

T7 RNA Polymerase Expression System for *Bacillus megaterium*



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An efficient alternative to common *Bacillus megaterium* expression systems: High yield protein expression with the T7 RNA polymerase expression system

MoBiTec offers this dual plasmid expression system as an easy-to-handle solution with pretransformed *Bacillus megaterium* protoplasts (MS941 carrying pT7-RNAP) ready for transformation and *Escherichia coli* / *Bacillus megaterium* shuttle vectors.

1. Introduction

1.1. General features of *Bacillus megaterium*

First described over 100 years ago, *B. megaterium* has recently been gaining more and more importance in scientific as well as industrial applications. The source of its significant name "*megaterium*" is its large size of the vegetative cells (over 10 µm) and its spores. The capability of sporulation has made *B. megaterium* an important tool for examining spore-mediated disease and cell development.

B. megaterium is able to grow on a wide variety of carbon sources and thus has been found in many ecological niches such as waste from meat industry or petrochemical effluents. Also, the degradation of persistent insecticides by *B. megaterium* has been documented (Saxena et al., 1987; Selvanayagam and Vijaya, 1989) offering potential applications as detoxifying agent. One of the genetic regulatory elements for carbon utilization is the xylose operon. It has been described by Rygus and Hillen (1991) and is used in the expression system MoBiTec is offering in this kit.

Further, several *B. megaterium* proteins are of importance. For example, a family of P₄₅₀ cytochrome monooxygenases is similar to eukaryotic P₄₅₀ playing a role in many diseases. Industrial applications of enzymes excreted by *B. megaterium* are diverse, starting from amylases used in bread industry to penicillin amidase which is used for the generation of new synthetic antibiotics.

An overview about the features of this unique organism is given in review articles as "Prime time for *Bacillus megaterium*" (Vary, 1994), "A short story about a big magic bug" (Bunk et al., 2010) and "*Bacillus megaterium* - from simple soil bacterium to industrial protein production host" (Vary et al., 2007).

1.2. *Bacillus megaterium* as expression host

In molecular biology, *B. megaterium* has been proven to be an excellent host for the expression of non-homologous DNA. Cloning vectors of the *B. megaterium* system (derivatives of the original pWH1520 (Rygus and Hillen, 1991; Malten et al., 2006; Biedendieck et al., 2007)) rely on the above mentioned xylose inducible expression system used as regulatory element. Further, a T7 RNA polymerase dependent promoter and terminator were introduced into a compatible vector.



In contrast to other bacilli strains *B. megaterium* has the advantage, that no alkaline protease is present. This fact enables excellent production and secretion of foreign proteins without degradation (Meinhardt et al., 1989; Rygus and Hillen, 1991). In addition, due to its Gram-positive character there are no endotoxins found in the cell wall. Furthermore, *B. megaterium* is able to stably maintain several extra-chromosomal DNA elements in parallel, so we can offer you this expression kit with two in parallel-replicating plasmids.

1.3. General features of the T7 RNA polymerase expression system

The T7 RNA polymerase (T7 RNAP) expression system originates from the bacteriophage DNA-dependent RNA polymerase. In 1985, the first described T7 RNAP expression system was developed for *Escherichia coli* (Tabor and Richardson, 1985). Advantages of this system are the stringent selectivity and the high transcriptional activity so that it is possible to lead to a saturation of the protein-synthesizing machinery in *E. coli*. Consequently, 50% or more of the total cellular protein can consist of the desired protein (Studier and Moffatt, 1986).

The T7 RNAP expression system for *B. megaterium* combines the features of this system in *E. coli* with the above mentioned regulation by the xylose operon. This system is based on two parallel-replicating plasmids: pT7-RNAP and pP_{T7} (Gamer et al. 2009). In addition to the *t7 rnap* gene under control of the strong *xyIA* promoter pT7-RNAP contains the genes for ampicillin and chloramphenicol resistance for easy selection in *E. coli* (Amp^R) and *B. megaterium* (Cm^R). pP_{T7} is responsible for the T7 RNAP-dependent expression of the target gene. Downstream of the T7 promoter it comprises a multiple cloning site with ten unique restriction enzyme cleaving sites. Additionally the plasmid comprises two resistances against ampicillin (in *E. coli*) and tetracycline (in *B. megaterium*).

Next to the pP_{T7} vector, we offer an expression vector that leads to secretion of expressed proteins into the surrounding medium. pP_{T7}-SP_{LipA} provides the n-terminal signal peptide LipA that uses the Sec-pathway for protein secretion.

Furthermore, a great obstacle in protein production and engineering, the cloning of genes coding for proteins that are toxic to the cloning host *E. coli*, can be overcome by means of the T7 expression system due to the absence of T7 RNAP in the cloning host.

For your convenience MoBiTec offers *B. megaterium* protoplasts pretransformed with pT7-RNAP, so that you just have to insert your gene of interest into pP_{T7} and transform the pretransformed protoplasts with this plasmid.

For control purposes the *gfp*-expressing vector pP_{T7}-GFP is included in the kit.



2. Summary of advantages

- *B. megaterium* is not pathogenic
- No endotoxins are found in the cell wall
- Tightly regulated and efficiently inducible *xylA* operon (up to 350-fold)
- Stable, high yield protein production
- Extracellular protein production possible with pP_{T7}-SPlipA
- No indication of proteolytic instability even up to 5 h after induction, since alkaline proteases such as e.g. in *B. subtilis* are not produced
- Suited for small to industrial-scale protein production
- Tightly regulated and efficiently inducible *xylA* operon / T7 RNA polymerase
- Suitable for cloning of toxic proteins in *E. coli*
- Easy transformation by use of pretransformed *B. megaterium* protoplasts
- Control vector with GFP sequence included in the kit



3. Application examples

As a model for the recombinant overproduction of intracellular proteins Gamer et al. (2009) used the green fluorescent protein (GFP) from *Aequoria victoria*. They showed that GFP was found to be the dominant cytosolic protein 1.5 hours after the induction of *t7* expression induced by xylose. In comparison to a common used *B. megaterium* xylose-inducible expression system they found 5.3 times more GFP with the T7 RNAP expression system (**Fig. 1**) compared to the native xylose inducible one. The overall productivity was more than six times enhanced ($12.8 \text{ mg l}^{-1}\text{h}^{-1}$ with the T7 RNAP expression system - $2 \text{ mg l}^{-1}\text{h}^{-1}$ with the xylose-inducible system).

Treatment of the *B. megaterium* host cells by rifampicin that is a selective inhibitor of bacteria DNA-dependent RNA-polymerases leads to a stabilization of the T7 RNAP and to constant amounts of overproduced protein. However, the positive effects on cytosolic amounts are contrasted by growth defects caused by the rifampicin treatment. On the other hand rifampicin treatment led to an increase of 31% in volumetric levansucrase activity 8 hours after induction.

Notably, limitations were shown in the overproduction of the extracellular levansucrase from *Lactobacillus reuteri*. Due to yet unknown reasons, the protein with N-terminal signal peptide LipA for the export via the Sec-pathway was not found in higher amounts compared to the production with a common used *B. megaterium* xylose-inducible expression system (Gamer et al., 2009).

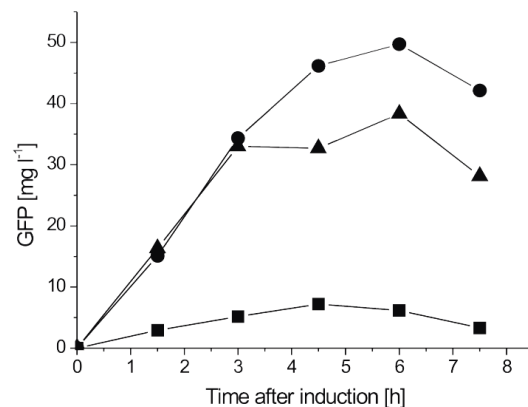


Fig. 1 Comparison of GFP amounts produced by variant expression systems. GFP produced by *B. megaterium* carrying the common xylose-regulated plasmid pRBBm34 (square), *B. megaterium* transformed with the T7 expression system plasmids (circle) and *B. megaterium* with the T7 expression system plasmids treated with rifampicin. (Gamer et al., 2009)



4. Protocols

4.1. Cloning the DNA fragment of interest

The pP_{T7} *E. coli* / *B. megaterium* shuttle vector is supplied as lyophilized DNA. Follow standard protocols for propagation of the plasmid in *E. coli*, plasmid mini preparation, restriction endonuclease cleavages and ligation of the DNA fragment of interest into the vector (Sambrook and Russell, 2001). After ligation of the insert, the vectors should be propagated in *E. coli* (*amp*^r) before transforming the *Bacillus* protoplasts (*tet*^r, *cm*^r).

4.2. General remarks on the handling of *B. megaterium*

Strains will grow well on rich media such as LB medium, plates and liquid, at 30 °C and 37 °C. Make sure to aerate liquid cultures well by vigorous agitation in baffled shaking flasks.

We found MS941 and derived strains to be asporogenic on common medium - they will die on plates, kept at 4 °C, within two weeks, so prepare glycerol stocks (30 % w/v) as a backup and streak the working cultures on fresh plates every 7 - 10 days.

Positive clones carrying the plasmids of interest can be selected by adding 10 µg/ml tetracycline and 4.5 µg/ml chloramphenicol to the growth medium.

To prove successful overexpression of the target gene harvest small samples of the culture just before and at intervals after induction of recombinant gene expression with xylose. To obtain crude extracts for gel analysis, the bacilli have to be lysed using lysozyme. Simple boiling of cells in sample buffer (Laemmli, 1970), which is quite convenient for *E. coli*, does not work with *B. megaterium*.

4.3. Transformation of *B. megaterium* protoplasts

For recombinant protein production *B. megaterium* protoplasts pretransformed with pT7-RNAP are transformed with the plasmids (pP_{T7}-derivatives) coding for the protein of interest. After transformation it is advisable to screen at least three different clones for protein production as the yield can vary among different clones.

Since *B. megaterium* cannot easily be transformed naturally, MoBiTec conveniently provides protoplasts of *B. megaterium*, which are ready for transformation. MoBiTec produces these protoplasts every second month. They can be used at least 2 months after date of arrival and have to be stored at -80 °C. The protoplast suspension is supplied in 5 aliquots of 0.5 ml each to prevent multiple freezing and thawing of protoplasts that are not used immediately. One aliquot is provided per transformation. It is advisable to use two of the vials for the control experiments as described below.

Below you can find a standard protocol for transformation of protoplasts.



Control Experiments:

1. Negative control: protoplasts without DNA

Note: *Each lot of protoplasts undergoes this test during our quality control as well.*

This is the control demonstrating that the protoplasts have not been contaminated but vital. Split the cells after transformation and incubation. You should get an empty plate without any colonies on the antibiotic (here: tetracycline/chloramphenicol) plate but a layer of cells on just chloramphenicol plates.

2. Positive control: protoplasts transformed with empty pP_{T7} plasmid (without insert) – not included in the kit! (alternatively with pP_{T7}-GFP)

This is your control for a successful transformation and should yield lots of colonies on antibiotic (here: tet/cm) plates. If this transformation works well, but you have problems with the plasmid containing your insert of interest, the problem most probably is associated with your construct.

Essential buffers are listed in chapter 5.

Transformation procedure:

1. Combine 500 µl of protoplast suspension and 3-5 µg of Plasmid DNA (DNA should be purified using a commercial preparation kit. Elute the DNA from the column using water) in a 15 ml tube, one for each transformation
2. Add 1.5 ml of PEG-P (room temperature RT), mix gently and incubate for 2 minutes at RT
3. Add 5 ml SMMP, mix by rolling the tube carefully
4. Harvest cells by gentle centrifugation (in e.g. a Heraeus Biofuge/Minifuge at 3,000 rpm (1,300 x g) for 10 minutes at RT), pour off supernatant immediately after centrifugation, supernatant does not have to be removed completely.
(Note: *do not check for a pellet - most of the time there will be none visible*)
5. Add 500 µl of SMMP to the rest of the supernatant
6. Incubate at 30 °C or 37 °C for 90 minutes with gentle shaking or rolling of tubes (max. 100 rpm) or for 45 min without followed by 45 min with shaking (300 rpm)
7. Prepare 2.5 ml aliquots of CR5-top agar in sterile tubes, one for each transformation
8. After outgrowth add all cells to 2.5 ml top agar, mix gently by rolling the tube between both hands (do not vortex!) and pour onto a prewarmed plate of LB containing the desired antibiotics
9. Incubate overnight at 30 °C or 37 °C - expect colonies of varying diameter because some will be covered with agar and others have easier access to air
(Note: *the colonies on the top of the agar surface will be shiny*)
10. Streak several different clones on fresh plates within two days

Note: *Protein production may vary among clones due to yet unknown reasons.*



4.4. Protein production

I. Test protein production

1. Grow the recombinant *B. megaterium* cells in LB medium including antibiotic (here: tet/cm) in baffled shaking flasks to an optical density at 578 nm (OD_{578nm}) of 0.3 - 0.4 at 37 °C and strong shaking (250 rpm)
2. Take a sample as control before induction
3. Induce the xylose inducible promoter by the addition of 0.5 % of (D)-xylose
4. Incubate at 37 °C and strong shaking (250 rpm)
5. Withdraw samples every 30 to 60 minutes until an OD_{578nm} of around 4 to 5 (depending on the growth medium) is reached (now, cells have entered the stationary phase). Take samples for OD_{578nm} -measurement and protein analysis. For extracellular protein analysis take 2 ml of cell culture. For intracellular protein analysis take 3 OD equivalents.
6. Centrifuge each sample to harvest cells and cell free supernatant
7. For extracellular protein analysis remove supernatant and store at 4 °C, for intracellular protein analysis completely remove supernatant and store cells at -20 °C.

II. Analysis of intracellular proteins

1. Resuspend cells in 30 µl of lysis buffer
2. Incubate for 30 min at 37 °C and 1,000 rpm. Vortexing every 10 minutes increases cell lysis
3. Centrifuge for 30 min at 4°C and 13,000 rpm to separate the insoluble fraction (pellet) from the soluble fraction (supernatant)
4. Mix 27 µl of supernatant (containing soluble proteins) with 13 µl of SDS sample buffer
5. Completely remove the supernatant. Resuspend the pelleted fraction in 30 ml of 8 % urea (w/v). Centrifuge for 30 min at 4°C and 13,000 rpm
6. Mix 27 µl of the supernatant (containing insoluble proteins) with 13 µl of SDS sample buffer
7. Heat each sample for 5 min at 95 °C
8. Load 7.5 µl of each sample (containing cells of 0.5 OD) onto an SDS-page gel

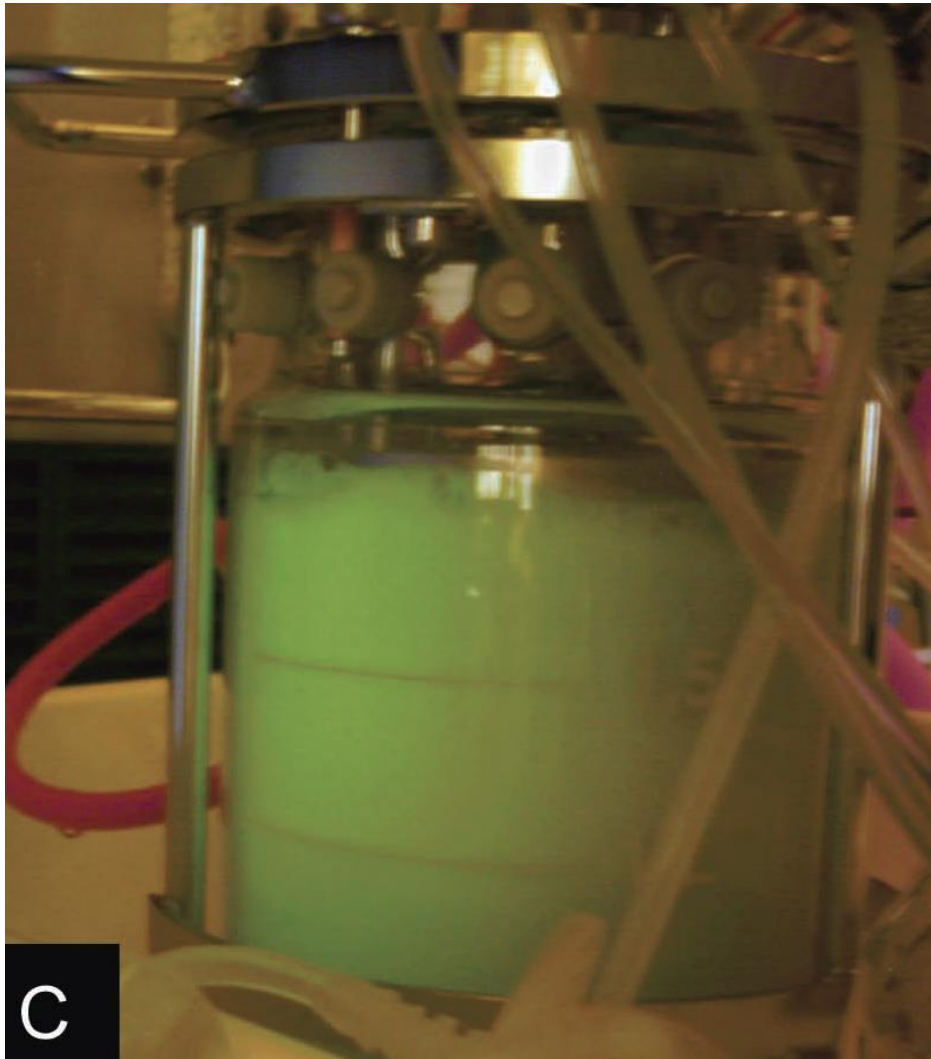
III. Ammonium sulfate precipitation of proteins in the cell-free supernatant

1. Add 600 mg of pestled ammonium sulfate to 1.5 ml of cell free supernatant and incubate for two hours at 4 °C and shaking
2. Centrifuge at 13,000 rpm and 4 °C for 30 minutes
3. Completely remove the supernatant, centrifuge again for 1 min and make sure the protein pellet is dry
4. Add 10 µl of 8 M urea (in 50 mM Tris-HCl, pH 7.5) and 5 µl SDS sample buffer to solve the proteins again
5. Spin shortly at 13,000 rpm, head to 99 % for 5 minutes and load onto a SDS polyacrylamide gel for analysis
6. Determine enzymatic activities with the appropriate assays (not included in the kit)
7. Perform Western blot using appropriate antibodies (not included in the kit)



IV. Scale up protein production

1. Grow larger culture and induce as indicated above
2. Harvest cells at the time point of maximal protein overproduction, as determined by the test experiment



B. megaterium carrying a plasmid coding for GFP-Strep fusion protein was grown in semi-defined minimal medium at 37°C initially in a batch phase with 4 g/L glucose. At the end of the batch phase an exponential feeding profile was started. GFP was visualized by a lamp emitting blue light and a yellow filter using a digital camera.



5. Materials

2 x AB3 (Antibiotic Medium No. 3, DIFCO)

- 7 g AB3 (Difco) in 200 ml deion. water
- autoclave 15 min

2 x SMM

solubilize in the given order!

- 1.16 g maleic acid (40 mM)
- 800 mg NaOH (80 mM)
- 2.03 g $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ (40 mM)
- 85.58 g sucrose (1 M)
- solubilize each component in deion. water
- mix and fill with deion. water to 250 ml
- sterilize by filtration

SMMP

- 2 x AB3 and 2 x SMM 1:1 (freshly prepared!)

PEG-P

- solubilize 20 g PEG-6000 with 1 x SMM and fill to 50 ml
- autoclave for 11 min

CR5-top-agar

prepare separately for 500 ml:

solution A

- 51.5 g sucrose
- 3.25 g MOPS
- 300 mg NaOH
- add to deionized water to 250 ml
- adjust pH to 7.3 with NaOH
- sterilize by filtration

solution B

- 2 g agar
- 100 mg casamino-acids
- 5 g yeast-extract
- add deionized water to 142.5 ml
- autoclave for 15 min

8 x CR5-salts

- 1.25 g K_2SO_4
- 50 g $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$
- 250 mg KH_2PO_4
- 11 g CaCl_2
- solubilize in 625 ml deion. water
- autoclave for 15 min

**12 % proline**

- 3 g L-proline
- add with deionized water to 25 ml
- sterilize by filtration

20 % glucose

- 5 g glucose
- add with deionized water to 25 ml
- sterilize by filtration or autoclave

for a 2.5 ml portion of CR5-top-agar add the following (in the given order!):

- 1.25 ml solution A
- 288 µl CR5-salts
- 125 µl 12 % proline
- 125 µl 20 % glucose

90 minutes after transformation:

- boil **solution B**
- add 713 µl to the provided CR5-top-agar
- immediately add the regenerated protoplasts and put onto prewarmed agar plates containing the corresponding antibiotic (here: tetracycline)

lysis buffer

- 100 mM Na₃PO₄
- 5 mg/ml lysozyme
- pH 6.5 (adjust with H₃PO₄)
- add 1 ml of a 1 M MgSO₄ solution and 2 µl HS-Nuclease (5 U/µl, cat.# GENUC10700-01*) per ml lysis buffer shortly before use

* HS-Nuclease is not available in the US and Canada. There it is available as TurboNuclease through Accelagen.



6. Vector maps

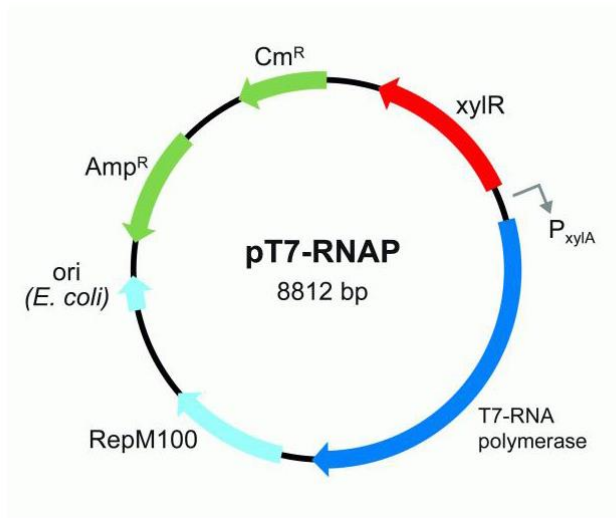


Fig. 2. Map of pT7-RNAP. Replicon derived from the rolling circle plasmid pBM100 264 of *B. megaterium* QM B1551 (RepM100); *E. coli* origin of replication (ori *E. coli*); resistance genes against ampicillin (Amp^R) and chloramphenicol (Cm^R); xylose inducible promoter (P_{xylA}) and its cognate repressor (xylR); sequence of the T7 RNA polymerase gene (T7-RNA polymerase).

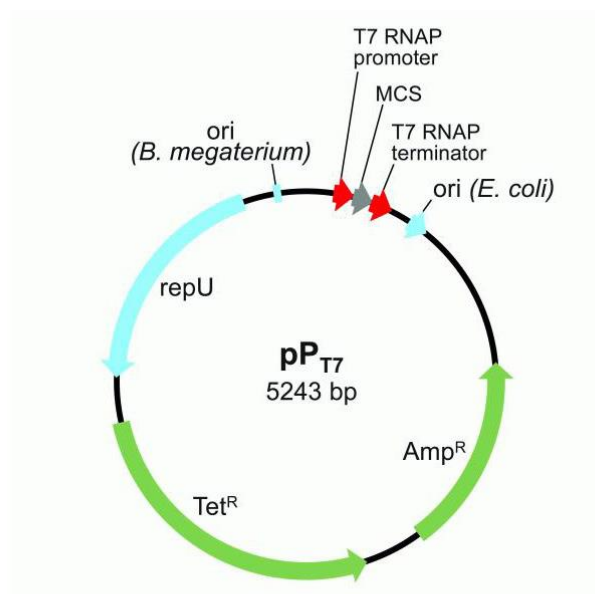


Fig. 3. Map of pP_{T7}. Replicon derived from *B. cereus* (repU); *B. megaterium* origin of replication (ori *B. megaterium*); T7 RNA polymerase promoter and terminator; multiple cloning site (MCS); *E. coli* origin of replication (ori *E. coli*); resistance genes against ampicillin (Amp^R) and tetracycline (Tet^R).

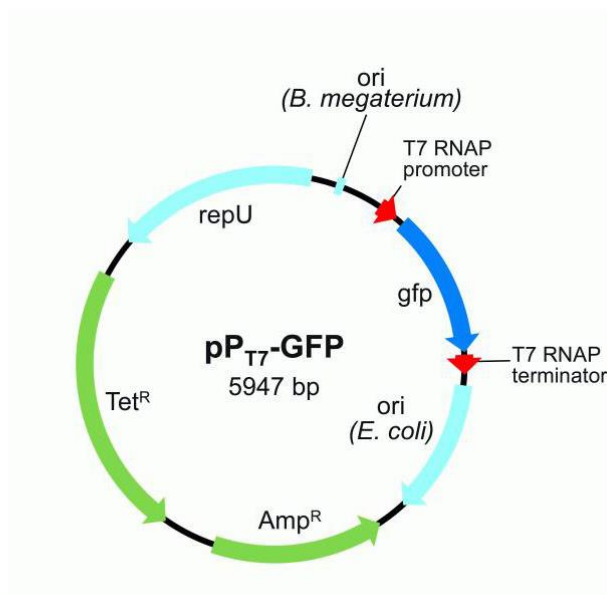


Fig. 4 Map of pP_{T7}-GFP. Replicon derived from *B. cereus* (RepU); *B. megaterium* origin of replication (ori *B. megaterium*); T7 RNA polymerase promoter and terminator; *gfp* (green fluorescent protein) gene sequence (*gfp*); *E. coli* origin of replication (ori *E. coli*); resistance genes against ampicillin (Amp^R) and tetracycline (Tet^R).

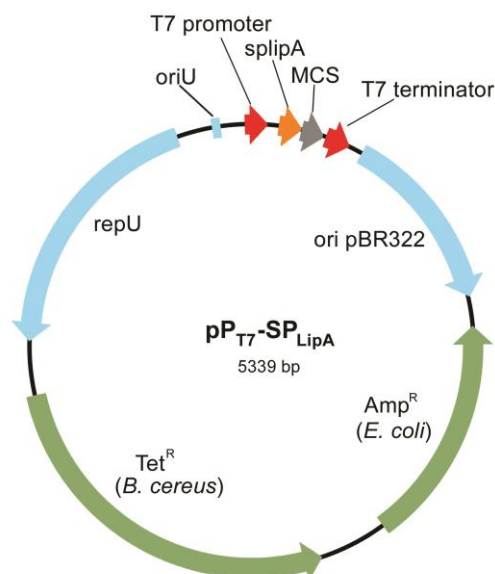


Fig. 5 Map of pP_{T7}-SP_{LipA}. Replicon derived from *B. cereus* (repU); T7 RNA polymerase promoter and terminator; multiple cloning site (MCS); signal peptide LipA (splipA); resistance genes against ampicillin (Amp^R) and tetracycline (Tet^R).

GTCTGTACGTTTCCTTAAGGCGAAATTAATACGACTCACTAT

T7 RNAP promoter

AGGGAGACCACAACGGTTTCCCGAATATTAATTAACCAAG

GAGGTGAAATGTACAATGGTCCAAACTAGTTCTGAAGATCTC

MCS

CGGAGCTCCCGGGATCCGGTACCGGCCGCATGCCGGC

STOP

TAATCGCGACCGGTAACTAGCATAACCCCTTGGGGCCTC

AgeI

TAAACGGGTCTTGAGGGGTTTTTGTCTAAAGGAGGAACTA

T7 RNAP terminator

TATCCGGTCCAATTTTGC

Fig.6 Incomplete sequence of pP_{T7}. T7 RNAP promoter and terminator are underlined. The multiple cloning site (MCS) is marked with a red bar. The last unique restriction site upstream of the T7 RNAP terminator is marked with a red rectangle. The stop codon (TAA) is illustrated by a dashed line.



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8. Order Information, Shipping and Storage

Order #	Description	Amount
BMEGT702	<i>Bacillus megaterium</i> protoplasts, strain MS941, pretransformed with pT7-RNAP	5 x 500 µl
BMEGT701	<i>Bacillus megaterium</i> high yield T7 gene expression kit, includes pretransformed protoplasts BMEGT702 (5 x 500µl), pP _{T7} cloning vector and pP _{T7} -GFP control vector (vectors lyophilized, 10 µg each)	1 Kit
BMEGT710	<i>Bacillus megaterium</i> pP _{T7} cloning vector, lyophilized	10 µg
BMEGT711	<i>Bacillus megaterium</i> pP _{T7} -SPlipA secretion vector, lyophilized	10 µg
BMEGT710C	<i>Bacillus megaterium</i> pPT7-GFP control vector, lyophilized	10 µg
BMEG50	<i>Bacillus megaterium</i> protoplasts, strain MS941	5 x 500 µl

Vectors are shipped at RT °C, protoplasts and kit on dry ice.

Store lyophilized vectors at 4 °C, reconstituted vectors at -20 °C, protoplasts at -80 °C

Vectors are *E. coli* / *B. megaterium* shuttle vectors

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