Independent regulation of H-NS-mediated silencing of the bgl operon at two levels: upstream by BglJ and LeuO and downstream by DnaKJ

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Silencing of the Escherichia coli bgl operon by the histone-like nucleoid-structuring protein H-NS occurs at two levels. Binding of H-NS upstream of the promoter represses transcription initiation, whilst binding within the coding region is also proposed to repress transcription elongation. The latter, downstream level of repression is counteracted by the protease Lon and, thus, silencing of the bgl operon is more effective in lon mutants. Transposon-mutation screens for suppression of this lon phenotype on bgl were performed and insertion mutations disrupting rpoS and crl were obtained, as well as mutations mapping upstream of the open reading frames of bglJ, leuO and dnak. In rpoS and crl mutants, bgl promoter activity is known to be higher. Likewise, as shown here, bgl promoter activity is increased in the bglJ and leuO mutants, which express BglJ and LeuO constitutively. However, BglJ and LeuO have no impact on downstream repression. A dnaKJ mutant was isolated for the first time in the context of the bgl operon. The mutant expresses lower levels of DnaK than the wild-type. Interestingly, in this dnaKJ::miniTn10 mutant, downstream repression of bgl by H-NS is less effective, whilst upstream repression by H-NS remains unaffected. Together, the data show that the two levels of bgl silencing by H-NS are regulated independently.

INTRODUCTION

The abundant histone-like nucleoid-structuring protein H-NS is a key regulator in the adaptation of Escherichia coli to its environment (Ussery et al., 1994; Schroder & Wagner, 2002) and it affects the expression of ~5% of the genes in E. coli K-12 (Hommais et al., 2001). The current view of the mechanism of H-NS-mediated repression is that H-NS binds as a dimer to AT-rich and planar-bent DNA sequences and then forms an extended oligomeric complex on the DNA, which represses transcription initiation when located next to a promoter. Efficient repression by H-NS often involves binding of H-NS to two close binding (or nucleation) sites and probably the formation of a DNA loop, by ‘zipping’ the two sites together (Dame et al., 2002; Dorman, 2004). In several H-NS-repressed systems analysed so far, the H-NS-mediated repression is relieved by specific mechanisms that disturb the formation of the repressing complex, for example by binding of a specific transcription factor or by a temperature-dependent change in the DNA bend (Dorman, 2004; Prosseda et al., 2004). To date, little is known about whether and how the activity of the H-NS protein itself and its capacity to form repressing complexes are modulated. One possibility is that the formation of heterodimers and hetero-oligomers between H-NS and its homologue StpA affects its activity (Williams et al., 1996; Johansson & Uhlin, 1999; Johansson et al., 2001). Interaction of H-NS with Hha and Hfq has also been reported (Kajitani & Ishihama, 1991; Nieto et al., 2000). Furthermore, it was found that hscA, encoding an HSP66 protein and DnaK homologue, modulates the H-NS-mediated repression of pilA and the bgl operon in an hns mutant with reduced levels of H-NS protein (Kawula & Lelivelt, 1994). H-NS may also be modulated by a post-translational modification with short-chain poly(R)-3-hydroxybutyrate (Reusch et al., 2002).

The E. coli bgl operon is one example of a system whose repression by H-NS is exceptionally specific. The bgl operon encodes the gene products for the fermentation of aryl β-D-glucosides, including a β-glucoside-specific permease EII_{Bgl} (or BglF) and a phospho-β-D-glucosidase BglB (Fig. 1b). Also encoded within the operon is the positive regulator and anti-terminator protein BglG, whose activity is regulated by phosphorylation and which, under inducing conditions, allows transcription elongation beyond two terminators (t1 and t2) within the operon (Amster-Choder & Wright, 1993; Görke, 2003).

Silencing of the bgl operon by H-NS occurs under all laboratory growth conditions tested (Prasad & Schaeffer, 1974; Reynolds et al., 1981; Lopilato & Wright, 1990).
Silencing involves two sites (Dole et al., 2004b): H-NS represses transcription initiation at the cAMP receptor protein (CRP)-dependent promoter by binding to an AT-rich and presumably bent upstream silencer sequence (Schnetz, 1995; Singh et al., 1995; Schnetz & Wang, 1996; Mukerji & Mahadevan, 1997). In addition, H-NS binds to a site located 600–700 bp downstream of the transcription-initiation site within the coding region of the first gene, bglG. Binding to this downstream silencer is likely to hinder transcription elongation (Schnetz, 1995; Dole et al., 2004b). Silencing of bgl can be relieved by mutations that disrupt the upstream H-NS-binding site or that improve the CRP-binding site (Reynolds et al., 1986; Schnetz & Rak, 1988; Singh et al., 1995). Recently, we found that the silencing by H-NS mediated via the downstream site (‘downstream repression’) is more efficient in lon mutants (Dole et al., 2004a), suggesting that it is modulated by the ATP-dependent Lon protease (Gottesman, 1996). Therefore, a bgl allele (bgl−CRP+) that escapes silencing in the wild-type due to a mutation that improves the CRP binding of the bgl promoter remains repressed in a lon mutant (Dole et al., 2004a).

In this work, we screened for suppressors of the more effective downstream repression of bgl by H-NS in a lon mutant. This transposon-mutagenesis screen yielded mutations in loci encoding products known to affect bgl operon expression, including letO, yjQ–bglJ, rpoS and crl. These mutations increase the bgl promoter activity, as shown here for BglJ and LeuO and as known for RpoS and Crl, and thus compensate indirectly for the more efficient downstream repression in the lon mutant. In addition, we isolated a suppressor mutant that carries a transposon insertion upstream of the dnaK open reading frame, which expresses decreased levels of DnaK. This mutant, which is novel in the context of bgl, was found to specifically affect downstream repression by H-NS.

Fig. 1. Mutagenesis screen for suppressors of efficient bgl operon silencing in lon mutants. (a) Plasmid pKESK18 was used to perform a transposon-mutagenesis screen to isolate suppressors of the more effective repression of bgl in lon mutants. Plasmid pKESK18 is a replication temperature-sensitive (rep+), pSC101 derivative, which carries a mini-Tn10 transposon (miniTn10) with a chloramphenicol-resistance gene (cam), the Tn10 transposase gene under control of the lambda P_R promoter and the lambda cl857 gene (see Methods). Transposition was induced by a temperature shift from 28 to 42°C and single miniTn10-Cam' transposon mutants were selected at 42°C on chloramphenicol plates and screened for Bgl- and Lac-positive mutants. Strains S764 (b) and S2103 (c) are lon mutants that carry an activated bgl operon and a fusion of the bgl regulatory region to the lac operon. The bgl operon is activated by a point mutation that improves the CRP-binding site (allele bgl−CRP+). (b) In strain S764, the bgl–lacZ fusion carries the same activated bgl promoter allele, bgl−CRP+, as is present at the bgl operon. Expression of this bgl–lacZ fusion is independent of BglG-mediated anti-termination, due to a deletion of the terminator bgl-t1 located in the leader of the operon. In addition, the bglG allele bglGort is present, carrying a mutated translation start codon (Dole et al., 2004a). (c) In strain S2103, the expression of the bglGort–lacZ fusion, which carries the downstream-regulatory site of bgl, is directed by the constitutive lacUV5 promoter. Both of these lon mutants are Bgl- and Lac-negative, due to more efficient bgl operon silencing by H-NS, whilst the phenotype is Bgl- and Lac-positive in the corresponding wild-type strains (b), strain S594; (c), strain S2101 (Dole et al., 2004a) and this study.

METHODS

Strains, plasmids and media. The genotypes of the E. coli strains used in this study are listed in Table 1. All experiments were performed using isogenic strains derived from E. coli K-12 strain S541 carrying a deletion of the bgl operon and the lacZ operon (Dole et al., 2002). Mutations were transduced by using phage T4G77 (Wilson et al., 1979). Integration of bgl-lacZ reporter-gene fusions into the chromosomal phage lambda attachment site attB was performed as described previously (Diederich et al., 1992; Dole et al., 2002). Plasmids were constructed according to standard techniques (Sambrook & Russell, 2001). Plasmid pKESK18 (see Fig. 1a) is a temperature-sensitive derivative of pSC101 (Hashimoto-Gotoh et al., 1981) with a kanamycin-resistance gene. The plasmid carries the phage lambda cl-857 allele encoding the temperature-sensitive lambda repressor, the Tn10 transposase gene under control of the
Transposon mutagenesis. Transposon-mutagenesis screens were performed by using pKESK18 (Fig. 1a) carrying a miniTn10-Cam′ transposon. In this plasmid, replication is temperature-sensitive and, also, expression of the transposase is repressed at 28 °C and induced at 42 °C. Thus, at 28 °C, the plasmid replicates, whilst the transposase is not expressed. Upon a temperature shift, expression of the transposase gene and thus transposition are induced, whilst replication of the plasmid stops, allowing the selection of transposon mutants on chloramphenicol plates at 42 °C. With this system, transposition takes place in approximately 1–5% of the cells and all of the mutants that we characterized carried single miniTn10-Cam′ transposition insertions. Transformants of strains S594 and S2103 with plasmid pKESK18 were grown at 28 °C in LB medium containing kanamycin and chloramphenicol. To select for transposon mutants, dilutions were plated on MacConkey lactose (Difco)/chloramphenicol plates and incubated at 42 °C. Mutants with a change in the lactose phenotype were restreaked and their Bgl phenotype was tested on bromothymol blue/salicyc indicator plates (Dole et al., 2002). In addition, the mutants were re-evaluated on M9 minimal plates containing 0·4% glycerol, 0·1% tryptone, 0·1% yeast extract (Difco), 0·2% Casamino acids and 20 μg 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside ml−1. Of mutants with a double phenotype, a random primer (S360, 5′-CGGTATCAACAGGGACACCAGGATTTATTTATTCT-3′) specific primer S156 of the sequence 5′-GGCCACGCGTCG-3′ and a nested miniTn10-specific primer (S357, 5′-GGCCACGCGTCGTTAATAGCCGTTATG-3′, or S358, 5′-GGGATCCAGGGGGTGCCGTCGTTAATAGCCGTTATG-3′) were used. The amplification products of this first PCR were re-amplified in a second PCR using a primer (S361, 5′-GGGGTCCGAGGCGGGTGCCGACTGATACGAGGATC-3′) and a miniTn10-specific primer (S357, 5′-GGCCACGCGTCGTTAATAGCCGTTATG-3′, or S358, 5′-GGGATCCAGGGGGTGCCGTCGTTAATAGCCGTTATG-3′) that matches to the ‘random primer’ S360 and a nested miniTn10-specific primer (S359, 5′-GGGATCCAGGGGGTGCCGTCGTTAATAGCCGTTATG-3′). The PCR products were gel-purified and sequenced with primer S359.

β-Galactosidase assays. For enzyme assays, cells were grown in M9 medium containing 1% (w/v) glycerol, 0·66% (w/v) Casamino acids (Difco) and 1 μg vitamin B1 ml−1, or in NB medium (Difco), as indicated. Cultures were inoculated to an OD600 of 0·1–0·15 from fresh overnight cultures grown in the same medium and grown at 37 or 30 °C as indicated. IPTG was added to this fresh culture, where indicated. Cells were harvested at an OD600 of 0·5. The β-galactosidase assays were performed as described previously (Miller, 1992; Dole et al., 2002). The enzyme activities were determined at least three times from at least two independent transformants or integration derivatives. SD values were < 10%.

DnaK Western analysis. Cultures were grown in LB at 30 °C to an OD600 of 0·5. IPTG (1 mM) was added where indicated. Cultures were stopped on ice. Then, cells were harvested by centrifugation and resuspended in SDS-PAGE sample buffer (Laemmli, 1970) at an OD600 of 0·05 per 10 μl sample buffer. A 5 μl (0·025 OD600) aliquot was separated by SDS-PAGE (12% gel) using an SE600 16 cm gel-electrophoresis unit (GE Healthcare). The gel was blotted onto a 0·45 μm pore size PVDF transfer membrane by using a TE70 semi-dry blotting apparatus (GE Healthcare). The blot was handled using a standard Western blotting protocol (Coligan et al., 2005). Monoclonal mouse antisera directed against DnaK (Stressgen Bioreagents) were used as the primary antibody at 1 μg ml−1; Alexa Fluor 680 rabbit anti-mouse IgG(H + L) (Molecular Probes) was used as the secondary antibody at a concentration of 0·5 μg ml−1. Visualization and quantification were done by using the Odyssey Imaging System (Li-Cor Biosciences) according to the instructions of the manufacturer.

RESULTS

Mutagenesis screen for suppression of more effective downstream repression of bgl by H-NS in lon mutants

In order to identify factors that suppress the more effective H-NS-mediated downstream repression of bgl in a lon mutant, we performed a transposon-mutagenesis screen using a modified miniTn10 system (Kleckner et al., 1991) present on the low-copy and replication temperature-sensitive plasmid pKESK18 (Fig. 1a; see Methods for details). To avoid mutations that map in cis to the operon, a double-phenotype screening strategy was used (Fig. 1b and c) (Dole et al., 2004a). Both of the lon mutant strains S764 and S2103 (containing allele lon-107::miniTn10-Tc′; Table 1), which were screened for suppressor mutations, carry a bgl operon allele (bgl-CRP+) whose promoter is activated by a point mutation improving the CRP-binding site. This bgl allele confers a Bgl-positive phenotype in the wild-type, but a Bgl-negative phenotype in the lon mutants (Fig. 1b and c) (Dole et al., 2004a). As a second reporter, the lon mutants carry bgl–lacZ fusions. In one strain (Fig. 1b, S764) the bgl promoter, including upstream- and downstream-regulatory elements, was fused to the chromosomal lac operon genes (Dole et al., 2004a). In this bgl–lacZ fusion, the same bgl promoter allele, Pbgl-CRP+, is present as at the bgl locus. In addition, the terminator t1 within the leader is deleted (Δt1) and translation of bglG is excluded by mutation of the translation start codon to avoid the necessity for anti-termination by BglG and crosstalk with the bgl operon. The second lon mutant strain (S2103) carries a bgl–lacZ fusion, which is specific for downstream repression by H-NS (Dole et al., 2004b). In this fusion, the bgl downstream-regulatory fragment, encompassing the bglG allele bglGorf (which cannot be translated), is inserted between the constitutive lacUV5 promoter and the lacZ gene (Fig. 1c). Both bgl–lac fusions direct a Lac-positive phenotype in the wild-type strains (S594 and S2101), but a...
### Table 1. E. coli K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>Construction†/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB7049</td>
<td>PA1/lacO dnaKJ lac-fl</td>
<td>Tomoyasu et al. (1998)</td>
</tr>
<tr>
<td>S544</td>
<td>bgl-CRP+ ΔlacZ-Y217 ara thi</td>
<td>Dole et al. (2002)</td>
</tr>
<tr>
<td>S594</td>
<td>bgl-CRP+ (Bgl+) ΔlacOP::[Spc' bgl-CRP+ Pbg1 Δ1 bglGorf] ara thi</td>
<td>Dole et al. (2004a)</td>
</tr>
<tr>
<td>S764</td>
<td>S594 lon-107::miniTn10 (II, lon-430)</td>
<td>Dole et al. (2004a)</td>
</tr>
<tr>
<td>S1075</td>
<td>S544 lon-107::miniTn10</td>
<td></td>
</tr>
<tr>
<td>S1553</td>
<td>S541 Δlon-510 sulA3</td>
<td>x T4G7T(S764) Te'</td>
</tr>
<tr>
<td>S2101</td>
<td>S544 attB::[Spc' lacUV5 (+95)bglGorf lacZ]</td>
<td>x pKESD49</td>
</tr>
<tr>
<td>S2103</td>
<td>S1075 attB::[Spc' lacUV5 (+95)bglGorf lacZ]</td>
<td>x pKESD49</td>
</tr>
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<td>S1729</td>
<td>S764 leuO-Y1::miniTn10-Cam' and leuO' (orientation I: TSD AE000118, 797–805; leuO coding region AE000118, 823–1767); 16 independent isolates, other strains are S1730, S1735, S1741, S1747–S1750, S1752, S1755, S1756 and S1760–S1763</td>
<td>Insertion mutants</td>
</tr>
<tr>
<td>S1733</td>
<td>S764 yjjQ/bglJ-Y5::miniTn10-Cam' and yjjQ'/bglJ' (I: TSD AE000507, 6928–6936; bglJ coding region maps AE000507, 7109–7786); two independent isolates, second strain is S1742</td>
<td>Insertion mutants</td>
</tr>
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<td>Dole et al. (2004b)</td>
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<td>Dole et al. (2004b)</td>
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<td>S1553 attB::[Spc' Pbg1(+25) lacZ] (pKEKB30)</td>
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Table 1. cont.

*Phgl and PlacUV5 describe the bgl and lacUV5 promoters, respectively, and bglGorf is a bglG mutant in which the translation-initiation codon and, in addition, ATG codons 3 and 27 were mutated to GCG (Dole et al., 2004a). For bgl–lacZ constructs integrated into attB, the relevant structure and the parent plasmid from which these fusions were derived are given. The bgl operon allele bgl-CRP* carries a C to T exchange in the CRP-binding site at position −66 (relative to the transcription start) that causes activation of the bgl promoter and, thus, this allele confers a Bgl-positive phenotype. For miniTn10 mutations, orientation I indicates that the cam gene is in the same orientation as the mutated gene, whilst in orientation II, it is inserted in the opposite direction. The target site duplication (TSO) or the position of the insertion site is also given. In the latter case, the position of the nucleotide located directly 3’ to miniTn10 is given for insertions in orientation I, whilst the position of the nucleotide located directly 5’ to miniTn10 is given for insertions in orientation II. All leuO and bgI mutants retain a wild-type copy of the respective gene as tested by PCR using gene-specific primers. Cam’ is used for chloramphenicol resistance, Tc’ for tetracycline resistance and Spc’ for spectinomycin resistance.

†Transductions [e.g. x T4GT7(S2142)] and integrations of bgl–lacZ reporter constructs (x pKESD49) were performed as described previously (Wilson et al., 1979; Diederich et al., 1992; Dole et al., 2002). For transduction of PAI/lacI dnaKJ lacP, LB/chloramphenicol plates containing 1 mM IPTG were used as selective plates.

Lac-negative phenotype in the lon mutant strains S764 and S2103 (Fig. 1b and 1c).

Mutagenesis of the lon mutant strain S764 carrying the complete bgl regulatory region fused to the lac operon yielded in total 25 transposon mutants with a clear double-phenotypic change to Bgl + and Lac +. Of these mutants, which were characterized by sequencing of the miniTn10 insertion site, 16 mapped in leuO, four mapped in yjjQ–bglJ, three in crl, one in rpoS and one in dnaK (Fig. 2a). Interestingly, the second mutagenesis screen for suppressors using the lon mutant strain S2103, which carries the lacUV5– bglGorf–lacZ reporter specific for downstream repression (Fig. 1c), yielded three insertion mutations with a clear double-phenotypic change to Bgl + and Lac +, all of which mapped at the dnaKJ locus (Fig. 2b). This result suggests that DnaK (and DnaJ) may affect downstream repression by H-NS, whilst the mutations mapping in leuO, bglJ, rpoS and crl may affect the promoter. In agreement with this is the fact that RpoS (together with Crl) is known to repress the bgl promoter (Schnetz, 2002). Thus, the miniTn10 insertion mutations mapping in crl and rpoS are likely to compensate indirectly for the more efficient, H-NS-mediated downstream repression in the lon mutant by increasing the promoter activity. The rpoS and crl mutants were not further analysed. The analysis of the other mutants is presented below. Another interesting result is that no hns mutant was isolated, although the bgl operon and the bgl–lacZ reporter constructs direct a Bgl- and Lac-positive phenotype in a lon hns double mutant (Dole et al., 2004a). A similar change in the spectrum of mutations that affect bgl was detected before in an rpoS background (Moorthy & Mahadevan, 2002).

Constitutive expression of BglJ and LeuO enhances the activity of the bgl promoter

All leuO::miniTn10 insertion mutants map upstream of the leuO open reading frame. In 15 of 16 mutants, the insertion site maps 26 bp upstream of an ATG that is equivalent to the translational start characterized for the Salmonella typhimurium leuO gene (Chen et al., 2004). In one mutant (leuO-Y18, strain S1739), the insertion site maps 139 bp upstream of leuO. Four mutants map within the putative yjjQ–bglJ operon at the 3’ end of the yjjQ open reading frame and upstream of bglJ. In allele yjjQ–bglJ-Y5 (which was isolated twice), the insertion maps 181 bp upstream of the putative translation start codon of bglJ, in allele Y6 it maps 37 bp upstream and in allele Y33 it maps 176 bp upstream. In all of these mutants, the orientation of the miniTn10 transposon may allow constitutive expression of leuO and bglJ, respectively, by the promoter of the chloramphenicol-resistance gene present within miniTn10-Cam’. Furthermore, PCR analyses revealed that, in all of these mutants, a copy of the wild-type leuO gene and yjjQ–bglJ operon is retained (this is not the case in the rpoS, crl or dnaK insertion mutants) (see Discussion).

The insertion mutations isolated at the bglJ and leuO loci are similar to mutations isolated before. It has also been shown before that constitutive expression of BglJ and LeuO, respectively, relieves silencing of bgl (Giel et al., 1996; Ueguchi et al., 1998). Here, we analysed whether the leuO and yjjQ–bglJ mutations relieve silencing of the bgl promoter, downstream silencing by H-NS or both levels of silencing. To this end, we used chromosomally encoded bgl–lacZ reporter constructs specific for promoter and downstream repression, respectively (Figs 3 and 4). The expression directed by these bgl–lacZ reporter constructs was tested in the wild-type, as well as in yjjQ–bglJ and leuO mutants, grown to the exponential phase (OD600 = 0.5) in minimal M9 glycerol medium.

The expression level of β-galactosidase directed by the bgl promoter–lacZ reporter (which carries the promoter fused at position +25 to lacZ) increased from 74 units in the wild-type to 225 units in the bglJ-Y5 mutant and to 675 units in the bglJ-Y6 mutant, demonstrating that, presumably, constitutive expression of bglJ activates the bgl promoter. The effect observed with allele yjjQ–bglJ-Y6 is bigger, possibly because the insertion element maps closer to bglJ (37 bp upstream). Likewise, in the leuO-Y1 mutant, the bgl promoter activity increased to 215 units, which suggests that
LeuO also activates the bgl promoter (Fig. 3a). In addition, the bgl and leuO genes were cloned under the control of the IPTG-inducible tac promoter in the pBR-derived plasmids pKEAP4 and pKEAP10 (see Methods for details). These plasmids were used for transformation of the wild-type strain carrying the bgl promoter–lacZ fusion. Induction of bgl expression with IPTG resulted in an increase of β-galactosidase expression directed by the bgl promoter to 570 units, whilst induction of leuO expression caused an increase to 735 units (Fig. 3a), confirming that BglJ and LeuO are activators of the bgl promoter and that the miniTn10 insertions cause constitutive expression of these genes. A bgl promoter allele (APbgl) that lacks the upstream silencer sequence necessary for H-NS-mediated repression was also tested. The activity of this promoter (APbgl) is approximately fourfold higher than that of the wild-type promoter (Dole et al., 2004b) (also compare Fig. 3a and b, 74 versus 300 units). The bglJ-Y6 mutation had no effect on the ΔPbgl promoter (320 units compared with 300 units in the wild-type), whilst the leuO-Y1 mutations caused a rather moderate 1·4-fold increase (to 425 units). These data suggest that constitutive expression of BglJ and LeuO activates the bgl promoter by counteracting the H-NS-mediated repression of the promoter.

The bgl–lacZ downstream reporter construct (which carries the bglGorf downstream regulatory region inserted between the lacUV5 promoter and lacZ as described above) was also analysed in the wild-type and the bglJ-Y6 and leuO-Y1 mutants. The β-galactosidase level directed by the downstream reporter (Fig. 4a) did not vary significantly between the wild-type (62 units), the yjQ–bglJ-Y6 mutant (69 units) and the leuO-Y1 mutant (75 units), demonstrating that LeuO and BglJ have no impact on downstream repression by H-NS. Thus, suppression of the more effective H-NS-mediated downstream repression in the lon mutant by expression of bglJ and leuO is indirect and compensated for by an increased promoter activity.

**In the dnaKJ::miniTn10 mutant, downstream repression by H-NS is specifically suppressed**

In both screening strategies (Fig. 1), insertion mutations mapping at the dnaKJ locus were isolated (Fig. 2). The single dnaKJ::miniTn10 mutant isolated in the first screen is identical to two of the three mutants isolated in the second screen, which was performed in the strain carrying the bgl–lacZ downstream reporter. These mutants carry a miniTn10 insertion that disrupts the dnaK promoter and that maps 63 bp upstream of the dnaK ATG start codon. In the other insertion mutation, miniTn10 also maps upstream of the dnaK open reading frame (26 bp upstream of the ATG) but, in addition, the mutant carries a small deletion of 29 bp. The phenotype of this latter mutant is weaker and this mutant was not further characterized.

The possible role of DnaKJ in bgl promoter and bgl downstream repression by H-NS and the modulation of the latter by Lon were addressed by using the same approach as described above for the leuO and bglJ mutants, i.e. by using bgl promoter and bgl downstream lacZ reporter constructs. The dnaKJ::miniTn10 mutants were grown in NB medium, because the mutants were also analysed in combination with lon, and lon mutants grow poorly in minimal medium.
First, we analysed whether the dnaK::miniTn10 mutant (allele dnaKJ-M2) affects the bgl promoter activity by using the bgl promoter–lacZ reporter described above (Fig. 3c). In the wild-type, this reporter directs the expression of 140 units of β-galactosidase activity when cells are grown to OD_{600}=0.5 in minimal M9 glycerol medium (a, b) or in NB medium (c, d). (a) In the wild-type (strain S1213), the bgl promoter directs 74 units of β-galactosidase activity. In the yjO–bglJ mutant, the promoter directs 225 units (allele bglJ-Y5, strain S1787) and 675 units (allele bglJ-Y6, strain S1799) of β-galactosidase activity. In the leuO–Y1 mutant (strain S1776), 215 units of β-galactosidase activity were detected. Upon overexpression of BglJ (+BglJ) and LeuO (+LeuO), the promoter activity increased to 735 and 735 units, respectively. Overexpression of BglJ and LeuO was accomplished by using plasmids pKEAP4 (BglJ) and pKEAP10 (LeuO), which carry the bglJ and leuO genes, respectively, under control of the inducible tac promoter. For induction of bglJ and leuO expression, 1 mM IPTG was added to the exponentially growing cultures. (b) Activity of the bgl promoter lacking the upstream silencer varied only slightly, from 300 units in the wild-type (strain S2111) to 320 units in the bglJ-Y6 mutant (strain S1801) and to 425 units in the leuO–Y1 mutant (strain S1777). (c, d) The activity of the promoter constructs did not differ significantly in the dnaK::miniTn10–Cam’ mutant. (c) When cells were grown in NB medium, the wild-type promoter directed 140 units in the wild-type strain (S1213), 165 units in the dnaK mutant (S2674), 135 units in the lon mutant (S1556) and 145 units in the lon dnaK double mutant (S2710). (d) The promoter lacking the upstream silencer directed 470 units in the wild-type background (S1211), 525 units in the dnaK mutant (S2676), 520 in the lon mutant (S1554) and 450 units in the lon dnaK double mutant (S2708). (e) The promoter activity was not affected in a PA1/lacO1 dnaK strain grown without or with 1 mM IPTG in LB medium at 30 °C. The promoter directed 80 units in the wild-type (S1213), 335 units in the dnaK::miniTn10 mutant (S2674), 90 units in the PA1/lacO1 dnaK strain (S2904) when grown without IPTG (−) and 75 units when grown with 1 mM IPTG (+).
The wild-type (strain S1195), 62 units of 

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bgl chloramphenicol-resistance gene located within the mini-

cassette (Tomoyasu et al., 1998). Thus, dnaKJ expression requires IPTG and DnaKJ levels are very low when cells are grown without IPTG. Downstream repression of bgl by H-NS was tested in the PA1/lacO dnaKJ strain using the downstream reporter (lacUV5–bglGorf–lacZ) as described above. As these cultures had to be grown in LB at 30 °C when grown without IPTG, the β-galactosidase assay for the wild-type and the dnaKJ::miniTn10 mutant was repeated at these conditions (Fig. 4c). In the wild-type, 156 units of β-galactosidase activity were detected and the activity increased to 335 units in the dnaKJ::miniTn10 mutant. In the PA1/lacO dnaKJ strain, 76 units of β-galactosidase activity were detected when IPTG for induction of the PA1/lacO1 promoter was present, i.e. high DnaKJ levels support downstream repression. In contrast, the expression level increased to 415 units when IPTG for induction of dnaKJ was omitted. This effect was specific for downstream repression. The activity of the Pbgl–lacZ promoter construct did not vary in the PA1/lacO dnaKJ strain when grown with or without IPTG (Fig. 3e). These data indicate that expression of dnaKJ is required for downstream repression by H-NS. To further strengthen this argument, the DnaK levels were analysed in a Western blot by using DnaK-specific antibodies (Fig. 5). The quantitative Western analysis demonstrated that DnaK levels are reduced by approximately twofold in the dnaKJ::miniTn10 mutant compared with the wild-type. In the PA1/lacO dnaKJ strain, DnaK levels were very low (fivefold lower than the wild-type) when cells were grown without IPTG, whilst the DnaK protein level was much higher upon induction with IPTG than in the wild-type strain (17-fold increase) (Fig. 5). Thus, the cellular DnaK protein levels correlate well with the differences in downstream repression of bgl by H-NS (Fig. 4c), i.e. downstream repression is more effective when DnaKJ is present.

Fig. 4. In dnaK mutants, downstream repression of bgl by H-NS is specifically reduced, whilst BglU and LeuO have no impact. The role of LeuO and BglU (a), as well as DnaK and Lon, on downstream repression of bgl was tested by using a bgl–lacZ downstream reporter construct that carries the bglG coding region, bglGorf, inserted between the lacUV5 promoter and the lacZ gene integrated into the chromosomal attB site. β-Galactosidase activities shown were determined for cultures grown to OD600 = 0.5 in minimal M9 glycerol medium at 37 °C (a), NB medium at 37 °C (b) or LB medium at 30 °C (c). (a) In the wild-type (strain S1195), 62 units of β-galactosidase activity were measured, which was not changed significantly in the bglU-Y6 mutant (strain S1797) and the leuO-Y1 mutant (strain S1773). (b) Wild-type cells (strain S1195) grown in NB medium expressed 95 units of β-galactosidase activity. In the dnaKJ mutant (strain S2712), the expression level increased by approximately twofold to 220 units. In the lon mutant (strain S1564), 40 units were detected, whilst in the dnaKJ lon double mutant (strain S2670), the expression level increased by approximately fourfold to 155 units. (c) When cells were grown at 30 °C in LB medium, 155 units were detected in the wild-type (S1195) and 335 units in the dnaKJ::miniTn10 mutant (S2712). In the PA1/lacO dnaKJ strain (S2888), the expression level was increased to 415 units when IPTG was omitted, and decreased to 76 units when dnaKJ expression was induced with IPTG.

chloramphenicol-resistance gene located within the mini-Tn10 transposon. To analyse whether an increase or a decrease in the cellular DnaK level affects downstream repression by H-NS, an additional dnaKJ mutant was used, in which the dnaKJ promoter is replaced by a lacI PA1/lacO

Fig. 5. The DnaK level is lower in the dnaKJ::miniTn10-Cam' mutant. A quantitative Western blot analysis of DnaK protein levels expressed in the wild-type (S1195), the dnaKJ::miniTn10 mutant (allele dnaKJ-M2; strain S2712) and the PA1/lacO dnaKJ strain (S2888) grown without IPTG (–IPTG) and with IPTG (+IPTG) at 30 °C in LB medium to OD500 = 0.5 was performed. The Western blot and the result of the quantitative analysis given as peak intensity, as well as relative protein levels compared with the wild-type (wt; set as 100 arbitrary units), are shown.
DISCUSSION

Silencing of bgl by H-NS occurs through binding of H-NS to two sites, located upstream and downstream of the promoter. The latter level of repression by H-NS also affects transcription elongation (Dole et al., 2004b). Here, we have shown that this downstream repression is modulated by DnaK, an HSP70 family chaperone (Dougan et al., 2002), and possibly DnaJ, the DnaK co-chaperone. Lon protease also affects the downstream repression (Dole et al., 2004a). DnaKJ (as Lon) has no impact on silencing of the promoter. Furthermore, we have shown that constitutive expression of transcription factors LeuO and BglJ relieves silencing of the bgl operon by countering repression of the promoter by H-NS through the upstream site, whilst BglJ and LeuO do not affect downstream repression. These data demonstrate that upstream and downstream repression of bgl by H-NS is regulated independently by LeuO and BglJ, as well as DnaKJ. Although upstream and downstream repression can be separated, silencing of bgl is significantly more effective when both silencers are present, indicating cooperativity between the two sites, as shown previously (Dole et al., 2004b).

Mutations characterized so far that relieve silencing (or repression) of the bgl operon by H-NS affect only one level of repression, the repression of the bgl promoter. Here, we have shown that the two transcription factors LeuO and BglJ also counteract the H-NS-mediated repression of the promoter. As, under laboratory growth conditions, the bgl operon is always repressed, the expression level of leuO and bglJ may be too low for activation of bgl. The regulation of bglJ has not been studied to date. Preliminary data from our lab indicate that it is repressed by H-NS. The expression of leuO is likewise repressed by H-NS, and not detectable during exponential growth (Klauck et al., 1997; Chen et al., 2004). However, leuO expression is induced in a ppGpp-dependent manner by starvation for branched-chain amino acids, and LeuO is necessary for resuming growth after such a starvation (Fang et al., 2000; Majumder et al., 2001). It is not known whether such a transient induction of leuO is sufficient to relieve silencing of bgl. The miniTn10 insertion mutants isolated in this work are similar to Tn10 mutants isolated before in the context of bgl (Giel et al., 1996; Ueguchi et al., 1998) and apparently direct constitutive expression of bglJ and leuO, respectively. Constitutive expression of leuO and bglJ results in activation of the bgl promoter. This explains the dominance of the bglJ and leuO mutants over the wild-type copy of the bglJ and leuO gene, respectively, also present in the strains, as they are repressed by H-NS. Similar leuO::Tn10 transposon mutants to those isolated here were isolated in different contexts and shown to enhance expression of cadA, encoding an acid-inducible lysine decarboxylase, as well as to reduce RpoS levels (Shi & Bennett, 1994; Klauck et al., 1997). The regulation of RpoS levels by LeuO is indirect and mediated through the small regulatory RNA DsrA, which, at low temperature, enhances translation of the rpoS mRNA and represses translation of the hns mRNA (Klauck et al., 1997; Lease & Belfort, 2000; Repoila & Gottesman, 2003). Thus, LeuO belongs to a regulatory network that involves RpoS, H-NS, Hfq and DsrA (Klauck et al., 1997; Repoila & Gottesman, 2003), and the bgl operon is clearly a system that is controlled by this network.

The second, downstream level of bgl operon repression by H-NS is not affected by LeuO, BglJ or RpoS/Crl. However, this level of repression is modulated by Lon (Dole et al., 2004a). In Lon-deficient mutants, the repression is more effective. This effect is independent of the H-NS homologue StpA (Dole et al., 2004a), a Lon target protein (Johansson & Uhlin, 1999; Johansson et al., 2001). In addition, as shown here, downstream repression is modulated by the heat shock-induced DnaKJ chaperone system. The dnaKJ mutants carry a miniTn10 insertion upstream of the dnaK open reading frame. Western analysis revealed that this mutant expresses lower levels of DnaK than the wild-type. Likewise, downstream repression is inefficient in a PA1/lacO dnaKJ strain when expression of dnaKJ is not induced with IPTG. These data imply that DnaKJ is required for efficient downstream repression, which indicates a modulation of H-NS activity by chaperones. Interestingly, the mutation of an HSP66 protein and DnaK homologue encoded by hscA was also found to have some effects on silencing by H-NS (Kawula & Leilvelt, 1994). Presently, it is not known whether Lon and the DnaKJ chaperone system affect downstream repression of bgl by H-NS directly or indirectly. One speculative possibility is that Lon and DnaKJ modulate the H-NS repressing complex or change the level of hns expression. However, Lon and DnaKJ specifically modulate downstream repression by H-NS, but not bgl promoter repression. In addition, we found no role for DnaKJ in silencing of proU by H-NS (data not shown). Therefore, Lon and DnaKJ may affect an additional process that is specific for downstream repression, e.g. modulation of the elongating RNA polymerase complex and its translocation along the DNA.

Results presented here are in agreement with and extend the finding that repression of the bgl operon is linked tightly to the regulatory network controlling the stress response in E. coli. Strictly controlled repression of bgl supports the idea that the utilization ofaryl β-D-glucosides is deleterious under certain conditions (Reynolds et al., 1981), whilst conservation of silencing of the bgl operon that is present in a majority of E. coli strains (G. Neelakanta and K. Schnetz, unpublished data) suggests an important role for bgl under specific conditions in nature.

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