Two-component systems are signal transduction devices found in all domains of life, and they are especially widespread in bacteria (91). These systems regulate diverse responses, including nutrient acquisition, energy metabolism, adaptation to environmental cues, complex developmental pathways, and host-pathogen interactions. Two-component systems are typically composed of a transmembrane sensor protein and a cytoplasmic transcriptional regulator. The transmembrane component harbors at least two domains: an input domain that senses the environmental stimulus and a cytoplasmic transmitter with histidine kinase activity that transforms the external stimulus into a cellular signal by autophosphorylation at a conserved histidine residue. The phosphorylated histidine is the source for phosphorylation of a conserved aspartic acid residue in the receiver domain of the transcriptional regulator. The phosphorylated transcription factor then mediates the cellular response, usually by differential expression of target genes. In a number of two-component systems, two histidine and two aspartic acid residues are present in four signaling domains that can be combined in several ways (91). On the basis of experimental evidence and protein sequence similarities, *Escherichia coli* and *Salmonella enterica* are thought to encode over 30 different two-component systems.

*S. enterica* can cause diseases ranging from gastroenteritis to typhoid fever in humans and other animals (73). Many virulence traits of *S. enterica* can be attributed to the presence of *Salmonella* pathogenicity islands (SPIs). The larger SPIs, SPI-1 and SPI-2, encode type III secretion systems (T3SS) (7). In *S. enterica* serovar Typhimurium, besides genes that are also found in the *Escherichia coli* genome, our screens identified *Salmonella*-specific genes regulated by RcsB, including *bapA, siiE, srfA*, and *srfB*. Here we show that the *srfABC* operon is negatively regulated by RcsB and by PhoP. In vivo studies using mutants with constitutive activation of the Rcs and/or PhoPQ system suggested that there is an overlap between these regulatory systems in the control of *Salmonella* virulence.

Genetic screens based on the use of MudJ-generated lac fusions permitted the identification of novel genes regulated by the Rcs signal transduction system in *Salmonella enterica* serovar Typhimurium. Besides genes that are also found in the *Escherichia coli* genome, our screens identified *Salmonella*-specific genes regulated by RcsB, including *bapA, siiE, srfA*, and *srfB*. Here we show that the *srfABC* operon is negatively regulated by RcsB and by PhoP. In vivo studies using mutants with constitutive activation of the Rcs and/or PhoPQ system suggested that there is an overlap between these regulatory systems in the control of *Salmonella* virulence.

The Rcs system was initially characterized in *E. coli* as a regulator of colanic acid capsule synthesis (9, 39, 82). The sensor protein RcsC, a hybrid histidine kinase, the intermediate phosphotransmitter RcsD (previously called YojN) (16, 84), and the transcriptional activator RcsB are the main components of the system, which also includes a second transcriptional activator, RcsA (83). RcsC has positive and negative regulatory effects on Rcs-regulated genes, and genetic data support the hypothesis that this protein has both kinase and phosphatase activities (18, 35, 56). RcsF is another component of the system and was originally proposed to be involved in RcsB phosphorylation (36). Recently, Majdalani et al. have shown that signaling proceeds through an ordered cascade, RcsF → RcsC → RcsD → RcsB (55). RcsF, rather than playing a role in RcsB phosphorylation, is critically involved in signal transduction from the cell surface to RcsC (55). The signals that activate the Rcs phosphorylase are largely unknown. Slejdeski and Gottesman (77) showed that osmotic upshift is an environmental signal that strongly but transiently induces colanic acid synthesis in *E. coli* in an rcsC- and rcsB-dependent manner. More recently, Hagiwara et al. (44) found

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that a combination of low temperature (20°C) and 0.4% glucose (or low temperature and zinc) is an effective stimulus for the Rcs activation. These authors also showed that, as far as signal transduction in response to glucose and zinc is concerned, the rcsF gene is also an essential component of the Rcs signaling system (44). A comprehensive review of the Rcs system has recently been published (54). In addition, recent evidence suggests that RcsB can receive a signal directly by accepting the phosphoryl group from acetyl phosphate (32).

Besides the transient or moderate effects exerted by environmental signals, mutations in some genes or overexpression of other genes can lead to permanent activation of the Rcs signal transduction pathway. Some examples in E. coli are mutations in mdoH (26) or pgsA (76) and overexpression of djlA (50). Overproduction of ResB also induces capsule synthesis that results in mucoidy (9). This is in agreement with the view that overproduction of the response regulator mimics the physiological phosphorylation response (1, 21, 48). In S. enterica serovar Typhimurium, mutations in the essential gene igaA result in mucoidy and reduced motility (10) and cause overgrowth in certain eukaryotic cell types (11).

The Rcs system has been shown to participate in additional physiological processes, including synthesis of flagella (10, 31), cell division control (12), regulation of invasion proteins, flagellin, and Vi antigen in S. enterica serovar Typhi (2, 89), synthesis of the E. coli outer membrane protein OsmC (23), expression of the E. coli tolQRA operon (19), and resistance to chlorpomazine-induced stress (20). Synthesis of certain extracellular polysaccharides in Erwinia amylovora and Klebsiella pneumoniae (5, 65) is also regulated through Rcs signaling. Proteus mirabilis (4, 43) and E. coli also use this system to regulate swarming (16, 46).

A role for the Rcs system in the control of Salmonella virulence has recently been described. Modest attenuation of Salmonella virulence at late stages of infection in mice was shown for RcsC' mutants (24). A more pronounced effect on acute infection has been reported for mutations in igaA (25) or rcsC (35, 64) that hyperactivate the Rcs system. This effect is partially suppressed by mutations that prevent colanic acid capsule synthesis (wca mutations) (35, 64), suggesting that overproduction of capsule is one of the causes of attenuation in these mutants. The fact that suppression by wca mutations is only partial suggests that additional Rcs-regulated genes may be involved in Salmonella virulence.

Several global searches of genes regulated by the Rcs system have been carried out recently in E. coli (29, 44, 67). However, the limited overlap in the genes identified by these studies suggests that other members of the E. coli Rcs regulon remain to be identified. To our knowledge, systematic searches for members of the Rcs regulon have not been carried out in S. enterica. The evolutionary relatedness between E. coli and S. enterica anticipates a high degree of overlap between the two Rcs regulons. However, differences between the E. coli and Salmonella genomes and the involvement of Rcs in Salmonella virulence also suggest that specific genes might exist in S. enterica. In this work, we describe several new members of the Rcs regulon in S. enterica serovar Typhimurium and confirm the existence of Salmonella-specific genes regulated by RcsB. Among these genes are srfA and srfB, which are part of the putative operon srfA-ABC. We show that RcsB and PhoP negatively regulate this operon. Interestingly, we provide evidence for functional overlap between these regulatory systems in the control of Salmonella virulence.

### MATERIALS AND METHODS

**Bacterial strains, bacteriophages, and strain construction.** S. enterica serovar Typhimurium strains used in this study are described in Table 1. Unless otherwise indicated, the strains were derived from the mouse-virulent strain ATCC 14028. Transductional crosses using phage P22 HT 105/1 int201 (74) were used for strain construction (57). To obtain phage-free isolates, transductants were purified by streaking on green plates (15). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

**Construction of S. enterica mutants by gene targeting.** Disruption and replacement of rcsA, rcsB, srb, or ssrB with a chloramphenicol resistance gene were performed as described previously (22). Briefly, the chloramphenicol resistance gene from plasmid pKD3 was PCR amplified with primers RcsA-P1 and RcsA-P2 for rcsA, with primers ResB-P1 and ResB-P2 for rcsB, with primers SsrP-P1 and SsrP-P2 for srb, and with primers SsaV-P1 and SsaV-P2 for ssrB. The sequences of the primers used are shown in Table 2. The PCR product was used to transform the wild-type strain carrying the PhoP constitutive strain ATCC 14028. Transductional crosses using phage P22 HT 105/1 int201 (74) were used for strain construction (57). To obtain phage-free isolates, transductants were purified by streaking on green plates (15). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

**Construction of an ssrB::zac fusion in the Salmonella chromosome.** The FRT site generated by excision of the antibiotic resistance cassette (22) was used to integrate plasmid pCE36 to generate a transcriptional lac fusion in ssrB (27).

**Chromosomal gene epitope tagging.** Addition of a 3xFLAG epitope tag at the 5' ends of the srfA, srfB, and srfC genes was carried out as described previously (88) using primers SrfA-P1Flag, SrfA-P2Flag, SrfB-P1Flag, SrfB-P2Flag, SrfC-P1Flag, and SrfC-P2Flag (Table 2).
Media and chemicals. The standard culture medium for *S. enterica* was Luria-Bertani (LB) broth. Solid LB medium contained 1.5% (final concentration) agar. Antibiotics were used at the following concentrations: kanamycin, 50 μg ml⁻¹; chloramphenicol, 20 μg ml⁻¹; and ampicillin, 100 μg ml⁻¹. For some experiments 40 μg ml⁻¹ 3-bromo-4-chloro-3-indolyl-beta-D-glucopyranoside (X-Gal) and 0.2% glucose or arabinose were added to LB medium. Motilify assays were carried out in LB medium prepared without yeast extract (37). Solid motility medium contained agar at a final concentration of 0.25%. For SPI-1-inducing conditions, *Salmonella* strains were grown overnight at 37°C in LB medium containing 0.3 M NaCl in static conditions. For SPI-2-inducing conditions, cells were grown overnight in LB medium were washed and diluted 1:100 with minimal medium at pH 5.8 (LPM) containing 80 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.8), 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 0.1% Casamino Acids, 38 mM glycerol, 337.5 μM K-HPO₄-K₂PO₄ (pH 7.4), and 8 μM MgCl₂, and incubated overnight at 37°C with shaking.

DNA amplification with PCR. Amplification reactions were carried out with a Perkin Elmer Gene-Ampl 2400 PCR system (Perkin Elmer Cetus, Foster City, CA). The final volume of reaction mixtures was 50 to 100 μl. The following concentrations of dNTPs were used: 200 μM primers, 1 μM Taq polymerase (Expand high-fidelity PCR system; Roche Diagnostics SL), 1 μl 10X buffer. The thermal program included the following steps: (i) initial denaturation for 2 min at 94°C; (ii) 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 to 3 min; and (iii) final incubation at 72°C for 7 min to complete extension.

**Plasmids.** Plasmid pBAD18 (Apr) is a member of the pBAD series of vectors performed with 0.2 ml of saline containing 10⁵ CFU. Bacteria were recovered from spleens 48 h after inoculation, and the CFU were enumerated on LB medium and on selective medium. A competitive index (CI) for each mutant was calculated by dividing the ratio of the mutant to the wild-type strain in the output (bacteria recovered from the host after infection) by the ratio of these strains in the input (initial inoculum) (6, 33, 85). The "cancelled-out" competitive index (COI) is the CI for mixed infections of double mutants with corresponding single mutant strains and was determined by dividing the ratio of a double mutant 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.

**Mutagenesis with MuL.** We employed the co-complementation procedure of Hughes and Roth (49), in which a defective *MuL* element is cotransduced with a *MuL* element that transiently provides transposition functions. *MuL* is the specialized transducing phage *MuL* (Ap Lac cts62) (13). *MuL* is a transposon-deficient Mu derivative that generates operon fusions that operate on insertion; this element was renamed *MuL* (14) is a transposition-deficient Mu derivative that generates operon fusions that operate on insertion; this element was renamed *MuL* by Hughes and Roth (49).

**Cloning and molecular characterization of *MuL* inserts.** Genomic DNA from each *MuL*-carrying isolate was digested with BamHI or XhoI and ligated with T4 DNA ligase to BamHI- or XhoI-digested plasmid SKH. The ligation mixtures were transformed into E. coli DH5α, and *MuL*-containing transfor- mants were selected on LB medium plates supplemented with kanamycin. The DNA sequence of the fusion junctions and the flanking DNA was obtained by sequencing with an automated DNA sequencer (Sistemas Genomicos, Valencia, Spain) using primer Mul, (87).

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

**Motility assays.** Liquid cultures were prepared in motility medium and incubated at 37°C with shaking. At the mid-exponential stage of growth, 5 μl of a 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.

**Western blotting and antibodies.** *Salmonella* strains were grown under SPI-1- or SPI-2-inducing conditions. The bacteria were then pelleted by centrifugation and resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Proteins were separated by SDS-PAGE on 10% acrylamide gels and electrophoretically transferred to nitrocellulose filters for Western blot analysis using anti-Flag M2 monoclonal antibodies (1:10,000; Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibodies (Bio-Rad) were used as secondary antibodies.

**Mouse mixed infections and determination of CIs and COIs.** Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were subjected to mixed infections. Groups of three or four animals were inoculated with two strains at a ratio of 1:1. Bacteria were grown overnight at 37°C in LB medium with shaking, diluted into fresh medium (1:100), and grown until the optical density at 600 nm was 0.3 to 0.6. Intraperitoneal inoculation was performed with 0.2 ml of saline containing 10⁵ CFU. Bacteria were recovered from spleens 48 h after inoculation, and the CFU were enumerated on LB medium and on selective medium. A competitive index (CI) for each mutant was calculated by dividing the ratio of the mutant to the wild-type strain in the output (bacteria recovered from the host after infection) by the ratio of these strains in the input (initial inoculum) (6, 33, 85). The “cancelled-out” competitive index (COI) is the CI for mixed infections of double mutants with corresponding single mutant strains and was determined by dividing the ratio of a double mutant strain to the corresponding single mutant in the output by the ratio of these strains in the input (6).

**Statistical analysis.** The CI or COI was expressed as the mean of at least three independent infections ± standard error. Student’s t test was used to analyze every COI with two null hypotheses: (i) the mean COI is not significantly different from 1 and (ii) the mean COI is not significantly different from the CI of the corresponding single mutant. P values of ≤0.05 were considered significant.

**RESULTS**

**Genetic screens for identification of *Salmonella* genes regulated by the Rcs system.** The initial goal of this work was identification of new genes regulated by the Rcs system in *Salmonella*. Given the involvement of the Rcs system in viru-
To carry out the first screen, we constructed strain SV4573, which shows the phenotypes associated with a lack of activation of the Rcs system. Two different, independent screens were devised with their expression under strong activation conditions with their expression with a lack of activation of the Rcs system. Ten thousand independent isolates carrying Mu\textsuperscript{d} mutation was introduced to prevent mucoidy, thereby facilitating comparison of colony colors in different media. Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.

For detection of IgaA-regulated loci, strain SV4573 was mutagenized with Mud\textit{J} to generate transcriptional \textit{lacZ} fusions. Ten thousand independent isolates carrying Mud\textit{J} inserts were patched in grids onto LB medium containing glucose and onto LB medium containing arabinose in the presence of the chromogenic indicator X-Gal. Color differences between LB medium containing glucose and LB medium containing arabinose suggested that there was regulation of the fusion by \textit{igaA}. Initially, 114 fusions were found to be differentially regulated. Reconstruction experiments and comparison of the \(\beta\)-galactosidase activities of the fusions in a wild-type background with the activities in an \textit{igaA5} background (25) allowed us to eliminate fusions whose phenotype was due to secondary mutations, insertion in the plasmid, or direct arabinose regulation. Finally, nine fusions were chosen for further study.

(ii) Identification of RcsB-regulated genes. Plasmid pIZ1589, containing \textit{rcsB} under \(P\text{BAD}\) promoter control, was transformed into wild-type strain ATCC 14028 (Table 1). Transformants carrying pIZ1589 were nonmucoid in glucose (when \textit{rcsB} was not expressed) and mucoid in arabinose (when \textit{rcsB} was expressed). This is in agreement with studies of \textit{E. coli} indicating that overexpression of \textit{rcsB} mimics overactivation of the system (9). Our second screen for Rcs-regulated genes is summarized in Fig. 1B. Plasmid pIZ1589 was introduced into strain SV4514 (\textit{gmm::MudQ}), yielding strain SV5090 (Table 1). The \textit{gmm} gene, also called \textit{wcaH}, is necessary for production of colanic acid capsule. The advantage of this screen is that the strain is nonmucoid in both glucose and arabinose.

**FIG. 1.** Identification of IgaA- and RcsB-regulated genes. (A) Diagram of the genetic screen for identification of IgaA-regulated genes in \textit{S. enterica} serovar Typhimurium. A plasmid carrying a wild-type \textit{igaA} allele under the control of an arabinose-inducible promoter (\(P\text{BAD}\)) was introduced into a strain harboring a null \textit{igaA} mutation. \textit{igaA} is expressed in the presence of arabinose as the sole carbon source but not in the presence of glucose. \textit{lac} transcriptional fusions were generated using Mud\textit{J}. The expression pattern of 10,000 independent fusions was monitored in glucose- and arabinose-containing LB medium plates supplemented with X-Gal. (B) Diagram of the genetic screen carried out to identify RcsB-regulated genes. A plasmid with a copy of the wild-type \textit{rcsB} gene under \(P\text{BAD}\) control was introduced into a strain harboring a null \textit{gmm} mutation. \textit{rcsB} is expressed in arabinose medium but not in glucose medium. \textit{lac} transcriptional fusions were generated, as described above, with Mud\textit{J}. The expression pattern of 20,000 independent fusions was monitored in glucose- and arabinose-containing LB medium plates supplemented with X-Gal. The \textit{gmm} mutation was introduced to prevent mucoidy, thereby facilitating comparison of colony colors in different media. Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.
media, making color comparisons easier. Twenty thousand MudJ-carrying isolates were analyzed in this second screen, and 17 fusions were chosen for further study.

To ascertain whether the MudJ insertions provided by the screens could be ascribed to previously known Rcs-regulated genes, two tests were carried out: (i) the abilities of the different MudJ insertions to suppress the mucoid phenotype of the igaA5 mutant were analyzed and (ii) the insertions were transduced into the wild type (strain ATCC 14028) and transductants were tested for motility. Four insertions obtained in the first screen were found to be suppressors of mucoidy, and transductional linkage analysis revealed that these insertions were linked to mnn (not shown). Three additional insertions, one from the first screen and two from the second, caused a loss of motility in an otherwise wild-type background, suggesting that they affected flagellar genes. Putative insertions in genes involved in colanic acid synthesis or motility were not characterized further.

Characterization of novel members of the Rcs regulon. Insertions that were suppressors of neither mucoidy nor motility were not linked to mnn. Among the insertions obtained in the screens, as well as the igaA5 mutation and a null rcsB or rcsC allele, were constructed. The β-galactosidase activities of these mutants indicated that all the genes studied were indeed regulated by RcsB and RcsC (Table 4).

The only exception was STM2176, whose regulation appears to be RcsC independent. RcsA is another component of the Rcs system known to participate in the expression of some but not all the genes regulated by RcsB (10). In a similar way, an rcsA mutation partially suppressed overexpression of yhhJ in an igaA5 mutant but failed to suppress overexpression of STM1491 and yiaD or to restore expression of other genes in an igaA5 background (Table 4). Altogether, these results suggest that for the RcsB regulated genes found in this work, RcsA regulates only some of the genes that are positively regulated by the Rcs system.

srfABC operon is negatively regulated by PhoP. Among the transcriptional units regulated by RcsB uncovered by our screens, the putative srfABC operon is of special interest. srfB was originally identified in a screen to find SsrB-regulated genes outside SPI-2, although the reported regulation by SsrB

### Table 3. IgaA- and RcsB-regulated fusions

<table>
<thead>
<tr>
<th>Insertion(s)</th>
<th>Gene</th>
<th>Induction ratio</th>
<th>Protein function and/or features</th>
<th>Putative transcriptional unit</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i36</td>
<td>trg</td>
<td>-129.6</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>trg</td>
<td>53, 81</td>
</tr>
<tr>
<td>i59, 170</td>
<td>srfB</td>
<td>-35.4</td>
<td>Unknown</td>
<td>srfABC</td>
<td>93</td>
</tr>
<tr>
<td>r74, r50</td>
<td>bapA</td>
<td>5.4</td>
<td>Large protein with repeats</td>
<td>bapABCD</td>
<td>51</td>
</tr>
<tr>
<td>r20, r44</td>
<td>ybiH</td>
<td>70.3</td>
<td>Outer membrane lipoprotein</td>
<td>ybiEFGH</td>
<td>29</td>
</tr>
<tr>
<td>r52</td>
<td>narH</td>
<td>-12.5</td>
<td>β-Subunit of nitrate reductase A</td>
<td>narGHJK</td>
<td>8, 79, 80</td>
</tr>
<tr>
<td>r55, r56</td>
<td>siiE</td>
<td>-3.3</td>
<td>Large secreted protein coded in SPI-4</td>
<td>siiABCDEF</td>
<td>63, 92</td>
</tr>
<tr>
<td>r71</td>
<td>dcuB</td>
<td>-1.9</td>
<td>Anaerobic C$_2$-dicarboxylate transporter</td>
<td>dcuBfumB</td>
<td>29, 38</td>
</tr>
<tr>
<td>r85</td>
<td>STM2176</td>
<td>-1.9</td>
<td>Glutathione θ-transferase</td>
<td>STM2179- STM2178- STM2177- STM2176- STM2175</td>
<td>58</td>
</tr>
<tr>
<td>r97</td>
<td>melB</td>
<td>-1.9</td>
<td>Melibiose uptake</td>
<td>melAB</td>
<td>62, 66, 70, 75</td>
</tr>
<tr>
<td>r111</td>
<td>yhlJ</td>
<td>-3.3</td>
<td>Transporter, ABC superfamily</td>
<td>yhlHyhhJ</td>
<td>72</td>
</tr>
<tr>
<td>r142, r171</td>
<td>srfA</td>
<td>-9.5</td>
<td>Similar to nuclear antigens</td>
<td>srfABC</td>
<td>93</td>
</tr>
<tr>
<td>r150</td>
<td>PSTL071</td>
<td>4.5</td>
<td>Unknown</td>
<td>PSTL071</td>
<td>68</td>
</tr>
<tr>
<td>r153</td>
<td>yiaD</td>
<td>17.7</td>
<td>Outer membrane lipoprotein</td>
<td>yiaD</td>
<td>29</td>
</tr>
<tr>
<td>r172</td>
<td>STM1491</td>
<td>7.4</td>
<td>ABC-type proline/glycine betaine transport, ATPase component</td>
<td>STM1494- STM1493- STM1492- STM1491</td>
<td>58</td>
</tr>
</tbody>
</table>

* a Insertions i36 to i74 were obtained in the first screen (with igaA4 expressed from a pBAD18 derivative), and insertions r20 to r71 arose from the second screen (with rcsB expressed from a pBAD18 derivative).

* b Induction ratios were determined as follows: igaA5/igaA$^-$ for genes positively regulated by RcsB (positive values) and igaA$^+$/igaA5 for genes negatively regulated by RcsB (negative values).

### Table 4. β-Galactosidase activities of the Rcs-regulated fusions in different genetic backgrounds

<table>
<thead>
<tr>
<th>Insertion in gene</th>
<th>Wild type</th>
<th>igaA5</th>
<th>igaA5 rcsA</th>
<th>igaA5 rcsB</th>
<th>igaA5 rcsC</th>
</tr>
</thead>
<tbody>
<tr>
<td>dcuB</td>
<td>1.818 ± 36</td>
<td>974 ± 33</td>
<td>919 ± 21</td>
<td>1.941 ± 12</td>
<td>1.922 ± 53</td>
</tr>
<tr>
<td>melB</td>
<td>15 ± 0.9</td>
<td>8 ± 0.4</td>
<td>12 ± 0.3</td>
<td>18 ± 0.4</td>
<td>16 ± 0.8</td>
</tr>
<tr>
<td>narH</td>
<td>138 ± 25</td>
<td>11 ± 5</td>
<td>6 ± 2</td>
<td>69 ± 14</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>siiE</td>
<td>22 ± 2</td>
<td>6 ± 0.1</td>
<td>6 ± 0.2</td>
<td>66 ± 4</td>
<td>68 ± 8</td>
</tr>
<tr>
<td>srfA</td>
<td>38 ± 0.3</td>
<td>4 ± 0.1</td>
<td>2 ± 0.1</td>
<td>86 ± 26</td>
<td>26 ± 0.3</td>
</tr>
<tr>
<td>srfB</td>
<td>248 ± 10</td>
<td>7 ± 0</td>
<td>7 ± 1</td>
<td>233 ± 0.8</td>
<td>225 ± 29</td>
</tr>
<tr>
<td>trg</td>
<td>648 ± 28</td>
<td>5 ± 0</td>
<td>5 ± 0.6</td>
<td>557 ± 72</td>
<td>588 ± 39</td>
</tr>
<tr>
<td>yhhJ</td>
<td>44 ± 3</td>
<td>12 ± 0.1</td>
<td>11 ± 0.4</td>
<td>49 ± 3</td>
<td>36 ± 0.1</td>
</tr>
<tr>
<td>yiaD</td>
<td>9 ± 0.1</td>
<td>159 ± 10</td>
<td>166 ± 0.9</td>
<td>8 ± 0.0</td>
<td>8 ± 0.3</td>
</tr>
<tr>
<td>ybiH</td>
<td>11 ± 0.1</td>
<td>713 ± 69</td>
<td>159 ± 27</td>
<td>7 ± 0.1</td>
<td>6 ± 0.2</td>
</tr>
<tr>
<td>STM1491</td>
<td>57 ± 1.3</td>
<td>420 ± 36</td>
<td>512 ± 12</td>
<td>56 ± 13</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>STM2176</td>
<td>15 ± 0.9</td>
<td>8 ± 0.1</td>
<td>8 ± 0.3</td>
<td>15 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>bapA</td>
<td>68 ± 5</td>
<td>369 ± 9</td>
<td>228 ± 5</td>
<td>45 ± 2</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>PSTL071</td>
<td>2 ± 0.3</td>
<td>9 ± 0.8</td>
<td>10 ± 0.2</td>
<td>2 ± 0</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

* a β-Galactosidase activities were determined using stationary-phase cultures in LB medium. The data are the means ± standard deviations of two independent experiments. Similar results were obtained when the assays were performed with exponential-phase cultures.

* b Activities are variable from one experiment to another with this insertion.
was only marginal (93). srfABC was proposed to constitute a multigene horizontal acquisition based on (i) a G+C content significantly higher than the genome average G+C content and (ii) the lack of E. coli homologues (93). A BLAST search revealed the presence of this putative operon in other Enterobacteriaceae, including Enterobacter sp. and Yersinia sp. Interestingly, the plant pathogen Pseudomonas syringae has an srfC ortholog coding for a protein that is secreted through a T3SS (69). Therefore, we decided to further explore the pattern of srfABC expression. Introduction of a 3×FLAG epitope at the 3′ end of srfA, srfB, or srfC permitted detection of C-terminally tagged proteins by Western blotting against the FLAG epitope. All products were the expected size (Fig. 2). Western blotting with anti-FLAG antibody showed that the igaA5 mutation inhibits synthesis of SrfA, SrfB, and SrfC and that this inhibition requires both RcsB and RcsC (Fig. 2).

Next, we examined the srfABC expression pattern in media that optimize invasivity (SPI-1-inducing conditions) or that imitate the intracellular milieu (SPI-2-inducing conditions). We took advantage of the lacZ transcriptional fusion created by the MuJ insertion in srfB to measure the levels of transcription under SPI-1-inducing conditions (LB medium) and SPI-2-inducing conditions (LPM) in different genetic backgrounds, including wild type, phoP, pho-24 (a mutation that causes constitutive activation of the PhoPQ system), hilA, and ssrB (Fig. 3A). As controls, the same culture conditions and genetic backgrounds were used to monitor expression of the SPI-1 gene prgH and the SPI-2 gene ssaV. As expected, a prgH::lac transcriptional fusion was expressed under SPI-1-inducing conditions and was subjected to HilA positive regulation and PhoP negative regulation (Fig. 3B). In turn, an ssaV::lac transcriptional fusion was expressed under SPI-2-inducing conditions, and its expression was dependent on PhoP and SsrB (Fig. 3B). As shown in Fig. 3A, the srfABC operon is expressed in LB medium and repressed in LPM, and a phoP null mutation leads to expression of the operon even in LPM. These results provide evidence that the srfABC operon is expressed under SPI-1-inducing conditions and is repressed un-

![FIG. 2. Regulation of srfA, srfB, and srfC by RcsB at the protein level. Extracts from ATCC 14028 (wild-type strain) derivatives expressing 3×FLAG-tagged SrfA, SrfB, or SrfC were resolved by 10% SDS-PAGE. The same amount of protein was loaded in each lane. Immuno blotting was performed with a monoclonal anti-FLAG antibody. rcsC54 and igaA5 are mutants with constitutive activation of the Rcs system. wt, wild type.](http://jb.asm.org/)

![FIG. 3. Transcriptional repression of srfABC by PhoP. Expression levels of srfB (A) and prgH and ssaV (B) were monitored with lacZ transcriptional fusions. Strains carrying the indicated mutations were cultured in the following media: LB medium for SPI-1 induction; LPM for SPI-2 induction; and LB medium containing EDTA for Mg²⁺ chelation. The β-galactosidase activities shown were measured in stationary-phase cultures, but similar results were obtained in exponential cultures. The data represent the averages and standard deviations from two experiments. wt, wild type.](http://jb.asm.org/)
der SPI-2-inducing conditions in a PhoP-dependent manner. In support of this conclusion, expression of the operon is also repressed in a pho-24 background under SPI-1-inducing conditions and when LB medium is supplemented with EDTA, an $\text{Mg}^{2+}$ chelator. In contrast, EDTA does not prevent transcription of the operon in a PhoP$^{+}$ background. The results obtained with the srfB::lacZ transcriptional fusion were confirmed at the protein level by detecting epitope-tagged SrfC by Western blotting (Fig. 4).

In vivo analysis reveals a partial overlap between the RcsB and PhoP regulons. Regulation of the srfA-ABC operon by RcsB and PhoP suggests the possibility that there is a genetic interaction between these systems. Since both systems regulate virulence in Salmonella, we investigated their hypothetical interaction in the mouse model of infection, taking advantage of a method that has been used previously for in vivo genetic analysis. BALB/c mice were infected intraperitoneally with a mixture of single and double mutants carrying igaA1 and pho-24 mutations, and a COI (6) was calculated. The COI (igaA1 pho-24 versus igaA1) was statistically different from the CI (pho-24 versus wild type) but also statistically different from 1 (Fig. 5). Similarly, the reciprocal COI (igaA1 pho-24 versus igaA1) was significantly different from 1 and from the CI (igaA1 versus wild type) (Fig. 5). These results suggest that there is a partial overlap between these regulatory systems in the control of certain virulence functions.

In support of this conclusion, we compared the expression of three representative genes (prgH [an SPI-1 gene], ssaV [an SPI-2 gene], and gmm [a capsule gene]) in PhoP-constitutive and Rcs-constitutive backgrounds. Note that overproduction of the colanic acid capsule is a relevant factor in the attenuation of Rcs constitutive mutants (35). Data in Table 5 show that both the PhoPQ and Rcs systems transcriptionally repress prgH. This result is in agreement with a previous report suggesting that the Rcs system negatively controls Salmonella invasion of epithelial cells (64). In contrast, ssaV is activated by PhoP but not by RcsB, and gmm is activated by RcsB but not by PhoP (Table 5). Altogether, these results support the view that there is partial overlap between Rcs and PhoPQ in the control of Salmonella virulence.

![FIG. 4. Regulation of SrfC-3×FLAG levels by PhoP. Strains carrying the indicated mutations were cultured in LB medium (for SPI-1 induction). LPM (for SPI-2 induction), and LB medium containing EDTA (for $\text{Mg}^{2+}$ chelation). 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. wt, wild type.](image)

![FIG. 5. Overlap of PhoPQ and Rcs systems in the control of virulence: analysis of the pho-24 igaA1 double mutant in mixed infections with either the pho-24 or igaA1 single mutant. The indices represented are CIs (single mutant versus wild type) and COIs (double mutant versus single mutant). The strains used in each mixed infection are represented by the relevant mutations, as indicated under the bars. COIs were compared to 1.0 and to the CI relevant in each case. COIs are significantly different from the corresponding CIs and from 1.0 ($P < 0.05$), wt, wild type.](image)

**DISCUSSION**

The Rcs system is present in enteric and nonenteric bacteria, including *E. coli*, *S. enterica*, *Vibrio cholerae*, *K. pneumoniae*, *E. amylovora*, *P. mirabilis*, and *Pseudomonas aeruginosa*. From its initial description as a regulator of capsule synthesis (39), a diversity of roles have been assigned to the Rcs signaling system (54). Of special interest is the involvement of the Rcs system in *Salmonella* virulence (25, 35, 64).

Here, we identified RcsB-regulated genes in *S. enterica* serovar Typhimurium by comparing the expression of MuJ-generated fusions with a basal level of Rcs activation and the expression of MuJ-generated fusions with strong activation. The latter was achieved by using either igaA mutations or rcsB overexpression. The results of our screens overlap only partially with those obtained for *E. coli* (29, 44, 67). This can be explained by differences between *E. coli* and *S. enterica* and also by the diverse methodologies employed (macroarray, microarray, transcriptional fusions) and the disparate conditions used for activation of the Rcs system (growth at 20°C in medium with glucose and $\text{ZnCl}_2$, DjlA overproduction, igaA mutations, rcsB overexpression).

Data in Table 4 confirm that all the genes identified in our screens are indeed regulated by RcsB. Several interesting details should also be noted. (i) STM2176 appears to be RcsC independent, in contrast to the remaining genes. (ii) *rcsB* and *igaA* are significantly different from 1 and from the CI (igaA1 versus wild type) (Fig. 5). These results suggest that there is a partial overlap between these regulatory systems in the control of certain virulence functions.

In support of this conclusion, we compared the expression of three representative genes (prgH [an SPI-1 gene], ssaV [an SPI-2 gene], and gmm [a capsule gene]) in PhoP-constitutive and Rcs-constitutive backgrounds. Note that overproduction of the colanic acid capsule is a relevant factor in the attenuation of Rcs constitutive mutants (35). Data in Table 5 show that both the PhoPQ and Rcs systems transcriptionally repress prgH. This result is in agreement with a previous report suggesting that the Rcs system negatively controls *Salmonella* invasion of epithelial cells (64). In contrast, ssaV is activated by PhoP but not by RcsB, and gmm is activated by RcsB but not by PhoP (Table 5). Altogether, these results support the view that there is partial overlap between Rcs and PhoPQ in the control of *Salmonella* virulence.

**TABLE 5. Partial overlap of Rcs and/or PhoPQ in the regulation of genes relevant for *Salmonella* virulence**

<table>
<thead>
<tr>
<th>lac fusion in gene</th>
<th>PhoPQ</th>
<th>RcsB</th>
</tr>
</thead>
<tbody>
<tr>
<td>prgH</td>
<td>120 ± 4</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>ssaV</td>
<td>7 ± 0.1</td>
<td>7 ± 0.2</td>
</tr>
<tr>
<td>gmm</td>
<td>2 ± 0.1</td>
<td>901 ± 26</td>
</tr>
</tbody>
</table>

*β-Galactosidase activities were determined using stationary-phase cultures in LB medium. The data are the means ± standard deviations of two independent experiments. Similar results were obtained when the assays were performed with exponential-phase cultures.*
rsC null mutations not only suppress repression of siiE by igaA5 but also increase siiE expression on their own. (iii) A similar effect is exerted by srfA by an rcsB mutation (but not by an rcsC mutation). These results suggest that under our experimental conditions, RcsB is not completely inactive in a wild-type background. These data could also indicate an RcsC-independent role for acetyl phosphate or another unknown donor in RcsB phosphorylation (28, 54).

Our screens unveiled genes already known to be members of the Salmonella Rcs regulon, including genes for colanic acid capsule synthesis, flagella, and chemotaxis (10). These genes served to validate our methodology but were not studied further. A second group included genes that have homologues in E. coli. None of them had previously been identified as a member of the Salmonella Rcs regulon, and only two, yiaD and yjbH, have been shown to be part of the E. coli Rcs regulon. E. coli yiaD and yjbH were found to be regulated by RscC in a screen of transcriptional fusions generated by λplacMu53 (29). In the same study, macroarray analysis identified yjbE, yjbF, and yjbG, the three open reading frames upstream of yjbH, as members of the RscC regulon (29). It has recently been shown that the yjb operon is involved in production of exopolysaccharides (28). Based on similarities to known genes, yiaD, yjbH, and yjbF are predicted to encode lipoproteins. Interestingly, many of the genes identified as members of the E. coli Rcs regulon in another study (44) are predicted to encode cell envelope-associated proteins, including three putative lipoproteins (OsmB, YajI, and YggG). All these genes are positively regulated by the system, as are yiaD and yjbH. This is in agreement with the involvement of the Rcs system not only in the production of capsule but also in the control of surface-related processes, such as swarming (4, 43, 46, 84) and biofilm formation (29). The remaining genes in this second group are negatively regulated by the system. The list includes dcuB and narH, suggesting that the Rcs system may also play a role in the control of anaerobic respiration. Interestingly FlhD and FlhC, which are negatively regulated by RcsB, have been shown to regulate a number of E. coli genes involved in anaerobic respiration (71), but dcuB and narH were not on the list. In addition, our data suggest that FlhD and FlhC do not regulate these genes in S. enterica (data not shown).

In previous work we showed that srfC mutants with constitutive activation of the Rcs system are severely attenuated for virulence in intraperitoneally inoculated BALB/c mice and that overexpression of the colanic acid capsule is one of the factors responsible for attenuation (35). However, we also presented evidence that there are additional factors (35). Hence, we were especially interested in Salmonella genes regulated by the Rcs system that are not present in related bacteria. The Salmonella-specific genes revealed by our genetic screens are bapA, siiE, srfA, and srfB. The product of bapA has recently been described as a cell surface protein required for biofilm formation in S. enterica serovar Enteritidis (51). Interestingly, the Rcs system is involved in biofilm formation in E. coli (29). siiE is located in SPI-4, a Salmonella-specific chromosomal segment (92) predicted to be an operon containing six open reading frames which encode a secreted protein (SiiE) and components of a type I secretion system (58). Conclusive evidence that this island is required for intestinal but not systemic infection in mice was recently provided (63).

srfA and srfB, together with srfC, appear to form a 7-kb operon that is part of a putative horizontally acquired DNA segment that was described as regulated by SsrB (93), a repressor encoded within SPI-2 that is required for SPI-2-encoded T3SS expression (17, 47). Here, we show that the srfABC operon is expressed in SPI-1-inducing conditions but not in SPI-2-inducing conditions. In fact, our experiments show that in addition to being repressed by RcsB, srfABC is repressed by PhoP. In contrast, we provide evidence that srfABC is not under the control of SsrB (Fig. 3 and 4).

The previously reported regulation of some PhoP-activated genes by RcsB suggested the existence of a regulatory circuit between the PhoPO and Rcs networks (86). However, in vivo studies demonstrated that attenuation of virulence caused by activation of the Rcs system is not related to loss of function of the PhoPO system (25). These experiments thus failed to show any overlap between the two systems for the control of virulence. However, we show that the srfABC operon is repressed by both RcsB and PhoP, providing examples of PhoP-repressed genes (prg genes) that are simultaneously repressed by RscB. On the basis of the latter results, we investigated the effect of a mutation that causes constitutive activation of the Rcs system (igaA1) and a mutation that causes constitutive activation of the PhoPO system (pho-24) in the mouse model. If virulence genes repressed by both systems exist, an epistatic effect should be detected in infections with double mutants. The main advantage of using igaA1 (CI, 0.006) in the virulence experiments instead of other more attenuated igaA mutations like igaA5 (CI, 0.0000586) (25) is that it can be expected to permit detection of complete additivity with pho-24 (CI, 0.00006), since with this system we are able to detect CI values as low as 0.0000004 but lower values are not accurately measured (25, 35; data not shown). The actual results (Fig. 5) are intermediate between additivity and epistasis and suggest that there is partial overlap between the Rcs and PhoPO systems in the control of Salmonella virulence. This interpretation is strengthened by the existence of virulence genes that are repressed simultaneously by both systems, including prgH (Table 5) and other SPI-1 genes (64), whereas other genes are under the control of one system but not the other. Examples of the latter class include an SPI-2 gene (ssaV) and a capsule gene (gmm) (Table 5). Further investigations can be expected to reveal more precisely the overlap between the systems and to identify coregulated genes important for the systemic phase of Salmonella infection.

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