university of groningen

University Medical Center Groningen

University of Groningen

Heterologous expression system comprising a b. subtilis promoter

Hamoen, Leendert; Kuipers, Oscar Paul; Lindner, Johanna

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hamoen, L., Kuipers, O. P., & Lindner, J. (2004). *Heterologous expression system comprising a b. subtilis promoter.* (Patent No. *WO2004015114*).

(54) Title: NOVEL BACTERIAL EXPRESSION SYSTEM

(57) Abstract: The present invention relates to expression cassettes suitable for the expression of a heterologous gene. Furthermore,
the invention relates to recombinant plasmids comprising such expression cassettes. Also, the invention relates to host cells com-
prising such cassettes or plasmids. Another embodiment relates to promoters for use in an expression cassette and in heterologous
expression systems. Finally, an embodiment relates to methods for the expression of a heterologous gene.

Novel bacterial expression system

The present invention relates to expression cassettes, to recombinant plasmids
comprising such expression cassettes, to host cells comprising such cassettes or
5   recombinant plasmids, to promoters for use in an expression cassette and use in
heterologous expression systems and to methods for the expression of a
heterologous gene.

Bacterial heterologous expression systems are universally used for the expression of
10   a variety of different heterologous genes. A heterologous gene is considered to be a
gene that does not originate from *Bacillus subtilis* or, if originating from *Bacillus
subtilis*, is not or not solely under the control of its native promoter. It may be a gene
of bacterial origin, found in either the bacterial cell that is also used as the host for
the expression system (e.g. in the case that over-expression of a native gene is
15   needed) or from another species. It may equally well be a gene of eukaryotic origin.
Bacterial heterologous expression systems may have a few known disadvantages
over eukaryotic heterologous expression systems, but for the expression of many
heterologous genes of both prokaryotic and eukaryotic origin, they provide very
versatile and inexpensive systems. With regard to the host cells of bacterial
20   expression systems, a difference is made between the Gram-positive and Gram-
negative bacteria. Gram-negative bacteria have both an inner and an outer
membrane. The outer membrane sometimes interferes negatively with transportation
of secreted proteins over the cell membrane. Gram-positive bacteria do suffer to a
much lesser extent from this problem, because they lack the outer membrane. A few
25   randomly chosen examples of Gram-positive bacteria suitable as host cell for
heterologous expression systems are e.g. bacteria belonging to the genera
*Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, Streptococcus,
Enterococcus, Staphylococcus, Bacillus, Sarcina, Ruminococcus* or *Listeria*.

30   A problem connected with all heterologous expression systems is the fact that (over)-
production of the heterologous expression product frequently interferes in a negative
way with the viability or growth characteristics of the host cell in which it is expressed.
Thus, when choosing a bacterial expression system, it has to be decided first, if the
heterologous protein to be expressed is detrimental or even toxic for the host cell. If
35   this is the case, the heterologous expression system would preferably have the
option of switching the heterologous expression on or off. But even if the
heterologous protein to be expressed is known to be only slightly or even not toxic at

all for the host cell, continuous expression of the heterologous gene after the moment that growth reaches the stationary phase would finally kill the host cell. In such a case, an expression system would be preferred that continuously synthesizes the desired heterologous protein, but only to a level that is directly linked to the growth

5   rate of the bacterium: if the growth rate slows down or halts, the heterologous expression level should preferably slow down or halt as well.

It is an objective of the present invention to provide a highly versatile expression system that offers a solution to the problems indicated above.

10

Surprisingly, it was found now that two genes coding for Single-stranded DNA Binding Proteins (SSB) found in i.a. the *B. subtilis* genome have promoter sequences that do have the characteristics that make them very suitable for a heterologous expression system with one or both of the desired characteristics.

15

One SSB-like gene, further referred to as *ssb*, maps at 358.6° of the *B. subtilis* genome and is flanked by the *rpsF* and *rpsR* genes coding for the ribosomal proteins S6 and S18, respectively (Figure IA). A structure similar to Rho-independent transcriptional terminators is situated downstream of the *rpsR* gene. Since no

20   terminator-like structure was found between the genes *rpsF, ssb* and *rpsR*, it is likely that these genes belong to an operon. The second *ssb*-like gene, *ywpH*, maps at 319.4 'and is flanked by another gene of unknown function (*ywpG*) and the *glcR* gene coding for a regulator involved in carbon catabolite repression (Figure 1B). Full details of the location and the sequence of the genes mentioned have been given in

25   F. Kunst et al., Nature 390: 249-256 (1997).

Transcriptional fusions of the potential promoter-containing fragments with a promoter less lacZ gene were constructed and integrated into the *B. subtilis* chromosome at an ectopic site. An ectopic site is a site at which the gene is not

30   found in the wild-type bacterium. Upstream regions of the *ssb* and *ywpH* genes, indicated as S 1, S2, R1 and Y1 were amplified by PCR using chromosomal DNA of *B. subtilis 168* as template. The PCR fragments were cloned into a promoter-screening vector and introduced via transformation into *B. subtilis 168,* selecting for kanamycin resistant transformants. The transformants were screened for an

35   amylase-deficient phenotype to confirm that the construct had integrated at the selected ectopic site, i.e. the *amyE* locus. (The *amyE* locus is merely an example of a

suitable integration site, because the *amyE* gene is a non-essential gene, and can
thus safely be used for the insertion of another gene.

The blue or white phenotypes of the resulting strains BIV7 (S1), 17 (S2), 8 (Rl), and
12 (Y1) on rich (TY) and minimal medium (MM) agar plates revealed promoter

5   activity only for the constructs in strain BIV 17, 8, 12 and 13, but not in BIV7. In
BIV12, promoter activity was detected only in MM indicating a medium-dependent
expression of the gene *ywpH* (Figure 1). Whereas *ywpH* was found to be transcribed
from a promoter directly upstream of the gene, no promoter activity could be detected
from the S 1 fragment comprising the 295 bp immediately upstream of the *ssb* start

10   codon. However, promoter activity was detected from the S2 fragment containing
also the complete *rpsF* gene and 274 bp upstream of its start codon indicating that
ssb is the second gene of an operon, which is constituted of the *rpsF*, *ssb*, and *rpsR*
genes. Clearly promoter activity was found also from the smaller Rl fragment
comprising a 274 bp upstream of the *rpsF* start codon only. These results indicate

15   that *ssb* is *co*transcribed and co-regulated with genes coding for ribosomal proteins,
thereby coupling the regulation of protein synthesis and DNA metabolism.

In order to study the expression of *ssb* and *ywpH* in more detail, strains containing
the S1-, R1- and Y1-lacZ fusions (BIV7, 8 and 12), respectively, were grown in TY
and MM and ß-galactosidase activity was examined as described by Miller et al.

20   (Miller, J. H. 1982. Experiments in molecular genetics. Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, N.Y.). Whereas no expression could be
detected from the S1 fragment under the conditions employed, the *ssb* operon
appeared to be strongly expressed from the *rpsF*-promoter in both rich and minimal
media. The highest values (between 200 and 300 Miller units per OD) were reached

25   during exponential growth (Figure 2A, B). When cells stopped growing the
expression dropped. There is still expression in the stationary phase but on a much
lower level.

In conclusion these results show directly growth-related characteristics of expression

30   behavior of the *ssb* operon, and thus of the *rpsF*-promoter. Therefore, this promoter
clearly is the promoter of choice for the expression of heterologous genes encoding
non-toxic or slightly toxic proteins. The level of expression is directly linked to the
growth rate of the bacterium. The protein encoded by the heterologous gene will be
expressed only during logarithmic growth. If the growth rate of the bacterium slows

35   down, e.g. as a result of decreased amounts of nutrients, the synthesis of the protein
encoded by the heterologous gene also decreases, thus avoiding growth retardation

or even killing of the host cell, i.e. the bacterium, by over-production of the heterologous gene.

One of the advantages of such an expression system is, that the host cells remain viable. Further expression can very easily be obtained by just adding more medium

5   to the cells, regardless the fact that the cells may have temporarily reached at any moment in time the stationary phase.

In contrast, no expression of the *ywpH* gene could be detected in cells of *B. subtilis* BIV12 grown in TY and only very low expression was observed in exponentially

10   growing cells in MM. However, expression of *ywpH* increased significantly, when cells growing in MM entered the stationary phase and reached its highest level within two hours thereafter (Figure 2C).

In conclusion: the *ywpH* promoter is only expressed during the stationary phase in MM. This promoter would clearly be the promoter of choice for the production of

15   proteins that are toxic for the host cell, because during logarithmic growth of the cells, growth is not at all hampered by expression of toxic heterologous protein. Genes encoding such proteins can safely be cloned downstream of the *ywpH* promoter since the cells can be grown in MM or rich medium to a high density without any heterologous gene expression. Only as soon as the stationary phase is reached and

20   cells are grown in MM, the heterologous toxic protein will be synthesized. Even more efficiently, cells can be grown in rich medium to high densities, i.e. the stationary phase, followed by further culturing in MM. This will then trigger the synthesis of the toxic protein. If needed, a small sample of cells can be taken out during logarithmic growth phase and kept frozen for use in further cultures.

25

The basic element of a heterologous expression system is the combination of the promoter and the heterologous gene to be expressed. This combination is in principle sufficient for obtaining the heterologous expression product, i.e. the protein encoded by the heterologous gene, as expressed under the influence of the *rpsF*-promoter or

30   the *ywpH*-promoter.

This basic element will be further referred to as an expression cassette.

It should be clear however, that usually this expression cassette will subsequently be cloned into a plasmid, and/or be inserted in the genome of the host.

The recombinant plasmid carrying the expression cassette can be used for the

35   transformation of the host bacterium. If a high-copy plasmid is chosen, this will generally lead to an enhanced expression, due to the fact that several copies of the expression cassette will be expressed at the same time in the host. This allows for a

further fine-tuning of the amount of expression product made. Such high-copy plasmids are known in the art. Instead of maintaining the expression cassette on a plasmid in the host, the expression cassette can be integrated, in one or more copies, in the genome of the host cell, e.g. through the process of homologous
5   recombination. A combination of the two systems is also feasible.

Thus a first embodiment of the present invention relates to an expression cassette comprising a promoter which is obtainable from the *Bacillus subtilis* genome wherein the promoter is located in the region between the stop codon of the *yyaF*-gene and
10   the start codon of the *rpsF*-gene.

Only one promoter is found in this region, and therefore the fact that the promoter according to the invention is located in the region between the stop codon of the *yyaF*-gene and the start codon of the *rpsF*-gene is an unequivocal identification of
15   the promoter.

As mentioned above, full details of the location and the sequence of the genes mentioned here have been given in F. Kunst et al., Nature 390: 249-256 (1997).

20   Genomes of microorganisms whose complete genome sequence is known were screened for SSB homologues and their gene organization using the NCBI database (http://www.ncbi.nhn.nih.gov). At this moment 69 complete sequences of bacterial genomes are available including 59 different species. Of these, 23 species show the *ssb* gene flanked by the *rpsF* and *rpsR* genes as was observed for *B. subtilis*. These
25   species mainly belong to the taxonomic group of the Firmicutes, like *B. subtilis* itself, to the Spirochaetales, Thermotogales and Aquificales.
This search revealed that the gene organization *rpsF ssb rpsR* is commonly found in these taxonomic groups.

30   The sequence of this region in *Bacillus subtilis* is given in figure 3. The desired promoter region, which is described in more detail below, can now easily be found on the genome of *Bacillus subtilis*, and subsequently be synthesized and cloned with the help of standard PCR techniques, well-known in the art. Such PCR- and cloning techniques are e.g. described in standard laboratory manuals such as in Dieffenbach
35   & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995) and in Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

The promoter of the expression cassette according to the present invention is obtainable from *Bacillus subtilis* as mentioned above, but was shown, as also stated above, to be present in the taxonomic group of the Firmicutes, like *B. subtilis* itself,

5   the Spirochaetales, Thermotogales and Aquificales. It can therefore easily be located and cloned from these taxons by screening the genome of any of the bacteria belonging to these taxons, using the sequence given in figure 3 as a probe. Such screening is also a standard technique, known in the art for years now, and also described in Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-

10   87969-447-5 (1995) and in Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

Therefore, promoters as described in the invention obtained from any of the taxonomic groups mentioned above are considered to be within the scope of the

15   present invention.

It is clear that any of the taxonomic groups mentioned above are also suitable for use as a host cell for expression cassettes according to the invention, because the promoter is present in, and thus recognized in these taxonomic groups.

20

Promoter sequences can be recognized by a few motives, such as their specific so-called –35 site, -10 site and the spacer between these sites, as well as the fact that at a certain distance from these sites of (very roughly) 50-65 nucleotides downstream of the –10 site a ribosome binding site motive is found. The distance between –10

25   site and RBS is however far from critical.

In a preferred form of this embodiment, the *rpsF* promoter of the heterologous expression system according to the invention has the following characteristics:

30   a) a nucleotide sequence TATAAT, preferably GTATAAT, TATAATA or
             GTATAATA, more preferably GGTATAAT, TATAATAT or GGTATAATAT at
             the –10 site,
             b) a nucleotide sequence TTGTAA, preferably GTTGTAA, TTGTAAA or
             GTTGTAAA, more preferably AGTTGTAA, TTGTAAAG or AGTTGTAAAG at

35   the–35 site,
             c) a spacer of 17 +/- 2 nucleotides, preferably 17 +/- 1 nucleotides, more
             preferably 17 nucleotides.

A spacer is understood to be the nucleic acid sequence separating the –35 sequence TTGTAA from the –10 sequence TATAAT. This spacer varies in length between 15 and 19 nucleotides.

5

In a more preferred form of this embodiment, the promoter comprises a nucleotide sequence as depicted in SEQ ID NO 1.

In principle, the Ribosome Binding Site (RBS) to be used is not critical, especially

10   because it is not an actual part of the promoter as such. Nevertheless, the RBS as found between the rpsF-promoter and the start codon of the rpsF gene would be a preferred RBS.

Therefore, in an even more preferred form, the promoter comprises a nucleotide

15   sequence as depicted in SEQ ID NO 2. This sequence differs from the sequence of SEQ ID NO 1 in that it additionally comprises the sequence downstream of the –10 region to include the Ribosome Binding Site (RBS) preceding the rpsF gene.

In order to obtain the most native form of the rpsF promoter, it is preferred to also use

20   the region between the RBS and the start-codon (preferably including the start codon) of the rpsF gene.

Thus, in a most preferred form, the promoter according to the invention comprises a nucleotide sequence as depicted in SEQ ID NO 3. This sequence differs from the

25   sequence of SEQ ID NO 2 in that it additionally comprises the sequence downstream of the RBS to include the ATG-codon of the rpsF gene.

As can be seen from figure 3, the DNA sequence located upstream of the –35 region comprises several AT-rich regions which will render the DNA helix more flexible. This

30   recurrent motive is a typical DNA bending motive.
Such DNA-bending motives make it possible for distant upstream sequences involved in transcription, to become spatially located close to the promoter region.
As shown in figure 5, several constructs have been made, in which the rpsF-promoter according to the invention is fused to a promoter-less ß-galactosidase

35   gene, and is preceded by stretches of nucleotides, of different length, as present upstream of the promoter sequence in the native situation. Figure 6 clearly shows that those constructs having longer stretches of native nucleotide sequences

upstream the −35 region still in place give a higher expression level compared to
those constructs having a shorter upstream −35 sequence.

This clearly shows, that the presence of these AT-rich regions enhances the
efficiency of the *rpsF*-promoter according to the invention.

5

Therefore, in a more preferred form of this embodiment, the promoter described in
the invention is preceded by a stretch of nucleotides having at least 1, preferably 2,
more preferably even three AT-rich regions, such as e.g. ATTT, ATTTA or TTATT
upstream of the −35 region.

10

Preferably, the stretch of nucleotides having AT-rich regions upstream of the −35
region comprises the nucleotide sequence as depicted in SEQ ID NO 4, more
preferably SEQ ID NO 5, even more preferably SEQ ID NO 6.

15   The sequence of the *ywpH* promoter is given in figure 4. Actually, the region of this
promoter that is of importance is referred to as a *comK*-box. *ComK*-boxes play an
important role in the competence-signal-transduction network. *ComK*-boxes have as
a common characteristic that they share the consensus motive AAAANNNNTTTT.
The following must be stressed here: a consensus motive is a motive statistically the
20   most prevalent motive found, when several of these motives are compared. This
does not mean that minor modifications are not tolerated. Occasionally, A/T-, A/G- or
A/C-transitions are found in the AAAA stretch of the motive and occasionally, T/A-,
T/G- or T/C-transitions are found in the TTTT-stretch. The TATT tetranucleotide
found instead of the TTTT sequence in the most upstream located *comK*-box of the
25   *ywpH* gene is a good example of such a transition. Therefore, a "*comK*-box" is
understood to have a sequence that does not differ at the "A"-side and/or the "T"-side
of the box in more than one nucleotide compared to the consensus sequence. As a
result, a *comK*-box as used in the present invention, i.e. falling under the definition of
a consensus *comK*-box, could e.g. have the sequence AAAANNNNTTTT,
30   ATAANNNNTTTT, AATANNNNTATT or AAAANNNNTATT. These examples are
of course not limiting.

Another characteristic of *comK*-boxes is that they are always found twice. The
presence of two *comK*-boxes is a prerequisite for their functionality.
35   The two *comK*-boxes in figure 4 are presented in bold letters.

The length of the spacer region separating the two *comK*-boxes is not critical. Values between 10 and 150 base pairs are however quite commonly found.

5   The two *comK*-boxes are located upstream of the gene to be expressed at a distance that is not critical. Roughly however, the distance between the *comK*-boxes and the gene to be expressed should not exceed 500 nucleotides. Typically, the distance would be between 20 and 200 nucleotides.

10   More information about the *comK*-boxes can be found e.g. at http://elmo.ims.u-tokyo.ac.jp/dbtbs/tfac/ComK.html.

Therefore, another embodiment of the invention relates to an expression cassette that comprises a promoter having two *comK*-boxes with a consensus sequence AAAANNNNNTTTT that are present twice within a mutual distance of between 10

15   and 150 nucleotides. Thus the sequence of this kind of promoter can be represented shortly as follows: AAAANNNNNTTTTTX_nAAAANNNNNTTTTT, wherein X_n represents a number of nucleotides between 10 and 150, and N and X can be A, T, G or C.

In a preferred form of this embodiment, the expression cassette according to the

20   invention comprises the promoter of the *ywpH* gene.
       This promoter comprises the nucleotide sequence as depicted in SEQ ID NO: 7.

In a more preferred form of this embodiment, the promoter is followed by the native downstream nucleotide sequence up to, and including, the *ywpH* RBS. This promoter

25   has the sequence as depicted in SEQ ID NO: 8.

In an even more preferred form of this embodiment, the promoter is followed by the native downstream nucleotide sequence up to, and including, the *ywpH* RBS and further extending to the start codon of the *ywpH* gene. This promoter has the

30   sequence as depicted in SEQ ID NO: 9.

Another embodiment of the invention relates to recombinant plasmids that comprise an expression cassette according to the invention. As mentioned above, such plasmids serve as a way of transforming the host cell. Such plasmids may be high-

35   copy or low-copy plasmids.
       Techniques for the transformation of bacterial cells, more specifically *Bacillus*-like cells are well-known in the art.

If desired, 5'- terminal and 3'-terminal parts of a bacterial gene encoding an e.g. non-essential gene can be added at the upstream and downstream end of the expression cassette according to the invention. This allows for homologous recombination with

5   the host cell's genome once the expression cassette optionally carried by the recombinant plasmid is brought into the host cell. The expression cassette will thus become stably integrated into the host's genome. Homologous recombination into a selectable non-essential gene highly facilitates the selection for integrants. As a selectable non-essential gene in *B. subtilis*, the *amyE* gene would be a very suitable

10   gene.

Still another embodiment of the invention relates to bacterial host cells comprising an expression cassette or a recombinant plasmid according to the invention. As mentioned above, this embodiment relates to both bacterial host cells comprising an

15   expression cassette located on a recombinant plasmid and bacterial host cells comprising an expression cassette integrated in the bacterial genome.

Again still another embodiment of the invention relates to promoters as described above for use in an expression cassette.

20

And still another embodiment of the invention relates to promoters as described above for use in a heterologous expression system.

Again another embodiment of the invention relates to methods for the heterologous

25   expression of a gene wherein those methods comprise the construction of an expression cassette according to the present invention. Examples of the construction of such an expression cassette are given in the Examples below.

Still other embodiments relate to the use of a promoter as described in the present

30   invention in an expression cassette and to the use of such a promoter in a heterologous expression system.

Finally, an embodiment of the present invention relates to methods for the heterologous expression of a gene, that comprise the transformation of a bacterium

35   with an expression cassette according to the invention.

Example 1

Transcription analysis of the _ssb_ and _ywpH_ genes.

5   For all PCR-reactions _B. subtilis_ 168 DNA was used as template. This strain can be
     obtained from the Bacillus Genetic Stock Center; Strains & Data, The Ohio State
     University, Department of Biochemistry, 484 West Twelfth Avenue, Columbus, Ohio
     43210, USA.

10   Expression of these two genes and localization of their respective promoters.
     Transcriptional fusions of the potential promoter-containing fragments with a
     promoter less _lacZ_ gene were constructed and integrated into the _B. subtilis_
     chromosome at an ectopic site: the alpha-Amylase E gene, _amyE_.
     Upstream regions of the _ssb_ and _ywpH_ genes, fragment S1 (from 295 bp upstream to
15   11 bp downstream of the start codon of _ssb_), S2 (from 602 bp upstream to 11 bp
     downstream of the start codon of _ssb_), R1 (from 274 bp upstream to 50 bp
     downstream of the start codon of _rpsF_), Y1 (from 282 bp upstream to 10 bp
     downstream of the start codon of _ywpH_) were amplified by PCR using Pwo DNA
     polymerase (Roche) and chromosomal DNA of _B. subtilis 168_ as template. The PCR
20   fragments were cloned into the SmaI-digested promoter-screening vector pBTK2
     (pBTK2 is a plasmid that carries 3'- and 5'-terminal parts of the _Bacillus subtilis amyE_
     gene, each flanking one side of a promoter less _lacZ_ gene. See for details: Meijer, W.
     J. J. 1995. Replication and maintenance of plasmids in _Bacillus subtilis_. Thesis,
     available through the University of Groningen, The Netherlands). Resulting plasmids
25   carrying the insert in the correct orientation were linearized and used to transform _B._
     subtilis 168, selecting for kanamycin resistant transformants. The transformants
     were screened for an amylase-deficient phenotype to confirm that the construct had
     integrated at the _amyE_ locus. The cloning and transformation were performed
     according to common techniques (Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989.
30   Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold
     Spring Harbor, N.Y., the supplier's manuals, and Bron, S. and G. Venema. 1972.
     Mutat.Res. 15:1-10).
     The blue or white phenotypes of the resulting strains BIV7 (S1), 17 (S2), _8 (RI),_ and
     12 (Y1) on rich (TY) and minimal medium (MM) agar plates (Meima, R., C.
35   Eschevins, S. Fillinger, A. Bolhuis, L. W. Hamoen, R. Dorenbos, W. J. Quax, J. M.
     van Dijl, R. Provvedi,1. Chen, D. Dubnau, and S. Bron. 2002. J.Biol.Chem. 277:
     6994-7001), respectively, containing 0.004 % X-Gal revealed promoter activity only

for the constructs in strain BIV 17, *8,* 12 and 13, but not in BIV7. In BIV12, promoter activity was detected only in MM indicating a medium-dependent expression of the gene *ywpH* (Figure 1). Whereas *ywpH* was found to be transcribed from a promoter directly upstream of the gene, no promoter activity could be detected from the S 1

5   fragment comprising the 295 bp immediately upstream of the *ssb* start codon. However, promoter activity was detected from the S2 fragment containing also the complete *rpsF* gene and 274 bp upstream of its start codon indicating that *ssb* is the second gene of an operon, which is constituted of the *rpsF*, *ssb,* and *rpsR* genes. Clearly promoter activity was found also from the smaller RI fragment comprising the

10   274 bp upstream of the *rpsF* start codon only. These results suggest that *ssb is cotranscribed* and co-regulated with genes coding for ribosomal proteins, thereby coupling the regulation of protein synthesis and DNA metabolism.

In order to study the expression of *ssb* and *ywpH* in more detail, strains containing the S1-, R1- and Y1-lacZ fusions (BIV7, *8* and 12), respectively, were grown in TY

15   and MM and ß-galactosidase activity was examined as described by Miller et al. (Miller, J. H. 1982. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Whereas no expression could be detected from the S1 fragment under the conditions employed, the *ssb* operon appeared to be strongly expressed from the *rpsF*-promoter in both rich and minimal

20   media. The highest values (between 200 and 300 Miller units per OD) were reached during exponential growth (Figure 2A, B). When cells stopped growing the expression dropped. There is still expression in the stationary phase but on a much lower level.

25   In conclusion these results show directly growth-related characteristics of expression behavior of the *ssb* operon, and thus of the *rpsF*-promoter.

In contrast, no expression of the *ywpH* gene could be detected in cells of *B. subtilis* BIV12 grown in TY and only very low expression was observed in exponentially

30   growing cells in MM. However, expression of *ywpH* increased significantly, when cells growing in MM entered the stationary phase and reached its highest level within two hours thereafter (Figure 2C).

In conclusion: the *ywpH* promoter is only expressed during the stationary phase in MM.

35

Below, the primers for, and the sequences of the S1, S2, R1 and Y1 constructs are given. Underlined regions reflect restriction sites.

S1: made with primers

ssb-1 Forward primer to amplify the *ssb* promoter region   GCG<u>AAGCTT</u>CCAAACATTGACGAAGAGTCT

ssb-2 Reverse primer to amplify the *ssb* promoter region   GCT<u>GGATCC</u>TCGGTTAAGCATAAGAAAGACC

Sequence:

5   GCTTCCAAACATTGACGAAGAGTCTAAAAAAGCAGTTATCGAGCGTTTCAACAAC

       GTTCTGACTTCTAACGGTGCGGAGATCACTGGAACAAAGGATTGGGGTAAACGT

       CGTCTTGCTTACGAAATCAACGATTTCCGCGACGGTTTCTACCAAATCGTAAACG

       TTCAATCTGACGCTGCGGCAGTTCAAGAATTTGACCGTCTAGCTAAGATCAGTG

       ACGATATCATTCGCCACATTGTTGTTAAAGAAGAAGAATAAGCAATTTTGAAATAT

10   ATAATGGTAAAAGGTGGTCTTTCTTATGCTTAACCGAGGAT

S2: made with primers

rps-1 Forward primer to amplify the *rpsF* promoter region   GCG<u>AAGCTT</u>GTGACTTTGAGCGGGGCTT

Ssb-2 Reverse primer to amplify the *ssb* promoter region   GCT<u>GGATCC</u>TCGGTTAAGCATAAGAAAGACC

Sequence:

15   GCTTGTGACTTTGAGCGGGGCTTCATTCGTGCTGAGACAGTTGCTTATGAGGAT

       CTTCTTGCGGGCGGCGGTATGGCAGGAGCTAAAGAGGCAGGAAAAGTCCGCCT

       TGAAGGGAAAGAATATGTGGTCCAAGACGGAGATGTTATTCATTTCCGATTTAAT

       GTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATATAAAATTGTGAG

       TAATAGAATTATTGCTCCTTGCCCATTATGGGCCGCTTAGTCCAAAAGGAGGTG

20   CAAACAGATGAGAAAGTACGAAGTTATGTACATTATCCGCCCAAACATTGACGAA

       GAGTCTAAAAAAGCAGTTATCGAGCGTTTCAACAACGTTCTGACTTCTAACGGTG

       CGGAGATCACTGGAACAAAGGATTGGGGTAAACGTCGTCTTGCTTACGAAATCA

       ACGATTTCCGCGACGGTTTCTACCAAATCGTAAACGTTCAATCTGACGCTGCGG

       CAGTTCAAGAATTTGACCGTCTAGCTAAGATCAGTGACGATATCATTCGCCACAT

25   TGTTGTTAAAGAAGAAGAATAAGCAATTTTGAAATATATAATGGTAAAAGGTGGT

       CTTTCTTATGCTTAACCGAGGAT

R1: made with primers:

Rps-1   Forward primer to amplify the *rpsF* promoter region   GCG<u>AAGCTT</u>GTGACTTTGAGCGGGGCTT

Rps-2   Reverse primer to amplify the *rpsF* promoter region   GCT<u>GGATCC</u>ATCTTCGTCAATGTTTGGGCG

30   Sequence:

GCTTGTGACTTTGAGCGGGGCTTCATTCGTGCTGAGACAGTTGCTTATGAGGAT
CTTCTTGCGGGCGGCGGTATGGCAGGAGCTAAAGAGGCAGGAAAAGTCCGCCT
TGAAGGGAAAGAATATGTGGTCCAAGACGGAGATGTTATTCATTTCCGATTTAAT
GTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATATAAAATTGTGAG
5   TAATAGAATTATTGCTCCTTGCCCATTATGGGCCGCTTAGTCCAAAAGGAGGTG
CAAACAGATGAGAAAGTACGAAGTTATGTACATTATCCGCCCAAACATTGACGAA
GATGG

Y1: made with primers

ywpH-1 Forward primer to amplify the *ywpH* promoter region CCC<u>AAGCTT</u>TCAAGCTGTCAATGCCG

ywpH-2 Reverse primer to amplify the *ywpH* promoter region CGC<u>GGATCC</u>GATTGAACATGCGATTCC

10

Sequence:
GCTTTCAAGCTGTCAATGCCGGAAAAAAAATTGAGCTTTCAGTGGTTTGCGTGG
GATGGCTCTTCCTATGTGCGCATGAATACGCAAAACTGGCTGACAAAGCAAATC
TTTTTCCGTTTTTTAAAAAGTACATATTTCTTCAAAGGAAAAAAGCAAAGATGTT
15   TTTAGCTGAAGGAAAAATGAAAACGAAAGATAAAAACAGAGGCTGAAAGCCATTT
TTAAGCGTTTTTCTTTTCTTGTTGCATCATTTACAATACATACAACCGCAAGGAGA
GGAGGAATCGCATGTTCAATCGGAT

Example 2

Cloning strategy _Bacillus subtilis_ integration/ multi copy plasmid with expression

5   cassette.

In this construct, the _rpsF_ promoter as described in the invention is used, but the

RBS is the _spoVG_ RBS as also found on the _Bacillus subtilis_ genome. The gene

cloned and expressed in this expression cassette is the _Clostridium perfringens_ beta-

toxin (Hunter et al.; Infect. & Immun. 61: 398-965 (1993))

10

1 PCR reactions:

1.1 amyE

Primers for amplifying the _amyE_ gene introducing a 5' _XbaI_ site and a 3' _SacI_ site

15   were as follows:

amyE-1: Forward primer: GCTCTAGACGAATTATATGGATGTGAC

amyE-2: Reverse primer: GCCTAGAGCTCGGATCTCCTTTTCCGATTG

Template: chromosomal DNA 168

Product size: 823 bp

20

1.2 Kanamycin resistance gene

Primers for amplifying the kanamycin resistance gene (same as in pUB110)

introducing 5' _PstI_ and _NruI_ sites and a 3' kpnI site

Kn-1: Forward primer: CAATCTGCAGTCGCGATGAGAATAGTGAATGGAC

25   Kn-2: Reverse primer: CGGGTACCTCAAAATGGTATGCGTTTTG

Template: pUB110 (Gryczan, T. J., Contente, S., Dubnau, D.; J. Bacteriol. 134: 318-

329 (1978), McKenzie, T., Hoshino, T., Tanaka, T., Sueoka, N.; Plasmid 15: 93-103

(1986)).

Product size: 794 bp

30

1.3 spoVG RBS

Artificially made:

GAAGATCTCTCGAGGGTACCTTGATACACTAATGCTTTTATATAGGGAAAAGGTGGTGAACTCATATGAATCGAG
CTTCTAGAGAGCTCCCATGGAACTATGTGATTACGAAAATATATCCCTTTTCCACCACTTGAGTATACTTAGCTC

35

GAAGATCTCTCGAGGGTACCTTGATACAC   CTAATGCTTTTATATAGGGAAAAGGTGGTG
                    CATGGAACTATGTGATTACGAAAATATATC   CCTTTTCCACCACTTGAGTATACTTAGCTC

Primers:

spoVG1   5'- GA<u>AGATCT</u>CTCGAGGG<u>TACC</u>TTGATACAC-3'

spoVG2   5'- CTATATAAAAGCATTAGTGTATCAAGGTAC-3'

5   *spoVG3*   5'-CTAATGCTTTTATATAGGGAAAAGGTGGTG-3'

spoVG4   5'-CTCGATT<u>CATATG</u>AGTTCACCACCTTTTCC-3'

spoVG RBS can be found on the *Bacillus subtilis* genome.

Because of a *BglII* site in this kanamycin gene, the *spoVG* RBS could not be cloned

10   with *BglII*, so instead of *BglII* a *PstI* site must be introduced:

spoVG5: Forward primer: GA<u>CTGCAG</u>CTCGAGGG<u>TACC</u>TTGATACAC

spoVG4: Reverse primer: CTCGATT<u>CATATG</u>AGTTCACCACCTTTTCC

Template: *spoVG* RBS

15   Product size: 75 bp

1.4 gnt terminator

Primers for amplifying 3 stop codons and the gnt-terminator introducing 5' *BamHI*

and *HpaI* sites and 3' *XbaI* and *StuI* sites.

20   Gntterm-1: Forward primer:

GC<u>GGATCC</u>A<u>GGCCT</u>AACTAATTAACCTGTATTAAAAACACG

Gntterm-2: Reverse primer: GC<u>TCTAGA</u>GT<u>TAAC</u>CTTCTGTTGTTTGGGATAG

Template: chromosomal DNA 168

Product size: 107 bp.

25

1.5 *Clostridium perfringens* beta-toxin (Btox)

Primers for amplifying Btox, introducing a 5' *PstI*, *NdeI* site and a 3' *BamHI* site.

B-toxoid3: 5'-AA<u>CTGCAG</u>AGATCT<u>CATATG</u>AAGAAAAAATTTATTTCATT-3'

30   Btoxoid2: CGC<u>GGATCC</u>TTAAATAGCTGTTACTTTGTGAG

Product size: About 1000 bp

1.6 The *rpsF* promoter without *rpsF* RBS

rpsF-3: 5'-G<u>GAATTC</u>CT<u>GCAG</u>GTGACTTTGAGCGGGGCTTC-3'

35   *rpsF*-4: 5'-CGTACTTTCTCATATGTTTGCACC-3'

Template: chromosomal DNA 168

Product size: 295 bp.

2 Cloning:

2.1 Cloning kanamycin in pBlueSK-

The kanamycin PCR product was digested with *PstI* and *KpnI* and ligated into the

5 likewise digested pBlueSK-. This resulted in pIV26.

2.2 Cloning *amyE* in pIV26

The *amyE* PCR product was subsequently digested with *XbaI* and *SacI* and ligated
into the likewise digested pIV26. This resulted in pIV27.

10

2.3 Cloning Btox in pIV27

The *Btox* PCR product was digested with *PstI* and *BamHI* and ligated into the
likewise digested pIV27. This resulted in pIV28.

15 ### 2.4 Cloning the gnt terminator in pIV28

The gnt terminator PCR fragment was digested with *XbaI* and *BamHI* and ligated into
likewise digested pIV28, which resulted in pIV29.

2.5 Cloning the *spoVG* RBS in pIV29

20 The *spoVG* PCR product was digested with *PstI* and *NdeI* and ligated into the
likewise digested pIV29. This resulted in pIV33.

2.6 Cloning the *spoVG*-RBS + Btox + terminator- cassettes in pTRKH2

Plasmid pIV33 was digested with *XbaI* and *PstI* and ligated into likewise digested

25 pTRKH2. This resulted in pIV69. pTRKH2 is described by O'Sullivan, D.J. and
Klaenhammer, T,R. in Gene 137: 227-231 (1993).

2.7 Cloning the *rpsF* promoter (with *spoVG* RBS) in pIV69

Plasmid pIV69 was digested with *StuI* and ligated to the blunt ended *rpsF* PCR

30 fragment. This resulted in pIV82.
This plasmid was used for transformation of *Bacillus subtilis*.

Bacillus subtilis was subsequently transformed with the construct of 2.7, and grown in
rich medium. As is clear from figure 7, large amounts of *Clostridium perfringens* beta-

35 toxin were expressed and excreted into the supernatant. The increase in formation of
insoluble material in the cell is probably due to too high a level of expression. The
expression level can easily be decreased by using a low copy vector, or by

integration of the expression cassette in the genome at a single integration site or at
a few integration sites.

So far, several other toxins, i.a. the Pasteurella multocida toxin (see i.a. EP409895),
the E. coli heat-labile toxin and the Staphylococcus aureus alfa-toxin have equally
successfully been expressed.

This shows that the expression cassettes according to the invention are very
versatile expression vectors.

<u>Example 3:</u>

<u>Cloning strategy double integration construct</u>

5   **1. PCR**

1.1 *amyE*back:

PCR for amplifying the 5' *amyE* region, introducing a HindIII and a XhoI site.

*amyE*back-1: CCC<u>AAGCTT</u>TCGACATGGATGAGCGATG

10   *amyE*back-2: GCAG<u>CTCGAG</u>GCTCCGGCGCAAATGCAG

Product size: 1256 bp

1.2 *amyE*front:

PCR for amplifying the 3' *amyE* region, introducing a SacI and a XbaI site.

15   *amyE*front-1: CGC<u>GAGCTC</u>AACAAAATTCTCCAGTCTTC

*amyE*front-2: CGG<u>TCTAGA</u>AGTTTTTAATTTGTGTGTTTCC

Product size: 634 bp

1.3 Chloramphenicol:

20   PCR for amplifying the chloramphenicol resistance gene, introducing a EcoRI and a

HindIII site.

cmR-1: CGG<u>GAATTC</u>TCATGTTTGACAGC

cmR-2: CGCG<u>AAGCTT</u>CCCAGTAGTAGGTTGAGGCC

Product size: 1310 bp

25

1.4 Terminator:

PCR for amplifying stop codons in all frames and the gnt terminator sequence,

introducing BamHI, SnaBI, HpaI and XbaI sites.

Gntterm-3: Forward primer:

30   GC<u>GGATCCTACGTA</u>AACTAATTAACCTGTATTAAAAACACG

Gntterm-2: Reverse primer: GC<u>TCTAGAGTTAAC</u>CTTCTGTTGTTTGGGATAG

Template: chromosomal DNA 168

Product size: 107 bp.

35   **1.5 *rpsF* promoter:**

PCR for amplifying the *rpsF* promoter, introducing a PstI and a NdeI site.

rpsF-3: 5'-G<u>GAATTCCTGCAG</u>GTGACTTTGAGCGGGGCTTC-3'

rpsF-5 5'-CGTACTTTCT<u>CATATG</u>TTTGCACC-3'

Template: 168 chromosomal DNA

Product size: 295 bp

5 **1.6 Btox:**

PCR for amplifying Btox, introducing a BamHI and NdeI site.

B-toxoid3: AA<u>CTGCAGAGATCTCATATG</u>AAGAAAAAATTTATTTCATT

Btoxoid2: CGC<u>GGATCC</u>TTAAATAGCTGTTACTTTGTGAG

Product size: 1037 bp

10

2 Cloning

2.1 *amyE*back in pBlueKS-

15   The *amyE*back PCR product was digested with HindIII and XhoI and ligated into the

likewise digested pBlueKS- to produce construct 1.

2.2 *amyE*front in construct 1.

The *amyE*front PCR product was digested with SacI and XbaI and ligated into

20   likewise digested 1 to produce construct 2.

2.3 Chloramphenicol in construct 2.

The cm PCR product was digested with EcoRI and HindIII and ligated in likewise

digested 2 to produce construct 3.

25

2.4 gnt terminator in construct 3.

The terminator PCR product was digested with BamHI and XbaI and ligated in

likewise digested 3 to produce construct 4.

2.5 *rpsF* promoter in construct 4.

30   The *rpsF* PCR product was digested with PstI and ligated into PstI-SmaI digested 4

to produce construct 5.

2.6 Btox in construct 5.

35   The Btox PCR product was digested with BamHI and NdeI and ligated into likewise

digested 5 to produce construct 6.

Legend to the figures.

Figure 1: Gene organization of the *ssb* operon (A) and the *ywpH* gene (B) in *B. subtilis* 168 with schematic representation of the constructed *lacZ* fusions. Black lines in bold type represent the PCR-amplified DNA fragments fused to the promoter less *lacZ* gene.

The presence or absence of β-galactosidase activity in the various constructs after growth in rich (TY) or minimal medium (MM) is indicated on the right site by a '+' and '-', respectively.

Figure 2: Expression of the *ssb* operon and the *ywpH* gene in *B. subtilis*.
Bacterial growth (open symbols) in TY (triangles) or minimal medium (circles) and expression of the transcriptional *lacZ* fusions (closed symbols) under the control of the *rpsF* promoter, in the strain BIV8 (A, B) or the *ywpH* promoter in the strain BIV12 (C) reflected as β-galactosidase activity per OD.

Figure 3: upstream region of the *rpsF* gene, indicating the location of the –10 and –35 regions and AT-rich stretches further upstream, as well as the RBS and the start codon of the *rpsF* gene.

Figure 4: upstream region of the *ywpH* gene, indicating the location of the two *comK* boxes (bold), the RBS (italic) and the 30 first nucleotides (including ATG start codon) of the *ywpH* gene (underlined).

Figure 5: schematic representation of constructs of various length comprising the *rpsF* promoter fused to the ß-galactosidase gene. The location of the –10 and –35 region, the RBS and the *rpsF* start codon as well as the length of the various upstream fragments are indicated.

Figure 6: schematic representation of the ß-galactosidase activity of the various constructs of figure 5.

Figure 7: Expression of Btox under the control of *rpsF* promoter on a high copy vector. Sup=supernatant, Sol=soluble fraction, Insol=insoluble fraction. BtoxCp=wild type Clostridium perfringens beta-toxin. T=growth time in hours.

Claims

1) Expression cassette, characterized in that said expression cassette
   comprises a promoter obtainable from the region between the stop codon of
5 the *yyaF* gene and the start codon of the *rpsF* gene as found on the *Bacillus
   subtilis* genome.

2) Expression cassette, characterized in that said expression cassette
   comprises a promoter having the following characteristics
10 a) a nucleotide sequence TATAAT, preferably GTATAAT or TATAATA at the
   –10 site,
   b) a nucleotide sequence TTGTAA, preferably GTTGTAA or TTGTAAA at the
   –35 site,
   c) a spacer of 17 +/- 2 nucleotides, preferably 17 +/- 1 nucleotides, more
15 preferably 17 nucleotides.

.3) Expression cassette according to claim 1 or 2, characterized in that the
   promoter comprises the nucleotide sequence as depicted in SEQ ID NO 1.

20 4) Expression cassette according to claim 1 or 2,, characterized in that the
   promoter comprises the nucleotide sequence as depicted in SEQ ID NO 2.

5) Expression cassette according to claim 1 or 2, characterized in that the
   promoter comprises the nucleotide sequence as depicted in SEQ ID NO 3.

25
6) Expression cassette according to claims 1-5, characterized in that the
   promoter is preceded by a stretch of nucleotides having at least 1, preferably
   2, more preferably even three AT-rich regions upstream of the –35 region.

30 7) Expression cassette according to claim 6, characterized in that said stretch of
   nucleotides having AT-rich regions upstream of the –35 region comprises the
   nucleotide sequence as depicted in SEQ ID NO 4, preferably in SEQ ID NO
   5, more preferably in SEQ ID NO 6.

35 8) Expression cassette, characterized in that said expression cassette
   comprises a promoter having a consensus sequence

AAAANNNNNTTTTX$_n$AAAANNNNNTTTT, wherein 10<n<150

9) Expression cassette according to claim 8, characterized in that the promoter comprises the nucleotide sequence as depicted in SEQ ID NO: 7, preferably in SEQ ID NO: 8, more preferably in SEQ ID NO: 9.

10) Recombinant plasmid characterized in that said plasmid comprises an expression cassette according to claims 1-9.

11) Bacterial host cell comprising an expression cassette according to claims 1-9 or a recombinant plasmid according to claim 10.

12) Use of a promoter as described in claims 1-9 in an expression cassette.

13) Use of a promoter as described in claims 1-9 in a heterologous expression system.

14) Method for the heterologous expression of a gene, characterized in that said method comprises the construction of an expression cassette according to claims 1-9.

15) Method for the heterologous expression of a gene, characterized in that said method comprises the transfection of a bacterium with an expression cassette according to claims 1-9.
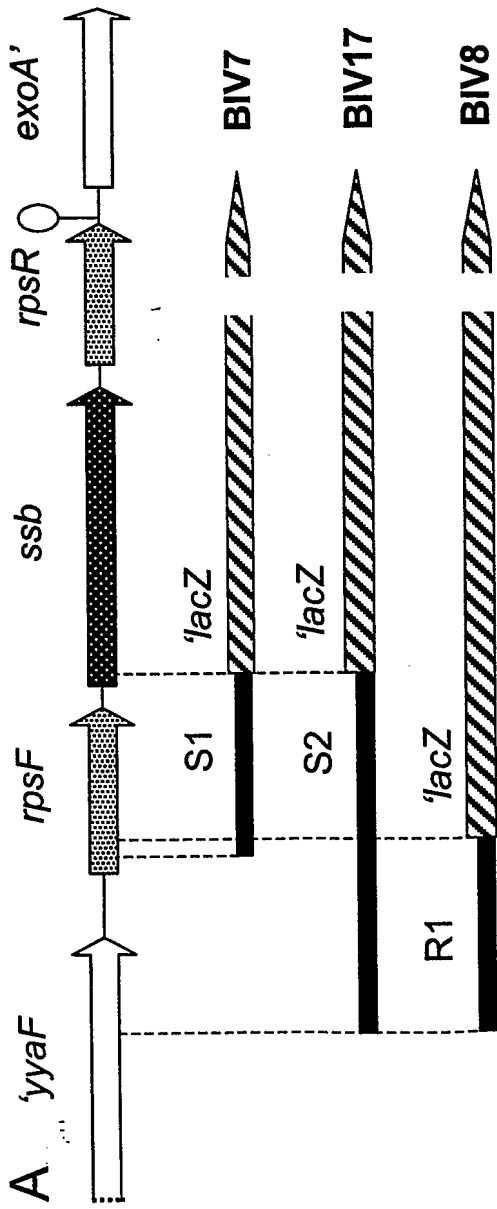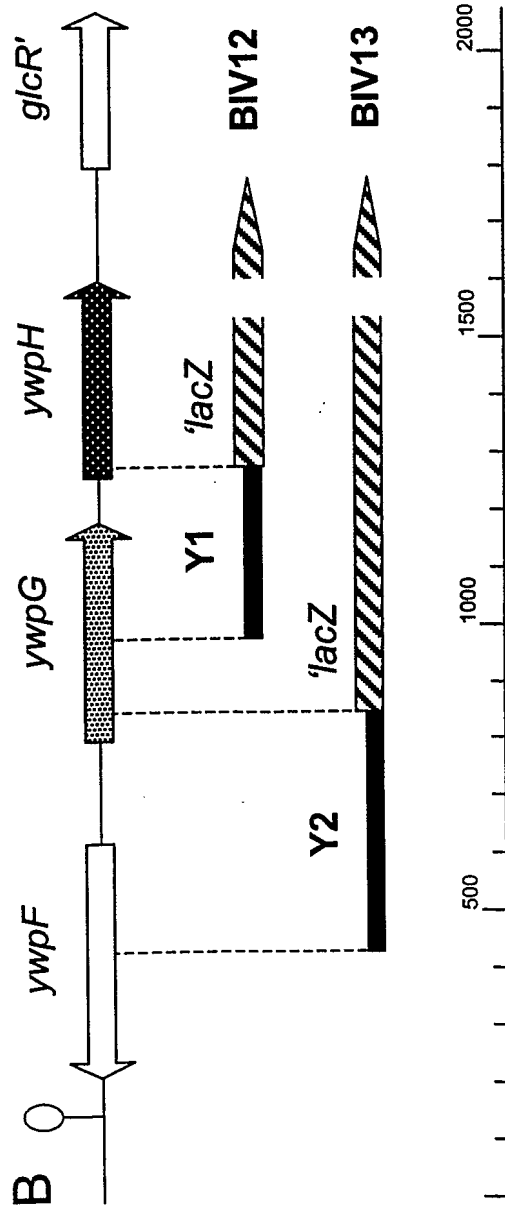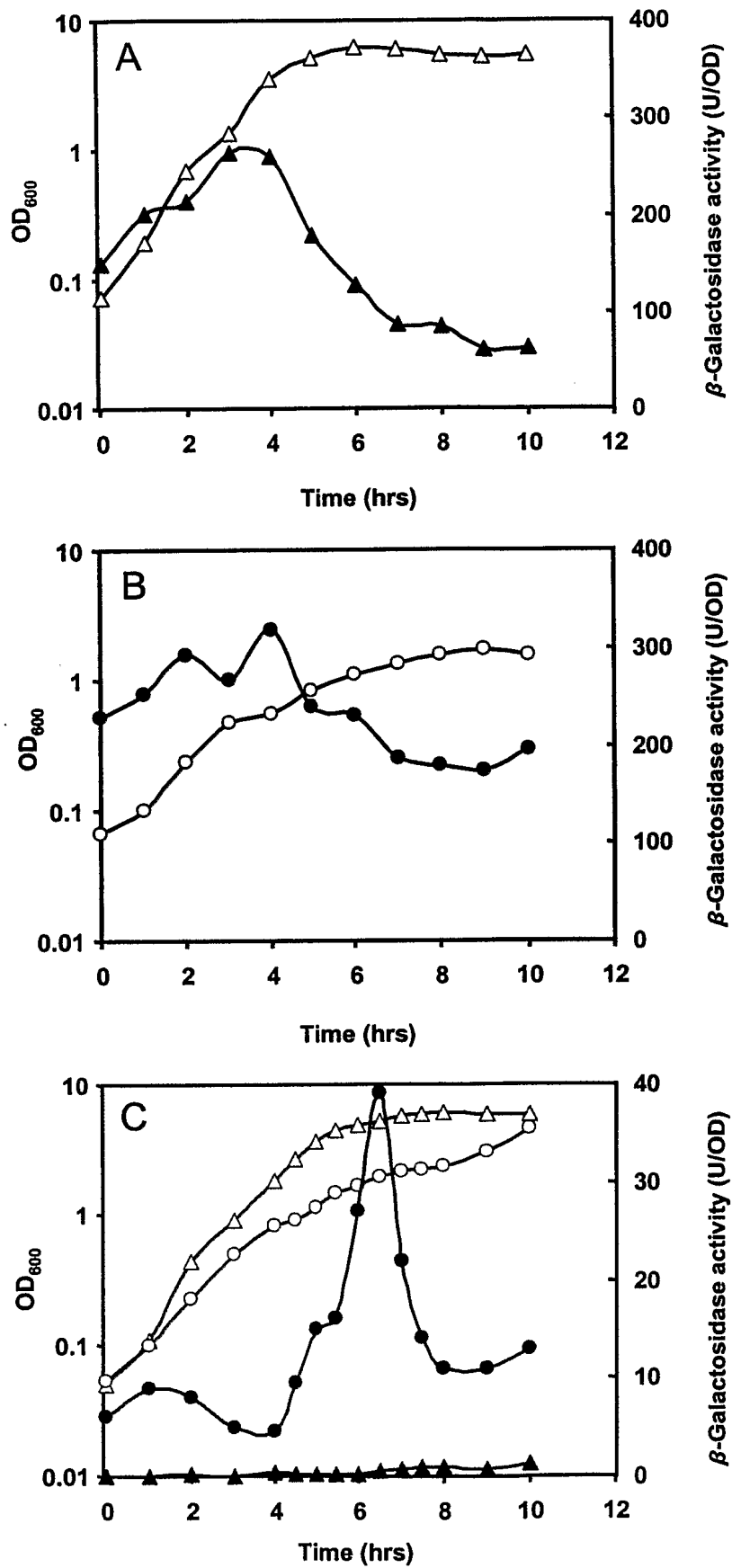
Figure 1.

Figure 2.

CTGAGTGTGCAGGAATTATTCATAGTGACTTTGAGCGGGGGCTTCA
                                        → Begin promoter construct

TTCGTGCTGAGACAGTTGCTTATGAGGATCTTCTTGCGGGCGGGCG

GTATGGCAGGAGCTAAAGAGGGCAGGAAAAGTCCGCCTTGAAGGGA

AAGAATATGTGGTCCAAGACGGAGATGTTATTCATTCCGATTTA
Stopcodon yyaF   -35   -10
ATGTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATA

TAAAATTGTGAGTAATAGAATTATTGCTCCTTGCCCATTATGGGC
                    RBS   Start codon rpsF
CGCTTAGTCCAAAAGGAGGTGCAAACAGAGTGGAAGTTACTGACGT

Figure 3.

TCAAGCTGTCAATGCCGGAAAAAAATTGAGCTTTCAGTGGT

TGCGTGGGATGGCTCTTCCTATGTGCGCATGAATACGCAAAA

CTGGCTGACAAAGCAAATCTTTTCCGTTTTT**AAAAAG**TACA

TATTTCTTCAAAGGAAAAAGC**AAAAG**ATG**TTTTT**AGCTGAAG

GAAAAATGAAAACGAAAGATAAAACAGAGGCTGAAAGCCATT

TTTAAGCCGTTTTCTTTTCTTGTTGCATCATTACAATACATACA

ACCGCAAGGAGGAGGAGGAATCGCCATG<u>TTCAATCAGGTCATGC</u>

<u>TTGTCGGACGT</u>

Figure 4.
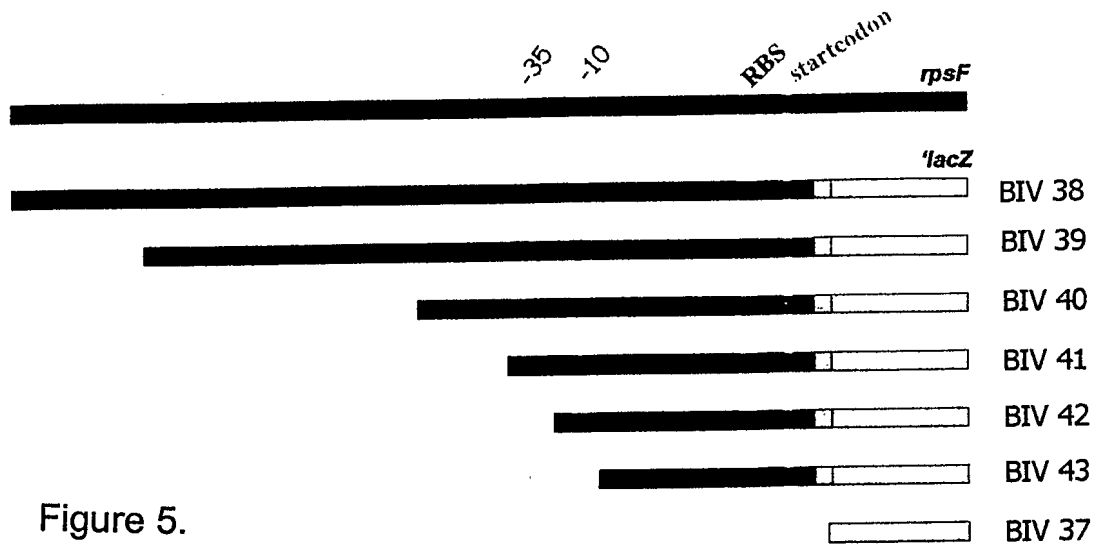
Figure 5.
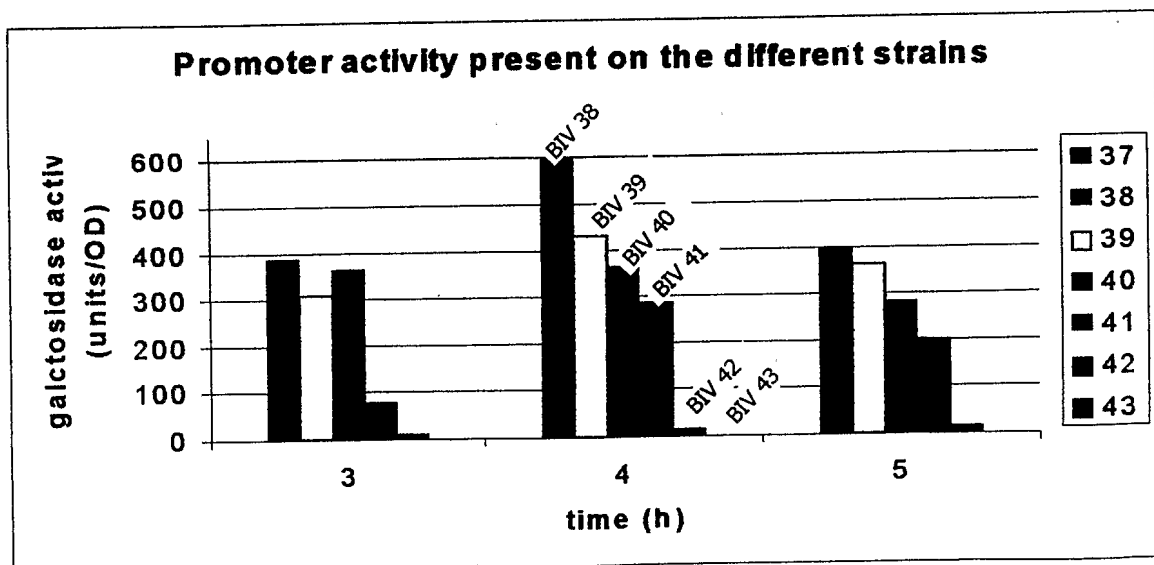

Figure 6.

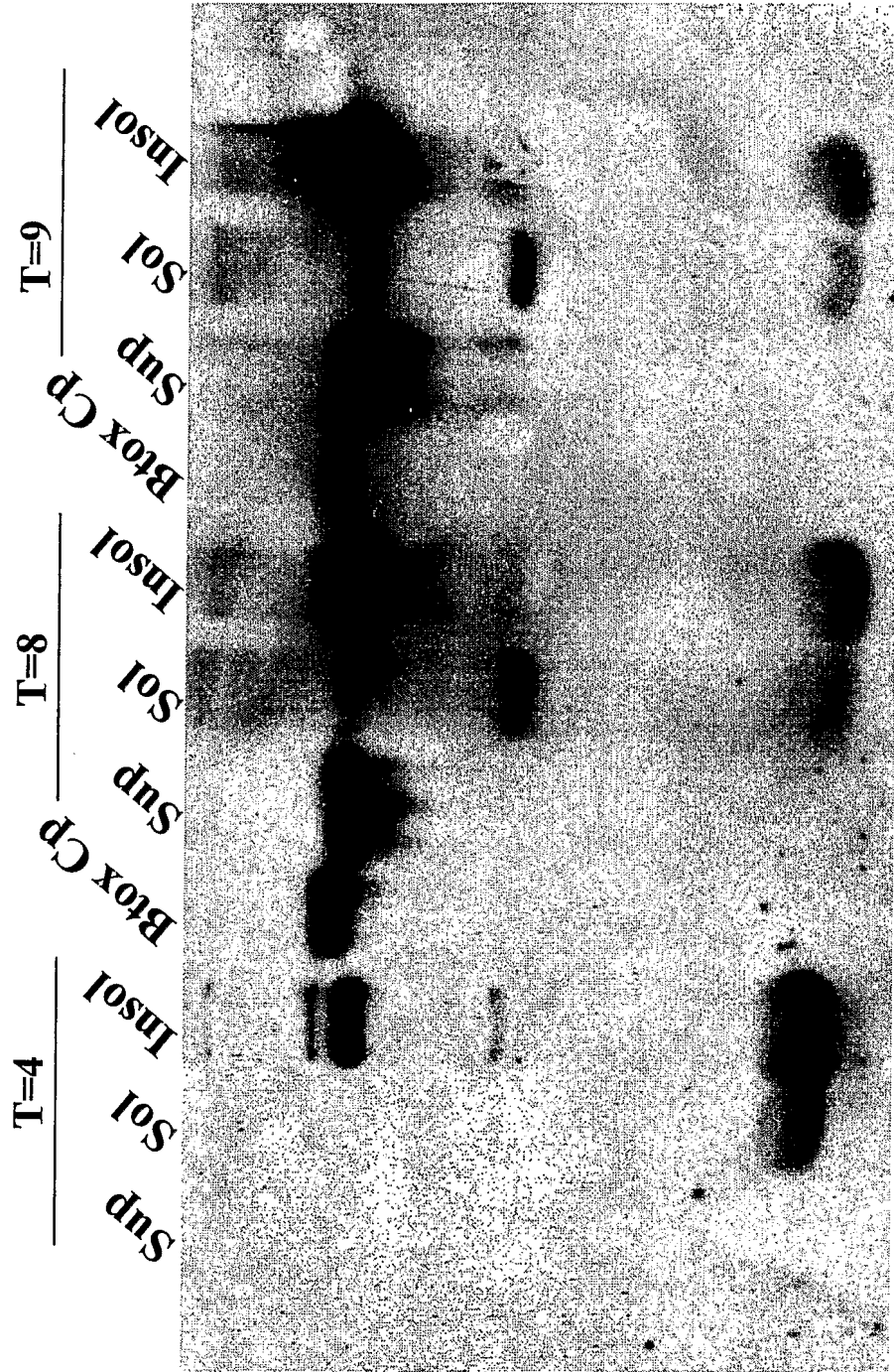Figure 7.

SEQUENCE LISTING

<110> akzo nobel n.v.

<120> novel bacterial expression system

<130> 2002.012

<160> 9

<170> PatentIn version 3.1

<210> 1

<211> 29

<212> DNA

<213> Bacillus subtilis

<400> 1
ttgtaaaggg acaagagctt tggtataat   29

<210> 2

<211> 94

<212> DNA

<213> Bacillus subtilis

<400> 2
ttgtaaaggg acaagagctt tggtataata taaaattgtg agtaatagaa ttattgctcc   60

ttgcccatta tgggccgctt agtccaaaag gagg   94

<210> 3

<211> 113

<212> DNA

<213> Bacillus subtilis

<400> 3
gatgcagttg taaagggaca agagctttgg tataatataa aattgtgagt aatagaatta   60

ttgctccttg cccattatgg gccgcttagt ccaaaaggag gtgcaaacag atg   113

<210> 4

<211> 20

<212> DNA

<213> Bacillus subtilis

<220>

<221> misc_feature

<222> (8)..(8)

<223> N

<220>

<221> misc_feature

<222> (13)..(14)

<223> N

<220>

<221> misc_feature

<222> (17)..(18)

<223> N

<220>

<221> misc_feature

<222> (20)..(20)

<223> N

<400> 4
atttaatnta tannatnnan   20

<210> 5

<211> 33

<212> DNA

<213> Bacillus subtilis

<220>

<221> misc_feature

<222> (6)..(6)

<223> N

<220>

<221> misc_feature

<222> (21)..(21)

<223> N

<220>

<221> misc_feature

<222> (26)..(27)

<223> N

<220>

<221> misc_feature

<222> (30)..(31)

<223> N

<220>

<221> misc_feature

<222> (33)..(33)

<223> N

<220>

<221> misc_feature

<222> (11)..(13)

<223> N

<400> 5
ttattnattt nnnatttaat ntatannatn nan   33

<210> 6

<211> 33

<212> DNA

<213> Bacillus subtilis

<400> 6
ttattcattt ccgatttaat gtataggatg cag   33

<210> 7

<211> 43

<212> DNA

<213> Bacillus subtilis

<400> 7
aaaagtacat atttcttcaa aggaaaaaag caaaagatgt ttt   43

<210> 8

<211> 154

<212> DNA

<213> Bacillus subtilis

<400> 8
aaaagtacat atttcttcaa aggaaaaaag caaaagatgt ttttagctga aggaaaaatg   60

aaaacgaaag ataaaaacag aggctgaaag ccatttttaa gcgtttttct tttcttgttg   120

catcatttac aatacataca accgcaagga gagg   154

<210> 9

<211> 166

<212> DNA

<213> Bacillus subtilis

<400> 9
aaaagtacat atttcttcaa aggaaaaaag caaaagatgt ttttagctga aggaaaaatg   60

aaaacgaaag ataaaaacag aggctgaaag ccatttttaa gcgtttttct tttcttgttg   120

catcatttac aatacataca accgcaagga gaggaggaat cgcatg   166