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BglJ-RcsB Heterodimers Relieve Repression of the *Escherichia coli bgl* Operon by H-NS▽†

G. Raja Venkatesh,‡ Frant Carlot Kembou Koungni,‡§ Andreas Paukner,‡ Thomas Stratmann, Birgit Blissenbach,¶ and Karin Schnetz*

Institute for Genetics, University of Cologne, Zülpicher Str. 47a, 50674 Cologne, Germany

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RcsB is the response regulator of the complex Rcs two-component system, which senses perturbations in the outer membrane and peptidoglycan layer. BglJ is a transcriptional regulator whose constitutive expression causes activation of the H-NS- and StpA-repressed *bgl* (aryl-β,D-glucoside) operon in *Escherichia coli*. RcsB and BglJ both belong to the LuxR-type family of transcriptional regulators with a characteristic C-terminal DNA-binding domain. Here, we show that BglJ and RcsB interact and form heterodimers that presumably bind upstream of the *bgl* promoter, as suggested by mutation of a sequence motif related to the consensus sequence for RcsA-RcsB heterodimers. Heterodimerization of BglJ-RcsB and relief of H-NS-mediated repression of *bgl* by BglJ-RcsB are apparently independent of RcsB phosphorylation. In addition, we show that LeuO, a pleiotropic LysR-type transcriptional regulator, likewise binds to the *bgl* upstream regulatory region and relieves repression of *bgl* independently of BglJ-RcsB. Thus, LeuO can affect *bgl* directly, as shown here, and indirectly by activating the H-NS-repressed *yjjQ-bglJ* operon, as shown previously. Taken together, heterodimer formation of RcsB and BglJ expands the role of the Rcs two-component system and the network of regulators affecting the *bgl* promoter.

The nucleoid-associated protein H-NS is a pleiotropic regulator that generally functions as a repressor (silencer) of transcription. The biological role of H-NS has been best studied in *Escherichia coli* and *Salmonella enterica* and includes control of stress responses, pathogenicity, and horizontal transfer of DNA (reviewed in references 15, 18, 44, and 54). In addition, H-NS has been proposed to be important in nucleoid organization (45). Numerous studies have addressed the mechanism of DNA binding and transcriptional regulation by H-NS. The protein supposedly binds as a dimer to specific nucleation sites usually located within an AT-rich sequence context. Then, H-NS forms extended complexes by polymerization along the DNA (now defined as "stiffening") and by building DNA–H-NS–DNA bridges ("bridging") (8, 35). Formation of such H-NS–DNA complexes next to promoters represses transcription by occluding RNA polymerase or, as shown in some cases, by trapping RNA polymerase at the promoter (44). In addition, H-NS-like proteins, such as StpA, can contribute to gene regulation and silencing (16, 42).

Repression (silencing) by H-NS can be relieved by various mechanisms (44, 54). Most commonly, repression by H-NS is relieved by the binding of specific transcriptional regulators, which compete with H-NS for binding or which restructure the H-NS–nucleoprotein complex. Other mechanisms include locus-specific changes of the DNA structure (bending) (17), enhancement of the transcription rate (43), and possibly direct modulation of H-NS activity by changes in the physiological conditions, such as osmolarity, temperature, and pH (35).

The *bgl* (aryl-β,D-glucoside) operon of *E. coli* is a classical example of a locus that is tightly repressed by H-NS. Efficient repression of *bgl* by H-NS involves synergistic binding of H-NS to regulatory elements located upstream of the promoter and downstream within the transcription unit (43). Historically, spontaneous mutations mapping in *cis* to the *bgl* promoter that relieve repression have attracted attention, and it has been speculated that such mutations are a means to control *bgl* expression at the level of the population under selective environmental conditions (36, 47). Later, it was found that repression of the *bgl* operon by H-NS can be relieved by the LysR-type transcription factor LeuO (see below) and by BglJ (25, 58). BglJ ia a transcription factor with a C-terminal helix-turn-helix motif of the LuxR type and is encoded in an operon together with YjjQ, another LuxR-type transcription factor proposed to be important for virulence of avian-pathogenic *E. coli* (APEC) (34, 55). Another prominent member of the family of LuxR-type transcription factors is RcsB, the response regulator of the complex Rcs (regulation of capsule synthesis) two-component system, which senses outer membrane stress and perturbations in the peptidoglycan layer (20, 31). RcsB is a pleiotropic transcription factor involved in the control of motility, cell division, outer membrane protein expression, capsule synthesis, acid stress response, and the small regulatory RNA RprA (5, 29, 39). RcsB, as a homodimer, activates transcription of several genes by binding upstream of the −35 promoter region, including *ftsA*, *osmC*, *osmB*, *bdm*, and *rprA* (2, 10, 23, 56). In addition, RcsB forms heterodimers with RcsA, which is likewise a LuxR-type transcription factor.

* Corresponding author. Mailing address: Institute for Genetics, University of Cologne, Zülpicher Str. 47a, 50674 Cologne, Germany. Phone: 49-221-4703815. Fax: 49-221-4705185. E-mail: schnetz@uni-koeln.de.

‡ G.R.V., F.C.K.K., and A.P. contributed equally.

§ Present address: Johns Hopkins University, School of Medicine, 725 N. Wolfe St. 607 PCTB, Baltimore, MD 21205.

¶ Present address: IMMIH, University Hospital of Cologne, Goldenfelsstr. 19-21, 50935 Cologne, Germany.

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TABLE 1. *E. coli* K-12 strains

Strain	Relevant genotype[a]	Construction/source
BW30270	MG1655 *rph*$^+$	Coli Genetic Stock Center no. 7925
KL788	λ$^-$ Thr-1 Δ(*gpt-lac*)*5 tsx-35 sulA3* e14$^-$ Rac-0 *rfbD1 mgl-51 recA441*(Ts) *relA1 rpsL31*(*strR*) *kdgK51 mtl-1 spoT1 thi-1 lexA71*::Tn*5 creC510* (stored as S1152)	Coli Genetic Stock Center no. 6218
M182 stpA::tet	Δ*lac74 galU galK strA stpA*::*tet* (stored as S159)	64
SU101	λ lysogen with P_{sulA} *lexA*-op+/+ *lacZ* fusion in JL1434	12
SU202	λ lysogen withP_{sulA} *lexA*-op408/+ *lacZ* fusion in JL1434	12
S1734	*yjjQ/bglJ*-Y6::miniTn*10*-cat (= $bglJ_C$) in S764	38
S524	CSH50 Δ*lacZ*-Y217 (*gpt-pro*)$^+$	14
S2176	S524 *yjjQ/bglJ*-Y6::miniTn*10*-cat (= $bglJ_C$)	S524 × T4*GT7* (S1734)
S2817	S524 *attB*::[Spec^r wt-P_{bgl}(+25) *lacZ*] (Bgl$^-$ and Lac$^-$)	S524 × pKEKB30
S2822	S524 *attB*::[Spec^r wt-P_{bgl}(+25) *lacZ*] $bglJ_C$ (Bgl$^+$ and Lac$^+$)	S2817 × T4*GT7* (S1734)
S2828	S2822 *rcsB*-2828::miniTn*10*-tet (Bgl$^-$ and Lac$^-$)	S2822 × λNK1323
S3918	S524 Δ*rcsB*::Spec^r	S524 × T4*GT7* (S3278)
S3919	S524 $bglJ_C$ Δ*rcsB*::Spec^r	S2176 × T4*GT7* (S3278)
S541	CSH50 Δ*lacZ*-Y217 Δ*bgl*-AC11	14
S3010	S541 Δ*hns*::kan_{KD4}	43
S3278	S541 Δ*rcsB*::Spec^r	× PCR S774/S775(pKESD8)
S3377	S541 Δ*rcsB*::Spec^r Δ(*yjjP-yjjQ-bglJ*)::cat_{KD3}	S3278 × PCR S783/S676 (pKD3)
S1185	S541 *sulA3*	13
S3360	S541 *sulA3 lexA71*::Tn*5*	S1184 × T4*GT7*(KL788) Kan^r
S3373	S3360 Δ$rcsB_{FRT}$	× PCR S819/S820 pKD3 × pCP20
S3384	S3360 Δ$rcsB_{FRT}$ Δ(*yjjP-yjjQ-bglJ*)$_{FRT}$	S3373 × PCR S783/S676 pKD3; × pCP20
S3434	S3384 *attB*::(Spec^r *lacI*^{q} T1 P_{sulA} *lexA*-op+/+ *lacZ*)	S3384/pLDR8 × pKES163
S3442	S3384 attB::(Spec^r *lacI*^{q} T1 P_{sulA} *lexA*-op408/+ *lacZ*)	S3384/pLDR8 × pKES164
S3974	BW30270 *ilvG*$^+$ (valine resistant)	BW30270/pKD46 × annealed oligonucleotides T96/T97
S4197	BW30270 *ilvG*$^+$ Δ*lacZ*	S3974 × pFDY217
T15	S4197 *rcsB*::kan_{KD4}	× PCR S819/S820(pKD4)
T70	S4197 Δ(*yjjP-yjjQ-bglJ*)::cat	× PCR S676/S783(pKD3)
T71	S4197 Δ$leuO_{FRT}$	× PCR T209/T210(pKD3); × pCP20
T314	S4197 Δ$leuO_{FRT}$ Δ(*yjjP-yjjQ-bglJ*)$_{FRT}$	T71 × T4*GT7* (T70); × pCP20
T568	T314 *attB*::(Spec^r P_{bgl} $t1_{RAT}$ *bglG lacZ*)	T314/pLDR8 × pKENV61
T576	T314 *attB*::(Spec^r P_{bgl}-*mut2* $t1_{RAT}$ *bglG lacZ*)	T314/pLDR8 × pKES220
T578	T314 *attB*::(Spec^r P_{bgl}-*mut3* $t1_{RAT}$ *bglG lacZ*)	T314/pLDR8 × pKES221
T580	T314 *attB*::(Spec^r P_{bgl}-*mut1* $t1_{RAT}$ *bglG lacZ*)	T314/pLDR8 × pKES222
T727	T314 *attB*::(Spec^r P_{bgl} $t1_{RAT}$ *bglG lacZ*) Δ$rcsB_{FRT}$	T568 × T4GT7 (T15); × pCP20
T729	T314 *attB*::(Spec^r P_{bgl} $t1_{RAT}$ *bglG lacZ*) Δhns_{FRT}	T568 × T4GT7 (S3010); × pCP20
T731	T314 *attB*::(Spec^r P_{bgl}-*mut2* $t1_{RAT}$ *bglG lacZ*) Δhns_{FRT}	T576 × T4GT7 (S3010); × pCP20
T733	T314 *attB*::(Spec^r P_{bgl}-*mut3* $t1_{RAT}$ *bglG lacZ*) Δhns_{FRT}	T578 × T4GT7 (S3010); × pCP20
T735	T314 *attB*::(Spec^r P_{bgl}-*mut1* $t1_{RAT}$ *bglG lacZ*) Δhns_{FRT}	T580 × T4GT7 (S3010); × pCP20
T757	T314 *attB*::(Spec^r P_{bgl} $t1_{RAT}$ *bglG lacZ*) Δhns_{FRT} *stpA*::tet	T729 × T4GT7 (M182 stpA::tet)

[a] JL1434 is *lexA71*::Tn*5* (Def)*sulA211* Δ*lac169*/F′ *lacI*^{q} *lacZ*ΔM15::Tn*9*. S764 is CSH50 *bgl*$^+$-C234 Δ*lacOP*::[SpecR P_{bgl}-C234(+54) $bglG_{orf}$] *lon*-107::miniTn*10*-tet *yjjQ/bglJ*-Y6::miniTn10-cat. *rcsB*-S2828 carries a miniTn*10*-cm insertion in *rcsB* with a target site duplication of 9 bp at positions 217 to 225 relative to the *rcsB* translation start site. *sulA3* carries a single-nucleotide A-to-G exchange 35 bp upstream of ATG. The construction of strains by procedures (×) including transduction with phage T4*GT7*, integration of reporter constructs into *attB*, replacement of genes with resistance cassettes, and deletion of the resistance cassettes using plasmid pCP20 was performed as described previously (9, 55, 61). Δ*lacZ* was introduced into strain S3974 by gene replacement using the *rep*(Ts) plasmid pFDY217, as described previously (3).

RcsA-RcsB heterodimers activate the capsule synthesis operons *cps* and *yjbEFGH*, positively autoregulate *rcsA* (21, 59), and repress *fhlCD*, encoding the master regulators of bacterial flagellum biogenesis (22). Furthermore, interaction of RcsB with the acid stress regulator GadE was recently described (5), and in *Salmonella enterica* serovar Typhi, interaction of RcsB with TviA was found to control Vi antigen synthesis (62).

LeuO is a pathogenicity determinant in *S. enterica* and is important for biofilm formation in *Vibrio cholerae* (32, 41, 57). It is a regulator of many genes, including those for outer membrane proteins, drug efflux, the small regulatory RNA DsrA, and the RNA-based immunity system CRISPR (28, 33, 53, 60). LeuO also activates expression of the *yjjQ-bglJ* operon (55). However, while the relevance of LeuO and YjjQ for pathogenicity indicates that their genes are expressed under certain *in vivo* conditions in the host environment, both the *leuO* gene and the *yjjQ-bglJ* operon are repressed by H-NS under laboratory growth conditions (6, 55).

In this work, we addressed the mechanism by which BglJ counteracts repression of the *bgl* promoter by H-NS. A screen for mutants in which derepression of *bgl* by BglJ is abrogated yielded an *rcsB* mutant, and we demonstrate here that the two-component response regulator RcsB is essential for BglJ to act as an H-NS antagonist at the *bgl* locus. Further analyses demonstrated that BglJ and RcsB form heterodimers and suggested that these heterodimers directly bind to the *bgl* upstream regulatory element (URE). In addition, we show that LeuO also binds to the *bgl* URE and directly activates the *bgl* promoter.

MATERIALS AND METHODS

Strains, plasmids, and media. All strains and plasmids used in this study are listed in Table 1. Cloning of plasmids, construction of strains by transduction, and gene replacement followed standard protocols (1, 9, 61), as briefly described in the supplemental material.

Transposon mutagenesis. Transposon mutagenesis using the phage λNK1323 miniTn*10*::tet transposon system was performed as described previously (40). Briefly, strain S2822 carrying the *bgl* promoter dual reporter constructs was infected with λNK1323 lysate (40), and transposon mutants were selected at 41°C on MacConkey lactose tetracycline plates. Lac-negative colonies were restreaked on MacConkey lactose tetracycline plates, as well as on BTB salicin tetracycline indicator plates (14), and Lac- and Bgl-negative mutants were further analyzed by a semirandom two-step PCR (ST-PCR protocol), as described previously (7, 38). In one of the mutants, the miniTn*10*::tet transposon mapped within the *rcsB* open reading frame (at position bp 225 relative to the translation start, with a 9-bp target site duplication, TACATCAAG). This allele was designated *rcsB*::miniTn*10*-tet and stored as strain S2828 (Table 1).

β-Galactosidase assay. Cultures were grown overnight in LB medium with antibiotics. Then, 8-ml cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.05 to 0.1 and grown to an OD_{600} of 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was added, where indicated, to a final concentration of 1 mM to the overnight and the exponential cultures for induction. The bacteria were harvested, and β-galactosidase activities were determined independently at least three times, as described previously (40). Standard deviations were less than 10%, unless otherwise indicated.

Coimmunoprecipitation. Coimmunoprecipitation to analyze the interaction of RcsB with BglJ was performed using transformants of strain S3377 [Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)] with plasmids expressing tagged variants of the RcsB and BglJ proteins. For expression of RcsB with a C-terminal hemagglutinin (HA) tag, strain S3377 was transformed with plasmid pKEAP38. For expression of BglJ with a C-terminal FLAG tag, plasmid pKERV10 was used. The untransformed strain S3377, single transformants (carrying either pKEAP38 or pKERV10), and the double transformants (carrying pKEAP38 and pKERV10) were grown overnight in LB without (empty strain) or with suitable antibiotics. Then, 100 ml of the same medium was inoculated from the overnight culture to an OD_{600} of 0.05 and grown to an OD_{600} of 0.3, when IPTG (1 mM final concentration) was added for induction of protein expression. Cells were harvested after 2 h of induction, pelleted by centrifugation, washed once with lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM EDTA, 10% glycerol), and again pelleted by centrifugation. The cells were resuspended in 1 ml lysis buffer (with 1 mM phenylmethylsulfonyl fluoride [PMSF] freshly added) and lysed by sonication. The cell lysates were cleared by centrifugation. A fraction of the lysate equivalent to 200 μg of soluble protein was diluted to a volume of 950 μl with lysis buffer and incubated with 5 μl rabbit anti-HA IgG (Sigma-Aldrich; H6908; 1:200 dilution for immunoprecipitation) for 3 h at 4°C in a tube rotator. Then, 5 mg of protein A-Sepharose beads (GE Healthcare) was added. The samples were incubated for 2 h at 4°C in a tube rotator to allow binding, and the beads were pelleted by centrifugation and washed 3 times with 1 ml lysis buffer. After the final wash, 50 μl Laemmli buffer (49) was added, and the proteins were separated on 12% SDS-PAGE gels and then blotted onto a PVDF membrane (GE Healthcare). For Western analysis, the membranes were blocked with 3% nonfat dry milk powder in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and incubated with rat anti-HA (Roche, 1:500) and mouse anti-Flag (Sigma-Aldrich, 1:5,000) antibodies. As secondary antibodies, IRDye 800CW-conjugated goat anti-mouse antibody (Li-Cor Bio-Sciences; 1:10,000) and IRDye 680-conjugated goat anti-rat antibody (Molecular Probes; 1:5,000) were used. The blots were scanned with an Odyssey imaging system (Li-Cor Biosciences).

DNase I footprinting. For DNase I footprinting, the *bgl* promoter and upstream region (positions −202 to +30 relative to the transcription start site) were amplified by PCR. For 5′-end labeling of the top strand with T4-polynucleotide kinase and [γ-^{32}P]ATP, primers T79 (5′ OH) and T110 (5′ phosphate) were used, while for labeling of the bottom strand, primers T109 (5′ phosphate) and T80 (5′ OH) were used. The binding reaction of LeuO (in the indicated amounts) to the labeled fragments (approximately 200,000 cpm) was performed at 30°C for 20 min in a volume of 20 μl in binding buffer (100 mM KCl, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 10% glycerol, 500 mM imidazole), 50 ng/μl bovine serum albumin (BSA), and 5 ng/μl herring sperm DNA. Then, 2 μl DNase I (Roche Molecular Biochemicals; 5 ng/μl in binding buffer) was added, and the reaction was stopped exactly 1 min later by the addition of 20 μl of phenol. The samples were extracted with chloroform-isoamyl alcohol, and the DNA was ethanol precipitated. The dried samples were resuspended in 6 μl of sequencing gel loading buffer (79% formamide, 0.1% [wt/vol] bromphenol blue, 0.1% [wt/vol] xylene cyanol, and 5 mM EDTA) and separated on 6% denaturing sequencing gels (6% Long Ranger [Lonza], 7 M urea, 0.8× Tris-borate-EDTA [TBE]) next to a sequencing ladder. The sequencing ladder was generated using the T7 sequencing kit (USB Corporation) and [α-^{32}P]dCTP, with the same primers used for generation of the *bgl* PCR fragment.

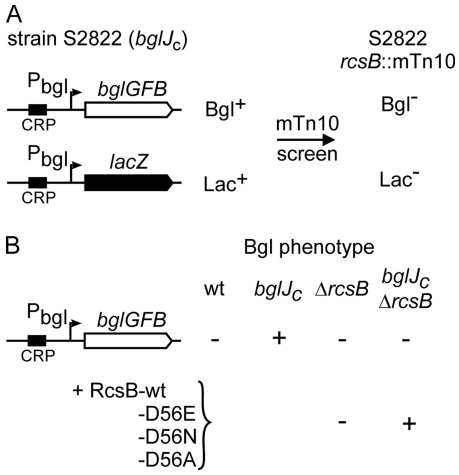

FIG. 1. Activation of the *bgl* operon by BglJ requires RcsB. (A) Schematic of a transposon mutagenesis screen for mutants in which activation (relief of H-NS-mediated repression) of *bgl* by BglJ is abrogated. Strain S2822 carries the *bgl* operon and a *bgl* promoter-*lacZ* fusion as dual reporters for *bgl* expression. In addition, the strain carries allele *yjjQ*/*bglJ*-Y6::mini*Tn*10-cat ($bglJ_c$) for constitutive expression of *bglJ*. A mini*Tn*10-tet (mTn10) transposon mutagenesis screen yielded Bgl- and Lac-negative mutants, one of which carried a transposon insertion in *rcsB* (assigned to strain S2828 [Table 1]). (B) RcsB is required for derepression of *bgl* by BglJ. The Bgl phenotypes of the *E. coli* K-12 wild type (wt) (strain S524) and its isogenic derivatives, which constitutively express *bglJ* ($bglJ_C$; strain S2176) or carry a deletion of *rcsB* (Δ*rcsB*; strain S3918), as well as the double mutant $bglJ_C$ Δ*rcsB* (strain S3919), was determined on BTB salicin indicator plates. Shown is complementation of the Δ*rcsB* mutants with plasmids encoding wild-type RcsB (pKETS6) or the RcsB mutants D56E (pKETS7), D56N (pKETS8), and D56A (pKES235).

RESULTS

Derepression of *bgl* by BglJ requires RcsB. Transcription factor BglJ relieves repression of the *bgl* operon by H-NS (25). To identify factors that are involved in derepression of *bgl* by BglJ, we performed a transposon mutagenesis screen using strain S2822, which carries the *bgl* operon and a *bgl* promoter-*lacZ* fusion as dual reporters (Fig. 1A). In addition, the strain constitutively expresses *bglJ* (referred to as $bglJ_c$ below) because of the insertion of a miniTn*10* transposon upstream of *bglJ* (allele *yjjQ*/*bglJ*-Y6::miniTn*10*) (38). Thus, this dual-reporter strain for monitoring activity of the *bgl* promoter is Bgl and Lac positive. Transposon mutants were screened for a Bgl- and Lac-negative phenotype, and one of the transposon mutations mapped in *rcsB* (Fig. 1A). To verify that a mutation of *rcsB* interferes with derepression of the *bgl* promoter by BglJ, an *rcsB* deletion was introduced into strain S2176, which carries the wild-type *bgl* operon and expresses *bglJ* constitutively ($bglJ_c$). Analysis of the Bgl phenotype on indicator plates demonstrated that the deletion of *rcsB* abrogates repression of *bgl* by BglJ (Fig. 1B). Further, complementation of the Δ*rcsB* mutant with plasmid pKETS6 carrying *rcsB* under the control of the IPTG-inducible *tac* promoter restored the Bgl-positive phenotype in the $bglJ_c$ strain, but not in the wild type. This suggests that both RcsB and BglJ are required to relieve the

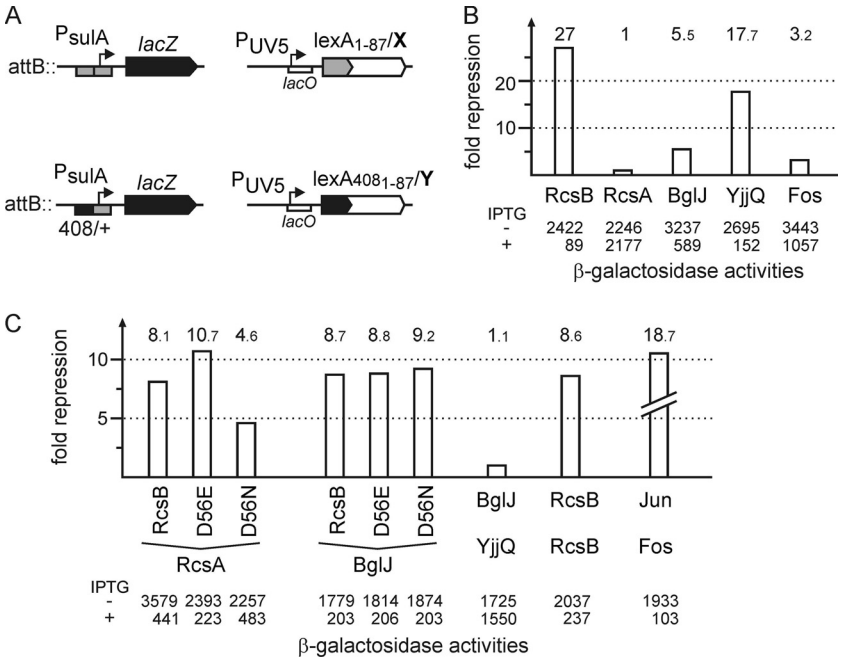

FIG. 2. Interaction of BglJ with RcsB and YjjQ. (A) In the LexA-based two-hybrid system, the *sulA* promoter-*lacZ* fusion with the wild-type LexA operator was used to analyze homodimerization, and the *sulA* promoter-*lacZ* reporter fusion with a hybrid $lexA_{408/+}$ operator served as a reporter for heterodimerization. For analysis of homodimerization, a fusion of the respective protein (X) to the wild-type LexA DNA-binding domain ($lexA_{1\text{-}87}$/X) was expressed from a plasmid under the control of the IPTG-inducible *lacUV5* promoter (P_{UV5}). For heterodimerization analysis, fusions of protein X to the wild-type LexA DNA-binding domain ($lexA_{1\text{-}87}$/X) and of protein Y to the LexA408 mutant DNA-binding domain ($lexA408_{1\text{-}87}$/Y) were coexpressed from compatible plasmids. (B) Analysis of homodimer formation in RcsB, RcsA, BglJ, and YjjQ was performed with transformants of strain S3434 with plasmids pKEMK17 ($lexA_{1\text{-}87}$-*rcsB*), pKES192 ($lexA_{1\text{-}87}$-*rcsA*), pKEAP30 ($lexA_{1\text{-}87}$-*bglJ*), pKEAP27 ($lexA_{1\text{-}87}$-*yjjQ*), and pMS604 ($lexA_{1\text{-}87}$-*fos*) as controls. Cultures were grown in LB tetracycline medium to an OD_{600} of 0.5. IPTG was added to 1 mM final concentration where indicated (+). The β-galactosidase activity was determined to monitor repression of the *sulA* promoter by the $LexA_{1\text{-}87}$-X fusion protein. The fold repression (indicated by the bars), as a measure for dimerization, was calculated as the ratio of the β-galactosidase activities measured without and with induction of the LexA fusion proteins. (C) Analysis of heterodimer formation was performed with strain S3442, which was cotransformed with plasmids coding for $LexA_{1\text{-}87}$-X and $LexA408_{1\text{-}87}$-Y fusions, respectively. The cultures were grown in LB with antibiotics, and IPTG was added where indicated. The fold repression of the *sulA* promoter-*lacZ* fusion with the hybrid *lexA* operator (*lexA*-op408/+) is a measure of heterodimerization (indicated by the bars). The following plasmids were used: $LexA_{1\text{-}87}$-RcsB (pKEMK17), $LexA_{1\text{-}87}$-RcsA (pKES192), $LexA_{1\text{-}87}$-BglJ (pKEAP30), $LexA_{1\text{-}87}$-YjjQ (pKEAP27), and $LexA_{1\text{-}87}$-Fos (pMS604) (12), as well as $LexA408_{1\text{-}87}$-RcsB (pKEAP28), $LexA408_{1\text{-}87}$-$RcsB_{D56E}$ (pKES150), $LexA408_{1\text{-}87}$-$RcsB_{D56N}$ (pKES151), $LexA408_{1\text{-}87}$-BglJ (pKEAP29), and $LexA408_{1\text{-}87}$-Jun (pDP804) (12) as controls.

H-NS-mediated repression of *bgl*. Interestingly, complementation of the Δ*rcsB* strain was also possible with RcsB mutants carrying exchanges in the conserved aspartate (residue 56) of the N-terminal receiver domain. Mutation RcsB-D56E mimics the active phosphorylated state, and mutations D56N and D56A mimic the inactive state of RcsB (27, 51). These results suggest that BglJ and RcsB act together independently of RcsB phosphorylation.

Interaction of the LuxR-type transcription factors RcsB, BglJ, and YjjQ. The LuxR-type response regulator RcsB is known to interact with RcsA and GadE (see the introduction). As derepression of the *bgl* operon by BglJ requires RcsB, we analyzed whether BglJ also interacts with RcsB and whether BglJ forms homodimers. In addition, we analyzed whether BglJ interacts with YjjQ (as BglJ and YjjQ are encoded in one operon [55]).

Interaction was tested using the bacterial LexA-based two-hybrid system (12) and by coimmunoprecipitation (see below). The LexA-based two-hybrid system is based on repression of the *sulA* promoter by LexA (Fig. 2A). The reporter for homodimer formation consists of the native *sulA* promoter fused to *lacZ*. In a *lexA* mutant, this promoter is constitutively active but can be repressed by fusion of the N-terminal LexA-DNA-binding domain ($LexA_{1\text{-}87}$) to a protein that forms homodimers (Fig. 2A) (12). The *sulA* promoter-*lacZ* reporter for heterodimer formation carries a hybrid *lexA* operator (op408/+) with a mutation in one half-site (Fig. 2A). This operator can only be bound by heterodimers in which one partner includes a $LexA_{1\text{-}87}$ wild-type DNA-binding domain and the other protein partner contains a $LexA408_{1\text{-}87}$ mutant DNA-binding domain (Fig. 2A) (12). For the current analysis of homodimer and heterodimer formation, the LexA-based two-hybrid-system was transferred to a Δ*rcsB* and Δ(*yjjP-yjjQ-bglJ*) strain background (see the supplemental material). In addition, plasmids that express fusions of the wild-type $LexA_{1\text{-}87}$ DNA-binding domain to BglJ, RcsB, YjjQ, and RcsA were constructed. RcsB and BglJ were also fused to the mutant $LexA408_{1\text{-}87}$ DNA-binding domain (Fig. 2 and Table 1).

In the homodimerization assay, induction of the $LexA_{1\text{-}87}$-RcsB fusion caused strong repression of the *sulA* promoter-*lacZ* reporter (in strain S3434), as expected (Fig. 2B). In contrast, the $LexA_{1\text{-}87}$-RcsA fusion caused no repression (Fig. 2B),

as anticipated from earlier studies, which suggested that RcsA forms heterodimers with RcsB but no homodimers (39). In comparison, the $LexA_{1-87}$-BglJ fusion protein caused very moderate repression, indicating that BglJ forms weak homodimers. However, the $LexA_{1-87}$-YjjQ fusion caused strong repression, suggesting efficient homodimer formation by YjjQ. As a control, a $LexA_{1-87}$-Fos fusion known for its low capacity for homodimer formation was included. Induction of this fusion resulted in only weak repression (Fig. 2B).

In the heterodimerization assay, coinduction of $LexA408_{1-87}$-RcsB with $LexA_{1-87}$-RcsA resulted in strong repression (Fig. 2C). This result is in agreement with earlier data suggesting that RcsA and RcsB form heterodimers (39). Coexpression of BglJ (fused to $LexA_{1-87}$) and RcsB (fused to $LexA408_{1-87}$) likewise resulted in strong repression, suggesting that BglJ and RcsB form heterodimers (Fig. 2C). Interestingly, YjjQ (fused to $LexA_{1-87}$) and BglJ (fused to $LexA408_{1-87}$) caused no repression (Fig. 2C), suggesting that the two LuxR-type transcription factors BglJ and YjjQ do not interact, although they are encoded in one operon. Since neither deletion of *yjjQ* nor plasmid-directed expression of YjjQ plays a role in regulation of the *bgl* operon (data not shown), YjjQ was not included in further analyses. As additional positive controls for heterodimerization, interaction analyses of $LexA_{1-87}$-Fos and $LexA408_{1-87}$-Jun, as well as of RcsB (using $LexA_{1-87}$-RcsB and $LexA408_{1-87}$-RcsB), were included, which, as expected, caused repression (Fig. 2C).

Furthermore, we analyzed whether heterodimer formation of RcsB with BglJ and RcsA, respectively, depends on phosphorylation of RcsB. Heterodimer formation of RcsA with RcsB-D56E was enhanced compared to that of wild-type RcsB, while it was reduced with the RcsB-D56N mutant (Fig. 2C). In contrast, heterodimer formation of BglJ and RcsB was not affected by the mutation of the presumptive RcsB phosphorylation site (Fig. 2C). These data indicate that interaction of RcsB with RcsA is modulated by phosphorylation of RcsB and thus by induction of the Rcs signaling cascade. In contrast, the interaction of RcsB with BglJ is presumably not affected by RcsB phosphorylation, in agreement with the complementation analysis shown above (Fig. 1B).

In a second set of experiments, heterodimer formation of BglJ with RcsB was analyzed by coimmunoprecipitation. To this end, compatible vectors for coexpression of BglJ-Flag and RcsB-HA in strain S3377 [Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)] were used. These plasmid-encoded BglJ-Flag and RcsB-HA proteins are functional, as tested by complementation of the respective mutants using *bgl* as a reporter (data not shown). Coimmunoprecipitation of cell lysates was performed with an HA tag-specific antibody (rabbit anti-HA IgG). For visualization of the proteins by Western blotting, fluorescent secondary antibodies were used, allowing simultaneous detection of the FLAG- and HA-tagged proteins in one gel (Fig. 3). Analysis of the cell lysates demonstrated that the proteins were well expressed (Fig. 3, lysates). After coimmunoprecipitation with an HA-specific antibody, RcsB-HA was detectable irrespective of whether it was expressed in the absence or presence of BglJ-FLAG (Fig. 3). However, BglJ-FLAG was precipitated only when it was coexpressed with RcsB-HA (Fig. 3). This demonstrated that the coimmunoprecipitation was specific and suggests that BglJ-FLAG interacts with RcsB-HA (Fig. 3).

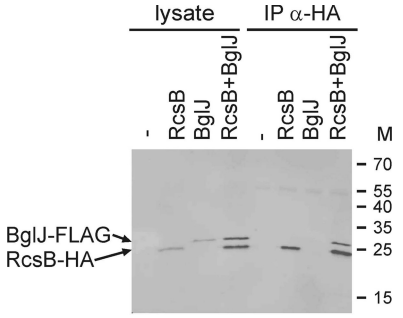

FIG. 3. Coimmunoprecipitation of BglJ-FLAG with RcsB-HA. (A) Coimmunoprecipitation of BglJ-FLAG with RcsB-HA was performed for lysates prepared from strain S3377 [Δ*rcsB* Δ(*yjjP-yjjQ-bglJ*)] (−) and for transformants of strain S3377 with plasmids pKEAP38 (RcsB-HA) (RcsB), pKERV10 (BglJ-FLAG) (BglJ), or both plasmids. Immunoprecipitation (IP) was performed with rabbit anti-HA IgG. The lysates and the coimmunoprecipitates were separated on SDS-PAGE and analyzed by Western blotting. For simultaneous detection of BglJ-FLAG and RcsB-HA, the Western blot was developed with rat anti-HA (α-HA) and mouse anti-FLAG as primary antibodies and fluorescence labeled anti-mouse and anti-rat secondary antibodies.

Mapping of a BglJ-RcsB box in the *bgl* regulatory region. The data indicate that BglJ-RcsB heterodimers relieve repression of *bgl* by H-NS by binding next to the *bgl* promoter. For RcsA-RcsB heterodimers, a consensus sequence (termed the RcsAB box) was proposed (39, 59). This RcsAB box is nonpalindromic (see Fig. S1 in the supplemental material) and is presumably recognized by binding of RcsB to one half-site and by binding of RcsA to the other half-site (22). Interestingly, within the *bgl* regulatory region, a perfect match to one half-site of the RcsAB box is located at positions −88 to −95 (relative to the transcription start site) (Fig. 4A). Assuming that the right half-site of the RcsAB box is bound by RcsB (see Fig. S1 in the supplemental material), this match indicates that the RcsB subunit of the BglJ-RcsB heterodimer may bind to this motif and that BglJ contacts adjacent base pairs.

To test the relevance of this presumptive BglJ-RcsB binding site, site-specific mutations were introduced in the most conserved bases matching the right half-site of the RcsAB box (Fig. 4A, mutant 1). In addition, the left half-site of the presumptive BglJ-RcsB box was mutated (Fig. 4A, mutant 2), and mutations in both half-sites were combined (Fig. 4A, mutant 3). The effect of these mutations on derepression of *bgl* by BglJ-RcsB was tested using a *bgl-lacZ* reporter construct that carries all elements required for repression by H-NS (Fig. 4B). However, expression of this reporter is independent of regulatory elements for sugar-specific regulation, as it carries a mutation of terminator t1 (43, 46). Note that sugar-specific regulation of the *bgl* operon is promoter independent and is mediated by the specific transcriptional antiterminator BglG, which allows transcription read-through at terminator t1. BglG activity is regulated by phosphorylation that is dependent on the availability of the specific substrate and other sugars (26).

For expression analyses, the *bgl-lacZ* reporter constructs with the putative wild-type and mutated BglJ-RcsB binding sites, respectively, were integrated at the λ-*attB* site of strain T314 [Δ*lacZ*, Δ(*yjjP-yjjQ-bglJ*), and Δ*leuO*, as LeuO also derepresses the *bgl* operon (see below)]. To analyze derepression of

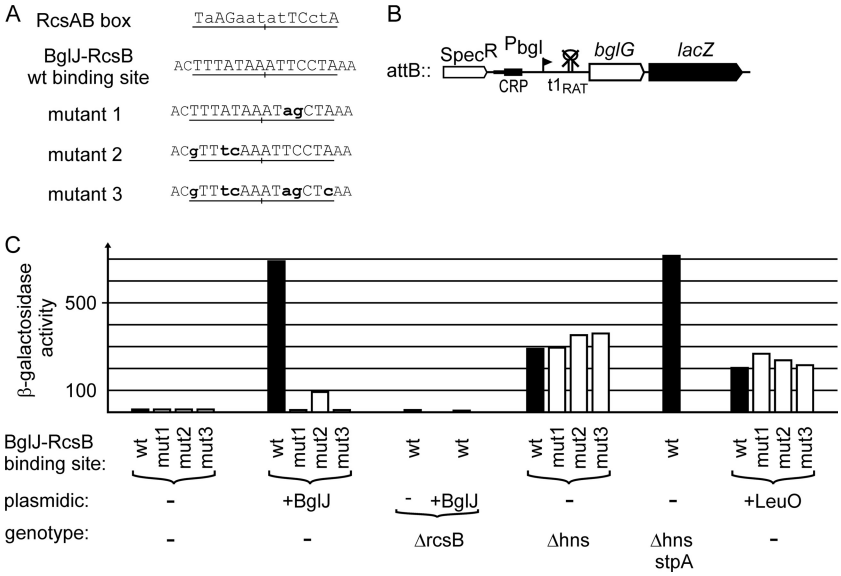

FIG. 4. Effect of mutation of the BglJ-RcsB binding site on derepression of *bgl* by BglJ-RcsB. The expression levels directed by *bgl* promoter-*lacZ* fusions (shown schematically in panel B) with wild-type and mutant BglJ-RcsB binding sites (A) were determined for exponential cultures grown in LB (with appropriate antibiotics and 1 mM IPTG) (C). (A) The BglJ-RcsB binding site is underlined, and mutations are indicated in lowercase boldface letters. (B) The *bgl-lacZ* fusions were integrated at the phage λ *attB* site (the strains are listed in Table 1). The *bgl* promoter (P_{bgl}) is indicated by a flagged arrowhead, the cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP) binding site is shown as a black box, and the mutation of terminator t1 ($t1_{RAT}$) is indicated by a crossed hairpin loop. (C) Expression levels were determined in strain T314 [Δ(*yjjP-yjjQ-bglJ*) Δ*leuO*] (−) transformed with the empty vector pKESK22, with plasmid pKETS1 for expression of BglJ in *trans* (+BglJ), or with plasmid pKEDR13 for expression of LeuO (+LeuO). The β-galactosidase activities with LeuO provided in *trans* were determined 6 times independently, as the standard deviation was up to 40%. In addition, the expression levels of the *bgl-lacZ* fusions were analyzed in transformants of Δ*hns*, Δ*rcsB*, and Δ*hns stpA* mutant derivatives, as indicated.

the *bgl-lacZ* reporter, BglJ was provided in *trans* using plasmid pKETS1 carrying *bglJ* under the control of the inducible *tac* promoter. Note that there is some ambiguity about the translation start codon of *bglJ*. Plasmid pKETS1 includes the most 5′ AUG, which maps within *yjjQ*. This plasmid directs the expression of active BglJ protein (see below), while plasmids pKETS9 and pKETS10, which include the second or third start codon, provide no functional BglJ (data not shown), suggesting that translation of the *bglJ* gene begins at the very first start codon of the open reading frame.

The *bgl-lacZ* reporter construct with the presumptive wild-type BglJ-RcsB box directed low levels of β-galactosidase activity, as expected (12 units) (Fig. 4C). When BglJ was provided in *trans*, expression increased 57-fold to 690 units (Fig. 4C). In a Δ*rcsB* mutant, expression was low (9 units), and expression remained low (8 units) when BglJ was provided in *trans*, demonstrating again that derepression of *bgl* by BglJ requires RcsB (Fig. 4C). Next, the expression levels directed by BglJ-RcsB binding-site mutants 1 to 3 (Fig. 4A) were tested in the absence or presence of BglJ. In the cases of mutants 1 and 3, which both carry exchanges corresponding to the conserved bases of the right half-site, induction of plasmid-encoded BglJ had no effect (10 to 13 units in all cases) (Fig. 4C). This demonstrates that mutations in the presumptive BglJ-RcsB binding site abrogate derepression of *bgl* by BglJ-RcsB. Interestingly, binding site mutant 2 also affected derepression of the *bgl* promoter-*lacZ* fusion by BglJ-RcsB, as the expression level increased only 8-fold, from 12 to 92 units, when BglJ was expressed (Fig. 4C). Mutant 2 carries mutations in the left half of the putative BglJ-RcsB box, which is presumably contacted by the BglJ subunit of the BglJ-RcsB heterodimer (Fig. 4A). Taken together, these data demonstrate that the putative BglJ-RcsB motif is important for derepression of *bgl* by BglJ-RcsB heterodimers.

As a further control, expression of the *bgl-lacZ* reporter constructs with the wild-type BglJ-RcsB box and its mutants was tested in isogenic Δ*hns* strains. Expression levels were high (290 to 360 units) (Fig. 4C), as expected, as H-NS represses the *bgl* promoter. Further, in the Δ*hns* mutant, activity was similarly high, irrespective of whether the BglJ-RcsB box was mutated, demonstrating that the site-specific mutations do not affect promoter activity or repression by H-NS (Fig. 4C). Interestingly, the expression level directed by the *bgl-lacZ* fusion was lower in the Δ*hns* mutant (290 units) than when plasmid-encoded BglJ was provided in the wild type (690 units). This indicated that the *bgl* promoter is not fully active in the *hns* mutant. In agreement with previous studies, which had demonstrated that StpA partially represses *bgl* in *hns* mutants (24, 42, 63), the expression level directed by the *bgl-lacZ* reporter was 715 units in the Δ*hns stpA* double mutant and thus similar to that upon derepression of *bgl* by BglJ-RcsB. However, growth of the Δ*hns stpA* double mutant was significantly slower than that of the *hns* mutant. Similarly, expression of plasmidic BglJ resulted in significantly slower growth in the *hns* mutant and caused a severe growth reduction in the *hns stpA* double mutant (data not shown). Therefore, we could not test whether BglJ-RcsB further enhances *bgl* promoter activity in the absence of H-NS and StpA. However, taken together, the data suggest that the BglJ-RcsB heterodimer binds within the up-

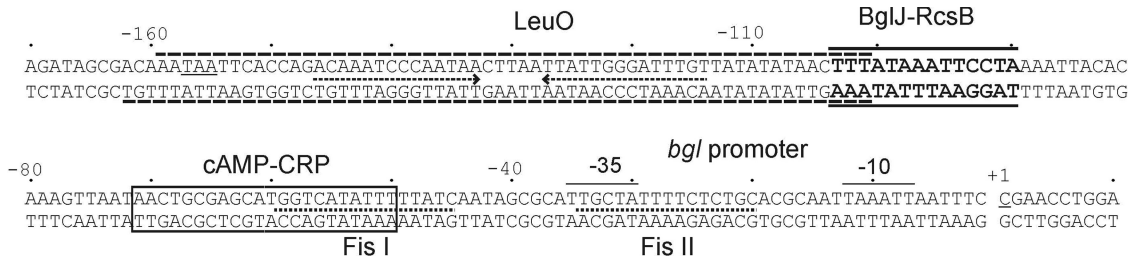

FIG. 5. Sequence of the *bgl* promoter and upstream regulatory region. Indicated are the −35, −10, and transcription start sites of the promoter; the CRP binding site (boxed) (48); and the Fis binding sites (dotted lines) (4), as well as the LeuO (dashed lines) and BglJ-RcsB (solid lines) binding sites characterized here. H-NS binds to the upstream regulatory element and the promoter, but the H-NS nucleation sites have so far not been mapped. The stop codon of the *phoU* gene located upstream of *bgl* is underlined, and the inverted arrows indicate an inverted repeat that may represent the *phoU* transcriptional terminator.

stream regulatory region and antagonizes repression of *bgl* by H-NS, and also by StpA.

Derepression of *bgl* by BglJ-RcsB and that by LeuO are independent of each other. In addition to BglJ, the LysR-type transcription factor LeuO abrogates H-NS-mediated repression of *bgl* (58). LeuO also activates the *yjjQ-bglJ* operon (55). To test whether derepression of *bgl* by LeuO is independent of BglJ and whether the mutations in the BglJ-RcsB binding site interfere with derepression of *bgl* by LeuO, expression levels were also tested with LeuO provided in *trans*. For this, plasmid pKEDR13 carrying *leuO* under the control of the inducible *tac* promoter was used. Induction of plasmid-encoded LeuO caused derepression of the *bgl-lacZ* fusion (directing 200 units of β-galactosidase activity) (Fig. 4C), demonstrating that LeuO activates *bgl* independently of BglJ-RcsB [the strain background is Δ(*yjjP-yjjQ-bglJ*) Δ*leuO*]. Similarly high expression levels were directed by the BglJ-RcsB binding-site mutants when LeuO was present (200 units for the wild type compared to 215 to 265 units for the RcsB site mutants) (Fig. 4C). This demonstrates that the mutations of the presumptive BglJ-RcsB box do not interfere with derepression of *bgl* by LeuO. However, LeuO did not cause full activation (approximately 200 units in the presence of LeuO compared to 690 units in the presence of BglJ), possibly because plasmid-directed expression of the pleiotropic LeuO affects the accuracy of the β-galactosidase assay (Fig. 4C). Nonetheless, the data suggest, that LeuO derepresses *bgl* by directly binding to the upstream regulatory region of the *bgl* promoter. Importantly, these data suggest that mutations in the presumptive BglJ-RcsB binding site do not abolish LeuO binding. To further validate this assumption, the LeuO binding site was mapped by DNase I footprinting using purified C-terminally His-tagged LeuO. The LeuO footprint showed protection of approximately 60 bp, extending from positions −101 to −160 relative to the *bgl* promoter transcription start site (see Fig. S2 in the supplemental material). Thus, the LeuO binding site maps just adjacent to the BglJ-RcsB site (Fig. 5). Extended footprints are typical of LeuO (11, 28, 60) and other LysR-type transcription factors (37). However, it remains unknown whether LeuO and BglJ-RcsB can bind simultaneously.

DISCUSSION

BglJ, a LuxR-type transcriptional regulator, and LeuO, a LysR-type transcriptional regulator, relieve repression of the *bgl* operon by H-NS. Here, we have shown that activation of *bgl* by BglJ depends on the two-component response regulator RcsB. BglJ and RcsB interact and presumably form heterodimers, as demonstrated by two-hybrid analysis and coimmunoprecipitation. These BglJ-RcsB heterodimers bind to the *bgl* URE, as suggested by site-specific mutation of a sequence motif, one half of which is related to the RcsA-RcsB consensus sequence. In addition, we have shown that LeuO likewise binds to the *bgl* URE and activates *bgl* directly. Taken together, the data suggest that binding of BglJ-RcsB and LeuO, respectively, interferes with formation of a repressing nucleoprotein complex by H-NS and thus results in activation of *bgl*. Furthermore, interaction of BglJ and RcsB extends the Rcs two-component signaling transduction system and the network of transcription regulators that affect the *bgl* operon (Fig. 6 shows a model).

The Rcs two-component system is widely conserved in enterobacteria and has an impact on biofilm formation and virulence in various species (29, 39). The response regulator RcsB functions both as a homodimer and as an RcsA-RcsB heterodimer. RcsB also interacts with GadE, which is likewise a LuxR-type transcription factor, and possibly with TviA in *S. enterica* (5, 62). Our finding that BglJ and RcsB also form a

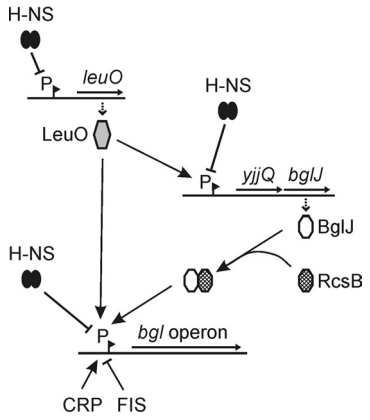

FIG. 6. Model illustrating regulation of the *bgl* promoter and regulatory interactions of the transcriptional regulators LeuO and BglJ-RcsB (for details, see Discussion). Pointed arrowheads indicate activation, and blunt arrowheads indicate repression. Promoters (P) are indicated by pointed flags. In addition to being controlled by H-NS, StpA, LeuO, and BglJ-RcsB, the *bgl* promoter is CRP dependent and is repressed by FIS (4, 48, 52).

heterodimer that acts as a transcriptional activator of the *bgl* operon expands the role of the Rcs system and underscores the notion that RcsB activity, in addition to being modulated by phosphorylation, is controlled by interaction with other transcriptional regulators. Furthermore, the activity of the heterodimer BglJ-RcsB is presumably independent of RcsB phosphorylation, while the activity of the RcsA-RcsB heterodimer is phosphorylation dependent (39). This extra level of combinatorial control of the response regulator RcsB is likely to have an impact on the regulatory repertoire attributable to the Rcs two-component signal transduction system.

There are several parallels between RcsA and BglJ. Both RcsA-RcsB and BglJ-RcsB heterodimers function as activators, or rather, as H-NS antagonists. BglJ-RcsB counteracts H-NS-mediated repression of the *bgl* operon (see above), while RcsA-RcsB activates the *cps-wza* and *yjb* operons, encoding enzymes for capsule synthesis, and the H-NS-repressed *rcsA* gene (21, 29, 39). Activation by RcsA-RcsB and BglJ-RcsB, respectively, involves binding sites that map 100 bp or more upstream of the transcription start site (see Fig. S1 in the supplemental material). A further parallel is that both the *rcsA* gene and the *bglJ* gene (within the *yjjQ-bglJ* operon) are repressed by H-NS. Expression of *rscA* is autoregulated, while expression of the *yjjQ-bglJ* operon is activated by the LysR-type transcription factor LeuO. Similarly, the complex regulation of the acid stress response gene *gadA*, which is activated by GadE and RcsB, involves repression by H-NS (5). This indicates that RcsB, with its interacting partners, may play an important role as an H-NS antagonist.

Furthermore, we demonstrated that LeuO directly binds to the *bgl* upstream regulatory region and relieves repression independently of BglJ-RcsB. Considering the fact that LeuO also relieves H-NS-mediated repression of the *yjjQ-bglJ* operon (55), this suggests that LeuO can affect *bgl* expression in two ways, directly by activating the *bgl* promoter and indirectly by activating expression of the *yjjQ-bglJ* operon. However, the *leuO* gene is also repressed by H-NS and only moderately induced by branched amino acid starvation in a ppGpp-dependent manner (19). The latter may not lead to sufficiently high expression levels of LeuO under laboratory conditions, as these stress conditions seem not to affect LeuO target genes. Accordingly, up-to-date analyses of regulation by LeuO have been performed with chromosomal or plasmidic alleles under the control of constitutive or inducible promoters (11, 28, 53, 55, 60).

Taken together, LeuO and BglJ-RcsB form a small regulatory network that relieves H-NS-mediated repression of the *bgl* operon (Fig. 6). However, expression of *leuO* and *bglJ* is repressed by H-NS, at least under laboratory growth conditions. As LeuO is a virulence factor in *S. enterica* (32, 57) and as YjjQ, which is coencoded with BglJ, is presumably important for infection by avian-pathogenic *E. coli* (34), it is conceivable that certain conditions in the host environment induce their expression, which in turn should also relieve repression of the *bgl* operon and allow its induction by substrate (aryl-β,D-glucosides). The *bgl* operon is a very tightly controlled locus which may possibly serve a very specialized function related to extraintestinal pathogenicity (50), in agreement with the finding that the *bgl* operon is induced in a septicemic strain when it infects the mouse liver (30).

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