Correlation of Antagonistic Regulation of leuO Transcription with the Cellular Levels of BglJ-RcsB and LeuO in Escherichia coli

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LeuO is a conserved and pleiotropic transcription regulator, antagonist of the nucleoid-associated silencer protein H-NS, and important for pathogenicity and multidrug resistance in Enterobacteriaceae. Regulation of transcription of the leuO gene is complex. It is silenced by H-NS and its paralog StpA, and it is autoregulated. In addition, in Escherichia coli leuO is antagonistically regulated by the heterodimeric transcription regulator BglJ-RcsB and by LeuO. BglJ-RcsB activates leuO, while LeuO inhibits activation by BglJ-RcsB. Furthermore, LeuO activates expression of bglJ, which is likewise H-NS repressed. Mutual activation of leuO and bglJ resembles a double-positive feedback network, which theoretically can result in bi-stability and heterogeneity, or be maintained in a stable OFF or ON states by an additional signal. Here we performed quantitative and single-cell expression analyses to address the antagonistic regulation and feedback control of leuO transcription by BglJ-RcsB and LeuO using a leuO promoter mVenus reporter fusion and finely tunable bglJ and leuO expression plasmids. The data revealed uniform regulation of leuO expression in the population that correlates with the relative cellular concentration of BglJ and LeuO. The data are in agreement with a straightforward model of antagonistic regulation of leuO expression by the two regulators, LeuO and BglJ-RcsB, by independent mechanisms. Further, the data suggest that at standard laboratory growth conditions feedback regulation of leuO is of minor relevance and that silencing of leuO and bglJ by H-NS (and StpA) keeps these loci in the OFF state.

Keywords: transcription regulator, nucleoid-associated protein, H-NS, H-NS antagonist, feedback regulation

INTRODUCTION

LeuO is a conserved and pleiotropic LysR-type transcription factor that has been best characterized in Escherichia coli and Salmonella enterica. LeuO functions both as activator and as repressor, and is presumably a tetramer, similar to other LysR-type regulators (Maddocks and Oyston, 2008; Guadarrama et al., 2014). LeuO is a master regulator with more than 100 target loci, and supposedly an important H-NS antagonist, since many LeuO-activated loci are H-NS repressed (Ueguchi et al., 1998; Chen et al., 2003; Chen and Wu, 2005; De la Cruz et al., 2007; Stoebel et al., 2008; Stratmann et al., 2008, 2012; Shimada et al., 2011; Dillon et al., 2012; Ishihama et al., 2016). In addition, genomics data revealed a significant overlap of co-regulation by LeuO and H-NS both in E. coli...
and in *S. enterica*, where 78 and 40%, respectively, of the LeuO targets are H-NS bound (Shimada et al., 2011; Dillon et al., 2012; Ishihama et al., 2016). H-NS represses transcription by formation of extended complexes on the DNA (Dillon and Dorman, 2010; Landick et al., 2015; Winardhi et al., 2015). For activation of H-NS repressed loci by LeuO several mechanisms have been proposed including alteration of the repressing H-NS nucleoprotein-complex, the prevention of spreading of the H-NS complex, and competition with H-NS for DNA binding (Chen and Wu, 2005; Shimada et al., 2011; Dillon et al., 2012). The biological role of LeuO is pleiotropic. LeuO is relevant for pathogenicity in *S. enterica*, for biofilm formation in *Vibrio cholerae* and *E. coli*, as well as the acid stress response and multidrug efflux in *E. coli* (Stoebel et al., 2008; Shimada et al., 2009, 2011; Dillon et al., 2012). Further, LeuO activates expression of the H-NS repressed genes coding for the CRISPR/Cas immunity system in *E. coli* and *S. enterica* (Pul et al., 2010; Westra et al., 2010; Medina-Aparicio et al., 2011). In accordance with the pleiotropic role of LeuO, transcription of *leuO* is tightly controlled. Under laboratory conditions the *leuO* gene is repressed by H-NS and by the H-NS paralog StpA, and thus the *leuO* gene is silent in *E. coli* and *S. enterica* (Klauck et al., 1997; Chen et al., 2001). Moderate upregulation of *leuO* expression was observed in stationary phase and under amino acid starvation (Fang and Wu, 1998; Fang et al., 2000; Majumder et al., 2001; Shimada et al., 2011; Dillon et al., 2012). In addition, positive autoregulation by LeuO and transcriptional coupling of *leuO* expression to expression of neighboring genes by DNA supercoiling has been reported (Fang and Wu, 1998; Chen et al., 2003). Furthermore, in *E. coli* *leuO* is activated by the heterodimeric transcription regulator BglJ-RcsB (Stratmann et al., 2012). Activation of *leuO* by BglJ-RcsB is inhibited by LeuO, and LeuO represses *leuO* transcription in *hns* and in *hns stpA* mutants (Figure 1A). Thus, LeuO is also a negative autoregulator (Stratmann et al., 2012). The *leuO* gene is preceded by at least two promoters (P1 and P2) which are repressed by H-NS and StpA and negatively autoregulated by LeuO in *hns stpA* mutants; the P2 promoter is activated by BglJ-RcsB (Stratmann et al., 2012). BglJ-RcsB is a heterodimer that activates transcription of various loci in *E. coli* (Venkatesh et al., 2010; Stratmann et al., 2012; Salscheider et al., 2014). BglJ-RcsB consists of RcsB, the response regulator of the Rcs two-component phosphorelay system (Majdalani and Gottesman, 2005), and BglJ, which has initially been found as an activator of the *bgl* operon (Giel et al., 1996). Further, BglJ-RcsB is active independent of phosphorylation of RcsB by the Rcs phosphorelay (Venkatesh et al., 2010; Stratmann et al., 2012; Pannen et al., 2016).

Intriguingly, activation of *leuO* by BglJ-RcsB is one element of a presumptive double-positive feedback loop, since LeuO in turn activates expression of the *yjjQ-bglJ* operon that is likewise H-NS repressed (Stratmann et al., 2008). This double-positive feedback loop is interlocked with a negative feedback loop which is based on negative autoregulation by LeuO (Figure 1). Such a network motif can function like a switch that is stable both in the OFF as well as in the ON state. Often an external signal locks such feedback loops in one state. Further, bi-stability resulting in population heterogeneity and oscillation can be based on interlocked positive and negative feedback loops (Angeli et al., 2004; Alon, 2007; Shoval and Alon, 2010). In this study we addressed the antagonistic regulation of *leuO* transcription by BglJ-RcsB and LeuO, which is presumably a crucial element in the complex control of *leuO* expression. For quantitative and single-cell expression analysis, we established a reporter fusion of the *leuO* promoter region (*P*leuO) to *mVenus* and expressed *bglJ* and *leuO in trans* using tightly controlled and gradually inducible plasmidic expression systems. Expression analyses of the *P*leuO *mVenus* reporter at steady state growth conditions revealed uniform expression. The level of *leuO* expression correlates with the relative cellular concentration of BglJ and LeuO. The data are in agreement with a straightforward model of antagonistic
regulation by the two regulators that act independently of each other.

RESULTS

Experimental System for Analyzing Regulation of leuO Expression by BglJ and LeuO

The regulation of leuO transcription by BglJ-RcsB and LeuO is an important element in the control of the LeuO master regulator. To address regulation of leuO transcription that is directed by at least two promoters (PleuO) in dependence of the concentrations of BglJ and LeuO, a suitable experimental system was established. First, the mVenus reporter gene (coding for the yellow fluorescent protein mVenus) was fused to the leuO promoter-regulatory region by replacement of the leuO gene resulting in allele PleuO mVenus, ∆leuO (Figure 1B). Second, BglJ and LeuO were ectopically expressed from two different sets of plasmids. In one plasmid set, bglJ was expressed under control of the IPTG-inducible lacUV5 promoter (P<sub>UV5</sub>) using low-copy plasmid pKETS26 (pSC origin of replication), and leuO was expressed under control of the arabinose-inducible P<sub>BAD</sub> promoter using the low to medium copy plasmid pKES302 (pBAD30-derived, p15A origin of replication). In the other plasmid set, bglJ was expressed under control of the P<sub>BAD</sub> promoter (pKES302, p15A-ori) and leuO under control of IPTG-inducible P<sub>Lac</sub> promoter (pKEHB27, pSC-ori). The genes encoding the AraC and the LacI regulators, respectively, are also carried on these plasmids. Additionally, the yjjQ-bglJ operon was deleted resulting in allele ∆(yjjQ-bglJ) to ensure that only plasmid-encoded BglJ is present in the cell. Note that RcsB is not limiting for activation of leuO and other loci by BglJ-RcsB (Salscheider et al., 2014; Pannen et al., 2016). Third, to allow controlled and finely tunable expression of bglJ and leuO directed by the arabinose-inducible P<sub>BAD</sub> promoter and the IPTG-inducible P<sub>UV5</sub> and P<sub>Lac</sub> promoters, respectively, additional modifications and mutations were introduced into the reporter strain (Figure 1B). The P<sub>UV5</sub> promoter is gradually induced over a range of inducer concentrations (IPTG) when the lactose permease gene lacY is deleted (Jensen et al., 1993). Therefore, the lacZYA operon and the lacI gene were deleted in the reporter strain resulting in allele ∆(lacI-lacZYA) (Table 1). Likewise, the arabinose regulon was modified to ensure a gradual induction of the P<sub>BAD</sub> promoter with arabinose, as described before (Khlebnikov et al., 2001; Kogenaru and Tans, 2014). Briefly, the P<sub>BAD</sub> promoter is known to have a stochastic behavior when induced with arabinose. This stochastic behavior is caused by the araE and araFGH genes encoding the arabinose transporters, because induction of the transporter genes by arabinose leads to a higher arabinose uptake and thus positive feedback (Siegele and Hu, 1997; Megerle et al., 2008). In addition, a negative feedback caused by fermentation of intracellular arabinose through the AraBAD enzymes leads to a non-graual induction (Siegele and Hu, 1997). To avoid the negative and positive feedback, the araC gene and the araBAD and araFGH operons were deleted. Further, the low affinity arabinose transporter araE was put under the control of constitutive promoter P<sub>cp8</sub>, as described (Khlebnikov et al., 2001; Kogenaru and Tans, 2014). The genotype of the resulting reporter strain U69 is PleuO mVenus ∆leuO ∆(yjjQ-bglJ) q(ΔaraE p<sub>cp8</sub> araE) ∆(araH-F) ∆(araC-araBAD) ∆(lacI-lacZYA) (Table 1). Using this strain the expression level of PleuO mVenus was measured by flow-cytometry to quantify the cellular fluorescence in the population. Further, to ensure steady state conditions, cultures were grown in nutrient-poor tryptone medium. In this medium cultures that were inoculated from fresh overnight cultures to OD<sub>600</sub> of 0.05 reached an OD<sub>600</sub> of about 0.7–1 after 5 h of growth.

Regulation of leuO Promoter by BglJ–RcsB and by LeuO

First, activation of the PleuO mVenus fusion by BglJ-RcsB was tested. To this end, the reporter strain U69 was transformed with low-copy plasmid pKETS26 carrying bglJ under control of the IPTG-inducible P<sub>UV5</sub> promoter (P<sub>UV5</sub> bglJ, pSC-ori), and with plasmid pKES302 carrying bglJ under control of the arabinose-inducible P<sub>BAD</sub> promoter (P<sub>BAD</sub> bglJ, p15A-ori), respectively (Figure 2). Expression of bglJ was either not induced or induced by gradually increasing inducer concentrations. The analysis of PleuO mVenus expression by flow-cytometry revealed that gradual induction of P<sub>BAD</sub> bglJ expression (plasmid pKES302) with 2 μM–50 μM arabinose resulted in full activation of PleuO mVenus even at the very low arabinose concentration of 2 μM (Figures 2B,C). Induction of P<sub>BAD</sub> bglJ with 100 μM arabinose or higher concentrations caused growth defects. However, induction of P<sub>UV5</sub> bglJ with IPTG concentration ranging from 10 μM to 100 μM led to a gradual increase in expression of PleuO mVenus and this increase was uniform in the population (Figures 2B,D). The presence of the P<sub>UV5</sub> bglJ or the P<sub>BAD</sub> bglJ plasmids per se did not cause a significant increase in expression of PleuO mVenus (Figures 2B–D). Likewise, IPTG or arabinose induction of transformants of the empty vectors pBAD30 and pKETS24, respectively, had no effect (Figure 2B). Taken together these data confirm activation of leuO transcription by BglJ-RcsB, they suggest that low cellular levels of BglJ are sufficient for activation, and that the P<sub>UV5</sub> bglJ plasmid is suitable for gradual induction of bglJ, while the P<sub>BAD</sub> bglJ plasmid is not suitable.

Second, autoregulation of PleuO mVenus by LeuO was analyzed using the leuO providing plasmids P<sub>UV5</sub> leuO (pKETS25, pSC-ori) and P<sub>Lac</sub> leuO (pKEHB27, pSC-ori) which carry leuO under control of the IPTG-inducible P<sub>UV5</sub> and P<sub>Lac</sub> promoters, respectively. In addition, a P<sub>BAD</sub> leuO plasmid (pKES303, p15A-ori) was used. The promoter P<sub>UV5</sub> (carrying the UV5 mutation in the—10 box and the lacI8 mutation in the CRP-binding site) is ~10 times weaker than the P<sub>Lac</sub> promoter (Lanzer and Bujard, 1988), while the tightly regulated P<sub>BAD</sub> leuO plasmid presumably directs similar levels of LeuO as the P<sub>Lac</sub> leuO plasmid considering that the P<sub>BAD</sub> promoter is approximately 3 fold weaker than P<sub>Lac</sub> and that the copy number of the P<sub>BAD</sub> plasmid (pKES303, p15A-ori) is ~3-fold higher than the copy number of the pSC-derived P<sub>Lac</sub> plasmid (Guzman et al., 1995). Flow cytometry revealed a slight increase in PleuO mVenus expression
at low levels of induction of plasmidic leuO (Figure 3). The data seem in agreement with weak positive autoregulation that was reported previously (Fang and Wu, 1998; Chen et al., 2003), but are statistically not significant (student’s t-test, P-value > 0.05).

### Antagonistic Regulation of the leuO Promoter by BglJ–RcsB and by LeuO

Next we addressed antagonistic regulation of P_{leuO} mVenUS by BglJ-RcsB and by LeuO. To this end, the P_{leuO} mVenUS

| Strain | Genotype | Reference/Construction |
|--------|----------|------------------------|
| BW27269 | Δ(araH-araF)572 in pKD3 | Khlebnikov et al., 2001 |
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| S974 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| S4197 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T17 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T1024 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T1037 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T1094 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T1095 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| T1241 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U1 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U9 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U11 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U15 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U16 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U20 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U47 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U49 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U51 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U53 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U55 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U57 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U59 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U61 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U62 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U63 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U65 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U67 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U69 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U76 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U92 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U93 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U94 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U95 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |
| U97 | Δlac1-lac2T946/T947 | Datsenko and Wanner, 2000 |

Alleles Δ(araC-araBAD) and Δ(lac-lacZYA) were constructed by homologous recombination, as described (Hamilton et al., 1989), using pREP plasmids pKETS27 and pKETS28, respectively. Transcriptional fusions of mVenUS to the leuO promoter (P_{leuO} mVenUS) and downstream of the leuO gene (P_{leuO} mVenUS::mVenUS) were constructed by Red-Gam mediated recombination, as described (Datsenko and Wanner, 2003). Red-Gam expression carried on plasmid pKD46 was induced with 10 mM arabinose. Plasmids pKES292 and pKES293 were used as templates for amplification of mVenUS-FRT-kan and mVenUS-FRT fragments. The oligonucleotides used for generating the PCR fragments are indicated by “PCR T547/T548.” Deletion of the lac genes in strain T1024 was constructed as described (Datsenko and Wanner, 2003) using oligonucleotides S911/S937 for generating the PCR fragment of pKD3 as template. Resistance cassettes flanked by FRT (pCP recombinase target) sites were deleted using temperature sensitive plasmid pCP20, as described (Datsenko and Wanner, 2003). The transfer of alleles by transduction using phage T4GT7 is indicated by “x T4GT7 (donor strain).” All alleles were confirmed by PCR, alleles P_{leuO} mVenUS in strain T1037, P_{leuO} mVenUS in strain T1094 and P_{leuO} mVenUS in strain T1095 were confirmed by sequencing. Further designations are cm = chloramphenicol resistance, kan = kanamycin resistance, FRT = FLP recombinase target site, repB = temperature sensitive replication.
reporter strain U69 was transformed with the two sets of **lewO** and **bglJ** expressing plasmids. First we analyzed antagonistic regulation of **lewO** transcription using the plasmid set, in which **bglJ** is expressed under control of the **P_BAD** promoter (**P_BAD bglJ**, pKES302) and **lewO** is expressed under control of the **P_tac** promoter (**P_tac lewO**, pKEHB27). Induction of **bglJ** expression with 2 μM–50 μM arabinose caused full activation of **P_lewO mVenus** (**Figure 4**), irrespective of the arabinose concentration, as shown above (**Figure 2**). Simultaneous induction of **lewO** by IPTG strongly reduced BglJ-RcsB-mediated activation of **P_lewO mVenus**, but even full induction of plasmidic **lewO** expression with 200 μM IPTG did not completely abrogate BglJ-RcsB-mediated activation (**Figure 4**). These results indicate that the level of BglJ provided by the **P_BAD bglJ** plasmid is above a threshold up to which LeuO can fully inhibit BglJ-RcsB activation. Since the **P_BAD bglJ** plasmid does not allow gradual activation, this plasmid set does not seem suitable for gradual induction of both regulators.

Second, we analyzed antagonistic regulation of **P_lewO mVenus** using the reverse set of plasmids that includes **P_UVS** **bglJ** (pKETS26) and **P_BAD lewO** (pKES303) (**Figure 5**). With this set of plasmids expression levels of BglJ are lower and gradual induction of **bglJ** by IPTG resulted in a gradual increase in activation of the **P_lewO mVenus** fusion by BglJ-RcsB (**Figure 5**, compare with data in **Figure 2**). Simultaneous gradual induction of plasmidic **P_BAD lewO** with arabinose and of **P_UVS** **bglJ** with IPTG led to a uniform decrease of expression of **P_lewO mVenus** in the whole population as compared to level of activation by BglJ-RcsB alone (**Figure 5**). Induction of **lewO** with an arabinose concentration of 50 μM was sufficient to completely abrogate activation by BglJ-RcsB (bottom right panel, **Figure 5B**). A plot of the median values of the flow cytometry results visualizes the gradual effects (**Figure 5A**).

Taken together, the data confirm that LeuO counteracts activation of the **lewO** promoter by BglJ-RcsB. Further, the data show that antagonistic regulation of the **lewO** promoters by LeuO and by BglJ-RcsB depends on the relative concentration of BglJ and LeuO, and the data indicate that BglJ-RcsB-mediated activation of **P_lewO mVenus** is inhibited by LeuO only if BglJ levels are rather low. The experimental data shown in **Figure 5** were used to describe **P_lewO activity** in dependence of the concentration of BglJ and LeuO by a thermodynamic model based on Michaelis-Menten kinetics. In this model it was assumed that BglJ and
LeuO regulate $P_{leuO}$ independently of each other. Fitting of the function to the experimental data was significant ($P$-value < 0.001) (function plotted in Figure 6).

Analysis of Feedback Regulation of $leuO$ via $yjjQ$–$bglJ$ and by LeuO

Next we addressed the relevance of the presumptive double-positive feedback regulation of $leuO$ and $bglJ$ by including the native gene of one of these two players, while providing the other one by the expression plasmid. In particular, we analyzed whether presence of the native $yjjQ$–$bglJ$ operon that is activated by LeuO results in enhanced $P_{leuO}$ $mVenus$ expression, when LeuO is provided in trans. Second, we tested whether the presence of native $leuO$ might affect activation of $P_{leuO}$ by BglJ-RcsB.

For determining whether activation of the H-NS repressed $yjjQ$–$bglJ$ operon by LeuO may yield sufficient BglJ protein for activation of $P_{leuO}$ we compared $P_{leuO}$ $mVenus$ expression in ($yjjQ$–$bglJ$)$^+$ strain U95 with expression in the isogenic $\Delta(yjjQ$–$bglJ)$ strain U69 (Figure 3). The data revealed no difference between wild-type $yjjQ$–$bglJ$ $^+$ strain U95 and $\Delta(yjjQ$–$bglJ)$ strain U69 suggesting that activation of $yjjQ$–$bglJ$ by LeuO is either too low to provide sufficient levels of BglJ for activation of $P_{leuO}$ $mVenus$ or that LeuO interferes with activation by BglJ-RcsB. Second, we analyzed whether the presence of native $leuO$ may affect activation of the $leuO$ promoter by BglJ-RcsB. For this analysis the $leuO$ gene was retained at its native locus and the fluorescence reporter gene $mVenus$ was inserted downstream of $leuO$ (as a transcriptional fusion) resulting in allele $P_{leuO}$ $leuO::mVenus$ in strain U97. Transformants of this strain with $bglJ$ carrying...
plasmid pKETS26 (PUV5 bgll, pSC-ori), were grown with IPTG concentrations ranging from 10 μM to 200 μM and PleuO leuO::mVenus expression was determined by flow cytometry. Comparison of the data obtained of PleuO leuO::mVenus with the data obtained for PleuO mVenus (ΔleuO) revealed no significant difference (Figures 2B,D). These data indicate that induction of the native leuO gene by BglJ does not provide sufficient LeuO to antagonize BglJ-RcsB-mediated activation of leuO.

Furthermore, we analyzed whether LeuO inhibits BglJ-RcsB-mediated activation of leuO transcription indirectly by downregulating BglJ-RcsB activity rather than by inhibiting activation of the leuO P2 promoter by BglJ-RcsB. To this end, activation of another BglJ-RcsB-activated promoter, the molR promoter (Salscheider et al., 2014), was analyzed in absence and presence of LeuO. BglJ was provided by PUV5 bgll plasmid pKETS26, and LeuO was provided by PBAD leuO plasmid pKES303. As control, transformants with the empty vectors were analyzed in parallel. Activity of the molR promoter was determined using a PmolR mVenus reporter fusion. The expression analyses demonstrate that LeuO neither does affect activation of PmolR by BglJ-RcsB nor does LeuO-mediated activation of the native yjjQ-bglJ operon present in strain U76 lead indirectly to activation of PmolR (Figure 7). We note that induction of the PBAD leuO with 50 μM arabinose resulted in slower growth to OD600 = 0.6 after 5 h as compared to OD600 = 1 which may explain the 1.5-fold reduce in basal expression of PmolR mVENUS in transformants of PBAD

**FIGURE 6 | Modeling of antagonistic regulation of leuO transcription by BglJ-RcsB and LeuO.** To describe the transcription rate directed by PleuO in dependence of the concentration of BglJ and LeuO, a thermodynamic model based on Michaelis-Menten kinetics was used. In this model it was assumed that BglJ and LeuO bind and regulate leuO transcription independently of each other. Median fluorescence values of flow cytometry data (Figure 5) were fitted to the function (bottom) describing leuO promoter activity in dependence of promoter occupancy by BglJ and LeuO. Fitting of the parameters to the experimental data by nonlinear regression according to (Fox and Weisberg, 2011) yielded P-values < 0.001. The data were plotted with Mathematica (Wolfram Research) using logarithmic scales for induction of plasmidic leuO with arabinose (ara) and of plasmidic bgll with IPTG.

**FIGURE 7 | Activation of the molR promoter (PmolR) by BglJ-RcsB is not affected by LeuO.** For determining activation of PmolR by BglJ-RcsB strain U76 was used that carries a replacement of the molR coding region by mVenus. Transformants of U76 with plasmids carrying PUV5 bgll (pKETS26) and PBAD leuO (pKES303) as well as control plasmids (pKETS24 and pBAD30) were grown in tryptone medium for 5 h. For induction (+) IPTG (100 μM) and arabinose (50 μM) were added. When harvested, the cultures had an OD600 of approximately 1, while induction of leuO resulted in slower growth to OD600 of approximately 0.6. Yellow fluorescence of three biological replicates was determined and expression levels are given in arbitrary units (a. u.).

**DISCUSSION**

In *E. coli* transcription of leuO is directed by at least two promoters, P1 and P2, which are repressed by H-NS and StpA. The P2 promoter requires activation by BglJ-RcsB, while LeuO inhibits activation of P2 by BglJ-RcsB. In addition, LeuO represses the leuO promoters in hns stpA mutants. Thus, leuO is antagonically regulated by BglJ-RcsB and LeuO. The characterization of leuO transcription using a leuO promoter-mVenus reporter fusion revealed that the antagonistic regulation of leuO transcription by LeuO and by BglJ-RcsB correlates to the relative cellular amounts of these regulators. The experimental data are in agreement with a theoretical model according to which LeuO and BglJ-RcsB regulate transcription independently. Further, data indicate that double-positive feedback regulation of leuO and bgll is of minor relevance, at least at the laboratory steady state conditions tested, since deletion of leuO and bgll, respectively, had no significant effect on the regulation of the leuO promoter reporter fusion by LeuO and BglJ-RcsB.

Activation of the leuO P2 promoter by the BglJ-RcsB heterodimer does not occur under standard lab conditions due to H-NS-mediated repression of the yjjQ-bglJ operon (Stratmann et al., 2008, 2012). To address the antagonistic regulation of leuO transcription by BglJ-RcsB and LeuO, we tested low to medium copy plasmids for gradual induction of bgll under control of the PUV5 and PBAD promoter, respectively. The data show that rather low amounts of BglJ are sufficient...
for full activation of the \textit{leuO} P2 promoter (Figures 2, 4, 5). Gradual activation of \textit{leuO} by BglJ-RcsB was observed only upon gradual induction of \textit{bglJ} provided by the low-copy \textit{P_{UV5}}, \textit{bglJ} plasmid, while \textit{bglJ} expression levels directed by the \textit{P_{BAD}} \textit{bglJ} plasmid turned out to be too high even when induced with just 2 \(\mu\)M arabinose, while induction with 100 \(\mu\)M arabinose caused growth defects. Likewise, we addressed autoregulation of \textit{leuO} transcription by gradual induction of \textit{leuO} carrying plasmids, which carry \textit{leuO} under control of the \textit{P_{UV5}}, \textit{P_{lac}}, and \textit{P_{BAD}} promoter, respectively. The data (Figure 3) indicate that positive autoregulation of \textit{leuO} that was reported previously (Fang and Wu, 1998; Chen et al., 2003; Stratmann et al., 2012) is negligible at steady state growth conditions.

Further experiments, with simultaneous gradual induction of \textit{bglJ} and \textit{leuO} revealed that the activity of the \textit{leuO} promoter correlates with the relative \textit{BglJ} and \textit{LeuO} concentrations (Figure 5). Interestingly, no switch-like response was observed. This might be plausible, because the distance of the \textit{LeuO} DNA-binding sites to the BglJ-RcsB DNA-binding site is more than 100 bp (Stratmann et al., 2012), and \textit{LeuO} and BglJ-RcsB presumably can bind simultaneously. Therefore, the \textit{LeuO}-mediated inhibition of activation by BglJ-RcsB is putatively not caused by competition for binding, but by another mechanism, as for example inhibition of RNA polymerase binding to \textit{leuO} promoter P2 or inhibition of transcription initiation at P2 by \textit{LeuO}. Such a mechanism of repression is supported by \textit{in vitro} DNA binding analyses, which revealed that \textit{LeuO} inhibits open complex formation by RNA polymerase at sites mapping next to \textit{leuO} promoter P1 and reduces open complex formation by RNA polymerase at sites close to P2 (Stratmann et al., 2012). A thermodynamic model based on Michaelis-Menten kinetics (Figure 6) supports the interpretation that antagonistic

### Table 2 | Plasmids

| Plasmid | Features | Reference, Construction |
|---------|----------|------------------------|
| pBAD30  | araC \textit{P_{BAD}} MCS ori-p15A amp | Guzman et al., 1995 |
| pK03    | FRT cm FRT oriRff amp | Datsenko and Wanner, 2000 |
| pK04    | FRT kan FRT oriRff amp | Datsenko and Wanner, 2000 |
| pK046   | \textit{P_{BAD}} \textit{F}_{-}\text{Red-recombinase amp} (rep\textsuperscript{16} ori-pSC) | Cherepanov and Wackernagel, 1995 |
| pCP20   | clg\textsubscript{7} \textit{F}_{-}\text{P}_{-}\text{flp}-recombinase cm amp (rep\textsuperscript{16} ori-pSC) | Dole et al., 2002 |
| pVS133  | mVenus (yfp variant) in pTrc99a | Stratmann et al., 2008 |
| pKESK10 | lacI \textit{P_{UV5}} bgG ori-pSC cm | V. Sourjik laboratory, Germany, and (Amann et al., 1988) |
| pKESK22 | lacI\textsuperscript{2} \textit{P}_{-}\textit{lac} MCS in ori-p15A kan | Dole et al., 2002 |
| pKETS1  | lacI\textsuperscript{2} \textit{P}_{-}\textit{lac} bgU in pKESK22 (ori-p15A kan) | Venkatesh et al., 2010 |
| pKETS5  | lacI\textsuperscript{2} \textit{P}_{-}\textit{lac} leuO in pKESK22 (ori-p15A kan) | Stratmann et al., 2012 |
| pKETS27 | chi-site polB\textsuperscript{8} \textit{D}_{-}\text{araDABC} yabol chi-site tetR (rep\textsuperscript{16} ori-pSC) | fragments flanking ara-C-BAD were amplified by PCR with T646/T647 and T648/T649, and cloned into a tetR rep\textsuperscript{15} ori-pSC vector, chi-sites were included to enhance homologs recombination |
| pKETS28 | chi-site cynX \textsuperscript{2} lacAYZI mhpR chi-site tetR (rep\textsuperscript{16} ori-pSC) | fragments flanking lacf-lacZYA were amplified by PCR with T650/T651 and T652/T653, and cloned into a tetR rep\textsuperscript{15} ori-pSC vector, chi-sites were included to enhance homologs recombination |
| pKES285 | pK03 with MCS (BarnHI SpeI EcoRI Sall) |
| pKES287 | pK04 with MCS (BarnHI SpeI EcoRI Sall) |
| pKES292 | mVenus (with enhanced RBS\textsuperscript{3}) in pK03 |
| pKES293 | mVenus (with enhanced RBS\textsuperscript{3}) in pK04 |
| pKES295 | mVenus fragment amplified by PCR with T146/T368 of pVS133, digested with BarnHI, EcoRI cloned into BarnHI, EcoRI-digested vector plasmid pKES285 |
| pKES296 | mVenus fragment cloned as pKES292, but into vector plasmid pKES287 |
| pKES297 | araC \textit{P_{BAD}} bgU in pBAD30 (ori-p15A amp) |
| pKES298 | araC \textit{P_{BAD}} leuO in pBAD30 (ori-p15A amp) |
| pKETS25 | lacI \textit{P_{UV5}} leuO ori-pSC cm |
| pKETS26 | lacI \textit{P_{UV5}} bgU ori-pSC cm |
| pKEHB27 | lacI\textsuperscript{2} \textit{P}_{-}\textit{lac} leuO ori-pSC cm |
| pKEHB28 | lacI\textsuperscript{2} \textit{P}_{-}\textit{lac} bgU ori-pSCori |
| pKEHB29 | araC \textit{P}_{-}\textit{ara} mVenus in pBAD30 (ori-p15A amp) |

\*The following abbreviations and genetic designations are used: FRT, Flp recombinase target site; MCS, multiple cloning site; genes coding for antibiotic resistance are designated as amp, ampicillin resistance, cm, chloramphenicol resistance, kan, kanamycin resistance. Origins of replications include ori-pSC (derived of low-copy plasmid pSC101), ori-p15A (derived of low to medium copy plasmid p15A), and P\textsubscript{R}-dependent oriRf.

\*\textsubscript{m} V. Sourjik was fused to the enhanced RBS (ribosomal binding site) that is derived of phage T7, gene 10 (Chins and Rangwala, 1989).
regulation by BglJ-RcsB and LeuO is mediated by independent mechanisms.

Previous data suggested that LeuO is controlled by interlocked double-positive and negative feedback control, because LeuO activates expression of the H-NS repressed yjjQ-bglJ operon (Stratmann et al., 2008). In the present study we analyzed whether activation of bglJ by LeuO may indirectly also turn on transcription of \( P_{\text{leuO}} \) mVenus (Figure 3) or \( P_{\text{molR}} \) mVenus as another BglJ-RcsB target (Figure 7), which was not the case indicating that activation of the native yjjQ-bglJ operon by LeuO does not yield sufficient BglJ. Likewise, expression analyses of an mVenus fusion downstream of the \( P_{\text{leuO}} \) coding region yielded the same results as the \( P_{\text{leuO}} \) mVenus reporter indicating that LeuO levels, when expressed from its native locus, remain too low to antagonize BglJ-RcsB. Taken together, double-positive feedback regulation of the \( P_{\text{leuO}} \) and yjjQ-bglJ loci is not relevant, at least at laboratory conditions, since the presence of the native \( P_{\text{leuO}} \) gene had no effect on BglJ-RcsB mediated activation of leuO that was triggered by plasmidic bglJ. Likewise the presence of native bglJ had no influence. Thus, the data suggest that repression of \( P_{\text{leuO}} \) by H-NS and StpA and of yjjQ-bglJ by H-NS dominates regulation of these loci and keeps them in the OFF state.

MATERIALS AND METHODS

Strains, Media, and Plasmids

Bacterial cultures of \( E. \ coli \) K-12 were grown in LB (10 g/l Bacto Tryptone, 5 g/l Bacto Yeast Extract, 5 g/l NaCl) or tryptone (10 g/l Bacto Tryptone, 5 g/l NaCl) media. Antibiotics were added with concentrations of 50 \( \mu \)g/ml ampicillin, 15 \( \mu \)g/ml chloramphenicol, and 25 \( \mu \)g/ml kanamycin. Strains, listed in Table 1, were constructed by transduction using phage T4GT7, by Red-Gam mediated gene deletion or gene replacement, and by homologous recombination, as described (Wilson et al., 1979; Hamilton et al., 1989; Datsenko and Wanner, 2000). Plasmids and their construction are listed in Table 2 and oligonucleotides are listed in Table 3. Standard molecular techniques, such as cloning, PCR, culture growth and induction of plasmid-provided genes, were performed according to standard protocols (Ausubel et al., 2005).

Flow Cytometry and Fluorescence Assay

For expression analyses by flow cytometry cultures of transformants were inoculated from fresh overnight cultures to an OD\(_{600}\) of 0.05 and grown for 5 h at 37°C in 10 ml tryptone medium containing antibiotics for selection of the plasmids. The
cultures were diluted to OD$_{600}$ of 0.1 and kept on ice prior to analysis by flow cytometry. Flow cytometry was performed on a BD FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 50,000 events were measured at a rate between 500 and 1000 events per second. The experiments were repeated at least twice and representative sets of data are shown.

Fluorescence directed by the $P_{molR}$ mVenus fusion was determined by fluorescence spectroscopy using a CLARIOstar plate reader (BMG LABTECH, Germany). Briefly, cultures were grown as for flow cytometry and the fluorescence of cells equivalent to 1.5 OD$_{600}$ was measured using yellow fluorescent proteins specific excitation (495–515 nm) and detection (540–620 nm) channels. The average obtained of three biological replicates was calculated and the standard deviation is less than 25%.

Theoretical Model

To describe the transcription rate directed by PleuO in dependence of the concentration of BglJ and LeuO, a thermodynamic model based on Michaelis-Menten kinetics was used. In this model it was assumed that BglJ and LeuO regulate PleuO independently of each other. The binding probabilities were defined as $B/(B_0+B)$ and $L/(L_0+L)$, where $B$ represents the concentration of BglJ in the cell, $B_0$ the BglJ concentration at which the promoter is half occupied, $L$ represents the concentration of LeuO and $L_0$ the LeuO concentration at which the promoter is half occupied. Since LeuO acts as a repressor and BglJ as an activator of the leuO promoter four different states with a different expression rate were described. The basal expression level directed by PleuO in absence of BglJ and LeuO was defined as $\eta_0$. In presence of LeuO and absence of BglJ, expression remains at a basal level defined as $\eta_0$. In presence of BglJ but absence of LeuO, the expression level is higher which is defined as $\eta_1$. When BglJ and LeuO are bound at the same time, the expression rate is defined as $\eta_0$, because high levels of LeuO inhibit activation by BglJ, when BglJ is provided by the low-copy $P_{UV5}$ bglJ plasmid. Taking these four different states into account the expression rate of leuO in dependence of LeuO and BglJ concentration was described as

$$\eta_{B_0,L_0}(B,L) = \frac{\eta_0 + \eta_0 \frac{L}{L_0} + \eta_1 \frac{B}{B_0} + \eta_0 \frac{L}{L_0} \frac{B}{B_0}}{(1 + \frac{B}{B_0})(1 + \frac{L}{L_0})}$$

The function was fitted to the median expression values determined by flow cytometry ($P_{UV5}$ bglJ, and $P_{RAD}$ leuO, Figure 5) using non-linear regression according to (Fox and Weisberg, 2011), which yielded a high fitting significance ($P$-value < 0.001).

AUTHOR CONTRIBUTIONS

HB contributed to the design of the work, acquired the data, and together with KS interpreted the data and drafted the work. KS conceived the project, contributed to the design of the work, and drafted the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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