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The RcsCDB Signaling System and Swarming Motility in *Salmonella enterica* Serovar Typhimurium: Dual Regulation of Flagellar and SPI-2 Virulence Genes[▽]†

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The Rcs phosphorelay is a multicomponent signaling system that positively regulates colanic acid synthesis and negatively regulates motility and virulence. We have exploited a spontaneously isolated mutant, IgaA(T191P), that is nearly maximally activated for the Rcs system to identify a vast set of genes that respond to the stimulation, and we report new regulatory properties of this signaling system in *Salmonella enterica* serovar Typhimurium. Microarray data show that the Rcs system normally functions as a positive regulator of SPI-2 and other genes important for the growth of *Salmonella* in macrophages, although when highly activated the system completely represses the SPI-1/SPI-2 virulence, flagellar, and fimbrial biogenesis pathways. The auxiliary protein RcsA, which works with RcsB to positively regulate colanic acid and other target genes, not only stimulates but also antagonizes the positive regulation of many genes in the *igaA* mutant. We show that RcsB represses motility through the RcsB box in the promoter region of the master operon *flhDC* and that RcsA is not required for this regulation. Curiously, RcsB selectively stimulates expression of the flagellar type 3 secretion genes *fliPQR*; an RcsAB box located downstream of *fliR* influences this regulation. We show that excess colanic acid impairs swimming and inhibits swarming motility, consistent with the inverse regulation of the two pathways by the Rcs system.

The Rcs phosphorelay signaling system appears to be exclusively present only in the family *Enterobacteriaceae* (30). This system was first identified by its role in the positive regulation of capsular polysaccharide or colanic acid synthesis in *Escherichia coli* but is now known to also regulate genes involved in the maintenance of cell wall integrity, cell division, the regulation of stationary-phase sigma factor σ^S, motility, and virulence (Fig. 1) (39). In response to unknown environmental signals, the hybrid kinase RcsC is activated to autophosphorylate a conserved His residue. From here, the phosphate is transferred first to an Asp residue within RcsC itself, then to a His residue in RcsD, and finally to the response regulator RcsB. RcsB~P (the phosphorylated or functionally active form of RcsB) associates with an unstable protein, RcsA, to bind to an RcsAB box in the promoter region of several genes (70), although not all genes require RcsA for regulation. Conditions that modestly induce the Rcs regulon include osmotic and other kinds of membrane stress, growth at low temperatures in the presence of glucose and zinc, and possibly growth on a solid surface (27, 39). Capsule synthesis also is reported to be activated by acetyl phosphate via RcsB independently of RcsC (20). Capsular polysaccharide helps cells survive drying (52) and apparently is important for later stages of biofilm formation (10).

While the Rcs system positively regulates capsule synthesis, it negatively regulates flagella synthesis (Fig. 1) (39). Swimming and swarming motility are both dependent on flagella (28, 36). In *E. coli* the Rcs system is thought to repress motility by controlling the master flagellar operon (18). Purified RcsA enhances the affinity of RcsB for the RcsAB box within the promoter region of *flhDC* (18); however, RcsA does not appear to affect the motility of *E. coli* in vivo unless expressed at high levels from a plasmid (20). Flagellar biogenesis in both *E. coli* and *S. enterica* involves the expression of more than 50 genes that are transcribed in operons of three temporal classes (8). The class 1 genes are included in the master operon *flhDC*, the proteins of which direct transcription of class 2 promoters, which encode structural and assembly proteins required for the biosynthesis of the base of the flagellar structure. FliA (σ^{28}) is made at this stage and regulates class 3 transcription, which produces the functional flagellar filament. Early in assembly, after the inner membrane/supramembrane (MS) ring and the cytoplasmic C ring are assembled, a flagellum-specific type 3 secretion (TTS) apparatus docks at the base of this structure and directs the export of most extracytoplasmic flagellar subunits comprising the rod, the external hook, and the filament. The flagellar TTS system shares sequence and structural similarities with secretion systems that export virulence proteins into host cells (38).

Mutations that activate the Rcs phosphorelay system are reported to attenuate virulence in *S. enterica*; the activating mutations were either in RcsC (25, 48) or in IgaA (14). A point mutation in *igaA* (*i*ntracellular *g*rowth *a*ttenuator) was first identified in a screen for *S. enterica* mutants able to proliferate in fibroblast cells (5); the mutant was attenuated for growth in macrophages. *igaA* encodes an inner membrane protein (14)

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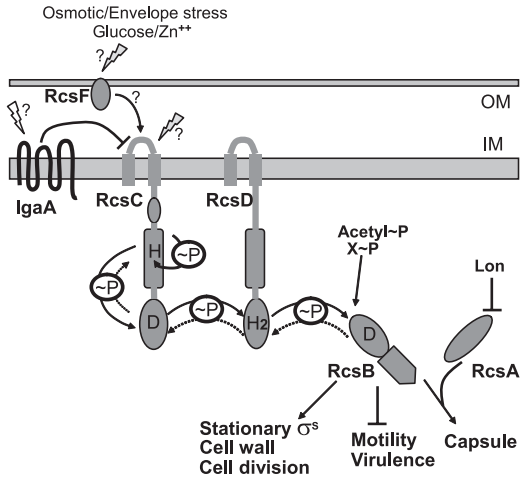

FIG. 1. Rcs signal transduction pathway. Phosphate (~P) transfer from the RcsC kinase via RcsD to the response regulator RcsB is indicated. H/H_2 and D refer to phosphorylated histidine and aspartate residues in the signaling proteins. Dotted lines indicate a reversal of the pathway due to the phosphatase activity of RcsCD. Acetyl~P or unknown phosphodonors (X~P) are implicated in phosphorylating RcsB independently of RcsC. Pathways positively (arrowhead) or negatively (bar head) influenced by RcsB~P alone, or in conjunction with the coregulator RcsA, are indicated. Lon protease degrades RcsA. RcsF and IgaA respond to the indicated known or unknown (?) signals to regulate RcsC. OM and IM, outer and inner membrane, respectively. See the text for details. Adapted from reference 39 with the permission of the publisher.

that is homologous to *yrfF* in *E. coli* and to *umoB* in *Proteus mirabilis* (15). The *igaA* mutant in *Salmonella* was mucoid and showed reduced motility, and the locus was shown to be essential (4). Lethality was suppressed by mutations in *rcsB*, *rcsC*, or *rcsD*, suggesting that IgaA represses the Rcs signaling system in both *E. coli* and *S. enterica* (Fig. 1) (4, 9, 14). In the absence of IgaA, an overactive Rcs signaling pathway is lethal, likely because of the inappropriate regulation of an Rcs regulon member(s). The role of IgaA in virulence apparently is to prevent the activation of the RcsCDB system during early stages of infection. We show in this study, however, that the Rcs system is required for the activation of some virulence genes required for later stages of infection.

Our study began with the isolation of a spontaneous mutant of *S. enterica* that was simultaneously mucoid and completely defective in both swimming and swarming motility. We mapped the mutation to *igaA*. A literature search revealed that the particular missense mutation we had isolated (T191P) had been reported earlier, and it was the strongest of the five mutations assayed for the induction of colanic acid synthesis (14). An initial microarray analysis of the *igaA* mutant revealed a 20- to 400-fold induction of genes for colanic acid synthesis and a similarly impressive repression of flagellar and SPI-1/SPI-2 (SPI is short for "*Salmonella* pathogenicity island") virulence genes. We therefore exploited this mutant, which apparently is nearly maximally activated for the Rcs signaling pathway, to identify a vast set of genes (~20% of the *Salmonella* genome) positively and negatively regulated by RcsB in both mid-log and stationary phases of growth. We also have identified RcsA-dependent and -independent targets of RcsB. We provide most of these microarray data as supplementary material, but we present the novel results showing dual regulation of SPI-2 and other virulence genes by the Rcs system. The experimental focus of this paper is on the regulation of flagellar genes and motility.

We report that RcsB regulates flagellar gene expression both negatively and positively. We show that the negative regulation occurs solely at the initiation of transcription of the master operon and operates through an RcsB box within the *flhDC* promoter; RcsA appears to have no effect on *flhDC* transcription. Positive regulation involves the three TTS genes *fliPQR*; RcsA does not promote this regulation but instead antagonizes it. An RcsAB box located downstream of *fliR* appears to be required for the elevated transcript levels of *fliPQR*. We also show that excess colanic acid inhibits swarming motility. Thus, while polysaccharides in general favor surface motility (28), the colanic acid polysaccharide does not.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and procedures. Strains are listed in Table 1. Bacteria were grown in L broth (LB) base (20 g/liter). LB swim plates were solidified with 0.3% agar, while LB swarm plates were supplemented with 0.5% glucose and were solidified with 0.6% agar (65). Eiken agar (Eiken Chemical Co., Tokyo, Japan) was used in most experiments. For preparation of RNA samples, cultures were harvested at an optical density at 600 nm (OD_{600}) of 0.6 (~2.5 h) for mid-log phase and at 6 h for stationary phase. The growth curve for wild-type bacteria has been published already (65). Strains with the *igaA* mutant allele had a slightly slower growth rate than the wild type and reached mid-log phase (OD_{600} of 0.6) with a delay of ~0.5 h; they were harvested for stationary phase by an equivalent delay. Procedures for motility assays, viewing bacteria on plates, and photography have been described previously (42). Antibiotics used in this study are ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), tetracycline (12.5 μg/ml), and kanamycin (25 μg/ml). Deletion of genes and regulatory regions was achieved by the one-step mutagenesis procedure (11) as described previously (68). Mutant combinations were constructed by P22 transduction where appropriate.

Mapping and identification of the *igaA* mutation. P22 was grown on TH341 carrying Tn*10*dCm (defective Tn*10*, lacking the transposase, and carrying chloramphenicol resistance [Cm^r]) and used to generate a random pool of Tn*10*dCm hops into the spontaneous mucoid mutant, which was transformed with pNK972, a plasmid expressing the transposase gene of Tn*10*. Transducing lysates prepared from ~25,000 Cm^r pooled colonies were crossed with the wild-type strain 14028, selecting for mucoid and nonmotile colonies resistant to Cm. One such transductant was named QW119, and it was crossed back with 14028 to determine the distance (~14 kb) between the Cm^r marker and the mutated locus using cotransduction frequencies. The Cm^r marker was located at a noncoding region between genes *bigA* and *yhfL* by using semirandom-primed PCR from both ends of the Cm coding sequence and subsequent sequencing of the PCR products. TH1793 carrying a tetracycline resistance (Tc^r) insertion in *dam*, 3.6 kb down from *bigA*, then was crossed with QW119, and the distance between *dam*::Tc^r and the mutated locus was determined to be ~11 kb by cotransduction frequencies, establishing the order of the markers as Cm^r–*dam*::Tc^r–mucoid locus. DNA sequencing around the predicted position of the mutation revealed a point mutation of A to C at nucleotide 571 in *igaA* (Table 1).

Introducing point mutations on the chromosome. Point mutations within the chromosomal RcsAB box sequence in *flhDC* were created as follows. The *flhDC* coding sequence first was replaced with *tetRA* (encoding tetracycline resistance genes [7]) by a modification of the one-step mutagenesis procedure (11). The modification involved using genomic DNA from TH338 (carrying *tetRA*) as the template to amplify the *tetRA* sequence using hybrid primers that included 40 to 50 nucleotides homologous to sequences flanking either side of the region to be deleted within *flhDC*, followed by 20 nucleotides homologous to sequences on either side of the *tetRA* cassette. The amplified PCR product was introduced into the wild-type strain as described previously (11), and the resulting strain, *flhDC*::*tetRA*, was named QW215. The region replaced by *tetRA* in *flhDC* started at the +1 transcription start site of *flhD* and extended to the termination codon TAA of *flhC*. Point mutations in the RcsAB box (Table 1) were introduced into QW215 by designing appropriate PCR primers carrying the mutations. The basic forward primer containing the RcsAB box was 5′CTCCGTTGTATGTCACGA

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description[a]	Mutation site[b]	Source or reference
Strains			
14028	Wild-type ATCC strain of *S. enterica* serovar Typhimurium		42
QW184	p_{wza}*wza*::*kan*	−478~1140 (1140)	This study
QW119	*igaA**	571 A'C	This study
QW200	*igaA** p_{wza}*wza*::*kan*		This study
QW220	*igaA** $p_{flhD(g8t)}$	−196 G'T	This study
QW221	*igaA** $p_{flhD(a9c)}$	−195 A'C	This study
QW222	*igaA** $p_{flhD(g8t,\ a9c)}$	−196 G'T, −195 A'C	This study
QW226	*igaA** $p_{flhD(g8t,\ a9c,\ c15a)}$	−196 G'T, −195 A'C, −189 C'A	This study
QW227	*igaA** $p_{flhD(c15a)}$	−189 C'A	This study
QW228	*igaA** $p_{flhD(g8t)}$ p_{wza}*wza*::*kan*		This study
QW229	*igaA** $p_{flhD(a9c)}$ p_{wza}*wza*::*kan*		This study
QW230	*igaA** $p_{flhD(g8t,\ a9c)}$ p_{wza}*wza*::*kan*		This study
QW231	*igaA** $p_{flhD(g8t,\ a9c,\ c15a)}$ p_{wza}*wza*::*kan*		This study
QW233	*igaA** $p_{flhD(c15a)}$ p_{wza}*wza*::*kan*		This study
QW327	Δ*rcsA*	99~539 (624)	This study
QW434	Δ*rcsB*	33~715 (651)	This study
QW398	Δ*rcsC*	1~2847 (2847)	This study
QW459	Δ*rcsD*	7~2322 (2670)	This study
QW329	Δ*rcsAB*		This study
QW436	Δ*rcsCD*		This sutdy
QW441	*igaA** Δ*rcsA*		This study
QW442	*igaA** Δ*rcsB*		This study
QW556	*igaA** Δ*RA*(*1-48*)	See Fig. 3	This study
TH341	F::Tn*10*dCm		K. Hughes
TH338	F::Tn*10*dTc		K. Hughes
TH1793	*dam*::Tn*10*dTc		K. Hughes
QW215	*flhDC*::*tetRA*	−243 of *flhD*(351)~579 of *flhC*(579)	This study
Phage/plasmids			
P22	HT12/4*int*103		42
pKD4	Source for kanamycin cassette		11
pKD46	Lambda red recombinase		11
pCP20	FLP recombinase		11
pNK972	pBR322 origin, Tn*10* transposase		K. Hughes

[a] Mutations indicated in parenthesis after p_{flhD} refer to base changes within the RcsAB box in the promoter region of *flhD*. Δ and :: refer to deletion of or insertion/substitution within the indicated gene or F episome.

[b] A minus refers to the nucleotides upstream of ATG, a tilde indicates the nucleotides included in the deletion (when the description indicates a deletion) or deletion/substitution (when the description indicates ::*kan* or ::*tetRA*), an apostrophe indicates mutation (i.e., 571 A'C means a mutation from A to C at nucleotide 571), and the number in parentheses is the total length of the gene from ATG to TAA. Positive values indicate residues after the start codon. For single-base-pair changes, the nucleotide at which the indicated change was introduced is shown. Note that deletions created by the Datsenko and Wanner method (11) leave behind a scar sequence of ~80 bp.

AGCTGACGAGTAGAGTTGCGTCGAATTAGGAAAAATCTTAGGCATT TGTAAAAATTGATGTA (the box is underlined). In mutant primers, the G, A, and C residues shown in boldface in the wild-type sequence were changed to T, C, and A, respectively, either singly or in combinations. Each of the forward primers, together with the reverse primer (5′GACATCATCCTTCCGCTGTT), was used to generate PCR products using wild-type *flhDC* as the template. The PCR products were electroporated into QW215 with pKD46 (a helper plasmid encoding phage lambda recombinase [11] to replace the *tetRA* cassette). Tet^s transformants were selected on media described previously (41) on which Tet^s colonies appear much bigger than Tet^r colonies; these were further verified by replica plating on tetracycline and unmodified LB plates. All point mutations finally were verified by DNA sequencing. The Cm^r-linked *igaA* allele was moved into the RcsAB box mutants by transduction and screening for mucoidy, and the construct was verified by DNA sequencing.

Microarray experiments and reverse transcription-PCR (RT-PCR). RNA preparation and microarray analyses were done as described previously (65). The microarray data in all tables represent two to five independent experimental repeats. A list of differentially expressed (DE) genes for each comparison (e.g., wild type compared to Δ*rcsB* at one growth phase) was selected by using the significance analysis of microarrays method, which assigns a score to each gene on the basis of the change in gene expression relative to the standard deviation of repeated measurements (63). Briefly, significance analysis of microarrays orders the genes by using a modified statistic *t* and declares a gene to be up- or down-regulated if the observed modified statistic *t* is above or below the global cutoff point, which is chosen to control the estimated median false discovery rates. The false discovery rate was set to 5%. Each DE list was further screened stringently according to the behavioral consistency of each DE gene in two genetic backgrounds ($RcsB^{++}$ [high levels of active RcsB] and Δ*rcsB*) and in two growth phases (mid-log phase and stationary phase).

RT-PCR was performed essentially as described previously (66), using a one-step RT-PCR kit (Qiagen). Briefly, DNase (Ambion)-treated RNA samples initially were diluted to 20 ng/μl and were further normalized against RT-PCR-amplified 30S rRNA as an internal control. An RT-PCR of 50 μl consisted of 10 μl buffer (5×), 1 μl of deoxynucleoside triphosphates (10 mM), 2 μl each of forward and reverse primers (25 μM), 1 μl of RT-PCR enzyme mix, and ~40 ng of RNA and RNase-free water supplemented to 50 μl. RT-PCR cycling conditions were as follows: 50°C for 35 min and 95°C for 15 min, followed by 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, and then an extra step of elongation at 72°C for 10 min.

RESULTS

IgaA(T191P) is strongly induced for colanic acid synthesis and simultaneously is repressed for flagellar, fimbriae, and SPI-1/SPI-2 virulence genes. A spontaneous mucoid mutant of *S. enterica* (QW119) (Table 1) was found to be completely

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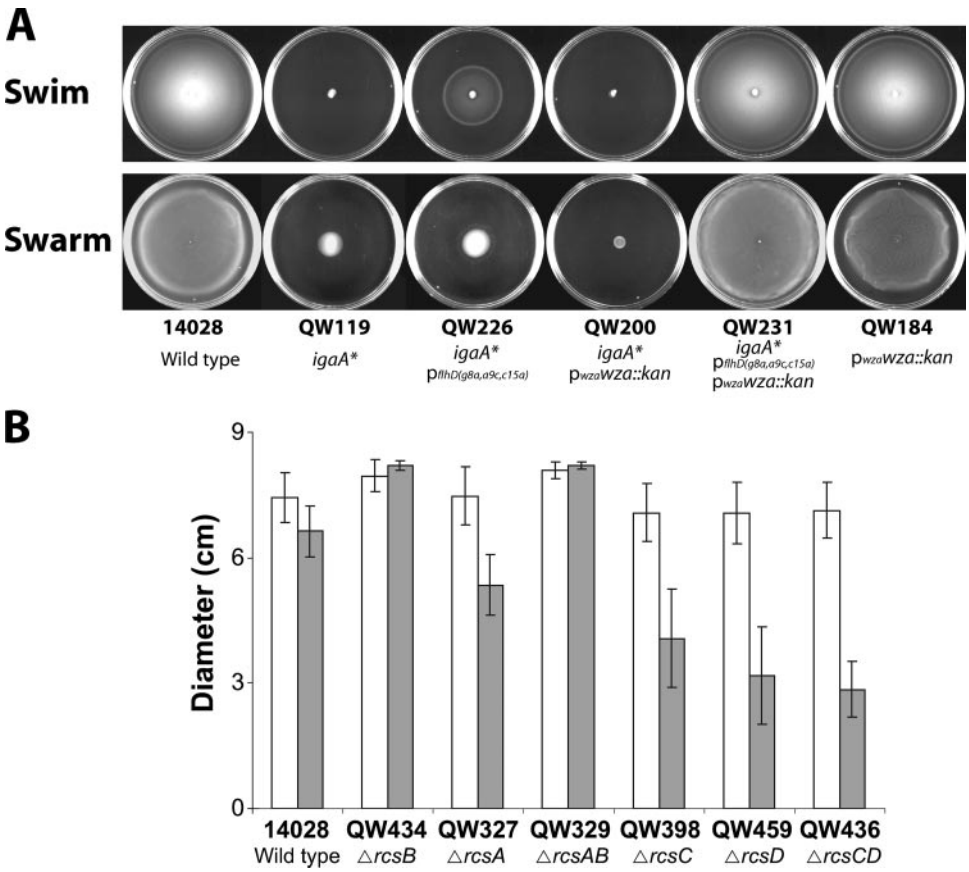

FIG. 2. Swimming and swarming motility of the Rcs signaling pathway and regulatory mutants. (A) The motility of the indicated mutant strains on 0.3% swim or 0.6% swarm Eiken agar. Mutations introduced in the promoter region of *flhD* are indicated in parenthesis after p_{flhD}. (B) The motility of the indicated mutant strains on 0.3% swim (white bars) or 0.6% swarm (gray bars) Eiken agar. Similar results were obtained with Bacto agar. The data are averages from three independent experiments conducted in triplicate. Error bars are standard deviations from the means. Plates in both panels were incubated at 37°C for 6 h.

defective in swimming and swarming motility (Fig. 2A). The mutation was mapped to the *igaA* locus by three-factor crosses, and sequence analysis identified the mutation as IgaA(T191P) (see Materials and Methods). We will refer to this allele as *igaA**. Consistent with the mutant phenotype, microarray analysis of this mutant showed a dramatic induction of colanic acid synthesis genes (20- to 400-fold) that are a major target of the Rcs signaling system (see Table S1 in the supplemental material). These data, along with experiments reported previously (14), suggest that the Rcs system is nearly maximally induced in the *igaA** strain QW119 (indicated by $RcsB^{++}$ in Table S1 in the supplemental material). Comparison of the $RcsB^{++}$ to Δ*rcsB* strains therefore should give us the whole range of genes positively and negatively regulated by RcsB. These microarray experiments showed that RcsB regulates nearly one-fifth of the *Salmonella* genome. Data for genes that are strongly regulated in both mid-exponential and stationary phases of growth are provided in Tables S1 and S2 in the supplemental material. A list of 445 other genes regulated in either one or the other growth phase (but not in both phases) is available from the authors upon request. We also have examined the contribution of RcsA to this regulation (see Tables S3 and S4 in the supplemental material). We first summarize the overall findings from these studies and then focus on the regulation of flagellar genes and motility.

Positive regulation of colanic acid and a subset of flagellar biosynthesis genes. Positively regulated genes are those for which the transcription levels are elevated in an $RcsB^{++}$ background or decreased in a Δ*rcsB* background. The most strongly positively regulated genes belong to the colanic acid biosynthesis cluster (see Table S1 in the supplemental material), followed by those associated with membrane and periplasmic functions. Among genes with known functions, inclusion of the class 2 flagellar genes *fliQ* and *fliR* (see Table S1 in the supplemental material) in the set of strongly induced genes is curious, because the rest of the flagellar genes are repressed by RcsB (see Table S2 in the supplemental material); *fliQR* are members of an export system specific for extracytoplasmic flagellar proteins (37).

Negative regulation of flagellar and virulence genes. Negatively regulated genes are those for which the transcription levels are decreased in an $RcsB^{++}$ background or increased in a Δ*rcsB* background. Among the most strongly negatively regulated genes are those encoding flagellar motility (up to 117-fold repression) (see Table S2 in the supplemental material). Besides the well-known flagellar biosynthetic pathway, the

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TABLE 2. Dual regulation of virulence and other genes by the Rcs system[a]

Gene/open reading frame and functional category[b]	WT/Δ*rcsB*[c]	WT/RcsB^{++}[d]	Function/description[e]
Pathogenicity (47)			
mig-14	4.0	2.4	Put. transcription activator
pagC	13	35	PhoP regulated; reduced macrophage survival
pagD	13	9.5	PhoP regulated
pagK	3.6	5.5	PhoPQ-activated gene
pgtE	3.4	2.9	Phosphoglycerate transport; outer membrane protein E
pipB	7.5	59	Pathogenicity island-encoded protein; SPI-3
sifA	16	38	Replication in macrophages
sifB	8.8	26	Translocated effector; translocated by SPI-2
spvA	2.0	4.4	*Salmonella* plasmid virulence; outer membrane protein
spvC	1.9	12	*Salmonella* plasmid virulence; hydrophilic protein
spvD	1.9	5.5	*Salmonella* plasmid virulence; hydrophilic protein
ssaB, *ssaC*, *ssaD*, *ssaE* (*ssaB*)	7.3–26	8.0–89	Secretion system apparatus
ssaG~*L*, STM1410 (*ssaG*)	12–30	17–150	Secretion system apparatus
ssaM~*Q*, *ssaV* (*ssaM*)	4.0–34	4.4–33	Secretion system apparatus
sscB, *sseF*, *sseG*	3.4–13	4.9–35	Secretion system chaperone
sseA~*E*, *sscA* (*sseA*)	13–19	42–58	Secretion system effector
sseJ	42	48	*Salmonella* translocated effector; regulated by SPI-2
ssrA, *ssrB* (*ssrA*)	3.6–5.4	9.7–11	Secretion system regulator; sensor component
STM0972	11	22	Homologous to secreted protein SopD
ugtL (*ugtL*)	13	16	Put. membrane protein; homology with chitinase
virK	7.3	6.4	Virulence gene; homologous to *virK* in *Shigella*
yscR, *ssaS*, *ssaT*, *ssaU* (*yscR*)	2.8–16	3.6–56	Homology with YscR of *Yersinia* secretion system
Fimbriae (8)			
fimA	74.3	37.2	Major type 1 subunit fimbrin (pilin)
fimI, *fimC*, *fimD*, *fimF* (*fimI*)	1.8–14	2.1–15	Fimbrial protein internal segment
fimW	9.8	8.5	Put. fimbrial protein
fimZ	15	15	Fimbrial protein Z; Put. transcriptional regulator
stdA	12	14	Put. fimbria-like protein
Membrane and periplasm (11)			
cvpA, *purF* (*cvpA*)	2.3–2.4	3.4	Membrane protein required for colicin V production
STM1689 (STM1689)	23	78	Put. inner membrane protein
STM2139	8.3	18	Put. inner membrane protein
STM2245	8.0	10	Put. outer membrane protein
STM4257~4262 (STM4257)	2.6–8.1	20–40	Put. inner membrane or exported
Others (14)			
carA, *carB* (*carA*)	3.0–3.4	8.2–13	Carbamoyl-phosphate synthetase
ilvB, *ilvN* (*ilvB*)	2.7	2.4–3.0	Acetolactate synthase I; large subunit, valine sensitive
STM1055 (STM1055)	2.4	2.3	Gifsy-2 prophage
STM1548	2.7	4.9	*S*-adenosylmethionine/tRNA-ribosyltransferase-isomerase
STM2137	4.1	4.7	Put. cytoplasmic protein
STM2138	9.5	28	Put. cytoplasmic protein
STM2287	17	38	Put. cytoplasmic protein
STM2585	4.3	7.3	Gifsy-1 prophage; similar to transposase
STM2778	7.6	25	Pseudogene; frameshift
STM2780	9.8	33	Homologue of *pipB*; Put. pentapeptide repeats
STM3133, STM3132 (STM3133)	2.1–3.6	3.7	Put. amidohydrolase

[a] The microarray data are for RNA prepared from cells harvested at stationary phase. Indicated transcription ratios are averages from two to five independent experiments. These ratios are expressed such that the numbers are >1 for both positively and negatively regulated genes, because it is easier to compare fold regulation using whole numbers rather than fractions. The range indicates low and high values for individual genes within the operon.

[b] Genes and open reading frames from the same operon are listed together; the first gene of the operon is indicated in parentheses. ~ indicates intervening genes in the operon. Operon prediction was based on data from reference 47 and from http://biocyc.org/STYP99287/NEW-IMAGE?object=Transcription-Units. Only genes significantly differentially expressed in an operon are listed. The genes are grouped under broad functional categories, and the total number in each category is indicated in parentheses.

[c] Δ*rcsB* refers to strain QW434, which contains a deletion of *rcsB*. Genes in this column are positively regulated by RcsB.

[d] RcsB^{++} refers to the *igaA* mutant strain QW119 with elevated RcsB levels and activity. Genes in this column are negatively regulated by RcsB.

[e] Functions and descriptions are based on data from reference 45; when several genes in an operon are listed, only the first one is described. Put., putative.

more recently identified motility genes (*mcpC*, STM3155, *yhjH*, etc. [21, 67]) also were repressed by RcsB. Equally striking was the repression of virulence genes encoded on the pathogenicity island SPI-1 (up to 270-fold) (see Table S2 in the supplemental material) as well as virulence genes encoded on the pathogenicity island SPI-2 (up to 150-fold) and genes for fimbriae (up to 37-fold) (Table 2). The latter class of genes (SPI-2 and *fim*) showed a novel regulation that is discussed separately below.

Dual regulation of SPI-2 and other virulence genes. The microarray data revealed a third class of genes that were both

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positively and negatively regulated depending of the activity level of RcsB (Table 2). The transcription ratios at normal levels of RcsB (wild type compared to Δ*rcsB*) show that RcsB is required for expression of the indicated genes, i.e., the genes are positively regulated by RcsB. The transcription ratios at elevated levels of RcsB (wild type compared to RcsB^{++}) show that the same genes are repressed when RcsB is highly active, i.e., negatively regulated by RcsB. The largest functionally related group of genes showing dual regulation included genes encoding the TTS system on the pathogenicity island SPI-2 (*ssa*, *ssb*, *sse*, etc.). This system is required for the intracellular survival of *Salmonella* and the remodeling of the *Salmonella*-containing vacuole (69). Genes for the SsrAB two-component signaling system encoded within SPI-2 also show dual regulation. This system controls the expression of components of the SPI-2 TTS apparatus as well as its translocated effectors. Other genes grouped under pathogenicity also are required for growth of *Salmonella* in macrophages. For example, the genes *pagC*, *pagD*, *ugtL*, *mig-14*, *virK*, *pgtE*, *pipB*, *sopD*, and *pagK* have functions associated with the bacterial envelope, and some have been directly implicated in virulence and resistance to antimicrobial peptides (50). Fimbrial genes (*fim*) belonged to the dual category as well; fimbriae are thought to play a role only during the intestinal phase of infection (32). RcsB recently has been reported to influence the piliation state of *E. coli* (57).

RcsA both stimulates and antagonizes a subset of RcsB-regulated genes. Earlier array studies do not distinguish between RcsA-dependent and RcsA-independent targets. In order to study these, we first compared gene expression of the wild type to that of an *rcsA* mutant but found no genes that were significantly differentially expressed. We therefore performed microarray analysis of the wild-type and RcsB^{++} strains with or without RcsA. RcsA had either a stimulatory or an antagonistic effect on the positive regulation of several genes by RcsB (some of these are indicated in Tables S1 and S2 in the supplemental material). The effect of RcsA on the negative regulation of genes by RcsB was less clear in that the effect either was small, not observed in all members of a regulon, or not consistently observed in all experimental repeats. The data showing all the positively regulated genes for which expression either was stimulated or antagonized by RcsA are presented in Tables S3 and S4 in the supplemental material, respectively.

RcsA was not required for the negative or the positive regulation of flagellar genes. However, an antagonistic effect was seen on the positive regulation of *fliPQR*; i.e., the absence of RcsA increased the positive stimulation by RcsB (see Tables S1 and S4 in the supplemental material). An antagonistic effect also was observed on the positive regulation of *rcsA*, the gene immediately downstream of *fliR* (see Tables S1 and S4 in the supplemental material). Note that the Δ*rcsA* strain QW327 used in these experiments had removed most of the gene, leaving ~100 bp at the beginning and at the end (Table 1); the *rcsA* transcript corresponding to residual regions of the gene is being detected in the microarrays. An antagonistic effect may result from competition between the two proteins for binding the RcsAB box and could be interpreted as a moderating influence of RcsA on the positive regulation of genes by RcsB.

TABLE 3. Effect of RcsAB box mutations on recovery of motility[a]

Strain	Genotype	Colony diameter (cm)	
		Swarm	Swim
14028	Wild type	8.3	7.8
QW184	p_{wza}*wza*::*kan*	8.2	7.8
QW119	*igaA**	—	—
QW200	*igaA** p_{wza}*wza*::*kan*	—	—
QW220	*igaA** $p_{flhD(g8t)}$	—	3.4
QW221	*igaA** $p_{flhD(a9c)}$	—	4.7
QW222	*igaA** $p_{flhD(g8t, a9c)}$	—	4.8
QW226	*igaA** $p_{flhD(g8t, a9c, c15a)}$	—	4.6
QW227	*igaA** $p_{flhD(c15a)}$	—	3.7
QW228	*igaA** $p_{flhD(g8t)}$ p_{wza}*wza*::*kan*	4.3	5.1
QW229	*igaA** $p_{flhD(a9c)}$ p_{wza}*wza*::*kan*	8.3	8.3
QW230	*igaA** $p_{flhD(g8t, a9c)}$ p_{wza}*wza*::*kan*	8.3	8.3
QW231	*igaA** $p_{flhD(g8t, a9c, c15a)}$ p_{wza}*wza*::*kan*	8.3	8.0
QW233	*igaA** $p_{flhD(c15a)}$ p_{wza}*wza*::*kan*	—	3.0

[a] Effect of RcsAB box mutations on the recovery of motility in *igaA** strains. Mutations within the RcsAB box TA**GG**AAAAAT**C**TTA found in the promoter region of *flhD* are indicated in parenthesis after p_{flhD}. Residue numbering is from the +1 transcription start site. Boldface residues represent conserved residues among four such boxes identified in *ftsA*, *osmC*, and two *flhD* variants in *E. coli* (18). The numbers in the Swarm and Swim columns refer to the diameter of the colonies, while the dash refers to no migration (swim) or movement (swarm) outwards from the point of inoculation.

Regulation of flagellar genes and motility. (i) Negative regulation of flagellar genes occurs through the RcsB-binding box in the master flagellar operon *flhDC*: excess colanic acid inhibits swarming motility. An RcsAB box was identified within a footprint of RcsAB proteins in the promoter region of *flhDC* in *E. coli* (18). An identical sequence (TAG**GA**AA/AAT**C**TTA) is present at a similar location in *S. enterica*, at +5 to +19 from the +1 transcription start site (71). We shall refer to this sequence as the RcsB box in *S. enterica*, since RcsA is not required for repression. To test if the inhibition of motility genes was entirely through repression of the *flhDC* master operon, we created point mutations in the GA (left half site) and C (right half site) residues of the RcsB box (in boldface), alone and in combinations, in the *igaA** strain QW119 (see Materials and Methods). These three residues were seen to be conserved between several RcsA-independent targets and an RcsA-dependent target (18). All of these mutant derivatives remained mucoid. While most of the mutant strains had regained swimming motility to various extents when assayed on 0.3% agar plates, none of them showed swarming motility on 0.6% agar plates (Table 3). Motility assays for representative mutants are shown in Fig. 2A. Of the five combinations of point mutants, the single mutants g8t (QW220) and c15a (QW227) showed the lowest recovery of motility, while all mutants that included the a9c mutation (QW221, QW222, and QW226) had recovered half the swimming motility of the wild type (Table 3 and Fig. 2A, compare QW119 to QW226). In order to test if mucoidy was interfering with motility, we introduced a deletion mutation of *wza*, the first gene in the colanic acid biosynthesis operon, into the *igaA** strain (resulting in strain QW200) as well as in all of its RcsB box mutant derivatives (resulting in strains QW228 to QW233). Mutation of *wza* abolished the mucoid phenotype of the mutants but did not reverse the motility defect in the *igaA** parent (Fig. 2A, compare QW200 to QW119). However, both swarming and

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swimming motility now could be observed in the RcsB box mutants (Table 3). The results confirmed that the a9 residue was critical, the g8 residue less critical, and the c15 residue not critical for RscB-mediated repression of motility (only QW231 is shown in Fig. 2A). A control strain with the *wza* deletion alone (QW184) swarmed as well as the wild type, showing that colanic acid was not required for swarming (also see reference 62). Gene expression was assayed only in the mutant with all three RcsB box residues altered (QW226); repression of motility operons was completely relieved in this strain, while the expression pattern of all other genes remained unchanged (some of the microarray data for this strain were reported previously [67]; the rest of the data are not shown). The motility defect of QW226, despite the relief of *flhDC* repression, demonstrates that the excess colanic acid still being produced by this strain impairs swimming and inhibits swarming (Fig. 2A, compare QW226 to QW231).

In an earlier study, we had reported that a Tn*10* insertion in *rcsD* (formerly *yojN*) in *S. enterica* serovar Typhimurium inhibited swarming on medium solidified with one commercial source of agar (Bacto) but not another (Eiken) (62). In light of the reported stimulation of swarming motility by insertion mutations in *rcsD* in both *E. coli* (60) and *P. mirabilis* (2), we decided to reexamine our results by creating specific deletions in each of the *rcs* genes by the method of Datsenko and Wanner (11) and by assaying the motility phenotypes of the resulting mutants (Fig. 2B). The Δ*rcsB* mutant (QW434) showed better swimming and swarming motility than the wild type, consistent with the negative effect of RcsB on the expression of *flhDC*. Δ*rcsA* (QW327) had no effect on swimming, also as expected; however, swarming motility was inhibited ~25%. A Δ*rscAB* double mutant (QW329), however, showed the same phenotype as the *rscB* mutant. Δ*rcsC* (QW398) and Δ*rcsD* (QW459) mutants showed a small inhibition of swimming but ~40 to 60% inhibition of swarming. A Δ*rcsCD* double mutant (QW436) was similar to the single mutants for swimming, but it was slightly worse at swarming. We attribute the modest inhibition of swimming motility in the *rcsC* and *rcsD* mutants to the loss of phosphatase activity of RcsCD and a consequent accumulation of RcsB~P due to phosphorylation from other phosphor donors (20, 39) (Fig. 1) (see Discussion). The more pronounced effect of these mutants on swarming motility is due to the development dynamics of the swarmer colony, in which motility initiates only after a substantial lag. Both mutants initiated swarming later than the wild type, the delay being longer for *rcsD* than for *rcsC*. A small initial delay was observed in the *rcsA* mutant as well (see Discussion).

In summary, our mutation data show that only conserved residues in the left half of the RcsB box are important for the repression of *flhDC* in *S. enterica* and that RcsB-mediated inhibition of motility occurs entirely through the repression of the *flhDC* promoter. Colanic acid is not required for swarming, and excess colanic acid inhibits swarming motility completely. Deletion of *rcsB* has a positive effect on motility, but deletion of *rcsC* or *rcsD* had a slightly negative effect on swimming motility and a more pronounced negative effect on swarming motility.

(ii) Positive regulation of flagellar genes *fliPQR*. The *fliL* flagellar operon encodes seven genes, *fliL* through *fliR* (Fig. 3A) (36). *rcsA* is located 281 bp after *fliR* (45). An RcsAB box,

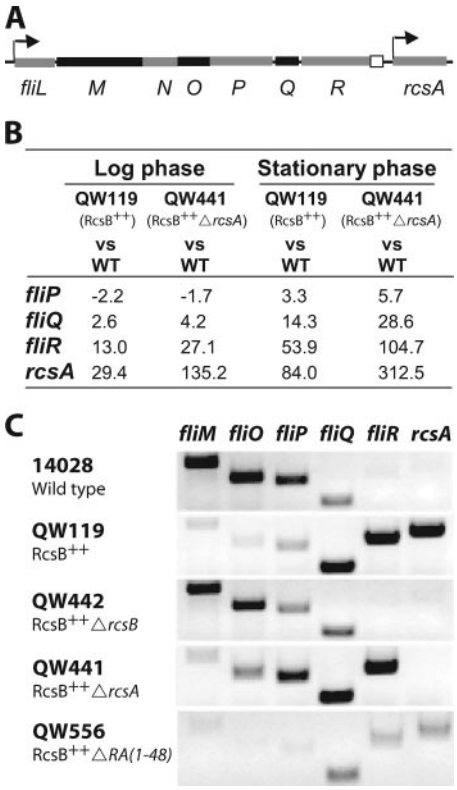

FIG. 3. Positive regulation of *fliPQR* by RcsB. (A) Schematic showing the *fliL* operon and *rcsA*. The lengths of the region corresponding to the individual genes within the operon are drawn approximately to scale with respect to each other. Arrows indicate the transcription start sites. The square box in the intergenic region between *fliR* and *rcsA* is the RcsAB box (not drawn to scale). (B) Microarray data showing positive or negative regulation of the indicated genes during mid-exponential and stationary phases of growth. QW119 is the *igaA** ($RcsB^{++}$) mutant, and QW441 its isogenic Δ*rcsA* derivative. The data are extracted from Table S4 in the supplemental material. (C) RT-PCR results for RNA isolated from strains indicated on the left. QW556 is deleted for residues 1 to 48 in the intergenic region between *fliR* and *rcsA*, starting at the first base pair after the stop codon of *fliR*. Primers pairs were designed to anneal within the coding region of the genes indicated at the top. RNA for the wild-type (WT) strain was isolated at mid-log phase, at which point flagellar gene expression is maximal, while that for the *igaA** strains was isolated at stationary phase, at which point the activity of the Rcs system is maximal.

homologous to the one identified in *E. coli*, occurs 18 bp downstream from the stop codon of *fliR*. The RcsA and RcsB proteins have been shown to bind to this box and to positively regulate *rcsA* expression in *E. coli* (70).

The maximum induction of *fliPQR* genes was seen at stationary phase in the $RcsB^{++}$ *igaA** strain QW119 (Fig. 3B) (note that *fliP* is not included in Table S1 in the supplemental material, because that table lists only genes upregulated in both exponential and stationary phases of growth). The stimulation of *fliQ* and *fliR* was strong (14- and 54-fold, respectively), and the stimulation of *fliP* was moderate in comparison (threefold). This stimulation was increased in the absence of *rcsA*, an effect also seen on expression of *rcsA* itself, as noted earlier (Fig. 3B; also see Table S4 in the supplemental material). Curiously, there appeared to be a reverse gradient of expression of these genes, in that the last gene in the operon

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was expressed at higher levels than the previous gene; thus, the order of expression levels was *fliR* > *fliQ* > *fliP* (Fig. 3B).

RT-PCR was used to confirm all three findings from microarray experiments; i.e., the elevated expression of only *fliPQR* in the RcsB^{++} mutant, the RcsB dependence of the response, and the increased stimulation of expression in the absence of RcsA. These results are presented in Fig. 3C, in which *fliM* is used as a representative early gene in the operon and *fliO* is used as the nearest neighbor of the positively regulated gene *fliP*. In the wild-type strain 14028, the gradient of gene expression follows proximal genes ≫ distal genes in the *fliL* operon; the *fliR* message is barely detected. In the RcsB^{++} strain QW119, however, the *fliM* transcript is barely detected, while expression of *fliQR* is elevated, along with some elevation of *fliP*. The increased expression of *rcsA* in the RcsB^{++} background (QW119) is consistent with the positive regulation of this gene by RcsB (see Table S1 in the supplemental material). In the absence of RcsB (QW442), transcription of *fliM* is restored due to the release of *flhDC* repression, and the positive regulation of both *fliR* and *rcsA* is abolished. In the absence of RcsA, however, the expression of *fliPQR* is enhanced further (Fig. 3B), and the reverse transcription gradient extends into *fliO* (Fig. 3C). Deletion of 48 bp containing the RcsAB box in the *fliR-rcsA* intergenic region decreases both the *fliPQR* and *rcsA* transcripts. Since RcsB binding to the RcsAB box is required for stimulation of *rcsA* transcription (70), the positive regulation of *fliPQR* likely also is promoted by RcsB binding here.

Given that the transcription of the *fliL* operon is repressed in the *igaA** background, it follows that *fliPQR* must be transcribed from an internal RcsB-dependent cryptic promoter in this operon or that a basal level of the transcript is being stabilized in the *igaA** background. The detailed nature of this positive regulation, which apparently does not affect motility as determined by similar levels of swimming and swarming observed for the wild type and *igaA** mutant QW231 (wherein *flhDC* repression is relieved and excess colanic acid synthesis is abrogated [Fig. 2A, compare 14028 to QW231]), will be explored in future studies.

DISCUSSION

In this study, we have exploited a spontaneously arising point mutant, IgaA(T191P) (or *igaA**), which is nearly maximally induced for the Rcs signaling system, to glean information about motility, virulence, and other genes regulated by this system in *S. enterica*. Microarray experiments revealed that the Rcs system regulates nearly one-fifth of the *S. enterica* genome under these conditions. Flagellar genes were the largest group of functionally related genes that were strongly negatively regulated, the other large group being the SPI-1 and SPI-2 virulence genes. Within the flagellar regulon, three flagellar genes, *fliPQR*, were selectively upregulated. A large set of SPI-2 and other virulence genes primarily involved in the growth of *Salmonella* in macrophages were activated or repressed, depending on the level of RcsB. We have followed up further only on the microarray results pertaining to motility.

The Rcs system and regulation of motility. (i) Repression of the flagellar regulon. A sequence identical to that of the RcsAB-binding box consensus derived from *E. coli* studies is present in *S. enterica* at an equivalent position within the *flhDC* promoter. We have called this the RcsB box in *S. enterica*, because microarray data did not show a requirement for RcsA in the repression of the flagellar genes by RcsB. These results are consistent with those of earlier studies on *S. enterica* that showed no effect of an *rcsA* mutation on the expression of *flhDC*::*lac* in an *igaA1* mutant background (4). They also are consistent with results of studies on *E. coli* that showed no effect of *rcsA* on motility when present as a single copy (20). We note that the RcsB box is part of a large region bound by the osmoregulator OmpR in *E. coli*; OmpR represses *flhDC* under conditions of high osmolarity (59). However, deletion of *ompR* does not affect motility under our experimental conditions (our unpublished data; also see reference 62).

Our mutational analysis showed that the conserved GA residues (+8 and +9) in the left half of the RcsB box were important for repression, while the C residue (+11) was not, as determined by assays for the restoration of motility (Table 3 and Fig. 2A). Microarray analysis of the triple-conserved-residue RcsB box mutant determined that transcription of the flagellar regulon was restored to wild-type levels (data not shown), showing that the repression of motility by RcsB is solely through *flhDC*.

(ii) Positive regulation of *fliPQR*. Microarray experiments showed that three class 2 flagellar genes, *fliPQR*, were positively regulated by RcsB (Fig. 3). These results were confirmed by RT-PCR experiments. A curious aspect of this regulation is what we have termed a reverse gradient of expression; i.e., expression levels in the order *fliR* > *fliQ* > *fliP*. Whether this regulation involves the initiation of transcription from an internal cryptic promoter and/or stabilization of a basal-level transcript remains to be determined. The *fliR-rcsA* intergenic region containing an RcsAB box appears to be important for the regulation (Fig. 3).

We had reported earlier that *fliQR* showed a different expression pattern from that of class 2 flagellar genes, in that unlike class 2 genes they continued to be expressed in stationary phase (65). Their expression during log phase also was not significantly affected in *flhDC* and *fliA* mutant backgrounds (our unpublished data). It appears, therefore, that these two genes can be regulated independently of flagellar operon control. The exact role of the *fliPQR* genes in flagellar export is not known, but along with *fliO* they are essential for flagellar biogenesis, and their gene products are found in the basal body (37). The existence of a natural FliR/FlhB fusion in *Clostridium* (51) suggests an association of FliR with the membrane protein FlhB, which plays a role in determining export substrate specificity (37). The positive regulation of these flagellar export-related genes by RcsB, for which other positively regulated functions are involved in responding to membrane stress, including capsule and other polysaccharide synthesis, suggests that perhaps FliPQR have a dual role in the cell. Thus, these proteins may participate in the export of flagellar components when conditions favor motility but export other molecules when conditions call for a sessile existence.

(iii) *rcs* genes and swarming. Deletion of *rcsB* had a positive effect on motility, consistent with a role of the Rcs system in the repression of motility (Fig. 2B). However, deletion of *rcsC* or *rcsD* had a slightly negative effect on swimming motility and a more pronounced negative effect on swarming motility. The

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motility phenotype of the *rcsC* mutant could be related to the dual function of RcsC as both a phosphatase and a kinase (39). The loss of phosphatase activity could result in the accumulation of RcsB~P due to cross-talk from other sources. Indeed, acetyl phosphate-mediated activation of RscB has been reported to delay transcription of the flagellar operon in an *rcsC* mutant in *E. coli* (20). A similar mechanism may be invoked to explain the phenotype of the *rcsD* mutant, since RcsD links RcsC and RcsB in the signaling pathway (Fig. 1). The more severe effect on swarming motility of both the *rcsC* and *rcsD* mutations is due to a longer lag in the initiation of swarming in these mutants. The *rcsA* mutation, which does not affect swimming, affects swarming at the initiation stage as well. We speculate that this may be due to an antagonistic effect of RcsA on the repression of *flhD*C, i.e., more inhibition in the absence of RcsA; we observed such an effect in one of three microarray experimental repeats. Swarming apparently is more sensitive to changes in the transcription profile of flagellar genes, likely because of other factors that influence the initiation of motility on the surface (28).

(iv) Colanic acid and swarming. Moist surface conditions are of paramount importance during swarming (28). Swarming bacteria often secrete special wetting agents and surfactants that aid in trapping sufficient moisture and decreasing surface tension to facilitate the spreading of the swarming colony. For example, *P. mirabilis* produces an acidic capsular polysaccharide (19, 54), *Serratia marcescens* and *Serratia liquefaciens* produce the surfactant serrawettin (35, 43, 44), *Pseudomonas aeruginosa* makes rhamnolipids (13), and *Bacillus subtilis* makes the surfactant surfactin (33, 46), all of which aid the advancement of the surface colony. In *E. coli* and *S. enterica*, the lipopolysaccharide is proposed to play this role (62). We therefore were curious to learn whether excess colanic acid would promote swarming. We have found that not only does excess colanic acid not promote swarming, but in fact it inhibits swarming while moderately inhibiting swimming motility. Consistent with our earlier findings that genes for colanic acid synthesis are not induced during swarming or growth on an agar surface (65), deletion of *wza* does not affect swarming (Fig. 2A). Thus, colanic acid is not required for swarming, is not induced during swarming, and does not promote swarming if overproduced. Inhibition of motility by excess colanic acid is consistent with the transcriptional shutdown of motility functions by the organism in an apparent preparation for a sessile existence. Our results are inconsistent with the finding that *cps* genes are induced upon growth on an agar surface in *E. coli* (17). It is possible that *E. coli* and *Salmonella* respond differently to the induction of these genes on surfaces.

The Rcs system and regulation of virulence genes. *S. enterica* encodes two virulence systems that operate at different steps of the infection cycle (31). The SPI-1 system is involved in the intestinal phase of infection and is essential for the invasion of nonphagocytic cells (22). The SPI-2 system is required for intracellular survival and proliferation and is essential for systemic infection (29, 58, 69). Consequently, the expression of these systems is differentially regulated: the expression of SPI-1 is stimulated by environmental cues (e.g., high osmolarity and low oxygen tension) present in the intestinal tract, while the expression of SPI-2 is stimulated by conditions present within host cells (e.g., low Mg^{2+} concentrations and acidic pH) (23). The results reported in this study suggest that the Rcs signaling system is an important player in fine-tuning the bacterial response to different environmental challenges. We discuss below only our new results with respect to SPI-2.

Dual regulation of SPI-2 genes. A majority of the genes showing dual regulation by RcsB in our studies, i.e., genes positively regulated at normal levels and negatively regulated at highly activated levels of RcsB, either belong to the SPI-2 group or are virulence determinants known to be involved in survival in macrophages, which are endowed with robust and varied antimicrobial defenses (55) (Table 2). The SPI-2-encoded TTS system and the two-component PhoPQ system are absolutely required to withstand these defenses (26, 34, 69). The SPI-2 effectors remodel the *Salmonella*-containing vacuole (SCV) by a variety of functions, which include an inhibition of various aspects of endocytic trafficking, an avoidance of NADPH oxidase-dependent killing, the induction of a delayed apoptosis-like host cell death, the control of SCV membrane dynamics, the assembly of a meshwork of F-actin around the SCV, an accumulation of cholesterol around the SCV, and interference with the localization of inducible nitric oxide synthase to the SCV (69). *ugtL*, *virK*, *mig-14*, and *pgtE* are PhoPQ-regulated genes that play a role in resistance to polymyxin B and/or cationic antimicrobial peptides (50). *pagC*, *pagD*, *pipB*, *sopD*, and *pagK* also are controlled by the PhoPQ signaling system (73). Positive regulation of all of these virulence genes by the Rcs system is consistent with the report that an *rcsC* mutant of *S. enterica* serovar Typhimurium was attenuated for systemic infection (12).

PhoPQ controls a large number of genes in *Salmonella* (47, 72, 73); why only a subset of these is affected by the Rcs system is not clear. We note that a connection between the Rcs and the PhoPQ systems has been seen in several studies (24, 27, 61). Both OmpR/EnvZ and PhoPQ have been implicated in the expression of SPI-2 genes and associated effectors by controlling the SsrAB system, which regulates transcription of the SPI-2 genes (3, 16). It appears from our studies that the Rcs system is the third signaling system that also regulates SsrAB (Table 2). Multiple regulation of the SPI-2 pathway may be designed to respond to different stimuli at different stages of infection (1). The *Salmonella ugd* gene, for example, which is required for the incorporation of 4-aminoarabinose in the lipopolysaccharide and resistance to the antibiotic polymyxin B, also is controlled by three two-component systems: PhoPQ, PmrAD, and RcsCDB systems (49, 61). The dual regulation of SPI-2 we have observed in an isolated system is only an indicator of the more complex regulation inside the bacterial host, where the Rcs system must activate SPI-2 gene expression in macrophages while repressing motility and SPI-1 virulence. The significance of the degree of RcsB activation for SPI-2 expression may be related to the different host signals encountered by the bacteria en route to their site of proliferation or within their specific niches. The IgaA protein must play an important role during these transitions.

How is the dual regulation of the virulence genes by RcsB achieved? Identification of promoters that are directly regulated by RcsB will allow a dissection of the molecular mechanism of this regulation. The 14-bp RcsAB box consensus (70) generally occurs in the promoter region but can be found as far upstream as 50 to 300 bp from the transcription start site (27).

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The consensus sequence therefore does not have enough sequence or position specificity to reliably predict which genes may be directly regulated. The identification of genes directly regulated by these regulators must await a more direct analysis, such as chromatin immunoprecipitation-on-chip.

Function of the Rcs regulon. The specific signals that activate the Rcs system are not known, although the general physiological conditions that trigger the system are accepted to be osmotic or other membrane stresses acting through RcsF and IgaA upstream of RcsC (6, 39, 40) (Fig. 1). *rcs* genes are not present in endosymbionts, which have only an intracellular existence. Consideration of the pathways that are the most strongly regulated suggests that the Rcs system initially evolved to protect the organism from osmotic changes in the environment as well as to support a sessile existence within biofilms. Colanic acid is reported to be required for maintaining a proper biofilm architecture (10, 53). Motility and pathogenicity (including SPI-1/SPI-2) are not required in such an environment, and the Rcs system is expected to be most activated in such a sessile state. A transition from a sessile to a motile state requires the system to be deactivated. Inside the host, motility and SPI-1 virulence are important for the intestinal phase of infection but then must be selectively turned off in the macrophages while still allowing SPI-2 gene expression. IgaA must be an important player in these transitions, which clearly are influenced by other signaling pathways as well.

S. enterica contains more than 30 two-component systems (64). Of these, the PhoPQ system has been reported to control as much as 2 to 3% of genes in *E. coli* and *S. enterica* (47, 72). This system often mediates its effects indirectly via activation of other regulatory systems, such as the PmrAB two-component system (1, 56). While we do not yet have information on which genes are directly regulated by RcsB, our analysis suggests that the RcsCDB signaling system interacts with the PhoPQ and SsrAB systems, shares signals and regulatory pathways with the EnvZ/OmpR system, and likely controls the largest genetic program identified to date.

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