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The Protease Lon and the RNA-Binding Protein Hfq Reduce Silencing of the *Escherichia coli bgl* Operon by H-NS

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The histone-like nucleoid structuring protein H-NS represses the *Escherichia coli bgl* operon at two levels. H-NS binds upstream of the promoter, represses transcription initiation, and binds downstream within the coding region of the first gene, where it induces polarity of transcription elongation. In *hns* mutants, silencing of the *bgl* operon is completely relieved. Various screens for mutants in which silencing of *bgl* is reduced have yielded mutations in *hns* and in genes encoding the transcription factors LeuO and BglJ. In order to identify additional factors that regulate *bgl*, we performed a transposon mutagenesis screen for mutants in which silencing of the operon is strengthened. This screen yielded mutants with mutations in *cyaA*, *hfq*, *lon*, and *pgi*, encoding adenylate cyclase, RNA-binding protein Hfq, protease Lon, and phosphoglucose isomerase, respectively. In *cyaA* mutants, the cyclic AMP receptor protein-dependent promoter is presumably inactive. The specific effect of the *pgi* mutants on *bgl* is low. Interestingly, in the *hfq* and *lon* mutants, the downstream silencing of *bgl* by H-NS (i.e., the induction of polarity) is more efficient, while the silencing of the promoter by H-NS is unaffected. Furthermore, in an *hns* mutant, Hfq has no significant effect and the effect of Lon is reduced. These data provide evidence that the specific repression by H-NS can (directly or indirectly) be modulated and controlled by other pleiotropic regulators.

The abundant histone-like nucleoid structuring protein H-NS is a key regulator in the adaptation of *Escherichia coli* to its environment. H-NS directly or indirectly affects the expression of ~5% of the genes in *E. coli* K-12, including pathogen determinants, several motility and adhesion systems, and proteins of the osmotic and acid stress responses, many of which are controlled by environmental signals (17). H-NS binds nonspecifically to DNA with a preference for bent and AT-rich DNA sequences, represses the transcription of most loci, and is present at very high cellular levels (~20,000 to 60,000 molecules per cell) (47). To date, little is known about how H-NS activity is modulated. Expression of the *hns* gene is growth rate regulated and autorepressed (6, 11). At the translational level, *hns* is repressed upon overproduction of the 87-nucleotide regulatory RNA DsrA. This repression depends on Hfq (25, 26, 49). In addition, H-NS activity may be modulated by its homologue, StpA, with which it can form heterodimers (18, 19, 53).

Among the H-NS-controlled loci, the repression of the *E. coli bgl* operon and the *proU* operon by H-NS is exceptionally specific. The *proU* operon encoding an uptake system for the osmoprotectants glycine and betaine is induced by osmotic stress. The *bgl* operon encodes the gene products for the fermentation of aryl-β,D-glucoside. Its gene products are the positive regulator and antiterminator BglG, the β-glucoside-specific permease EII^{Bgl} (or BglF), and the phospho-β,D-glucosidase BglB (see Fig. 2). The *bgl* operon is silent under all laboratory growth conditions (35, 39). Both the *proU* and *bgl* operons are repressed ~50- to 100-fold by H-NS, and in both systems, regulatory elements located upstream and downstream of the promoter are required for the H-NS-mediated repression (9, 20, 21, 43, 48). We have shown that repression of the *bgl* operon by H-NS occurs at two levels (Fig. 1). H-NS represses transcription initiation at the cyclic AMP (cAMP) receptor protein (CRP)-dependent promoter by binding to an AT-rich and presumably bent upstream silencer sequence (32, 43, 46, 48). In addition, H-NS binds to a downstream silencer located within the coding region of the first gene, *bglG*, ~600 to 700 bp downstream of the transcription initiation site, where it induces a Rho-dependent polarity of transcription (9).

In addition to H-NS, which is essential for *bgl* operon silencing, the operon is affected by other pleiotropic regulators. Constitutive expression of *leuO* and *bglJ* relieves silencing of *bgl* (14, 51). The *leuO* gene encodes a pleiotropic transcription factor that controls, e.g., *dsrA* (37), while *bglJ* encodes a putative transcription factor of the LuxR family of unknown function. Furthermore, RpoS contributes to repression of the operon (8, 33, 44), while the H-NS homologue StpA and the RNA chaperone Hfq are involved in regulation of *bgl* in an *hns* mutant that expresses a truncated H-NS protein (12, 50). Silencing of the *bgl* operon can also be relieved by in *cis* mutations that disrupt the upstream silencer or make the CRP-binding site more similar to the consensus CRP-binding site (40, 45, 48).

In this work, we report that the RNA chaperone Hfq and the protease Lon reduce silencing of the *bgl* operon. While in all screens to date, mutants were isolated in which silencing of *bgl* is relieved, these factors were characterized in a reverse screen for mutants in which silencing of *bgl* is strengthened. Further characterization of the *hfq* and *lon* mutants revealed that the H-NS-mediated silencing of *bgl* via the downstream silencer was more efficient, while silencing of the *bgl* promoter was

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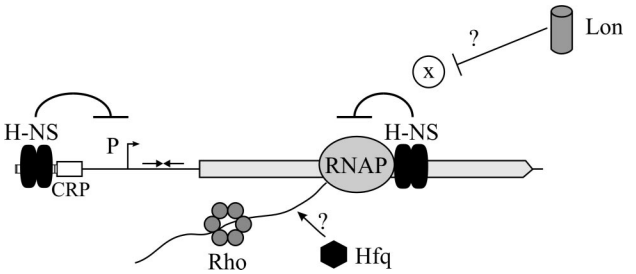

FIG. 1. Model of the H-NS-mediated repression of the *bgl* operon at two levels. H-NS binds upstream of the promoter and represses transcription initiation. In addition, H-NS binds within the coding region of the first gene, approximately 600 to 700 bp downstream of the transcription initiation site, where it induces a Rho-dependent polarity (9). Hfq and Lon reduce the H-NS-induced polarity (this work).

unchanged. Furthermore, in an *hns* null mutant background, mutations in *hfq* and *lon* had little effect on *bgl*, which suggests that Hfq and Lon directly or indirectly control H-NS to induce polarity at this specific locus.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of the *E. coli* strains used in this study are given in Table 1. All experiments were performed by using isogenic strains derived from *E. coli* K-12 CSH50 [*ara* Δ(*gpt-lac*) *ara thi*] (27). Mutations were transduced by using phage T4*GT7* (54). Integration of *bgl-lacZ* reporter gene fusions into the chromosomal phage λ attachment site *attB* was performed as described previously (7, 8). Strains S581 and S594 carry replacements of the *lac* promoter by the *bgl* promoter, including the upstream and downstream regulatory elements. They were constructed by site-specific recombination using plasmids pKES50 and pKES51, respectively, according to Dabert and Smith (4). These plasmids carry a fragment encompassing the *lacI* gene, followed by an Ω spectinomycin resistance cassette (36), the *bgl* regulatory region, and the *lacZ* gene. Within the *bgl* fragment terminator, *t1* was deleted (from positions +55 to +120 relative to the transcription start) and translation of *bglG* was excluded by mutation of the translation start codon and codon 3 of *bglG* (ATG to GCG). The *lacI-bgl-lacZ* cassette is flanked by chi sites (5′GCTGGTGG) in the proper orientation to enhance site-specific recombination (4). Recombinants were selected on spectinomycin plates, and correct replacement was tested by PCR.

Plasmids were constructed according to standard techniques (42). Site-specific mutations and fusion of *bgl* and *lac* sequences were introduced by PCR. All regions of plasmids that were derived from PCR fragments were sequenced. The relevant structures of the plasmids are schematically shown in the figures. Details of constructions and compiled sequences of the plasmids are available upon request. Media and plates were used as described previously (8). Where necessary, antibiotics were added to final concentrations of 25-μg/ml kanamycin, 50-μg/ml ampicillin, 15-μg/ml chloramphenicol, and 50-μg/ml spectinomycin.

Transposon mutagenesis. The transposon mutagenesis screen was performed using λ phage NK1323, which carries a mini-Tn*10* transposon with a tetracycline resistance marker (23). Due to several mutations, the λ phage does not replicate in wild-type *E. coli* and it cannot integrate into the genome of *E. coli* as a prophage (23). In addition, the transposase gene is encoded outside of the mini-Tn*10* on the λ phage and thus was lost along with the other λ sequences. Therefore, after infection, single transposition events of the mini-Tn*10* transposon into the chromosome can be selected. Transduction experiments and direct sequencing of the chromosomal DNA confirmed that all mutants isolated were due to single transposition events (data not shown).

In the mutagenesis screen, strains S581 and S594, respectively, were infected with λ NK1323 and plated onto MacConkey lactose-tetracycline plates. Mutants with a change in the lactose phenotype were restreaked, and their Bgl phenotype was tested on bromthymol blue-salicin indicator plates (8). For mutants with a double phenotype change, the insertion position of the mini-Tn*10* transposon on the chromosome was determined by sequencing of chromosomal DNA using the mini-Tn*10*-specific primer S156 with the sequence 5′-GATGATAAAAGGCAC CTTTGGTCA. To confirm the integration site, the mutated gene fragment carrying the mini-Tn*10* insertion of some mutants was amplified with gene-specific primers and the integration site was sequenced using the Tn*10*-specific primer S156.

Determination of β-galactosidase activities. For enzyme assays, cells were grown in M9 medium containing 1% (wt/vol) glycerol, 0.66% (wt/vol) Casamino Acids (Difco), and 1-μg/ml vitamin B_1 or in NB medium (Difco) as indicated. Cultures were inoculated from fresh overnight cultures grown in the same medium. Cells were harvested after approximately 3 h of growth at 37°C at an optical density at 600 nm (OD_{600}) of 0.5. The β-galactosidase assays were performed as described previously (27). The enzyme activities were determined at least three times from at least two independent transformants or integration derivatives. Standard deviations were <10%.

RESULTS

A mutagenesis screen for identification of factors involved in *bgl* operon regulation. In order to identify factors that regulate the *bgl* operon in addition to H-NS, we performed a transposon mutagenesis screen using a phage λ mini-Tn*10* system (23). In one approach, mutants were isolated that cause derepression of the silent wild-type *bgl* operon. In a second, reverse screen, we screened for mutants in which expression of an active *bgl* operon is down-regulated. To avoid mutations that map in *cis* to the operon, a double-phenotype screening strategy was established (Fig. 2). We constructed strains that carry the *bgl* operon and a fusion of the *bgl* regulatory region to the *lac* operon at its natural chromosomal locus. In this fusion, the *lac* promoter was replaced by the *bgl* promoter, including the upstream and downstream negative regulatory elements. In the *bgl-lac* fusion, the terminator gene *t1* was deleted (from nucleotides +55 to +120 relative to the transcription start, Δ*t1*) and translation of *bglG* was excluded by mutation of the translation start codon and an additional ATG (codon 3) to GCG (*bglGorf*) to render expression independent of BglG-mediated antitermination and to avoid cross talk between expression of the *bgl-lac* fusion and the *bgl* operon.

In the screen for mutations that activate the operon, strain S581 was used, in which both the *bgl* operon and the *bgl-lac* operon fusion carry the wild-type *bgl* promoter. This screen yielded six mutations, which were Bgl and Lac positive and which all mapped in *hns* (data not shown). The reverse screen for mutations reducing expression of *bgl* was performed with strain S594, in which expression of both the *bgl* operon and the *bgl-lac* fusion is activated by the identical point mutation improving the *bgl* CRP-binding site. This mutation is a C-to-T exchange at position −66 relative to the transcription start, which generates the conserved TGTGA motif in the promoter distal half-site of the CRP-binding site. This strain is therefore Bgl and Lac positive. Sixteen transposon mutants of this strain with a double-phenotypic change to Bgl^- Lac^- (from a total of more than 50,000 mutants) were isolated and characterized by sequencing of the mini-Tn*10* insertion site. One of the mutations carried by these mutants mapped in *cyaA*, five mapped in *pgi*, seven mapped in *lon*, and three mapped in the *miaA-hfq* locus (Fig. 2).

Gene *cyaA* codes for adenylate cyclase. Due to a lack of cAMP, the CRP-dependent *bgl* promoter is likely to be inactive and thus the *bgl* operon and the *bgl-lac* fusion are not expressed in the *cyaA1405*::mini-Tn*10* mutant. This mutation was not further analyzed. Lon is a highly conserved ATP-dependent protease that degrades abnormally folded proteins during heat shock and starvation. It also degrades some proteins specifically (15), including protein StpA, which is 67% similar to

TABLE 1. Characteristics of the *E. coli* K-12 strains used in this study

Strain	Genotype[a]	Construction[b] or reference
AM111	MC4100 *hfq1*::Ω(Km^r)	31, 50
AM112	MC4100 *hfq2*::Ω(Km^r)	31, 50
CSH50	*bgl*^{o} Δ*(lac-pro) ara thi*	27
CY307	*zcb-222*::Tn*10 pyrD34 relA1 spoT1 metB1-Hfr (argF→lac)*	CGSC#6428
JT4000	Pro$^+$ Tc^s Δ*lon-510*	Susan Gottesman
KL788	λ$^-$ *Thr-1* Δ*(gpt-lac)5 tsx-35 sulA3 el4*$^-$ *Rac-0? rfbD1? mgl-51 recA441* (Ts) *relA1? rpsL31 (strR) kdgK51? mtl-1 spoT1? thi-1? lexA71*::Tn*5 creC510?*	CGSC#6218
M182 *stpA*::Tc^r		55
PD32	MC4100 *hns-206*::Ap^r Str^r	6
RH90	RH90 = MC4100 *rpoS359*::Tn*10*	24
SG1039	Δ*lac proC* mutant *zaj-403*::*tet*	Susan Gottesman
W3110	λ$^-$ F$^-$ IN(*rrnD-rrnE*)	CGSC#4474 (2)
S486	CSH50 *bgl*^{o} (Bgl$^-$) (*gpt-lac*)$^+$	8
S539	CSH50 Δ*bgl*-AC11 (*gpt-lac*)$^+$	8
S541	CSH50 Δ*bgl*-AC11 (*gpt-lac*)$^+$ Δ*lacZ*-Y217	8
S544	CSH50 *bgl*-CRP$^+$ (Bgl$^+$) (*gpt-lac*)$^+$ Δ*lacZ*-Y217	8
S572	S544 (*gpt-lac*)$^+$	×$^-$ T4*GT7*(W3110), Lac$^+$ Pro$^+$
S581	S486 *bgl*^{o} Δ*lacOP*::(Spc^r P*bgl* Δ(+55 to 120) *bglGorf*)	× pKES50
S594	S572 *bgl*-CRP$^+$ Δ*lacOP*::(Spc^r *bgl*-CRP$^+$ P*bgl*Δ(+55 to 120) *bglGorf*)	× pKES51
S690	S539 *hns-206*::Ap^r	× T4*GT7*(PD32), Ap^r
S706	S594 *hns-206*::Ap^r	× T4*GT7*(PD32), Ap^r
S749	S594 *lon-110*::mTn*10* (orientation I, position *lon*:1537)	This work
S750	S594 *miaA21*::mTn*10* (I, *miaA*:50)	This work
S751	S594 *pgi-30*::mTn*10* (II, *pgi*:1325)	This work
S752	S594 *pgi-28*::mTn*10* (II, *pgi*:1270)	This work
S753	S594 *pgi-29*::mTn*10* (identical to *pgi-28*::mTn*10*)	This work
S754	S594 *lon-113*::mTn*10* (II, *lon*:1976)	This work
S755	S594 *pgi-27*::mTn*10* (II, *pgi*:509)	This work
S756	S594 *cyaA1405*::mTn*10* (I, *cyaA*:2296)	This work
S757	S594 *hfq-21*::mTn*10* (I, *hfq*:96)	This work
S759	S594 *lon-108*::mTn*10* (I, *lon*:1360)	This work
S760	S594 *lon-112*::mTn*10* (I, *lon*:1960)	This work
S762	S594 *lon-111*::mTn*10* (same as S749)	This work
S763	S594 *miaA22*::mTn*10* (II, *miaA*:733)	This work
S764	S594 *lon-107*::mTn*10* (II, *lon*:430)	This work
S765	S594 *pgi-26*::mTn*10* (I, *pgi*:500)	This work
S766	S594 *lon-109*::mTn*10* (as *lon-108*::mTn*10*)	This work
S778	S539 *hfq-21*::mTn*10*	× T4*GT7* (S757), Tc^r
S794	S539 *lon-107*::mTn*10*	× T4*GT7*(S764), Tc^r
S1048	S594 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1097	S541 *attB*::[Spc^r *lacUV5 t1-L bglG lacZ*]	8
S1105	S539 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1132	S1079 *rpoS359*::Tn*10*	× T4*GT7*(RH90), Tc^r
S1182	S541 *zcb-222*::Tn*10 pyrD34*	× T4*GT7*(S1179), Tc^r
S1185	S1182 *sulA3 pyrD*$^+$ (a *lon-107*::mTn*10* derivative of this strain is nitrofurantoin resistant) (13)	× T4*GT7*(CY307), Ura$^+$
S1189	S541 *attB*::[Spc^r *lacUV5 bgl-t1L bglGorf lacZ*]	9
S1193	S541 *attB*::[Spc^r *lacUV5* (+95)*bglG lacZ*]	9
S1195	S541 *attB*::[Spc^r *lacUV5* (+95)*bglGorf lacZ*]	9
S1211	S541 *attB*::[Spc^r P*bgl*(−76 to +25) *lacZ*]	9
S1213	S541 *attB*::[Spc^r P*bgl*(+25) *lacZ*]	9
S1215	S541 *attB*::[Spc^r *bgl-CRP*$^+$ P*bgl*(+25) *lacZ*]	9
S1218	S1185 *proC* mutant *zaj*::Tn*10*	× T4*GT7*(SG1039), Tc^r
S1219	S1189 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1223	S1193 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1225	S1195 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1258	S1195 *hns*::Ap^r	9
S1311	S1097 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1371	S1195 stpA::Tc^r	× T4*GT7*(M182 *stpA*::Tc^r)
S1379	S541 Δ*lon-510 sulA3 clpP*::Cm^r *stpA*::Tc^r *attB*::[Spc^r *lacUV5* (+95)*bglGorf lacZ*]	× T4*GT7*(M182 *stpA*::Tc^r)
S1416	S1132 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1422	S539 *pgi-30*::mTn*10*	× T4*GT7*(S751), Tc^r
S1424	S539 *pgi-26*::mTn*10*	× T4*GT7*(S765), Tc^r
S1432	S541 Δ*lon*-510 *sulA3 clpP*::Cm^r *hns*-206::Ap^r *attB*::[Spc^r *lacUV5* (+95)*bglGorf lacZ*]	× pKESD49
S1553	S1218 Δ*lon*-510 *proC*$^+$	× T4*GT7*(JT4000), Pro$^+$ Tc^s
S1554	S1553 *attB*::[Spc^r P*bgl*(−76 to +25) *lacZ*]	× pKEKB25
S1556	S1553 *attB*::[Spc^r P*bgl*(+25) *lacZ*]	× pKEKB30

Continued on following page

TABLE 1—*Continued*

Strain	Genotype[a]	Construction[b] or reference
S1558	S1553 *attB*::[Spc^r *bgl-CRP*$^+$ P*bgl*(+25) *lacZ*]	× pKEYK1
S1562	S1553 *attB*::[Spc^r *lacUV5* (+95)*bglG lacZ*]	× pKESD48
S1564	S1553 *attB*::[Spc^r *lacUV5* (+95)*bglGorf lacZ*]	× pKESD49
S1582	S1213 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1584	S1211 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1586	S1215 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1588	S1217 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1624	S541 *attB*::[Spc^r *lacUV5 bgl*(1–173)*t1-L lacZ*]	× pKESD44
S1642	S1553 *attB*::[Spc^r *lacUV5 bgl*(1–173)*t1-L lacZ*]	× pKESD44
S1654	S594 *hfq2*::Ω(Km^r)	× T4*GT7*(AM112), Km^r
S1656	S539 *hfq2*::Ω(Km^r)	× T4*GT7*(AM112), Km^r
S1676	S1624 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r
S1680	S1553 *attB*::[Spc^r *lacUV5 t1-L bglG lacZ*]	× pKESD20
S1684	S1553 *attB*::[Spc^r *lacUV5 t1-L bglGorf lacZ*]	× pKESD28
S1767	S1371 *hns*:*amp*	× T4*GT7*(PD32), Ap^r
S1828	S1258 *hfq1*::Ω(Km^r)	× T4*GT7*(AM111), Km^r

[a] Strains carrying the silent wild-type operon, designated *bgl*^{o}, are Bgl$^-$ (for nomenclature see reference 39). *bgl-CRP*$^+$ (a C-to-T exchange in the CRP binding site at position −66 relative to the transcription start) causes activation of the *bgl* operon. In the t1-L terminator mutant, 3 bases in the stem-loop structure of the terminator are mutated (8). P*bgl* and *lacUV5* represent the *bgl* and *lacUV5* promoters, respectively, and *bglGorf* represents a *bglG* mutant, in which the translation initiation codon was mutated. The coordinates of cloned fragments are given in brackets, where relevant. For mini-Tn*10* mutations, the orientation (I or II) and the position of the insertion site are given relative to the translation start of the mutated gene (e.g., *lon*:1360). Orientation I indicates that the Tn*10 tetA* gene is in the same orientation as the mutated gene. Tn*10* usually generates a target site duplication of 9 bp. The position of the nucleotide located directly 3′ to mini-Tn*10* is given for insertions in orientation I, while the position of the nucleotide located directly 5′ to mini-Tn*10* is given for insertions in orientation II. Tc^r, tetracycline resistant; Ap^r ampicillin resistant; Km^r, kanamycin resistant; and Spc^r, spectinomycin resistant.

[b] For strains that were obtained from the *E. coli* Genetic Stock Center, the respective strain number is given as a reference. ×, in strains S581 and S594, the *lac* promoter was replaced by the *bgl* regulatory region by site-specific recombination using plasmids pKES44 and pKES51, respectively, according to Dobert and Smith (4). Transductants (using T4*GT7*) were selected as indicated. In case of the transduction of *lon* alleles, which map at 9.8 min, phenotype analysis or PCR confirmed that the *lac* locus, which is located at 7.8 min, was not cotransduced. Integrations into *attB* were performed as described previously (8).

the H-NS protein (18, 19). Gene *pgi* codes for the enzyme phosphoglucose isomerase, which catalyzes the isomerization of glucose-6-phosphate to fructose-6-phosphate. The genes *miaA* and *hfq* code for the tRNA modification enzyme Δ(2)-isopentenylpyrophosphate transferase (MiaA) and the host factor for replication of RNA phage Qβ (Hfq), respectively. Hfq, a 15-kDa protein with ~30,000 molecules per cell (1), has an RNA binding/chaperone activity (28, 29, 38, 56). It is involved in the regulation of translation by the regulatory RNAs DsrA and OxyS, and it interferes with translation of the *ompA* mRNA (31, 49, 52). The MiaA-mediated modification of tRNAs affects translation efficiency and is required for translation of the virulence-related transcriptional regulator VirF (10).

In *hfq* and *lon* mutants, expression of the *bgl-lacZ* fusion is specifically down-regulated. For the quantification of the effect of mutations in the *miaA-hfq*, *lon*, and *pgi* loci, we determined the β-galactosidase expression level directed by the *bgl-lac* fusion, which carries the *bgl* promoter derivative activated by the improved CRP-binding site (Fig. 3). As a control, we measured the influence of mutations in *miaA-hfq*, *lon*, and *pgi* on the expression of the wild-type *lac* operon, which was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 3). β-Galactosidase assays were performed with cultures grown to the exponential phase (OD_{600} of 0.5) in M9 minimal medium containing glycerol and Casamino Acids.

In the *lon* mutants (alleles 107, 108, 110, 112, and 113), the expression level of the *bgl-lac* fusion is approximately three- to fourfold lower (between 80 and 105 U) than in the wild-type strain background (315 U) (Fig. 3). The mutation *lon-107*, which carries the mini-Tn*10* insertion closest to the 5′ end of the gene (Fig. 2), was chosen for further analysis. This mutation did not significantly reduce (less than 1.3-fold) expression of the *lac* operon (Fig. 3). These data show that mutations in *lon* specifically down-regulated expression of *bgl*.

The two *pgi* mutations *pgi-26* and *pgi-30* caused a weak (1.5- to 1.7-fold) decrease in the expression of the *bgl-lac* fusion, while the expression level of the *lac* operon was also weakly (1.3-fold) reduced (Fig. 3). Because of this minimal weak effect on *bgl*, the *pgi* mutant was not analyzed further.

The three mutations mapping in the *miaA-hfq* loci (*miaA21*, *miaA22*, and *hfq-21*) caused a specific decrease in the expression of the *bgl-lac* fusion (Fig. 3). Genes *miaA* and *hfq* are part of the complex *amiB-mutL-miaA-hfq-hflX* operon, which has several promoters directing expression of the different genes of the operon to various degrees (50). Out of the three mutants isolated in the screen, two mutations (*miaA21* and *miaA22*) map in the *miaA* gene and one (*hfq-21*) maps in the *hfq* gene (Fig. 2). To determine whether these mutations affect *bgl* expression due to the mutation of *miaA* or *hfq* or polar effects on downstream genes, their effect was compared to that caused by the two previously described mutations *hfq1*::Ω and *hfq2*::Ω (50). In *hfq1*::Ω, an Ω cassette carrying a kanamycin resistance marker flanked by Rho-independent transcriptional terminators (36) is inserted towards the 5′ end of the *hfq* gene and thus causes an Hfq-negative phenotype. In *hfq2*::Ω the Ω cassette is inserted towards the 3′ end of the *hfq* gene, which allows expression of an active Hfq protein. Both mutations have the same polar effects on expression of the downstream genes (50). The mutation *hfq1*::Ω had a negative effect on expression of the *bgl-lac* fusion very similar to that of *hfq-21* (85 and 95 U, respectively, compared to 315 U in the wild-type), while *hfq2*::Ω had no significant effect on *bgl* expression (256 U) (Fig. 3), which demonstrates that Hfq reduces *bgl* silencing. The

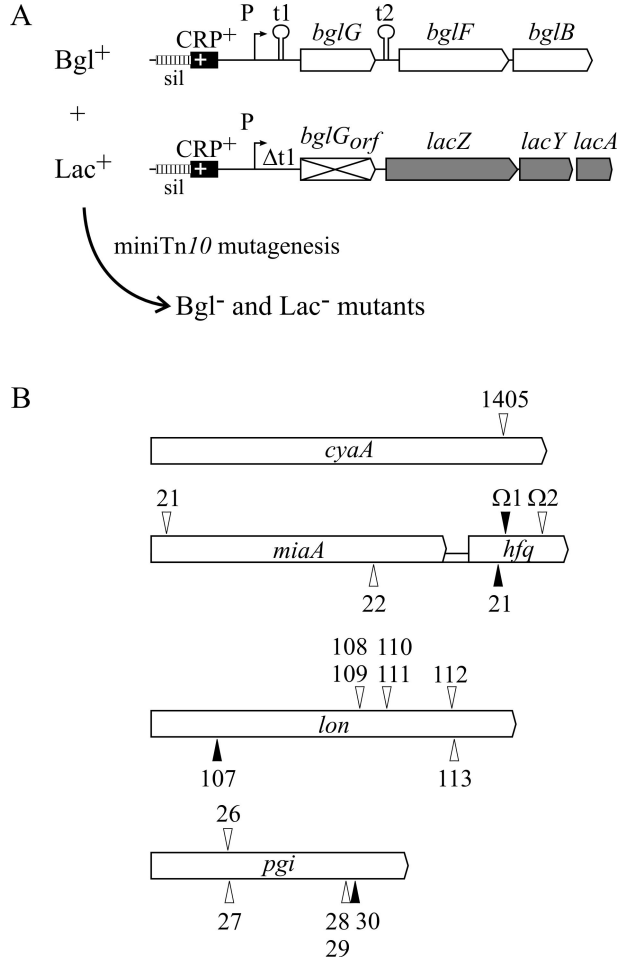

FIG. 2. Mutagenesis screen for mutants with reduced expression of the *bgl* operon. (A) Mutagenesis strategy to isolate in *trans* mutations affecting expression of the *bgl* operon. Strain S594 carries an activated *bgl* operon and a fusion of the *bgl* regulatory region to the *lac* operon. In both the *bgl* operon and the *bgl-lac* fusion, the *bgl* promoter is activated by an improved CRP-binding site, and thus strain S594 is Bgl^+ Lac^+. Expression of the *bgl-lac* fusion is independent of BglG-mediated antitermination at terminator t1, due to a deletion (Δ*t1*) of the terminator (including positions +55 to +120 relative to the transcription start site). In addition, translation of *bglG* was excluded by mutation of the translation start codon and codon 3 to GCG (*bglGorf*). After mini-Tn*10* mutagenesis, mutants with a double-phenotype change to Bgl^- Lac^- were isolated. (B) Schematic representation of the mini-Tn*10* insertion mutants obtained in the screen. The insertion sites were sequenced using a Tn*10*-specific primer (Materials and Methods and Table 1). Arrowheads above the mutated gene depict insertions in orientation I, while arrowheads underneath the gene were used for insertions in orientation II. Solid arrowheads represent the alleles used throughout the study. The *hfq* alleles Ω1 and Ω2 (50) were also used.

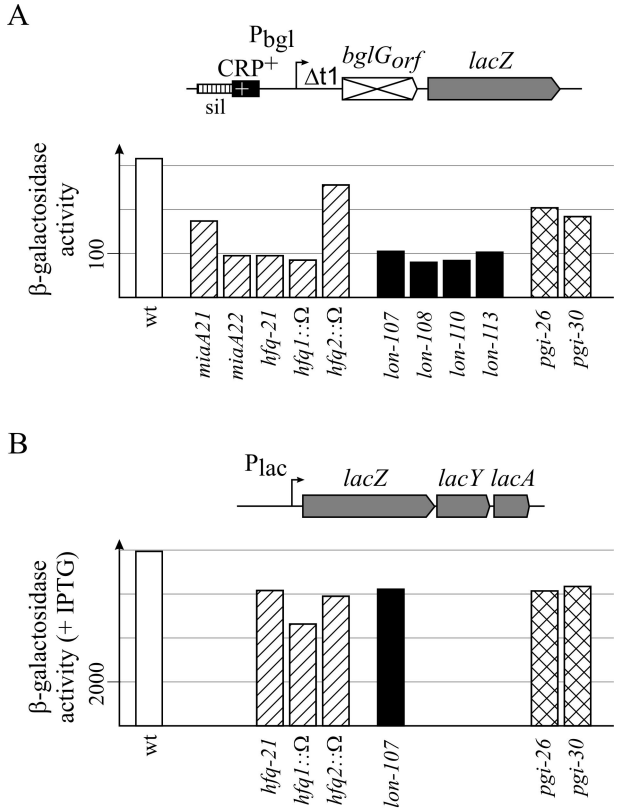

FIG. 3. Mini-Tn*10* mutants of *miaA-hfq* and *lon* specifically reduce expression of a *bgl-lac* fusion. (A) The expression level directed by the *bgl-lac* fusion (see Fig. 1 for details) was determined in the wild-type (wt) strain (S594) and its mini-Tn*10* derivatives (strains S749, S754, S759, S762, S764, S751, S765, S750, S757, S763, S1048, and S1654). (B) As a control, the effect of some of the mutations on the wild-type *lac* operon was also determined (strains S539, S794, S1422, S1424, S778, S1105, and S1656). Cultures were grown in minimal M9 medium containing glycerol and Casamino Acids to an OD_{600} of 0.5.

effect of the mutation *miaA22*, which maps very close to the *hfq* gene, was similar to *hfq-21* (both 95 U), while the effect of *miaA21*, which maps at the 5′ end of the *miaA* gene, was weaker (175 U) (Fig. 3). This suggests that these two mutations cause a down-regulation of *bgl* expression due to their polar effects on *hfq*. The expression of the *lac* operon was also moderately (1.3- to 1.7-fold) reduced in the *hfq-21* and *hfq1*::Ω mutants (Fig. 3). However, in contrast to the *bgl-lacZ* fusion, the expression of the *lac* operon was similarly (1.3-fold) reduced in the *hfq2*::Ω mutant, which expresses a functional Hfq protein (Fig. 3). Taken together, these data suggest that Hfq also reduces silencing of *bgl*. Further analyses were performed using the *hfq-21* and *hfq1*::Ω alleles. For comparison, expression of the *bgl-lac* fusion construct that is activated by an improved CRP-binding site was also determined in an *hns* background. Mutation of the *hns* gene resulted in an approximately sevenfold increase (to 1,940 U) in the expression level of the *bgl-lacZ* fusion, while expression of the wild-type *lac* operon was lowered twofold (to 2,860 U) in the *hns* mutant. This decrease in *lac* operon expression may be due to poor growth of the *hns* mutant in minimal medium. Since the other mutants also showed a reduced growth rate in minimal medium, all further experiments were performed with cultures grown in NB or Luria-Bertani (LB) medium, as indicated.

The *bgl* promoter is not affected by Hfq and Lon. Silencing of the *bgl* operon requires H-NS, which represses the expression at two levels. H-NS hinders transcription initiation, and H-NS binds 600 to 700 bp downstream of the transcription start, where it induces polarity of transcription (9). To further analyze the regulation of the *bgl* operon by Lon and Hfq, we

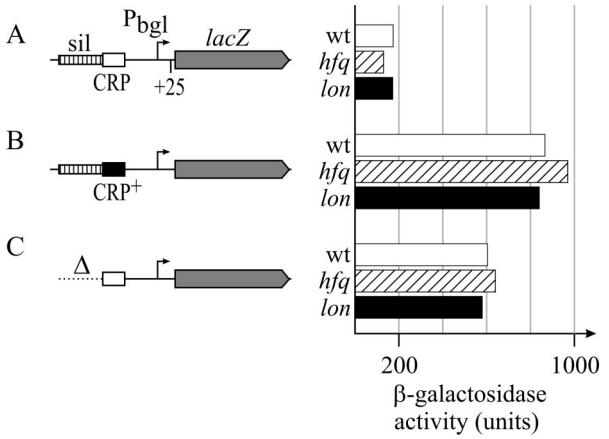

FIG. 4. Effect of Hfq and Lon on the *bgl* promoter. The activity of the wild-type *bgl* promoter (wt) and activated derivatives, which carry a deletion (Δ, including position −77 and upstream) of the upstream silencer (sil), and an improved CRP-binding site (CRP^+; a C-to-T exchange at position −66) were determined in *hfq*, *lon*, and *pgi* mutants by using *lacZ* gene fusion 25 bp downstream of the transcription start side and compared to the expression level in the wild-type strain tested previously (9). Cultures were grown in NB medium to an OD_{600} of 0.5. (A) Wild-type *bgl* promoter. Shown are results for strains S1213 (wild type [wt]), S1582 (*hfq1*::Ω), and S1556 (Δ*lon-510 sulA3*). (B) *bgl* promoter activated by an improved CRP-binding site (CRP^+). Shown are results for strains S1215 (wild type), S1586 (*hfq1*::Ω), and S1558 (Δ*lon-510 sulA3*). (C) *bgl* promoter activated by deletion of the upstream silencer (Δ). Shown are results for strains S1211 (wild type), S1584 (*hfq1*::Ω), and S1554 (Δ*lon-510 sulA3*).

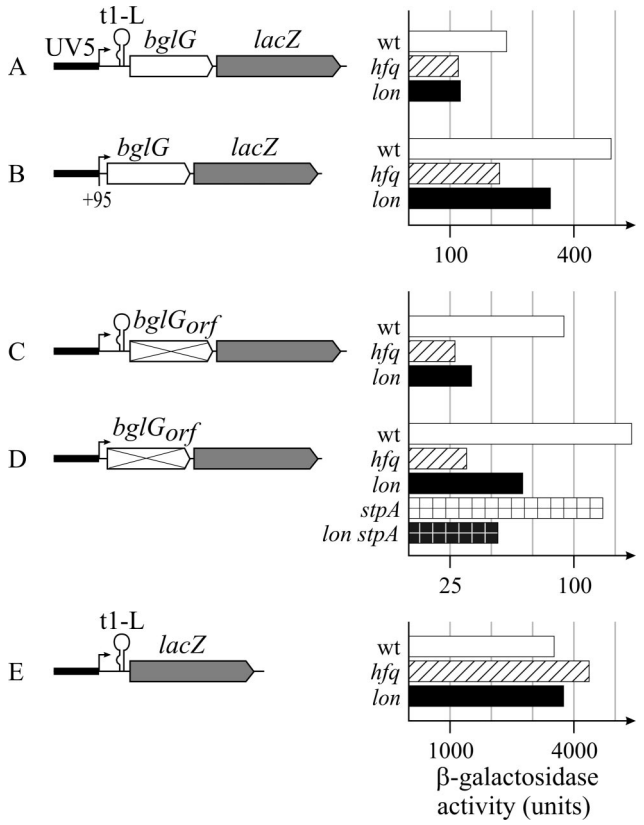

FIG. 5. Effect of Hfq and Lon on repression of *bgl* via the downstream silencer. The expression level directed by the *bgl*-downstream silencer-*lacZ* reporter constructs (9) was determined in *hfq*, *lon*, and *pgi* mutants and compared to the expression level in the wild-type strain tested previously (9). The expression of these *bgl-lacZ* fusions is directed by the constitutive *lacUV5* promoter. Cultures were grown in NB medium to an OD_{600} of 0.5. These constructs carry the following insertions between the *lacUV5* promoter and the *lacZ* gene. (A) The complete downstream region including the leader (with the *tl-L* terminator mutant) and gene *bglG* fragment (S1097, wild-type) (9), S1311 (*hfq1*::Ω), S1680 (Δ*lon-510 sulA3*), S1132 (*rpoS359*::Tn*10*), and S1416 (*hfq1*::Ω, *rpoS359*::Tn*10*). (B) The *bglG* gene fragment (from position +95) (S1193, wild type), S1223 (*hfq1*::Ω), and S1562 (Δ*lon-510 sulA3*). (C) Same as A, but translation of *bglG* excluded due to mutations of codons 1, 3, and 27 to GCG (S1189, wild-type) (9), S1219 (*hfq1*::Ω), and S1684 (Δ*lon-510 sulA3*). (D) Same as B, but translation of *bglG* excluded due to mutations of codons 1, 3, and 27 to GCG (S1195, wild-type), S1225 (*hfq1*::Ω), S1564 (Δ*lon-510 sulA3*), S1371 (*stpA*::Tc^r), and S1379 (Δ*lon-510 sulA3 clp*::Cm^r *stpA*::Tc^r). (E) *bgl* leader (from positions +1 to +176) including the *tl-L* terminator mutant (S1624, wild type), S1676 (*hfq1*::Ω), and S1642 (Δ*lon-510 sulA3*).

used a series of chromosomally encoded *bgl-lacZ* reporter gene fusions, which carry either the promoter and upstream regulatory region (Fig. 4) or the downstream regulatory region (Fig. 5). Expression of the latter is directed by the constitutive *lacUV5* promoter (9). The role of Lon in *bgl* regulation was studied in strains in which a deletion of the *lon* gene (Δ*lon-510*) was combined with a mutation of *sulA* (*sulA3*), encoding the cell division inhibitor SulA. The SOS-induced SulA is normally degraded by Lon. However, in *lon* mutants, the block of cell division remains and the cells are SOS sensitive (15, 41). In our hands, the growth rate and reproducibility of enzyme assays were significantly improved with the Δ*lon sulA3* double mutant compared to those with the single *lon-107* mutant (data not shown). For the analyses of the role of Hfq in regulation of the *bgl* operon allele, *hfq1*::Ω was used.

To determine the effect of Hfq and Lon on the *bgl* promoter, we used *bgl* promoter-*lacZ* fusions, in which the *lacZ* gene was fused at position +25 (relative to the transcription start) (Fig. 4). The expression of three *bgl* promoter constructs carrying either the wild-type promoter, an allele with a deletion of the upstream silencer, or an allele with the improved CRP-binding site was determined from cultures grown to the exponential phase (OD_{600} of 0.5) in NB medium. The expression level directed by the wild-type *bgl* promoter is approximately 3.5-fold lower than that of the promoter that lacks the upstream silencer (175 and 600 U, respectively) (Fig. 4A and C), while the improvement of the CRP-binding site leads to an ~5-fold increase in the promoter activity (to 865 U) (Fig. 4B) (9). In the *hfq*::Ω1 and Δ*lon sulA3* mutants, the expression level directed by the wild-type *bgl* promoter and its two activated derivatives was not significantly altered (Fig. 4). These results demonstrate that neither Hfq nor Lon affects transcription initiation at the *bgl* promoter.

Hfq and Lon affect the *bgl* operon via the downstream silencer. The second level of repression of the *bgl* operon by H-NS occurs at a downstream silencer located within the *bglG* coding region (9). To analyze whether Hfq and Lon (directly or indirectly) affect this level of regulation, a set of *bgl*-downstream silencer-*lacZ* reporter constructs were used (9). In these reporter constructs, expression is directed by the constitutive *lacUV5* promoter. Between the *lacUV5* promoter and the *lacZ* reporter, fragments were inserted that encompass the

complete downstream region, including the leader and the *bglG* gene (from positions +1 to +971), the *bglG* gene alone (+95 to +971), or, as a control, the leader only (+1 to +176) (9). To prevent transcription termination at terminator t1 located in the leader, the *tl-L* terminator mutant was used, in which 3-base exchange in the left stem of the terminator hairpin structure disrupts the terminator (8). In addition, variants of those fusions carrying the *bglG* gene were used in which the translation of *bglG* is prevented by mutation of the translation start codon and two additional ATG codons (codons 3 and 27) to GCG. When translation of *bglG* is excluded, the repression by H-NS via the downstream silencer is more efficient (9).

A construct in which the *lacUV5* promoter is followed by the *bgl* leader with the *t1-L* terminator mutant, the *bglG* gene, and the *lacZ* reporter gene directs 240 U of β-galactosidase activity in the wild-type strain (Fig. 5A) (9). In the *hfq1*::Ω and *lon sulA3* mutants, the expression level of this *lacUV5–t1-L–bglG–lacZ* fusion decreased approximately twofold to 120 and 125 U, respectively (Fig. 5A). The expression level of a reporter construct carrying a *bglG* fragment inserted between the *lacUV5* promoter and the *lacZ* gene decreased from 490 U in the wild type to 220 U in the *hfq* mutant. However, the decrease in the *lon* mutant (to 370 U) was less significant (Fig. 5B). The effects of Hfq and Lon on the corresponding reporter constructs, in which translation of *bglG* is excluded, were higher. The expression level of the *lacUV5–t1-L–bglGorf–lacZ* reporter construct decreased 2.5- to 3-fold in the *hfq* and *lon* mutants (Fig. 5C). Ninety-five units of β-galactosidase activity was detected in the wild type, 28 U was detected in the *hfq1*::Ω mutant, and 44 U was detected in the Δ*lon-510 sulA3* mutant (Fig. 5C). Likewise the expression level of the *lacUV5-bglGorf-lacZ* reporter construct decreased two- to threefold from 135 U in the wild type to 35 U in the *hfq* mutant and 57 U in the *lon* mutant (Fig. 5D). A control carrying the *bgl* leader, including the *t1-L* terminator mutant (positions +1 to +179), which lacks the downstream H-NS binding site, was not affected in the *hfq* and *lon* mutants (Fig. 5E). These data show that Hfq and Lon control the expression of the *bgl* operon via the downstream regulatory region located within *bglG*. Their effects are stronger when the *bglG* gene is not translated, which indicates that access to the mRNA is important in this process. Repression by H-NS via the downstream silencer is also more efficient when *bglG* is not translated (9).

Hfq affects the *bgl* operon independent of RpoS. Hfq is required for translation of the *rpoS* mRNA, and RpoS levels are thus low in *hfq* mutants (31). In addition, RpoS is known to repress the *bgl* operon approximately two- to threefold in the exponential phase (8, 33, 44). If Hfq affected *bgl* indirectly via RpoS, *bgl* expression should increase in the *hfq* mutant. However, here we found that the expression of *bgl* decreases in *hfq* mutants. In addition, previous data suggest that RpoS affects the *bgl* promoter (8), while here we found that Hfq acts via the downstream silencer. This suggests that Hfq affects the *bgl* operon independent of RpoS. To confirm this, the expression level directed by the *lacUV5–t1-L–bglG–lacZ* construct was also determined in an *rpoS* mutant and an *hfq rpoS* double mutant. The results support the assumption that Hfq reduces silencing of *bgl* independent of RpoS (data not shown).

H-NS is epistatic over Hfq and Lon. H-NS induces a Rho-dependent polarity of transcription within *bglG* (9). Here we found that Hfq and Lon also act via the *bglG* coding region. One possibility is that Hfq and Lon act on a different level of regulation from H-NS. Another possibility is that these proteins directly or indirectly counteract H-NS to induce polarity. For example, Lon and Hfq could reduce the activity or action of H-NS or another protein involved in this process. If this were the case, then the mutation of *hfq* or *lon* should have no effect on *bgl* when tested in an *hns* mutant. To address this question, the expression level of the downstream silencer reporter construct (*lacUV5-bglGorf-lacZ*), which carries the nontranslatable *bglG* coding region, was tested in *hfq hns* double and *lon sulA3 hns* triple mutants and compared to the expression level directed in the *hns* mutant (Fig. 6).

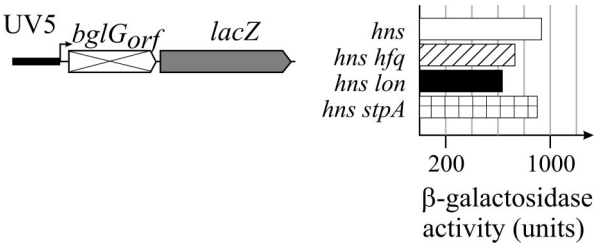

FIG. 6. Effect of Hfq and Lon via the downstream silencer in an *hns* mutant. The expression level directed by the *bgl*-downstream silencer-*lacZ* reporter constructs carrying the *bglG* coding region (Fig. 5D) was determined in *hfq hns*, *lon hns*, and *stpA hns* mutants and compared to the expression level in the *hns* strain tested previously (9). Cultures were grown in NB medium to an OD_{600} of 0.5. The *hfq hns* double mutant was grown in LB medium. β-Galactosidase activity is shown for the following strains from top to bottom: S1258 (*hns-206*::Ap^r) (9), S1828 (*hfq1*::Ω *hns-206*::Ap^r), S1432 (Δ*lon-510 sulA3 hns-206*::Ap^r), and S1767 (*stpA*::Tc^r *hns-206*::Ap^r).

The expression level of the *lacUV5-bglGorf-lacZ* fusion has been shown to increase approximately sevenfold in the *hns* mutant (from 135 U to 905 U) (9), while it decreased approximately twofold in the *lon* mutant and approximately fourfold in the *hfq* mutant. Since the *hns hfq* double mutant grew very poorly in NB medium, the β-galactosidase activity expressed in this strain was determined from cultures grown in LB medium. The expression levels directed in the wild type and the *hns* and *hfq* single mutants were similar when cultures were grown in NB or LB medium (data not shown). In the *hns hfq* double mutant, 730 U of β-galactosidase activity was determined and in the *hns* Δ*lon sulA3* triple mutant 640 U was determined, as compared to 905 U in the *hns* mutant (Fig. 6). Thus, the effect of Hfq and Lon was reduced in the *hns* mutant. This suggests that Hfq and Lon control a protein or process that is required for H-NS to induce polarity. In case of Lon, the H-NS homologue StpA is a candidate. StpA is degraded by Lon (18, 19), and StpA plays a role in silencing of *bgl* by a truncated H-NS variant (12). We therefore analyzed whether StpA is important for the stimulation of *bgl* expression by Lon and determined the expression level of the downstream silencer reporter *lacUV5-bglGorf-lacZ* in *stpA* and *stpA lon sulA3* mutants (Fig. 5D). In the *stpA* mutants, the expression level was unchanged compared to the level of expression in the wild-type and *lon sulA3* strains (Fig. 5D), suggesting that StpA is not the protein substrate via which Lon affects *bgl*. The mutation of *stpA* in the *hns* mutant likewise had no effect (Fig. 6).

DISCUSSION

H-NS silences the *bgl* operon at two levels: it represses transcription initiation, and it induces polarity within the transcription unit. Here we presented evidence that the latter level of silencing by H-NS, the induction of polarity, is reduced by RNA chaperone Hfq and by protease Lon. However, the H-NS-mediated silencing of the *bgl* promoter is not altered by these proteins. Our results suggest that specificity in the control by the pleiotropic regulator H-NS can be achieved by other pleiotropic factors, which control or modulate the action of H-NS at a specific locus, as shown here for Hfq and Lon, which counteract only the second level of silencing of *bgl* by H-NS.

The *bgl* operon is silent under laboratory growth conditions. To date, only mutants in which silencing of the operon is overcome have been isolated. Silencing can be relieved by mutations that map in *cis* to the promoter and interfere with its repression by H-NS, or they map in the *hns* gene (5, 39, 40). Constitutive expression of the *leuO* or *bglJ* gene, which encode transcription factors, relieves silencing by unknown mechanisms (14, 51). In this work, a reverse transposon mutagenesis screen for mutants was performed, in which expression of a *bgl* operon derivative was reduced. This screen yielded mutations mapping in the *cyaA*, *miaA-hfq*, *lon*, and *pgi* loci (Fig. 2). The mutation of *cyaA* presumably impairs the CRP-dependent promoter. In *pgi* mutants, the glycolytic pathway is blocked, leading to the accumulation of glucose-6-phosphate. It has been shown that this accumulation stimulates the RNase E-dependent destabilization of the *ptsG* mRNA (22, 30). The *ptsG* gene encodes the glucose permease enzyme $IICB^{Glc}$, which belongs to the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) (34). The use of a *bgl-lacZ* reporter construct revealed that the specificity of the *pgi* mutant for *bgl* is low (Fig. 3), and the mutant was not analyzed further.

Mutation of *hfq* and *lon*, respectively, had no effect on the *bgl* promoter. In these mutants, the second level of *bgl* silencing by H-NS, the induction of polarity downstream of the promoter, was more efficient. The use of double mutants revealed that in an *hns* mutant, Hfq plays no role and Lon plays a rather reduced role: i.e., H-NS is epistatic over Hfq and also Lon. What could be the mechanisms by which expression of *bgl* is reduced in these mutants? H-NS binds approximately 600 to 700 bp downstream of the transcription initiation site, within the coding region of the first gene, *bglG*, and represses expression beyond *bglG*. The repression is more efficient when the *bglG* mRNA is not translated, suggesting that access of an RNA-binding protein, such as, e.g., termination factor Rho, or an RNase to the *bglG* mRNA is important for H-NS to induce polarity (9). In the *hfq* and *lon* mutants, the expression is further reduced, indicating that Hfq and Lon reduce the silencing by H-NS. The highly conserved ATP-dependent protease Lon degrades nonfunctional proteins and some proteins specifically (15). In case of the *bgl* operon, Lon could degrade a protein required for H-NS to induce polarity. In a *lon* mutant, this negative regulator protein would be stabilized and accumulate to higher levels, causing low *bgl* expression. As a candidate, we tested StpA (a protein with 67% similarity to the H-NS protein), which is a specific Lon substrate (18, 19) and which is involved in the repression of *bgl* by a truncated H-NS protein (12). When we determined the expression level of the *bgl*-downstream silencer-*lacZ* reporter construct in *stpA* and *lon sulA3 stpA* mutants, no difference compared to the $stpA^+$ strains was observed (Fig. 5). Thus, StpA is likely not to be the specific substrate via which Lon affects *bgl*.

Hfq is an RNA chaperone that affects the stability and translation of several RNAs (3, 28, 29, 38, 56, 57). For example, Hfq binds to and stabilizes the small regulatory DsrA RNA (3). The *dsrA* gene is induced at low temperature, allowing its product, the DsrA RNA, to induce translation of the *rpoS* mRNA in exponentially growing cells under these conditions (49). DsrA, when overproduced, represses translation of *hns* by an Hfq-dependent mechanism (26, 49). However, it is not clear whether this is relevant under physiological conditions (38). At 30°C, a weak (1.5-fold) relief of the H-NS-mediated repression of the *rcsA* gene by Hfq/DsrA was observed (49). Whether at 37°C Hfq affects H-NS levels via DsrA is not known. In the case of specific reduction of the H-NS-mediated downstream silencing of *bgl* by Hfq, several mechanisms seem plausible. First, specific competition experiments suggest that the affinity of H-NS to the upstream silencer is higher than to the downstream silencer. Thus, a small reduction of H-NS amounts in the *hfq* mutant could result in the specific reduction of downstream silencing by H-NS. Second, Hfq could affect downstream silencing indirectly: e.g., via another small regulatory RNA. Third, Hfq could bind to the *bglG* mRNA and change the secondary structure of the *bglG* mRNA and/or reduce access of Rho or an RNase and thus counteract H-NS-mediated polarity.

To date, the biological meaning of *bgl* operon silencing is not understood. The operon is present in approximately 70% of *E. coli* isolates, and in all of the strains in which it is present, its silent state is conserved (G. Neelakanta and K. Schnetz, unpublished observations). This and its complex regulation suggest that the expression of the operon is disadvantageous under some conditions, while it is required and induced under certain physiological and environmental conditions. Irrespective of these open questions, the *bgl* operon is a valuable tool for the understanding of specific repression by H-NS. How specificity in the control by the pleiotropic regulator H-NS is achieved is largely unknown. The *bgl* operon is controlled at two levels by H-NS. Here we have shown that one of these levels of silencing by H-NS is modulated by the RNA chaperone Hfq and by the protease Lon, while the other level—the silencing of the promoter—is unaffected by these proteins. This finding suggests that the action of H-NS at a specific locus can be modulated by other pleiotropic regulators. Thus, locus-specific control by H-NS may be the result of the combination of H-NS with various sets of pleiotropic factors. A similar combinatorial mechanism has been discussed for the specific regulation by RpoS, the second key regulator of the adaptation response (16).

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