

Genetic Analysis of the RcsC Sensor Kinase from *Escherichia coli* K-12

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The Rcs two-component pathway is involved in the regulation of capsule production in *Escherichia coli*. RcsC is predicted to be the sensor component of this two-component pathway, and in this study we present the first genetic data that support the role of RcsC as a hybrid sensor kinase.

Bacteria respond to changes in the environment largely through the activity of specialized signaling systems called two-component pathways. These pathways are characterized by the presence of conserved communication domains that are involved in signaling via the intermolecular transfer of phosphoryl groups. These domains, called the transmitter domain (H1) and the receiver domain (D1), contain well-conserved amino acid residues, histidine and aspartate, respectively, that are directly implicated in the phosphotransfer. This phosphorelay between transmitter and receiver domains (His→Asp) is the basis of all two-component pathways (7, 12).

In *Escherichia coli* K-12 the Rcs two-component pathway controls the expression of the *cps* operon, encoding the proteins required for the production and secretion of acidic extracellular polysaccharide colanic acid (9). Genetic studies have indicated that this pathway is composed of the RcsC sensor protein, the YojN HPT-containing protein, and the RcsB response regulator (see Fig. 2) (2, 14, 15). Analysis of the amino acid sequence of the RcsC sensor protein suggests that it is a hybrid kinase, as the protein contains well-conserved H1 and D1 domains (see Fig. 2 and 3). Only two other prokaryotic sensors with similar H1-D1 arrangements have been characterized, i.e., the VirA sensor protein of *Agrobacterium tumefaciens* and the LuxN sensor from *Vibrio harveyi* (1, 3, 8). In VirA the D1 domain is not required for signaling and this domain has a regulatory role whereby it appears to inhibit the kinase activity of VirA (4). On the other hand, the D1 domain of LuxN has been shown to play a direct role in the transfer of the phosphoryl group from LuxN to the response regulator LuxO via the HPT-containing protein LuxU (8). Interestingly, the HPT-containing protein YojN has recently been implicated in the Rcs phosphorelay, suggesting that the D1 domain of RcsC is directly involved in the Rcs phosphorelay (15). In this study we present the first genetic data to directly implicate specific amino acid residues, H463 and D859, in the activity of RcsC. In silico analysis predicts that these residues are involved in the Rcs phosphorelay, and we describe the role of RcsCH463 and RcsCD859 in the regulation of *cpsB-lacZ* expression. Our data support the hypothesis that RcsC has both kinase and phosphatase activities, and we discuss the implications of our data

for the current model describing the complex Rcs phosphorelay, as proposed by Takeda and colleagues (15).

Induction of *cpsB-lacZ* expression by DjlA overproduction requires the RcsC protein. It has been previously shown that the moderate overproduction of DjlA, an inner membrane DnaJ-like protein (6), induces the expression of the *cps* operon in an *rscC*-dependent manner (10, 16). Therefore, DjlA overproduction can be used as a signal to facilitate a genetic analysis of the activity of the RcsC protein. However, we first needed to show that a plasmid-borne copy of *rscC* would respond to DjlA overproduction in the expected way. Therefore, we amplified the *rscC* gene using *Pfu* DNA polymerase and the oligonucleotide pair DC121 and DC132. The amplified PCR product was digested with *SacI* and *XbaI* and ligated into the appropriately digested pTRC99a vector (Amersham Pharmacia Biotech). Plasmid DNA was isolated from a single transformant, and the insert was completely sequenced to confirm the presence of the *rscC* gene. This plasmid, pPSG980, was then transformed into *E. coli* K-12 strains PSG1031 (*rscC*⁺ *cpsB-lacZ*) and PSG1038 (*rscC52::Tn10 cpsB-lacZ*) carrying DjlA-overproducing plasmid pPSG961-31. In pPSG961-31 DjlA production is under the control of the *araBAD* promoter (5). As expected, in strain PSG1031, DjlA overproduction resulted in a substantial increase in *cpsB-lacZ* expression (Fig. 1). In contrast, DjlA overproduction had no effect on *cpsB-lacZ* expression in the strain carrying mutant *rscC*, PSG1038, confirming that the RcsC sensor is required for the observed increase in *cpsB-lacZ* expression (Fig. 1) (10). However, when a copy of the *rscC* gene is provided in *trans*, *cpsB-lacZ* expression in PSG1038 was seen to increase following DjlA overproduction. This confirms that DjlA induces *cps* expression through the RcsC protein and permits the use of DjlA overproduction as a tool for the analysis of RcsC.

The conserved signaling residues of RcsC, H463 and D859, are essential for induction of *cpsB-lacZ* expression. In silico examination of the RcsC amino acid sequence predicts that this protein is a hybrid sensor kinase as it contains both transmitter (H1) and receiver (D1) domains (Fig. 2B). These domains are characterized by the presence of conserved amino acids, histidine and aspartate, respectively, often associated with the transfer of phosphoryl groups during signaling. To identify these conserved residues in RcsC, we aligned the amino acid sequence of this protein with the amino acid sequences of well-characterized transmitter and receiver domains from other proteins (Fig. 3). This alignment clearly

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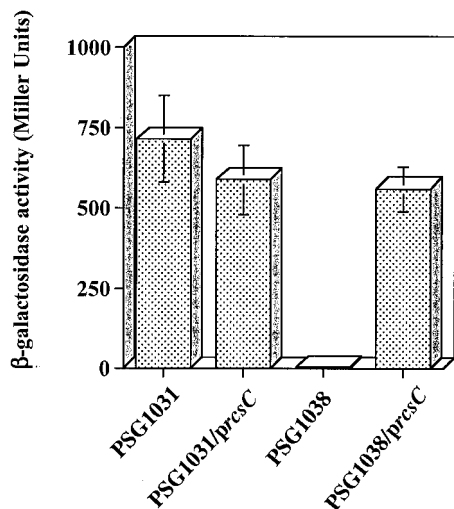


FIG. 1. Induction of *cpsB-lacZ* expression by DjlA overproduction requires an intact copy of *rcsC*. PSG1031 (*rcsC*⁺ *cpsB-lacZ*) and PSG1038 (*rcsC52::Tn10 cpsB-lacZ*) were transformed with pPSG961-31 (*paraBAD-djlA*) and, where indicated, pPSG980 (p *rcsC*⁺). Cells were cultured overnight at 30°C on LB agar plates in the presence of 0.2% (wt/vol) L-arabinose. To determine the level of *cpsB-lacZ* expression, cells were harvested and assayed for β -galactosidase activity. The results shown are the means of five independent measurements, and the error bars represent standard deviations.

predicts that H463 and D859 are the conserved residues in the H1 and D1 domains of RcsC, respectively. Therefore, we predict that these residues are directly involved in the Rcs phosphorelay. To test this, we individually mutated H463 and D859 to the nonphosphorylatable amino acid glutamine (Q), resulting in RcsCH463Q and RcsCD859Q (encoded by plasmids

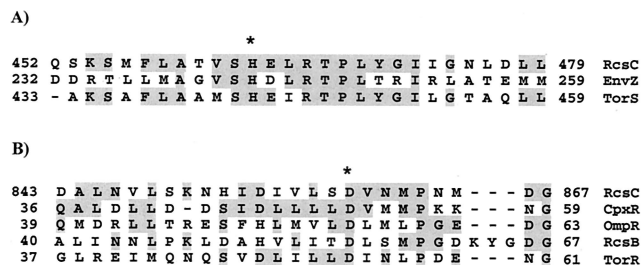


FIG. 3. Identification of the amino acids predicted to be involved in the RcsC phosphorelay. (A) Alignment of the histidine-containing region (i.e., the H box) of the transmitter domain (H1) from *E. coli* sensor proteins EnvZ, TorS, and RcsC. Asterisk, position of the His residue known to be phosphorylated in EnvZ and TorS, which aligns with H463 in RcsC. (B) Alignment of the receiver domain (D1) of RcsC with the receiver domains of CpxR, OmpR, RcsB, and TorR. Asterisk, Asp residue that has been shown, either genetically or biochemically, to be phosphorylated in CpxR, OmpR, RcsB, and TorR. This residue clearly aligns with D859 in RcsC. Both alignments were carried out using the ClustalW algorithm at the European Bioinformatics Institute (<http://www.ebi.ac.uk>).

pPSG980H463Q and pPSG980D859Q, respectively). Mutagenesis, using the Stratagene QuikChange kit, was carried out according to the manufacturer's instructions. To introduce point mutations into *rcsC*, we used pPSG980 as a template and oligonucleotide pairs DC190 and DC191 (*rcsCH463Q*) and DC188 and DC189 (*rcsCD859Q*) (Table 1). Plasmid DNA was recovered from each mutant, and the *rcsC* allele was recloned into pTRC99a, completely sequenced to verify the presence of the desired mutation, and then transformed into strain PSG1038 (*rcsC52::Tn10 cpsB-lacZ*) containing pPSG961-31. A single transformant was restreaked onto Luria-Bertani (LB) agar plates, with or without added L-arabinose, and incubated at 30°C overnight. Cells were then harvested and washed in 1× phosphate-buffered saline, and the level of *cpsB-lacZ* expression was determined by β -galactosidase activity assays. The results show that RcsCH463Q and RcsCD859Q could not induce *cpsB-lacZ* expression in response to DjlA overproduction (Fig. 4A). Therefore H463 and D859 are required for the DjlA-mediated activation of RcsC. Indeed, the inactivity of the RcsCH463Q protein is the first genetic evidence that supports the role of RcsC as a kinase. Moreover, the data obtained with the RcsCD859D protein is in agreement with recent results showing that the HPT-containing protein YojN is involved in the Rcs phosphorelay (15).

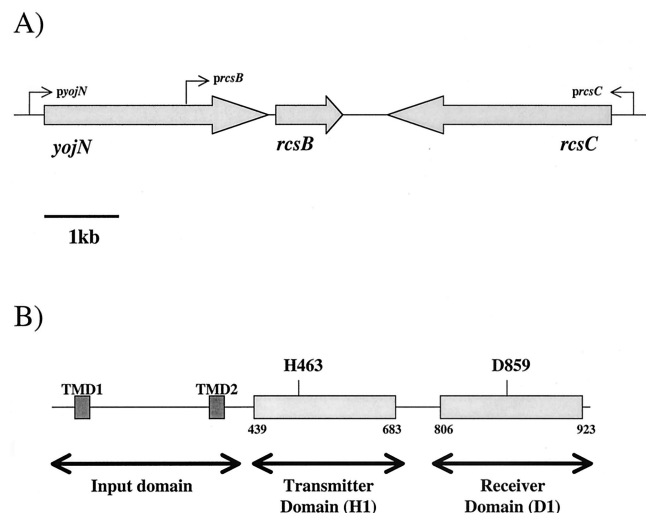


FIG. 2. *yojN rcsCB* genetic locus. (A) Arrangement of genes in the *yojN rcsCB* locus in *E. coli* K-12. Arrows, approximate positions of promoters implicated in the expression of each gene. (B) The modular architecture of RcsC. The input domain spans the cytoplasmic membrane and contains two transmembrane domains (TMD). The numbers flanking the transmitter domain and receiver domain represent the predicted amino acid sequence limits of each signaling domain based on in silico homology searches.

TABLE 1. Oligonucleotides used during this study

Oligonucleotide	Sequence
DC121.....	ATAGCGAGCTCGTACAACCCTGAAAGCCTCG
DC132.....	ATAGCTCTAGACTACGAATCCCGCGATTTCCTGACC
DC188.....	GATATCGTGCTTAGCAACGTC AACATGCC
DC189.....	GGCATGTTGACGTTGCTAAGCACGATATC
DC190.....	CCACCGTCAGTCAAGAGCTGCGAACGC
DC191.....	GCGTTCGACGCTCTTGACTGACGGTGG
DC210.....	CTAGAACAAAAAATCATCTCAGAAGAGATCTGTGA
DC211.....	AGCTTCACAGATCCTCTTCTGAGATGAGTTTTGTGTT
DC212.....	TCGAGCCATGGTCAGAGCATTGGCGTTAGTGC ^a
DC213.....	ATAGCTCTAGAGAAATCCCGGATTTCCTGACC ^b

^a The *NcoI* site is underlined.

^b The *XbaI* site is underlined.

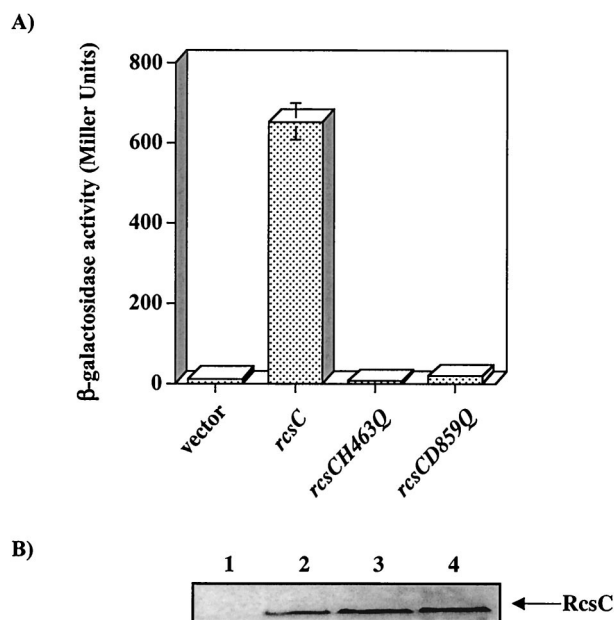


FIG. 4. RcsCH463 and RcsCD859 are required for the induction of *cpsB-lacZ* expression associated with DjlA overproduction. (A) PSG1038 (*rcsC52::Tn10 cpsB-lacZ*)/pPSG961-31 (*paraBAD-djlA⁺*) was transformed with either pTRC99a (vector) or derivatives carrying different mutant alleles of *rcsC*, *rcsC⁺* (pPSG980), *rcsCH463Q* (pPSG980H463Q), or *rcsCD859Q* (pPSG980D859Q). Cells were cultured for 20 h at 30°C on LB agar supplemented with 0.2% L-arabinose and assayed for β -galactosidase activity. The results shown are the means of four independent measurements, and the error bars represent standard deviations. In some cases the error bars are too small to be shown on the graph. (B) PSG1038 (*rcsC52::Tn10 cpsB-lacZ*) cells carrying plasmids expressing different *c-myc*-tagged alleles of *rcsC* were grown as outlined in the text and collected by centrifugation, and 0.2 *A*₆₀₀ equivalents were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody that recognizes the *c-myc* epitope. Lane 1, pBMM101 (vector); lane 2, pBMM102 (*rcsC-myc*); Lane 3, pBMM103 (*rcsCH463Q-myc*); Lane 4, pBMM105 (*rcsCD859Q-myc*). The arrow indicates the RcsC band that cross-reacts with the anti-*myc* monoclonal antibody.

RcsC mutants accumulate in the cell to the same level as RcsC. To verify the negative effect of RcsC mutants, we needed to confirm that the mutant proteins accumulated in the cell to the same level as wild-type RcsC. Unfortunately, anti-RcsC antibodies are not available and several attempts to raise these antibodies in our laboratories failed. Therefore, a vector that allowed the addition of a *c-myc* epitope tag to the C termini of the wild-type and mutant proteins was constructed. This vector, pBMM101, was constructed from expression vector pTRC99a as follows. Two complementary oligonucleotides were synthesized (DC210 and DC211; Table 1) such that when these oligonucleotides anneal they form a small region of double-stranded DNA, with a 5' *Xba*I-compatible end and a 3' *Hind*III-compatible end, that encodes the *c-myc* epitope, Leu Glu Gln Lys Leu Iso Ser Glu Glu Asp Leu. The annealed oligonucleotides were then ligated with pTRC99a and electroporated into *E. coli* XL1-Blue. The plasmid DNA from several of the resulting transformants was sequenced, and a clone was selected and named pBMM101. Thus, proteins can be C-terminally tagged with the *c-myc* epitope by subcloning the genes encoding these proteins into the *Nco*I-*Xba*I sites of pBMM101.

The different *rcsC* alleles were amplified by PCR using *Pfu* DNA polymerase, and the PCR products were then ligated into pBMM101, resulting in plasmids pBMM102 (*rcsC-myc*), pBMM103 (*rcsCH463Q-myc*), and pBMM104 (*rcsCD859Q-myc*). These plasmids were transformed into PSG1038/pPSG961-31 cells and tested for their ability to induce *cpsB-lacZ* expression in response to DjlA overproduction. The results were identical to those in Fig. 3A, indicating that the *c-myc* epitope tag had no effect on the activity of the RcsC protein (data not shown). However, under the growth conditions used for our biochemical analyses, i.e., no added IPTG, it was not possible to detect any of the RcsC proteins after immunoblotting and hybridization with anti-*myc* monoclonal antibodies, even though it is clear from the β -galactosidase assays that RcsC-*myc* is being produced (data not shown). Therefore, cells were induced with 1 mM IPTG for 120 min at 37°C and samples were taken for immunoblotting. A band at the correct predicted molecular mass (approximately 100 kDa) was observed in all samples tested, except the vector control (Fig. 4B). Moreover, the intensities of this band in all lanes were similar, confirming that the mutations have no apparent effect on the inherent stability of RcsC. Interestingly, even under these IPTG-inducing conditions the mutant *rcsC* alleles were unable to complement the *rcsC52::Tn10* mutation (data not shown). Therefore, we conclude that the mutations must be interfering with the normal signaling activity of RcsC.

RcsC phosphatase activity requires D859 but is largely independent of H463. Previous studies have suggested that RcsC has both positive and negative regulatory effects on the expression of the *cps* operon (2, 14). This is consistent with the RcsC sensor having both kinase and phosphatase activities. Therefore, in the presence of an activating signal, RcsC kinase activity would result in an increase in the level of phospho-RcsB and an increase in *cps* expression. On the other hand, in the absence of an activating signal, the phosphatase activity of RcsC would result in a net dephosphorylation of phospho-RcsB, leading to low levels of *cps* expression. It is possible to isolate *rcsC* mutant strains that have high levels of *cps* expression in the absence of a signal, e.g., strains carrying *rcsC137*, and it has been suggested that these mutants have lost the phosphatase activity associated with RcsC (2). This is supported by the observation that the *rcsC137* allele is a recessive mutation that can be complemented with a wild-type copy of *rcsC* supplied in *trans*. We can use this observation to determine whether the *rcsC* mutants constructed during this study have retained some phosphatase activity. Strain SG20907 (*rcsC137 cpsB-lacZ*) (a gift from Susan Gottesman, National Institutes of Health) was transformed with either pPSG980 or the plasmids encoding the different *rcsC* alleles, and the cells were assayed for β -galactosidase activity. As expected, in the presence of the vector alone there is a high level of *cpsB-lacZ* expression, i.e., 1,175 U (Fig. 5). However, when *rcsC* is supplied in *trans* from pPSG980, the level of *cpsB-lacZ* expression is reduced 70-fold, confirming that wild-type *rcsC* can complement the *rcsC137* mutation. Expression of the *rcsCH463Q* allele also reduced the level of *cpsB-lacZ* expression, indicating that RcsCH463Q still retains some phosphatase activity, although the levels of phosphatase are apparently reduced compared to those for the wild-type protein. In contrast, expression of *rcsCD859Q* failed to complement *rcsC137*, suggesting that

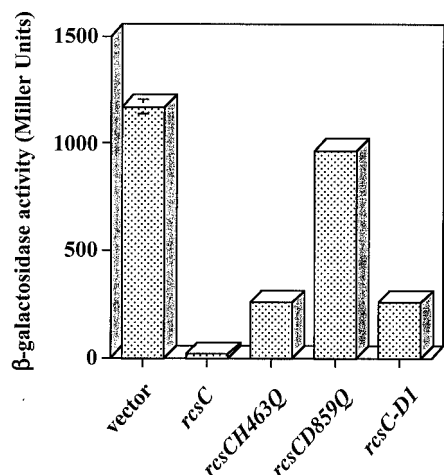


FIG. 5. RcsCD859 is required for complementation of the *rcsC137* allele. SG20907 (*rcsC137 cpsB-lacZ*) was transformed with either pTRC99a (vector) or derivatives carrying different *rcsC* alleles, as indicated. Cells were grown on LB agar at 30°C for 20 h and assayed for β -galactosidase activity. The results shown are the means of two independent measurements, and the error bars represent standard deviations. In some cases the error bars are too small to be shown on the graph.

RcsCD859 does not have phosphatase activity. This indicates that D859 is important for RcsC phosphatase activity. In support of this, we cloned the coding sequence for the D1 domain of RcsC (RcsC-D1) into pTRC99a, resulting in plasmid pMMG104, and found that the RcsC-D1 domain, alone, is sufficient for complementation of the *rcsC137* allele, suggesting that the phosphatase activity of RcsC is localized to the receiver domain (Fig. 5).

Effect of RcsCH463Q and RcsCD859Q on in vivo signaling by the wild-type RcsC protein. Our results indicate that RcsCH463Q and RcsC-D1 have a phosphatase activity that is unaffected by DjIA overproduction (Fig. 4A and data not shown). Therefore, these mutant RcsC proteins should have a negative effect on signaling by the wild-type RcsC protein. PSG1031 (*rcsC⁺ cpsB-lacZ*) was transformed with plasmids expressing the different alleles of *rcsC*, and the level of β -galactosidase activity in the cell was assayed in the presence of DjIA overproduction. The expression of the different *rcsC* alleles is controlled by the *trc* promoter, and, under noninducing conditions (i.e., in the absence of IPTG), the expression of *rcsCH463Q*, *rcsCD859Q*, and *rcsC-D1* had no effect on RcsC signaling (Fig. 6). This is in contrast to the complementation results obtained using *rcsC137*, where the presence of either *rcsCH463Q* or *rcsC-D1* complemented *rcsC137* under noninducing conditions (Fig. 5). However, when IPTG (isopropyl- β -D-thiogalactopyranoside) was added to 100 μ M, the expression of *rcsCH463Q* and *rcsC-D1* significantly reduced the level of *cpsB-lacZ* expression, supporting our hypothesis that both RcsCH463Q and RcsC-D1 retain the phosphatase activity of RcsC (Fig. 6). However, the expression of *rcsCD859Q* failed to reduce *cpsB-lacZ* expression in the presence or absence of IPTG, confirming that RcsCD859Q has no phosphatase activity.

We used both *rcsC137* and DjIA overproduction as tools to determine whether mutant derivatives of RcsC can interfere

with normal signaling. From these results it is interesting that the level of mutant RcsC proteins required to perturb signaling is dependent on the signal used to activate the pathway. In the *rcsC137* background, basal levels of expression of the *rcsC* mutant alleles from plasmids pPSG980H463Q and pMMG104 affected signaling (Fig. 5). On the other hand, when DjIA overproduction is used as the activating signal, higher levels of expression of *rcsCH463Q* and *rcsC-D1* (achieved by the addition of 100 μ M IPTG) are required before any effect on *cpsB-lacZ* expression is observed (Fig. 6). This difference in sensitivity can be simply explained by the fact that, in the latter case, DjIA (the signal) is overproduced. Therefore, assuming that DjIA interacts with RcsC, it is not unreasonable to expect that higher levels of both RcsCH463Q and RcsC-D1 will be required to interfere with the normal interaction between DjIA and RcsC. The fact that the overproduction of RcsCD859Q has no effect on *cpsB-lacZ* expression (Fig. 6) can be attributed to the fact that RcsCD859Q does not appear to have any phosphatase activity (Fig. 5).

Intermolecular complementation of RcsC mutants in vivo.

We were interested in testing whether the *rcsCH463Q* and *rcsCD859Q* alleles could complement each other in vivo. To carry out this experiment, both alleles need to be expressed at the same time in PSG1038 (*rcsC52::Tn10 cpsB-lacZ*). Plasmid pTRC99a, used to construct both pPSG980H463Q and pPSG980D859Q, carries the *bla* gene, encoding ampicillin resistance. Therefore, the *rcsCD859Q* allele was cloned into the pEH3 vector, which carries the *cat* gene and which encodes resistance to chloramphenicol, resulting in plasmid pBMM110. Both pPSG980H463Q and pBMM110 have pUC-based origins, and cells carrying both plasmids can be selected on medium containing both ampicillin and chloramphenicol. In this experiment, constitutive 5- to 10-fold overproduction of DjIA was achieved using kanamycin resistance-encoding

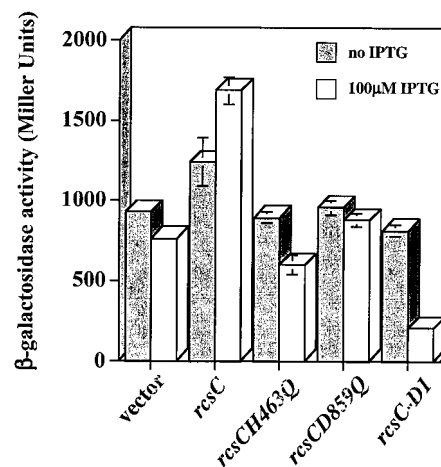


FIG. 6. RcsCH463Q and RcsC-D1 interfere with normal signaling from the RcsC protein. PSG1031 (*rcsC⁺ cpsB-lacZ*)/pPSG961-31 (*paraBAD-djIA*) was transformed with either pTRC99a (vector) or derivatives carrying different mutant alleles of *rcsC*, as indicated. Cells were cultured for 20 h at 30°C on LB agar supplemented with 0.2% L-arabinose and assayed for β -galactosidase. The results shown are the means of four independent measurements, and the error bars represent standard deviations. In some cases the error bars are too small to be shown on the graph.

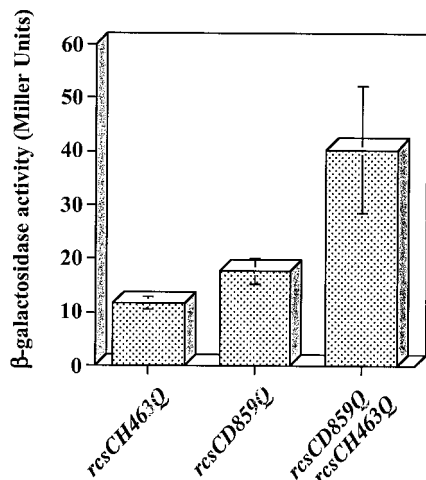


FIG. 7. In vivo intermolecular complementation between *rcsCH463Q* and *rcsCD859Q*. PSG1038 (*rcsC52::Tn10 cpsB-lacZ*)/pPSG957 (*djlA⁺*) was transformed with either pPSG980H463Q (*rcsCH463Q*), pBMM110 (*rcsCD859Q*), or both plasmids together (*rcsCH463Q rcsCD859Q*). Cells were cultured for 20 h at 30°C on LB agar and assayed for β -galactosidase activity. The results shown are the means of four independent measurements, and the error bars represent standard deviations.

pACYC-based plasmid pPSG957 (unpublished data). Therefore, PSG1038 carrying the pPSG957 plasmid was transformed with pPSG980H463Q, pBMM110, or both plasmids together, and the cells were tested for their ability to induce *cpsB-lacZ* expression in the presence of DjlA overproduction. As expected the presence of either the *rcsCH463Q* or the *rcsCD859Q* allele resulted in no increase in *cpsB-lacZ* expression (Fig. 7). However, when both alleles were expressed together, there was a small increase (approximately threefold) in the level of *cpsB-lacZ* expression, suggesting that RcsCH463Q and RcsCD859Q show some intermolecular complementation (Fig. 7). Unfortunately, due to the fact that both pPSG980H463Q and pBMM110 have the same type of origin, this system does not permit quantitative estimations of the level at which intermolecular complementation occurs. Moreover, the relatively large error bars seen in Fig. 7 might be indicative of bacterial populations that contain different relative levels of each plasmid. However, these data can be used to infer that RcsC is probably active as a multimer (most likely a dimer) and that, during signaling, phosphoryl groups can be transferred between H463 and D859 on different RcsC monomers.

Conclusions. RcsC is predicted to be a hybrid sensor kinase, and computer analyses implicates RcsCH463 and RcsCD859 in signaling. In this study we show, for the first time, that RcsCH463 and RcsCD859 are required for the induction of *cps* expression. Therefore, as for LuxN in *Vibrio harveyi*, both the transmitter domain (H1) and the receiver domain (D1) of RcsC are required for signaling, and this supports the role of RcsC as a hybrid kinase. We propose that under activating conditions, e.g., DjlA overproduction, dessication, or increased

osmolarity (11, 13), RcsC autophosphorylates on the conserved His residue (H463) in its transmitter domain, H1 (Fig. 5). This phosphoryl group is then transferred to the conserved Asp residue (D859) in the receiver domain, D1, of RcsC. RcsC has also been shown to repress *cps* expression, and we have shown that this negative activity appears to be localized to the D1 receiver domain and requires the D859 residue. We propose that this negative effect on *cpsB-lacZ* expression is, in fact, a phosphatase activity associated with the RcsC sensor protein, and biochemical studies to support this model are under way.

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