# Multiple level regulation of the *Escherichia coli bgl* operon

**Inaugural-Dissertation**

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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(M & S Copy Druckhaus, Köln)

2001

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Tag der mündlichen Prüfung:  3 December 2001.

**¥æ§-ÕæÕæâ**

(to my parents)

# Acknowledgements

First and foremost I thank Karin Schnetz for her thorough guidance, help, criticisms and allround support. I learnt a lot from her.

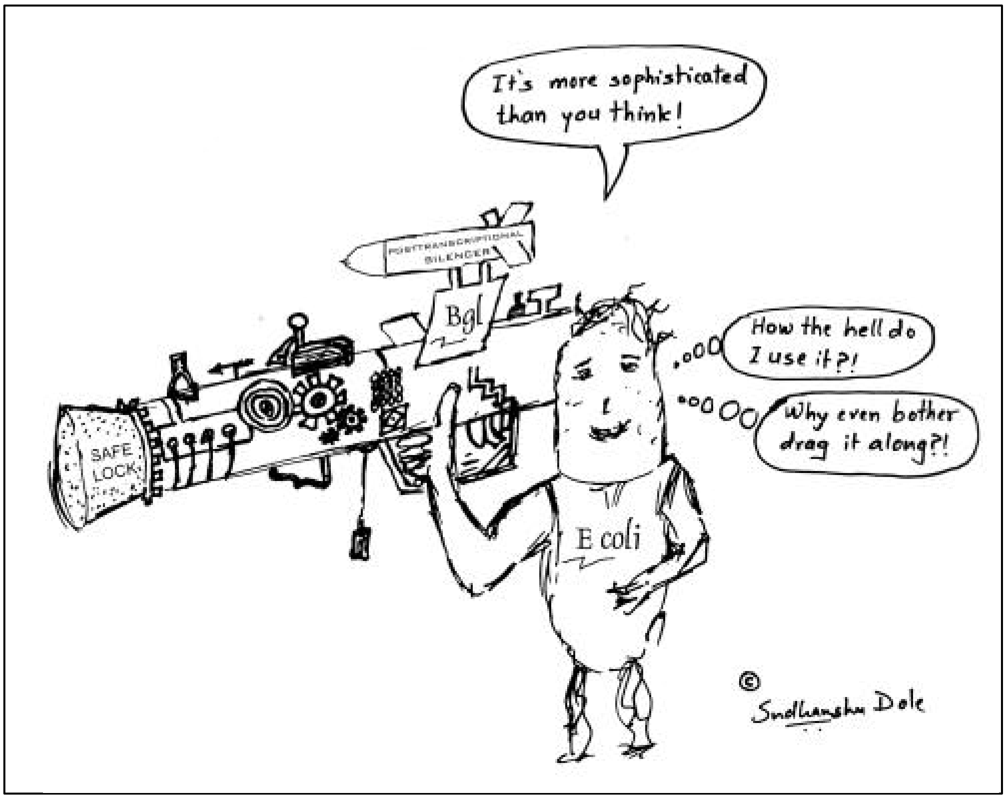
I thank all the Schnetz lab members, past and present, for a wonderful co-operative atmosphere, especially Sandra Kühn who was always ready to help with a smile. Oliver Hofmann assisted in performing some of the enzyme assays. Mass spectroscopic analysis of proteins was performed with assistance from service laboratory of Center for Molecular Medicine, Cologne.

I thank professors of my graduate programme, especially Maria Leptin, Jonathan Howard, Matts Paulsson, Benno Müller-Hill and Michael Melkonian for their guidance and help, Robert Wilson for technical suggestions, Klaus Reiners and Matthias Krämer for administrative help.

Financial support from DFG Graduiertenkolleg "Genetik zellulärer Systeme" and SFB is gratefully acknowledged.

My parents and family were a tremendous source of support and encourangement. Without my wife Vandana, everything would have been impossible and meaningless.

Sudhanshu Dole.


It's more sophisticated than you think!
POSTTRANSCRIPTIONAL SILENCER
Bgl
SAFE LOCK
E coli
How the hell do I use it?!
Why even bother drag it along?!
© Sudhanshu Dole

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## Abbreviations

| | |
|---|---|
| bp | base pairs |
| BTB | bromothymol blue |
| cAMP | 3', 5'-cyclic adenosine-monophosphate |
| CRP | catabolite regulator protein |
| cpm | counts per minute |
| DTT | dithiothreitol |
| EDTA | ethylene-diamine-tetraacetic acid |
| FIS | factor for inversion stimulation |
| H-NS | histone-like nucleoid-structuring protein |
| IPTG | isopropyl-β,D-thiogalactopyranoside |
| kDa | kilo Dalton |
| NaTCA | sodium trichloroacetate |
| OD | optical density |
| ONPG | o-nitrophenyl-β,D-galactopyranoside |
| PCR | polymerase chain reaction |
| PNPG | p-nitrophenyl-β,D glucopyranoside |
| rpm | revolutions per minute |
| wt | wild type |

## Zusammenfassung

Das *bgl*-Operon von *Escherichia coli* wird auf mehreren Ebenen reguliert. Das 'Silencing' wird zusätzlich zur bekannten Repression/Aktivierung des Promotors durch die Limitierung von BglG bei niedrigen Expressionsraten und die Regulation der *bglG*-mRNA-Stabilität durch H-NS und andere pleiotrope Faktoren gesteuert.

Sequenzen, die 'Upstream' und 'Downstream' des Promotors kartieren, sind für das Silencing essentiell. Die Funktion des Downstream Silencer war bisher ungeklärt. Ein neuer post-transkriptioneller Mechanismus der Regulation des *bgl* Operons wurde aufgedeckt. Entscheidend dabei ist die Regulation der *bglG*-mRNA-Stabilität. So reprimiert das H-NS Protein die Expression über den 'downstream silencer' 14-fach, in dem es die *bglG*-mRNA Stabilität vermindert. Diese Repression ist höher als die Repression des *bgl*-Promotors (und damit des Operons) durch Bindung von H-NS an die Upstream-Silencer-DNA-Sequenz. Die Stabilität der 'Leader'-RNA wird nicht durch H-NS verändert.

Die von den drei Genen *pgi*, *lon* und *hfq* kodierten Proteine, wurden als Faktoren identifiziert, die für die Expression des (durch De-Repression des Promotors) aktivierten *bgl*-Operons notwendig sind. Weitere Analysen zeigten, dass Hfq unabhängig vom Promotor und der Termination/Antitermination im Leader wirkt. Für den positiven Effekt von Hfq ist die für *bglG* kodierende Region notwendig. Wahrscheinlich wird die *bglG*-mRNA Stabilität durch Hfq, Pgi und translatierende Ribosomen erhöht.

Das Protein BglG bewirkt die transkriptionelle Antitermination im Leader des Operon. Es wurde gezeigt, dass die zelluläre Konzentration von BglG bei niedrigen Transkriptionsraten limitierend ist. Dies hat zur Folge, dass die Antitermination (und damit Expression des Operons) erst ab einer Schwellenwert-Konzentration von BglG erfolgt, wodurch der Grad des Silencings und der Aktivierung des Operons signifikant verstärkt wird.

Früheren Arbeiten ließen vermuten, dass zusätzlich zu H-NS andere Faktoren für die Ausbildung eines Nukleoproteinkomplexes notwendig sind, durch den der Promotor reprimiert wird. In *in-vitro*-Bindestudien wurde H-NS als einziges Protein identifiziert, das an den Upstream Silencer und/oder die Promotorregion bindet. Mit dem hier charakterisierten Mechanismus des *bgl*-Silencings durch die Downstream-Silencer-Sequenz, lassen sich die früheren Ergebnisse und deren Widersprüche erklären.

## Summary

The *Escherichia coli bgl* operon was shown, in the present work, to be regulated at multiple levels viz. repression/activation of the *bgl* promoter, amplification of *bgl* silencing/activation by limitation of antiterminator BglG at low expression levels and regulation of the *bglG* mRNA stability by H-NS and other pleiotropic factors.

Silencer sequences upstream and downstream of the *bgl* promoter were previously shown to be essential for silencing, but the role of the downstream silencer was not understood. A new post-transcriptional mechanism of the *bgl* operon regulation via the downstream silencer was discovered. Regulation of the *bglG* mRNA stability emerged as a prominent theme. The H-NS protein was found to downregulate the *bgl* expression at the downstream silencer sequence by reducing the *bglG* mRNA stability. This downregulation was up to 14 fold and was found to be more than the H-NS mediated downregulation of the *bgl* promoter, and thus the operon, via the upstream silencer. H-NS was found to decrease the half life of the *bglG* mRNA whereas it did not affect the stability of the *bgl* leader mRNA.

Proteins encoded by three genes, namely *pgi*, *lon* and *hfq* were found to be required for expression of the *bgl* operon when the promoter was de-repressed. Further analysis showed that the role of Hfq protein is independent of the *bgl* promoter regulation and termination/antitermination. The *bglG* sequence, which is part of the downstream silencer, was found to be essential for the positive regulatory effect of Hfq. The *bgl* mRNA stability is presumably positively regulated by Hfq, Pgi and the translating ribosomes.

In the promoter downstream region, transcriptional termination is prevented by the antiterminator protein BglG. It was found that cellular levels of BglG are limiting and the requirement for a BglG threshold could amplify the silencing or activation of the *bgl* operon.

Unknown Cellular factors in addition to H-NS were previously postulated to be essential to form a nucleoprotein complex at the silencers flanking the *bgl* promoter. Only H-NS was found to bind, *in vitro*, to the upstream silencer and promoter region of the *bgl* operon. The mechanism by which the downstream sequences contribute to *bgl* silencing discovered in the present work could explain the previously proposed requirement of additional cellular factors for *bgl* silencing.

# II. Introduction

The paradigm of gene regulation in prokaryotes is specific regulators like the Lac repressor regulating expression of the *lac* operon at the transcriptional level. However, increasing evidence points to regulation of a large numbers of prokaryotic genes by pleiotropic gene regulators at various levels of the gene expression including transcription, RNA modification/stability, translation and protein stability. In *Escherichia coli*, nucleoid-associated proteins like the histone-like nucleoid structuring protein (H-NS) have been shown to affect the expression of approximately 5% of the total number of proteins (Hommais et al., 2001). The mechanisms by which H-NS and other pleiotropic proteins regulate gene expression may well be diverse and are not completely understood. One of the operons which is directly and indirectly regulated by a multitude of pleiotropic regulators, including H-NS and others, is the *bgl* operon.

## 1. The cryptic *bgl* operon of *Escherichia coli*.

In *E. coli*, the utilization of β-glucosidic sugars like salicin, arbutin and esculin requires expression of the cryptic *bgl* (β-glucoside utilization) operon (figure 1a, b). Cryptic or silent genes have been defined as genes that are not expressed during the normal life cycle of an organism (Hall et al., 1983). However, their expression can be caused by mutations that abolish the silent state. The *bgl* operon was first discovered by isolating spontaneous mutants of *E. coli* K12 which were capable of metabolizing β-glucosides (Schaefler and Maas, 1967). The *bgl* operon (schematically shown in figure 1a) contains six genes (*bglG*, *F*, *B*, *H*, *I* and *K* ) which are expressed from a single promoter. Of these genes, the first three genes are sufficient for utilization of the β-glucosides (Prasad and Schaefler, 1974; Mahadevan et al., 1987; Schnetz et al., 1987). Two rho-independent transcriptional terminators, *bglt1* and *t2*, flank the first gene *bglG* encoding the antiterminator BglG (Mahadevan and Wright, 1987; Schnetz et al., 1987; Schnetz and Rak, 1988). The BglG activity is regulated by the catabolic state of the cell as well as presence of β-glucosides and other sugars as discussed below. When active, the BglG protein causes antitermination at the terminators *t1* and *t2* and allows transcriptional readthrough (Houman et al., 1990; Aymerich and Steinmetz, 1992). The second gene *bglF* codes for a membrane transport protein EnzymeII$^{Bgl}$ and specifically transports β-glucosides across the cell membrane and

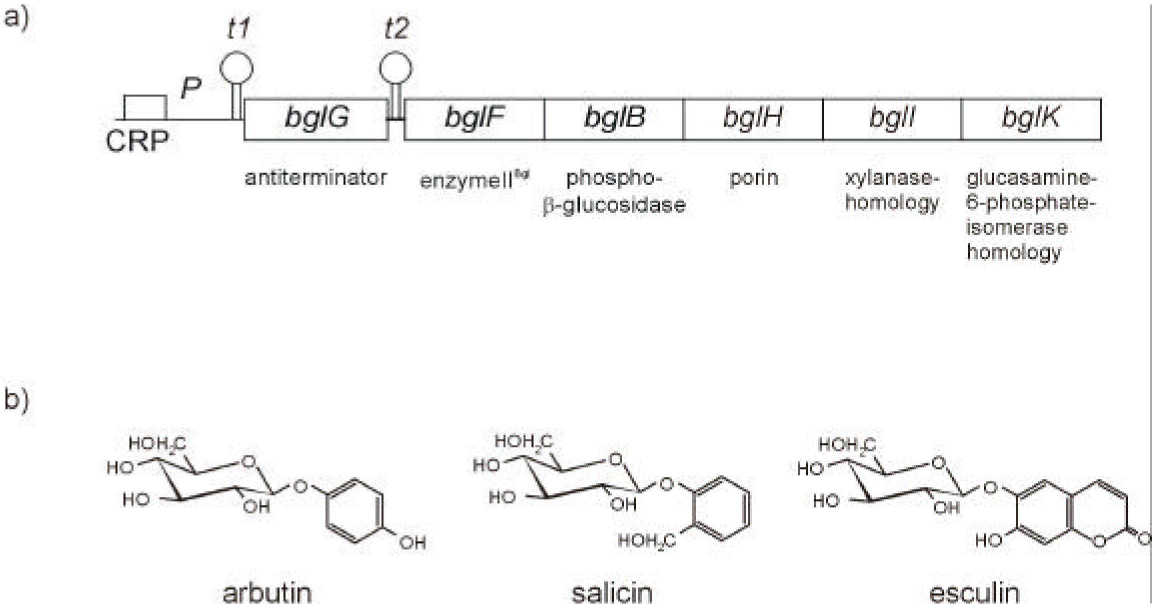


**Figure 1. The *E. coli bgl* operon.** a) The operon contains six genes of which the first three are sufficient for β-glucoside utilization (Prasad and Schaefler, 1974; Mahadevan et al., 1987; Schnetz et al., 1987). Two rho-independent transcriptional terminators, *bglt1* and *t2* are also shown. b) Chemical structures of β-glucosides arbutin, salicin and esculin (Rimpler, 1990).

negatively regulates the BglG activity (Schnetz et al., 1987; Saier et al., 1988). The third gene *bglB* codes for the enzyme phospho-β-glucosidase which catalyzes the first hydrolysis step of β-glucoside utilization (Prasad and Schaefler, 1974; Mahadevan et al., 1987; Schnetz et al., 1987).

## 2. Silencing and activation of the *bgl* operon.

The *bgl* promoter consists of the –10 and –35 conserved motifs and a catabolite regulator protein (CRP) binding site upstream of the –35 box (Reynolds et al., 1981; Reynolds et al., 1986). The *wt bgl* promoter is silent i.e. there is no significant transcription and the cells are phenotypically Bgl$^-$. After further incubation on indicator plates containing salicin, spontaneous mutants (papillae) arise which are phenotypically Bgl$^+$. These spontaneous mutants are integrations of insertion elements (*eg* IS1 and IS5) in the AT-rich region upstream of the promoter, integrations in the region downstream of the promoter, deletions of promoter upstream regions, point mutations like mutation *C234* (a single bp change from C to T at position –66 relative to transcription start) which improves the CRP binding site and causes CRP binding with higher affinity (figure 2) (Reynolds et al., 1986; Schnetz and Rak, 1988; Lopilato and Wright, 1990; Schnetz and Rak, 1992; Schnetz, 1995; Singh et al., 1995).

There are also *trans* activating mutations *eg* in the *hns* gene (Defez and de Felice, 1981; Higgins et al., 1988), which encodes the histone like nucleoid structuring protein H-NS (see below), in the gyrase genes *gyrA* and *gyrB* (minor effect) (DiNardo et al., 1982), mutations causing overexpression of the *bglJ* gene which codes for a protein with homology to the UhpA-LuxR family of transcriptional activators (Giel et al., 1996) and mutations causing overexpression of the *leuO* gene which encodes a putative LysR-like DNA-binding protein (Ueguchi et al., 1998).

Flanking the *bgl* promoter are two silencer sequences. The AT-rich upstream silencer is located 5' to the CRP binding site and the downstream silencer encompasses the *bgl* leader and the *bglG* sequence. It has been shown that both the upstream and the downstream silencer sequences are required for *bgl* silencing (Schnetz, 1995). The operon is active in the absence of either of the silencers. Similarly other active promoters, *eg* the *lac* promoter, are silenced when placed in context of the two silencers (Schnetz, 1995). Thus, it is the presence of the *bgl* promoter in context of these two silencer sequences which makes it cryptic. It was proposed that a silencing nucleoprotein complex forms at these silencers (Schnetz, 1995; Caramel and Schnetz, 1998). H-NS is an essential component of the complex. Formation of the repressing nucleoprotein complex presumably prevents access to the promoter by CRP and RNA polymerase (figure 2). Protein FIS (factor for inversion

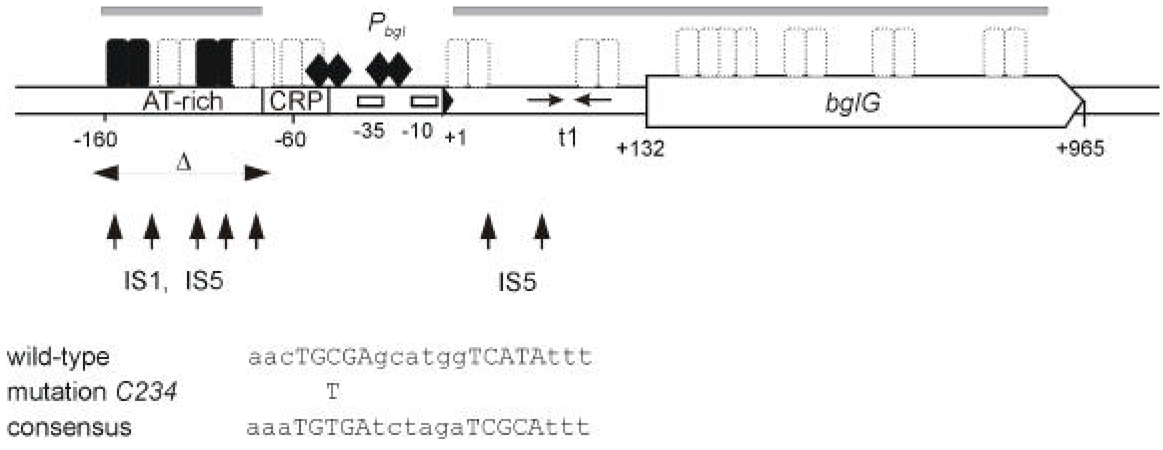


**Figure 2. Silencing and activation of the *bgl* promoter.** c) The *wt bgl* promoter ($P_{bgl}$) is silenced in context of the upstream and downstream silencer sequences (gray bars). The CRP binding site (CRP), terminator *t1* and the first gene *bglG* are shown. According to the nucleoprotein repressor complex model (Schnetz, 1995; Caramel and Schnetz, 1998), H-NS (black ovals) binds the upstream AT-rich sequence and presumably promotes the formation of a silencing nucleoprotein complex (white ovals) involving Fis (black quadrangles) and possibly other unknown cellular factors. Spontaneous mutations in *cis* which can relieve silencing include insertions (IS1 and IS5) in the silencers, deletions of the silencers (Δ), or point mutations like CRP binding site mutation *C234* which improves the CRP binding site. Numbers indicate nucleotide position relative to transcription start (Reynolds et al., 1986; Lopilato and Wright, 1990; Schnetz and Rak, 1992; Schnetz, 1995; Singh et al., 1995).

stimulation, see below) may form a part of this complex (Caramel and Schnetz, 2000). Furthermore, it was reported that *in vitro*, the *wt bgl* promoter is active (Schnetz and Wang, 1996). It could be, like its activated alleles (the improved CRP binding site mutant and the allele activated by partial deletion of the upstream silencer), transcribed in single round transcription assays by RNA polymerase from negatively supercoiled templates. In this case the transcription was CRP independent. When relaxed DNA templates were used, *in vitro* transcription became CRP-cAMP dependent but the levels of transcription from the *wt* and the active promoters were similar. H-NS rendered both the *wt* and the activated promoters CRP-dependent. Further addition of crude cell extracts was necessary for specific repression of the *wt bgl* promoter in the presence of CRP-cAMP. The repression of the improved CRP binding site promoter allele by crude cell extracts was reversible by addition of CRP-cAMP. Thus, it was postulated that H-NS forms a essential part of the repressing nucleoprotein complex but cellular factors in addition to H-NS are required for formation of the complex.

## 3. Regulation of the BglG activity.

The key element in the substrate-specific regulation is the antiterminator protein BglG encoded by the first gene of the operon (Prasad and Schaefler, 1974; Mahadevan and Wright, 1987; Mahadevan et al., 1987; Schnetz et al., 1987; Schnetz and Rak, 1988). BglG mediates specific antitermination at two rho-independent terminators, located in the leader of the operon and in the inter-cistronic region between the *bglG* and the *bglF* genes, respectively (Mahadevan and Wright, 1987; Schnetz et al., 1987; Schnetz and Rak, 1988). Active BglG dimer binds to the mRNA at a BglG-binding motif which forms alternatively to and overlapping with the transcriptional terminator *t1* loop and prevents formation of the latter (Houman et al., 1990; Aymerich and Steinmetz, 1992; Amster-Choder and Wright, 1992). Thus, BglG binding mediated prevention of the terminator loop formation allows transcriptional readthrough or antitermination (Houman et al., 1990; Aymerich and Steinmetz, 1992) (figure 3). BglG also binds to a similar target sequence in the second terminator *bgl t2* region (Amster-Choder and Wright, 1993). BglG activity is controlled by reversible phosphorylation both negatively and positively in response to the availability of the specific substrate and the catabolic state of the cell, respectively. In the absence of β-glucosides, the sugar-specific permease enzymeII$^{Bgl}$, which is encoded by the

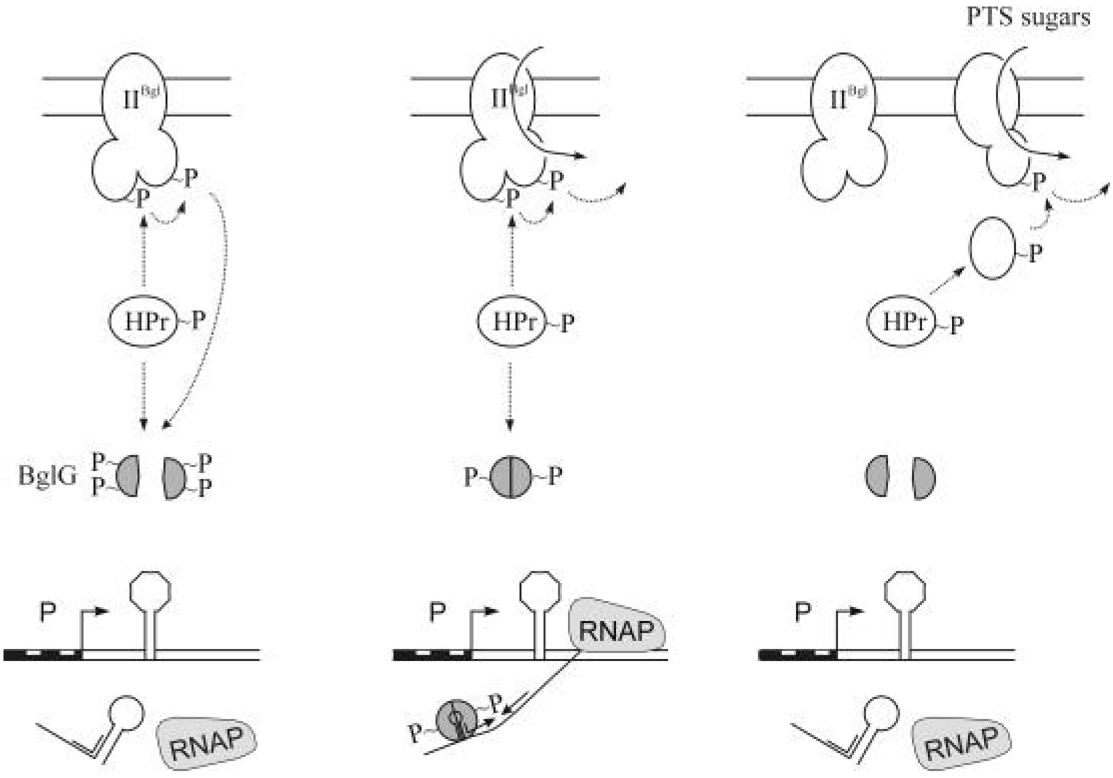


**Figure 3.** b**-glucoside specific regulation of the *bgl* operon.** Left, in the absence of β–glucosides, the transport protein II$^{Bgl}$ is phosphorylated (~P) by Hpr, a component of the PTS system. This phosphate is transferred to cause phosphorylation of the BglG protein (gray semicircles) and thus inactivating the latter. Hpr also phosphorylates BglG directly. Center, in the presence of β-glucosides, the transport protein II$^{Bgl}$ transfers the phosphate to the incoming β-glucosides and not to BglG. This form of BglG, phosphorylated only by Hpr is active, it dimerizes and binds to the BglG binding mRNA motif overlapping with the *t1* loop and causes antitermination. Right, in the presence of excess of β-glucosides or other PTS sugars, all the phosphate is drained away from Hpr, and BglG becomes totally unphosphorylated and inactive (Mahadevan and Wright, 1987; Schnetz et al., 1987; Schnetz and Rak, 1988; Amster-Choder et al., 1989; Schnetz and Rak, 1990; Amster-Choder and Wright, 1992; Görke and Rak, 1999). Figure outline modified according to (Görke and Rak, 1999)

second gene *bglF* of the operon, catalyzes phosphorylation of BglG which presumably prevents its dimerization to the active form (Schnetz and Rak, 1988; Amster-Choder et al., 1989; Amster-Choder and Wright, 1990; Schnetz and Rak, 1990; Amster-Choder and Wright, 1992). In the presence of β-glucosides and absence of other PTS-carbohydrates, BglG is phosphorylated at a second site and thereby activated by HPr (Görke and Rak, 1999). HPr is a main component of the phospho-*enol*-pyruvate-dependent phosphotransfer system (PTS) that mediates and controls the uptake and fermentation of carbohydrates (figure 3) (Postma et al., 1993; Saier and Reizer, 1994).

## 4. Factors involved in regulation of the *bgl* operon.

Besides the positive regulator BglG, no specific regulators of the *bgl* operon are known. Proteins involved in regulation of the *bgl* operon which were known previously or were discovered during the course of this work are briefly described below. All these proteins are non-specific or pleiotropic regulators of *E. coli* gene expression.

**H-NS:** This is a small (15.4kDa) histone like protein involved in structuring of the *E. coli* chromsome´s higher order structure called the nucleoid (Drlica and Rouvière-Yaniv, 1987; Tupper et al., 1994; Williams and Rimsky, 1997; Dorman et al., 1999). H-NS can bind non-specifically to DNA with a preference to AT-rich and curved sequences (Bracco et al., 1989; Yamada et al., 1991; Owen-Hughes et al., 1992; Tupper et al., 1994; Zuber et al., 1994). H-NS binding has been shown to compact DNA (Spassky et al., 1984; Spurio et al., 1992), change DNA topology (Higgins et al., 1988; Owen-Hughes et al., 1992; Mojica and Higgins, 1997) and to constrain negative DNA supercoils *in vitro* (Tupper et al., 1994). Besides its structural function, it is known to be involved in regulation of various genes and operons besides the *bgl* operon, *eg* CFA/I fimbrial operon (Jordi et al., 1992), *proU* which encodes an osmo-protective glycine-betaine system (Higgins et al., 1988; Lucht et al., 1994; Fletcher and Csonka, 1995) and the rRNA operon *rrnB* (Tippner et al., 1994; Afflerbach et al., 1998). H-NS regulates the *hns* gene itself (Dersch et al., 1993; Ueguchi et al., 1993; Falconi et al., 1993). In most cases, H-NS inhibits the transcription of its target genes by either changes in the DNA topology and/or by preventing RNA polymerase or transcriptional activators from accessing the DNA (Higgins et al., 1988). H-NS regulated genes *eg* those encoding virulence factors, pathogenic determinants, etc. often respond to environmental signals such as temperature, pH, osmolarity and Oxygen availability (Atlung and Ingmer, 1997). H-NS mediated regulation of the *bgl* operon may also be subject to environmental perturbations. It was shown (Khan and Isaacson, 1998) that the *bgl* operon may be activated in pathogenic *E. coli in vivo* (inside the host) where specifically activating environmental conditions may exist.

**Fis:** Factor for inversion stimulation (Fis) was discovered as a protein that stimulates site specific DNA inversion (Johnson and Simon, 1985; Kahmann et al., 1985; Huber et al., 1985). It is a homodimer of 11.2 kDa subunits. It can bind to and bend DNA (Finkel and Johnson, 1992). Fis expression is growth rate regulated and dramatically increases for a short period in the early exponential growth phase in a rich medium (Ball et al., 1992; Nilsson et al., 1992; Ninnemann et al., 1992). Fis was shown to bind *in vitro* to the *bgl* upstream silencer and promoter regions, it could compete *in vitro* with CRP for binding to the *wt bgl* promoter but not with RNA polymerase. It was also shown that Fis can prevent *in vitro* transcription from a *wt bgl* promoter (Caramel and Schnetz, 2000). Fis is known to be involved in various other processes like regulation of DNA replication at *oriC* (Gille et al., 1991; Filutowicz et al., 1992), phage λ excision (Ball and Johnson, 1991), and modulation of DNA topology (Schneider et al., 1997). Fis stimulates transcription of stable RNA promoters (Nilsson et al., 1990; Ross et al., 1990) and regulates a set of RpoS dependent genes including *aldB*, *proP*, *glnQ*, *mglA*, *xylF* and *sdhA* (Xu and Johnson, 1995a; Xu and Johnson, 1995b). Transcription of the *fis* gene itself is subject to auto-regulation (Ninnemann et al., 1992). Fis regulates the *crp* gene encoding the catabolite regulator protein (González-Gil et al., 1998), *hns* gene (Falconi et al., 1996) and the *hupA* and *hupB* genes encoding HU (Claret and Rouvière-Yaniv, 1996).

**StpA:** It is 67% similar to the H-NS protein (Johansson and Uhlin, 1999) and can functionally substitute H-NS in several cases, although it displays unique properties of its own (Zhang and Belfort, 1992; Zhang et al., 1996; Sonden and Uhlin, 1996). StpA has RNA chaperone activity (Zhang et al., 1995) and it can also bind DNA (Sonnenfield et al., 2001). Expression of StpA is negatively regulated by H-NS but stimulated by the leucine-responsive protein Lrp (Sonden and Uhlin, 1996). The StpA and H-NS proteins can form heteromers (Williams et al., 1996). In one study where the H-NS protein was truncated for its C-terminal DNA binding domain, it was found that StpA is required for *bgl* repression and was proposed to act as an adapter for binding of the truncated H-NS to DNA (Free et al., 1998), while in another analysis RpoS has been identified to be required for silencing by the truncated H-NS protein and the involvement of StpA was not confirmed (Ohta et al., 1999). StpA has also been reported to have a negative effect on *bgl* expression in a *wt hns* background (Free et al., 1998). It was shown that StpA is susceptible to proteolysis by the Lon protease in the absence of H-NS but was stable in the presence of H-NS. (Johansson and Uhlin, 1999; Johansson et al., 2001).

**Lon:** It is a ATP-dependent protease and Lon monomer consists of an ATPase domain and a proteolytic domain. It forms ring-like multiple layered polymers which are the active forms of the protein (Gottesman, 1996; Wickner et al., 1999). Lon expression is upregulated on heat shock and starvation stress where it is involved in the proteolysis of inactivated or misfolded proteins (Baker et al., 1984; Goff et al., 1984; Phillips et al., 1984; Kuroda et al., 2001). Lon specifically degrades proteins like cell division inhibitor SulA (Goff et al., 1984), the F-plasmid dependent protective protein CcdA (Van Melderen et al., 1994), the λ phage life cycle regulatory proteins N and Xis (Maurizi, 1987; Leffers and Gottesman, 1998), the DNA damage checkpoint effector, UmuD protein (Diez-Gonzalez et al., 1998) and RcsA which regulates capsular polysaccharide synthesis (Stout et al., 1991). As described above, Lon degrades StpA protein in the absence of H-NS (Johansson and Uhlin, 1999). Lon has been reported to bind DNA specifically (Fu et al., 1997).

**Hfq:** Host factor for Qβ phage replication is a abundant 15kDa protein and is required for replication of the RNA genome of the Qβ (August and Shapiro, 1968; Franze de Fernandez et al., 1968; Franze de Fernandez et al., 1972). Hfq functions by destabilizing an RNA secondary structure on the 3' end of the positive strand of Qβ RNA. In addition Hfq binds poly(A) RNA (Carmichael, 1975; Senear and Steitz, 1976) and *oxyS* RNA (Zhang et al., 1998). Several mRNAs in *E. coli* are targeted for degradation by Hfq, possibly by increasing polyadenylation (Hajnsdorf and Regnier, 2000) or by interfering with the ribosome binding (Vytvytska et al., 2000). Efficient translation of RpoS in *E. coli* (Muffler et al., 1996) and *Salmonella typhimurium* (Brown and Elliott, 1996) requires Hfq. The mRNAs of *E. coli* genes *ompA*, *miaA*, *mutS* and *hfq* are destabilized by Hfq (Tsui et al., 1997; Vytvytska et al., 1998). The Hfq protein can be copurified with H-NS and overexpression or mutation of Hfq can mask some *hns* phenotypes (Shi and Bennett, 1994).

**RpoS:** It is the alternative sigma factor of *E. coli* RNA polymerase and is required for transcription of many genes expressed during the onset of stationary growth phase

(Loewen and Hengge-Aronis, 1994). RpoS is also required for expression of genes involved in adaptation to stresses like starvation, high osmolarity, heat shock, peroxide stress, and UV exposure (Hengge-Aronis, 1993). RpoS expression is regulated at multiple levels and Hfq is required for translation of the *rpoS* mRNA. As described before, RpoS was reported to be necessary for silencing of the *bgl* operon by truncated H-NS.

## 5. Open questions and possible approaches.

What additional factors besides H-NS are required for formation of the proposed silencing nucleoprotein complex?

They could presumably bind to the silencer/*bgl* DNA sequences and thus could be purified based on this presumed *bgl* DNA binding property and identified. Another possible approach to identify factors involved in *bgl* silencing was a genetic one. Random mutagenesis screens could be performed and mutants defective in *bgl* silencing i.e. those in which the *wt bgl* operon is expressed could be identified using a suitable selection strategy. Also a reverse screen could be carried out in which mutations causing downregulation of an active *bgl* operon could be identified.

It has been shown that H-NS can specifically bind to the upstream *bgl* silencer DNA (Wahle and Schnetz, unpublished results). This supports the role of the upstream silencer in formation of the repressor complex. However, H-NS can not specifically bind to the downstream silencer (Wahle and Schnetz, unpublished results). What is then the mechanism of silencing by the downstream silencer?

It is possible that H-NS binds specifically to the upstream silencer along with other unknown factors and this repressor complex may "spread over" on to the downstream regions. However other mechanisms of downstream silencer mediated silencing are possible.

Regulation of the *bgl* operon expression was usually studied using plasmidic reporter gene constructs for quantitative analysis. It is possible that the *bgl* operon regulation on multicopy plasmids is different than that on the chromosome (natural situation) *eg* due to limiting levels of positive or negative regulators.

In order to assess this possibility expression on plasmidic and chromosomal systems could be compared.

## 6. Aim of the thesis

The aim of this thesis was to study following aspects of the *Escherichia coli bgl* operon regulation

- Identification of cellular factors involved in the *bgl* operon regulation in addition to H-NS and to study their role.
- Analysis of the role of the silencer sequence downstream of the *bgl* promoter.

# III. Results

## 1. Biochemical characterisation of proteins binding to the upstream silencer region of the *bgl* operon.

According to the silencing nucleoprotein complex model (Schnetz, 1995; Schnetz and Wang, 1996; Caramel and Schnetz, 1998) the H-NS protein is an essential component of the repressing complex. However *in vitro* data indicated that H-NS is not sufficient to repress the *wt bgl* promoter (Schnetz and Wang, 1996). Additional cellular components were required to specifically prevent transcription from the *wt bgl* promoter *in vitro* (Schnetz, 1995). To characterize *E. coli* proteins which in addition to H-NS may bind to the upstream *bgl* silencer a biochemical approach was taken.

The principle of the method based on DNA affinity chromatography is shown in figure 4. Biotinylated DNA fragment including the upstream silencer region

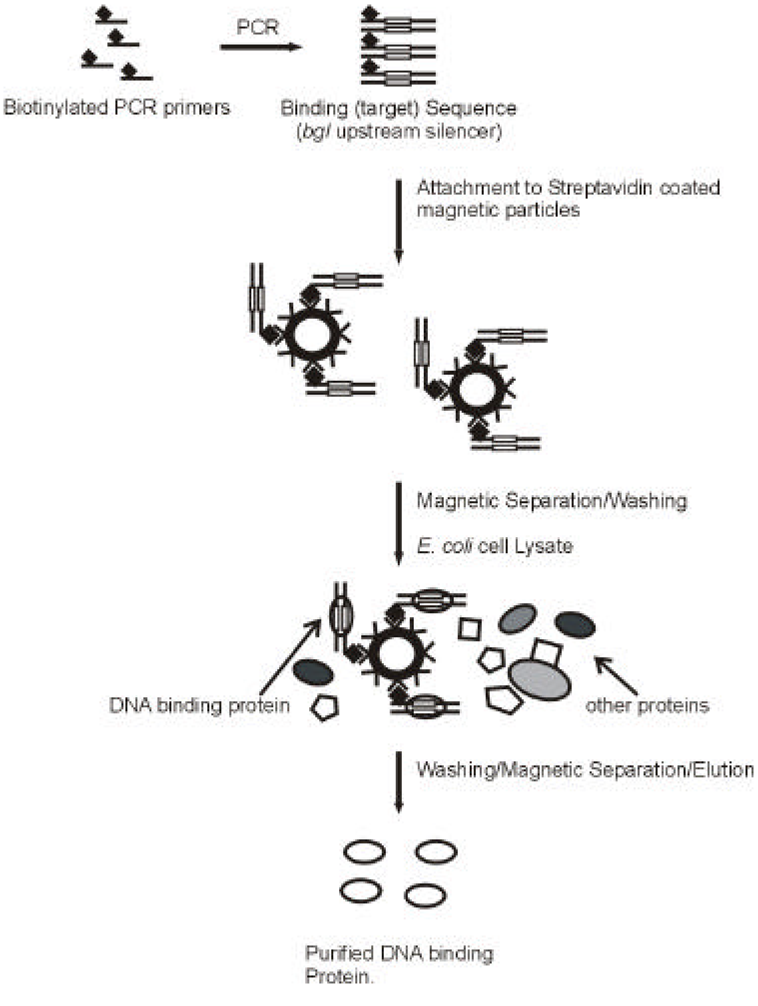


**Figure 4: Principle of DNA affinity chromatography**

corresponding to the *bgl* operon nucleotide positions –191 to +79 (with the transcriptional start site as +1) was used as a "bait" for protein binding. As a negative control a similarly sized biotinylated DNA fragment corresponding to the *lacZ* gene was used (figure 5). These fragments were then allowed to bind to Streptavidin coated magnetic beads. After washing off unbound DNA, cell extracts made from *wt* (S541 = $\Delta$*bgl*-AC11 (*gpt-lac*)$^{+}$ ) or *hns* cells (S102 = *hns::Ap*$^{R}$) were added. In another experiment the cell extracts used were pre-purified and concentrated for DNA-binding proteins, by binding to phosphocellulose and washing off the non-binding proteins. After allowing the cellular proteins to bind to the DNA attached to the magnetic beads, unbound and nonspecifically bound proteins were washed off. The specifically bound proteins were subsequently eluted using a high salt (700mM KCl) containing buffer (see Materials and Methods for details). These eluted proteins were then precipitated with trichloroacetic acid, resuspended and separated on SDS-PAGE gels. The gels were silver stained. Specific bands (proteins) which bound only the *bgl* DNA but not the control *lacZ* DNA were cut out, in gel digested with Trypsin and the resulting peptides were analysed using MALDI-TOF mass spectroscopy in cooperation with the service laboratory at Cologne molecular medicine center. The resulting peptide mass data was then analysed using the Profound tool at the Prowl server (http://prowl.rockefeller.edu/) to identify the corresponding *E. coli* protein.

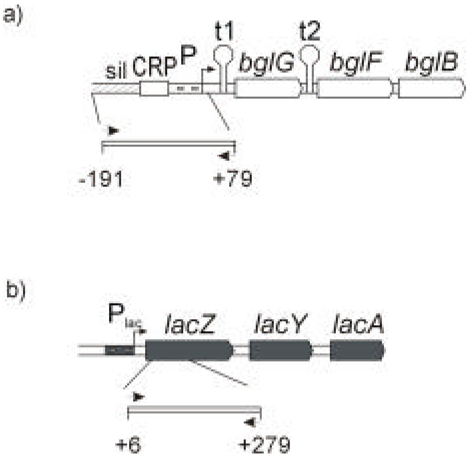


**Figure 5: Schematic representation of the *bgl* and *lacZ* DNA fragments used in the affinity chromatography as "baits".** Numbers indicate nucleotide position with +1 indicating transcription start site for *bgl* (a) and first nucleotide of the structural gene for *lacZ* (b).

A specific protein band of approximate molecular weight 16 kDa (figure 6, lane 2, black arrow) that could bind only the *bgl* DNA was identified as H-NS protein of *E. coli.* Additional minor differential bands (arrowheads in figure 6) were not

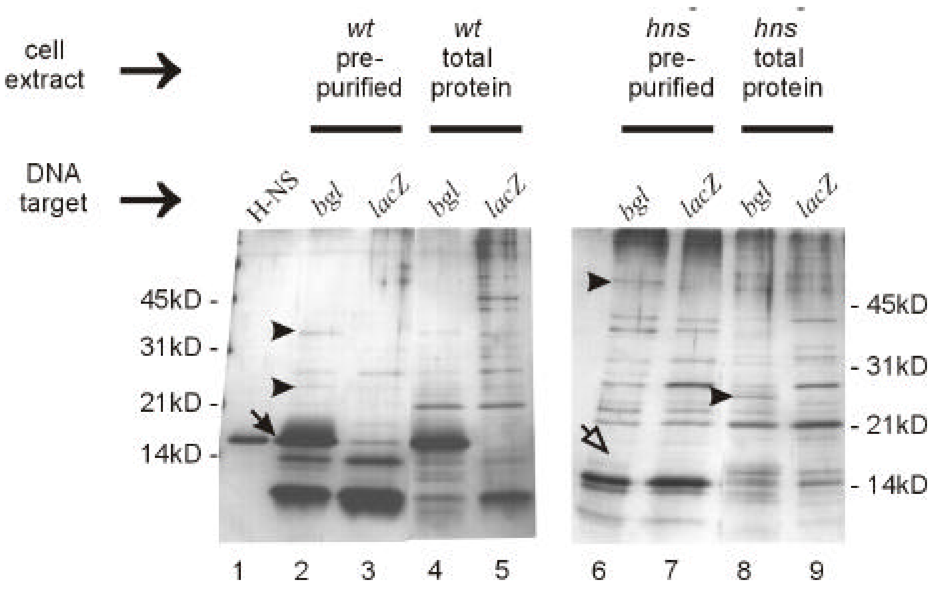


**Figure 6: H-NS binds to *bgl* DNA.** Proteins purified by the DNA affinity chromatography were separated by SDS-PAGE and the gel was silver stained. Cell extracts used were made either from *wt* (S541) or *hns* (S102) strains and either used directly (total protein) or were prepurified over phosphocellulose as described in the text. The DNA "baits" used were *bgl* (lanes 2, 4, 6 and 8) or negative control *lacZ* (lanes 3, 5, 7 and 9). Position of the H-NS band is indicated (black arrow, lane 2) which is missing in lane 6 (white arrow). Pure H-NS protein was loaded in lane 1. Positions of the molecular weight markers are indicated.

reproducible. The 16 KDa H-NS band was observed to be binding the *bgl* fragment both from total and phosphocellulose prepurified *wt* cell extracts. On SDS-PAGE gels this band was found to run at exactly the same position as that of pure H-NS (figure 6, lane 1). The H-NS band was absent in the negative control with *lacZ* DNA fragment (figure 6, lanes 3 and 5). Additionally, the 16 kDa H-NS band was also absent when cell extracts made from a *hns*$^-$ *E. coli* strain were allowed to bind the *bgl* DNA (*eg* figure 6, lane 6, white arrow). Thus, it was confirmed that H-NS specifially binds to the *bgl* region used in this experiment. However, no additional proteins could be reproducibly identified that bind specifically to this *bgl* region. Possible reasons for this result are that

- the additional proteins binding to the *bgl* DNA may bind outside of the fragment used in this experiment. Downstream sequences contribute to *bgl* silencing *in vivo.*
- the *in vitro* conditions of protein concentration, salt concentration, temperature and pH used in this experiment may not reflect those found *in vivo* and therefore some proteins did not bind the DNA *in vitro*.

- the unknown proteins may bind the *bgl* DNA with a weaker affinity or may form a stoicheometrically minor part of the repressing nucleoprotein complex and thus could not be purified in concentrations sufficient for detection.
- some proteins involved in *bgl* regulation, *eg* FIS are growth phase regulated and may be present in insignificant amounts in the cell extracts used.
- only H-NS protein binds to the *bgl* DNA fragment tested and the requirement of additional cellular factors for *in vitro* repression of the *bgl* promoter has alternative reasons. This possibility is further substantiated in following sections.

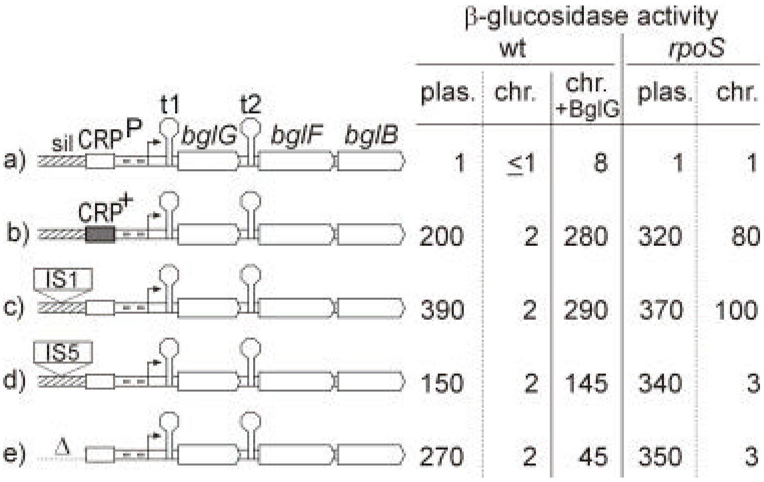
## 2. Silencing and activation of the *Escherichia coli bgl* operon are amplified by the limitation of BglG-mediated antitermination at low transcription rates.

(This section, in part, is in revision for publication to Molecular Microbiology. Some of the experiments in figures 7 and 11 were carried out by Sandra Kühn.)

Regulation of the *bgl* operon has been studied quantitatively, mostly using plasmidic constructs. It is possible that regulation of the *bgl* operon on multicopy plasmids is different compared to that on the chromosome (natural situation) *eg* due to limiting levels of regulatory factors. To asses this possibility, the *bgl* operon expression was studied using a chromosomal system and compared with regulation of the same constructs on the plasmids. The chromosomal system consisted of either the natural *bgl* operon constructs or strains in which the relevant constructs were integrated in to the chromosomal phage λ attachment site *attB* mediated by the λ *attP* sequence as described in materials and methods (Diederich et al., 1992).

### 2.1 Low activation of the chromosomal *bgl* operon .

Expression levels of the *wt bgl* operon and of a set of activated derivatives were determined when encoded on the chromosome or on plasmids (figure 7). The activated *bgl* operon derivatives included alleles that carries a point mutation improving the CRP-binding site (figure 7b, exchange C-66T), an integration of IS1 (figure 7c), an integration of IS5 (figure 7d), and a deletion



| | β-glucosidase activity | | | | |
|---|---|---|---|---|---|
| | wt | | | rpoS | |
| | plas. | chr. | chr. +BglG | plas. | chr. |
| a) | 1 | ≤1 | 8 | 1 | 1 |
| b) | 200 | 2 | 280 | 320 | 80 |
| c) | 390 | 2 | 290 | 370 | 100 |
| d) | 150 | 2 | 145 | 340 | 3 |
| e) | 270 | 2 | 45 | 350 | 3 |

**Figure 7: The expression of chromosomally encoded, activated *bgl* operon derivatives is down-regulated by limitation of BglG and by RpoS.** Phospho-β-glucosidase activities (encoded by *bglB*) directed by (a) the wild-type *bgl* operon and (b to e) activated derivatives were determined when encoded on plasmids (p15A origin of replication, (Chang and Cohen, 1978)) (plas.) or on the chromosome (chr.). Cultures were grown in minimal glycerol medium containing the β-glucoside salicin. To provide anti-terminator BglG *in trans* (+BglG) transformants of plasmid pKESK10 were grown in medium containing in addition IPTG. Strains S541 (Δ*bgl*) and S887 (Δ*bgl*, *rpoS*) were transformed with plasmids (a) pFDX733 (Schnetz et al., 1987), and its Bgl$^+$ derivatives (b) pFDX733-C234 (Schnetz and Rak, 1992), (c) pKESD62 (*bgl::IS1*-R1243), (d) pFDX733-H3 (Schnetz and Rak, 1992), and (e) pFDY446 (*bgl-D2*). Chromosomal strains used were (a) S524 (b) S544, (c) S1245 (d) S1365, (e) S1367, as well as the respective *rpoS* mutants (a) S1071, (b) S1079, (c) S1250, (d) S1384, and (e) S1286.

of the upstream silencer (figure 7e), respectively. The chromosomal *wt bgl* operon and two of the activated derivatives (improved CRP-binding site and integration of IS1, figure 7 b and c) map at the natural locus. The alleles that are activated by integration of IS5 (figure 7d) and by deletion of the upstream silencer (figure 7e) were integrated in to the chromosomal phage lambda attachment site *attB* of strain S541 (relevant genotype is Δ*bgl*, Δ*lacZ*) (figure 7, chr.). Expression levels of plasmid encoded *bgl* operon alleles were determined of transformants of strain S541 (figure 7, plas.).

The *wt bgl* operon expressed low to undetectable levels of phospho-β-glucosidase activity both when plasmid or chromosomally encoded (figure 7a, 1 unit and ≤1unit). Activation of the *bgl* promoter caused a strong increase (>100 fold) in the expression level when plasmid encoded (150 to 390 units, Figure 7 plas.), as reported previously (Reynolds et al., 1981; Schnetz et al., 1987; Schnetz and Rak, 1988; Lopilato and Wright, 1990; Schnetz and Rak, 1992; Schnetz, 1995). Unexpectedly, in the chromosomal system the expression levels of the activated *bgl* operon derivatives were low (2 units) (figure 7 b to e, chr.).

## 2.2 Construction of a *bgl-lacZ* reporter system

To be independent of the β-glucoside specific negative regulation of the anti-terminator BglG by enzymeII$^{Bgl}$, *bgl-lacZ* reporter gene constructs were constructed (figure 8). The *lacZ* gene was fused 3' to the *bglG* gene of the *wt bgl* operon and of activated derivatives. Again expression levels were determined when encoded on

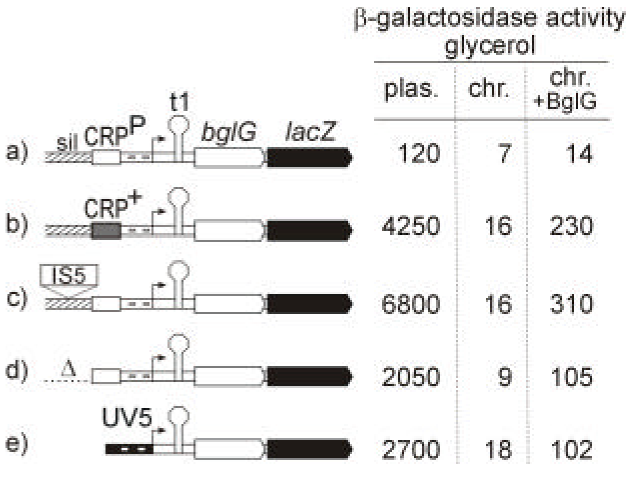


**Figure 8: *bgl-lacZ* reporter constructs are expressed at low levels when encoded on the chromosome due to limitation of BglG-mediated antitermination**. The β-galactosidase activity directed by *bgl-lacZ* reporter constructs carrying the (a) silent wild-type *bgl* promoter, (b to d) activated *bgl* promoter derivatives, or the constitutive *lacUV5* promoter (e), were determined when encoded on plasmids (plas.) or when integrated in to the *attB*–site (chr.). BglG (+BglG) was provided *in trans* by transformation with plasmid pKESK10 and induction of *bglG* by IPTG. Cultures were grown in minimal M9 glycerol medium. Tested were (plas.) transformants of S541 with (a) pKESD8, (b) pKESD9, (c) pKESD12, (d) pKESD11, and (e) pKESD20 and (chr.) strains carrying integrations of the identical reporter constructs in to *attB* (a) S940, (b) S1052, (c) S1054, (d) S942, and (e) S1095.

plasmids (figure 8, plas.) and after integration in to the chromosomal *attB*-site (figure 8, chr.).

In the plasmidic system, the expression of the *bgl-lacZ* derivatives directed by activated *bgl* promoter alleles was significantly increased (17 to 57 fold, 2050 to 6800 units) in comparison to the *wt bgl* promoter (120 units) (figure 8, compare a to b-d, plas.). However, when these *bgl-lacZ* reporter constructs were integrated in to the chromosome the expression levels directed by the activated promoter alleles were again unexpectedly low (9 to 16 units) as compared to the *wt bgl* promoter (7 units) (figure 8, chr.).

### 2.3 Low chromosomal expression is not *bgl* promoter-specific

To test whether low chromosomal expression may be caused by a down-regulation of the de-repressed *bgl* promoter alleles in the chromosomal environment, a *lacUV5* promoter lacking the *lac* operator was introduced. This constitutive *lacUV5* promoter (from position -40 to +1) was fused to the transcription start of the *bgl* operon (position +1). In the plasmidic system, the *lacUV5* promoter directed about 2700 units of β-galactosidase activity (figure 8e, plas.). However, when this *lacUV5-bglt1-bglG-lacZ* construct was integrated in to the chromosome the β-galactosidase activity was again very low (18 units) (figure 8e, chr.). Thus, low chromosomal expression was found to be not promoter-specific.

### 2.4 Antiterminator BglG is limiting to expression when encoded on the chromosome.

The possible reason for high levels of activation on plasmids but not on the chromosome may be that in the plasmidic system a negative regulator of *bgl* expression becomes limiting. Alternatively, in the chromosomal system a positive regulator, encoded by the *bgl* operon itself may become limiting due a reduction in gene dosage. The obvious candidate for the latter possibility was the antiterminator protein, BglG. *Eg* BglG is required for antitermination at terminator *bgl-t1* in the *lacUV5-bglt1-bglG-lacZ* construct (figure 8e). To test whether BglG is limiting to expression of the activated *bgl* operon derivatives and *bgl-lacZ* reporter constructs when encoded on the chromosome, a low copy plasmid pKESK10 (a pSC101 derivative) was introduced. This plasmid encodes the *bglG* gene under control of the *lacUV5-lac* operator promoter. In addition, plasmid pKESK10 carries the *lacI* gene to allow regulation of plasmid encoded *bglG* expression which can be induced by IPTG addition.

The introduction of pKESK10 and induction by IPTG of *bglG* expression *in trans* had little effect on the expression level of the chromosomally encoded *bgl-lacZ* construct carrying the *wt bgl* promoter (figure 8a, +BglG). Interestingly, the expression of the *wt bgl* operon (figure 7a, +BglG) increased to 8 units, i.e. to a value that is higher than the activity of the de-repressed *bgl* derivatives in the absence of extra BglG protein (figure 7b to e, chr.). Furthermore, providing BglG *in trans* led to a strong increase (20 to 140 fold) in the expression levels of activated *bgl* operon derivatives (figure 7b to e, +BglG). In contrast, the expression levels of the plasmid encoded activated *bgl* operon derivatives increased less than 1.5 fold when BglG was provided *in trans* (unpublished lab results). Likewise, the expression levels of the chromosomally encoded *bgl-lacZ* reporter constructs carrying de-repressed *bgl* promoter alleles or the constitutive *lacUV5* promoter increased significantly (5 to 20 fold) when pKESK10 encoded *bglG* expression was induced (figure 8b to e, +BglG). Similar results were obtained when BglG was expressed at higher levels *in trans* using a high copy plasmid, demonstrating that BglG levels provided by plasmid pKESK10 are saturating (unpublished lab results). These data show that the amounts of BglG synthesized by the de-repressed *bgl* operon and *bgl-lacZ* reporter constructs is limiting for expression when these are encoded on the chromosome.

## 2.5 Mutation of the *bgl* terminator *t1* to render expression BglG independent results in high chromosomal expression levels.

To provide further evidence that BglG-mediated antitermination is limiting to expression, the terminator *t1* was mutated in the *bgl-lacZ* reporter constructs (figure 9 and 10). Antitermination by BglG is mediated by specific binding of BglG to a secondary RNA structure that overlaps and forms alternatively to the terminator stem-loop structure, thus preventing termination (figure 9) (Houman et al., 1990; Aymerich and Steinmetz, 1992). Four site-specific mutants of terminator *t1* were constructed. Mutation *DG100* was described previously to render *bgl* operon expression constitutive and was used as a control (Mahadevan and Wright, 1987). In addition, a terminator mutant with three base exchanges in the left stem of the terminator hairpin

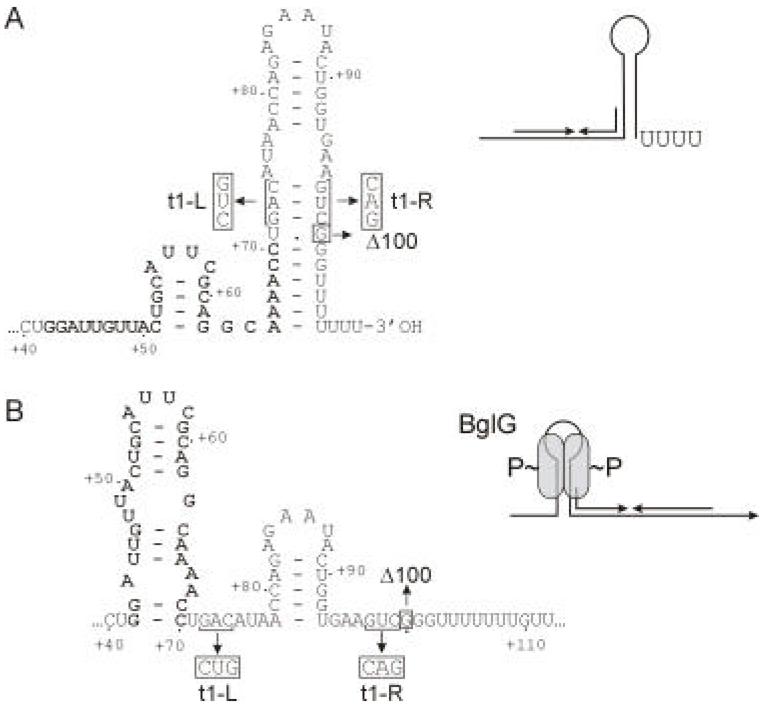


**Figure 9: Terminator *bgl-t1* and mechanism of antitermination by BglG.** Shown are the secondary structures and schemes of the rho-independent terminator *t1* (A) and the mode of antitermination by BglG (B) (Mahadevan and Wright, 1987; Schnetz et al., 1987). A: Mutation Δ*G100* confers a constitutive phenotype and is likely to disrupt the terminator (Mahadevan and Wright, 1987). Mutations *t1-L* and *t1-R* disrupt terminator *t1* (see Figure 10). These mutations complement each other and in the double mutant (*t1-LR*) the termination is restored (see Figure 10). B: Antiterminator protein BglG binds to an alternative secondary structure that forms alternatively to and the terminator loop and thus prevents termination (Houman et al., 1990).

(mutation *t1-L*), a mutant with three base exchanges in the right stem (*t1-R*), and the double mutant (*t1-LR*) in which the mutations in the left and right stem of the terminator compensate each other, were constructed. None of these mutations is expected to disrupt the BglG-binding motif (figure 9 b).

The effect of the mutation of terminator *t1* on chromosomal expression levels was tested using the *bgl-lacZ* reporter construct that carries the constitutive *lacUV5* promoter (figure 10). Terminator mutations *t1-L* (figure 10 b), *t1-R* (figure 10 c), and Δ*G100* (figure 10 e), resulted in high chromosomal expression levels (100 to 150 units). In these mutants providing BglG *in trans* did not lead to a significant further increase of in the β-galactosidase activity (figure 10 b, c, and e, +BglG). Expression

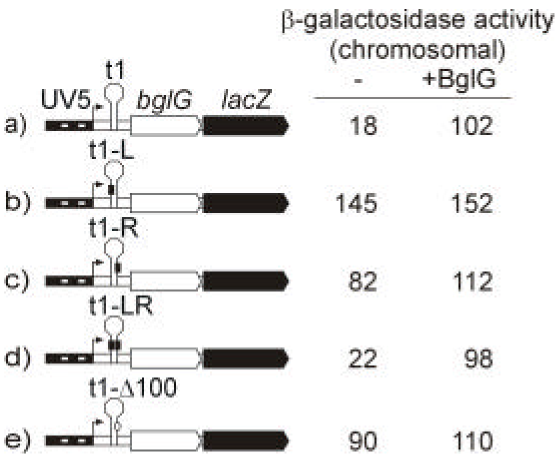


**Figure 10: Mutation of terminator *t1* results in high expression levels of chromosomal *lacUV5-bgl-lacZ* reporter constructs.** The effect of terminator mutations *t1-L*, *t1-R*, the double mutant *t1-LR*, and Δ*G100* was determined in β-galactosidase assays after the constructs were integrated in to the *attB*-site of strain S541 (Δ*bgl*, Δ*lacZ*). Cultures were grown in minimal glycerol medium. To test whether expression of *bglG in trans* increases expression, the strains were transformed with pKESK10 and grown in the presence of IPTG (+BglG). Tested were strains (a) S1095, (b) S1097, (c) S1099, (d) S1101, and (e) S1103.

of the double mutant (*t1-LR*) was low in the absence of additional BglG protein and induction of *bglG* expression *in trans* caused an increase of the expression level similar to the *wt* terminator construct (compare Figure 10 a and d). In case of terminator mutation *t1-L* (figure 10b) the β-galactosidase activity (150 units) was ~1.5 fold higher than in case of the other constructs (figure 10). This small effect may relate to *eg* a changed RNA-stability and was not followed further.

## 2.6 A moderate (3-fold) effect of RpoS on the transcription rate can result in an up to 50 fold increased expression levels of activated *bgl* operon derivatives

Silencing of the *bgl* operon requires H-NS, as an essential component, and may involve StpA, Hfq, RpoS, and Fis (see introduction). The effect of RpoS (using a *rpoS359*::Tn10 mutant) was tested on expression of the *bgl* operon (figure 7) and of the BglG-independent *bgl-lacZ* reporter constructs carrying the mutated terminator *t1-L* (figure 11). The expression levels of the chromosomal *bgl* operon derivatives activated by an improved CRP-binding site and by integration of IS1, respectively, increased 40 to 50 fold in the *rpoS* mutant (figure 7b and c). The *wt* operon (as observed previously (Ohta et al., 1999) and derivatives activated by integration of IS5 or deletion of the upstream silencer remained low in the *rpoS* mutant (figure 7a, d and e). Interestingly, these two activated alleles were also expressed at significantly lower levels when BglG protein was provided *in trans* (figure 7d and e). RpoS had a minor effect (less than 1.5 fold) on the plasmid encoded *bgl* operon (figure 7). In contrast, the expression level of all BglG-independent chromosomal *bgl-lacZ* reporter constructs carrying the *wt* or activated promoters increased 2.5 to 3 fold (figure 11). RpoS had a similar

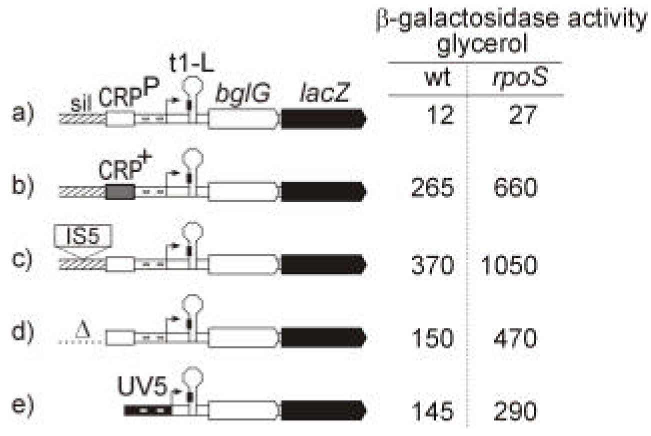


**Figure 11: BglG-independent *bgl-lacZ* reporter constructs are down-regulated 3 fold by RpoS.** The terminator mutation *t1-L* was introduced in to *bgl-lacZ* reporter constructs carrying the wild-type *bgl* promoter and activated promoter derivatives. β-galactosidase activities were determined in the wild-type strain background and a *rpoS359*::Tn10 mutant from cultures grown in minimal M9 glycerol medium. (a) S1142 and S1155 (= S1142 *rpoS359*::Tn10), (b) S1144 and S1157, (c) S1148 and S1161 and (d) S1146 and S1159.

effect on these *bgl-lacZ* reporter constructs when plasmid encoded (unpublished lab results). Thus, an approximately 3 fold contribution of RpoS to regulation of the *bgl* operon could be either amplified in to a strong (~50 fold) increase in the expression level of the *bgl* operon or remained undetectable if the initial transcriptional level was low. The *lacUV5* promoter *bgl-lacZ* construct was regulated two-fold by RpoS (figure 11e) indicating that RpoS affects the *bgl* operon indirectly and moderately at the *bgl* promoter.

In Glucose minimal medium, catabolite regulation of the CRP mediated *bgl* promoter was seen. Also, the catabolite control of BglG mediated antitermination was found to be significant in glucose medium as previously known (unpublished lab results) (Görke and Rak, 1999; Gulati and Mahadevan, 2000).

## 3. A mutagenesis screen to identify factors involved in regulation of the *bgl* operon.

Regulation of the *bgl* operon is known to occur via at least two phenomena, namely, silencing of the *bgl* promoter and substrate specific transcriptional termination/antitermination. According to the nucleoprotein repressor complex model, (Schnetz, 1995; Schnetz and Wang, 1996; Caramel and Schnetz, 1998). H-NS and possibly more unknown proteins are required for silencing of the *bgl* promoter. In order to identify these unknown additional proteins a genetic screening strategy based on random transposon (miniTn10) mediated mutagenesis was carried out as follows.

The mutagenesis screen was done in two ways. First mutagenesis was performed and mutants causing de-repression of the *wt bgl* operon were screened. Secondly, a reverse screen was performed for mutations which downregulated active *bgl* operon expression. To avoid mutations in *cis*, a double phenotype screen strategy was used. This strategy yielded mutants with double phenotypic change that have a in *trans* mutation.

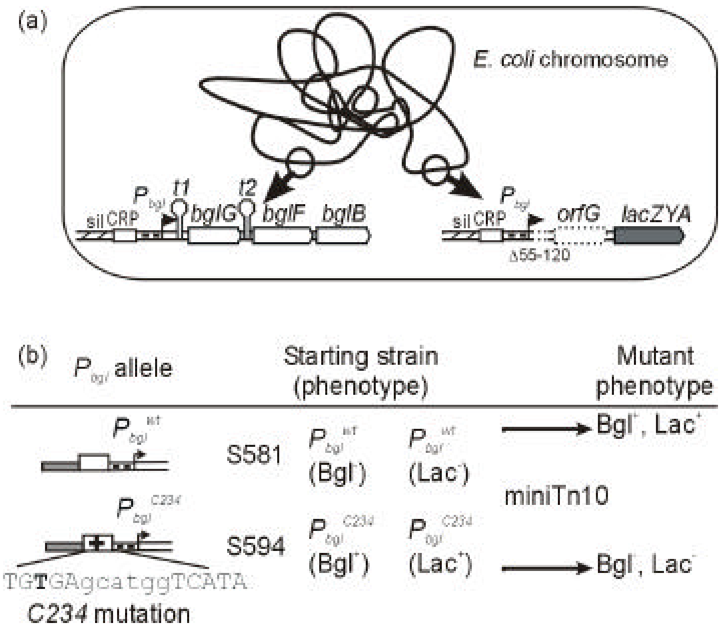


**Figure 12: Mutagenesis screen strategy** (a) The starting tester strain of *E. coli* has the natural *bgl* operon. It also has the *lac* promoter-operator region replaced by a cassette consisting of the upstream *bgl* silencer, the *bgl* promoter and the downstream silencer. In this reporter the *bgl* leader sequence is deleted for terminator *t1* (spanning the nucleotides +55 to +120 relative to the transcription start) and the *bglG* gene is mutated to *orfG*. (b) In one experiment a tester strain (S581) carrying the silent *wt bgl* promoter at both the positions (phenotype Bgl$^-$, Lac$^-$) was mutagenised and screened for a double phenotypic conversion to (Bgl$^+$, Lac$^+$). All such Tn10 insertion mutations mapped in the *hns* locus. In a reverse screen, a tester strain (S594) carrying an activated *bgl* promoter allele (allele *C234*) at both positions was mutated and screened for a phenotypic conversion from Bgl$^+$, Lac$^+$ to Bgl$^-$, Lac$^-$. This strategy yielded mutants carrying transposon insertions in the *pgi*, *lon* and *hfq* and *cyaA* loci.

For introducing mutations, a λ phage vehicle carrying a miniTn10 transposon with a tetracycline resistance marker was used (Kleckner et al., 1991). The λ phage vehicle contains nonsense mutation in the λ repressor gene and a deletion of the phage integration system. Thus, it can only introduce the miniTn10 in to the *E. coli* chromosome but can not enter the lytic cycle of growth nor can it become a prophage. The gene coding for the transposase enzyme required for Tn10 insertion in to the chromosome is encoded on sequences outside the miniTn10 itself and thus lost along with other λ sequences. Therefore the miniTn10 transposon can transpose in to the chromosome only once.

A starting tester strain of *E. coli* (S581) was constructed. This strain (figure 12) has the natural *wt bgl* operon ($Bgl^-$). It also has the *lac* promoter-operator region of the *lac* operon replaced by a cassette consisting of the upstream *bgl* silencer, the *bgl* promoter with the *bgl* leader sequence and the *bglG* gene. This *bgl* leader sequence has a deletion spanning the nucleotides +55 to +120 relative to the transcription start. Thus, the terminator *t1* is deleted. In addition, the start codon and two additional ATG (codon 3 and 27) of *bglG* are mutated (ATG to GCG). Therefore the *bglG* gene (*orfG*) can be transcribed but not translated. The fusion of the *wt* (silenced) *bgl* promoter renders the *lac* operon silent. Thus, the tester strain has a starting phenotype of $Bgl^-$ , $Lac^-$. After transposition, the tester strain mutants (Tetracycline resistant) were plated on to MacConkey Lactose Tetracycline plates and those mutants with a change in phenotype from $Lac^-$ (white colonies) to $Lac^+$ (red colonies) were purified and restreaked on BTB Salicin plates to score for the Bgl phenotype change from $Bgl^-$ (white colonies) to $Bgl^+$ (orange colonies). From a total of more than 50,000 mutants scored, 6 mutants showed a double phenotypic change from $Bgl^-$, $Lac^-$ to $Bgl^+$, $Lac^+$. The insertion position of miniTn10 transposon on the chromosome was determined by direct genomic sequencing using a miniTn10 specific primer (S156) or by sequencing of the PCR amplified mutated region. All the 6 mutants above were found to map in the *hns* locus. The insertion of miniTn10 in the *hns* locus presumably causes a disruption of the *hns* gene and absence of active H-NS in these cells leads to activation of the *bgl* promoter as known before (Higgins et al., 1988).

A reverse strategy was adopted to identify those genes required for the expression of the *bgl* operon when the promoter has been activated by a mutation in the *bgl* silencer region. For this experiment a double reporter tester strain (S594) was constructed. It was similar to the tester strain described above except that an activated allele of the *bgl* promoter was used both at the *bgl* operon and the *lac* operon loci. This *bgl* promoter allele has mutation C234 (see introduction), which improves the CRP binding site and thus derepresses the *bgl* promoter (figure 12). The starting phenotype of this strain was Bgl$^+$, Lac$^+$. It was mutagenised by random miniTn10 insertion and mutants with double phenotypic conversion to Bgl$^-$, Lac$^-$ were identified. A total of 16 such mutants were identified from more than 1,00,000 mutants screened. The insertion position of miniTn10 transposon on the chromosome was determined by direct genomic sequencing using a miniTn10 specific primer (S156) or by sequencing of the PCR amplified mutated region. The mutated/disrupted genes were (strain number)

- *cyaA* (1 mutant) : mutation number 152 (S756)
- *pgi* (5 mutants) : mutation numbers 111 (S751), 112 (S752), 123 (S753), 132 (S755), 194 (S765)
- *lon* (7 mutants) : mutation numbers 108 (S749), 124 (S754), 166 (S759), 172 (S760), 183 (S762), 187 (S764), 204 (S766)
- *miaA/hfq* (3 mutants) : mutation numbers 110 (S750), 155 (S757), 184 (S763)

The miniTn10 insertion positions in the *lon* and *miaA/hfq* loci are shown in figure 13.

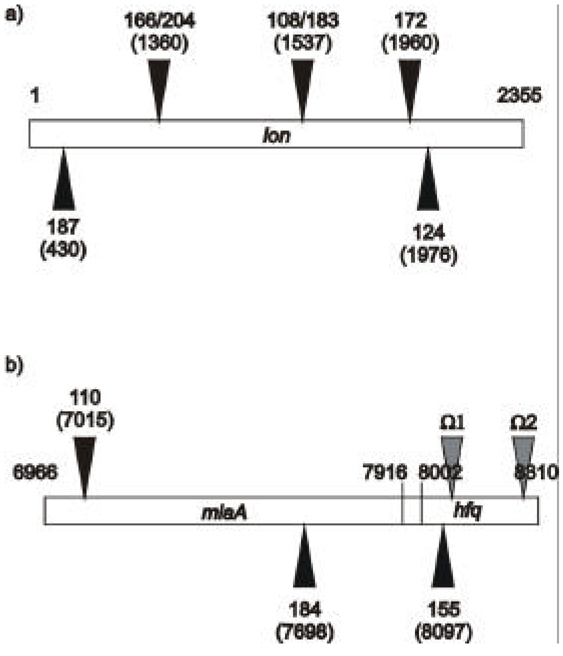
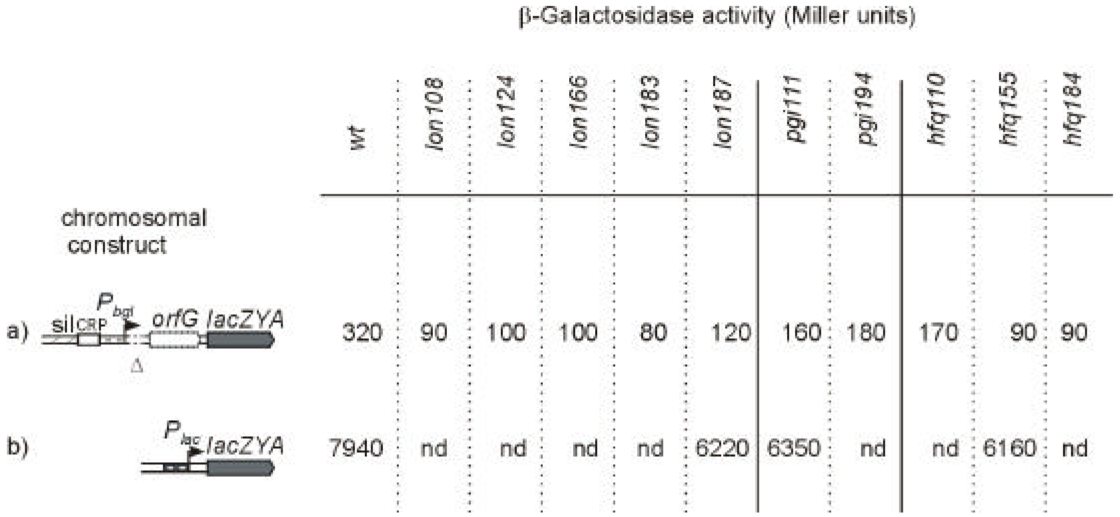


**Figure 13. The miniTn10 insertion positions in the *lon* and *miaA/hfq* loci.** Mutation numbers are indicated. Numbers in brackets indicate the miniTn10 insertion position relative to the *lon* structural gene nucleotide position, (a) and according to nucleotide numbering in Genebank entry Primary acc No.: gb|AE000489|ECAE000489 (b). Previously described *hfq* mutations, *W1* and *W2* are also shown (b).

From the 16 mutants, one mapped in the *cyaA* gene which codes for the adenylate cyclase enzyme catalyzing production of cyclic AMP (cAMP). Due to a lack of cAMP in this strain the active CRP-cAMP complex can not form and bind the CRP binding site of the *bgl* promoter thereby causing low or no expression. This mutation was not further analyzed. Mutations obtained in the other three loci *(pgi, lon* and *hfq)* were further analyzed to determine the specificity of their effect on the expression of the *bgl* operon.

The tester strain (S594) was used to quantitate the effect of these mutations on the expression of the *Pbgl*$_{C234}$*-D(55-120)-orfG-lacZYA* construct by measuring the β-Galactosidase activity. For comparison the effect of the mutations on the expression of the *wt lac* operon (strain S539) was also measured (figure 14).



| chromosomal construct | wt | lon108 | lon124 | lon166 | lon183 | lon187 | pgi111 | pgi194 | hfq110 | hfq155 | hfq184 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| a) | 320 | 90 | 100 | 100 | 80 | 120 | 160 | 180 | 170 | 90 | 90 |
| b) | 7940 | nd | nd | nd | nd | 6220 | 6350 | nd | nd | 6160 | nd |

**Figure 14: Mutations in *lon, pgi* and *hfq* genes specifically downregulate the *bgl* expression.** Expression levels of a) the C234-P$_{bgl}$ *D(55-120) orfG lacZ* construct (S594) and b) the *wt lac* operon (S539) were measured in various mutantion backgrounds obtained during the screen.

## 3.1 The *pgi* mutation specifically downregulates *bgl* expression.

The *pgi* gene codes for the enzyme Phosphoglucoisomerase of the glycolysis pathway. This mutation results in a decreased expression of the *bgl* operon construct as shown in figure 14. However, expression levels of the *wt lac* operon were not significantly altered by the *pgi* mutation. Thus, *pgi* mutation specifically downregulates the *bgl* operon expression. It was recently shown that in a *pgi* mutant, glycolysis is blocked and this accelerates RNaseE mediated degradation of the *ptsG* gene transcript. The *ptsG* gene codes for a transport protein for Glucose uptake and is a part of the PTS transport system (Kimata et al., 2001).

### 3.2 The *lon* mutation specifically downregulates *bgl* expression.

Lon is a ATP dependent protease and it has been shown that Lon can specifically degrade StpA protein of *E. coli* which is 67% similar to the H-NS protein (Johansson and Uhlin, 1999) (see introduction).

As seen in figure 14, different *lon* mutations obtained during the screen resulted in approximately 3 fold lower expression of the *bgl-lacZ* construct. These mutations did not significantly alter expression of the *wt lac* operon. Thus, *lon* specifically downregulates *bgl* expression. One of the *lon* mutations, *lon187* which maps closest to 5´end of the *lon* gene was selected for further analysis (figure 13). The *lon* mutation can affect expression of the *bgl* operon in following way. Lon protein presumably degrades a protein which has a negative effect on expression of the *bgl* operon. Thus, in a *lon* mutant this negative regulator protein is stabilized and can accumulate to higher levels causing low *bgl* expression. As mentioned above protein StpA is a possible candidate for this negative regulator. Although the role of StpA in *bgl* regulation is not clear, it is possible that StpA can exert its negative role only when present in high levels as may be found in *lon* mutant cells. In order to determine whether the effect of Lon protease is due to accumulation of StpA in the cells, attempts were made to construct a *lon*, *stpA* double mutant strain by T4 phage mediated transduction. However, all such attempts failed. The probable reason may be that in the double mutant cells, levels of the cell division negative regulator protein SulA are high resulting in complete inhibition of cell division and thus causing a lethal phenotype. Experiments using a *sulA* mutant strain to combine the *lon* and *stpA* mutations indicated that the positive effect of Lon on *bgl* expression is independent of StpA (Klingen and Schnetz, unpublished results).

### 3.3 The *hfq* mutations specifically downregulate *bgl* expression.

Gene *hfq* codes for Host Factor for replication of RNA phage Qβ (Hfq). It is a 15KDa protein with ~30000 molecules/cell (Ali et al., 1999). Hfq has a RNA binding/chaperone activity. It has been shown to bind to and decrease the stability of *ompA* mRNA (Vytvytska et al., 1998; Vytvytska et al., 2000). Hfq also binds to the *rpoS* mRNA and is essential for the translation of this mRNA (Muffler et al., 1996; Zhang et al., 1998). All the mutations mapping in the *hfq* region had a specific negative effect on expression of the *bgl-lacZ* construct of strain S594 (figure 14). There was no significant effect on the expression of the *wt lac* operon.

### 3.4 The *hfq155* is a true *hfq*⁻ mutation.

The *hfq* gene is a part of the complex *amiB-mutL-miaA-hfq-hflX* operon. Regulation of this operon is not completely understood. It has been shown to have multiple promoters which cause expression of different genes of the operon to various degrees (Tsui et al., 1994). Out of the three mutations in this operon isolated during the screen, two mapped in the *miaA* gene and one (*hfq155*) mapped in the *hfq* gene (figure 13). Thus, it was necessary to determine whether these mutations affected *bgl* expression due to a defect in expression of the *hfq* gene or other genes of this operon. Effect of mutation *hfq155* on *bgl* expression was compared to that caused by two previously described mutations *hfq1::W* and *hfq2::W*. Both these mutations are insertions of a Ω cassette containing kanamycin resistance marker and strong

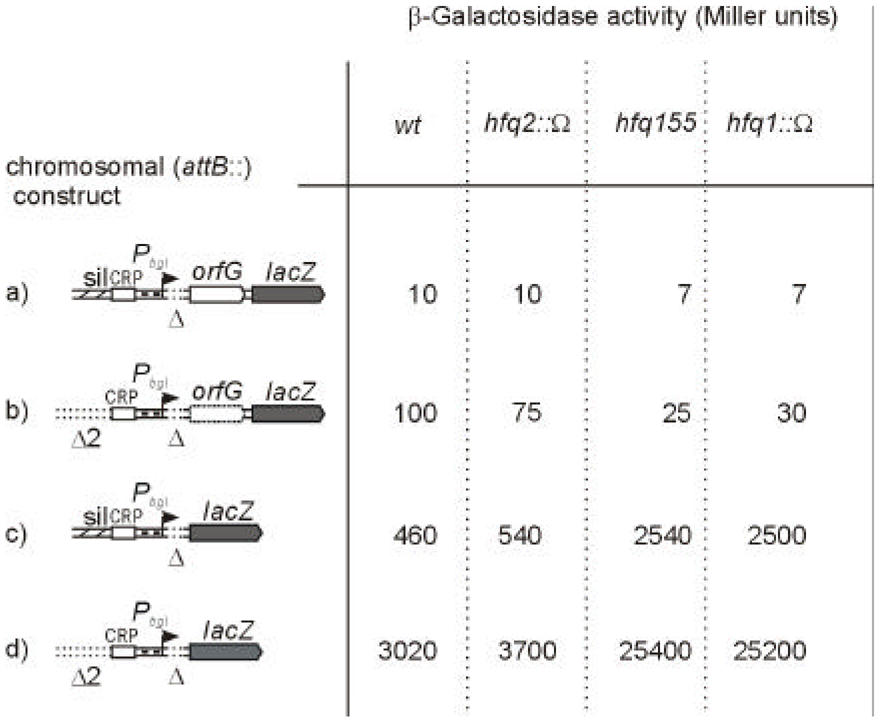


**Figure 15: Mutation *hfq155* affects the *bgl* expression similar to *hfqW1*.** The effect of mutations *hfq155*, *hfqW1* and *hfqW2* was determined in β-Galactosidase assays on the constructs integrated in to the chromosomal *attB* site. All the constructs have a deletion spanning +55 to +120 relative to transcription start. In constructs a and c the *wt bgl* promoter is present while constructs b and d contain a *bgl* promoter allele activated by deletion of the upstream silencer (D2). Furthermore, in constructs a and b the start codon and two additional ATG (codon 3 and 27) of *bglG (orfG)* are mutated (ATG to GCG). In constructs c and d, the *bglG* gene is absent and is replaced by *lacZ*. Strains used were in the order *wt, hfq2::W, hfq155, hfq1::W* a) S397, S841, S891, S833 b) S403, S843, S893, S835 c) S387, S837, S790, S829 d) S393, S839, S792, S831.

rho-independent transcriptional terminators. In *hfq1::W*, the Ω cassette is inserted towards the 5´-end of the *hfq* gene thus causing a Hfq$^-$ phenotype. In *hfq2::W* the Ω cassette is inserted towards the 3´-end of *hfq* gene resulting in a active Hfq expression. However, both mutations have the same polar effects on expression of the downstream genes (Tsui et al., 1994) (figure 13). As shown in figure 15, mutation *hfq2::W* had no significant effect on *bgl* expression. Whereas, mutation *hfq1::W* had a negative effect on *bgl* expression very similar to that of *hfq155*. Thus, it was concluded that *hfq155* mutation and presumably other mutations obtained during the screen in the same region are indeed phenotypically Hfq$^-$. Although they may still differentially affect expression of other genes in this locus, it is their effect on *hfq* expression that results in lower *bgl* expression. Thus, *hfq* mutation causes specific downregulation of *bgl* expression. One of the *hfq* mutations, *hfq155* which maps in the *hfq* gene was selected for further analysis.

### 3.5 The effect of *hfq* on *bgl* expression is independent of the *bgl* promoter, termination/antitermination and RpoS.

Hfq is required for translation of the *rpoS* mRNA (Muffler et al., 1996). RpoS is the alternative stationary phase specific sigma factor of *E. coli* RNA polymerase and its expression is upregulated during starvation and upon transition in to the stationary phase of *E. coli* growth. RpoS-RNA polymerase holoenzyme causes transcription and expression of certain starvation stress induced and stationary phase specific genes involved in adaptation of the cell to changes in the environmental conditions (Hengge-Aronis, 1996).

Hfq possibly binds to *rpoS* mRNA and changes its tertiary structure to facilitate ribosome binding and translation. In the absence of Hfq, 5'-end of the *rpoS* mRNA forms a closed loop structure preventing ribosomal access to the ribosome binding sequence (*RBS*) on the mRNA. Presumably, Hfq binding prevents formation of this loop structure and opens up the 5' mRNA end of the mRNA making the *RBS* accessible (Muffler et al., 1996; Zhang et al., 1998).

Mutation of the *rpoS* gene can positively affect *bgl* expression (Ohta et al., 1999). In the *hfq* mutants, the RpoS expression is low. To substantiate that the effect of Hfq on *bgl* expression is independent of RpoS, *bgl* expression was measured in *hfq*, *rpoS* and *hfq*,*rpoS* double mutant strains (figure 16). In this experiment *wt* (a) and

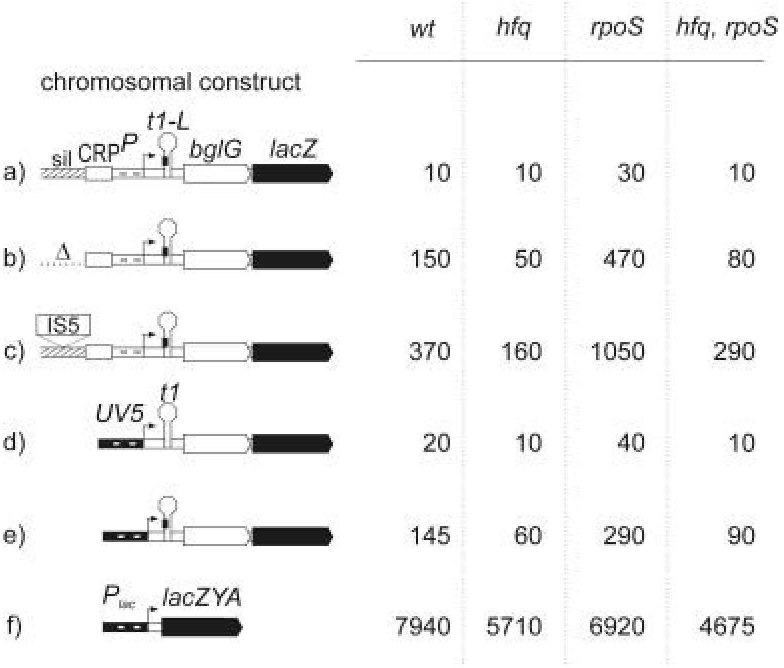


**Figure 16: Hfq effect is independent of the promoter, termination/antitermination and RpoS.** The β-Galactosidase expression levels of various chromosomally integrated (*attB:*) constructs (a to e) and the *wt lac* operon (f) were measured in *wt*, *hfq*, *rpoS* and *hfq*, *rpoS* mutation backgrounds. Strains used were in the order *wt*, *hfq*, *rpoS* and *hfq,rpoS* a) S1142, S1402, S1155, S1404 b) S1146, S1406, S1159, S1408 c) S1148, S1410, S1161, S1412 d) S1095, S960, S1130, S1414 e) S1097, S1311, S1132, S1416 f) S539, S1418, S1107, S1420.

activated (*D2* and *IS5*-insertion) *bgl* promoter alleles were used (figure 16, constructs b and c, respectively). Additionally, a heterologous constitutive promoter (*lacUV5*) was used instead of the *bgl* promoter (construct d). In construct e, the terminator *t1-L* mutation (described in results section 2) was used to render the expression independent of BglG mediated antitermination. In all the cases, *rpoS* mutation positively affected *bgl-lacZ* expression (compare columns *wt* and *rpoS*). A significant negative effect of *hfq* mutation was seen only in b, c, d, and e (compare columns *wt* and *hfq*). It should be noted that in *hfq* mutant cells, low level RpoS expression is possible. Thus, to determine the effect of absence of only Hfq in a constant RpoS background, expression levels in *rpoS* and *hfq*, *rpoS* double mutant strain should be compared (columns *rpoS* and *hfq*, *rpoS*). Such an analysis showed that when Hfq was absent with no possible change in RpoS activity (nil), the *bgl* expression was downregulated upto 3 fold for the *wt bgl* promoter construct (a) and for the other constructs carrying an active promoter (b and c). Thus, it can be concluded that the negative effect of *hfq* mutation on *bgl* expression is independent of RpoS.

The analysis in figure 16 also shows that the Hfq effect is independent of the *bgl* promoter. Even when a heterologous *lacUV5* promoter was used (d) instead of the *bgl* promoter (a, b and c), a similar *hfq* effect was observed. Also the disruption or deletion of the upstream *bgl* silencer sequence in b, c and d did not change the *hfq* effect. In construct d the *wt* terminator *t1* is present while in construct e, the terminator *t1-L* mutation prevents the transcriptional terminator loop formation. Both these constructs show a similar fold *hfq* effect. It is possible that in construct c, the expression increases if extra BglG is provided in *trans*. However, it can be concluded that the effect of *hfq* mutation on *bgl* expression is independent of the upstream *bgl* silencer, the *bgl* promoter and termination/antitermination at *t1*. There was no significant Hfq effect on expression of the control *lac* operon (construct f).

### 3.6 Effect of *hfq* depends on the presence of *bglG* sequence.

The effect of *hfq* on the expression of various *bgl* constructs was studied as in figure 17. All the constructs had a heterologous *lacUV5* promoter and *t1-L* mutation since *hfq* effect was shown to be independent of the promoter and antitermination.

The *lacZ* reporter gene was fused downstream of the *bglG* gene as a transcriptional fusion (constructs a to e) in which translation of the *lacZ* mRNA is facilitated by its own ribosome binding site (*RBS lacZ*). In construct b, the start codon and two additional ATG (codon 3 and 27) of *bglG* are mutated (ATG to GCG). Therefore, the *bglG* gene (*orfG*) can be transcribed but not translated. The constructs c, d and e had 15, 5 or none of the 5´codons of the *bglG* gene, respectively. In c and d, a stop codon was added at the ends of the truncated *bglG* genes.

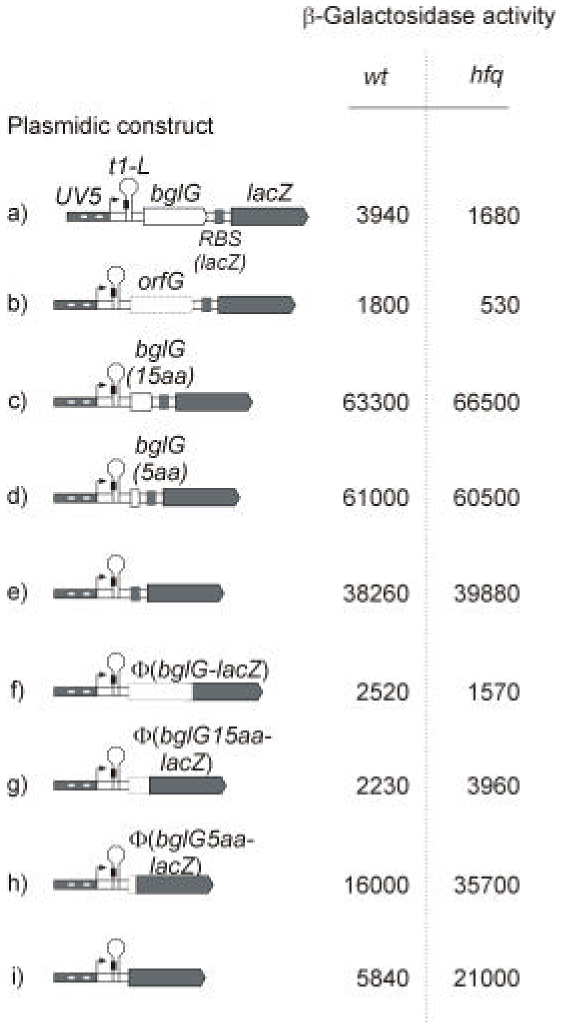
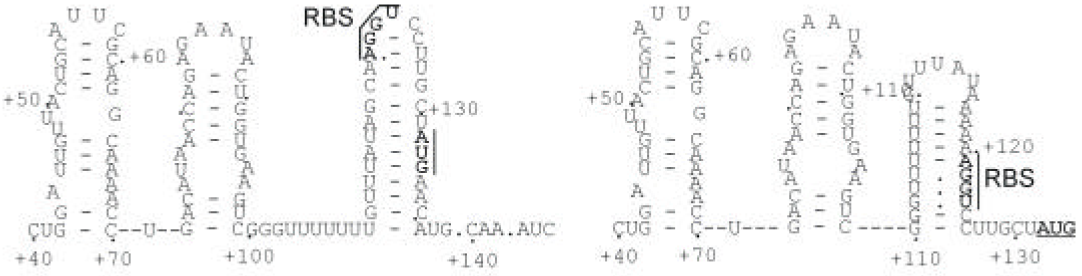


**Figure 17: The Hfq effect is *bglG* sequence dependent.** The β-Galactosidase expression levels from various plasmidic constructs were measured in *wt* and *hfq* strains. The plasmids used were a) pKESD28, b) pKESD47, c) pKESD44, d) pKESD42, e) pKESD36, f) pKESD39, g) pKESD45, h) pKESD43 and i) pKESD24.

**The mRNA structures of the (*bglG-lacZ*) translational fusion constructs.**
Computer analysis of the mRNA structures of (*bglG-lacZ*) translation fusions was performed using the mfold software (http://bioinfo.math.rpi.edu/~mfold/rna/) (Zucker et al., 1999) The mRNAs of translational fusion constructs carrying all or the first 15 amino acids of BglG had a structure (left) with three stem loops, the first loop is the BglG binding domain, the second loop is the remnant of the terminator *t1* loop prevented from forming completely and the third loop containing the ribosome binding site (RBS) and the start codon (AUG) of *bglG*. This closed structure can possibly downregulate translation of BglG. In contrast, the mRNA of a translational fusion construct carrying only the first 5 amino acids of BglG had a open structure (right) with the first two stem loops similar to the closed structure and the third loop shifted upstream such that the RBS and start codon of *bglG* are accessible.



closed structure

open structure

In constructs f to i, the *lacZ* gene was fused to the *bglG* gene as a translational fusion so that LacZ is translated as a fusion protein along with BglG. In f, the entire *bglG* gene without the last stop codon is fused to the 5´-end of *lacZ* gene. In constructs g, h and i only 15, 5 or none of the 5´codons of the *bglG* gene are present and are translationally fused to the *lacZ* gene. In i, *lacZ* gene is translated using the *RBS* of *bglG* gene.

The expression levels of these constructs were measured in *wt* and *hfq* cells. Absence of the entire *bglG* gene sequence caused a upregulation of expression in *wt* cells (figure 17, compare *wt* expression of c, d, e to a and h, i to f). Thus, the *bglG* gene was shown to cause a polar effect on expression of the downstream (*lacZ*) gene. This negative polar effect was higher in construct b, when *orfG* was not translated.

The negative effect of *hfq* on *bgl* expression was seen only when the entire *bglG* sequence was present (constructs a and f). The effect was also seen when only *orfG* was present but not translated (b). However, in all other cases when complete *bglG* sequence was absent the *hfq* effect was lost (c, d and e) or reversed (g, h, i). Thus, it was concluded that *hfq* effect depends on the presence of complete *bglG* sequence. It should be noted that in construct b, when *orfG* is not translated *hfq* still downregulated the expression. Thus, the effect of *hfq* on *bgl* expression is independent of *bglG* translation.

Moreover, Hfq positively affects *bgl* expression both when transcriptional (a) and translational fusion (f) reporter constructs are used. Thus, Hfq protein plays its regulatory role before the translation process. Since, the Hfq effect is independent of the promoter, it probably does not affect transcription initiation. Hfq is a known RNA binding protein and has RNA chaperone activity and it is conceivable that it affects either the stability or the structure of *bgl* mRNA.

## 4. H-NS regulates expression of the *bgl* operon at multiple levels.

The histone-like protein H-NS is essential for silencing of the *bgl* operon (Defez and de Felice, 1981; Higgins et al., 1988). H-NS represses the CRP/cAMP dependent *bgl* promoter, where it is likely to bind to a AT-rich silencer sequence located upstream of the CRP-binding site (Schnetz, 1995; Schnetz and Wang, 1996; Mukerji and Mahadevan, 1997) and putatively forms an extended repressing nucleoprotein complex (Caramel and Schnetz, 1998). The *wt bgl* operon is activated in a *hns* mutant strain presumably due to de-repression of the *wt bgl* promoter. Indeed, *in vitro* DNA binding studies using mobility shift and DNaseI protection (footprinting) experiments indicated that H-NS specifically binds to the *bgl* upstream silencer DNA (Wahle and Schnetz, unpublished results).

It has been shown that sequences downstream of the *bgl* promoter contribute significantly to silencing of the *bgl* operon (Schnetz, 1995). The mechanism of this observation is not clear. As shown in results section 2, requirement of threshold BglG levels contributes towards regulation of the *bgl* operon. However, additional mechanisms of regulation acting at the promoter downstream sequences are possible.

Expression of different chromosomally integrated (*attB*) *bgl* constructs was upregulated by *hns* mutation. As shown in figure 18, *hns* mutation increased expression by 26 and 7 folds, respectively when *wt* and an a de-repressed allele of the *bgl* promoter directed the expression of a *lacZ* reporter construct. (figure 18, constructs a and b, respectively). The effect of H-NS on the *bgl* promoter and the upstream silencer element is shown in figure 18, constructs g - j. The expression of these constructs was measured in NB medium unlike constructs a - f which were measured in LB2Y medium. This was to avoid trace amounts of glucose which may be present in LB2Y medium and which can cause catabolite repression of the *bgl* promoter. In constructs g - j, the *bgl* sequence only upto +25 (relative to transcription start) is present. In construct g, the upstream silencer element is present and *hns* mutation upregulates expression of this construct 4 fold. When the upstream silencer is deleted (figure 18, construct h) or when the CRP binding site is improved (construct i), *bgl* expression in the *wt* strain is 4 to 5 fold high as compared to the *wt* promoter construct (construct g). However, *hns* mutation does not change the expression levels of constructs h and i significantly. In construct j, the upstream silencer is deleted as well as the CRP site is improved. It was seen that these two independent mutations

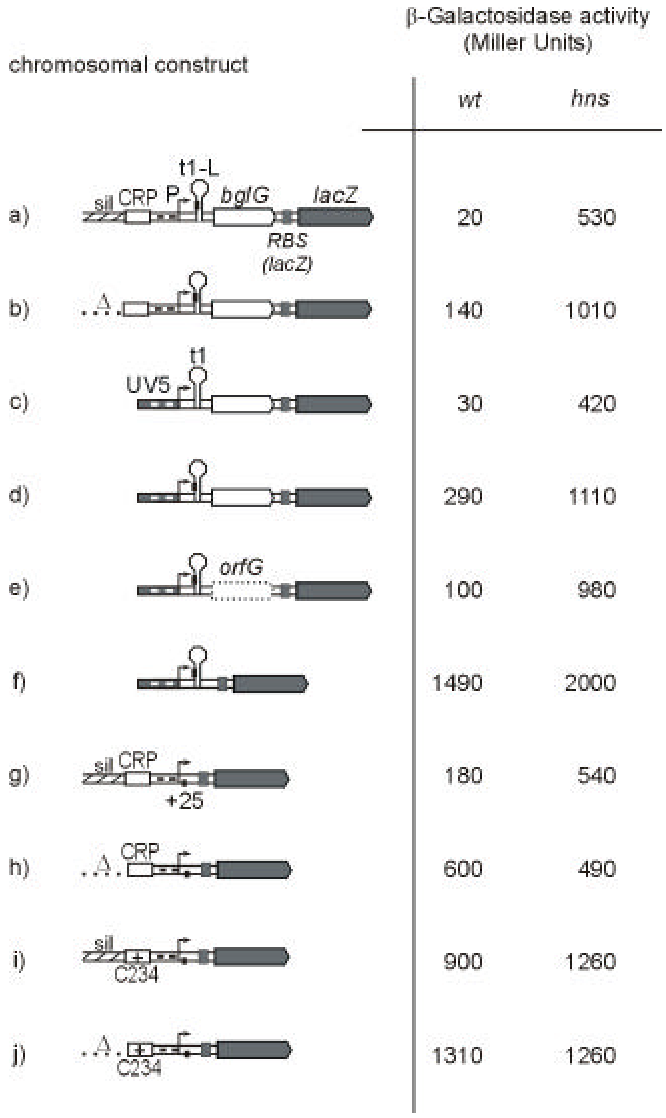


**Figure 18: H-NS mediated downregulation of *bgl* expression via upstream and downstream silencers.** The β-Galactosidase activity directed from various *bgl* constructs was measured in *wt* and *hns* strains. Cultures were grown in LB2Y medium (a-f) or NB medium (g-j). Strains used were in the order *wt*, *hns* a) S1142, S1467 b) S1146, S1469 c) S944, S1307 d) S1097, S1309 e) S1189, S1252 f) S1191, S1254 g) S1213, S1471 h) S1211 S1473 i) S1215, S1475 j) S1217, S1477.

known to de-repress the *bgl* promoter have an additive de-repressive effect on the *bgl* promoter since the expression levels of construct j in the *wt* strain is higher than constructs h and i which have the individual mutations. Expression of construct j is also not significantly altered in the *hns* strain. Thus, it can be concluded that H-NS mediated repression of the *bgl* promoter operates via the upstream silencer. When the upstream silencer is deleted (construct h) or when binding of H-NS to the upstream silencer is presumably counteracted by improved binding of CRP (construct i), H-NS has no effect on the *bgl* promoter.

When the *bgl* promoter was replaced by a constitutive *lacUV5* promoter and the upstream silencer was absent (figure 18, construct c), *hns* mutation caused a upregulation by 14 fold. This construct has the *wt* terminator *t1*, therefore it is possible that in the presence of extra BglG in *trans* the expression levels are higher both in the *wt* and the *hns* cells. Still, this indicates that H-NS has a negative effect on *bgl* expression in addition to that on the *bgl* promoter. When the terminator *t1* was mutated to *t1-L* (figure 18, construct d), *hns* mutation caused upregulation of expression by 4 fold showing that H-NS mediated downregulation is independent of the termination/antitermination. In figure 18, construct e, the start codon and two additional ATG (codon 3 and 27) of *bglG* are mutated (ATG to GCG) such that the *bglG* gene (*orfG*) can be transcribed but not translated. Expression of this construct is upregulated 10 fold in the *hns* mutant. Thus, the negative regulation by H-NS is enhanced in the absence of translation of *bglG*. However, when the *bglG* gene is deleted (construct f), the *hns* mutation has no effect on the *lacZ* expression. Thus, the H-NS protein possibly exerts its negative regulatory effect on expression of the *bgl* operon at multiple levels: In addition to its known role in repression of the *bgl* promoter it acts on the sequences downstream of the promoter. The *bglG* gene sequence is essential for this downstream role of H-NS. Possible mechanisms of H-NS mediated downregulation at the downstream sequences are

- prevention of transcription initiation by DNA binding. However, it has been shown that H-NS does not specifically bind DNA sequences downstream of the *bgl* promoter (Wahle and Schnetz, unpublished results).
- inhibition of transcription elongation through the leader and *bglG* sequence
- RNA destabilization

The effect of H-NS is stronger when *bglG* is not translated (*orfG*) (figure 18, construct e). To test the possibility whether H-NS effects *bgl* mRNA stability, S1 nuclease protection studies were carried out as described below. The H-NS mediated repression via the upstream silencer is maximum 4 fold (construct g). Interestingly, H-NS mediated downregulation of the *bgl* operon via the downstream silencer can be upto 14 fold (construct c). Thus, it can be concluded that the majority of the H-NS mediated downregulation of the *bgl* operon is via the downstream silencer. It should be noted that when the terminator *t1* is mutated thus rendering the *bgl* expression independent of BglG mediated antitermination (construct d), effect of H-NS is 4 fold. Thus, H-NS mediated downregulation of *bgl* expression via the downstream silencer is presumably enhanced due to limiting BglG levels.

## 4.1 HNS mediated regulation on plasmids.

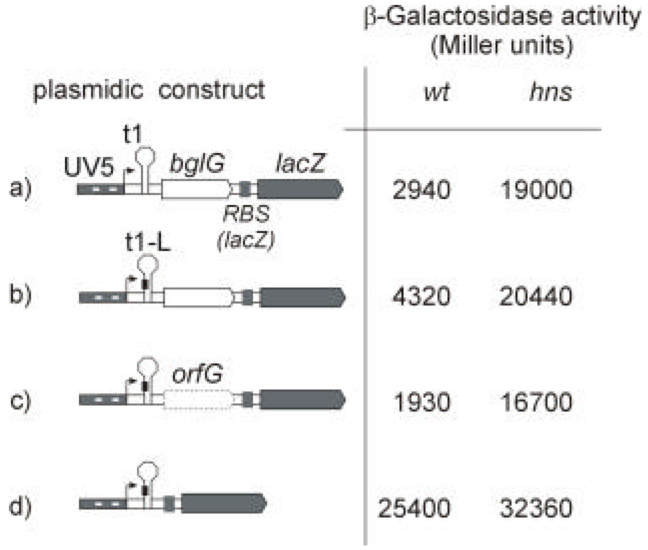


**Figure 19: H-NS mediated regulation of plasmid encoded *bgl* constructs.** The *wt* (S541) and *hns* (S614) strains were transformed with plasmids with the shown *bgl-lacZ* reporter constructs. Cultures were grown in LB2Y medium and the β-Galactosidase activities measured. Plasmids used were pKESD20 (a), pKESD28 (b), pKESD47 (c) and pKESD36 (d).

The negative regulation by H-NS via the downstream silencer was possibly due to *bgl* RNA destabilization. It was necessary to use plasmidic constructs for RNA analysis in order to obtain RNA yields sufficient for detection. Regulation of plasmid encoded *bgl* constructs by H-NS was found to be similar to that of chromosomally encoded constructs (figure 19). Expression of the plasmidic *bgl* construct with the constitutive *lacUV5* promoter and the *wt* terminator *t1* was upregulated 6 fold in *hns* strain (construct a). A similar effect was seen when terminator *t1* was mutated to *t1-L* (construct b). In construct c, the start codon and two additional ATG (codon 3 and 27) of *bglG* are mutated (ATG to GCG) such that the *bglG* gene (*orfG*) can be transcribed but not translated. Expression of this construct was upregulated 10 fold in the *hns* mutant. However when the *bglG* gene was deleted (construct d), the *hns* mutation had no effect on *bgl* expression. Thus, plasmids like pKESD28 (figure 19, construct b) could be used for further analysis.

## 4.2 H-NS causes destabilization of the *bglG* mRNA.

S1 nuclease protection assay was used to quantitate *bgl* mRNA levels. The amounts of *bgl* mRNA from plasmidic construct pKESD28 (figure 19, b) were quantitated in *wt* and *hns* strains. The outline of S1 nuclease protection assay experiment is shown in figure 20. S1 nuclease is a single strand specific endonuclease and it can not act on double stranded RNA, DNA or RNA-DNA hybrids. The *bgl* mRNA was probed at four different regions namely, in the leader and at three

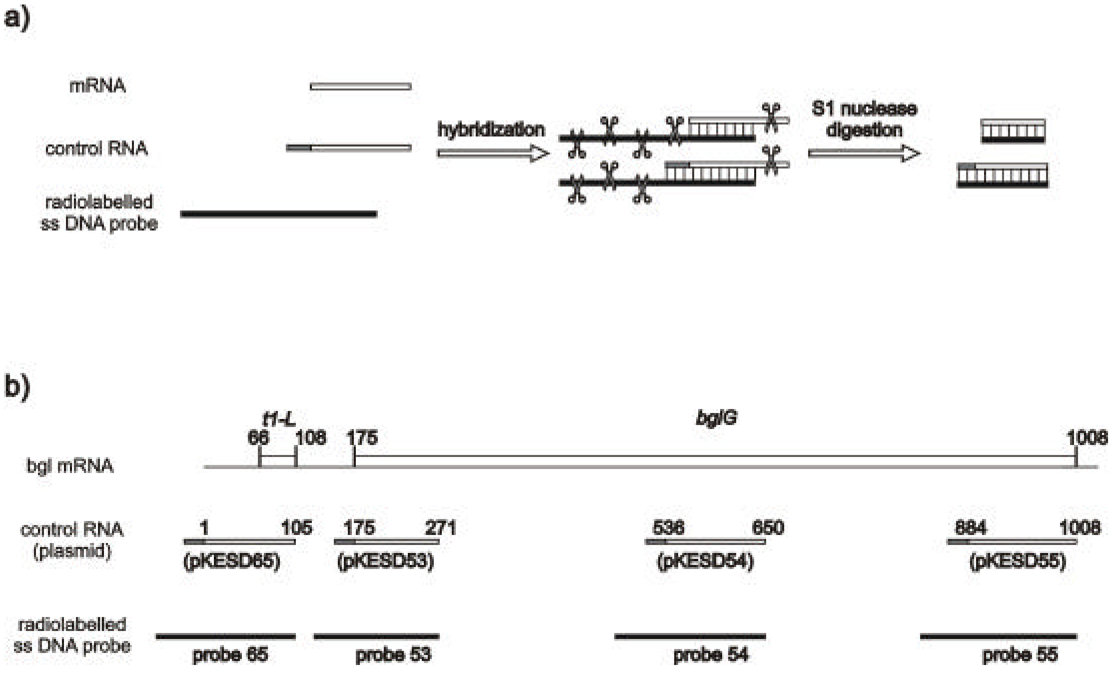


**Figure 20: Principle of S1 nuclease protection assay.** a) Experimental and control RNAs are hybridized together with the single stranded radiolabeled DNA probe. The non hybridized RNAs and probe regions are digested using S1 nuclease. The protected probe fragments are run on a sequencing gel. b) The *bgl* mRNA was probed at four regions, in the leader and at three positions along the *bglG* gene. The *bgl* sequences present in the control RNAs are shown by the nulcleotide positions relative to transcription start.

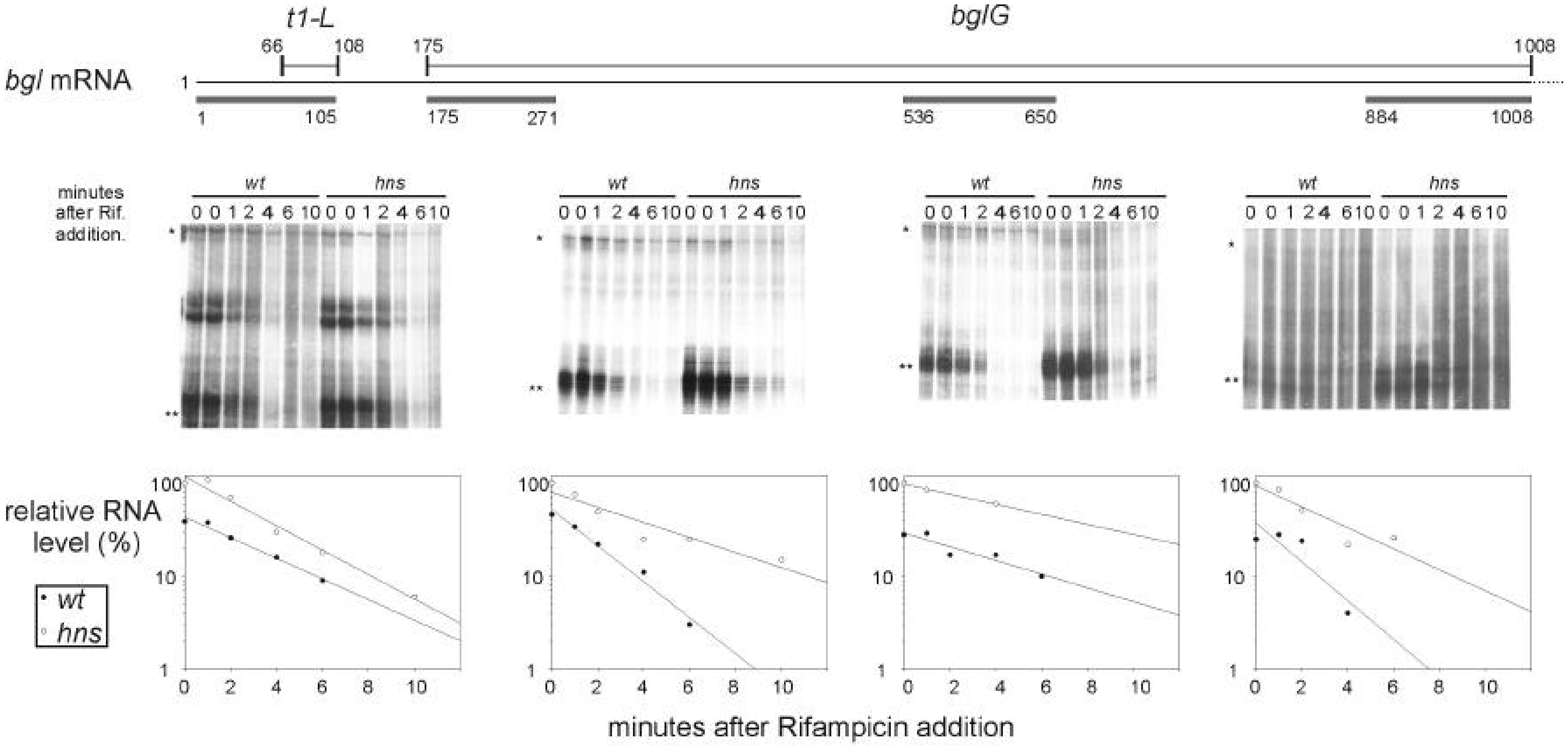
positions along the *bglG* gene (figure 20, b). For accurate quantitation of mRNA, internal recovery control RNAs were used (figure 20, a). The control RNAs normalize differences in RNA yields, differences in labeling efficiencies, variations in hybridization efficiencies and other experimental and autoradiography variations. These control RNAs correspond to the indicated sequences of the *bgl* operon with additional non-*bgl lac* operator sequences at their 5' ends (gray regions in figure 20). The DNA fragments corresponding to the control RNAs were cloned in a high copy plasmid (pKESD65, pKESD53, pKESD54 and pKESD55, see figure 20, b) such that a IPTG inducible *tac* promoter directed their transcription. The non-*bgl* sequences at the 5´-ends correspond to the *lac* operon leader sequence. For control RNA preparations total RNAs were prepared using the hot phenol method from *wt* strain (S541) transformed individually with the above four plasmids and induced with 1mM IPTG. Fixed amounts of each control total RNA (100µg each) were added during the preparation of pKESD28 RNA from *wt* (S541= Δ*bgl* Δ*lacZ*) and *hns* (S614= Δ*bgl* Δ*lacZ hns::*$Ap^R$) strains as described in materials and methods. Briefly, *wt* or *hns* strain transformed with pKESD28 was grown in LB2Y medium till $OD_{600}$ ~ 0.5.

Rifampicin was added (time 0) to 100µg/ml stopping new transcription initiation and 20ml samples were taken at different time points. These were quickly added to a pre-heated phenol, SDS mix containing 100µg each of the four control RNAs. The RNAs were then further extracted and purified as described in materials and methods. The DNA in these preparations was digested using RNase free DNaseI. Finally, 200µg total RNA from each sample was used for S1 analysis. The plasmids pKESD65, pKESD53, pKESD54 and pKESD55 were also used to synthesize single stranded internally radiolabeled ($P^{32}$) DNA probes using suitable primers and T7-DNA polymerase. Thus, each probe could hybridize to the entire control RNA from that plasmid and also to a corresponding shorter sequence on the *bgl* mRNA. After hybridization, S1 nuclease digestion was performed such that the nonhybridized single stranded regions of the DNA probe as well as any unhybridized RNA are digested away. When run on urea:acrylamide sequencing gels two bands corresponding to the radioactive probe protected by the control RNA (larger band) and probe protected by the *bgl* mRNA (smaller band) were seen (for details see materials and methods).

The band intensities were then quantitated using a Fuji BAS1000 phosphorimager and the gels were also auto-radiographed. The experimental band intensities were normalized with the control band intensity from the same lane. After correcting for background in each lane and for the $OD_{600}$ of the culture, the intensities were expressed as percentage of the normalized intensity of experimental band for that gel from *hns* cells at time 0 of rifampicin addition (100%). Figure 21 shows results of such an experiment. Averages of the normalized band intensity values from two independent experiments were plotted against time after rifampicin addition and these plots were used to calculate the half life values of the *bgl* mRNA (figure 21).

It was observed that the half life of the leader part of the *bgl* mRNA was not significantly different in *wt* (3.3minutes) and *hns* (2.8minutes) strains. However, the mRNA half life of the three *bglG* regions were approximately half in *wt* cells as compared to that in the *hns* cells. Thus, it can be concluded that in *wt* cells presence of H-NS causes a 2-fold reduction in the stability of *bglG* mRNA. However, there is no effect of H-NS on the stability of the *bgl* leader RNA.

H-NS can differentially affect the stabilities of different parts of the same RNA due to various reasons. It is possible that H-NS can directly interact with only



| | mRNA half life (minutes) | | | |
|---|---|---|---|---|
| *wt* | 3.3 | 1.5 | 3.5 | 1.5 |
| *hns* | 2.8 | 3.8 | 6.0 | 2.9 |

**Figure 21: Quantitation of *bgl* mRNA using S1 nuclease protection assay**. Total RNA was isolated from *wt* and *hns* cells transformed with pKESD28 (*lacUV5 t1-L bglG lacZ*) at indicated time points after Rifampicin addition as described in the text. Four probes were used which hybridized with different parts of the mRNA in the leader and along *bglG* (grey bars). After S1 assay the undigested probes were run on sequencing gels. The respective gels show control (*) and experimental (**) bands. These were quantitated using Fuji BAS1000 phosphorimager and the experimental band intensities were normalized with the control band intensities from the same lane. After correcting for background in each lane and for the $OD_{600}$ of the culture the intensities were expressed as percentage of the normalized intensity of experimental band for that gel from *hns* cells at time 0 of Rifampicin addition (100%). Average values from two independent experiments were plotted against time after Rifampicin addition and mRNA half lives were calculated from these plots. Numbers for the mRNA structure indicate nucleotide position relative to transcription start. For further details see text and materials and methods.

part of the mRNA making it unstable. It is also possible that there are endonulease sites between the leader and *bglG* parts of the mRNA and after endonucleolytic cleavage the leader mRNA is stable whereas the *bglG* part which may interact with H-NS is destabilized. Results obtained using the probe hybridizing with the 3'-end of *bglG* mRNA were not accurate and should be reconfirmed using a different primer.

The differential effect of H-NS on the leader mRNA and *bglG* mRNA is in agreement with the *in vivo* results in figure 18. The *hns* mutation had an effect on *bgl* expression only when the *bglG* sequence was present (figure 18, constructs a, b, c, d and e) but not when the *bglG* sequence is deleted (construct f) although the *bgl* leader sequence is present.

# IV. Discussion

## 1. Multiple levels of the *bgl* operon regulation.

In the present work the *bgl* operon of *E. coli* was shown to be regulated at multiple levels as shown schematically in figure 22. The *bgl* promoter is repressed by formation of a nucleoprotein complex of which H-NS forms an essential and dominant component. Formation of the repressor complex involves the upstream silencer and the promoter regions. In the promoter downstream region two additional levels of regulation operate. The BglG protein causes antitermination at the terminator *t1* and threshold cellular levels of BglG are required for efficient antitermination. This threshold can amplify silencing and activation of the operon at low expression levels. A new dominant theme of *bgl* regulation is regulation of mRNA stability. H-NS causes destabilization of the *bglG* mRNA and other factors like Hfq, Pgi and translating ribosomes also play a part in regulating the *bgl* mRNA level. Evidences for each level of regulation are discussed below.

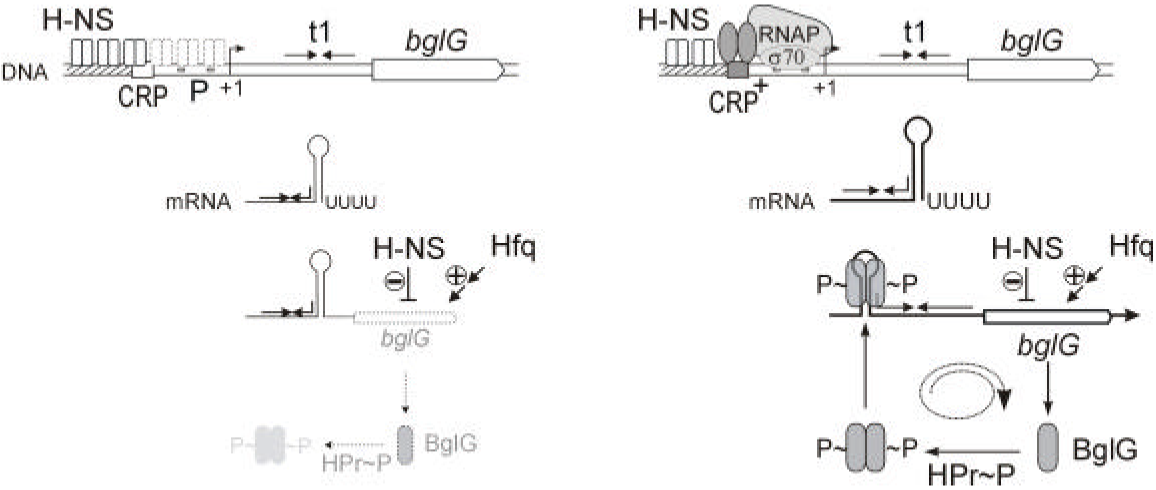


**Figure 22. A new model showing multiple levels of the *bgl* operon regulation.** Left, the *wt bgl* operon is silenced. H-NS, possibly alongwith other proteins like FIS binds the upstream silencer and the promoter regions causing transcriptional repression. Basal levels of transcription initiation events mostly result in transcriptional termination at *t1*. A leakthrough may result in low transcription of the downstream sequences. H-NS causes destabilization of such *bglG* mRNA and eclipses a presumed positive Hfq effect on the mRNA stability and further reduces the expression. Background levels of BglG protein which may result are not sufficient for efficient antitermination. Right, in the case of an activated *bgl* promoter allele (*eg* improved CRP site allele) CRP binding with higher affinity destabilizes the H-NS containing repressor complex. RNA polymerase (RNAP) can gain access to the promoter and transcription initiates. The resulting higher BglG expression causes efficient antitermination resulting in still higher BglG amounts thus starting an expression amplification loop. H-NS and Hfq still act negatively and positively on the mRNA stability, respectively. The translating ribosomes and Pgi presumably also cause mRNA stabilization.

## 2. Repression of the promoter.

It has been proposed (Caramel and Schnetz, 1998), that H-NS binds the upstream AT-rich sequence and presumably promotes the formation of a silencing nucleoprotein complex involving Fis and possibly other unknown cellular factors (Schnetz, 1995). In the present work an attempt was made to identify the additional cellular factors required for silencing of the *bgl* promoter using DNA affinity chromatography (results section 1). Proteins specifically binding to the *bgl* upstream silencer and promoter region, as found from this experiment, included only H-NS. This confirmed the role of H-NS in *bgl* promoter silencing via direct binding to the *bgl* DNA fragment used. However, no other proteins could be identified which played a similar role. As discussed in the results section 1, this may have been a limitation of the biochemical approach used. Alternatively, as substantiated from further results, only H-NS may bind to the upstream *bgl* silencer and the *bgl* promoter DNA, causing transcriptional repression. The requirement of additional factors for *bgl* silencing proposed earlier (Schnetz, 1995) might have to do with regulation of the *bgl* operon at other physical locations like the downstream silencer and at different levels *eg* at post-transcriptional level.

## 3. Amplification of *bgl* silencing and activation by BglG cellular threshold at low expression levels.

The downstream *bgl* silencer region consists of the non-coding, *bgl* leader sequence and the *bglG* gene sequence. The most prominent feature of the leader sequence is the transcriptional terminator *t1*. As described in results section 2, unlike on plasmids, the *bgl* operon could not be highly activated when present on the chromosome, by the promoter de-repressing mutations. On a high copy plasmid, such mutations resulted in upto 200-fold activation whereas when the same *bgl* constructs were present on the chromosome the activation was only upto two folds. This observation implied that a reduction in the copy number from the plasmidic to the chromosomal system was responsible for this phenomenon. It was possible that this maybe due to a titration effect on a negative regulatory factor, which became limiting when the *bgl* operon was plasmid encoded. Alternatively, a positive regulatory factor, which has to be encoded by the *bgl* operon itself, could become limiting due to low expression levels when encoded on the chromosome. The obvious candidate for the latter possibility was the positive regulator and antiterminator protein BglG. To test this possibility, extra BglG was provided in *trans* to a chromosomal de-repressed *bgl* promoter construct causing increased activation upto 60 fold (results section 2.4). Thus, high cellular levels of BglG protein, which could be achieved in the plasmidic system but not from a chromosomal construct, were required for high expression of an activated *bgl* operon. Thus BglG is limiting to *bgl* operon expression when the transcription rate remains below a threshold, but not when the operon is transcribed at higher levels. High expression levels are obtained (a) when additional BglG protein is provided *in trans*, (b) when the expression is rendered BglG-independent by mutation of the terminator, or (c) when the copy number is increased (from a chromosomal to a plasmidic system). Interestingly, this threshold level of BglG required for high expression could be reached even in a chromosomal system by a small further activation of the promoter.

A model is shown in figure 22. Basal level of transcription that is initiated at the repressed *bgl* promoter mostly terminates in the leader at terminator *bgl-t1* (Fig. 22, left) (Mahadevan and Wright, 1987; Schnetz and Rak, 1988). If basal amounts of *bglG*-mRNA that are transcribed due to the inherent leakiness of terminator *bgl-t1* are insufficient to allow synthesis of enough BglG protein for antitermination then the expression remains low (Fig. 22, left). If the transcription rate is higher the basal BglG amounts may exceed a threshold that allows antitermination (Fig. 22, right). Once antitermination occurs, BglG amounts increase and the remainder of the operon can be expressed at high levels by antitermination at terminator *t2*. Possible mechanisms leading to limitation of BglG may involve (a) inefficient activation of BglG by HPr mediated phosphorylation (Görke and Rak, 1999) and dimerization (Amster-Choder and Wright, 1992) at low cellular concentrations and/or (b) low efficiency of BglG synthesis possibly due to instability of the *bglG* mRNA and the low translation efficiency. Expression of BglG *in trans* or an increase in the copy number would overcome these limitations and lead to full expression. In a *rpoS* mutation, the three fold upregulation of the *bgl* promoter was sufficient to result in a sufficiently high BglG expression, which in turn caused efficient antitermination resulting again in higher BglG levels and so on (results section 2.6). The threshold of BglG required for starting the expression amplification loop shown in figure 22 can amplify silencing or activation of the operon. The leader (terminator *t1*) part of the downstream silencer, thus contributes to the *bgl* operon regulation.

## 4. Positive regulatory factors of the *bgl* operon expression.

A genetic mutation screen (results section 3) for genes required for high *bgl* expression yielded three new candidate genes *hfq*, *pgi* and *lon*. Hfq is a known RNA binding protein and has RNA chaperone activity. Although Hfq is essential for translation of the *rpoS* mRNA, the positive effect of Hfq on *bgl* expression was independent of RpoS. It was shown that the positive effect exerted by Hfq is at the *bglG* gene sequence. It is conceivable that Hfq specifically binds to the *bglG* mRNA and stabilizes it. Direct binding of Hfq to the *bglG* mRNA needs to be confirmed experimentally. Recently it has been suggested that *pgi* mutation leads to a block in the glycolytic pathway and catabolic utilization of glucose. This was also shown to accelerate the RNaseE mediated degradation of *ptsG* mRNA. It was proposed that *pgi* mutation could lead to a general response in which activities of various RNases are upregulated resulting in specific downregulation of the genes whose mRNAs are susceptible to the respective RNases (Kimata et al., 2001). The *bgl* operon could be specifically downregulated by such a response caused by the *pgi* mutation. It would be interesting to further characterize this response and to determine if the genes downregulated by it belong to a particular functional category.

The *lon* gene mutation also caused downregulation of an activated *bgl* operon. It is possible that Lon, which is a protease, specifically downregulates a *bgl* repressor protein and thus in the absence of Lon, the repressor protein activity is higher causing *bgl* downregulation. One possible candidate for such a repressor was StpA and Lon could regulate *bgl* expression via StpA (Johansson and Uhlin, 1999). This possibility could not be tested since the *lon* and *stpA* mutations could not be combined using phage mediated transductions. In such double mutants, the levels of cell division inhibitor SulA, which is degraded by Lon, are perhaps too high and cause a lethal phenotype. It was possible to combine the *lon* and *stpA* mutations in a *sulA* background showing that the positive effect of Lon on *bgl* expression is independent of StpA (Klingen and Schnetz, unpublished results). Thus, precise mechanism of the Lon effect remains to be determined.

The genetic mutation screen for genes required for silencing of the *bgl* operon yielded only *hns* mutants (results section 3) thus supporting the hypothesis that H-NS is the dominant negative regulator of the *bgl* operon. Other possible negative regulators may not exist or these factors are non-redundant and mutation of the respective gene may cause a lethal phenotype. It is also possible that the effects of some of the negative regulatory factors, direct or indirect, are smaller compared to that of H-NS and may escape detection in the mutagenesis screen *eg* the negative effect of RpoS on *bgl* expression can remain unobserved until the antiterminator BglG level threshold is crossed.

### 5. Regulation of the *bgl* mRNA stability.

It was shown that H-NS downregulates the *bgl* expression via the downstream silencer even in the absence of the upstream silencer sequences (results section 4). This downstream effect of H-NS was upto 14 fold and was more than the known role of H-NS i.e. binding to the upstream silencer sequences and repression of the *bgl* promoter (upto 4 fold, in the absence of the downstream silencer). The downstream effect of H-NS was dependent on the *bglG* sequence and it was shown that in a *hns* mutant the mRNA half life of the *bglG* part of the *bgl* mRNA (measured at three different locations along the *bglG* sequence) was approximately 2 fold higher compared to that in a *wt* strain. However, *hns* mutation did not affect the stability of the *bgl* leader mRNA (results section 4.2). It remains to be determined whether H-NS can directly bind to the *bgl* mRNA and decrease its stability or whether it is an indirect effect.

At this point an interesting picture of the *bgl* regulation via the downstream silencer sequence emerges. First of all, the terminator *t1* prevents transcriptional readthrough unless the cellular levels of antiterminator BglG are sufficiently high. Even when there is efficient antitermination, the *bglG* mRNA is destabilized in the presence of H-NS. Indeed, this post-transcriptional role of H-NS is more prominent than its previously known role, namely, transcriptional silencing of the *bgl* promoter via the upstream silencer. Two more genes *pgi* and *hfq* possibly play a role in regulation of the *bgl* mRNA stability. Thus, post-transcriptional regulation of the *bgl* expression by alteration of the mRNA stability emerges as a prominent level of *bgl* regulation. The roles of H-NS, Hfq and Pgi in such a system need to be further analyzed. It is necessary to determine whether some of these proteins can directly bind to the *bgl* mRNA thus possibly changing its structure and/or susceptibility to the RNA degrading machinery. It will also be interesting to determine exactly which components of the RNA degradosome are specifically involved in *bgl* mRNA degradation. It is possible that H-NS, a negative regulator of the *bgl* mRNA stability and Hfq which presumably regulates it positively, act as antagonists. The RNA protective role played by the translating ribosomes is also important. It is the changes in relative levels of these, which may decide the *bgl* mRNA stability and the operon activity. The *pgi* mutation may also feed in to this system indirectly by influencing activities of the various RNA degradosome components.

What kind of environmental or other conditions may change the relative levels of these factors leading to activation of the *wt bgl* operon? Activation of the *wt bgl* operon in a pathogenic *E. coli* strain *in vivo* (in the host) may be caused by such a change in the relative levels of various pleiotropic regulators (Khan and Isaacson, 1998).

Furthermore, it is possible that there are factors/proteins binding directly to the downstream silencer DNA causing transcriptional downregulation. It has been shown that H-NS does not specifically bind to the leader or *bglG* DNA (Wahle and Schnetz, unpublished results). However downregulation of transcription elongation via secondary structure formation can not be ruled out.

One of the important questions that remains to be answered is the significance and mechanism of evolutionary maintenance of the silent *bgl* operon. It has been suggested that the *bgl* operon is kept silent in order to avoid the effects of certain toxic β-glucosides present in nature (Reynolds et al., 1981). It is possible that the *wt bgl* operon is expressed under certain environmental conditions *eg* those found inside a host (Khan and Isaacson, 1998). However this possibility needs further examination. Otherwise, there are no known *in vitro* (outside the host) conditions which result in activation of the *bgl* operon. In the current work no specific regulators of the *bgl* operon expression were found. Proteins like H-NS, StpA, Hfq, RpoS and Lon are non-specific or pleiotropic regulators of *E. coli* gene regulation. It is possible that under certain environmental conditions, yet unknown, the relative levels of these regulators change in such a way as to allow expression of the *bgl* operon considering the amplification in the *bgl* operon activity by the requirement of cellular threshold of the antiterminator BglG.

# V. Materials and methods

## 1. Chemicals, enzymes and other materials

Chemicals and enzymes were purchased unless otherwise specified from commercial sources. Oligonucleotides were purchased from Eurogentec or Invitrogen life technologies.

## 2. Media and agar plates

| | | |
|---|---|---|
| LB2Y medium (1l) | 10g | Bacto Tryptone (Difco) |
| | 10g | Yeast Extract (Difco) |
| | 5g | NaCl |
| | (for plates 15g Bacto Agar, Difco) | |
| NB medium (1l) | 8g | Bacto NB broth, dehydrated (Difco)<br>(3g Bacto Beef extract, 5g Bacto peptone) |
| MacConkey lactose indicator plates (1l) | 40g | MacConkey Agar (Difco) |
| | 10g | Lactose |
| BTB salicin indicator plates (Schaefler, 1967) | 15g | Bacto Agar (Difco) |
| | 1g | Yeast-Extract (Difco) |
| | 1g | Tryptone (Difco) |
| | 5g | NaCl |
| | add 900ml $H_2O$, autoclave | |
| add sterile: | | |
| | 1ml | 1M $MgSO_4$ |
| | 1ml | 0.1M $CaCl_2$ |
| | 1ml | Vitamin B1 (stock solution 1mg/ml, filter sterilized) |
| | 0.5ml | $FeCl_3$ 1mM |
| | 20ml | 10% (w/v) Casaminoacids |
| | 50ml | sugar ( 10% Salicin) |
| | 10ml | bromthymol blue stock solution (2% BTB in 50% Ethanol, 0.1N NaOH) |
| M9 Medium (Miller, 1972) | 20 x M9: | 140g $Na_2HPO_4$ x 2 $H_2O$ |
| | | 60g $KH_2PO_4$ |
| | | 20g $NH_4Cl$ |
| | | $H_2O$ to 1l |

M9 Medium (prepare from sterile solutions):

| | |
|---|---|
| 20 x M9 | 50ml |
| 0.1M $CaCl_2$ | 1ml |
| 1 M $MgSO_4$ | 1ml |
| 1mM $FeCl_3$ | 0.5ml |
| carbon source 1% final concentration: | |
| 20% Glucose | 50ml |
| or 80% Glycerin | 12.5ml |
| if required: | |
| 1mg/ml Vitamin B1 | 1ml |

| | | |
|---|---|---|
| 10% casamino acids | | 66ml |
| $H_2O$ | final volume | 1l |

Antibiotics were added to the final concentrations as shown below

| | | |
|---|---|---|
| ampicillin | stock 50mg/ml in 50% ethanol | final conc. 50µg/ml |
| chloramphenicol | stock 30mg/ml in ethanol | final conc. 15 µg/ml |
| kanamycin | stock 10mg/ml in $H_2O$ | final conc. 25 µg/ml |
| rifampicin | stock 100mg/ml in methanol | final conc. 100 µg/ml |
| spectinomycin | stock 50mg/ml in 30% ethanol | final conc. 50µg/ml |

**Table 1: Synthetic oligonucleotides used in the present work**

| name[a] | sequence[b] | description[c] |
|---|---|---|
| S6 | *AACCCGACTTCACCAGTATTC* | *bgl*: +554 to +534 |
| S83 | CCGCTCGA GGATCCTCTGTTACAGGTCACTAATACCATCTAA | lambda *attP* |
| S84 | CCGGTCGACAGATCTGAAATCAAATAATGATTTTATTTTGACTG | lambda *attP* |
| S92 | CAAGAGGAATATGACTTAAGAGTTCG | *bgl*: +792 to +757 |
| S100 | CATCGTAACCGTGCATCTGCCA | *lac*: +330 to +309 |
| S107 | GGATCTTCACCTAGATCCTTTTGGTCGA | pFDX733: 8632 to 8651 |
| S123 | TGTGGAATTGTGAGCGGATA | sequencing primer - *tacOP* |
| S125 | P-*GGATAAACTGCTGGCGGG* | *bgl*: +260 to +277 |
| S127 | P-CATGATTACGGATTCACTGGCCGT | *lac*: +67 to +44 |
| S140 | P-*GTTAUGTCAGGTTTTGCCTGCGAA* | *bgl*: +9 to +56 |
| S141 | P-CATCUGCCAGTTTGAGGGGACGACGA | *lac*: +317 to +292 |
| S156 | GATGATAAAAGGCACCTTTGGTCA | MiniTn10 primer |
| S172 | CCGAAGCTTGAATTCGCATATAAGGAAAAGAGAGAATGGCTA | *miaA/hfq* |
| S174 | GCGC GTCGACAGGCTTTACACTTTATGCTT | *PlacUV5*:-41 to -21 |
| S175 | TCCAGGTTCGTCCACACATTATACGAGCCGGAA | *PlacUV5*: +1 to –22 |
| S176 | AATGTGTGGA*CGAACCTGGATGTTCGTTATAA* | *PlacUV5*:-10 region<br>*bgl*: +1 to +22 |
| S184 | ATAACCAGAGAATACTGGTGAACAGGGGTTTTTTTGTTTATAAAA | *bgl*: +75 to +119 |
| S185 | TTCACCAGTATTCTCTGGTTATCAGAGGTTTTGCCTGCGAATGCA | *bgl*: +96 to +51 |
| S186 | ATAACCAGAGAATACTGGTGAAGTCGGGT | *bgl*: +75 to +103 |
| S187 | TTCACCAGTATTCTCTGGTTATGTCAGGTT | *bgl*: +96 to +67 |
| S188 | CCTGACATAACCAGAGAATACTGGTGAAGTCGGTTTTTTTGTTTA | *bgl*: +69 to +113 |
| S189 | P-*GCAAAGAACAC*ATGACCATGATTACGGATTCACTGGCCGT | *bgl*: +955 to +965<br>*lac*: +39 to +67 |
| S190 | AATCTAGA GCAAGGACCTTTTTTATAAACAA | *bgl*: +131 to +108 |
| S204 | AATCTAGA TTA*GATTTGCATGTTCATAGCAAGGA* | *bgl*: +146 to +124 |
| S205 | *ATGAACATGCAAATC*ATGACCATGATTACGGATTCA | *bgl*: +132 to +146<br>*lac*: +39 to +59 |
| S206 | GATTTGCATGTTCATAGCAAGGACCTT | *bgl*: +146 to +120 |
| S207 | AATCTAGA TTA*CACCACAACATTATTGTTGAGAA* | *bgl*: +176 to +154 |
| S208 | *CACCACAACATTATTGTTGAGAA* | *bgl*: +176 to +154 |
| S209 | *CTCAACAATAATGTTGTGGTG*ATGACCATGATTACGGATTCA | *bgl*: +156 to +176<br>*lac*: +39 to +59 |
| S215 | TTCCGGCTCGTATAATGTGTGG*AAGTCGGGTTTTTTTGTTTATAAAAAAGGT* | *bgl*: +95 to +124 |
| S220 | GCGGATCC*ATGAACATGCAAATCACCAAAATTCTCA* | *bgl*: +132 to +159 |

**Table 1: Synthetic oligonucleotides used in the present work**

| name[a] | sequence[b] | description[c] |
|---|---|---|
| S221 | AAGCTAGC*CAATTCCGCGCCCCATGACGA* | *bgl*: +226 to +205 |
| S222 | GGGGATCC*TGGGATATCCAGCGGCTTTACCCGA* | *bgl*: +492 to +516 |
| S223 | TTGCTAGC*TGGCAATAAAGCCCACTTCATCTTTCGGTA* | *bgl*: +606 to +577 |
| S224 | GGGGATCC*ACCCGCAAGCATGGCAATGT* | *bgl*: +841 to +860 |
| S225 | TTGCTAGC*GTGTTCTTTGCGCACGCGCTCTA* | *bgl*: +965 to +943 |
| S251 | AAGCTAGC*AGCAAGGACCTTTTTTATAAACAAAAAAACCCGA* | *bgl*: +131 to +98 |

a: Name of the oligo in lab collection

b: Sequence is shown from 5' to 3' end. P indicates 5' phosphate, U indicates biotinylated Uridine, restriction enzyme sites are underlined, *bgl* sequences are in italics.

c: Wherever relevant *bgl* and *lac* operon homologies are indicated. Numbering relative to transcription start of the respective operon. Additional descriptions and strategies when used for cloning are documented in lab records.

**Table 2: *E. coli* K-12 strains used in the present work**

| **strain** | **relevant genotype or structure$^a$** | **construction$^b$ / reference** |
|---|---|---|
| W3110 | $\lambda^-$ F$^-$ *bgl°* IN(*rrnD-rrnE*) (=CGSC#4474) (=S48) | (Bachmann, 1996) |
| CSH50 | *bgl°* Δ(*lac-pro*) *ara thi* (=S49) | (Miller, 1972) |
| PD32 | MC4100 *hns-206::*Ap$^R$ str$^R$ (=S102) | (Dersch et al., 1993) |
| R1243 | CSH50 *bgl*::*IS1*-R1243 (Bgl$^+$) (=S157) | (Schnetz and Rak, 1992) |
| S162 | CSH50 Δ*bgl*-AC11 | (Caramel and Schnetz, 1998) |
| S228 | CSH50 *DbglGFBHIK-AC11 DlacZ-Y217 pro*$^+$ | lab collection |
| S278 | S162 *Dbgl-AC11 D(argF-lac)U169 Pro*$^+$ | x T4*GT7*(MC4100), lab collection |
| S387 | S228 *attB*::[SpecR $wtP_{bgl}$ +54-*bglG-lacZ*] | x pKES15, lab collection |
| S393 | S228 *attB*::[SpecR $D2P_{bgl}$ +54-*bglG-lacZ*] | x pKES18, lab collection |
| S397 | S228 *attB*::[SpecR $wtP_{bgl}$ +54-*orfG-lacZ*] | x pKES20, lab collection |
| S403 | S228 *attB*::[SpecR $D2P_{bgl}$ +54-*orfG-lacZ*] | x pKES23, lab collection |
| S484 | CSH50 *bgl° D(argF-lac)U169 Pro*$^+$ | x T4*GT7*(MC4100) , lab collection |
| S486 | S484 *bgl°* (*gpt-lac*)$^+$ Lac$^+$ | x T4*GT7*(W3110) , lab collection |
| S524 | S486 Δ*lacZ*-Y217 | lab collection |
| S539 | S278 Δ*bgl*-AC11 (*gpt-lac*)$^+$ Lac$^+$ | x T4*GT7*(W3110) |
| S541 | S539 Δ*bgl*-AC11 Δ*lacZ*-Y217 | lab collection |
| S544 | *bgl-CRP* Δ*lacZ*-Y217 | Bgl$^+$ mutant of S524 |
| S572 | CSH50 (pro-lac)$^+$ *bglR::C234* (CAP-site mutant) | x T4*GT7*(S48) |
| S581 | S486 *bgl°* ΔlacOP::(spec$^R$ $wtP_{bgl}$ +55-*orfG*) | x pKES44 (Dabert and Smith, 1997) |
| S594 | S572 *bgl-CAP*$^+$*-C234 DlacOP*::(spec$^R$ *CAP+-C234* $P_{bgl}$ +55-*orfG*) | x pKES51 (Dabert and Smith, 1997) |
| S614 | S541 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S749 | S594 *lon108* | results section 3, figure 13 |
| S750 | S594 *hfq110* | results section 3, figure 13 |
| S751 | S594 *pgi111* | results section 3 |
| S752 | S594 *pgi112* | results section 3 |
| S753 | S594 *pgi123* | results section 3 |
| S754 | S594 *lon124* | results section 3, figure 13 |
| S755 | S594 *pgi132* | results section 3 |
| S756 | S594 *cyaA152* | results section 3 |
| S757 | S594 *hfq155* | results section 3, figure 13 |
| S759 | S594 *lon166* | results section 3, figure 13 |
| S760 | S594 *lon172* | results section 3, figure 13 |
| S762 | S594 *lon183* | results section 3, figure 13 |
| S763 | S594 *hfq184* | results section 3, figure 13 |
| S764 | S594 *lon187* | results section 3, figure 13 |
| S765 | S594 *pgi194* | results section 3 |
| S766 | S594 *lon204* | results section 3, figure 13 |
| S770 | S541 *pgi111* | x T4*GT7*(S751) |
| S772 | S541 *hfq155* | x T4*GT7*(S757) |
| S776 | S541 *lon187* | x T4*GT7*(S764) |
| S777 | S541 *pgi*194 | x T4*GT7*(S765) |
| S778 | S539 *hfq155* | x T4*GT7*(S772) |
| S790 | S387 *hfq155* | x T4*GT7*(S772) |
| S792 | S393 *hfq155* | x T4*GT7*(S772) |
| S794 | S539 *lon187* | x T4*GT7*(S776) |
| RH90 | MC4100 *rpoS359::Tn10* (=S810) | (Lange and Hengge-Aronis, 1991) |
| MC4100 | F$^-$ *bgl°* Δ(*argF-lac*)U169 *araD139 deoC1 flb5301 relA1 rpsL150 ptsF25 rbsR* (=S101) | (Casadaban, 1976) |
| AM111 | MC4100 *hfq1::W* (=S812) | (Muffler et al. 1996) |
| AM112 | MC4100 *hfq2::W* (=S813) | (Muffler et al. 1996) |
| S829 | S387 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S831 | S393 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S833 | S397 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S835 | S403 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |

**Table 2: *E. coli* K-12 strains used in the present work**

| strain | relevant genotype or structure$^a$ | construction$^b$ / reference |
|---|---|---|
| S837 | S387 *hfq2::W* (Kan$^R$) | x T4*GT7*(AM112) |
| S839 | S393 *hfq2::W* (Kan$^R$) | x T4*GT7*(AM112) |
| S841 | S397 *hfq2::W* (Kan$^R$) | x T4*GT7*(AM112) |
| S843 | S403 *hfq2::W* (Kan$^R$) | x T4*GT7*(AM112) |
| S885 | S541 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S887 | S541 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S891 | S397 *hfq155* | x T4*GT7*(S772) |
| S893 | S403 *hfq155* | x T4*GT7*(S772) |
| S940 | S541 *attB*::[SpecR *wt*$P_{bgl}$ *t1 bglG lacZ*] | x pKESD8 |
| S942 | S541 *attB*::[SpecR *bgl-D2* $P_{bgl}$ *t1 bglG lacZ*] | x pKESD11 |
| S944 | S541 *attB*::[SpecR *lacUV5 t1 bglG lacZ*] | x pKESD20 |
| S960 | S944 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1052 | S541 *attB*::[SpecR *bgl-CRP* $P_{bgl}$ *t1 bglG lacZ*] | x pKESD9 |
| S1054 | S541 *attB*::[SpecR *bgl*::*IS5-H3* $P_{bgl}$ *t1 bglG lacZ*] | x pKESD12 |
| S1071 | S524 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1079 | S544 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1095 | S541 *attB*::[SpecR *lacUV5 t1 bglG lacZ*] | x pKESD20 |
| S1097 | S541 *attB*::[SpecR *lacUV5 t1-L bglG lacZ*] | x pKESD28 |
| S1099 | S541 *attB*::[SpecR *lacUV5 t1-R bglG lacZ*] | x pKESD29 |
| S1101 | S541 *attB*::[SpecR *lacUV5 t1-LR bglG lacZ*] | x pKESD30 |
| S1103 | S541 *attB*::[SpecR *lacUV5 t1-DG100 bglG lacZ*] | x pKESD31 |
| S1107 | S539 *rpoS359::Tn10* | x T4*GT7*(RH90) |
| S1112 | S614 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1130 | S1095 *rpoS359::Tn10* | x T4*GT7*(RH90) |
| S1132 | S1097 *rpoS359::Tn10* | x T4*GT7*(RH90) |
| S1142 | S541 *attB*::[SpecR *wt*$P_{bgl}$ *t1-L bglG lacZ*] | x pKESK11 |
| S1144 | S541 *attB*::[SpecR *bgl-CRP*$P_{bgl}$ *t1*-L *bglG lacZ*] | x pKESK12 |
| S1146 | S541 *attB*::[SpecR *bgl-D2*$P_{bgl}$ *t1*-L *bglG lacZ*] | x pKESK13 |
| S1148 | S541 *attB*::[SpecR *bgl*::*IS5-H3*$P_{bgl}$ *t1*-L *bglG lacZ*] | x pKESK14 |
| S1155 | S1142 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1157 | S1144 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1159 | S1146 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1161 | S1148 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1189 | S541 *attB*::[SpecR *lacUV5 t1-L orfG lacZ*] | x pKESD47 |
| S1191 | S541 *attB*::[SpecR *lacUV5 t1-L RBS-bglG-RBS-lacZ lacZ*] | x pKESD36 |
| S1211 | S541 *attB*::[SpecR $P_{bgl}$*(-76 to +25) lacZ*] | x pKEKB25 |
| S1213 | S541 *attB*::[SpecR *wt*$P_{bgl}$*+25 lacZ*] | x pKEKB30 |
| S1215 | S541 *attB*::[SpecR *bgl-CRP*$P_{bgl}$*+25 lacZ*] | x pKEYK01 |
| S1217 | S541 *attB*::[SpecR *bgl-CRP*$P_{bgl}$*(-76 to +25) lacZ*] | x pKEYK02 |
| S1245 | S541 *bgl*::*IS1*-R1243 [Bgl$^+$] | x T4*GT7*(R1243) |
| S1250 | S887 *bgl*::*IS1*-R1243 [Bgl$^+$] *rpoS359*::*Tn10* | x T4*GT7*(R1243) |
| S1252 | S1189 *hns:: amp*$^R$ | x T4*GT7*(PD32) |
| S1254 | S1191 *hns:: amp*$^R$ | x T4*GT7*(PD32) |
| S1307 | S944 *hns:: amp*$^R$ | x T4*GT7*(PD32) |
| S1309 | S1097 *hns:: amp*$^R$ | x T4*GT7*(PD32) |
| S1311 | S1097 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1365 | S541 *attB*::[SpecR *bgl*::*IS5-H3 bglGFB*] | x pKESD63 |
| S1367 | S541 *attB*::[SpecR *bgl-D2 bglGFB*] | x pKESD64 |
| S1384 | S1365 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1386 | S1367 *rpoS359*::*Tn10* | x T4*GT7*(RH90) |
| S1402 | S1142 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1404 | S1155 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1406 | S1146 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1408 | S1159 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1410 | S1148 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1412 | S1161 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |

**Table 2: *E. coli* K-12 strains used in the present work**

| strain | relevant genotype or structure[a] | construction[b] / reference |
|---|---|---|
| S1414 | S964 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1416 | S1132 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1418 | S539 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1420 | S1107 *hfq1::W* (Kan$^R$) | x T4*GT7*(AM111) |
| S1422 | S539 *pgi111* | x T4*GT7*(S770) |
| S1424 | S539 *pgi194* | x T4*GT7*(S777) |
| S1467 | S1142 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S1469 | S1146 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S1471 | S1213 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S1473 | S1211 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S1475 | S1215 *hns::Ap*$^R$ | x T4*GT7*(PD32) |
| S1477 | S1217 *hns::Ap*$^R$ | x T4*GT7*(PD32) |

a: The relevant genotype of the constructed CSH50 derivatives refers to the *bgl*, *lac*, *hfq*, *lon, pgi, cyaA*, *hns* and *rpoS* loci. Mutations causing activation of the silent *bgl* operon include *bgl-CRP* (a C to T exchange in the CRP binding site at position –66, relative to the transcription start), *bgl*::*IS1-R1243* (integration of *IS1* in orientation II generating a target site duplication from -88 to -80), *bgl::IS5-H3* (an integration of IS5 in orientation II generating a target site duplication from position –92 to –89), and *bgl-D2* (a deletion of the upstream silencer, extending from position –77). For terminator mutations *t1-L*, *t1-R*, *t1-LR*, and *DG100* see figure 9. (Strain numbers in brackets refer to lab-stocks.)

b: Transductants (using T4*GT7*) of Bgl$^+$ alleles were selected on minimal Salicin B1 plates, of *rpoS359*::*Tn10 on* LB tetracycline plates, on minimal glucose B1 plates (Pro$^+$), or on minimal lactose plates (Lac$^+$). Integrations in to *attB* were performed as described(see material and methods). (Diederich et al., 1992)

**Table 3: Plasmids used in the present work**

| name | relevant structure/description[a] and replicon/resistance[b] | source/construction[c]/ reference |
|---|---|---|
| pLDR8 | lambda repressor, temperature sensitive allele *cI-857*; *int* under control of $\lambda$ $P_R$, pSC101 rep-ts, kan | (Diederich et al., 1992) |
| pFDX733 | *wt bgl* operon, kan | (Schnetz et al., 1987) |
| pFDX733-C234 | *bgl-C234* allele of *bgl* operon, kan | Lab collection |
| pFDX733-H3 | *IS5-H3* allele of *bgl* operon, kan | Lab collection |
| pFDY167 | *wt-*$P_{bgl}$ *t1 bglG lacZ*, kan | Lab collection |
| pFDY167-H3 | *IS5-H3-*$P_{bgl}$ *t1 bglG lacZ*, kan | Lab collection |
| pFDY217 | *lacI lacOP lacY*, pSC101 rep-ts, tet | Lab collection |
| pFDY241 | *lacUV5 t1 bglGFB*, kan | Lab collection |
| pFDY446 | *bgl-D2* allele of *bgl* operon, kan | Lab collection |
| pFDY558 | *C234-*$P_{bgl}$ *t1 bglG lacZ*, kan | Lab collection |
| pFDY560 | *D2-*$P_{bgl}$ *t1 bglG lacZ*, kan | Lab collection |
| pHP45 omegon | *aadA bla T4-t32a* terminator, pBR | (Prentki and Krisch, 1984) |
| pKEKB25 | *attP* $P_{bgl}$ (*bgl*: –76 to +25) *lacZ* | Lab collection |
| pKEKB30 | *attP wt-*$P_{bgl}$*+25 lacZ* | Lab collection |
| pKES15 | *attP wt-*$P_{bgl}$*+54 lacZ* | Lab collection |
| pKES18 | *attP D2-*$P_{bgl}$*+54 lacZ* | Lab collection |
| pKES20 | *attP wt-*$P_{bgl}$*+54 orfG lacZ* | Lab collection |
| pKES23 | *attP D2-*$P_{bgl}$*+54 orfG lacZ* | Lab collection |
| pKES44 | *chi lacI-q wt-*$P_{bgl}$*+54 lacZ chi* | Lab collection |
| pKES51 | *chi lacI-q C234-*$P_{bgl}$*+54 orfG lacZ chi* | Lab collection |
| pKESD7 | *attP wt-*$P_{bgl}$ *bglG lacZ*, kan | V: pFDY167-SalI, phosphatase<br>F: Lambda *attP* PCR S83/S84, XhoI/Sal I |
| pKESD8 | *attP wt-*$P_{bgl}$ *bglG lacZ* | V: pKESD07 BglII, phosphatase<br>F: pHP45Omegon BamHI (2056bp fragment containing spc/str res gene *aadA*) |
| pKESD9 | *attP C234-*$P_{bgl}$ *bglG lacZ* | V: pKESD08 SalI/XhoI<br>F: pFDY558 SalI/XhoI |
| pKESD11 | *attP* Δ2-$P_{bgl}$ *bglG lacZ* | V: pKESD08 SalI/XhoI<br>F: pFDY560 SalI/XhoI |
| pKESD12 | *attP IS5-H3-*$P_{bgl}$ *bglG lacZ* | V: pKESD08 SalI/XhoI<br>F: pFDY167-H3 SalI/XhoI |
| pKESD20 | *attP lacUV5 t1 bglG lacZ* | V: pKESD08 salI/AflII,phosphatase<br>F: PCR template pFDY241 S174/S175, PCR template pKESD8 S176/S92, PCR template products 1+2 S174/S92, SalI/AflII |
| pKESD24 | *attP lacUV5 t1-L lacZ* (translational fusion in to *bglG*-ATG) | V: pKESD23 SalI/Eco81I, phosphatase<br>F: PCR template pFDX733 S174/S185, PCR template pFDX733 S186/S100, PCR template products1+2 S174/S100, SalI/Eco81I |
| pKESD28 | *attP lacUV5 t1-L bglG lacZ* | V: pKESD20 SalI/AflIII, phosphatase<br>F: PCR template pFDX733 S174/S185, PCR template pFDX733 S186/S100, PCR template products 1+2 S174/S92, SalI/AflII |
| pKESD29 | *attP lacUV5 t1-R bglG lacZ* | V: pKESD20 SalI/AflIII, phosphatase<br>F: PCR template pFDX733 S174/S187, PCR template pFDX733 S184/S100,<br>PCR template products 1+2 S174/S92, SalI/AflII |

**Table 3: Plasmids used in the present work**

| **name** | **relevant structure/description[a] and replicon/resistance[b]** | **source/construction[c]/ reference** |
|---|---|---|
| pKESD30 | *attP lacUV5 t1-LR bglG lacZ* | V: pKESD20 SalI/AflIII, phosphatase<br>F: PCR template pFDX733 S174/S185, PCR template pFDX733 S184/S100, PCR template products 1+2 S174/S92 SalI/AflII |
| pKESD31 | *attP lacUV5 t1-DG100 bglG lacZ* | V: pKESD20 SalI/AflIII, phosphatase<br>F: PCR template pFDX733 S174/S187, PCR template pFDX733 S188/S100, PCR template products 1+2 S174/S92 SalI/Eco81I |
| pKESD36 | *attP lacUV5 t1-L +130 lacZ* | V: pKES15 XbaI/SalI, phosphatase<br>F: PCR template pKESD28 S94/S190 SalI/XbaI |
| pKESD39 | *attP lacUV5 t1-L F(bglG 278aa-lacZ)* | V: pKESD28 Eco81I/FspI, phosphatase<br>F: PCR template pFDX232 S189/S100 Eco81I |
| pKESD42 | *attP lacUV5 t1-L bglG (5aa) lacZ* | V: pKES15 SalI/XbaI, phosphatase<br>F: PCR template pKESD28 S94/S204 SalI/XbaI |
| pKESD43 | *attP lacUV5 t1-L F(bglG 5aa-lacZ)* | V: pKESD28 SalI/Eco81I, phosphatase<br>F: PCR template pKESD28 S94/S206, PCR template pKESD28 S205/S100, PCR template products 1+2 S94/S100 SalI/Eco81I |
| pKESD44 | *attP lacUV5 t1-L bglG (15aa) lacZ* | V: pKES15 with SalI/XbaI, phosphatase<br>F: PCR template pKESD28 S94/S207, SalI/XbaI |
| pKESD45 | *attP lacUV5 t1-L F(bglG 15aa-lacZ)* | V: pKESD28 SalI/Eco81I, phosphatase<br>F: PCR template pKESD28 S94/S208, PCR template pKESD28 S209/S100, PCR template products 1+2 S94/S100, SalI/Eco81I |
| pKESD47 | *attP lacUV5 t1-L orfG lacZ* | V: pKESD28 salI/AflII phosphatase,<br>F: PCR template pKESD28 S92/S185, PCR template pFDY487S186/S92, PCR template products 1+2 S92/S94, SalI/AflII |
| pKESD48 | *attP lacUV5 +95 bglG lacZ* | V: pKESD28 SalI/AflII, phosphatase<br>F: PCR template pKESD28 S94/S214, PCR template pKESD28 S215/S92, PCR template products 1+2 S94/S92, SalI/AflII |
| pKESD49 | *attP lacUV5 +95 orfG lacZ* | V: pKESD28 SalI/AflII, phosphatase<br>F: PCR template pKESD28 S94/S214, PCR template pKESD47 S215/S92, PCR template products 1+2 S94/S92, SalI/AflII |
| pKESD53 | *lacI* $P_{tac}$ *bglG* gene fragment ( *bglG* nt 1-95) *rrnB-T1,T2*, pBR, amp | V: pFDY157 BamHI/Nhe1, phosphatase<br>F: PCR template pFDX733 S220/S221, BamHI/NheI |
| pKESD54 | *lacI Ptac bglG* gene fragment (*bglG* nt361-475) *rrnB-T1,T2*, pBR, amp | V: pFDY157 BamHI/Nhe1, phosphatase<br>F: PCR template pFDX733 S222/S223, BamHI/NheI |
| pKESD55 | *lacI Ptac bglG* gene fragment(*bglG* nt709-834) *rrnBT1/T2*, pBR, amp | V: pFDY157 BamHI/Nhe1, phosphatase<br>F: PCR template pFDX733 S224/S225, BamHI/NheI |
| pKESD62 | *IS1-*$P_{bgl}$ *t1 bglGFB*, kan | V: pFDX733 SalI/AflII, phosphatase<br>F: PCR template genomic DNA strain S157 S107/S92, SalI/AflII |
| pKESD63 | *attP IS5-H3-*$P_{bgl}$ *t1 bglGFB* | V: pKESD08 SalI/XhoI, phosphatase<br>F: pFDX733-H3 SalI/XhoI |
| pKESD64 | *attP D2-*$P_{bgl}$ *t1 bglGFB* | V: pKESD08 SalI/XhoI, phosphatase F: pFDY446 SalI/XhoI |

**Table 3: Plasmids used in the present work**

| name | relevant structure/description[a] and replicon/resistance[b] | source/construction[c]/ reference |
|---|---|---|
| pKESD65 | *lacI Ptac lacO bgl:+1-131 t1-L,* pBR, amp | V: pFDY157 BamHI/NheI, phosphatase<br>F: PCR template pKESD46 S123/S251, BamHI/NheI |
| pKESK10 | *lacI lacUV5 lacO bglG*, pSC101,cm | Lab collection |
| pKESK11 | *wt*-$P_{bgl}$ *t1-L bglG lacZ* | Lab collection |
| pKESK12 | *C234*-$P_{bgl}$ *t1-L bglG lacZ* | Lab collection |
| pKESK13 | *D2*-$P_{bgl}$ *t1-L bglG lacZ* | Lab collection |
| pKESK14 | *IS5-H3*-$P_{bgl}$ *t1-L bglG lacZ* | Lab collection |
| pKEYK01 | *attP C234*-$P_{bgl}$*+25 lacZ* | Lab collection |
| pKEYK02 | *attP C234*-$P_{bgl}$ *(-76 to +25) lacZ* | Lab collection |

a: The relevant genotype is shown. Mutations causing activation of the silent *bgl* operon include *bgl-C234* (a C to T exchange in the CRP binding site at position –66, relative to the transcription start), *bgl*::*IS1-R1243* (integration of *IS1* in orientation II generating a target site duplication from -88 to -80), *bgl::IS5-H3-H3* (an integration of IS5 in orientation II generating a target site duplication from position –92 to –89), and *bgl-D2* (a deletion of the upstream silencer, extending from position –77). For terminator mutations *t1-L*, *t1-R*, *t1-LR,* and *DG100* see figure 9.

b: Plasmids when not stated carried pACYC (pA15) replication origin (Chang and Cohen, 1978) and kanamycin (kan), spectinomycin resistance markers. Those carrying pSC101 replication origin (Hashimotoh-Gotoh et al., 1981) or pBR replication origin (Bolivar, 1978) and chloramphenicol (cm) or tetracyclin (tet) resistance markers are indicated.

c: This table gives a short description of the plasmid construction. The first line (V:) indicates the vector fragment. The second line (F:) indicates the insert fragment preparation. PCR reactions are indicated in the order; PCR, template DNA and primers used. For further details see methods. Detailed plasmid construction descriptions are documented in lab records and all sequences are compiled in Vector NTi. All the PCR fragments cloned were verified for absence of PCR introduced errors by sequencing.

## 3. General Methods

The molecular biology methods like restriction and other enzyme reactions, PCR, plasmid DNA purification, SDS-PAGE chromatography, sequencing gels and auto-radiography were performed as described (Sambrook et al., 1989) or according to the manufacturer's instructions, unless otherwise stated. Large scale preparation of plasmid DNA were performed using the plasmid maxi kit (Qiagen) according to manufacturers instructions.

## 4. TNE-method (Serghini et al., 1989)

Isolation of plasmid DNA for analytical purposes was performed according to the TNE method

TNE buffer: 10mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0
100mM NaCl

- centrifuge 1.5ml of an overnight culture for 2 minutes 13000 rpm
- decant the supernatant, re-suspend the pellet in 100µl TNE buffer
- add 1 volume of Phenol-Chloroform-isoamyl alcohol, vortex very briefly (1-2 seconds)
- centrifuge for 5 minutes at 13000 rpm
- transfer the upper aqueous phase in to a new tube
- precipitate DNA with 2.5 volumes 100% ethanol, mix by inverting the tube
- centrifuge for 10 minutes at 13000 rpm
- wash the pellet with 80% ethanol
- centrifuge for 5 minutes at 13000 rpm
- decant the supernatant and dry the pellet under vacuum
- dissolve DNA pellet in 50µl $H_2O$
- for restriction enzyme analysis use 8µl (low copy plasmids) and 2µl (high copy plasmids), add RNase before loading DNA on to agarose gel
- store plasmid DNA at 4°C or -20°C

## 5. Plasmid isolation using $CsCl_2$ gradient method (for pure supercoiled plasmid DNA preparation)

buffer P1: 50mM Tris-HCl, pH 8.0
10mM EDTA pH 8.0
buffer P2: 0.2N NaOH
1% SDS (w/v)
buffer P3: 3M potassium-acetate, pH 5.5
CsCl/TE/Ethidium-bromide stock: 1:1:1/20 (*eg* 50g CsCl, 50ml TE, 2.5ml Ethidium-bromide 10mg/ml)
Butanol ($H_2O$/CsCl saturated)

- grow 800ml over night culture
- centrifuge the overnight culture: *eg* J6-Beckman-centrifuge at 4200 rpm for 20 minutes, 4°C
- resuspend bacterial pellet in buffer P1, use 10ml per 100ml culture
- incubate for 5 minutes at room temperature
- add buffer P2 (10ml/100ml culture volume), mix gently, incubate for 5 minutes on ice
- add buffer P3 (10ml/100ml culture volume), mix thoroughly with a pipette, incubate for at least 5 minutes

- centrifuge for 15-20 minutes at 10000rpm, 4°C, *eg* GSA/Sorvall
- filter the supernatant to remove particles
- precipitate DNA by adding 0.7 volume isopropanol
- centrifuge for 15 minutes at 10000 rpm and 4°C
- air-dry the pellet
- re-dissolve the pellet in 3-4ml TE-buffer, pH8.0
- add CsCl to DNA / TE solution (1.1g CsCl per ml DNA solution), dissolve CsCl carefully, clear turbid solutions by centrifugation
- add Ethidium-bromide (10mg/ml stock, 50µl per 1ml DNA solution)
- ultra centrifuge, *eg* 4h using Beckman TLN100 rotor, 100,000rpm
- recover supercoiled plasmid band with a syringe
- run a second gradient (fill up centrifuge tube with CsCl/TE/Ethidium-bromide stock
- recover supercoiled plasmid DNA band with a syringe
- extract several times with Butanol ($H_2O$/CsCl saturated) to remove Ethidium-bromide (till solution is colorless and one additional time)
- precipitate DNA by adding 4 volumes 50% iso-propanol
- wash DNA pellet with 80% ethanol, dry pellet
- re-suspend DNA in 400µl TE (pH 8.0)
- measure $OD_{260}$ (dilute the DNA, for example 1:50 or 1:100).
  1 $OD_{260}$ = 50µg/ml

### 6. Preparation of competent cells and transformation ($CaCl_2$ method)

TEN buffer: 20mM Tris-Hcl pH 7.5, 1mM EDTA, 50mM NaCl

- grow cells in 50ml LB2Y medium till $OD_{600}$=0.3
- centrifuge and resuspend the cell pellet in 25ml ice cold 0.1M $CaCl_2$
- incubate on ice for 20 minutes
- centrifuge again and resuspend pellet in 2ml ice cold 0.1M $CaCl_2$
- use 100µl of these cells for transformation
- add 10-50ng of the plasmid to be transformed in to 50µl of TEN buffer and cool on ice.
- add 100µl of competent cells and incubate on ice for 20 minutes
- heat-shock at $42^{o}$C for exactly 2 minutes
- incubate on ice for 10 minutes
- add to 1ml LB2Y medium and shake at $37^{o}$C for 1 hour
- plate 100µl on suitable selective plates

### 7. Plasmids and DNA fragments

A brief description of plasmid constructions can be found in table 2. Details of plasmid constructions are documented in the lab records and sequences compiled in Vector NTi.

A series of plasmids starting with pKESD7 were constructed which have a pACYC (p15A) replication origin (figure 23). They have the λ phage attachment site, *attP*, cloned in to them to allow for λ integrase mediated recombinational insertion in to the *attB* site of the *E. coli* chromosome (Diederich et al., 1992). These plasmids also have a Ω cassette which contains the spectinomycin resistance gene, *aadA* and strong transcriptional terminators at its 3' end. As seen in figure 23, plasmid pKESD8 has convenient restriction enzyme sites which could be used to replace the *bgl* construct with a different construct. For integration into the chromosomal *attB* site, all these plasmids were then cut with BamHI and the originless fragment containing the spectinomycin resistance gene and the *bgl* construct was used for insertion in to the *attB* site as described below.

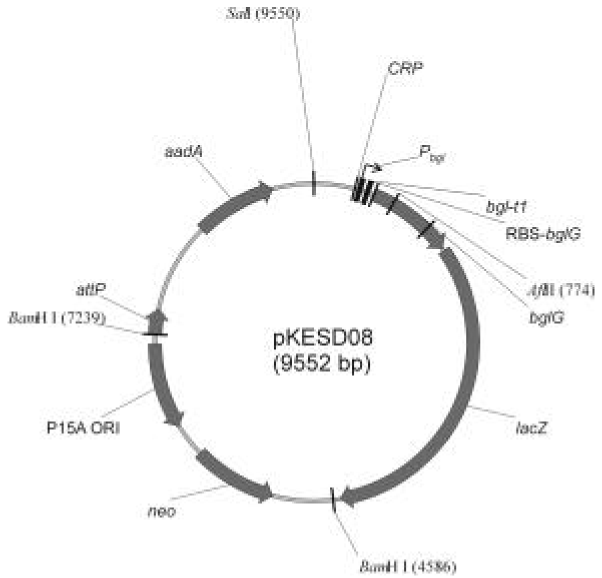


**Figure 23: Schematic representation of plasmid pKESD8**. This plasmid and later plasmids based on the same principle carry a λ phage attachment site *attP* for integration in to the *E. coli* genome. Resistance markers for spectinomycin (*aadA*) and kanamycin (*neo*) are shown. Plasmid pKESD8 has a $wtP_{bgl}$-*bglG-lacZ* construct. This contruct can be conveniently replaced with a different construct to generate a series of plasmids used in this study. Some of the restriction sites which could be used for this purpose are shown.

## 8. Integration of plasmids in the *attB* site of *E. coli* chromosome (Diederich et al., 1992)

- transform the recipient strain with helper plasmid pLDR8 and select on LB2Y kanamycin plates at 28°C
- inoculate 3-4ml LB2Y kanamycin medium in glass tubes from a fresh single colony of the strain/pLDR8-plates
- shake culture over night at 28°C
- inoculate 25ml LB2Y kanamycin medium in 100ml Erlenmeyer flask with 1.25ml (1:20 dilution) of the fresh over night culture
- shake culture at 37°C for 90minutes
- prepare competent cells using the $CaCl_2$ method
- cut 5µg of the plasmid containing the construct to be integrated with BamHI (10U) per 100µl over night at 37°C
- run on agarose gel and extract the originless fragment using Qiagen gel extraction kit
- use 10ng BamHI-fragment for re-ligation in 20µl total volume
- transform the competent cells prepared as above using 10µl (=1/2) of BamHI religation reaction
- plate: 2x 0.2ml on LB2Y spectinomycin plates and 1x 0.2ml on MacConkey lactose spectinomycin plates

- incubate plates at 42°C over night

testing of clones via PCR:

- pick a colony and resuspend the cells in 100µl $H_2O$
- use the PCR-primer: S93/S164: to test the attB/P`-side
  S95/S96: to test the attP/B`-side
  S95/S164: to see integrations of dimers
  suitable primers to test the fragment

## 9. Biochemical purification of *bgl* DNA binding proteins

**Preparation of *E. coli* cell extracts** (modified from Schnetz, 1995)

CE medium: 100mM potassium phosphate, pH 6.2, 10g/l yeast extract, 1mg/ml thiamine
extraction buffer: 12% Glycerol, 20mM Tris-HCl, pH 7.9, 2mM EDTA, 1mM DTT
high salt lysis buffer: 12% Glycerol, 20mM Tris-HCl, pH 7.9, 1mM DTT, 1mM PMSF, 700mM KCl

- prepare ovenight cultures in CE medium using a fresh colony
- inoculate 500ml CE medium using the overnight culture to a starting $OD_{600}$ of 0.1.
- grow cultures with shaking at $37^0$C till $OD_{600}$ of 0.8
- put on ice and add 125ml pre-chilled 20mM Tris-Cl, pH 7.9
- centrifuge at 5000rpm, 20 minutes, 4°C
- wash with 50ml extraction buffer and centrifuge the cells again
- resuspend cells in 4.5ml high salt lysis buffer containing 1mg/ml Lysozyme
- incubate on ice with occassional swirling
- sonicate the cells on ice ( 2 x 10 second pulses)
- ultracentrifuge at 20000rpm for 30 minutes, $4^o$C (Beckman TLS 55 rotor)
- collect supernatant and add 10mM $CaCl_2$ (final concentration)
- add Micrococcal nuclease (Roche) to 1000U/ml final concentration
- incubate at room temperature for 30 minutes
- add 50mM EGTA pH 8.0 solution to 2mM final concentration
- purify the protein solution over a PD10 column (Amersham Pharmacia Biotech) using extraction buffer containing 2mM EDTA and 1mM PMSF for equilibration of the column and for elution
- measure protein concentration using Bio-Rad protein assay kit, according to manufacturer´s instructions
- aliquot protein solution, quick freeze in liquid $N_2$ and store at –80°C

**Phosphocellulose purification of cell extract**

- dilute the cell extract prepared as above to 30mM KCl by adding extraction buffer and 2.5M KCl
- wash activated phosphocellulose thrice with 3 volumes each of extraction buffer containing 1M KCl. (1ml of phosphocellulose binds approximately 10-60mg protein)
- equilibrate phosphocellulose thrice in 3 volumes of extraction buffer containing 30mM KCl
- add cell lysate to phosphocellulose, allow binding for 30 minutes at 4°C, with slow shaking

- wash thrice in 3 volumes of extraction buffer containing 30mM KCl
- elute with 1 volume extraction buffer containing 800mM KCl
- repeat elution if necessary
- desalt eluted proteins with Nap5 column (Amersham Pharmacia Biotech)
- determine protein concentration using Bio-Rad protein assay kit, according to manufacturer's instructions

**DNA Affinity chromatography**

B&W buffer: 5mM Tris-HCl, pH 7.5, 0.5mM EDTA, 1M NaCl
protein binding buffer: 12% glycerol, 20mM Tris-HCl, pH7.9, 2mM EDTA, 1mM DTT, 100mM KCl
elution buffer: 12% glycerol, 20mM Tris-HCl, pH7.9, 2mM EDTA, 1mM DTT, 700mM KCl

- prepare DNA fragments by PCR using Pwo polymerase (Roche Molecular Biochemicals), plasmid pKESD08 as template, primer pairs S125/S140 (*bgl*) and S127/S141(*lacZ*)
- purify the resulting PCR fragments using the PCR purification kit (Qiagen) and concentrate if required by vaccum drying (Speedvac, Savant). Determine final DNA concentration by measuring $OD_{260}$
- use 100µl Streptavidin coated magnetic beads (10mg/ml) (Dynabeads M-280, Dynal) per reaction. DNA binding capacity of the beads is 1pmol/mg
- at each of the following steps magnetically separate beads from the washing buffer using the magnetic separator (Roche Molecular Biochemicals)
- remove supernatant and wash thrice with 100µl 1x B&W buffer
- add 25pmoles of respective DNA fragment in 1x B&W buffer (100µl) to the beads and allow DNA binding by slowly rotating overnight at room temperature.
- remove supernatant and wash thrice with 100µl 1x B&W buffer
- wash thrice with 100µl 1x protein binding buffer
- wash thrice with 100µl elution buffer
- wash thrice with 100µl 1x protein binding buffer
- add 100µl (50µg/100µl) protein solution in 1x protein binding buffer (for this dilute the original cell extract with 5x protein binding buffer and distilled water)
- incubate for 10 minutes at room temperature with slow rotation
- remove supernatant and wash thrice with 100µl 1x protein binding buffer
- perform the last washing in a fresh tube to avoid proteins bound to the tube walls
- elute twice by incubating in 10µl elution buffer for 10 minutes each
- wash thrice with 100µl elution buffer
- wash thrice with 100µl 1x protein binding buffer
- beads with attached DNA fragments can now be reused for fresh protein binding
- to the elutes (20µl) add equal volume of 12% trichloroacetic acid and incubate on ice for 30 minutes
- centrifuge at 13000rpm, 4°C for 30 minutes
- wash with cold acetone (20µl) and re-centrifuge for 10 minutes

- resuspend in SDS-PAGE loading dye and load on to 15% SDS-PAGE gel (Sambrook et al., 1989)

**Silver staining and protein identification**

- fix gel in 50% methanol, 5% acetic acid for 20 minutes
- wash for 10 minutes in 50% methanol
- wash for 10 minutes in distilled water
- incubate in 0.02% Sodium thio-sulfate for 1minute
- rinse twice with distilled water
- put in chilled 0.1% silver nitrate, incubate for 20 minutes at 4°C
- rinse twice with distilled water
- add developer solution (0.04% formaldehyde, 2% $Na_2CO_3$), keep gently shaking till bands develope sufficiently. If required replace the colored developer solution with fresh solution
- stop the reaction with 5% acetic acid
- store in 1% acetic acid solution at 4°C
- cut the desired bands with scalpel and use them for in-gel Trypsin digestion and mass spectroscopic analysis

**10.** b **-galactosidase assay** (Miller, 1972)

| | |
|---|---|
| Z buffer: | 100mM Na-phosphate pH 7.0, 10mM KCl, 1mM $MgSO_4$, 100µg/ml chloramphenicol |
| 1 M $Na_2CO_3$ | |
| ONPG: | 4mg/ml in 0.1M phosphate buffer pH 7.0 |
| SDS: | 0.1% (w/v) |
| chloroform | |

day 1:

- prepare overnight cultures (3-4 ml) in minimal media M9 with necessary supplements as required *eg* glycerol or other carbon source, casamino acids, thiamine, antibiotics etc.

day 2:

- measure the $OD_{600}$ of the overnight cultures diluted 1:5 or 1:10
- inoculate 10ml cultures in M9 media (containing 1mM IPTG; 0.5% (w/v) salicin if required) to an $OD_{600}$ of 0.15 and let them grow at 37°C till $OD_{600}$ = 0.5
- when $OD_{600}$ reaches 0.5 stop cultures on ice
- to perform enzyme assay prepare dilutions from the cooled cultures in ice cold Z buffer
- add one drop of 0.1% (w/v) SDS and two drops of chloroform to the probes on ice. Vortex the probes for at least 15 seconds and then immediately pre-incubate them for 5 minutes at 28°C
- start the reaction by adding 0.2ml ONPG with a multipette and mix
- stop the reaction after 30 minutes by adding 0.5ml 1M $Na_2CO_3$ (or later, when the color turns to a strong yellow, note exact time)
- centrifuge the probes for 5-10 minutes and then measure the $OD_{420}$
- calculate the enzyme activity in Miller units as

$$\text{Miller units} = \frac{OD_{420} \ \text{x dilution factor x } 1000}{OD_{600} \ \text{x time (minutes)}}$$

**11. ß-Glucosidase-assay** (modified from (Schnetz and Rak, 1988)

Z buffer: 60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 100µg/ml chloramphenicol

1 M $Na_2CO_3$

PNPG: 8mg/ml in 0.1M phosphate buffer pH7.0

day 1:

- prepare overnight cultures (3-4 ml) in minimal media M9 with necessary supplements as required *eg* glycerol or other carbon source, casamino acids, thiamine, antibiotics etc.

day 2:

- measure the $OD_{600}$ of the overnight cultures diluted 1:5 or 1:10
- inoculate 10ml cultures in M9 media (containing 1mM IPTG; 0.5% (w/v) Salicin if required) to an $OD_{600}$ of 0.15 and let them grow at 37°C till $OD_{600}$ = 0.5
- when $OD_{600}$ reach 0.5 stop cultures on ice
- to perform enzyme assay prepare dilutions from the cooled cultures in ice cold Z buffer.
- pre-incubate them for 5 minutes at 37°C
- start the reaction by adding 0.2 ml PNPG to the probes with a multipette and mix
- stop the reaction after 30 minutes by adding 0.5ml 1M $Na_2CO_3$ (or earlier, when the color turns to a strong yellow, note exact time). Centrifuge the probes for 5-10 minutes and then measure the $OD_{410}$
- calculate the enzyme activity in as

$$\text{units} = \frac{OD_{410} \text{ x dilution factor x 1000}}{OD_{600} \text{ x time (minutes)}}$$

**12. Transposition using the mini Tn10 (Tet) (l 1323)** (Kleckner et al., 1991)

- inoculate 4ml LB2Y with a colony of the strain to be mutagenised
- incubate overnight at 37°C -stationary (without shaking)
- next day-inoculate 9.5ml LB2Y with 0.5ml of the overnight culture and keep stationary at 37°C for 2 hours
- shift it to 37°C shaker-1hour
- centrifuge at 3000rpm, 15 minutes. Resuspend in 1ml LB2Y containing 1mM IPTG + 10µl 1M $MgSO_4$
- make 1:10 dilution of the phage λ1323 (5 x $10^{10}$ PFU/ml) using LB2Y containing 1mM IPTG + $MgSO_4$
- mix 200µl of phage dilution + 200µl of resuspended culture – keep at 37°C, stationary for 15 minutes
- add 2ml LB2Y with 1mM IPTG –37°C shake for 90 minutes
- make 1:10 dilution (1:2 dilution if high colony count required) of this and
- plate 100µl each on indicator tetracyclin plates.
- incubate at 41°C.

**13. Transduction with Phage T4GT7** (Wilson et al., 1979; Plakidou et al., 1984)

T4-Topagar

| | |
|---|---|
| 6g | Bacto-Agar (Difco) |
| 10g | Bacto-Tryptone (Difco) |
| 8g | NaCl |

| | |
|---|---|
| 2g | Tri-Natriumcitrate-Dihydrate |
| 3g | Glucose |
| ad 1l | $H_2O$ |

T4GT7-plate lysate

- incubate 100µl overnight culture with T4GT7-lysate ($10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ ml) for 20 minutes at room temperature
- transfer bacteria-phage mixture in to 10ml reaction tubes containing 1ml LB2Y
- add 3ml T4-topagar, mix carefully by rolling the tube
- pore on to LB-plates, incubate plates at 37°C for 8-14h only
- take a plate which shows an almost confluent lysis
- pipette 1ml LB2Y on to plate, scratch top-agar off and transfer to 10ml glass tubes
- extract at least twice with chloroform, use glass pistils to carefully mix top-agar with chloroform
- determine phage titer, expected multiplicity of infection = $10^{11}$ phages/ml

T4GT7-Transduction

- mix 100-200µl of a fresh overnight culture with T4GT7 lysate (*eg* 2µl, 0.5µl , 0.1µl), incubate for 20 minutes at room temperature
- plate 1 x 100 µl, and 1 x 10 µl on to the respective plates for selection of the transductants
- re-streak colonies several times to get rid of contaminating T4GT7 phages

### **14. RNA preparation by hot phenol method** modified from (Schnetz, 1995)

| | |
|---|---|
| solution I: | 50mM glucose |
| | 25mM Tris-HCl pH8.0 |
| | 10mM ETDA |
| | 2mg/ml lysozyme (add fresh) |
| TM: | 10mM Tris-HCl pH7.5 |
| | 10mM $Mg_2SO_4$ |
| Mg-Saline: | 0.85% NaCl |
| | 10 mM $MgSO_4$ |

- grow 20ml culture to $OD_{600}$ of 0.5 to 0.8, harvest in ice
- pellet cells by centrifugation (5000 rpm, 10 minutes)
- wash with Mg-Saline
- resuspend cell pellet in 200µl solution I with lysozyme and incubate for 5 to 10 minutes on ice
- transfer cell suspension to 2ml screw cap tube
- add 500µl phenol, 250µl 0.5 M Na-acetate pH5.2, and 20µl 6% SDS, make sure tubes are tightly closed, vortex vigorously and incubate for 6 minutes at 80°C, mix every minute
- centrifuge (3-5 minutes, 12000rpm), transfer aqueous phase to fresh tube
- extract 2 to 3 times with chloroform/isoamylalcohol (24:1), transfer aqueous phase to a new tube each time
- precipitate nucleic acids by the addition of 1/20 volume 3M $NH_4$-Acetate pH5.2 (or 3M Na-acetate) and 2 volumes ethanol (incubate for 5 minutes or overnight at -20°C or on ice)
- centrifuge for 10 minutes, 12000 rpm
- wash with 80% ethanol and centrifuge for another 5 minutes, 12000rpm
- dry under vacuum

- re-suspend nucleic acids in 200µl TM-buffer
- add 10U DNase (RNase free) and incubate for 1hour at 37°C
- extract once with phenol, followed by two chloroform:isoamylalcohol (24:1) extractions
- precipitate RNA by adding 1/10 volume 3M $NH_4$-acetate pH5.2 and 2.5 volume ethanol
- repeat DNase treatment once
- resuspend RNA pellets in 200µl DEPC treated $H_2O$
- measure $OD_{260}$ of an appropriate dilution
- the quality of the RNA preparation can be checked by loading 5µg of RNA (in 50% formamid, heated 65°C) on to a 4% acrylamide:bis (19:1), 7M Urea, 1x TBE gel
- stain the gel with Ethidium-bromide 0.5µg/ml

**15. RNA preparation for half life determination.** modified from (Appleman et al., 1998)

| | |
|---|---|
| TM: | 10 mM Tris-HCl pH 7.5<br>10 mM $Mg_2SO_4$ |
| Rifampicin stock: | 50mg/ml in methanol (store in dark at -20°C) |

(Control RNA for S1 experiment-start with 200ml LB2Y culture, OD~0.5, centrifuge and proceed according to Hot Phenol method)

- grow LB2Y culture to $OD_{600}$ of 0.5 (100ml culture for RNA half-life determination experiment)
- optional: add rifampicin to final concentration of 100µg/ml
- transfer 5ml culture (optional: use different time points after Rifampicin addition starting 2 minutes before Rifampicin addition = -2 minute sample) to 50ml tubes containing 750µl non buffered, water saturated phenol, 250µl 6% SDS (and optional 200µg each of the control RNAs) preheated to 70°C.- mix vigorously and keep at 70°C for at least 5 minutes
- centrifuge (3-5 minutes, 12000 rpm), transfer aqueous phase to an new tube
- extract 2 times more with chloroform/isoamylalcohol (24:1), transfer aqueous phase to a new tube each time
- precipitate nucleic acids by the addition of 1/20 volume 3M $NH_4$-Acetate, pH 5.2 (or 3M Na-acetate) and 2 volumes of ethanol (incubate for 5 minutes or overnight at -20°C or on ice)
- centrifuge for 10 minutes, approx. 12000rpm
- wash with 80% ethanol and centrifuge for another 5 minutes, 12000rpm
- dry the pellet under vacuum
- re-suspend nucleic acids in 50µl TM-buffer
- add 2.5 units DNase (RNase free) and incubate for 1 hour at 37°C
- extract once with phenol, followed by two chloroform:isoamylalcohol (24:1) extractions
- precipitate RNA by adding 1/10 volume 3M $NH_4$-acetate pH 5.2 and 2.5 volumes of ethanol
- repeat DNase treatment once
- resuspend RNA pellets in 50µl DEPC treated $H_2O$
- measure $OD_{260}$ of an appropriate dilution

- the quality of the RNA preparation can be checked by loading 5µg of RNA (in 50% formamid, heated 65°C) on to a 4% acrylamide:bis (19:1), 7M Urea, 1x TBE gel
- stain the gel with Ethidium-bromide 0.5µg/ml

### 16. S1 nuclease assay

DNA Probe labeling (modified from T7 DNA polymerase sequencing kit protocol, Amersham Pharmacia Biotech)

annealing buffer: 1M Tris-HCl (pH 7.5), 100mM MgCl2, 160mM DTT
labeling mix-dATP: 1.375µM each dCTP, dGTP and dTTP, 333.5mM NaCl
chase mix: 840µM each dATP, dCTP, dGTP and dTTP, 40mM Tris-HCl (pH 7.5), 50mM NaCl

- denature 2µg plasmidic DNA ($CsCl_2$ purified supercoiled DNA) by mixing with 8µl 2M NaOH (total volume 40µl)
- vortex gently and incubate at room temperature for 10 minutes
- add 4µl 3M Sodium acetate pH 4.8, and 50µl 100% Ethanol
- quick freeze in liquid nitrogen and allow to slowly thaw at –20°C
- centrifuge, remove supernatant and air dry pellet
- add 10µl distilled water, 2µl primer (5pmol/µl), 2µl annealing buffer
- mix and incubate at 37°C for 30 minutes
- add 3µl labeling mix-dATP, 1µl $\alpha^{32}$p dATP(Amersham, specific activity 3000Ci/mMol ), 2µl diluted T7 DNA polymerase (1.6U/µl)
- mix and incubate at room temperature for 5 minutes
- add 19µl of this solution to 10.6µl of prewarmed chase mix
- incubate at 37°C for 1 hour
- stop reaction by extracting with phenol/chloroform/isoamyl alcohol (25:24:1)
- extract twice with chloroform/isoamyl alcohol (24:1)
- remove unincorporated radioactivity by loading on to a NAP-5 column (Pharmacia) and elute in 1ml distilled water
- measure radioactive incorporation by measuring Cerenkov counts of 1µl

S1 reaction: modified from (Schnetz, 1995)

Na-TCA buffer: 3M Na-trichloroacetate, 50mM PIPES pH 7.0, 5mM EDTA
S1 digestion buffer: 250mM NaCl, 40mM Na-acetate pH 5.5, 1mM $ZnCl_2$, 20µg/ml denatured herring sperm DNA

- mix 200µg RNA with radiolabeled probe corresponding to half the labeling reaction (approx. 300,000 cpm)
- quick freeze in liquid nitrogen and vacuum dry
- resuspend in 40µl Na-TCA buffer
- incubate at 65°C for 5 minutes and transfer to 45°C for at least 4 hours
- to 20µl of this reaction add 190µl digestion buffer containing 500U/ml S1 nuclease
- incubate at 37°C for 30 minutes
- stop by extracting with phenol/chloroform/isoamyl alcohol (25:24:1)
- extract twice with chloroform/isoamyl alcohol (24:1)
- precipitate with 0.1 volume 3M $NH_4$-acetate and 2.5 volume 100% ethanol
- wash with 80% ethanol
- resuspend in 9µl sequencing gel loading dye and load 3µl on the gel after pre-heating at 80°C for 2 minutes

- on the same gel, as molecular weight marker, run sequencing ladder prepared according to T7 DNA polymerase sequencing kit protocol, Amersham Pharmacia Biotech

**17. DNA sequencing**

Sequence analysis was done using the Big Dye terminator cycle sequencing kit (ABI Prism) according to manufacturer's instructions and using an automated DNA sequencer.

**18. Genomic DNA sequencing**

- prepare genomic DNA using the Qia-amp DNA mini kit (Qiagen)
- perform sequencing PCR using 16µl Terminator ready dye mix (ABI Prism), 3µg genomic DNA, 10pmoles primer S156, distilled water to 40µl. (genomic sequencing protocol from Big Dye protocol, ABI prism)
- purify the PCR reaction using centri-sep column (Princeton separations, Inc.)
- determine sequence using an automated DNA sequencer

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Teilpublikationen:

Sudhanshu Dole, Sandra Kühn, and Karin Schnetz (2001). Post-transcriptional enhancement of *Escherichia coli bgl* operon silencing by limitation of BglG-mediated antitermination at low transcription rates. In revision for submission to Mol. Microbiol.

Datum: Unterschrift:

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