Construction and analysis of novel controllable expression vectors for *Bacillus subtilis*

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Contents

Zusammenfassung

Das Gram-positive Bakterium *Bacillus subtilis* spielt eine wichtige Rolle im Bereich der Landwirtschaft, der Medizin und der Ernährung und bei der Produktion rekombinanter Proteine. Momentan werden etwa 60% aller kommerziell verfügbaren technischen Enzyme von *Bacillus*-Spezies produziert. Außerdem sind eine Vielzahl von Informationen über die Transkription, die Translation, die Protein-Faltung, die Sekretions-Mechanismen, die genetische Manipulation und die Fermentation im industriellen Maßstab verfügbar. Dem steht gegenüber, dass effiziente und preiswerte Expressions-Vektoren bislang fehlen. Um diese Lücke zu schließen, wurden ein Glycin-induzierbares und ein Lysin-autoinduzierbares Expressionssystem entwickelt. Ferner wurden IPTG-induzierbare Expressions-Vektoren konstruiert und analysiert, die Überexpression und Reinigung von Proteinen erlauben. Weiterhin wurde ein Promoter-Testvektor entwickelt, der die Analyse von sehr starken Promotoren sowie von mRNA stabilisierenden Elementen erlaubt, um die Menge an Transkript und die mRNA-Stabilität zu erhöhen und damit eine höhere Produktion an rekombinanten Proteinen zu gewährleisten.

Während der Entwicklung der Glycin- und Lysin-induzierbaren Vektoren wurde mittels Northern-Blot bestätigt, dass in beiden Fällen zunächst ein kurzes Transkript, genannt Riboswitch, synthetisiert wird, welches nach Zugabe von Glycin und nach Entfernen von Lysin in ein längeres Transkript umgewandelt wird. Um die Expressionsstärke nach Induktion zu quantifizieren, wurden die beiden Promotoren mit ihren Riboswitches an das *lacZ*-Reportergen fusioniert. Im Falle des Glycin-Systems wurde der Promotor optimiert und die Produktion von HtpG, Pbp4* und α-Amylase als Modellproteine analysiert. Diese Ergebnisse zeigten, dass der Glycin-Riboswitch erfolgreich für die regulierte Produktion von sowohl intra- als auch extrazellulären Proteinen und der Lysin-Riboswitch als autoinduzierbares System verwendet werden können. Im letzteren Fall beginnt die Produktion der rekombinanten Proteine, wenn die Lysin-Konzentration in der Zelle unter einen Schwellenwert gefallen ist.

Außerdem wurden sechs verschiedene neuartige IPTG-induzierbare Expressions-Vektoren für *B. subtilis* konstruiert. Während der erste Vektor die intrazelluläre Produktion von rekombinanten Proteinen erlaubt (pHT01), enthält der zweite ein starkes Sekretions-Signal (pHT43). Der dritte Vektor erlaubt das Anfügen des c-Myc Epitop-Tags (pHT10), und die restlichen drei Vektoren das Anfügen der His- (pHT08) und Strep-Reinigungs-Tags (pHT09

und pHT24). Die Anwendung aller sechs Vektoren wurde durch das Einklonieren geeigneter Reportergene und die nachfolgende Überproduktion ihrer Proteine gezeigt.

Zum schnellen Screening starker Promotoren wurde ein neuer Promotor-Test Vektor entwickelt mit dem *bgaB*-Gen als Reportergen, welches ein Blau-Weiß Screening erlaubt. Dieser Vektor enthält den *lacO*-Operator upstream von der Ribosomen-Bindungsstelle (RBS) und downstream von einer DNA-Sequenz mit Erkennungssequenzen für verschiedene Restriktionsenzyme. Er enthält außerdem das Gen für den Lac-Repressor, der die IPTG-kontrollierte Expression der Promotoren erlaubt und somit die Klonierung auch starker Promotoren in *E. coli*, sowie die regulierte Expression von *bgaB* in *B. subtilis* ermöglicht.

Insgesamt 85 verschiedene und P_{groE}-modifizierte σ^A-abhängige Promotoren wurden in pHT06 kloniert and analysiert. Es zeigte sich, dass DNA-Sequenzen um den Transkriptionsstart, die -10-, die -15- und die -35-Region und upstream vom Promotor dessen Stärke beeinflussen. Um die Aktivität der verschiedenen Promotoren zu vergleichen, wurden die BgaB-Aktivitäten bestimmt und Northern-Blot-Experimente durchgeführt. Die Messungen einiger neuer Kombinationen von Core-Promotoren und UP-Elemente zeigten, dass die β-Galaktosidase-Aktivitäten jeweils bis zu 13-fach bzw. 43-fach gesteigert werden konnten im Vergleich mit dem bereits starken P_{grac}-Promotor. Wurden beide Elemente kombiniert, dann wurde eine etwa 690-fache Aktivität, wieder bezogen auf den P_{grac}-Promotor, gemessen, und die Synthese an BgaB-Protein erreichte bis zu 30% des gesamten zellulären Proteins.

Die mRNA-stabilisierenden Elemente wurden mit einem vergleichbaren experimentellen Ansatz untersucht. Zunächst wurden 17 verschiedene Stem-Loop-Strukturen am 3'-Ende der mRNA analysiert. Keine dieser DNA-Sequenzen zeigte einen signifikanten Einfluss auf die Menge an synthetisiertem Protein. Dann wurden 5'-mRNA-stabilisierende Elemente analysiert, und zwar eine starke RBS, ein *lacO*-kontrollierbares stabilisierendes Element (genannt CoSE) und die Spacer-Regionen zwischen RBS und CoSE. Diese Ergebnisse zeigten, dass das CoSE-Element zusammen mit einem geeigneten Spacer und einer starken RBS die Genexpression 9-fach steigern konnte, wieder bezogen auf den P_{grac}-Promotor. Dies führtezn bis zu 26% an rekombinantem Protein und einer Halbwertszeit der mRNA von mehr als 60 min. Eine Kombination von starken Promotoren und stabilisierenden Elementen führtezn bis zu 42% an rekombinantem Protein als Anteil am Gesamtprotein.

Summary

The gram-positive bacterium *Bacillus subtilis* is well-known for its contributions to agricultural, medical, and food biotechnology and for the production of recombinant proteins. At present, about 60% of the commercially available technical enzymes are produced by *Bacillus* species. Furthermore, a large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation has been acquired. But so far, efficient and inexpensive expression vectors for *B. subtilis* are still missing. To fill this gap, a glycine-inducible expression system and a lysine-autoinducible one were explored and IPTG-inducible expression plasmids that allow overexpression and purification of proteins were constructed and analyzed. Furthermore, a technique with a useful promoter-probe plasmid to analyze strong promoters in *B. subtilis* was established, which allowed to study promoter and mRNA stabilizing elements to enhance the transcript level and mRNA stability leading to higher production of recombinant protein.

During the development of the glycine-inducible and lysine-autoinducible expression plasmids, the presence of a small transcript termed riboswitch corresponding to the 5' UTR in the absence of L-glycine or presence of L-lysine and its conversion into the full-length transcript after addition of the L-glycine or removal of L-lysine was confirmed by Northern blot. Next, the promoter and downstream riboswitch was fused to the *lacZ* reporter gene to measure glycine or lysine-dependent induction. The production potential for the glycine system was analyzed in detail, and the promoter strength improved by using HtpG, Pbp4* and α-amylase as model proteins. In summary, the glycine riboswitch can be used successfully for regulatable production of both intra- and extracellular proteins, and the lysine riboswitch can be applied as an auto-inducible expression system allowing production of recombinant proteins when the L-lysine concentration within the growth medium falls below a threshold value.

Six commercially available novel plasmid-based IPTG-inducible expression plasmids for *B. subtilis* were constructed, too. While the first vector allows intracellular production of recombinant proteins (pHT01), the second provides a strong secretion signal (pHT43). The third vector allows addition of the c-Myc epitope tag (pHT10), and the remaining three vectors provide the purification tags His (pHT08) and Strep (pHT09 and pHT24). The versatility of all six vectors was verified by insertion of appropriate reporter genes and by demonstrating high level production of their proteins.

To develop a simple technique for rapid screening of strong promoters in *B. subtilis*, a new promoter-probe plasmid (pHT06) using the *bgaB*-encoded β-galactosidase was constructed allowing blue/white screening. This promoter-probe plasmid contains the *lacO* operator, upstream of the ribosome binding site (RBS) and downstream of a multiple cloning sites (MCS), and *lacI* coding for the Lac repressor, which allowed promoters to be controlled by IPTG, facilitating strong promoters to be cloned in *E. coli* and regulated expression of *bgaB* in *B. subtilis*.

A total of 85 different synthetic and *groE*-modified σ^A-dependent promoters were introduced into pHT06 and analyzed. Sequences around the transcriptional start site, the -10 region, the -15 region, the -35 region and the upstream region turned out to influence the promoter strength. BgaB activities and Northern blot analyses were used to measure the activity of the different promoters. The measurements of some new combinations of core promoters and UP elements on gene expression revealed that the β-galactosidase activity expression levels could be increased up to 13-fold and the mRNA levels up to 43-fold as compared to the strong P_{grac} promoter. If both elements were combined, an activity roughly 690 times higher than that obtained with the P_{spac} promoter were obtained, and synthesis of BgaB, under control of these promoters, could reach up to 30% of the total cellular protein.

The mRNA stabilizing elements were also analyzed by using a similar experimental approach. First, seven-teen different 3'-mRNA terminal stem-loops have been investigated, which did not significantly influence neither the amount of protein produced nor the mRNA stability. Second, the 5'-mRNA stabilizing elements including a strong RBS, the *lacO* Controllable Stabilizing Element (CoSE) and the spacer between the RBS and CoSE were examined. The results demonstrated that CoSE together with an appropriate spacer and a strong RBS could increase gene expression 9-fold as compared to the P_{grac} promoter, reaching up to 26% of total cellular protein and a half-life of the mRNA of more than 60 min. A combination of strong promoters and stabilizing elements showed that recombinant protein synthesis levels of up to 42% of the total cellular protein could be obtained.

1 Introduction

Bacillus species have been major workhorses in industrial microbiology since a very long time [56, 136]. The development of strains and production strategies has been influenced by the application of molecular biology techniques to strain development. *Bacillus* species are attractive industrial microorganisms due to several reasons such as high growth rate, their capacity to secrete proteins into the medium and their GRAS (generally regarded as safe) status with the Food and Drug Administration for some species including *B. subtilis*, the best-studied Gram-positive bacterium today. Expression systems for the production of recombinant proteins produced intra- and extracellularly have been reviewed recently [97, 163].

Bacillus subtilis is generally considered to have a great industrial potential for production and secretion of proteins of clinical interest like interferon [113], insulin [109], pathogenic antigens [2], and toxins [154], or technical enzymes of great industrial interest like proteases [63], α-amylase [66], and lipases [63]. The major advantages of *B. subtilis* as compared to other host production systems are high-cell-density growth and secretion of synthesized proteins into the cultivation medium, which facilitates isolation and purification of recombinant proteins during downstream processing [14, 17, 88]. High-level production of recombinant proteins as a prerequisite prior to subsequent purification has become a standard technique. Important applications of recombinant proteins are: (i) immunization, (ii) biochemical studies, (iii) three-dimensional analysis of the protein, and (iv) biotechnological and therapeutic use. Production of recombinant proteins involves cloning of the appropriate gene into an expression vector under the control of an inducible promoter. The selection of a particular expression system requires a cost breakdown in terms of design, process and other economic considerations.

1.1 Controllable expression systems in *B. subtilis*

Expression systems for recombinant proteins rely on inducible promoter systems. Based on the inducing signal, promoters can be grouped into three classes: Those of class I are activated by adding a chemical compound which acts as an inducer. Cells are grown first in the absence of the inducer to the mid-logarithmic growth phase followed by addition of the inducer, a chemical compound such as IPTG or xylose. Expression of the recombinant gene in the absence of inducer is prevented by a repressor protein which can be inactivated by the added inducer such as the LacI repressor by IPTG or the XylR repressor by xylose [55]. Promoters

of class II are activated by a temperature shift, either up or down. Cells are grown at 30-37^{o}C and shifted for induction of the promoter system either to high (40-45^{o}C) or low temperature (20-15^{o}C) [103, 122]. And promoters of class III are auto-inducible that direct low levels of expression in the lag and log phase, and much higher levels in the stationary phase. Their induction relies on the intracellular concentration of a metabolite such as the promoter of *aprE* encoding for subtilisin E [71]. If this metabolite is present in excess, it will prevent expression of those genes involved in its own synthesis. During growth, the metabolite will be consumed by the cell, and if its concentration falls beyond a threshold value, the structural genes involved in its synthesis will be induced. And this class also includes promoters belonging to σ^{B}-dependent promoters such as of *gsiB*, encoding for a general stress protein [94].

1.1.1 Published controllable expression system

In order to produce homologous or heterologous proteins, several systems for inducible gene expression in *B. subtilis* have been developed. The starch-inducible amylase promoter is frequently used for production of heterologous proteins in which the desired protein is fused to the α-amylase promoter and leader peptide, which efficiently drives secretion of the protein produced into the culture medium [2, 63]. Several prophage derived heat-inducible gene expression systems that show very tight control of gene expression have been described. However, the levels of expression upon maximum induction are relatively low compared to those of other inducible gene expression systems [29, 63, 88]. The series of plasmid-based expression vectors pHCMCs has been constructed allowing stable intracellular expression of recombinant proteins in *B. subtilis* cells. These expression vectors are based on the recently described *Escherichia coli* - *B. subtilis* shuttle vector pMTLBs72 that uses the theta mode of replication [156]. Three different controllable promoters have been inserted into the shuttle vector: P_{gsiB} that can be induced by heat, acid shock, and by ethanol, and P_{xylA} and P_{spac} that respond to the addition of xylose and IPTG, respectively. All recombinant vectors exhibited full structural stability [104]. An inducible gene expression system based on the regulation machinery of *E. coli Tn10*-encoded tetracycline resistance has been shown to be functional in *B. subtilis* [42]. This system has been reported to generate 100-fold-increased expression upon induction with tetracycline; however, considerable basal levels of expression are observed. A more tightly regulated variant of this system has been developed, but it appeared to generate lower maximal levels of expression upon induction [42]. Furthermore, a well-known system is the *xylA* system, in which a gene of interest is fused to the xylose-inducible *xylA* promoter,

which is integrated at the *amyE* locus of the *B. subtilis* chromosome, has been reported to generate very high transcription activity upon xylose induction, whereas the basal level of expression is low [10, 79]. Recently, the SURE system, a SUbtilin-Regulated Expression system for *B. subtilis* has allowed strict control of gene expression by addition of subtilin. In this system, the *spaRK*-dependent signal transduction is used to control P_{spaS}-driven gene expression. Several multicopy expression vectors carrying subtilin-responsive promoter elements, which facilitated both transcriptional and translational promoter-gene fusions, have been constructed [14]. Very recently, a maltose-inducible expression vector in *B. subtilis* has been developed and characterized. The vector permitted β-galactosidase expression at a high level (maximum activity, 8.16 U/ml) when induced and its expression was markedly repressed by glucose. This provided a potential expression system for cloned genes in *B. subtilis* [98]. Finally, the well-known *E. coli lac* repressor-based expression system has been functionally implemented in *B. subtilis* as follows [117, 173].

1.1.2 IPTG-inducible expression system

The well-known *E. coli lac* repressor-based expression system has been functionally implemented in *B. subtilis* using a two-plasmid system, which allowed isopropyl-β-D-thiogalactopyranoside (IPTG)-controlled gene expression in the latter species. This system was reported to exhibit no expression in the absence of the inducer, while very high levels of expression (10 to 15% of the total cellular protein) were observed after IPTG induction [55, 85, 117].

1.1.2.1 Chromosomal integration systems

This control mechanism is used in an expression system that employs the hybrid P_{spac} promoter, which is composed of the *B. subtilis* phage SPO-1 promoter and the *E. coli lac* operator, in which IPTG-mediated derepression leads to transcription activation and yields high levels of gene expression. These plasmids allow the insertion of any kind of genetic information into the *B. subtilis* chromosome. The *amyE* locus, coding for a nonessential α-amylase, is used in most cases for ectopic integration [55, 161, 173].

1.1.2.2 Plasmid-based systems

Two plasmid-based expression vectors have been constructed in which one plasmid allows intracellular production of recombinant proteins while the other directs the proteins into the culture medium. Both vectors use the strong promoter, P_{grac}, which is composed of the *B. subtilis* g*roE* promoter preceding the *groESL* operon (codes for the essential heat shock

proteins GroES and GroEL) of *B. subtilis* fused to the *E. coli lac* operator allowing their induction by addition of IPTG. While the background level of expression of these expression cassettes was very low in the absence of the inducer, an induction factor of about 1300 was measured upon addition of IPTG [117]. We and others observed that the *groE* promoter of *B. subtilis* is a strong promoter most probably due to the presence of an UP element [57, 96]. Based on these observations, this promoter was used to study whether it could drive expression of recombinant genes. Since the *groE* promoter is constitutive and high-level production of many recombinant proteins can be deleterious to the cells, a regulatory element had to be added. The *lac* operator (*lacO*) of *E. coli* K12 was chosen, which had already been successfully used in combination with different promoters such as P_{spac} [173].

1.1.3 Riboswitches-based expression systems

Each cell must regulate the expression of hundreds of different genes in response to changing environmental or cellular conditions. The majority of these sophisticated genetic control factors are proteins, which monitor metabolites and other chemical cues by selectively binding to targets. It has been discovered that RNA can also form precise genetic switches and that these elements can control fundamental biochemical processes. Riboswitches are a type of natural genetic control element that uses untranslated sequence in the 5' region of mRNAs to form a binding pocket for a metabolite that regulates expression of that gene.

During the last years the great importance of RNA for regulating gene expression in all organisms has become obvious. Consequently, several recent approaches aim to utilize the outstanding chemical properties of RNA to develop artificial RNA regulators for conditional gene expression systems. A combination of rational design, *in vitro* selection and *in vivo* screening systems has been used to create a versatile set of RNA-based molecular switches. These tools rely on diverse mechanisms and exhibit activity in several organisms, so they have been developmed recently in the application of engineered riboswitches for gene regulation *in vivo* [6]

1.1.3.1 General characteristics of riboswitches

As mentioned, riboswitches are metabolite binding domains located within the 5' untranslated regions (UTR) of some mRNAs which are involved in gene regulation [159, 166]. Allosteric rearrangement of mRNA structures is mediated by metabolite binding resulting in modulation of gene expression, and a change in expression with increasing ligand concentration, ranging from between 7-fold and 1,200-fold has been observed [50, 91, 95, 167] (Fig. 1.1).

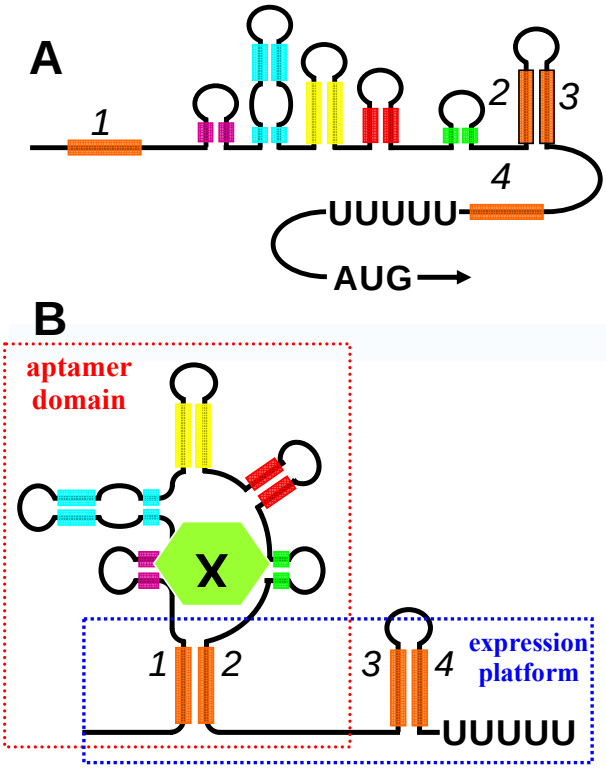


Fig. 1.1. Model of gene regulation by a typical riboswitch. (A) When the cellular concentration of metabolite is too low to occupy the riboswitch binding site, the transcription is completed, the biosynthetic and/or transport proteins are expressed; (B) when the cellular concentration of metabolite is high, the metabolite binds to the riboswitch and leads to formation of an intrinsic terminator, the metabolite biosynthetic or transport protein is not produced [41].

Riboswitches are conceptually divided into two parts: the aptamer and the expression platform (Fig. 1.1B). The aptamer directly binds the metabolite, and undergoes structural changes in response. These structural changes affect the expression platform, which is the mechanism by which gene expression is regulated. Expression platforms typically turn off gene expression in response to the metabolite, but some turn it on [37, 86, 159].

In the past year, three newly confirmed riboswitch classes have been reported (Fig. 1.2) [148, 159]. The first of these, the regulation of transcription termination, is utilized by nearly every riboswitch class and typically involves metabolite-dependent formation of a terminator stem, which prevents transcription elongation and inhibits gene expression (Fig. 1.2A). Two exceptions are the adenine and glycine riboswitch, wherein metabolite binding prevents terminator stem formation and activates gene expression [91-93, 148]. Second, the regulation of translation initiation is less widely utilized and involves altering the accessibility of the SD sequence (Fig. 1.2B). In this case, metabolite binding masks the SD sequence within a secondary structure to prevent ribosome binding and thereby inhibit gene expression. Interestingly, riboswitches in Gram-negative bacteria seemingly prefer regulation of translation initiation, whereas Gram-positive bacteria favour transcription termination, a correlation that probably reflects the higher frequency of polycistronic genes in Gram-positive

bacteria [108, 167]. For example, the TPP sensing riboswitch can terminate transcription of downstream genes in Gram-positive bacteria, suppress translation initiation in Gram-negative bacteria [135]. A third expression platform that can be utilized by riboswitches to affect gene expression is the regulation of RNA processing events. A conceptually simplistic manifestation of this expression platform is represented by the GlcN6P riboswitch, for which ligand binding induces catalytic self-cleavage of the mRNA and inhibition of gene expression (Fig. 1.2C) [168]. However, it seems unlikely that the aptamer and expression platform (ribozyme) are separable functionalities, as they are for other riboswitches. Interestingly, the discovery of TPP-dependent riboswitches in eukaryotic genes has unveiled other possibilities for riboswitch control of RNA processing [83, 152]. For instance, the presence of TPP aptamers within introns or 3' untranslated regions (UTRs) suggests that riboswitches might regulate splicing or 3' end formation, respectively [135, 148, 159].

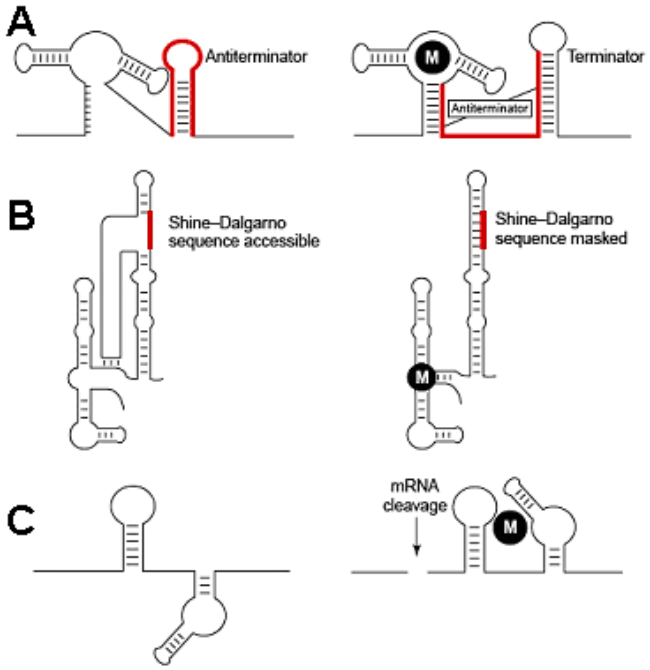


Fig. 1.2. Mechanisms of riboswitch function. (A) Transcription termination induced by metabolite (M) binding to nascent RNA, as observed for a guanine riboswitch; (B) translation initiation modulated by metabolite-dependent sequestration of a SD sequence, as observed for a TPP riboswitch; (C) RNA processing regulated by metabolite-dependent self-cleavage, as observed for a GlcN6P riboswitch [148].

1.1.3.2 The glycine riboswitch

It has been suggested that about 2% of the *B. subtilis* genes are regulated via riboswitches [93], and three riboswitches have been studied so far. One of these riboswitches precedes the *lysC* gene [24]. The second is the *gcvT* operon involved in the degradation of L-glycine if the concentration is high within the cell [5, 93]. Both of these riboswitches operate by opposite mechanisms. The third are the members of the GlcN6P class of riboswitch which are self-cleaving ribozymes; they are activated when they are bound with the sugar-phosphate compound [148].

The tricistronic *gcvT-gcvPA-gcvPB* operon codes for enzymes involved in the degradation of L-glycine if its concentration is high within the cell. The *gcvT* operon will be transcribed when the L-glycine concentration within the cell is high, and the metabolite will bind to a tandem riboswitch. The glycine riboswitch consisting of two strikingly similar aptamers, connected by a short linker region present upstream of glycine catabolism and efflux genes in a wide variety of bacteria. The glycine riboswitch binds L-glycine to regulate three glycine metabolism genes by activation via inhibition of premature termination of transcription, to use L-glycine as an energy source (type of regulation as Fig. 1.2A) [5, 93, 148, 159].

1.1.3.3 The lysine riboswitch

The *lysC* gene of *B. subtilis* encodes the inphase overlapping genes for the α- and β-subunits of a lysine-responsive aspartokinase II [24]. The lysine riboswitch (also called *L-box*) binds L-lysine to regulate lysine biosynthesis, catabolism and transport. The *lysC* gene is induced when the L-lysine concentration is low within the cell and the metabolite-free riboswitch favors formation of an anti-terminator structure. If the concentration of L-lysine is high in the cell, transcription of the lysine operon is initiated but terminated after a transcript of about 270 nucleotides has been synthesized. This 5' region of the lysine transcript is not translated, but forms a complicated secondary structure which is stabilized by L-lysine. This in turn leads to the formation of a terminator structure which causes the RNAP to dissociate from the DNA template and to release the transcript into the cytoplasm. This regulatory principle has been designated as riboswitch-mediated control of gene expression (type of regulation as Fig. 1.2A) [51, 153].

1.2 Purification of recombinant proteins synthesized in *B. subtilis*

An important application of gene technology is the overproduction of different proteins that can be utilized as pharmaceutical agents, as antigens for the production of antibodies, or as tools for structural and functional analyses. To separate one single protein from a complex mixture of proteins, while maintaining biological function, one can maintain biological function by controlling the pH, the temperature and the ionic strength (salt concentration) [11]. Many different proteins, domains, or peptides can be fused with the target protein. The advantages of using fusion proteins to facilitate purification and detection of recombinant proteins are well-recognized. The most frequently used and versatile systems are: Arg-tag, calmodulin-binding peptide, cellulose-binding domain, DsbA, c-Myc-tag, glutathione S-transferase, FLAG-tag, HAT-tag, His-tag, maltose-binding protein, NusA, S-tag, SBP-tag,

Strep-tag, and thioredoxin [155]. Vectors allowing production of His-tagged proteins in *B. subtilis* have been published [142].

1.2.1 General protein purification strategy

To purify the protein of interest (separate it from other proteins in a mixture) one can take advantage of its general and specific properties: its native surface charge using ion exchange chromatography, its unique shape and size using gel filtration column chromatography, and its biological activity using affinity chromatography. These steps are sometimes applied in succession: first ion exchange chromatography to separate other proteins that have a different charge from the protein of interest, next gel filtration to separate all other proteins with a different size/shape than the protein of interest, and finally affinity chromatography to separate, based on biological activity, which is usually highly specific for the protein/enzyme of interest. In some cases, an enzyme may be purified to homogeneity (completely purified) using affinity chromatography alone since it is so effective at separating a specific enzyme from all other proteins in a mixture [11, 155].

1.2.2 Purification of His-tagged proteins

This protein purification system is based on the remarkable selectivity of the unique Ni-NTA (nickel-nitrilotriacetic acid) resin for recombinant proteins carrying a small affinity tag consisting of 6 to 10 consecutive histidine residues, termed the His-tag. The high affinity of the Ni-NTA resins for His-tagged proteins or peptides is due to both the specificity of the interaction between histidine residues and immobilized nickel ions and to the strength with which these ions are held to the NTA resin. NTA has a tetradentate chelating group that occupies four of six sites in the nickel coordination sphere. The metal is bound much more tightly than to a tridentate chelator such as IDA (imidodiacetic acid), which means that nickel ions made the proteins be very strongly bound to the resin. This allows more stringent washing conditions, better separation, higher purity, and higher capacity without nickel leaching [97, 121, 155].

1.2.3 Purification of Strep-tagged proteins

The Strep-tag is a selected eight-amino acid peptide (sequence: WSHPQFEK) with high specificity and affinity towards streptavidin; the Strep-tag has been developed as an alternative tool [139, 140]. Its sequence was derived by selection from a genetic peptide library [139]. The Strep-tag was bound at the same surface pocket where biotin, the natural ligand of streptavidin, gets complexed [138].

The Strep-tag can be genetically fused up- or downstream of the reading frame of any gene and expressed as fusion protein. The Strep-tag system can be used to purify functional Strep-tag proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria. Because of its small size, the Strep-tag generally does not interfere with the bioactivity of the fusion partner [67].

1.3 Features for overproduction of proteins in *B. subtilis*

Control of gene expression can occur at the transcriptional or/and translation level (Fig. 1.3). Furthermore, gene expression can be controlled at the level of degradation of their mRNAs. Different levels of gene expression are the result of varying frequencies of transcription and translation initiations. General features for overproduction of proteins are a high transcription rate (with strong promoters), low mRNA degradation rate (including mRNA stabilizing elements) and high translation rate (with strong RBS) [70, 77].

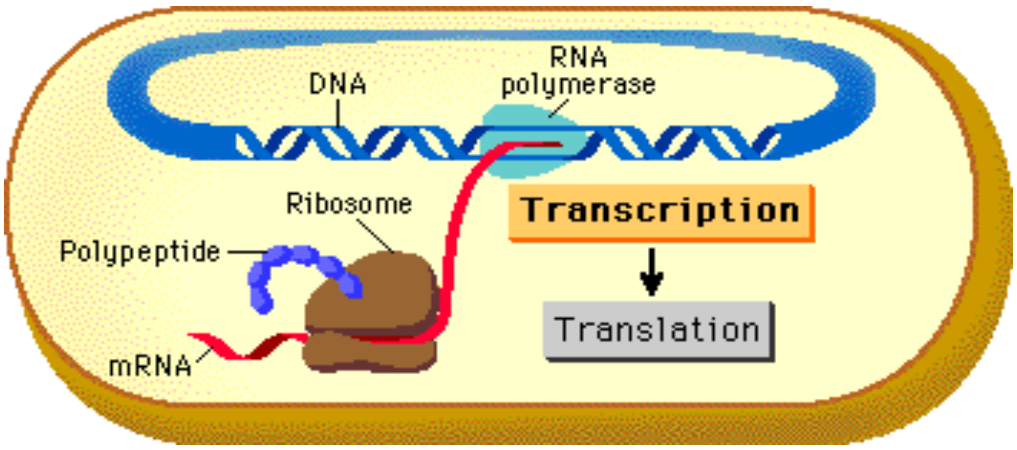


Fig. 1.3. Transcription and translation in a prokaryotic cell. Transcription and translation are coupled; that is, translation begins while the mRNA is still being synthesized [12].

1.3.1 Elements of a strong σ^A-dependent promoter in *B. subtilis*

Many housekeeping genes expressed during vegetative growth contain a typical σ^A-dependent promoter, which is characterized by a -35 TTGACA consensus sequence and the -10 TATAAT hexanucleotide core elements and sometimes an UP element, in which, several weakly conserved A and T residues are present upstream of the -35 region (-36 to -70). The two hexanucleotide core elements are usually separated by a 17-nucleotide spacer sequence, and transcription is initiated around five nucleotides downstream of the -10 box (Fig. 1.4). Changes in their distance and in bases (even one single base) within these sequences can cause subtle to drastic changes in promoter activity. Altogether, some 4,000 genes are part of

the σ^A regulon of *B. subtilis,* although their relative expression may vary significantly depending primarily, but not exclusively, on the actual sequence of the -35 and -10 elements [57, 101, 119].

1.3.1.1 The core promoter

The core promoter is the area from the -35 region to the transcription start site, which contains the canonical hexameric -35 box (essential for RNAP holoenzyme binding) and -10 box (essential for transcription initiation after binding has occurred), centred ~10 and 35 bp upstream of the transcription start site. Bacteria have a multisubunit RNA polymerase (RNAP) with a conserved subunit composition. The core enzyme is composed of β, β', ω and two α subunits. Association of a σ subunit with the core enzyme forms the holoenzyme and determines the specificity of promoter utilization. Most RNAP holoenzyme molecules present during logarithmical growth contain the σ^A factor [89, 96, 101, 119].

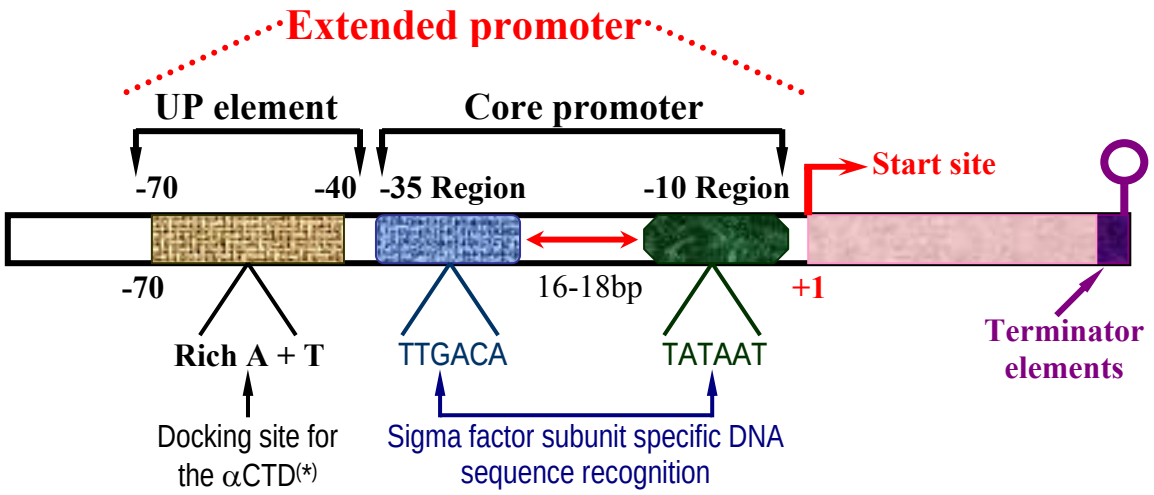


Fig. 1.4. Elements of the housekeeping σ^A-dependent promoter. (*) C-terminal domain of the RNAP α subunit.

1.3.1.2 The UP element

The UP element, located immediately 5' to the -35 element, has a recognizable pattern of AT-rich sequences. It enhances RNAP binding by complexing with the C-terminal domain of α subunits and stimulates transcription initiation [57, 129]. In most cases, however, transcription of weak promoters is enhanced by regulatory proteins that act by binding to cognate and specific DNA sequences located upstream of the promoter and stimulating one or more steps of transcription initiation [89].

The upstream promoter regions (-36 to -80) of *B. subtilis* σ^A-dependent promoter sequences are enriched for short A and T tracts, suggesting that UP elements may be common for σ^A-dependent *B. subtilis* promoters [57, 96].

1.3.2 Messenger RNA stablizing elements

Currently, at least 15 RNases are known in *E. coli* and at least 10 in *B. subtilis* [28]. The rate of mRNA decay is an important element in the control of gene expression (Fig. 1.5). Given the absence of 5' to 3' exoribonucleolytic activities in prokaryotes, both endoribonucleases and 3' to 5' exoribonucleases are involved in chemical decay of mRNA. As the 3' to 5' exoribonucleolytic activities are readily inhibited by stem-loop structures which are usual at the 3' ends of bacterial messages, the rate of decay is primarily determined by the rate of the first endonucleolytic cleavage within the transcripts, after which the resulting mRNA intermediates with a 5'-monophosphate end is created, to which 3' to 5' exoribonucleases have greater affinity than the 5'-triphosphate end of the initial transcribed product. Successive cleavage events result in mRNA fragments with accessible 3'-ends, which are rapidly degraded by 3'-5'-exoribonucleases to oligonucleotides [35, 124]. Final turnover of mRNA oligonucleotides to mononucleotides is accomplished by oligoribonuclease [43] (Fig 1.6). During the steps of mRNA decay, stable RNA structures pose formidable barriers to the 3' to 5' exonucleases [3, 146].

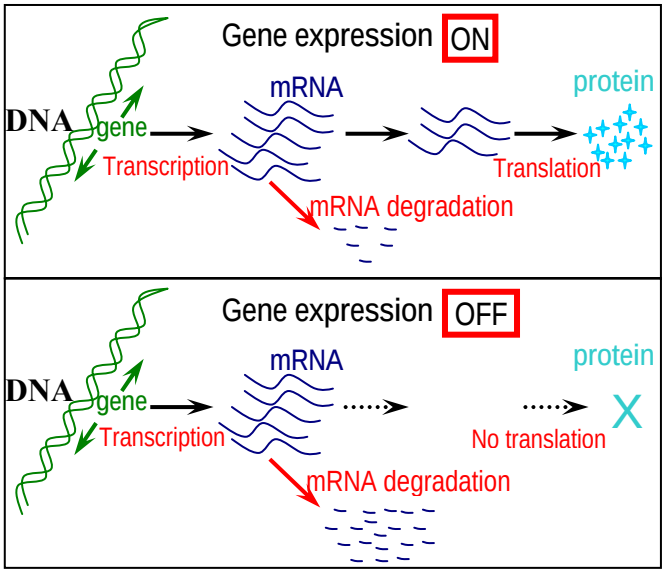


Fig. 1.5. Novel mechanism in control of gene expression. When the rate of mRNA degradation is low, most mRNA molecules are translated (gene expression is ON; upper panel). When the rate of mRNA degradation is high, most mRNA molecules are degraded without translation (gene expression is OFF; lower panel).

While 3'-terminal stem-loop structures play an important role as 5' stabilizers, 5'-proximal secondary structures or events such as ribosome stalling, regulatory protein binding, and ribosome binding can act as 5' stabilizers [7, 8, 44, 45, 53, 54, 134, 145].

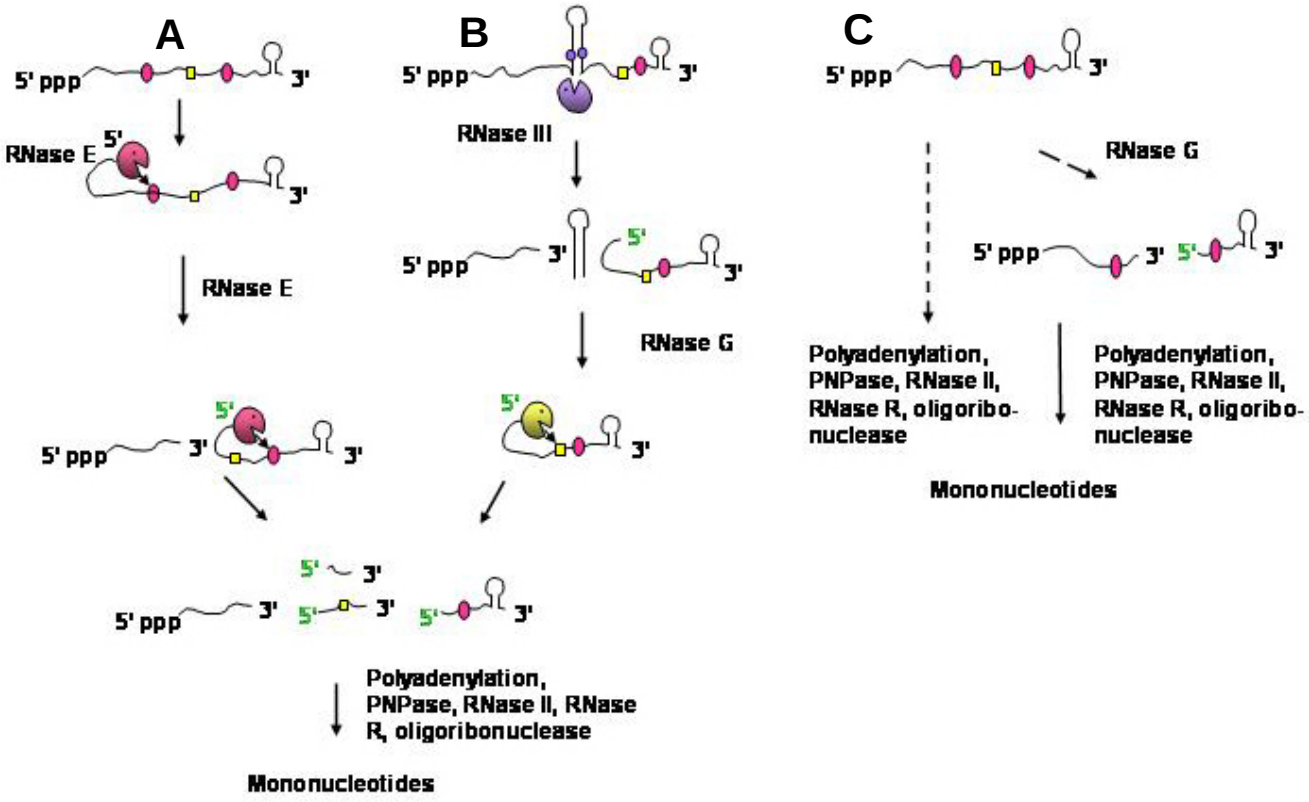


Fig. 1.6. Model for mRNA decay in *E. coli*. (A) mRNA decay is initiated by the binding of RNase E to the 5' terminus of the transcript, followed by cleavage at an internal site (⬮); (B) a polycistronic transcript is cleaved in an intergenic region (●) by RNase III. For some transcripts (C) degradation does not involve any endonucleolytic cleavages but is carried out primarily by exonucleolytic attack by enzymes such as PNPase or RNase II. RNase G (restriction site ▫) does not bind efficiently to 5' termini that contain a triphosphate so it is hypothesized that it primarily cleaves degradation products that have been generated by either RNase E or RNase III. Dotted lines indicate inefficient pathways, 5'-triphosphates are shown in black while 5'-monophosphates are shown in green. Oligoribonuclease is necessary to degrade short oligoribonucleotides (4-7 nt) that are resistant to both PNPase and RNase II. This model is based on data published in [110].

1.3.3 Strong ribosome binding site

Translation in bacteria is initiated by interaction of the 3' end of the 16S rRNA, which is part of the small ribosomal subunit, with the Shine Dalgarno sequence also called RSB. In *B. subtilis*, the consensus RBS sequence is AAAGGAGG, which is separated from the start codon by an about 7-nucleotide spacer sequence. The most frequently used start codon is

ATG (78%), but TTG (13%), GTG (9%), and CTG (<1%) are also used as translation starts [127].

High-level expression is not only dependent upon a strong regulatable promoter and 5’ and 3’ mRNA stabilizers, but also on an efficient RBS sequence. One important example for a 5’ stabilizer has been described as part of the *gsiB* transcript, where a strong RBS enhances the half-life of the original transcript [74]. And so far, it was found that the 3' end of the *cry* gene of *Bacillus thuringiensis* conferred increased stability on other mRNAs in both *E. coli* and *B. subtilis* [169].

1.4 Aims of the doctoral thesis

The major objections of the present doctoral thesis are as follows:

(i) Development of a glycine-inducible expression system.

(ii) Development of an autoinducible expression system relying on the consumption of L-lysine within the cell.

(iii) Construction of a strong IPTG-inducible expression system based on the strong *groESL* promoter.

(iv) Further enhancement of gene expression by optimizing the UP element, the region of the transcriptional start site and the half-life of the mRNA.

2 Materials and methods

2.1 Bacterial strains, plasmids, oligonucleotides, antibiotics and media

2.1.1 Bacterial strains

The bacteria strains used in the course of this work are listed in the Tab. 2.1.

Tab. 2.1. Bacterial strains used in this work

Name	Description	Reference
DH10B (*E. coli*)	F-, *mcrA*, Δ(*mrr*, *hsdRMS*, *mcrBC*), φ80d (*lacZΔM15*, Δ*lacX74*), *deoR*, *recA1*, *araD139*, Δ(*ara*, *leu*)$_{7697}$, *galK*, λ$^-$, *rpsL*, *endA1*, *nupG*	Bethesda Research laboratories
XL1 Blue (*E. coli*)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
1012	*leuA8 metB5 trpC2 hsdRM1*	[132]
AM01	1012 *amyE ::cat* (Cm^R)	[99]
PT02	AM01 *gcv::neo* (Cm^R and Neo^R)	*
PT05	1012 *gcvT::neo* (Neo^R)	*
PT17	AM01 *amyE::P$_{groE}$-Rib-lacZ* (Spec^R)	*
PT21	AM01 *amyE::P$_{gcv(-10\ consensus)}$-lacZ* (Spec^R)	*
PT22	AM01 *amyE::P$_{gcv(-35\ consensus)}$-lacZ* (Spec^R)	*
PT23	AM01 *amyE::P$_{gcv(-10\ and\ -35\ consensus)}$-lacZ* (Spec^R)	*
PT40	AM01 *Δ gcv::neo* (Cm^R and Neo^R)	*
PT41	AM01 *Δ lysC::neo* (Cm^R and Neo^R)	*
PT42	AM01 *amyE::P$_{lysC}$-lacZ* (Spec^R)	*
PT43	PT41 *amyE::P$_{lysC}$-lacZ* (Neo^R and Spec^R)	*
PT44	AM01 *amyE::P$_{lysC}$-Δter-laZ* (Spec^R)	*
PT45	PT41 *amyE::P$_{lysC}$-Δter-laZ* (Neo^R and Spec^R)	*
PT46	AM01 *amyE::P$_{gcv}$-lacZ* (Spec^R)	*
PT47	PT40 *amyE::P$_{gcv}$-lacZ* (Neo^R and Spec^R)	*
PT48	AM01 *amyE::P$_{gcv}$-Δter -lacZ* (Spec^R)	*
PT49	PT40 *amyE::P$_{gcv}$-Δter -lacZ* (Neo^R and Spec^R)	*

Strains marked with an asterisk (*) were constructed during this work.

2.1.2 Plasmids

The plasmids used during this work are listed in the Tab. 2.2.

Tab. 2.2. Plasmids used during this work

Name	Description	Reference
pDG1728	contains the promoter-less *lacZ* allowing integration at *amyE*	[52]
pMUTIN-*ydrB*	template for *lacZ* reporter gene	[75]
pMUTIN-*gfp*+	template for *gfp*+ reporter gene	[75]
pBgaB	template for t_0 terminator of phage λ	[99]
pX-*bgaB*	template for *bgaB* reporter gene	[79]
pHCMC01	pMTLBs72 with *trpA* transcriptional terminator, resistant to Cm (Cm^R)	[104]
pNDH09	template for *srtA* gene of *L. monocytogenes*	[106]
pNDH33	template for P_{groE} promoter	[117]
pNDH37	template for *amyQ* signal sequence	[117]
pBluescript IIKS	*lacZ*, f1 *ori*, Amp^R, T7 and T3 promoter	Stratagene
pCT105	pBR322 + *celA*, template for *celA*	[30]
pCT208	pBR322 + *celB*, template for *celB*	[30]
pT02Z	pT05-*lacZ* with wild-type promoter P_{lysC}-lys riboswitch	*
pT05Z	pHCMC01 with *lacZ* reporter gene	*
pT05-*lacZ*	pT05Z with t_0 terminator of phage λ	*
pT12	pDG1728 with wild-type promoter P_{gcv}-gly riboswitch	*
pT13	pDG1728 with wild-type promoter P_{lysC}-lys riboswitch	*
pT17	pDG1728 with wild-type promoter P_{groE}-gly riboswitch	*
pT20	Contains neomycin cassette for knockout of *gcv* operon	*
pT21	pDG1728 with promoter $P_{gcv(-10\ consensus)}$- gly riboswitch	*
pT22	pDG1728 with promoter $P_{gcv(-35\ consensus)}$- gly riboswitch	*
pT23	pDG1728 with promoter $P_{gcv(-10\ and\ -35\ consensus)}$- gly riboswitch	*
pT24	pHT01 containing *bgaB* fused to promoter P_{gcv}-gly riboswitch	*

pT25	pHT01 containing *bgaB* fused to consensus promoter P_{gcv}-gly riboswitch	*
pT27-*htpG*	pHT01 containing *htpG* fused to promoter P_{gcv}-gly riboswitch	*
pT27-*pbpE*	pHT01 containing *pbpE* fused to promoter P_{gcv}-gly riboswitch	*
pT27-*amyQ*	pHT43 containing *amyQ* fused to promoter P_{gcv}-gly riboswitch	*
pT28-*htpG*	pHT01 containing *htpG* fused to consensus promoter P_{gcv}-gly riboswitch	*
pT28-*pbpE*	pHT01 containing *pbpE* fused to consensus promoter P_{gcv}-gly riboswitch	*
pT28-*amyQ*	pHT43 containing *amyQ* fused to consensus promoter P_{gcv}-gly riboswitch	*
pT30	pHT01 with wild-type promoter P_{gcv}-gly riboswitch	*
pT31	pHT01 with consensus promoter P_{gcv}-gly riboswitch	*
pT32	pHT43 with wild-type promoter P_{gcv}-gly riboswitch	*
pT33	pHT43 with consensus promoter P_{gcv}-gly riboswitch	*
pT37	Contains neomycin cassette for knockout whole *gcv* operon	*
pT39	Contains neomycin cassette for knockout of *lysC* operon	*
pT40	pDG1728 with promoter P_{gcv}-*Δter*–gly riboswitch	*
pT41	pDG1728 with promoter P_{lysC}-*Δter*–lys riboswitch	*
pHT01	derivative of pNDH33 with deletion of a 117-bp direct repeat	*
pHT06	promoter-probe plasmid to identify and screen promoters	*
pHT36	plasmid for investigate terminal stabilizing elements	*
pHT43	derivative of pNDH37 with the deletion of a 117-bp direct repeat	*
pHT08	pHT01 with 8x His tag at the N terminus	*
pHT08-*yhcS*	pHT08 with *yhcS* gene of *B. subtilis*	*
pHT08-*srtA*	pHT08 with *srtA* gene of *L. monocytogenes*	*
pHT09	pHT01 with Strep-tag at the N terminus	*

pHT09-*gfp*+	pHT09 with *gfp* reporter gene	*
pHT10	pHT01 with c-Myc epitope-tag at the N terminus	*
pHT10-*ywbN*	pHT10 with *ywbN* gene of *B. subtilis*	*
pHT24	pHT01 with Strep-tag at the C terminus	*
pHT24-*gfp*+	pHT24 with *gfp* reporter gene	*
pHT42	pHT08 with Strep-tag at the C terminus	*
Plasmids for the construction of promoter elements		
Plasmid	Name of promoters/ **oligos** required for hybridization	Reference
pHT57	P57/ **P57F & P57R**	*
pHT58	P58/ **P58F & P58R**	*
pHT59	P59/ **P59F & P59R**	*
pHT60	P60/ **P60F & P60R**	*
pHT61	P61/ **P61F & P61R**	*
pHT62	P62/ **P62F & P62R**	*
pHT68	P68/ **P68F & P68R**	*
pHT69	P69/ **P69F & P69R**	*
pHT70	P70/ **P70F & P70R**	*
pHT71	P71/ **P71F & P71R**	*
pHT72	P72/ **P72F & P72R**	*
pHT73	P73/ **P73F & P73R**	*
pHT74	P74/ **P74F & P74R**	*
pHT75	P75/ **P75F & P75R**	*
pHT76	P76/ **P76F & P76R**	*
pHT77	P77/ **P77F & P77R**	*
pHT78	P78/ **P78F & P78R**	*
pHT79	P79/ **P79F & P79R**	*
pHT80	P80/ **P80F & P80R**	*
pHT81	P81/ **P81F & P81R**	*
pHT82	P82/ **P82F & P82R**	*

pHT83	P83/ **P83F & P83R**	*
pHT84	P84/ **P84F & P84R**	*
pHT85	P85/ **P85F & P85R**	*
pHT86	P86/ **P86F & P86R**	*
pHT87	P87/ **P87F & P87R**	*
pHT88	P88/ **P88F & P88R**	*
pHT89	P89/ **P89F & P89R**	*
pHT90/ pHT95	P90/ P95/ **P90F & P90R**	*
pHT91/ pHT96	P91/ P96/ **P91F & P91R**	*
pHT92/ pHT97	P92/ P97/ **P92F & P92R**	*
pHT93/ pHT98/	P93/ P98/ **P93F & P93R**	*
pHT94/ pHT99	P94/ P99/ **P94F & P94R**	*
pHT100	P100/ **P100F & P100R**	*
pHT251	P251/ **P251F & P251R**	*
pHT252	P252/ **P252F & P252R**	*
	Intermediate PCR products required for generation of promoters	
	Template/ **Name of oligos**	PCR products
	P_{grac}/ **S102R1 & ON76F**	P_{groE} 01
	P_{grac}/ **S104R1 & ON76F**	P_{groE} 02
	P_{grac}/ **S203R1 & ON76F**	P_{groE} 03
	P_{grac}/ **S206R1 & ON76F**	P_{groE} 04
	P_{grac}/ **S207R1 & ON76F**	P_{groE} 05
	P_{grac}/ **S211R1 & ON76F**	P_{groE} 06
	Hybridization of **S250F & S250R**	P_{groE} 07
	P_{grac}/ **S228R & ON76F**	P_{groE} 08
	P_{grac}/ **S229R & ON76F**	P_{groE} 09
	P_{lepA}/ **S221F & S224R**	P_{lepA} 224*
Plasmids for the study of stabilizing elements		
Plasmid	Templates/ **oligos** / name of stabilize element PCR products	Reference
pHT102	P_{groE} 01/ **S102R2 & ON76F**/ S102	*
pHT103	P_{groE} 01/ **S103R2 & ON76F**/ S103	*
pHT104	P_{groE} 02/ **S104R2 & ON76F**/ S104	*
pHT105	P_{groE} 02/ **S105R2 & ON76F**/ S105	*

pHT106	P_{grac}/ **S106F & S106R**/ S106	*
pHT107	P106/ **S107R & ON76F**/ S107	*
pHT108	P106/ **S108R & ON76F**/ S108	*
pHT109	P106/ **S109R & ON76F**/ S109	*
pHT201	P_{groE} 01/ **S201R2 & ON76F**/ S201	*
pHT202	P_{groE} 01/ **S202R2 & ON76F**/ S202	*
pHT203	P_{groE} 03/ **S202R2 & ON76F**/ S203	*
pHT204	P_{groE} 03/ **S201R2 & ON76F**/ S204	*
pHT205	P_{groE} 03/ **S104R2 & ON76F**/ S205	*
pHT206	P_{groE} 04/ **S206R2 & ON76F**/ S206	*
pHT207	P_{groE} 05/ **S105R2 & ON76F**/ S207	*
pHT208	P_{groE} 05/ **S208R2 & ON76F**/ S208	*
pHT209	P_{groE} 02/ **S208R2 & ON76F**/ S209	*
pHT210	P_{groE} 06/ **S210R2 & ON76F**/ S210	*
pHT211	P_{groE} 06/ **S211R2 & ON76F**/ S211	*
pHT212	P_{groE} 06/ **S212R2 & ON76F**/ S212	*
pHT213	P_{groE} 06/ **S213R2 & ON76F**/ S213	*
pHT214	P_{groE} 01/ **S214R2 & ON76F**/ S214	*
pHT215	P_{groE} 01/ **S215R2 & ON76F**/ S215	*
pHT221	P_{lepA}/ **S221R & S221F**/ S221	*
pHT222	P_{spac} 222*/ **S223F & S212R2**/ S222	*
pHT223	P_{groE} 06/ **S223F & S212R2**/ S223	*
pHT224	P_{lepA} 224*/ **S221F & S212R2**/ S224	*
pHT225	P_{groE} 06/ **S223F & S213R2**/ S225	*
pHT228	P_{groE} 08/ **ON76F & S104R2**/ S228	*
pHT229	P_{groE} 09/ **ON76F & S104R2**/ S229	*
pHT250	P_{groE} 07/ **S250F & S212R2**/ S250	*

The name, description and references of the plasmids are given. Plasmids marked with an asterisk (*) were constructed during this work.

2.1.3 Oligonucleotides

The oligonucleotides used during this work are listed in the Tab. 2.3.

Tab. 2.3. Oligonucleotides used in the course of this work

Name	Sequence (5' to 3')	Description
ON01	CTAATACGACTCACTATAGGGAGAaaggacagagaaacacctcatgta	3’ end of gly-riboswitch probe
ON02	ATATGAGCGAATGACAGCAAGG	5’ end of gly-riboswitch probe
ON03	CTAATACGACTCACTATAGGGAGAagcattaatgacaagcagatag	3’ end of *gcvT* probe
ON04	GACCTGTATAAGGAATATGGAGGA	5’ end of *gcvT* probe
ON05	TAGATGGAGCTCAGAACGCCGTTATTTGACCTGT	5’ end of *gcvT* gene
ON06	CGCTGACCGCGGCTTCATCAATAAACGCAA	3’ end of *gcvT* gene
ON07	GGCCATCTCGAGGGCGCTTTACGTTTGATTATG	5’ end of *gcvPB* gene
ON08	GGCCATGGTACCGCCTCGTATCTGAGCACTG	3’ end of *gcvPB* gene
ON09	GGCCATGAATTCTTCAAACTCTGGAATTGCTAATG	5’ end of P_{gcv}
ON10	GGCCATGGATCCTTCCTCCTTTATCAACGGCGCAGCT	3’ end of P_{gcv}-riboswitch
ON11	ggagattctttattataagaatTGTCCATAACAGCATGAAAATATG	recombinant primer for P_{groE}- riboswitch
ON12	TCGTTCGAATTCAGCTATTGTAACATAATCGGTACG	5’ end of P_{groE}
ON13	GGAATTGTTATCCGCTCACAATTCCACAATTCTTATAATA	3’ end of P_{groE}
ON14	GATGTAAGATATTGCTATAATATGTCCATAACAGCATGAAAATATGAG	5’ end of -10 consensus
ON15	GAGTATGTATTTGATGTAAGATATTGCTATAATATGTCCATAACAGC	3’ end of -10 consensus
ON16	GAGTTTGACATTGATGTAAGATATTGCTATAGTATGTCC	5’ end of -35 consensus
ON17	GCATATAGTGATGATGGTAGGATATGAGTTTGACATTGATGTAAGATATTGCTATA	3’ end of -35 consensus
ON18	GAGTTTGACATTGATGTAAGATATTGCTATAATATGTCCATAACAGCATG	5’ end of -35 and -10 consensus
ON19	GGCCATGAGCTCTTCAAACTCTGGAATTGCTAATG	5’ end of P_{gcv} of *B. subtilis*

ON20	GGCCATTGATCAATGATTCAAAAACGAAAGCGGACAG	5’ end of signal sequence
ON21	GGCCATGGATCCTACGGCTGATGTTTTTGTAATCGG	3’ end of signal sequence
ON22	GGCCAGGATCCTTTCCCCTTTATCACACCTCATGTAAAATGAAGGTTCTC	3’ end of gly-riboswitch without terminator
ON23	GGCCATGAGCTCCACTGTGACACAAGGGAAGC	5’ end of *yqhH* gene
ON24	GGCCATGGATCCAATGAATACAGAAATGATCTACGATG	3’ end of *yqhH* gene
ON25	GGCCATAGATCTCTAATTTCATAGTTAGATCGTGTTATATGG	5’ end of lys-riboswitch probe
ON26	gctaatacgactcactatagggaCTCTCATTGCTTATCAATTAATCATCAT	3’ end of lys-riboswitch probe
ON27	CGCCAGAATTACAGATATCGACACTTC	5’ end of *lysC* probe
ON28	gctaatacgactcactatagggTATACTCTTCAAGCACCGCAACGG	3’ end of *lysC* probe
ON29	GGCCATGAGCTCTGATCGGTGATCCGCTGG	5’ end of *uvrC* gene
ON30	GGCCATGGATCCTATCAGATCTTATTTAAAAGGACAACAT	3’ end of *uvrC* gene
ON31	GGCCATCTCGAGTCGCTTCACGATGCA	5’ end of *yslB* gene
ON32	GGCCATGGTACCCTCACCAACGTAAGCG	3’ end of *yslB* gene
ON33	GGCCATGAATTCACAAATTGCAAAAATAATGTTGTC	5’ end of P_{lysC}
ON34	GGCCATGGATCCCATGTATTACCACCCTTTACATTTTG	3’ end of P_{lysC}
ON35	GGCCATGGATCCTTCTCCCTTTCCTCTCATTGCTTATCAATTAATCATCA	3’ end of P_{lysC} without terminator
ON36	GGCCATTGATCAACAAATTGCAAAAATAATGTTGTC	5’ end of P_{lysC}
ON37	GGCCATTCTAGACATGTATTACCACCCTTTACATTTTG	3’ end of P_{lysC}
ON38	GCAGGATCCAAGGAGGAATCTAGAATGGAAGTTACTGACGTAAGATTACG	5’ end of *lacZ* gene
ON39	GGCCATACTAGTTTATTTTTGACACCAGACCAACTGG	3’ end of *lacZ* gene
ON40	GGCCATGCTAGCGATCTCTGCAGTCGCGATGAT	5’ end of t_0 terminator
ON41	GGCCATGGTACCGGGCAACGTTCTTGCCA	3’ end of t_0 terminator
ON42	GCCATCTCGAGGGTAACTAGCCTCGCCGATCC	5' *amp-ColE1*
ON43	GCCATCTTAAGCATGCGTATTGGGCGCTCTTCCG	3' *amp-ColE1*

ON44	GGCCATGGATCCATGAATGTGTTATCCTC	5' end of *bgaB*
ON45	GGCCATGACGTCCTAAACCTTCCCGGCTTCATCA	3' end of *bgaB*
ON46	GGCCATGGATCCATGGCGAAAAAAGAGTTTAAAGCAGAGTC	5' end of *htpG*
ON47	GGCCATGACGTCTTACACCATGACCTTGCAAATATTGTTCG	3' end of *htpG*
ON48	GGCCATGGATCCATGAAGCAGAATAAAAGAAAGCATC	5' end of *pbpE*
ON49	GGCCATGACGTCTTACTACTTCGTACGGACCGCTTCT	3' end of *pbpE*
ON50	GGCCATGGATCCATGATTCAAAAACGAAAGCGGACAG	5' end of *amyQ*
ON51	GGCCATGACGTCTTATTTCTGAACATAAATGGAGACG	3' end of *amyQ*
ON52	GGCCATAGATCTGCAAACACTGTGTCAGCGGCA	5' end of *celA*
ON53	GGCCATGACGTCTTAATAAGGTAGGTGGGGTATGCTC	3' end of *celA*
ON54	GGCCATGGATCCGAAGGGTCATATGCTGATTTGGCAG	5' end of *celB*
ON55	GGCCATGACGTCTTATTTATACGGCAACTCACTTATGC	3' end of *celB*
ON56	GATCTATGCGCGGAAGCCATCACCATCACCATCACCATCACG	5’ end of 8xHis-tag
ON57	GATCCGTGATGGTGATGGTGATGGTGATGGCTTCCGCGCATA	3’ end of 8xHis-tag
ON58	GATCTATGAATTGGAGCCATCCGCAATTTGAAAAAG	5’ end of Strep-tag
ON59	GATCCTTTTTCAAATTGCGGATGGCTCCAATTCATA	3’ end of Strep-tag
ON60	CGAACAAAAACTTATTAGCGAAGAAGATCTTTAATAACACGT	5’ end of c-Myc-tag
ON61	GTTATTAAAGATCTTCTTCGCTAATAAGTTTTTGTTCGACGT	3’ end of c-Myc-tag
ON62	CTGGAGCCATCCGCAATTTGAAAAATAAACGT	5’ end of Strep-tag
ON63	TTATTTTTCAAATTGCGGATGGCTCCAGACGT	3’ end of Strep-tag
ON64	GGCCAGATCTATGGGAGGCTTTAAATTGATCGATACG	5' end of *yhcS* gene
ON65	GGCCATAGATCTAGAATGAAGAAAAGCCGCAGGCACT	3' end of *yhcS* gene
ON66	GGCCATGGATCCATGATTAGTATATTTATTGCAGAAGA	5' end of *desR* gene
ON67	GGCCATGACGTCTTATTTAAACCAGCCTTTTTCTTTTG	3' end of *desR* gene
ON68	GGCCAGATCTATGAGTGGTCATGAAACAATCG	5' end of *srtA* gene
ON69	GGCGGATCCCGTTTTCGGCAGTTTCGTAGGGAAATATTTATTC TCTAGTT	3' end of *srtA* gene
ON70	GGCCATAGATCTATGAAGCCATCGAAAAAGGATGAAAAAG	3' end of *ywbN* gene
ON71	GGCCATAGATCTTGATTCCAGCAAACGCTGGGC	5' end of *ywbN* gene
ON72	GGCCATAGATCTATGGCTAGCAAAGGAGAAGAACT	5' end of *gfp* gene

ON73	GGCCATAGATCTTTTGTAGAGCTCATCCATGCCA	3' end of *gfp* gene
ON74	GGCCATGACGTCTTATTTGTAGAGCTCATCCATGCCA	3' end of *gfp* gene
ON75	GCTAATACGACTCACTATAGGGAACATTAATATCGCGCCAGT TAACATG	3’ end of *bgaB* probe
ON76F	TCGTTCGGTACCAGCTATTGTAACATAATCGGTACG	5’ end of P_{groE}
ON77	GGCCATGGTACCAAAGGAGGTAAGGATCCATGAATGTGTTAT CCTC	5’ end of *bgaB* for pHT02
ON78	GGCCATGACGTCCTAAACCTTCCCGGCTTCATCA	3' end of *bgaB* for pHT02
ON79	GATCACTAGTTAACCGCGGAATTGTGAGCGGATAACAATTCC CATATAAAGGAGGAAG	MCS for pHT06
ON80	GATCCTTCCTCCTTTATATGGGAATTGTTATCCGCTCACAATTC CGCGGTTAACTAGT	MCS for pHT06
P57F	CTAGTTGACATTGGAAGGGAGATATGTTATTATAAGAATTGC	5’ end of P57
P57R	AATTCTTATAATAACATATCTCCCTTCCAATGTCAA	3’ end of P57
P58F	CTAGTTGAAATTGGAAGGGAGATATGTTATAATAAGAATTGC	5’ end of P58
P58R	AATTCTTATTATAACATATCTCCCTTCCAATTTCAA	3’ end of P58
P59F	CTAGAAAATTTTTTAAAAAATCACTTGAAATTGGAAGGGAGA TTCTTTATTATAAGAATTGC	5’ end of P59
P59R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAAGTGATTT TTTAAAAAATTTT	3’ end of P59
P60F	CTAGAAAATTTTTTATCTTATCACTTGAAATTGGAAGGGAGAT TCTTTATTATAAGAATTGC	5’ end of P60
P60R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAAGTGATAA GATAAAAAATTTT	3’ end of P60
P61F	CTAGAAAATTTTTTATCTTATCAGTTGAAATTGGAAGGGAGAT TCTTTATTATAAGAATTGC	5’ end of P61
P61R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAACTGATAA GATAAAAAATTTT	3’ end of P61
P62F	CTAGAAAATTTTTTATCTTACTACTTGAAATTGGAAGGGAGAT TCTTTATTATAAGAATTGC	5’ end of P62
P62R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAAGTAGTAA GATAAAAAATTTT	3’ end of P62
P63F	CTAGAAAATTTTTTATCTTATCTCTTGAAATTGGAAGGGAGAT TCTTTATTATAAGAATTGC	5’ end of P63
P63R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAAGAGATAA GATAAAAAATTTT	3’ end of P63
P64F	CTAGAAAATTTTTTAAAAAATCTCTTGAAATTGGAAGGGAGA TTCTTTATTATAAGAATTGC	5’ end of P64
P64R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAAGAGATTT TTTAAAAAATTTT	3’ end of P64
P65F	CTAGTAGACAAACTATCGTTTAACATGTTATACTATAATATGC	5’ end of P65

P65R	ATATTATAGTATAACATGTTAAACGATAGTTTGTCTA	3’ end of P65
P66F	CTAGTTGACACTTTATCTTCCATCTGGTATAATAAATAGAGC	5’ end of P66
P66R	TCTATTTATTATACCAGATGGAAGATAAAGTGTCAA	3’ end of P66
P67F	CTAGTTGACAAATATTATTCCATCTATTACAATAAATTCAGC	5’ end of P67
P67R	TGAATTTATTGTAATAGATGGAATAATATTTGTCAA	3’ end of P67
P68F	CTAGAAAATTTTTTAAAAAATCTCTTGACATTGGAAGGGAGA TATGTTATAATAAGAATTGC	5’ end of P68
P68R	AATTCTTATTATAACATATCTCCCTTCCAATGTCAAGAGATTT TTTAAAAAATTTT	3’ end of P68
P69F	CTAGAAAATTTTTTAAAAAATCTCTTGACATTGGAAGGGAGA TTCTTTATAATAAGAATTGC	5’ end of P69
P69R	AATTCTTATTATAAAGAATCTCCCTTCCAATGTCAAGAGATTT TTTAAAAAATTTT	3’ end of P69
P70F	CTAGTTGAAATTGGAAGGGAGATTCTTTATTATAAGAATTGC	5’ end of P70
P70R	AATTCTTATAATAAAGAATCTCCCTTCCAATTTCAA	3’ end of P70
P71F	CTAGTTGACATTGGAAGGGAGATTCTTTATTATAAGAATTGC	5’ end of P71
P71R	AATTCTTATAATAAAGAATCTCCCTTCCAATGTCAA	3’ end of P71
P72F	CTAGTTGAAATTGGAAGGGAGATTCTTTATAATAAGAATTGC	5’ end of P72
P72R	AATTCTTATTATAAAGAATCTCCCTTCCAATTTCAA	3’ end of P72
P73F	CTAGTTGAAATTGGAAGGGAGATATGTTATTATAAGAATTGC	5’ end of P73
P73R	AATTCTTATAATAACATATCTCCCTTCCAATTTCAA	3’ end of P73
P74F	CTAGTTGAAATTGGAAGGGAGATTTGTTATTATAAGAATTGC	5’ end of P74
P74R	AATTCTTATAATAACAAATCTCCCTTCCAATTTCAA	3’ end of P74
P75F	CTAGTTGAAATTGGAAGGGAGAATGTTTATTATAAGAATTGC	5’ end of P75
P75R	AATTCTTATAATAAACATTCTCCCTTCCAATTTCAA	3’ end of P75
P76F	CTAGTTGAAATTGGAAGGGTTTATGTTTATTATAAGAATTGC	5’ end of P76
P76R	AATTCTTATAATAAACATAAACCCTTCCAATTTCAA	3’ end of P76
P77F	CTAGTTGAAATTGGAATATAGATTCTTTATTATAAGAATTGC	5’ end of P77
P77R	AATTCTTATAATAAAGAATCTATATTCCAATTTCAA	3’ end of P77

P78F	CTAGTTGACATTGGAAGGGAGATTCTTTATAATAAGAATTGC	5’ end of P78
P78R	AATTCTTATTATAAAGAATCTCCCTTCCAATGTCAA	3’ end of P78
P79F	CTAGTTGACATTGGAAGGGAGATATGTTATAATAAGAATTGC	5’ end of P79
P79R	AATTCTTATTATAACATATCTCCCTTCCAATGTCAA	3’ end of P79
P80F	CTAGTTGACATTGGAATATAGATATGTTATAATAAGAATTGC	5’ end of P80
P80R	AATTCTTATTATAACATATCTATATTCCAATGTCAA	3’ end of P80
P81F	CTAGTTGAAATTGGAATATTTTATGTTTATTATAAGAATTGC	5’ end of P81
P81R	AATTCTTATAATAAACATAAAATATTCCAATTTCAA	3’ end of P81
P82F	CTAGTTGACATTGGAATATAGATTCTTTATTATAAGAATTGC	5’ end of P82
P82R	AATTCTTATAATAAAGAATCTATATTCCAATGTCAA	3’ end of P82
P83F	CTAGTTGACATTGACAGGGAGATTCTTTATTATAAGAATTGC	5’ end of P83
P83R	AATTCTTATAATAAAGAATCTCCCTGTCAATGTCAA	3’ end of P83
P84F	CTAGTTGACATTGGAATATAGATATGTTATAATAAGAATAGC	5’ end of P84
P84R	TATTCTTATTATAACATATCTATATTCCAATGTCAA	3’ end of P84
P85F	CTAGTTGAATCTTTACAATCCTATTGATATAATCTAAGCTGC	5’ end of P85
P85R	AGCTTAGATTATATCAATAGGATTGTAAAGATTCAA	3’ end of P85
P86F	CTAGTTGACATTGGAATATAGATATGTTATAATAAGAATAGG GC	5’ end of P86
P86R	CCTATTCTTATTATAACATATCTATATTCCAATGTCAA	3’ end of P86
P87F	CTAGTTGACATTGGAATATTTTATGTTTATAATAAGAATTGC	5’ end of P87
P87R	AATTCTTATTATAAACATAAAATATTCCAATGTCAA	3’ end of P87
P88F	CTAGTTGACATTGGGTATAAGATTTGTTATAATAAGAATAGC	5’ end of P88
P88R	TATTCTTATTATAACAAATCTTATACCCAATGTCAA	3’ end of P88
P89F	CTAGTTGACATTGGTTTAAAGATATGTTATAATGGGTATAGC	5’ end of P89
P89R	TATACCCATTATAACATATCTTTAAACCAATGTCAA	3’ end of P89
P90F	CAAAAGAATGATGTAAGCGTGAAAAATTTTTTATCTTA	5’ end of P90

P90R	CTAGTAAGATAAAAAATTTTTCACGCTTACATCATTCTTTTGGTAC	3’ end of P90
P91F	CAAAAGAATGATAAAAGCGTGAAAAATTTTTTAAAAAA	5’ end of P91
P91R	CTAGTTTTTTAAAAAATTTTTCACGCTTTTATCATTCTTTTGGTAC	3’ end of P91
P92F	CTCACAAAAAAAGTGAGGATTTTTTTATTTTTGTA	5’ end of P92
P92R	CTAGTACAAAAATAAAAAAATCCTCACTTTTTTTGTGAGGTAC	3’ end of P92
P93F	CAACCCAGATATGATAGGGAACTTTTCTCTTTCTTGTTA	5’ end of P93
P93R	CTAGTAACAAGAAAGAGAAAAGTTCCCTATCATATCTGGGTTGGTAC	3’ end of P93
P94F	CTGTCAACATGAGAATTCTTATCATCAATTTTTGAAAA	5’ end of P94
P94R	CTAGTTTTCAAAAATTGATGATAAGAATTCTCATGTTGACAGGTAC	3’ end of P94
P100F	CTAGAAAATTTTTTAAAAAATCTCTTGACATTGGAAGGGAGATATGTTATTATAAGAATTGC	5’ end of P100
P100R	AATTCTTATAATAACATATCTCCCTTCCAATGTCAAGAGATTTTTTAAAAAATTTT	3’ end of P100
P222F	GGCCATGGTACCAGGCCTTACACAGCCCAGTCCAG	5’ end of P222
P222R1	TGTGGAATTGTGATCCGCTCACAATCCACAATACACATTATGCCACACCTTGTAGAT	3’ end of P222
P251F	CTAGAAAATTTTTTAAAAAATCTCTTGACAAATATTATTCCATCTATGATTATAAATTCAGC	5’ end of P251
P251R	TGAATTTATAATCATAGATGGAATAATATTTGTCAAGAGATTTTTTAAAAAATTTT	3’ end of P251
P252F	CTAGAAAATTTTTTAAAAAATCTCTTGACACTTTACAATCCTAATGATATTATCTAAGCTGC	5’ end of P252
P252R	AGCTTAGATAATATCATTAGGATTGTAAAGTGTCAAGAGATTTTTTAAAAAATTTT	3’ end of P252
S102R2	GCCATGGATCCTTCCTCCTTTAATTGGTATCCGCTCACAATTCCACAA	3’ end of S102
S103R2	GGCAGGATCCTTCCTCCTTTAATTGAATTGTTATCCGCTCACAATT	3’ end of S103
S104R	GGAATTGTTATCCGCTCACAATTCCCCTATTCTTATAATAAAG	3’ end of S104
S104R2	GGCCATGGATCCTTCCTCCTTTAATTGGGAATTGTTATCCGCTCACA	3’ end of S104
S105R2	GGCAGGATCCTTCCTCCTTTAATTGGGGAATTGTTATCCGCTCACAATT	3’ end of S105
S106F	GGCCATACTAGTGTACCAGCTATTGTAACATAATCGGTACG	5’ end of S106
S106R	GCTACCGCGGTATTCTTATAATAAAGAATCTCCCTTCC	3’ end of S106
S107R	GGCAGGATCCTTCCTCCTTTAATTGCGGAATTGTTATCCGCTCACAATT	3’ end of S107

S108R	GGCAGGATCCTTCCTCCTTTAATTCGCGGAATTGTTATCCGCT CACAATT	3’ end of S108
S109R	GGCAGGATCCTTCCTCCTTTAATTCCGCGGAATTGTTATCCGC TCACAATT	3’ end of S109
S201R2	GGCAGGATCCTTCCTCCTTTAATTTGGAATTGTTATCCGCTCA CAATT	3’ end of S201
S202R2	GGCAGGATCCTTCCTCCTTTAATTTGTGGAATTGTTATCCGCT CACAATT	3’ end of S202
S206R2	GGCAGGATCCTTCCTCCTTTAATTGGGAATTGTGATCCGCTCA CAA	3’ end of S206
S208R2	GGCAGGATCCTTCCTCCTTTAGTTGGGGAATTGTTATCCGCTC ACAATT	3’ end of S208
S210R2	GGCAGGATCCTTCCTCCTTTAATTGGTTGAATTGATAGAATCT AGTTGTTGTGGAATTGTGATCCGCTCAC	3’ end of S210
S211R2	GGCAGGATCCTTCCTCCTTTAATTGGGAATTGTGATCCGCTCA CAA	3’ end of S211
S212R2	GGCAGGATCCTTCCTCCTTTAATTGGTGTTGGTTGTGGAATTG TGATCCGCTCACA	3’ end of S212
S213R2	GGCAGGATCCTTCCTCCTTTAATTGGTGTTGGTTGTTGTTGTG GAATTGTGATCCGCTCACA	3’ end of S213
S214R2	GGCAGGATCCTTCCTCCTTTAATTGGTGTTGGTTGTGGAATTG TTATCCGCTCACA	3’ end of S214
S215R2	GGCAGGATCCTTCCTCCTTTAATTGGTGTTGGTTGTTGTTGTG GAATTGTTATCCGCTCACA	3’ end of S215
S221R	GGCAGGATCCTTCCTCCTTTAATTAAACGCAAAATACACTAGC TTAGAT	3’ end of S221
S223F	CCGAGGTACCAAAATTTTTTAAAAAATCTCTTGACATTGGAAG GGAGATTCTTTATAATAAGAATTGTGG	5’ end of S223
S228R	GGAATTGTTATCCGCTCACAATTCCGCTATTCTTATAATAAAG AATCTC	3’ end of S228
S229R	GGAATTGTTATCCGCTCACAATTCCGCAATTCTTATAATAAAG AATCTC	3’ end of S229

ON, oligonucleotide; P, oligonucleotides for promoter elements; S**,** oligonucleotides for stabilizing elements; the DNA sequences recognized by restriction enzyme are underlined.

2.1.4 Antibiotics

The antibiotics used in this work are listed in Tab. 2.4.

Tab. 2.4. List of antibiotic solutions used in this work

Antibiotic	**Concentration of stock solution (mg/ml)**	**Dissolved in**	**Final concentration (μg/ml)**
Ampicillin	50 - 100	70% ethanol	50
Chloramphenicol	20	100% ethanol	10
Rifampicin	100	50% methanol	100
Neomycin	10	water	10
Spectinomycin	100	water	100

2.1.5 Media

LB medium (1 % (w/v) trypton, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl).

Spizizen minimal medium (SMM) [149] supplemented with 50 µg/µl L-tryptophan, 50 µg/µl L- methionine and 50 µg/µl L-leucine. When necessary, the medium was supplemented with L-lysine at a final concentration of 300 µg/ml or L-glycine at a final concentration of 10 mM.

Antibiotics (Table 2.4), 2 % (w/v) insoluble starch, 0.5 % CMC (w/v), 1.6 % X-gal and 0.01 or 0.1 or 1 mM IPTG were added when necessary.

Agar was added to 1.5 % (w/v) to prepare plates.

2.2 Enzymes, antibodies, biochemicals, chemicals and kits

2.2.1 Enzymes

Roche: alkaline phosphatase, T7 RNA polymerase, DNase I.

Merk: proteinase K.

Sigma: RNase A, lysozyme.

Fermentas: restriction enzymes, taq DNA polymerase, Deepvent DNA polymerase.

2.2.2 Antibodies

All the antibodies used are given in Tab. 2.5 with their final dilutions.

Tab. 2.5. Antibodies used in this work

Name	From organism	Dilution	Reference
α-AmyQ	*Bacillus amiloliquefaciens*	1 : 15 000	V. Kontinen
α-GFP	*Aequoria victoria*	1 : 5000	Clontech
α-HtpG	*Bacillus subtilis* 1012	1 : 10 000	S. Schwab
α-PBP*	*Bacillus subtilis* 1012	1 : 10 000	[176]
α-His	synthetic	1 : 5000	Qiagen
α-Strep	synthetic	1 : 5000	IBA
α-c-Myc	synthetic	1 : 5000	Sigma

2.2.3 Biochemicals and chemicals

Amersham: Amonium persulphate, hyperfilm ECL.

Fermentas: DNA ladder, RNA ladder and protein ladder.

Pierce: Luminol substrate.

Roche: blocking reagent, chemiluminescent substrate CPD-Star, protease inhibitor, RNAase inhibitor, Xgal, ONPG, DTT and IPTG.

Roth: acetic acid, agar, agarose, aqua phenol, chloroform, diethylpyrocarbonate (DEPC), Ethidium bromide (Et.Br), isopropanol, L-lysine, L-glycine, L-Tryotophan, L-methionine, L-leucine, pepton, potassium acetate, potasium phosphate, polyacrylamide, sodium phosphate, sodium chloride, starch, MOPS, sodium dodecyl sulphate, TEMES, Tris, yeast extract.

2.2.4 Kits

Epicentre: Fast-Link™ DNA Ligation Kit.

Qiagen: PCR purification Kit, gel-extraction Kit, midi purification Kit and Ni-NTA Spin (50) Kit.

IBA: Strep-Tactin Spin Column Kit.

2.3 General methods

2.3.1 PCR and colony PCR

The technique of PCR (Polymerase Chain Reaction) is used to produce a large number of copies of a DNA sequence, e.g., a gene, to provide a sufficient amount for cloning [131]. This became effective by the isolation of a thermostable DNA polymerase from *Thermus aquaticus* [130]. During the PCR, DNA is denatured at high temperature and specific oligonucleotide primers are annealed and elongated at lower temperature in a cyclic manner.

Colony PCR is based on standard PCR using total DNA from colonies as template to allow the rapid detection of recombinant clones.

2.3.2 Cloning

All the steps necessary for cloning were carried out as described by using standard methods [133]. Preparation of competent *E. coli* cells and transformation were carried out as standard heat shock transformation [68] or electroporation [36]; PCR for screening of plasmids and preparation of plasmid DNA by the alkaline lysis method with SDS have been described [13, 69]. The correct DNA sequence of all inserts into plasmids was verified by sequencing carried out by the SeqLab, and only plasmids with the correct DNA sequence were used in further experiments.

2.3.3 Growth and collection of samples

During this work, *B. subtilis* strains were grown in LB medium or SMM with the appropriate antibiotic(s) when necessary in a waterbath shaker (~200 rpm) at 37°C. Overnight cultures in 5 ml medium in glass tubes were transferred partially to Erlenmeyer flasks containing medium to an OD_{578} of 0.05-0.08. When an OD_{578} of 0.8 (in LB medium) or 0.2 (in SMM) was reached (set as t = 0), the culture was divided into two subcultures where one was further grown in the absence and the other in the presence of the inducer, 0.01 mM or 0.1 mM or 1 mM for IPTG or 10 mM for L-glycine or 300 µg/ml for L-lysine. Aliquots were removed and centrifuged, and either the pellet and/or the culture supernatant were collected. Further samples were taken at different points of time after induction as indicated in the experiments. Normally, a certain amount of cells was collected corresponding to 1.2 of OD_{578} for β-galactosidase assays, or 2.5 of OD_{578} for protein analyses and 10 of OD_{578} for RNA analyses.

2.4 Northern blot experiments

Northern blot analysis was performed as described [65, 128].

2.4.1 Isolation of total RNA from *B. subtilis*

B. subtilis cells were grown and induced as described in 2.3.3. Especially, *B. subtilis* strains 1012, PT43, PT45, PT47, PT49 and *B. subtilis* 1012/pHT plasmids were induced by removal of L-lysine or 10 mM L-glycine or 0.1 mM IPTG and/or 100 µg/ml of rifampicin were added when necessary. The cells were then killed by addition of "killing buffer" (5 mM $MgCl_2$, 20 mM NaN_3, 20 mM Tris-HCl; pH7.5). Total RNA was extracted using the protocol for isolation of RNA from yeast with modifications [126]. The cell walls of the cells were digested by lysozyme (1 mg/ml) on ice for 5 min before extraction of the RNA. The samples were heated at 95°C for 5 min before addition of phenol.

2.4.2 Electrophoresis of RNA and vacuum blot transfer to membranes

RNA samples were separated on 1.2% agarose gels and the transfer occurred onto Nylon-membranes. The transfer was carried out with the help of the Vacuum-Blot-Annex (VacuGene™X1, Pharmacia).

2.4.3 Transcriptional labelling of RNA probes

Pairs of primers ON44/ON75 were used to amplify an internal part of the *bgaB* gene. The primer pairs ON01/ON02 and ON25/ON26 were used to generate the PCR product

corresponding to the *lysC*-riboswitch and *gcv*-riboswitch region and ON03/ON04 and ON27/ON28 to an internal part of *lysC* and *gcv,* respectively. These amplicons harbour a T7 promoter at the 3' end and were used as templates for *in vitro* transcription according to the instructions of the manufacture [128].

2.4.4 Cleaning of DIG-labelling RNA probes

When the DIG-labelled-antisense-RNA was used at the beginning for hybridization experiments, a very strong background was detected on the X-ray-film. The more RNA probe was used, the more the background was decreased. While this phenomenon has been observed, its reasons are not known. Therefore, the RNA probes were purified routinely before they were used in hybridization experiments. All the steps were carried as in the hybridization procedures, but the blank membranes were used.

2.4.5 Hybridization of membrane-bound RNA with RNA probes

This experiment was carried out as described [128].

2.4.6 Stripping of RNA probes

This experiment was carried out as described [128].

2.5 SDS-PAGE, Western blot analysis and rapid purification of proteins

2.5.1 Extraction of denatured total cell lysate from *B. subtilis*

For the extraction of denatured cell lysate from *B. subtilis* cells (2.5 of OD_{578}) prepared as in 2.3.3 were resuspended in 100 µl of lysis buffer (15% (w/v) sucrose, 50 mM Tris/HCl; pH 7.2) containing 2.5 mg/ml lysozyme and incubated at 37°C for 5 min. Then, 50 µl of 3x sample loading buffer (0.135 M Tris/HCl, 30% glycerol, 3% SDS, 0.03% bromophenol blue, 0.15 M DTT) were added to the suspension and frozen until they would be used. Before the samples were used, they had been heated for 5 min at 95°C and 15 µl of each sample were used for SDS-PAGE.

2.5.2 Measurement of protein concentrations

The method of Bradford was used for the measurement of the protein concentrations from cell extracts [81].

2.5.3 Precipitation of proteins from the culture supernatant

Protein from cultured supernatant was collected by the TCA method. One volume of 40% TCA was mixed with three volumes of culture supernatant, incubated on ice for 10 min and

centrifuged (12,000 rpm at 4°C for 10 min). The pellet was then washed twice with ice-cold acetone and dried at room temperature. The pellet was dissolved in water and loading buffer for SDS-PAGE was added.

2.5.4 Protein electrophoresis using discontinuous SDS-PAGE

The electrophoretic separation of proteins according to their molecular mass was performed as first described by Laemmli [84].

2.5.5 Immunoblot analysis

In order to immunochemically detect proteins using antibodies, the proteins were transferred, after their electrophoretic separation, onto a nitrocellulose membrane using electroblotting [158]. The electrophoretic transfer of proteins to nitrocellulose membranes was achieved by "Semi-Dry-Blotting" between graphite plate electrodes in a "Fast-Blot" apparatus (Biorad). The procedure for detection of labelled proteins followed the instruction of ECL Western blot (Amersham Biociences).

To corroborate the versatilities of the expression vectors pT and pHT for the overproduction of proteins, *B. subtilis* 1012 harbouring plasmids with the heat shock gene *htpG* [143] (pT27-*htpG,* pT28-*htpG* and pHT01-*htpG*) and the gene *pbpE* coding for a penicillin binding protein (pT27-*pbpE,* pT28-*pbpE* and pHT01-*pbpE*) were analysed by immunoblot. *B. subtilis* strain 1012 carrying one of these two vectors was grown either in LB medium or SMM [149] at 37°C to the mid-exponential growth phase. Then, the cultures were divided into subcultures where one was further grown untreated while the others were induced with 10 mM L-glycine or 1 mM IPTG. These cells were further grown, and samples were collected as described in 2.3.3 and prepared (see under 2.5.1). Equal amounts of proteins (0.025 of OD_{578}) were applied per lane, and the blots were probed with α-HtpG or α-Pbp4*.

To confirm expression of α-amylase in the culture medium, cells of *B. subtilis* 1012 containing the plasmids pT27-*amyQ* or pT28-*amyQ* or pHT43-*amyQ* were grown as described in 2.3.3. Aliquots were taken immediately before addition of 10 mM L-glycine or 1 mM IPTG (0 h) and 2 to 4 h after induction. Cells were pelleted by centrifugation and equal amounts of supernatant were analyzed, and the blots were probed with α-AmyQ.

In addition, to confirm the presence of the 10-amino-acid epitope-tag (c-Myc, pHT10-*ywpN* and pHT10-*gfp*) or the 8-amino-acid His (8xHis, pHT08-*yhcS* and pHT08-*srtA*) and Strep

(pHT09-*gfp* and pHT24-*gfp*) purification tags, a Western blot was carried and the monoclonal antibodies α-c-Myc, α-His or α-Strep were used.

All other cells were grown as described under 2.3.3; samples were applied on polyacrylamide gels and transferred to membranes for immunoblot detection as described in this section.

2.5.6 Purification of proteins with His-tag and Strep-tag

In order to allow purification of recombinant proteins from bacterial lysates by single-step of affinity chromatography, the proteins were fused with two different purification tags, namely the His- and the Strep-tag. The purification procedure can be divided into three stages: preparation of the cell lysate, binding of the recombinant protein to the affinity column, washing, and elution of the recombinant protein. The method of single-step purification of His-tagged proteins is described in [121] and that of Strep-tagged proteins in [67].

2.6 Visualization and measurement of reporter gene expression

2.6.1 Visualization of extracellular enzyme activity (α-amylase) on plates

Single colonies of the *B. subtilis* strains carrying plasmid pT27-*amyQ* or pT28-*amyQ* were grown for 24 h on LB or SMM plates with or without 10 mM L-glycine and 2% insoluble starch and stained with I2/KI solution [107]. Pictures were recorded using a digital camera.

2.6.2 Observation of the strength of promoters on X-gal plates

Blue colonies can be seen on X-gal LB plates containing IPTG. However, to see the difference of the promoter strength, low amount of IPTG should be added. Especially, to screen for strong promoters, 0.01 mM IPTG was applied and the plates with *B. subtilis* strains could be observed between 10 – 30 h for blue and white colonies. To compare *B. subtilis* strains carrying different plasmids, cells were transferred to new plates with various concentrations of IPTG (0, 0.0025, 0.005, 0.01, 0.025, 0.05 and 0.1 mM) and incubated at 30^{o}C or 37^{o}C. Each sample was replicated twice. The grey values (density) of colonies were determined using QuantityOne programme (Biorad) and represents the strength of promoters; higher values mean stronger promoters.

2.6.3 Measurement of the β-galactosidase activity

2.6.3.1 β-galactosidase BgaB

Blue colonies from LB-Xgal plates were used for determination of β-galactosidase activity. *B. subtilis* strains 1012 carrying pT or pHT with the *bgaB* reporter gene were grown, and

samples collected as described under 2.3.3. The activity was determined at 55°C as described [99] with the exception that the BgaB activity was measured in a microtiterplate reader (VersaMax, Molecular Devices). One unit is defined as $\Delta A_{420}*OD_{578}^{-1}*min^{-1}$ and displayed as units/OD_{578} for all the results, in which one OD_{578} is defined as the optical density of the samples used in the assay, A_{420} is the absorbance of the samples measured by the microtiterplate reader and, min indicates the incubation time of the plate at 55°C.

2.6.3.2 β-galactosidase LacZ

B. subtilis strains PT17, PT21, PT22, PT23, PT42 to PT49 and 1012 carrying pT02Z were grown and samples were collected according to section 2.3.3. β-galactosidase activity were measured at 405 nm at 28°C as described elsewhere [164]. One unit is defined as $V_{max}*OD_{578}^{-1}$, in which OD_{578} is defined as the optical density of the samples used in the assay and V_{max} is the maximum kinetic rate reported as miliOD/minute. The data were displayed as units/OD_{578} for all the results.

2.7 Construction of plasmids and strains

2.7.1 Construction of expression vectors based on the glycine riboswitch

To allow integration of transcriptional fusions at the *amyE* locus, the integration vector pDG1728 was used. This vector contains a promoter-less *lacZ* gene sandwiched between *amyE*-front and *amyE*-back [52] and was used as a backbone for the construction of several plasmids. pT12 contains the wild-type promoter of the *gcv* operon and the complete riboswitch amplified by PCR using the oligos ON9 and ON10 and DNA of strain 1012 as template inserted into pDG1728. pT17 harbours the promoter of the *groE* operon (ON12/ON13; template pNDH33) fused to the riboswitch (ON10/ON11; template DNA 1012); both PCR products were fused using recombinant PCR. pT21 carries the -10 consensus region of the *gcv* promoter and was obtained by using the mutagenic primer ON15 together with ON9 to amplify the promoter region and ON10 and ON14 to amplify the riboswitch followed by recombinant PCR to fuse both amplicons. pT22 contains the -35 consensus sequence and was constructed in a similar way using ON9 and the mutagenic primer ON17 to amplify the promoter region and ON10 and ON16 for the riboswitch. pT23 carries the complete consensus sequence obtained with the primer pairs ON9 and ON17 for the promoter and ON10 and ON18 for the riboswitch region. To study the influence of the transcriptional terminator on the regulation of *gcv*, the region corresponding to the right arm of the inverted repeat was removed using the primer pair ON9 and ON22 resulting in pT40. ON22 contains

the SD sequence of *gcv* to ensure translation of *lacZ*. All amplicons were verified by DNA sequencing. The resulting plasmids pT12, pT17, pT21, pT22, pT23, pT40 were transformed into the *B. subtilis* strain AM01 selecting for spectinomycin-resistant cells and screening for chloramphenicol sensitivity, and strain PT46, PT17, PT21, PT22, PT23 and PT48 were kept for further studies. The transcriptional fusion from plasmids pT12 and pT40 were also transformed into strain PT40 yielding PT47 and PT49 [116].

The following constructs allow expression of recombinant genes from two plasmid vectors: pHT01 carrying either the promoter-less *bgaB* or *htpG* or *pbpE* gene and pHT43 carry the promoter-less *amyQ* gene including its own signal sequence. The wild-type *gcv* promoter together with the riboswitch (ON10/ON19; template pT12) was fused to *bgaB* (pT24), *htpG* (pT27-*htpG*), *pbpE* (pT27-*pbpE*) and *amyQ* (pT27-*amyQ*). The consensus promoter and the downstream riboswitch was fused (ON10/ON19; template pT23) to *bgaB* (pT25), *htpG* (pT28-*htpG*), *pbpE* (pT28-*pbpE*) and *amyQ* (pT28-*amyQ*) as well. Insertion of the wild-type promoter without (ON10/ON19, template pT12) and with the signal sequence of *amyQ* (ON20/ON21; template pNDH37) into pHT01 resulted into pT30 (Fig. 2.1 A) and pT32 (Fig. 2.1 B), respectivety. Replacement of the wild-type promoter by the consensus promoter resulted in pT31 (ON10/ON19; template pT23) and pT33 (ON20/ON21; template pNDH37), respectively [116].

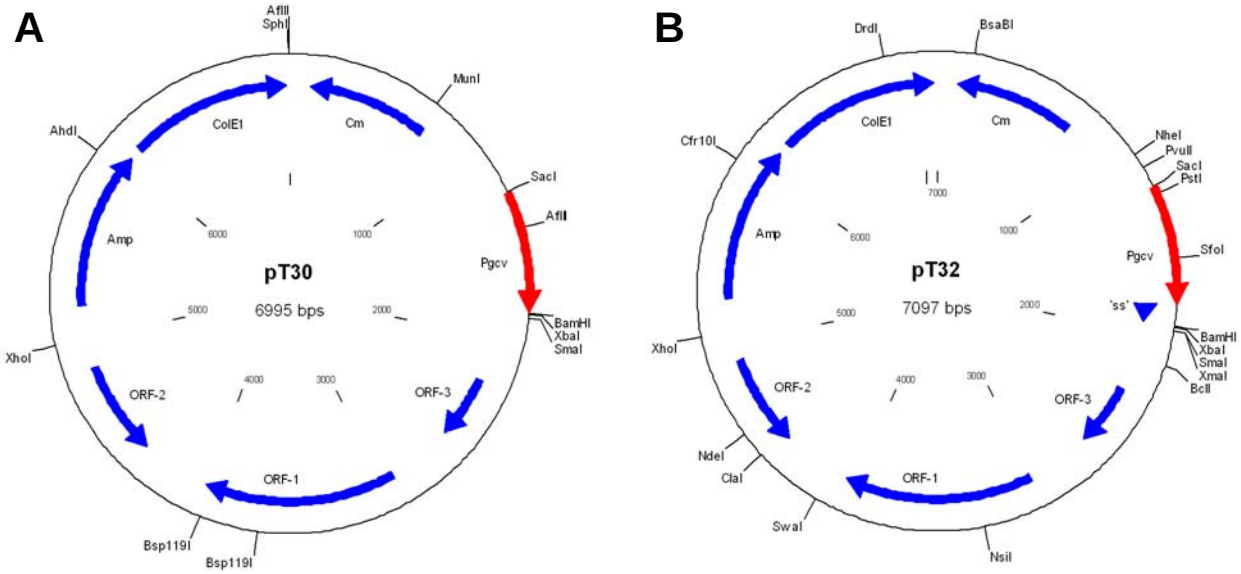


Fig. 2.1. Genetic and restriction map of the expression vector pT30 and the expression-secretion vector pT32. (A) pT30 with the wild-type P_{gcv} promoter; (B) pT32 with the wild-type P_{gcv} promoter fused to the coding region for the signal sequence.

2.7.2 Construction of expression vectors based on the lysine riboswitch

To check for the function of the riboswitch, it was transcriptionally fused to the *lacZ* gene present in the integration vector pDG1728 [52]. The region coding for the riboswitch including its promoter was generated by PCR using the primer pair ON33/ON34 and DNA of strain 1012 as template. The amplicon was cleaved with *Eco*RI and *Bam*HI and ligated into pDG1728 treated with the same enzymes. The resulting plasmid pT13 was transformed into the *B. subtilis* strain AM01 selecting for spectinomycin-resistant cells and screening for chloramphenicol sensitivity, and strain PT42 was kept for further studies. The transcriptional fusion was also transformed into strain PT41 yielding PT43.

To study the influence of the transcriptional terminator on the regulation of *lysC*, the region corresponding to the right arm of the inverted repeat was removed using the primer pair ON33 and ON35 resulting in pT41. ON35 contains the SD sequence of *lysC* to ensure translation of *lacZ*. pT41 was transformed into strain AM01 and all following steps were as described for pT13. Strain PT44 is a derivative of AM01 and PT45 of PT41, both carrying the transcriptional fusion with the partial deletion of the terminator.

To test whether the lysine system can be used as an auto-inducible expression system, plasmid pT02Z was constructed allowing expression of the *lacZ* gene. The coding sequence of *lacZ* gene was amplified using ON38/ON39 with pMUTIN-*ydrB* as template, cleaved with *Xba*I and *Bam*HI and inserted into pHCMC01 treated with the same enzymes resulting in pT05Z. Next, the terminator t_0 was amplified using ON40/ON41 with pBgaB as template and inserted into pT05Z at *Nhe*I and *Kpn*I sites resulting in pT05-*lacZ* (Fig. 2.2). The region coding for the riboswitch including its own promoter was generated by PCR using the primer pair ON36/ON37 and DNA of strain 1012 as template. The amplicon was fused with the *lacZ* gene from pT05-*lacZ* at the *Bcl*I and *Xba*I restriction sites resulting in pT02Z. The correct plasmid was then transformed into *B. subtilis* 1012, and the expression level of β-galactosidase was measured in the absence and presence of different concentrations of L-lysine.

2.7.3 Construction of structurally stable plasmids

It has been observed that the two plasmids pNDH33 and pNDH37 [117] exhibit some structural instability in *E. coli* [105]. When the complete DNA sequence of these two plasmids was analysed, an 117-bp sequence was detected occurring twice. This sequence in derived for the 3' end of the *lacI* gene and occurs as a direct repeat. To increase the stability

of these two plasmids in *E. coli,* one copy of the 117 bp direct repeat containing the 3' end of *lacI* was removed as follows. The DNA region containing the *bla* gene and the origin of replication derived from the pBR322 vector was amplified using oligonucleotides ON42 and ON43 with pMTLBs72 as template. The resulting 2010-bp amplicon was cleaved with *Xho*I and *Afl*II and ligated with pNDH33 or pNDH37 treated with the same enzymes resulting pHT01 and pHT43, respectively (Fig. 2.3).

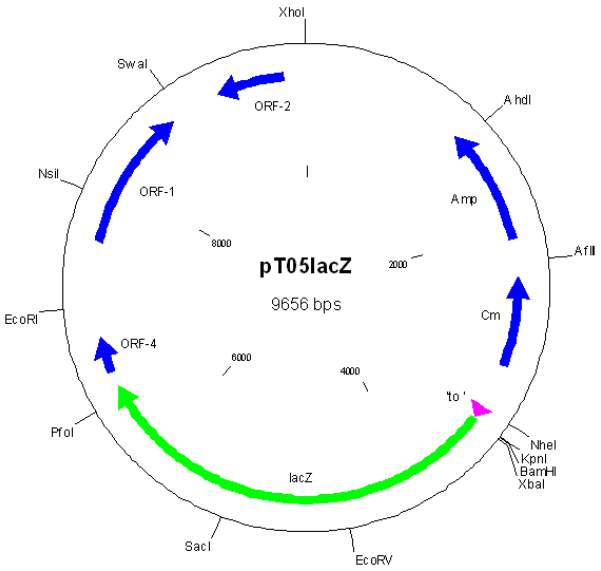
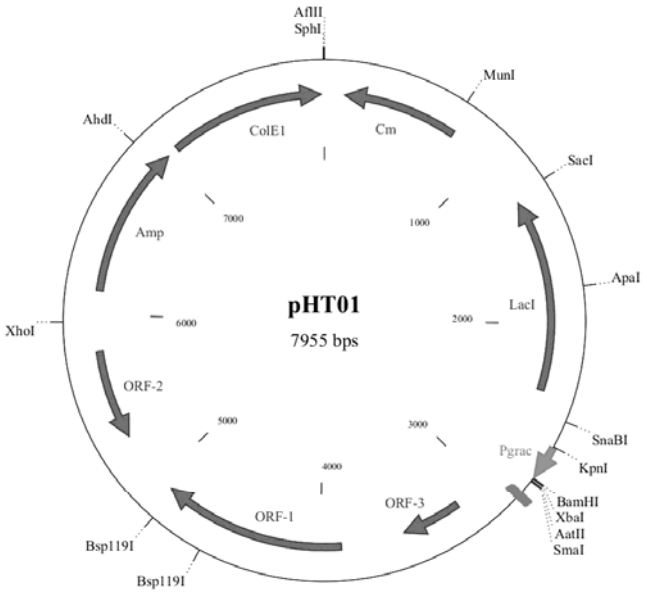


Fig. 2.2. Genetic and restriction map of the expression vector pT05-*lacZ*. This vector has been derived from pHCMC01 containing the *lacZ* reporter gene and the t_0 terminator of phage λ.

To test whether the removal of the repeated region has any influence on the control of gene expression, the following plasmids were constructed. The coding sequences for BgaB, HtpG, Pbp4* and α-amylase AmyQ containing its own signal sequence were amplified using ON44/ON45 with pX-*bgaB* [79], ON46/ON47 and ON48/ON49 with chromosomal DNA of *B. subtilis* 1012 and ON50/ON51 with pKTH10 [112] as template, cleaved with *Bam*HI and *Aat*II and inserted into pHT01 treated with the same enzymes resulting in pHT01-*bgaB*, pHT01-*htpG*, pHT01-*pbpE* and pHT43-*amyQ* respectively. The genes *celA* and *celB* were amplified using ON52/ON53 with pCT105 and ON54/ON55 with pCT208 as template [30]. The amplicons were cleaved with *Bgl*II/*Aat*II for *celA* and *Bam*HI/*Aat*II for *celB* and inserted into pHT43 treated with *Bam*HI and *Aat*II resulting pHT43-*celA* and pHT43-*celB*, respectively.



pHT01 P*grac*-lacO-RBS-GGATCCTCTAGAGTCGACGTCCCCGGG - Terminator
*Bam*HI *Xba*I *Aat*II *Sma*I

pHT08 ATGCGCGGAAGCCATCACCATCACCATCACCATCACGGATCC --------
M R G S H H H H H H H H *Bam*HI

pHT09 ATGAATTGGAGCCATCCGCAATTTGAAAAAGGATCCTCTAGA ---------
M N W S H P Q F E K *Bam*HI *Xba*I

pHT24 GGATCCTCTAGAGTCGACGTC -- Strep-Tag -- TAACCCCGGGGC -------
*Bam*HI *Xba*I *Aat*II Stop codon

pHT10 GGATCCTCTAGAGTCGACGTC -- c-Myc -- TCTTTAATAACACGT -------
*Bam*HI *Xba*I *Aat*II Stop codon

pHT43 ATG -- SamyQ -- GGATCCTCTAGAGTCGACGTCCCCGGGGCA -------
M *Bam*HI *Xba*I *Aat*II *Sma*I

Fig. 2.3. Genetic and restriction map of the expression vector pHT01. Indicated are three *orf*s (*orf-1*, *orf-2* and *orf-3*) derived from the *Bacillus* plasmid pBS72, the ampicillin (Amp) and chloramphenicol (Cm) resistance marker active in *E. coli* and *B. subtilis,* repectively, the replication region (*rep*) of pMB1, the *lacI* gene and the P_{grac} promoter followed by a MCS and a strong transcription terminator indicated as bar. Below the plasmid map, the DNA sequences of the MSC of pHT01, of the His-tag of pHT08, of the Strep-tag of pHT09, the MCS preceding the Strep-tag in pHT24 and the c-Myc-tag in pHT10 and the MCS of the expression-secretion vector pHT43.

To allow detection and rapid isolation of recombinant proteins, the coding region for three different tags were inserted into pHT01. We have chosen the 10-amino-acid epitope-tag c-Myc [39], and the 8-amino-acid His [64] and Strep [73] purification tags. First, the coding regions for the His- and Strep-tag including the start codon were inserted into the *Bam*HI site of pHT01 downstream of the SD sequence using the two pairs of complementary oligonucleotides ON56/ON57 and ON58/ON59, respectively, resulting in the novel plasmids pHT08 and pHT09. The coding regions for c-Myc and Strep including a stop codon allowing

their fusion to the 3' end of any gene of interest were inserted into pHT01 at the *Aat*II site using two pairs of complementary oligonucleotides each (ON60/ON61 for c-Myc and ON62/ON63 for Strep) resulting in pHT10 and pHT24, respectively (Fig. 2.3). Next the gene *yhcS* was amplified using ON64/ON65, with *B. subtilis* 1012 chromosomal DNA as template and the core region of *srtA* (termed as *srtA*) of *L. monocytogenes* [125] using ON65/ON67 and pNDH09 [106] as template; the amplicons were cleaved by appropriate enzymes and then inserted into pHT08 yielding pHT08-*yhcS* and pHT08-*srtA*. The gene *ywbN* and *gfp*+ were amplified using ON68/ON69 with *B. subtilis* 1012 chromosomal DNA and ON70/ON71 with pMUTIN-*gfp*+ [75] as template and inserted into pHT10 and pHT24 resulting pHT10-*ywbN*, pHT10-*gfp* and pHT24-*gfp*. The gene *gfp*+ was also amplified using ON70/ON72 and then inserted into pHT09 resulting pHT09-*gfp*.

2.7.4 Construction of the promoter-probe vector pHT06

The promoter-probe plasmid, pHT06, basal on *bgaB*, was constructed for the identification and screening of promoters was constructed as follows. The BgaB-encoding region was amplified by PCR using plasmid pX-*bgaB* [79] as template with primers ON77 and ON78. The amplified fragment was cloned into the pHT01 [105] at *Kpn*I/*Aat*II site resulting pHT02. The *lacO* sequence with restriction sites for *Spe*I and *Sac*II was inserted at the *Bam*HI restriction site using the two complementary oligonucleotides ON79 and ON80 resulting in pHT06 (Fig. 2.4). *E. coli* or *B. subtilis* carrying the promoter-probe plasmid pHT06 will give white colonies on IPTG-X-gal LB plates.

2.7.5 Construction of plasmids to identify elements of strong promoters

Construction of plasmids pHT59 to pHT100 (Tab. 2.2): To generate a library of promoters, core promoters and some full-length promoters were introduced into plasmid pHT06 between *Spe*I and *Sac*II using two complementary oligonucleotides (see on Tab. 2.3). Some upstream regions of core promoters were introduced into plasmids pHT70 or pHT80 between *Kpn*I and *Spe*I; the number of plasmids corresponds to the number of the oligos used (Tab. 2.2 and 2.3). As examples, promoter P88 was assembled from the two complementary oligonucleotides P88F and P88R, ligated into plasmid pHT06 at *Spe*I and *Sac*II resulting in plasmid pHT88. Transformants were first screened by blue/white colonies and then verified by colony PCR. Other synthetic promoters were introduced into this plasmid at the MCS by similar approaches.

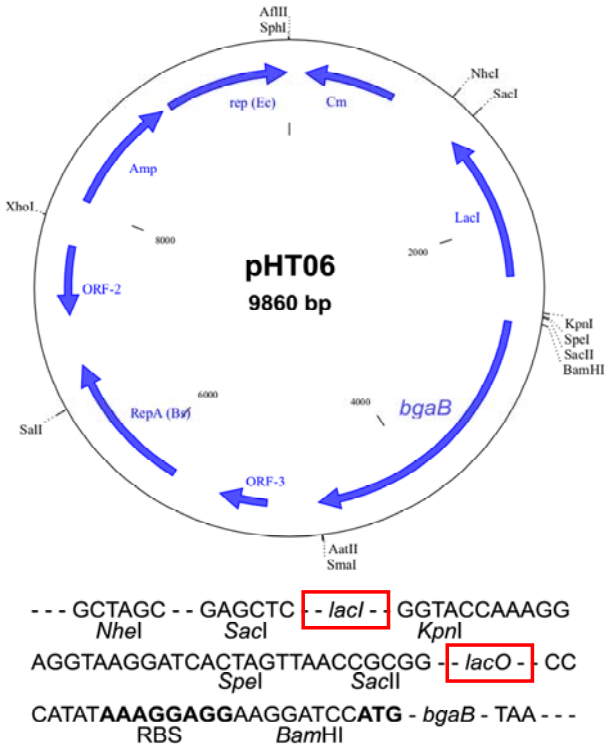


Fig. 2.4. Genetic and restriction map of the promoter-probe plasmid pHT06.

2.7.6 Construction of plasmids allowing the analysis of 3' stabilizing elements

To investigate the influence of 3'-stablizer elements on the expression of the *bgaB* reporter gene, plasmid pHT36 was constructed by removing the *trpA* transcriptional terminator from pHT01-*bgaB*. The coding regions for ORF-3 and ORF-1 were amplified by PCR using pMTLBs72 as template and the primer pair S36F and S36R. The *Aat*II/*Cla*I-treated PCR fragment was cloned into the 7917 bp *Aat*II/*Bsp*119I-treated fragment of pHT01-*bgaB* resulting in pHT36 (Fig. 2.5).

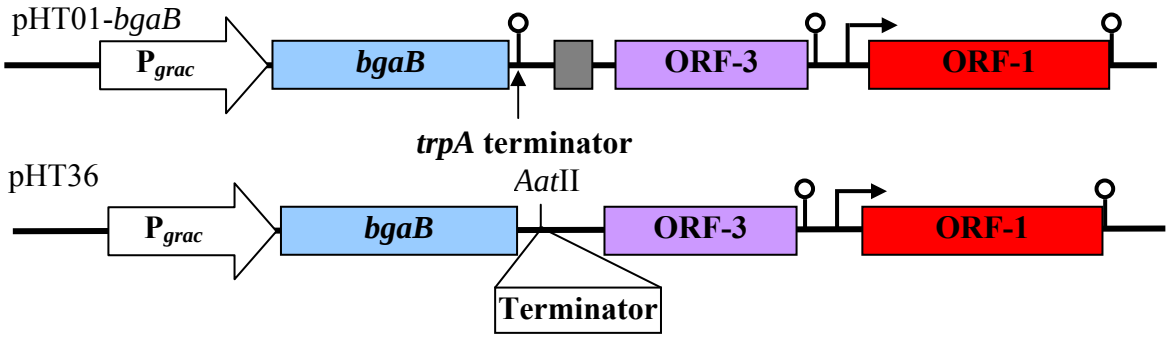


Fig. 2.5. Genetic features of the expression vector pHT01-*bgaB* and plasmid pHT36 to investigate 3' stabilizer elements.

2.7.7 Construction of plasmids to study mRNA stabilizing elements

Construction of a plasmid with 3' stabilizing elements: Two complementary oligonucleotides were hybridized to generate a terminal stabilizing element and these terminators were introduced into plasmid pHT36 at *Aat*II resulting in plasmids pHT110 to pHT124 with numbers corresponding to the numbers of their primers (Tab. 2.2 and 2.3). As an example, the element S124 was generated by hybridization of the two complementary oligonucleotides S124F and S124R; this hybridized product was ligated into pHT36 at *Aat*II. The correct transformants were screened by using colony PCR, and candidate plasmids were further analysed by restriction enzymes and DNA sequencing.

Construction of other plasmids with names numbered larger than pHT100 (Tab. 2.2): Other promoters with stabilizing elements numbered larger than 100 (Tab. 2.3) were generated by PCR and then introduced into pHT06 at the MCS region. As an example, P212 promoter with S212 was generated by two PCR steps. First, the promoter *groE* with *lacO* fragment, called P_{groE}_211* was amplified using the two primers ON76F and S211R1 and the promoter P_{grac} in pNDH33 as template. The PCR products were then purified by the gel extraction kit (Qiagen). Second, the promoter with S212 was amplified using two oligos ON76F and S212R2. The PCR fragments were digested with *Kpn*I and *Bam*HI and then ligated into pHT06 treated with the same enzymes, resulting in pHT212. The right transformants were also screened by blue/white colonies and using colony PCR, candidate plasmids were further analysed by restriction analysis and sequencing (see Fig. 2.6 for more detail).

2.7.8 Construction of the knockout strains to study glycine expression system

To delete the *gcv* structural genes (*gcvT, gcvPA, gcvPB*) from the *B. subtilis* chromosome (Fig. 2.7 A), about 300-bp-regions up- and downstream of the operon were amplified using ON5/ON6 and ON7/ON8 and chromosomal DNA of strain 1012 as template, and the two amplicons were used to sandwich a neomycin resistance marker as shown in Fig. 2.7, and all fragments were cloned into pBluescript 2KT (+) resulting in plasmid pT20 (Fig. 2.7 B). The recombinant plasmid was used to transform strain 1012, neomycin-resistant colonies were selected and several candidates were checked for correct replacement of the *gcv* operon by PCR and by the presence of the neomycin resistant marker. One positive strain, PT05, was kept for further studies. The *gcv::neo* marker was transferred into AM01 resulting in strain PT02 to facilitate subsequent isolation of integrants after transformation with the pDG1728 derivatives.

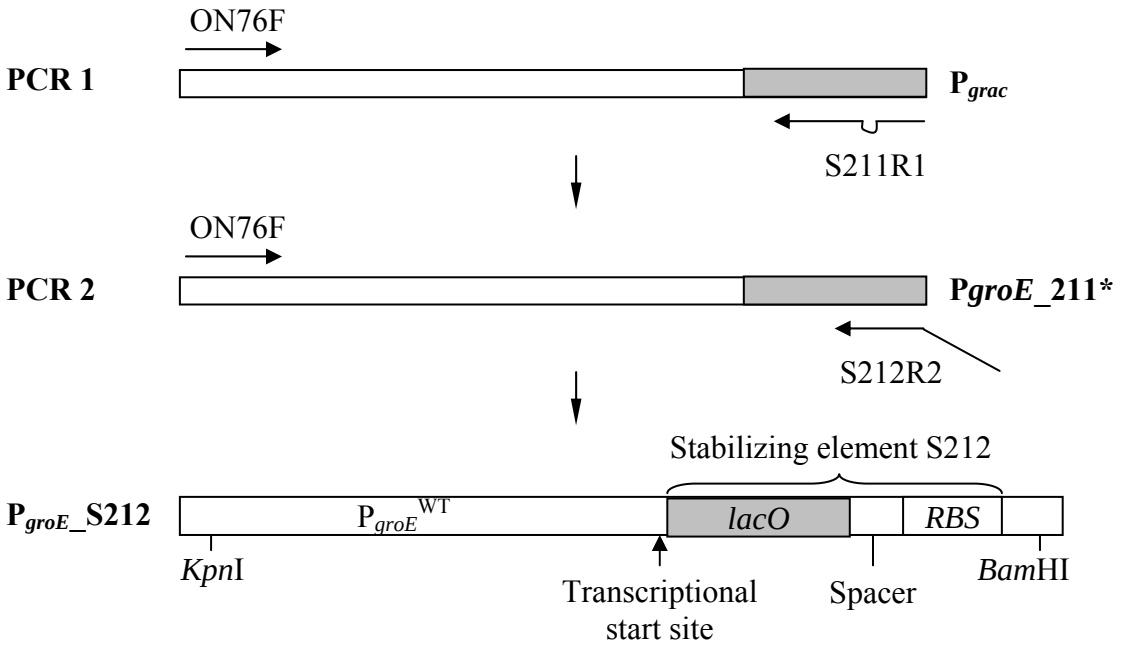


Fig. 2.6. Schematic representation of the construction of the promoter P*groE*_S212, P212. Positions of transcriptional start site, stabilizing element, spacer and ribosome binding site are indicated; grey box represent the *lacO* operator.

To completely delete the *gcv* operon (including the riboswitch and the structure genes) from *B. subtilis* chromosome, an about 300-bp region upstream of the *gcv* operon (on *yqhH*) (Fig. 2.7 A) was amplified using ON23/ON24 and chromosomal DNA of strain 1012 as template, and the amplicon was inserted into pT20 between the *Sac*I and *Bam*HI restriction sites resulting in plasmid pT37 (Fig. 2.7 C). The neomycin resistance marker was amplified using ON23/ON8 and plasmid pT37 as template, and all fragments were used to transform strain AM01. Neomycin-resistant colonies were selected and checked for correct replacement of the whole *gcv* operon by PCR and by the presence of the neomycin resistant marker. One positive strain, PT40, was kept for further studies.

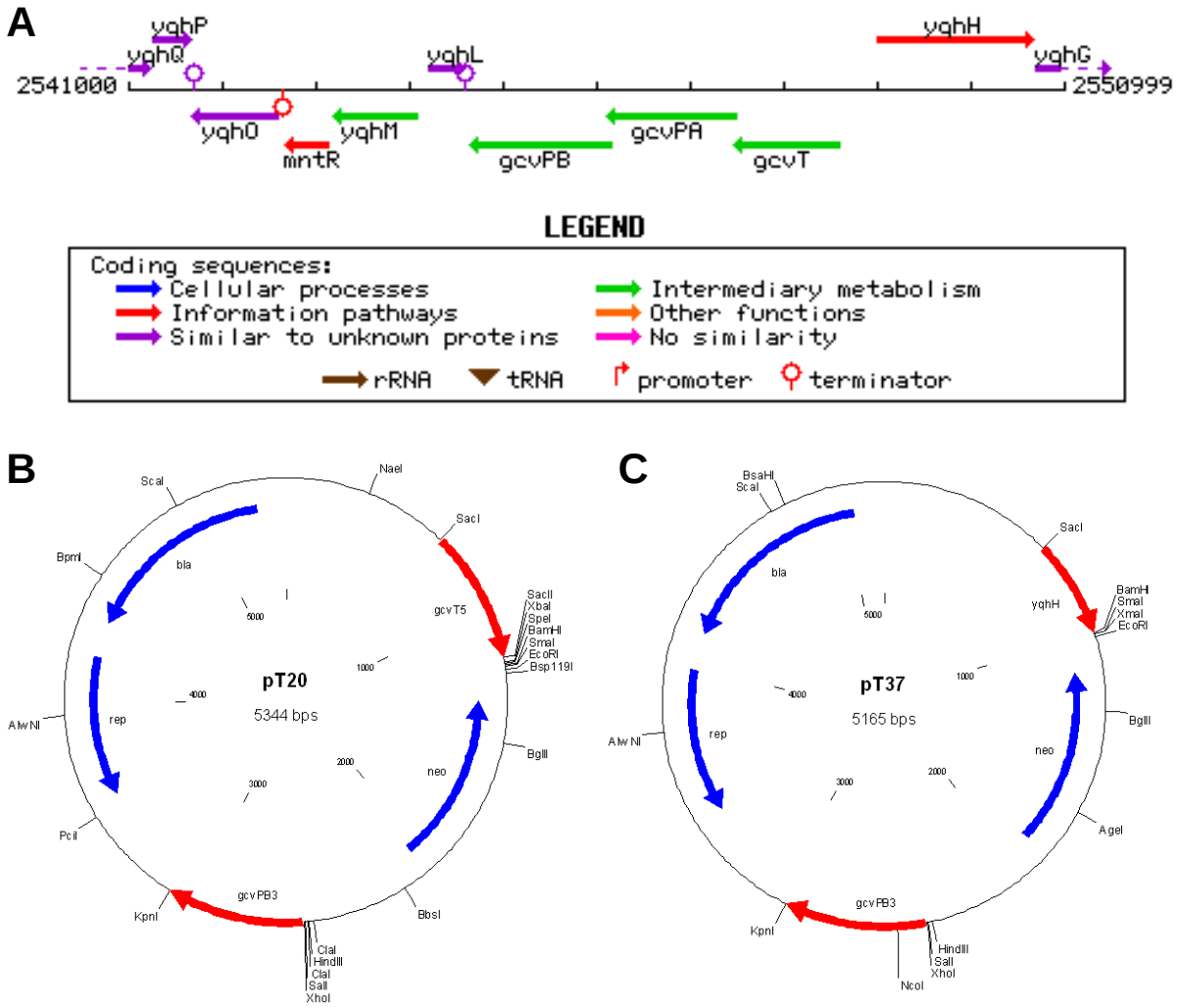


Fig. 2.7 Genetic and restriction map of the *gvc* operon and of the plasmids pT20 and pT37 which have been constructed for the deletion of the *gcv* operon. (A) The coding regions of the *gcv* operon on *B. subtilis* chromosome, *gcvPB* (glycine decarboxylase subunit 2), *gcvPA* (glycine decarboxylase subunit 1) and *gcvT* (aminomethyltransferase) are shown; (B) plasmid pT20 used to delete the *gcv* structural genes (*gcvT, gcvPA, gcvPB*) from the *B. subtilis* chromosome; (C) plasmid pT37 used to delete the whole *gcv* operon including *gcv*-riboswitch and the *gcv* structural genes from the *B. subtilis* chromosome.

2.7.9 Construction of *lysC* knockout strains to study lysine expression system

The complete *lysC* gene including its leader region was deleted from the chromosome as follows. The *lysC* gene is flanked by the genes *yslB* and *uvrC* (Fig. 2.8 A); about 300 bp from both genes were amplified separately using the oligonucleotide pair ON29/ON30 for *uvrC* and ON31/ON32 for *yslB* and chromosomal DNA of *B. subtilis* strain 1012 as template. Both

amplicons were treated with the enzymes *Sac*I and *Bam*HI for *uvrC* and *Kpn*I and *Xho*I for *yslB*, and the DNA fragments were cloned into plasmid pT20 flanking the *neo* gene resulting in pT39. In the last step during the construction of the *lysC* knockout, the flanking regions of *lysC* including the *neo* marker were amplified using ON29 and ON32 and plasmid pT39 as template. The linear DNA fragment was transformed into strain AM01, and transformants were selected on LB plates containing neomycin. Chromosomal DNA was isolated out of several neomycin-resistant transformants and analysed for replacement of the *lysC* gene by the *neo* gene by PCR. All of them turned out to correct, and one transformant (strain PT41) was kept for further studies (Fig. 2.8 B).

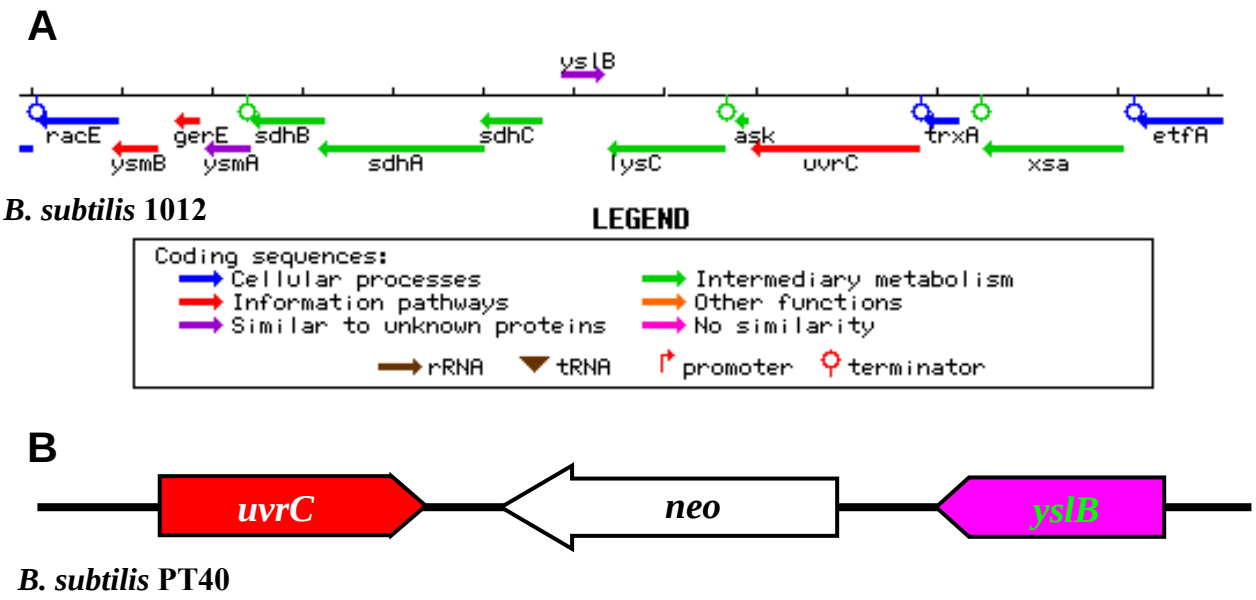


Fig. 2.8. Display of the chromosomal regions of the knockout strains PT41. (A) Chromosomal region of the *lysC* gene in *B. subtilis* 1012; (B) construction of strain PT41.

3 Results

This work was focused on the construction of novel controllable expression systems and to identify features important for overproduction of recombinant proteins in *B. subtilis*. First, the L-glycine and L-lysine amino acid-responsive riboswitches that regulate gene expression are used to construct glycine-inducible and lysine-autoinducible expression vectors; and IPTG-inducible expression plasmids that allow expression and purification of proteins were also constructed. Second, a protocol with a useful promoter-probe plasmid to analyze strong promoters in *B. subtilis* was established. Using this new technique, promoter elements and mRNA stabilizing elements were studied to enhance the transcriptional level and mRNA stability leading to higher protein production levels.

3.1 Exploring glycine controllable expression systems

This part describes the development of a glycine-inducible expression system for *B. subtilis*. This is the first report that a naturally occurring riboswitch can be used for controllable overproduction of recombinant proteins using the inexpensive inducer L-glycine. First, the presence of a small transcript corresponding to the 5' UTR in the absence of L-glycine which is converted into the full-length transcript after addition of the amino acid was confirmed by Northern blot. Next the *lacZ* reporter gene was fused with the promoter and the riboswitch and glycine-dependent induction was demonstrated. Furthermore, to obtain higher levels of recombinant proteins, the promoter strength was enhanced, and the HtpG, Pbp4* and α-amylase were used as model proteins.

3.1.1 The glycine degrading *gcv* operon is strongly induced after addition of L-glycine

3.1.1.1 Demonstration of the transcription attenuation model of the three genes *gcvT-gcvPA-gcvPB in-vivo*

The model of transcription attenuation suggested by Mandal and coworkers [93] predicts the synthesis of a short transcript in the presence of low L-glycine concentrations, while the full-length mRNA should appear after raising the concentration to 10 mM. To prove this prediction, *B. subtilis* 1012 cells were grown in minimal medium to the mid-logarithmic growth phase in the complete absence of L-glycine at 37^{o}C. Then, L-glycine was added to a final concentration of 10 mM and growth was continued. Aliquots were withdrawn immediately before adding L-glycine and at different time points after addition of this amino acid. Total RNA was prepared and subjected to Northern blotting, which was probed with two different antisense RNAs, one complementary to the riboswitch and the second to the *gcvT*

gene, the first gene of the tricistronic operon. When antisense RNA complementary to the riboswitch was used to probe the Northern blot, a short transcript with a length of about 200 nucleotides was predominating, while three additional very faint larger bands were present, too (Fig. 3.1A). Already 5 min after increasing the L-glycine concentration, the full-length transcript could be detected (about 4 kb) including two additional smaller bands (Fig. 3.1A). These could arise either from premature transcription termination, from internal processing, they could represent degradation products or a mixture of these possibilities. If the second probe, complementary to the *gcvT* gene, was used the short transcript was not detected as to be expected, while the overall pattern was comparable (Fig 3.1B). Most interestingly, the amount of the band representing the attenuation product decreased slowly over time followed by a further increase (Fig. 3.1A). One possibility to explain this surprising result could be that the attenuation product is very stable.

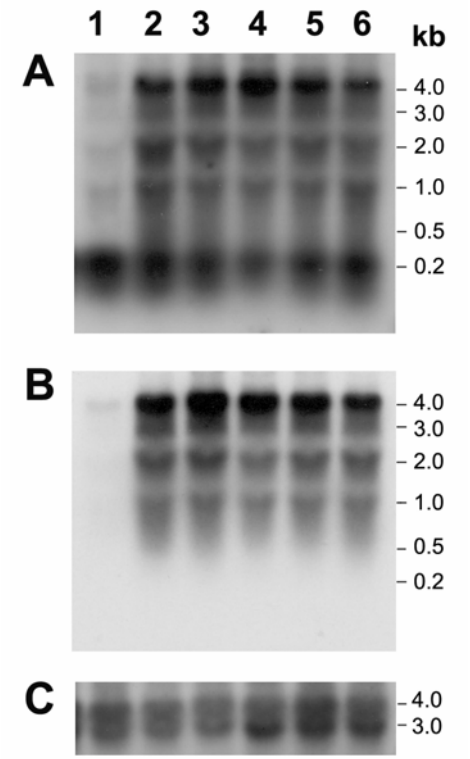


Fig. 3.1. Northern blot analysis of the *gcv* operon. Total RNA was prepared from *B. subtilis* wild-type strain 1012 grown in minimal medium at 37°C before (lane 1) and 5, 10, 30, 60 and 90 min (lanes 2-6) after addition of 10 mM L-glycine. The Northern blot was probed with antisense RNA complementary to the riboswitch (A), the *gcvT* gene (B), and *dnaK* (C) which served as a loading control. 20 micrograms of RNA were applied per lane. Transcript size was determined by comparison with RNA size marker as indicated.

3.1.1.2 To analyse for enhanced stability of the *gcv* riboswitch RNA

To examine this possibility experimentally, cells were grown to the mid-exponentially growth phase, and *de novo* synthesis of RNA was inhibited by the addition of rifampicin. As shown in Fig. 3.2B, the attenuation product was undetectable after 2 min indicating that an extended half-life does not explain the appearance of this band in Fig. 3.1A. Another possibility to explain this unexpected finding could involve processing of the full-length transcript.

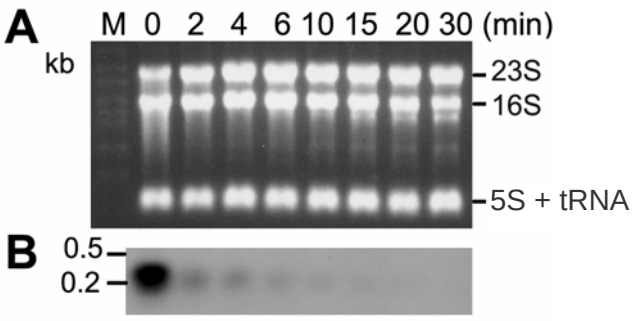


Fig. 3.2. Northern blot analysis for enhanced stability of the *gcv* riboswitch RNA. Cells of *B. subtilis* strain 1012 were grown in minimal medium at 37°C to the mid-exponential phase. Then, rifampicin was added at a final concentration of 100 µg/ml to inhibit further transcription. Total RNA was prepared just before addition of rifampicin (lane 0) and 2, 4, 6, 10, 15, 20 and 30 min (lanes number 2-30) after addition of rifampicin. (A) The total RNAs were separated on an agarose gel and stained with Et.Br, and the ribosomal RNAs served as a loading control; (B) the Northern blot was probed with antisense RNA complementary to the riboswitch.

3.1.1.3 To analyse for processing of the full-length transcript

To examine this possibility, the rifampicin experiment was repeated in a slightly different way. Cells were grown first in absence and then in presence of L-glycine, and rifampicin was added 2.5 min later. Total RNA was prepared 30 min before addition of the amino acid (t = -30), immediately after supplementation with L-glycine (t = 0) and at different time points after addition of rifampicin as indicated in Fig. 3.3.

The Northern blot was probed with anti-riboswitch (Fig. 3.3A) and anti-*gcvT* RNA (Fig. 3.3B), and the X-ray film was overexposed to detect low abundance transcripts, too. While the full-length transcript and its putative degradation products could be detected already in the absence of L-glycine as described above, there was a dramatic increase upon addition of L-glycine, and both the full-length and the riboswitch RNA decayed rapidly after inhibition of transcription (Fig. 3.3A). When the Northern blot was probed with anti-*gcvT*, the result was different. Here, the full-length RNA was more stable (Fig. 3.3B). While no full-length RNA could be detected 20 min after addition of rifampicin when the blot was probed with anti-riboswitch RNA, this transcript was still present, though in reduced amounts, using anti-*gcvT* RNA (compare Fig. 3.3A and 3.3B). The only interpretation of this result is that indeed processing of the full-length transcript occurs to remove the riboswitch RNA. It follows that only the high molecular weight transcript in the Fig. 3.3A represents full-length RNA, while the corresponding transcript in the Fig. 3.3B corresponds to a processing product. The

difference in molecular weight between these two RNA species cannot be resolved under the experimental conditions used here. In summary, the continued synthesis of the riboswitch RNA neither results from an enhanced half-life nor from processing of the full-length transcript.

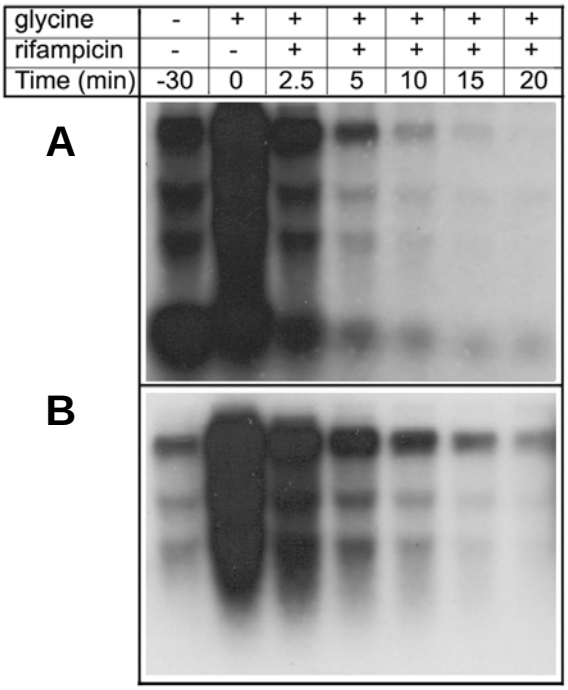


Fig. 3.3. The *gcv* encoded attenuation product is rather unstable and its continued synthesis during the induction phase does not result from processing. Cells of *B. subtilis* strain 1012 were grown in minimal medium at 37°C to the mid-exponential phase, cells were induced with glycine and then rifampicin was added at a final concentration of 100 µg/ml to inhibit further transcription. Total RNA was prepared 30 min before addition of L-glycine (t = -30), immediately after addition of the inducer (t = 0) and rifampicin (t = 2.5) at the time points indicated. The Northern blot was probed with antisense complementary to the riboswitch (anti-riboswitch) (A) and with anti-*gcvT* RNA (B).

3.1.1.4 Detection of the DNA sequence coding for the transcriptional terminator results in the disappearance of the riboswitch RNA

Base on published data [93], it can be expected that removal of the transcriptional terminator located at the 3' end of the riboswitch will result in constitutive expression of *gcv* operon independent of the absence or presence of L-glycine in the growth medium. But will the riboswitch RNA be synthesized in such a deletion mutant? To answer this question, the coding region of the terminator was deleted as described in the Methods section 2.7.1, and the deletion derivative was fused to the *lacZ* reporter gene and integrated at the *amyE* locus in strain PT40 with a complete deletion of the *gcv* operon (strain PT49). As a control, the wild-type promoter-riboswitch region was fused to *lacZ* and integrated ectopically in the same strain (PT47).

Next, both strains were incubated in minimal medium. When the cultured cells reached the mid-log growth phase, L-glycine was added as described. Aliquots were withdrawn just before adding of the L-glycine (t = 0) and at different points after induction and probed with anti-riboswitch RNA. When the operon fusion carrying the wild-type riboswitch was analysed, only the leader transcript was present when the cells grew in the absence of L-glycine. Addition of L-glycine resulted in the appearance of additional bands as described above (Fig. 3.4A). Next, expression was analysed with a truncated the transcriptional terminator. As to be expected expression was constitutive and the riboswitch RNA was not produced (Fig. 3.4B). These results clearly indicate that the terminator plays an important role in transcription termination and release of the riboswitch RNA, and the *gcv*-riboswitch is also synthesised if fused to a foreign gene. This observation opens the possibility to use the *gcv*-riboswitch for controlled expression of recombinant gene.

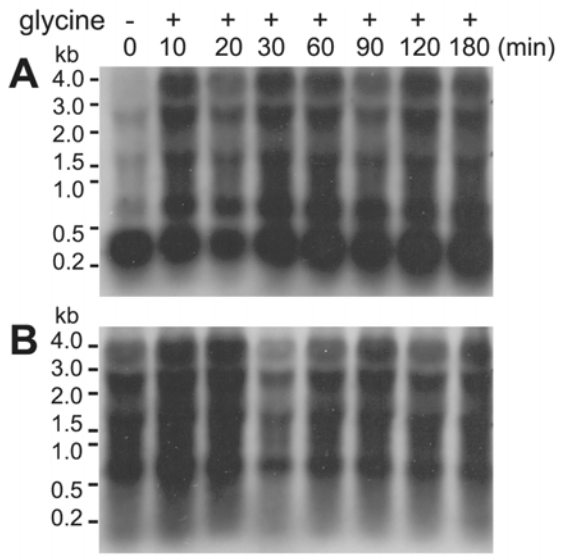


Fig. 3.4. In the absence of the transcriptional terminator the riboswitch RNA is absent. (A) Strain PT47 carrying the transcription terminator; (B) strain PT49 devoid of part of the terminator. The two strains were first grown in minimal medium at 37°C to the mid-exponential phase (t = 0), cells were induced with 10 mM L-glycine and then total RNA was prepared at the time points indicated and probed with anti-riboswitch RNA.

3.1.2 Expression of the reporter gene *lacZ* fused to glycine riboswitch

3.1.2.1 To determine the optimal L-glycine concentration resulting in highest induction factor

Based on these results, the promoter activity was measured in the absence and presence of L-glycine. The *lacZ* gene was fused to the riboswitch including the σ^A-dependent promoter, and the transcriptional fusion was integrated into the *B. subtilis* chromosome at the *amyE* locus using strain PT02 as recipient which carries a deletion of the *gcv* operon resulting in PT46 as described in the Methods section 2.7.1.

First, the different L-glycine concentrations (from 0.1 to 100 mM) were tested to determine the optimal concentration resulting in the highest induction factor. Cells were grown in minimal medium to the mid-exponential growth phase. Then, the culture was split into six subcultures, where one was incubated without further treatment, while the others were induced with different L-glycine concentrations as indicated in Fig. 3.5. Aliquots were withdrawn from all cultures for determination of the enzymatic activity. 50 mM L-glycine resulted in the highest β-galactosidase activities, which increased dramatically within the first hour after addition of L-glycine to about 48 units followed by a further increase to approximately 65 units 6 h after induction. Since the difference in the enzymatic activity at later times between 10 and 50 mM was only minor (Fig. 3.5), the 10 mM of L-glycine was used in all subsequent experiments.

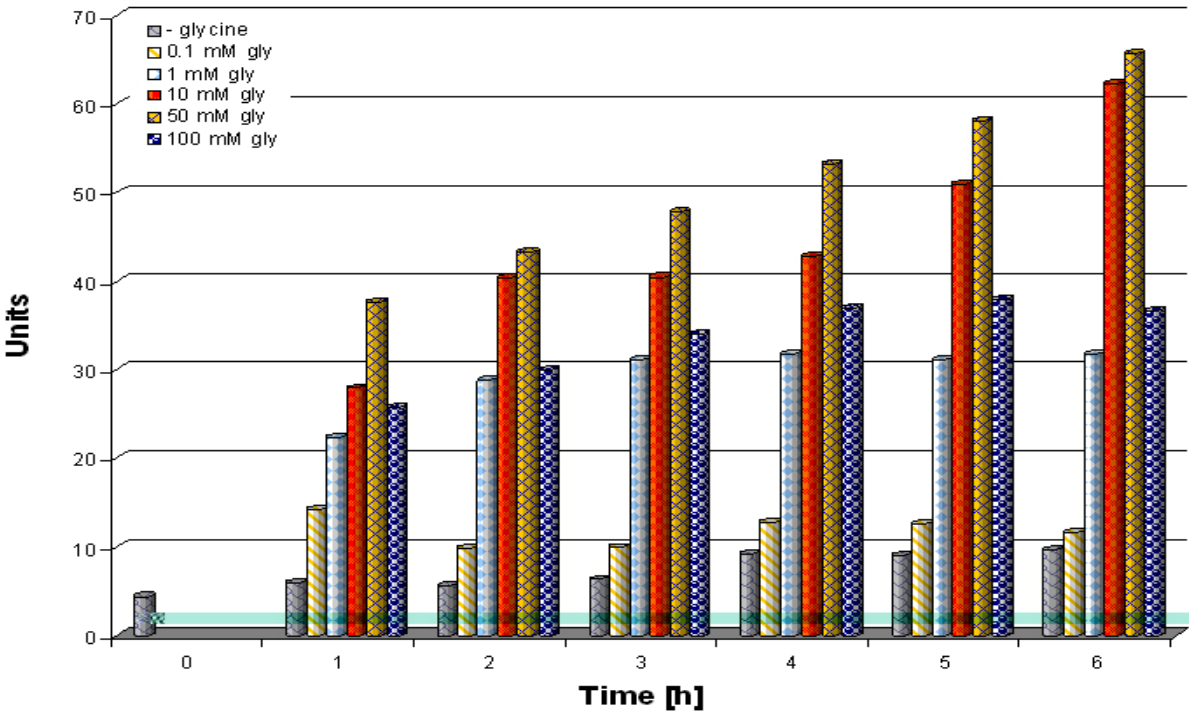


Fig. 3.5. Full induction of the glycine-dependent riboswitch RNA occurs after addition of a L-glycine concentration of 0.1-100 mM. Cells of strain PT46 were grown in minimal medium in the absence of L-glycine at 37°C to the mid-exponential growth phase. Then, the culture was split into six subcultures, where one was further grown in the absence and the others in the presence of L-glycine at the concentrations indicated. Aliquots were removed at the time points indicated for determination of the β-galactosidase activities.

3.1.2.2 The effect of culture medium on β-galactosidase activity

To follow induction more closely and to compare the increase in β-galactosidase activity in minimal and LB medium, the experiment was repeated. The β-galactosidase activity started to increase slightly 10 min after addition of L-glycine and reached the maximum level after about 2 h in LB medium (Fig. 3.6A). Since the enzymatic activity of the culture grown in the absence of L-glycine also started to increase after about 60 min, an induction factor of 2.3 can be calculated after 240 min (Fig. 3.6A). When the same experiment was carried out in minimal medium the induction levels were higher but reached about the same values after 2 to 4 h (Fig. 3.6B). In contrast to the culture grown in LB medium, the β-galactosidase activity did not increase over time resulting in an induction factor of 10.

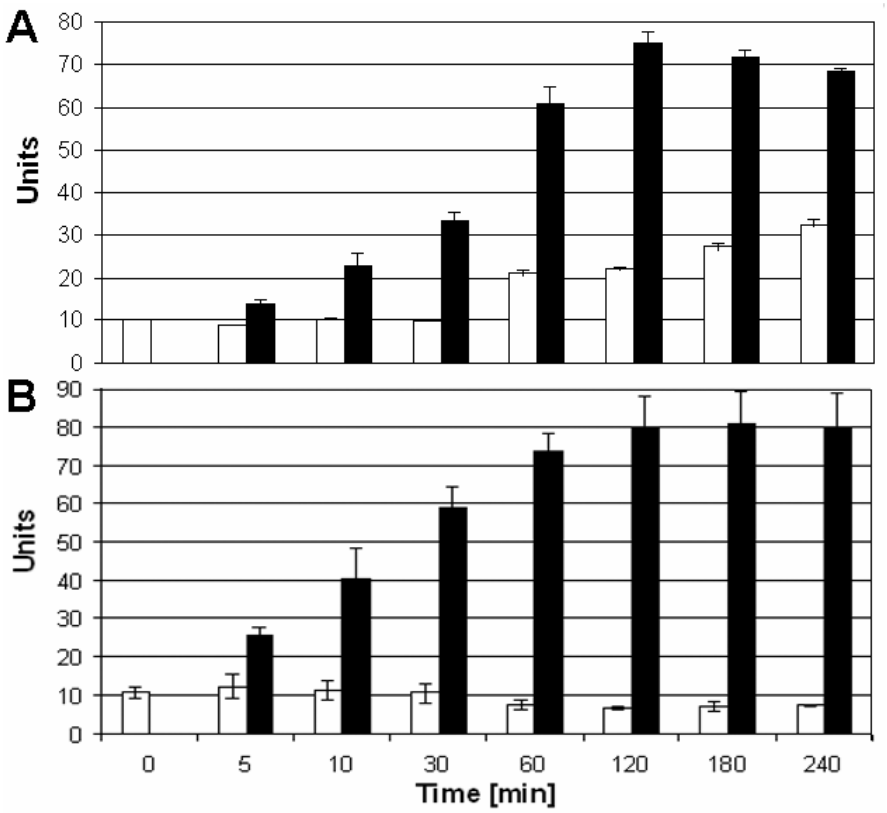


Fig. 3.6. The riboswitch of the *gcv* operon confers glycine-inducibility to the *lacZ* reporter gene. The transcriptional fusion present in plasmid pT12 was integrated at the *amyE* locus in strain PT02 which carries a deletion of the *gcv* operon. Cells were grown in LB (A) or in minimal medium (B) to the mid-exponential growth phase. Aliquots were withdrawn immediately before (t = 0) and at the time points indicated after addition of 10 mM L-glycine. The enzymatic activities are expressed in relative β-galactosidase units. The reporter enzyme activities were measured from cultures without added L-glycine (open bars) and at the time point indicated after addition of 10 mM L-glycine (closed bars).

These results prove that glycine-inducible expression of *lacZ* can be obtained.

3.1.3 Does the growth temperature influence the expression level?

Next, I asked whether the growth temperature will influence the basal level of expression through the stability of the attenuation terminator. The stability of RNA secondary structure is temperature dependent. This is used to regulate initiation of translation by several genes such as the *rpoH* gene of *E. coli*, the ROSE element of *Bradyrhizobium japonicum* and the *prfA* gene of *L. monocytogenes* [26, 26, 72, 72, 102, 102]. To find out whether the growth temperature will influence the basal expression level or the expression level after increasing the L-glycine concentration or both, cells were grown at three different temperatures, and the β-galactosidase activity was measured. Since the cells were grown up to 45°C, *lacZ* could not be used as a reporter gene since its activity is highly unstable at high temperatures [177]. Instead, we fused the *bgaB* gene to the riboswitch coding for the heat-stable β-galactosidase (pT24) [62], and the fusion is expressed from a plasmid assumed to be present in about four copies [156]. Cells were grown at three different temperatures (30, 37 and 45°C) in LB medium till the mid-logarithmic growth phase was reached (t = 0). Then, the cultures were divided while one subculture was further grown without addition of L-glycine while 10 mM L-glycine was added to the second. The basal levels measured after 2 h in all three uninduced control cultures grown at three different temperatures did not vary considerably, and the induced levels remained comparable in the 30 and 37°C cultures, too (Fig. 3.7A and B), but was reduced in the 45°C culture (Fig. 3.7C). These results revealed that growth from 30 to 45°C did not influence the basal level of expression, while the high temperature reduced the induced level.

3.1.4 Analysis of mutant *gcv* promoters

A comparison of the DNA sequence of the σ^A-dependent promoter directing expression of the *gcv* operon with that of the consensus sequence [57, 58] revealed that the -35 region deviates in four and the -10 region in only one position from the consensus sequence (Fig. 3.8). In addition, there is an extended -10 region present. Such variants of the -10 element have been reported to increase the strength of the promoter, and they are often accompanied by a weak -35 element as shown in the present case [57].

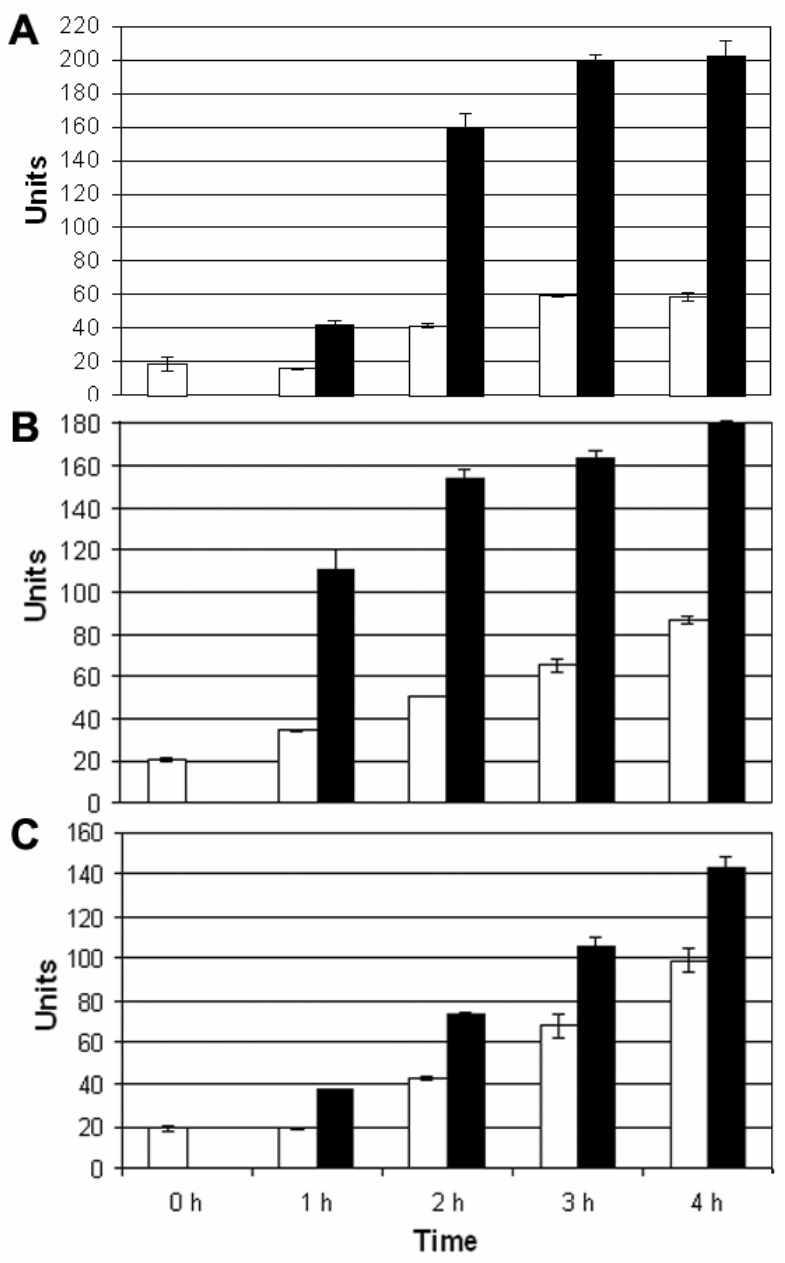


Fig. 3.7. Influence of the growth temperature on the basal and induced level of the reporter enzyme. Cells were grown in LB medium at 30°C (A), 37°C (B) and 45°C (C). Aliquots were withdrawn for β-galactosidase determinations before (0 h) and at different times after addition of 10 mM L-glycine (1 to 4 h) from the uninduced (open bars) and induced (closed bars) cultures.

5'-**aTGtat** TTGATGTAAGATATTGC **TATAgT**-3'	*gcv* promoter (pT12)
5'-**TTGACA** N17 **TATAAT**-3'	consensus sequence
5'-**TTGACA** TTGATGTAAGATATTGC **TATAgT**-3'	-35 consensus (pT22)
5'-**aTGtat** TTGATGTAAGATATTGC **TATAAT**-3'	-10 consensus (pT21)
5'-**TTGACA** TTGATGTAAGATATTGC **TATAAT**-3'	-35 and –10 consensus (pT23)

Fig. 3.8. DNA sequences of the wild-type *gcv* promoter, the consensus sequence of the σ^A housekeeping promoter and of three promoter mutants. TG represents the extended -10 region.

The question was asked whether altering the two elements of the *gcv* promoter will lead to a stronger expression level. To this end, both the -35 and the -10 regions were mutated separately and both elements together to the consensus sequence as shown in Fig. 3.8. All three strains were grown in LB and in minimal medium (only the data for minimal medium are shown) as described in 2.3.3, and L-glycine was added when the cells had reached the mid-log growth phase. Aliquots were taken for determination of β-galactosidase activities as shown in Fig. 3.9. When the promoter with the -10 consensus region was analyzed, the basal levels were comparable with those measured with the wild-type promoter (compare Figs. 3.6 and 3.9A). Addition of 10 mM L-glycine led to reduced expression suggesting that the *gcv* promoter with a -10 consensus sequence is weaker relative to the wild-type promoter. At least, the mutation to the consensus sequence did not improve the promoter strength. When the promoter with the -35 consensus element was analyzed both the basal and the induced levels were significantly increased, and an induction factor of about 10 was measured (Fig. 3.9B). In summary, the alterations in the -35 region improved the promoter strength, but at the expense of the basal level. When both the -35 and the -10 regions were changed to the consensus sequence, the basal level was increased more than 10-fold as compared to the wild-type promoter (compare Figs. 3.6 and 3.9C), and the induced level was slightly decreased as compared to the promoter with the -35 consensus sequence, leading to an induction factor of about 3.5. In conclusion, while the –10 consensus element did not led to a promoter improvement, the –35 consensus increased the promoter strength about 5-fold.

3.1.5 Fusion of the *groE* promoter to the riboswitch

Can the *gcv* promoter be replaced by a foreign promoter without affecting the induction behaviour? The strong *groE* promoter [117] was fused to the riboswitch and to *lacZ* and the operon fusion was integrated at the *amyE* locus resulting in PT17 (see 2.7.1). As already observed for the consensus *gcv* promoter, the basal level was rather high, and the induced level reached values about 10-fold higher after 3 h of induction (Fig. 3.10A). In conclusion, the promoter can be exchanged without impairing the induction behaviour. In another experiment, the *gcv* promoter-riboswitch region was moved to the plasmid pHT01 fusing it to the *bgaB* reporter gene (pT24). Here, the results from Figs. 3.7B and 3.10B were compared; in both cases, cells were grown at 37^{o}C, *bgaB* was used as reporter gene and the fusion is expressed from a plasmid assumed to be present in about four copies [156]. In the first case, cells were grown in LB medium and the second case is minimal medium. The basal level was significantly lower when the reporter gene was expressed in LB medium.

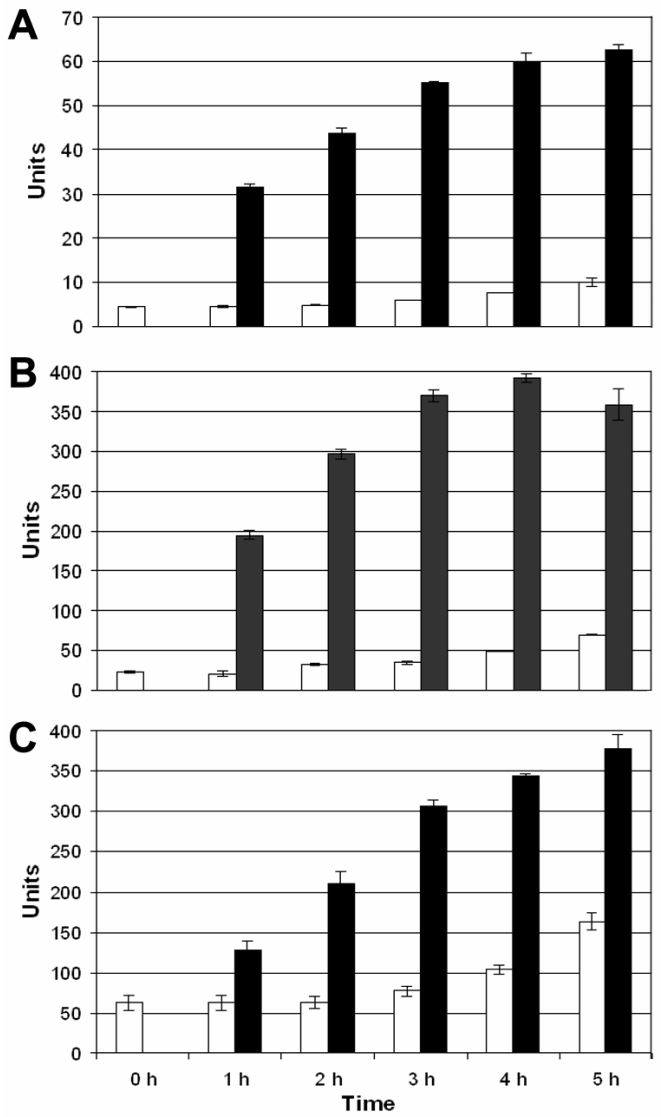


Fig. 3.9. Analyses of mutant *gcv* promoters. The β-galactosidase activities expressed from promoters with the -10 consensus element (A), the -35 consensus element (B) and both consensus elements (C) were analyzed with cells grown in minimal medium. See legend to Fig. 3.6 for further explanations.

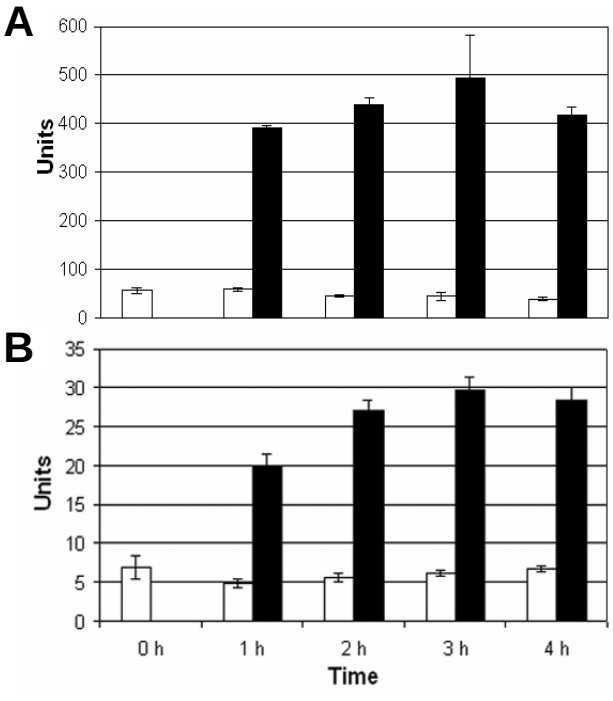


Fig. 3.10. Expression of reporter genes fused to the *groE* promoter integrated in the chromosome and to the *gcv* promoter located on a plasmid. (A) The *groE* promoter was fused to the riboswitch and to *lacZ* and the operon fusion was integrated at the *amyE* locus, see legend to Fig. 3.6 for further explanations; (B) the wild-type *gcv* promoter was fused to the *bgaB* gene present on a plasmid. Cells were grown in minimal medium; see legend to Fig. 3.7B for further explanations.

3.1.6 Synthesis of recombinant proteins from plasmid-based expression systems

In the last series of experiments, the amount of several recombinant proteins were analyzed which can be produced using the glycine riboswitch with the wild-type (pT27) and the consensus promoter (pT28) using the vector plasmids pHT01 and pHT43. As model proteins, we have chosen two intra- and one extracellular protein (see in 2.7.1).

3.1.6.1 Synthesis of intracellular recombinant proteins

The intracellular protein is encoded by *htpG* heat shock gene [143] and *pbpE* coding for a penicillin binding protein [118]. Synthesis of both proteins was analyzed in LB and in minimal medium, before and 2 and 4 h after glycine-induction by separation of the whole proteins on an SDS-PAGE followed by Coomassie staining (Figs. 3.11 and 3.12).

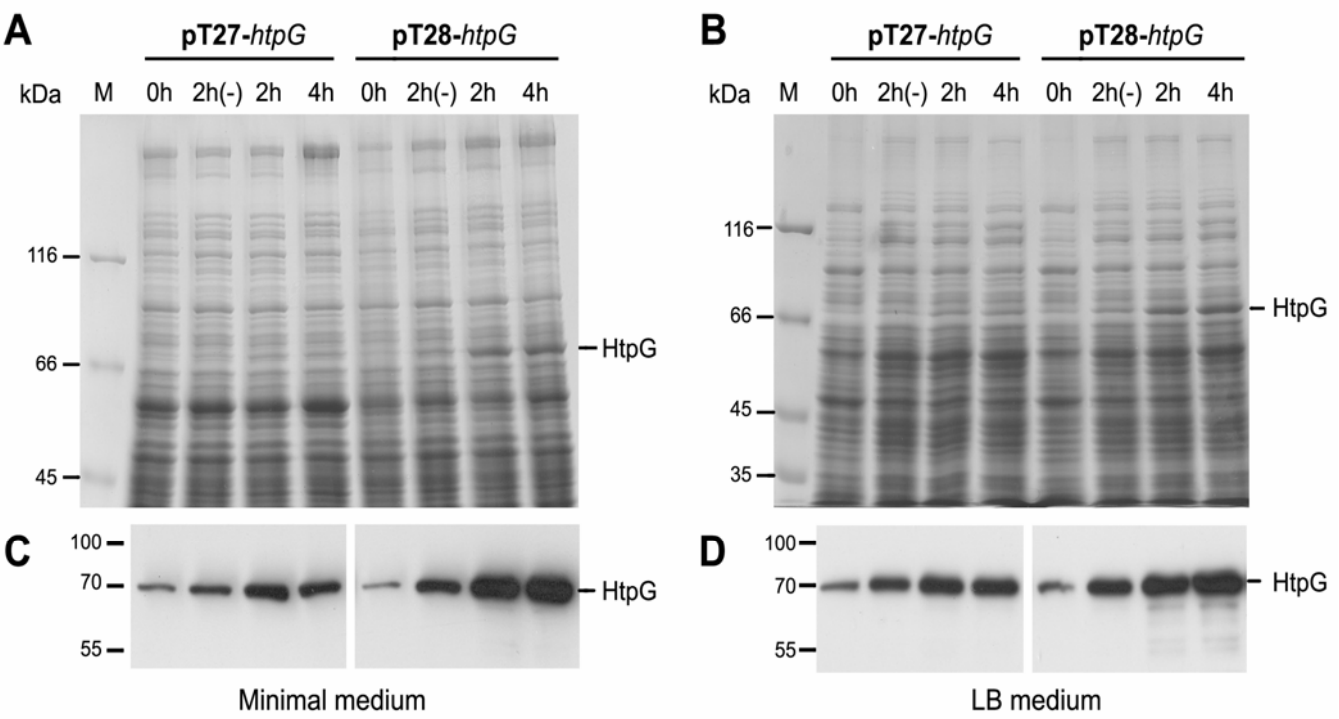


Fig. 3.11. Identification of the *htpG* gene product. *B. subtilis* strain PT05 carrying either pT27-*htpG* or pT28-*htpG* were grown in either minimal (A) or LB medium (B) at 37°C to mid-log, divided into two subcultures, where one was further grown untreated and the second treated with 10 mM L-glycine. Aliquots were taken before addition of L-glycine (0 h), two hours later from the untreated (2 h (-)) and 2 and 4 h later from the induced cultures (2 h and 4 h). Cells were lysed by lysozyme and 100 µg of protein were loaded per lane on an 10% SDS-PAGE. After gel electrophoresis, the proteins were stained with Coomassie blue. Immunoblot analysis of HtpG present in the cells grown in minimal (C) and LB (D) medium.

While no HtpG-specific and Pbp4*-specific band could be detected when the gene was fused to the wild-type promoter in both minimal and LB medium after addition of L-glycine (Figs. 3.11A, B and 3.12A, B), a band with the molecular weight of HtpG and Pbp4* became visible with the consensus promoter in both media (Figs. 3.11A, B and 3.12A, B). To use a more sensitive method allowing detection of even small amounts of protein, immunoblots were carried out. Here, HtpG and Pbp4* were shown to be present in all samples even before induction, but its amount increased significantly after addition of L-glycine (lanes 2 h and 4 h in Figs. 3.11C, D and 3.12C, D). It has to be mentioned that there is a low level expression of *htpG* and *pbpE* from its chromosomal location. The highest amount of HtpG and Pbp4* could be detected when the gene was transcribed from the consensus promoter and the cells were cultivated in minimal medium. These data are in agreement with measurement of the β- galactosidase activities.

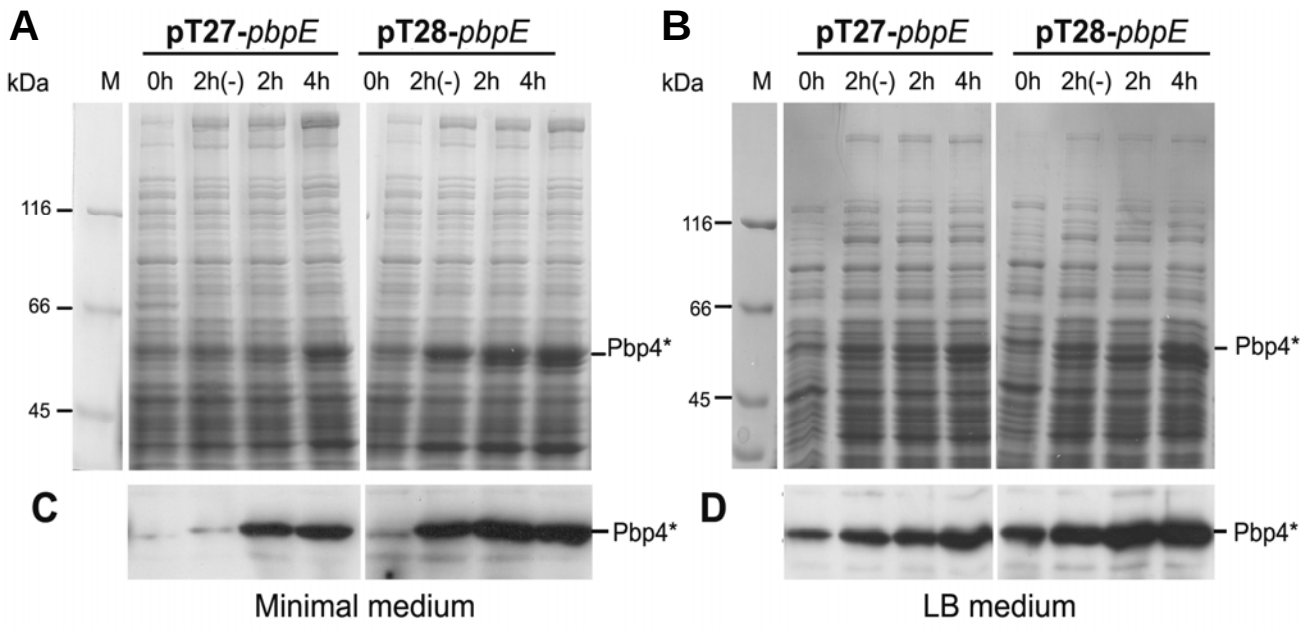


Fig. 3.12. Identification of the *pbpE* gene product. *B. subtilis* strain PT05 carrying either pT27-*pbpE* or pT28-*pbpE* were grown and treated as described in the legend to Fig. 3.11. Coomassie blue staining of Pbp4* present in cells grown in minimal (A) and LB medium (B). Immunoblot analysis of Pbp4* present in the cells grown in minimal (C) and LB (D) medium.

3.1.6.2 Synthesis of extracellular recombinant proteins

Synthesis of extracellular proteins coded for by the *amyQ* gene [112] was analyzed in minimal and in LB medium, before and 2 and 4 h after glycine-induction. Production of

α-amylase was analyzed first by checking for halo formation on minimal and LB medium containing starch. As to be expected small colonies were formed on minimal and larger ones on LB medium (Fig. 3.13). The size of the halo was influenced by the promoter used and by the absence or presence of L-glycine. It turned out that the largest amount of α-amylase was secreted when the *amyQ* gene was fused to the consensus promoter in the presence of 10 mM L-glycine (Fig. 3.13). Next, this result was verified by applying equal amounts of protein collected from the supernatants of the two strains grown in the two media to SDS-PAGE analysis. As can be seen from Fig. 3.14, α-amylase was produced in both media and from both promoters, but the largest amount of enzyme was secreted in LB medium using the consensus promoter (Fig. 3.14B) which could be confirmed by Western blots (Fig. 3.14D).

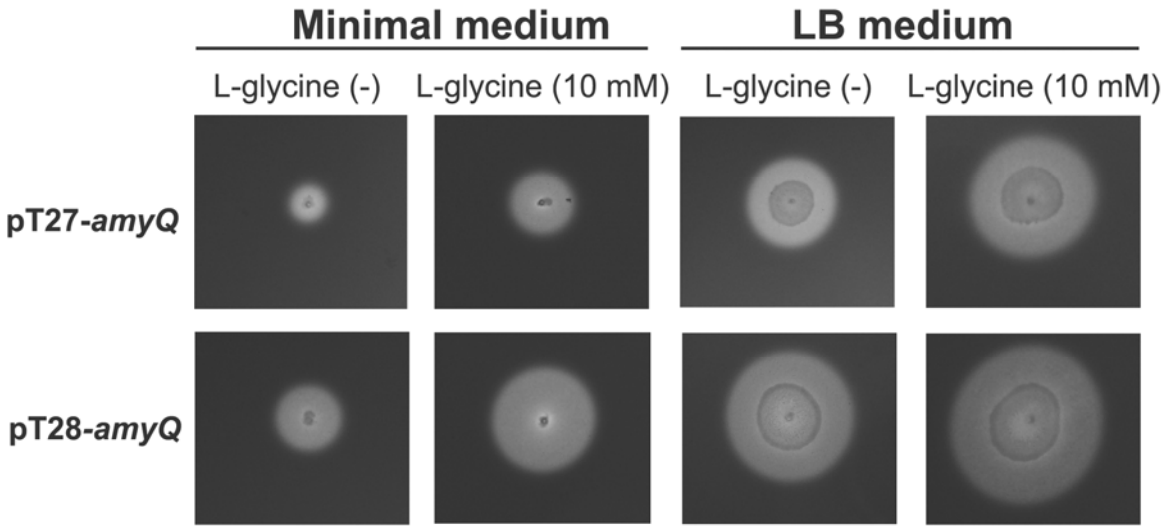


Fig. 3.13. Production of α-amylase by single colonies as verified by halo formation on plates containing starch. Single colonies were analyzed on either LB or minimal medium in the absence or presence of 10 mM L-glycine.

These results clearly demonstrate that the glycine riboswitch can be used for regulatable production of both intra- and extracellular proteins. Four plasmid-based expression vectors have been constructed where two allow intracellular production of recombinant proteins (pT30 and pT31), while the other two direct the proteins into the culture medium (pT32 and pT33) (see in 2.7.1). Both vectors pT30 and pT32 use the wild-type P_{gcv} promoter while the other two pT31 and pT33 use the consensus promoter allowing their induction by addition of L-glycine (Fig. 2.1).

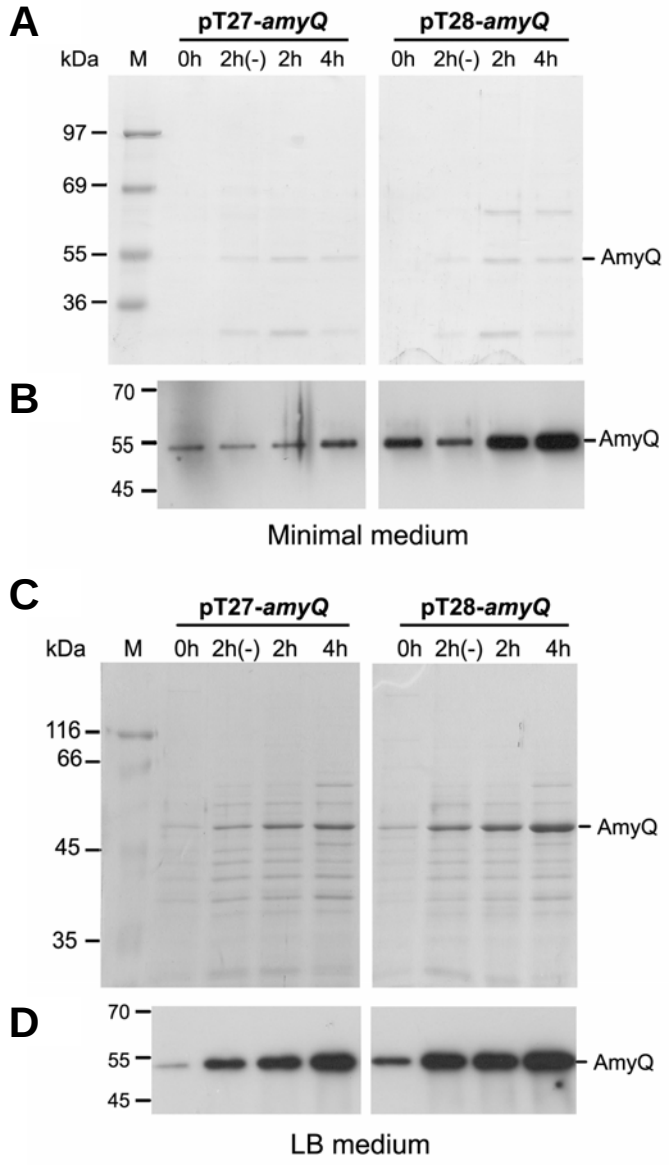


Fig. 3.14. Identification of α-amylase. Cells carrying the plasmids pT27-*amyQ* or pT28-*amyQ* were grown in either minimal (A) or LB medium (B) in the absence (0 h, 2 h (-)) or after addition of 10 mM L-glycine (2 and 4 h). Cells were pelleted by centrifugation and equal amounts of supernatant were analyzed by a 10% SDS-PAGE stained with Coomassie blue. In addition, the α-amylase produced was visualized by immunoblotting in minimal medium (C) and in LB medium (D).

3.2 Exploring lysine controllable expression systems

This part describes the development of a lysine auto-inducible expression system for *B. subtilis*. First, a detailed transcriptional analysis of the lysine-responsive and riboswitch-regulated *lysC* operon of *B. subtilis*, the presence of a small transcript corresponding to the 5' UTR in a high concentration of L-lysine which is converted into the full-length transcript after remove or in the presence of a low concentration of the amino acid was confirmed and carried out by Northern blot. Next, the *lacZ* reporter gene was fused with the promoter and the riboswitch and lysine-dependent induction was demonstrated. Furthermore, it was shown that using system can be used as the auto-inducible expression system based on different L-lysine concentrations.

3.2.1 Transcriptional analysis of the *lysC* gene

As reported above, expression of the *lysC* gene is regulated by a lysine-responsive riboswitch which directly senses the amount of L-lysine present within the cell [51, 153]. To test for the switch in transcription following removal of L-lysine, a Northern blots was carried out using two different antisense RNAs, one which detects the riboswitch and the full-length transcript and the other the full-length transcript only. *B. subtilis* strain 1012 was grown in minimal medium supplemented with 300 µg/ml of L-lysine. When cells reached the mid-exponential growth phase, they were sedimented by centrifugation, washed twice in minimal medium without L-lysine and resuspended in the original volume without L-lysine. Aliquots were withdrawn immediately before centrifugation (t = 0) and up to 180 min later. When the riboswitch probe was used, only an about 0.27 kb transcript was present when the L-lysine concentration was high (Fig. 3.15A). Upon removal of the L-lysine, the full-length 1.6-kb transcript became detectable already after 5 min with no further increase up to 180 min (Fig. 3.15A). When the same RNA preparations were challenged with anti-*lysC* RNA, only the full-length transcript became apparent after removal of the L-lysine (Fig. 3.15B). In conclusion, removal of L-lysine from the growth medium changes the transcription profile from the transcription attenuation product to the full-length product within 5 min. Surprisingly, the 0.27-kb riboswitch persists for up to at least 180 min as the dominant transcript. Two different possibilities were envisaged to explain for the continued presence of the riboswitch RNA, stability or processing.

3.2.1.1 Analysis for enhanced stability of the riboswitch RNA

To analyse for enhanced stability of the riboswitch RNA, cells of strain 1012 were grown in minimal medium to the mid-exponential growth phase and rifampicin was added to inhibit further transcription. Aliquots were removed just before addition of the antibiotic and up to 10 min later. As can be seen from Fig. 3.16B, the 0.27-kb transcript decayed rapidly with a half-life of 2-4 min. Therefore, increased stability of the riboswitch RNA under these growth conditions can be excluded.

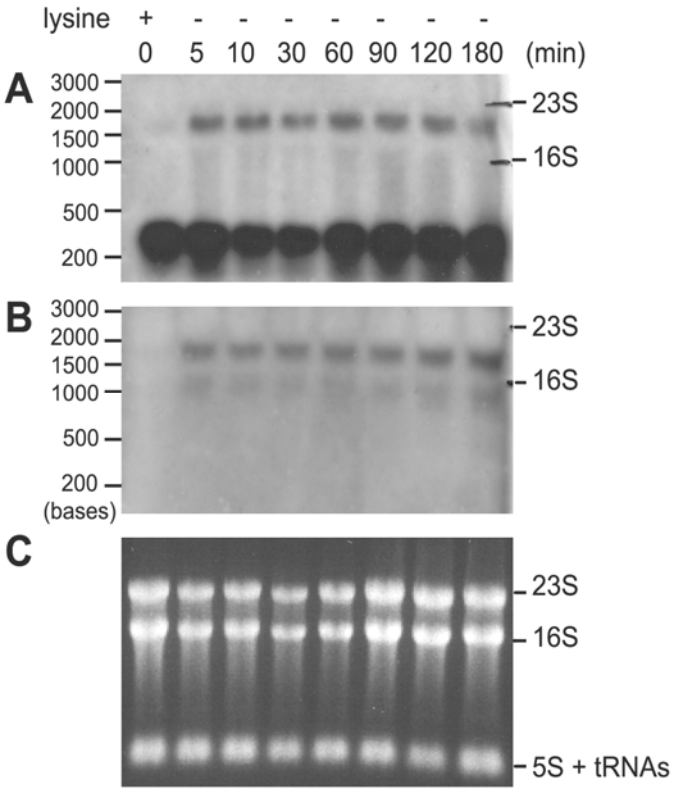


Fig. 3.15. Northern blot analysis of the *lysC* gene of *B. subtilis*. Cells of strain 1012 were grown in minimal medium in the presence of 300 µg/ml L-lysine till the mid-exponential growth phase. Then, the cells were sedimented by centrifugation, washed twice in minimal medium without L-lysine and finally resuspended in the same medium. Aliquots were removed for total RNA preparation just before the centrifugation step (t = 0) and at the time points indicated. The Northern blot was probed with (A) anti-riboswitch RNA; (B) anti-*lysC* RNA. (C) an Et.Br stained gel is shown that comparable amounts of RNA were applied per lane.

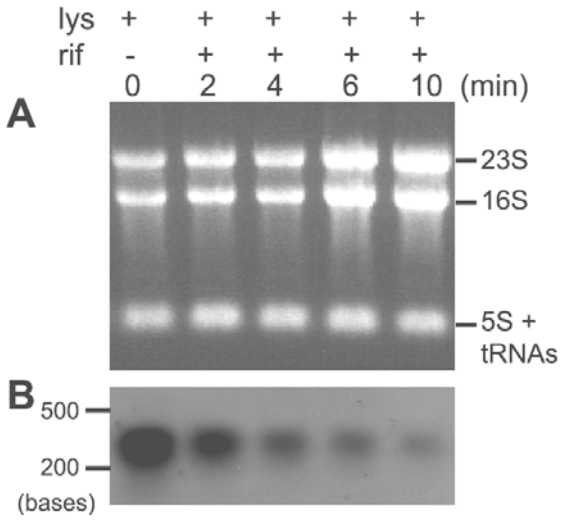


Fig. 3.16. The riboswitch RNA is unstable. Cells of strain 1012 were incubated in minimal medium in the presence of 300 µg/ml L-lysine. Then, rifampicin was added at a final concentration of 100 µg/ml. Aliquots were removed just before addition of the antibiotic (t = 0) and up to 10 min later. (A) Et.Br stained gel; (B) Northern blot probed with anti-riboswitch RNA.

3.2.1.2. Analysis for processing of the full-length transcript

Next, the possibility was investigated whether the riboswitch RNA may be derived from the full-length 1.6-kb transcript by processing. In this experiment, cells were grown again in minimal medium supplemented with L-lysine followed by its removal as described. Rifampicin was added immediately after resuspension of the cells in L-lysine-free medium, and aliquots were taken 30 min before removal of the L-lysine and up to 20 min in the absence of added L-lysine. When the RNA preparations were probed with anti-riboswitch RNA, a dramatic increase in the amount of the leader transcript can be observed which

rapidly decayed over time as described above (Fig. 3.17A). When the same RNAs were probed with anti-*lysC* RNA, a tiny band was present before removal of the L-lysine, followed by a significant increase at t = 0 and a rapid decrease in the presence of rifampicin (Fig. 3.17B). 5 min after addition of the antibiotic, no 1.6-kb transcript is visible. This result clearly argues against processing as a possibility for the presistance of the riboswitch RNA in the absence of L-lysine. There is only one possibility and that is continued synthesis.

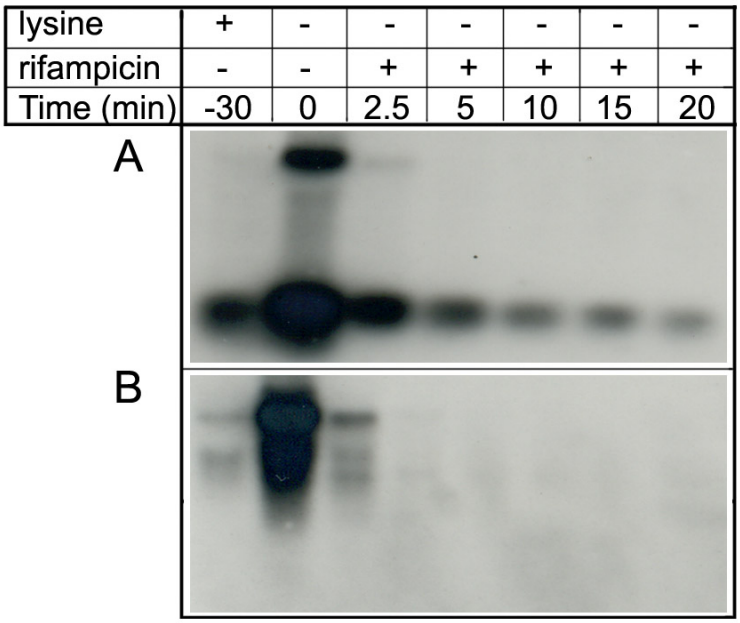


Fig. 3.17. The riboswitch RNA is not produced by processing. Strain 1012 was grown as described in minimal medium with added L-lysine and cells were sedimented, washed as described and resuspended in L-lysine-free minimal medium. Then, rifampicin was added at a final concentration of 100 µg/ml at t = 0. Aliquots were removed 30 min before transfer into the L-lysine-free medium (t = -30) and at the time points indicated and hybridized to the riboswitch riboprobe (A) and *lysC* riboprobe (B).

3.2.2. Deletion of the DNA sequence coding for the transcriptional terminator results in the disappearance of the riboswitch RNA

It has been published that point mutations within the leader region of the *lysC* gene results in constitutive expression [24, 174]. Based on these results it can be expected that removal of the transcriptional terminator will also result in constitutive expression of *lysC* independent of the presence or absence of L-lysine in the growth medium. But what happens to the riboswitch RNA? Will it be synthesized in such a deletion mutant? To answer this question, the coding region of the terminator was deleted as described in the Methods section, and the deletion derivative was fused to the *lacZ* reporter gene and integrated at the *amyE* locus in strain PT41

with a complete deletion of the *lysC* region (strain PT45). As a control, the wild-type promoter-riboswitch region was fused to *lacZ* and integrated ectopically in the same strain (PT43).

Next, both strains were incubated in minimal medium supplemented with L-lysine. When the culture reached the mid-log growth phase, the L-lysine was removed as described. Aliquots were withdrawn just before removal of the L-lysine (t = 0) and at different time points after removal and probed with anti-riboswitch RNA. When the operon fusion carrying the wild-type riboswitch was analysed, only the leader transcript was present when the cells grew in the presence of L-lysine. Removal of L-lysine resulted in the appearance of additional bands, where the smaller ones most probably represent degradation products (Fig. 3.18A). In contrast to strain 1012 where the maximum amount of the full-length transcript became already apparent after 5 min, it took about 60 min with the *lacZ* transcript. In addition, the riboswitch RNA was present all the times as described before. In conclusion, this result demonstrates that the riboswitch is also synthesised if fused to a foreign gene and that such a fusion can change the induction behaviour. This observation opens the possibility to use the lysine riboswitch for controlled expression of recombinant genes (see Discussion). Next, expression was analysed with a truncated transcriptional terminator. As to be expected expression was constitutive and the riboswitch RNA was not produced (Fig. 3.18B). This result clearly indicates that the terminator plays an important role in transcription termination and the release of the riboswitch RNA

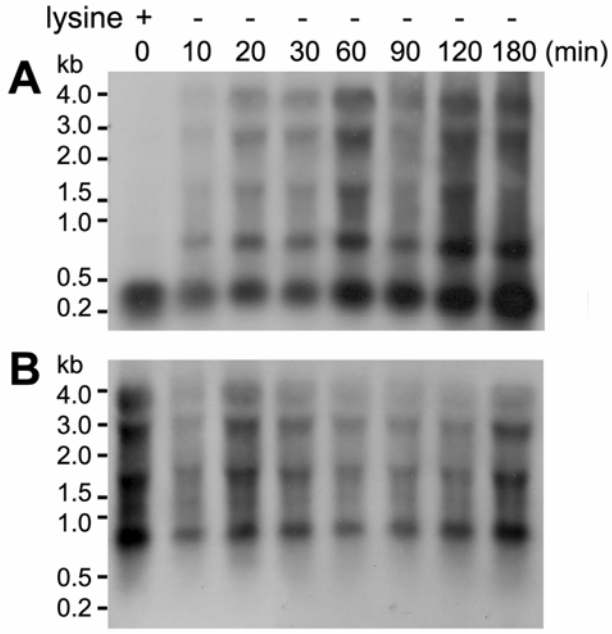


Fig. 3.18. In the absence of the transcriptional terminator the riboswitch RNA is absent. (A) Strain PT43 carrying the transcription terminator and (B) strain PT45 devoid of part of the terminator were first grown in minimal medium supplemented with L-lysine followed by centrifugation, washing and resuspension in L-lysine-free minimal medium at t = 0. Total RNA was prepared at the time points indicated and probed with anti-riboswitch RNA.

3.2.3 Removal of L-lysine from the growth medium leads to an about ten-fold induction of *lacZ*

The results obtained with the Northern blot shown in Fig. 3.18A prompted me to quantify the induction of the *lacZ* reporter gene after removal of the L-lysine. Cells of strain PT42 were grown in minimal medium supplemented with 300 µg/ml L-lysine to mid-log, washed and divided into two subcultures. While one was further grown in the presence of L-lysine, the second was incubated in its absence. Aliquots were removed for determination of β-galactosidase activities just before centrifugation (t = 0) and 2, 3 and 4 h later. While about 4 units of β-galactosidase activity were measured before centrifugation which increased to about 30 units upon further growth in the presence of L-lysine, 130 units were found after 1 h of growth in the absence of the amino acid which increased to approximately 270 units upon further growth (Fig. 3.19A). In summary, removal of L-lysine from the growth medium resulted in an about 10-fold induction of the *lacZ* gene.

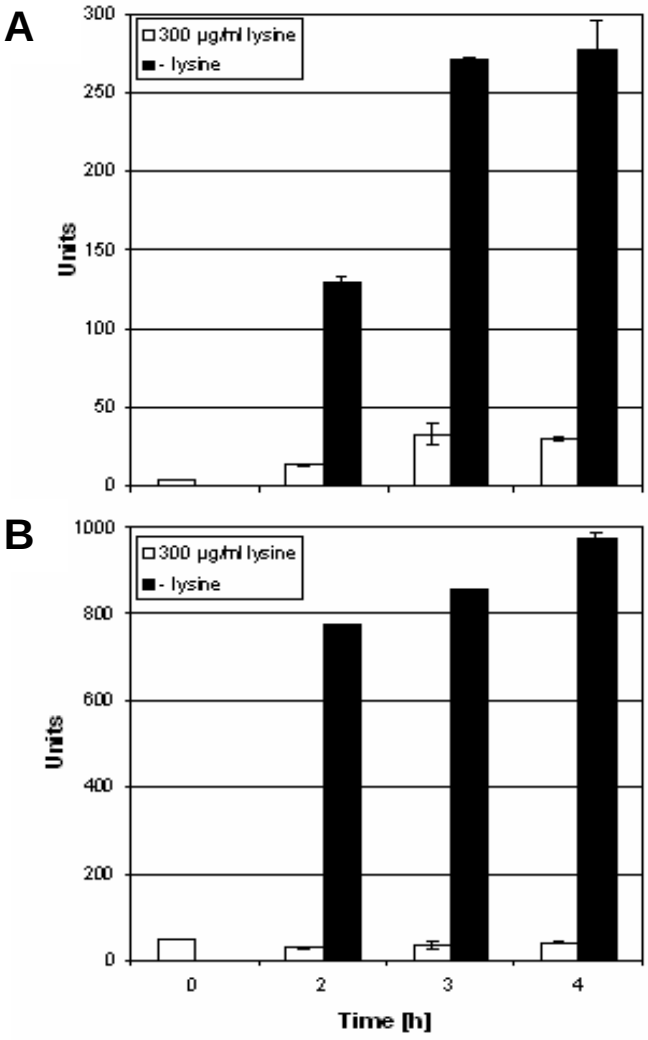


Fig. 3.19. Analysis of the promoter-riboswitch region fused to *lacZ*. Strain PT42 with the wild-type *lysC* region (A) and strain PT43 carrying a deletion of *lysC* (B), and both containing the promoter-riboswitch region of *lysC* fused to *lacZ* were grown in minimal medium supplemented with L-lysine, centrifuged, washed and split into two cultures as described in the legend to Fig. 3.15. Aliquots were removed for measurement of β-galactosidase activity at the time points indicated.

Next, I checked whether the induction behaviour will be influenced by the presence of the wild-type *lysC* gene. The experiment was repeated with strain PT43 carrying a complete deletion of the *lysC* operon including its promoter and riboswitch region. As can be seen from Fig. 3.19B, the basal level was increased from 30 to 40 units in the presence of L-lysine while removal of the amino acid resulted in 770 to 970 units. In conclusion, deletion of the wild-type *lysC* copy from the chromosome increased the induction factor to 25.

In another experiment, to answer the question what extend inactivation of the transcription terminator will influence the expression level of the *lacZ* reporter gene, strains PT44 and PT45 with and without the wild-type chromosomal copy of *lysC* and the transcriptional fusion between the promoter region and the riboswitch with the truncated terminator sequence and *lacZ* were analysed. While close to 600 units were measured in strain PT44 in the presence of L-lysine, there was a slight increase after its removal from 720 about 1000 units in both the presence and absence of L-lysine (Fig. 3.20A). A slight increase was already seen in the wild-type background (Fig. 3.19A) and seems to be the result of growth to a higher cell density. But most importantly, there is no difference in the β-galactosidase activities between the two cultures confirming the constitutive expression of *lacZ* in the absence of a functional transcription terminator. When the operon fusion was analysed in strain PT45, no significant difference was found as compared to the isogenic wild-type strain (Fig. 3.19B). From this result I infer that the presence of a wild-type riboswitch does not influence the expression level at a second copy with a truncated transcriptional terminator.

3.2.4 The auto-inducible expression systems

From the result shown in Fig. 3.19A, removal of L-lysine from the growth medium resulted in an about 10-fold induction of the *lacZ* gene. Next, the whole of cassette was transferred onto the promoter- probe plasmid pT05-*lacZ* as described under Materials and methods 2.7.2 resulting in pT02Z and checked for the influence of the induction factor. *B. subtilis* strain 1012 carrying pT02Z was grown in minimal medium with L-lysine as described, and the culture was divided into two subcultures when cells reached mid-exponential growth. Where one was further incubated in the presence of L-lysine, the other was grown in the absence of L-lysine, the β-galactosidase activities in the supplemented medium increased from 43 units at t = 0 to 56 units at t = 270 min (Fig. 3.21A). When the L-lysine has been removed, the enzymatic activity increase within the first 15 min to 270 units and reached its maximum value after 90 min with 520 units (Fig. 3.21A) resulting in an induction factor of about 12.

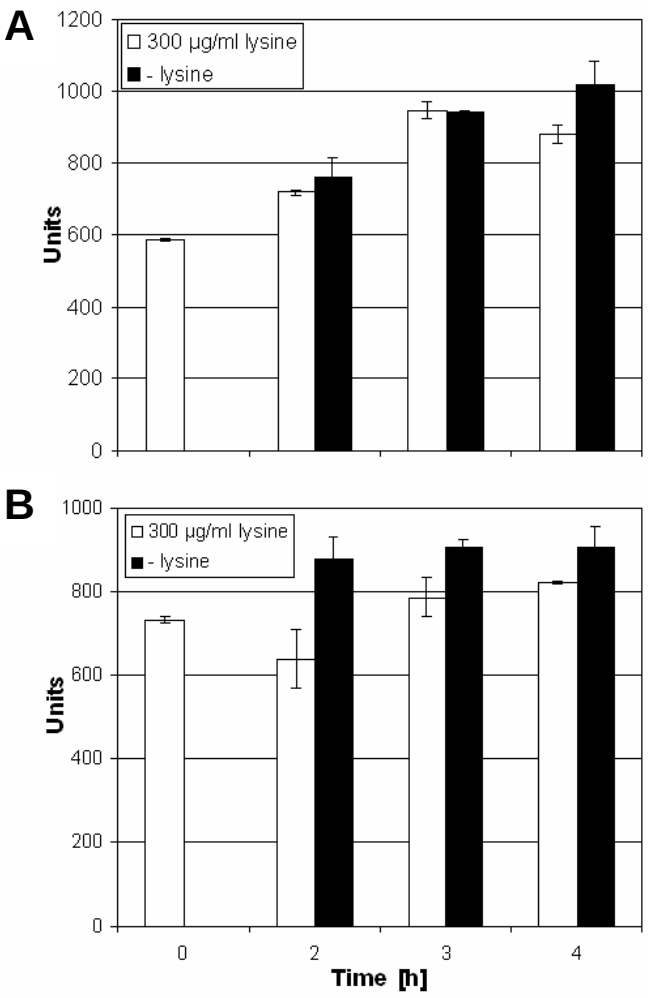


Fig. 3.20. Analysis of the promoter-riboswitch region fused to *lacZ* in the absence of the transcriptional terminator. Strain PT44 (A) and strain PT45 (B) were grown and treated as described in the legend to Fig. 3.19.

Next, different L-lysine concentrations (from 2.5 to 20 µg/ml) were tested to determine the optimal concentration resulting in the highest induction factor and for the purpose of creating an auto-inducible system. Cells were grown in minimal medium supplemented with 300 µg/ml L-lysine to mid-log, washed and divided into seven subcultures. Where one was further grown in the absence of L-lysine, the others were incubated in the presence of different L-lysine concentrations as indicated in Fig. 3.21B (2.5, 5, 7.5, 10, 15, and 20 µg/ml). Aliquots were removed for determination of β-galactosidase activities after centrifugation 0.5, 1, 2, 4, 6, 8, 10 and 24 h. 5 µg/ml L-lysine resulted in the high induction factor and could be used for auto-induction (Fig. 3.21B).

In conclusion, a novel auto-inducible expression system for *B. subtilis* and related species allowing the regulated expression of recombinant genes has constructed. This system is based on the low concentration of L-lysine in the mid-exponential growth phase leading to the concomitant induction of the recombinant gene.

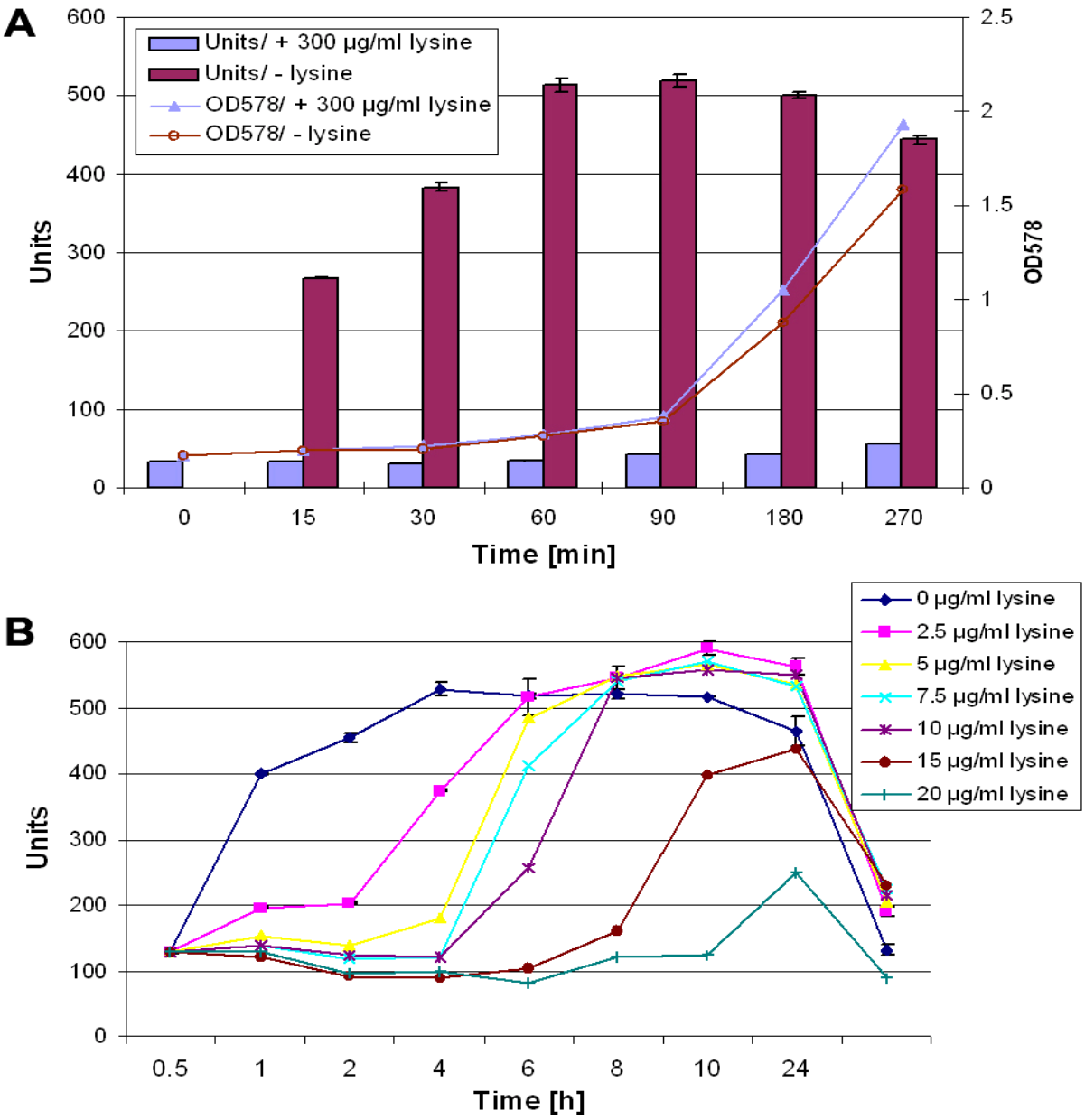


Fig. 3.21. β-Galactosidase activity of *lacZ* reporter gene located on a vector plasmid. Strain 1012 contains the vector plasmid pT02Z with the wild-type *lysC* region (P_{lysC}-riboswitch) fused to *lacZ* was grown in minimal medium supplemented with L-lysine, centrifuged, washed and split into different cultures. (A) To analyse the promoter-riboswitch region fused to *lacZ* on the vector plasmid, cells were washed and split into two cultures as described in the legend to Fig. 3.15; (B) full induction of the lysine-dependent riboswitch RNA occurs after addition of a L-lysine concentration of 2.5-20 µg/ml. Cells were washed and divided into seven subcultures with different L-lysine concentrations (0, 2.5, 5, 7.5, 10, 15, and 20 µg/ml). Aliquots were removed for measurement of β-galactosidase activity at the time points indicated.

3.3 Construction of plasmids allowing detection and single-step purification of recombinant proteins expressed in *B. subtilis*

Six novel plasmid-based IPTG-inducible expression vectors were constructed for *B. subtilis*. While one vector allows intracellular production of recombinant proteins, the second provides secretion. The third vector allows addition of the c-Myc epitope tag, and the remaining three vectors provide the purification tags His and Strep. The versatility of all six vectors was proven by insertion of appropriate reporter genes and demonstration of regulated overexpression. Recombinant proteins with a His- or Strep-tag could be purified to near homogeneity in a single step.

3.3.1. Removal of a 117-bp direct repeat results in structural stability of expression vectors in *E. coli*

During the work with the two novel expression and secretion vectors pNDH33 and pNDH37 [117], respectively, in *E. coli,* these vectors were observed to suffer from structural instability. After transformation of these two cloning vectors, small and large colonies appeared on the plates. Analysis of plasmids from small colonies revealed the expected size, while those from large colonies carried smaller plasmids allowing faster growth. To find out the reason for this structural instability, we investigated the DNA sequence of both vector plasmids for the presence of a direct repeat. Direct repeats have been reported to frequently result in deletions removing the DNA located between the direct repeats and one copy of the repeat [4]. Indeed, inspection of the DNA revealed the presence of a 117 bp segment occurring twice in the DNA and carrying the 3' end of the *lacI* gene (data not shown), where the second copy is located between the *Sph*I and *Drd*I sites in Fig. 1 of [117]. The second copy was removed as described under Materials and methods 2.7.3 yielding the derivative plasmids pHT01 and pHT43 (Fig. 2.3). Both plasmids resulted in colonies of equal size after transformation into *E. coli*. To compare the structural stabilities of the former plasmid pNDH33 with the new plasmid pHT01 in *E. coli*, the derivatives containing the *bgaB* reporter gene coding for a heat-stable β-galactosidase was used [61].

The experiment started from one blue colony formed on LB plates containing X-Gal and 1 mM of IPTG, which were grown overnight in selective LB medium. The plasmid DNA from these overnights and from serial dilutions and new overnights were analyzed up to about 80 generations. While pHT01-*bgaB* remained stable, a smaller plasmid derivative of pNDH33-*bgaB* became apparent already after about 40 generations and predominated

thereafter (Fig. 3.22). The size of the smaller plasmid was determined to be 3.4 kb which is in perfect agreement with recombination between the two direct repeats where the smaller recombination product carries the *bla* gene and the origin of replication, while the *bgaB* gene has been deleted. When cells were analyzed for their phenotype on X-Gal plates after about 80 generations, more than 99% of them exhibited a blue phenotype when carrying pHT01-*bgaB*, while only 0.2% of those inheriting pNDH33-*bgaB* showed blue colonies already after 40 generations. It was concluded that indeed the 117 bp direct repeat is responsible for the structural instability of the plasmids pNDH33 and pNDH37. Both, pHT01 and pHT43 will substitute pNDH33 and pNDH37 as expression and expression-secretion vectors, respectively.

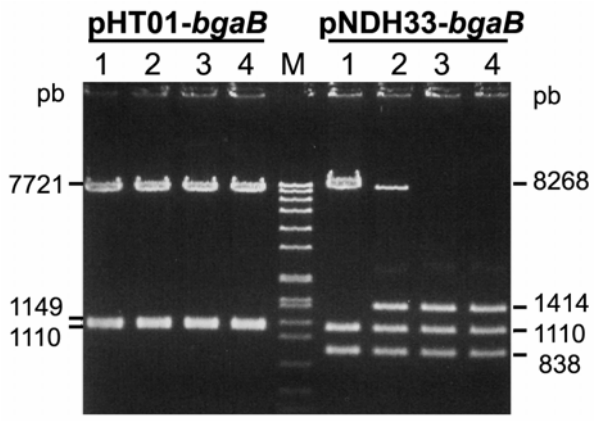


Fig. 3.22. Analysis of the structural stability of two different plasmids. Single colonies of *E. coli* strain XL1 Blue carrying pHT01-*bgaB* and pNDH33-*bgaB*, respectively, were grown overnight in LB medium at 37°C in the presence of ampicillin. Plasmids were prepared from these overnight cultures, cut with *Afl*II and *Bgl*I and analyzed by agarose gel electrophoresis (lane 1). About 1,000 cells of the overnight were used to prepare a new overnight, etc. Lane 1 through 4 represent the plasmids obtained after about 20, 40, 60, and 80 generations.

3.3.2. Expression levels of the new expression vectors

To compare the expression and secretion levels of the new with the previous vector plasmids, first, two reporter genes *htpG* and *pbpE* were fused to the strong P_{grac} promoter in pHT01 resulting in pHT01-*htpG* and pHT01-*pbpE*, where *htpG* codes for a heat shock protein of so far unknown function [61], and *pbpE* for the penicillin-binding protein 4* [118]. *B. subtilis* strain 1012 carrying these recombinant plasmids and the already described pHT01-*bgaB* were grown in LB medium at 37°C to mid-log and IPTG was added at 1 mM. Aliquots were withdrawn just before adding IPTG (Fig. 3.23A, lanes 1, 4 and 7), 2 (lane 2, 5 and 8) and 4 h (lanes 3, 6 and 9) and analyzed for the production of the recombinant proteins by SDS-PAGE and Coomassie blue staining. In all three cases, a prominent protein band can be visualized corresponding to the expected size of the recombinant protein (Fig. 3.23A). It also becomes

clear that 4 h of induction did not lead to a further increase in the amount of recombinant proteins as compared to 2 h of induction.

To further verify overexpression of the recombinant proteins, after addition of the inducer, 0.15 OD_{578} were applied per lane except for lanes C4 to C6 where only 0.025 OD_{578} were added. Western blots were carried out using antibodies raised against Pbp4* and HtpG; antibodies against BgaB are not available. When the Western blot was probed with αPbp4*, a very weak band could be detected in the uninduced culture (Fig. 3.23B, lane 7) which dramatically increased after addition of IPTG to the growing cells (lanes 8 and 9). A similar result was obtained when the Western blot was probed with αHtpG (compare Fig. 3.23C, lane 4 with lanes 5 and 6 where only 0.025 OD_{578} were added). These data clearly demonstrate that the new expression vector pHT01 results in overexpression of recombinant proteins comparable to the results obtained with pNDH33 [117].

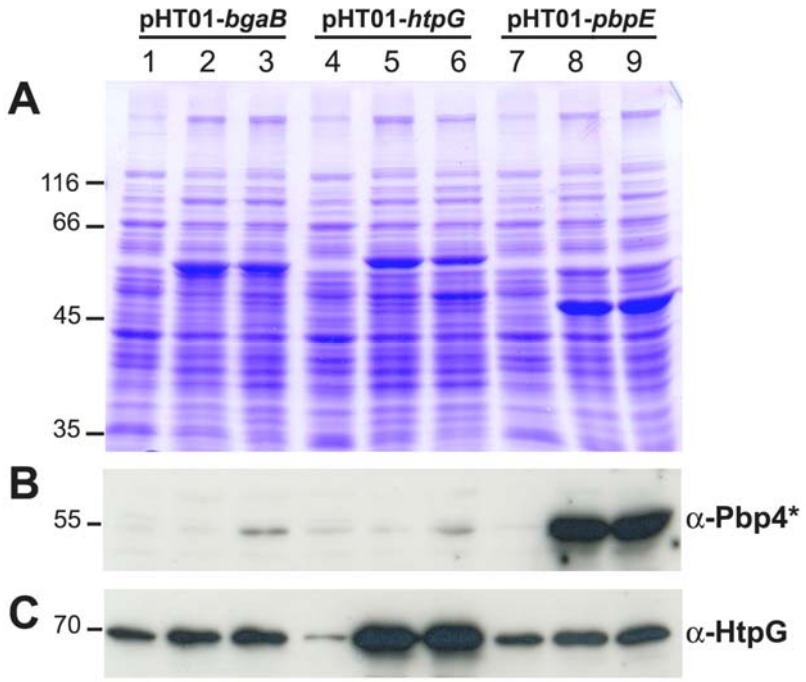


Fig. 3.23. Overexpression of three different proteins fused to the P_{grac} promoter. *B. subtilis* 1012 cells carrying the recombinant plasmids pHT01-*bgaB*, -*htpG* and -*pbpE* were grown in LB medium at 37°C to mid-log. Then, IPTG was added at 1 mM and cells were incubated for another 4 h. Aliquots were withdrawn immediately before adding ITPG (lanes 1, 4 and 7) and 2 (lanes 2, 5 and 8) and 4 h (lanes 3, 6 and 9) after addition of the inducer. 0.15 OD_{578} were applied per lane except for lanes C4 to C6 where only 0.025 OD_{578} were added. (A) Coomassie blue staining of the 10% SDS-PAGE; (B) and (C) Western blots probed with αPbp4* and αHtpG, respectively.

Next, the new expression-secretion vector pHT43 was compared with pNDH37 using three different reporter genes, *celA*, *celB* and *amyQ*, where the first two code for cellulases and the latter for an α-amylase [30, 112]. As described before, *B. subtilis* cells were grown in LB medium at 37°C to the mid-log phase (t = 0) followed by induction with 1 mM of IPTG, and the supernatants were analyzed for the presence of the secreted recombinant protein. While none of the three proteins was present in detectable amounts before addition of IPTG (Fig. 3.24, lane 0), additional protein bands started to appear 1 h after induction (lane 1) and increased up to about 5 h (lane 5). While the amounts of CelA and CelB appeared to be comparable with both vectors, significantly higher amounts of AmyQ were synthesized with the new vector pHT43 (compare lanes 5 in Fig. 3.24C). In conclusion, both vectors produce at least comparable amounts of exoenzymes. Why the amount of AmyQ expressed from pHT43 is higher than the amount synthesized with pNDH37 is unknown.

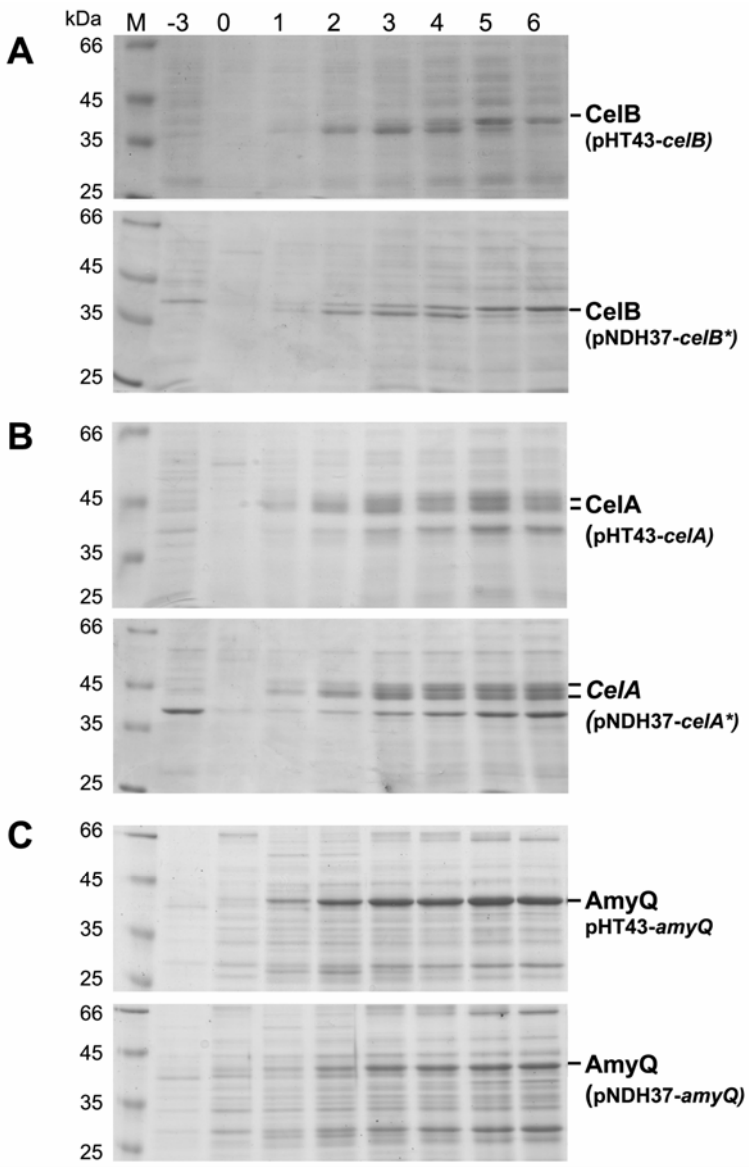


Fig. 3.24. Secretion of different exoenzymes. *B. subtilis* 1012 cells carrying either pHT43-*celB*, -*celA* or -*amyQ* were grown in LB medium as described before. Supernatant was removed just before adding 1 mM IPTG (lane 0), and 1 to 6 h later (lanes 1 to 6). 300 µl of supernatant was applied per lane of a 10% SDS-PAGE which was stained with Coomassie blue. The data for the plasmids pNDH37-*amyQ*, pNDH37-*celB* and pNDH37-*celA* were taken from [117].

3.3.3. Incorporation of the epitope tag c-Myc

Preparation of monoclonal antibodies against proteins is a time-consuming task taking several months. To reduce the time involved, epitope tags have been developed which can be added either to the N- or to the C-terminus of any protein. We constructed a derivative of the vector pHT01 where the coding region for the c-Myc tag [39] was inserted in such a way that it can be added to the C-terminus of any recombinant protein of interest resulting in pHT10. Next, the coding regions of two different genes were fused in-frame to c-Myc. We have chosen the genes *ywbN* and *gfp* coding for a putative sortase [111] and for the GFP [141]. Synthesis of both proteins was induced by addition of IPTG as described before using the recombinant plasmids pHT10-*ywbN* and pHT10-*gfp*. While both proteins were not detectable in the absence of IPTG (Fig. 3.25A, lanes 1 and 4), they became detectable 2 h after induction of the cells with IPTG (lanes 2 and 5) and did not increase in their amount when analyzed after 4 h (lane 3 and 6) as described above for different proteins.

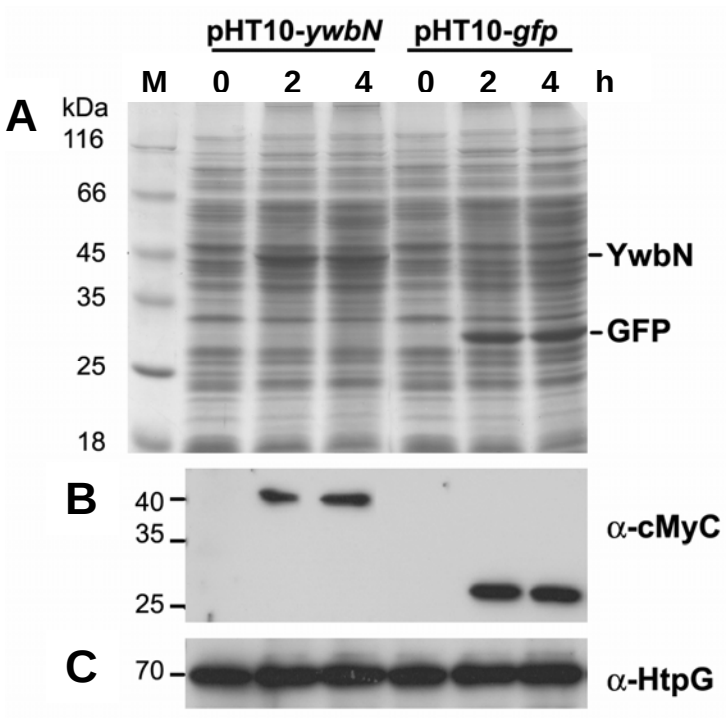


Fig. 3.25. Detection of two different proteins carrying the c-Myc epitope tag by immunoblotting. *B. subtilis* 1012 inheriting pHT10-*ywbN* and pHT10-*gfp* were grown in LB medium and treated with 1 mM IPTG. Aliquots were taken before addition of IPTG (t = 0) and 2 and 4 h after addition (t = 2 and 4). 0.15 OD_{578} were lysed and analyzed by SDS-PAGE (A) and by a Western blot of the gel was probed with α-cMyc (B), α-HtpG as a loading control (C).

Next, the presence of the c-Myc tag was analyzed by Western blot using monoclonal antibodies. In each case, one band became visible with the expected molecular mass (Fig. 3.25B); HtpG served as a loading control (Fig. 3.25C). In summary, the vector plasmid

pHT10 allows overproduction of c-Myc-tagged recombinant proteins and their easy detection by immunoblotting.

3.3.4. Addition of the His- and Strep-tags

To improve the versatility of the expression vector by allowing single-step purification of recombinant proteins, two different purification tags were decided, namely the His- and the Strep-tag. While the His-tag is widely used in conjunction with metal chelate resins [64], the Strep-tag has been developed as an alternative tool [139, 140]. The Strep-tag, an eight-amino acid peptide (sequence: WSHPQFEK), has a high specificity and affinity towards streptavidin. Its sequence was derived by selection from a genetic peptide library [140] and later found to occupy the same pocket of streptavidin where biotin normally becomes complexed [138]. Both tags allow a one-step purification of recombinant proteins using affinity chromatography. The coding region for eight histidine residues was inserted immediately downstream of the P_{grac} promoter in such a way that the His-tag is located at the N-terminus of the recombinant protein (pHT08). The 8-amino-acid Strep-tag was inserted into pHT01 in such a way to allow N- and C-terminal fusions (pHT09 and pHT24, respectively).

3.3.4.1. Addition of the His-tag

The functionality of both vectors was checked by first fusing the coding region of the genes *yhcS* and *srtA* coding for a putative *B. subtilis* sortase and the core region of the *L. monocytogenes* sortase A, respectively [111, 125] to the His purification tag present in pHT08. Both plasmids were transformed into *B. subtilis* 1012, and cells were grown in the absence of IPTG to the mid-log phase. Then, production of the recombinant proteins was induced by addition of IPTG and production of the protein was followed by applying samples on a SDS-PAGE followed by Coomassie blue staining and by Western blotting using anti-His antibodies (Fig. 3.26). In both cases, 2 h of induction turned out to be sufficient for high level of production of the recombinant proteins (Fig. 3.26A and C lane T and 2). Next, both proteins were purified using Ni-NTA columns as described [39] (Fig. 3.26C, lanes E1 and E2). Both proteins could be purified to near homogeneity as demonstrated in Fig. 3.26C. The recombinant proteins were confirmed by Western blot (Fig. 3.26B and D). It was concluded that the expression vector pHT08 is suitable for overproduction and single step purification of recombinant proteins with a His-tag at their N-terminus.

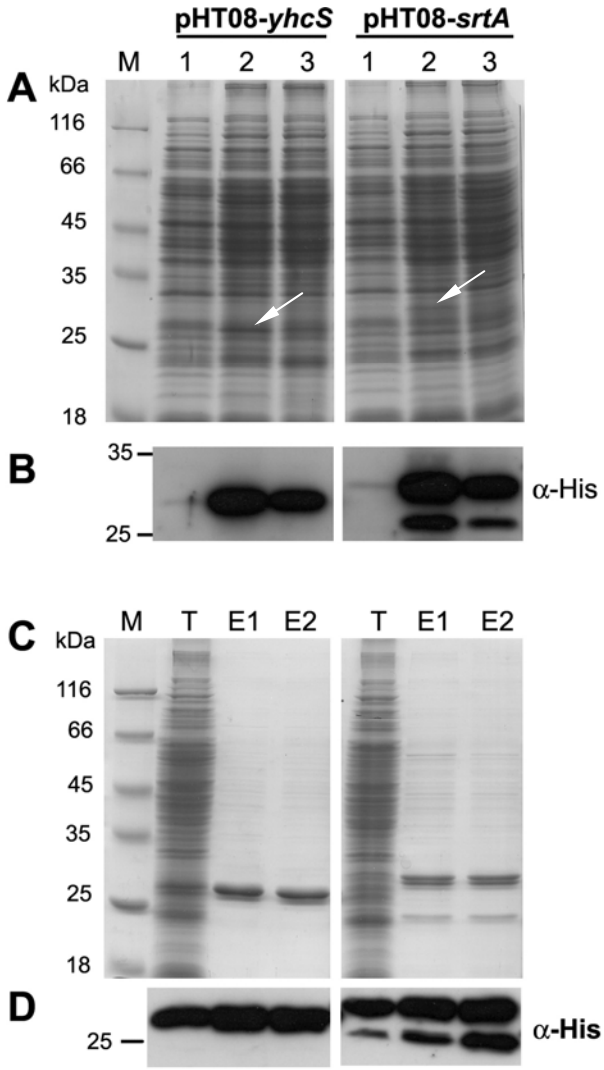


Fig. 3.26. Overproduction and affinity purification of proteins with a His-tag. *B. subtilis* 1012 carrying pHT08-*yhcS* and pHT08-*srtA* were grown in LB medium to mid-log, and production of the recombinant proteins was induced by addition of 1 mM IPTG. (A) Cells were lysed and aliquots were analyzed by SDS-PAGE (lane 1, before induction; lane 2 and 3, after 2 and 4 h induction); (B) Western blot analysis using α-His; (C) Cells were lysed and aliquots were analyzed by SDS-PAGE (lane T, total protein), the cellular extracts were applied to appropriate affinity columns, washed and the bound protein was eluted. E1 and E2 stand for the first and second elution step; and (D) Western blot analysis using α-His.

3.3.4.2. Addition of the Strep-tag

The coding region for the Strep-tag was inserted into pHT01 in such a way to allow its fusion either to the N- or C-terminus of the recombinant protein resulting in pHT09 and pHT24, respectively. To prove the functionality of both vectors, the coding region of *gfp* was translationally fused to the Strep-tag resulting in pHT09-*gfp* and pHT24-*gfp*. Both proteins could be produced after IPTG induction though the strain where the Strep-tag was fused to the C-terminus produced higher amounts of GFP (Fig. 3.27). With both tags, GFP could be purified to near homogeneity using an affinity column with immobilized streptavidin.

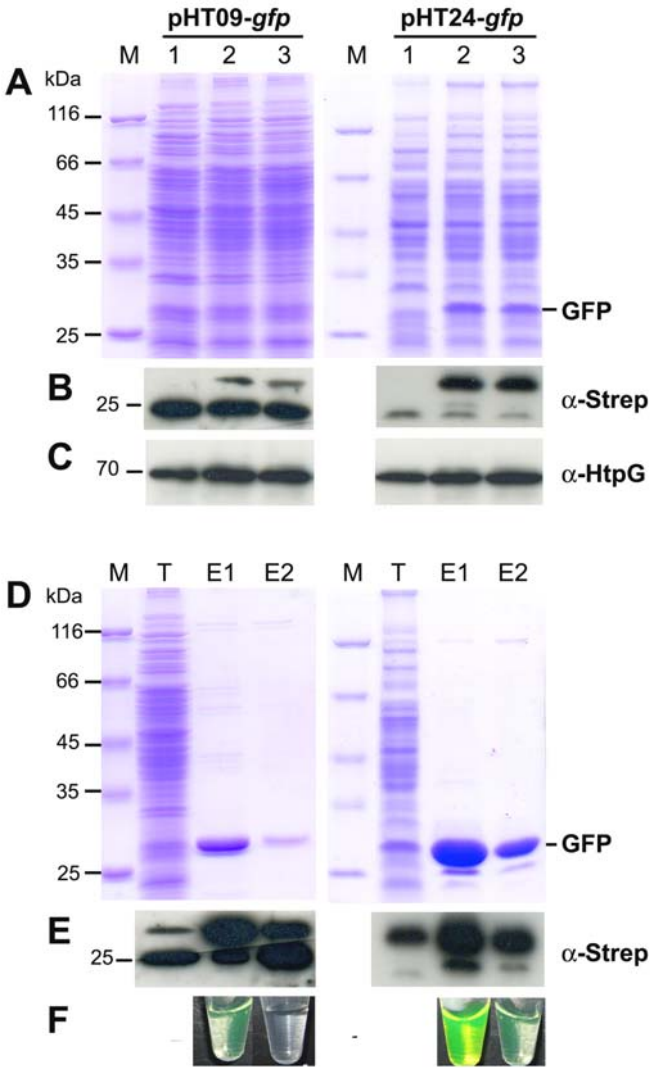


Fig. 3.27. Overproduction and affinity purification of proteins with a Strep-tag. *B. subtilis* 1012 carrying pHT09-*gfp* and pHT24-*gfp* were grown in LB medium to mid-log, and production of the recombinant proteins was induced by addition of 1 mM IPTG. (A) Cells were lysed and aliquots were analyzed by SDS-PAGE (lane 1, before induction; lane 2 and 3, after 2 and 4 h induction); (B) Western blot analysis using α-Strep; (C) α-HtpG as loading control. (D) Cells were lysed and aliquots were analyzed by SDS-PAGE (lane T, total protein), the cellular extracts were applied to appropriate affinity columns, washed and the bound protein was eluted. E1 and E2 stand for the first and second elution step; (E) Western blot analysis using α-Strep; (F) Eppendorf tubes contain aliquots after elution.

3.4 Establishment of a simple method for identification and screening of strong and efficient promoters

To be able to screen for strong promoters easily, a promoter-probe vector has been developed, which uses the *bgaB*-encoded β-galactosidase as reporter enzyme. Furthermore, this vector carries the *lacO* operator, the *lacI* gene encoding the Lac repressor and a MCS allowing insertion of promoters. The *lacO* and *lacI* elements convert any promoter into a controllable promoter, which can be induced by IPTG. Therefore, it should be able to insert even very strong promoters into this new promoter-test vector to be cloned them in the absence of IPTG in *E. coli*. The following chapters first describe construction of the promoter-probe vector and then analyze their induction in relation of the IPTG concentration added to the medium.

3.4.1 Construction of the promoter-probe vector pHT06

In order to screen for strong promoters in *B. subtilis*, the promoter-probe vectors pLacZ [176], pBgaB [99], pNDH05 and pT05Z have been used in the past. Plasmid pNDH05 is similar to pHT06 but does not contain *lacO* and *lacI* and in pT05Z *bgaB* was replaced by *lacZ* to screen for promoter strength. However, we failed at the very first step, to clone promoters P88, P89 and some others in *E. coli* using pNDH05 or pT05Z (data not shown). The reason for this failure is most probably overproduction of the *bgaB* or *lacZ* encoded enzymes leading to death of the *E. coli* transformants. To overcome this problem, the *lacO* operator and the *lacI* gene were introduced to control expression of *bgaB* in both *E. coli* and *B. subtilis* (2.7.4) resulting in the novel promoter-probe plasmid pHT06 (Fig. 2.4). This plasmid was constructed based on the backbone of plasmid pMTLBs72 [156] and its derivative pHT01 [105] as described in Material and methods (2.7.4).

3.4.2 The use of the promoter-probe vector pHT06 for cloning and analysis of strong promoters

In order to check whether plasmid pHT06 will accept strong promoters, the two synthetic promoters P88 and P89 were introduced. As to be expected, the cloning step in *E. coli* is rather simple by allowing to screen for blue colonies on X-gal plates in the low concentration of IPTG. Plasmids were then extracted for restriction enzyme analysis, DNA sequencing and transformation into *B. subtilis*. The BgaB activity of the *B. subtilis* strains harbouring those plasmids was measured. The activity was higher than that from the strong promoter P_{grac}, pHT01-*bgaB* [105], which served as a reference in all the following experiments. Based on this positive finding, more than 80 different promoters were introduced into this plasmid to analyse for the influence of different promoter elements (Fig. 3.29A and part 3.5) and stabilizing elements (Fig. 3.29B and part 3.6). This result indicates that the first problem of cloning and maintaining strong promoters has been successfully solved.

3.4.3 Linearity of BgaB activity and IPTG concentration

Next, it was attempted to measure the activity of different promoters induced at 1 mM and 0.1 mM of IPTG to compare their strength, which did not work properly (data not shown). But while analyzing with the libraries, it was found that there was a correlation between BgaB activity and IPTG concentration. To compare the promoter activities, the linear range between the IPTG concentration and the β-galactosidase activity was investigated, using the three different strong promoters P69, P78 and P223 and P_{grac} as a reference [117]. The results

revealed a linear range for all promoters with an IPTG concentration from 0.0025 to 0.025 mM added to the culture medium (Fig. 3.28). Furthermore, it turned out that the weaker promoters had an extended linear range. P69 and P78 could be induced with 0.05 mM of IPTG and P_{grac} with up to 0.1 mM. The data also showed that using high concentrations of IPTG, for example 1 mM for very weak promoters such as P_{lepA}, P85 and P70, better resolution between the different promoters was obtained (data not shown). These results further demonstrate that the second problem to compare the strength of different promoters has been solved.

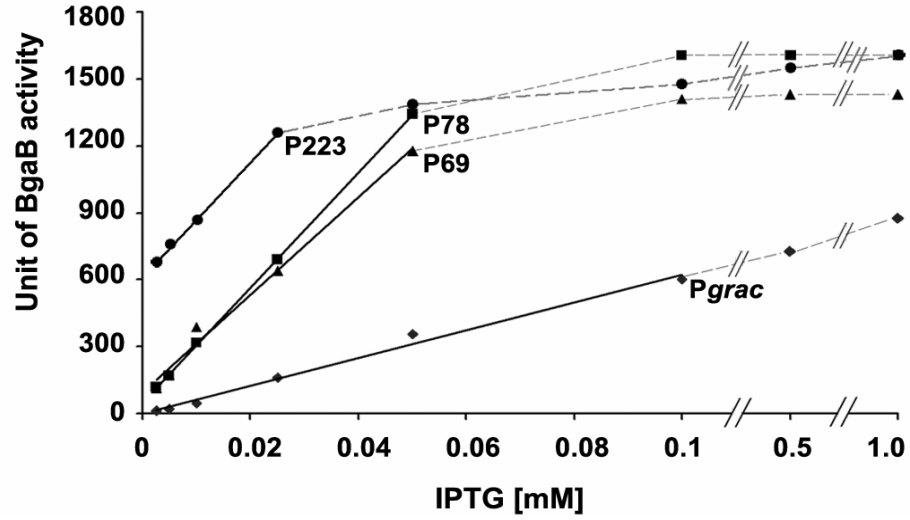


Fig. 3.28. Linear range between IPTG concentration and BgaB activity. The samples were collected 2 h after IPTG addition (0.0025, 0.005, 0.01, 0.025, 0.05, 0.1 and 1 mM). The promoters P_{grac}, P69, P78, P223 were analyzed. The data represent the averages of three independent experiments.

3.4.4 Observation and measurement of the promoter strength

All promoters from the library were analyzed on X-gal plates containing different concentrations of IPTG. Plates were incubated at 37°C, and pictures were taken by a digital camera (Fig. 3.29). As to be expected, the colour of the colonies could be distinguished at concentrations of IPTG between 0.0025 and 0.025 mM. Representative colonies on plates with 0.01 or 0.1 mM IPTG are shown in Fig. 3.29C and the activity of promoters from plates with 0.01 mM IPTG is easier to be distinguished based on the colour. Higher concentrations of IPTG did not allow to resolve the strength of strong promoters, but resulted in a better resolution for weak promoters. Based on these results, the concentration of 0.01 mM IPTG was chosen for further investigations. Fig. 3.29A and 3.29B exhibit the activity of different

promoters on X-gal plate with 0.01 mM IPTG and bluer colour indicates stronger promoters. In addition, the pictures (Fig. 3.29A, 3.29B) were analysed by the QuantityOne programme (Biorad) and the intensity of the blue colour was converted into grey values, which represent the strength of the promoters; higher values represent stronger promoters. From these plate experiments, the strength of the promoters could be visualized based on the intensity of the blue colour of the colonies (Fig. 3.29) and classified based on the grey value of colonies (Fig. 3.30 and data not shown).

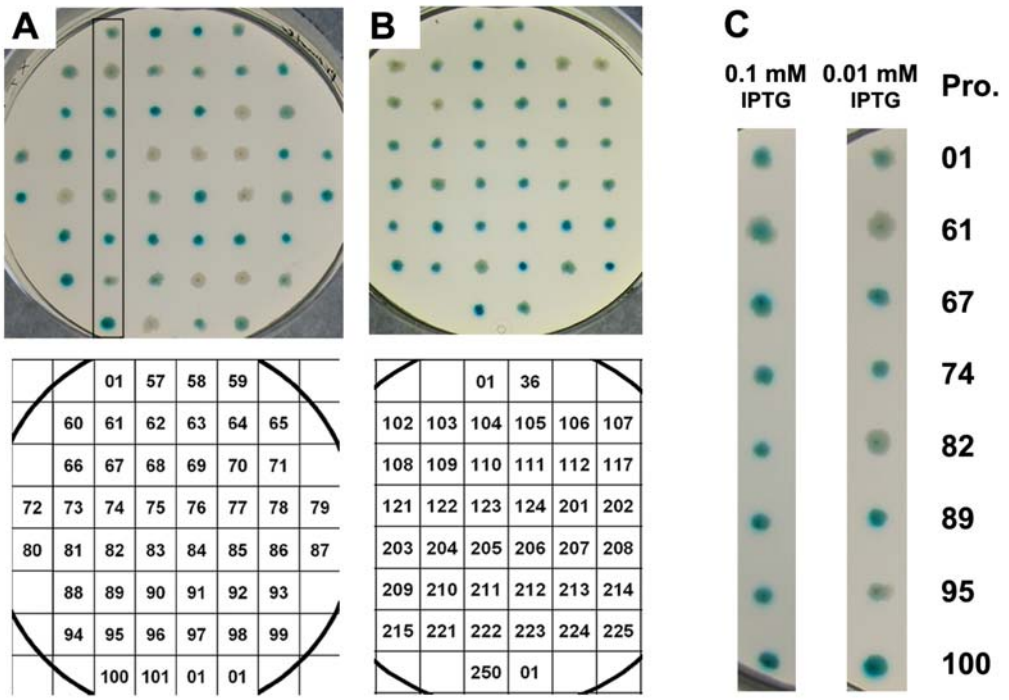


Fig. 3.29. Screening the strength of strong promoters on LB plates. All clones were spotted on LB X-gal plates containing 0.01 mM IPTG and incubated at 37°C for 16 h (A and B). Different clones are shown to study the influence of promoter elements (A) and 5'-mRNA stabilizing elements (B). Below the plates with the colonies, the numbers of the different promoters are given, which will be described below in detail. (C) Influence of IPTG concentrations (0.01 and 0.1 mM) on the development of blue colour of the colonies; the colonies from (A) indicated by a rectangle were displayed grown in the presence of two different IPTG concentrations as indicated.

In addition, all promoters were also evaluated by BgaB measurement 2 and 4 h after addition of IPTG. The strength of the promoters was almost in agreement with the data obtained from the colour of the colonies (Fig. 3.30 and Fig. 3.33B). A major difference between the plate and the liquid assays is that reading of the blue colour on plates occurred during stationary

growth phase, while determination of the β-galactosidase activity used cells in the exponential growth phase. This might explain some of the slight differences. Fig. 3.30A and Fig 3.30B showed a good agreement of grey values and BgaB activities of strong σ^A-dependent promoters in *B. subtilis*. Moreover, the strength of the promoter P223 was 23 times higher than P_{grac} 4 h after addition of 0.01 mM IPTG and P85 was 5 times weaker than P_{grac}. From these data, it can be inferred that choosing the appropriate concentration of IPTG within a linear range is important for a successful analysis. It also turned out that incubation of the plates at 30°C yielded better results than incubation at 37°C, and there were no differences between the promoters when the plates were kept from 10 – 30 h in the incubator (data not shown).

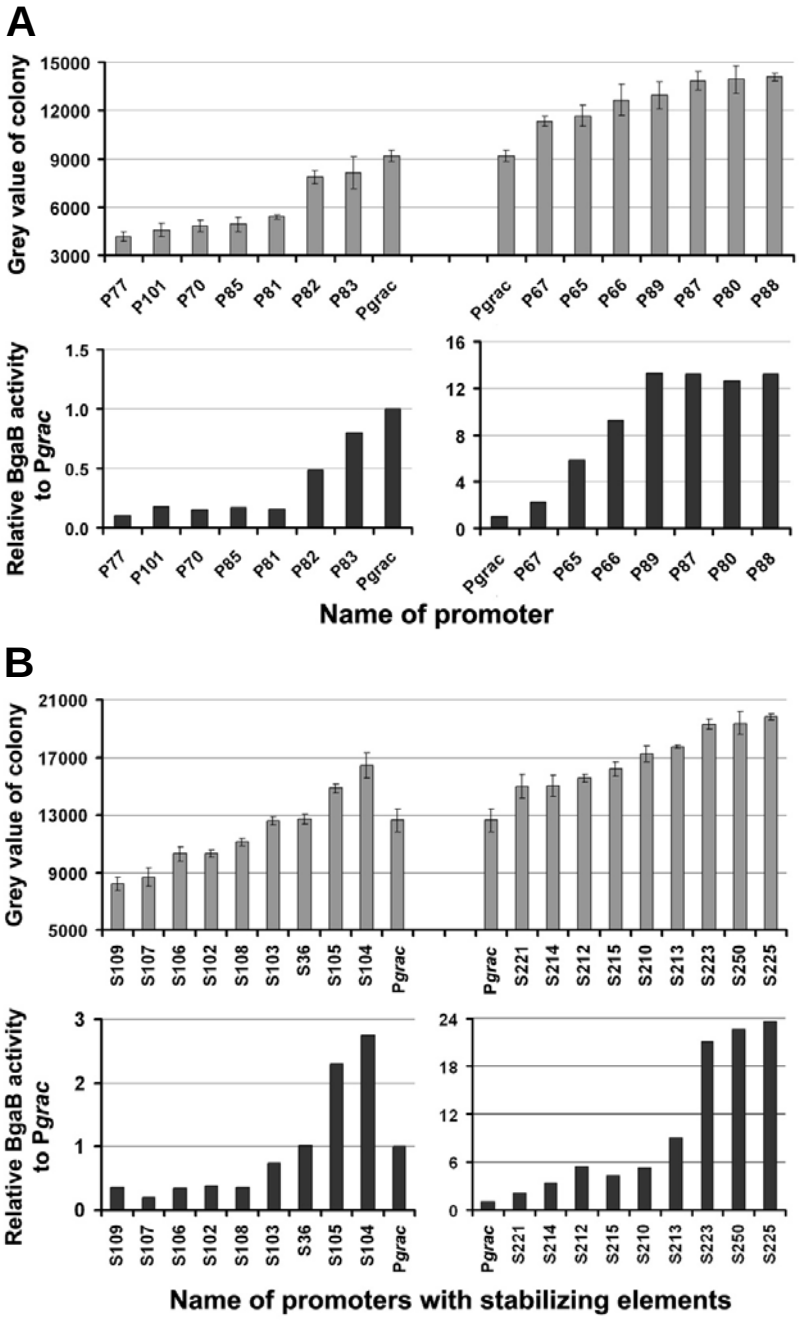


Fig. 3.30. Analysis of promoter strength based on grey values of colonies and their BgaB activity. Samples were arranged in increasing grey values. Labels of partial promoters in A and B here are comparable with A and B in Fig. 3.29. The intensity of colonies from pictures was analyzed by the QuantityOne programme (Biorad). The data shown here were the averages of at least two independent plates or BgaB measurements 2 h after IPTG induction. The two or more values differed among themselves by <15% for samples with activities 10 times higher than those obtained with the P_{grac} promoter and <5% for other samples.

In summary, these results clearly show that screening of a library of different promoters can be done first on LB plates containing 0.01 mM IPTG and based on this analysis, appropriate colonies can be selected for further analysis. The plate method developed here saves time as it circumvents analysis of each promoter by the time-consuming β-galactosidase measurement.

3.5 Elements of strong σ^A-dependent promoters in *B. subtilis*

A total of 40 different synthetic and *groE*-modified σ^A-dependent promoters (Fig 3.31) were introduced into the promoter-probe plasmid, pHT06, and analyzed. Strong promoters could be screened for and classified based on the strength of promoters as described before. The further analysis focused on the influence of five different on the promoter strength: (1) the transcriptional start site, (2) the -10 region, (3) the -15 region, (4) the -35 region and (5) the upstream region. BgaB activities and Northern blot analyses were carried out to measure the activity of the different promoters. The analysis of combinations of core promoters and UP elements on gene expression revealed that the β-galactosidase activity expression levels could be increased up to 23-fold and the mRNA levels up to 43-fold as compared to the strong P_{grac} promoter, and expression of *bgaB*, under control of these new promoters, could reach up to 30% of the total cellular protein.

3.5.1 P_{grac} is a strong σ^A-dependent promoter for *B. subtilis*

One of the new synthetic strong promoters called P_{grac} was constructed using the *B. subtilis* promoter *groE* and the *lacO* operator from *E. coli* (Fig. 3.32A). Expression of *bgaB*, *htpG* and *pbpE* under control of this promoter resulted in 10%, 12% and 16%, respectively, of recombinant protein of the total cellular protein, and this promoter is 30 and 60 times stronger than P_{xylA} and P_{spac} based on the BgaB activity [117]. Northern blot analysis demonstrated that the amount of *bgaB* mRNA expressed from P_{grac}, (plasmid pHT01 [105]) is 10 and 18 times higher than P_{spac} and P_{lepA}, respectively (Fig. 3.32B). These data demonstrate that P_{groE} is indeed a strong promoter and the question was raised which genetic element(s) is responsible for the high expression level. To be able to dissect and to analyse the different promoter elements separately, the new promoter-probe plasmid pHT06 was used that facilitated generation and analysis of promoter libraries. Plasmids with different *groE*-modified σ^A-dependent promoters were generated (Fig 3.31 for promoter sequences). The grey values of transformants, BgaB activities (Fig. 3.29 and 3.30) and the Northern blot analyses (Fig. 3.33 and 3.34) were used to determine the activity of these promoters.

```
Core promoters          -35                          -10       +1
P65 (Pc2mu)             TAGACAAACTATCGTTTAACATGTTATACTATAATATGCG
P66 (PlacUV*)           TTGACACTTTATCTTCCATCTGGTATAATAAATAGAGCGG
P67 (PaprE*)            TTGACAAATATTATTCCATCTATTACAATAAATTCAGCGG
P85 (PlepA)             TTGAATCTTTACAATCCTATTGATATAATCTAAGCTGCGG

P70 (PgroE)             TTGAAAATTGGAAGGGAGATTCTTTATTATAAGAATTGCGG
P57 (-35, -15)          ----C--------------ATG------------------
P58 (-15, -10)          ------------------ATG----A-------------
P71 (-35)               ----C-----------------------------------
P72 (-10)               -------------------------A-------------
P73 (-15)               ------------------ATG------------------
P74 (-15)               --------------------TG------------------
P75 (-15)               ------------------A-G------------------
P76 (-15)               ---------------TTTA-G------------------
P77 (-22)               ------------TAT--------------------------
P78 (-35, -10)          ----C--------------------A-------------
P79 (-35, -15, -10)     ----C--------------ATG----A-------------
P80 (-35, -15, -10)     ----C-------TAT----ATG----A-------------
P81 (-22, -15)          ------------TATTTTA-G--------------------
P82 (-35, -22)          ----C-------TAT--------------------------
P83 (-35, -25)          ----C----AC------------------------------
P87 (-35, -15, -10)     ----C-------TATTTTA-G-----A-------------
P88 (-35, -15, -10)     ----C-----GTATA----ATG----A--------A----
P89 (-35, -15, -10)     ----C-----TTTAA----ATG----A--GG-T--A----
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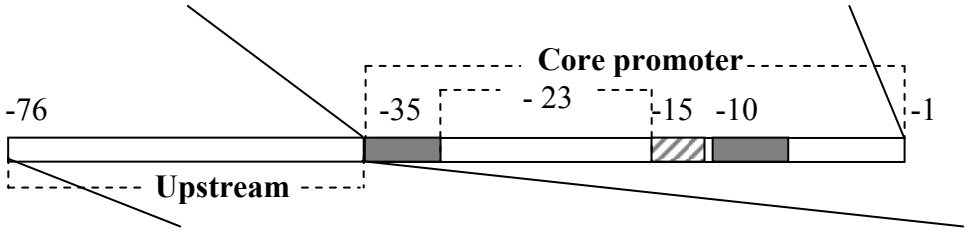


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Upstream region of core promoter                          -36
P60 (groEWT)            AAAGGAGGTAAGGATCACTAGAAAATTTTTTATCTTATCAC
P59 (groE-42)           ---------------------------------AAAA-----
P61 (groE-36)           ------------------------------------------G
P62 (groE38)            ----------------------------------------CT--
P63 (groE37)            ------------------------------------------T-
P64 (groE-37_-42)       ---------------------------------AAAA---T-
P70/P80 (HT06)          ACCGGAATTAGCTTGGTACCAAAGGAGGTAAGGATCACTAG
P95/P90 (groE-36-38)    AAAAGAATGATGTAAGCGTGAAAAATTTTTTATCTTACTAG
P96/P91 (groE-36-38-42) ---------------------------------AAAA-CT-G
P97/P92 (hag)           CCTCACAAAAAAAGTGAGGATTTTTTTATTTTTGTACTAGT
P98/P93 (lepA)          ACCCAGATATGATAGGGAACTTTTCTCTTTCTTGTTACTAG
P99/P94 (c2mu)          TGTCAACATGAGAATTCTTATCATCAATTTTTGAAAACTAG

Full promoter           UP    -35                         -10       +1
P64  AAAATTTTTTAAAAAATCTCTTGAAAATTGGAAGGGAGATTCTTTATTATAAGAATT
P91  AAAATTTTTTAAAAAACTAGTTGACAATTGGAATATAGATATGTTATAATAAGAATT
P68  AAAATTTTTTAAAAAATCTCTTGACAATTGGAAGGGAGATATGTTATAATAAGAATT
P69  AAAATTTTTTAAAAAATCTCTTGACAATTGGAAGGGAGATTCTTTATAATAAGAATT
P100 AAAATTTTTTAAAAAATCTCTTGACAATTGGAAGGGAGATATGTTATTATAAGAATT
P252 AAAATTTTTTAAAAAATCTCTTGACACTTTACAATCCTAATGATATTATCTAAGCT
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Fig. 3.31. Sequence of modified promoters based on the promoter of the *groE* operon (P70). Only chances are indicated within promoters P57-P89 from P70, and within promoters P59-P64 from P60.

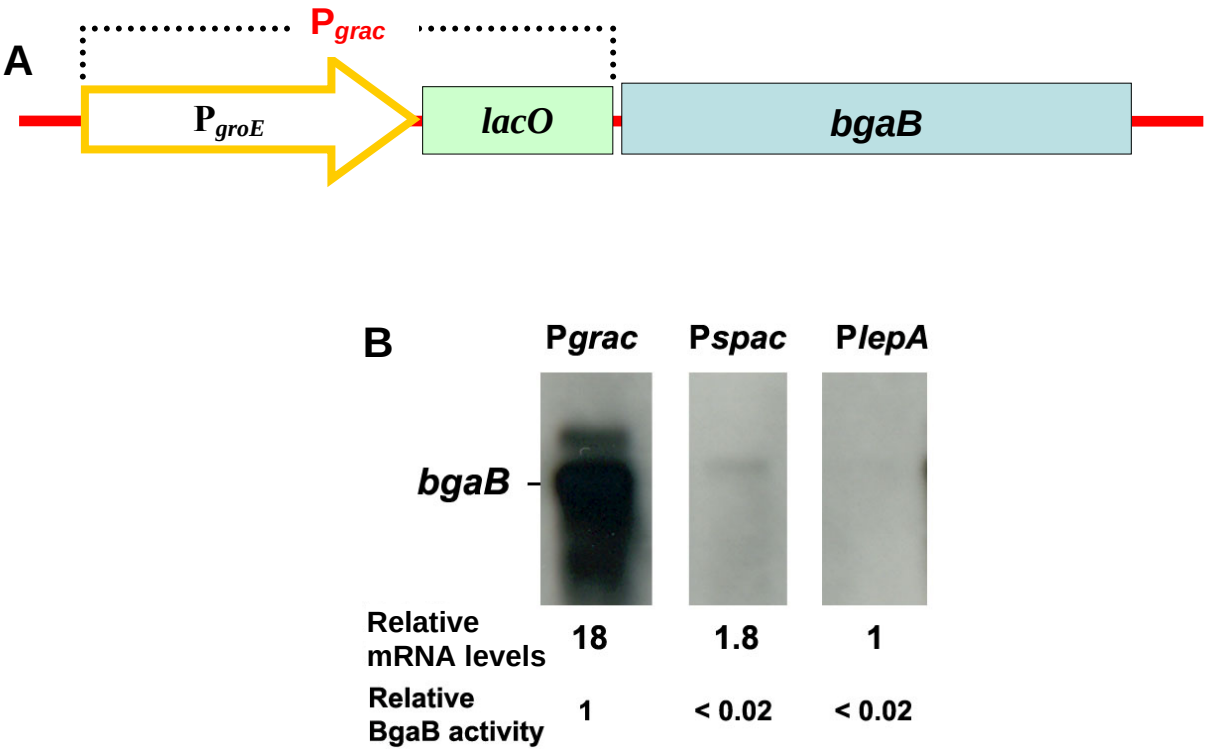


Fig. 3.32. The P_{grac} promoter. (A) Genetic features of the P_{grac} promoter; (B) Northern blot analyses of different promoters as indicated. Relative mRNA expression levels were calculated by the QuantityOne programme (Biorad), BgaB activities were measured using 1 mM IPTG.

3.5.2 Influence of UP elements on gene expression

It has been reported that UP elements can enhance the transcription initiation in *B. subtilis* [21, 96]. To answer the question whether the upstream region of the *groE* promoter functions as an UP element stimulating the core promoter activity in P_{grac}, different promoters were compared. The BgaB activity of the full-length P_{groE} promoter (UP element and core promoter) in P01 and P229 (changing the T in P01 to a C in P229 at +3) were compared with the P_{groE} core promoter lacking the UP element in P70 and a weak promoter such as P_{lepA} in P85 (Tab. 3.1). The relative BgaB activity shows that the complete promoters P01 and P229 lead to activities 10 times higher than that expressed from promoter P70 without the UP element (Tab. 3.1). These results demonstrate that the UP element of P_{groE} plays an important role in the strength of the P_{grac} promoter. This result further indicates that the expression level is largely dependent on the strength of the promoter. This was also the case for the weak promoter P85 (Tab. 3.1), where the BgaB activity is 5 times lower than that measured with P_{grac}.

Tab. 3.1. Influence of the upstream region of the core promoter P_{groE} on the BgaB activity.

	Promoters	Relative BgaB activity		
		0 h	2 h	4 h
UP + Core elements	P01 (P*grac*)	1	1	1
	P229 (P01 T+3C)	0.8	0.99	0.98
Core element	P70 (P*groE*)	0.17	0.11	0.10
	P85 (P_{lepA})	0.25	0.19	0.18

Next, different upstream regions of P_{groE} and of several other promoters were compared (Tab. 3.2). The UP element mutant *C2mu* derived from a phage ϕ29 promoter has been shown experimentally to enhance the promoter activity 1.6-fold as compared to the *C2* wild type promoter [96]. The UP element of the *hag* gene could also be shown to stimulate transcription from both σ^D- and σ^A-dependent promoters in *B. subtilis* [21]. These UP elements were fused to the *groE* core promoter, and the strength of these promoters was compared with those of the *lepA* and *groE* promoters. The activity of the upstream region of the core *groE* promoter (P95) with modifications (because of the introduction of a restriction enzyme site) was comparable with that of *C2mu* (P99), which indicates that the *groE* promoter contains a strong UP element. When the DNA sequence TCTT located at -42 to was mutated to AAAA (promoter P96), its activity increased two-fold. When the UP elements of the *lepA* and *hag* promoters were fused to the *groE* core promoter (P97 and P98), the activities turned out to be 4-fold lower (Tab. 3.2, left panel). By fusing the UP elements of the *groE* and *C2mu* promoters to the strong core promoter P80 (Tab. 3.2, P90, P91 and P94), the BgaB expression levels of those UP elements from *groE* (pHT90 and pHT91) were relevant to *C2mu* (P94). However, when the UP elements of the *lepA* and *hag* promoters were fused to the strong core promoter P80 (Tab. 3.2, P92 and P93), the activities were not much lower as in case of fusing with the weaker core promoter P70 (P97 and P98).

Tab. 3.2. Influence of the upstream region of core promoters on the BgaB activity.

UP element	Promoter	Relative BgaB activity		Promoter	Relative BgaB activity	
		2 h	4 h		2 h	4 h
groE^{wt1}	P01	1.00	1.00	P01	1.00	1.00
(-)	P70	0.11	0.10	P80	12.66	14.99
groE^{-36-38}	P95	0.41	0.50	P90	12.95	16.14
groE$^{-36-38-42}$	P96	0.91	1.01	P91	15.65	15.63
l*epA*	P97	0.11	0.15	P92	11.63	13.34
hag	P98	0.15	0.18	P93	12.95	16.14
C2mu	P99	0.43	0.46	P94	15.36	16.80

Cells were induced with 0.01 mM IPTG for 2 and 4 h; small superscript numbers indicate modification sites when compared with the wild type upstream sequence of the *groE* promoter. Data are given for the *groE* core promoter P70 (left panel) and P80 (right panel) in the absence (-) or presence of different UP elements. P_{grac} was called P01 here; *lepA*, *hag* and *C2mu*, represent upstream sequences of the *lepA, hag* and the mutated of phage ϕ29 *C2* promoters.

To have a closer look on the strong *groE* UP element, it was modified and fused with the core promoter region of the *groE* promoter (P70) and the strength of derivative promoters was measured. The UP element was enriched for short A and T tracts based on the consensus sequence (-54-nAnnnnnTnnnAAAAnnnTn-36) [57]. The modified *groE* promoters effected the amount of mRNA produced (Fig. 3.33A), the BgaB activity using the plate assay (Fig. 3.33B) and the BgaB activity after 2 h and 4 h of induction (Fig 3.33B). Shorter UP regions seem not to affect the expression level of BgaB when compared to the promoter P01 and P60 (*groE*^{WT2}) (Fig 3.33B). The amount of mRNA produced from P60 was slightly higher as compared to P01 30 min after addition of IPTG (Fig. 3.33A) for unknown reasons. This result was in total agreement with the finding in *E. coli* that the RNAP holoenzyme initially binds to a DNA region covering 75-80 bp, extending from –55 to +20 [82]. Single changes at -38, TC → CT (P62) and at -37, A → T (P63) resulted in reduction of the BgaB expression level. Changes at -36, C → G (P61) or a combination of -36 and -38 (P95) reduced the activity by at least a factor of two, while changes at -42, from TCTT → AAAA increased the activity when compared P95/P96 (Tab. 3.2) and P60/P59 and P63/P64 (Fig. 3.33). Especially, modification of this promoter (P64) based on the consensus sequence above resulted in two times higher BgaB activity and five times higher mRNA levels as compared to the wild-type sequence of P01 and P60 (Fig. 3.33). From this result, one can conclude that the modified nucleotide sequence of the *groE* UP element, -55-AAAATTTTTTAAAAAATCTC-36 is an active UP element for σ^A-dependent promoters, at least in this case of *groE* promoter.

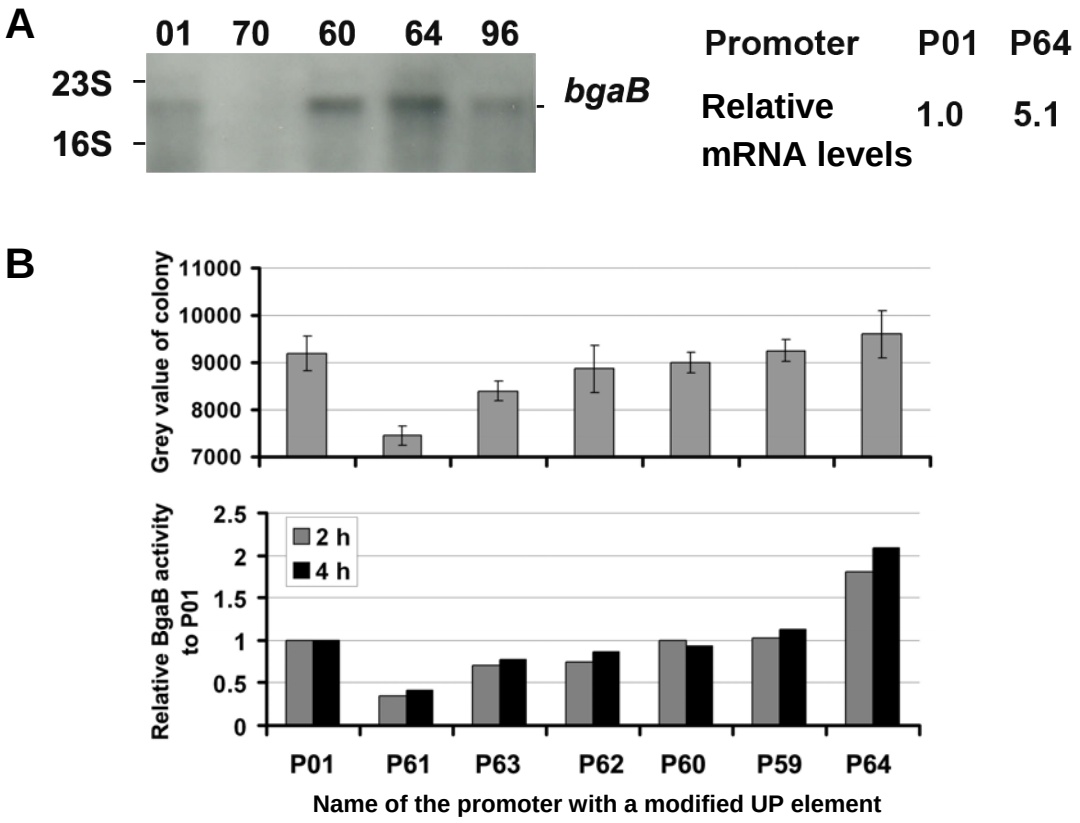


Fig. 3.33. Analysis of *bgaB* expression under control of different promoters with modified UP elements. (A) Northern blot analysis; (B) grey values of colonies (partially extracted from Fig. 3.29), and relative BgaB activities. Relative mRNA expression levels were calculated by the QuantityOne programme (Biorad).

3.5.3 Influence of the +1 region on gene expression

To investigate the influence of transcriptional start site region on the expression level, constructs with different nucleotides at the +1, +2 and +3 positions were changed or introduced (Tab. 3.3). The BgaB expression level increased by 20% when changing the T in P01 to an A in P205 at +1, while it produced only a slight increase when combined with the strong promoter P80 resulting in P84. Addition of two additional CC (pHT106) or GG (pHT86) nucleotides resulted in a more than 3-fold reduction of the BgaB expression level. By changing the U residue at +3 in P205 to G in P104, the BgaB expression level was increased more than twice, most probably by enhancing the mRNA stability.

Tab. 3.3. Influence of the +1 region on the expression of *bgaB*.

Promoter	Original promoter	Sequence	Relative BgaB activity	
			2 h	4 h
P01	P01	+1-UGUGG-*lacO*-	1.0	1.0
P205	P01	+1-AGUGG-*lacO*-	1.2	1.2
P104	P01	+1-AGGGG-*lacO*-	2.7	2.8
P106	P01	+1-ACCGCGG-*lacO*-	0.3	0.4
P80	P80	+1-TGCGG-*lacO*-	12.7	15.0
P84	P80	+1-AGCGG-*lacO*-	13.4	14.7
P86	P80	+1-AGGGCGG-*lacO*-	1.5	1.9

Promoters P104, P106 and P205 are different from P01 (P_{grac}), P84 and P86 are different from P80 as indicated in the sequence. Potential transcriptional start sites are underlined.

3.5.4 Influence of the core promoter on gene expression levels

In this part, the core *groE* promoter was modified and compared to the wild-type sequence (Fig. 3.31). I also analysed the following published promoters: (i) *C2* derived from the lysogenic phage ϕ29 [96] (in P65); (ii) the *E. coli lacUV5* promoter carrying a promoter-UP mutation [59] (in P66); and (iii) an *aprE* derivative promoter [160] (in P67). These promoters exhibited a two- to eleven-fold higher BgaB activity as compared to P01 (Tab. 3.4). The modifications of the *groE* core promoter with changes to reduce G residue within the -23 region (from -26 to -21) resulted in P77, P81, P80 and P82, the BgaB expression levels reduced slightly as compared to the original promoter P70, P76, P79 and P71 (Tab. 3.4)

3.5.4.1 Influence of the -10 and -35 regions on the BgaB activity

RNAP from diverse bacteria recognizes the same housekeeping-dependent promoters with the TTGACA (-35) and TATAAT (-10) consensus elements, as defined for the *E.coli* RNAP, indicating conserved features of this class of eubacterial promoters [57, 101]. To determine the influence of the -35 region on the BgaB activity in *B. subtilis*, the -35 region of P_{groE}^{WT} TTGAAA (P70) was replaced by the TTGACA consensus sequence (P71). When the BgaB activity of this new promoter P71 was measured, it was increased about 7-fold in *B. subtilis* and 2-fold in *E. coli* (Tab. 3.4). In the case of the -10 region, the TATTAT sequence in P70 was changed to the TATAAT consensus one (P72). The activity of BgaB was increased about 10-fold in *B. subtilis,* but only twice in *E. coli* (Tab. 3.4). When both consensus sequences were combined in the promoter P78, the amount of transcript was increased 38.8-fold (Fig.

3.34) and the relative BgaB activity 96-fold (Tab. 3.4). These results are in agreement with the consensus hexanucleotide core elements of strong promoters [57, 101].

Tab. 3.4. BgaB measurement of core promoter regions in *B. subtilis* and in *E. coli*.

Promoter	Relative activity in *B. subtilis*			Promoter	Relative activity in *B. subtilis*		
	0 h	2 h	4 h		0 h	2 h	4 h
P57 (-35, -15)	13.9	12.1	17.9	P79 (-35, -15, -10)	18.0	14.6	18.1
P58 (-15, -10)	11.5	13.8	16.8	P80 (-35, -15, -10)	15.4	12.7	15.0
P65 (*C2*)	7.2	5.9	6.7	P82 (-35, -22)	0.6	0.5	0.6
P66 (*lacUV5*)	11.6	9.3	11.2	P83 (-35, -25)	0.7	0.8	0.8
P67 (*aprE*)	1.4	2.2	2.6	P87 (-35, -15, -10)	5.3	13.2	16.0
P77 (-22)	0.17	0.08	0.07	P88 (-35, -15, -10)	9.9	13.2	16.0
P78 (-35, -10)	5.6	8.3	9.6	P89 (-35, -15, -10)	11.5	13.3	16.7
					Relative activity in *E. coli*		
P70 ($groE^{WT}$)	0.17	0.11	0.10	P70 ($groE^{WT}$)	0.75	0.80	0.68
P71 (-35)	0.88	0.73	0.91	P71 (-35)	1.19	1.65	2.46
P72 (-10)	1.03	1.25	1.58	P72 (-10)	1.18	1.92	2.64
P73 (-15)	5.79	8.34	9.40	P73 (-15)	1.37	1.79	2.36
P74 (-15)	1.52	1.55	1.49	P74 (-15)	1.07	0.59	0.51
P75 (-15)	0.26	0.19	0.13	P75 (-15)	0.94	0.26	0.22
P76 (-15)	0.28	0.21	0.16	P76 (-15)	0.73	0.38	0.28
P81 (-22, -15)	0.21	0.10	0.08	P81 (-22, -15)	0.71	0.30	0.24

3.5.4.2 Influence of the -15 region on the BgaB activity in *B. subtilis* and *E. coli*

Another set of mutations, located in the -15 region (between positions -20 and -13) was generated. It is known that the -15 region ($^{-20}$TTTATGTT^{-13}) shown to exert high level of expression in *E. coli* [89] as in P76 and P81 exhibited only a slight effect in *E. coli* and none in *B. subtilis* (P76 and P81; Tab. 3.4). Further studies identified sequence elements, including the dinucleotide TG at position -15 and -14 (designated the extended -10 region), which is the most highly conserved DNA sequence in promoters from gram-positive bacteria but not from *E. coli* [49, 57, 101]. In the present study, changes at position -15 and -14 of the dinucleotide CT (P70) to TG (P74) had an apparent effect in increasing the activity 15 times when compared to P74/P70 in *B. subtilis* and not at all in *E. coli* (Tab. 3.4). This result is in agreement with the dinucleotide TG at position -15 and -14 having a more pronounced effect on transcription in *B. subtilis* than in *E. coli* [59]. An A residue at the -16 position seems to be

more important in increasing the promoter strength in *B. subtilis* (5-fold increase; compare P73 and P74) than in *E. coli* (3-fold), ATG at the -16 to -14 region (P73) increased the activity 94-fold as compared to the core *groE* promoter P70, while moving the ATG region upstream of the -17 to -15 region in P75 resulted in an about 40-fold drop in the BgaB activity (compare P73 with P75; see the DNA sequence in Fig. 3.31 and the relative BgaB activity in Tab. 3.4). These results suggest that promoters with the -15 ATG extended region could enhance the *bgaB* expression more than 94-fold as compared to the wild-type core promoter of *groE*, and 8-fold higher than P_{grac}.

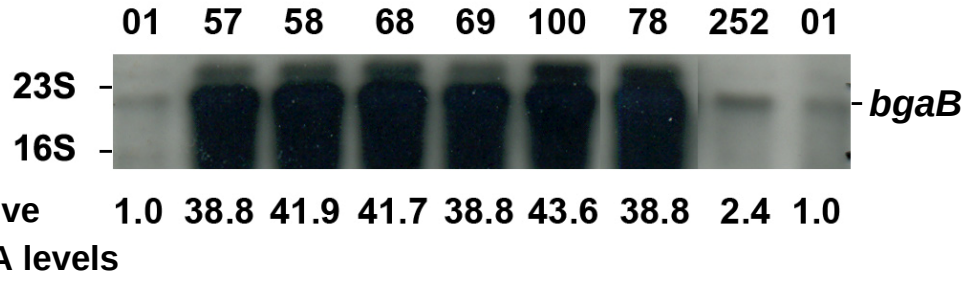


Fig. 3.34. Northern blot analysis of *bgaB* expression under control of different promoters. These promoters are derivatives of the wild-type *groE* promoter and carry either mutations of the core region or are combined with UP elements or both. Relative mRNA expression levels were calculated by QuantityOne programme (Biorad).

3.5.4.3 Analysis of combinations of the -10, the -15 and the -35 regions

The combination of the -35 or the -10 consensus sequence with the -15 ATG region resulted in promoters P57 and P58 (see Fig. 3.31 for sequences), and both of them were analysed by Northern blot (Fig. 3.34) and measurement of the BgaB activity (Tab. 3.4). The results revealed a 38- to 42-fold higher mRNA level when they were compared with P_{grac} (Fig. 3.34), and 12-fold higher BgaB activity (Tab. 3.4). The promoter with a combination of both the -35 and -10 consensus sequence with the -15 ATG region was named P79, and some derivatives called P80, P87, P88 and P89 (sequences in Fig. 3.31). When their relative BgaB activities were measured, they were 150 to 180 times higher as compared to P_{groE}^{WT} (P70) (Tab. 3.4). Clearly, the strong *groE* promoters include not only the TTGACA (-35) and TATAAT (-10) hexanucleotide core elements but also an ATG trinucleotide at position -16, -15, -14.

3.5.5 Combinations of UP elements and core promoters

The previous results already showed that a modified UP element of the *groE* promoter, (P64) enhanced expression of *bgaB*. Will this improved UP element further increase the activity of already strong promoters? To answer this question experimentally, this strong UP element was combined with the core promoter regions from P79, P78 and P57 resulting in P68, P69 and P100. The BgaB activities of these promoters are presented in Tab. 3.5, the amount of BgaB protein in Fig. 3.35, of the transcripts in Fig. 3.34. The new promoters did not significantly enhance *bgaB* expression when compared with P57, P78 and P79. This result indicates that a combination of a strong UP element and a strong core promoter does not further increase expression. A core promoter itself is sufficient to obtain high gene expression levels. However, the influence of the two new promoters P68 and P100 with the -15 ATG region revealed that the β-galactosidase activity levels could be increased up to 13-fold and the mRNA levels up to 43-fold as compared to the strong P_{grac} promoter. This corresponds to an about 690-fold increase when compared with the well-known P_{spac} promoter, and expression of *bgaB* under control of these promoters reached up to 30% of the total cellular protein (Fig. 3.35, P68, P100). I also checked for the production of another intracellular recombinant proteins (Pbp4*). Expression of *pbpE* under control of the promoter P100 reached up to 38% of the total cellular protein (Fig. 3.43). Those data also corroborated previous observations that the -15 region plays an important role in increasing the strength of the *groE* promoter.

Tab. 3.5. BgaB measurement of consensus promoters in *B. subtilis*.

Promoter	**Original core promoter**	**Relative BgaB activity**		
		0 h	**2 h**	**4 h**
P01 (*grac*-full)	P_{groE}	1.0	1.0	1.0
P68 (UP*, -35, -15, -10)	P_{groE} (P79)	9.8	12.3	15.8
P69 (UP*, -35, -10)	P_{groE} (P78)	10.3	10.0	13.0
P100 (UP*, -35, -15)	P_{groE} (P57)	10.8	14.8	16.0
P252 (UP*, -35, -15, -10)	P_{lepA} (P85)	1.5	1.1	1.2

UP*: UP element from P64 was used for this experiment.

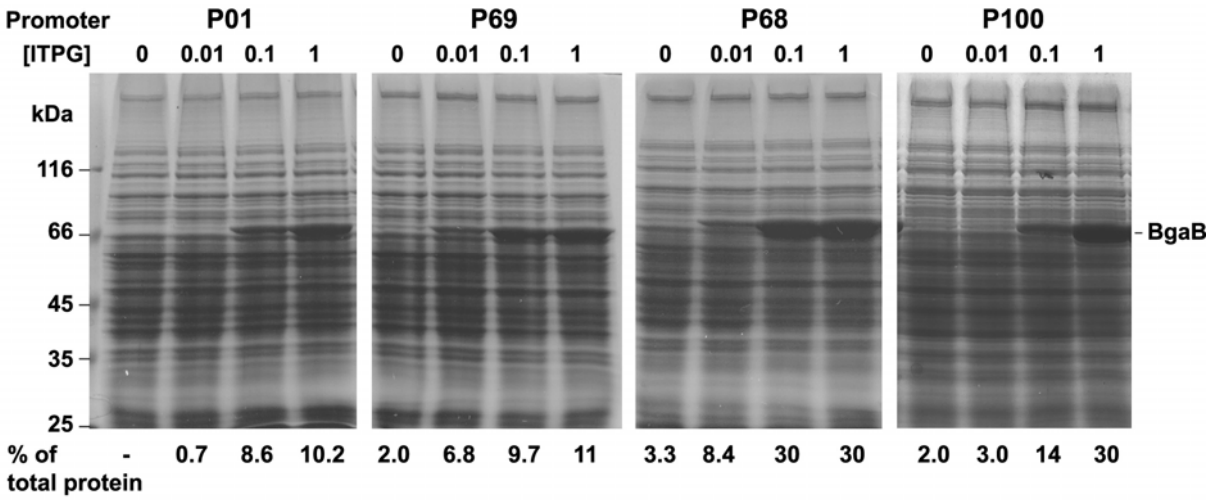


Fig. 3.35. Production of BgaB from different promoters. Different concentrations of IPTG were applied as indicated, and proteins were separated by 10% SDS-PAGE. The percentage of the expressed BgaB protein was calculated by AlphaEaseFC (Alpha Innotech) programme.

In case of the weak promoter *lepA*, even though the core promoter region of the *lepA* promoter (Fig. 3.31, P85) has a good -15 and -10 region ($^{-15}$TGA**TATAAT**$^{-7}$), the expression level is rather low when compared with the corresponding modified P_{groE} (Tab. 3.4, P72 and P74). Promoter P252 was constructed by a combination of a strong UP element, the -35 and -15 region of P100 and the core *lepA* promoter. This new promoter improved the *lepA* promoter about 5-fold when compared to P85 (Tab. 3.5) at 0.01 mM IPTG, and the mRNA levels increased up to 2.4-fold as compared to the strong P_{grac} promoter (Fig. 3.34). However, when the BgaB activity induced with 1 mM IPTG was determined with P252, it turned out to be 60 times higher than that expressed from P85 and P_{lepA} wild-type (data not shown) as in the case of P_{grac} (Fig. 3.32).

3.6 Using stabilizing elements to enhance the protein expression level in *B. subtilis*

In order to improve the productivity of *B. subtilis*, efforts have focused on the development of protease deficient strains [170] and the identification of efficient regulatory elements at the transcriptional (see in Results part at 3.5), the translational and the protein secretion levels [172], but the potentiality of transcript stabilization has hardly been investigated [32]. Decay of mRNA plays an important role in gene expression control. The main factors affecting mRNA stability in bacteria are translation initiation frequency, codon usage and RNA secondary structures. Efficient translation initiation and consequent immediate ribosomal protection from degradation stabilizes the mRNA and is achieved by selection of ribosomal

binding sites lacking inhibitory secondary structural elements. Stable hybrid mRNAs might be constructed by implementation of efficient 5' and 3' stabilizing sequences as a barrier against exonucleases [124, 150].

In this work, mRNA stabilizing elements were also analyzed by using a similar experimental approach. First, 17 different 3'-mRNA terminal stem-loops have been investigated. Second, the 5'-mRNA stabilizing elements including a strong RBS, the *lacO* controllable stabilizing element (CoSE) and the spacer between RBS and CoSE were examined (Fig. 3.36). The results demonstrated that the CoSE together with an appropriate spacer and a strong RBS could increase the protein expression level 9-fold as compared to the P_{grac} promoter, resulting in up to 26% of the total cellular protein and a half-life of the mRNA of more than 60 min. A combination of strong promoters and stabilizing elements revealed that recombinant protein expression levels up to 42% of the total cellular protein could be obtained.

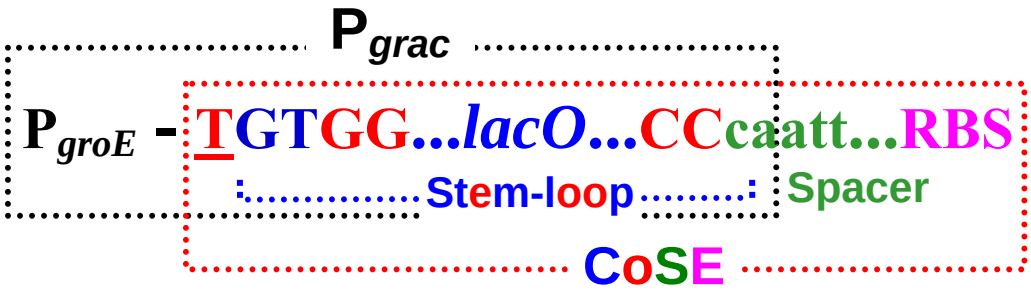


Fig. 3.36. Genetic features of the P_{grac} promoter.

3.6.1 3'-mRNA terminal stem-loops

Protection of the 3' end has been less well studied for *B. subtilis* than for *E. coli*. *E. coli* transcription terminators and other stable secondary structures inhibit the progress of both PNPase and RNase II. The *cryIAa* transcription terminator serves as a stabilizer of exonucleases heterologous RNAs in *B. subtilis* [169], suggesting that this phenomenon may be shared by both organisms even though PNPase is the only major exonuclease they have in common [28]. To further examine the specificity of the enhancement effect of the *trpA* terminator fragment at the 3' end, plasmid pHT36 was constructed by removing the *trpA* transcriptional terminator from pHT01-*bgaB* (see in 2.7.6, Fig. 2.5 for genetic features, and Fig 3.37 for the pedigree of plasmids). Then, terminal stabilizing elements were introduced into pHT36 at the *Aat*II restriction site, and the cells were cultured for the measurement of β-galactosidase activity (Tab. 3.6). When the OD_{578} reached to 0.8, 0.1 mM IPTG was added and cells further incubated for 30 min. Then, rifampicin was added and samples were taken

just before addition of rifampicin (0) and 5, 10, 15, 20 30, 45 and 60 min thereafter. Total RNA was prepared, separated through an 1.2% agarose gel, blotted and probed with anti-*bgaB* RNA. Densitometric quantification was performed using the QuantityOne programme. The mRNA half-life was calculated by setting the control value (t = 0) to 100% (Fig. 3.38 and Tab. 3.6).

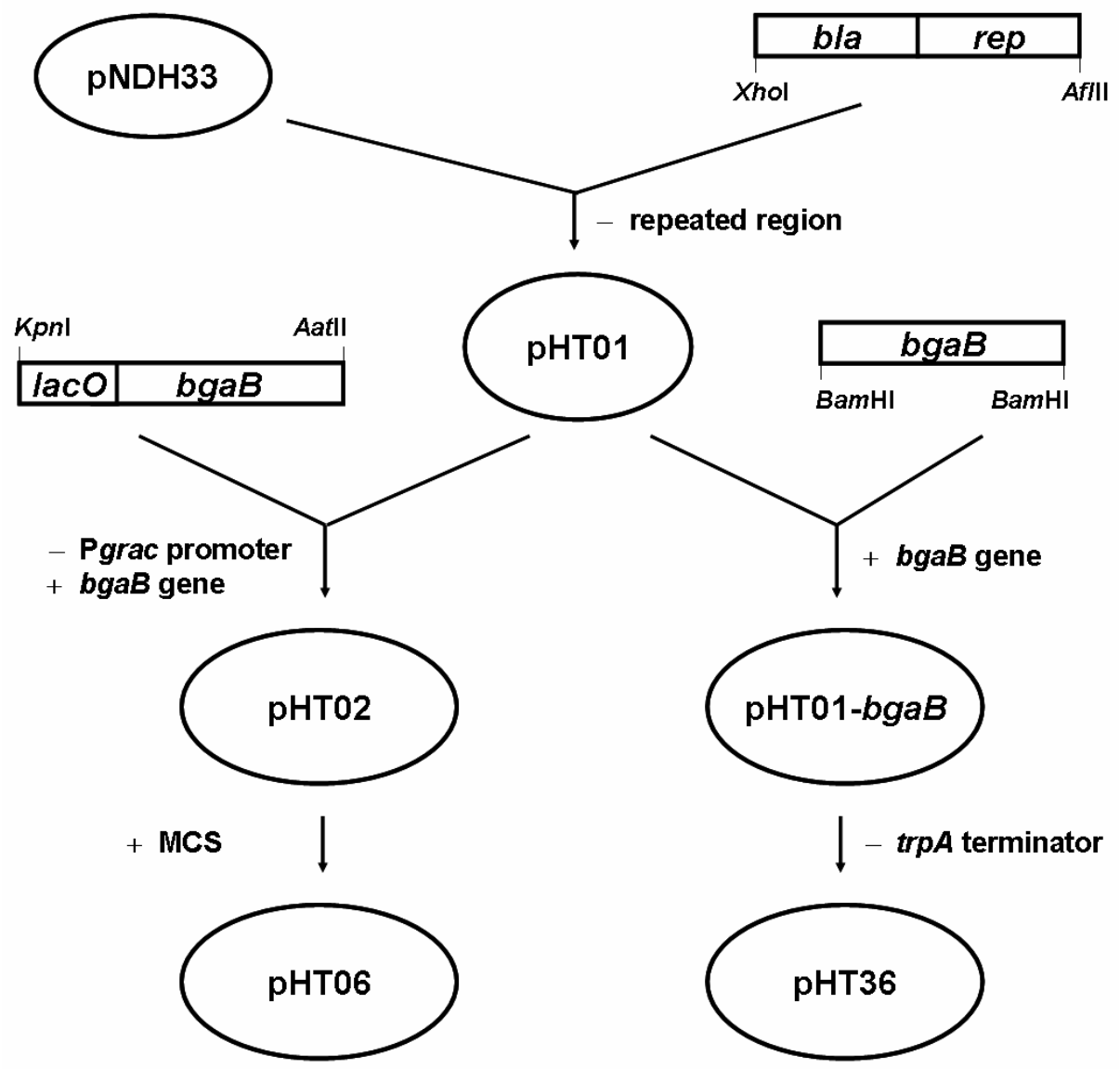


Fig. 3.37. Relation between the plasmids pNDH33, pHT01, pHT06 and pHT36. -, removal of the repeated 117-bp region; P_{grac} promoter or *trpA* terminator; +, addition of *bgaB* gene or MCS.

Tab. 3.6. Sequences of 3'-mRNA terminal stem-loops and their influence on mRNA stability and BgaB activity in *B. subtilis*.

Name	Origine	ΔG kcal/mol	Relative BgaB activity		Sequence of the 3'-mRNA terminal stem-loops
			2 h	4 h	
S01	*trpA*^{WT}	-15.7	1.00	1.00	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUUUUUUC
S36	*orf-3*	-13.8	0.71	0.58	CAACAAAGGCUGAGACAGACUCCAAACGAGUCUGUUUUUUUAA
S110	*skfA*	-23.3	1.07	0.96	GACGUCUUUGAGAAUAGGGAGUUGAGCGUAUUUGCUUAUACUCCUUAUUUUCUUUUUUUU
S111	*	-21.5	1.00	0.92	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCUUUUUC
S112	*	-24.3	0.83	0.84	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCCUUUUUC
S113	*	-27.1	0.79	0.81	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCCCUUUUUC
S114	*	-29.9	0.78	0.79	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCCCCUUUUUC
S115	*trpA*^{WT}	-15.7	0.95	0.98	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUUUUUUC
S116	**	-20.5	0.78	0.65	GACGUGAAAAAAGCCCGCUCAUUAGGCGGGCUGCCCCGGGGACGUC
S117	*	-26.2	0.99	0.82	GACGUCCCCGGGCAGCCCGCCUAAUGAGCGGGCUGCCCCUUUUUC
S118	*	-27.7	0.82	0.83	GACGUCCCCGGGGCAGCCUGCCUAAUGAGCGGGCUGCCCCUUUUUC
S119	*	-27.1	1.22	1.35	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCCCUUUC
S120	*	-27.1	1.17	1.20	GACGUCCCCGGGGCAGCCCGCCUAAUGAGCGGGCUGCCCUUC
S121	*	-30.0	0.91	0.74	GACGUCUAGCCCGGGUAAGCUUCGGCCGAGCCCGCCUAAUGAGCGGGCUCGGCCUUUUUC
S122	*	-30.0	0.91	0.94	GACGUCUAGCCCGGGUAAGCUUCGGCCGAGCCCGCCUAAUGAGCGGGCUCGGCCUUUC
S123	*htpG*	-23.0	0.72	0.68	GACGUCAAAAAGGAAUCGUCUCAUAAGGAGACGAUUCCUUUUUUUC
S124	*dnaK*	-20.0	0.75	0.89	GACGUCAGAAAGUCAAAGUCAGGCAUCUCUUGGCUUUGACUUUUUUUC

S36 were generated by removal of *trpA* termininator from S01 (plasmid pHT01) and the terminator of the transcriptional unit is at the end of *orf-3* on plasmid pHT01. All stabilizing elements (terminators), from S110 to S124 were introduced into plasmid pHT36 at *AatII* site (underline). *trpA*^{WT}, wild type *E. coli trpA* terminator; *, modification of *E. coli trpA* terminator; **, reversed complement of S115. *skfA*, stem-loop between *B. subtilis skfA* and *skfB* with additional of poly T. *htpG* and *dnaK* are terminators of *B. subtilis htpG* and *dnaK* genes. The free energy was calculated by RNAfold programme at 37°C.

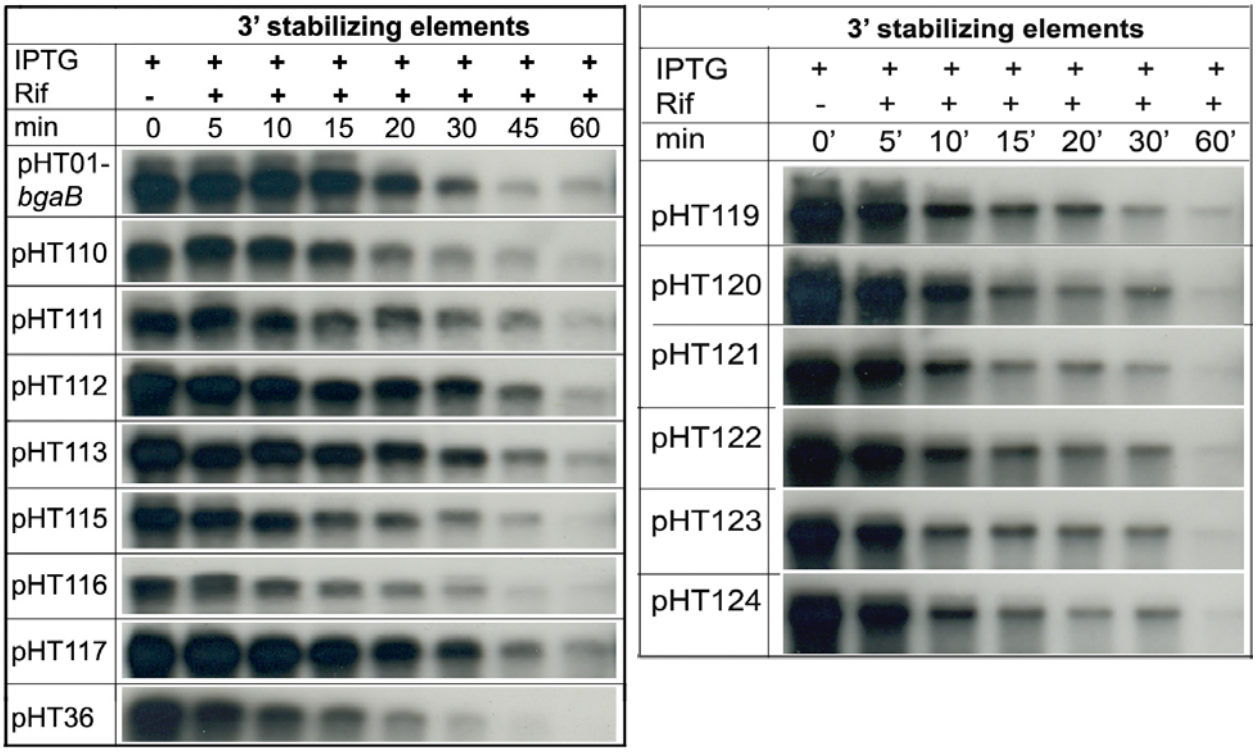


Fig. 3.38. Rifampicin experiment for determination of mRNA stability. The plasmid pHT36 carries different transcription terminators. The cells were cultured in LB medium at 37°C, and when an OD_{578} of 0.8 was reached, 0.1 mM IPTG was added and the incubation continued for 30 min. Then rifampicin (100 μg/ml final concentration) was added. Total RNA was prepared at the time points indicated and probed with anti-*bgaB* RNA. Densitometric quantification was performed using the QuantityOne programme (Biorad). The mRNA half-life was calculated by setting the value of control (t=0) to 100%.

The marked differences between the plasmid pHT01-*bgaB* (S01) and pHT36 (S36) presented in Fig. 2.5, in which S01 contains the *E. coli trpA* terminator and a truncated *orf-4* (function unknown) [156], while these sequences are absent from S36. The expression level from S36 was slightly lower than that from S01 (Tab. 3.6 and Fig. 3.38). S01 and S115 use the same *E. coli trpA* terminator, but S115 does not contain the truncated *orf-4*. The expression levels of these two constructs were comparable. These results indicate that the *E. coli trpA* terminator slightly influenced both the BgaB activity and mRNA stability.

The data in Tab. 3.6 indicate that lowering the free energy in S113 and S121 resulted in a slight reduction of the BgaB activity. However, after removal of some T-residues (Tab. 3.6; S119, S120 and S122), the BgaB activity increased. It follows that leaky transcription of the

downstream genes, *orf-3* (function unknown) and *orf-1* (Fig. 2.5), will increase BgaB expression. This phenomenon might explain the result of leaky expression of *orf-1*, which is supposed to code for RepA, and by increasing the amount of RepA the copy number of the plasmid is increased, too [156, 157]. The *orf-1* product (RepA) displays homology to DnaA chromosomal initiators and carries a DNA binding (HTH) motif and the intergenic region encompasses repeated sequences (iterons) and a *dnaA* box [156, 157]. It has been proposed that binding of RepA to the iterons is a prerequisite for initiating plasmid replication [33]. One might exploit this characteristic of the plasmid to construct a copy-number control regulated expression plasmid for *B. subtilis* like the *E. coli* pBAC/*oriV* vector [165].

In general, the expression levels of *bgaB* with differently modified *trpA* stem-loop terminators or some natural stem-loop terminators of *skfA*, *htpG* and *dnaK* were similar. Decreasing or increasing the free energy of stem-loop structures did not increase significantly the BgaB activity (30% increase or decrease), and the average half-life of the *bgaB* transcripts is 30 min, similar to S01 from pHT01-*bgaB*. In summary, data from these experiments do not recommend using these 3'-mRNA terminal stem-loop structures to stabilize mRNAs for enhancing protein production.

3.6.2 The 5' stem-loop structure (*lacO* stabilizing element)

It is known that the 5'-end of transcript plays an important role as a stabilizing element [15, 16, 38, 90, 146]. It was asked whether the *lacO* operator can be used to both to control expression of recombinant genes and to act as a 5' stabilizing element. The *lacO* of P_{grac} (Fig. 3.36) was used for further modifications. The different promoters with modification of the *lacO* stem-loop structure were inserted into the promoter-probe plasmid pHT06.

To increase the free energy (ΔG) of the *lacO* stem-loop structure, I modified some nucleotides outside of the *lacO* terminus resulting in S102 (ΔG = -6.3) and S103 (ΔG = -2.8) (S01 from P_{grac}; ΔG = -9.8) (Fig. 3.39 and Tab. 3.7). The results showed that a higher free energy of the stem-loops in S102 and S103 lowered the BgaB activity and reduced the mRNA stability (Tab. 3.7 and Fig. 3.39). As mentioned before, a high GC at the +1 region decreased the BgaB activity (Fig. 3.9 and Tab. 3.7, S106, S107, S108 and S109) due to reduction of the amount of mRNA molecules (data not shown), though the mRNA stability of S109 increased up to 60 min. Keeping ΔG around - 9.8 in the cases of S201, S205, S228 and S229 did not change the BgaB expression level significantly. In contrast, the *bgaB* mRNA stability was decreased to 13.9 min (S201) and 18.7 min (S205), and kept at about the same level in the case of S1 (29.8

min), S228 (26 min) and S229 (30 min) (Fig.3.39, Tab. 3.7). This result indicates that there might be interferences between the *lacO* stem-loop and the stabilizing RBS.

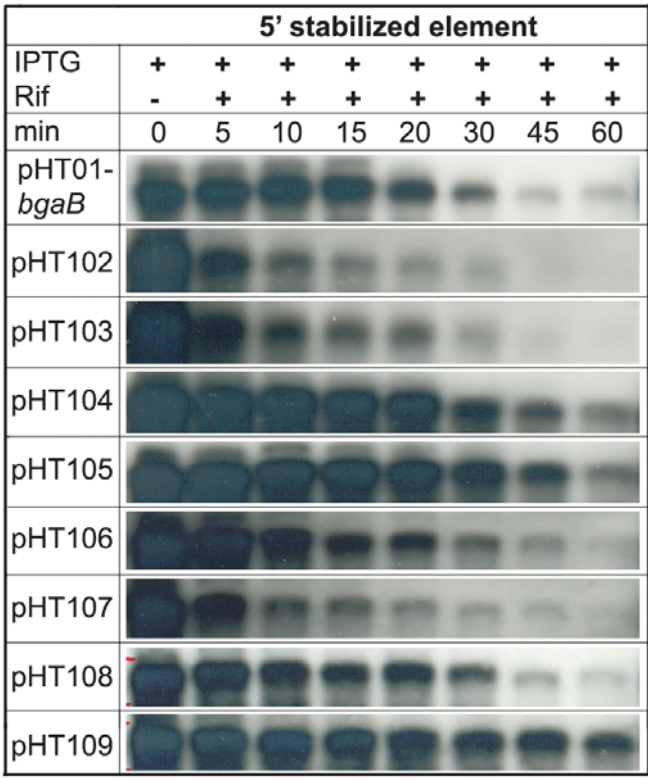
Based on published data [146], a decrease in free energy of the stem-loop at the 5'-terminus should increase the mRNA stability. Addition of some nucleotides outside *lacO,* S104 (-12.9), S105 (-16.4), S203 (-14.1), S207 (-16.9), S208 (ΔG = -16.9) and S209 (-16.1) turned out to increase the mRNA stability from 32 to 60 min. The stabilizing elements S104, S105, S203, S209 could increase the BgaB activity 1.5- to 2.8-fold as compared to S01, while S207 and S208 exerted the same influence as S01 (Tab. 3.7). Unexpectedly, S202 (ΔG = -14.4) reduced both the mRNA (21 min) and the BgaB (0.7) expression level (Tab. 3.7, S202). This result indicates that increased mRNA stability is not always accompanied by an increase in the BgaB expression level, which reinforces the assumption that there is an influence between the stem-loop and the RBS leading to a lower BgaB expression level. On the other hand, analysis of modified nucleotides inside *lacO* to decrease the free energy of the stem-loop as seen with S206 (ΔG = -13.7) and S211 (ΔG = -11) did not increase the mRNA stability, but the BgaB activity was enhanced more than 2-fold as compared to the P_{grac} promoter (Tab. 3.7). The modification inside *lacO* especially draw my attention because of a lower background level (data not shown) as compared to the other sequences, and it will be easy to decrease the free energy of the stem-loop by addition of other nucleotides outside *lacO* for further study of protein expression.

It can be concluded that a lower free energy of the *lacO* stem-loop at the 5'-terminus of transcripts resulted in a higher mRNA stability, even there were some exceptions, but the BgaB activity is still not very high. This result suggests a putative interference between the stem-loop structure and the stabilizing RBS element. I decided to investigate the interference between the strong RBS and the spacer region between the *lacO* stem-loop and the RBS. Generally, the promoters S104, S105, S206, S209 and S211 could be used for further investigations. However, only the promoters S206 and S211 have been used for further modifications of *lacO* because they revealed a lower background and the possibility to decrease the free energy below -16.6, while avoiding too many GCs at the transcriptional start site region.

Tab. 3.7. Sequences of 5'-mRNA stabilizing elements and their influence on mRNA stability and BgaB activity in *B. subtilis*

Name	ΔG kcal/mol	Half-life of mRNA (min)	Relative BgaB activity		Stem-loop sequence	Spacer and RBS
			2 h	4 h		
S01	-9.8	29.8	1.00	1.00	UGUGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S102	-6.3	14.9	0.38	0.49	UGUGGAAUUGUGAGCGGAUAACAAUUC	-----AAUUAAAGGAGG
S103	-2.8	16.4	0.74	0.90	UGUGGAAUUGUGAGCGGAUAACAAUU	-----AAUUAAAGGAGG
S104*	-12.9	**49.0**	**2.75**	**2.79**	AGGGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S105	-16.4	**62.9**	**2.29**	**1.77**	AGGGGAAUUGUGAGCGGAUAACAAUUCCCC	--AAUUAAAGGAGG
S201	-10.5	13.9	0.91	1.02	UGUGGAAUUGUGAGCGGAUAACAAUUCCA	---AAUUAAAGGAGG
S202	-14.4	21.1	0.70	0.73	UGUGGAAUUGUGAGCGGAUAACAAUUCCACA	-AAUUAAAGGAGG
S203	-14.1	39.0	1.57	1.49	AGUGGAAUUGUGAGCGGAUAACAAUUCCACA	-AAUUAAAGGAGG
S205	-8.7	18.7	1.18	1.18	AGUGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S206	-13.7	**27.4**	**2.33**	**2.47**	AGUGGAAUUGUGAGCGGAUCACAAUUCCC	---AAUUAAAGGAGG
S207	-16.9	36.4	1.09	1.03	UGGGGAAUUGUGAGCGGAUAACAAUUCCCC	--AAUUAAAGGAGG
S208	-16.9	46.1	0.91	1.06	UGGGGAAUUGUGAGCGGAUAACAAUUCCCC	--AACUAAAGGAGG
S209	-16.4	**41.1**	**2.12**	**2.71**	AGGGGAAUUGUGAGCGGAUAACAAUUCCCC	--AACUAAAGGAGG
S211	-11.0	**31.9**	**2.25**	**2.24**	UGUGGΔAUUGUGAGCGGAUCACAAUUCCC	---AAUUAAAGGAGG
S228*	-9.0	26.0	1.20	1.21	AGCGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S229*	-9.8	30.0	0.99	0.98	UGCGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S106*	-9.0	30.8	0.34	0.39	ACCGCGGAAUUGUGAGCGGAUAACAAUUCCC	---AAUUAAAGGAGG
S107	-15.7	15.6	0.20	0.21	ACCGCGGAAUUGUGAGCGGAUAACAAUUCCGC	--AAUUAAAGGAGG
S108	-17.5	29.4	0.36	0.44	UCCGCGGAAUUGUGAGCGGAUAACAAUUCCGCG	-AAUUAAAGGAGG
S109	-21.0	60.8	0.35	0.54	ACCGCGGAAUUGUGAGCGGAUAACAAUUCCGCGGAAUUAAAGGAGG	

All stabilizing elements were fused with the promoter *groE*. The free energy was calculated by the RNAfold programme at 37°C. Relative BgaB activities were compared with those measured with pHT01-*bgaB* which was set as 1. Densitometric quantifications were performed using the QuantityOne programme. The mRNA half-life was calculated by setting the control value (t=0) to 100%. *, Construction of plasmid is described in 3.5.



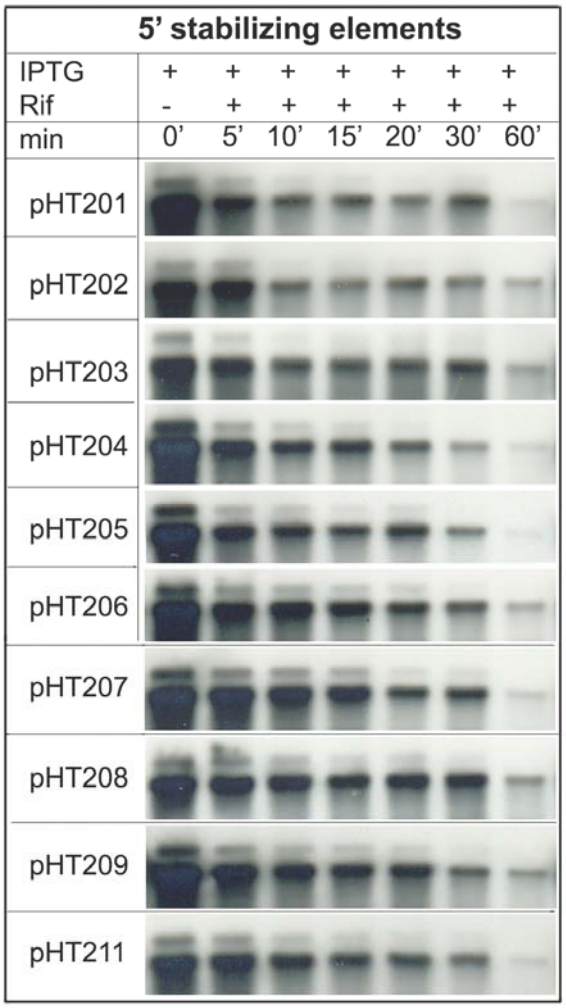


Fig. 3.39. Rifampicin experiment for determination of mRNA stability. All *lacO* variants have been inserted into plasmid pHT06. The cells were grown and treated as described in the legend to Fig. 3.38.

3.6.3 Influence of a strong RBS

It has been reported that a strong RBS could function as an mRNA stabilizing element and enhance the protein expression level [7, 8, 32, 44, 45, 53, 54, 74, 134, 145, 146]. The strong *B. subtilis gsiB* RBS [74] was evaluated again in this work. The promoter *groE* and *lepA* were fused with the strong *gsiB* RBS, then the recombinant promoters were introduced to pHT06, in which *lacO* was removed. I was unable to construct promoters S219 and S220 (Tab. 3.8) and other strong promoters (not shown), indicating that P_{groE} was already a very strong promoter in *E. coli*, which causes lethal overproduction of BgaB when combined with a strong RBS. This result also indicates that when using stabilizing elements (RBS and 5'-stem-loop structures), a strong promoter must be controlled to facilitate cloning in *E. coli* and expression later in *B. subtilis*. This could explain the failure to use known stabilizing elements

for overproduction of recombinant protein. To study controllable stabilizing elements for *B. subtilis* including strong RBSs for overproduction of proteins is the main task of part 3.6.3.

When the wild type RBS of *lepA* was replaced by the strong *gsiB* RBS, the BgaB activity was more than 100-fold higher, and the BgaB protein reached about 2.8% of the total cellular protein (Fig. 3.42), while it was undetectable when synthesized from the *lepA* RBS (data not shown). In addition, replacement of the RBS increased the half-life of the *bgaB* transcript from 6 to 17 min (Fig. 3.41). These findings are in agreement with the observation that the stability of the *gsiB* mRNA depends on the strong RBS [74]. It further indicates that the *gsiB* RBS can be used to enhance the stability of the *bgaB* transcript significantly and most probably of any transcript.

Tab. 3.8. BgaB activities and half-lifes of *bgaB* mRNA in *B. subtilis* equipped with different RBS sequences.

Name	**Original promoter**	**Relative BgaB activity**		**Half-life of *bgaB* mRNA (min)**
		2 h	**4 h**	
S219	P_{groE}	-	-	-
S220	P_{groE}	-	-	-
lepA (S02)	From pHCMC02	< 0.02	< 0.02	6.04
S221	P_{lepA}	2.1	3.97	17.12
Name	**RBS**	**Sequences from transcriptional start site to RBS**		
S219	(P_{groE}) *groE*^{WT} RBS	TGTGACTATTG**AGGAGGT**TGGATCC		
S220	(P_{groE}) *gsiB* RBS	TGTGAAATT**AAAGGAGG**AAGGATCC		
lepA (S02)	(P_{lepA}) *lepA*^{WT} RBS	TAGTGTATTTTGCGTTTAATAGT**AGGAG**TGAGGATCC		
S221	(P_{lepA}) *gsiB* RBS	TAGTGTATTTTGCGTTTAATT**AAAGGAGG**AAGGATCC		

The sequence S02 corresponds to the wild-type *lepA* promoter-RBS of pHCMC02 [104]. This table gives the names of the promoters and of the RBSs used. Potential transcriptional start sites and RBS are underlined. *B. subtilis* cells were induced with 1 mM IPTG.

3.6.4 Influence of the spacer length between the *lacO* stem-loop and the RBS

From the results shown in 3.6.2, and 3.6.3, it can be concluded that the spacer length between the *lacO* operator and the RBS is an important factor, which contributes to mRNA stability and overexpression of recombinant proteins. To screen for the optimal spacer, two DNA sequence sets were analysed one based on S202 (S214 and S215) and the other on S211 (S213

and S210) using a *lacO* sequence with a free energy ΔG of about -14.5. Interestingly, the spacer did not only influence the mRNA stability, but the *bgaB* expression level (Tab. 3.9 and Fig. 3.40). Spacer lengths of 13, 19 and 29 nucleotides were tested to optimize BgaB expression. A spacer of 19 nucleotides (S213) seems to be optimal for the production of BgaB, in that the BgaB activity could be increased up to 9-fold as compared to the original spacer and the half-life of the mRNA was enhanced to more than 60 min (Fig. 3.40).

The two promoters P212 and P01 are identical, but differ in their stabilizing elements including the stem-loop and the spacer region. With P212, the BagB protein accumulated to about 30% of the total cellular protein as compared to about 10% with P01 (Fig 3.42). These data indicate that spacers of 13 to 29 nucleotides located between *lacO* and RBS greatly enhanced the stability of the mRNA and the protein expression level.

Tab. 3.9. Sequence of stabilizing elements with different spacer lengths.

Name	ΔG	Sequence of controllable stabilizing elements
S202	-14.4	UGUGGAAUUGUGAGCGGAUAACAAUUCCACAaauuAAAGGAGG (Spacer 4)
S214	-14.6	UGUGGAAUUGUGAGCGGAUAACAAUUCCACAaccaacaccaauuAAAGGAGG (Spacer 13)
S215	-14.6	UGUGGAAUUGUGAGCGGAUAACAAUUCCACAacaacaaccaacaccaauuAAAGGAG (Spacer 19)
S212	-14.9	UGUGGΔAUUGUGAGCGGAUCACAAUUCCACAaccaacaccaauuAAAGGAGG (Spacer 13)
S213	-14.9	UGUGGΔAUUGUGAGCGGAUCACAAUUCCACAacaacaaccaacaccaauuAAAGGAG (Spacer 19)
S210	-14.9	UGUGGΔAUUGUGAGCGGAUCACAAUUCCACAacaacuagauucuaucaauucaaccaauuAAAGGAGG (Spacer 29)

S202 was used to construct S215 and S215; S211 was used to construct S212, S213 and S210.

3.6.5 Combinations of strong stabilizing elements with different promoters

The strong controllable stabilizing elements (CoSE) S212 and S213 were fused to the two weak promoters P_{lepA} (resulting in P224) and P_{spac} (P222) and the two strong promoters P69 (P223 and P225) and P100 (P250) (see description in Tab. 3.10). To assay for functionality of CoSE, the combinations of CoSEs and promoters were inserted into the promoter-probe plasmid pHT06. Next, these recombinant plasmids were transformed into *B. subtilis* 1012, and the BgaB activities were measured (Tab. 3.10). While the activity was extremely low in

the absence of CoSE, it increased 45-fold when P_{lepA} and P_{spac} were combined with CoSE S212 (P224 and P222) as compared to P_{lepA} (in pHCMC02) and P_{spac} (in pHCMC05). The half-life of the mRNAs also increased (Tab. 3.10 and Fig. 3.41, P_{lepA}/P224 and P_{spac}/P222). The expression level of the BgaB protein under control of P224 (P_{lepA}-CoSE) reached up 7% of the total cellular protein (with did not result in the appearance of any additional band with a molecular mass of BgaB, in the case of P_{lepA}) (Fig. 3.42, S224).

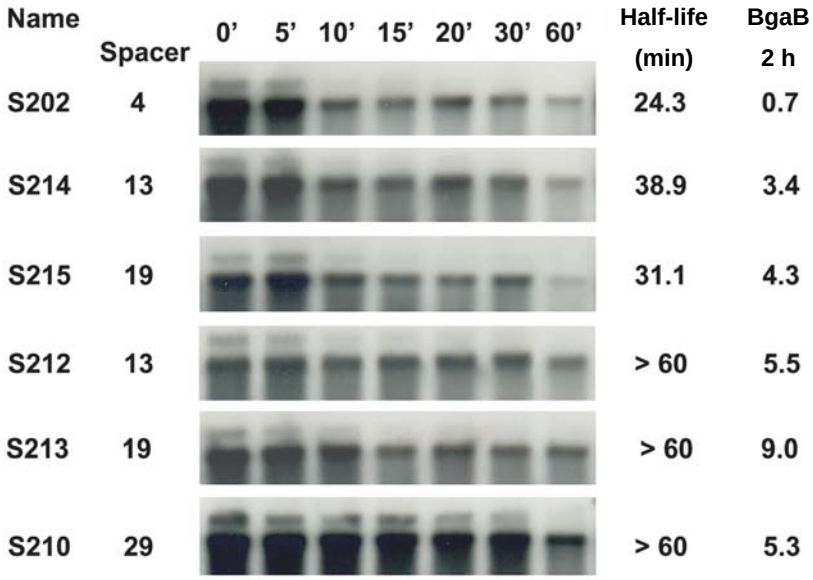


Fig. 3.40. Rifampicin experiment for determination of mRNA stability and relative BgaB activity. The plasmid pHT06 carried the different promoters with modifications of the spacer length between *lacO* and RBS. The cells were grown and treated as described in the legend to Fig. 3.38.

The products expressed from the three recombinant promoters P223, P225 and P250 were analysed. The BgaB activity was enhanced 2- to 3-fold as compared to the original promoters (P68 and P100) and to a more than 23-fold level as compared to strong promoter P_{grac} (Tab. 3.10). I also looked directly for the production of the BgaB reporter protein using the different recombinant promoters P223 and P250, and compared them to P01 (P_{grac}^{WT}) and P212 (P_{groE}^{WT}-CoSE). As shown in Fig. 3.42, P250 led to the production of BgaB up to 35.5% of the total cellular protein, and P223 could even overproduce BgaB up to 42% of the total cellular protein. While BgaB protein produced from the strong P_{grac} in P01 produced 9.2%, P_{groE}^{WT}-CoSE (P212) produced 26.7%, P68 and P100 (see in Fig 3.35) 30% of the total cellular protein. These results strongly indicate that CoSE could help to enhance significantly both mRNA stability and the protein level.

Tab. 3.10. Combination of stabilizing elements with different promoters (P_{spac}, P_{lepA}, P69 and P100).

Promoter	Description	Relative BgaB activity		Half-life
		2 h	4 h	
P01	P_{grac} in pHT01-*bgaB*	1.0	1.0	29.4
P_{lepA}	From pHCMC02	< 0.02	< 0.02	6.04
P224	P_{lepA} with S212	0.67	0.89	> 60
P_{spac}	From pHCMC05	< 0.02	< 0.02	5.3
P222	P_{spac} with S212	0.48	0.92	20.9
P69	P69 (UP*, -35, -10)	10.0	13.0	
P223	P69 with S212	21.02	33.01	36.8
P225	P69 with S213	23.63	41.60	32.9
P100	P100 (UP*, -35, -15)	14.8	16.0	
P250	P100 with S212	22.53	34.55	> 60

UP*: The UP element was modified from P64; -35, -10, -15, and the core promoter was modified at the -35, -15 or/and -10 region.

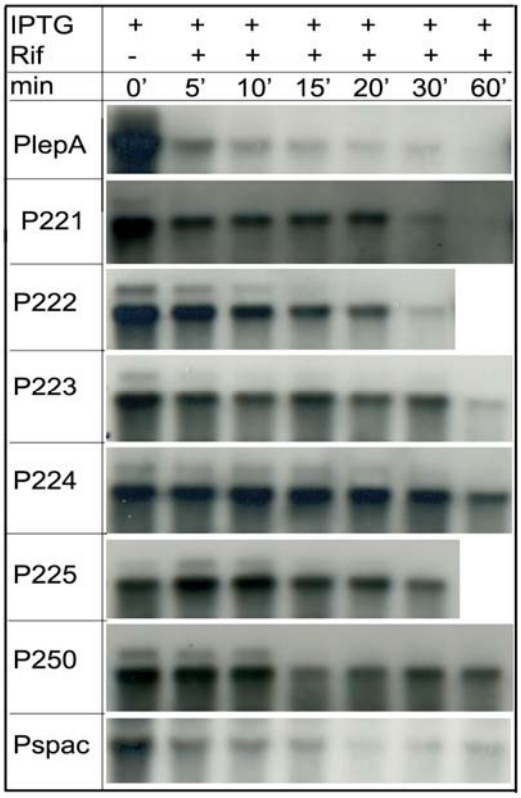


Fig. 3.41. Rifampicin experiment for the determination of mRNA stability. The plasmid pHT06 carried different promoters. The cells were grown and treated as described in the legend to Fig. 3.38.

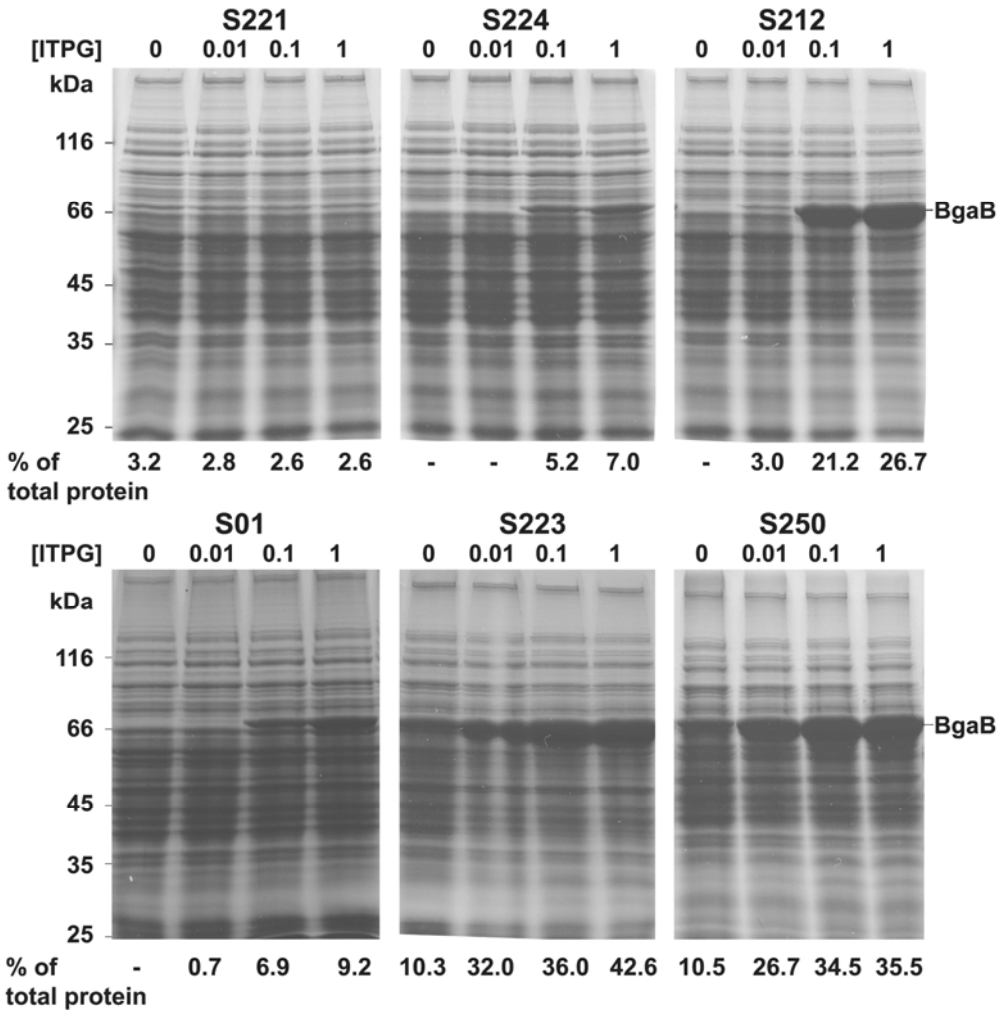


Fig. 3.42. SDS-PAGE of some plasmids with stabilizing elements or recombinant promoters. Different concentrations of IPTG were applied as indicated. The percentage of the produced BgaB in total cellular protein was calculated by the AlphaEaseFC programme (Alpha Innotech).

To analyse expression of two additional genes, *pbpE* and *htpG* were fused to the strong promoter P250, carrying the stabilizing element S250. Gene *pbpE* was additionally fused to P100 to allow a comparison. Expression from these promoters result in up to 38%, 28% and 26% recombinant protein of total cellular protein (Fig. 3.43). This result indicates that those promoters and stabilizing elements can be used not only for BagB, but also for other proteins.

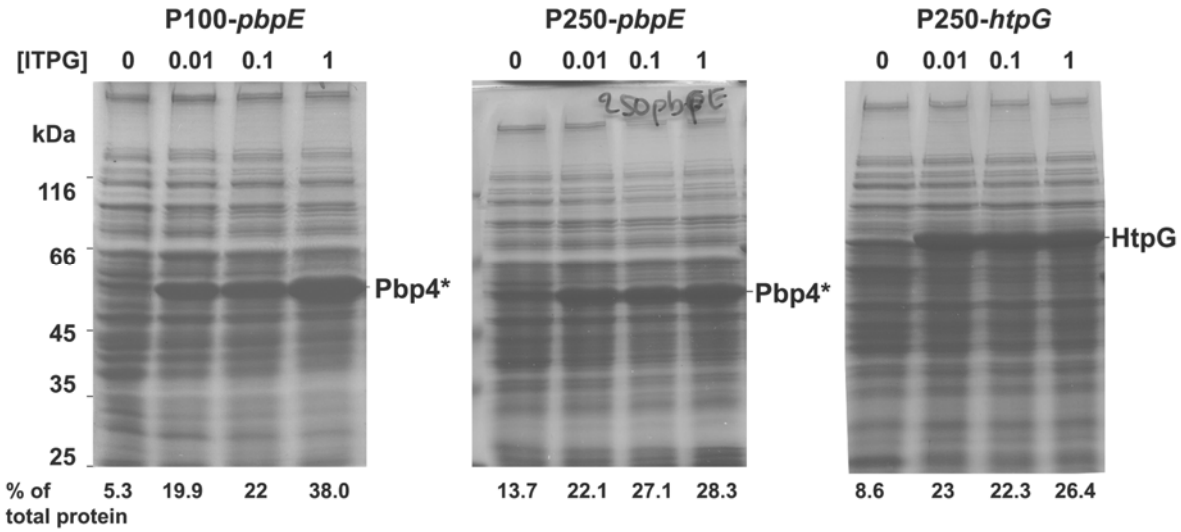


Fig. 3.43. SDS-PAGE of *pbpE* and *htpG* fused with promoters P100 and P250. Different concentrations of IPTG were applied as indicated. The percentage of the expressed recombinant proteins Pbp4* and HtpG was calculated by the AlphaEaseFC programme (Alpha Innotech).

4. Discussion

4.1 Riboswitches in expression systems

Expression systems based on strong and tightly regulated promoters play an important role in modern biotechnology (reviewed recently in [97, 163]). The first inducible system for *B. subtilis* was described by Yansura and Henner [173] based on the artificial *spac* promoter which is under the negative control of the LacI repressor. Subsequently, induction systems have been constructed based on sucrose [178], phosphate-starvation [87], T7 polymerase [29], xylose [79], stationary phase [70] and citrate [40] as inducer. From all of these different systems, only the IPTG- and the xylose-inducible ones are widely used. The advantages and disadvantages of these different expression systems would be discussed elsewhere [144]. New alternative systems allowing both intra- and extracellular expression are being searched for, which should be easy to use, very efficient and inexpensive. Two of them use the amino acids L-glycine and L-lysine as inducer.

4.1.1 The glycine system

It has been suggested that about 2% of the *B. subtilis* genes are regulated via riboswitches [93]. If *B. subtilis* cells grow in the presence of a high L-glycine concentration, they activate expression of the *gcv* operon whose enzymes degrade L-glycine to 5-10-methylene-tetrahydrofolate, ammonia and CO_2 [78]. Activation of the *gcv* operon occurs by L-glycine itself, which binds to a tandem riboswitch as first suggested and then experimentally proven [5, 93]. Based on this mechanism, we asked whether it can be converted into an expression-secretion system for *B. subtilis* and related species. In a first experiment, Northern blot was used to prove that indeed only a short transcript, the attenuation product, was synthesized in the absence of added L-glycine corresponding to the 5' UTR. Upon increasing the L-glycine concentration, full-length transcripts were present already after 5 min. These data fully confirm the *in vitro* data of Mandal and coworkers [93] that a low L-glycine concentration leads to transcription attenuation due to the formation of a rho-independent terminator. Most interestingly, the attenuation product did not disappear after L-glycine induction. An extended half-life of this short transcript could be excluded by the rifampicin experiment, as well as its production by processing. But processing indeed occurs, and seems to increase the stability of the 3' terminal product coding for the three enzymes. The question remains what might be the function of the riboswitch RNA under inducing conditions? Two possibilities were envisaged, not mutually exclusive. First, the riboswitch RNA could be translated. Indeed, a reasonable

Shine-Dalgarno sequence can be deduced from its primary sequence (AAGGAG) followed by an open reading frame encoding a potential 25-residues peptide. If this peptide is really synthesized, it might exert a regulatory role as described, e.g., for phage λ CIII and *B. subtilis* SpoVM peptides, which both inhibit the proteolytic activity of the ATP-dependent metalloprotease FtsH [31, 60]. Another example are the pentapeptides encoded by the *phr* genes, which inhibit their cognate phosphatases [115]. Second, the riboswitch RNA could act as a nc (non-coding) RNA. More than 60 ncRNAs have been described in *E. coli*, where most of them exert a regulatory function [46, 151].

These results suggest that the riboswitch regulatory region can be used to express recombinant genes. To evaluate its potential, the *lacZ* reporter gene was fused to the riboswitch. The *lacZ* gene offers a simple and fast way to measure promoter strength. First I investigated which L-glycine concentration yielded full induction. It turned out that 10-50 mM L-glycine resulted in an up to 6-fold increase in the β-galactosidase activity, and all subsequent experiments have been carried out using 10 mM of L-glycine. If this induction factor is compared to those obtained with the most used systems, an induction factor of about 40 was measured with xylose and an about 15-fold induction with IPTG, respectively [55]. Minimal medium yielded a higher induction factor than LB medium due to a higher basal level caused by the unknown L-glycine concentration present in this complex medium. Next, the promoter strength should be increased, and this was obtained when the wild-type -35 element was replaced by its consensus sequence. We noticed that both the basal and the induced level were increased. A further improvement of the -10 region was not possible suggesting that this element is already optimal. Experiments are in progress to reduce the basal level by (i) increasing the number of U residues immediately downstream of the terminator structure (in the wild-type sequence, five U residues are present) and (ii) to increase the strength of the terminator structure to obtain a more efficient transcription termination in the absence of added L-glycine (Fig. 4.1). The U residues immediately downstream of the terminator weaken the interaction between the transcript and the RNAP, and three to seven U residues have been described with natural terminators. We asked, whether the stability of the terminator structure could be influenced by the growth temperature, which in turn would influence the basal level of expression. This turned out to be the case, but only at 45°C; no significant difference could be measured between the two cultures grown at 30°C and 37°C, two temperatures which are normally used for the production of recombinant proteins.

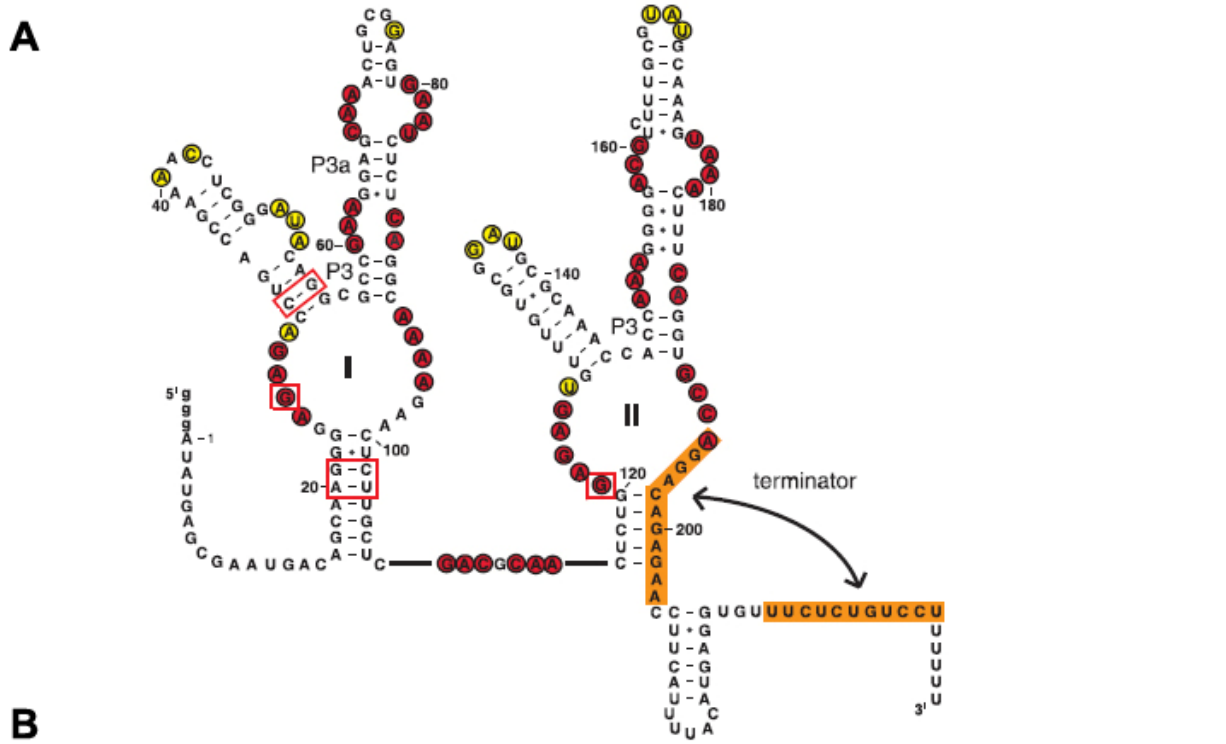


Fig. 4.1. The glycine-responsive riboswitch. (A) Secondary structure [93]; (B) primary structure; (C) primary structure with a potential open reading frame.

The production of two recombinant proteins was also checked, one present in the cytoplasm (HtpG and Pbp4*) and the other secreted into the medium (α-amylase). In both cases,

detectable amounts of the recombinant protein on Coomassie blue stained SDS-PAGE were only present when the genes were fused to the consensus promoter. While the medium did not significantly influence the amount of HtpG and Pbp4* protein produced, higher amounts of α-amylase were secreted into the medium when the cells were grown in LB medium indicating that less protein is secreted from cells grown in minimal medium.

In conclusion, these experiments demonstrate that the glycine tandem riboswitch can be used to obtain regulatable expression of recombinant proteins, both intra- and extracellularly. These data have already been published [116].

4.1.2 The lysine system

The lysine-responsive *lysC* gene as a second example for a riboswitch regulated gene was studied, which becomes induced at low L-lysine concentrations within the cytoplasm [51, 153]. Northern blot experiments were carried out to study the switch in transcription of *lysC* upon removal of L-lysine from the medium. The transcription pattern changed as to be expected from the short riboswitch RNA to the full-length transcript (Fig. 3.15A). Surprisingly, the riboswitch RNA not only continued to be synthesized for at least 3 h, but remained predominant. We could show convincingly that the riboswitch RNA does neither result from increased stability nor from processing of the full-length transcript. Using *in vitro* single-round transcription with *E. coli* RNAP in the absence of L-lysine, the riboswitch RNA could be detected, but it corresponded to only ~36% of the total transcript yield [51, 153]. Under *in vivo* conditions, the riboswitch RNA accumulated to more than 90% of the total *lysC*-specific transcript (Fig. 3.15A). When the right arm of the transcriptional terminator was removed, the riboswitch RNA failed to be synthesized as to be expected. When the β-galactosidase activity was measured, it turned out to be inducible in the presence of the wild-type terminator (Fig. 3.19), but constitutive when the truncated terminator was present (Fig. 3.20). This finding is surprising since all the sequence information to fold the anti-terminator is still present. This result indicates that formation of the active riboswitch able to bind L-lysine needs the sequence information of the complete transcriptional terminator. But these results cannot exclude the possibility that the coding region for *lacZ* interferes with the ability to fold the anti-terminator structure. A detailed mutational analysis has to be carried out to identify those sequences needed to build the active riboswitch.

Three important questions arise. By which mechanism continued synthesis of the riboswitch is ensured? What is the biological function of the riboswitch RNA during expression of *lysC*?

Why the level of the full-length mRNA is very low after removal of L-lysine? Theoretically, the riboswitch RNA could serve as a reservoir for L-lysine. This possibility is rather unlikely because of two reasons. First, the amount of riboswitch RNA molecules though not quantified should be too low to act as a storage devise for L-lysine. Second, the short half-life strongly argues against the storage hypothesis. Based on the finding that the amount of riboswitch RNA remains more or less constant, independent of the L-lyine concentration, one can ask whether it could be translated. A close inspection of the RNA sequence revealed a reasonable Shine-Dalgarno sequence (AGAAAGatGG) and a downstream GTG start codon followed by 23 sense codons as already suggested for the glycine riboswitch. Alternatively, the riboswitch RNA could serve as a ncRNA [46, 151] influencing translation of one or more transcripts (Fig. 4.2). Further experiments have to be carried out to test these possibilities. If the riboswitch RNA exerts a function within the cell, which has never been reported so far, low L-lysine concentrations will allow for transcription attenuation only for 5-10% of the riboswitch RNA molecules based on the results shown in Fig. 3.19A, and no specific mechanism will be needed. Alternatively, through less likely, an unknown metabolite could stabilize a secondary structure different from the one recognized by L-lysine thereby favouring transcription attenuation. Furthermore, *B. subtilis* produces three aspartate kinases, I, II, and III, regulated by different end products; the gene *dapG* coding for aspartate kinase I, the gene *lysC* coding for aspartate kinase II are regulated by diaminopimelate and lysine, respectively, and the *yclM* gene coding for aspartate kinase III is regulated by threonine and lysine [25, 48, 80]. To our surprise, the band of full-length transcript turned out to be rather low. What could be the reason for their relatively low amount the *lysC* mRNA? As mentioned, *B. subtilis* codes for a total of three aspartate kinase iso enzymes. Why there are three enzymes (as also described for *E. coli* [114]) is not known. The possibility exists that there is no need for a significant increase in the amount of aspartate kinase II, since kinase I or III or both are present in sufficient amounts. It will be interesting to analyse transcription of *dapG* and *yclM* under condition of high and low L-lysine concentrations. It will also be interesting to delete *dapG* or *yclM* and both genes together, and study the influence of these deletion derivatives on the expression of *lysC*. Perhaps, deletion of *dapG* and *yclM* together will significantly enhance expression of *lysC* under condition of L-lysine depletion.

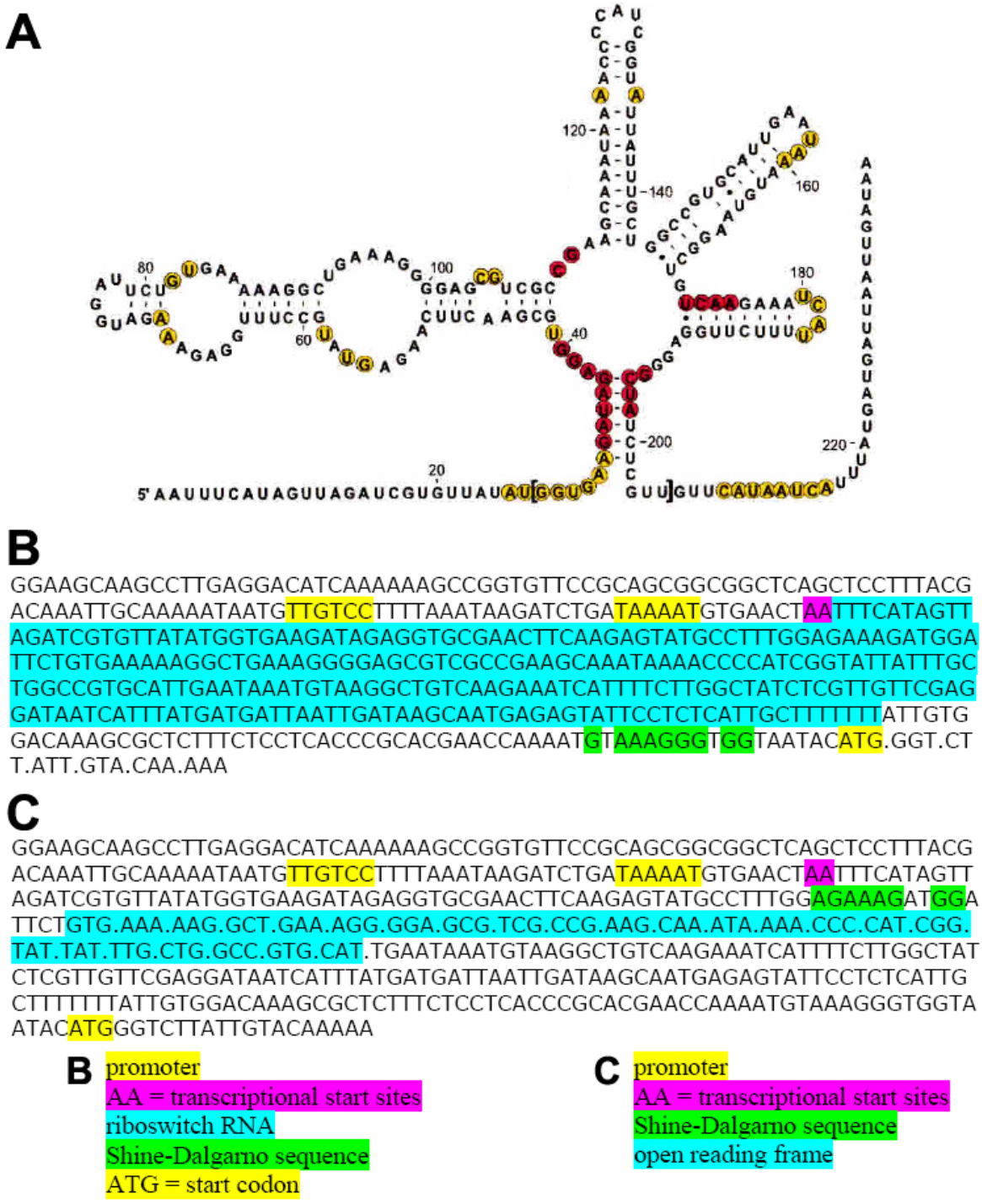


Fig. 4.2. The lysine-responsive riboswitch. (A) Secondary structure [153]; (B) primary structure; (C) primary structure with a potential open reading frame.

Analysis of the transcriptional fusion between the promoter region of *lysC* and *lacZ* indicates that it can be used for controlled expression of recombinant genes as already mentioned. Since removal of L-lysine by centrifugation is only feasible with small volumes, expression in large volumes requires a different and less time-consumable method. One possibility is to start

growth in an appropriate concentration of L-lysine, which, after consumption, leads to autoinduction of the recombinant gene. We have tested this hypothesis and obtained only a 3-fold induction after 4 h of growth starting with 5 µg/ml L-lysine (Fig. 3.21B). An alternative method would involve regulated expression of an artificial aptamer able to quickly sequester the L-lysine upon induction. There are already several examples where engineered riboswitches have been used to obtain regulated gene expression (reviewed in [6]) including an artificial system for *B. subtilis*, where expression of recombinant genes can be induced by the addition of theophylline [6], but none of them has used a natural riboswitch so far.

4.2 A promoter-probe plasmid for screening promoters in *B. subtilis*

The promoter-probe vector pHT06 based on a multi-copy theta-replication plasmid [156, 157] can replace the single-copy pBgaB [99] for screening of strong promoters, which allows heat stable β-galactosidase gene expresson in the cytoplasm. Plasmid pHT06 can be used as a promoter-probe vector by introducing DNA fragments at *Bam*HI, *Nhe*I, *Sac*I, *Kpn*I, *Spe*I, and *Sac*II or as controllable promoter-test vector as done in this work. In addition, one can use the feature of *lacO* to control expression in *E. coli* during cloning, but not in *B. subtilis* by replacing *lacI* by the promoter at *Sac*II, *Nhe*I and *Sac*I. As an example, a library of synthetic promoters was introduced, which had various strengths leading to different expression levels. In this work, promoters P88 and P89 were chosen to demonstrate the utility of plasmid pHT06 for cloning and screening strong promoters successfully.

Two obstacles were overcome during this work: (i) cloning and (ii) conditions to compare the strength of promoters by applying a linear range of IPTG concentrations. First, while it is difficult to use the available promoter-probe vectors pNDH05, pT05Z (this work), pLacZ [176] or pBgaB [99] for cloning of strong promoters, which causes lethal overproduction of recombinant proteins in *E. coli*, this problem can be avoided by using the new promoter-test plasmid pHT06. Second, there is little information about the correlation of IPTG concentrations and β-galactosidase activity. Actually, we faced this problem when all the promoters from the libraries were checked at 1 mM and then at 0.1 mM IPTG (data not shown). It turned out that almost all strong promoters conferred nearly the same activity. For example, the activity of P88, P89 and P_{grac} were nearly the same at 1 mM of IPTG after 2 or 4 h of induction and at 0.01 mM P88 and P89 were about 10 times higher thanthat of P_{grac}. This useful promoter-probe vector could largely solve problems to screen strong promoters by observation of the coulor of colonies on X-gal plates.

The low copy number and structural stable vector pHT06 together with the expression vector pHT01 [105] would become interesting for anyone who wants to establish a library of promoters or genes. With these available techniques, one could generate mutant libraries using the error-prone PCR method [27] with these new plasmids to identify and study elements of promoters in *B. subtilis* or gene functions.

4.3 Improve the productivity of *B. subtilis*

B. subtilis produces and secretes large amount of various proteins into the culture medium [120]. For the last two decades numerous attempts have been made to use this bacterium as an efficient host for the expression of recombinant proteins [147]. To improve the productivity of *B. subtilis,* we aimed to identify efficient regulatory elements enhancing transcription from expression plasmids. Published data [105, 117] and those presented in Fig. 3.32 prove that the P_{grac} promoter is indeed a strong promoter, which consists not only of the strong P_{groE} promoter, but also of the *lac* operater with a dual function: control of transcription and acting as a stabilizing element. Further efforts have been focused on the development of elements for increasing the promoter strength and for identification of mRNA-stabilizing elements in overproduction of proteins.

4.3.1 Strong promoters in *B. subtilis*

A compilation and analysis of *B. subtilis* σ^A-dependent promoter sequences [57] indicates that promoter recognition depends on the following consensus sequences: (i) the transcription start site, which should be a purine, (ii) the -10 region TATAAT, (iii) a six nucleotide spacer between the +1 and the -10 region, (iv) a TnTG sequence from -17 to -14, (v) the -35 region TTGACA, (vi) a 17 nucleotide spacer between the -10 and the -35 region and (vii) an upstream region containing An and Tn from -70 to -36 called UP element. Based on this information, I focused on sequences at transcriptional start site, the -10 region, the -15 region, the -35 region and the UP element upstream of the *groE* promoter aiming to construct stronger promoters. To analyse the elements for a strong promoter, the newly constructed promoter-probe plasmid pHT06 was used. A total of 85 different synthetic and *groE*-modified σ^A-dependent promoters (Fig. 3.31) were cloned into pHT06 and analysed by measuring the BgaB activities, the amount and stability of the *bgaB* transcripts and the amount of BgaB produced.

The promoter recognition by the bacterial RNAP holoenzyme involves interactions not only between core promoter elements and the sigma subunit, but also between a DNA element

upstream of the core promoter (UP element) and the α subunit. DNA binding by the α subunit can increase transcription dramatically [47]. UP elements are AT-rich sequences located upstream of the -35 element of some promoters [20, 47]. They act as binding sites for the αCTD of the α subunit of the RNAP [13, 52]. The promoter P_{groE} precedes the *groESL* operon coding for the GroEL chaperonin [137] and is a strong promoter because of a potential UP element (Tab. 3.1), and its activity is comparable with the mutant *C2mu* UP element [96]. The UP element of *groE* was modified based on the consensus sequence. The *groE* UP element sequence from P64 (-55-AAAATTTTTTAAAAAATCTC-36) served as an active UP element also for other σ^A-dependent promoters, and resulted in a 2-fold increase in the BgaB activity and a 5-fold higher mRNA expression as compared to P_{grac} (P01) [105, 117]. This agrees well with the alignment of 236 *B. subtilis* σ^A-dependent promoters [57] demonstrating that the -54-nAnnnnnTnnnAAAAnnnTn-36 sequence represents an UP element of σ^A-dependent *B. subtilis* promoters.

As mentioned, UP elements increase the affinity between the RNA polymerase and the downstream core promoter. But, as shown here, when the core promoter provides already a high affinity, it cannot be further increased by addition of an UP element. It follows that an UP element increases the strength of only those promoters with a low affinity for the RNA polymerase. To ensure a high transcription rate, nature has evolved two possibilities: a strong core promoter or a weak core promoter with an UP element.

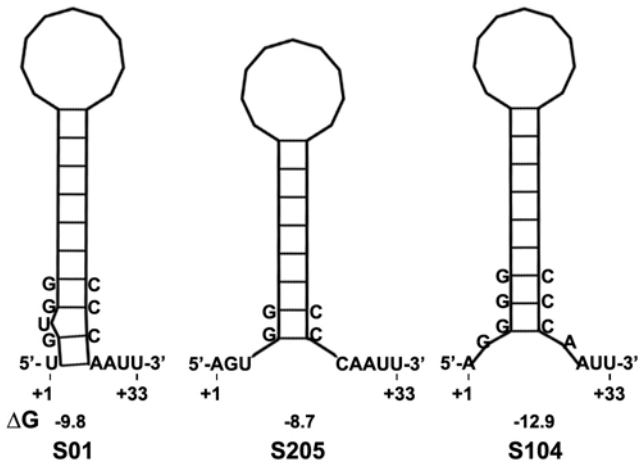


Fig. 4.3. Secondary structure of the *lacO* stem-loop of S01 (in P_{grac}), S104 (in P104) and S205.

When the UGU at transcriptional start site region was changed to AGG (in P104, Tab. 3.3), the BgaB expression level was more than doubled. It can be concluded that the new potential transcriptional start site confers increased stability to the transcript. When the U was replaced by an A residue (P205), this increases the predicted free energy from -12.9 to -8.7 (Fig. 4.3)

and reduced the mRNA stability. All these data point to the possibility to use the *lacO* as a 5' stabilizing element.

One set of mutations, located between positions -35 and -10, was created and tested. It turned out that mutation of the 'extended -10 region' (named -15 region in this thesis) was very important (Tab. 3.4, P73). The term 'extended –10 promoters' refers to the TG motif, 1 base upstream of the –10 region, which plays a vital role in several *E.coli* promoters [76]. In *B. subtilis,* it is termed the -16 region with the sequence 5'-RTRTG-3' [100]. Then, a more comprehensive analysis of 142 promoters, all with experimentally determined transcription start sites, confirm that the –16 region (TRTG) is conserved [57]. Based on this analysis, the -15 region TTCT in the P_{groE}^{WT} promoter was replaced by TATG, in P73, and the BgaB activity increased up to 94-fold. This result strengthens those published by Voskuil, indicating that the -16 region TRTG motif (R = purine) appears to be a basic element present in many gram-positive bacterial promoters [162]. We and others noticed that the -16 region TRTG motif was essential in some promoters [23, 89, 162, 171], especially in weak promoters [59] or promoters that lack an UP element [18, 19], or promoters that lack an identifiable –35 region [9, 22, 76, 162]. Promoters with a combination of UP elements and consensus core promoter elements (-15 ATG and -10 consensus; -15 ATG and -35 consensus; or -15 ATG and -10 and -35 consensus) resulted in the production of BgaB protein reaching levels of up to about 30% of the total cellular protein, especially with those promoters containing the -15 ATG triplet.

4.3.2 Role of messenger RNA stabilizing elements in overproduction of proteins

Gene expression levels are mainly determined by the efficiency of transcription, mRNA stability and the frequency of mRNA translation. Transcription (in 4.3.1) and translation has been the subject of intense optimization in recombinant expression systems. Stability of the mRNA transcript is however rarely addressed. But gene expression is controlled by the decay of mRNA [123, 124]. Messenger RNA stability has been shown to play an important role in the regulation of gene expression, and much needs to be learned about the elements that function as mRNA stability determinants. Four different elements with stabilizing potentialities will be discussed separately, namely: (i) a 3' mRNA terminal stem-loop; (ii) the 5' *lacO* stem-loop; (iii) a strong RBS; (iv) the spacer length between the 5' *lacO* stem-loop and the RBS.

3’ mRNA terminal stem-loop structures

We found that the terminal stem-loop at the 3’ end of the mRNA did not correlate with mRNA stability (Tab. 3.6, Fig. 3.38). This is in contrast to findings of Wong *et al.* (1986), who concluded, based on their data, that the 3’ end of the *cry* gene of *B. thuringiensis* (Fig. 4.4) conferred increased stability on other mRNAs in both *E. coli* and *B. subtilis* [169]. In another publication, addition of the *cry* transcription terminator at the 3' end of the *lacZ* gene did not confer any increase in its stability and produced only marginal effects on the final β-galactosidase activity [70]. This finding indicates that such a 3’ stabilizing element does not act by itself, but is influenced by the sequence of the transcript in a way, which is not yet understood at the molecular level. One possibility would be the formation of alternative secondary structures. A spacer region, inserted between the 3’ end of the mRNA and the *cry* element could alleviate this problem. Such a spacer region, which by itself will form a stem-loop structures could protect the *cry* hairpin structure or any other additional secondary structure. The average half-life of the *bgaB* transcripts was 30 min in this study with modified versions of the *trpA* terminator or the natural terminators of the *skfA*, *htpG* and *dnaK* genes. These results indicate that the 3’-mRNA terminal stem-loop structures did not further increase the half-life of mRNAs as compared to P01 with the $trpA^{WT}$ terminator (ΔG = -15.7 kcal/mol) with a half-life about 30 min. Wong [169] employed the *penP* gene with a half-life of 2.0-2.8 min to study the influence of the *cry* terminator in both *E. coli* and *B. subtilis*. The data in Tab. 3.6 indicate that the *E. coli trpA* terminator had only a slight influence on the BgaB activity. These results are consistent with the results of Wong [169], who had tested the terminators from two additional bacterial genes that encode stable mRNAs, the *lpp* gene of *E. coli* and the *ery* gene from the *S. aureus*. The mRNA half-lifes for these transcripts were reported to be 11.5 and >22 min, respectively. However, no enhancement on the expression of the *penP* gene was obtained; the *penP-lpp* and *penP-ery* fusions expressed *penP* at the same level as did the native *penP* gene in both *E. coli* and *B. subtilis.* These findings indicate that the half-life of a transcript and its translation efficiency do not necessarily correlate. This is surprising and further indicates that there might be an additional mechanism(s) influencing the amount of protein to be discovered.

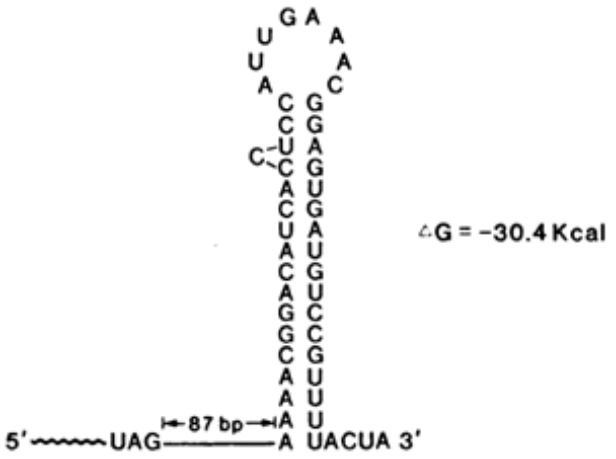


Fig. 4.4. Secondary structure of the potential 3'-end of the *cry* mRNA [169].

We noted that after removal of some T residues (U residues in Tab. 3.6), the BgaB activity increased. This means that leaky transcription termination of the upstream located gene *orf-1* (called *repA*) encoding the plasmid replication initiator [156] would increase BgaB expression. Increasing the amount of RepA in turn should lead to an increase in the copy number of the plasmids with a concomitant enhanced production of the BgaB protein due to the gene dosin effect. However, there is a transcriptional terminator just before *orf-1*; only a small amount of the transcript could reach to *orf-1* due to overriding the transcriptional terminator. One might exploit this feature to conditionally increase the copy number of plasmid for protein overproduction purpose. On the other hand, the exoribonuclease PNPase activity is demonstrated in *B. subtilis* by Deutscher and Reuven [34] and shown to be the predominant degradative activity in cell extracts with poly(A) or poly(U) RNA as a substrate.

The 5' *lacO* stem-loop structure

The results shown in Tab. 3.7 and Fig. 3.39 point to the importance of a stem-loop as exemplified by *lacO.* Therefore, the effect of the predicted *lacO* stem-loop on mRNA stability was analysed. When constructs with decreased free energy of *lacO* by changing nucleotides inside of *lacO* (Fig.4.5, S211) were tested, the BgaB activity increased more than 2-fold as compared to the P_{grac} promoter (Tab. 3.7, Fig. 3.39, S206 and S211). This is consistent with the idea that the major determinant for mRNA stability is a blocked 5'-end [146]. The *lacO* stem-loop increased the BgaB activity >2-fold, but there was no clear effection mRNA stability. Therefore, it would be of interest to determine whether a strong RBS and a spacer between the 5' stem-loop and the RBS caused an effect in the half-life of the mRNA.

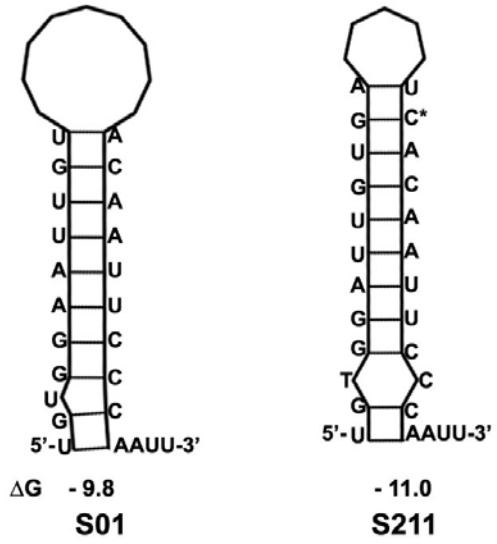


Fig. 4.5. Secondary structure of the 5' stem-loop from S01 (in P_{grac}) and S211

Influence of a strong RBS on mRNA stability

We found that the strong RBS of *gsiB* correlated with mRNA stability and expression of the reporter gene (Tab. 3.8 and Fig. 3.41 and Fig. 3.42), when it was fused to P_{lepA}. This is in agreement with the findings of *B. thuringiensis cryIIIA* mRNA [1] and *gsiB* mRNA [74] that mRNA stability can depend on a strong RBS, which is located four (*cryIIIA*) or nine (*gsiB*) nucleotides away from the 5'-end. These results suggest that a strong RBS enhance mRNA stability when positioned close the 5' end, most probably by impairing binding of an endonuclease.

The spacer length between 5'the stem-loop and the RBS

The experiments shown in Fig. 3.39 and 3.40 demonstrated that even a highly stable 5'-terminal structure could not confer stability without an adequate distance between the 5'end of the structure and the RBS. The spacer of a length of 13 to 29 bp not only influenced the mRNA stability, but also the BgaB protein expression level (Tab. 3.9 and Fig. 3.40). We and other propose that the binding of ribosomes at an RBS that is located too close to the 5'-terminal structure will result in perturbation of the structure. Based on crystallography studies [175], 15 nts upstream of the initiation codon are protected by the ribosome bound in a ternary complex. In the case of this study, a spacer length of 19 nucleotides in the case of S213 can be considered as optimal for production of BgaB, in that the BgaB activity could be increased up to 9-fold as compared to the P_{grac} promoter with a half-life of the mRNA of more than 60 min (Fig. 3.40). These data suggests that this spacer is short enough to allow ribosome binding without being inhibited by the formation of the protective 5'-terminal

structure. Once the spacer is increased to 29 nts (S110, Fig. 3.40), ribosome binding no longer affected secondary structure formation, resulting in a very stable mRNA.

It can be concluded that the *lacO* stem-loop, a Controllable Stabilizing Element (CoSE) and elements of strong σ^A-dependent P_{groE} promoter can be used for overproduction of recombinant proteins in *B. subtilis.* To evaluate its potential, CoSE was combined with other promoters, and all constructs could increase the half-life of the mRNA and increase the BgaB activity and the yield of BgaB, PBP4* and HtpG proteins. Promoter P223 consists of CoSE S212 and the strong promoter P68 able to overproduce BgaB up to 42% of total cellular protein (Fig. 3.42). While BgaB protein from strong P_{grac} 9.2% [105, 117]; from P_{groE}^{WT}-CoSE, S212, 26.7% (Fig. 3.42); from P68 and P100 (see in Fig 3.35), BgaB up to 30% of total cellular protein. The other recombinant proteins, PBP4*, P100 could overproduce this protein up to 38% and P250 up to 28% in total cellular protein (16% in P_{grac}); HtpG, P250 could overproduce this protein up to 26% in total cellular protein (12% in P_{grac}). On the other hand, CoSE could be extended to other bacteria. See in Fig. 4.6, the positive effect on BgaB expression in *E. coli*, the result shown that S01 (pHT01) is weaker than S212 and S221 is weaker than S224, which is similar the result in *B. subtilis*.

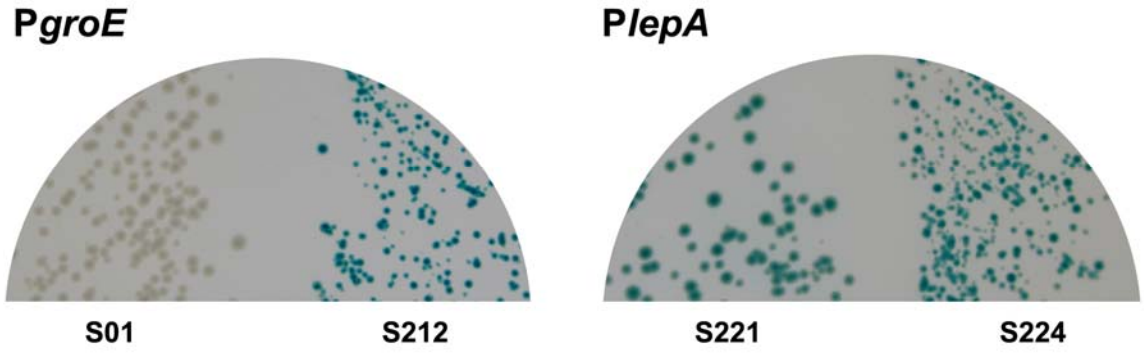


Fig. 4.6. Using CoSE in *E. coli.* Cell with different plasmids/stabilizing element were stretched on X-gal LB plate with 0.01 mM IPTG.

In summary, all of strong promoters and CoSE explored in this study are extremely useful for improvement expression system in both *B. subtilis* and *E. coli*.

4.4 Outlook

B. subtilis is used to produce and to secrete large amounts of various proteins into the culture medium since the last two decades [120]. Then, numerous attempts have been made to convert this bacterium into an efficient host for the expression of recombinant genes [147]. At present, about 60% of the commercially available enzymes are produced by *Bacillus* species; and a large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation has been acquired as mentioned in the 'Introduction' of this thesis and in [56, 97, 163, 170]. But more knowledge on cellular functions and development of better production systems is still needed [163]. Based on the results in this thesis, a key question is how the glycine-inducible and the lysine-autoinducible expression systems can be improved for industrial production and how strong promoters with CoSE as a useful tool can be applied for the downstream processing.

(i) One may improve the glycine-inducible system by fusing the CoSE at downstream of the riboswitch and to combine it with a strong promoter. This system can be used for overproduction of recombinant proteins using the inexpensive inducer glycine.

(ii) As discussed, there is the possibility to improve the expression level of the lysine-responsive riboswitch-regulated *lysC* gene by deleting the two other genes coding for aspatokinase iso enzymes. These experiments are in progress.

(iii) One possibility to reduce basal level of expression in *E. coli* in to use shuttle vectors with a low copy number in *E. coli* and a high copy number in *B. subtilis.*

(iV) Based on the strong promoter P68, P100, S212, S223 or S250 expression vectors with purification tags and secretion vectors can be developed.

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6 List of abbreviations and symbols

Abbreviation	Denotation
Aa	amino acid(s)
A_{260}	absorption at a wavelength of 260 nm
A_{280}	absorption at a wavelength of 280 nm
Amp^R	resistant to ampicillin
amyQ	gene coding for protein α-amylase (AmyQ)
APS	ammoniumperoxodisulfate
α-	alpha, indicating antibodies against something (except α-amylase) e.g, α-AmyQ means antibodies against AmyQ
αCTD	C-terminal domain of the RNA polymerase α subunit
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
bgaB	coding for the heat stable reporter β-gaclactosidase (BgaB) in *G. stearothermophilus*
bp	base pairs
B. subtilis	*Bacillus subtilis*
cat	gene coding for chloraphenicol-acetytransferase
celA	gene coding for cellulase A (CelA) from *C. thermocellum*
celB	gene coding for cellulase B (CelB) from *C. thermocellum*
Cm^R	resistant to chloramphenicol
CMC	carboxy methyl cellulose
C. thermocellum	*Clostridium thermocellum*
^oC	degrees centigrade
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
Δ	deletion
E. coli	*Escherichia coli*
Et.Br	ethidium bromide
et al.	et alteri
g	gram
GFP	green fluorescent protein
gly	L-glycine
kb	kilobase
kDa	kilo-Dalton
LB	Luria-Bertani

lysC	gene code for the α- and β-subinits of a lysine-responsive aspartokinase II
h	hour(s)
HCl	hydrocloride acid
htpG	gene coding for hight temperature protein G (HtpG)
IAA	isoamylalkohol
IPTG	isopropyl-ß-D-thiogalactoside
lacZ	gene coding for the reporter β-gaclactosidase (LacZ) in *E. coli*
l	liter
MCS	Multiple cloning sites
min	minute(s)
mg	milligram
ml	mililiter
mM	milimole
MOPS	morpholiopropanesulfonic acid
mRNA	messenger RNA
µg	microgram
µl	microliter
Neo	neomycin
nt	nucleotide(s)
ON	oligonucleotide
ONPG	ortho-nitrophenyl-β-galactoside
OD_{578}	optical Density at a wavelength of 578 nm
PBS	phosphate-buffer saline
pbpE	gene coding for penicillin-binding protein PBP4* in *B. subtilis*
P_{grac}	an IPTG inducble promoter, a hybrid promoter of P_{groE} and *lac* operator
pmol	picromole
P_{spac}	an IPTG-inducible promoter, a hybrid promoter of the phage SPO-1 and the *lacO*
P_{gsiB}	(glucose stavation inducible) σ^B-dependent general stress promoter
P_{gcv}	a glycine-inducible promoter, a promoter and 5'UTR of the *gcvT* operon
P_{lysC}	a lysine-inducible promoter, a promoter and 5'UTR of the *lysC* operon
RBS	ribosome binding site

Rif	rifampicin
RNA	ribonucleic acid
RNAP	RNA polymerase
rpm	revolution or round per minute
RT	room temperature
S. aureus	*Staphylococcus aureus*
sec	second
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
srtA	gene coding for SrtA (e.i. of *L. monocytogenes*)
TEMED	N,N,N`,N`-tetramethylenethylendiamide
TCA	trichloroacetic acid
Tris	tri-(hydroxymethyl)-aminomethane
Tween-20	polyoxyethylensorbitane monlaurate
U	units (enzyme activity)
UTR	untranslated region
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
yhcS	gene coding for protein YhcS in *B. subtilis*
ywpE	gene coding for protein YwpE in *B. subtilis*

Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, März 2007

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