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Role of RcsF in Signaling to the Rcs Phosphorelay Pathway in *Escherichia coli*†

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The *rcs* phosphorelay pathway components were originally identified as regulators of capsule synthesis. In addition to the transmembrane sensor kinase RcsC, the RcsA coregulator, and the response regulator RcsB, two new components have been characterized, RcsD and RcsF. RcsD, the product of the *yojN* gene, now renamed *rcsD*, acts as a phosphorelay between RcsC and RcsB. Transcription of genes for capsule synthesis (*cps*) requires both RcsA and RcsB; transcription of other promoters, including that for the small RNA RprA, requires only RcsB. RcsF was described as an alternative sensor kinase for RcsB. We have examined the role of RcsF in the activation of both the *rprA* and *cps* promoters. We find that a number of signals that lead to activation of the phosphorelay require both RcsF and RcsC; epistasis experiments place RcsF upstream of RcsC. The RcsF sequence is characteristic of lipoproteins, consistent with a role in sensing cell surface perturbation and transmitting this signal to RcsC. Activation of RcsF does not require increased transcription of the gene, suggesting that modification of the RcsF protein may act as an activating signal. Signals from RcsC require RcsD to activate RcsB. Sequencing of an *rcsC* allele, *rcsC137*, that leads to high-level constitutive expression of both *cps* and *rprA* suggests that the response regulator domain of RcsC plays a role in negatively regulating the kinase activity of RcsC. The phosphorelay and the variation in the activation mechanism (dependent upon or independent of RcsA) provide multiple steps for modulating the output from this system.

Expression of colanic acid, the major exopolysaccharide of *Escherichia coli* K-12, is a highly regulated process. Initial screens for activators of this pathway identified RcsC, RcsB, and RcsA as the major components of the pathway (17). RcsC and RcsB are members of a two-component signal transduction pathway; RcsC is the transmembrane sensor kinase, and RcsB is the cytoplasmic response regulator (38). However, unlike standard two-component systems, the Rcs pathway also involves an additional cytoplasmic coregulator, RcsA. The RcsA/RcsB heterodimer is necessary for activation of capsule gene expression (*cps*); under conditions in which either RcsA accumulates or RcsC is activated, colonies overproduce colanic acid, obvious as a mucoid phenotype on plates (23, 39). RcsA is itself subject to rapid degradation by the Lon protease (41).

Recent work has demonstrated that a large number of operons in addition to *cps* are also subject to regulation by the RcsC/RcsB system. RcsB/RcsA stimulates both *cps* and transcription of *rcsA* itself; it also inhibits the transcription of the *flhDC* flagellar master switch (10, 14, 43, 44). Furthermore, other promoters have been identified which are dependent upon RcsB but do not require RcsA. These include *ftsZ*, *osmC*, *osmB*, *bdm*, and the small RNA RprA (2, 8, 13, 16, 24). Array studies suggest that the set of genes regulated may be considerably larger (12, 20, 29).

Sequence comparisons of RcsC to other sensor kinases reveal that it is a transmembrane hybrid sensor kinase. At the N terminus are two transmembrane segments, separated by a 273-amino-acid periplasmic domain. After the second transmembrane domain comes an N-terminal kinase domain that includes a conserved histidine residue (His 479) that is the predicted phosphorylation site and is known to be essential for function (5). In addition to this domain, there is a C-terminal domain with homology to response regulators; this contains a conserved aspartate residue (Asp 875), also essential for function (5). RcsB is a traditional response regulator. The N-terminal response regulator domain contains a conserved aspartate that is likely to be the site of phosphorylation; the C-terminal domain contains a helix-turn-helix motif. The presence of the extra response regulator domain on RcsC posed a problem in understanding how this system works. Phosphotransfer in all other systems has been observed to proceed from His→Asp→His→Asp. In some cases, hybrid sensor kinases carry an additional histidine phosphotransmitter domain not present in RcsC (for instance, in ArcB); in other cases, a separate protein acts as the phosphotransmitter (21, 27). Takeda et al. suggested that there was a missing component in this Rcs system that acts as a phosphorelay between the response regulator domain of RcsC and RcsB (40). Their search led them to the discovery of RcsD (formerly YojN), a putative transmembrane protein with a histidine phosphotransmitter domain (HPt) similar to that found in ArcB (40). The *rcsD* gene is in an operon with and immediately upstream of *rcsB* and encodes an 890-amino-acid protein with a predicted molecular mass of 100 kDa (Fig. 1A). Takeda et al. established that RcsD is essential in the signaling from RcsC to RcsB and demonstrated phosphate transfer in vitro between RcsD and RcsB (40).

Another component of the *rcs* pathway is RcsF. It was ini-

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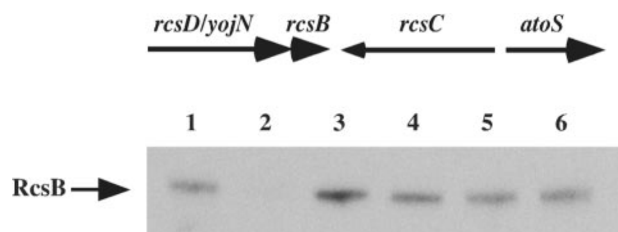

FIG. 1. Genetic organization of the *rcsB rcsD rcsC* region and test of polarity of *rcsD* mutations on *rcsB*. The top panel is a schematic representation of the *rcs* gene organization, with arrows indicating the direction of transcription. The bottom panel is a Western blot analysis with an anti-RcsB antibody showing steady-state amounts of RcsB at mid-exponential growth. Lane 1, MC4100; lane 2, DH311 (*rcsB*); lane 3, DJ480 (parental); lane 4, DH339 (*rcsD542*); lane 5, DH351 (*rcsD541*); lane 6, DH368 (*rcsD543*).

tially isolated as a multicopy suppressor of an *ftsZ* temperature-sensitive phenotype, leading to the demonstration that the *ftsA1* promoter is regulated by RcsB (15, 16). RcsF was recently independently identified as a multicopy activator of the promoter of the small regulatory RNA, RprA, and its RcsB-dependent promoter (24). RcsF is a small protein (14 kDa) believed to contain an N-terminal transmembrane domain and no homology to sequences in the database. Gervais and Drapeau originally proposed that RcsF represents an RcsC-independent alternative for phosphorylation of RcsB (15).

In this work, we establish the epistasis through the *rcs* phosphorelay pathway, and in particular we address the role of RcsF and confirm and extend previous findings on the role of RcsD in this pathway. To this end, we have used the RcsA-dependent *cps* promoter and the RcsA-independent *rprA* promoter. In both cases, the data indicate that signaling proceeds through an ordered cascade, RcsF→RcsC→RcsD→RcsB, and that RcsF, rather than playing a role in direct phosphorylation of RcsB, plays a critical role in signal transduction from the cell surface to RcsC.

MATERIALS AND METHODS

Media and chemicals. Luria-Bertani (LB) Lennox from KD Medical (Gaithersburg, MD) and tryptone broth (TB) media were used as indicated. Antibiotics and *o*-nitrophenyl-β-D-galactopyranoside were all from Sigma (St. Louis, MO). Oligonucleotides for the DH strain constructions and pNM plasmids are from Operon (Huntsville, AL).

Strain construction. The strains used are listed in Table 1 or described below. DH324 (Δ*rcsF*) was constructed by first deleting the *rcsF* open reading frame (ORF), replacing it with a PCR-amplified chloramphenicol resistance plus sucrase cassette (*cat-sacB*) using recombineering (45). The cassette was then removed by this same technique with a single-stranded oligonucleotide, counterselecting for sucrose resistance for loss of the *cat-sacB* cassette as described in supplemental materials (supplemental materials and methods). The unmarked *rcsF* deletion was verified by sequencing.

To move the unmarked *rcsF* deletion into other strains, the *cat-sacB* cassette was transduced by phage P1 into CAG18447, a *proAB*::*Tn*10 strain (36), to link Δ*rcsF* to the *proAB* marked mutation, selecting for Cm^r and screening for Tet^r (strain NM110). P1 grown on NM110 was then used to transduce the Δ*rcsF*::*cat-sacB* and *proAB*::*Tn*10 mutations into DH300 (*rprA-lacZ*) selecting for Tet^r and screening for Cm^r (DH303). P1 grown on NM12 (described in the supplemental materials) was used to transduce the unmarked *rcsF* deletion into DH303, selecting for growth on minimal glucose plates (Pro$^+$) and screening for loss of Cm^r and loss of Tet^r to create DH324. Three *rcsD* alleles are used in this work. All three delete the same 541 nucleotides at the 5′ end of *rcsD* (*yojN*) but have somewhat different sequences inserted. Strain DH351 (Δ*rcsD541*) was constructed according to the method of Datsenko and Wanner (7), using pKD13 as template and the ΔyojN.F and ΔyojN.R primers listed in supplementary table S1. The strain containing the Kan^r cassette is DH339 (*rcsD542*::Kan^r); the Kan^r cassette was then eliminated by the Flp recombinase (7) to create DH351, and

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Comment(s) or reference[a]
DJ480	MG1655 Δ(*argF-lac*)*U169*	Parental to all DH strains
DH300	*rprA-lacZ*	24
DH311	*rprA-lacZ rcsB*::Kan^r	24
DH312	*rprA-lacZ rcsC*::Kan^r	24
DH324	*rprA-lacZ* Δ*rcsF1*	See Materials and Methods
DH333	*rprA-lacZ* Δ*rcsF1 rcsC137 zei-10*::Cm^r	This study
DH339	*rprA-lacZ* Δ*rcsD542*::Kan^r	This study
DH351	*rprA-lacZ* Δ*rcsD541*	See Materials and Methods
DH354	*rprA-lacZ* Δ*rfa-1*::Cm^r	This study
DH355	*rprA-lacZ* Δ*rfa-1*::Cm^r *rcsC*::Tet^r	This study
DH356	*rprA-lacZ* Δ*rfa-1*::Cm^r Δ*rcsF1*	This study
DH357	*rprA-lacZ* Δ*rfa-1*::Cm^r Δ*rcsF1 rcsC*::Tet^r	This study
DH358	*rprA-lacZ* Δ*rcsD541* Δ*rfa-1*::Cm^r	This study
DH366	*rprA-lacZ rcsC137 zei-10*::Cm^r	This study
DH368	*rprA-lacZ* Δ*rcsD543*::Kan^r	This study
DH369	*rprA-lacZ rcsC137 zei-10*::Cm^r Δ*rcsD543*::Kan^r	See Materials and Methods
DH371	*rprA-lacZ rcsC137 zei-10*::Cm^r Δ*rcsD543*::Kan^r	See Materials and Methods
SG20781	*cpsB-lacZ lon*$^+$	Parental to all VS and MH1-3 strains (3)
SG20803	*cpsB-lacZ rcsC137 ompC*::*Tn*5	3; parental to MH4
MH1	*cpsB-lacZ rfa-1*::Cm^r *rcsF118*::Kan^r	This study
MH2	*cpsB-lacZ rcsD339*::Kan^r	This study
MH3	*cpsB-lacZ rcsD339*::Kan^r *rfa-1*::Cm^r	This study
MH4	*cpsB-lacZ rcsC137 ompC*::*Tn*5 *rcsF118*::Kan^r	This study
VS20299	*cpsB-lacZ rcsC10*::Kan^r	18
VS20324	*cpsB-lacZ rcsF118*::Kan^r	18; *rcsF* allele from reference 15
VS20302	*cpsB-lacZ rfa-1*::Cm^r	This study

[a] Strains made by P1 transduction for this work are indicated by "This study"; strains constructed in more complicated ways are indicated by "See Materials and Methods,"where the specifics of the construction are described.

polarity on RcsB was tested in a Western blot (Fig. 1B). Only a modest decrease in RcsB amounts was found.

Strain DH369 (Δ*rcsD543 rcsC137*) was constructed by sequential P1 transductions (see supplemental materials). Starting with DH300 (*rprA-lacZ*), the *rcsC137* mutation was introduced by selecting for the closely linked *zei*-10::Cm^r marker to yield a very mucoid strain (DH366). This strain was then transduced with P1 grown on the *rcsD543*::Kan^r mutant strain, selecting for kanamycin resistance. Because of the proximity of *rcsD* and *rcsC* on the chromosome and to make sure we retained the *rcsC137* mutation, several colonies were cross-streaked on a background of phage SY7 (*rcsD*$^+$) (3). Three of 12 colonies became mucoid in the presence of the phage; these were assumed to still carry the *rcsC137* allele. Complementation was confirmed by transforming these candidates with a pBAD-*rcsD* plasmid, which also restored mucoidy, and by sequencing the *rcsC137* mutation; one was designated DH369.

Strain DH371 was made in a multistep procedure (see supplemental materials). First, recombineering was used to insert a Cm^r cassette to delete and replace a 4.5-kb region extending from the 5′ end of *rcsD* to the region at the 3′ of *rcsC* where the *rcsC137* mutation is located. Second, from an *rcsD*::Kan^r strain, a PCR product spanning this same deleted region was generated by using a primer that introduces the *rcsC137* mutation into the product. This PCR product now carries the *rcsD543*::Kan^r deletion-insertion and incorporates the *rcsC137* point mutation as well as flanking regions of homology upstream and downstream of the deletion. Third, recombineering was used to replace the Cm^r cassette in the first strain by the PCR product carrying the Kan^r cassette and screening for the loss of Cm^r. This mutation was transduced by P1 into a DH300 (*rprA-lacZ*) background selecting for Kan^r and complemented to check for mucoidy with the pBAD-*rcsD* plasmid, and the *rcsC137* mutation was sequenced. All the other DH, MH, and VS strains listed in Table 1 were constructed by P1 transduction into the appropriate wild-type or mutant background.

Cloning of *rcsF*. Amplification of *rcsF* from the chromosome of *E. coli* was done using forward primer 5′-GGAATTCCTCGAGATGCGTGCTTTACCG and reverse primer 5′-CGCGGATCCGGAAACTGCTCATTTCGCCGT (IDT, Coralville, IA). AB1157 (*E. coli* Genetic Stock Center no. 1157) cells were grown overnight in LB medium at 37°C and diluted 1:100 in water, and 1 μl was used for a 50-μl PCR using *Taq* polymerase (Promega, Madison, WI). The PCR product was cloned into Invitrogen's TOPO2.1 plasmid (Carlsbad, CA) according to the manufacturer's specifications. Plasmids containing *rcsF* were digested and subcloned into pTrc99a from Amersham Pharmacia (Piscataway, NJ) using EcoRI and BamHI restriction enzymes (Promega, Madison, WI). The resulting plasmid construct is designated pMH300.

Cloning of *rcsD*. The pNM501 arabinose-inducible *rcsD* plasmid was constructed by restriction-less cloning or recombineering (6, 45). The Expand High Fidelity PCR system from Roche (Basel, Switzerland) was used for inverse PCR according to the manufacturer's specifications. The pBAD24 plasmid (19) was used as template for inverse PCR along with forward primer 5′-GGTAGGAG TGAAAAGCGGGTCGTGGCCGTTGTCTCTTTCTGACGCATGAATTCC TCCTGCTAGCCCAAAAAAAC-3′ and reverse primer 5′-GAAAAATACAT CAGCGACATTGACAGTTATGTCAAGAGCTTGCTGTAGGTACCCGGG GATCCTCTAGAGTCG-3′) to generate a linear pBAD24 vector with homologies to the start and end of the *rcsD* ORF. The PCR product was digested with 2 U of NcoI enzyme (NEB, Beverly, MA) for 2 h at 37°C to linearize the template and reduce the background in subsequent steps. The mix was purified with a Wizard PCR purification kit from Promega (Madison, WI) and checked on a gel for a single band of 4.5 kb. One-hundred nanograms of this DNA was electroporated into NM300 (MG1655, mini-λ-Tet^r) after induction of the λ functions (6), selecting for Amp^r transformants. Plasmids from 6 of 16 Amp^r clones contained an insert of the appropriate size. All six were used to transform DH351, an *rprA-lacZ rcsD* strain, to test for complementation on lactose MacConkey plates with ampicillin. The *rcsD* mutant strain is somewhat Lac$^+$ compared to the Lac$^-$ *rcsD*$^+$ strain (see Table 4); all of the clones were complemented without any arabinose, returning the strain to Lac$^-$. However, when the plasmid was introduced into either the *rcsD* mutant host or a (Lac$^-$) *rcsD*$^+$ host, DH300, the level of expression was increased in the presence of arabinose (data not shown), suggesting that higher levels of RcsD may allow signaling to RcsB, either via RcsC or via cross-talk, even in the absence of an inducing signal. The junction borders were confirmed by sequencing for two of these plasmids. They were identical, and one was designated pNM501.

β-Galactosidase assays. For the *rprA-lacZ* fusion, β-galactosidase kinetic assays were performed in a microtiter plate format and read in a SpectraMax250 plate reader as described previously (24). These specific activity units are about 1/25th of a Miller unit. For the *cps-lacZ* fusion, the standard Miller assay was used (26).

RNA extraction and fluorescent RT-PCR. RNA extraction was performed with the hot-phenol procedure described previously (25). Prior to reverse transcription-PCR (RT-PCR), to reduce or eliminate residual genomic DNA, RNA samples were treated with Ambion's Turbo DNase (Austin, TX) according to the manufacturer's specifications. The RNA samples were extracted once with phenol, precipitated, and resuspended in diethyl pyrocarbonate water. First-strand cDNA synthesis was performed on 1 μg of this RNA in a 20-μl volume as follows: 4 μl Superscript 5× RT buffer, 2 μl deoxynucleoside triphosphates (10 mM stock), 2 μl dithiothreitol (100 mM stock), 0.2 μl bovine serum albumin (10 mg/ml stock), and 0.2 μl random hexamer primer (2 pmol stock). The mix was heated to 70°C for 5 min to denature the RNA and transferred to 50°C for 30 min to allow the primer to hybridize to the RNA. One microliter of Superscript III enzyme was added (Invitrogen, Carlsbad, CA) and incubated at 50°C for 30 min. The PCRs were performed in triplicates using the SYBR Green PCR kit from QIAGEN (Valencia, CA) according to the manufacturer's specifications and run in the Opticon II real-time cycler from MJ Research (Waltham, MA). The *ompA* mRNA was used as an endogenous control in all samples. Specific primers for *rcsF* and *ompA* (control) were designed using Oligo 6.8 software (MBI, Cascade, CO) to generate a 120-nucleotide fragment and have similar annealing temperatures (supplemental materials, Table S1). Relative quantification of the RT-PCR results is described by Pfaffl (33) and by R. Soong, J. Ruschoff, and K. Tabiti (2000) in a Roche Diagnostics internal publication. The threshold cycle parameter (C_T) values for *ompA* were averaged and came out to 14.05, a number that was used in subsequent calculations. The comparative expression level (CEL) is $2^{-\Delta\Delta C_T}$ and is derived as follows: first, the equation $\Delta C_T = C_T$ (target) $- C_T$ (*ompA* normalizer 14.05) is calculated for each sample. Second, from among those samples, the negative control DH324 is chosen as the baseline signal. The next step is to calculate the equation $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (baseline DH324). Finally, that number is used for calculating the CEL.

Western blot of RcsB. For Western blots of RcsB, cells were grown to an optical density at 600 nm of ~0.5, and 1 ml of culture was immediately transferred to a tube containing trichloroacetic acid (TCA), 5% final concentration. Tubes were incubated on ice for 10 min and centrifuged for 10 min. Supernatants were removed, and pellets were washed once with cold acetone, resuspended in sodium dodecyl sulfate sample buffer, boiled for 5 min, and loaded proportionally to their respective optical densities. The 10% NuPAGE gels (Invitrogen, CA) were run and transferred onto a nitrocellulose membrane, blocked with 5% dry milk (Bio-Rad, CA), and probed with anti-RcsB antibody (Animal Research Center, Arizona State University) (Fig. 1).

Sequencing the *rcsC137* mutation. The *rcsC* gene from the genome of strain SG20803 was amplified in fragments of 750 bp using Promega's *Taq* polymerase (Madison, WI) according to the manufacturer's specifications. Both strands of each fragment were sequenced and assembled to cover the 2.8-kb *rcsC* gene in its entirety. Note that the originally published sequence for *rcsC* showed an initiation site that is 16 amino acids after that now currently defined by comparison with the sequence of *rcsC* in other organisms. Numbering of RcsC amino acids in this work uses the longer 949-amino-acid protein.

RESULTS

RcsF is required to sense signals. Previously, we had isolated *rcsF* in a screen of multicopy plasmids that stimulated the activity of the *rprA-lacZ* fusion. In that screen, several other plasmids were also isolated that activated *rprA-lacZ* activity; all were dependent upon RcsB (24). We tested the dependence of these plasmids on RcsF and RcsC for activation of the *rprA-lacZ* fusion. We had previously found that an insertion in *rcsC* led to a modest increase in expression of the *rprA-lacZ* fusion (24), presumably a reflection of cross-talk to RcsB and loss of the phosphatase activity of RcsC. None of the plasmids were able to further stimulate the fusion in the *rcsC* deletion, suggesting that they were all dependent upon RcsC (data not shown). This is consistent with previous observations of RcsC dependence of signaling by osmotic shock, mutations in the cell surface, and overexpression of some proteins (4, 11, 31, 37). We investigated whether RcsF was also required for these signals.

An *rcsF* mutant was constructed and introduced into both

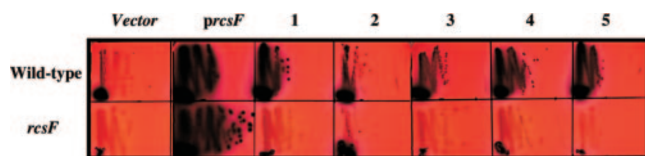

FIG. 2. Multicopy inducers of *rprA-lacZ* fusion require RcsF. A set of previously isolated plasmids (24) was used to transform DH300 (wild-type) and DH324 (*rcsF*) strains carrying an *rprA-lacZ* fusion. Plasmid-bearing cells were streaked on MacConkey lactose-ampicillin plates and incubated at 37°C. A representative gene is listed, and plasmids are described fully in Table 1 of reference 24. Lane 1, p*wzxC*; lane 2, p*dnaK*; lane 3, p*tolB*; lane 4, p*spot42*; and lane 5, p*yeiU*.

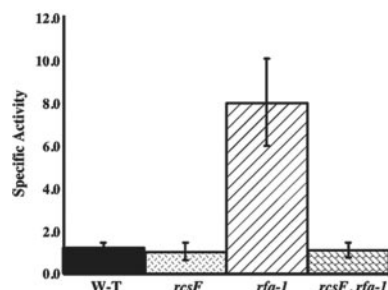

FIG. 3. Signaling through RcsF in an *rfa* mutant. Strains DH300 (wild-type), DH324 (*rcsF*), DH354 (*rfa-1*), and DH356 (*rfa-1 rcsF*) were grown overnight and diluted into LB media at 37°C. From an optical density at 600 nm of 0.1 and onward, samples were taken at 25-min time intervals and sampled in a kinetic microtiter plate assay as described in Materials and Methods. Bars represent the average specific activity over the length of sampling and are an average of three separate experiments. W-T, wild type.

the *rprA-lacZ* fusion strain and the *cps-lacZ* fusion. This mutation had a modest effect in reducing the basal level of expression of the fusions (Fig. 2, vector panels, compare top and bottom, and Table 2, compare VS20324 to SG20781). As expected, the plasmid carrying *rcsF* stimulated expression of the fusion and complemented the *rcsF* mutant (Fig. 2). All other plasmids failed to activate the *rprA-lacZ* fusion in the *rcsF* mutant background (Fig. 2). These results demonstrate that signaling from these plasmids is dependent upon RcsF as well as RcsC.

Deep rough mutants (*rfa*) carry mutations in the lipopolysaccharide biosynthesis pathway that result in constitutive *cps* expression and mucoid colonies on plates (31), affording us another way to induce the Rcs pathway. We introduced an *rfa-1* mutation into a wild-type or an *rcsF* null strain of *E. coli* carrying a single-copy chromosomal *cps-lacZ* or *rprA-lacZ* fusion and measured the β-galactosidase activity. As shown in Table 2 (VS20302 and MH1), activity of the *cps-lacZ* fusion increased significantly in the *rfa-1* mutant; all of this increase was abolished in an *rcsF* mutant. Similarly for *rprA-lacZ*, in the *rfa-1* strain activity of the fusion is high, but in the *rcsF rfa-1* mutant the fusion was not activated above basal levels (Fig. 3). As previously shown for *cps* synthesis (31), signaling to both *rprA* and *cps* also required RcsC; an *rfa-1 rcsC* double mutant expressed the fusions at the level of the *rcsC* mutant (data not shown). Therefore, as for the plasmids, RcsF is necessary for signal transmission in the *rfa-1* mutant, both for *cps-lacZ* and for *rprA-lacZ*.

TABLE 2. Regulation of expression of *cps-lacZ* fusion

Strain	Relevant genotype				Units of β-galactosidase[a] in tryptone broth
	rfa	*rcsF*	*rcsC*	*rcsD*	
SG20781	+	+	+	+	1 ± 0.2
VS20299	+	+	−	+	0.6 ± 0.1
VS20324	+	−	+	+	0.4 ± 0.2
MH2	+	+	+	−	0.7 ± 0.3
VS20302	−	+	+	+	54 ± 4
MH1	−	−	+	+	0.3 ± 0.3
MH3	−	+	+	−	0.4 ± 0.1
SG20803	+	+	*C137*	+	455 ± 113
MH4	+	−	*C137*	+	447 ± 54
SG20781(p*rcsF*$^+$)	+	+++	+	+	79 ± 10
VS20299(p*rcsF*$^+$)	+	+++	−	+	0.5 ± 0.1
MH2(p*rcsF*$^+$)	+	+++	+	−	0.6 ± 0.3

[a] Average of three independent Miller assays.

***rfa* mutations activate the phosphorelay without affecting the transcription of *rcsF*.** How do *rfa-1* mutants lead to activation of the RcsC/RcsB phosphorelay via RcsF? Do they increase the synthesis of RcsF or somehow change its activity? The isolation of plasmids containing *rcsF* as a multicopy activator suggested that increased synthesis was sufficient for activation (15, 24). We confirmed this using a multicopy isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *rcsF* plasmid. As expected, induction of *rcsF* expression leads to an increase in activity from both the *rprA-lacZ* fusion and from the *cps-lacZ* fusion (Fig. 4A and B).

If the *rfa-1* mutant activates the Rcs relay by increasing transcription from the *rcsF* promoter, we would not expect it to lead to further activation of the phosphorelay when *rcsF* is made from a foreign promoter. We tested this using the multicopy IPTG-inducible *rcsF* plasmid to transform an *rcsF* and an *rcsF rfa-1* strain carrying the *rprA-lacZ* fusion. We measured the *lacZ* activity in both of these strains using either no IPTG or 1 mM IPTG (Fig. 4C). In the double *rcsF rfa-1* mutant strain, *rcsF* activation of *rprA-lacZ* was still enhanced by the *rfa-1* mutation both in the absence and presence of IPTG (Fig. 4C, compare column 2 to 3 and column 4 to 5). Therefore, the effect of *rfa-1* is independent of the *rcsF* promoter, and we conclude that the *rfa* mutant is not activating expression of the Rcs system by affecting the transcription of *rcsF*. It may act to affect its translation or, more likely, its activity.

To confirm this result with *rcsF* in its normal context, we used RT-PCR to measure the levels of *rcsF* transcription from the chromosome in a wild-type strain compared to an *rfa-1* strain. While we could detect the increased *rcsF* transcription from a multicopy plasmid, the *rfa-1* mutant showed no increase in *rcsF* transcripts over an *rfa*$^+$ host (Table 3).

RcsF is upstream of RcsC and RcsD in the signaling pathway. RcsC is the sensor kinase for the Rcs phosphorelay and has been shown to be necessary for activation of the system by osmotic shock, some mutants, and other signals (4, 11, 31, 37). RcsD (formerly YojN) has been shown to be essential for RcsC→ RcsB signaling (40). As indicated in Fig. 1, *rcsD* is the upstream gene in an operon with *rcsB* with convergent orientation to *rcsC*. There is a high similarity in domain organization between RcsD and the histidine kinase RcsC with two crucial

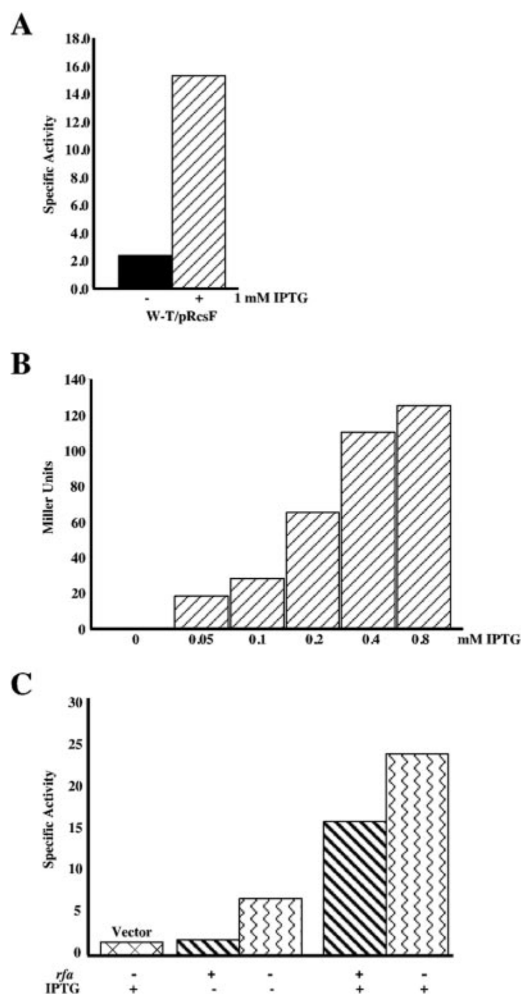

FIG. 4. Dose-dependent stimulation by RcsF and *rfa* acts independently of *rcsF* transcription. A) DH300 (*rprA-lacZ*) cells carrying pMH300 (p_{tac}-*rcsF*) were grown without or with IPTG as indicated. A kinetic β-galactosidase assay was performed, and bars represent an average of three assays. B) SG20781 (*cps-lacZ*) cells carrying pMH300 were grown in TB medium with increasing concentrations of IPTG and assayed in a standard Miller assay. Each bar represents the average of three independent assays. C) DH324 (*rcsF*) and DH356 (*rcsF rfa-1*) strains carrying pMH300 were grown in the absence or presence of 1 mM IPTG as indicated. The first bar is DH356 with a vector control grown in 1 mM IPTG shown for basal levels. Cultures were assayed three times in a kinetic β-galactosidase assay and averaged. W-T, wild type.

differences: RcsD lacks the His autophosphorylation site, and the C-terminal receiver domain is replaced by a predicted phosphotransfer (HPt) domain (40). We asked whether RcsF is upstream or downstream of RcsC and RcsD in the signaling pathway.

The relative positions of RcsF, RcsC, and RcsD in the signaling pathway were tested using epistasis experiments, activating the system initially by RcsF overproduction from a plasmid in cells carrying *rcsC* or *rcsD* mutants. An *rcsC*::Kan^r insertion mutant was used to inactivate *rcsC*. Two *rcsD* insertion-deletion derivatives were created and tested for polarity on *rcsB* as described in Materials and Methods. About a twofold decrease in RcsB levels was seen in the presence of the

TABLE 3. Relative mRNA levels of *rcsF* by RT-PCR

Variable	DH300 (wild type)	DH324 (Δ*rcsF*)	DH354 (*rfa-1*::Cm^r)	DH300/p*rcsF* plus IPTG (wild type/ p*rcsF*)
C_T	21.8	33.8	22.3	15.9
ΔC_T[a]	7.75	19.75	8.25	1.85
$\Delta\Delta C_T$[b]	−12.0	0	−11.5	−17.9
CEL[c]	4096	1	2896	244589

[a] $\Delta C_T = C_T$ (target) $- C_T$ (*ompA* normalizer 14.05).
[b] $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (baseline DH324).
[c] The comparative expression level is $2^{-\Delta\Delta C_T}$.

rcsD mutations (Fig. 1), confirming previous results suggesting that RcsB can be expressed from sequences within *rcsD* (38). Generally, the *rcsD541*::Kan allele was used in the experiments described below.

The results of such experiments for the *cps-lacZ* fusion are shown in Table 2 and for the *rprA-lacZ* fusion in Fig. 5A. At a concentration of 1 mM IPTG, both fusions are expressed at a significant level, as shown above. In contrast, in both the *rcsC* and *rcsD* mutants, induction of *rcsF* expression from the plasmid fails to stimulate either fusion above basal level (Table 2, compare SG20781 p*rcsF* to VS20299 and MH2, each carrying p*rcsF*; Fig. 5A, compare column 2 to columns 4 and 6). Thus, RcsF is dependent upon both RcsC and RcsD for signaling to *rprA* and *cps* promoters.

In a second test, the epistasis between *rfa-1* and *rcsD* was determined. If the effect of an *rfa-1* mutant is upstream of *rcsF*, and *rcsD* is downstream of *rcsF*, *rfa-1* signaling should be abolished in an *rcsD* mutant. This is what was observed (Table 2). The high-level expression of *cps-lacZ* in an *rfa-1* mutant (VS20302) was abolished when an *rcsD* mutation was introduced (MH3). A similar effect was seen for *rprA-lacZ* (Table 4, compare DH354 to DH358), although the high basal level of expression of the *rprA-lacZ* fusion in the absence of *rcsD* (DH351) masks some of the effect.

If RcsF acts upstream of RcsC to activate the RcsC histidine kinase, a mutant form of *rcsC* that is already active should be independent of RcsF. We used the *rcsC137* mutation to test this prediction. Previously isolated as an *rcsC* recessive mutation that strongly activated the *cps-lacZ* fusion (17), *rcsC137*

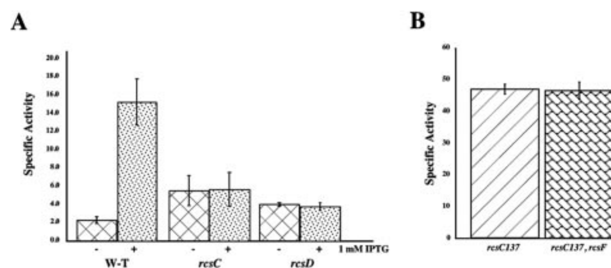

FIG. 5. Epistasis of *rcsD* and *rcsC* to *rcsF*. A) DH300 (wild-type), DH312 (*rcsC*), and DH351 (*rcsD*) cells harboring the pMH300 plasmid were grown in LB media in the absence or presence of IPTG as indicated. Kinetic β-galactosidase assays were performed, and the average of three assays is graphed. B) DH366 (*rcsC137*) and DH369 (*rcsC137 rcsF*) were grown in LB media and assayed in a kinetic β-galactosidase assay. Bars represent the averages of two independent experiments. W-T, wild type.

TABLE 4. Effect of *rcsD* mutations on *rprA-lacZ* expression

Strain	Relevant genotype	β-Galactosidase expression (sp act)[a]
DH300	Wild type	1.3 ± 0.3
DH351	*rcsD541*	4.9 ± 0.6
DH354	*rfa-1*	9.2 ± 0.4
DH358	*rfa-1 rcsD541*	6.4 ± 0.4
DH366	*rcsC137*	37 ± 2.6
DH369	*rcsC137 rcsD543*	3.1 ± 0.5
DH371	*rcsC137 rcsD543*	1.9 ± 0.1
DH371/pBAD-*rcsD*$^+$	*rcsC137 rcsD543*/pBAD-*rcsD*$^+$	25 ± 3.0 (without arabinose) 18.5 ± 3.7 (with arabinose)

[a] Average of at least two independent kinetic β-galactosidase experiments.

was sequenced and found to carry a single-nucleotide mutation in the codon GCT, changing it to GTT, which results in changing Ala_{904} to Val in the response regulator domain of RcsC. The mutation leads to constitutive and unregulated phosphorylation of RcsB (data not shown). Previously, we had noted in Northern blots that the amounts of RprA RNA increased by about 30-fold in an *rcsC137* mutant over the wild type (24), consistent with activation of the Rcs phosphorelay for *rprA* as well as for *cps*. Isogenic strains carrying *rcsC137*, with and without an *rcsF* mutant, and either the *rprA-lacZ* fusion (Fig. 5B) or the *cps-lacZ* fusion (Table 2) were assayed. As expected, introduction of the *rcsC137* allele significantly increased expression of both the *rprA-lacZ* fusion (Fig. 5B, column 1) and the *cps-lacZ* fusion (Table 2, SG20803), consistent with previous experiments. The presence of the *rcsF* null mutation had no effect on expression, confirming that *rcsC* is epistatic to *rcsF* (Fig. 5B, column 2; Table 2, MH4).

Testing epistasis between *rcsC137* and *rcsD* was complicated by their tight linkage. Therefore, strains were constructed both by P1 transduction and by linear transformation, as described in Materials and Methods (also see supplemental materials). In both cases, the *rcsC137 rcsD* double mutant lost the mucoid characteristic of the *rcsC137* parent, consistent with epistasis of the *rcsD* mutant.

We further quantitated this by assay of the *rprA-lacZ* fusion in the double mutant. The increased expression of *rprA-lacZ* in the *rcsC137* strain was totally abolished by the *rcsD* mutant, reducing fusion expression to that found for the *rcsD* mutant alone (Table 4). Introduction of a plasmid expressing RcsD from an arabinose-inducible promoter complemented the *rcsD* defect, restoring activity to about half of that seen in the *rcsC137 rcsD*$^+$ case in the absence or presence of arabinose (Table 4). The twofold decrease in activity may reflect some polarity of the *rcsD* insertion mutation on *rcsB* (Fig. 1).

Possible cross-talk in the Rcs phosphorelay. The phenotype of an *rcsC* or *rcsD* mutant is very close to that of a wild-type strain for the *cps-lacZ* fusion in the absence of any inducing treatment (Table 2). However, we noted previously that the wild-type basal level for the *rprA-lacZ* fusion is significantly elevated in an *rcsC* mutant (24); we find a similar, not quite as marked elevation in the *rcsD* mutant strain (Table 4). In the experiments reported here, the normal basal level of the *rprA-lacZ* fusion in a wild-type strain is about 1.5 U, while it is about 6 and 4 U for the *rcsC* and *rcsD* mutant strains, respectively. One interpretation of these results is that there is phosphorylation of RcsB from other sources (cross-talk); in the absence of the specific phosphotransfer and phosphatase proteins (RcsD and RcsC, respectively), this phosphate cannot be removed, leading to higher expression of the fusion. This would also suggest that low levels of RcsB-phosphate give a detectable signal for *rprA-lacZ* but not for *cps-lacZ*. Whether this is a characteristic of RcsA-independent promoters or is specific to *rprA* is not known. Deletion of two genes necessary for synthesis of acetyl-phosphate, a small molecule known to be able to phosphorylate various response regulators, *ackA* and *pta*, did not affect the elevated basal level of the fusion in the *rcsC* mutant (data not shown), suggesting that cross-talk must come from another, as yet unidentified, source.

DISCUSSION

While many two-component systems and phosphorelays have been studied, in many cases we know relatively little about how signals are transmitted to the sensor kinase. The data we present here establish RcsF as an important component of the Rcs phosphorelay and place it upstream of RcsC for signaling both to the *cps* promoter and the *rprA* promoter. The dependence on RcsF was true in *rfa* mutants (Fig. 3) and in cells carrying a number of multicopy plasmids that activate the Rcs system (Fig. 2). In studies by other labs, mutations in *pgsA*, necessary for synthesis of acidic phospholipids, induced the Rcs system, as did growth of wild-type cells at low temperature in the presence of excess Zn^{2+}; in both cases, induction required both RcsF and RcsC (20, 35). Therefore, a variety of perturbations to the cell surface and/or environmental signals act via RcsF to activate RcsC.

Our experiments also show that the induction of the signal via RcsF is independent of the normal transcriptional signals for RcsF (Fig. 4C) and does not result in increased transcription of *rcsF* (Table 3). Nonetheless, overproduction of RcsF in the absence of another inducing signal is sufficient to activate the phosphorelay (Fig. 4A and B). These data are most consistent with a model in which RcsF interacts, directly or indirectly, with RcsC to activate the phosphorelay (Fig. 6). Under the usual inducing conditions, changes in the RcsF structure or modifications to the protein may increase this interaction; overproduction may drive the interaction in the absence of this proposed structural change. Alternatively, overproduced RcsF may otherwise perturb the cell to provide a signal, which then acts through RcsC, much as the other plasmids in Fig. 2 do.

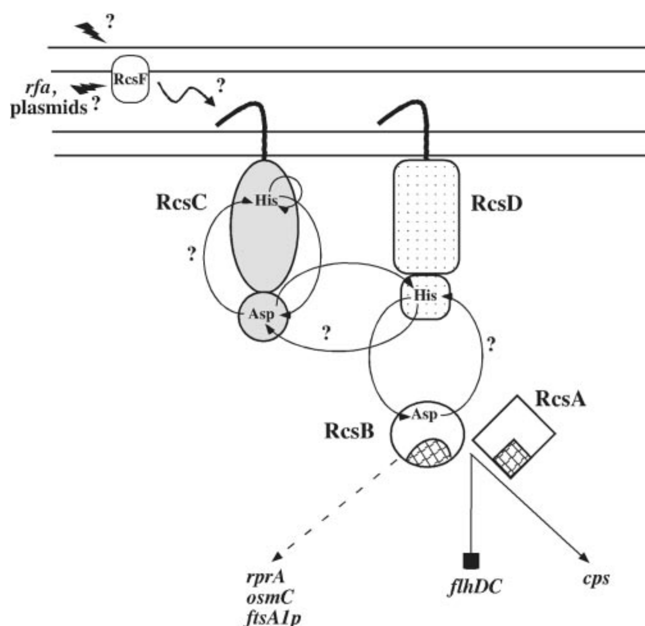

FIG. 6. Model of the Rcs signaling pathway. The schematic diagram is not to scale but shows the signaling flow through the Rcs cascade. Question marks indicate experimentally unresolved issues. RcsF localization to the outer membrane is according to in silico predictions based on the amino acid sequence and A. Jacq (personal communication; see Discussion). Amino acids involved in the phosphorelay are indicated. The shaded areas of RcsA and RcsB indicate the DNA-binding domains of these proteins. A dashed arrow points to genes that are RcsB dependent but RcsA independent. A blocked line indicates a gene repressed by an RcsA-RcsB heterodimer, and a solid arrow points to genes that require RcsA and RcsB for activation (see the text for details).

Some, but not all, of the plasmids in Fig. 2 encode membrane proteins, and we do not currently know how directly any of them act on RcsF to induce the Rcs signaling cascade.

RcsF has the sequence characteristics of an outer membrane lipoprotein and includes disulfide bonds that need to be maintained by interactions with DsbA (22). Palmitate labeling and density fractionation confirm that RcsF is an outer membrane lipoprotein (A. Jacq, personal communication). As an outer membrane protein, it may be poised to sense perturbations in the cell surface caused by osmotic shock or mutation of lipopolysaccharide synthesis, although the mechanism of this sensing remains unknown. If it interacts directly with RcsC, that interaction is most likely to take place in the periplasm (Fig. 6). We note that another lipoprotein, NlpE, has been implicated in the signaling pathway to Cpx (9, 30). The Cpx sensor kinase and RcsC have another characteristic in common: both are activated by interaction of the bacteria with surfaces, although possibly at different steps in the attachment process (12, 30). Finally, it is intriguing that overproduction of another putative lipoprotein, YpdI, has been found to activate the Rcs phosphorelay independently of RcsF (34); overproduction of a lipoprotein-specific chaperone, LolA, has also been found to activate the Rcs phosphorelay (4), although its dependence on RcsF or YpdI is not known. These findings suggest that lipoproteins may play important roles in the upstream sensing of signals for membrane sensor kinases; exactly what those signals are and how they are sensed is not yet understood.

RcsF was first identified by Gervais and Drapeau by its ability, in multicopy, to activate transcription from the RcsB-dependent *ftsA1p* promoter (15, 16). They suggested that RcsF is capable of phosphorylating RcsB directly or indirectly but in an RcsC-independent manner, based on measurements of colanic acid in an *rcsC* mutant carrying an *rcsF* plasmid. In our tests of epistasis between *rcsF* and *rcsC*, we unequivocally demonstrate that RcsF is upstream of and dependent upon RcsC. One possible explanation for the results of Gervais and Drapeau comes from our observation that an *rcsC* null mutant does increase activation of the very sensitive *rprA-lacZ* fusion. Possibly the increased colanic acid they measured reflected this same modest increase in activation of *cps* that the *cps-lacZ* fusion is not sensitive enough to detect.

The elevated basal level of the *rprA-lacZ* fusion observed in an *rcsC* or *rcsD* strain suggests that the Rcs system is capable of integrating signals from other sources. We are assuming that, in the absence of RcsD and RcsC, phosphorylation of RcsB by other means cannot be reversed, as it presumably is in the wild-type case. Therefore, dephosphorylation of RcsB may proceed through RcsD to RcsC. This is in agreement with studies on the dephosphorylation of response regulators ArcA and TorR, both of which have complex upstream signaling sensor kinases; dephosphorylation in these cases was shown to require the phosphotransfer domains as well as receiver domains in the sensor kinase (1, 32). We do not currently know which systems might provide the cross-talk phosphorylation of RcsB. ArcB kinase was able to phosphorylate RcsD in vitro (40), and we have also observed EnvZ and FixL phosphorylation of RcsB in vitro (M. Heck, G. Gupte, and V. Stout, unpublished data). An in vivo genome-wide analysis by Hagiwara et al. found evidence of cross-talk between the PhoQ/PhoP and Rcs systems (20). Other mechanisms for cooperation between a response regulator like PhoP and RcsB are also possible, however. For instance, PhoP appears to promote activation of an RcsB-dependent promoter of *ugd* under some conditions by binding to a site upstream of the RcsB site (28). Similarly, interactions between RcsA and RcsB can activate *cps* transcription under conditions that are not known to activate the RcsC cascade (stabilization of RcsA in a *lon* mutant) (39). Thus, any mechanism for improving RcsB binding to its target might bypass the requirement for phosphorylation. However, it is not clear why *rcsD* or *rcsC* mutants would improve the effect of cooperative binding by another protein, for instance, in activating *rprA* transcription. Therefore, we currently favor phosphorylation of RcsB from an unknown source as the explanation for increased expression of *rprA* in *rcsC* and *rcsD* null mutants.

rcsC137 leads to high-level activation of both capsule synthesis and RprA synthesis. We found that this mutation changes Ala_{904} to Val. Alanine is absolutely conserved in RcsC proteins from different species; Ala or Gly is found at this position in 80% of response regulators (42). *rcsC137* is recessive to wild-type *rcsC* and can also be complemented by the response regulator domain of *rcsC*, consistent with the sequence localization and suggesting that *rcsC137* is a loss-of-function mutant (3, 5). However, it seems unlikely that *rcsC137* is solely a mutant that has lost phosphatase activity. If so, we

would have expected the same low level of activity of *cps* and *rprA* seen with an *rcsC* null mutation (Fig. 5), which must certainly have also lost phosphatase activity. Therefore, this domain of RcsC must also act negatively in some other way; we suggest that it may negatively regulate the activity of the histidine kinase as well as play a necessary role in dephosphorylation. A number of other mutations isolated for increased *cps-lacZ* expression also map to this domain but had less dramatic effects than *rcsC137* (M. Heck and V. Stout, unpublished results). Deletion of the domain will not itself lead to activation of RcsB, since it also plays an essential role in the phosphorelay. Under conditions where no signal is activating the Rcs phosphorelay, any phosphorylation of RcsB from other sources will have little effect, due to reversal of the phosphorylation through RcsD to RcsC. Under activating conditions, the flow of phosphate is reversed. Whether activation of the kinase activity of RcsC mimics the effect of an *rcsC137* mutation, relieving the negative regulation by the D1 domain, or works by another pathway that overcomes this negative effect is not yet known. Also not known is whether there are environmental conditions under which cross-talk is not efficiently reversed and becomes physiologically important.

In summary, our results show that regulation of the RcsA-dependent *cps* promoter and the RcsA-independent *rprA* promoter are completely parallel in their dependence on the components of the Rcs phosphorelay, although the sensitivities of the promoters may be somewhat different. All activating signals we have tested here are dependent upon both RcsF and RcsC. RcsF thus plays an important upstream role in transmitting many signals to RcsC, and the activity, rather than the transcription, of RcsF appears to be controlled by a far-upstream signal, such as that created by an *rfa-1* mutant. This paradigm for upstream signaling to a sensor kinase by a lipoprotein may be preserved for other phosphorelays as well.

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