BglJ-RcsB heterodimers relieve repression of the *Escherichia coli* bgl operon by H-NS

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ABSTRACT

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 \textit{bgl} (aryl-\(\beta\),D-glucoside) operon in \textit{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 which presumably bind upstream of the \textit{bgl} promoter, as suggested by mutation of a sequence motif related to the consensus sequence for RcsA-RcsB heterodimers. Heterodimerization of BglJ-RcsB and relieve of H-NS mediated repression of \textit{bgl} by BglJ-RcsB is apparently independent of RcsB phosphorylation. In addition, we show that LeuO, a pleiotropic LysR-type transcriptional regulator, likewise binds to the \textit{bgl} upstream regulatory region and relieves repression of \textit{bgl} independently of BglJ-RcsB. Thus, LeuO can affect \textit{bgl} directly, as shown here, and indirectly by activating the H-NS repressed \textit{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 \textit{bgl} promoter.
INTRODUCTION

The nucleoid associated protein H-NS protein is a pleiotropic regulator which generally functions as 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 (15,18,44,54). 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. This protein supposedly binds as 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 and others 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 the H-NS activity by changes of 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 which 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 which 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 in 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 is 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. RcsA-RcsB heterodimers activate 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 Typhi*, interaction of RcsB with TviA was found to control Vi antigen synthesis (62).

LeuO is a pathogenicity determinant in *S. enterica*, and important for biofilm formation in *Vibrio cholerae* (32,41,57). It is a regulator of many genes including outer membrane proteins, drug efflux, 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 at 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 H-NS antagonist at the *bgl* locus. Further analyses demonstrate that BglJ and RcsB form heterodimers and suggest 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.
MATERIAL AND METHODS

Strains, plasmids and media

All strains and plasmids 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 supplement.

Transposon mutagenesis

Transposon mutagenesis using the phage λNK1323 miniTn10::tet transposon system was performed, as described (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 semi-random, two-step PCR (ST-PCR protocol), as described (7,38). In one of the mutants the miniTn10::tet transposon mapped within the rcsB open reading frame (at position 225 bp relative to the translation start with a 9 bp target site duplication TACATCAAG). This allele was assigned rcsB::miniTn10-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 OD600=0.05 to 0.1 and grown to OD600=0.5. IPTG was added to a final concentration of 1 mM to the overnight and the exponential culture for induction, where indicated. The bacteria were harvested and β-galactosidase
activities were determined at least three times independently, as described (40). Standard deviations were less than 10%, unless otherwise indicated.

Co-immunoprecipitation

Co-immunoprecipitation to analyze the interaction of RcsB with BglJ was performed using transformants of strain S3377 (ΔrcsB Δ[yjjP-yjjQ-bglJ]) with plasmids expressing tagged variants of the RcsB and BglJ proteins. For expression of RcsB with a C-terminal HA-tag strain S3377 was transformed with plasmid pKEAP38. For expression of BglJ with C-terminal FLAG-tag plasmids 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 were inoculated from the overnight-culture to OD600=0.05, and grown to OD600=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 pH7.5, 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 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) were added. The samples were incubated for 2 hrs at 4°C in a tube rotator to allow binding, 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, 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% non fat dry milk powder in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and incubated with rat anti-HA (Roche, 1:500) and mouse anti-Flag antibody (Sigma-Aldrich, 1:5000). As secondary antibodies IRDye 800CW conjugated goat anti-mouse antibody (Li-Cor BioSciences, 1:10000) and IRDye 680 conjugated goat anti-rat antibody (Molecular Probes, 1:5000) 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 (position -202 to +30 relative to the transcription start) was amplified by PCR. For 5'-end labeling of the top strand with T4-polynucleotide kinase and [γ-³²P]-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 (amounts as indicated in the figure) to the labeled fragments (approximately 200000 cpm) was performed at 30 °C for 20 minutes in a volume of 20 µl in binding buffer (100 mM KCl, 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 mM DTT, 10 % glycerol, 500 mM Imidazol) and 50 ng/µl BSA, 5 ng/µl herring sperm DNA. Then 2 µl DNase I (Roche Molecular Biochemicals, 5 ng/µl in binding buffer) were added and the reaction was stopped exactly 1 minute 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% formamid, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol, and 5 mM EDTA), and separated on 6% denaturing
sequencing gels (6% Long Ranger™, Lonza, 7 M urea, 0.8 x TBE) next to a sequencing ladder. The sequencing ladder was generated using the T7 sequencing kit (USB Corporation) and [α-32P]-dCTP, with the same primers as used for generation of the bgl PCR fragment.
RESULTS

Derepression of bgl by BglJ requires RcsB

Transcription factor BglJ relieves repression of the bgl operon by H-NS (25). To identify factors which 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, this strain constitutively expresses bglJ (named bglJc in the following) because of the insertion of a miniTn10 transposon upstream of bglJ (allele yjjQ/bglJ-Y6::miniTn10) (38). Thus, this dual reporter strain for monitoring activity of the bgl promoter is Bgl and Lac positive. Transposon mutants were screened for Bgl and Lac negative mutants, and one of these mutants 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 (bglJc). Analysis of the Bgl phenotype on indicator plates demonstrated that the deletion of rcsB abrogates depression of bgl by BglJ (Fig. 1B). Further, complementation of the ΔrcsB mutant with plasmid pKETS6 encoding rcsB under control of the IPTG inducible tac promoter restored the Bgl positive phenotype in the bglJc strain but not in the wild-type. This suggests that both RcsB and BglJ are required to relieve the H-NS mediated repression of bgl. Interestingly, complementation of the ΔrcsB strain was also possibly with RcsB mutants carrying exchanges in the conserved aspartate (residue 56) of the N-terminal receiver domain. Mutation RcsB-D56E mimics the active phosphorylated 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 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 co-immunoprecipitation (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 (LexA1-87) to a protein which forms homodimers (Fig. 2A) (12). The sulA promoter-lacZ reporter for heterodimer formation carries a hybrid lexA408+/ operator with a mutation in one half-site (Fig. 2A). This operator can only be bound by heterodimers, in which one partner includes a LexA1-87 wild-type DNA-binding domain and the other protein partner contains a LexA4081-87 mutant DNA-binding domain (Fig. 2A) (12). For the current analysis of homodimer and heterodimer formation of BglJ, YjjQ and RcsB, the LexA-based two-hybrid-system was transferred to a ΔrcsB and Δ(yjjP-yjjQ-bglJ) strain background (see Supplement). In addition, plasmids were constructed which express fusions of the wild-type LexA1-87 DNA-binding domain to BglJ, RcsB, YjjQ, and to RcsA, as control. RcsB and BglJ were also fused to the mutant LexA4081-87 DNA-binding domain (Fig. 2, Table 1).
In the homodimerization assay induction of the LexA$_{1-87}$-RcsB-fusion caused a strong repression of the $sulA$ promoter-$lacZ$ reporter (in strain S3434), as expected (Fig. 2B). In contrast, the LexA$_{1-87}$-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 a 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 control, a LexA$_{1-87}$-Fos fusion known for its low capacity of homodimer formation was included. Induction of this fusion resulted in weak repression only (Fig. 2B).

In the heterodimerization assay co-induction of LexA$_{408-1-87}$-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). Co-expression of BglJ (fused to LexA$_{1-87}$) and RcsB (fused to LexA$_{408-1-87}$) 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 LexA$_{408-1-87}$) 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 LexA$_{408-1-87}$-Jun, as well as of RcsB (using LexA$_{1-87}$-RcsB and LexA$_{408-1-87}$-RcsB) were included, which, as expected, caused repression (Fig. 2C).
Further, 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 as compared to wild-type RcsB, while 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 co-immunoprecipitation. To this end, compatible vectors for co-expression of BglJ-Flag and RcsB-HA in strain S3377 (ΔrcsB and 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 reporter (data not shown). Co-immunoprecipitation 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 co-immunoprecipitation 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 co-expressed with RcsB-HA (Fig. 3). This demonstrated that the co-
immunoprecipitation was specific and suggests that BglJ-FLAG interacts with RcsB-HA (Fig. 3).

Mapping of a BglJ-RcsB box in the bgl regulatory region

The data indicate that BglJ-RcsB heterodimers relieve repression of \textit{bgl} by H-NS by binding next to the \textit{bgl} promoter. For RcsA-RcsB heterodimers, a consensus sequence (termed RcsAB box) was proposed (39,59). This RcsAB box is non-palindromic (Fig. S1) and presumably recognized by binding of RcsB to one half-site and by binding of RcsA to the other half-site (22). Interestingly, within the \textit{bgl} regulatory region a perfect match to one half-site of the RcsAB box is located at position -88 to -95 (relative to the transcription start) (Fig. 4A). Assuming that the right half-site of the RcsAB box is bound by RcsB (see also Fig. S1), this match indicated 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 (mutant 1 in Fig. 4A). In addition, the left half-site of the presumptive BglJ-RcsB box was mutated (mutant 2 in Fig. 4A), and mutations in both half-sites were combined (mutant 3 in Fig. 4A). The effect of these mutations on derepression of \textit{bgl} by BglJ-RcsB was tested using a \textit{bgl}-\textit{lacZ} reporter construct, which 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 \textit{bgl} operon is promoter-independent and mediated by the specific transcriptional antiterminator BglG, which allows transcription read-


through at terminator t1. BglG activity is regulated by phosphorylation in dependence 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 (ΔlacZ, Δ(yjjP-yjjQ-bglJ), and ΔleuO, as LeuO also de-represses the bgl operon, see below). To analyze derepression of the bgl-lacZ reporter, BglJ was provided in trans using plasmid pKETS1 carrying bglJ under control of the inducible tac promoter. Please note that there is some ambiguity about the translation start codon of bglJ. Plasmid pKETS1 includes the most 5'-prime 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 the expression increased 57-fold to 690 units (Fig. 4C). In an ΔrcsB mutant expression was low (9 units) and expression remained low (8 units) when BglJ was provided in trans demonstrating again that de-repression of bgl by BglJ requires RcsB (Fig. 4C). Next, the expression levels directed by the BglJ-RcsB binding-site mutants 1 to 3 (Fig. 4A) were tested in the absence or presence of BglJ. In case 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, also binding site mutant 2 affected de-repression of the bgl promoter-lacZ fusion by BglJ-RcsB, as the expression level increased merely 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 de-repression of bgl by BglJ-RcsB heterodimers.

As further control, expression of the bgl-lacZ reporter constructs with the wild-type BglJ-RcsB box and its mutants was tested in isogenic ∆hns strains. Expression levels were high (290 to 360 units, Fig. 4C), as expected, as H-NS represses the bgl promoter. Further, in the ∆hns mutant the activity was similarly high, irrespective of whether the BglJ-RcsB box was mutated or not, demonstrating that the site-specific mutations do not affect the promoter activity or repression by H-NS (Fig. 4C). Interestingly, the expression level directed by the bgl-lacZ fusion was lower in the ∆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 ∆hns stpA double mutant and thus similarly high as upon derepression of bgl by BglJ-RcsB. However, growth of the ∆hns stpA double mutant was significantly slower then of the hns mutant. Similarly, expression of plasmidic BglJ resulted in significant slower growth in the hns mutant, and caused a severe growth reduction in the hns stpA double mutant (data not shown). Therefore, it could not be tested whether
BglJ-RcsB further enhances the 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 upstream regulatory region and antagonizes repression of bgl by H-NS and also by StpA.

De-repression of bgl by BglJ-RcsB and LeuO is 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 de-repression of bgl by LeuO is independent of BglJ and whether the mutations in the BglJ-RcsB binding site interfere with de-repression of bgl by LeuO, expression levels were in addition tested with LeuO provided in trans. For this, plasmid pKEDR13 carrying leuO under 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 ∆(yjjP-yjjQ-bglJ, ∆leuO). Similarly high expression levels were directed by the BglJ-RcsB binding site mutants, when LeuO was present (compare 200 units wild-type, 215 to 265 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 (compare approximately 200 units in the presence of LeuO with 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 de-represses 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-terminal His-tagged LeuO. The LeuO footprint showed a protection of approximately 60 bp, extending from position -101 to -160 relative to the {\textit{bgl}} promoter transcription start site (Fig. S2). Thus, the LeuO binding site maps just adjacent to the BglJ-RcsB site (Fig. 6). Extended footprints are typical of LeuO (11,28,60) and other LysR-type transcription factors (37). However, it remains open 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 co-immunoprecipitation. These BglJ-RcsB heterodimers bind to the bgl upstream regulatory element (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 which affect the bgl operon (for a model see Fig. 6).

The Rcs two-component system is widely conserved in enterobacteria and has impact on biofilm formation and virulence in various species (29,39). The response regulator RcsB functions both as a homodimer and as 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 heterodimer which 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 the 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 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 bgl operon (this work), 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 which map 100 base pairs or more upstream of the transcription start (Fig. S1). A further parallel is that both the rcsA gene and bglJ gene (encoded 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 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 a dual way, 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 on regulation by LeuO have been performed with chromosomal or plasmidic alleles under control of constitutive or inducible promoters (11,28,53,55,60).

Taken together, LeuO and BglJ-RcsB form a small regulatory network which 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 Salmonella enterica (32,57), and as YjjQ which is co-encoded 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 possibly may serve a very specialized function related to extraintestinal pathogenicity (50) in agreement with the finding that the bgl operon is induced in a septicimic strain when infecting mouse liver (30).

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Table 1: E. coli K12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>relevant genotype</th>
<th>construction / source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW30270</td>
<td>MG1655 rph+</td>
<td>Coli Genetic Stock Center #7925</td>
</tr>
<tr>
<td>KL788</td>
<td>λ Thr-1, Δ(gpt-lac)5, tex-35, sulA3, e14, Rac-07, rfbD17, mgl-51, recA441(s), relA1?, rpsL31(strR), kdgK51?, mtl-1, spoT1?, thi-1?, lexA71::Tn5, creC5107? (stored as S1152)</td>
<td>Coli Genetic Stock Center #6218</td>
</tr>
</tbody>
</table>

M182 stpA::tet ΔlacZ74 galU galK strA stpA::tet (stored as S159) (64)
SU101   λ lysogen with P_{lux} lexA-op+ ΔlacZ fusion in JL1434 (12)
SU202   λ lysogen with P_{lux} lexA-op408+ ΔlacZ fusion in JL1434 (12)
S1734   yjQ::bgU-Y6::miniTn10-cat (ΔbgU) in S764 (38)
S524    is CSH50 ΔlacZY217 (gpt-pro+) (14)
S2176   is S524 yjQ::bgU-Y6::miniTn10-cat (ΔbgU) S524 x T4GT7 (S1734)
S2817   is S524 attB::[Spec^R ΔyjjP-yjjQ-bglJ::cat] T4GT7 (S2176) S524 x pKEK830
S2822   is S524 attB::[Spec^R ΔyjjP-yjjQ-bglJ::cat] T4GT7 (S2176) S2817 x T4GT7 (S1734)
S2828   is S524 rcsB-2828::miniTn10-tet (ΔbgL and Lac') S2822 x ANK123
S3918   is S524 ΔrcsB::Spec^" T4GT7 (S3278)
S3919   is S524 bgU::ΔrcsB::Spec^" T4GT7 (S3278)
S541    is CSH50 ΔlacZY217 ΔbgI-AC11 (14)
S3010   is S541 Δhns::kan^R x PCR S774/S775 (pKE5D8)
S3278   is S541 ΔrcsB::Spec^" x PCR S783/S767 (pKD3)
S3377   is S541 ΔrcsB::Spec^" Δ(yjQ:: bgU-Y6::cat) T4GT7 (S3278) S3278 x PCR S783/S767 (pKD3)
S1185   is S541 ΔsulA3
S3360   is S541 sulA3 lexA71::Tn5 S1184 x T4GT7 (KL786) Kan^R
S3373   is S3360 ΔrcsB^R T4GT7 x PCR S819/S820 pKD3 x pCP20
S3384   is S3360 ΔrcsB^R Δ(yjQ:: bgU-Y6::cat) x PCR S783/S766 pKD3; x pCP20
S3343   is S3384 attB::[Spec^R ΔyjjP-yjjQ-bglJ::cat] T4GT7 x pES163
S3442   is S3384 attB::[Spec^R ΔyjjP-yjjQ-bglJ::cat] T4GT7 x pES164
S3974   is BW30270 ivG" (valine resistant) BW30270/pK946 x annealed oligonucleotides T96/T97
S4197   is BW30270 ivG" ΔlacZ S4197 x pFDY217
T15     is S4197 rcsB::kan^R T4GT7 x PCR S819/S820 pKD4; x pCP20
T70     is S4197 ΔyjQ:: bgU-Y6::cat T4GT7 x PCR S767/S763 (pKD3)
T71     is S4197 ΔyjQ:: bgU-Y6::cat T4GT7 x PCR T209/T210 (pKD3); x pCP20
T314    is S4197 ΔyjQ:: bgU-Y6::cat T4GT7 x PCR T209/T210 (pKD3); x pCP20
T568    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T576    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T578    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T580    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T727    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T729    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T731    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T733    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T735    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20
T757    is S314 attB::[Spec^R ΔyjQ:: bgU-Y6::cat] T4GT7 x PCR T209/T210 (pKD3); x pCP20

* JL134 is lexA71::Tn5 (Def); sulA21 ΔlacZ::F' lacI^H lacZΔM15::Tn9
S764 is CSH50 bgI"-C234 ΔlacOP::[SpecR P_{bgI} C234(+54) bgI\_op] lon-107::miniTn10-tet
yjQ::bgU-Y6::miniTn10-cat
rcsB-2828 carries a miniTn10-cm insertion in rcsB with a target site duplication of 9 bp at position
217 to 225 relative to the rcsB translation start
sulA3 carries a single nucleotide A to G exchange 35 bp upstream of ATG
ΔlacZ was introduced into strain S3974 by gene replacement using rep^" plasmid pFDY217, as
9 described (3).
**Figure legends**

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 reportors for *bgl* expression. In addition, this strain carries allele *yjjQ*/∥*bglJ*-Y6::mini*Tn*10-cat (*bglJ*) for constitutive expression of *bglJ*. A mini*Tn*10-tet transposon mutagenesis screen yielded Bgl and Lac negative mutants, one of which carried a transposon insertion in *rcsB* (assigned as strain S2828, Table 1). (B) RcsB is required for de-repression of *bgl* by BglJ. The Bgl phenotypes of *E. coli* K12 wild-type (wt, strain S524) and its isogenic derivatives which constitutively express *bglJ* (*bglJC*, strain S2176), or carry a deletion of *rcsB* (∆rcsB strain S3918), as well as the double mutant *bglJC* ∆rcsB (strain S3919) was determined on BTB salicin indicator plates. Complementation of the ∆rcsB mutants with plasmids encoding wild-type RcsB (pKETS6), or the RcsB mutants D56E (pKETS7), D56N (pKETS8), and D56A (pKES235).

Fig. 2  Interaction of BglJ with RcsB and YjjQ (A) In the LexA-based two-hybrid system the sulA promoter-*lacZ* fusion with wild-type LexA operator (lexA-op+/+) was used to analyze homodimerization and the sulA promoter-*lacZ* reporter fusion with a hybrid lexA408/+ operator serves as reporter for heterodimerization. For analysis of homodimerization a fusion of the respective protein (X) to the wild-type LexA DNA-binding domain (lexA1-87-X) was expressed from a plasmid under control of the IPTG inducible lacUV5 promoter (P_{UV5}). For heterodimerization analysis fusions of protein X to the wild-type LexA DNA-binding domain (LexA1-87-X) and protein Y to the LexA408 mutant DNA-binding domain (LexA4081-87-Y) were co-expressed from compatible plasmids. (B) Analysis of homodimer formation of RcsB, RcsA, BglJ, and YjjQ, was performed with transformants of strain S3434 with plasmids pKEMK17 (lexA1-87-rcsB), pKES192 (lexA1-87-rcsA), pKEAP30 (lexA1-87-bglJ), pKEAP27 (lexA1-87-yjjQ), and pMS604 (lexA1-87-fos) as control. 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$_{1-87}$-X fusion protein. The fold repression (indicated as 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 co-transformed with plasmids coding for LexA$_{1-87}$-X and LexA$_{408-1-87}$-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 for heterodimerization (indicated as bars). The following plasmids were used: LexA$_{1-87}$-RcsB (pKEMK17), LexA$_{1-87}$-RcsA (pKES192), LexA$_{1-87}$-BglJ (pKEAP30), LexA$_{1-87}$-YjjQ (pKEAP27), and LexA$_{1-87}$-Fos (pMS604) (12), as well as LexA$_{408-1-87}$-RcsB (pKEAP28), LexA$_{408-1-87}$-RcsB$_{D56E}$ (pKES150), LexA$_{408-1-87}$-RcsB$_{D56N}$ (pKES151), LexA$_{408-1-87}$-BglJ (pKEAP29), and LexA$_{408-1-87}$-Jun (pDP804) (12), as control.

Fig 3 Co-immunoprecipitation of BglJ-FLAG with RcsB-HA. (A) Co-immunoprecipitation of BglJ-FLAG with RcsB-HA was performed of lysates prepared from strain S3377 (ΔrcsB Δ(yjjP-yjjQ-bglJ) "-") and transformants of strain S3377 with plasmids pKEAP38 (RcsB-HA) (RcsB), pKERV10 (BglJ-FLAG) (BglJ), or both plasmids. Immunoprecipitation was performed with rabbit anti-HA IgG. The lysates and the co-immunoprecipitates 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 and mouse anti-FLAG as primary antibodies and fluorescence labeled anti-mouse and anti-rat secondary antibodies.

Fig. 4 Effect of mutation of the BglJ-RcsB binding-site on de-repression of bgl by BglJ-RcsB. The expression level directed by bgl promoter-lacZ fusions (schematically shown in A) with wild-type and mutant BglJ-RcsB binding sites (B) was determined of exponential cultures grown in LB (with appropriate antibiotics and 1 mM IPTG) (C). The bgl-lacZ fusions were integrated at the phage λ attB site (strains listed in Table 1). Expression
levels were determined in strain T314 (Δ[yjjP-yjjQ-bglJ] ΔleuO) (indicated as “-”) 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 level of the bgl-lacZ fusions was analyzed in transformants of Δhns, ΔrcsB, and Δhns ΔstpA mutant derivatives, as indicated.

Fig. 5 Sequence of the bgl promoter and upstream regulatory region. Indicated are the -35, -10 and transcription start site of the promoter, the CRP-binding site (48), Fis binding sites (4), as well as LeuO and BglJ-RcsB 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 inverted arrows indicate an inverted repeat which may represent the phoU transcriptional terminator.

Fig. 6 Model illustrating regulation of the bgl promoter and regulatory interactions of the transcriptional regulators LeuO and BglJ-RcsB (for details see discussion). Pointes arrowheads indicate activation and blunt arrowheads indicate repression. In addition to being controlled by H-NS, StpA, LeuO and BglJ-RcsB the bgl promoter is CRP-dependent, and repressed by FIS (4,48,52).

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