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Transcriptional regulation by BglJ–RcsB, a pleiotropic heteromeric activator in *Escherichia coli*

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ABSTRACT

The bacterial Rcs phosphorelay signals perturbations of the bacterial cell envelope to its response regulator RcsB, which regulates transcription of multiple loci related to motility, biofilm formation and various stress responses. RcsB is unique, as its set of target loci is modulated by interaction with auxiliary regulators including BglJ. The BglJ–RcsB heteromer is known to activate the HNS repressed *leuO* and *bgl* loci independent of RcsB phosphorylation. Here, we show that BglJ–RcsB activates the promoters of 10 additional loci (*chiA*, *molR*, *sfsB*, *yecT*, *yqhG*, *ygiZ*, *yidL*, *ykiA*, *ynbA* and *ynjI*). Furthermore, we mapped the BglJ–RcsB binding site at seven loci and propose a consensus sequence motif. The data suggest that activation by BglJ–RcsB is DNA phasing dependent at some loci, a feature reminiscent of canonical transcriptional activators, while at other loci BglJ–RcsB activation may be indirect by inhibition of HNS-mediated repression. In addition, we show that BglJ–RcsB activates transcription of *bgl* synergistically with CRP where it shifts the transcription start by 20 bp from a position typical for class I CRP-dependent promoters to a position typical for class II CRP-dependent promoters. Thus, BglJ–RcsB is a pleiotropic transcriptional activator that coordinates regulation with global regulators including CRP, LeuO and HNS.

INTRODUCTION

RcsB is the response regulator of the Rcs phosphorelay which is conserved in *Enterobacteriaceae*. RcsB plays a pleiotropic role in the control of biofilm formation and motility as well as in the general stress response (1,2). Correspondingly, RcsB controls transcription initiation at multiple loci. It does so either as homodimer or together with auxiliary transcriptional regulators including RcsA, BglJ, GadE, MatA and others (1,3–6). Interaction of RcsB with the auxiliary factors extends its regulatory repertoire and represents a unique mechanism of modulation of transcriptional control in bacteria, where heteromeric transcription factors are rare.

RcsB and its auxiliary regulators all belong to the same family of transcriptional regulators carrying a characteristic C-terminal helix-turn-helix DNA-binding domain of the FixJ-type (also named LuxR, NarL or UhpA-type) (7,8). The N-terminal domain of RcsB corresponds to a receiver domain typical of two-component response regulators, which becomes phosphorylated by the Rcs phosphorelay in response to perturbations of the outer membrane and peptidoglycan layer (2,9). Perception of these extracellular signals requires the lipoprotein RcsF and the sensory and transmitter proteins RcsC and RcsD (10,11). The N-terminal domains of the auxiliary regulators RcsA (11) and BglJ are related to a receiver domain, but their alignment suggests that BglJ lacks a aspartic acid phosphorylation site.

Genetic and two-hybrid analyses conducted for RcsA and BglJ, as well as molecular analyses for GadE, suggest that RcsB interacts with these auxiliary proteins and possibly forms heterodimers or heterooligomers (1,3,4). Accordingly, the DNA-binding motifs of RcsB homodimers, RcsB–RcsA heteromers and BglJ–RcsB heteromers are similar in only one half, which may be bound by RcsB (4,12,13). RcsA and BglJ are completely dependent on RcsB (1,4). The activity of the heteromeric RcsA–RcsB depends on phosphorylation of RcsB, while BglJ–RcsB is active independently of RcsB phosphorylation (4). An additional level of control of the RcsB-heteromers is based on the regulation of the genes encoding the auxiliary proteins. Expression of *rcsA* and *bglJ* is repressed by HNS, a global repressor and nucleoid structuring protein (14,15). Regulation of the *gadE* gene is part of the complex acid stress response network and involves multiple regulators (16).

In this study, we focused on the RcsB auxiliary protein BglJ. BglJ is an RcsB-dependent transcriptional regulator which was initially identified as activator of the *bgl* (aryl-β,D-glucoside) operon in *Escherichia coli* (17). At the *bgl*

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locus, the BglJ–RcsB binding site was mapped to a position ~100 bp upstream of the promoter (4). The *bgl* promoter is repressed by HNS and StpA, and binding of BglJ–RcsB within the regulatory region of the promoter abrogates repression by HNS (4,18,19). More recent studies demonstrated that BglJ–RcsB also activates the transcription of the *leuO* gene encoding the pleiotropic transcriptional regulator and HNS antagonist LeuO (20–22). Expression of the *leuO* gene is likewise repressed by HNS and StpA (20,23). The *leuO* gene has two promoters: the distal *leuO* P1 promoter becomes fully active in an *hns stpA* double mutant, whereas the proximal *leuO* P2 promoter is activated by BglJ–RcsB. The BglJ–RcsB binding site is situated ~70 bp upstream of this P2 promoter (20). Furthermore, activation of the *leuO* P2 promoter by BglJ–RcsB is inhibited by LeuO acting as negative autoregulator (20). As both of the characterized BglJ–RcsB activated loci, *bgl* and *leuO*, are repressed by HNS and StpA, BglJ–RcsB is a presumptive HNS and StpA antagonist. Beyond that, microarray data suggest that BglJ–RcsB regulates ~30 additional loci (20).

Here, we analyzed the activation of 10 target loci by BglJ–RcsB and we define a consensus DNA-binding motif. Our data suggest that BglJ–RcsB activates transcription of HNS repressed and non-HNS repressed loci by several mechanisms. At some promoters the mechanism corresponds to that of 'classical' bacterial activators, which bind upstream of the core promoter and interact with RNA polymerase. In addition, BglJ–RcsB is a transcriptional activator that can function as HNS antagonist. Furthermore, we found that BglJ–RcsB at the *bgl* operon activates a P2 promoter which is located 20 bp upstream of the known CRP-dependent *bgl* promoter. Both BglJ–RcsB and CRP are required for transcription activation of the newly identified P2*bgl*. The data suggests that BglJ–RcsB acts synergistically with CRP resulting in a shift of the transcription start site and abrogation of HNS mediated repression of *bgl*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strains and plasmids were constructed applying standard techniques. Details on their genotype and relevant structure, respectively, as well as their construction are given in the supplement (see Supplementary Table S1 for strains, Supplementary Table S2 for plasmids and Supplementary Table S3 for oligonucleotides). Briefly, for cloning fragments were amplified by PCR using primers carrying restriction sites at their 5′ ends (Supplementary Table S3). The integrity of the constructed plasmids was analyzed by sequencing of the cloned fragments and by restriction analyses of the overall plasmid structure. Site-specific mutations were introduced by PCR based methods using either combined chain reaction (24) or overlapping PCR fragments, as described earlier (4,20). Promoter *lacZ* reporter constructs were integrated into the phage Lambda *attB* site using a plasmid based *attB* integrations system, as described (25,26). Genomic mutations and deletions were introduced by Red-Gam mediated recombination (27) or transferred by transduction using phage T4*GT7* (28). All mutations and insertions were validated by PCR using primers flanking the respective alleles. Antibiotics were added for selection to final concentrations of 25 μg/ml kanamycin, 50 μg/ml ampicillin, 50 μg/ml spectinomycin and 12.5 μg/ml tetracycline, where necessary.

Expression analysis

For expression analysis of promoter *lacZ* reporter fusions, β-galactosidase assays were performed of exponential cultures grown in LB medium to an optical density of 0.5 at 600 nm, as described (20,29). The assay was repeated at least three times from independent cultures, and the standard deviation of the values (in arbitrary Miller Units) was <15% unless otherwise indicated. For qRT-PCR analyses RNA was isolated from cultures grown in LB medium to the exponential phase (optical density 0.5 at 600 nm) using the bacterial RNAprotect and RNeasy MiniKit system (Qiagen). Then cDNA was synthesized with the SuperScript III First Strand Synthesis Kit (Invitrogen) using 1 mg of RNA and random hexameric oligonucleotides as primers. Subsequently, quantitative PCR was carried out with gene specific oligonucleotide primers (Supplementary Table S3), SYBR Green I and a C1000 touch thermal cycler with optical reaction module CFX96 (Bio-Rad), as described (20). Data were normalized to *rpoD*, encoding the housekeeping sigma 70 subunit, as reference gene using the CFX Manager Software 2.1 (Bio-Rad) and applying a $\Delta\Delta C_t$ algorithm. For each locus, the relative expression was calculated as the ratio of the value obtained for the RNA isolated from mutant strains (T1166, T1048) as compared to the data obtained for RNA isolated from the reference strain (T75).

Mapping of transcription start sites by 5′-RACE

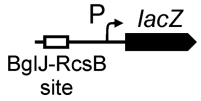
5′ RACE analysis was performed, as described (20,30). Briefly, RNA was isolated from strains T75, T1166 and T1048, as described earlier. Six microgram of the RNA was treated with tobacco acid pyrophosphatase (TAP; Epicentre Biotechnologies). After phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation the RNA adapter oligonucleotide T268 was ligated to the TAP-treated RNA and to the same amount of untreated RNA using RNA ligase (New England Biolabs) premixed with RNase inhibitor SuperaseIn (Ambion). The RNA adapter ligations were incubated overnight at 17°C, the samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), the RNA was precipitated with ethanol, and resuspended in 20 μl H_2O. Half of the RNA samples were used for cDNA synthesis using random hexameric oligonucleotide primers and the SuperScript III First Strand Synthesis Kit (Invitrogen). The cDNA was amplified by PCR using the RNA adapter specific DNA primer T265, gene specific primers (Supplementary Table S3) and PlatinumTaq Polymerase (Invitrogen). PCR products were digested with EcoRI and XbaI, separated by agarose gel electrophoresis, and cloned into the EcoRI and XbaI digested vector pUC12. The

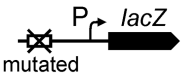
fragments of several clones were sequenced for mapping of the primary transcription start sites.

RESULTS

Regulation of the presumptive target promoters by BglJ–RcsB

BglJ–RcsB is a presumptive pleiotropic activator, as in a microarray analysis roughly 30 loci were found to be upregulated upon ectopic expression of BglJ in a *rcsB*$^+$ background but not in an isogenic *ΔrcsB* mutant (20). To analyze whether transcription of these putative target loci is indeed activated by BglJ–RcsB, in a first step, we constructed *lacZ* transcriptional fusions of the promoters of 10 putative target loci. In these constructs. the cloned fragments (ranging in size from 168 to 348 bp) encompass the intergenic region in between the target gene and the gene mapping upstream to include the promoter and possible regulatory sites (Figure 1; Supplementary Table S2). The selected loci include *chiA* encoding endochitinase, *yehH* (*molR*) presumably encoding molybdate metabolism regulator, and the loci *ykiA*, *ynbA*, *yqhG*, *sfsB*, *ynjI*, *yidL*, *ygiZ* and *yecT*. The latter genes encode proteins of unknown or predicted function (Table 1). The promoter *lacZ* fusions were integrated into the genome of strain T314 carrying deletion Δ*yjjP-yjjQ-bglJ* that encompasses *bglJ*. In addition, isogenic strains carrying a constitutively expressed *bglJ* allele (*bglJ*$_C$) were constructed. Constitutive expression of allele *bglJ*$_C$ is driven by a miniTn10 insertion upstream of *bglJ* (31). As a further control expression was analyzed in an isogenic *bglJ*$_C$ Δ*rcsB* background. Furthermore, all strains that were constructed carry a *lacZ* deletion and a deletion of the BglJ–RcsB activated *leuO* gene encoding the pleiotropic regulator and HNS antagonist LeuO (20,21), although we found later that the presence of *leuO* has little influence on the BglJ–RcsB targets (data not shown). The results of expression analyses in the *bglJ* deletion and *bglJ*$_C$ strain backgrounds as well as in the *bglJ*$_C$ Δ*rcsB* background demonstrate that all 10 promoter *lacZ* fusions are specifically activated in the presence of BglJ, ranging from a 4- to 73-fold activation, and that the activation by BglJ is RcsB dependent (Figure 1).





	β-gal. activity			fold act.	β-gal. activity	
	-	*bglJ*$_C$ Δ*rcsB*	*bglJ*$_C$		-	*bglJ*$_C$
P_{chiA} -115 to +95	6	9	66	11x		n.t.
P_{molR} -185 to +42	13	14	280	21x	18	18
P_{sfsB} -129 to +39	3	3	12	4x	3	3
P_{yecT} -112 to +120	<1	1	48	48x		n.t.
P_{ygiZ} -182 to +63	330	418	1340	4x	490	490
P_{yidL} -142 to +39	60	56	234	4x	51	51
P_{ykiA} -222 to +45	18	16	77	4x	25	18
P_{ynbA} -169 to +43	24	17	1760	73x	18	17
P_{ynjI} -271 to +37	33	29	320	10x	37	38
P_{yqhG} -221 to +127	25	21	1470	58x		n.t.

Figure 1. Specific activation of target gene promoters by BglJ–RcsB. Left panel: The expression level of chromosomal promoter *lacZ* fusions (coordinates given relative to the transcription start site) was determined in the *bglJ* deletion background (allele Δ*yjjP-yjjQ-bglJ*) (−), in isogenic *bglJ*$_C$ strains expressing BglJ constitutively, and in isogenic *bglJ*$_C$ *ΔrcsB* mutant derivatives. The factor of activation by BglJ–RcsB (fold act.) was calculated by dividing the β-galactosidase activity (β-gal. activity) determined in the *bglJ*$_C$ strain by the activity determined in the absence of BglJ (−). Right panel: BglJ–RcsB binding sites were mutated (mutations given in Figure 2), and the expression level of the mutant promoter *lacZ* fusions was determined in the absence (−) and presence of BglJ (*bglJ*$_C$) using isogenic *bglJ* deletion (Δ*yjjP-yjjQ-bglJ*) and *bglJ*$_C$ strains. For the seven promoters analyzed, the mutation of the binding site abrogated their activation by BglJ–RcsB. Cultures were grown in LB medium to the exponential phase and harvested at OD_{600} = 0.5; β-galactosidase activities are given in arbitrary units (29); n.t. is not tested.

Mapping of the promoters activated by BglJ–RcsB

To further characterize the regulation of the presumptive BglJ–RcsB target promoters, we mapped the transcription start sites of the 10 loci by 5′–RACE analyses of primary transcript. For this we isolated RNA from strain T75, carrying the *bglJ* deletion (allele Δ*yjjP-yjjQ-bglJ*) and of strain T1166 carrying the constitutive *bglJ*$_C$ allele. In the presence of BglJ (expressed from allele *bglJ*$_C$) a specific 5′-RACE product was obtained for each of the 10 loci, as analyzed by agarose gel electrophoresis. In the absence of BglJ the respective 5′-RACE product was apparent but very weak. These results support the conclusion that the analyzed loci are activated by BglJ–RcsB within their chromosomal context. To precisely map the transcription start sites, the 5′-RACE products obtained of the *bglJ* deletion and the *bglJ*$_C$ strains were cloned, and for each locus several independent clones were sequenced. For *chiA* the previously mapped transcription start site at genome position 3467918 (coordinates as in *E. coli* K12 MG1655 reference genome sequence NC_000913) (32) was confirmed. The map positions of the newly identified transcription start sites of the other nine loci are summarized in Figure 2.

Mapping of BglJ–RcsB binding sites

For characterization of the presumptive BglJ–RcsB binding sites and definition of a consensus binding motif, we took an iterative approach. First, we scanned the sequence of the target promoter regions for similarity to the two known BglJ–RcsB binding sites mapped at the *bgl* and *leuO* loci (4,20) using the program MEME

Table 1. Function of analyzed BglJ–RcsB target loci

Gene(s)	Function	References
chiA	Endochitinase (897 aa)	(32)
ynbAB	YnbA predicted diacylglycerol choline-phosphotransferase (201 aa)YnbB inner membrane protein (298 aa)	(33,34)
ynjI	Inner membrane protein (346 aa)	(34)
yecT	Unknown function (162 aa)	(33)
yqhGH	YqhG unknown function (308 aa)YqhH predicted lipoprotein (85 aa)	(33)
ygiZ	Inner membrane protein (110 aa)	(34)
sfsB	SfsB/Nlp transcriptional regulator, Ner-like protein (92 aa)	(33,35)
ykiA	Unknown function (113 aa)	(33)
yidL	Predicted AraC-type transcription regulator (307 aa)	(33)
molR (*yehH*)	Molybdate metabolism regulator (disrupted in K12)	(33)

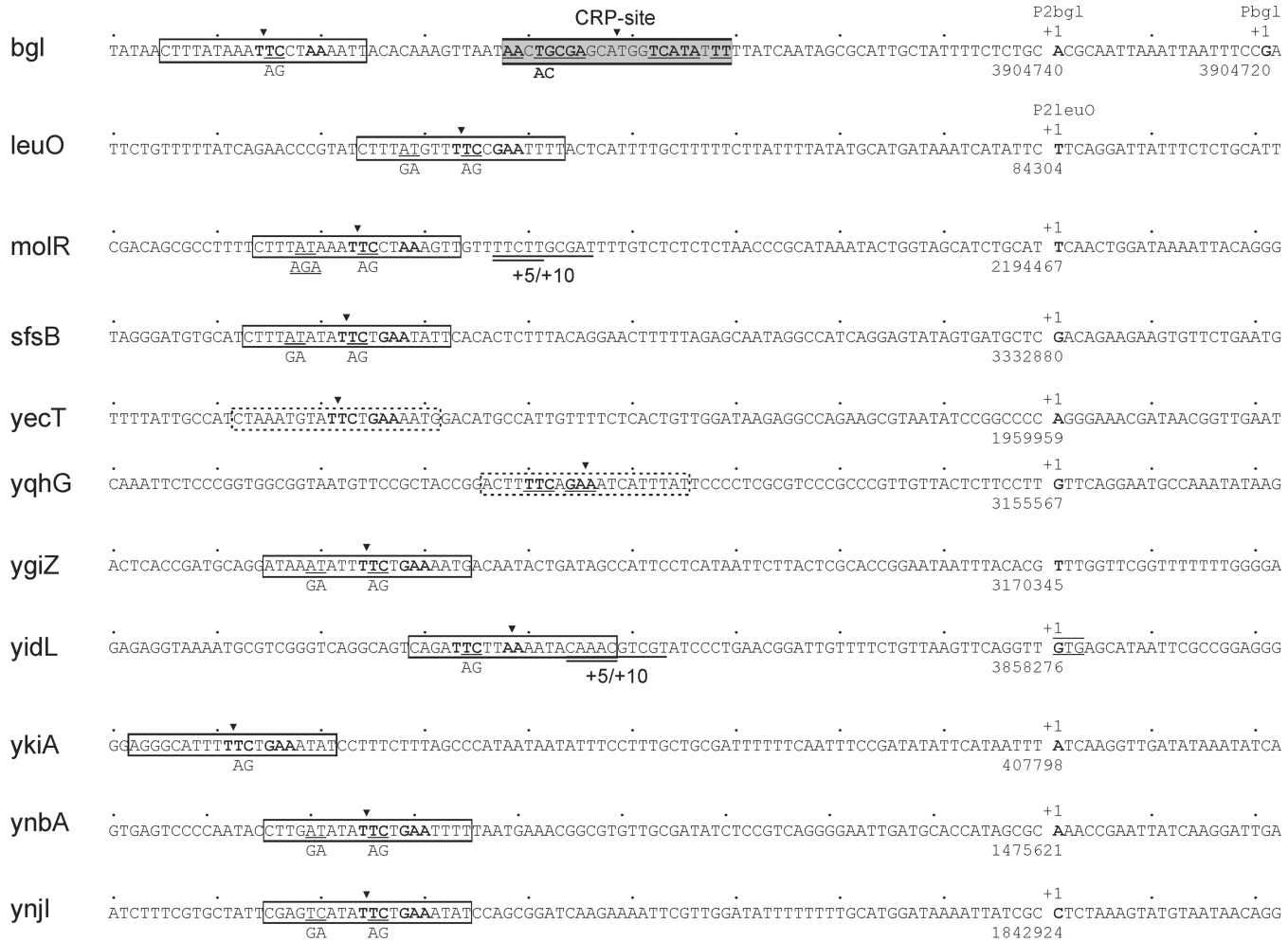


Figure 2. Sequence of BglJ–RcsB activated promoters and their BglJ–RcsB binding sites. The transcription starts sites (+1) were mapped by 5′-RACE of RNA isolated from strain T1166 constitutively expressing BglJ, as well as of RNA isolated from strain T75 carrying a *bglJ* deletion (allele Δ*yjjP-yjjQ-bglJ*). The positions of the transcription start sites refer to the MG1655 genome sequence NC_000913. For 5′-RACE analysis of *bgl* also RNA of the Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* mutant strain T1048 was used. This revealed the transcription start site mapping at position 3904720 in the *bglJ* deletion background (T75, Δ*yjjP-yjjQ-bglJ*) and Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* (T1048), i.e. the absence of BglJ, while the transcription start site at position 3904740 is used in the BglJ positive strain T1166. The BglJ–RcsB activated *leuO* P2 promoter was mapped previously (20). BglJ–RcsB binding sites marked by boxes with solid lines were mapped by mutagenesis in this work (*molR*, *sfsB*, *yecT*, *ygiZ*, *yidL*, *ykiA*, *ynbA* and *ynjI*) or previously [*bgl*, *leuO* (4,20)]. Site-specific mutations are indicated underneath the sequence. Insertions of 5 and 10 bp were generated by duplication of the underlined sequences (+5/+10). Predicted BglJ–RcsB binding sites are marked by boxes with dashed lines (*yecT* and *yqhG)*. The transcription start site of the *yidL* promoter maps to the first base of a GTG codon (underlined) that is annotated as translation start site (33).

SUITE (36). This approach yielded a putative BglJ–RcsB consensus motif, with a significant score (P-value $< 10^{-7}$) in eight loci. Mutagenesis of this predicted binding motif in the promoter *lacZ* fusions of five loci including *molR*, *sfsB*, *ygiZ*, *ynbA* and *ynjI*, indeed completely abrogated their activation by BglJ–RcsB. This was demonstrated by chromosomal integration of the mutant promoter *lacZ* fusions and expression analyses in the absence and presence of BglJ (Figure 1). The mutations are depicted in Figure 2. Mutagenesis of a predicted motif which overlapped with the core promoter of *ykiA* as well as mutagenesis of predicted motifs at *yidL* and *chiA* mapping ~150 bp upstream of the transcription start site did not affect their activation by BglJ–RcsB (not shown). Next, 50 bp DNA sequences encompassing the seven known BglJ–RcsB binding sites (including the five ones mapped here) were used to define a sequence logo using MEME SUITE (36). With this motif a putative binding site with a significant score was identified upstream of the *yqhG* promoter (Figure 2), but no significant hits were identified next to the promoters of *ykiA*, *yidL* and *chiA*. Therefore, we manually scanned the sequence upstream of these promoters for a match to the most conserved TTc–AA bases of the motif. Candidate sites for the *ykiA* and *yidL* promoters were mutated (Figure 2). These mutations resulted in the loss of activation of the promoter *lacZ* fusions by BglJ–RcsB (Figure 1). No BglJ–RcsB box was characterized or predicted next to the *chiA* promoter. Finally, the sequences of the nine BglJ–RcsB binding sites characterized in this and previous work were used to define a sequence logo shown in Figure 3 (36). This BglJ–RcsB motif was used to search the K12 genome sequence using the program FIMO (36). By this search all experimentally characterized BglJ–RcsB binding sites were confirmed (Figure 3). Further binding sites were predicted for *yecT* and *yqhG* as well as for the putative target loci *rhsA* and *rhsB* (20). The binding sites shown in Figure 3 scored among the top 14 of all sites that were predicted in the genome sequence. However, the score was lower for the binding site shown for *ykiA* (experimentally verified) and for *yqhG* (predicted). Additional motifs were predicted for other putative BglJ–RcsB targets. However, as their score was lower they require experimental validation to be shown. Interestingly, part of the BglJ–RcsB motif (including the central wwTTCykA sequence) corresponds well to the right half of the consensus sequence for the RcsA–RcsB binding site TaAGaa|tatTCctA (12), indicating that this part may be bound by the RcsB subunit. If this was indeed the case then the BglJ–RcsB motif would be oriented with RcsB facing the promoter at most promoters expect for P*yidL* and P*yqhG*.

bgl	c**TTTATA**aa**TTCctAAa**ATT	3904825 - 3904806
leuO	c**TTTATG**tt**TTCcgAAt**TTT	84238 - 84257
molR	c**TTTATA**aa**TTCctAAa**GTT	2194391 - 2194410
sfsB	c**TTTATA**ta**TTCtgAAt**ATT	3332803 - 3332822
ygiZ	a**TAAATA**tt**TTCtgAAa**ATG	3170420 - 3170401
yidL	G**TTTGTA**tt**TTAAgAAt**CTG	3858213 - 3858234
ykiA	a**GGGCAT**tt**TTCtgAAa**TAT	407710 - 407729
ynbA	c**TTGATA**ta**TTCtgAAt**TTT	1475546 - 1475565
ynjI	c**GAGTCA**ta**TTCtgAAa**TAT	1842999 - 1842980
predicted		
rhsA	**ATAAATA**tt**TTCctAAt**TAT	3760091 - 3760110
rhsB	**CTAAATA**ta**TTCctAAa**AAT	3617112 - 3617131
yecT	**CTAAATG**ta**TTCtgAAa**ATG	1959881 - 1959900
yqhG	**ATAAATG**tt**TTCtgAAa**AGT	3155532 - 3155513

Figure 3. BglJ–RcsB consensus sequence. Top: Sequence logo for the BglJ–RcsB binding site. The logo was generated by MEME SUITE (36) using 120 bp sequences encompassing the mapped BglJ–RcsB binding sites of the loci shown in the middle. Middle: Sequences of the BglJ–RcsB binding sites mapped by site-specific mutagenesis and their genome position in the MG1655 reference genome sequence NC_000913. Bottom: BglJ–RcsB binding sites which were predicted in the *E. coli* K12 MG1655 genome by the program FIMO (36) using the BglJ–RcsB logo (top) as input. All BglJ–RcsB binding sites except *ykiA* and *yqhG* scored among the top 14 hits in the FIMO search of the *E. coli* K12 MG1655 genome sequence. Letters in bold indicate a match to the consensus sequence motif, with upper case letters used for highly conserved bases, and lower case letters for ambiguous conserved bases.

Regulation of BglJ–RcsB targets by HNS/StpA

BglJ–RcsB was first characterized as activator of the HNS repressed loci *bgl* and *leuO* (4,20). Therefore, we had assumed that BglJ–RcsB acts as HNS antagonist, activating gene expression indirectly by inhibiting repression by HNS. To address the correlation between activation of a promoter by BglJ–RcsB and repression by HNS we tested for some of the loci whether they are also repressed by HNS. For quantitative expression analyses, qRT-PCR was performed of RNA isolated from strain T75 carrying the *bglJ* deletion (allele Δ*yjjP-yjjQ-bglJ*), from the isogenic $bglJ_C$ mutant (T1166) and from an isogenic Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* triple mutant derivative (T1048). In the latter strain (T1048), the *bglJ* gene was deleted, as the HNS repressed *yjjQ-bglJ* operon would otherwise be expressed. The normalized qRT-PCR values confirm that all tested loci are activated when BglJ is present (Figure 4). For the *molR* locus the expression level in *the bglJ* deletion strain and the Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* mutant was similar (Figure 4) suggesting that *molR* is not repressed by HNS and StpA. In contrast, expression of *yecT* and *ynjI* was moderately increased in the Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* and strongly increased in the $bglJ_C$ background (Figure 4), indicating that these loci are to some extent directly or indirectly regulated by HNS and/or StpA, while BglJ–RcsB activates them significantly. Two further BglJ–RcsB activated loci, *chiA* and *yidL*, were expressed at similarly high levels in the Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* mutant as in the $bglJ_C$ mutant (Figure 4). The *chiA* promoter is known to be repressed by HNS (32). The data imply that *yidL* is also HNS repressed. Taken together the data suggest, that activation by BglJ–RcsB occurs both at non-HNS repressed promoters as well as at promoters repressed by HNS and/or StpA.

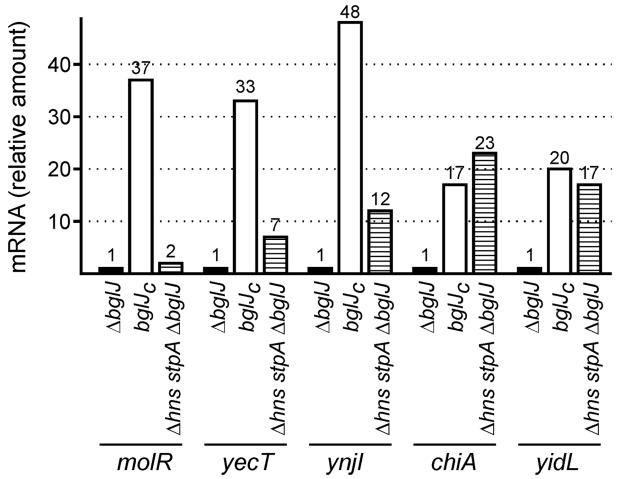


Figure 4. BglJ–RcsB activated genes and their co-regulation by HNS/StpA. For expression analysis of BglJ–RcsB activated genes, a qRT-PCR analysis was performed of RNA isolated from Δ*yjjP-yjjQ-bglJ* deletion strain T75 (indicated as Δ*bglJ*), from strain T1166 ($bglJ_C$) expressing BglJ constitutively and from the Δ*hns stpA* Δ*yjjP-yjjQ-bglJ* mutant T1048 (indicated as Δ*hns stpA* Δ*bglJ*). The qRT-PCR data were normalized to *rpoD*, and for each gene the expression level (indicated as mRNA, relative amount) was calculated relative to the data obtained for RNA isolated from strain T75 (Δ*yjjP-yjjQ-bglJ*), which was set as 1.

Synergistic regulation of bgl by BglJ–RcsB and CRP–cAMP

We previously characterized a sequence motif required for BglJ–RcsB-mediated activation of the *bgl* operon (4). Intriguingly, the center of this presumptive BglJ–RcsB binding site maps 96 bp upstream of the transcription start site of the HNS-repressed and CRP-dependent *bgl* promoter, P*bgl*, and thus further upstream than in case of the other BglJ–RcsB activated promoters (Figure 2). To test, whether BglJ–RcsB indeed activates transcription initiation at P*bgl* we performed a 5′-RACE analysis of RNA isolated from strain T1166 expressing *bglJ* constitutively. As controls, we analyzed RNAs isolated from strain T75 (Δ*yjjP-yjjQ-bglJ*), in which *bgl* transcription is repressed, and from a *Δhns stpA* Δ*yjjP-yjjQ-bglJ* mutant (T1048) in which the *bgl* promoter is active. These 5′-RACE analyses confirmed the transcription start of the known *bgl* promoter P*bgl* in the *bglJ* deletion strain and the Δ*hns stpA ΔyjjP-yjjQ-bglJ* triple mutant (Figure 2). Furthermore, it revealed a new BglJ-dependent transcription start site mapping 20 bp upstream of the start site of the known promoter. Consequently, the BglJ–RcsB binding site is located at position −76 relative to the transcription start site of the newly identified P2 promoter (Figure 2).

The previously identified *bgl* promoter (P*bgl*) is activated by the CRP–cAMP complex with the center of the CRP-binding site located at the canonical -61.5 position of type I CRP-dependent promoters (37). However, this CRP-binding site maps at position -41.5 relative to the BglJ–RcsB activated P2 promoter, and thus at a position typical for class II CRP-dependent promoters. To test whether CRP indeed is required for BglJ–RcsB activation of P2*bgl* or whether this promoter is CRP-independent, we determined expression of a *bgl-lacZ* reporter fusion in the *bglJ* deletion background (allele Δ*yjjP-yjjQ-bglJ*), a BglJ-positive strain ($bglJ_C$) and isogenic Δ*crp* derivatives of these strains (Figure 5A). As expected (4), expression levels were low in the *bglJ* deletion background (15 units) and high (354 units) in the BglJ expressing strain (Figure 5A). However, in the isogenic *crp* mutants expression levels were very low (5 units) irrespective of the absence or presence of BglJ (Figure 5A). These data suggest that activation of P2*bgl* by BglJ–RcsB is CRP-dependent.

Furthermore, complementation of the Δ*crp* mutants with plasmid pDCRP encoding CRP under control of its native promoter (40) restored activation of *bgl* transcription by BglJ–RcsB (Figure 5A). However, complementation with a plasmid encoding CRP-H159L did not restore activation by BglJ–RcsB, respectively (Figure 5A). The CRP-H159L mutant protein carries a substitution in the activation region 1 (AR1) of CRP and is defective in activation of class I promoters and several class II CRP-dependent promoters (40–42). These data further support the notion that CRP is required for activation of *bgl* transcription by BglJ–RcsB, and they demonstrate that the AR1 of CRP is important for this activation. In addition, we tested complementation of the Δ*crp* mutants with CRP-H19L and CRP-K101E mutants. These mutations map in the AR2 of CRP required for activation of open complex formation by RNA polymerase at the synthetic *PmelR* derived −41.5 CC promoter and other class II promoters (40–42). However, in the *bgl* system complementation with CRP-H19L and CRP-K101E mutants restored activation by BglJ–RcsB (Figure 5A). These data suggest that AR2 of CRP is not involved in co-activation of P2*bgl* by BglJ–RcsB and CRP.

As a further experiment to address co-regulation of *bgl* transcription by BglJ–RcsB and CRP we constructed chromosomal *bgl-lacZ* fusions with a mutated CRP-binding site, a mutated BglJ–RcsB-site, and with both sites mutated (Figure 5B). Expression of these fusions and their activation by BglJ–RcsB was tested in the absence and presence of BglJ (using strains carrying allele Δ*yjjP-yjjQ-bglJ* and $bglJ_C$, respectively). In addition, isogenic *crp* mutant derivatives were used (Figure 5B). The expression analysis revealed that activation of *bgl* by BglJ–RcsB requires both an intact BglJ–RcsB binding site and an intact CRP-binding site (Figure 5B). Furthermore, expression analysis in an isogenic Δ*hns stpA* derivative (carrying deletions of *bglJ* and *leuO*) was performed to monitor the influence of the site-specific mutations on *bgl* transcription independently of repression by HNS and StpA. These data demonstrate that the mutation of the BglJ–RcsB binding site does not influence the activity of the *bgl* promoters (Figure 5C). However, the expression levels directed by the *bgl-lacZ* fusions with mutant CRP-binding sites were 3-fold lower, which suggests that the CRP dependence of *bgl* transcription is moderate (3-fold) in the absence of HNS. This later observation is in agreement with *in vitro* transcription data (43). Taken together we conclude that

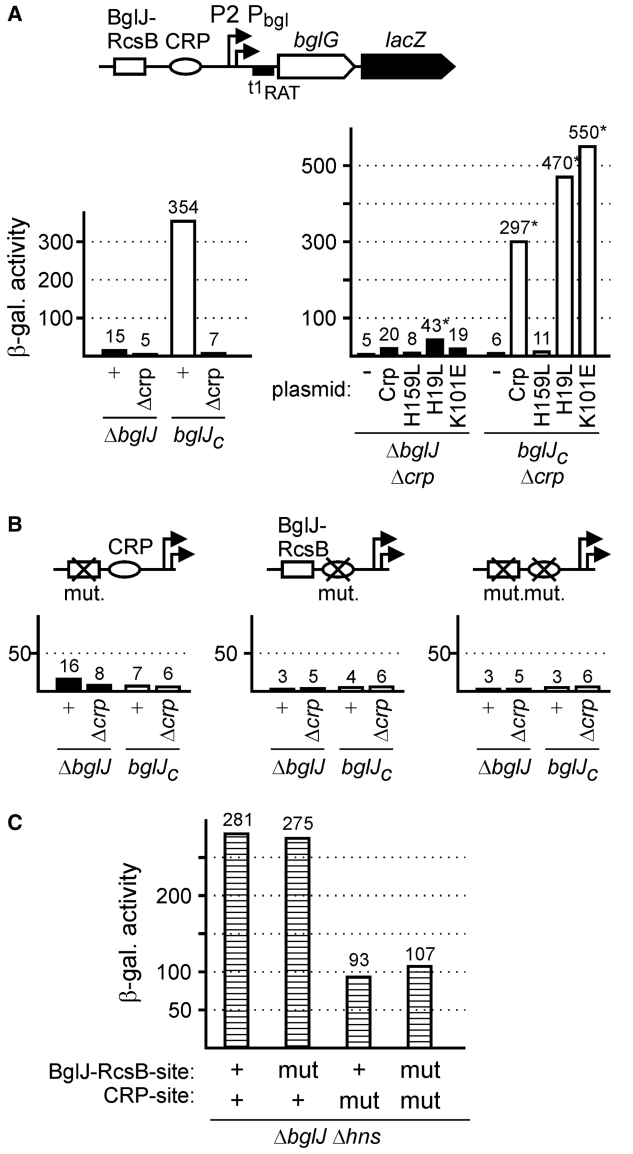


Figure 5. Synergistic regulation of *bgl* by BglJ–RcsB and cAMP-CRP. **(A)** Regulation of the P2*bgl* and P*bgl* promoters by BglJ–RcsB and CRP was analyzed by determining the expression level of a chromosomal *bgl-lacZ* fusion in strain T568 carrying the *bglJ* deletion (allele Δ*yjjP-yjjQ-bglJ*, indicated as Δ*bglJ*), its isogenic Δ*crp* derivative, as well as in isogenic derivatives carrying allele $bglJ_C$ for constitutive expression of BglJ (left panel in A). The Δ*crp* mutants were complemented with plasmids pDCRP, pDCRP-H159L, pKES324 (CRP-H19L), pKES328 (CRP-K101E) and the empty vector pDctrl as control (−) (right panel in A). The *bgl-lacZ* reporter fusion carries all elements required for repression by HNS, and a mutation of terminator t1 ($t1_{RAT}$) to render expression independent of substrate-specific regulation by the operon encoded transcriptional antiterminator BglG (38,39). The standard deviation of β-galactosidase activities marked with an asterisk (*) is 40%. **(B)** Expression of *bgl-lacZ* fusions with mutation of the BglJ–RcsB binding site, the CRP-binding site and mutations of both sites was likewise determined in the four genetic backgrounds, i.e. in the absence and presence of BglJ and CRP, respectively. **(C)** Expression levels directed by *bgl-lacZ* fusions with wild-type and mutant BglJ–RcsB and CRP-sites, respectively, as determined in *hns* mutant strains T729 (wild-type binding sites, indicated as +), T735 (BglJ–RcsB-site mutated), T1543 (CRP-site mutated) and T1545 (both sites mutated).

BglJ–RcsB and CRP synergistically activate the *bgl* P2 promoter and relief repression by HNS and StpA.

BglJ–RcsB, a canonical transcriptional activator and an HNS antagonist

The center of the BglJ–RcsB binding sites maps 65–68 bases upstream of the transcription start site in case of *ynbA*, *ynjI*, *molR*, *ygiZ* and *sfsB*. At *leuO* the BglJ–RcsB binding site maps 57 bases upstream of the transcription start and thus roughly one helical turn closer (Figure 2), while the distance at *bgl* and *ykiA* is 76 and 79 bases, respectively, roughly corresponding to one additional helical turn (Figure 2). This may indicate that activation by BglJ–RcsB is DNA phasing dependent and that the mechanism of activation is by recruitment of RNA polymerase similar to canonical transcriptional activators. However, at *yidL* the center of the BglJ–RcsB binding site maps out of phase at 52 bp upstream of the transcription start site. Furthermore, some of the BglJ–RcsB target promoters are repressed by HNS, including *bgl* (Figure 5) (4) and *yidL* (Figure 4), indicating that activation by BglJ–RcsB can be indirect by inhibition of HNS mediated repression. To test whether activation by BglJ–RcsB is sensitive to DNA phasing, we chose the non-HNS repressed *molR* promoter and the HNS repressed *yidL* promoter, and constructed derivatives with 5 and 10 bp sequence duplications in between the Bgl–RcsB binding site and the core promoter (Figures 2 and 6A). In parallel, we tested a series of 6 bp insertion that map within the regulatory region of the HNS represses *bgl* locus (Figure 6B). Activation of the *molR* promoter by BglJ–RcsB was abrogated upon insertion of 5 bp, but restored to 25% of the wild-type level upon insertion of 10 bp (Figure 6A). In contrast, activation of the HNS repressed *yidL* promoter was similarly reduced to ~50% of the wild-type level upon insertion of 5 and 10 bp. These data suggest that DNA phasing is important in case of activation of the *molR* promoter but not in case of the HNS repressed *yidL* promoter. In contrast, for the HNS repressed *bgl* promoter a 6 bp linker insertion mapping in between the BglJ–RcsB binding site and the CRP-binding site completely abrogated activation, as did insertions mapping within the BglJ–RcsB binding site (Figure 6B). Thus, DNA phasing seems to be crucial in case of the *bgl* promoter which is HNS repressed and synergistically activated by BglJ–RcsB and CRP.

DISCUSSION

BglJ is an auxiliary regulator that activates a specific set of target loci together with RcsB but independent of RcsB phosphorylation (4,20). Previously, BglJ–RcsB was identified as activator of the HNS repressed *leuO* and *bgl* loci (4,17,20). In this work, we characterized the activation of additional loci by BglJ–RcsB. Mapping of the BglJ–RcsB binding sites at seven loci and analysis of helical turn dependence at three loci suggest that BglJ–RcsB activates transcription by several mechanisms. First, at the *molR* promoter BglJ–RcsB may act similar to canonical activators which bind upstream of the core promoter and

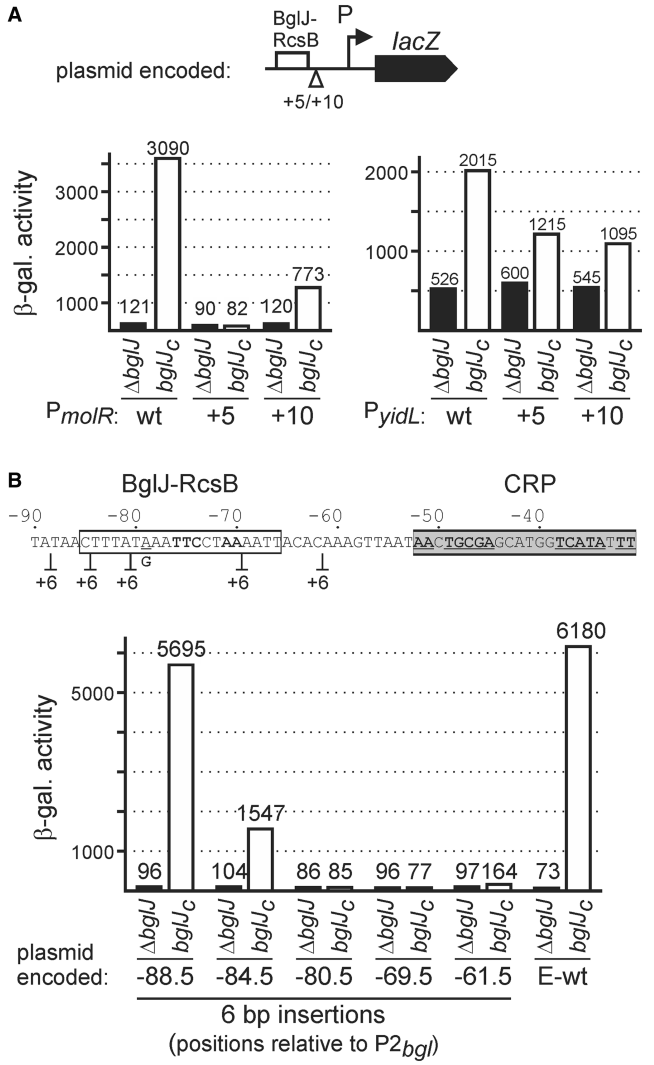


Figure 6. DNA phasing dependence of activation by BglJ–RcsB. **(A)** Activation of plasmid encoded *PmolR* and *PyidL lacZ* fusions as well as derivatives carrying 5 bp (+5) and 10 bp (+10) sequence duplications in between the BglJ–RcsB binding site and the core promoter was tested in the *bglJ* deletion strain T23 (Δ*bglJ*) and the isogenic $bglJ_C$ strain T1030. **(B)** Activation of plasmid encoded *bgl lacZ* fusions carrying the *bgl* promoter region with a single nucleotide exchange at position −79 generating an EcoRI site (E-wt), as well as derivatives with 6 bp CAATTG linker insertions was tested in strain S541 and its isogenic $bglJ_C$ derivative S3910.

interact with RNA polymerase. Second, at the *yidL* promoter BglJ–RcsB may act as HNS antagonist and activate transcription indirectly by preventing HNS-mediated repression. Third, at the HNS-repressed *bgl* operon BglJ–RcsB activates transcription synergistically with CRP. This synergistic activation involves a shift of the transcription start site by 20 bp from the *bgl* promoter (P*bgl*) to a novel P2 promoter (P2*bgl*). Taken together our data demonstrate a pleiotropic and versatile role of the BglJ–RcsB transcriptional activator that functions in the context of simple and more complex promoters, and that acts antagonistically as well as synergistically with additional transcription factors, such as HNS, CRP and LeuO.

The mapping of the asymmetric BglJ–RcsB consensus motif in relation to the transcription start site at several promoters (Figures 2 and 3) suggests a DNA phasing and orientation dependent positioning, with the half-site of the BglJ–RcsB motif that is presumably RcsB bound mapping closer to the promoter at most of the analyzed promoters, except *yidL* and *yqhG* (Figure 2). Furthermore, activation of *molR* and *bgl* by BglJ–RcsB turned out to be DNA phasing dependent (Figure 6) suggesting that the mechanism of transcription activation by BglJ–RcsB involves protein–protein interactions and thus resembles that of canonical activators (37). In addition, some promoters are regulated by BglJ–RcsB and other pleiotropic factors in an antagonistic or synergistic way. At the *yidL* promoter BglJ–RcsB and HNS control transcription antagonistically. At this locus the BglJ–RcsB binding site is inverted and maps out of phase. Activation of *yidL* transcription by BglJ–RcsB is independent of DNA phasing (Figure 6) and thus may be mediated by inhibition of HNS mediated repression. The *leuO* gene is another example for antagonistic regulation that involves BglJ–RcsB. Activation of the HNS and StpA repressed *leuO* gene by BglJ–RcsB is inhibited by LeuO (20). At the *bgl* operon, which is controlled by HNS, StpA, CRP, and LeuO, BglJ–RcsB and CRP presumably act synergistically to overcome repression by HNS (Figure 5). Synergistic activation of a transcriptional regulator together with CRP has been shown at several loci including the *ara* locus by AraC and CRP, *malE* by MalT and CRP, *rha* by RhaS/RhaR and CRP and *asc* by two CRP dimers, among others (37,44–47). However, at the *bgl* locus BglJ–RcsB shifts transcription initiation from a class I CRP dependent promoter to a promoter with the center of the CRP-binding site mapping at position −41.5, which is typical of class II CRP-dependent promoters (Figures 2 and 5). Interestingly though, for synergistic activation of *bgl* by BglJ–RcsB and CRP, the CRP AR1 but not the AR2 is important (Figure 5). This is in contrast to other class II CRP dependent promoters where CRP stimulates open complex formation by RNA polymerase (40–42). Thus, the synergistic activation of *bgl* by BglJ–RcsB and CRP apparently involves a novel mechanism.

The interaction of RcsB with various auxiliary transcriptional regulators may indicate that RcsB serves as a hub at which various signals are integrated by several means, such as induction of the RcsF-RcsCD phosphorelay and/or interaction with auxiliary factors (1,2). The characterization of BglJ–RcsB targets in this work adds an additional set of loci to the Rcs regulon. Remarkably, beyond *leuO*, *bgl* and *chiA*, most of the BglJ–RcsB targets are of unknown or predicted function, with many of them being conserved and/or encoding membrane proteins (Table 1). Furthermore, putative BglJ–RcsB targets include the complex *rhsA* and *rhsB* loci (Figure 3), among others (20). The *rhs* loci encode hydrophilic proteins with repetitive sequence elements (48,49), which presumably play a role in intercellular competition (50). Taken together, the spectrum of target loci indicates that the BglJ–RcsB regulon is important under conditions different from the standard laboratory conditions. Correspondingly, the *yjjQ-bglJ* locus has

been implicated in *E. coli* pathogenicity (51). Yet, up to date it is not known under which physiological conditions BglJ is present in the cell. Transcription of the *yjjQ-bglJ* operon is repressed by HNS under standard laboratory growth conditions (15). The *leuO* gene encoding the only known activator of the *yjjQ-bglJ* operon, is likewise repressed by HNS and StpA under standard laboratory conditions (20).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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