Bacillus subtilis
P*grac*01 Expression Vectors

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 proteins directly into the culture medium
- MoBiTec host strain for secretory protein production available: *B. subtilis* WB800N, an eightfold extracellular protease deficient strain.
- MoBiTec host strains for intracellular protein production available

1.2. P*grac*01 inducible expression vectors

- P*grac*01 vectors are structurally and segregationally stable
- Convenient cloning due to *B. subtilis* / *E. coli* shuttle vectors
- Expression of the gene of interest is controlled by the IPTG inducible promoter P*grac*01 and the corresponding repressor encoded by lacI
- P*grac*01 vectors allow highly efficient extra- and intracellular production of recombinant proteins
- MoBiTec P*grac*01 vectors with 8xHis-, Strep- and c-Myc-tag available
- Control vectors available

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 proteins directly into the culture medium in large scale. A large body of information is available concerning genome sequence, transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation.

With the *B. subtilis* P*grac*01 expression vectors MoBiTec offers an easy to handle tool for *B. subtilis* for high yield intracellular protein production and also for secretion of heterologous proteins into the culture medium in high amount.

3. The P*grac*01 Expression Vectors

The P*grac*01 expression vectors are *B. subtilis* / *E. coli* shuttle vectors, constructed for cloning in *E. coli* and high level 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). Expression of the gene of interest is regulated by the Pgrac01 promoter fused to the lac operator allowing the induction by addition of ITPG. While the background level of expression of these expression cassettes is very low in the absence of the inducer, an induction factor of about 1,300 was measured using the *bgaB* reporter gene (Phan *et al*., 2005). The amount of recombinant protein produced after addition of IPTG may represent 10% and 13%, respectively, of the total cellular protein (demonstrated when fusing the *htp*G and *pbp*E genes to the *gro*E promoter; Phan *et al*., 2005). High level secretion of α-amylase and cellulase A and B of *Clostridium thermocellum* was demonstrated. An efficient Shine-Dalgarno (SD) sequence as well as a multiple cloning site (BamHI, XbaI, AatII, SmaI) were also inserted. To obtain secretion of recombinant proteins, the signal sequence of the *amyQ* gene (encoding for α-amylase) was fused to the SD sequence of pHT01, thereby constructing pHT43. A further construct pHT1464 with improved signal sequence of *amyQ* was constructed in the same way, by introducing a modified version of the signal sequence.

Vectors for intracellular protein production:

- pHT01: IPTG inducible expression vector
- pHT08: same as pHT01 with C-terminal encoded 8xHis tag
- pHT09: same as pHT01 with C-terminal encoded Strep tag
- pHT10: same as pHT01 with C-terminal encoded c-Myc tag

Vectors for secretory protein production:

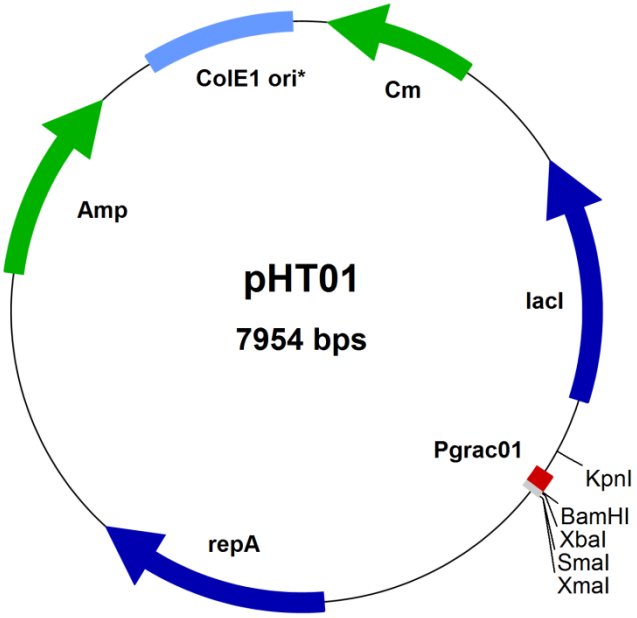
- pHT43: same as pHT01 with signal sequence of *amyQ* for protein export
- pHT1464: same as pHT01 with improved signal sequence of *amyQ* for extended protein export

Control vectors

The following positive control vectors validated for recombinant production in *B. subtilis* are, in combination with a regular *B. subtilis* vector, available:

- pHT01-bgaB : for intracellular production of β-galactosidase after addition of IPTG
- pHT10-gfp+: for intracellular production of gfp+ after addition of IPTG
- pHT43-amyQ: for expression and secretion of α-amylase after addition of IPTG

3.1. Vector Map pHT01



	Type	Start	End	Name	Description
	Selectable Genetic Marker	763	113	Cm	Chloramphenicol resistance (*B. subtilis*)
	Gene	2378	1296	lacI	lacI repressor gene
	Promoter	2724	2799	Pgrac01	P*grac*01 promoter
	Terminator	2831	2856	(not shown)	Region for transcription termination
	Gene	3881	4915	repA	replication gene A
	Selectable Genetic Marker	6136	6996	Amp	Ampicillin resistance gene (*E. coli*)
	Origin of replication	7259	7913	ColE1*	Origin of replication (*E. coli*); ColE1 incompatibility group

The P*grac* promoter region consists of the *gro*E promoter, the *lac*O operator and the SD$_{gsiB}$ (Shine-Dalgarno sequence of the *gsiB* gene). A part of the promoter region and the cloning sites are shown below. The complete DNA sequence of pHT01 is available on request.

GAAAAGAATGATGTAAGCGTGAAAAATTTTTTATCTTATCACTTGAAATTGGAAGGGAGATTCTTTATTAT
-35 region **-10 region**

AAGAATTGTGGAATTGTGAGCGGATAACAATTCCCAATTAAAGGAGGAAGGATCCTCTAGAGTCGACGTC
lacO **SD*gsiB*** **BamHI** **XbaI** **AatII**

CCCGGGGCAGCC

SmaI

3.2. Location of tags in pHT01 derivatives

Location of the 8xHis tag in pHT08:

8xHis tag

P*groE* – lacO - SD*gsiB*-TGCGCGGAAGC CAT CAC CAT CAC CAT CAC CAT CAC GGATCCTCTAGAGTCGACGTC
BamHI XbaI AatII

CCCGGGGCAGCC
SmaI

Location of the Strep tag in pHT09:

Strep tag

P*groE* – lacO - SD*gsiB*-ATGAAT TGG AGC CAT CCG CAA TTT GAA AAA GGATCCTCTAGAGTCGACGTC
BamHI XbaI AatII

CCCGGGGCAGCC
SmaI

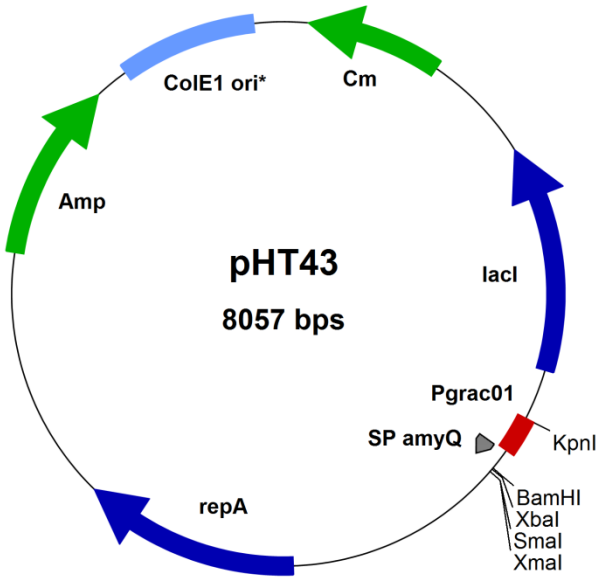
Location of the c-Myc tag in pHT10:

c-Myc tag

P*groE* – lacO - SD*gsiB*-GGATCCTCTAGA GTCGACGTC GAA CAA AAA CTT ATT AGC GAA GAA GAT CTT
BamHI XbaI

TAATAACACGTC

3.3. Vector Map pHT43



	Type	Start	End	Name	Description
	Selectable Genetic Marker	763	113	Cm	Chloramphenicol resistance (*B.subtilis*)
	Gene	2379	1297	lacI	lacI repressor gene
	Promoter	2723	2800	Pgrac	P*grac*01 promoter
	Signal Peptide	2809	2901	SP amyQ	signal peptide of α-amylase
	Terminator	2934	2959	(not shown)	Region for transcription termination
	Gene	3881	4915	repA	replication gene A
	Selectable Genetic Marker	6136	6996	Amp	Ampicillin resistance gene (*E. coli*)
	Origin of replication	7259	7913	ColE1*	Origin of replication (E. coli); ColE1 incompatibility group

The P*grac*01 promoter region consists of the *gro*E promoter, the *lac*O operator and the SD_{gsiB} (Shine-Dalgarno sequence of the *gsiB* gene). A part of the promoter region, the amyQ signal peptide (SP_{amyQ}) and the multiple cloning site is shown below. The complete DNA sequence of pHT43 is available on request.

SP*amyQ*

GCGGATAACAATTCCCAATTAAAGGAGGAAGGATCA **ATG** ATT CAA AAA CGA AAG CGG ACA GTT TCG

lacO SD*gsiB* M I Q K R K R T V S

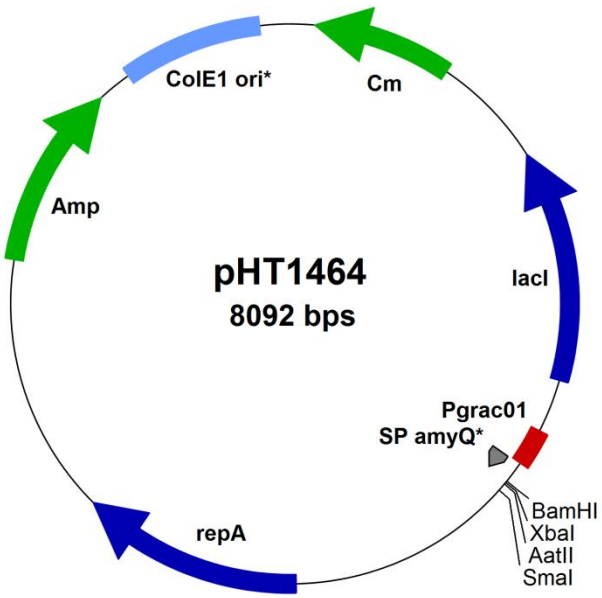
TTC AGA CTT GTG CTT ATG TGC ACG CTG TTA TTT GTC AGT TTG CCG ATT ACA AAA ACA TCA GCC

F R L V L M C T L L F V S L P I T K T S A

GTAGGATCCTCTAGAGTCGACGTCCCCGGGGCAGCC

BamHI XbaI AatII SmaI

3.4. Vector Map pHT1464



	Type	Start	End	Name	Description
	Selectable Genetic Marker	763	113	Cm	Chloramphenicol resistance (*B. subtilis*)
	Gene	2379	1297	lacI	lacI repressor gene
	Promoter	2629	2802	Pgrac01	P*grac*01 promoter
	Signal Peptide	2809	2904	SP amyQ*	modified signal peptide of α-amylase
	Terminator	2969	2994	(not shown)	Region for transcription termination
	Gene	4019	5053	repA	replication gene A
	Selectable Genetic Marker	6274	7134	Amp	Ampicillin resistance gene (*E. coli*)
	Origin of replication	7296	7950	ColE1*	Origin of replication (E. coli); ColE1 incompatibility group

The P*grac*01 promoter region consists of the *gro*E promoter, the *lac*O operator and the SD_{gsiB} (Shine-Dalgarno sequence of the *gsiB* gene). A part of the promoter region, the improved amyQ signal peptide (SP_{amyQ*}), and the multiple cloning site is shown below. The complete DNA sequence of pHT1464 is available on request.

SP*amyQ**

GCGGATAACAATTCCCAATTAAAGGAGGAAGGATCA ATG ATT CAA AAA CGA AAG CGG ACA GTT TCG

lacO SD*gsiB* M I Q K R K R T V S

TTC AGA CTT GTG CTT ATG TGC ACG CTG TTA TTT GTC AGT TTG CCG ATT ACA AAA GCA TCA GCT

F R L V L M C T L L F V S L P I T K A S A

GCT GGATCCATGTCTAGAGTCGACGTCTGGAGCCATCCGCAATTTGAAAAATAAACGTCCCCGGGGCA

BamHI XbaI AatII SmaI

4. *Bacillus subtilis* Host Strains

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

For intracellular protein production:

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

For secretion vectors:

- 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 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 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*

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 OD600 (optical density at 600 nm) of the overnight culture and inoculate 50 ml HS medium to an OD600 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 5µg/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 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 cells 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

8. Induction with IPTG and Sample Analysis

- 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 to one portion (t = 0)
- Collect samples at different time points for analysis (t = 1,…)

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 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 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 antiserum

8.2. Precipitation of proteins from culture supernatant

- 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
  2x Laemmli: 125 mM Tris/HCl (pH 6.8), 4 % SDS, 20 % glycerine,
  10 % mercaptoethanol, 0.04 % bromphenol blue

9. References

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10. Order Information, Shipping and Storage

Order#	Product	Quantity
PBS001	pHT01 vector, with P*grac*01 promoter	10 µg
PBS001C	pHT01-bgaB control vector, available only in combination with regular vector	10 µg
PBS002	pHT43 vector for secretory protein production, with P*grac*01 and *amyQ* signal sequence	10 µg
PBS002C	pHT43-amyQ control vector, available only in combination with regular vector	10 µg
PBS003	pHT08 vector, with P*grac*01 and 8xHis tag at N-terminus	10 µg
PBS004	pHT09 vector, with P*grac*01 and Strep tag at N-terminus	10 µg
PBS005	pHT10 vector with P*grac*01 and c-Myc tag at C-terminus	10 µg
PBS005C	pHT10-gfp+ control vector, available only in combination with regular vector	10 µg
PBS010	pHT1464 vector for secretory protein production, with P*grac*01 and improved *amyQ** signal sequence	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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