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Isolation and Characterization of *rcsB* Mutations That Affect Colanic Acid Capsule Synthesis in *Escherichia coli* K-12

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Regulation of colanic acid polysaccharide capsule synthesis in *Escherichia coli* requires the proteins RcsC and RcsB, in addition to several other proteins. By sequence similarity, these two proteins appear to be members of the two-component sensor-effector regulatory family found in bacteria. The present study characterizes the functional domains of RcsB. We have isolated mutations in *rcsB* that are able to suppress an *rcsC* "up" mutation (i.e., leading to increase in *cps* transcription) that normally results in constitutive expression of the capsule. In addition, constitutive capsule mutations in *rcsB* have been isolated. From the characterization of the mutants and by analogy to the three-dimensional structure of CheY, we have begun to define different domains of RcsB and to assign functions to them. A few of the constitutive capsule mutations were localized in an acidic pocket that has been proposed to play a crucial role in phosphorylation of RcsB. As seen in other two-component systems, an aspartate-to-glutamate substitution at the presumed site of phosphorylation of RcsB resulted in constitutive capsule expression. Lastly, several of our *rcsB* mutants were found to be allele specific (*rcsC137* specific) for *rcsC*, suggesting a physical as well as functional interaction between RcsC and RcsB proteins.

Capsule production is a characteristic shared among many microorganisms. This extracellular layer of polysaccharide functions in many ways to aid in the survival of bacteria in various hostile environments. Among the capsule's varied functions are evasion of host immune defenses, prevention of desiccation, and microcolony formation, which includes providing a nutrient-rich environment for bacteria (5, 6). *Escherichia coli* can synthesize over 70 different capsules (17). One of these capsules, colanic acid or M antigen, causes cells to become extremely mucoid (10). Colanic acid polysaccharide capsule synthesis in *E. coli* is encoded by the *cps* gene cluster, whose products function in polymerization, transport, and modification of the polysaccharide (24, 36).

Based on genetic studies, sequence analysis, and preliminary biochemical characterization, a model for regulation of capsule synthesis has been proposed elsewhere (14). According to this model, there are two pathways that regulate the synthesis of colanic acid capsular polysaccharide. Lon and RcsA compose one regulatory pathway. Lon is an ATP-dependent protease that degrades several proteins, including RcsA, which is a positive regulator of capsule synthesis (40). RcsA is, therefore, limiting for capsule expression. The second point of control is provided by a two-component, environmentally responsive, regulatory pair of proteins (14, 38). Sequence similarity identifies the two essential components of this pathway, RcsC and RcsB, as sensor and effector proteins, respectively (12, 38). By analogy to other two-component regulators, RcsC may be a membrane kinase protein that activates RcsB by phosphorylating it and deactivates RcsB by dephosphorylating it. In most of the well-studied cases, the sensor also dephosphorylates the effector (19). RcsC may be phosphorylating RcsB only after receiving a certain environmental signal(s). The phosphorylated and activated RcsB protein may then stimulate transcription of the target *cps* genes (14, 38). Although it is known that RcsB is absolutely required for capsule expression, phosphorylated RcsB activates the *cps* genes to the maximum level only when RcsA is present (3, 14, 38). Both RcsA and RcsB have been shown to be members of a family of regulatory proteins defined as the LuxR group (39). Members of this family bind DNA and alter transcription by activating or repressing it. In addition, recent studies have suggested that RcsA and RcsB form heterodimers (21). These results suggest that RcsA and RcsB may bind the *cps* promoter region together and activate transcription.

RcsF, a putative membrane protein, has been proposed to be a kinase that may exert its action through RcsB, possibly by phosphorylation. However, RcsF has no significant sequence similarity with any known sensor proteins (9). The presence of a multicopy $rcsC^+$ plasmid in cells carrying a multicopy $rcsF^+$ plasmid markedly reduces the level of mucoidy (9), which may indicate that RcsC acts as a phosphatase enzyme that dephosphorylates RcsB, thereby inactivating it. A mutation in *rcsC*, *rcsC137*, supports this proposed phosphatase activity of RcsC. *rcsC137* is a recessive mutation which results in increased expression of the *cps* genes. It has been suggested (38) that this mutant lacks phosphatase activity and, therefore, is locked in a conformation that leads to constitutive activation of RcsB. In this model, RcsB is phosphorylated by other sensor kinases by cross-talk or perhaps by a low-level kinase activity that is still present in the mutant RcsC protein. Therefore, RcsC may exert both a positive effect and a negative effect on expression of the *cps* genes through RcsB. The results from our biochemical experiments suggest that RcsB can be phosphorylated by RcsC and another sensor protein, FixL, by cross-talk phenomena (unpublished results).

From the model, we propose that RcsB has three functions, namely, interaction with RcsC, interaction with RcsA, and interaction with the *cps* promoter. Because of the crucial functions of RcsB in activating *cps* expression, it was critical to

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant genotype	Source or reference
MC4100-derived strains		
SG20250		12
SG20644	*lon-100 rcsA62 zed-650*::Tn*10 cpsB10*::*lac*-Mu-*imm*λ	3
SG20699	*lon-100 rcsB28*::ΔTn*10 cpsB10*::*lac*-Mu-*imm*λ	3
SG20780	Δ*lon-510 cpsB10*::*lac*-Mu-*imm*λ	3
SG20781	*cpsB10*::*lac*-Mu-*imm*λ	3
SG20803	*rcsC137 ompC*::Tn*5 cpsB10*::*lac*-Mu-*imm*λ	3
SG21101	*lon-100 rcsB28*::ΔTn*10*	3
SG21168	*rcsC10*::Δ*kan*	S. Gottesman
VS2032	Δ*lon510 rcsA51*::Δ*kan*	35
VS20183	*rcsB62*::Δ*kan lon100 cps-3*(E)::Tn*10*	37
VS20189	*rcsB62*::Δ*kan lon510 cpsB10*::*lac*-Mu-*imm*λ	SG20780 + P1(VS20183)
VS20190	*rcsA51*::Δ*kan cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(VS2032)
VS20191	*rcsB62*::Δ*kan cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(VS20183)
VS20260	*rcsA62* (*rcsA**) *zed-650*::Tn*10 cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(SG20644)
VS20299	*rcsC10*::Δ*kan cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(SG23003)
VS20300	*rcsB11*::ΔTn*10 rcsC137 ompC*::Tn*5 cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(SG12025)
VS20303	*rcsA62 rcsB62*::Δ*kan zed-650*::Tn*10 cpsB10*::*lac*-Mu-*imm*λ	VS20260 + P1(VS20183)
VS20305	*rcsB11*::ΔTn*10 rcsC137 ompC*::*Tn5*	MC4100 + P1(SG12025)
VS20555	*imm*^{21} *rcsB101*::*lacZ*	36
VS20322	*rcsC rcsB cpsB10*::*lac*-Mu-*imm*λ	This work
VS20323	*rcsA rcsB cpsB10*::*lac*-Mu-*imm*λ	VS20190 + P1(SG20699)
VS20324	*rcsF cpsB10*::*lac*-Mu-*imm*λ	SG20781 + P1(FG118)
VS20325	*rcsF rcsB cpsB10*::*lac*-Mu-*imm*λ	VS20324 + P1(SG20699)
Strains from other backgrounds		
C600	*thr leu thi lacY supE rfbD fhuA*	3
SG12025	*rcsB11*::ΔTn*10 rcsC137 ompC*::Tn*5*	3
N4956	*thr leu* r$^-$ m$^+$ *thi lacY supE rfbD fhuA*	3
LE30	F$^-$ *mutD5 rpsL azi galU95*	7
FG118	*rcsF*::Δ*kan*	9
Phage derivatives		
SB19i	*imm*^{21} *rcsB*$^+$	3
B53	*imm*^{21}	S. Gottesman
B482	*imm*^{21}	S. Gottesman
SY14	*imm*λ *c*I857 *rcsB14*::ΔTn*10*	3
Plasmids		
pJB100	*rcsB*$^+$ *bla*$^+$ (pBR322 + *rcsB*$^+$)	3
pJB201	*rcsC*$^+$ *bla*$^+$ (pUC19 + *rcsC*$^+$)	3
pVS102	pACYC184 + *rcsB*$^+$	37
pWPC101	pACYC184 + *rcsBD56N*	S. Gottesman

investigate structure-function relationships of this effector protein. In this work, we characterize the functional domains of RcsB by genetic analyses. Two categories of mutations were isolated based on their effect on *cps* expression. We have taken advantage of the three-dimensional model of CheY in characterizing the different *rcsB* mutations.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth conditions. The *E. coli* K-12 strains, plasmids, and bacteriophages used in this work are listed in Table 1. All strains are derivatives of MC4100 (34) unless otherwise noted. Generally, cells were grown in Luria-Bertani (LB) broth at 37°C with shaking or on LB or tryptone broth (TB) agar plates. When necessary, media were supplemented with antibiotics at the following concentrations: ampicillin, 125 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 15 μg/ml. *rcsA*, *rcsB*, and *rcsC* mutations were introduced into the strains by P1 transduction with mini- or full Tn*10* or Tn*5* transposons that were closely linked or inserted into these genes. P1 transductions were performed as described by Miller (25). Strains containing the *rcsC* multicopy plasmid pJB201 could be grown to an optical density at 600 nm of only 0.4 before the cells began to lyse. Strains containing the *cpsB10*::*lacZ* fusions were used to monitor capsule expression from the *cps* genes.

In addition to the strains described above, an *rcsC rcsB cpsB*::*lacZ* strain was constructed. To make this strain, SG21168 (*rcsC10*::Δ*Kan*) was lysogenized with phage SY14 (*rcsB*::Δ*Tn10*). This phage is a λD69 derivative and, therefore, is Int$^-$ and unable to lysogenize by site-specific recombination at *att*. Lysogenization of this phage occurs by homologous recombination with the *rcsB* region of the chromosome. The resulting lysogens were screened for resistance to kanamycin (Kan^r) and tetracycline (Tet^r). A Kan^r Tet^r isolate was grown at 42°C to early log phase to induce the SY14 phage, plated on LB-kanamycin agar, and incubated at 42°C. Kan^r colonies were tested for tetracycline resistance. Loss of SY14 was confirmed by cross-streaking against an SY phage as described by Silhavy et al. (34). A P1 lysate on this SY^s Kan^r Tet^r strain was made and used to transduce SG20781 (*cpsB*::*lacZ*) to Kan^r and was subsequently tested for Tet^r. The strain's genotype was confirmed by complementing the mutations with an *rcsC137 rcsB*$^+$ phage, and the strain was labeled VS20322.

Isolation of λ*rcsB*^{mut}. A λ*rcsB*$^+$ transducing phage (SB19) has been described previously (3). SB19 was mutagenized by growing the phage in a *mutD* strain (LE30) as described previously (7, 34). Twelve pools of mutagenized λ*rcsB* lysate were made (34). Primary screening of the mutagenized λ*rcsB* phage lysates was performed by screening the mutagenized λ*rcsB* lysates on TB agar plates overlaid with top agar containing VS20191 (*rcsB lon*$^+$ *cpsB*::*lacZ*) cells and 20 μl of 40-μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Wild-type λ*rcsB* (SB19) phage produced white plaques in this background assay. Mutant phages that yielded light, medium, or blue plaques were purified by picking up single plaques in TMG buffer (34) and vortexing to make a phage suspension. This suspension was spotted onto top agar containing the VS20191 strain and streaked with a platinum wire having a fine ball-shaped end until a homogeneous population of plaques was achieved. Plate lysate stocks were then made from well-isolated plaques by a protocol described by Silhavy et al. (34). A total of 90

TABLE 2. Characterization of *rcsB* up mutations

Mutant no.	Mutation	Fold increase[b]	Capsule expression in the following host strains[a]:						
			VS20191 (*rcsB*)	SG20781 (*rcsB*$^+$)	VS20322 (*rcsC rcsB*)	VS20323 (*rcsA rcsB*)	VS20300 (*rcsC137 rcsB*)	VS20189 (*lon rcsB*)	VS20325 (*rcsF rcsB*)
SB19		1.0	−	−	−	−	+++	+++	−
B73	D10N	1.0	+++	++++	++++	+++	+++	+++	+++
B36	D11N	3.9	+++	++	++	+++	++	+++	+
B81	D11A	6.5	++++	++++	++++	++++	+++++	+++	++++
B60	H12Y	2.0	+++	+++	++	++	++	+++	+
B26	F17L	1.8	+++	+++	++	++	++++	+++	++
B23	I19V	1.9	++	++	+	++	++	+++	+
B30	D56E	3.6	++++	++++	++++	++++	++++	+++	++++
B66	P113L	1.7	+++	+++	++	++++	+++	+++	++
B70	P117S	2.0	+++	++++	+++	+++	+++++	++++	++++
B82	P117L	8.3	++++	+++	+++	++++	+++	+++	+++
B88	K118E	1.6	++	+	+	+	++	+++	+
B8	A119T	7.4	+++	++	+++	++	+++++	+++	+++
B53	A119V	1.6	++	+	+	++	+++++	+++	+
B91	K187R	3.5	++++	++++	+++	+++	++++	++	+++
B33	E196K	1.4	++	+	++	++	+++	++	+
B46	D198N	2.8	+++	+++	+++	+++	+++	++	+++
B63	S206F	2.9	+++	+++	++	++	+++++	++++	++

[a] All strains are *cpsB*::*lacZ*, and the levels of *cps*::*lacZ* expression were estimated visually by plaque phenotypes on TB agar containing X-Gal. −, white; +, very light blue; ++, light blue; +++, blue; ++++, dark blue; +++++, very dark blue.

[b] Fold increase indicates increase in specific units of β-galactosidase (24) in comparison to those for wild-type *rcsB*. Cells were grown in tryptone broth at 37°C. The results are averages of at least three determinations.

λ*rcsB* mutants were isolated and designated *rcsB* "up" mutants (i.e., resulting in increased levels of *cps* transcription).

The mutagenized λ*rcsB* phage lysate pools were also subjected to primary screening in an *rcsC137 rcsB cpsB*::*lacZ* strain (VS20300). Mutant phage isolates that produced lighter blue plaques than those of the wild type on TB X-Gal plates were purified by restreaking. Lysates of isolated plaques were made as described for *rcsB* up mutants. A total of 52 λ*rcsB* mutants were isolated and labeled *rcsB* "down" mutants (i.e., resulting in decreased levels of *cps* transcription).

Transfer of mutations from lambda to the bacterial chromosome. To obtain the newly created λ*rcsB* mutations in single copy in a cell so that more-consistent regulation results could be obtained, the *rcsB* mutations were transferred to the chromosome by lysogenization. Since the λD69 derivatives carry chromosomal DNA at the *Bam*HI site within the *int* gene, these phages are Int$^-$ and cannot lysogenize by site-specific recombination into the attachment site for λ (26). Therefore, selection for lysogens with the SB19 phage carrying the *rcsB* gene should result in a single crossover event leading to insertion of the phage into the chromosome by homologous recombination. The up mutants were lysogenized into VS20191 (*rcsB62*::Δ*kan cpsB*::*lacZ*), and the down mutants were lysogenized into strain VS20300 (*rcsB11*::ΔTn*10 rcsC137 ompC*::Tn*5 cpsB*::*lacZ*) according to the lysogenization procedure described by Silhavy et al. (34). In general, the *rcsB* down mutant lysogens were unstable; in a few of the lysogens, up to 5% of the colonies that grew on TB plates containing X-Gal had mutated to a lighter color, and many had mutation frequencies of approximately 1%, which were calculated as ratios of numbers of lighter blue plaques to the total numbers of plaques. The mutation frequency was much higher when the lysogens were streaked on rich medium, such as LB, and there was a high number of mutants when they were streaked from stabs. Therefore, when lysogens were streaked from stored cultures, they were restreaked several times in order to achieve a relatively homogeneous population before subjecting the strains to β-galactosidase assays.

β-Galactosidase assays. β-Galactosidase assays of the λ*rcsB*^{mut} lysogens were performed as described by Miller (25). For each mutant lysogen, the assay was performed in triplicate, and the average β-galactosidase activities were calculated. Approximately 33 λ*rcsB*^{mut}/*rcsB cpsB*::*lacZ* lysogens that showed β-galactosidase values significantly greater than those of the wild-type parent lysogen were retained as *rcsB* up mutants. In addition, one lysogen that had the same specific units of β-galactosidase that the wild type (D10N) did was retained, since this mutant had yielded medium blue plaques. Similarly, approximately 21 λ*rcsB*^{mut}/*rcsC137 rcsB cpsB*::*lacZ* lysogens demonstrating β-galactosidase values that were less than that of the λ*rcsB*$^+$ lysogen were selected as *rcsB* down mutants.

DNA manipulations. The mutant *rcsB* genes were amplified from lysates by using the primers B18N2 (5′-GTGCAACTGGCGCAGG), which is located approximately 200 bp upstream of the putative RpoN-like promoter, and C19G8 (5′-ATCCGGCATTCAGTGC), which is located downstream of the *rcsB* gene, by PCR. The amplified DNA product was purified from the primers with a PCR purification kit (Qiagen). For each mutant, two reactions were performed, and the forward and reverse strands of the gene were sequenced to eliminate the possibility of identifying mutations due to PCR. Sequencing was performed with an Applied Biosystems 373 DNA sequencer. The sequences were analyzed with the Gap program of the University of Wisconsin Genetics Computer Group (8) software package.

Characterization of plasmid pWPC101. pWPC101 was constructed previously by site-directed mutagenesis (11) in order to replace the aspartate residue at amino acid 56 of RcsB with asparagine. To confirm this change, pWPC101 was sequenced by the dideoxy chain termination method (32) by using the Sequenase version 2.0 sequencing kit (U.S. Biochemicals). Transformation of the plasmid into various strains was performed by the method described by Cheung et al. (4). Transformants were selected on LB ampicillin plates that were spread with 20 μl of 40-mg/ml X-Gal solution. Plasmid pVS102 carrying the wild-type *rcsB*$^+$ gene was used as a control.

RESULTS

Isolation of *rcsB* up mutations. It has been shown that null mutations in *rcsB* eliminate synthesis of colanic acid capsule and *cps*::*lacZ* expression in either *lon* or *rcsC137* strains (13). These mutations can be complemented by a λ*rcsB*$^+$ transducing phage. To isolate mutations in *rcsB*, λ*rcsB*$^+$ was mutagenized in an *mutD* strain (LE30) and *rcsB* up mutants that stimulated *cpsB*::*lacZ* expression in an *rcsB lon*$^+$ background (VS20191) were isolated. The mutation frequencies ranged from 0.45 to 1.6%, as estimated by the ratio of the number of darker-blue plaques to the total number of plaques as expected from *mutD* mutagenesis. Approximately 90 of these mutants that produced plaques that were darker blue than wild-type *rcsB* plaques in this primary screening were lysogenized in an *rcsB lon*$^+$ *cpsB*::*lacZ* strain (VS20191). These lysogens were assayed for β-galactosidase activity in order to quantitate the effect of the mutation on the transcription of the *cps* genes. Approximately 33 *rcsB* mutations which resulted in a significant increase in *cps* transcription compared to that of the wild type were retained for further study. The fold increase column in Table 2 shows the increase in the β-galactosidase values of the *rcsB* up mutants in comparison to those of the wild-type *rcsB* (SB19) lysogen. In general, the β-galactosidase values correlated with plaque color, although there were a few exceptions, e.g., mutant B73 (carrying the D10N mutation) demon-

strated no change in β-galactosidase value in spite of its strong plaque phenotype. These exceptions may be related to the instability of the lysogens. The instability of the *rcsB* down lysogens could be due to the excision of the prophage which results in chromosomal DNA that looks like the parental strain (VS20300).

Sequencing of *rcsB* up mutants. Sequencing of the λ*rcsB* up mutants was performed in order to locate the mutations. All of the *rcsB* up mutants subjected to sequencing contained point mutations within the open reading frame (ORF) of *rcsB* (Table 2). There were 17 different mutations among the 34 mutants that were sequenced, and several of the mutations were independently isolated several times. Mutations that were isolated more than once included D10N, D11N, F17L, I19V, D56E, P113L, P117S, A119T, A119V, D198N, and S206F. One of the most interesting mutations was D56E, since this is the proposed site of phosphorylation. When the same mutation was isolated more than once, only one mutant was chosen for further study. Because all of the point mutations were located within the 651 bp of the ORF of *rcsB*, the levels of the mutant RcsB proteins were expected to be very similar to that of the wild type. In fact, no significant differences in the amounts of RcsB were observed (data not shown). Therefore, the increases in *cps* expression levels in these mutants are not due to increases in the levels of transcription or translation of *rcsB* in the cell.

In addition to the point mutations identified by sequencing, analysis also showed that there was an error in the published *rcsB* sequence (38). The correct sequence should have an additional cytosine residue at nucleotide 799 with respect to the published *rcsB* sequence. This C residue lies downstream of the ORF of *rcsB* within one of the two direct repeats found between the *rcsB* and *rcsC* genes (9, 38).

Secondary screening of *rcsB* up mutants. The *rcsB* up mutants were subjected to several secondary screens by streaking the selected individual λ*rcsB* up mutant phages on specific strains carrying mutations in the regulatory genes related to capsule expression (Table 2). The rationale for using the individual strains is given below.

Screening in an *rcsB*$^+$ *cpsB*::*lacZ* strain (SG20781) was performed to test for dominance. If the mutations were recessive, white plaques would be obtained due to suppression by *rcsB*$^+$. However, as expected for gain-of-function mutations, all of the *rcsB* up mutations were found to be dominant to wild-type *rcsB*$^+$, as seen in Table 2. Some of the mutations appeared to be codominant (D11N, P117L, K118E, A119T, A119V, and E196K). These results suggest that the *rcsB* up mutations stimulate *cps*::*lac* expression by (i) attaining a more active form due to enhanced interaction of RcsB with RcsC, (ii) enhanced interaction of RcsB with RcsA, or (iii) improved interaction of RcsB with the *cps* promoter.

For one of the secondary screens, an *rcsC rcsB cpsB*::*lacZ* (VS20322) strain was used in order to distinguish between mutations that were RcsC independent (blue on LB plus X-Gal) and those that were RcsC dependent (lighter blue on LB plus X-Gal) (Table 2). In the RcsC-dependent mutations, RcsB may be phosphorylated more efficiently or dephosphorylated less efficiently by RcsC compared to wild-type RcsB. The mutations, were classified as RcsC dependent or RcsC independent based on comparison with screening in the *rcsB cpsB*::*lacZ* strain. These *rcsB* up mutations may only be partially dependent on *rcsC*. The partial dependence on *rcsC* could be explained by cross-talk phenomena. Thus, in the absence of *rcsC*, it is possible that RcsB is phosphorylated by other sensor kinase proteins, although to a lesser extent. *rcsB* up mutants D11N, H12Y, F17L, I19V, P113L, P117L, K118E, A119V, K187R, and S206F were found to be RcsC dependent, whereas D10N, D11A, D56E, P117S, A119T, E196K, and D198N were RcsC independent. We believe that although the *rcsA* or *rcsC* dependence of certain *rcsB* up alleles is variable by only a small degree in most cases, these differences were significant.

The *rcsB* up mutants were subjected to screening in an *rcsC137 rcsB cpsB*::*lacZ* strain (VS20300). *rcsC137* increases the levels of *cpsB*::*lacZ* expression and capsule synthesis in *lon*$^+$ hosts. Studies with *rcsA rcsC137* and *rcsB rcsC137* double mutants have indicated that both *rcsA*$^+$ and *rcsB*$^+$ are essential for high-level expression in *rcsC137* hosts, suggesting that the *rcsC137* gene product does not act as a bypass for the RcsA-RcsB pathway but that it may affect the activities of these proteins. Although some residual capsule synthesis is observed in an *rcsC137 rcsA*-null mutant strain, *rcsC137 rcsB*-null mutants eliminate capsule expression (3). This suggests that RcsC primarily exerts its action through RcsB. RcsC137 is presumably mutated such that it lacks phosphatase activity and, hence, may be locked in a state that leads to constitutive activation of RcsB (38). Screening in the *rcsC137 rcsB cpsB*::*lacZ* strain was performed to test whether any of the *rcsB* up mutations were specific for rcsC$^+$. Since the *rcsC137* mutation results in increased capsule expression with wild-type *rcsB*, a decrease in the level of *cpsB*::*lacZ* expression in an *rcsC137 cpsB*::*lacZ* strain with an *rcsB* up mutant phage suggests that the particular *rcsB* mutation is specific for *rcsC*$^+$. As shown in Table 2, the D11N, H12Y, and P117L mutations appeared to result in a decrease in *cpsB*::*lacZ* expression compared to expression in the *rcsC*$^+$ *cpsB*::*lacZ* strain. Therefore, these may represent *rcsB* mutations that are allele specific for *rcsC*$^+$. As expected, these three mutations are RcsC dependent (Table 2).

The *rcsB* up mutations were also subjected to secondary screening in an *rcsA rcsB cpsB*::*lacZ* strain (VS20323) to determine which of the *rcsB* up mutations are dependent on RcsA. Most of the *rcsB* up mutations were found to be RcsA independent except for H12Y, F17L, K118E, A119T, K187R, and S206F (Table 2).

Screening of *rcsB* up mutations was also performed with a *lon rcsB cpsB*::*lacZ* strain (VS20189). Since Lon, an ATP-dependent protease, degrades RcsA, the level of expression of the *cps* genes normally increases dramatically in *lon* mutant hosts (41). This pathway of stimulation does not appear to require activated RcsB, because *cpsB*::*lacZ* stimulation is independent of *rcsC* (41). Therefore, RcsC-RcsB interaction may not be required for increased capsule expression in *lon* mutations. However, because *lon* mutant cells still require RcsA-RcsB interaction for increased *cps* expression, *rcsB* up mutations mutated for improved interaction with RcsA should show increased *cps* transcription in the *lon rcsB cpsB*::*lacZ* strain. This class of *rcsB* mutations cannot be distinguished from wild-type *rcsB*, since wild-type *rcsB* also results in increased capsule expression in a *lon* mutant. Therefore, no change in *cpsB*::*lacZ* expression suggests that the mutations are independent of RcsA, which in fact was the case for most of the *rcsB* up mutations (Table 2). Only the S206F, I19V, P117S, K118E, and A119V mutations appeared to be dependent on RcsA by this screening. Two of these mutations (K118E and S206F) appeared to be dependent by screening in the *rcsA* mutant strain, but the other three did not. It is important to note that the plaques in the *lon* strain had an unusual morphology in that they were much larger and diffused; hence, it was difficult to interpret the plaque color confidently. In this case, therefore, only a downrating of at least two degrees or an uprating of two degrees should be considered significant. At this stage, we do not have a good explana-

tion for the K187R mutation that results in a decrease in the level of *cpsB*::*lacZ* expression by two degrees; this is being investigated further. These differences indicate that the system is complex and that the two screenings may be affected by different components involved in capsule regulation.

Lastly, the *rcsB* up mutations were subjected to secondary screening in an *rcsF rcsB cpsB*::*lacZ* strain (VS20325). RcsF has been proposed to act as a kinase of RcsB (9); therefore, it was important to test the mutations for dependence on RcsF. Most of the mutations were dependent on RcsF, with only six (D10N, D11A, D56E, P117S, A119T, and D198N) being independent (Table 2). Interestingly, the mutations that were RcsF dependent were also dependent on RcsC. This suggests that RcsF may act on RcsB at sites that are similar to those that are involved in interaction with RcsC.

Isolation of *rcsB* down mutations. Primary screening of the pools of mutagenized λ*rcsB*$^+$ phage in an *rcsC137 rcsB cpsB*::*lacZ* strain (VS20300) on LB agar containing X-Gal yielded plaque phenotypes that were lighter blue than those of wild-type λ*rcsB*$^+$. These phenotypes indicated suppression of the constitutive *rcsC137* mutation by mutant *rcsB*.

λ*rcsB*^{mut} plaques demonstrating phenotypes of a lighter blue color than that of the wild type were purified as *rcsB* down mutations. The mutation frequency ranged from 5 to 14%, which was much higher than expected. This may have been due in part to difficulty in distinguishing wild-type blue plaques from those that were only slightly reduced in the intensity of blue. Because of this, it is possible that some wild-type phages were mistakenly categorized as mutant phages. Therefore, isolation of the down mutations was comparatively more difficult than that of the up mutations.

As predicted, there were many more *rcsB* down mutations with white plaque phenotypes than those with light-blue phenotypes, since phages carrying an *rcsB* null mutation will fail to complement the *rcsC137 rcsB cpsB*::*lacZ* host. These white mutations were not tested further, under the assumption that they represented complete inactivation of the *rcsB* gene.

The pool of λ*rcsB* mutations which suppressed the *rcsC137* mutation in the primary screening was lysogenized in the *rcsC137 rcsB cpsB*::*lacZ* strain. β-Galactosidase assays of each of the lysogens were performed to quantitate the effect of the mutation on *cps* transcription. Twenty-one *rcsB* mutations that demonstrated a decrease in the level of *cps* transcription compared to that of the wild type were retained for further study.

Sequencing of *rcsB* down mutations. Among the 21 *rcsB* down mutations that were subjected to sequencing, only 3 mutations that contained mutations within *rcsB* were identified. All three of these had substitutions within the ORF of *rcsB*. Mutant A58 contains a point mutation which results in the substitution of serine for proline at amino acid no. 92 of RcsB (P92S), mutant A61 has a valine-to-alanine substitution at amino acid no. 85 (V85A), and mutant A38 contains a substitution of proline for leucine at amino acid residue no. 205 (L205P) (Table 3). Presumably, the other 18 isolates had mutations somewhere else on the phage or were unmutated phages. This supports the idea that the higher mutation frequency seen in the screening for down mutations was due to false-positive results.

None of the *rcsB* up and down mutations were found in the regulatory region of the *rcsB* gene, indicating that the phenotypic changes are due to alterations in the conformation of the RcsB protein rather than expression of the *rcsB* gene. Quantification of the amount of RcsB protein in the mutant lysogens did not show any significant differences in RcsB protein levels compared to those of the wild type (data not shown).

TABLE 3. Characterization of *rcsB* down mutations

Mutant no.	Mutation	β-Galactosidase[b]	Capsule expression in host strains carrying the following genes[a]:		
			rcsC137 rcsB	*lon rcsB*	*rcsA* rcsB*
SB19 (WT)		238	+++	++	+++
A38	L205P	21	++	++	++
A58	P92S	13	++	+	++
A61	V85A	1.5	++	+	++

[a] All strains were *cpsB*::*lacZ*, and the levels of *cps*::*lacZ* expression were estimated visually by plaque phenotypes on TB agar containing X-Gal. +, very light blue; ++, light blue; +++, blue.

[b] Specific units of β-galactosidase (25) in *rcsC137 rcsB cpsB*::*lacZ* lysogens. Cells were grown in tryptone broth at 37°C. The results are averages of at least three determinations.

Secondary screening of *rcsB* down mutations. The *rcsB* down mutations were screened in two strains to categorize them into different groups: those showing interaction with RcsA, interaction with RcsC, and interaction with the promoter.

The first strain used for secondary screening of the down mutations was *lon rcsB cpsB*::*lacZ* (VS20189). The rationale for using this strain was described under the secondary-screening results for the *rcsB* up mutations. This screen should distinguish *rcsB* mutations that are mutated for RcsC interaction from those affecting either RcsA interaction sites or *cps* promoter interaction sites. The P92S and V85A mutations demonstrated decreased *cps* expression in this strain compared to that of the wild type (Table 3), and therefore, they are likely to be altered in their ability to interact with RcsA. The *cpsB*::*lacZ* expression level of the L205P mutation is unchanged in this strain.

The second screening was performed in *rcsA62 rcsB cpsB*::*lacZ*. *rcsA62* is a dominant allele encoding RcsA*, which increases the level of capsule expression even in *lon*$^+$ hosts. RcsA* differs from wild-type RcsA in the substitution of methionine 145 by valine (39). It has been demonstrated that RcsA* is more stable than RcsA in *lon*$^+$ hosts only when RcsB is present, suggesting that RcsA* interacts better with RcsB (14, 39). Therefore, if the *rcsB* down mutation affects RcsB-RcsC interaction, no change from the wild-type phenotype would be seen. In this screening, the level of *cpsB*::*lacZ* expression in all three of the mutations was less than that of the wild type (Table 3). Therefore, the results from the screening of the P92S and V85A mutations in this strain support the earlier interpretation that these mutations are affected in their interaction with RcsA. Classification of the L205P mutations is not clear. In summary, based on the secondary-screening results, the mutations were assigned to two different categories, with L205P in one group and the P92S and V85A mutations in the other.

Phenotypic effect of the RcsBD56N mutation. Because we obtained mutations of the up class at residue 56 (D56E), we wanted to examine the effect of another mutation at this position that had been constructed previously. This mutation was obtained by site-directed mutagenesis and resulted in a substitution of the conserved aspartic acid residue at amino acid 56 of RcsB with asparagine (D56N) (11). By analogy to other effector proteins, this is the presumed site of phosphorylation (1, 20). To analyze the phenotypic effect of this mutation, the plasmid carrying this mutated *rcsB*, pWPC101, was transformed into four different strains. The results of these transformations are shown in Table 4. The *rcsC137* mutation has been shown to increase the level of capsule expression even in *lon*$^+$ hosts (3). When *rcsC137 rcsB* and *rcsC137 rcsB cpsB*::*lacZ*

TABLE 4. Phenotypic effect of the RcsBD56N mutation on capsule expression[a]

Host strain and genotype	Phenotype with:	
	pVS102 (*rcsB*$^+$)	pWPC101 (*rcsB-D56N*)
VS20305 (*rcsC137 rcsB cps*$^+$)	Mucoid	Nonmucoid
VS20189 (Δ*lon rcsB62*::Δ*kan cps*::*lacZ*)	Dark blue	White
VS20190 (*rcsA51*::Δ*kan cps*::*lacZ*)	Dark blue	White
VS20300 (*rcsC137 rcsB11*::Tn*10* *ompC*::Tn*5 cps*::*lacZ*)	Blue	White

[a] Color phenotype determined by visual inspection of colonies growing on LB agar with ampicillin and X-Gal.

cells were transformed with a wild-type *rcsB* gene (pVS102), the level of capsule expression was high, whereas cells transformed with the mutant *rcsB* gene (pWPC101) showed no capsule expression. Similar results were seen when a *lon rcsB cpsB*::*lacZ* strain was transformed with the two plasmids; the wild-type *rcsB* gene allowed high levels of *cps* expression, and the mutant *rcsB* gene did not. It has been shown previously that in cells carrying *rcsB*$^+$ on a multicopy plasmid, the requirement for RcsA for capsule synthesis can be partially bypassed (3). A phenotypic effect similar to those in the *rcsC137* and *lon* strains was observed in the *rcsA* strain; although wild-type *rcsB* resulted in elevated *cps* expression, mutant *rcsB* had no positive effect on *cps* expression. From these results, it is evident that the D56N form of RcsB fails to activate transcription from the *cps* genes, whereas wild-type *rcsB* retains this ability.

DISCUSSION

We have isolated *rcsB* mutations that both decrease and increase the level of *cps* transcription (down and up mutations, respectively) compared to that of the wild-type gene. The *rcsB* down mutations suppressed an *rcsC* up (*rcsC137*) mutation, whereas the *rcsB* up mutations stimulated capsule expression in a wild-type background. Based on secondary screening in different mutant strains and sequence analysis, the mutations were categorized into different groups. According to the model, these functions include (i) interaction with RcsC, (ii) interaction with RcsA, and (iii) interaction with the *cps* promoter. In our interpretation, some of the mutations are affected in their interactions with RcsC and some of these demonstrate allele specificity.

RcsB shares sequence similarity in its N-terminal domain with effectors such as CheY, OmpR, NtrC, PhoB, VirG, FixJ, AlgR, DegU, UhpA, and NarL. This domain also includes the two conserved aspartic acid residues (amino acids 11 and 56 of RcsB) (38). RcsB has more extended sequence similarity in its C-terminal domain to the subfamily of regulators comprised of FixJ, NarL, DegU, and UhpA (14, 38).

Because of the conservation among the many response regulators, the domains defined by the *rcsB* mutations were compared to the known crystallographic structure of CheY, the effector protein involved in chemotaxis regulation (Fig. 1). CheY, which is made up of 128 amino acid residues, is compactly folded in a (β/α)5 manner, where all five β-strands are in a parallel β-sheet, with the topology β2β1β3β4β5. Much of the two outermost strands (β2 and β5) is solvent accessible (42).

Sequencing of the mutations revealed that the mutations were localized into four domains of RcsB. The first domain included amino acids 10 to 19 and 56. Upon alignment of RcsB with CheY by using the Gap program of the Genetics Computer Group software (8), D10 and D11 of RcsB aligned with the D12 and D13 residues of CheY, respectively (Fig. 1). These two aspartic acid residues are highly conserved among response regulators and, from the crystallographic structure of CheY, are known to contribute to the formation of the acidic pocket by projecting toward the site of phosphorylation. The D11A mutation of RcsB may confer a conformational change similar to that of the D13K mutation of CheY (1, 2), both of which result in constitutive phenotypes. It is possible that these two mutant proteins assume the active form without being phosphorylated. This is supported by the secondary screening showing that this mutation was *rcsC* independent. It is very likely that the D10N mutation has the same effect as that of the D11A mutation on conformation.

```
                  +++     + +
RcsB    1  ..MNNMNVIIADDHPIVLFGIRKSLEQIEWVNVVGEFEDSTALINNLPKLDA   50
           : ::::||    :   :|  |   : : | | |:||   ::| |
CheY    1  MADKELKFLVVDDFSTMRRIVRNLLKELGF.NNVEEAEDGVDALNKLQAGGY   51

                +                                 -     -
RcsB   51  HVLITDLSMPGDKYGDGITLIKYIKRH..FPSLSIIVLTMNNNPAILSAVLD   100
            ::|:|: ||      ||: |:|:|:    :  | ::::|:      : |::
CheY   52  GFVISDWNMPN...MDGLELLKTIRADGAMSALPVLMVTAEAKKENIIAAAQ   100

                        +   +++
RcsB  101   LDIEGIVLKQGAPTDLPKALAALQKGKKFTPESVSRLLL...           138
           : : | |:|      |   |  :    :
CheY  101   AGASGYVVKPFTAATLEEKLNKIFEKLGM                       130
```

FIG. 1. Sequence alignment of RcsB and CheY. The amino terminus of RcsB was aligned with the entire amino acid sequence of CheY (K02175) by using the Gap program of the Genetics Computer Group Sequence Analysis Package (8). Amino acids are designated by the single-letter code. Solid vertical line, identical amino acid residue; colon, conserved amino acid residue. The groups of conserved amino acids are as follows: nonpolar (ILMVAFW), polar uncharged (STY), aromatic (FWY), amide (NQ), basic (HKR), acidic (DE), and no group (G, C, and P). + and −, the amino acids that were mutated in the *rcsB* up and down mutations, respectively.

Based on secondary screening, the D10N and D11A mutations appeared to be constitutively active in promoting transcription of the *cps* genes in all of the strains. It is possible that the D11A form causes or allows formation of the active and phosphorylated conformation of the protein because of the nonpolar nature of alanine. Unexpectedly, when the D11 residue was changed to asparagine, it had a phenotype very different from that of the D11A mutation. The D11N mutation was still responsive to RcsC and RcsF, indicating that this change does not greatly alter the ability of RcsB to interact with RcsC or RcsF. This suggests that in the D11N mutation, the acidic pocket was still able to function. Interestingly, this mutant results in decreased *cps* expression in the *rcsC137* strain compared with that of wild-type *rcsC*, suggesting that it interacts better with wild-type RcsC than RcsC137. This indicates that D11N is specific for *rcsC*$^+$ (RcsB-RcsC interaction) and, hence, supports the proposed RcsC interaction function of RcsB. In brief, we speculate that D10 and D11 may be involved in forming an acidic pocket similar to CheY and probably aid in the interaction of RcsB with RcsC and/or RcsF. In addition, these two residues must play a critical role in RcsB function, since the D10N and D11N mutations were independently isolated several times.

The other mutations that are close to the domain defined by the D10 and D11 mutations, H12Y, F17L, and I19V, should be more distal to the acidic pocket, and as expected, they resulted in increased capsule expression (as seen by β-galactosidase values) to a lesser degree than that of the D11 mutations. Similarly to the D11N mutation, all three of these mutations are dependent on RcsC and RcsF. In addition, the H12Y mutation appears to be *rcsC137* specific for RcsC interaction, since it shows decreased expression in the *rcsC137* strain in-

stead of increased expression in the presence of wild-type RcsB.

The final mutation that defines the first domain was an alteration of the conserved aspartate residue, D56, that aligns with the known site of phosphorylation of CheY, D57 (31) (Fig. 1). This aspartate residue is conserved throughout the family of bacterial response regulatory proteins sharing sequence similarity at the amino-terminal region (28). This conserved aspartate has also been shown to be the site of phosphorylation in several response regulators, including VirG (18) and NtrC (19), and is presumed to play an important role in regulating responses to diverse environmental signals. By analogy to CheY, all of the *rcsB* mutations in the first domain are located in or distal to the acidic pocket of RcsB (Fig. 1). This is equivalent to the clustering of the CheY mutations near the site of phosphorylation (1, 22). This implies that the three aspartic acid residues of RcsB (D10, D11, and D56) are important for the protein, such that alterations of these residues may allow the protein to resemble the phosphorylated form of the protein.

Although phosphorylation is the required primary event in the activation of CheY (15, 16) and, presumably, all response regulators, phosphorylation and activation can be unlinked. The two most interesting types of phosphorylation mutations are (i) phosphorylatable but not active and (ii) nonphosphorylatable but constitutively active. A good example of the latter category is the RcsB D56E mutation, which appeared to be constitutive in all of the strains in which it was screened, indicating that it does not need RcsC, RcsF, or RcsA for its activity. Substitution of glutamate for the conserved aspartate has also been examined in other response regulators: CheY (1), VirG (29), and NtrC (20). In all of these cases, the change to glutamate resulted in constitutivity. In the nitrogen regulatory system, NtrC acquires a phosphate group from NtrB-phosphate (19, 27) and NtrC-P catalyzes ATP hydrolysis and, thereby, isomerization of closed complexes between the σ^{54} holoenzyme and the *glnA* promoter to open complexes (33). The site of phosphorylation of NtrC is aspartate 54 (19), and when it is changed to glutamate, this mutant NtrC activates transcription constitutively, in the absence of NtrB. The D54E substitution inactivates the phosphate-accepting activity of NtrC but mimics phosphorylation by allowing NtrC to hydrolyze ATP and to function as a transcriptional activator (20). Thus, the D56E form of RcsB, which activates transcription of the *cps* genes constitutively, will be interesting to test for phosphorylation by RcsC in vitro. We presume that it will be nonphosphorylatable.

The conserved aspartate residue of NtrC has also been mutated to alanine and asparagine. These forms of NtrC are not detectably phosphorylated in vitro and cannot activate transcription in vivo (20). This is similar to the in vivo results seen for the D56N form of RcsB encoded by pWPC101. The D56N form of RcsB needs to be tested for phosphorylation directly. It is likely that RcsB-D56N cannot be phosphorylated and, therefore, cannot activate transcription.

The second domain of RcsB is defined by the down mutations V85A and P92S and compares to CheY mutations that are located in the loop following the β4 strand that describe a spatially contiguous surface adjacent to the active site (30, 35). These two mutations flank the conserved T87 residue that has been proposed to function in CheY as the proton donor to the active site by contacting D57 in the presence of magnesium ions (23). Since our activity assays were performed in vivo with the V85A and P92S mutations and resulted in a very low level of capsule expression, these mutations must have a low level of activity even in the presence of Mg^{2+}. It is possible that these two residues help T87 contact D56 in RcsB, as with CheY. This domain may also be involved in interacting with RcsA, since these mutations resulted in a reduced level of *cps* expression even in a *lon* mutant strain.

The third domain included mutations that spanned amino acids 113 to 119. Although the CheY structure was used as a paradigm in assigning structure-function relationships for RcsB, the sequence similarity between these two proteins diverges after the conserved K109 residue. Therefore, the effect of mutations in this third domain on the structure of RcsB is less clear. It is possible that the domain defined by mutations in this part of the protein constitutes a hinge region between the phosphorylation and the DNA binding sites such that it functions in coordinating the phosphorylation signal to the DNA-binding domain. The extent of the bend in the hinge region may dictate the level of communication between the input domain (N terminus) and output domain (C terminus). The presence of two proline residues in this putative hinge region is likely to contribute to the beta turn in the secondary structure, thereby allowing formation of a hinge. The proline residues at 113 and 117 are clearly critical residues for RcsB function, since mutations in these residues were independently isolated several times. These proline mutations are RcsA independent, whereas the wild-type RcsB is dependent on RcsA for maximal *cps* expression. From these results, we speculate that RcsA may be involved in communicating between the input and the output domains.

The fourth and last domain defined by the mutations ranged from amino acids 187 to 206 and included both up and down mutations, near the C terminus of the protein. This domain lies just outside the domain (171 to 185) that has been proposed to have the helix-turn-helix motif involved in DNA binding (39). Although no mutations were found in the putative helix-turn-helix region, the fourth domain defined by five mutations may have context effects on the helix-turn-helix motif. Among these mutations, only D198N is constitutive, suggesting that this mutation may already be in the conformationally active form of the protein, without being phosphorylated. The other four mutations respond to at least one other regulator, indicating that they have a more minor effect on the protein's conformation than D198N. The *rcsB* down mutation, L205P, may be either an RcsC interaction mutation or a promoter interaction mutation. It is difficult to distinguish between these two categories, since screening in an *rcsC* strain will not reveal any additional information because, normally, the level of capsule expression is low in cells carrying a null mutation in *rcsC*. It is interesting that both up and down mutations were isolated in this region.

Surprisingly, we did not obtain a mutation at K109 of RcsB, which is an absolutely conserved residue among the response regulators. K109 from the β5 strand in CheY is critical for contacts with the acidic pocket residues; K109 has its side chain in a fully extended configuration and forms a firm hydrogen bond to the carboxyl group of D57 (31, 42).

In general, most of the up mutations were found to be independent of RcsA, as seen by screening in an *rcsA rcsB cps*::*lacZ* strain, which suggests that RcsA may act essentially as an accessory factor in stimulating capsule expression. There has been genetic evidence suggesting that RcsA may act either directly or indirectly through RcsB to activate transcription of the *cps* genes (3, 39). Previous work has also suggested that RcsA acts by directly interacting with RcsB. In fact, recently RcsA has been shown to form heterodimers with RcsB (21). Genetic evidence also suggests that RcsA and RcsB together form multimers and activate transcription of the *cps* genes (32). There is preliminary evidence suggesting that RcsA alone

can bind the promoter region of the *cps* genes (37a). From these observations, we speculate that RcsA may have two roles: first, it interacts with the *cps* promoter by itself and/or interacts with phosphorylated RcsB and helps RcsB bind the promoter region to highly stimulate capsule expression; second, RcsA may affect RcsC-RcsB interaction. The fact that most of the *rcsB* up mutations are RcsA independent suggests that the mutated form of RcsB may interact very well with RcsC and, therefore, that RcsA is not needed in the RcsB-RcsC interaction.

Experiments are currently under way to biochemically characterize some of the *rcsB* down and up mutations to aid in determining the functions of the various domains of RcsB. This involves performing in vitro and in vivo phosphorylation assays. It will also be interesting to analyze the up mutations that appeared to be constitutive in the in vitro transcription and gel mobility shift assays. We would expect them to bind very well to the *cps* promoter region without being phosphorylated.

Lastly, demonstration of the allele specificity of some of the *rcsB* up mutations for *rcsC* argues that RcsB and RcsC interact not only functionally but also physically. The search for more clearly defined allele-specific suppressors of *rcsB* up mutations represents the next step in this project. Physical interaction based on the lock-and-key mechanism can be demonstrated if an allele-specific mutation in *rcsB*, which results in an increased level of *cps* transcription, could be suppressed by a compensatory mutation in *rcsC*, thereby decreasing the level of *cps* transcription.

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