* for 10 min, 4°C), the bacterial pellet was processed using the Qiagen gDNA kit (catalog no. 19060/10243; Qiagen). DNA was finally suspended in 200 μl of deionized water, resulting in a total amount in the range of 60 to 90 μg. This DNA was further fragmented by sonication before its use in the hybridization assays. Labeling of cDNA and gDNA with Alexa Fluor 647 and Alexa Fluor 555 fluorescence molecules, respectively, was conducted as described previously (21) using exo-Klenow enzyme and the BioPrime Plus array CGH indirect genomic labeling system (catalog no. 18096-011; Invitrogen). Labeled cDNA and gDNA were further purified, and the amount of incorporated labeling was estimated in a NanoDrop ND-1000 UV–visible-light spectrophotometer. Hybridization conditions, data acquisition, normalization, and statistical analysis are described in detail in the supplemental material.

RT-PCR assays. RT-PCR was performed using a one-step RT-PCR kit (Qiagen) as previously described (55). Briefly, DNase (Ambion)-treated RNA samples were diluted to 20 ng/μl and were further normalized against the RT-PCR-amplified *ompA* product, used as an internal control. The RT-PCR was carried out with a final volume of 25 μl consisting of 5 μl buffer (5×), 1 μl of deoxynucleoside triphosphates (10 mM), 3 μl each of the forward and reverse primers (5 μM), 1 μl of RT-PCR enzyme mix, and ~20 ng of RNA and RNase-free water supplemented to 25 μl. The RT-PCR cycling conditions were as follows: 50°C for 35 min and 95°C for 15 min, followed by 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s and then an extra step of elongation at 72°C for 10 min. Oligonucleotides used in these RT-PCR assays are listed in Table S1 in the supplemental material.

β-Galactosidase assays. The levels of β-galactosidase enzyme derived from the *wcaH21*::Mu*dJ* and *spvA103*::Mu*dJ* transcriptional fusions were determined using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure, as described previously (44). Bacteria were grown overnight at 37°C in LB broth or ISM-glycerol medium under shaking conditions (180 rpm), until a final OD_{600} of ~3.0 (LB medium) or ~1.2 to 1.4 (ISM) was reached. Cultures were further diluted 1:100 in fresh medium and further incubated for 6 h, the time at which β-galactosidase activity was measured. In the case of RcsB variants expressed from plasmids under the arabinose-inducible P_{BAD} promoter, bacteria were grown overnight in ISM-2% glucose (OD_{600}, ~1.2 to 1.4) and later washed once in ISM with no sugar added. These bacteria were then added to either ISM-2% glucose or ISM-glycerol-0.05% arabinose medium (final OD_{600}, ~0.05) and further incubated for 16 h, the time at which the β-galactoside activity was measured. The experiments were repeated at least three times.

Virulence assays. To assess the virulence capacity of IgaA-deficient strains with distinct levels of expression of the RcsCDB system, the protocol of Beuzón and Holden involving a 1:1 mixture of mutant and wild-type strains was used (2). Three female BALB/c mice (6 to 8 weeks old) were challenged per mixture using the intraperitoneal route. The experiment was repeated twice using 2×10^5 CFU of each strain. The competitive index assay results were calculated from the numbers of viable bacteria counted in liver and spleen at 48 h postinfection.

Statistical analysis. Data were analyzed by one-way analysis of variance using Prism version 5.0 (GraphPad Software, Inc.). Differences in the values with a P of <0.05 were considered statistically significant.

Accession numbers. The characteristics and configuration of the Salgenomics microarray were deposited in the MIAME database (http://www.ebi.ac.uk/miamexpress) under accession number A-MEXP-846. Gene expression data were deposited in the Array Express database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-1612.

RESULTS

Definition of the serovar Typhimurium Rcs regulon in a high-osmolarity minimal medium. The Rcs regulon in *E. coli* has been extensively studied (23, 30, 46). In the case of serovar Typhimurium, a recent study reported microarray data of wild-type, *rcsC*, *rcsD*, *rcsB*, and *rcsA* strains exposed to sublethal concentrations of polymyxin B (20). The report of that study listed 26 serovar Typhimurium genes regulated by the RcsCDB system in an RcsA-independent manner (21 positively and 5 negatively). However, neither the identities of RcsB- and RcsA-coregulated genes nor the transcriptome profiles were provided. In another recent study, approximately 20% of the serovar Typhimurium genome was claimed to be under the control of RcsB in bacteria growing in nutrient-rich medium (55). In that study, a strain expressing an IgaA(T191P) mutant protein was used as a control of activation of the RcsCDB system.

In our attempt to dissect the modulation exerted by IgaA on the RcsCDB system, we performed genome expression profiling of serovar Typhimurium strains with defects in this attenuator and grown under conditions not tested in previous studies. We examined the effect of the R188H change in IgaA (allele *igaA1*), the first-characterized mutation that revealed a functional link between IgaA and the RcsCDB system (6, 7, 17). Bacteria were grown in the high-osmolarity minimal medium ISM (58), in which the Rcs response is more pronounced when IgaA is altered. Thus, the *igaA1* mutant expressed the colanic capsule gene *wcaH*, positively regulated by the RcsCDB system, at ~15-fold higher levels in ISM than in LB medium (Fig. 1). Our microarray study also included "mucoid" strains lacking IgaA which have recently been characterized (42). Two of these IgaA-defective strains with a functional RcsCDB system are MD0842-J4 (*igaA2*::KIXX *rcsC67′*) and MD0855-J10 [*igaA2*::KIXX Δ(*ompC′-micF*)], which spontaneously suppressed IgaA essentiality by diminishing the protein levels of RcsC and RcsD, respectively (42). In total, the iso-

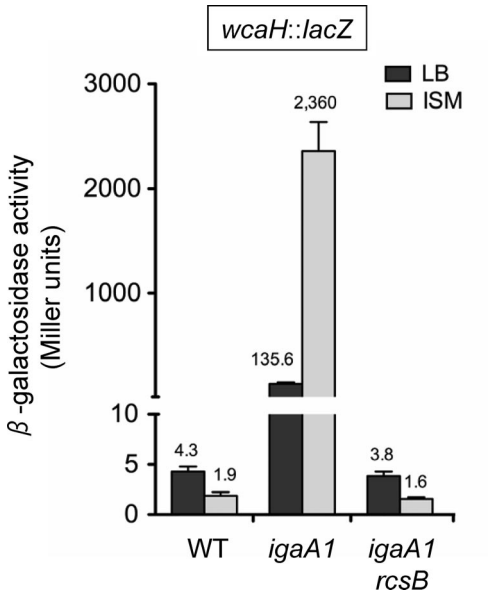

FIG. 1. Growth of serovar Typhimurium under high-osmolarity conditions augments the Rcs response resulting from defects in the attenuator IgaA. The expression of the colanic acid gene *wcaH* (*gmm*), positively regulated by the Rcs system, was monitored in bacteria grown in nutrient-rich LB medium (dark-gray bars) or ISM-glycerol minimal medium of high osmolarity (light-gray bars). The isogenic strains used were wild-type SV4407 (*wcaH21*::Mu*dJ*), MD0099 (*igaA1 wcaH21*::Mu*dJ*), and MD0870 (*igaA1 rcsB70 wcaH21*::Mu*dJ*). These strains are abbreviated in the figure as WT, *igaA*, and *igaA1 rcsB*, respectively. Bacteria were grown to an OD_{600} of ~2.5 to ~3.0 (LB medium) or ~1.2 to ~1.4 (ISM-glycerol medium). β-Galactosidase activities correspond to the means and standard deviations from a minimum of three independent experiments. Mean values are also shown on top of the bars.

genic strains used in our microarray studies included the wild type; *igaA1*, *rcsB*, *rcsA*, *igaA1 rcsB*, and *igaA1 rcsA* strains; MD0842-J4; and MD0855-J10 (Table 2). The microarray Salgenomics, designed on the genome sequence of the virulent serovar Typhimurium strain SL1344 (33), was constructed for this purpose (see the supplemental material). Only those gene expression changes with values of fourfold or higher ($\log_2$ ratio, ≤ -2 or ≥ 2) were considered significant.

Identification of IgaA-regulated genes. The effect of the IgaA(R188H) mutation in the Rcs regulon was assessed in the wild-type, *igaA1*, *rcsB*, and *igaA1 rcsB* strains. Expression profiles were processed in the form of comparisons of a mutant and the wild type or of two mutants (Table 2). A total of 71 genes displaying changes in expression from the wild-type strain of at least fourfold were identified in the *igaA1* mutant. Of these, 33 were upregulated (Table 3) and 38 downregulated (Table 4). Most of the upregulated genes ($n = 22$), are involved in colanic acid capsule synthesis (*wza-wzb*, *wcaABC-DEF-gmd-wcaGHI-manC*, *cpsG-wcaJ*, *wzxC-wcaKLM*, and the regulatory gene *rcsA*) (30, 55). The *yjbEFGH* operon, recently described to be required for the synthesis of a novel exopolysaccharide in *E. coli* (22), was also upregulated in the serovar Typhimurium *igaA1* mutant (Table 3). So, ~80% (26/33) of the genes highly upregulated in the *igaA1* mutant are involved in the synthesis of capsular exopolysaccharides. Other genes induced in the *igaA1* mutant and previously known to be regulated by RcsCDB are *osmB*, *ygaC*, and *yebE* (3, 23, 30, 55). On the other hand, *metB-metF*, two genes involved in methionine biosynthesis, were identified in our study as new genes positively regulated by the RcsCDB system.

Among the 38 downregulated genes in the *igaA1* mutant, 26 were flagellum and chemotaxis genes (Table 4). Unlike in a recent study that analyzed the response in LB medium of an *igaA*(*T191P*) mutant with high Rcs activity (55), no reduced expression of virulence genes present in *Salmonella* pathogenicity islands 1 and 2 or of fimbrial genes was noted. Interestingly, other genes repressed in our *igaA1* mutant are related to ammonia metabolism and nitrate/nitrite respiration (*glnK*, *glnP*, *napA*, *napD*, *nirB*), transport of maltose (*malE*, *lamB*), and uptake/metabolism of glycerol-3-phosphate (*glpT*, *glpA*). In addition, a pronounced repression of *spvA* and *spvB*, two *Salmonella*-specific genes required for virulence (29), was detected in the *igaA1* mutant with $\log_2$ ratios in the range of -4 or -5, equivalent to ~15- to 30-fold changes (Table 4). A search in the literature for genes previously assigned to Rcs regulons of different enterobacteria revealed that among the 71 genes identified with altered expression in the *igaA1* mutant, 13 were not reported before as genes regulated by the RcsCBD system. These include *yebE*, *dsrB*, *ypeC*, *metB*, *metF*, *spvB*, *spvA*, *glnK*, *glnP*, *napA*, *napD*, *glpA*, *yhbU*, and *nirB* (Tables 3 and 4).

The gene expression profile of the *igaA1* mutant was then compared to those of the *igaA1 rcsB* and *rcsB* strains. Among the 71 genes having altered expression in the *igaA1* mutant compared to that in wild-type bacteria (Tables 3 and 4), 68 of them did not display any significant change in expression in the *igaA1 rcsB* and *rcsB* strains (Fig. 2A and B). It is noteworthy that the *yciGFE* operon and the *Salmonella*-specific genes STM1862 (*pagO*), STM1863, and STM1864 were downregulated only in the mutants lacking RcsB, irrespective of the

TABLE 2. List of the tables in the supplemental material showing serovar Typhimurium genes with altered expression caused by mutations in *igaA*, components of the RcsCDB system, and/or the coregulator RcsA[a]

Table in the supplemental material	Strains used for transcriptome comparison[b]
S2	All strains
S3	*igaA1* mutant vs *igaA1 rcsB* mutant
S4	*rscB* mutant vs wild type
S5	*igaA1 rcsB* mutant vs wild type
S6	*igaA1* mutant vs *igaA1 rcsA* mutant
S7	*igaA1 rcsA* mutant vs wild type
S8	*rcsA* mutant vs wild type
S9	*igaA2*::KIXX *rcsC′* mutant vs wild type
S10	*igaA2*::KIXX (Δ*ompC′-micF*) mutant vs wild type
S11	*igaA1* mutant vs *igaA2*::KIXX *rcsC′* mutant
S12	*igaA1* mutant vs *igaA2*::KIXX (Δ*ompC-micF*) mutant
S13	*igaA2*::KIXX (Δ*ompC-micF*) mutant vs *igaA2*::KIXX *rcsC′* mutant

[a] Altered expression is indicated by a P value of ≤ 0.05 and a $\log_2$ ratio that was less than or equal to -2 or ≥ 2.

[b] Strain designations for the indicated genotypes are SL1344 (wild type), SV4450 (*igaA1* mutant), SV4379 (*rcsA* mutant), SV4406 (*rcsB* mutant), SV4343 (*igaA1 rcsA* mutant), MD0862 (*igaA1 rcsB* mutant), MD0842-J4 (*igaA2*::KIXX *rcsC′* mutant), and MD0855-J10 [*igaA2*::KIXX (Δ*ompC′ -micF*) mutant]. See also Table 1. Genes with differential expression in the comparison of the *igaA1* mutant and the wild type are shown in Tables 3 and 4.

TABLE 3. Genes in the serovar Typhimurium *igaA1* mutant with increased expression relative to that in the wild-type strain

Systematic gene name	Gene	Product	Log_2 ratio for the indicated comparison[a]					Reference(s) if the product was previously assigned to the Rcs regulon[b]
			igaA1 mutant vs WT	*igaA1 rcsB* mutant vs WT	*rcsB* mutant vs WT	*igaA1* mutant vs *igaA1 rcsA* mutant	*igaA1 rcsA* mutant vs WT	
STM1633	STM1633	Putative periplasmic binding protein	2.3	−1.9	−2.2	0.1	2.2	55
STM1705	*osmB*	Lipoprotein B	3.0	−0.9	−0.05	−1.0	4.1	23, 30, 55
STM1880	*yebE*	Putative inner membrane protein	2.2	−0.06	0.1	1.3	0.9	
STM1982	*rcsA*	Colanic acid capsular regulator A	3.6	−1.6	−1.2	2.1	1.7	23, 55
STM1983	*dsrB*	Hypothetical protein STM1983	2.4	−0.7	−0.9	1.2	1.2	
STM2080	*udg*	UDP-glucose/GDP-mannose dehydrogenase	2.5	−0.8	−1.1	2.5	−0.2	23, 55
STM2099	*wcaM*	Putative colanic acid biosynthetic protein	3.3	−0.6	−1.0	3.0	0.5	23, 30, 55
STM2100	*wcaL*	Putative glycosyl transferase	6.4	0.1	0.9	2.0	4.4	30, 55
STM2101	*wcaK*	Putative galactokinase	6.6	−0.6	−0.1	3.2	3.3	30, 55
STM2102	*wzxC*	Putative colanic acid exporter	5.4	−0.1	−0.2	2.9	2.7	23, 55
STM2103	*wcaJ*	UDP-glucose lipid carrier transferase	5.6	−0.9	−0.2	2.9	2.8	30, 55
STM2104	*cpsG*	Phosphomannomutase	7.0	−0.9	0.5	3.2	3.8	30, 55
STM2105.S	*manC*	Mannose-1-phosphate guanylyl transferase	8.0	−0.5	0.5	3.1	4.6	23, 55
STM2106	*wcaI*	Putative glycosyl transferase	8.1	−0.1	1.0	3.2	4.7	23, 30, 55
STM2107	*wcaH*	GDP-mannose mannosyl hydrolase	8.3	−0.8	1.0	3.6	4.5	23, 30, 55
STM2108	*wcaG*	GDP-fucose synthetase	8.0	−0.6	−0.4	3.5	4.4	23, 30, 55
STM2109	*gmd*	GDP-D-mannose dehydratase	8.6	−1.7	−1.2	3.7	4.3	23, 30, 55
STM2110	*wcaF*	Putative acyl transferase	7.6	−0.7	−0.9	4.0	3.6	23, 30, 55
STM2111	*wcaE*	Putative transferase	6.9	−0.7	0.1	4.1	2.7	23, 30, 55
STM2112	*wcaD*	Putative colanic acid polymerase	6.5	−0.3	−0.5	4.7	2.0	23, 30, 55
STM2113	*wcaC*	Putative glycosyl transferase	4.6	−0.6	−0.1	4.1	0.8	23, 30, 55
STM2114	*wcaB*	Putative acyl transferase	5.5	−0.2	−0.2	3.6	1.9	30, 55
STM2115	*wcaA*	Putative glycosyl transferase	4.3	0.03	2.1	3.9	0.4	23, 30, 55
STM2117	*wzb*	Putative phosphotyrosine protein phosphatase	5.8	−0.9	−0.4	5.0	0.9	23, 55
STM2118	*wza*	Putative outer membrane polysaccharide export protein	5.1	−0.7	−0.7	5.1	0.2	23, 55
STM2407	*ypeC*	Putative periplasmic protein	2.2	−0.7	−0.6	−0.02	2.4	
STM2801	*ygaC*	Putative cytoplasmic protein	3.0	−1.5	−2.3	0.4	2.9	23, 55
STM4100	*metB*	Cystathionine gamma synthase	2.4	0.2	0.5	−0.4	3.0	
STM4105	*metF*	5,10-Methylenetetrahydrofolate reductase	2.5	0.5	0.2	0.4	2.1	
STM4222.S	*yjbE*	Putative outer membrane protein	5.9	−0.2	−0.1	4.3	1.7	20, 22, 23, 55
STM4223	*yjbF*	Putative outer membrane lipoprotein	5.9	−0.5	0.3	3.2	2.6	22, 23, 55
STM4224	*yjbG*	Putative periplasmic protein	5.0	−0.7	−0.9	3.6	1.6	22, 23, 55
STM4225	*yjbH*	Putative outer membrane lipoprotein	3.2	−0.2	−0.9	2.4	1.0	22, 23, 27, 55

[a] Each gene was represented by a single 70-mer oligonucleotide spotted in two separate sections of a microarray slide (see the supplemental material for details). For the statistical analyses, the two spots were considered different entities. The table lists only those genes identified in the comparison of the *igaA1* mutant and the wild type (WT), both of whose *P* values were ≤0.05 and whose two log_2 ratios were ≥2. For simplification of this table, only the average of the two log_2 ratios for each gene is shown (see Table S2 in the supplemental material for a complete list of the log_2 ratios and *P* values).

[b] No reference indicates no previous assignment.

functional status of IgaA (*igaA1 rcsB* and *rcsB* mutants) (Fig. 2B; see also Tables S3, S4, and S5 in the supplemental material). Such observations may indicate that these genes are maximally expressed with low levels of RscB~P. Other genes, such as *spvA* and *spvB*, were, however, downregulated to a large extent in the three strains tested, namely, the *igaA1*, *igaA1 rcsB*, and *rcsB* mutants (Table 4; Fig. 2B). To further confirm these observations, RT-PCR was used as an alternative method to monitor the expression of representative genes not described before as regulated by the RcsCDB system (*spvA*, *metB*, *ypeC*, *glnK*, *glnP*, *napA*, *glpA*, and *nirB*). Control genes known to form part of the Rcs regulon, such as *wcaH* and *ygaC*, were also included. The results of these RT-PCR assays were in agreement with the microarray data, with expression profiles for the *igaA1* mutant being clearly opposite to those of the wild-type, *igaA1 rcsB*, and *rcsB* strains, except in the expression of *spvA* (Fig. 3). Taken together, these studies demonstrated that in bacteria growing at high osmolarity, IgaA antagonizes the activity of RcsB~P for most genes that are targeted by this response regulator.

Identification of genes coregulated by RcsA and RcsB. RcsA coregulates with RcsB the expression of colanic acid capsule genes and a distinct exopolysaccharide synthesized by the products of the *yjbEFGH* operon (22, 39, 55). To define the effect of *igaA* mutations on the contribution of RcsA in serovar Typhimurium, we monitored transcription profiles in *igaA1*, *igaA1 rcsA*, and *rcsA* strains. The genes displaying altered transcription were differentiated into two classes on the basis of the strict requirement of the coregulator RcsA for expression. The first class contained genes that were entirely dependent on

TABLE 4. Genes with decreased expression in the serovar Typhimurium *igaA1* mutant versus the wild-type strain

Systematic gene name	Gene	Product	Log$_2$ ratio for the indicated comparison[a]					Reverence(s) if the product was previously assigned to the Rcs regulon
			igaA1 mutant vs WT	*igaA1 rcsB* mutant vs WT	*rcsB* mutant vs WT	*igaA1* mutant vs *igaA1 rcsA* mutant	*igaA1 rcsA* mutant vs WT	
PSLT039	*spvB*	Hydrophilic protein	−4.0	−4.5	−4.2	−0.9	−3.1	
PSLT040	*spvA*	Outer membrane protein	−5.1	−5.2	−4.6	−1.8	−3.2	
STM0462	*glnK*	Nitrogen regulatory protein P-II 2	−2.6	0.6	0.7	−2.8	−0.04	
STM0829	*glnP*	Glutamine ABC transporter permease component	−2.5	0.3	0.1	−1.7	−0.9	
STM1172	*flgM*	Antiflia factor	−2.9	−1.6	−1.2	0.4	−3.5	55
STM1174	*flgB*	Flagellar basal body rod protein	−3.0	−0.9	−1.0	1.5	−5.0	20, 55
STM1175	*flgC*	Flagellar basal body rod protein	−3.9	−2.1	−2.0	1.6	−5.8	46, 55
STM1176	*flgD*	Flagellar basal body rod modification protein	−3.6	−1.9	−1.8	1.6	−5.5	20, 55
STM1177	*flgE*	Flagellar hook protein	−3.5	−1.8	−1.4	1.2	−5.1	55
STM1178	*flgF*	Cell-proximal portion of basal body rod	−3.1	−1.7	−1.7	1.8	−5.3	55
STM1179	*flgG*	Flagellar basal body rod protein	−3.4	−1.5	−1.5	0.4	−3.9	46, 55
STM1181	*flgI*	Flagellar P-ring protein precursor	−2.5	−1.3	−1.7	1.3	−4.0	46, 55
STM1182	*flgJ*	Flagellar biosynthesis protein	−2.5	−1.2	−0.6	0.2	−2.8	55
STM1183	*flgK*	Flagellar hook-associated protein	−3.1	−1.5	−1.5	1.1	−4.4	55
STM1916	*cheY*	Chemotaxis regulator	−3.0	−1.5	−1.5	0.8	−4.0	55
STM1917	*cheB*	Chemotaxis-specific methylesterase	−2.5	−1.5	−1.2	1.1	−3.9	55
STM1919	*cheM*	Methyl-accepting chemotaxis protein II	−3.1	−1.4	−1.4	−0.06	−3.2	55
STM1921	*cheA*	Chemotaxis sensory histidine protein kinase	−2.7	−1.0	−1.4	−0.07	−2.8	55
STM1922	*motB*	Flagellar motor protein	−2.2	−1.2	−0.9	1.4	−3.8	55
STM1956	*fliA*	Flagellar biosynthesis sigma factor flia	−2.7	−1.7	−1.2	1.2	−4.1	55
STM1959	*fliC*	Flagellar biosynthesis protein	−3.1	−1.3	−1.5	1.9	−5.3	55
STM1960	*fliD*	Flagellar hook-associated protein	−3.2	−1.2	−0.8	0.3	−3.8	55
STM1961	*fliS*	Flagellar protein flis	−2.2	−0.8	−0.9	0.7	−3.2	55
STM1962	*fliT*	Possible flid export chaperone	−2.3	−0.7	−1.1	0.8	−3.3	55
STM1970	*fliG*	Flagellar motor protein	−2.2	−1.5	−0.8	0.06	−2.5	55
STM1971	*fliH*	Flagellar assembly protein	−2.5	−1.2	−0.9	0.09	−2.7	55
STM1973	*fliJ*	Flagellar protein	−2.3	−1.3	−1.4	0.2	−2.6	55
STM1974	*fliK*	Flagellar hook length control protein	−2.1	−1.4	−1.2	0.1	−2.4	55
STM1975	*fliL*	Flagellar biosynthesis protein	−2.3	−1.3	−1.4	0.1	−2.5	55
STM2259	*napA*	Periplasmic nitrate reductase	−3.7	0.7	0.8	−0.4	−3.3	
STM2260	*napD*	Periplasmic nitrate reductase	−2.7	0.6	0.2	0.1	−2.9	
STM2283	*glpT*	Sn-glycerol-3-phosphate transport protein	−2.2	−0.05	−0.2	0.2	−2.3	23
STM2284	*glpA*	Sn-glycerol-3-phosphate dehydrogenase large subunit	−2.8	0.7	0.7	0.2	−3.1	
STM2771	*fljB*	Flagellar biosynthesis protein	−3.1	−1.3	−1.5	0.9	−2.0	55
STM3274	*yhbU*	Putative protease	−2.3	0.8	0.2	−0.6	−1.5	
STM3474	*nirB*	Nitrite reductase large subunit	−2.5	0.6	0.2	0.4	−3.1	
STM4229	*malE*	Periplasmic maltose-binding protein	−2.4	−1.7	−1.4	−0.8	−1.7	46
STM4231	*lamB*	Maltoporin precursor	−2.7	−2.1	−1.6	−0.5	−2.3	46

[a] Each gene was represented by a single 70-mer oligonucleotide spotted in two separate sections of a microarray slide (see the supplemental material for details). For the statistical analyses, the two spots were considered different entities. The table lists only those genes identified in the comparison of the *igaA1* mutant and the wild type, both of whose *P* values were ≤0.05 and whose two log$_2$ ratios were ≤2. For simplification of this table, only the average of the two log$_2$ ratios for each gene is shown (see Table S2 in the supplemental material for a complete list of log$_2$ ratios and *P* values).

[b] No reference indicates no previous assignment.

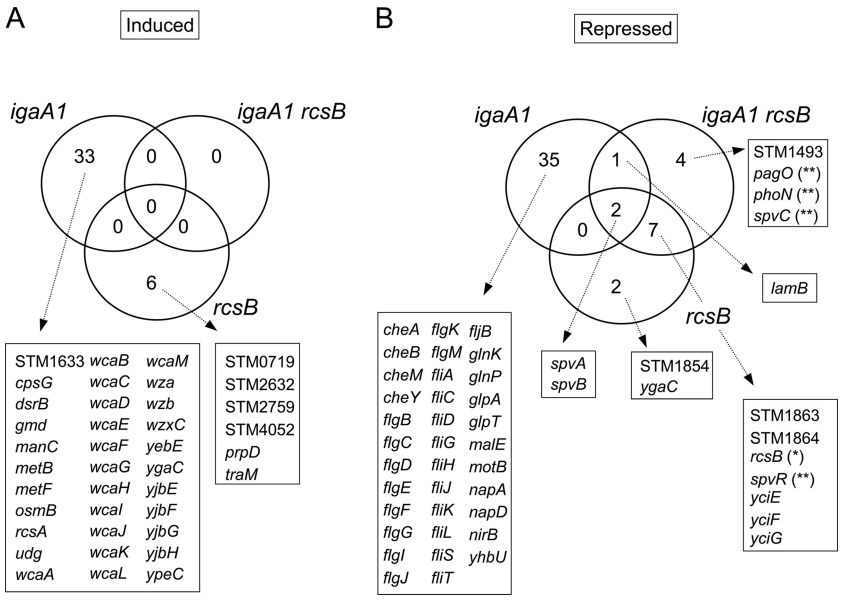

FIG. 2. The attenuator IgaA controls part of the Rcs regulon. The Venn diagrams show genes induced (A) or repressed (B) in *igaA1*, *igaA1 rcsB*, and *rcsB* strains upon comparison of their respective gene expression profiles with that of the wild-type strain. Only genes displaying changes with a $\log_2$ ratio that was ≤ -2 or ≥ 2 are shown. Some genes, such as STM1863-STM1864 or *yciGFE*, are downregulated when RcsB is not functional but not induced in the *igaA1* mutant with a strong RcsCDB response. Other genes, such as *spvAB*, display a complex regulatory pattern since they are positively regulated by RcsB (repressed in the *igaA rcsB* and *rcsB* strains) but also downregulated when the RcsC→RcsD→RcsB phosphorelay is stimulated (*igaA1* strain). The asterisk after *rcsB* indicates that the gene is knocked out in the two mutants (*igaA1 rcsB* and *rcsB* mutants). Genes labeled with two asterisks indicate cases in which, for some of the comparisons shown, one of the $\log_2$ ratios of the gene duplicates was less than or equal to -2 while the other value was close to -2. Thus, STM1862 (*pagO*) and *phoN* could be assigned to the category of genes repressed by RcsB: their $\log_2$ ratios in the comparison of the *rcsB* mutant with the wild type were $-2.96/-1.69$ and $-2.4/-1.8$, respectively. Likewise, *spvC* and *spvR* could be assigned to the category of genes repressed in the *igaA1*, *igaA1 rcsB*, and *rcsB* strains. *spvC* had $\log_2$ ratios of $-2.8/-1.1$ and $-2.7/-1.5$ in the comparisons of the *igaA1* mutant with the wild type and of the *rcsB* mutant with the wild type, respectively. *spvR* had $\log_2$ ratios of $-2.2/-1.4$ in the comparison of the *igaA1* mutant with the wild type (see Table S2 in the supplemental material for a complete list of genes).

RcsA, i.e., with altered expression in *igaA1 rcsA* and *igaA1* strains. This class included the positively regulated colanic acid genes *wcaM*, *wza-wzb*, and *wcaA-wcaB-wcaC-wcaD*; the *yjbE-FGH* operon; and *yebE* (Table 3; see also Table S6 in the supplemental material). Within this category, but negatively regulated, were *glnK* and *glnP* (Table 4; see also Table S6 in the supplemental material). To our knowledge, this result represents the first evidence of RcsA being involved in negatively regulating metabolic genes. In a second category, we classified the genes in the transcriptome of the *igaA1 rcsA* strain with altered expression levels compared with those in the wild type. Within this class, we identified upregulated genes, such as the operons *wcaE-wcaF-gmd-wcaG-wcaH-wcaI* and *cpsG-wcaJ-wzxC-wcaK-wcaL*, involved in colanic acid synthesis (Table 3; see also Table S7 in the supplemental material). This result suggests that RcsB~P alone, probably at high levels as a consequence of the *igaA1* mutation, can drive the expression of a certain proportion of colanic acid capsule genes in an RcsA-independent manner. Other conditions leading to enhanced RcsB~P levels, such as the expression of the constitutive mutant allele *rcsC137* or the expression of $rcsB^+$ in multicopy, have previously been shown to render the expression of colanic genes independent of RcsA (4). A detailed view of our expression profiles revealed, however, that this set of colanic capsule genes (*wcaE-wcaF-gmd-wcaG-wcaH-wcaI* and *cpsG-wcaJ-wzxC-wcaK-wcaL*) are expressed in the *igaA1* mutant to a higher extent than in the *igaA1 rcsA* strain (Table 3; see also Table S6 in the supplemental material). Lastly, note that some of the RcsB-regulated genes expressed in an $IgaA^+$ (wild-type) background, such as *yciGFE* (Fig. 2; see also Table S4 in the supplemental material), also require RcsA for expression (see Table S8 in the supplemental material). All together, these data indicate that RcsA controls part of the Rcs regulon of serovar Typhimurium and that genes belonging to this class can be further subdivided by their capacity to be expressed by RcsB~P in the absence of this coregulator.

A decreased amount of RcsC or RcsD results in different Rcs responses. We have recently shown that serovar Typhimurium can spontaneously suppress the requirement of the essential protein IgaA by acquiring deletions affecting the integrity of the *rcsCDB* locus (42). Two of the characterized suppressor strains lacking IgaA, MD0842-J4 and MD0855-J10, retain activity in the RcsCDB system since both display increased expression of the colanic acid gene *wcaH*, produce altered levels of the flagellins FliC and FljB and the cell division proteins FtsA and FtsZ, and are mucoid on plates (42). As a result of deletions affecting the regulatory regions of *rcsC* and *rcsD*, MD0842-J4 and MD0855-J10 mutants produce small amounts of RcsC and RcsD, respectively (42). To obtain insights into how the RcsCDB system could operate in the absence of IgaA, we monitored genome expression profiles in MD0842-J4 and MD0855-J10. The two mutants showed

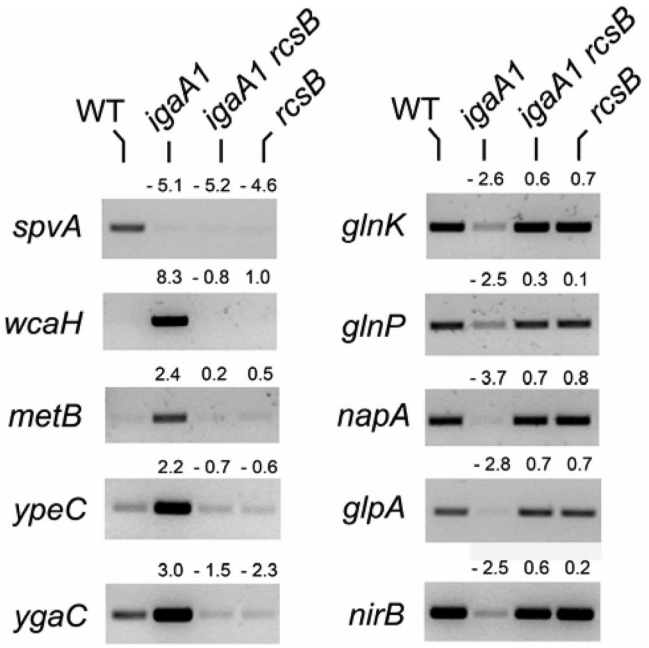

FIG. 3. Altered expression in the *igaA1* mutant of genes not previously assigned to the RcsCDB regulatory system. Shown are the results of RT-PCR assays of representative genes that the microarray experiments identified as up- or downregulated in the *igaA1* mutant (Tables 3 and 4). *wcaH*, required for synthesis of the colanic acid capsule, and *ygaC* were used as controls for genes positively regulated by the RcsCDB system. Note that, in concordance with the microarray data, *spvA* is downregulated in the *igaA1* mutant and in strains lacking RcsB. RNA was isolated from bacteria grown in ISM-glycerol medium at an OD_{600} of ~1.2 to ~1.4. Numbers indicate the average $\log_2$ ratios shown in Tables 3 and 4 for each of the indicated strains (*igaA1*, *igaA1 rcsB*, and *rcsB* strains) and relative to values for the wild type (WT).

changes similar to those observed in the *igaA1* mutant and indicative of the activity of the RcsCDB system, including the upregulation of colanic capsule genes and repression of flagellar genes (see Tables S9 to S13 in the supplemental material). Nonetheless, several new features differentiated their Rcs responses. The IgaA-defective strain MD0842-J4, with low levels of RcsC, showed reduced expression of colanic capsule genes compared to that of the *igaA1* mutant (see Table S11 in the supplemental material). This result was consistent with the higher expression of a *wcaH*::*lacZ* transcriptional fusion in the *igaA1* mutant than in MD0842-J4 (42). Unlike the *igaA1* mutant, the MD0842-J4 mutant, however, expressed *spvAB* at wild-type levels (see Table S9 in the supplemental material). In contrast, the IgaA-defective strain MD0855-J10, which has low levels of RcsD, displayed reduced *spvAB* expression (see Table S10 in the supplemental material). As expected by the pronounced mucoidy displayed by MD0855-J10 on plates, the expression of the colanic capsule genes in this mutant was comparable to that observed in the *igaA1* mutant (see Table S12 in the supplemental material). Globally, these data indicate that a decrease in RcsC significantly reduces the responsiveness of the RcsCDB system, even in the absence of the attenuator IgaA. This effect is evident in the case of colanic acid genes, which are induced only at intermediate levels, and the *spvAB* genes, whose expression remains unaltered. Conversely, the RcsCDB system can reach high activation levels with decreased amounts of RcsD, leading to high expression of colanic acid genes and repression of the *spvAB* genes.

A decreased level of RcsC or RcsD in IgaA-deficient strains has different impacts on serovar Typhimurium virulence. An inverse correlation between RcsCDB activity and the capacity of serovar Typhimurium to cause disease in the mouse acute-infection model has previously been shown (17, 45). The microarray data obtained with the IgaA-deficient strains MD0842-J4 and MD0855-J10 revealed differences in *spvAB* expression, and as previously mentioned, these two strains display Rcs responses of different intensities (42). Since both MD0842-J4 and MD0855-J10 are mucoid, it was of interest to test whether these strains behave differently in the mouse typhoid virulence model. Intraperitoneal-challenge assays showed that the MD0855-J10 strain, with a higher Rcs response (42), was highly attenuated but that the MD0842-J4 strain, with lower Rcs activity, was virulent (Table 5). We included the MD0835-J1 mutant as a control strain, which also lacks IgaA but harbors a deletion of the *apbE′-ompC-micF-rcsD-rcsB-rscC′* region (42). This MD0835-J1 mutant behaved as a virulent strain (Table 5); therefore, we discarded the possibility that the attenuation of the MD0855-J10 mutant, which harbors an *ompC-micF-rcsD′* deletion (42), was due to the lack of *ompC* and/or *micF*. These data reinforce the idea of IgaA modulating virulence exclusively at the stage of controlling the responsiveness of the RcsCDB system. In IgaA-defective bacteria, virulence can differ significantly depending on whether the relative amount of either RcsC or RcsD is compromised.

***spvA* expression is positively controlled by RcsB but affected negatively by the RcsC→RcsD→RcsB phosphorelay.** The microarray data uncovered an unexpected complexity in the regulation of the virulence genes *spvAB.* These genes were downregulated in the *igaA1* mutant upon activation of the RcsCDB system but were also repressed in bacteria lacking RcsB (Table 4; Fig. 2). Furthermore, *spvAB* displayed altered expression in IgaA-deficient bacteria but only in the mutant having reduced levels of RcsD, MD0855-J10 (see Table S10 in the supplemental material). Based on these observations, we hypothesized that *spvAB* could be subjected to positive regulation by dephosphorylated RcsB. In this context, any increase in the RcsB~P/RcsB ratio could therefore lead to *spvAB* repression. This postulate predicts that normal *spvAB* expression levels should be restored in the *igaA1* mutant upon inactivation of either RcsC or RcsD. To test this hypothesis, expression of an *spvA*::*lacZ* transcriptional fusion was monitored in the wild-type, *igaA1*, *igaA1 rcsB*, *rcsB*, *igaA rcsC*, *rcsC*, *igaA1 rcsD*, *igaA1 rcsA*, *rcsA*, *rcsD*, MD0842-J4, and MD0855-J10 strains. All of the mutants were characterized in previous studies (6, 17, 42), except those containing the *rcsD* null mutation, which we generated for this work. The *rcsD* deletion encompassed the entire *rcsD* open reading frame except the last 130 nucleotides at the 3′ end, which were left intact to preserve intact the 5′ regulatory region of *rcsB*. Control assays revealed that this deletion does not affect *rcsB* transcription (see Fig. S1 in the supplemental material). Figure 4 shows that *spvA* transcription decreases significantly when the level of the RcsC→RcsD→RcsB phosphorelay is high (*igaA1*, *igaA1 rcsA*, and MD0855-J10 strains). Interestingly, *rcsC* and *rcsD* null mutations, but not

TABLE 5. Virulence of IgaA-deficient mutants of serovar Typhimurium having distinct activation levels of the RcsCDB system

Strain mixture	Status of the Rcs system[a]	Competitive index[b]	
		Liver	Spleen
MD0835-J1 [*igaA2*::KIXX Δ(*apbE′-rcsC′*)] × WT	Inactive	0.63 ± 0.11	0.82 ± 0.12
MD0842-J4 [*igaA2*::KIXX *rcsC67′*] × WT	Intermediate	0.65 ± 0.03	0.92 ± 0.11
MD0855-J10 [*igaA2*::KIXX Δ(*ompC′-micF*)] × WT	High	0.06 ± 0.02	0.07 ± 0.01

[a] As indicated by the relative levels of expression of the *wcaH21*::*lacZ* transcriptional fusion in the MD0835-J1, MD0842-J4, and MD0855-J10 strains (see reference 42).

[b] Competitive index values were calculated as described previously (2) after intraperitoneal challenge of BALB/c mice with a dose of 2×10^5 CFU per strain. Liver and spleen were extracted at 48 h postinfection. Shown are the medians and standard deviations corresponding to results from two independent experiments.

the *rcsA* mutation, restored wild-type levels of expression of *spvA* in the *igaA1* background (Fig. 4). This result suggested that the repression of *spvA* observed in the *igaA1* mutant requires an active RcsC→RcsD→RcsB phosphorelay but is independent of RcsA. Since the loss of RcsC or RcsD alone did not have any effect on *spvA* transcription, but RcsB was essential for such expression, we conclude that dephosphorylated RcsB may contribute to positively regulate this virulence gene.

Production of a nonphosphorylatable RcsB(D56Q) variant enhances *spvA* expression. Data shown in Fig. 4 suggested that dephosphorylated RcsB could play a role in the positive regulation of *spvA*. However, other interpretations are also possible, such as that *spvA* was activated at low RcsB~P levels but repressed at high levels of RcsB~P or that RcsB was phosphorylated by other phospho-donors, such as acetyl-phosphate in the *rcsC* or *rcsD* mutant. To address this point, we used plasmids in which $RcsB^+$ and a nonphosphorylatable RcsB (D56Q) variant were produced from the arabinose-inducible P_{BAD} promoter. To confirm the activities and correct expression of these two RcsB versions, they were first analyzed in an *igaA rcsB* strain. As expected, $RcsB^+$ but not RcsB(D56Q) restored mucoidy in bacteria grown in medium supplemented with arabinose (Fig. 5A). $RcsB^+$ or RcsB(D56Q) were then produced in wild-type, *igaA1*, *igaA1 rcsB*, and *rcsB* strains carrying the *spvA*::*lacZ* transcriptional fusion (Fig. 5B). When *spvA* expression was compared in media containing either glucose or arabinose, two phenomena were observed. Unlike with what was observed in bacteria grown with glycerol as the only carbon source (Fig. 4), low *spvA* expression was detected in all of the different strains grown in glucose-containing medium (see the legend to Fig. 5). This effect did not impair our analysis of the relative changes in *spvA* expression upon the production of RcsB from plasmid (arabinose versus glucose). The expression of the nonphosphorylatable RcsB(D56Q) variant, but not of $RcsB^+$, drastically increased *spvA* expression (Fig. 5B). This effect, noticeable in strains lacking endogenous RcsB, was, however, more pronounced in strains with an $rcsB^+$ background. Thus, when RcsB(D56Q) was produced in wild-type and *igaA1* strains, *spvA* expression increased up to 22- and 90-fold, respectively (Fig. 5B). In silico analysis of the promoter regions of *spvA* and *spvR*, the latter encoding a positive regulator of the *spvABC* operon (59), did not reveal any sequence close to the consensus RcsAB box (56; data not shown). Taken together, these observations unequivocally demonstrated the capacity of dephosphorylated RcsB to stimulate *spvA* expression. In addition, they indicated that RcsB~P may also be required to achieve proper *spvA* expression levels.

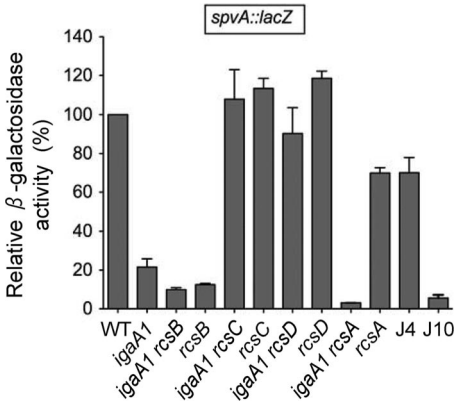

FIG. 4. Expression of the serovar Typhimurium virulence gene *spvA* requires RcsB but is impaired upon stimulation of the RcsCDB system. *spvA* expression was monitored in bacteria grown in ISM-38 mM glycerol (OD_{600}, ~1.2 to ~1.4). The following isogenic strains were used: SV4312 (*spvA103*::Mu*dJ*), MD1368 (*igaA1 spvA103*::Mu*dJ*), MD1369 (*igaA1 rcsB70 spvA103*::Mu*dJ*), MD1370 (*rcsB70 spvA103*::Mu*dJ*), MD2008 (*igaA1 rcsC52 spvA103*::Mu*dJ*), MD2007 (*rcsC52 spvA103*::Mu*dJ*), MD2028 (*igaA1* Δ*rcsD69 spvA103*::Mu*dJ*), MD2027 (Δ*rcsD69 spvA103*::Mu*dJ*), MD1396 (*igaA1 rcsA51 spvA103*::Mu*dJ*), MD1397 (*rcsA51 spvA103*::Mu*dJ*), MD1371–MD0842-J4 (J4) (*igaA3*::Cam^r *rcsC67′ spvA103*::Mu*dJ*), and MD1372–D0855-J10 (J10) [*igaA3*::Cam^r Δ(*ompC′-micF*) *spvA103*::Mu*dJ*]. The genotype of each of these strains is abbreviated in the figure. The β-galactosidase activities correspond to the means and standard deviations from a minimum of three independent experiments and are shown as relative to the wild-type level (100%). The β-galactosidase activity of the wild-type strain (WT) had a mean value of 131.6 Miller units, as determined from a total of nine independent experiments. Note that *spvA* expression is repressed in strains with high activity of the RcsC→RcsD→RcsB phosphorelay (*igaA1* mutant, *igaA1 rcsA* mutant, and MD0855-J10) and in those lacking RcsB (*igaA1 rcsB* and *rcsB* mutants).

DISCUSSION

The RcsCDB system is a complex regulatory system playing an important role in biofilm formation, virulence, and adaptation to envelope stress (34, 39). Genome expression studies, in fact, show that most of the genes of the Rcs regulon encode proteins related to envelope homeostasis (20, 23, 30, 31, 46, 55). Here, we also studied the role of IgaA as an attenuator of the RcsCDB system with a strategy based on genome expression profiling. On this occasion, we used a large number of serovar Typhimurium *igaA* mutants and exposed the bacteria to high osmolarity, a stimulus known to exacerbate the activity

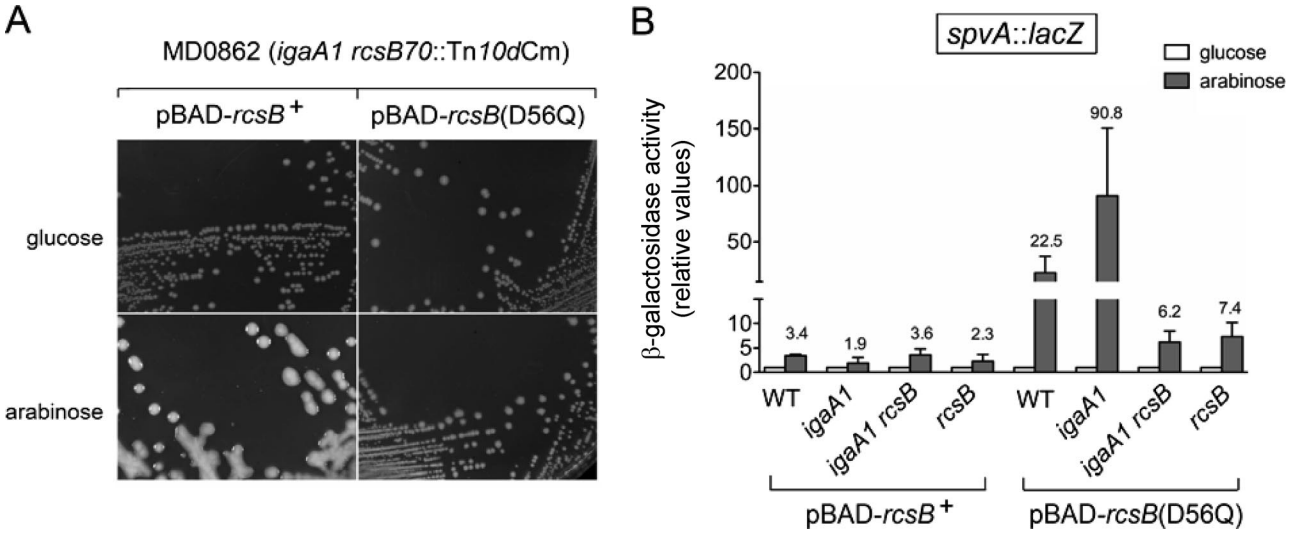

FIG. 5. Expression of a nonphosphorylatable RcsB(D56Q) variant enhances *spvA* expression to a higher extent than wild-type RcsB$^+$. These RcsB proteins were produced from plasmids under the arabinose-inducible P_{BAD} promoter (Table 1). (A) Control experiment showing that the expression of wild-type RcsB$^+$ but not that of the nonphosphorylatable RcsB(D56Q) variant restores the activity of the RcsCDB system, as denoted by the mucoidy displayed by bacteria on plates. (B) Expression of *spvA* in wild-type, *igaA1*, *igaA1 rcsB*, and *rcsB* strains producing either RcsB$^+$ or RcsB(D56Q). For every strain, the β-galactosidase activity measured under inducing conditions (16 h in ISM-0.05% arabinose-38 mM glycerol) is relative to that obtained in glucose-containing medium (16 h in ISM-2% glucose), which was normalized to 1. The β-galactosidase activity in Miller units measured for each strain in ISM-2% glucose was 7.4 (wild-type [WT] P_{BAD}-*rcsB*$^+$ strain), 6.2 (*igaA1* P_{BAD}-*rcsB*$^+$ strain), 3.0 (*igaA1 rcsB* P_{BAD}-*rcsB*$^+$ strain), 17.3 (*rcsB* P_{BAD}-*rcsB*$^+$ strain), 9.6 [wild-type P_{BAD}-*rcsB*(D56Q) strain], 4.8 [*igaA1* P_{BAD}-*rcsB*(D56Q) strain], 9.3 [*igaA1 rcsB* P_{BAD}-*rcsB*(D56Q) strain], and 9.2 [*rcsB* P_{BAD}-*rcsB*(D56Q) strain]. Data correspond to the means and standard deviations from three independent experiments.

of the RcsCDB system. Under these particular growth conditions, 71 genes in the *igaA1* mutant, 60 genes in the IgaA-defective MD0842-J4 strain (low RcsC levels), and 47 genes in the IgaA-defective MD0855-J10 mutant (low RcsD levels) displayed changes in expression higher than fourfold. The analysis of functions equally affected in the three mutants defines an Rcs regulon of at least 89 genes, approximately 2% of the serovar Typhimurium genome.

In qualitative terms, the Rcs regulon characterized in this work is similar to that reported in previous studies, with most genes encoding envelope and motility or chemotaxis functions (23, 30, 46). However, our study uncovered new regulatory features and identified up to 13 novel genes regulated by the RcsCDB system (Tables 3 and 4). Some of these new genes encode hypothetical proteins, while others are involved in processes related to glycerol-3-phosphate metabolism, methionine biosynthesis, ammonia metabolism, or nitrate/nitrite respiration. Some of these processes, such as those linked to the metabolism of ammonia, nitrate, and nitrite, are repressed upon activation of the RcsCDB system. These observations suggest that the role of the RcsCDB system might extend beyond the control of motility, chemotaxis, and envelope components to other scenarios related to metabolic readjustments in response to stress.

Our data also revealed that the RcsB~P activity present in wild-type bacteria may be sufficient to account for maximal expression of genes such as STM1862 (*pagO*)-STM1863-STM1864 and *yciGFE*. Other cases in which higher expression is detected in the wild type than in the *rcsB* mutant are known for the *gadAB* genes in *E. coli*, required for glutamate-dependent acid resistance (9), and for *srfA* and *siiE* in serovar Typhimurium (27). More recently, STM1841, a serovar Typhimurium gene of unknown function, was also shown to have a 30-fold-higher induction in the wild type than in the *rcsB* mutant (55). These observations reflect that some genes of the Rcs regulon may require only low RcsB~P levels to be maximally expressed but that the rest are gradually upregulated as the relative RcsB~P levels increase in response to stimuli.

We were also interested in dissecting the contribution of RcsA to the Rcs regulon of serovar Typhimurium. *rcsA* was highly expressed in the *igaA1* mutant, confirming the autoregulation proposed to act via an RcsAB box present in the *rcsA* promoter (18, 56). Two classes of RcsA-regulated genes, one entirely dependent on this coregulator, were identified. However, we did not observe any correlation of these two categories with the magnitude of changes observed in the absence/presence of RcsB or upon induction of the RcsCDB system by mutations in IgaA. A representative example is that of the *yciGFE* genes, maximally expressed in the wild-type strain (i.e., not induced in the *igaA1* mutant), but displaying an absolute dependence on both RcsB and RcsA for expression.

Another objective of this study was to analyze how the Rcs regulon could be affected by a decrease in the relative levels of RcsC and RcsD. To our knowledge, no precedent exists for this type of analysis at the genome level with reduced amounts of the sensor protein(s) of a two-component system. Colanic acid genes were induced, albeit to different extents, in three distinct mucoid serovar Typhimurium strains having augmented Rcs responses: MD0842-J4, MD0855-J10, and the *igaA1* mutant (42). Based on this parameter, the intensity of the RcsCDB response was graded in an *igaA1* mutant > MD0855-J10 > MD0842-J4 order. Interestingly, phenotypic

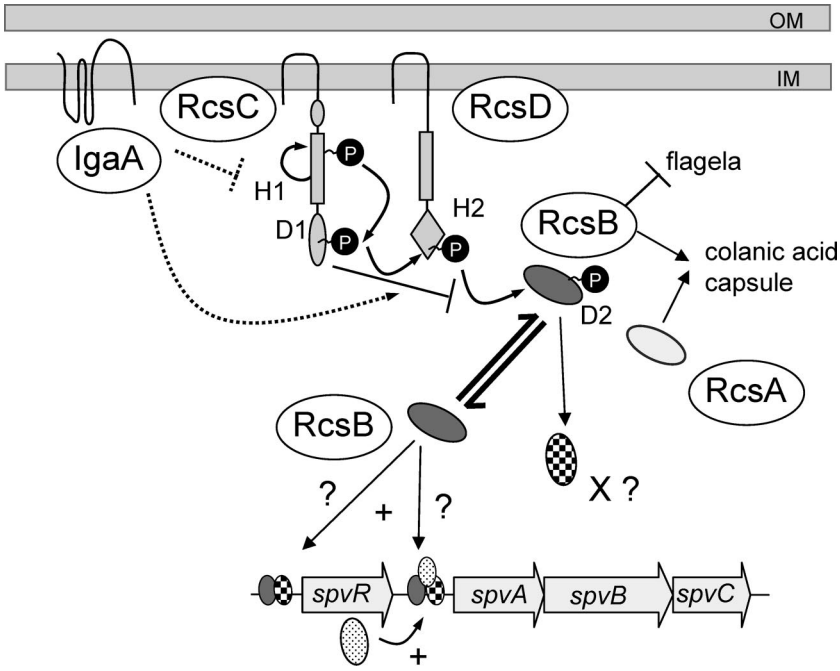

FIG. 6. Model showing the putative role of nonphosphorylated RcsB in the positive regulation of *spvA* expression. This regulation could be hypothetically exerted on the *spvABC* regulatory region or, indirectly, via the dedicated activator SpvR. The model also highlights the requirement for this process of RcsB~P, probably stimulating the production of an unknown regulatory factor (X) that may act as a coregulator together with dephosphorylated RcsB. The model also includes two alternate mechanisms of attenuation of the RcsC→RcsD→RcsB phosphorelay by IgaA, either by inhibition of autophosphorylation in the H1 kinase domain of RcsC or stimulation of the phosphatase activity associated with the D1 receiver domain of RcsC (dotted lines). This role of IgaA may be essential to fine-tune the switch between RcsB~P and nonphosphorylated RcsB and, as a consequence, to dictate the intensity at which the Rcs regulon is expressed.

traits performed with IgaA-deficient bacteria showed virulence attenuation only for the MD0855-J10 strain (Table 5). Since both the MD0842-J4 and MD0855-J10 strains upregulate colanic acid capsule genes and the *yjbEFGH* operon, we see as unlikely a negative effect of exopolysaccharide overproduction on virulence (28, 45). On the other hand, MD0842-J4 and MD0855-J10 displayed different levels of expression of the virulence genes *spvAB*, a change that could explain the observed phenotypes. Obviously, alterations in the expression and/or activities of other virulence factors may also contribute to the attenuation of the MD0855-J10 mutant. Overall, the genome expression data collected from the IgaA-deficient strains demonstrate that the strong Rcs responses resulting from the absence of this attenuator can be differentiated.

The regulation exerted on the *spv* genes by the RcsCDB system was also of interest. The transcriptional activity of *spvA* as registered in different genetic backgrounds revealed a positive regulation of RcsB on this virulence gene. It is noteworthy that *spvA* expression was found to be normal in *rscC* and *rcsD* mutants, which indicated that an intact RcsC→RcsD→RcsB phosphorelay was not required for this regulation. This observation, however, contrasted with the reduced *spvA* expression linked to the stimulation of the RcsCDB system. This finding prompted us to consider an involvement of dephosphorylated RcsB. The data obtained with the nonphosphorylatable RcsB (D56Q) variant demonstrated that RcsB does not need to be phosphorylated to promote *spvA* expression (Fig. 5B). To our knowledge, these results are the first evidence sustaining the role of dephosphorylated RcsB as a regulator of gene expression. Precedents for response regulators that retain activity independently of phosphorylation are nonetheless known for NblR in *Synechococcus* sp. (48), HP1043 in *Helicobacter pylori* (50), and FrsZ in *Myxococcus xanthus* (25). In our model, there is, however, a feature not described in the previous cases. RcsB (D56Q) causes a more-pronounced positive effect on *spvA* when it is produced in an *rcsB*$^+$ background, which suggests that both RcsB~P and dephosphorylated RcsB may be required for attaining proper *spvA* expression. This double requirement was tentatively integrated into a model in which RcsB~P is proposed to regulate positively a hypothetical coregulatory protein that could act together with dephosphorylated RcsB on *spv* promoters (Fig. 6). This hypothetical RcsB-coregulator complex may act on the promoter of *spvR*, the *spvABC* operon, or both (58). Future work is required to discern which of these scenarios is correct. An additional unexpected finding was the low expression of *spvA* registered in glucose-containing medium compared to that under conditions in which glycerol was the only carbon source. Although the basis of these differences is currently unknown, this new level of regulation supports the idea of an intricate regulatory circuit operating on the *spvABC* genes. Besides the control exerted on these genes by RpoS (59), the dedicated regulator SpvR (58), polynucleotide phosphorylase (60), and the RcsCDB system (this study), catabolic repression may be relevant.

In summary, the diminished *spvA* expression observed in the *igaA1* mutant can be a consequence of the high RcsB~P/RcsB ratio existing upon stimulation of the RcsC→RcsD→RcsB phosphorelay. IgaA may then ensure proper *spvA* expression

by facilitating the switch of RcsB~P to the dephosphorylated state. In a more general role, IgaA could be responsible for constantly adjusting the RcsB~P/RcsB ratio in response to the amount and/or type of signals perceived by the bacteria. However, how could IgaA ultimately perform this hypothetical function? Given its location as an integral inner membrane protein, IgaA has the potential to interact with membrane components of the Rcs system as the primary sensor RcsC. Following this, IgaA might attenuate the autophosphorylation taking place in the H1 kinase domain or, alternatively, stimulate the phosphatase activity at the receiver D1 domain of RscC (12, 26, 39) (Fig. 6). The possibility of IgaA also modulating the autophosphorylation rate of RcsB in the presence other phospho-donors, such as acetyl-phosphate (26), cannot be discarded with the available data. Work directed to explore these diverse mechanisms is in progress.

ACKNOWLEDGMENTS

We thank Josep Casadesús (Universidad of Seville, Spain) and members of the F. García-del Portillo lab for their valuable comments. We are also grateful to Roberto Serrano, Marta Godoy (Genomics Unit, Centro Nacional de Biotecnología-CSIC, Madrid, Spain), and Juan Carlos Oliveros (Bioinformatics Unit, Centro Nacional de Biotecnología-CSIC, Madrid, Spain) for their assistance in the microarray experiments and data analyses. We also thank the Wellcome Trust Sanger Institute (Hinxton, Cambridge, United Kingdom) for the public availability of the serovar Typhimurium SL1344 sequences.

This work was supported by grants from the Ministry of Science and Innovation of Spain (GEN2003-20234-C06-01, BIO2007-67457-C02-01, and CSD2008-00013-INTERMODS). J.F.M. was supported by a fellowship from the Programa de Formación de Personal Investigador of the Ministry of Science and Innovation.

REFERENCES

1. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. **9:**143–152.
2. **Beuzón, C. R., and D. W. Holden.** 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. Microbes Infect. **3:**1345–1352.
3. **Boulanger, A., A. Francez-Charlot, A. Conter, M. P. Castanie-Cornet, K. Cam, and C. Gutierrez.** 2005. Multistress regulation in *Escherichia coli*: expression of *osmB* involves two independent promoters responding either to σ^S or to the RcsCDB His-Asp phosphorelay. J. Bacteriol. **187:**3282–3286.
4. **Brill, J. A., C. Quinlan-Walshe, and S. Gottesman.** 1988. Fine-structure mapping and identification of two regulators of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **170:**2599–2611.
5. **Calva, E., and R. Oropeza.** 2006. Two-component signal transduction systems, environmental signals, and virulence. Microb. Ecol. **51:**166–176.
6. **Cano, D. A., G. Dominguez-Bernal, A. Tierrez, F. Garcia-del Portillo, and J. Casadesus.** 2002. Regulation of capsule synthesis and cell motility in *Salmonella enterica* by the essential gene *igaA*. Genetics **162:**1513–1523.
7. **Cano, D. A., M. Martinez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesus, and F. Garcia-Del Portillo.** 2001. *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infect. Immun. **69:**6463–6474.
8. **Castanie-Cornet, M. P., K. Cam, and A. Jacq.** 2006. RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. J. Bacteriol. **188:**4264–4270.
9. **Castanie-Cornet, M. P., H. Treffandier, A. Francez-Charlot, C. Gutierrez, and K. Cam.** 2007. The glutamate-dependent acid resistance system in *Escherichia coli*: essential and dual role of the His-Asp phosphorelay RcsCDB/AF. Microbiology **153:**238–246.
10. **Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata.** 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high-frequency-transducing lysate. Virology **50:**883–898.
11. **Chen, M. H., S. Takeda, H. Yamada, Y. Ishii, T. Yamashino, and T. Mizuno.** 2001. Characterization of the RcsC→YojN→RcsB phosphorelay signaling pathway involved in capsular synthesis in *Escherichia coli*. Biosci. Biotechnol. Biochem. **65:**2364–2367.
12. **Clarke, D. J., S. A. Joyce, C. M. Toutain, A. Jacq, and I. B. Holland.** 2002. Genetic analysis of the RcsC sensor kinase from *Escherichia coli* K-12. J. Bacteriol. **184:**1204–1208.
13. **Clavel, T., J. C. Lazzaroni, A. Vianney, and R. Portalier.** 1996. Expression of the *tolQRA* genes of *Escherichia coli* K-12 is controlled by the RcsC sensor protein involved in capsule synthesis. Mol. Microbiol. **19:**19–25.
14. **Conter, A., R. Sturny, C. Gutierrez, and K. Cam.** 2002. The RcsCB His-Asp phosphorelay system is essential to overcome chlorpromazine-induced stress in *Escherichia coli*. J. Bacteriol. **184:**2850–2853.
15. **Costa, C. S., M. J. Pettinari, B. S. Mendez, and D. N. Anton.** 2003. Null mutations in the essential gene *yrfF* (*mucM*) are not lethal in *rcsB*, *yojN* or *rcsC* strains of *Salmonella enterica* serovar Typhimurium. FEMS Microbiol. Lett. **222:**25–32.
16. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
17. **Dominguez-Bernal, G., M. G. Pucciarelli, F. Ramos-Morales, M. Garcia-Quintanilla, D. A. Cano, J. Casadesus, and F. Garcia-del Portillo.** 2004. Repression of the RcsC-YojN-RcsB phosphorelay by the IgaA protein is a requisite for *Salmonella* virulence. Mol. Microbiol. **53:**1437–1449.
18. **Ebel, W., and J. E. Trempy.** 1999. *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. J. Bacteriol. **181:**577–584.
19. **Ebel, W., G. J. Vaughn, H. K. Peters III, and J. E. Trempy.** 1997. Inactivation of *mdoH* leads to increased expression of colanic acid capsular polysaccharide in *Escherichia coli*. J. Bacteriol. **179:**6858–6861.
20. **Erickson, K. D., and C. S. Detweiler.** 2006. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. Mol. Microbiol. **62:**883–894.
21. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103–118.
22. **Ferrieres, L., S. N. Aslam, R. M. Cooper, and D. J. Clarke.** 2007. The *yjbEFGH* locus in *Escherichia coli* K-12 is an operon encoding proteins involved in exopolysaccharide production. Microbiology **153:**1070–1080.
23. **Ferrieres, L., and D. J. Clarke.** 2003. The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. Mol. Microbiol. **50:**1665–1682.
24. **Francez-Charlot, A., M. P. Castanie-Cornet, C. Gutierrez, and K. Cam.** 2005. Osmotic regulation of the *Escherichia coli bdm* (biofilm-dependent modulation) gene by the RcsCDB His-Asp phosphorelay. J. Bacteriol. **187:**3873–3877.
25. **Fraser, J. S., J. P. Merlie, Jr., N. Echols, S. R. Weisfield, T. Mignot, D. E. Wemmer, D. R. Zusman, and T. Alber.** 2007. An atypical receiver domain controls the dynamic polar localization of the *Myxococcus xanthus* social motility protein FrzS. Mol. Microbiol. **65:**319–332.
26. **Fredericks, C. E., S. Shibata, S. Aizawa, S. A. Reimann, and A. J. Wolfe.** 2006. Acetyl phosphate-sensitive regulation of flagellar biogenesis and capsular biosynthesis depends on the Rcs phosphorelay. Mol. Microbiol. **61:**734–747.
27. **Garcia-Calderon, C. B., J. Casadesus, and F. Ramos-Morales.** 2007. Rcs and PhoPQ regulatory overlap in the control of *Salmonella enterica* virulence. J. Bacteriol. **189:**6635–6644.
28. **Garcia-Calderon, C. B., M. Garcia-Quintanilla, J. Casadesus, and F. Ramos-Morales.** 2005. Virulence attenuation in *Salmonella enterica rcsC* mutants with constitutive activation of the Rcs system. Microbiology **151:**579–588.
29. **Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen.** 1993. Molecular analysis of *spv* virulence genes of the *Salmonella* virulence plasmids. Mol. Microbiol. **7:**825–830.
30. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.** 2003. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J. Bacteriol. **185:**5735–5746.
31. **Hinchliffe, S. J., S. L. Howard, Y. H. Huang, D. J. Clarke, and B. W. Wren.** 2008. The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic yersiniae. Microbiology **154:**1117–1131.
32. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
33. **Hoiseth, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
34. **Huang, Y. H., L. Ferrieres, and D. J. Clarke.** 2006. The role of the Rcs phosphorelay in Enterobacteriaceae. Res. Microbiol. **157:**206–212.
35. **Ize, B., I. Porcelli, S. Lucchini, J. C. Hinton, B. C. Berks, and T. Palmer.** 2004. Novel phenotypes of *Escherichia coli tat* mutants revealed by global gene expression and phenotypic analysis. J. Biol. Chem. **279:**47543–47554.
36. **Kaldalu, N., R. Mei, and K. Lewis.** 2004. Killing by ampicillin and ofloxacin induces overlapping changes in *Escherichia coli* transcription profile. Antimicrob. Agents Chemother. **48:**890–896.
37. **Kelley, W. L., and C. Georgopoulos.** 1997. Positive control of the two-component RcsC/B signal transduction network by DjlA: a member of the

DnaJ family of molecular chaperones in *Escherichia coli*. Mol. Microbiol. **25:**913–931.
38. **Laub, M. T., and M. Goulian.** 2007. Specificity in two-component signal transduction pathways. Annu. Rev. Genet. **41:**121–145.
39. **Majdalani, N., and S. Gottesman.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu. Rev. Microbiol. **59:**379–405.
40. **Majdalani, N., M. Heck, V. Stout, and S. Gottesman.** 2005. Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. J. Bacteriol. **187:**6770–6778.
41. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones & Barlett, Boston, MA.
42. **Mariscotti, J. F., and F. Garcia-del Portillo.** 2008. Instability of the *Salmonella* RcsCDB signalling system in the absence of the attenuator IgaA. Microbiology **154:**1372–1383.
43. **Mascher, T., J. D. Helmann, and G. Unden.** 2006. Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol. Mol. Biol. Rev. **70:**910–938.
44. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45. **Mouslim, C., M. Delgado, and E. A. Groisman.** 2004. Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. Mol. Microbiol. **54:**386–395.
46. **Oshima, T., H. Aiba, Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, and T. Mizuno.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol. Microbiol. **46:**281–291.
47. **Parker, C. T., A. W. Kloser, C. A. Schnaitman, M. A. Stein, S. Gottesman, and B. W. Gibson.** 1992. Role of the *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. **174:**2525–2538.
48. **Ruiz, D., P. Salinas, M. L. Lopez-Redondo, M. L. Cayuela, A. Marina, and A. Contreras.** 2008. Phosphorylation-independent activation of the atypical response regulator NblR. Microbiology **154:**3002–3015.
49. **Sailer, F. C., B. M. Meberg, and K. D. Young.** 2003. beta-lactam induction of colanic acid gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **226:**245–249.
50. **Schar, J., A. Sickmann, and D. Beier.** 2005. Phosphorylation-independent activity of atypical response regulators of *Helicobacter pylori*. J. Bacteriol. **187:**3100–3109.
51. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
52. **Shiba, Y., Y. Yokoyama, Y. Aono, T. Kiuchi, J. Kusaka, K. Matsumoto, and H. Hara.** 2004. Activation of the Rcs signal transduction system is responsible for the thermosensitive growth defect of an *Escherichia coli* mutant lacking phosphatidylglycerol and cardiolipin. J. Bacteriol. **186:**6526–6535.
53. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659–669.
54. **Tierrez, A., and F. Garcia-del Portillo.** 2004. The *Salmonella* membrane protein IgaA modulates the activity of the RcsC-YojN-RcsB and PhoP-PhoQ regulons. J. Bacteriol. **186:**7481–7489.
55. **Wang, Q., Y. Zhao, M. McClelland, and R. M. Harshey.** 2007. The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar Typhimurium: dual regulation of flagellar and SPI-2 virulence genes. J. Bacteriol. **189:**8447–8457.
56. **Wehland, M., and F. Bernhard.** 2000. The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. J. Biol. Chem. **275:**7013–7020.
57. **West, A. H., and A. M. Stock.** 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26:**369–376.
58. **Wilson, J. A., T. J. Doyle, and P. A. Gulig.** 1997. Exponential-phase expression of *spvA* of the *Salmonella typhimurium* virulence plasmid: induction in intracellular salts medium and intracellularly in mice and cultured mammalian cells. Microbiology **143:**3827–3839.
59. **Wilson, J. A., and P. A. Gulig.** 1998. Regulation of the *spvR* gene of the *Salmonella typhimurium* virulence plasmid during exponential-phase growth in intracellular salts medium and at stationary phase in L broth. Microbiology **144:**1823–1833.
60. **Ygberg, S. E., M. O. Clements, A. Rytkonen, A. Thompson, D. W. Holden, J. C. Hinton, and M. Rhen.** 2006. Polynucleotide phosphorylase negatively controls *spv* virulence gene expression in *Salmonella enterica*. Infect. Immun. **74:**1243–1254.
61. **Zhou, L., X. H. Lei, B. R. Bochner, and B. L. Wanner.** 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J. Bacteriol. **185:**4956–4972.