Journal of Bacteriology, Sept. 2007, p. 6635–6644 Vol. 189, No. 18
0021-9193/07/$08.00+0 doi:10.1128/JB.00640-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Rcs and PhoPQ Regulatory Overlap in the Control of *Salmonella enterica* Virulence[▽]

Clara B. García-Calderón, Josep Casadesús, and Francisco Ramos-Morales*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain

Received 24 April 2007/Accepted 28 June 2007

Genetic screens based on the use of Mu*d*J-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.

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) that in *S. enterica* confer the ability to invade nonphagocytic cells and to survive and proliferate within the phagosome (45, 52, 90, 94). The PhoPQ two-component system has long been known as a master regulator of virulence in *S. enterica*. Both its inactivation (by null mutations in *phoP* or *phoQ*) and its hyperactivation (by a gain-of-function mutation in *phoQ*) result in strong virulence attenuation in mice (30, 34, 60, 61). Mg^{2+} and Ca^{2+} have been identified as the physiological signals detected by the sensor kinase PhoQ. The prevalent model suggests that *Salmonella* identifies its environment within the host by monitoring Mg^{2+} levels via the PhoQ protein (40). A low Mg^{2+} concentration is an indication of an intracellular environment and leads to activation of the PhoPQ system. In turn, a high Mg^{2+} concentration serves as a hallmark of an extracellular environment and leads to inactivation of the system. Accordingly, PhoP-activated genes (*pag* genes) are turned on inside host cells, whereas PhoP-repressed genes (*prg* genes) are expressed outside host cells. Only a fraction of PhoP-regulated genes are involved in *Salmonella* virulence. These genes appear to have been acquired by horizontal gene transfer (40). Other two-component systems that contribute to the regulation of *Salmonella* virulence are PmrA-PmrB, RcsC-RcsD-RcsB, OmpR-EnvZ, SsrA-SsrB, and SirA-BarA (for a review, see reference 3). Certain interactions between these regulatory systems have been shown. For instance, a subset of PhoP-activated genes are regulated via PmrA-PmrB (41, 78), while PhoP directly regulates SsrB-SsrA, a regulatory system whose activation is necessary for expression of the SPI-2-encoded T3SS (7).

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 phosphorelay are largely unknown. Sledjeski 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

* Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Avda Reina Mercedes, 6, 41012 Sevilla, Spain. Phone: 34 95 455 7107. Fax: 34 95 455 7104. E-mail: framos@us.es.
▽ Published ahead of print on 6 July 2007.

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

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 RcsB 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 chlorpromazine-induced stress (20). Synthesis of certain exopolysaccharides 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 *srfABC*. 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.

TABLE 1. Strains of *S. enterica* serovar Typhimurium used in this study

Strain[a]	Description	Source or reference
ATCC 14028	Wild type	ATCC
55130	*pho-24* (PhoP constitutive)	E. A. Groisman
SV4439	*rcsC52*::Mu*d*Q	10
SV4514	*gmm*::Mu*d*Q	This study
SV4530	*igaA1*	25
SV4535	*igaA3*::Cm^r *yojN*::KIXX	Laboratory stock
SV4573	*igaA3*::Cm^r/pNG1166	This study
SV4608	*trg*::Mu*d*J	This study
SV4676	*srfB*::Mu*d*J	This study
SV4757	*rcsC54* (Rcs constitutive)	35
SV4773	*igaA5*	25
SV4918	*yjbH*::Mu*d*J	This study
SV4919	*siiE*::Mu*d*J	This study
SV4920	*melB*::Mu*d*J	This study
SV4921	*narH*::Mu*d*J	This study
SV4922	*dcuB*::Mu*d*J	This study
SV4923	STM2176::Mu*d*J	This study
SV4924	*yhhJ*::Mu*d*J	This study
SV5049	Δ*rcsB*::Cm^r	This study
SV5090	*gmm*::Mu*d*Q/pIZ1589	This study
SV5091	*srfA*::Mu*d*J	This study
SV5092	*yiaD*::Mu*d*J	This study
SV5093	Δ*rcsA*::Cm^r	This study
SV5094	*bapA*::Mu*d*J	This study
SV5095	PSLT071::Mu*d*J	This study
SV5106	STM1491::Mu*d*J	This study
SV5190	*srfA*::3×FLAG	This study
SV5191	*srfB*::3×FLAG	This study
SV5192	*srfC*::3×FLAG	This study
SV5303	*prgH*::*lacZ*	J. López-Garrido
SV5373	Δ*hilA*	J. López-Garrido
SV5452	Δ*ssrB*::Cm^r	This study
SV5470	*ssaV*::*lacZ*	This study

[a] Derivatives of some of the strains were used as indicated in the text.

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*, *ssrB*, or *ssaV* 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 RcsB-P1 and RcsB-P2 for *rcsB*, with primers SsrB-P1 and SsrB-P2 for *ssrB*, and with primers SsaV-P1 and SsaV-P2 for *ssaV*. The sequences of the primers used are shown in Table 2. The PCR product was used to transform the wild-type strain carrying the Red recombinase expression plasmid pKD46. When necessary, the antibiotic resistance cassette introduced by the gene-targeting procedure was eliminated by recombination with plasmid pCP20 (22).

Construction of an *ssaV*::*lac* 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 *ssaV* (27).

Chromosomal gene epitope tagging. Addition of a 3×FLAG epitope tag at the 3′ 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).

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

TABLE 2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5′–3′)
RcsA-P1	CAATTCGGCCCTGCGCTTTCAACCACTCGCGTACCAGAAAGTGTAGGCTGGAGCTGCTTC
RcsA-P2	GAGGTACATTGCCAGTCCGGATGTCTCAGCGCATGTTAACCATATGAATATCCTCCTTAG
RcsB-P1	ATTATTGCCGATGACCACCCGATTGTACTGTTCGGTATTCGTGTAGGCTGGAGCTGCTTC
RcsB-P2	AGAGAGATAGTTGAGCAGCGCGATATCATTCTCTACGCCCCATATGAATATCCTCCTTAG
SsrB-P1	AATATGACCAATGCTTAATACCATCGGACGCCCCTGGTTAGTGTAGGCTGGAGCTGCTTC
SsrB-P2	TACTTAATATTATCTTAATTTTCGCGAGGGCAGCAAAATGCATATGAATA TCCTCCTTAG
SsaV-P1	CATCATCGACAAATAAAATTTCTGGAGTCGCAATGCGTTCGTGTAGGCTGGAGCTGCTTC
SsaV-P2	CAATTCATTCTTCATTGTCCGCCAACTCCTCTTCGCTAAGCATATGAATATCCTCCTTAG
SrfA-P1Flag	CCACGCGGCAATTCCGTTGACGTTTAAAAAGATAGGTGCCGACTACAAAGACCATGACGG
SrfA-P2Flag	GACGCTCTGTTTGTAATCACACAGATTGACCAACATAAAACATATGAATATCCTCCTTAG
SrfB-P1Flag	CAGCCACTACTGGATAGATAGTGGGAGTGTATACCTGAAAGACTACAAAGACCATGACGG
SrfB-P2Flag	CCCACTCGATAACAGCCTGGGTGGTGTTTAACGGTTTTGCATATGAATATCCTCCTTAG
SrfC-P1Flag	CCGACGTAGACAGAGCGCAATTAATTGCCCTGATAGCCGACTACAAAGACCATGACGG
SrfC-P2Flag	GATAAGTTTCCCCGTCGCATTGCTGTTTTCGCTTTTCTGACATATGAATATCCTCCTTAG
RcsB5’	AGCGGAATTCAGGAGGAATACATGAACAATATGAACG
RcsB3’	GTGAAAGCTTGTCGACAAGCGATTTATTCTTTGTCTG
MuL	CGAATAATCCAATGTCCTCC

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 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 0.2% glucose or arabinose were added to LB medium. Motility 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 from overnight cultures 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 $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 0.1% Casamino Acids, 38 mM glycerol, 337.5 μM K_2HPO_4-KH_2PO_4 (pH 7.4), and 8 μM $MgCl_2$ and incubated overnight at 37°C with shaking.

DNA amplification with PCR. Amplification reactions were carried out with a Perkin Elmer Gene-Amp 2400 PCR system (Perkin Elmer Cetus, Foster City, CA). The final volume of reaction mixtures was 50 to 100 μl, and the final concentration of $MgCl_2$ was 1 mM. Reagents were used at the following concentrations: deoxynucleoside triphosphates, 200 μM; primers, 1 μM; and *Taq* polymerase (Expand high-fidelity PCR system; Roche Diagnostics SL), 1 U per reaction mixture. 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 (Ap^r) is a member of the pBAD series of vectors and permits tight regulation of cloned genes via the arabinose-inducible P_{BAD} promoter (42). Plasmid pNG1166 is a pBAD18 derivative which carries the *igaA* gene under control of the P_{BAD} promoter (10). Plasmid pIZ1589 was constructed as follows. Genomic DNA from strain ATCC 14028 was PCR amplified using primers RcsB5′ and RcsB3′ (Table 2), which introduce EcoRI and HindIII sites, respectively. The amplified DNA fragment was digested with EcoRI and HindIII for oriented cloning of the *rcsB* gene on pBAD18. Ligation mixtures were used to transform *E. coli* DH5α, with selection of Ap^r transformants on LB medium-ampicillin plates. One of the transformants was the source of pIZ1589, which carries the *rcsB* gene under the control of P_{BAD}.

Mutagenesis with Mu*d*J. We employed the *cis*-complementation procedure of Hughes and Roth (49), in which a defective Mu*d*J element is cotransduced with a Mu*d*1 element that transiently provides transposition functions. Mu*d*1 is the specialized transducing phage Mu*d*1 (Ap Lac cts62) (13). Mu*d*I1734[KmLac] (14) is a transposition-deficient Mu derivative that generates operon fusions upon insertion; this element was renamed Mu*d*J by Hughes and Roth (49).

Cloning and molecular characterization of Mu*d*J inserts. Genomic DNA from each Mu*d*J-carrying isolate was digested with BamHI or XhoI and ligated with T4 DNA ligase to BamHI- or XhoI-digested pBluescript SKII. The ligation mixtures were transformed into *E. coli* DH5α, and Mu*d*J-containing transformants 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 Genómicos, 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.

β-Galactosidase assays. Levels of β-galactosidase activity were determined for exponential- and stationary-phase cultures in LB medium as described previously (59), using the $CHCl_3$-sodium dodecyl sulfate (SDS) permeabilization procedure.

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 10^5 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-

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

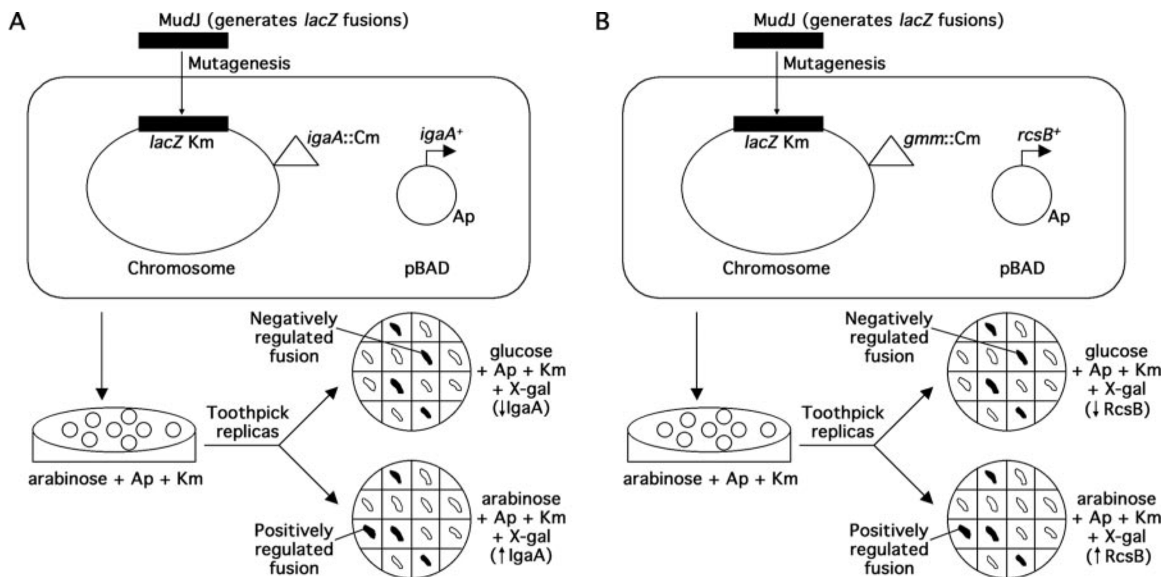

FIG. 1. Identification of IgaA- and RcsB-regulated genes. (A) Diagram of the genetic screen for identification of IgaA-regulated genes in *S. enterica* serovar Typhimurium. A plasmid carrying a wild-type *igaA* allele under the control of an arabinose-inducible promoter (P_{BAD}) was introduced into a strain harboring a null *igaA* mutation. *igaA* is expressed in the presence of arabinose as the sole carbon source but not in the presence of glucose. *lac* transcriptional fusions were generated using Mu*d*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 *rcsB* gene under P_{BAD} control was introduced into a strain harboring a null *gmm* mutation. *rcsB* is expressed in arabinose medium but not in glucose medium. *lac* transcriptional fusions were generated, as described above, with Mu*d*J. The expression pattern of 20,000 independent fusions was monitored in glucose- and arabinose-containing LB medium plates supplemented with X-Gal. The *gmm* mutation was introduced to prevent mucoidy, thereby facilitating comparison of colony colors in different media. Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.

lence, we were particularly interested in identifying genes that were *Salmonella* specific. For this purpose, we generated random transcriptional *lac* fusions in the *Salmonella* chromosome and compared their expression under strong activation conditions with their expression with a lack of activation of the Rcs system. Two different, independent screens were devised (Fig. 1).

(i) Identification of IgaA-regulated genes. The product of *igaA* has a negative effect on the activation of the Rcs system. Null mutations in *igaA* are lethal. Mutants with some point mutations, like *igaA1* and *igaA5*, are viable but show mucoidy and a lack of motility. *rcsC*, *rcsD*, and *rcsB* mutations suppress these phenotypes, suggesting that they are due to overactivation of the Rcs system (10, 25). Therefore, genes regulated by RcsB are expected to be regulated by IgaA in the opposite way. To carry out the first screen, we constructed strain SV4573 (Table 1), in which the null mutation *igaA3*::Cm^r is complemented by a plasmid-borne, wild-type *igaA* allele expressed from an arabinose-dependent promoter (10, 25). In solid LB medium with arabinose, *igaA* is expressed and the strain forms nonmucoid colonies. In LB medium with glucose, the strain shows the phenotypes associated with a lack of *igaA*. Although null mutations in *igaA* are lethal (10, 25), we observed that patching of colonies growing in LB medium arabinose onto LB medium containing glucose allowed residual growth that was useful for the purpose of the screen. Mucoidy was observed in the presence of glucose, confirming the occurrence of reduced *igaA* expression and concomitant Rcs activation. The procedure is summarized in Fig. 1A.

For detection of IgaA-regulated loci, strain SV4573 was mutagenized with Mu*d*J to generate transcriptional *lacZ* fusions. Ten thousand independent isolates carrying Mu*d*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 *igaA*. Initially, 114 fusions were found to be differentially regulated. Reconstruction experiments and comparison of the β-galactosidase activities of the fusions in a wild-type background with the activities in an *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 *rcsB* under P_{BAD} promoter control, was transformed into wild-type strain ATCC 14028 (Table 1). Transformants carrying pIZ1589 were nonmucoid in glucose (when *rcsB* was not expressed) and mucoid in arabinose (when *rcsB* was expressed). This is in agreement with studies of *E. coli* indicating that overexpression of *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 (*gmm*::Mu*d*Q), yielding strain SV5090 (Table 1). The *gmm* gene, also called *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

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

TABLE 3. IgaA- and RcsB-regulated fusions

Insertion(s)[a]	Gene	Induction ratio[b]	Protein function and/or features	Putative transcriptional unit	Reference(s)
i36	*trg*	−129.6	Methyl-accepting chemotaxis protein	*trg*	53, 81
i59, i70	*srfB*	−35.4	Unknown	*srfABC*	93
i74, r50	*bapA*	5.4	Large protein with repeats	*bapABCD*	51
r20, r44	*yjbH*	70.3	Outer membrane lipoprotein	*yjbEFGH*	29
r52	*narH*	−12.5	β-Subunit of nitrate reductase A	*narGHJI*	8, 79, 80
r55, r56	*siiE*	−3.3	Large secreted protein coded in SPI-4	*siiABCDEF*	63, 92
r71	*dcuB*	−1.9	Anaerobic C_4-dicarboxylate transporter	*dcuBfumB*	29, 38
r85	STM2176	−1.9	Glutathione *S*-transferase	STM2179- STM2178- STM2177- STM2176- STM2175	58
r97	*melB*	−1.9	Melibiose uptake	*melAB*	62, 66, 70, 75
r111	*yhhJ*	−3.3	Transporter, ABC superfamily	*yhiHyhhJ*	72
r142, r171	*srfA*	−9.5	Similar to nuclear antigens	*srfABC*	93
r150	PSLT071	4.5	Unknown	PSLT071	68
r153	*yiaD*	17.7	Outer membrane lipoprotein	*yiaD*	29
r172	STM1491	7.4	ABC-type proline/glycine betaine transport, ATPase component	STM1494- STM1493- STM1492- STM1491	58

[a] Insertions i36 to i74 were obtained in the first screen (with *igaA* expressed from a pBAD18 derivative), and insertions r20 to r172 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).

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

media, making color comparisons easier. Twenty thousand Mu*d*J-carrying isolates were analyzed in this second screen, and 17 fusions were chosen for further study.

To ascertain whether the Mu*d*J 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 Mu*d*J 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 *gmm* (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 cloned and sequenced. To identify the loci where the Mu*d*J element had inserted, chromosomal fragments containing the Mu*d*J Km^r gene were cloned in pBluescript SKII. DNA sequencing was performed using the MuL primer (87). Genes positively regulated by RcsB (or negatively regulated by IgaA) and genes negatively regulated by RcsB (or positively regulated by IgaA) were identified. Data for these genes and their products are shown in Table 3.

Null mutations in *rcsC* or *rcsB* suppress mucoidy and the lack of motility of an IgaA$^-$ mutant (10). Therefore, these mutations are expected to suppress the positive or negative effect of the *igaA* mutation on the expression of all genes found in our screens. Triple mutants bearing one of the Mu*d*J 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 *yjbH* 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 4. β-Galactosidase activities of the Rcs-regulated fusions in different genetic backgrounds

Insertion in gene	β-Galactosidase activity (Miller units)[a]				
	Wild type	*igaA5*	*igaA5 rcsA*	*igaA5 rcsB*	*igaA5 rcsC*
dcuB	1,818 ± 36	974 ± 33	919 ± 21	1,941 ± 12	1,922 ± 53
melB	15 ± 0.9	8 ± 0.4	12 ± 0.3	18 ± 0.4	16 ± 0.8
narH	138 ± 25	11 ± 5	6 ± 2	69 ± 14	76 ± 16
siiE	22 ± 2	6 ± 0.1	6 ± 0.2	66 ± 4	68 ± 8
srfA	38 ± 0.3	4 ± 0.1	2 ± 0.1	86 ± 26	26 ± 0.3
srfB	248 ± 10	7 ± 0	7 ± 1.1	233 ± 0.8	225 ± 29
trg	648 ± 28	5 ± 0	5 ± 0.6	557 ± 72	588 ± 39
yhhJ	44 ± 3	12 ± 0.1	11 ± 0.4	49 ± 3	36 ± 0.1
yiaD	9 ± 0	159 ± 10	166 ± 0.9	8 ± 0	8 ± 0.3
yjbH	11 ± 0.1	773 ± 69	159 ± 27	7 ± 0.1	6 ± 0.2
STM1491	57 ± 1.3	420 ± 36	512 ± 12	56 ± 13	84 ± 4
STM2176	15 ± 0.9	8 ± 0.1	8 ± 0.3	15 ± 1	9 ± 1
bapA[b]	68 ± 5	369 ± 9	228 ± 3	45 ± 2	98 ± 3
PSLT071	2 ± 0.3	9 ± 0.8	10 ± 0.2	2 ± 0	2 ± 0

[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.

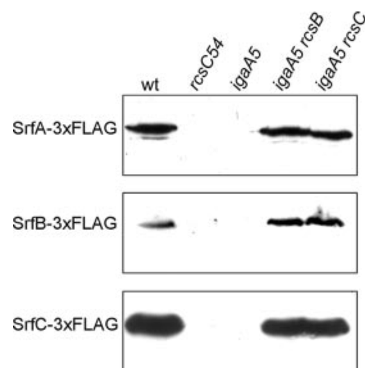

FIG. 2. Regulation of *srfA*, *sfrB*, and *srfC* by RcsB at the protein level. Extracts from ATCC 14028 (wild-type strain) derivatives expressing 3×FLAG-tagged SrfA, SrfB, or SrfC were resolved by 10% SDS-PAGE. The same amount of protein was loaded in each lane. Immunoblotting was performed with a monoclonal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

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 Mu*d*J 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-

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

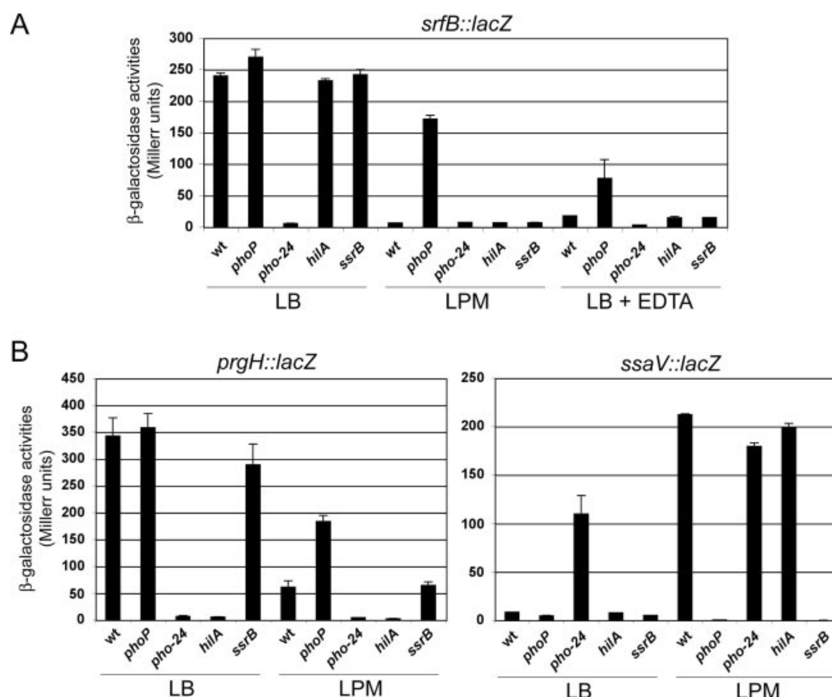

FIG. 3. Transcriptional repression of *srfABC* by PhoP. Expression levels of *srfB* (A) and *prgH* and *ssaV* (B) were monitored with *lacZ* transcriptional fusions. Strains carrying the indicated mutations were cultured in the following media: LB medium for SPI-1 induction; LPM for SPI-2 induction; and LB medium containing EDTA for Mg^{2+} 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.

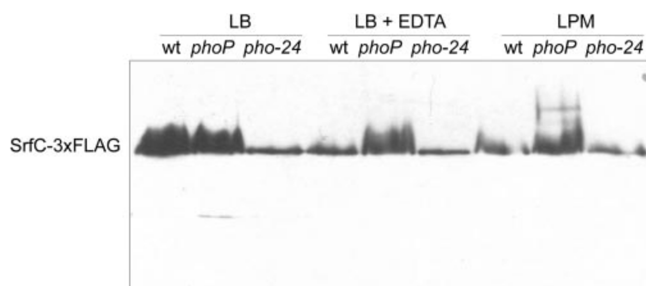

FIG. 4. Regulation of SrfC-3×FLAG levels by PhoP. Strains carrying the indicated mutations were cultured in LB medium (for SPI-1 induction), LPM (for SPI-2 induction), and LB medium containing EDTA (for Mg^{2+} chelation). Protein extracts were resolved by 10% SDS-PAGE. The same amount of protein was loaded in each lane. Immunoblotting was performed with a monoclonal anti-FLAG antibody. wt, wild type.

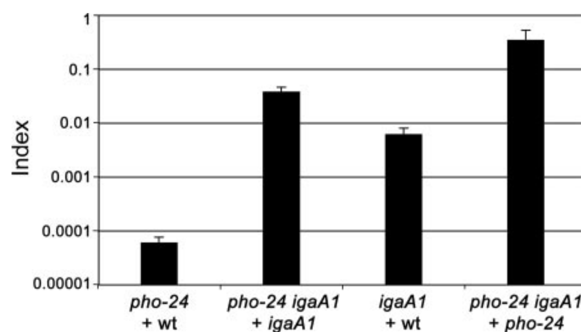

FIG. 5. Overlap of PhoPQ and Rcs systems in the control of virulence: analysis of the *pho-24 igaA1* double mutant in mixed infections with either the *pho-24* or *igaA1* single mutant. The indices represented are CIs (single mutant versus wild type) and COIs (double mutant versus single mutant). The 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.

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 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 *srfABC* 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 *pho-24*) 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.

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 Mu*d*J-generated fusions with a basal level of Rcs activation and the expression of Mu*d*J-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 $ZnCl_2$, 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

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

lac fusion in gene	β-Galactosidase activity (Miller units)[a]		
	Wild type	*igaA5*	*pho-24*
prgH	120 ± 4	1 ± 0	3 ± 0
ssaV	7 ± 0.1	7 ± 0.2	103 ± 13
gmm	2 ± 0.1	901 ± 26	2 ± 0.1

[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.

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

rcsC null mutations not only suppress repression of *siiE* by *igaA5* but also increase *siiE* expression on their own. (iii) A similar effect is exerted on *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 RcsC in a screen of transcriptional fusions generated by λp*lac*Mu53 (29). In the same study, macroarray analysis identified *yjbE*, *yjbF*, and *yjbG*, the three open reading frames upstream of *yjbH*, as members of the RcsC 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 *rcsC* 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 regulator 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 PhoPQ 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 PhoPQ system (25). These experiments thus failed to show any overlap between the two systems for the control of virulence. However, we show that the *sfrABC* operon is repressed by both RcsB and PhoP, providing examples of PhoP-repressed genes (*prg* genes) that are simultaneously repressed by RcsB. 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 PhoPQ 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, 0.0000036) (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 PhoPQ 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.

ACKNOWLEDGMENTS

We are grateful to F. García-del Portillo for helpful discussions and to E. A. Groisman and J. López-Garrido for the generous gift of some strains.

This work was supported by grants BIO2004-04355-CO2-02 (to J.C.) and SAF2004-00227 (to F.R.-M.) from the Ministry of Education and Science of Spain and the European Regional Development Fund. C.G.-C. is a Ph.D. student supported by a predoctoral grant from the Spanish Ministry of Education and Science.

REFERENCES

1. **Aguilar, P. S., A. M. Hernandez-Arriaga, L. E. Cybulski, A. C. Erazo, and D. de Mendoza.** 2001. Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. EMBO J. **20:**1681–1691.
2. **Arricau, N., D. Hermant, H. Waxin, C. Ecobichon, P. S. Duffey, and M. Y. Popoff.** 1998. The RcsB-RcsC regulatory system of *Salmonella typhi* differ-

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

entially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. Mol. Microbiol. **29:**835–850.

3. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. **9:**143–152.
4. **Belas, R., R. Schneider, and M. Melch.** 1998. Characterization of *Proteus mirabilis* precocious swarming mutants: identification of *rsbA*, encoding a regulator of swarming behavior. J. Bacteriol. **180:**6126–6139.
5. **Bereswill, S., and K. Geider.** 1997. Characterization of the *rcsB* gene from *Erwinia amylovora* and its influence on exopolysaccharide synthesis and virulence of the fire blight pathogen. J. Bacteriol. **179:**1354–1361.
6. **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.
7. **Bijlsma, J. J., and E. A. Groisman.** 2005. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. Mol. Microbiol. **57:**85–96.
8. **Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy.** 1989. Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the alpha and beta subunits in iron binding and electron transfer. Mol. Gen. Genet. **218:**249–256.
9. **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.
10. **Cano, D. A., G. Domínguez-Bernal, A. Tierrez, F. García-del Portillo, and J. Casadesús.** 2002. Regulation of capsule synthesis and cell motility in *Salmonella enterica* by the essential gene *igaA*. Genetics **162:**1513–1523.
11. **Cano, D. A., M. Martínez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesús, and F. García-del Portillo.** 2001. *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infect. Immun. **69:**6463–6474.
12. **Carballès, F., C. Bertrand, J. P. Bouché, and K. Cam.** 1999. Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Mol. Microbiol. **34:**442–450.
13. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76:**4530–4533.
14. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-mu bacteriophage transposons. J. Bacteriol. **158:**488–495.
15. **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.
16. **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.
17. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30:**175–188.
18. **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.
19. **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.
20. **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.
21. **Cotter, P. D., C. M. Guinane, and C. Hill.** 2002. The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. Antimicrob. Agents Chemother. **46:**2784–2790.
22. **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.
23. **Davalos-Garcia, M., A. Conter, I. Toesca, C. Gutierrez, and K. Cam.** 2001. Regulation of *osmC* gene expression by the two-component system *rcsB-rcsC* in *Escherichia coli*. J. Bacteriol. **183:**5870–5876.
24. **Detweiler, C. S., D. M. Monack, I. E. Brodsky, H. Mathew, and S. Falkow.** 2003. *virK*, *somA* and *rcsC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. Mol. Microbiol. **48:**385–400.
25. **Domínguez-Bernal, G., M. G. Pucciarelli, F. Ramos-Morales, M. García-Quintanilla, D. A. Cano, J. Casadesús, and F. García-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.
26. **Ebel, W., G. J. Vaughn, H. R. Peters, 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.
27. **Ellermeier, C. D., A. Janakiraman, and J. M. Slauch.** 2002. Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153–161.
28. **Ferrières, 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.
29. **Ferrières, 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.
30. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
31. **Francez-Charlot, A., B. Laugel, A. Van Gemert, N. Dubarry, F. Wiorowski, M. P. Castanie-Cornet, C. Gutierrez, and K. Cam.** 2003. RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. Mol. Microbiol. **49:**823–832.
32. **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.
33. **Freter, R., P. C. O'Brien, and M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect. Immun. **34:**234–240.
34. **Galán, J. E., and R. Curtiss III.** 1989. Virulence and vaccine potential of *phoP* mutants of *Salmonella typhimurium*. Microb. Pathog **6:**433–443.
35. **García-Calderón, C. B., M. García-Quintanilla, J. Casadesús, and F. Ramos-Morales.** 2005. Virulence attenuation in *Salmonella enterica rcsC* mutants with constitutive activation of the Rcs system. Microbiology **151:** 579–588.
36. **Gervais, F. G., and G. R. Drapeau.** 1992. Identification, cloning, and characterization of *rcsF*, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *ftsZ84* in *Escherichia coli* K-12. J. Bacteriol. **174:**8016–8022.
37. **Gillen, K. L., and K. T. Hughes.** 1991. Negative regulatory loci coupling flagellin synthesis to flagellar assembly in *Salmonella typhimurium*. J. Bacteriol. **173:**2301–2310.
38. **Golby, P., D. J. Kelly, J. R. Guest, and S. C. Andrews.** 1998. Transcriptional regulation and organization of the *dcuA* and *dcuB* genes, encoding homologous anaerobic C4-dicarboxylate transporters in *Escherichia coli*. J. Bacteriol. **180:**6586–6596.
39. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162:**1111–1119.
40. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835–1842.
41. **Gunn, J. S., and S. I. Miller.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178:**6857–6864.
42. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. **177:**4121–4130.
43. **Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes.** 1995. A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. Mol. Microbiol. **17:**1167–1175.
44. **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.
45. **Hansen-Wester, I., and M. Hensel.** 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect. **3:**549–559.
46. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. USA **91:**8631–8635.
47. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. **30:**163–174.
48. **Hirakawa, H., K. Nishino, T. Hirata, and A. Yamaguchi.** 2003. Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J. Bacteriol. **185:**1851–1856.
49. **Hughes, K. T., and J. R. Roth.** 1984. Conditionally transposition-defective derivative of Mu *d*1(Amp Lac). J. Bacteriol. **159:**130–137.
50. **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.
51. **Latasa, C., A. Roux, A. Toledo-Arana, J. M. Ghigo, C. Gamazo, J. R. Penadés, and I. Lasa.** 2005. BapA, a large secreted protein required for biofilm

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA

formation and host colonization of *Salmonella enterica* serovar Enteritidis. Mol. Microbiol. **58:**1322–1339.
52. **Lostroh, C. P., and C. A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microbes Infect. **3:**1281–1291.
53. **Macnab, R. M.** 1996. Flagella and motility, p. 123–145. *In* F. C. Neidhardt, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
54. **Majdalani, N., and S. Gottesman.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu. Rev. Microbiol. **59:**379–405.
55. **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.
56. **Majdalani, N., D. Hernandez, and S. Gottesman.** 2002. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. Mol. Microbiol. **46:**813–826.
57. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones & Barlett, Boston, MA.
58. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
59. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
60. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
61. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2490.
62. **Mizushima, K., S. Awakihara, M. Kuroda, T. Ishikawa, M. Tsuda, and T. Tsuchiya.** 1992. Cloning and sequencing of the *melB* gene encoding the melibiose permease of *Salmonella typhimurium* LT2. Mol. Gen. Genet. **234:**74–80.
63. **Morgan, E., J. D. Campbell, S. C. Rowe, J. Bispham, M. P. Stevens, A. J. Bowen, P. A. Barrow, D. J. Maskell, and T. S. Wallis.** 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **54:**994–1010.
64. **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.
65. **Nassif, X., N. Honore, T. Vasselon, S. T. Cole, and P. J. Sansonetti.** 1989. Positive control of colanic acid synthesis in *Escherichia coli* by *rmpA* and *rmpB*, two virulence-plasmid genes of *Klebsiella pneumoniae*. Mol. Microbiol. **3:**1349–1359.
66. **Okada, T., K. Ueyama, S. Niiya, H. Kanazawa, M. Futai, and T. Tsuchiya.** 1981. Role of inducer exclusion in preferential utilization of glucose over melibiose in diauxic growth of *Escherichia coli*. J. Bacteriol. **146:**1030–1037.
67. **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.
68. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848–852.
69. **Petnicki-Ocwieja, T., D. J. Schneider, V. C. Tam, S. T. Chancey, L. Shan, Y. Jamir, L. M. Schechter, M. D. Janes, C. R. Buell, X. Tang, A. Collmer, and J. R. Alfano.** 2002. Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. tomato DC3000. Proc. Natl. Acad. Sci. USA **99:**7652–7657.
70. **Prestidge, L. S., and A. B. Pardee.** 1965. A second permease for methyl-thio-beta-D-galactoside in *Escherichia coli*. Biochim. Biophys. Acta **100:**591–593.
71. **Pruss, B. M., J. W. Campbell, T. K. Van Dyk, C. Zhu, Y. Kogan, and P. Matsumura.** 2003. FlhD/FlhC is a regulator of anaerobic respiration and the Entner-Doudoroff pathway through induction of the methyl-accepting chemotaxis protein Aer. J. Bacteriol. **185:**534–543.
72. **Saurin, W., M. Hofnung, and E. Dassa.** 1999. Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J. Mol. Evol. **48:**22–41.
73. **Scherer, C. A., and S. I. Miller.** 2001. Molecular pathogenesis of salmonellae, p. 265–333. *In* E. A. Groisman (ed.), Principles of bacterial pathogenesis. Academic Press, San Diego, CA.
74. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
75. **Schmitt, R.** 1968. Analysis of melibiose mutants deficient in alpha-galactosidase and thiomethylgalactoside permease II in *Escherichia coli* K-12. J. Bacteriol. **96:**462–471.
76. **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.
77. **Sledjeski, D. D., and S. Gottesman.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **178:**1204–1206.
78. **Soncini, F. C., and E. A. Groisman.** 1996. Two-component regulatory systems can interact to process multiple environmental signals. J. Bacteriol. **178:**6796–6801.
79. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52:**190–232.
80. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151:**1320–1325.
81. **Stock, J. B., and M. G. Surette.** 1996. Chemotaxis, p. 1103–1129. *In* F. C. Neidhardt, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
82. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659–669.
83. **Stout, V., A. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman.** 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. **173:**1738–1747.
84. **Takeda, S., Y. Fujisawa, M. Matsubara, H. Aiba, and T. Mizuno.** 2001. A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC $\rightarrow$ YojN $\rightarrow$ RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. Mol. Microbiol. **40:**440–450.
85. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
86. **Tierrez, A., and F. García-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.
87. **Torreblanca, J., S. Marqués, and J. Casadesús.** 1999. Synthesis of FinP RNA by plasmids F and pSLT is regulated by DNA adenine methylation. Genetics **152:**31–45.
88. **Uzzau, S., N. Figueroa-Bossi, S. Rubino, and L. Bossi.** 2001. Epitope tagging of chromosomal genes in *Salmonella*. Proc. Natl. Acad. Sci. USA **98:**15264–15269.
89. **Virlogeux, I., H. Waxin, C. Ecobichon, J. O. Lee, and M. Y. Popoff.** 1996. Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi*: *rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. J. Bacteriol. **178:**1691–1698.
90. **Waterman, S. R., and D. W. Holden.** 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell. Microbiol. **5:**501–511.
91. **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.
92. **Wong, K. K., M. McClelland, L. C. Stillwell, E. C. Sisk, S. J. Thurston, and J. D. Saffer.** 1998. Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a *Salmonella* pathogenicity island located at 92 minutes on the chromosome map of *Salmonella enterica* serovar typhimurium LT2. Infect. Immun. **66:**3365–3371.
93. **Worley, M. J., K. H. Ching, and F. Heffron.** 2000. *Salmonella* SsrB activates a global regulon of horizontally acquired genes. Mol. Microbiol. **36:**749–761.
94. **Zhou, D., and J. Galán.** 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. **3:**1293–1298.

Downloaded from http://jb.asm.org/ on February 26, 2016 by USE/BCTA.GEN UNIVERSITARIA