Bacillus subtilis
Constitutive Expression System

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1. Features

1.1. Recombinant protein production with *Bacillus subtilis*

- Non-pathogenic and considered as a GRAS organism (generally regarded as safe)
- *B. subtilis* has no significant bias in codon usage
- It can secrete functional extracellular proteins directly into the culture medium
- MoBiTec host strain for secretional protein production available: *B. subtilis* WB800N, an eightfold extracellular protease deficient strain
- MoBiTec host strains for intracellular protein production available

1.2. P*grac* constitutive expression vectors

- P*grac* vectors are structurally and segregationally stable
- Inducer-free high-level production of the target protein in *B. subtilis*
- Convenient cloning due to *B. subtilis* / *E. coli* shuttle vectors
- Strong repression of the target gene expression in *E. coli lacI*^{q} strains
- For subsequent purification of the protein of interest, the P*grac* constitutive expression vectors encode for 8xHis- or Strep tag II
- Control vector available (expressing β-galactosidase)

2. 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. At present, about 60% of the commercially available enzymes are produced by *Bacillus* species. *B. subtilis* is advantageous in that it is non-pathogenic, does not have significant bias in codon usage, and is capable of secreting functional extracellular proteins directly into the culture medium in large scale. A lot of information is available concerning genome sequence, transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation.

With the *B. subtilis* Constitutive Expression System MoBiTec offers an easy-to-handle tool for high yield protein production with *B. subtilis*.

3. The P*grac* Constitutive Expression Vectors

The P*grac* Constitutive Expression Vectors are *B. subtilis* / *E. coli* shuttle vectors, constructed for cloning in *E. coli lacI*^{q} strains and high-level constitutive expression of heterologous proteins with *B. subtilis*.
The vectors are structurally and segregationally stable, using the theta-mode of replication within *B. subtilis* (Jannière *et al*., 1990; Titok *et al*., 2003). These constitutive expression vectors were derived from IPTG inducible expression vectors by deleting the *lacI* gene, encoding for the lacI repressor protein. The operator sites lacOI and lacO3 (binding sites for the lacI repressor) were both retained in the vector. For cloning with *E. coli*, the existence of the operator sites allows repressing of the gene of interest in *lacI*^{q} strains in the absence of IPTG (Tran *et al*., 2017). This is important, since constitutive expression in *E. coli* is mostly not desired and may create problems depending on the type of recombinant protein.
By using the *bgaB* reporter gene (encoding for β-galactosidase) within the constitutive expression system, high amounts of β-galactosidase with an activity of $14x10^4$ units are produced in *Bacillus subtilis* 1012. The amount of recombinant protein produced may represent up to 13% of the total cellular protein (Tran *et al*., 2017, Phan *et al*., 2005).
For easy purification of the protein of interest the constitutive expression vectors are available with 8xHis tag or Strep tag II.

Vectors for constitutive protein production:

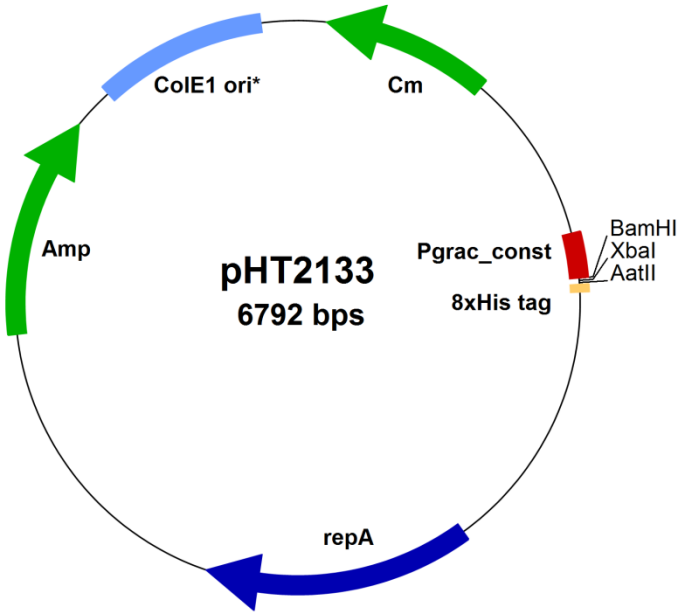
- pHT2133: constitutive expression vector with C-terminal encoded 8xHis tag
- pHT2134: constitutive expression vector with C-terminal encoded Strep tag

Control vector

The following positive control vector validated for constitutive recombinant production in *B. subtilis* is only available in combination with a regular *B. subtilis* vector:

- pHT2071: for constitutive intracellular production of β-galactosidase

3.1. Vector Map pHT2133

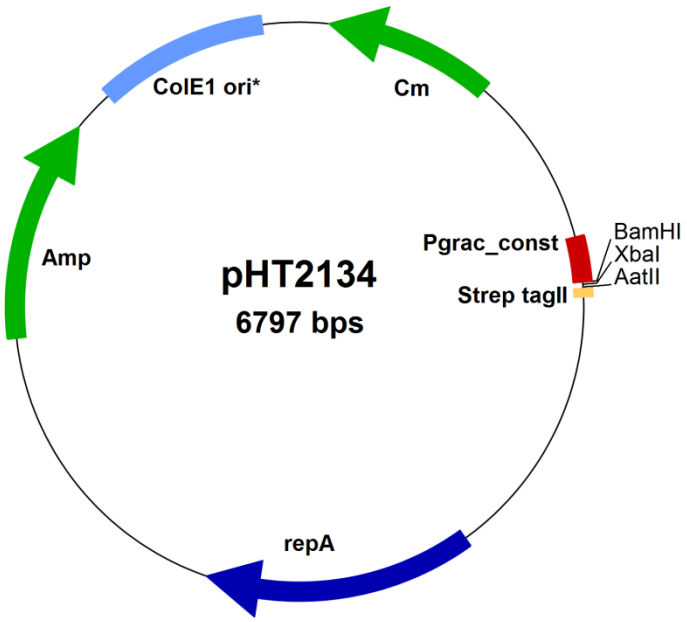


	Type	Start	End	Name	Description
	Selectable Genetic Marker	761	111	Cm	Chloramphenicol resistance (*B. subtilis*)
	Promoter	1429	1602	Pgrac_const	Constitutive promoter
	Tag	1627	1656	8x His tag	8x His tag
	Terminator	1669	1694	(not shown)	Region for transcription termination
	Gene	2719	3753	repA	Replication gene A
	Selectable Genetic Marker	4974	5834	Amp	Ampicillin resistance gene (*E. coli*)
	Origin of replication	5996	6650	ColE1*	Origin of replication (*E. coli*); ColE1 incompatibility group

The sequence of the ribosome binding site (RBS), the multiple cloning site (MCS), and the position of the 8x His tag in pHT2133 is shown below. The complete vector DNA sequence is available at our website.

5' AAAGGAGGAAGGATCCATGTCTAGAGTCGACGTCGCT – 8xHis tag – TAACGTCCCCGGGGC 3'
RBS BamHI XbaI AatII SmaI

3.2. Vector Map pHT2134



	Type	Start	End	Name	Description
	Selectable Genetic Marker	763	113	Cm	Chloramphenicol resistance (*B.subtilis*)
	Promoter	1431	1604	Pgrac_const	Constitutive promoter
	Tag	1629	1661	Strep tag II	Strep tag II
	Terminator	1674	1699	(not shown)	Region for transcription termination
	Gene	2724	3758	repA	Replication gene A
	Selectable Genetic Marker	4979	5839	Amp	Ampicillin resistance gene (*E. coli*)
	Origin of replication	6001	6655	ColE1*	Origin of replication (E. coli); ColE1 incompatibility group

The sequence of the ribosome binding site (RBS), the multiple cloning site (MCS), and the position of the Strep tag in pHT2134 is shown below. The complete vector DNA sequence is available at our website.

5' AAAGGAGGAAGGATCCATGTCTAGAGTCGACGTC -Strep tag II- AACGTCCCCGGGGC 3'
RBS BamHI XbaI AatII SmaI

4. *Bacillus subtilis* Host Strains

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

For intracellular expression:

- 1012 wild type: *leuA8 metB5 trpC2 hsdRM1* (commonly used)
- 168 Marburg: *trpC2* (Trp^-)

For expression and secretion:

- WB800N: *nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg cm::neo;* NeoR
  Please note that WB800N carries resistance to neomycin!

5. Storage and Handling Instructions

Storage and handling of plasmids

Plasmids are supplied lyophilized. Upon receipt, add 100 µl distilled water (final DNA 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.
Please note that we strongly recommend using E. coli *lacI*^{q} strains for cloning. Without adding IPTG, expression of the gene of interest is repressed in these strains by the genomicly encoded lacI repressor.

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 or LB medium. Under optimal conditions the doubling time of *E. coli* is 20 min, 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* chloramphenicol (5 µg/ml)
E. coli ampicillin (50 µg/ml)

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). *Bacillus subtilis* can be transformed via natural competence or by electroporation.

7.1. Protocol A - Natural Competence

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

Preparation of competent *Bacillus subtilis* cells

- Prepare an overnight culture of the appropriate recipient cells in 5 ml HS medium at 37 °C under vigorous shaking. For inoculation we recommend using one single colony grown on an LB agar plate
- Measure the OD_{600} (optical density at 600 nm) of the overnight culture and inoculate 50 ml HS medium to an OD_{600} of 0.05; incubate under vigorous shaking at 37 °C
- Record the growth curve
- Immediately at transition of exponential to stationary growth phase start taking samples of 10 ml, each 15 min
- 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

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 plastic reaction tube, add 10 µl of 0.1 M EGTA, and incubate for 5 min at room temperature
- Add 1 µg 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 an plastic reaction tube
- Centrifuge, discard supernatant carefully and resuspend the cells into the final supernatant remaining on the pellet
- Plate on selective 2xYT medium containing 5 µg/ml chloramphenicol
- Incubate at 37 °C overnight

7.2. Protocol B - Electroporation

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 OD_{600} 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/µl)
- 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

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) casamino acids
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) casamino acids
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
2xYT medium:	16 g tryptone 10 g yeast extract 5 g sodium chloride (NaCl)
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
2x Laemmli buffer	125 mM Tris/HCl, pH 6,8 4% SDS 20% glycerin 10% β-mercaptoethanol 0,04% bromphenol blue

8. Heterologous Protein Production

- Grow appropriate *B. subtilis* strain overnight in fresh 2xYT or LB medium
- Inoculate into fresh 2xYT medium to an OD_{600} of 0.15 and let grow at 37 °C while vigorously shaking
- Cultivation time, cell growth and best time for harvesting depends on the gene cloned and the protein produced. We recommend a cultivation time of 4-10 h (OD_{600} 0.8 – 1.5)

8.1. 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 reaction tubes containing 1 ml of cell suspension, supplemented with lysozyme (250 µg/ml) 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

- Take 100 µl of the preparation as first total protein sample (T1)
- Remove cell debris 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
- Analyse samples by immunoblotting with specific antibodies

8.2. Precipitation of proteins from culture supernatant

- For production of a secretory protein, your gene of interest must bring its own signal sequence; there is no signal sequence included in any of the constitutive expression vectors
- Collect proteins 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 2x Laemmli buffer and denature at 95 °C for 10 min for SDS-PAGE

9. References

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10. Order Information, Shipping and Storage

Order#	Product	Quantity
PBS008	*Bacillus subtilis* constitutive expression vector pHT2133	10 µg
PBS009	*Bacillus subtilis* constitutive expression vector pHT2134	10 µg
PBS008C	*Bacillus subtilis* control vector pHT2071 for the constitutive expression system, available only in combination with regular vector	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		

11. Contact and Support

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These vector systems have been developed in the laboratory of Wolfgang Schumann at the Institute of Genetics, University of Bayreuth, Germany.

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