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Interaction of the RcsB Response Regulator with Auxiliary Transcription Regulators in *Escherichia coli*[*]

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The Rcs phosphorelay is a two-component signal transduction system that is induced by cell envelope stress. RcsB, the response regulator of this signaling system, is a pleiotropic transcription regulator, which is involved in the control of various stress responses, cell division, motility, and biofilm formation. RcsB regulates transcription either as a homodimer or together with auxiliary regulators, such as RcsA, BglJ, and GadE in *Escherichia coli*. In this study, we show that RcsB in addition forms heterodimers with MatA (also known as EcpR) and with DctR. Our data suggest that the MatA-dependent transcription regulation is mediated by the MatA-RcsB heterodimer and is independent of RcsB phosphorylation. Furthermore, we analyzed the relevance of amino acid residues of the active quintet of conserved residues, and of surface-exposed residues for activity of RcsB. The data suggest that the activity of the phosphorylation-dependent dimers, such as RcsA-RcsB and RcsB-RcsB, is affected by mutation of residues in the vicinity of the phosphorylation site, suggesting that a phosphorylation-induced structural change modulates their activity. In contrast, the phosphorylation-independent heterodimers BglJ-RcsB and MatA-RcsB are affected by only very few mutations. Heterodimerization of RcsB with various auxiliary regulators and their differential dependence on phosphorylation add an additional level of control to the Rcs system that is operating at the output level.

In bacteria, two-component signaling systems play a central role in modulating the intracellular response to specific extracellular signals (1). In canonical two-component systems, signal sensing by the input domain of the sensor kinase triggers its autophosphorylation at a conserved histidine residue. From there, the phosphoryl group is transferred to a conserved aspartate residue within the receiver domain of the response regulator, whose phosphorylation affects its output function (1). The Rcs phosphorelay is a complex two-component signal transduction system in *Enterobacteriaceae* that was originally identified as a regulatory system of colanic acid capsule biosynthesis in *Escherichia coli* (2–4). Its complexity is evident at the levels of signal sensing, signal transfer, and signaling output. Sensing of perturbations of the bacterial cell wall and outer membrane proteins involves the lipoprotein RcsF and the inner membrane protein IgaA that are acting upstream of the inner membrane sensor kinase RcsC and the phosphotransfer protein RcsD (5–7). Intriguingly, signal sensing mediated by RcsF involves a switch in its localization. RcsF is exposed to the cell surface by the outer membrane β-barrel protein assembly machinery, but upon perturbation of outer membrane protein assembly or lipoprotein transport machinery, newly synthesized RcsF is no longer surface-exposed and is then able to trigger Rcs signaling from the periplasm via IgaA (7).

The complexity of the output that is generated by the Rcs phosphorelay via the response regulator RcsB is likewise high and involves additional protein components. RcsB is a transcriptional regulator that acts as a homodimer or by interaction with auxiliary transcriptional regulators, including RcsA, GadE, and BglJ (3, 8, 9). RcsB and its auxiliary partners all belong to the FixJ/NarL family of transcriptional regulators characterized by a conserved C-terminal DNA-binding domain family (3, 10, 11). The interaction of RcsB with the auxiliary partners alters the DNA binding specificity (8, 9, 12) and thus extends the regulatory repertoire of the Rcs system to the control of multiple loci related to motility and biofilm formation, various stress responses, cell surface components, and additional functions (3, 4). As a homodimer, RcsB activates or represses transcription of several loci, including *rprA*, encoding the small regulatory RNA RprA (3, 13). The heterodimer RcsA-RcsB activates loci coding for exopolysaccharide production and represses transcription of *flhDC*, encoding the flagella master regulator (14–16). GadE-RcsB activates expression of the *gadA* gene, encoding a glutamate decarboxylase important in the acid-stress response, whereas phosphorylated RcsB represses expression of *gadA* as a homodimer by binding to a different site (8). BglJ-RcsB activates expression of >10 loci, including *leuO* encoding a pleiotropic LysR-type transcription regulator (9, 17, 18).

The flexibility of the Rcs output is increased because only some of the RcsB heterodimers depend on RcsB phosphorylation. Transcriptional activation by RcsB-RcsB homodimers and RcsA-RcsB heterodimers depends on RcsB phosphorylation and thus on induction of the Rcs signaling cascade (3). In contrast, BglJ-RcsB and GadE-RcsB heterodimers are active independent of RcsB phosphorylation (8, 9). Thus, there is a dual control of RcsB activity, by phosphorylation via the Rcs phosphorelay and by interaction with auxiliary partners all belonging to the FixJ/NarL family of transcriptional regulators. To date, heterodimerization of bacterial response regulators in addition to RcsB has only been described for BldM and WhiI in the filamentous bacteria *Streptomyces* (19).

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In this study, we analyzed whether RcsB interacts with additional FixJ/NarL auxiliary partners, and we initiated a characterization of amino acid residues of RcsB relevant for this interaction. A two-hybrid analysis for interaction of RcsB with the 18 members of the FixJ/NarL family present in *E. coli* K12 allowed us to expand the RcsB regulon by two interaction partners, MatA (also named EcpR) and DctR. MatA activates expression of fimbria synthesis genes, named *mat* in newborn meningitis- and septicemia-associated *E. coli* (NMEC)[2] and named *ecp* (for *E. coli* common pili) in enterohemorrhagic *E. coli* and other *E. coli* (20–23). Our data suggest that this activation is mediated by MatA-RcsB heterodimers independently of RcsB phosphorylation. DctR plays a role in protection against organic acids that are metabolic products prevalent at high cell densities under growth in acidic conditions (24–26). However, no target gene of DctR is known. Further, we identified amino acids within the receiver domain of RcsB that are presumably important for interaction with specific auxiliary partners. Our data show that transcriptional activation by RcsB homodimers and RcsA-RcsB heterodimers, those depending on RcsB phosphorylation, is affected by mutation of amino acid residues belonging to the "active quintet" coordinating phosphorylation as well as conserved and further amino acid residues in close proximity to the phosphorylation site. In contrast, the activity of the phosphorylation-independent heterodimers BglJ-RcsB and MatA-RcsB is disturbed by only very few of the specific RcsB mutants that were analyzed, including RcsB-I14A with a mutation mapping in helix α1 and K180A mapping in the DNA-binding domain.

Experimental Procedures

Bacterial Strains, Plasmids, and Media—*E. coli* K12 strains and their construction are described in Table 1. Strain construction included transductions with phage T4*GT7* and phage P1*vir* (27, 28), integration of reporter constructs at the attachment site *attB* (29, 30), and generation of chromosomal replacements and deletions by Red-Gam-mediated recombination (31). Mutants were analyzed by allele-specific PCRs, whereas replacements of the *matA* and *rcsA* promoters, respectively, were in addition characterized by sequencing. The plasmids and their construction are listed in Table 2, and the sequences of the oligonucleotides are shown in Table 3. Bacterial cultures were grown in LB medium (5 g/liter yeast extract, 10 g/liter tryptone, 5 g/liter NaCl). Antibiotics were added to 50 μg/ml ampicillin and 25 μg/ml kanamycin, respectively, if required.

RNA Isolation and Microarray Analysis—For identification of target genes that are regulated by MatA/EcpR or DctR, *E. coli* strain BW30270 was transformed with plasmids pKEDP30 (encoding MatA), pKEDP31 (encoding DctR), and pKESK22 (control plasmid), respectively. Overnight cultures of these transformants were used to inoculate a 15-ml culture to an A_{600} of 0.05 in LB medium supplemented with kanamycin. The cultures were grown in a shaker at 37 °C until A_{600} = 0.3. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cultures were harvested after 30 min of further incubation using RNAprotect bacteria reagent (Qiagen, Hilden, Germany). Total RNA was isolated using the RNeasy minikit system, including an on-column DNase I treatment according to the manufacturer's instructions (Qiagen, Germany). For each strain background, three biological replicates of the RNA preparations were used for microarray analysis, which was performed by the Cologne Center for Genomics using Affymetrix GeneChip *E. coli* Genome 2.0 microarrays, as described (17). Differential expression levels were calculated as -fold change relative to the empty vector control.

β-Galactosidase Assay—For expression analyses of promoter *lacZ* fusions, β-galactosidase assays were carried out as described (28). Briefly, exponential cultures were inoculated from a fresh overnight culture to an A_{600} of 0.05 in LB medium, which contained appropriate antibiotics in the case of transformants. Where indicated, IPTG (1 mM final concentration) was added to the overnight and the exponential culture for induction. Bacteria were grown at 37 °C and harvested on ice at an A_{600} of 0.5. The β-galactosidase assays were repeated from at least three independent biological replicates.

Motility Assays—For motility assays, 3 μl of a fresh overnight culture grown in LB medium, which in the case of transformants was supplemented with 25 μg/ml kanamycin and 1 mM IPTG, was pipetted to the center of freshly poured LB soft agar plates (0.2% agar). In the case of transformants, the plates were supplemented with 25 μg/ml kanamycin and 0.2 mM IPTG. The motility assay plates were incubated for 5 h at 37 °C and then scanned using an Epson Perfection V700 Photo transparency scanner.

Structure Prediction of the RcsB Receiver Domain—The RcsB protein consists of 216 amino acids with the N-terminal receiver domain comprising residues 1–124 and the C-terminal DNA-binding domain comprising residues 144–209 (3). The structure of the DNA-binding domain was solved for RcsB from *Erwinia amylovora* (PDB code 1P4W) (32), which is 92% identical to *E. coli* RcsB. The structure of the RcsB receiver domain has not been solved. Therefore, we used structural modeling by Phyre2 (33), I-TASSER (34), and HHPred (35), using the *E. coli* K12 RcsB amino acid sequence from residue 1 to 125 as a query. For Phyre2, the highest scoring model was based on the crystal structure of NarL from *Mycobacterium tuberculosis* (PDB code 3EUL) (36) and a 26% protein identity. The model is shown under "Results." For I-Tasser, the model was based on the crystal structure of NarL from *E. coli* (PDB code 1A04) (37, 38), and for HHPred, the model was based on a response regulator from *Aurantimonas* sp. S185-9A1 (PDB code 3CZ5). Structures of receiver domains exhibit a conserved $(\alpha\beta)_5$ fold (39). Accordingly, the models of the RcsB receiver domain are very similar in the well ordered $(\alpha\beta)_5$ fold but show variations in the loops that connect the α helices and β sheets.

Results

RcsB Interacts with MatA (EcpR) and DctR—The response regulator RcsB is known to interact with auxiliary regulators, such as RcsA, BglJ, and GadE, which likewise carry a FixJ/NarL-

[2] The abbreviations used are: NMEC, meningitis- and septicemia-associated *E. coli*; UPEC, uropathogenic *E. coli*; IPTG, isopropyl β-D-1-thiogalactopyranoside; PDB, Protein Data Bank.

TABLE 1

E. coli K12 and UPEC strains

The *E. coli* K12 strains and the UPEC strain used in this study are listed with their genotype and the source or construction of the strain. For construction of strains by transduction with phage T4*GT7* and P1*vir*, respectively, the number of the source strain is given in parentheses. In the cases of generation of deletions or replacement by Red-Gam-mediated recombination, the fragments were generated by PCR with the indicated oligonucleotide primers and DNA templates (x primer1/primer2 (template plasmid)). Plasmid pCP20 containing the *flp* gene was used for site-specific deletion of the resistance cassettes (x pCP20). Furthermore, promoter *lacZ* fusions were integrated at the attachment site *attB* using helper plasmid pLDR8 (encoding Int) and the religated BamHI fragments of the indicated plasmids (x plasmid number). Constructed and transduced alleles were analyzed by PCR using oligonucleotide primers given in Table 3.

Strain	Genotype	Source/Construction
***E. coli* K12 strains**		
BW30270	MG1655 *rph*$^+$	CGSC #7925
S3974	BW30270 *ilvG*$^+$ (non-motile)	Ref. 9
T1241	BW30270 *ilvG*$^+$ (motile)	BW30270 x T4*GT7* (S3974)
U89	BW30270 *ilvG*$^+$ Δ*rcsB*$_{cmR}$	T1241 x T4*GT7* (T13)
S4197	BW30270 *ilvG*$^+$ Δ*lacZ* (non-motile)	Ref. 9
T21	S4197 Δ*rcsB*$_{FRT}$	Ref. 17
T768	S4197Δ(*rcsDB-rcsC*)$_{cmR}$	S4197/pKD46 x PCR T433/T329 (pKD3)
T903	S4197 Δ*rcsB*$_{FRT}$ P$_L$-*rcsA*$_{kanR}$	Ref. 58
JW1224	Δ*(araD-araB)567* Δ*lacZ4787* (::rrnB-3), λ$^-$ Δ*galU745*$_{kanR}$ *rph1* Δ*(rhaD-rhaB)568 hsdR514*	CGSC #9110 (59)
UPEC *E. coli* strain		
CFT073	Uropathogenic *E. coli*	Ref. 60
E. coli* K12 strains with an integration of a promoter *lacZ* fusions at *attB		
S3432	CSH50 Δ*lacZ* Δ*bgl sulA*3 *lexA71*::Tn5 Δ*rcsB*$_{FRT}$ attB::(Spec^R *lacI*^{q} P$_{sulA}$+/+ *lacZ)*	Ref. 9
S3434	CSH50 Δ*lacZ* Δ*bgl sulA*3 *lexA71*::Tn5 Δ*rcsB*$_{FRT}$Δ(*yjjP-yjjQ-bglJ*)$_{FRT}$ attB::(Spec^R *lacI*^{q} P$_{sulA}$+/+ *lacZ)*	Ref. 9
S3440	CSH50 Δ*lacZ* Δ*bgl sulA*3 *lexA71*::Tn5 Δ*rcsB*$_{FRT}$ attB::(Spec^R *lacI*^{q} P$_{sulA}$408/+ *lacZ*)	Ref. 9
S3442	CSH50 Δ*lacZ* Δ*bgl sulA*3 *lexA71*::Tn5 Δ*rcsB*$_{FRT}$Δ(*yjjP-yjjQ-bglJ*)$_{FRT}$ attB::(Spec^R *lacI*^{q} P$_{sulA}$408/+ *lacZ*)	Ref. 9
T572	S4197 attB::(Spec^R P$_{leuO}$ *lacZ*) Δ*rcsB*$_{FRT}$ *bglJ*$_C$	Ref. 17
T2023	S4197 attB::(Spec^R P$_{rprA}$ *lacZ*)	S4197/pLDR8 x pKES299
T1052	S4197 attB::(Spec^R P$_{rprA}$ *lacZ*) Δ*rcsB*$_{FRT}$	T21/pLDR8 x pKES299
T2041	S4197 attB::(Spec^R P$_{rprA}$ *lacZ*) Δ*galU*$_{kanR}$	T2023 x P1*vir* (JW1224)
T2037	S4197 attB::(Spec^R P$_{wza}$ *lacZ*)	S4197/pLDR8 x pKES260
T2039	S4197 attB::(Spec^R P$_{wza}$ *lacZ*)$_{kanR}$P$_L$-*rcsA*	T2037 x T4*GT7* (T903)
T864	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ*rcsB*$_{FRT}$	T21/pLDR8 x pKES260
T927	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ*rcsB*$_{FRT}$ P$_L$-*rcsA*$_{FRT}$	T864 x T4*GT7* (T903) x pCP20
T921	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ(*rcsDB-rcsC*)$_{cmR}$	T864 x T4*GT7* (T768)
T963	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ(*rcsDB-rcsC*)$_{FRT\ FRT}$P$_L$-*rcsA*	T921 x T4*GT7* (T903) x pCP20
T2042	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ*galU*$_{kanR}$	T2037 x P1*vir* (JW1224)
T2044	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ*galU*$_{FRT}$	T2042 x pCP20
T2045	S4197 attB::(Spec^R P$_{wza}$ *lacZ*) Δ*galU*$_{FRT\ kanR}$P$_L$-*rcsA*	T2044 x T4GT7 (T903)
T1749	S4197 attB::(Spec^R P$_{matA}$ *lacZ*)	S4197/pLDR8 x pKEDP49
T1747	S4197 attB::(Spec^R P$_{matA}$ *lacZ*) Δ*rcsB*$_{FRT}$	T21/pLDR8 x pKEDP49
T1986	S4197 attB::(Spec^R P$_{matA}$ *lacZ*)$_{FRT\ FRT}$P$_L$-*matA*	T1749/pKD46 x PCR OA83/OA84 (pKES263) x pCP20
T1987	S4197 attB::(Spec^R P$_{matA}$ *lacZ*) Δ*rcsB*$_{FRT\ FRT}$P$_L$-*matA*	T1747/pKD46 x PCR OA83/OA84 (pKES263) x pCP20

TABLE 2

Plasmids and their relevant features

Plasmids that were used or constructed are listed with their name, relevant features, and source or construction. For plasmids that were constructed by cloning of PCR fragments, the primers used for amplification of the fragment and the cloning vector are given. The PCR fragments were amplified from plasmids carrying the wild-type gene or from the *E. coli* K12 genome. Mutagenesis of *rcsB* was conducted by fusion PCR using oligonucleotide primers (listed in Table 3) that carry the specific mutation and flanking primers T106 and T358; the mutated nucleotides are indicated. Derivatives of plasmids pDP804 and pMS604 carry translational fusions of the indicated genes to *lexA(1–87)WT* and *lexA(1–87)408*, respectively.

Plasmid	Features	Source/Construction
pCP20	cI_{857} λ-P_R *flp pSC101* rep^{ts} *bla*	Ref. 61
pKD46	*araC Para* γ-β-*exo in pSC101* rep^{ts} *bla*	Ref. 31
pLDR8	cI_{857} λ-P_R *int pSC101* rep^{ts} *neo*	Ref. 29
pKD3	FRT *cat* FRT oriRγ *bla*	Ref. 31
pKD13	FRT *neo* FRT oriRγ *bla*	Ref. 31
pKES263	FRT *neo* FRT P_L oriRγ *bla* in pKD13	PCR T464/T465 from phage λ
pKES148	MCS *lacZ attP aadA* p15A *neo*	Ref. 9
pKES260	P_{wza} *lacZ attP aadA* p15A *neo*	PCR T460/T461 in pKES148
pKES299	P_{rprA} *lacZ attP aadA* p15A *neo*	PCR T563/T564 in pKES148
pKES268	P_{lacUV5} MCS *lacZ attP aadA* p15A *neo*	Ref. 18
pKEDP49	P_{matA} *lacZ attP aadA* p15A *neo*	PCR T908/T909 from CFT073 in pKES268
pDP804	P_{lacUV5} lexA(1–87)408-*jun* p15A *bla*	Ref. 40
pKEAP28	P_{lacUV5} lexA(1–87)408-*rcsB* in pDP804	Ref. 9
pKEAP29	P_{lacUV5} lexA(1–87)408-*bglJ* in pDP804	Ref. 9
pKEDP58	P_{lacUV5} lexA(1–87)408-*dctR* in pDP804	PCR OA29/OA30 in pDP804
pKEDP59	P_{lacUV5} lexA(1–87)408-*matA* in pDP804	PCR OA27/OA28 in pDP804
pKEDP60	P_{lacUV5} lexA(1–87)408-*rcsA* in pDP804	PCR OA25/OA26 in pDP804
pMS604	P_{lacUV5} lexA(1–87)WT-*fos* ColE1 *tet*	Ref. 40
pKEAP30	P_{lacUV5} lexA(1–87)WT-*bglJ*	Ref. 9
pKEMK17	P_{lacUV5} lexA(1–87)WT-*rcsB*	Ref. 9
pKES192	P_{lacUV5} lexA(1–87)WT-*rcsA*	Ref. 9
pKEAP27	P_{lacUV5} lexA(1–87)WT-*yjjQ*	PCR S691/S692 in pMS604
pKEMK1	P_{lacUV5} lexA(1–87)WT-*dctR*	PCR S956/S957 in pMS604
pKEMK2	P_{lacUV5} lexA(1–87)WT-*fimZ*	PCR S960/S961 in pMS604
pKEMK3	P_{lacUV5} lexA(1–87)WT-*malT*	PCR S964/S965 in pMS604
pKEMK4	P_{lacUV5} lexA(1–87)WT-*matA*	PCR S966/S967 in pMS604
pKEMK5	P_{lacUV5} lexA(1–87)WT-*narL*	PCR S968/S969 in pMS604
pKEMK6	P_{lacUV5} lexA(1–87)WT-*narP*	PCR S970/S971 in pMS604
pKEMK7	P_{lacUV5} lexA(1–87)WT-*sdiA*	PCR S972/S973 in pMS604
pKEMK8	P_{lacUV5} lexA(1–87)WT-*uhpA*	PCR S974/S975 in pMS604
pKEMK9	P_{lacUV5} lexA(1–87)WT-*uvrY*	PCR S976/S977 in pMS604
pKEMK10	P_{lacUV5} lexA(1–87)WT-*ygeK*	PCR S978/S979 in pMS604
pKEMK11	P_{lacUV5} lexA(1–87)WT-*yhjB*	PCR S980/S981 in pMS604
pKEMK12	P_{lacUV5} lexA(1–87)WT-*yahA*	PCR S982/T10 in pMS604
pKEMK15	P_{lacUV5} lexA(1–87)WT-*evgA*	PCR S958/S959 in pMS604
pKEMK16	P_{lacUV5} lexA(1–87)WT-*gadE*	PCR S962/S963 in pMS604
pKES177	P_{lacUV5} lexA(1–87)WT-*csgD*	PCR S885/S886 in pMS604
pBAD24	*araC* P_{BAD} pBR *bla*	Ref. 62
pKEDP51	*araC* P_{BAD} *matA*	PCR T691/T692 in pBAD24
pKEDP57	*araC* P_{BAD} *dctR*	PCR T694/T695 in pBAD24
pKESK22	$lacI^q$ P_{tac} p15A *neo*	Ref. 54
pKETS6	$lacI^q$ P_{tac} *rcsB* in pKESK22	Ref. 9
pKETS7	$lacI^q$ P_{tac} *rcsB*-D56E (GAT → GAG)	Ref. 9
pKES229	$lacI^q$ P_{tac} *rcsB*-D66A (GAT → GCG)	This work
pKES230	$lacI^q$ P_{tac} *rcsB*-H77A (CAT → GCG)	This work
pKES231	$lacI^q$ P_{tac} *rcsB*-I14A (ATA → GCA)	This work
pKES232	$lacI^q$ P_{tac} *rcsB*-M88A (ATG → GCG)	This work
pKES234	$lacI^q$ P_{tac} *rcsB*-Y64A (TAC → GCC)	This work
pKES235	$lacI^q$ P_{tac} *rcsB*-D56A (GAT → GCG)	This work
pKES271	$lacI^q$ P_{tac} *rcsB*-D11A (GAC → GCC)	This work
pKES272	$lacI^q$ P_{tac} *rcsB*-P60A (CCT → GCT)	This work
pKES273	$lacI^q$ P_{tac} *rcsB*-G67A (GGC → GCC)	This work
pKES274	$lacI^q$ P_{tac} *rcsB*-T87A (ACT → GCT)	This work
pKES275	$lacI^q$ P_{tac} *rcsB*-K109A (AAA → GCA)	This work
pKES276	$lacI^q$ P_{tac} *rcsB*-K180A (AAA → GCA)	This work
pKEDP47	$lacI^q$ P_{tac} *rcsB*-D62G (GAT → GGT)	This work
pKESL111	$lacI^q$ P_{tac} *rcsB*-L95A (CTT → GCT)	This work
pKESL112	$lacI^q$ P_{tac} *rcsB*-L99A (TTG → GCG)	This work
pKESL113	$lacI^q$ P_{tac} *rcsB*-D100A (GAT → GCT)	This work
pKESL114	$lacI^q$ P_{tac} *rcsB*-E104A (GAA → GCA)	This work
pKESL115	$lacI^q$ P_{tac} *rcsB*-I106A (ATC → GCC)	This work
pKESL116	$lacI^q$ P_{tac} *rcsB*-L108A (CTG → GCG)	This work
pKESL117	$lacI^q$ P_{tac} *rcsB*-T114A (ACC → GCC)	This work
pKESL118	$lacI^q$ P_{tac} *rcsB*-K118A (AAA → GCA)	This work
pKESL119	$lacI^q$ P_{tac} *rcsB*-S96A (AGT → GCT)	This work
pKESL120	$lacI^q$ P_{tac} *rcsB*-D115A (GAT → GCT)	This work

type DNA-binding domain (3, 8, 9). In the *E. coli* K12 genome, in total, 18 proteins with a FixJ/NarL-type DNA-binding domain are encoded. Here we investigated whether RcsB interacts with any other member of these FixJ/NarL-type proteins. To this end, we performed interaction studies using the bacterial LexA-based one-hybrid and two-hybrid system (40). The one-hybrid reporter for examining homodimer formation consists of the native *sulA* promoter fused to *lacZ* (Fig. 1*A*). Only homodimers of proteins fused to the wild-type DNA-binding domain (amino acid residues 1–87) of the LexA repressor

TABLE 3

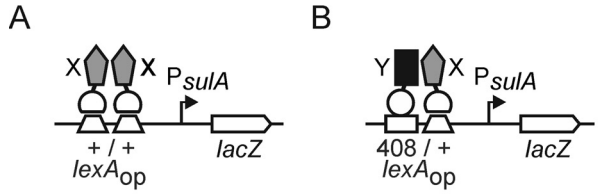
Oligonucleotides

The sequences of oligonucleotides used as PCR primers and for mutagenesis are shown, along with a brief description of their use. Bases of the oligonucleotide that match the template are shown in capital letters, whereas other bases are in lowercase letters. Restriction sites are underlined.

Name	Nucleotide sequence	Use
T106	cagggatcctctagattaGTCTTTATCTGCCGGACTTAAGGTCAC	*rcsB* cloning
T358	gaccgaattcTTGCTGTAGCAAGGTAGCCTATTACATG	*rcsB* cloning
T329	gagaacattgcggtaacacgcttttaccgctaccttaaccacactGTGTAGGCTGGAGCTGCTTCG	*rcsDB-rcsC* deletion
T433	ggtaagagtctggaatttcacactgtacccttatactgccctatCATATGAATATCCTCCTTAGTTCCTATTCC	*rcsDB-rcsC* deletion
T460	agcagtcgacCTCACATTATCCCTGAATTAAAAGTGG	P*wza* cloning
T461	agcgtctagattaCATCATTGTTTATTTATCACTTTGGCAG	P*wza* cloning
T464	agcagtcgacCTCTCACCTACCAAACAATGCCC	P_L cloning
T465	agcaggatccTCATGGTGGTCAGTGCGTCC	P_L cloning
T466	aatacctacgaacatcttccaggatactcctgcagcgaaatattGTGTAGGCTGGAGCTGCTTCG	Replacement of P*rcsA* by P_L
T467	cataccctcactcaatgcgtaacgataattcccctttacctgaaTCATGGTGGTCAGTGCGTCC	Replacement of P*rcsA* by P_L
T563	agcagtcgacAATTGATATTTGCTTGCTCTTCCCC	P*rprA* cloning
T564	agcgtctagaCCGTGAGCTAATAGTAGGCATACGG	P*rprA* cloning
T691	agcagaattcAATTACAGGTTTGGAAAGTAGTGACATG	*matA* cloning
T692	agcgTCTAGATTACTGAACCAACTTATATATTTTTGAGTACAGC	*matA* cloning
T694	agcagaattcGTCCGCACCAGGAGTCGG	*dctR* cloning
T695	agcgTCTAGATCACACCAGATAATCAATATGCTGATG	*dctR* cloning
T908	agcagtcgacGCCATCGTTCCTGTGACAACTG	P$matA_{CFT073}$ cloning
T909	agcgtctagaTTGCCATGTCACTACTTTCCAAACC	P$matA_{CFT073}$ cloning
OA25	agcactcgagTCAACGATTATTATGGATTTATGTAGTTACAC	LexA (1–87)408 *rcsA* cloning
OA26	agcgagatcttTTAGCGCATGTTGACAAAAATACC	LexA (1–87)408 *rcsA* cloning
OA27	agcactcgagACATGGCAAAGTGATTACAGTAGGGAC	LexA (1–87)408 *matA* cloning
OA28	agcgagatcttTTACTGAACCAACTTATATATTTTTGAGTACAGCTT	LexA (1–87)408 *matA* cloning
OA29	agcactcgagTTTCTTATAATTACCAGGGATACGATGTTC	LexA (1–87)408 *dctR* cloning
OA30	agcgagatcttTCACACCAGATAATCAATATGCTGATG	LexA (1–87)408 *dctR* cloning
OA83	tcttcaatgacagctcatcatagttttatattctatcccttaGTGTAGGCTGGAGCTGCTTCG	Replacement of P*matA* by P_L
OA84	actgtaatcactttgccatgtcactactttccaaacctgtaaTCATGGTGGTCAGTGCGTCC	Replacement of P*matA* by P_L
S691	ttctgcagTTGCCAGGATGCTGCAAAA	LexA (1–87)WT *yjjQ* cloning
S692	ttctcgagACTCTCAATACCGATACTACTCATGACG	LexA (1–87)WT *yjjQ* cloning
S885	ccatctgcagTTTAATGAAGTCCATAGTATTCATGGTCATAC	LexA (1–87)WT *csgD* cloning
S886	ccatctcgagTTATCGCCTGAGGTTATCGTTTGC	LexA (1–87)WT *csgD* cloning
S956	ccatctgcagTTTCTTATAATTACCAGGGATACGATGTTC	LexA (1–87)WT *dctR* cloning
S957	ccatctcgagTCACACCAGATAATCAATATGCTGATG	LexA (1–87)WT *dctR* cloning
S958	ccatggatcctgcagAACGCAATAATTATTGATGACCATCCT	LexA (1–87)WT *evgA* cloning
S959	ccatggatcctcgagTTAGCCGATTTTGTTACGTTGTGC	LexA (1–87)WT *evgA* cloning
S960	ccatctgcagAAACCAACGTCGGTGATCATTATG	LexA (1–87)WT *fimZ* cloning
S961	ccatctcgagTTATATTAATTCGTATAATTTGGCGTAGTCGAT	LexA (1–87)WT *fimZ* cloning
S962	ccatggatcctgcagATTTTTCTCATGACGAAAGATTCTTTTCTT	LexA (1–87)WT *gadE* cloning
S963	ccatggatcctcgagCTAAAAATAAGATGTGATACCCAGGGTGAC	LexA (1–87)WT *gadE* cloning
S964	ccatctgcagCTGATTCCGTCAAAACTAAGTCGTCC	LexA (1–87)WT *malT* cloning
S965	ccatctcgagTTACACGCCGTACCCCATCAT	LexA (1–87)WT *malT* cloning
S966	ccatctgcagACATGGCAAAGTGATTACAGTAGGGAC	LexA (1–87)WT *matA* cloning
S967	ccatctcgagTTACTGAACCAACTTATATATTTTTGAGTACAGCTT	LexA (1–87)WT *matA* cloning
S968	ccatctgcagAGTAATCAGGAACCGGCTACTATCCTG	LexA (1–87)WT *narL* cloning
S969	ccatctcgagTCAGAAAATGCGCTCCTGATG	LexA (1–87)WT *narL* cloning
S970	ccatctgcagCCTGAAGCAACACCTTTTCAGGT	LexA (1–87)WT *narP* cloning
S971	ccatctcgagTTATTGTGCCCCGCGTTGTT	LexA (1–87)WT *narP* cloning
S972	ccatctgcagCAGGATAAGGATTTTTTCAGCTGG	LexA (1–87)WT *sdiA* cloning
S973	ccatctcgagTCAAATTAAGCCAGTAGCGGCC	LexA (1–87)WT *sdiA* cloning
S974	ccatctgcagATCACCGTTGCCCTTATAGACGAT	LexA (1–87)WT *uhpA* cloning
S975	ccatctcgagTCACCAGCCATCAAACATGCG	LexA (1–87)WT *uhpA* cloning
S976	ccatctgcagATCAACGTTCTACTTGTTGATGACCAC	LexA (1–87)WT *uvrY* cloning
S977	ccatctcgagTCACTGACTTGATAATGTCTCCGCAT	LexA (1–87)WT *uvrY* cloning
S978	ccatctgcagATGGGGGCCGAACTCGTAAA	LexA (1–87)WT *ygeK* cloning
S979	ccatctcgagTTATATAGTGCAAACACCCATACGTAAAGC	LexA (1–87)WT *ygeK* cloning
S980	ccatctgcagCAAATAGTCATGTTTGACAGGCAGTCA	LexA (1–87)WT *yhjB* cloning
S981	ccatctcgagTCAGGAGGAGATATTTAACATCATTGC	LexA (1–87)WT *yhjB* cloning
S982	ccatctgcagAATTCATGTGATTTTCGTGTTTTTCTG	LexA (1–87)WT *yahA* cloning
T10	ccatagatcttTCAACCACCTGCTTTCATTACCC	LexA (1–87)WT *yahA* cloning

(LexA(1–87)WT) are able to bind to the *lexA* operator and repress P*sulA lacZ* expression. For analyzing heterodimer formation, the *sulA* promoter carries a hybrid *lexA* operator (408/+) with a mutation in one half-site (Fig. 1*B*) (40). Only heterodimers, in which one partner is fused to LexA(1–87)WT and the other partner is fused to the 408 mutant DNA-binding domain (LexA(1–87)408), are able to bind the hybrid operator and repress P*sulA lacZ* expression (40).

The two-hybrid assays for heterodimer formation of RcsB with any of the additional 17 other FixJ/NarL-type proteins encoded in *E. coli* K12 were conducted in the *lexA* Δ*rcsB* strain S3440 or S3442 carrying the *sulA* 408/+ hybrid promoter fused to *lacZ* (Fig. 1 and Table 4). The reporter strain was co-transformed with plasmid pKEAP28 harboring RcsB fused to the LexA(1–87)408 DNA-binding domain, together with a plasmid harboring one of the other FixJ/NarL-type proteins fused to the wild-type LexA(1–87)WT DNA-binding domain (Fig. 1*B*). The co-induction of LexA(1–87)408-RcsB with LexA(1–87)WT-MatA as well as the co-induction of LexA(1–87)408-RcsB with LexA(1–87)WT-DctR resulted in a 9- and 12-fold repression, respectively, suggesting that RcsB forms heterodimers with both MatA and DctR (Fig. 1*C*). This repression was similar to previous results obtained for BglJ-RcsB and RcsA-RcsB heterodimerization (shown for comparison in Fig. 1*C*) (9). Beyond



C

fusion to $LexA_{1-87}$408			fusion to $LexA_{1-87}$wt				
	hetero-dimerization		RcsB	BglJ	RcsA	MatA	DctR
RcsB	repression		9	9	8	9	12
	IPTG	-	2037	1779	3579	2200	1892
		+	237	203	441	254	164
BglJ	repression		7		1	1	1
	IPTG	-	1809	NT	1840	1752	1823
		+	252		1741	1908	1921
RcsA	repression					1	1
	IPTG	-	NT	NT	NT	1606	1636
		+				1612	2021
MatA	repression						1
	IPTG	-	NT	NT	NT	NT	1433
		+					1372

D

	homo-dimerization		fusion to $LexA_{1-87}$wt RcsB	BglJ	RcsA	MatA	DctR
	repression		27	5	1	1	1.5
	IPTG	-	2422	3237	2246	3184	3010
		+	89	589	2177	2836	1924

FIGURE 1. **Homo- and heterodimer formation by RcsB, BglJ, RcsA, MatA, and DctR.** The LexA two-hybrid system exploits repression of the *sulA* promoter by dimeric LexA (40). Expression is repressed in cases when a fusion of a protein (×) to the LexA-DNA-binding domain forms homodimers (*A*). For analysis of heterodimerization, a *sulA* promoter variant carrying a hybrid *lexA* 408/+ operator is used, which is repressed by heterodimers of proteins X and Y that are fused to the LexA(1–87)WT and LexA(1–87)408 DNA-binding domains, respectively (*B*). The LexA fusion proteins were expressed from compatible plasmids under the control of the IPTG-inducible *lacUV5* promoter. *C*, heterodimer formation by RcsB, BglJ, RcsA, MatA, and DctR. The -fold repression of the *lexA*$op_{408/+}$ *sulA* promoter *lacZ* fusion, as a measure of heterodimerization, is calculated as the ratio of expression values (given in *smaller type*) directed by the P*sulA lacZ* reporter when bacteria are grown without and with induction, respectively, of LexA fusion protein expression. Strain S3440 (Δ*rcsB*) or S3442 (Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)) were co-transformed with plasmids encoding for LexA(1–87)-X and LexA(1–87)408-Y fusions, respectively. The following plasmids were used: pKEMK17 (LexA(1–87)-RcsB), pKEAP30 (LexA(1–87)-BglJ), pKES192 (LexA(1–87)-RcsA), pKEMK4 (LexA(1–87)-MatA), and pKEMK1 (LexA(1–87)-DctR) as well as pKEAP28 (LexA(1–87)408-RcsB), pKEAP29 (LexA(1–87)408-BglJ), and pKEDP59 (LexA(1–87)408-MatA). The cultures were grown to A_{600} of 0.5 in LB medium supplemented with ampicillin and tetracyclin. IPTG was added where indicated. Values for RcsB, BglJ, and RcsA homo- and heterodimer analysis are taken from Ref. 9. *D*, homodimer formation of RcsB, BglJ, RcsA, MatA, and DctR. The -fold repression, as a measure for dimerization, was calculated as the ratio of the β-galactosidase activities determined of cultures grown without and with induction of the LexA fusion proteins. Strain S3434 (Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)) was transformed with plasmids pKEMK17 (LexA(1–87)-RcsB), pKEAP30 (LexA(1–87)-BglJ), and pKES192 (LexA(1–87)-RcsA), respectively. Strain S3432 (Δ*rcsB*) was transformed with plasmid pKEMK4 (LexA(1–87)-MatA) and pKEMK1 (LexA(1–87)-DctR). Cultures were grown in LB tetracycline medium to A_{600} of 0.5 without and with 1 mM IPTG.

MatA, DctR, RcsA, and BglJ, heterodimerization between RcsB and any of the other FixJ/NarL type proteins was weaker or could not be observed at all (Table 4) (see below).

Because RcsB interacts with RcsA, BglJ, MatA, and DctR, these proteins may form heterodimers in other combinations as well. To analyze such a potential mutual interaction between BglJ, RcsA, MatA, and DctR, all other pairs of combinations were tested by the two-hybrid assay (Fig. 1*C*). However, the co-expression of none of these protein pairs resulted in repression of the *sulA* 408/+ hybrid promoter *lacZ* reporter (Fig. 1*C*). These results suggest that neither BglJ nor RcsA nor MatA nor DctR forms a heterodimer with any other of these four proteins.

RcsB forms homodimers, whereas for the known auxiliary regulators BglJ and RcsA no or only weak homodimer formation was detected by the one-hybrid assay (9). Here we tested homodimer formation of MatA, DctR (Fig. 1*D*), and the other FixJ/NarL-type proteins (Table 4). Homodimer formation was analyzed in the Δ*rcsB* reporter strain S3432 or S3434, carrying the wild-type *sulA* promoter *lacZ* fusion (Fig. 1*B*). Neither the induction of LexA(1–87)WT-MatA nor the induction of LexA(1–87)WT-DctR resulted in repression of P*sulA lacZ* expression, suggesting that neither MatA nor DctR forms homodimers (Fig. 1*D*). The other FixJ/NarL type proteins tested, except GadE, all formed homodimers (Table 4). For GadE, we could detect neither homodimer nor heterodimer formation, at least under standard growth conditions used in this study. In the case of GadE, it is possible that its interaction with RcsB might be acid stress-dependent. For EvgA, the data indicate that it forms homodimers, as expected, and heterodimers with RcsB (Table 4). However, EvgA plays a pleiotropic role in activating genes related to acid resistance, osmotic adaptation, and drug resistance (41), and we observed a slow culture growth upon induction of LexA(1–87)WT-EvgA fusions. Therefore, the implication of a possible interaction of RcsB with EvgA was not analyzed further. Taken together, the one-hybrid and two-hybrid analyses suggest that RcsB interacts with RcsA, BglJ, MatA, and DctR, respectively, and that these auxiliary partners do not form homodimers. The data for the remaining FixJ/NarL-type proteins (except for EvgA, GadE, and YgeK) suggest that these form homodimers but no or only weak heterodimers with RcsB, indicating that those proteins that form heterodimers with RcsB do not form homodimers, and *vice versa*.

Activation of the mat Promoter by MatA (EcpR) Depends on RcsB—Our two-hybrid results suggest that the FixJ/NarL-type proteins MatA (EcpR) and DctR form heterodimers with RcsB, whereas they do not form homodimers. However, no target loci of DctR (25) are known, whereas MatA (EcpR) activates the *mat* (*ecp*) operon in NMEC, enterohemorrhagic *E. coli*, and other *E. coli* but not in *E. coli* K12 (20, 22, 23). To analyze the relevance of the MatA-RcsB and DctR-RcsB protein interaction in gene regulation, we first needed to identify target genes that may serve as reporters for DctR and MatA, respectively. For this, we performed a microarray analysis and compared the transcriptome of *E. coli* K12 strain BW30270 harboring plasmids encoding MatA (pKEDP30) and DctR (pKEDP31), respectively, with the same strain harboring vector pKESK22, as control. However, this microarray analysis did not reveal any specific target locus, neither of MatA nor of DctR in *E. coli* K12. Therefore, a reporter system for analysis of the relevance of the DctR-RcsB protein interaction remains to be established. For

TABLE 4

Homo- and heterodimerization of RcsB and other FixJ/NarL-type proteins encoded by the *E. coli* K12 genome

Homodimerization of fusions of FixJ/NarL-type proteins to the LexA(1–87) wild-type DNA-binding domain was analyzed in strain S3432, whereas homodimerization of LexA(1–87)WT-YjjQ was analyzed in strain S3434 and other strains. Heterodimerization of RcsB fused to LexA(1–87)408 was analyzed in strain S3440 co-transformed with a plasmid expressing a fusion of the indicated FixJ/NarL-type protein to LexA(1–87) wild type. The -fold repression of the *sulA* promoter-*lacZ* reporter is a measure of dimerization and is calculated as the quotient of the β-galactosidase activity obtained without induction (−IPTG) and with induction of expression of the fusion proteins (+IPTG).

Fusion to LexA(1–87)WT	Homodimerization			Heterodimerization with LexA(1–87)408-RcsB		
	β-Galactosidase activity			β-Galactosidase activity		
	−IPTG	+IPTG	-Fold repression	−IPTG	+IPTG	-Fold repression
EvgA[a]	672	93	7.2	2108	278	7.6
FimZ	1874	122	15.4	2080	612	3.4
NarL	2626	150	17.5	1987	575	3.5
NarP	2256	120	18.8	2037	702	2.9
UhpA	2684	109	24.6	2171	409	5.3
UvrY	2860	116	24.7	2255	591	3.8
YgeK	3164	1057	3.0	1838	1781	1.0
CsgD	3145	167	18.8	2084	1113	1.9
GadE	3141	2273	1.4	1787	564	3.2
SdiA	1037	124	8.4	2302	1407	1.6
YhjB	3159	107	29.5	2225	1085	2.0
YjjQ	2695	152	17.7[b]	2180	595	3.7
MalT	2182	112	19.5	2142	1557	1.4
YahA/PdeL[a]	414	101	4.1	2126	732	2.9

[a] For these proteins, a repression could be observed even without induction by IPTG.
[b] Values taken from Ref. 9.

MatA, it is known that activation of the H-NS-repressed *mat* operon in NMEC strain IHE3034 and other *E. coli* strains depends on the specific sequence of the *mat* promoter and regulatory region (23). The sequence of the *mat* regulatory region is divergent between the *E. coli* lineages B2, D, and E as compared with the *E. coli* A and B1 lineages, whereas the nucleotide sequence of the *matABCDEF* coding region is highly conserved among all *E. coli* strains (23). To establish a suitable reporter system, we constructed a *lacZ* fusion of the *matA* promoter and regulatory region of UPEC strain CFT073 encompassing positions −552 to +68 relative to the T1 transcription start site, as described before (23). The nucleotide sequence of the *matA* promoter and regulatory region is almost identical in UPEC strain CFT073 and NMEC strain IHE3034, which both belong to the B2 *E. coli* lineage (23).

The P$matA_{CFT073}$ *lacZ* reporter for analysis of regulation by RcsB and MatA was integrated into the chromosome of the *E. coli* K12 Δ*lacZ* strain S4197 (*rcsB*$^+$) and an isogenic Δ*rcsB* strain (yielding strains T1749 and T1747, respectively). In addition, isogenic P_L-*matA* and P_L-*matA* Δ*rcsB* strains were constructed (yielding strains T1986 and T1987). In the two latter strains, the native *mat* promoter in the K12 genome was replaced by the phage λ P_L promoter, for constitutive expression of *matA*. To analyze regulation by MatA and RcsB, these reporter strains were transformed either with the control plasmid pKESK22 or plasmid pKETS6 carrying *rcsB* under control of the IPTG-inducible *tac* promoter (Fig. 2*A*). The $matA_{CFT073}$ promoter *lacZ* fusion directed basal expression levels of 167 units in the *rcsB*$^+$ strain T1749 that was transformed with the control plasmid (Fig. 2*A*). In this strain (T1749), RcsB is expressed by the native chromosomal *rcsB* gene, whereas the chromosomal *matA* gene is not expressed, because its K12-specific promoter is silenced by H-NS. In the isogenic Δ*rcsB* strain (T1747), the expression level was 135 units, and thus it was slightly lower than in the *rcsB*$^+$ strain (Fig. 2*A*). Complementation of the Δ*rcsB* strain (T1747) with plasmid-encoded *rcsB* using plasmid pKETS6 caused a moderate increase of expression to 319 units (Fig. 2*A*). In the presence of MatA encoded by allele P_L-*matA*, the expression level of the P$matA_{CFT073}$ *lacZ* reporter increased to 1367 units in the *rcsB*$^+$ strain T1986 (Fig. 2*A*). However, in the P_L-*matA* Δ*rcsB* strain (T1987), the activity of the P*mat lacZ* reporter was very low (89 units) (Fig. 2*A*). Complementation of this P_L-*matA* Δ*rcsB* strain with *rcsB* using plasmid pKETS6 restored expression of P*mat lacZ* to 2468 units (Fig. 2*A*). The data are in accordance with previous results showing that MatA (EcpR) activates the *mat* operon and that RcsB is required for *mat* operon expression (22, 23). Taken together, the results confirm that both proteins, MatA and RcsB, are required for activation of the $matA_{CFT073}$ promoter, and they indicate that a MatA-RcsB heterodimer activates the *mat* promoter.

MatA-RcsB Activates the mat Promoter Independently of Phosphorylation and Inhibits Motility—The activity of RcsA-RcsB and RcsB homodimers is phosphorylation-dependent, whereas BglJ-RcsB and GadE-RcsB are active independent of RcsB phosphorylation (3, 4, 8, 9, 18). Therefore, we assessed whether the activity of MatA-RcsB depends on RcsB phosphorylation. To this end, the P*mat lacZ* reporter strain T1987 (Δ*rcsB* P_L-*matA*) was transformed with plasmids encoding RcsB mutants RcsB-D56E (pKETS7), mimicking phosphorylated RcsB, and RcsB-D56A (pKES235), mimicking non-phosphorylated RcsB (42). Upon complementation of this reporter strain (T1987) with *rcsB*-D56E and *rcsB*-D56A, expression levels increased to 3207 and 2177 units, respectively (Fig. 2*A*). The moderate difference in activation of P*mat* by the RcsB-D56 mutants and by wild-type RcsB (2468 units) suggests that transcriptional activation of the $matA_{CFT073}$ promoter by MatA-RcsB heterodimers is not (or is only weakly) dependent on RcsB phosphorylation. These results are in accordance with expression studies in NMEC strain IHE3034, where deletion of the Rcs phosphorelay genes *rcsC* and *rcsD* did not affect *mat* expression (22).

Recently, it was shown that ectopic expression of *matA* impairs the swimming behavior of *E. coli NMEC* strain

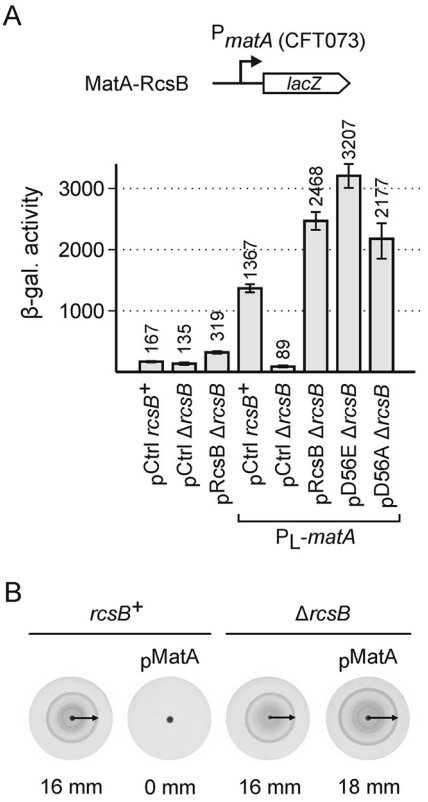


FIGURE 2. **MatA-RcsB activates the *matA*CFT073 promoter and inhibits motility.** *A*, β-galactosidase expression levels directed by the $matA_{CFT073}$ promoter *lacZ* reporter were determined in *rcsB*$^+$ strain T1749, Δ*rcsB* strain T1747, *rcsB*$^+$ P$_L$-*matA* strain T1986, and P$_L$-*matA* Δ*rcsB* strain T1987. These strains were either transformed with control plasmid pKESK22 (pCtrl) or plasmids pKETS6 (pRcsB), pKET7 (pD56E), and pKES235 (pD56A), respectively. Cultures for β-galactosidase assays were grown to A_{600} of 0.5 in LB medium, supplemented with 1 mM IPTG and 25 mg/ml kanamycin. *B*, motility was determined of wild-type strain T1241 and Δ*rcsB* strain U89 and of transformants of these strains ectopically expressing MatA under the control of P*tac* using plasmid pKEDP30 (pMatA). Overnight cultures were grown in LB medium, which was supplemented with 1 mM IPTG and 25 mg/ml kanamycin for growth of the transformants. Three μl of each culture was spotted on the center of a soft agar plate (0.2% agar), supplemented with 0.2 mM IPTG and 25 mg/ml kanamycin in the case of transformants, and the plates were incubated at 37 °C for 5 h. The plates were scanned, and the motility radii that are indicated by *arrows* were measured in mm. *Error bars* represent S.D. The images of the plates have been scaled to 25% of the original size.

IHE3034 and K12 strain MG1655, presumably by repression of the *flhDC* operon encoding the master regulator of flagella synthesis, $FlhD_4C_2$ (43). Here we tested whether this repression of motility by ectopically expressed MatA likewise depends on RcsB. To this end, 3 μl of an overnight culture of motile *E. coli* K12 wild-type strain (T1241) was spotted to the center of a soft agar plate (0.2% agar), and the motility radius was measured after growth of 5 h at 37 °C. When the motile K12 wild-type strain T1241 was transformed with a plasmid containing *matA* under control of the *tac* promoter (pKEDP30), its motility on soft agar plates (supplemented with 0.2 mM IPTG for *matA* induction and kanamycin for plasmid selection) was completely abolished (Fig. 2*B*). The motility of an isogenic Δ*rcsB* strain (U89) was similar to that of the wild-type strain T1241 (16 mm), and this Δ*rcsB* strain remained motile upon additional expression of *matA* (18 mm) (Fig. 2*B*). These data demonstrate that inhibition of motility by MatA requires RcsB, indicating that MatA-RcsB heterodimers repress motility.

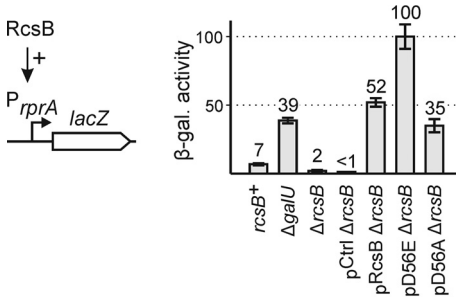


FIGURE 3. **Validation of a P*rprA-lacZ* fusion as a reporter system of RcsB activity.** Expression levels directed by the chromosomal P*rprA lacZ* fusion were determined in *rcsB*$^+$ strain T2023, Δ*galU* strain T2041, and Δ*rcsB* strain T1052. The strains were either untransformed or complemented with *rcsB*, encoded by plasmid pKETS6 (pRcsB). RcsB derivatives D56E (pD56E) and D56A (pD56A) were expressed from plasmids pKETS7 and pKES235, respectively. Cultures for β-galactosidase assays were grown in LB medium to an A_{600} of 0.5, which was supplemented with 1 mM IPTG and 25 μg/ml kanamycin in the case of the transformants. *Error bars* represent S.D.

Characterization of Amino Acid Residues of RcsB That Affect Its Activity as Homo- and Heterodimer—Our results suggest that MatA-RcsB heterodimers are active independent of RcsB phosphorylation, as shown previously for BglJ-RcsB and GadE-RcsB (8, 9, 18). In contrast, the activity of RcsA-RcsB heterodimers and RcsB homodimers is phosphorylation-dependent (3, 4). Phosphorylation of response regulators at the conserved aspartic acid residue is considered to induce a structural change that stabilizes the active form (39, 44). This presumptive structural change is apparently not relevant for the RcsB-heterodimers that are independent of RcsB phosphorylation, indicating that their active form is more stable and/or that they interact differently. Receiver domains of two-component response regulators typically exhibit a $(\beta\alpha)_5$ topology with five parallel β sheets in the center surrounded by two α helices on the one and three on the other side (39). To identify amino acids of the receiver domain of RcsB that are important for its activity as homodimer and heterodimer, respectively, we performed an alanine mutagenesis of amino acid residues specified below and tested the activity of these RcsB mutants using suitable promoter *lacZ* reporter fusions. For measuring MatA-RcsB activity, the P$matA_{CFT073}$ P$_L$-*matA* Δ*rcsB* Δ*lacZ* strain T1987 was used, as described above. For BglJ-RcsB, we used a P*leuO* $bglJ_C$ Δ*rcsB* Δ*lacZ* reporter strain (T572), in which *bglJ* is expressed constitutively (17). For analyzing the RcsB homodimer activity, we constructed a P*rprA lacZ* fusion, as described (13), and for analyzing RcsA-RcsB activity, we constructed a fusion of the RcsA-RcsB-activated P*wza* to *lacZ* (45).

First, we analyzed the suitability of the P*rprA lacZ* fusion as reporter for analyzing mutant RcsB homodimers. The reporter for RcsB homodimer activity, P*rprA lacZ*, was integrated into the chromosome of Δ*lacZ* strain S4197, resulting in strain T2023 (Fig. 3). In this *rcsB*$^+$ strain T2023, the P*rprA lacZ* was poorly expressed, as expected (7 units; Fig. 3). Rcs signaling is known to be induced in Δ*galU* strains, which cannot produce UDP-D-glucose (46). Accordingly, P*rprA lacZ* expression increased to 39 units upon deletion of *galU* (strain T2041) (Fig. 3). In the isogenic Δ*rcsB* strain (T1052) P*rprA lacZ* expression was very low (2 units; Fig. 3), whereas expression increased to 52 units upon complementation with plasmidic *rcsB* using

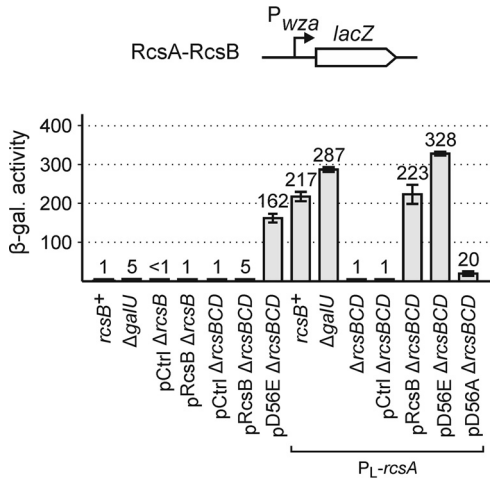


FIGURE 4. **Establishing P*wza-lacZ* as reporter system of RcsA-RcsB activity.** The expression levels of the chromosomally encoded P*wza lacZ* fusion were determined in *rcsB*$^+$ strain T2037, Δ*galU* strain T2042, Δ*rcsB* strain T864, Δ*rcsBCD* strain T921, P$_L$-*rcsA* strain T2039, P$_L$-*rcsA* Δ*galU* strain T2045, and P$_L$-*rcsA* Δ*rcsBCD* strain T963. Expression levels were determined of non-transformed strains or of strains complemented with plasmidic *rcsB*, expressed from plasmid pKETS6 (pRcsB), pKET7 (pD56E), and pKES235 (pD56A), respectively. Cultures for β-galactosidase assays were grown to A_{600} of 0.5 in LB medium, which was supplemented with 1 mM IPTG and 25 μg/ml kanamycin in the case of transformants. *Error bars* represent S.D.

pKETS6 and induction of *rcsB* expression by IPTG (Fig. 3). Upon complementation with RcsB mutant D56E, mimicking the phosphorylated form, the expression of P*rprA lacZ* increased to 100 units, whereas complementation with the RcsB-D56A mutant plasmid resulted in lower activation of the P*rprA* promoter (35 units) (Fig. 3). Thus, complementation with plasmidic *rcsB* overcomes the requirement for induction of the Rcs signaling cascade, whereas mutation of the RcsB phosphorylation site still affects the activation of P*rprA* by RcsB homodimers. Accordingly, the P*rprA lacZ* Δ*rcsB* strain is suitable for analysis of the activity of RcsB alanine mutants.

Second, for analyzing the activity of RcsA-RcsB heterodimers, a chromosomal P*wza lacZ* fusion was used as reporter. The *wza* promoter is derived from the RcsA-RcsB regulated capsular exopolysaccharide (EPS) biosynthesis gene cluster *wza-wca* (45). Expression analyses of this P*wza* reporter revealed that strain P_L-*rcsA* Δ*rcsBCD* Δ*lacZ* (T963) is suitable. Briefly, the P*wza lacZ* reporter was not expressed (1 unit) in the *rcsB*$^+$ strain T2037 (Fig. 4). Induction of Rcs signaling by deletion of *galU* (T2042) induced P*wza lacZ* expression slightly, to 5 units (Fig. 4). Complementation of the isogenic Δ*rcsB* strain (T864) with plasmidic *rcsB* did not confer an increase in expression (1 unit), whereas complementation of the isogenic Δ*rcsBCD* strain T921 induced the reporter slightly (5 units), suggesting that the Δ*rcsBCD* background in which RcsB cannot be dephosphorylated by the RcsCD phosphorelay might be more suitable. Further, the data suggest that induction of Rcs signaling or plasmidic *rcsB* expression is not sufficient for activation of the *rcsA* gene, which itself is H-NS-repressed and positively autoregulated by RcsA-RcsB (3, 47). Therefore, the *rcsA* promoter was replaced by the phage λ P_L promoter, causing constitutive expression of *rcsA* (allele P_L-*rcsA*). This resulted in activation of P*wza lacZ* to 217 units in the *rcsB*$^+$ P_L-*rcsA* strain T2039 (Fig. 4). Additional deletion of *galU* (strain T2045) caused a further increase of expression to 287 units (Fig. 4). For the RcsB mutant characterization, complementation of P*wza lacZ* P_L-*rcsA* Δ*rcsBCD* (T963) with plasmidic *rcsB* was tested. In the P_L-*rcsA* Δ*rcsBCD* strain (T963), the P*wza* promoter was inactive (1 unit), as expected, whereas complementation with plasmidic *rcsB* (pKETS6) activated the P*wza* promoter (223 units) (Fig. 4). Complementation with RcsB mutant D56E resulted in a further increase of P*wza* activity to 323 units, whereas complementation with RcsB-D56A caused only a weak activation of P*wza* (20 units), confirming that activation of P*wza* by RcsA-RcsB in the reporter strain T963 remains RcsB phosphorylation-dependent (Fig. 4).

For identification of amino acid residues of RcsB that are important for RcsB activity, we mutated (i) amino acids residues of the active quintet that is coordinating phosphorylation; (ii) additional highly conserved amino acid residues adjacent to the active quintet; (iii) presumably surface-exposed residues, including residues of the α1 helix, which has been identified as the dimerization surface in NarL-type response regulators, and the α4-β5-α5 surface, which is the dimerization interface of PhoB-type response regulators; and (iv) residues in the helix-turn-helix DNA-binding domain (39, 44, 48). Because the structure of the RcsB receiver domain has not been solved, sequence alignments and comparison of structural models of the RcsB receiver domain (see "Experimental Procedures") with conserved features of response regulators (39) were used to choose amino acid residues for alanine scanning mutagenesis. The models of the RcsB receiver domain suggest that the active quintet comprises amino acid residue Asp-56 (phosphorylation site) (3) as well as Asp-10, Asp-11, Thr-87, and Lys-109 (Fig. 5*A*). The three aspartate residues of the presumptive active quintet (Asp-10, Asp-11, and Asp-56) coordinate the metal ion that is essential for the phosphoryl group chemistry and hence for receiver domain function (39). Of the active quintet, alanine substitutions of residues Asp-11, Asp-56, Thr-87, and Lys-109 were analyzed. In addition, conserved amino acid residues Pro-60 and Gly-67 as well as residue Met-88 were exchanged to alanine (Fig. 5*A*). At the Met-88 position, a small amino acid (alanine or glycine) is conserved (39). Furthermore, the structural model (Fig. 5*B*) was used to choose presumably surface-exposed residues mapping in α4-β5-α5 (Leu-95, Ser-96, Leu-99, Asp-100, Glu-104, Ile-106, Leu-108, Thr-114, Asp-115, and Lys-118) as well as six additional amino acid residues (Ile-14, Asp-62, Tyr-64, Asp-66, Arg-76, and His-77), including Ile-14 mapping in the α1 helix.

For RcsB mutant analyses the reporter strains (T1052 for RcsB-RcsB, T963 for RcsA-RcsB, T572 for BglJ-RcsB, and T1987 for MatA-RcsB) were transformed with plasmids coding for wild-type RcsB (pKETS6) as well as the RcsB mutants (Fig. 6). To allow data comparison, the expression levels directed by the respective *lacZ* reporter fusions were normalized to those obtained in the presence of the active RcsB derivative D56E, which were defined as 100% (Fig. 6). A brief summary of the data is as follows. First, mutation of residues of the active quintet, including Asp-56, Asp-11, Thr-87, and Lys-109 had the highest impact on the phosphorylation-dependent RcsA-RcsB heterodimer and on RcsB-RcsB homodimers. Among these mutants, only K109A had a strong impact on activation by BglJ-

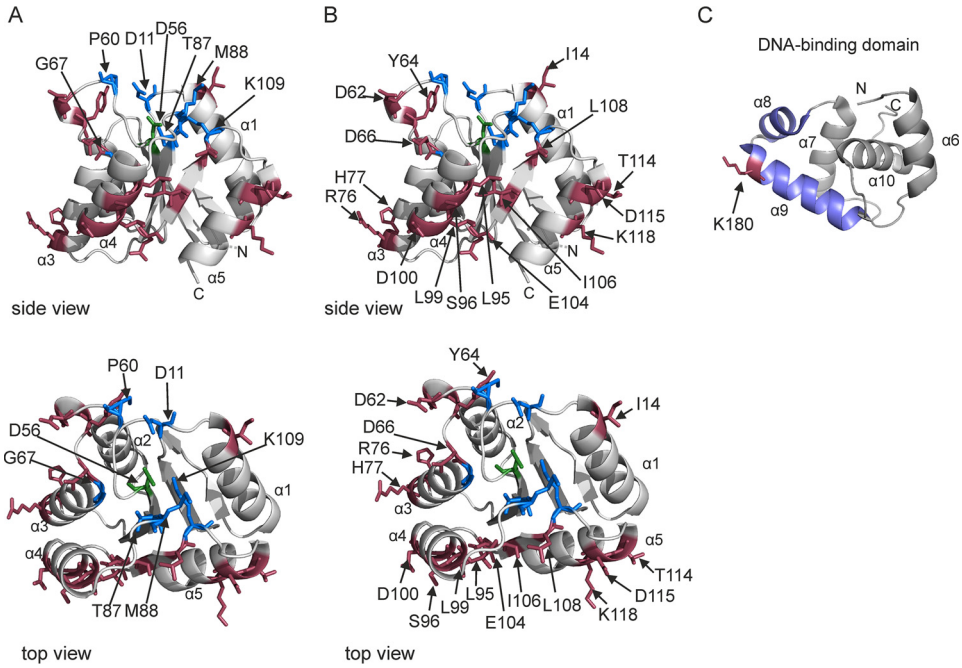


FIGURE 5. **Structure model of the receiver and helix-turn-helix domains of RcsB.** The structure model of the RcsB receiver domain predicted by the Phyre2 server (57) on the basis of the crystal structure (PDB code 3EUL) of *M. tuberculosis* NarL (36). *Colored amino acids* were replaced by alanine. *Blue*, active site residues Asp-11, Thr-87, and Lys-109 and highly conserved residues Pro-60, Gly-67, and Met-88. *Green*, the phosphorylation site Asp-56. *Pink*, presumably surface-exposed residues. For structure presentation, we used the PyMOL Molecular Graphics System, version 1.7.4 (Schrödinger, LLC). *A* and *B* show the same *side* and *top views* of the RcsB receiver domain. In *A*, residues of the active quintet and conserved residues of RcsB are *labeled*, whereas in *B*, other surface-exposed residues that were mutated are *labeled*. *C*, structure of the RcsB helix-turn-helix domain of *E. amylovora* (PDB code 1P4W) (32), which is 92% identical to *E. coli* RcsB. α helices 8 and 9, which bind to the DNA, are shown in *blue*. Residue Lys-180, which was mutated, is labeled K180.

RcsB and MatA-RcsB (Fig. 6). Second, mutation G67A strongly impaired RcsB-RcsB, RcsA-RcsB, and BglJ-RcsB, but not MatA-RcsB, whereas P60A impaired only the phosphorylation-dependent RcsB-RcsB and RcsA-RcsB (Fig. 6). In contrast, M88A resulted in high activity of all RcsB homo-/heterodimers (Fig. 6). Third, of the mutations of presumptive surface-exposed residues of α4-β5-α5, those that are located close to the phosphorylation site (including L95A, I106A, L108A, and D115A) reduced the activity of RcsB and RcsA-RcsB but had little to no impact on BglJ-RcsB and MatA-RcsB (Fig. 6). Fourth, of the six additional presumptive surface-exposed amino acid residues, only mutation of Ile-14 impaired the activity of all dimers, whereas mutation of Asp-62, Tyr-64, Asp-66, Arg-76, and His-77 affected RcsB-RcsB, RcsA-RcsB, and BglJ-RcsB and had no effect on MatA-RcsB (Fig. 6). Fifth, mutation of Lys-180 in the helix-turn-helix DNA-binding domain impaired all dimers (Fig. 6).

Taken together, RcsB homodimers and RcsA-RcsB heterodimers, both depending on RcsB phosphorylation, are impaired by mutations of residues Asp-11, Asp-56, Pro-60, Gly-67, Thr-87, Lys-109 (active quintet and highly conserved) as well as the surface-exposed Ile-14, Asp-62, Tyr-64, Asp-66, Arg-76, His-77, Leu-95, Ile-106, Leu-108, and Asp-115. For the activity of BglJ-RcsB dimers, amino acids Ile-14, Tyr-64, Gly-67, Lys-109, and Lys-180 have a crucial role and Asp-62 and Arg-76 are important. For the activity of MatA-RcsB, mutations of amino acids Ile-14, Lys-109, and Lys-180 are relevant. These data suggest that a structural change that is presumably elicited by phosphorylation and considered to stabilize the active form controls RcsB-RcsB and RcsA-RcsB activity and that the heterodimers of BglJ-RcsB and MatA-RcsB are intrinsically active.

Discussion

The interaction of the transcriptional response regulator RcsB with auxiliary proteins modulates the DNA binding specificity, adds a further level of output control to the Rcs system, and expands the regulatory role of RcsB in *E. coli*. RcsB functions as homodimer and in interaction with the auxiliary response regulator-like proteins RcsA and BglJ of the FixJ/NarL-family as well as with GadE (3, 8, 9). Our two-hybrid data suggest that RcsB in addition forms heterodimers with MatA (also known as EcpR) and DctR, which likewise belong to the FixJ/NarL family of transcriptional regulators (Fig. 7). Further data suggest that the activation of the *matA* promoter and repression of motility (23, 43) is mediated by the MatA-RcsB heterodimer independently of RcsB phosphorylation, whereas no target gene of DctR-RcsB could be identified. An alanine scanning mutagenesis of the RcsB receiver domain revealed that the majority of mutations in the vicinity of the phosphorylation site Asp-56 impair the activity of the phosphorylation-dependent RcsB-RcsB homodimer and RcsA-RcsB heterodimer but have little effect on the BglJ-RcsB and MatA-RcsB heterodimers, which are phosphorylation-independent. These residues are presumably affected by the structural change that is elicited by phosphorylation. Mutation of very few amino acid residues within the receiver domain, including Ile-14 and Lys-109, impaired or reduced the activity of all of the RcsB heterodimers that we tested. Further, mutation of residues Asp-62, Gly-67, Tyr-64, and Arg-76 significantly impaired the activity

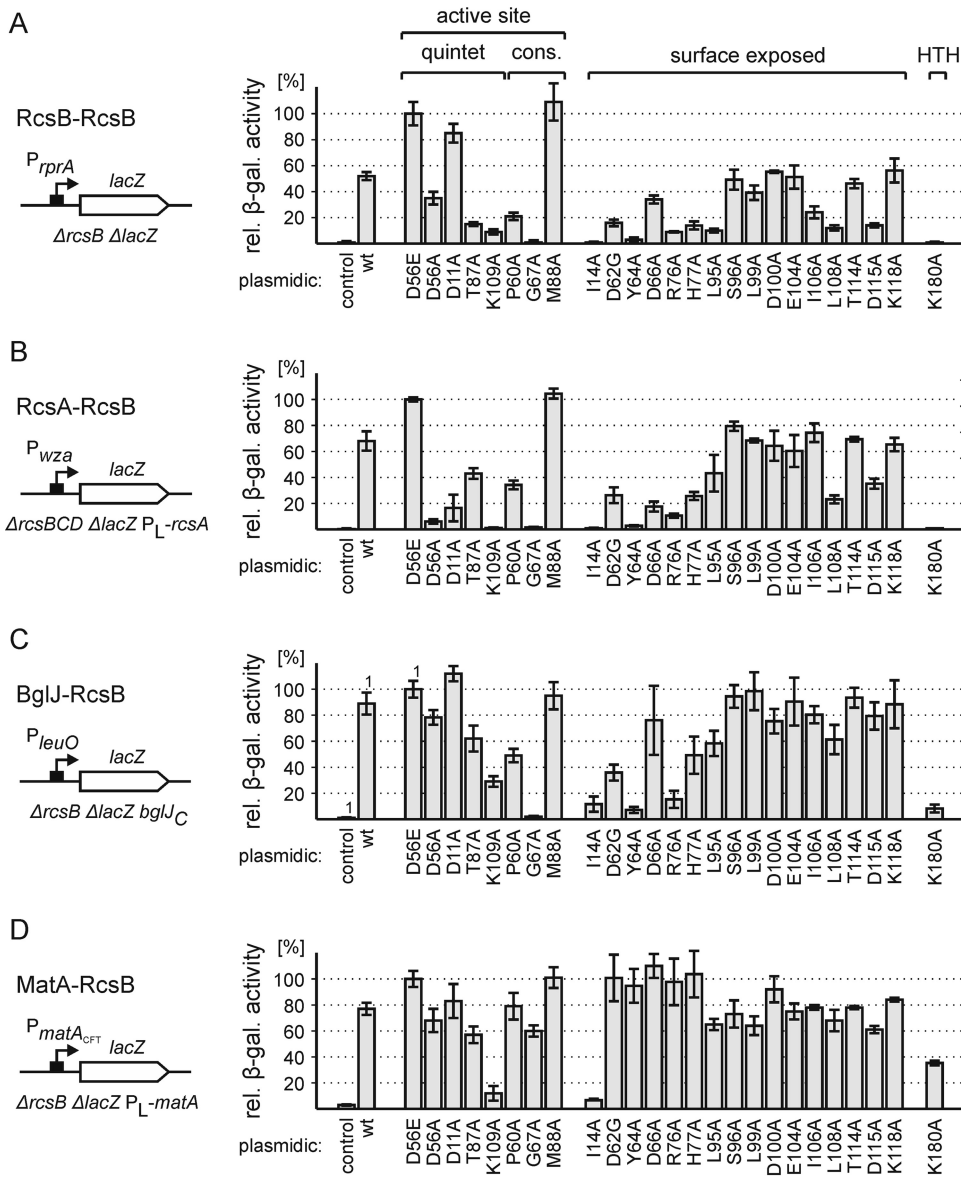


FIGURE 6. **Activation by RcsB mutants as RcsB-RcsB homodimer and as RcsA-RcsB, BglJ-RcsB, and MatA-RcsB heterodimers.** The activity of RcsB mutants with exchanges of residues of the active quintet and conserved residues, surface-exposed residues, and a mutation mapping in the DNA-binding domain was analyzed using reporters for RcsB-RcsB (*A*, strain T1052), RcsA-RcsB (*B*, strain T963), BglJ-RcsB (*C*, strain T572), and MatA-RcsB (*D*, strain T1987). The strains were transformed with empty plasmid pKESK22, as control, and plasmids expressing wild-type and mutant RcsB under control of the IPTG-inducible *tac* promoter. The pKETS6-derived RcsB plasmids are listed in Table 2. β-Galactosidase expression levels were normalized to the values obtained for RcsB-D56E, which was defined as 100%. Values of *bars marked with 1* are from Ref. 17. Cultures for β-galactosidase assays were grown to A_{600} of 0.5 in LB medium supplemented with 1 mM IPTG and 25 mg/ml kanamycin. *Error bars* represent S.D.

of BglJ-RcsB, RcsA-RcsB, and RcsB-RcsB but had little effect on MatA-RcsB. These data suggest that the stability and/or interaction surface of RcsB with its auxiliary partner proteins varies.

The auxiliary interaction partners of RcsB, which include RcsA, BglJ, MatA, DctR, and GadE, all belong to the family of FixJ/NarL-type helix-turn-helix DNA-binding proteins. These auxiliary proteins do not form or only weakly form homodimers, and they do not interact among each other, as analyzed by a two-hybrid assay. Other members of the FixJ/NarL family present in *E. coli* K12 form homodimers but no heterodimers with RcsB. The interaction of RcsB with the auxiliary proteins alters the DNA binding specificity because the DNA-binding motifs of RcsB homodimers and heterodimers with RcsA, BglJ, and MatA are similar in one half-site, which is presumably RcsB-bound, whereas the DNA sequence of the other half-site varies (9, 12, 18, 23, 49). Note that mutation of RcsB residue Lys-180 in the helix-turn-helix motif renders RcsB and its heterodimers inactive and that acetylation of this residue inhibits RcsB DNA binding (50, 51).

Heterodimerization of response regulators in bacteria is rare. Currently, it has been described for BldM and WhiI in the filamentous bacteria *Streptomyces* (19). BldM and WhiI are atypical and orphan response regulators, which are presumably not phosphorylated by cognate sensor kinases. Rather, their expression is controlled by alternative σ factors. Notably, BldM and WhiI likewise belong to the FixJ/NarL family of transcriptional regulators. The BldM homodimer and BldM-WhiI heterodimers play key roles in the morphological differentiation of

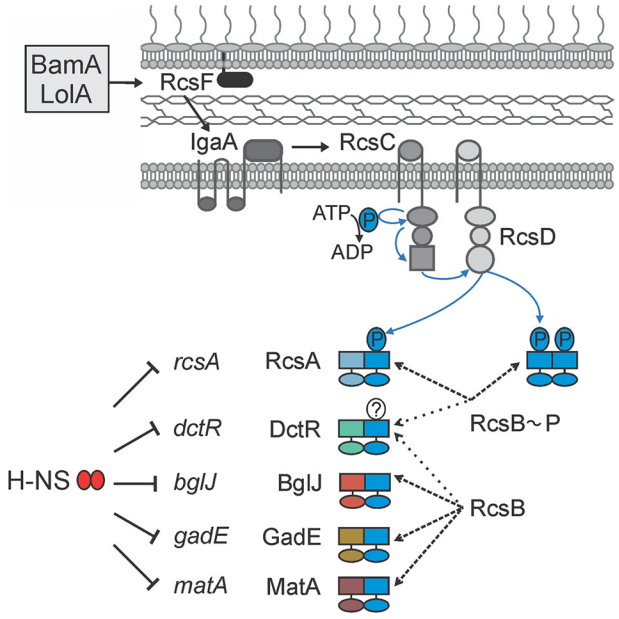


FIGURE 7. **Model of the complex Rcs phosphorelay.** The lipoprotein RcsF monitors the lipoprotein transport by LolA and the outer membrane protein assembly by BamA. Upon perturbations, RcsF activates the sensor kinase RcsC via IgaA (7). Upon induction of the Rcs phosphorelay and autophosphorylation of RcsC, the phosphate is transferred via the receiver domain of RcsC to the histidine transfer domain of RcsD and from there to the receiver domain of RcsB. Regulation of target genes by RcsB homodimers and by RcsA-RcsB heterodimers is dependent on RcsB phosphorylation. RcsB also forms heterodimers with DctR, BglJ, GadE, and MatA. Transcriptional activation by heterodimers of RcsB with BglJ, GadE, and MatA is RcsB phosphorylation-independent. For DctR-RcsB, it is not known whether the activity is phosphorylation-dependent. The genes *rcsA*, *dctR*, *bglJ*, *gadE*, and *matA*, which are encoding the auxiliary regulators of RcsB, are all repressed by the nucleoid-associated global repressor H-NS, and activation of their expression provides an additional level of control of the Rcs output.

Streptomyces. BldM homodimers activate transcription of group I genes required for early stages of development, whereas BldM-WhiI heterodimers regulate expression of group II genes involved in the late stages of development. Correspondingly, the BldM consensus DNA-binding site is palindromic, whereas the BldM-WhiI consensus DNA-binding site is asymmetric with one half-site matching the BldM consensus sequence (19). This example demonstrates that heterodimerization of a response regulator is a means to control a developmental program (19).

RcsB and its auxiliary proteins all carry a FixJ/NarL-type helix-turn-helix DNA-binding domain. For other response regulators of this family, such as the homodimeric NarL, VraR, and DesR, it was shown that the DNA-binding domain is buried or inhibited by the non-phosphorylated form of the receiver domain (48, 52, 53). The phosphorylation of the receiver domain induces an interdomain structural change that releases the DNA-binding domain, allowing its dimerization and/or DNA binding. Our RcsB mutant analysis indicates that the activities of the phosphorylation site-dependent RcsB-RcsB and RcsA-RcsB may be regulated by such a structural change because mutations of residues in the vicinity of the phosphorylation site impaired their activity. In addition, Ile-14 (located in the α1 helix) impaired the activity, whereas the mutation of additional presumably surface-exposed residues in the $\alpha4\beta5\alpha5$ surface had no effect. In contrast, for the activity of BglJ-RcsB and MatA-RcsB, only a few residues were found to be important; these are Ile-14 (located in the α1 helix) and Lys-109 next to the active center for both heterodimers and further residues (Asp-62, Gly-67, Tyr-64, and Arg-76) for BglJ-RcsB. These data indicate that the presumptive phosphorylation-induced structural change of the receiver domain and an interdomain interaction are irrelevant for heterodimers formed by RcsB with BglJ and MatA, respectively. Furthermore, the data indicate that RcsB and its auxiliary proteins interact via a surface that includes helix α1, similar to NarL and related proteins (48, 53), because the mutation of RcsB Ile-14 to alanine renders all dimers inactive.

How is the interaction of RcsB with the auxiliary proteins regulated? One of the main elements in this control is the availability of the interaction partners. Expression of all of the corresponding genes is repressed by the nucleoid-associated and global repressor protein H-NS (22, 47, 54, 55) (Fig. 7). Still, little is known about the induction of these genes. The *rcsA* gene is autoregulated by RcsA-RcsB (12, 56), but our data indicate that induction of Rcs signaling is not sufficient to induce expression of the RcsA-RcsB target *cps*/*wza*, suggesting that the cellular RcsA concentration is still low, at least at 37 °C. Further, the RcsA protein is a substrate of the ATP-dependent Lon protease, which adds a further level of control on RcsA (14). The *bglJ* gene is the second gene of the H-NS repressed *yjjQ-bglJ* operon that can be activated by the pleiotropic transcription regulator LeuO (54). However, the *leuO* gene is also repressed by H-NS at standard laboratory growth conditions. Although the *leuO* gene can be activated by BglJ-RcsB and the mutual regulation of *leuO* and *bglJ* resembles a double-positive feedback regulation, inducing conditions are not known (17). The *dctR* gene is encoded in the acid stress island and presumably activated by YdeO, a regulator of genes involved in the cellular response to acid resistance. This is in agreement with the role of DctR in protection against metabolic end products under acidic conditions (26). The *matA* gene is the first gene of the *mat* (*ecp*) operon that encodes a conserved fimbrial adhesin in *E. coli*. Expression of this locus is induced at low temperature, acidic pH, and high acetate concentration in some lineages of *E. coli*, including B2 strains, whereas in other strains, *mat* is not expressed due to variations in the promoter region (23). Taken together, it seems that the activity of the RcsB heterodimers is largely controlled by the regulation of the auxiliary partners. Further, it is an open question whether the auxiliary partners do compete for RcsB under certain conditions.

Author Contributions—D. P. conducted most of the experiments, analyzed the results, and wrote the paper with K. S. M. F. conducted experiments on homodimer and heterodimer formation and analyzed the results. L. G. conducted experiments on RcsB mutants. K. S. conceived the project and wrote most of the paper.

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