Bacillus subtilis
P*grac100* Expression Vectors

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

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***Bacillus subtilis* Expression Vectors**

Plasmid-based expression vectors for highly efficient intracellular production of recombinant proteins in *Bacillus subtilis*.

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:

i. It is non-pathogenic and is considered as a GRAS organism (generally regarded as safe), which means it can be used for food production;
ii. It has no significant bias in codon usage;
iii. Due to only one membrane 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);
iv. A large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation and large-scale fermentation has been acquired.

But there are also two obstacles reducing the use of *B. subtilis*: (i) production of a number of extracellular proteases which recognize and degrade secreted heterologous proteins, and (ii) stable vector plasmids. The first obstacle, relevant only when secretion vectors are being used, has been largely solved by the construction of protease-deficient strains. And the second has been completely overcome by introducing plasmids using the theta-mode of replication such as those derived from the natural plasmids pAMβ1 and pBS72 (Jannière *et al.*, 1990; Titok *et al.*, 2003).

Using the *E. coli – B. subtilis* shuttle vector pMLBs72, which has been shown to replicate stably in both species (Titok *et al.*, 2003), several new expression vectors based on established induction systems were developed by Nguyen *et al.* (2005). These led to the first MoBiTec pHT expression systems (Nguyen *et al.*, 2007), based on the strong promoter of the groESL operon which was fused to the lac operator resulting in the Pgrac promoter (Phan *et al.*, 2006). It could be demonstrated that this system can result in up to 16% recombinant protein of the total cellular protein.

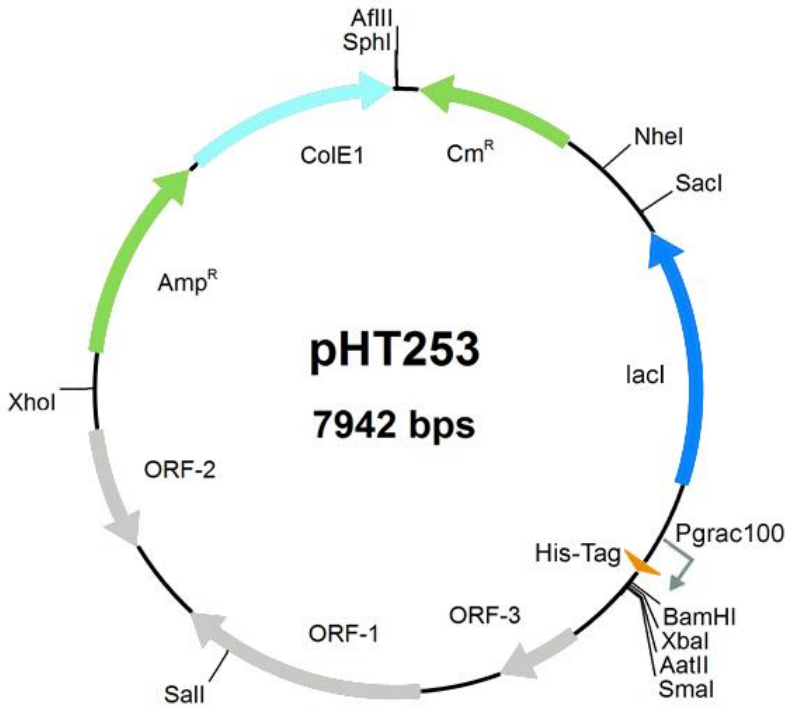
To develop the present *Bacillus subtilis Pgrac100* Expression System, Phan *et al.* (2011) improved the wild-type *groESL* promoter first by systematically altering its different DNA elements namely the UP element, the −35, −15 and −10 elements and the transcriptional start site, and second to combine the improved elements into different single promoters. As result the amount of recombinant protein could be enhanced up to about 30%.

2. The P*grac100* Vectors

All P*grac100* vectors use the strong promoter preceding the *groESL* operon of *Bacillus subtilis* with improved regulatory elements fused to the *lac* operator allowing their induction by IPTG. Nucleotides were optimized at the conserved regions of the *groESL* promoter including the UP element, the −35 and the −15 region. Combination of these changes into one promoter enhanced the amount of recombinant proteins accumulating intracellularly up to about 30% of the total cellular protein of *B. subtilis* (Phan *et al.*, 2011). In addition, the target proteins could be also expressed efficiently in *E. coli* in some cases.

3. Vector Maps

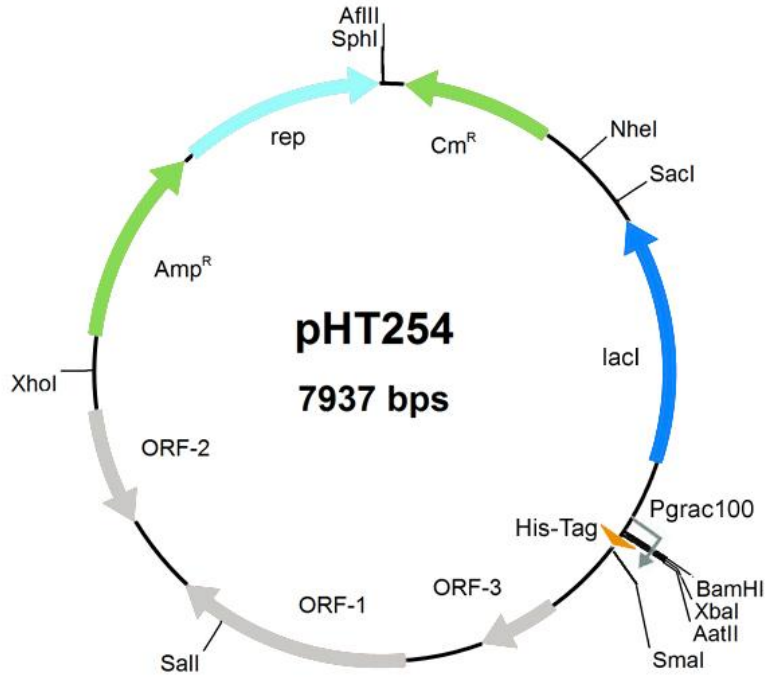
3.1 Map of pHT253



Sequence of the promoter region and MCS on pHT253:

AAAGGAGGAAGGATCT**ATG**-8xHis-tag-GGATCCATGTCTAGAGTCGACGTCCCCGGGGCAGCC
RBS *Bam*HI *Xba*I *Aat*II *Sma*I

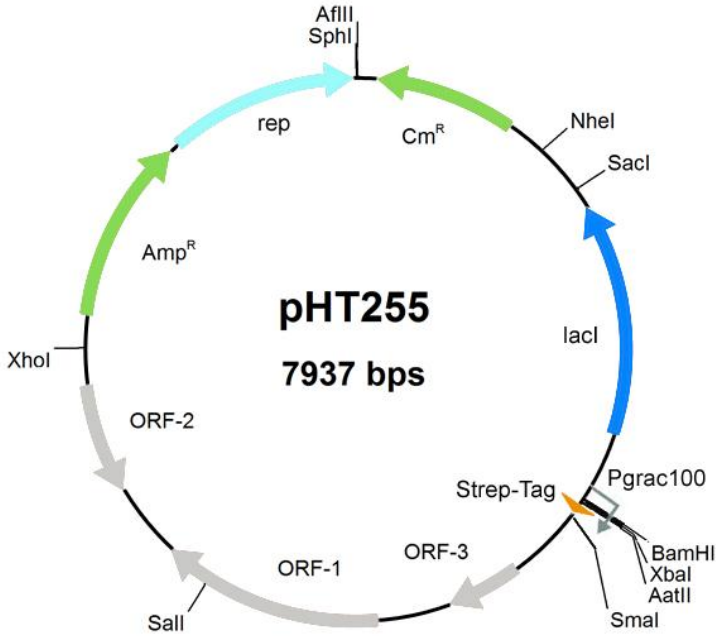
3.2 Map of pHT254



Sequence of the promoter region and MCS on pHT254:

AAAGGAGGAAGGATCC**ATG**TCTAGAGTCGACGTCGCT-8xHis-tag-**TAA**CGTCCCCGGGGCAGCC
RBS *Bam*HI *Xba*I *Aat*II *Sma*I

3.2 Map of pHT254



Sequence of the promoter region and MCS on pHT255:

AAAGGAGGAAGGATCC**ATG**TCTAGAGTCGACGTCGCT-Strep-tag-**TAA**CGTCCCCGGGGCAGCC
RBS *Bam*HI *Xba*I *Aat*II *Sma*I

4. *Bacillus subtilis* Host Strains

The following *Bacillus subtilis* strains suitable as hosts for gene expression are available from MoBiTec:

For intracellular expression:

- AS1: 1012 *hrcA*::*neo* (producing strain for enhancing solubility of intracellular protein Schulz and Schumann, 1996, and Phan *et al.*, 2006)
- 1012 wild type: *leuA8 metB5 trpC2 hsdRM1*
- 168 Marburg: *trpC2* (Trp^-)

For extracellular expression:

- WB800N: *nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg cm::neo;* NeoR

Note: *The neomycin marker of AS1 and WB800N is usually not required.*

5. Storage and Handling Instructions

Storage and handling of plasmids

Plasmids are supplied lyophilized. Upon receipt, add 100 µl distilled water (final concentration 0.1 µg/µl) and incubate at 50 °C for 5 minutes. Vortex for 1 minute and store at -20 °C.

All plasmids of this system are *E. coli* / *B. subtilis* shuttle vectors.

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. Streak 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.

6. 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, of *B. subtilis* 30 min.

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

Antibiotics: *B. subtilis* chloramphenicol (5 µg/ml)
E. coli ampicillin (50 µg/ml)

Order information:

Order#	Product	Quantity
CB-J902-100GAM	2xYT medium broth	100 g
CB-0230-100GAM	chloramphenicol	100 g
CB-0339-25GAM	ampicillin sodium salt	25 g
CB-J859-100GAM	tryptone	100 g
CB-J851-100GAM	casamino acids	100 g
CB-0241-1KGAM	sodium chloride (NaCl)	1 kg

7. 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).

7.1 Protocol A:

The following protocol is adopted from Klein *et al.* (1992). Immediate usage of freshly prepared competent cells usually results in higher transformation rates.

1.1 Preparation of competent *Bacillus subtilis* cells

- Culture appropriate recipient cells in 5 ml HS medium at 37 °C overnight
- 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 to identify the time point(s) yielding high level competent cells
- Discard the non- or low competent aliquots

1.2 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 2 ml Eppendorf 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 Eppendorf tubes)
- If glass tubes were used, transfer cell suspension into an Eppendorf tube
- Centrifuge, discard supernatant carefully and resuspend the cells into the final supernatant remaining on the pellet
- Plate on selective 2xYT medium
- Incubate at 37 °C overnight

7.2 Protocol B:

Electroporation of *B. subtilis* (modified from Zhang *et al.*, 2011)

- Culture *B. subtilis* in 2xYT medium overnight
- Dilute 100-fold with 2xYT medium
- Grow culture to an OD600 of 0.2
- Then supplement culture with 1% DL-threonine, 2% glycine, 0.1% tryptophan and 0.03% Tween 80
- Grow while shaking for 1 h
- Cool on ice for 20 min
- Spin at 5000 x g for 10 min at 4 °C
- Wash twice with electroporation buffer
- Resuspend in electroporation buffer at 1/100 of the original culture volume
- Add 100 µl cell to an ice-cold 2 mm cuvette
- Add 2 µl DNA (25 ng/ml)
- Shock by a single 12.5 kV/cm pulse (Gene Pulser; Bio-Rad), resistance 200 Ω, capacitance 25 µF
- Immediately add 1 ml 2xYT broth containing 0.5 M sorbitol and 0.38 M mannitol
- Incubate at 37 °C for 3 h
- Spread on selective 2xYT plates

7.3 Media and solutions

2xYT medium:	16 g tryptone 10 g casamino acids 5 g sodium chloride (NaCl)
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 M $MgCl_2$
0.05 ml 1 M $CaCl_2$
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

Electroporation buffer

0.5	M	trehalose
0.5	M	sorbitol
0.5	M	mannitol
0.5	mM	$MgCl_2$
0.5	mM	K_2HPO_4
0.5	mM	KH_2PO_4
		pH 7.4

filter-sterilize and store frozen

8. Induction with IPTG

- Grow 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 induce with 1 mM IPTG (CB-0487-1GAM) to one portion (t = 0)
- Collect samples at different time points for analysis (t = 1, 2, 3,…)

9. Sample analysis

9.1 Preparation of soluble and insoluble cell extracts from *B. subtilis*

- 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 Eppendorf tubes containing 1 ml of cell suspension, supplemented with lysozyme (250 µg/ml, CB-0663-5GAM), on ice

- *Alternatively, cells can be disrupted by beat 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

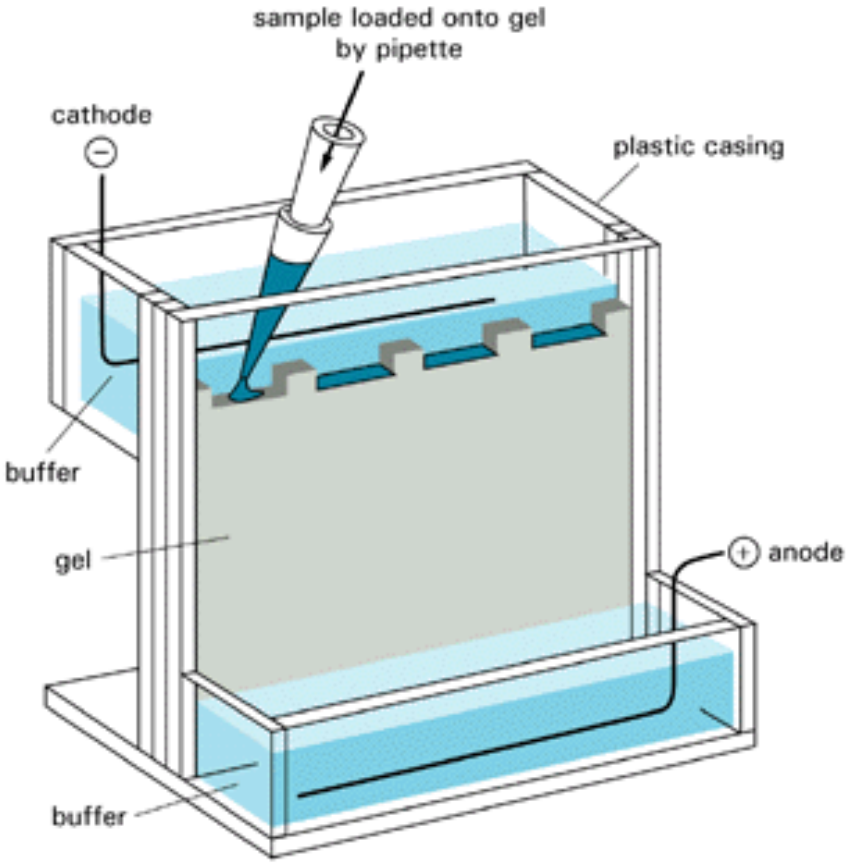
- Take 100 µl of the preparation as first total protein sample (T1)
- Remove remained cells by centrifugation at 4,300 x g, 10 min, 4 °C
- Take 100 µl of the supernatant for the second total protein sample (T2)

- Spin at 8.200 x g (10 min, 4 °C) to separate into insoluble (I) and soluble (S) protein fractions.
- Per sample use the amount of protein corresponding to 0.025 of OD_{600} for separation by SDS-PAGE
- Analyze samples by immunoblotting with specific antiserum

Order#	Product	Quantity
CB-0487-1GAM	IPTG	1 g
CB-0663-5GAM	lysozyme, egg white	5 g

9. 2 Precipitation of proteins from culture supernatant

- Collect protein from cultured supernatant by TCA method
- Mix 1 volume of 40 % TCA with 3 volumes of culture supernatant
- Incubate on ice for 10 min
- Centrifuge at 12,000 x g at 4 °C for 10 min (until the supernatant is clear)
- Wash pellet twice with ice-cold acetone and dry at room temperature
- Dissolve pellet in water and loading buffer for SDS-PAGE



10. References

Anagnostopoulos, C. and Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. 81:741-746.

Jannière, L., Bruand, C. and Ehrlich, S.D. (1990). Structurally stable *Bacillus subtilis* cloning vectors, Gene 87:53-61.

Klein, C., Kaletta, C., Schnell, N. & Entian, K.D. (1992) Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl Environ Microbiol 58: 132–142.

Nguyen, D.H., Nguyen, Q.A., Ferreira, R.C., Ferreira, L.C.S., Tran, L.T. and Schumann, W. (2005). Construction of plasmid-based expression vectors for *Bacillus subtilis*. Plasmid; 2005 Nov; 54(3): 241-8. Epub 2005 Jul 11.

Nguyen,H.D., Phan,T.T.P. and Schumann,W. (2007) Expression vectors for the rapid purification of recombinant proteins in *Bacillus subtilis*. Curr. Microbiol., 55, 89-93.

Phan, T.T.P., Nguyen, H.D. and Schumann, W. (2006). Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *Bacillus subtilis*. Protein Expr. Purif. 46, 189–195.

Phan, T.T.P., Nguyen, H.D. and Schumann, W. (2011). Development of a strong intracellular expression system for *Bacillus subtilis* by optimizing promoter elements. J. Biotechnol. (2011)

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

Schulz, A. and Schumann, W. (1996) *hrcA*, the first gene of the *Bacillus subtilis dnaK* operon encodes a negative regulator of class I heat shock genes. J. Bacteriol., 178, 1088-1093.

Titok, M.A., Chapuis, J., Selezneva, Y.V., Lagodich, A.V., Prokulevich, V.A., Ehrlich, S.D. and Jannière, L. (2003). *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector, Plasmid 49: 53-62.

Weber, M.H.W., Marahiel, M.A., Bacterial cold shock responses, Science Progress 86 (2003) 9–75. *Bacillus subtilis* Marburg, Mol. Gen. Genet. 170 (1979) 117–122.

Zhang,G., Bao,P., Zhang,Y., Deng,A., Chen,N. and Wen,T. (2011) Enhancing electro-transformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal. Biochem., 409, 130–137.

11. Order Information, Shipping and Storage

Order#	Product	Quantity
PBS013	pHT253 plasmid with promoter P*grac*100 and 8xHis-tag at the N-terminus	10 µg
PBS014	pHT254 plasmid with promoter P*grac*100 and 8xHis-tag at the C-terminus	10 µg
PBS015	pHT255 plasmid with promoter P*grac*100 and Strep-tag at the C-terminus	10 µg
Shipped at RT. Lyophilized plasmid DNA can be stored at 4 °C. Once the DNA has been dissolved in sterile water or buffer we recommend storage at -20 °C.		
PBS020	*Bacillus subtilis* strain 1012wt	1 ml
PBS021	*Bacillus subtilis* strain 168 Marburg	1 ml
PBS022	*Bacillus subtilis* strain WB800N (for secretion vectors)	1 ml
PBS026	*Bacillus subtilis* strain AS1	1 ml
Shipped on dry ice; store at -80 °C		

12. Contact and Support

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These vector systems were initially constructed in the laboratory of Wolfgang Schumann at the Institute of Genetics, University of Bayreuth, Germany, Germany, and continue to develop in the laboratory of Hoang Duc Nguyen at the Center for Bioscience and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh City.

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