

The SURE Gene Expression System  
for *Bacillus subtilis*



**Mo Bi Tec**  
MOLECULAR BIOTECHNOLOGY



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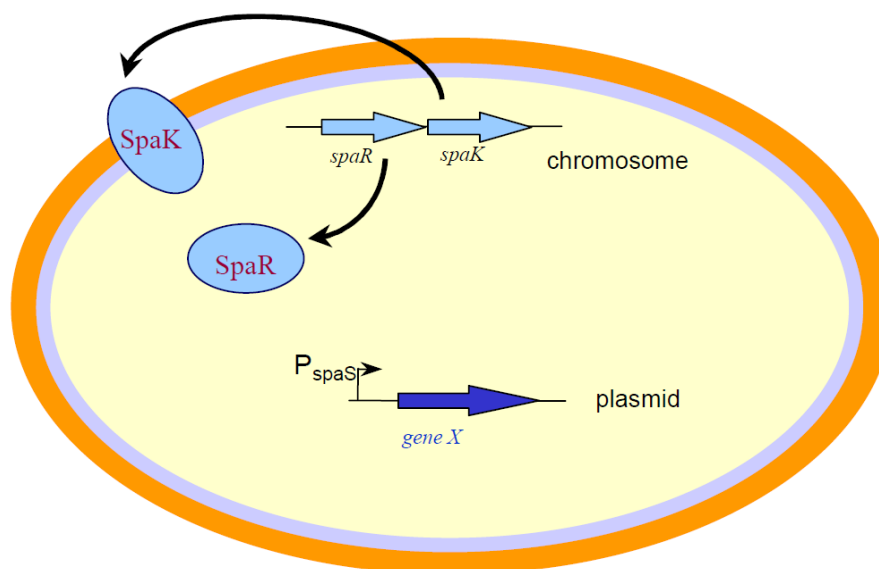
## 1. Introduction

Gram-positive bacteria are well known for their contributions to agricultural, medical and food biotechnology and for the production of recombinant proteins. Among them, *Bacillus subtilis* has been developed as an attractive host because of several reasons:

- *Bacillus subtilis* is non-pathogenic and is considered as a GRAS organism (generally regarded as safe).
- There is no significant bias in codon usage.
- It is capable of secreting functional extracellular proteins directly into the culture medium (at present, about 60% of the commercially available enzymes are produced by *Bacillus* species).
- A large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation and large-scale fermentation has been acquired.

**Subtilin** is a small peptide antibiotic of 32 amino acids produced by *Bacillus subtilis*. Subtilin production and regulation is encoded in the chromosome by a cluster of 9 genes that are transcribed from two promoters. Subtilin regulates/activates its own biosynthesis via a two component regulatory system.

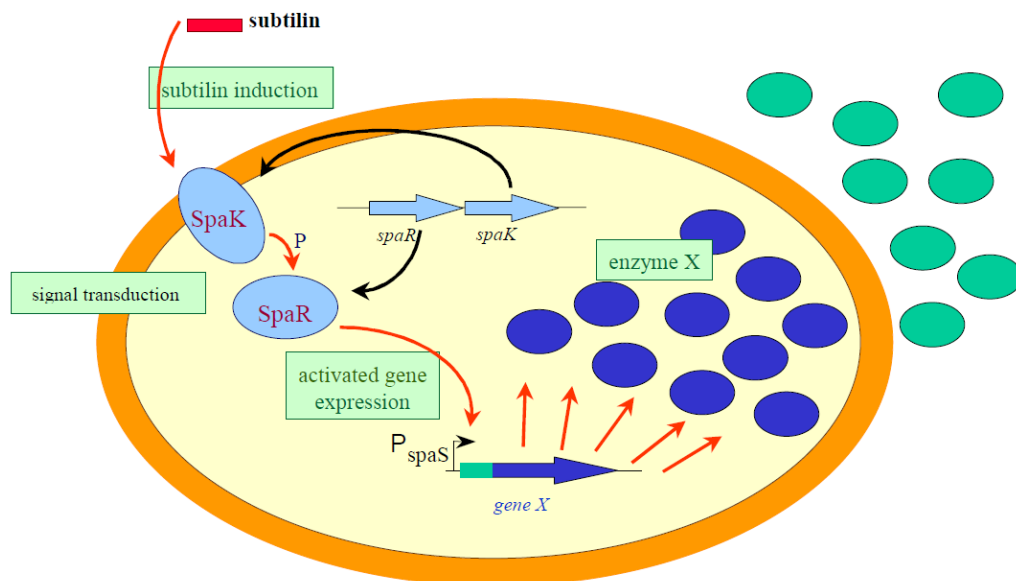
For the **SURE** system, **SUBtilin Regulated gene Expression**, developed by the company **NIZO food research bv** (The Netherlands), the genes of the regulatory components called SpaR (response regulator) and SpaK (membrane sensor histidine kinase) were isolated and placed on the chromosome of a *B. subtilis* host strain. One of the subtilin-regulated promoters is located upstream of a multiple cloning site into which the gene of interest can be cloned (*gene X*). Figure 1 shows the basic components of the **SURE** system.



**Figure 1:** Components of the **SURE** system, further explained in Fig. 2



Upon addition of subtilin the system is activated and the protein or interest is produced. It accumulates either intracellularly (blue), or is secreted (green) into the medium (see Figure 2).



**Figure 2:** Induction of gene expression in **SURE** after addition of subtilin to the culture (P = Phosphorylation)

### Advantages of SURE:

- Tightly controlled gene expression in a bacterium with long history of biotechnology
- Genome sequence available (Kunst *et al.*, 1997)
- Longstanding genetic engineering experience
- Complete set of genetic engineering tools available
- Potentially useful for cloning of genes with toxic products
- Can be used for the identification of essential genes after insertion into the chromosome upstream of the gene in question
- Potential for secretion of gene products
- Controlled gene expression for metabolic engineering

**Bacillus subtilis** sequence information: <http://genolist.pasteur.fr/SubtiList/>

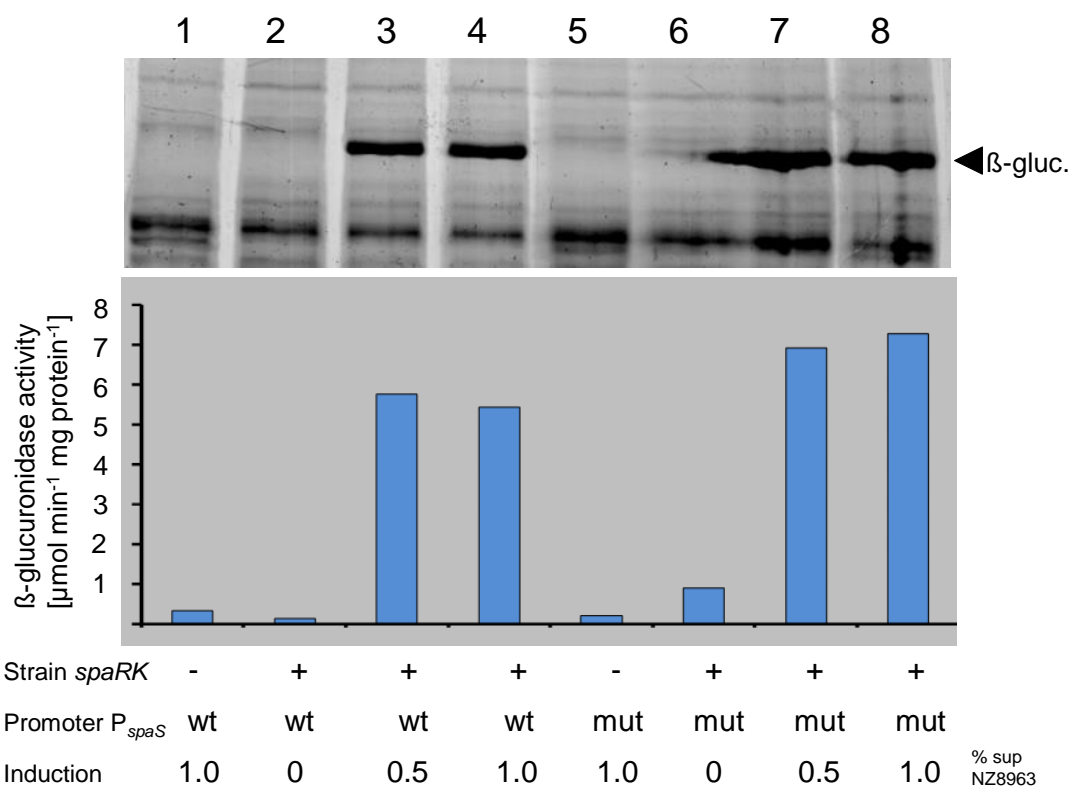
### Further resources:

- The *Bacillus subtilis* centered wiki [SubtiWiki](#): A community-curated consensual annotation that is continuously updated
- [SubtiPathways](#) is a model of *Bacillus subtilis* metabolism and regulation in SBML/SBGN (Systems Biology Markup Language/ Graphical Notation).



### Example:

Expression of the intracellular reporter gene  $\beta$ -glucuronidase (GUS) of *Escherichia coli* in *Bacillus subtilis*:



**Figure 3:** GUS expression in *B. subtilis* using the **SURE** system. 0.5 % and 1.0 %, respectively, of the supernatant of a subtilin producing strain NZ8963 were added. The figure shows both the SDS-PAGE analysis and an activity assay of the expression of the *gusA* gene of *E. coli* in *B. subtilis*. The plasmids pNZ8904 and pNZ8906 correlate to the plasmids pNZ8911 and pNZ8910 with the *gusA* gene coupled to the *spaS* promoter as translational fusion, respectively. Lanes: (1) Control strain NZ8901 + pNZ8904, (2) – (4) NZ8900 + pNZ8904, (5) Control strain NZ8901 + pNZ8906, (6) – (8) NZ8900 + pNZ8906; induction is indicated in % (vol/vol) of supernatant of strain NZ8963

First experiments have been conducted which show that **SURE** can also be used for the secretion of proteins. Corresponding products will be added once available.

In addition to the SURE gene expression system for *Bacillus subtilis*, MoBiTec offers further expression systems for *Bacillus subtilis*, as well as for *Bacillus megaterium*, *Lactococcus lactis*, yeast, and other prokaryotic and eukaryotic hosts. Please visit our website <http://www.mobitec.com> for details, or contact us at [info@mobitec.com](mailto:info@mobitec.com).



## 2. Strains and Plasmids of the SURE System

| Plasmids | Remarks  |
|----------|--|
| pNZ8901  | <b>SURE</b> expression vector, P <sub>spaSmut</sub> , Cm <sup>R</sup><br>High promoter activity, but some leakage.<br>Suitable for the production of non-toxic proteins.   |
| pNZ8911  | <b>SURE</b> expression vector, P <sub>spaS</sub> , Cm <sup>R</sup><br>No promoter activity without subtilin induction, lower expression than P <sub>spaSmut</sub> . Suitable for the production of potentially toxic proteins. |
| pNZ8902  | <b>SURE</b> expression vector, P <sub>spaSmut</sub> , Em <sup>R</sup><br>High promoter activity, but some leakage.<br>Suitable for the production of non-toxic proteins.   |
| pNZ8910  | <b>SURE</b> expression vector, P <sub>spaS</sub> , Em <sup>R</sup><br>Some promoter leakage without subtilin induction, caused by the vector; lower expression than pNZ8911.   |

| Strains                  | Remarks  |
|--------------------------|--|
| <i>E. coli</i>           | <b>MC1061</b><br>Intermediate cloning host (6). F <sup>-</sup> <i>araD139</i> $\Delta$ <i>ara-leu</i> 7696 $\Delta$ ( <i>lac</i> )X74 <i>galU galK hsdR2 mcrA mcrB1 rspL</i>   |
| <i>Bacillus subtilis</i> | <b>NZ8963</b><br>Wild type, subtilin producing strain (ATCC 6633)  |
|                          | <b>NZ8900</b><br><i>Bacillus subtilis</i> 168 strain with <i>spaR</i> and <i>spaK</i> integrated into the chromosome at the <i>amyE</i> locus. Host strain for subtilin inducible gene expression in <i>B. subtilis</i> . <i>amyE::spaRK</i> , Km <sup>R</sup> |
|                          | <b>NZ8901</b><br><i>B. subtilis</i> 168 with kanamycin gene integrated into the chromosome at the <i>amyE</i> locus.<br>Negative control strain. 168, <i>amyE::Km<sup>R</sup></i>  |

### Storage and handling of plasmids

Plasmids are supplied lyophilized. Upon receipt, add 100  $\mu$ l distilled water or buffer (10 mM Tris/HCL pH8.5) to a final concentration of 0.1  $\mu$ g/ $\mu$ l and incubate at 50 °C for 5 minutes. Vortex for 1 minute and store at -20 °C. Please note that all plasmids of this system are *E. coli* / *B. subtilis* shuttle vectors. We recommend *E. coli* MC1061 for plasmids deriving from Gram-positive bacteria.

### Storage and handling of *Bacillus* strains

The *Bacillus* strains are supplied as frozen cultures and shipped on dry ice. Store the stock at -80 °C. For propagation remove tube from freezer, scratch off some material from the surface of the frozen stock using a sterile loop. Replace stock immediately. Streak cell material onto an LB plate, seal the plate with parafilm and incubate at 37 °C overnight. *Bacillus* plates can be stored at 4 °C for 1 month. Use fresh bacteria for transformation.



### 3. Growth Conditions

Detailed protocols for *E. coli* and *Bacillus* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).

*B. subtilis* and *E. coli* can be grown aerobically at 37 °C in 2xYT medium (Bagyan *et al.*, 1998). Under optimal conditions the doubling time of *E. coli* is 20 min and of *B. subtilis* 30 min.

2xYT medium:                    16 g tryptone  
    10 g yeast extract  
    5 g sodium chloride (NaCl)  
 Add distilled water to 1000 ml, autoclave at 121 °C for 15 min

|              |                    |            |                 |             |
|--------------|--------------------|------------|-----------------|-------------|
| Antibiotics: | <i>B. subtilis</i> |            | <i>E. coli</i>  |             |
|              | chloramphenicol    | (5 µg/ml)  | chloramphenicol | (10 µg/ml), |
|              | erythromycin       | (5 µg/ml)  | erythromycin    | (150 µg/ml) |
|              | kanamycin          | (10 µg/ml) |                 |             |

| Order#         | Product                | Amount |
|----------------|------------------------|--------|
| CB-J902-500GAM | 2xYT medium broth      | 500 g  |
| CB-J859-500GAM | tryptone               | 500 g  |
| CB-J851-500GAM | casamino acids         | 500 g  |
| CB-0241-1KGAM  | sodium chloride (NaCl) | 1 kg   |
| CB-0230-100GAM | chloramphenicol        | 100 g  |
| 0219-10GAM     | erythromycin           | 10 g   |
| 0408-10GAM     | kanamycin sulfate      | 10 g   |
| J637-500GAM    | agar, bacteriological  | 500 g  |



## 4. Transformation of *Bacillus subtilis*

Detailed protocols for *E. coli* and *Bacillus* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).

The following protocol is adopted from Klein *et al.*, 1992.

### 4.1. Preparation of Competent *Bacillus subtilis* Cells

- Overnight culture of the appropriate recipient cells in 5 ml HS medium at 37 °C
- Inoculate 50 ml HS medium with 0.5 ml of the overnight culture; incubate under vigorous shaking at 37 °C
- Record the growth curve
- Take samples of 10 ml each when cells reach the stationary phase at 15 min intervals
- Add 1 ml of sterile glycerol (87%), mix and leave for 15 min on ice
- Fractionate into 1 ml aliquots, freeze in liquid nitrogen and store at -80 °C
- Check one aliquot from each time point with a reference plasmid DNA (see below) to identify the time point(s) yielding high level competent cells; discard the non- or low competent aliquots

### 4.2. Transformation of Competent *Bacillus subtilis* Cells

- Thaw one aliquot at 37 °C
- Use these cells to inoculate 20 ml LS medium
- Shake cells slowly in a 30 °C water bath to obtain maximal competence (about 2 h)
- Take 1 ml aliquots into a glass tube or a 2 ml plastic reaction tube, add 10 µl of 0.1 M EGTA (CB-0732-10GAM), and incubate for 5 min at room temperature
- Add plasmid or chromosomal DNA and incubate for 2 h at 37 °C while well shaking (well mixing is important when using plastic reaction tubes)
- If glass tubes were used, transfer cell suspension into a 2 ml plastic reaction tube
- Centrifuge, discard supernatant carefully, and resuspend the cells in the residual liquid remaining on the pellet
- Plate on selective 2xYT medium (see page 7)
- Incubate at 37 °C overnight





## 5. Media and Solutions

10x S-base (Spizizen's salt):

2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
14 g K<sub>2</sub>HPO<sub>4</sub>  
6 g KH<sub>2</sub>PO<sub>4</sub>  
1 g sodium citrate  
add distilled water to 100 ml and autoclave  
add 0.1 ml 1M MgSO<sub>4</sub> after autoclaving

HS medium:

66.5 ml distilled water  
10 ml 10x S-base  
2.5 ml 20 % (w/v) glucose  
5 ml 0.1 % (w/v) L-tryptophan  
1 ml 2 % (w/v) casein  
5 ml 10 % (w/v) yeast extract (Difco)  
10 ml 8 % (w/v) arginine, 0.4 % histidine  
autoclave all components separately  
tryptophan solution: sterile filtration

LS medium:

80 ml distilled water  
10 ml 10x S-base  
2.5 ml 20 % (w/v) glucose  
0.5 ml 0.1 % (w/v) L-tryptophan  
0.5 ml 2 % (w/v) casein  
5 ml 2 % (w/v) yeast extract (Difco)  
0.25 ml 1 mM MgCl<sub>2</sub>  
0.05 ml 1 mM CaCl<sub>2</sub>  
autoclave all components separately  
tryptophan solution: sterile filtration

0.1 M EGTA

dissolve 3.8 g EGTA in 50 ml distilled water  
adjust the pH to 7.2 using 10 N NaOH  
add distilled water to 100 ml; autoclave



## 6. Preparation of Subtilin for Induction

- Inoculate fresh overnight culture of *B. subtilis* NZ8963 into fresh 2xYT medium at an optical density at 600 nm ( $OD_{600}$ ) of 0.15
- Collect supernatant at culture  $OD_{600} = 1.0$  and heat for 10 min at 80 °C to eliminate residual living *B. subtilis* NZ8963 cells

## 7. Activation of the *spaS* / *spaSmut* Promoter by Subtilin

- Grow the appropriate *B. subtilis* strain overnight in fresh 2xYT medium
- Inoculate into fresh 2xYT medium to an  $OD_{600}$  of 0.15
- When culture reaches  $OD_{600}$  0.7 – 0.8, split into 2 portions and add supernatant of the fresh overnight culture of NZ8963 to one portion (optimum amount to be determined empirically. Suggested range 0.1, 0.2, 0.5, 1, 1.5, 2 % (v/v))
- Take samples at different time points for analysis

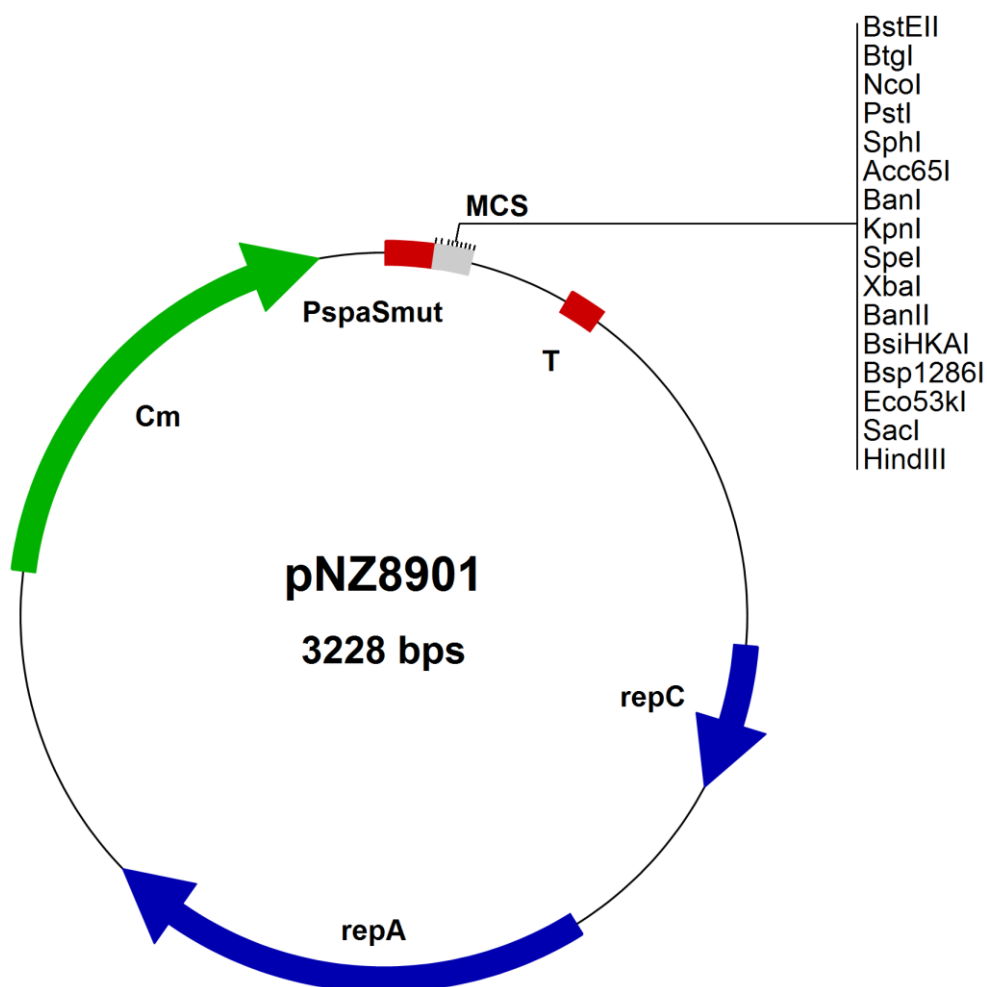
## 8. Sample Analysis for Intracellular Proteins

- Harvest cells by centrifugation (10 min, 6,000 x g, 4 °C)
- Wash and resuspend in 50 mM sodium phosphate buffer (pH 7.0) at an  $OD_{600}$  of 10
- Disrupt cells by ultrasonication (12 W, 6 x 15 pulses with 15 sec intervals) in 1.5 ml reaction tubes containing 1 ml of cell suspension, supplemented with lysozyme (250 µg/ml, CB-0663-5GAM), on ice
- Alternatively, cells can be disrupted by bead beating:
  - disrupt three times with glass beads (0.1 mm in diameter) (1 g/ml of cell suspension) in an orbital mixer at 180 V, with the mix kept on ice for 3 min between each disruption
- Remove cell debris (and glass beads) by centrifugation at 430 x g, 10 min, 4 °C
- Use the amount of protein corresponding to 0.025 of  $OD_{600}$  per sample for separation by SDS-PAGE

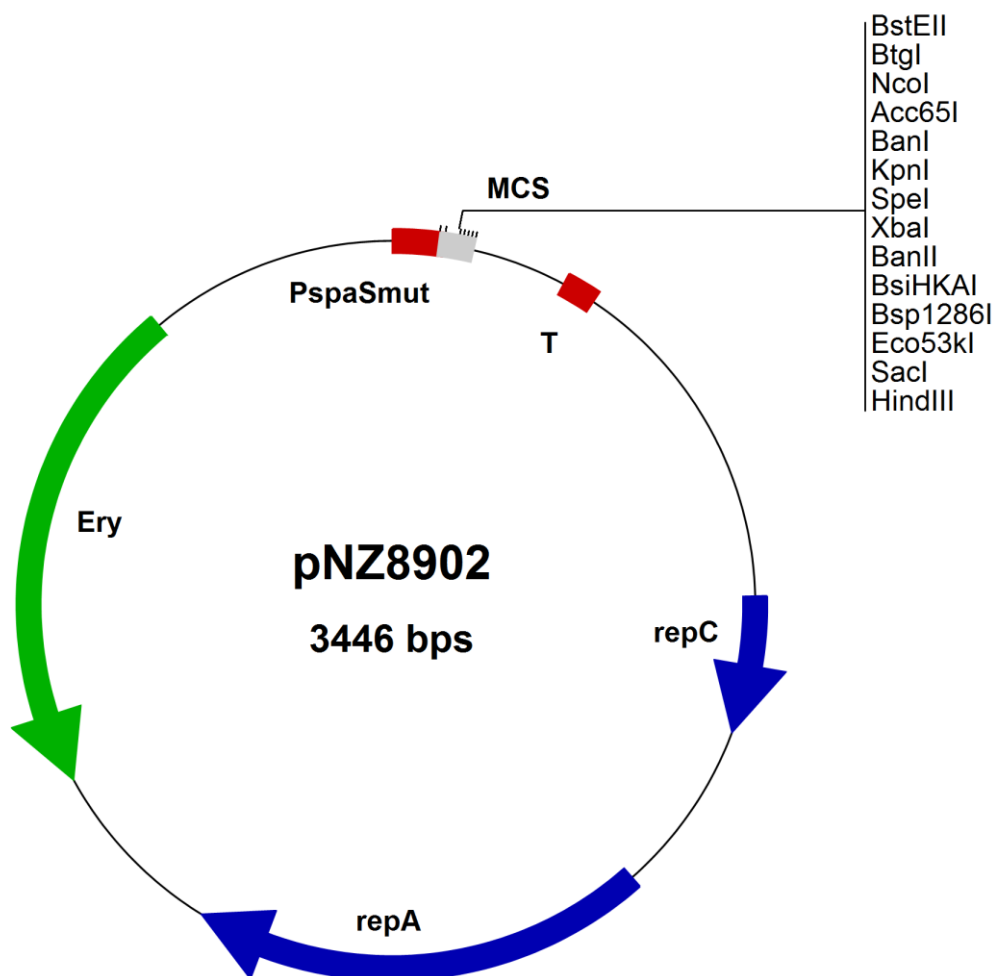


## 9. Vector Maps

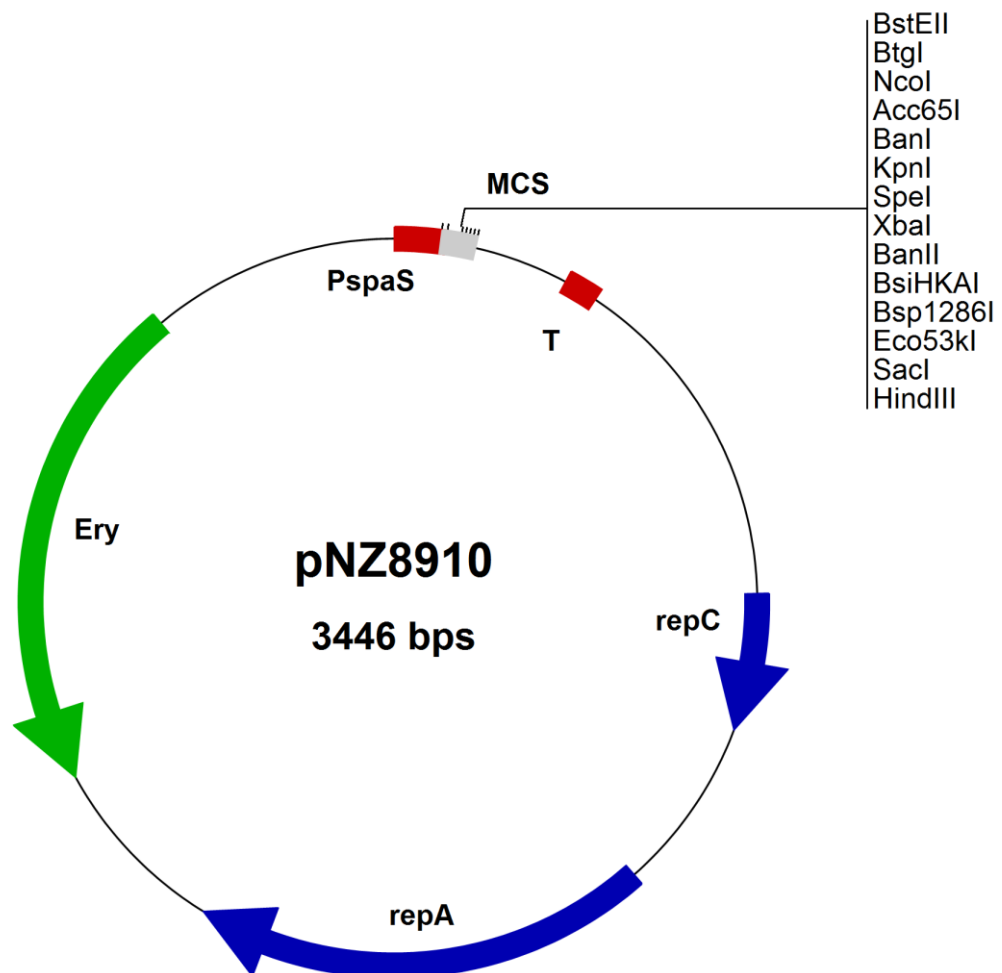
High-resolution maps and sequences of the vectors are available at [www.mobitec.com](http://www.mobitec.com)



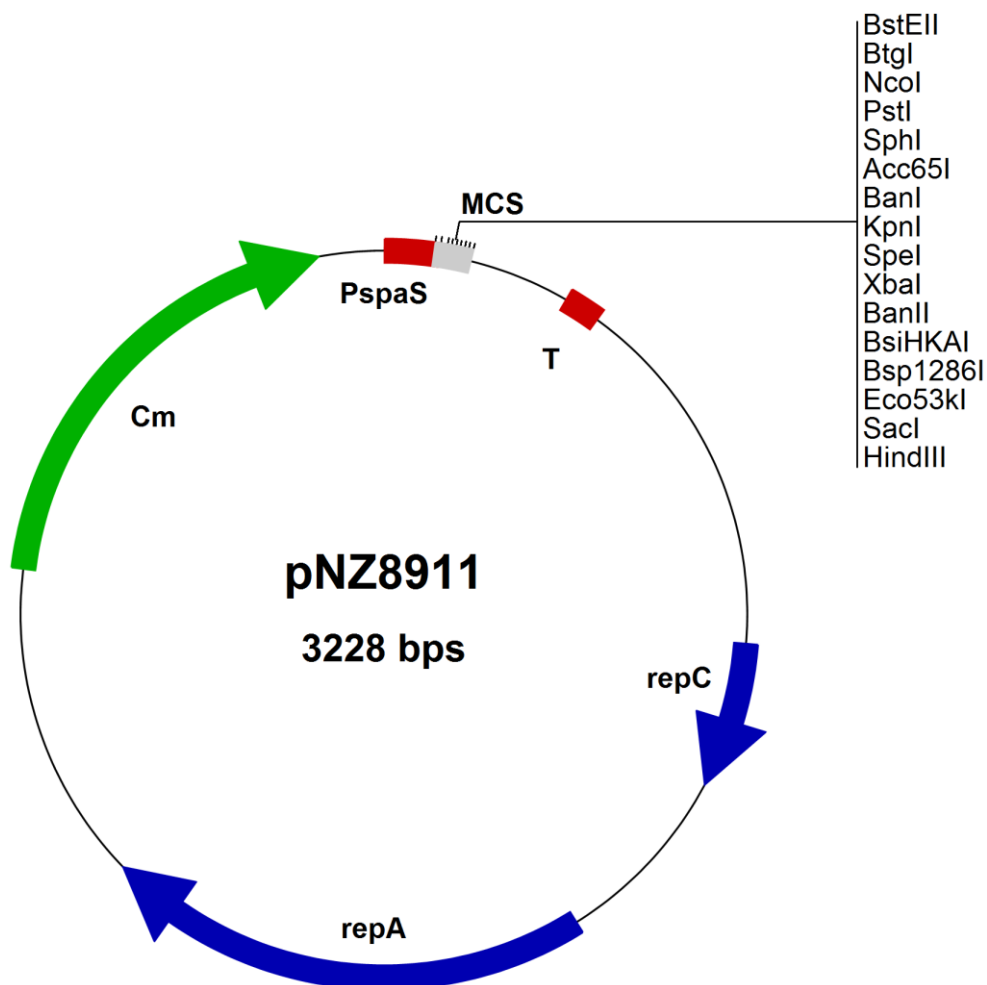
|  | Type                      | Name     | Start | Stop | Description  |
|--|---------------------------|----------|-------|------|--|
|  | Promoter                  | PspaSmut | 1     | 79   | Subtilin-regulated promoter (high expression activity)           |
|  | Terminator                | T        | 270   | 322  |  |
|  | Region                    | MCS      | 71    | 124  | Multiple Cloning Site  |
|  | Gene                      | repC     | 849   | 1058 | Replication gene C   |
|  | Gene                      | repA     | 1327  | 2025 | Replication gene A   |
|  | Selectable Genetic Marker | Cm       | 2485  | 3135 | Chloramphenicol resistance ( <i>B.subtilis</i> / <i>E.coli</i> ) |



|  | Type                      | Name     | Start | Stop | Description   |
|--|---------------------------|----------|-------|------|---|
|  | Promoter                  | PspaSmut | 1     | 79   | Subtilin-regulated promoter (high expression activity)        |
|  | Terminator                | T        | 270   | 322  |   |
|  | Region                    | MCS      | 71    | 124  | Multiple Cloning Site   |
|  | Gene                      | repC     | 849   | 1058 | Replication gene C  |
|  | Gene                      | repA     | 1327  | 2025 | Replication gene A  |
|  | Selectable Genetic Marker | Ery      | 3064  | 2309 | Erythromycin resistance ( <i>B.subtilis</i> / <i>E.coli</i> ) |



|  | Type                      | Name  | Start | Stop | Description   |
|--|---------------------------|-------|-------|------|---|
|  | Promoter                  | PspaS | 1     | 79   | Subtilin-regulated promoter (medium expression activity)      |
|  | Terminator                | T     | 270   | 322  |   |
|  | Region                    | MCS   | 71    | 124  | Multiple Cloning Site   |
|  | Gene                      | repC  | 849   | 1058 | Replication gene C  |
|  | Gene                      | repA  | 1327  | 2025 | Replication gene A  |
|  | Selectable Genetic Marker | Ery   | 3064  | 2309 | Erythromycin resistance ( <i>B.subtilis</i> / <i>E.coli</i> ) |



|  | Type                      | Name  | Start | Stop | Description  |
|--|---------------------------|-------|-------|------|--|
|  | Promoter                  | PspaS | 1     | 79   | Subtilin-regulated promoter (medium expression activity)         |
|  | Terminator                | T     | 270   | 322  |  |
|  | Region                    | MCS   | 71    | 124  | Multiple Cloning Site  |
|  | Gene                      | repC  | 849   | 1058 | Replication gene C   |
|  | Gene                      | repA  | 1327  | 2025 | Replication gene A   |
|  | Selectable Genetic Marker | Cm    | 2485  | 3135 | Chloramphenicol resistance ( <i>B.subtilis</i> / <i>E.coli</i> ) |



## 10. References

### General references:

**Bagyan, I., Casillas-Martinez, L. and Setlow, P.** (1998). The *katX* Gene, Which Codes for the Catalase in Spores of *Bacillus subtilis*, Is a Forespore-Specific Gene Controlled by  $\zeta$ F, and KatX Is Essential for Hydrogen Peroxide Resistance of the Germinating Spore; *J Bacteriol.* 180(8), 2057–2062

**Klein, C., Kaletta, C., Schnell, N. and Entian K.-D.** (1992). Analysis of Genes Involved in Biosynthesis of the Lantibiotic Subtilin; *Applied and Environmental Microbiology*, Jan. 1992, 132-142

**F. Kunst, N. Ogasawara, I. Moszer, <146 other authors>, H. Yoshikawa, A. Danchin** (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*; *Nature* 390, 249-256

**Sambrook, J. and Russel, D.W.** (2001) *Molecular Cloning: A laboratory manual*

### References on SURE:

1. **Bongers, R. S., J. W. Veening, M. Van Wieringen, O. P. Kuipers, and M. Kleerebezem** (2005) Development and characterization of a subtilin-regulated expression system in *Bacillus subtilis*: strict control of gene expression by addition of subtilin; *Applied and Environmental Microbiology* 71, 8818-8824

2. **Kleerebezem, M.** (2004) Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis; *Peptides* 25, 1405-1414

3. **Kleerebezem, M., R. Bongers, G. Rutten, W. M. de Vos, and O. P. Kuipers** (2004) Autoregulation of subtilin biosynthesis in *Bacillus subtilis*: the role of the spa-box in subtilin-responsive promoters; *Peptides* 25, 1415-1424

4. **Mierau, I., and M. Kleerebezem** (2005) 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*; *Applied Microbiology and Biotechnology* 9, 1-13



## 11. Order Information, Shipping and Storage

| Order#         | Product   | Amount |
|----------------|---|--------|
| VS-ELS10610-01 | NICE <sup>®</sup> /SURE <i>E. coli</i> host Strain MC1061 | 1 ml   |
| PBS023         | <i>Bacillus subtilis</i> strain NZ8963                    | 1 ml   |
| PBS024         | <i>Bacillus subtilis</i> strain NZ8900                    | 1 ml   |
| PBS025         | <i>Bacillus subtilis</i> strain NZ8901                    | 1 ml   |
| PBS031         | pNZ8901 vector, lyophilized plasmid DNA                   | 10 µg  |
| PBS032         | pNZ8902 vector, lyophilized plasmid DNA                   | 10 µg  |
| PBS033         | pNZ8910 vector, lyophilized DNA                           | 10 µg  |
| PBS034         | pNZ8911 vector, lyophilized DNA                           | 10 µg  |

Plasmids are shipped at room temperature (RT), strains on dry ice. Lyophilized plasmid DNA can be stored at 4 °C. We recommend storage at -20 °C, once the DNA has been dissolved in sterile water or buffer.

## 12. Related Products

| Order#         | Product                | Amount |
|----------------|------------------------|--------|
| CB-J902-500GAM | 2xYT medium broth      | 500 g  |
| CB-J859-500GAM | tryptone               | 500 g  |
| CB-J851-500GAM | casamino acids         | 500 g  |
| CB-0241-1KGAM  | sodium chloride (NaCl) | 1 kg   |
| CB-0230-100GAM | chloramphenicol        | 100 g  |
| 0219-10GAM     | erythromycin           | 10 g   |
| 0408-10GAM     | kanamycin sulfate      | 10 g   |
| CB-0339-25GAM  | ampicillin sodium salt | 25 g   |
| CB-0663-5GAM   | lysozyme, egg white    | 5 g    |
| 0732-10GAM     | EGTA                   | 10 g   |
| J637-500GAM    | agar, bacteriological  | 500 g  |

## 13. Contact and Support

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