The SURE Gene Expression System for *Bacillus subtilis*

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MOLECULAR BIOTECHNOLOGY

Contents

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, **SU**btilin **R**egulated gene **E**xpression, 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.

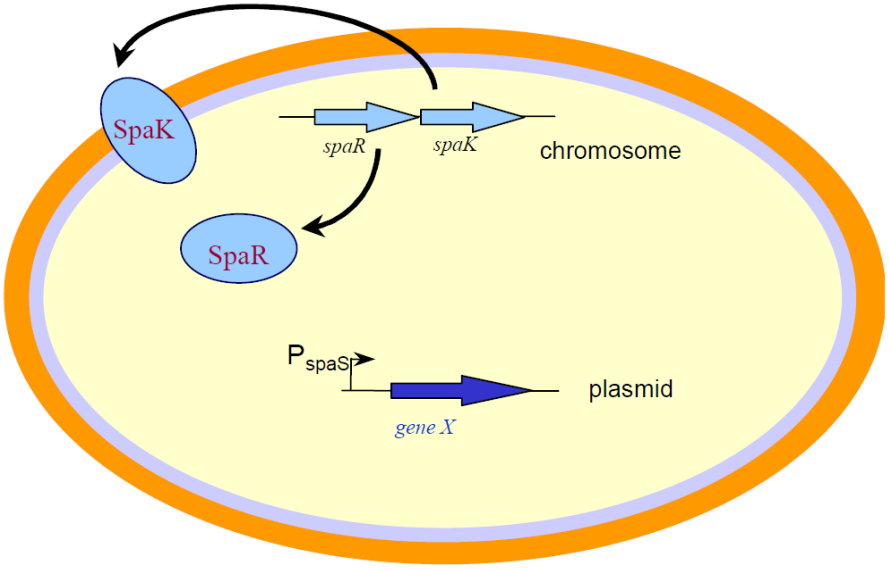


Figure 1: Components of the **SURE** system, further explained in Fig. 2

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).

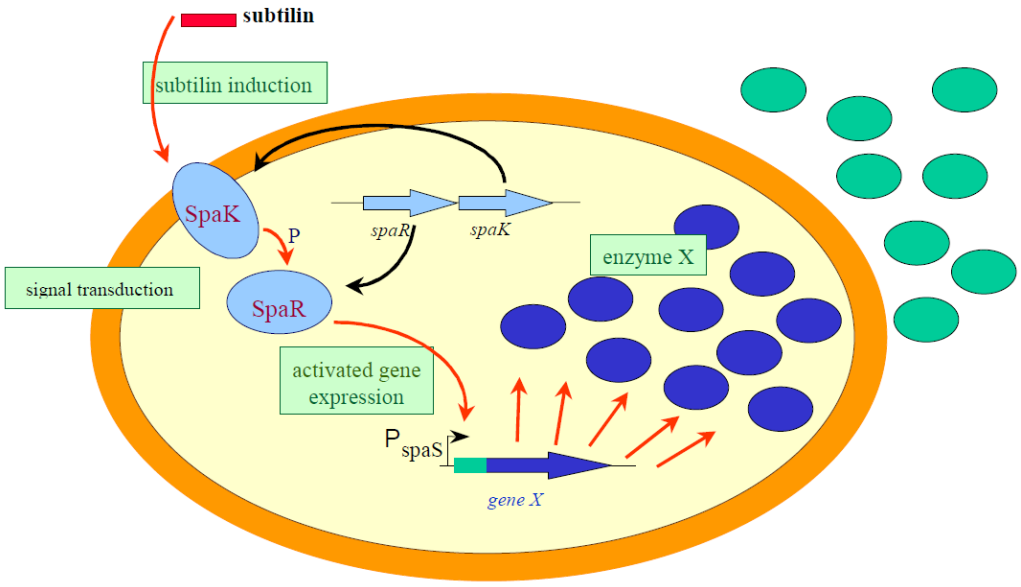


Figure 2: Induction of gene expression in **SURE** after addition of subtilin to the culture (P = Phosphorylation)

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 β-glucuronidase (GUS) of *Escherichia coli* in *Bacillus subtilis*:

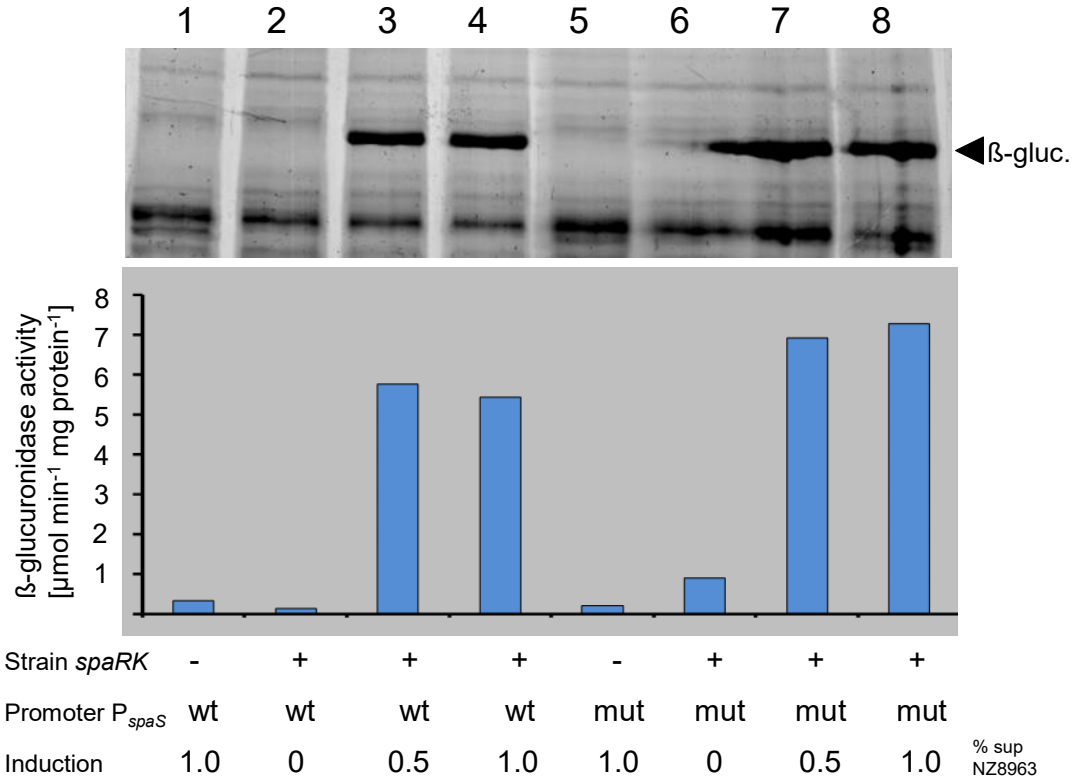


Figure 3: GUS expression in *B. subtilis* using the **SURE** system. 0.5 % and 1.0 %, respectively, of the supernatant of a subtilin producing strain NZ8963 were added. The figure shows both the SDS-PAGE analysis and an activity assay of the expression of the *gusA* gene of *E. coli in B. subtilis*. The plasmids pNZ8904 and pNZ8906 correlate to the plasmids pNZ8911 and pNZ8910 with the *gusA* gene coupled to the *spa*S 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.

2. Strains and Plasmids of the SURE System

Plasmids	Remarks
pNZ8901	**SURE** expression vector, P*spaS*mut, Cm^R High promoter activity, but some leakage. Suitable for the production of non-toxic proteins.
pNZ8911	**SURE** expression vector, P*spaS*, Cm^R No promoter activity without subtilin induction, lower expression than P*spaS*mut. Suitable for the production of potentially toxic proteins.
pNZ8902	**SURE** expression vector, P*spaS*mut, Em^R High promoter activity, but some leakage. Suitable for the production of non-toxic proteins.
pNZ8910	**SURE** expression vector, P*spaS*, Em^R Some promoter leakage without subtilin induction, caused by the vector; lower expression than pNZ8911.

Strains		Remarks
E. coli	**MC1061**	Intermediate cloning host (6). F^- *araD*139 Δ*ara-leu*)7696 Δ(lac)X74 *galU galK hsdR2 mcrA mcrB1 rspL*
Bacillus subtilis	**NZ8963**	Wild type, subtilin producing strain (ATCC 6633)
	NZ8900	*Bacillus subtilis* 168 strain with *spa*R and *spa*K integrated into the chromosome at the *amyE* locus. Host strain for subtilin inducible gene expression in *B. subtilis*. *amyE::spaRK*, Km^R
	NZ8901	*B. subtilis* 168 with kanamycin gene integrated into the chromosome at the *amyE* locus. Negative control strain. 168, *amyE*::Km^R

Storage and handling of plasmids
Plasmids are supplied lyophilized. Upon receipt, add 100 µl distilled water or buffer (10 mM Tris/HCL pH8.5) to a final concentration of 0.1 µg/µl 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:

B. subtilis		*E. coli*	
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	$(NH_4)_2SO_4$
14 g	K_2HPO_4
6 g	KH_2PO_4
1 g	sodium citrate

add distilled water to 100 ml and autoclave
add 0.1 ml 1M $MgSO_4$ 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 $MgCl_2$
0.05 ml	1 mM $CaCl_2$

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* / *spaS*mut 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

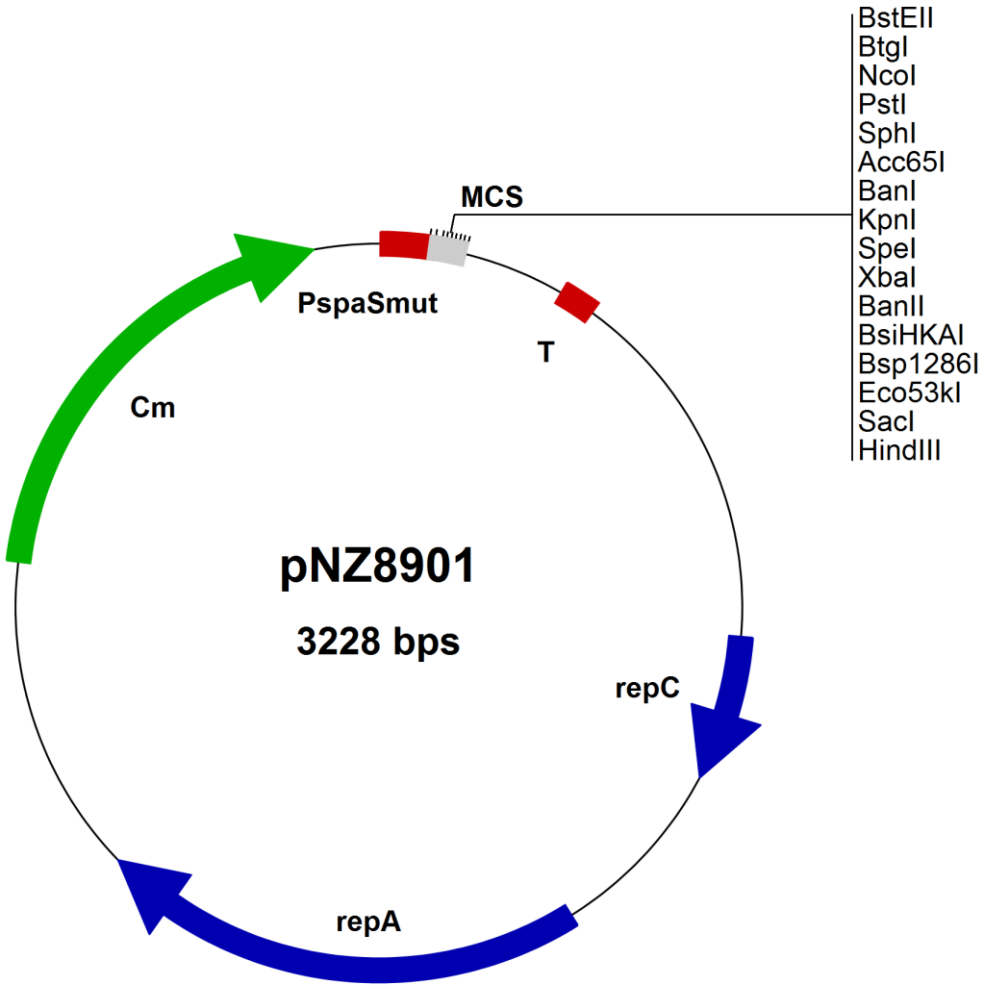
8. Sample Analysis for Intracellular Proteins

- 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:

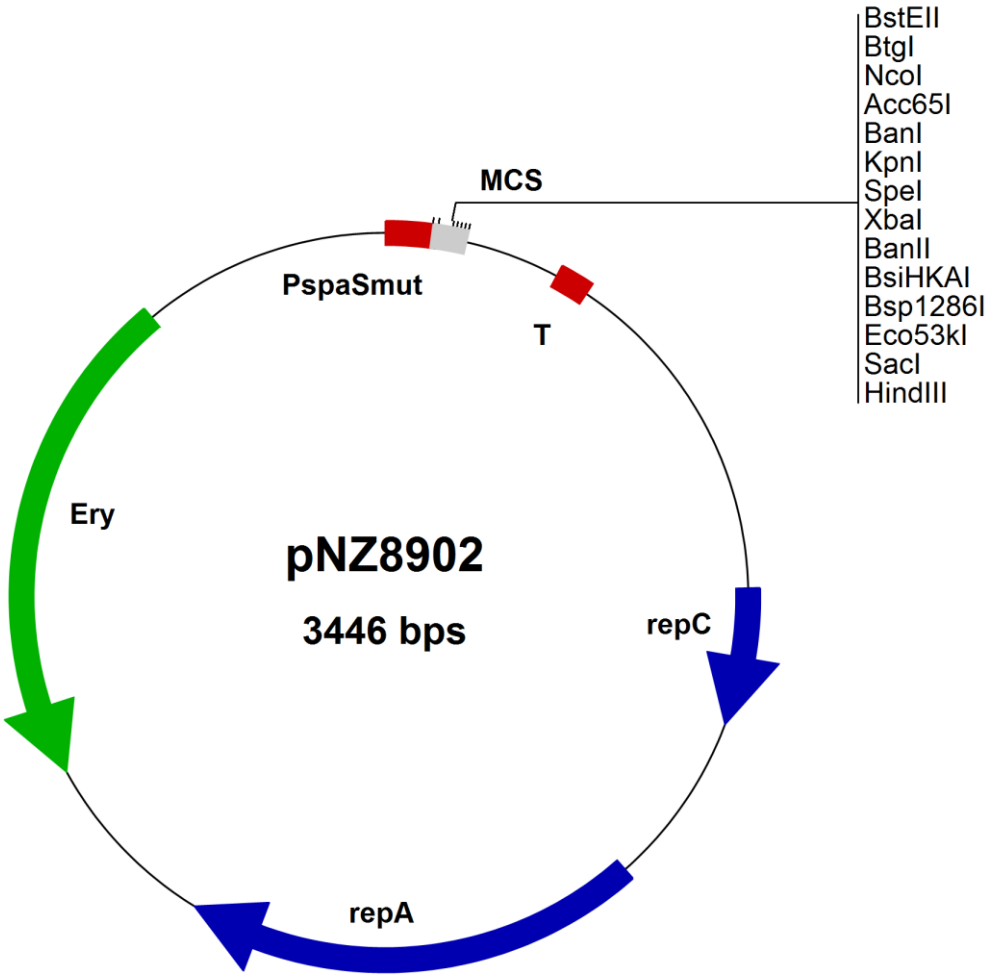
  disrupt three times with glass beads (0.1 mm in diameter) (1 g/ml of cell suspension) in an orbital mixer at 180 V, with the mix kept on ice for 3 min between each disruption
- Remove cell debris (and glass beads) by centrifugation at 430 x g, 10 min, 4 °C
- Use the amount of protein corresponding to 0.025 of OD_{600} per sample for separation by SDS-PAGE

9. Vector Maps

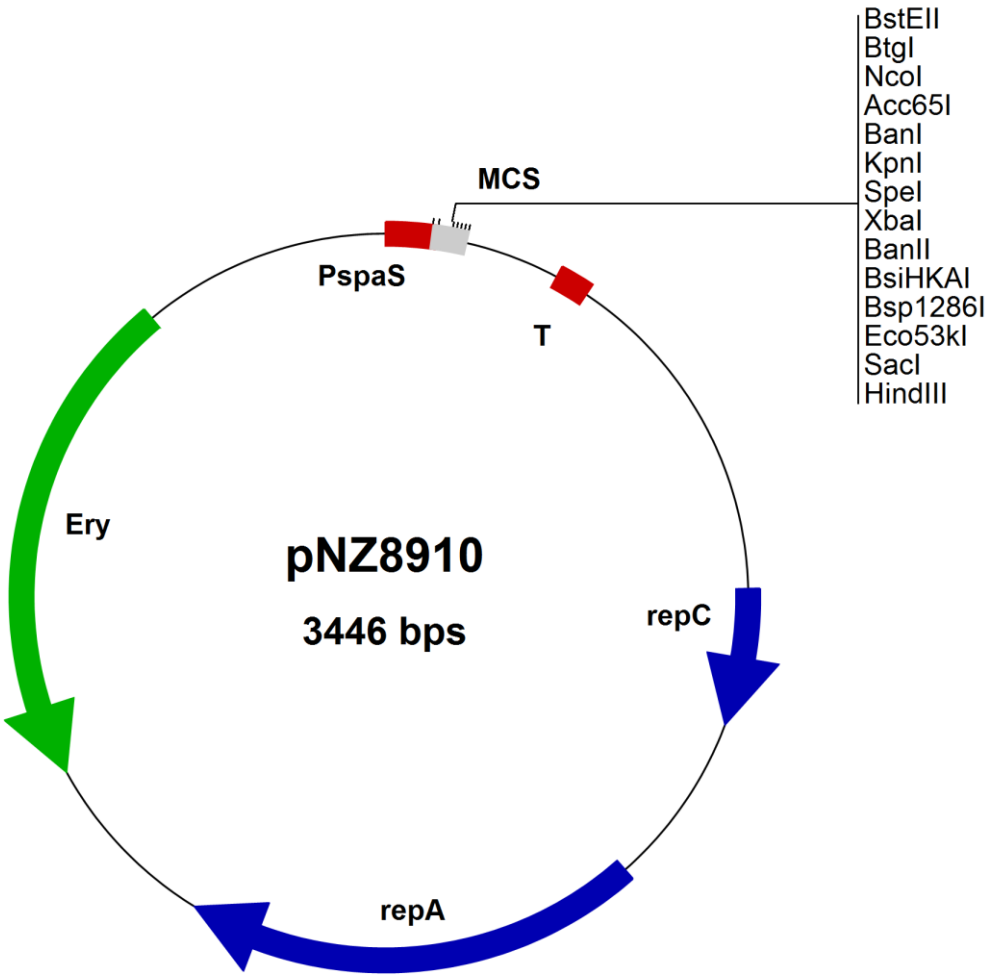
High-resolution maps and sequences of the vectors are available at www.mobitec.com



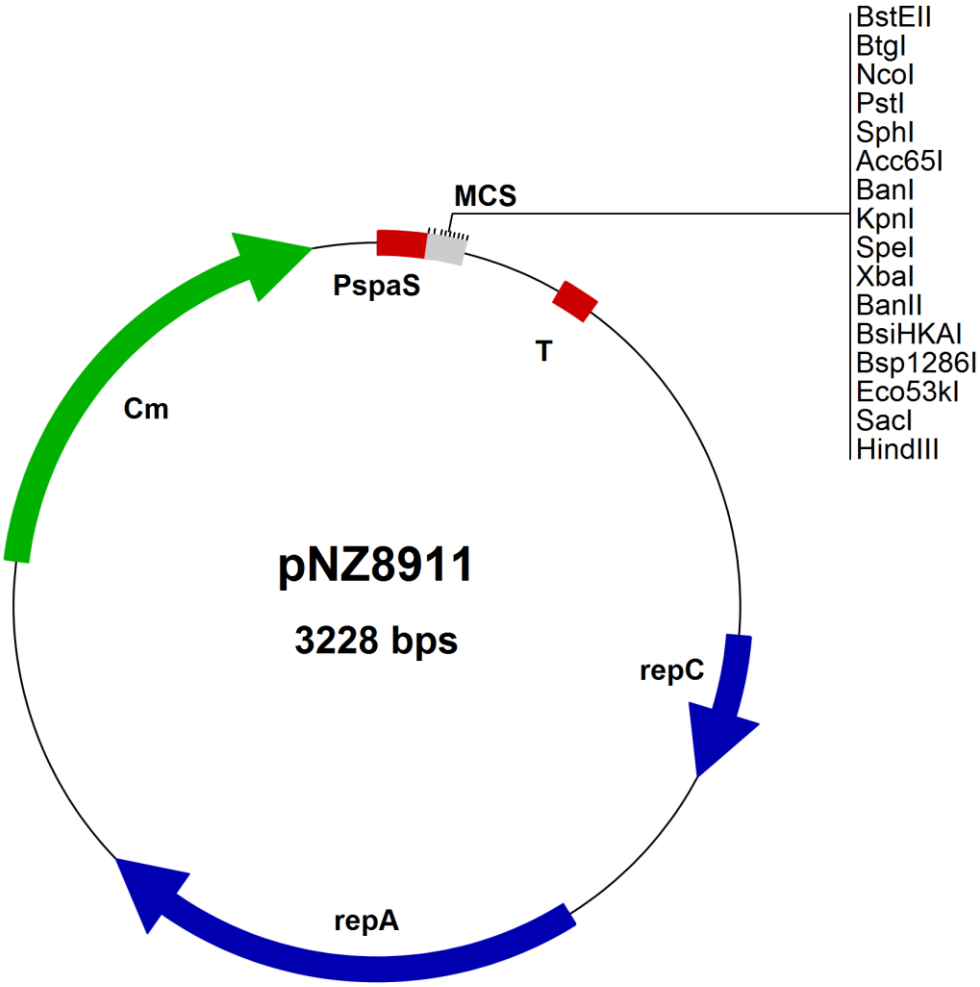
	Type	Name	Start	Stop	Description
	Promoter	PspaSmut	1	79	Subtilin-regulated promoter (high expression activity)
	Terminator	T	270	322	
	Region	MCS	71	124	Multiple Cloning Site
	Gene	repC	849	1058	Replication gene C
	Gene	repA	1327	2025	Replication gene A
	Selectable Genetic Marker	Cm	2485	3135	Chloramphenicol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



	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 (*B.subtilis* /*E.coli*)



	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 (*B.subtilis* /*E.coli*)



	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 (*B.subtilis* /*E.coli*)

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 ςF, 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®/SURE *E. coli* host Strain MC1061	1 ml
PBS023	*Bacillus subtilis* strain NZ8963	1 ml
PBS024	*Bacillus subtilis* strain NZ8900	1 ml
PBS025	*Bacillus subtilis* 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

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