

Bacillus subtilis
Expression Vectors



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***B. subtilis* Expression Vectors**

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



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);
- ii. It has no significant bias in codon usage;
- iii. 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 heterologous proteins, and (ii) stable vector plasmids. The first obstacle 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 pAM β 1 and pBS72 (Janni \acute{e} re *et al.*, 1990; Titok *et al.*, 2003).

The construction and use of four different expression vectors, based on the *E.coli-B. subtilis* shuttle vector pMTLBS72, exhibiting full structural stability was published in 2005 (Nguyen *et al.*, 2005).

The two new vectors pHT01 and pHT43 allow high-level expression of recombinant proteins within the cytoplasm, where pHT43 directs the recombinant proteins into the medium. Both vectors are based on the strong σ^A -dependent promoter preceding the *groE* operon of *B. subtilis* which has been converted into an efficiently controllable (IPTG-inducible) promoter by addition of the *lac* operator.

Derivatives of pHT01 are available either with a 8xHis tag (pHT08), a *Strep tag* (pHT9) or a c-Myc tag (pHT10).

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

- pHT01-bgaB
- pHT43-amyQ
- pHT10-gfp+

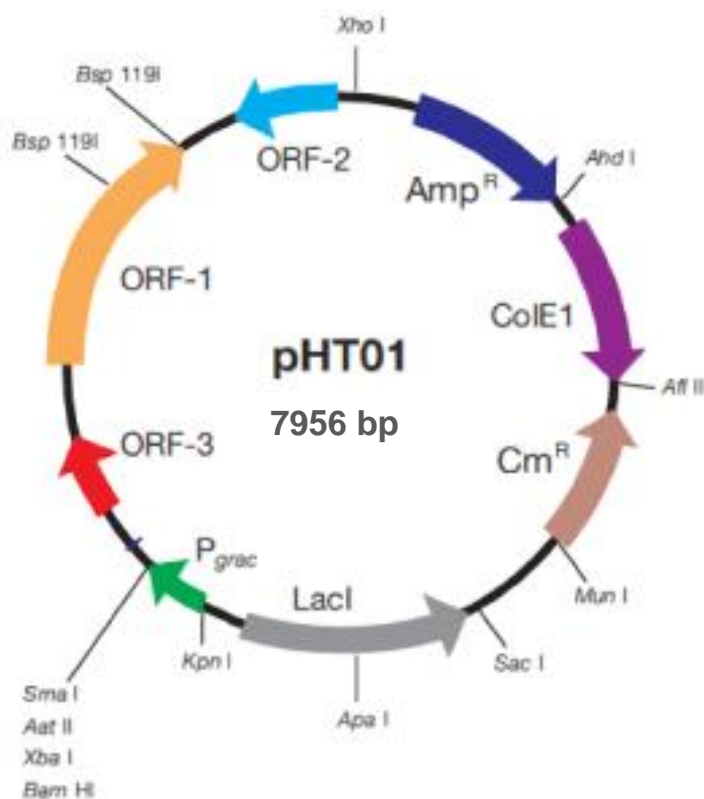


The pHT Vectors

All vectors use the strong promoter preceding the groESL operon of *Bacillus subtilis* fused to the lac operator allowing their induction by addition of IPTG. 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 htpG and pbpE genes to the groE promoter; Phan *et al.*, 2005). High level secretion of amyQ 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 (BamH I, Xba I, Aat II, Sma I) were also inserted. To obtain secretion of recombinant proteins, the coding region for the signal peptide of the amyQ gene encoding an α -amylase was fused to the SD sequence of pHT01, thereby constructing pHT43.



Vector Map pHT01



P_{grac}: *P_{grac}* promoter (consisting of the *groE* promoter; the *lacO* operator and the *gsi*BSD sequence)

ColE1 ori: *ColE1* origin

Amp^R: ampicillin resistance

lacI: *lacI* gene (*lac* repressor)

Cm^R: chloramphenicol resistance

Complete DNA sequence is available on our website.

**gaaaagaatgatgtaagecgtgaaaaatttttatctttatcac
TTGAAAttggaaggagattcttTATTaagaattgt**

-35

-10

ggAATTGTGAGCGGATAACAATTcccaatt

lacO

aaaggaggaaggatcctctagagtcgacgtccccggggcagcc

RBS

***Bam*HI**

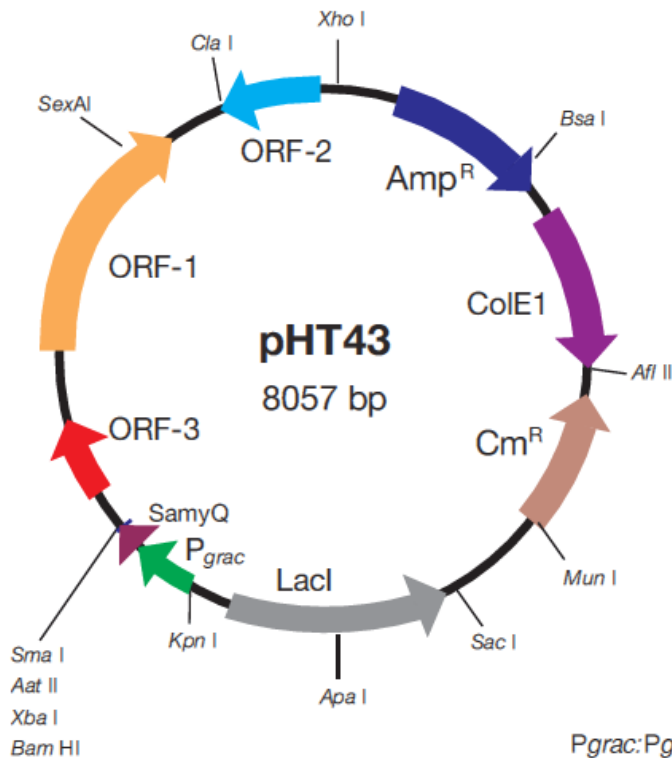
***Xba*I**

***Aat*II**

***Sma*I**



Vector Map pHT43



Pgrac: *Pgrac* promoter (consisting of the *groE* promoter, the *lacO* operator and the *gsIB* SD sequence)
ColE1 ori: *ColE1* origin
Amp^R: ampicillin resistance
lacI: *lacI* gene (*lac* repressor)
Cm^R: chloramphenicol resistance
SamyQ: *amyQ* signal sequence

Complete DNA sequence is available on our website.

GCGGATAACAATTcccaattaaaggaggaaggatca
lacO **RBS**

atgattcaaaaacgaaagcggacagtttctggttcagacttgtgcttatg

M I Q K R K R T V S F R L V L M

tgcacgctgttatttgtcagtttgccgattacaaaacatcagccg

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

taggatcctctagagtcgacgtccccggggcagcc

BamHI XbaI AatII SmaI



Location of the tags in the pHT01 derivatives

Location of the 8xHis tag in pHT08:

8xHis tag

*Bam*H I *Xba* I

Sma I

Pgrac-lacO-RBS-tgcgcggaagccatcaccatcaccatcaccatcacGGATCC TCTAGAgtcgacgtcCCCGGG

Location of the *Strep* tag in pHT09:

Strep tag

*Bam*H I *Xba* I

Sma I

Pgrac-lacO-RBS-atgaattggagccatccgcaatttgaaaaaGGATCC TCTAGAgtcgacgtcCCCGGG

Location of the *c-Myc* tag in pHT10:

*Bam*H I *Xba* I

c-Myc tag

Pgrac-lacO-RBS-GGATCC TCTAGAgtcgacgtcgaacaaaaacttattagcgaagaagatctttaataacaagtc

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 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!

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.

Please note that 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.



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-J902-500GAM	2xYT medium broth	500 g
CB-0230-100GAM	chloramphenicol	100 g
CB-0230-500GAM	chloramphenicol	500 g
CB-0339-100GAM	ampicillin sodium salt	100 g
CB-0339-25GAM	ampicillin sodium salt	25 g
CB-J859-100GAM	tryptone	100 g
CB-J859-1KGAM	tryptone	1 kg
CB-J859-500GAM	tryptone	500 g
CB-J851-100GAM	casamino acids	100 g
CB-J851-1KGAM	casamino acids	1 kg
CB-J851-500GAM	casamino acids	500 g
CB-0241-1KGAM	sodium chloride (NaCl)	1 kg

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. Please note that immediate usage may result in higher transformation rates.

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



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 cups)
- 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

2. Media and solutions

10x S-base (Spizizen's salt): 2 g (NH₄)₂SO₄
 14 g K₂HPO₄
 6 g KH₂PO₄
 1 g sodium citrate
 add distilled water to 100 ml and autoclave
 add 0.1 ml 1M MgSO₄ 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 MgCl₂
 0.05 ml 1 M CaCl₂
 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



Induction with IPTG

- grow appropriate *B. subtilis* strain overnight in fresh 2xYT medium
- inoculate into fresh 2xYT medium to an OD₆₀₀ of 0.15
- when culture reaches OD₆₀₀ 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,...)

Sample analysis

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 OD₆₀₀ 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 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 OD₆₀₀ 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

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



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Order Information, Shipping and Storage

Order#	Product	Quantity
PBS001	pHT01 vector, lyophilized plasmid DNA	10 µg
PBS001C	pHT01-bgaB control vector, lyophilized plasmid DNA, available only in combination with regular vector	10 µg
PBS002	pHT43 vector, lyophilized plasmid DNA	10 µg
PBS002C	pHT43-amyQ control vector, lyophilized plasmid DNA, available only in combination with regular vector	10 µg
PBS003	pHT08 vector, lyophilized plasmid DNA	10 µg
PBS004	pHT09 vector, lyophilized plasmid DNA	10 µg
PBS005	pHT10 vector, lyophilized plasmid DNA	10 µg
PBS005C	pHT10-gfp+ control vector, lyophilized plasmid DNA, 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.		

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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