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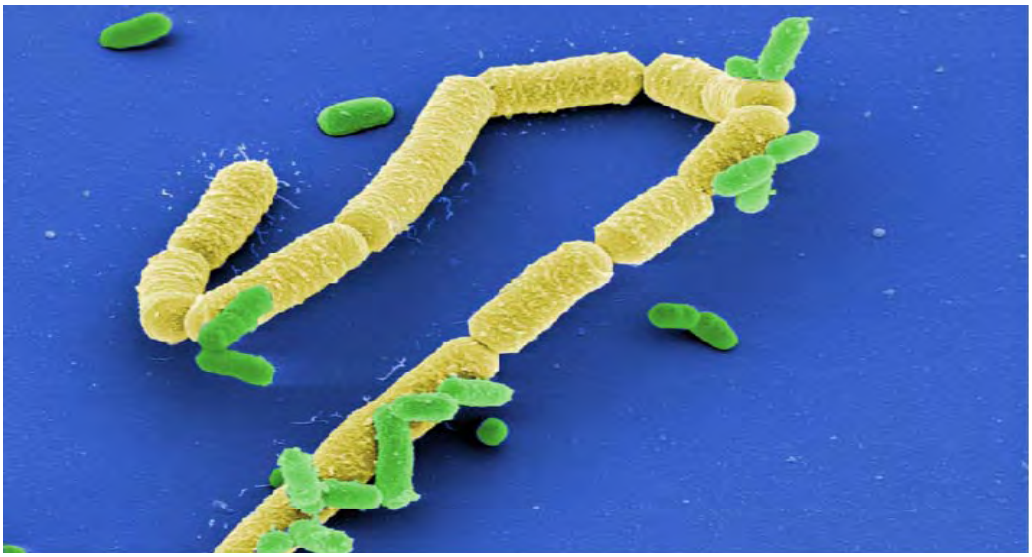
Bacillus Megaterium
Protein Expression System



Electron microscope image of *Bacillus megaterium* and *Escherichia coli*,
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Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Content

An efficient alternative to *E. coli*: Stable protein expression with high yield - suited not only for industrial scale.

MoBiTec offers this expression system as an easy-to-handle kit with *E. coli/B. megaterium* shuttle vectors and - to be ordered separately - *B. megaterium* protoplasts ready for transformation.

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 the significant name "*megaterium*" was the large size of the vegetative cells (over 1 µm) and the 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 documented has been the degradation of persistent insecticides by *B. megaterium* (Sexana *et al*., 1987; Selvanay-agam 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.

Several *B. megaterium* proteins are of importance. A family of P-450 cyto-chrome monooxygenases e.g. is similar to eukaryotic P-450 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 generation of new synthetic antibiotics.

A comprehensive overview about the features of this unique organism is given in Patricia S. Vary's review article "Prime time for *Bacillus megaterium*" (1994).

1.2. *Bacillus megaterium* as expression host

In molecular biology, *B. megaterium* has proven to be an excellent host for the expression of non-homologous DNA. All cloning vectors of the *Bacillus megaterium* system (which are both derivatives of the original Rygus & Hillen pWH1520; Malten *et al.*, 2004; Barg *et al.*, 2005; Biedendieck *et al.*) rely on the above mentioned xylose operon used as regulatory element.

In contrastto other bacilli strains *B. megaterium* hasthe advantage, thatnone of the alkaline proteases are present. This fact enables an excellent cloning and expression offoreign proteinswithoutdegradation (Meinhardt *et al.*, 1989; Rygus & Hillen, 1991). In addition, there are noendotoxinsfound inthe cellwall.

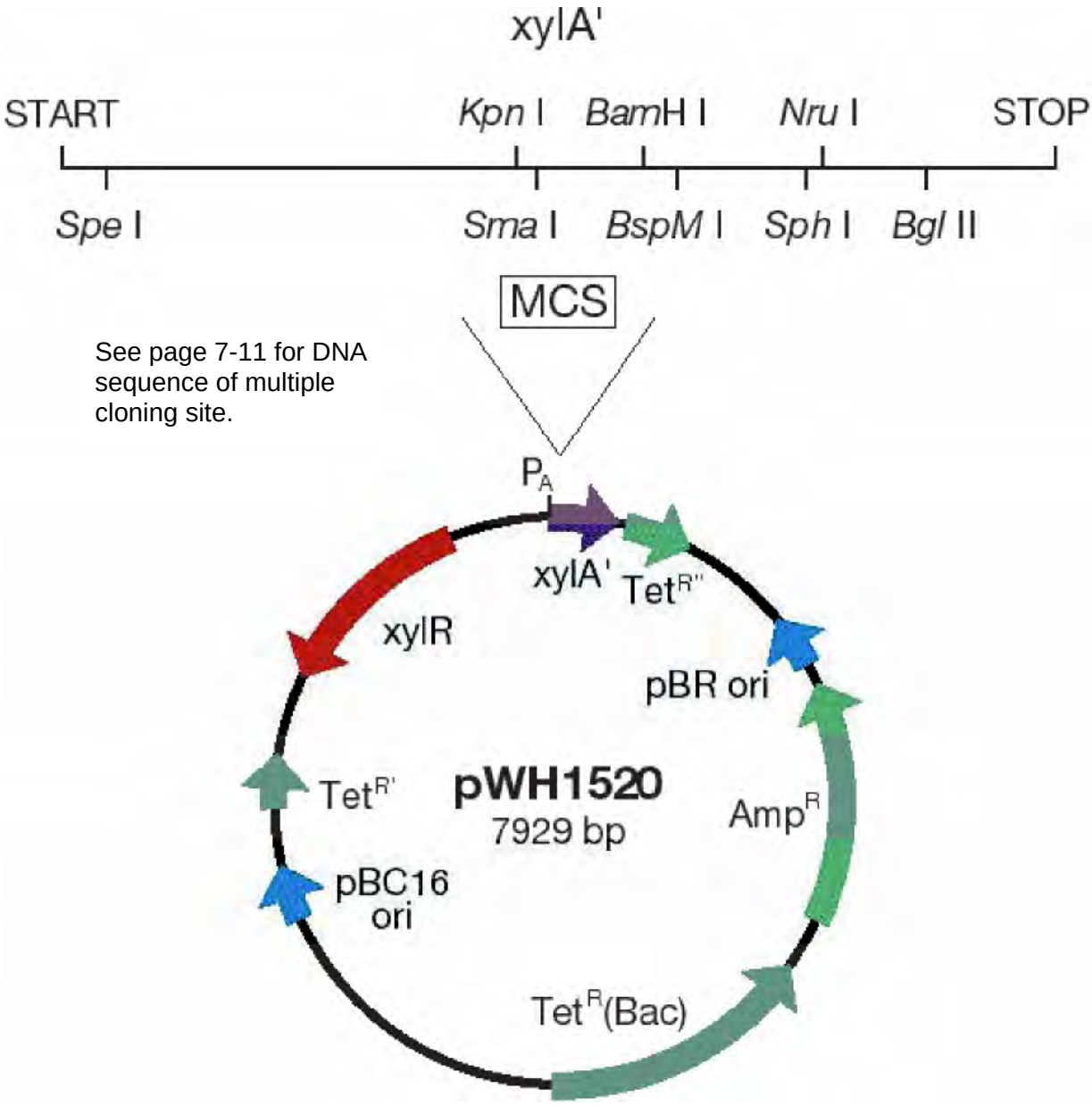
Protein yields are exceptionally good, also if inexpensive substrates are used.

Recombinant plasmids are structurally and segregationally stable. The *B. megaterium* glucose dehydrogenase gene (*ghd*) e.g. has been cloned back into *B. megaterium* and remained stable without selective pressure over a period of three weeks with daily subculturing (Meinhardt *et al.*, 1989).

Several proteins have successfully been overproduced in *B. megaterium* (see chapter 3). Rygus and Hillen (1991) describe cloning and expression of the four protein encoding genes *lacZ* from *E. coli*, *gdh* from *B. megaterium*, *mro* (mutarotase) from *Acinetobacter* and human *puk* (a urokinase-like plasminogen activator, rscuPA). Using the xylose operon the genes were 130- to 350-fold induced without proteolysis. Such a system offers unique possibilities for the industrial production of proteins and is of great interest to manufacturers in the biomedical field. In a diagnostic test for AIDS, the HIV coat protein is commercially produced by *B. megaterium* (Ginsburgh *et al.*, 1989).

2. Summary of advantages

- Stable, high yield protein production
- Suited for small to industrial-scale protein production
- Tightly regulated and efficiently inducible *xylA* operon (up to 350-fold)
- No endotoxins are found in the cell wall
- 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
- Extended polylinker downstream of promoter allows versatile cloning
- Additional *Bsr*GI site enables cloning without modifying the N-terminus
- Plasmids available for either intracellular or extracellular production
- Easy purification and detection of either 6xHis, Strep-tagged or Strep-/6xHis-double-tagged target proteins
- Removable purification tags due to TEV and Factor Xa sites
- Compatible with all *Bacillus subtilis* vectors



See page 7-11 for DNA sequence of multiple cloning site.

Fig. 1
Map of the original pWH 1520. Shuttle vector for *E. coli/B. megaterium*. Tet^R (Bac), tetracycline resistance *Bacillus*; $Tet^{R'}$, $Tet^{R''}$, tetracycline resistance, interrupted; Amp^R, ampicillin resistance; XylR, xylose-dependent repressor; XylA', xylose isomerase, gene incomplete; P_A, xylA promoter; MCS, multiple cloning site; pBC16 ori, *Bacillus* origin of replication; pBR, ColE1 origin of replication.

All vector maps and the complete DNA sequences are available for download on our internet web page http://www.mobitec.com.

3. Application Examples

Proteins successfully overproduced with the *B. megaterium* system are:

- β-Galactosidase (LacZ)[1]
- Catabolite control protein (CcpA)[2,3]
- Clostridium difficile toxin A[4]
- Cobaltochelatase (CbiX)[5]
- Dextransucrase[6]
- Endolevanase (LevB)[7]
- Glucose dehydrogenase (GdhA)[1]
- Heat shock protein (HPr) from PTS (phosphotransferase sugar transport system)[8]
- Human single-chain urokinase-like plasminogen activator (rscuPA)[1]
- Levansucrase[9]
- Mutarotase (Mro)[1]
- Neopullulanase[10]
- Translocation ATPase of the preprotein translocase (SecA)[11]
- Trehalose repressor (TreR)[12]

Protein yield:

Protein yields vary depending on the protein expressed. Rygusand Hillen (1991) have observed, that e.g. Gdh and Mro accumulated to 20% and 30% of the total soluble protein, respectively. The time dependence of the induced expression of these enzymes is shown in Fig. 2.

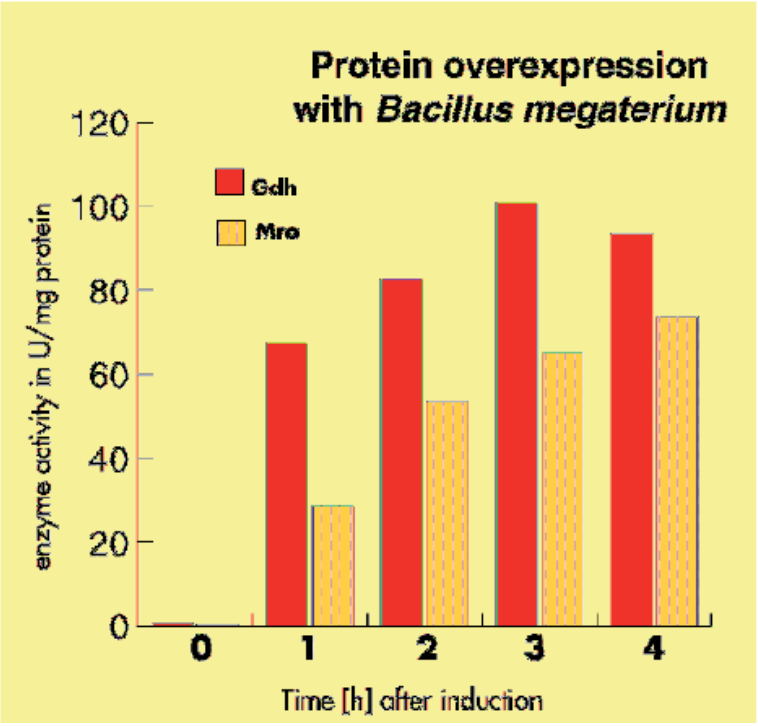


Fig. 2
Time dependence of induced expression of the enzymes Gdh (glucose dehydrogenase) and Mro (mutarotase) in *Bacillus megaterium*. Enzymatic activity given in U/mg protein.

4. Features of the *B. megaterium* expression vectors

All plasmids contain the strong *xylA* promoter originating from *Bacillus megaterium*. Transcription fromthis promoterisxylose inducible.Afterxylose addition, the xylose repressor coded by the *xylR* gene on the plasmids is released from P_{xylA} and transcription is initiated.

The most convenient cloning sites for insertion of DNA fragments carrying heterologous genes are located in the reading frame of *xylA* (see sequences). Therefore, any protein can be expressed using one out of three functionally different fusion strategies.

A transcriptional fusion requires that the gene of interest carries its own ribosome binding sequence (RBS) and translation initiation codon. Such a DNA fragment can be fused into any of the available restriction sites within *orf1*. Whether the resulting transcriptional fusion leads to expression of the gene of interest, which is independent from *orf1* expression, depends on the location of the newly created *orf1* stop codon with respect to the start codon of the gene of interest (inserting a target gene in one of the polylinker's restriction sites - not in frame with *orf1* - may create a new stop codon for translation termination of *orf1*). If these stop and start codons are close together, translational coupling may occur, in which the ribosomes translating the *orf1* reading frame would terminate at its newly created stop codon, generating a locally high concentration of ribosomes, so that translation initiation at the proximal target gene's start codon would be more efficient compared to a construct in which the *orf1* translation terminates farther away from the start codon.

On the other hand, if the *orf1* reading frame continues for a long distance into the reading frame of the gene of interest, the ribosomes translating the created *orf1* fusion protein might inhibit initiation of translation of the protein of interest. Therefore, it is advisable to pay attention to placement of a stop codon when constructing the gene fusion. Taken together, although a transcriptional or operon fusion is constructed, the efficient translation of the *orf1* reading frame, and any fusion there of created by the insertion, is likely to, positively or negatively, influence the translation efficiency of the gene of interest.

Alternatively, a truncated version of the gene of interest, lacking its own start codon, may be fused in frame to the *orf1* reading frame to create a translational or protein fusion. This will result in expression of a chimeric protein consisting of the signal peptide enabling secretion of the fusion protein (SP_{lipA}-containing plasmids only), the purification tags (6xHis, Strep or Strep/6xHis) and additional amino acids specified by the *orf1* encoding sequence, followed by the sequence encoded by the gene of interest (target protein).

Using the *Bsr*GI restriction site directly before the ATG start codon enables cloning without changing the N-terminus of the protein of interest. Target genes can be directly inserted after the signal peptidase restriction site using the *Kas* I, *Nar* I or *Sfo* I site allowing a complete removal of the signal peptide and the Strep-tag.

It is important to note that the multiple cloning site and its reading frame are identical in plasmids encoding for a purification tag and in the corresponding plasmids without tag starting from *BstB*I (e.g. pSTREP1525 and pSTREP-HIS1525) . Hence, a parallel cloning strategy of the gene of interest for tagging with 6xHis, Strep or double-tagging with Strep and 6xHis is possible. The Strep II sequence upstream the MCS (and the 6xHis tag downstream the MCS) allow convenient purification and detection of the expressed 6xHis, Strep or Strep/6xHis-tagged target proteins. For 6xHis tagging of the target protein, the *orf1* stop codon has to be used.

For expression of target proteins tagless plasmids for intracellular expresssion (pWH1520, pMM1522) and extracellular expression (pMM1525) are available, as well as constructs with 6xHis tags (pHIS1522, pC-His1622 & pHIS1525), Strep tags (pSTREP1522, pC-Strep1622 & pSTREP1525), 6xHis/Step double-tags (pSTREPHIS1525) and constructs with TEV protease site (pN-His-TEV1622, pN-Strep-TEV1622) and Factor Xa restriction sites (pN-Strep-Xa1622).

DNA sequences of the multiple cloning sites can be found in figures 3 to 7. Please find the order information in chapter 8.

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Fig. 3 Sequence of (incomplete) *xylA* gene including multiple cloning site. With pWH1520, gene fusions (translational fusions) as well as operon fusions (transcriptional fusions) are possible, depending on the cloning site and reading frame chosen (details see chapter 4).

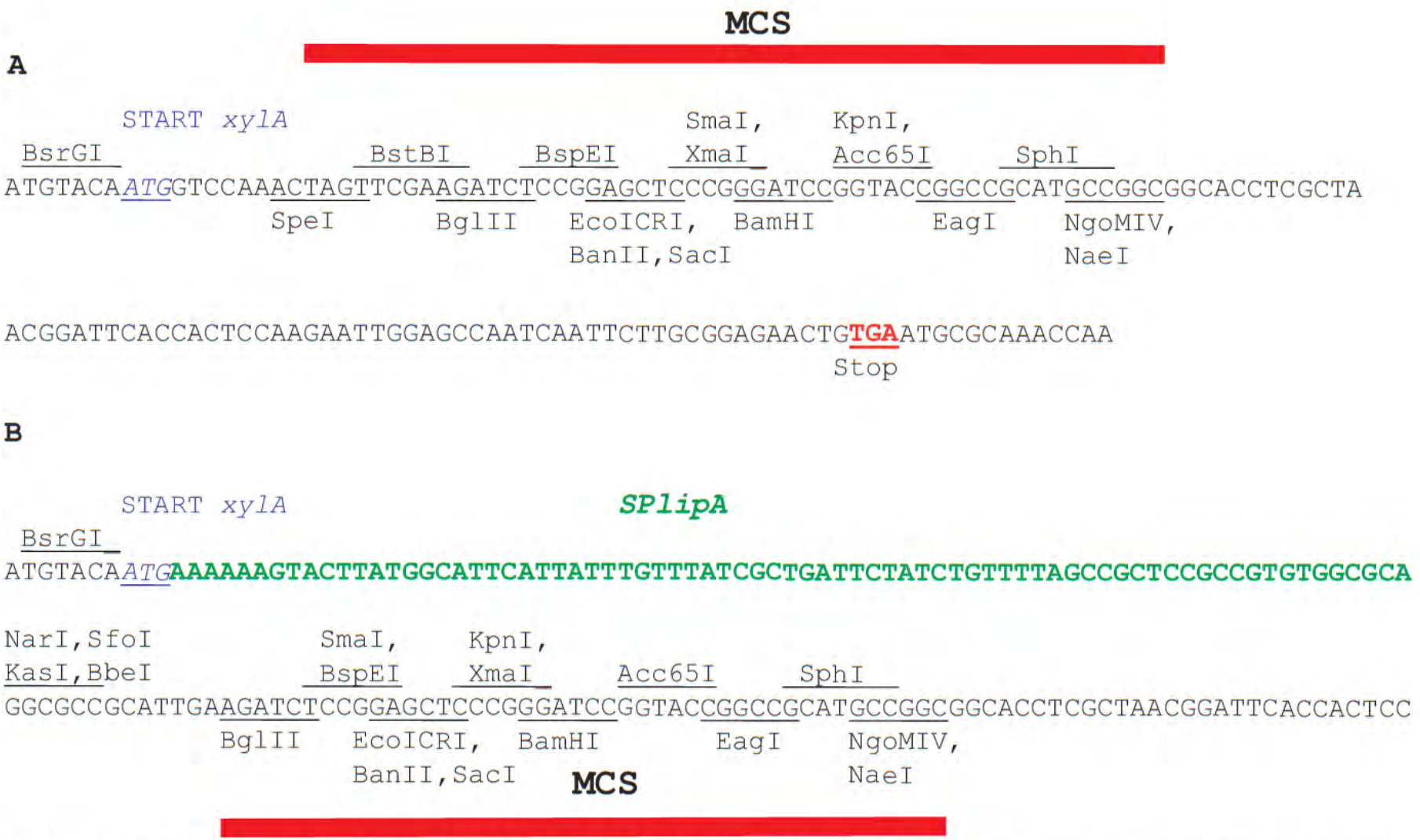

Fig. 4 Sequence of the multiple cloning sites (MCS) of pMM1522 (A) and pMM1525 (B). SP_{lipA}, signal peptide sequence (extacellular esterase LipA). With pMM1522/pMM1525, gene fusions (translational fusions) as well as operon fusions (transcriptional fusions) are possible, depending on the cloning site and reading frame chosen (details see chapter 4).

Fig. 5 Sequence of the multiple cloning sites (MCS) of pHIS1522 (A) and pHIS1525 (B). SP_{lipA}, signal peptide sequence (extacellular esterase LipA). With pHIS1522/pHIS1525, gene fusions (translational fusions) as well as operon fusions (transcriptional fusions) are possible, depending on the cloning site and reading frame chosen (details see chapter 4).

A

START orf1 SP_{lipA}

ATGTACT*ATG*AAAAAAGTACTTATGGCATTCATTATTTGTTTATCGCTGATTCTATCTGTTTTAGCCGCTCCGCCGTCTGGCGCA

BstBI — Strep-tag — NarI,SfoI / KasI,BbeI — BglII — BspEI — EcoICRI, BanII, SacI — SmaI, XmaI — BamHI — KpnI, Acc65I

GGAGCCGCATTCGAATGGAGCCACCCGCAGTTTGAAAAAATCGAAGGGCGCCAGATCTCCGGAGCTCCCGGGATCCGGTAC

SphI — EagI — NgoMIV, NaeI — **Stop** — NruI

CGGCCGCATGCCGGC*TAA*ACCTCGCGAACGGA

B

START orf1 SP_{lipA}

ATGTACT*ATG*AAAAAAGTACTTATGGCATTCATTATTTGTTTATCGCTGATTCTATCTGTTTTAGCCGCTCCGCCGTCTGGCGCA

BstBI — Strep-tag — NarI,SfoI / KasI,BbeI — BglII — BspEI — EcoICRI, BanII, SacI — SmaI, XmaI — BamHI — KpnI, Acc65I

GGAGCCGCATTCGAATGGAGCCACCCGCAGTTTGAAAAAATCGAAGGGCGCCAGATCTCCGGAGCTCCCGGGATCCGGTAC

SphI — EagI — NgoMIV, — AgeI — 6xHis-tag — **Stop**

CGGCCGCATGCCGGCCTGAGAGGATCGCATCACCATCACCATCAC*TAA*CCGGTCCAAGAAT

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Fig. 6 Sequence of orf1 with the multiple cloning sites of pSTREP1525 (A) and pSTREP-HIS1525 (B). SP_{lipA}, signal peptide sequence (extracellular esterase LipA). With pSTREP1525/pSTREPHIS1525, gene fusions (translational fusions) as well as operon fusions (transcriptional fusions) are possible, depending on the cloning site and reading frame chosen (details see chapter 4).

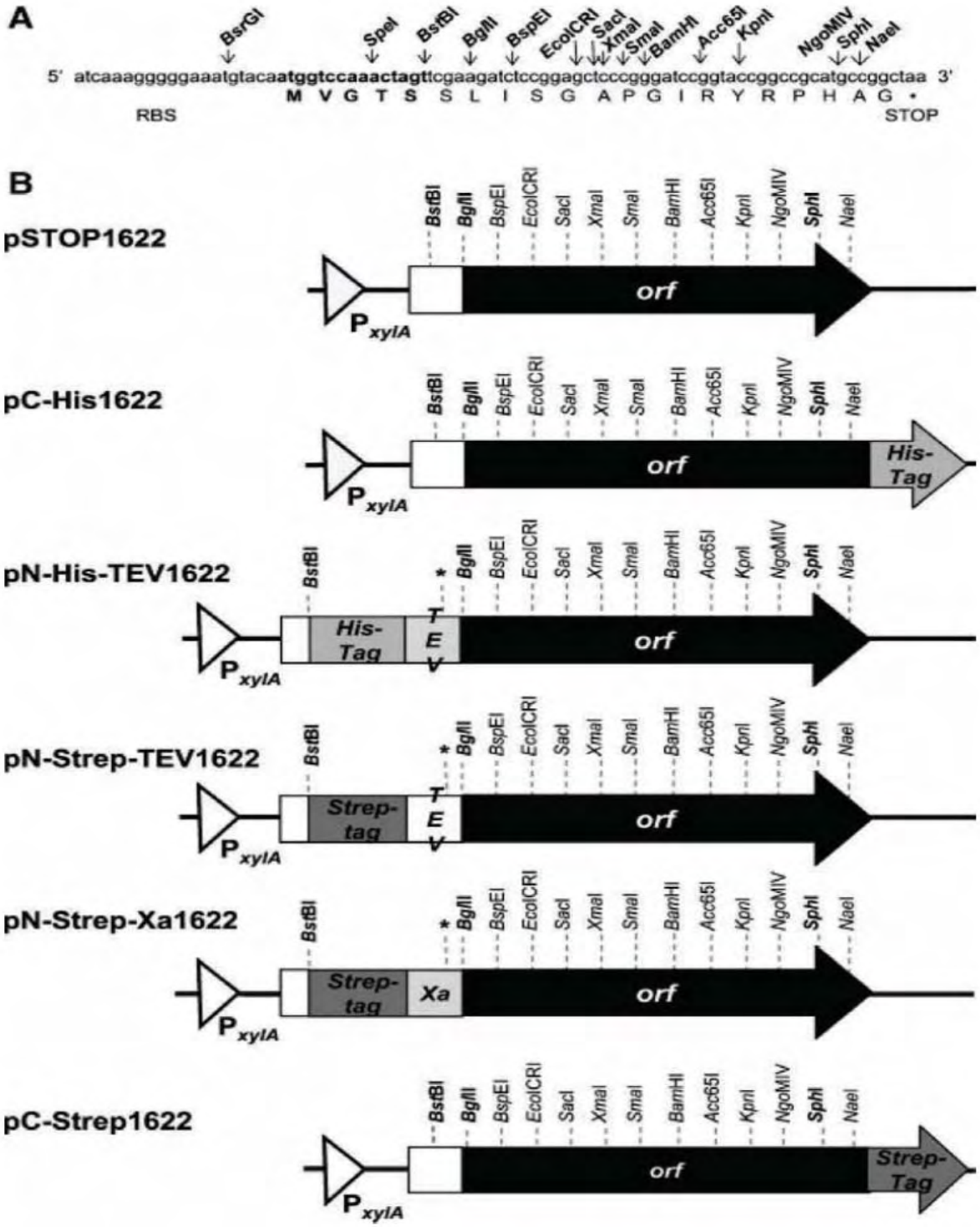

Fig. 7 Series of expression plasmids for intracellular production of tagged proteins in *B. megaterium*. All plasmids of the 1622 series are based on the shuttle vector pSTOP1522 (Malten *et al.*, 2006). A: DNA sequence of ribosome binding site (RBS) and multiple cloning site (MCS) of the expression plasmid pSTOP1622. The coding sequence of an open reading frame (ORF) comprising the multiple cloning site is marked in bold. B: Scheme of expression plasmids based on pSTOP1622. All shown expression plasmids allow parallel cloning of genes of interest into the identical multiple cloning site from *Bgl*II (marked in bold) to *Nae*I. Restriction site *Nar*I is indicated by a star (*).$P_{PxylA,}$ promoter of *xylA*; TEV, tobacco etch virus protease cleavage site; Xa, Factor Xa protease cleavage site.

5. Protocols

5.1. Cloning the DNA fragment of interest

The *E. coli/B. megaterium* shuttle vectors are supplied as lyophilized DNA. Follow standard protocols for propagation of the plasmid in *E. coli*, minipreps, restriction endonuclease cleavages and ligation of the DNA fragment of interest into the vector (Sambrook *et al.*, 1989). After ligation of the insert, the vectors should be propagated in *E. coli* before transforming the *Bacillus* protoplasts.

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

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

We found WH320 and derivated strains to be asporogenic - they will die on plates, kept at 4 °C within two weeks, so prepare DMSO/glycerol stocks as a backup and streak the working cultures on fresh plates every 7 - 10 days.

For secretion of proteins into the culture medium when using plasmids with a SP_{lipA} signal sequence, we recommend *B. megaterium* strain MS941 as host (order # BMEG50, Wittchen & Meinhardt, 1995).

Positive clones can be selected for by adding 5 to 10 mg/l tetracycline to the growth medium.

To check for successfu l overexpression harvest small samples of the culture just before and at intervals after induction. To obtain crude extracts for gel analysis, the bacilli have to be lysed using lysozyme or sonication or other more harsh methods. Simple boiling of cells in sample buffer (Laemmli, 1970) which is quite convenient for *E. coli* does not work with *Bacillus megaterium*.

5.3. Transformation of *B. megaterium* protoplasts

For protein expression the plasmids with the insert coding for the protein of interest is transformed into *B. megaterium*.

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 protoplast transformation, which is a modification of the method from Puyet *et al.* (1987).

Control Experiments:

1. Negative control: protoplasts only without DNA

   This is the control demonstrating, that the protoplasts have not been contaminated. You should get an empty plate without colonies on the antibiotic (tet) plate.

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

2. Positive control: protoplasts transformed with empty plasmid (without insert) - not included in the kit!

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

   Essential buffers are listed in chapter 6.

Transformation procedure:

(1) Combine 500 µl of protoplast suspension and 5 µg of DNA (in SMMP, see chapter 6) in one 12 ml tube for each transformation
(2) Add 1.5 ml of PEG-P, incubate 2 minutes at room temperature (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 for 10 minutes at RT), pour off supernatant immediately after centrifugation; note: do not check for a pellet - most of the time there will be none visible and the pellet may be fragile
(5) Add 500 µl SMMP
(6) Incubate at 37 °C for 90 minutes with gentle shaking or rolling of tubes (max. 100 rpm)
(7) Prepare 2.5 ml aliquots of CR5-top agar in sterile tubes in a waterbath (max. 43 °C)
(8) After outgrowth add 50 to 200 µl of cells to 2.5 ml top agar, mix gently by rolling the tube between both hands (do not vortex!) and pour on a prewarmed plate of LB containing the desired antibiotics
(9) Incubate overnight at 37 °C - expect colonies of varying d iameter because some will be covered with agar and others have easier access to air (Remark: the colonies on the top of the agar surface will be shiny)
(10) Streak on fresh plates within two days

5.4. Protein production

The strong, tig htly regulated promoter as well as the repressor gene from the *B. megaterium* xylose operon were used to construct the xylose inducible high-level expression vector pWH1520 as well as its improved derivatives. The multiple cloning site downstream of the promoter allows versatile cloning of genes under its transcriptional control.

Relevant restriction sites are indicated in the sequence maps in Fig. 3 to 7 on pages 10 to 14.

I. Test protein expression

(1) Grow the transformed *B. megaterium* cells in LB medium (+Tc) to an optical density at 600 nm (OD_{600}) of 0.3 at 37 °C

(2) Take a sample as control before induction

(3) Induce the xylose promoter by addition of 0.5% (D)-xylose

(4) Incubate at 37 °C

(5) Withdraw samples every 30 to 60 minutes until an OD_{600} of 1.5 is reached (i.e. the cells enter the stationary phase)

(6) Centrifuge each sample to harvest cells

(7) Resuspend cells in sonication buffer to a final concentration of 0.01 OD/ml

(8) Sonicate 3 times in short bursts (20 seconds) at 50 W; allow sample to cool for 20 seconds between each burst

(9) Centrifuge lysate to separate the insoluble fraction (pellet) from the soluble fraction (supernatant)

(10) Dilute the insoluble fraction in sonication buffer to a final concentration of 0.02 OD/ml

(11) In order to determine in which fraction the protein of interest is found, use 10 - 15 µl of each fraction (soluble and insoluble), and use standard protocols to perform an SDS-PAGE (Sambrook *et al.*, 1989)

(12) Determine enzymatic activities with the appropriate assays (not included in the kit)

(13) Perform Western blot using appropriate antibodies (not included in the kit)

II. Scale up protein production

(14) Grow larger culture and induce as indicated above

(15) Harvest cells at the time point of maximal protein overproduction, as determined by the test experiment

III. Acetone precipitation of proteins in culture medium

(16) Add 12 ml acetone (-20 °C) to 3 ml of culture medium and incubate overnight at -20 °C

(17) Centrifuge at 5,000 rpm and 4 °C for 15 minutes

(18) Remove supernatant completely and dry tube at 37 °C for 10 minutes

(19) Resuspend pellet in 500 µl of deionized water and transfer in 1.5 ml spin tubes

(20) Centrifuge at 13,000 rpm and 4 °C for 10 minutes

(21) Remove supernatant completely using a small pipette

(22) Dry pellet for 5 minutes at 35 °C under vacuum (speed vac)

(23) Add 10 µl of 8 M urea (in 50 mM Tris-HCl, pH 7.5) and 10 µl SDS sample buffer

(24) Spin shortly at 13,000 rpm and load a 10 µl sample (corresponding to 1.5 ml of culture medium) onto a SDS polyacrylamide gel for analysis

For your convenience and in order to provide a positive control, the following vectors validated for recombinant production and one-step affinity purification of *L. reuteri* levansucrase from growth medium using a *B. megaterium* expression system are available:

Basic secretion vector	Final secretion vector	Encoded protein	Order #
pHIS1525	pRBBm15	LevΔ773His	BMEG13C
pSTREP1525	pRBBm13	StrepLevΔ773	BMEG14C
pSTREPHIS1525	pRBBm16	StrepLevΔ773His	BMEG15C

Please note that these vectors are available only in combination with a regular *B*. *megaterium* expression vector!

For background information regarding the controls please see p. 23.
Malten M., *et al*.

6. Materials

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

prepare as 2x concentrated medium: 7 g in 200 ml H_2O autoclave for 15 minutes

2x SMM

1M	sucrose
40 mM	maleic acid, disodium salt
40 mM	$MgCl_2$
pH 6.5	

autoclave for 12 minutes (should not get brownish)

SMMP

mix equal volumes of 2x SMM and 2x AB3; prepare freshly before use

Antibiotics

Ampicillin	100 µg/ml finalconcentration (for *E. coli*)
Tetracycline	10 µg/ml final concentration (for *B. megaterium*)

PEG-P

40 % (w/v) PEG6000 in 1x SMM
autoclave for 12 minutes

LB plates

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
agar	15 g
add 1 L	

adjust pH to 7.5 with sodium hydroxide

Sonication buffer

Tris-HCl	10 mM, pH 7.5
NaCl	200 mM
β-mercaptoethanol	5 mM (add just before usage)

CR5 topagar for 500 ml: components a)-c)

component a)

51.50 g sucrose
3.25 g MOPS
0.33 g NaOH

add 250 ml
adjust to pH 7.3 with NaOH and
sterilize by filtration

component b)

2.0 g agar
0.1 g casamino acids
5.0 g yeast extract

add 142.5 ml
autoclave for 20 minutes
500 ml bottle, include stir bar

After autoclaving, combine the two components a) and b) after they have cooled down to 50 °C. Then add the following:

component c)

57.5 ml 8x CR5 salts *
25.0 ml 12 % proline (w/v; sterilize by filtration)
25.0 ml 20 % glucose (w/v; sterilize by filtration)

Aliquot in sterilized containers - contaminates easily.

***CR5 salts 8x stock:**

1.25 g K_2SO4
50.00 g $MgCl_2 \times 6\ H_2O$
0.25 g KH_2PO4
11.00 g $CaCl_2 \times 2\ H_2O$

add 625 ml H_2O
autoclave for 20 minutes

Adjust to 42 - 43 °C in a waterbath, add bacteria and poor mixture onto agar plates.

The recipe on the previous page yields the following final concentrations in the CR5 topagar (per litre):

component a)

sucrose	103.00 g/l
MOPS	6.50 g/l
NaOH	0.66 g/l

adjust to pH 7.3 and
and sterilize by filtration

component b)

agar	4.0 g/l
casamino acids	0.2 g/l
yeast extract	10.0 g/l

autoclave for 20 minutes

component c)

K_2SO_4	0.25 g/l
$MgCl_2$ x 6 H_2O	10.00 g/l
glucose	10.00 g/l
proline	6.00 g/l
KH_2PO_4	0.05 g/l
$CaCl_2$	2.20 g/l

sterilize glucose and proline by filtration;
autoclave other components for 20 minutes

7. References

General:

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8 Order Information, Shipping and Storage

Order#	Product	Quantity
BMEG02	*Bacillus megaterium* WH320 protoplasts	5 x 500 µl
BMEG50	*Bacillus megaterium* MS941 protoplasts	5 x 500 µl
shipped on dry ice; store at -80 °C		
BMEG03	*Bacillus megaterium* vector pWH1520, lyophilized DNA	5 µg
BMEG10	*Bacillus megaterium* vector, pMM1522, lyophilized DNA	10 µg
BMEG11	*Bacillus megaterium* vector, pMM1525, lyophilized DNA	10 µg
BMEG12	*Bacillus megaterium* vector, pHIS1522, lyophilized DNA	10 µg
BMEG13	*Bacillus megaterium* vector, pHIS1525, lyophilized DNA	10 µg
BMEG14	*Bacillus megaterium* vector, pSTREP1525	10 µg
BMEG15	*Bacillus megaterium* vector pSTREPHIS1525	10 µg
BMEG20	*Bacillus megaterium* vector pC-His1622	10 µg
BMEG21	*Bacillus megaterium* vector pC-Strep1622	10 µg
BMEG22	*Bacillus megaterium* vector pN-His-TEV1622	10 µg
BMEG23	*Bacillus megaterium* vector pN-Strep-TEV1622	10 µg
BMEG24	*Bacillus megaterium* vector pN-StrepXa1622	10 µg
BMEG25	*Bacillus megaterium* vector, pSTOP1622, lyophilized DNA.	10 µg
shipped at RT; store at 4 °C		

Levansucrase expression positive control vectors:

Order#	Product	Quantity
BMEG13C	Levansucrase expression positive control, HIS-Tag	10 µg
BMEG14C	Levansucrase expression positive control, STREP-Tag	10 µg
BMEG15C	Levansucrase expression positive control, STREPHIS-Tag	10 µg
shipped at RT; store at 4 °C		

Enzymes for removal of purification tags:

Order#	Product	Quantity
EP0504	Factor Xa Protease (Ile-Glu-Gly-Arg)	250 µg
shipped at RT °C; store at 4 °C		
PR-ETA10010-01	MobiTEV Protease, recombinant, His-Tag	1000 U
PR-ETA10010-05	MobiTEV Protease, recombinant, His-Tag	10 x 1000 U
shipped at -20 °C; store at -70 °C		

9 Contact and Support

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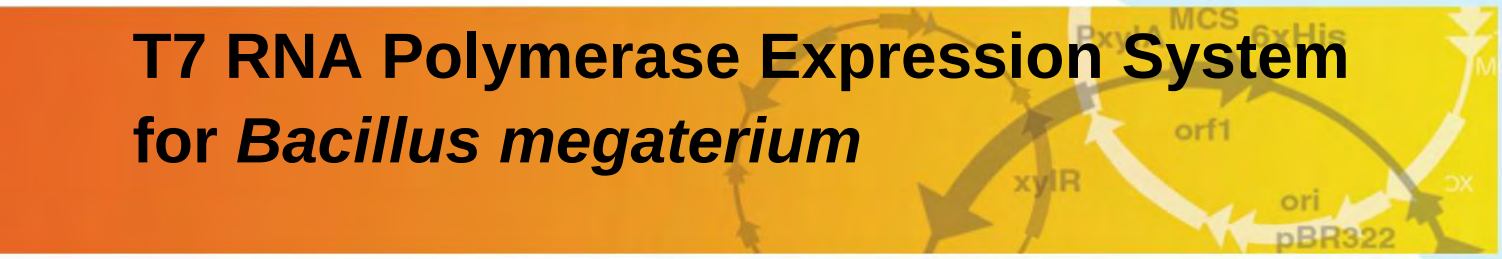
T7 RNA Polymerase Expression System for *Bacillus megaterium*



01.2010

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Content

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 *B. megaterium* protoplasts ready for transformation and *E. coli / B. 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 the significant name "*megaterium*" was the large size of the vegetative cells (over 1 µm) and the 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 documented has been the degradation of persistent insecticides by *B. megaterium* (Sexana *et al*., 1987) 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.
Several *B. megaterium* proteins are of importance. A family of P450 cytochrome monooxygenases e.g. 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.
A comprehensive overview about the features of this unique organism is given in Patricia S. Vary's review article "Prime time for *Bacillus megaterium*" (1994).

1.2. *Bacillus megaterium* as expression host

In molecular biology, *B. megaterium* has proven to be an excellent host for the expression of non-homologous DNA. All cloning vectors of the *Bacillus megaterium* system rely on the above mentioned xylose operon used as regulatory element and the bacteriophage T7 RNA polymerase.
In contrast to other bacilli strains *B. megaterium* has the advantage, that none of the alkaline proteases are present. This fact enables an excellent cloning and expression of foreign proteins without degradation (Meinhardt *et al.*, 1989; Rygus & Hillen, 1991). In addition, 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* (Tabour 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 *Bacillus 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 xylA promoter pT7-RNAP contains the genes of *ampicillin* and *chloramphenicol* 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 to ampicillin (in *E.coli*) and tetracycline (in *B. megaterium*).

For your convenience MoBiTec offers *B. megaterium* protoplasts pretransformed with pT7-RNAP, so that you just have to insert your gene of interest into pPT7 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

- Stable, high yield protein production
- Suited for small to industrial-scale protein production
- Tightly regulated and efficiently inducible xylA operon / T7 RNA polymerase
- No endotoxins are found in the cell wall
- 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
- 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 overexpression 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. 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) and the overall productivity was more than six times enhanced (12.8 mg $l^{-1}h^{-1}$ with the T7 RNAP expression system – 2mg $l^{-1}h^{-1}$ with the xylose-inducible system).

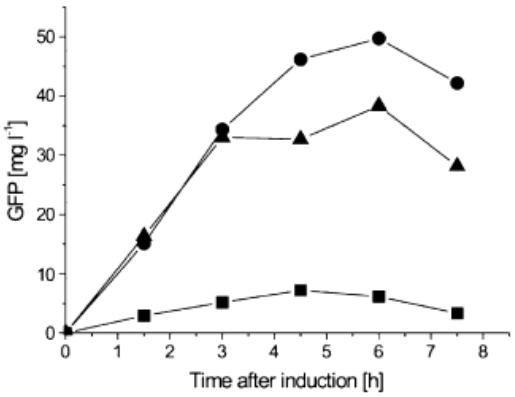


Fig. 1 Comparison of GFP amounts produced by variant expression systems. GFP produced by *B. megaterium* carrying the common xylose-regulated Plasmide pRBBm34 (square), *B. megaterium* transformed with the T7 expression system plasmids (circle) and *B.megaterium* with the T7 expression system plasmids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

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 overexpressed protein. However, the positive effects on cystolic 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 overexpression of the extracellular protein 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 expression with a common used *B. megaterium* xylose-inducible expression system (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*, minipreps, restriction endonuclease cleavages and ligation of the DNA fragment of interest into the vector (Sambrook *et al.*, 1989). After ligation of the insert, the vector should be propagated in *E. coli (*Amp^R*)* before transforming the *B. megaterium* protoplasts.

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

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

We found MS941 and derivated strains to be asporogenic - they will die on plates, kept at 4 °C within two weeks, so prepare DMSO/glycerol stocks as a backup and streak the working cultures on fresh plates every 7 - 10 days.

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

To check for successful overexpression harvest small samples of the culture just before and at intervals after induction. To obtain crude extracts for gel analysis, the bacilli have to be lysed using lysozyme or sonication or other more harsh methods. Simple boiling of cells in sample buffer (Laemmli, 1970) which is quite convenient for *E. coli* does not work with *Bacillus megaterium*.

4.3. Transformation of *B. megaterium* protoplasts

For protein expression the plasmid pP_{T7} with the insert coding for the protein of interest is transformed into with pT7-RNAP pretransformed protoplasts of *B. megaterium*.

After transformation it is advisable to screen at least three different clones for protein expression as the yield can vary among 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.

Control Experiments:

1. Negative control: protoplasts only without DNA

This is the control demonstrating, that the protoplasts have not been contaminated. You should get an empty plate without colonies on the antibiotic (tet) plate.

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

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 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 5 µg of DNA
(in SMMP, see chapter 6) in one 12 ml tube for each transformation
(2) Add 1.5 ml of PEG-P, incubate 2 minutes at room temperature (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 for 10 minutes at RT), pour off supernatant immediately after centrifugation;
note: do not check for a pellet - most of the time there will be none visible and the pellet may be fragile
(5) Add 500 µl SMMP
(6) Incubate at 37 °C for 90 minutes with gentle shaking or rolling of tubes
(max. 100 rpm)
(7) Prepare 2.5 ml aliquots of CR5-top agar in sterile tubes in a waterbath
(max. 43 °C)
(8) After outgrowth add 50 to 200 µl of cells to 2.5 ml top agar, mix gently by rolling the tube between both hands (do not vortex!) and pour on a prewarmed plate of LB containing the desired antibiotics
(9) Incubate overnight at 37 °C - expect colonies of varying d iameter because some will be covered with agar and others have easier access to air
(Remark: the colonies on the top of the agar surface will be shiny)
(10) Streak on fresh plates within two days

4.4. Protein production

The multiple cloning site downstream of the *p*romoter allows versatile cloning of genes under its transcriptional control.
Relevant restriction sites are indicated in the sequence map in Fig. 5 on page 14.

I. Test protein expression

(1) Grow the transformed *B. megaterium* cells in LB medium (+Tc, +Cm) to an optical density at 578 nm of 0.4 at 37 °C

(2) Take a sample as control before induction

(3) Induce the xylose promoter by addition of 0.5% D-xylose (w/v)

(4) Incubate at 37 °C

(5) Withdraw samples every 30 to 60 minutes until an OD_{600} of 1.5 is reached (i.e. the cells enter the stationary phase)

(6) Centrifuge each sample to harvest cells

(7) Resuspend cells in sonication buffer to a final concentration of 0.01 OD/ml

(8) Sonicate 3 times in short bursts (20 seconds) at 50 W; allow sample to cool for 20 seconds between each burst

(9) Centrifuge lysate to separate the insoluble fraction (pellet) from the soluble fraction (supernatant)

(10) Dilute the insoluble fraction in sonication buffer to a final concentration of 0.02 OD/ml

(11) In order to determine in which fraction the protein of interest is found, use 10 - 15 µl of each fraction (soluble and insoluble), and use standard protocols to perform an SDS-PAGE (Sambrook *et al.*, 1989)

(12) Determine enzymatic activities with the appropriate assays (not included in the kit)

(13) Perform Western blot using appropriate antibodies (not included in the kit)

II. Scale up protein production

(14) Grow larger culture and induce as indicated above

(15) Harvest cells at the time point of maximal protein overproduction, as determined by the test experiment

III. Acetone precipitation of proteins in culture medium

(16) Add 12 ml acetone (-20 °C) to 3 ml of culture medium and incubate overnight at -20 °C

(17) Centrifuge at 5,000 rpm and 4 °C for 15 minutes

(18) Remove supernatant completely and dry tube at 37 °C for 10 minutes

(19) Resuspend pellet in 500 µl of deionized water and transfer to 1.5 ml spin tubes

(20) Centrifuge at 13,000 rpm and 4 °C for 10 minutes

(21) Remove supernatant completely using a small pipette

(22) Dry pellet for 5 minutes at 35 °C under vacuum (speed vac)

(23) Add 10 µl of 8 M urea (in 50 mM Tris-HCl, pH 7.5) and 10 µl SDS sample buffer

(24) Spin shortly at 13,000 rpm and load a 10 µl sample (corresponding to 1.5 ml of culture medium) onto a SDS polyacrylamide gel for analysis

5. Materials

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

prepare as 2x concentrated medium: 7 g in 200 ml H_2O; autoclave for 15 minutes

2x SMM

1M sucrose
40 mM maleic acid, disodium salt
40 mM $MgCl_2$
pH 6.5

autoclave for 12 minutes (should not get brownish)

SMMP

mix equal volumes of 2x SMM and 2x AB3; prepare freshly before use

Antibiotics

Ampicillin 100 µg/ml final concentration (for *E. coli*)
Chloramphenicol 4.5 µg/ml final concentration (for *B. megaterium*)
Tetracycline 10 µg/ml final concentration (for *B. megaterium*)

PEG-P

40 % (w/v) PEG6000 in 1x SMM
autoclave for 12 minutes

LB plates

Bacto-tryptone 10 g
Bacto-yeast extract 5 g
NaCl 10 g
agar 15 g
add 1 L
adjust pH to 7.5 with sodium hydroxide

Sonication buffer
Tris-HCl 10 mM, pH 7.5
NaCl 200 mM
β-mercaptoethanol 5 mM (add just before usage)

CR5 topagar for 500 ml: components a)-c)

component a)

51.50 g sucrose
3.25 g MOPS
0.33 g NaOH
add 250 ml H_2O

adjust to pH 7.3 with NaOH and sterilize by filtration

component b)

2.0 g agar
0.1 g casamino acids
5.0 g yeast extract
add 142.5 ml H_2O

autoclave for 20 minutes in a 500 ml bottle, include stir bar

After autoclaving, combine the two components a) and b) after they have cooled down to 50 °C. Then add the following:

component c)

57.5 ml 8x CR5 salts *
25.0 ml 12 % proline (w/v; sterilize by filtration)
25.0 ml 20 % glucose (w/v; sterilize by filtration)

Aliquot in sterilized containers - contaminates easily.

***CR5 salts 8x stock:**

1.25 g K_2SO4
50.00 g $MgCl_2$ x 6 H_2O
0.25 g KH_2PO4
11.00 g $CaCl_2$ x 2 H_2O

add 625 ml H_2O
autoclave for 20 minutes

Adjust to 42 - 43 °C in a waterbath, add bacteria and pour mixture onto agar plates.

The recipe on the previous page yields the following final concentrations in the CR5 topagar (per litre):

component a)

sucrose 103.00 g/l
MOPS 6.50 g/l
NaOH 0.66 g/l

adjust to pH 7.3 and
sterilize by filtration

component b)

agar 4.0 g/l
casamino acids 0.2 g/l
yeast extract 10.0 g/l

autoclave for 20 minutes

component c)

K_2SO_4 0.25 g/l
$MgCl_2$ x 6 H_2O 10.00 g/l
glucose 10.00 g/l
proline 6.00 g/l
KH_2PO_4 0.05 g/l
$CaCl_2$ 2.20 g/l

sterilize glucose and proline by filtration;
autoclave other components for 20 minutes

6. Vector maps

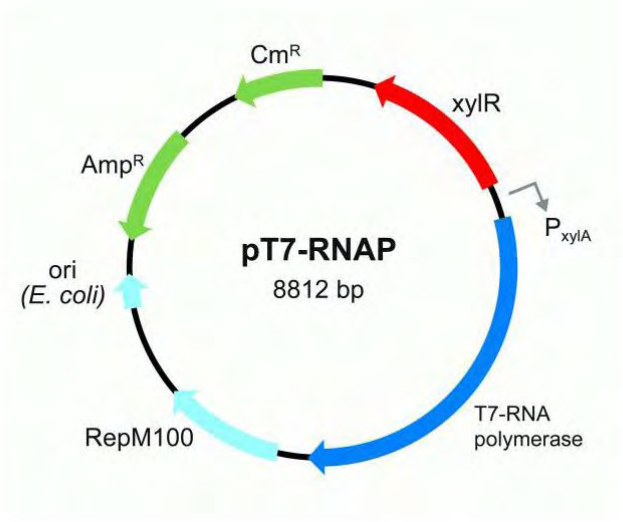


Fig. 2 Map of pT7-RNAP. replicon derived from the rolling circle plasmide pBM100 264 *of B. megaterium* QM B1551 (RepM100); *E. coli* origin of replication (ori *E. coli*); resistances for ampicillin (Amp^R) and chloramphenicole (Cm^R); xylose promoter (P_{xylA}) and its cognate repressor (xylR); sequence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

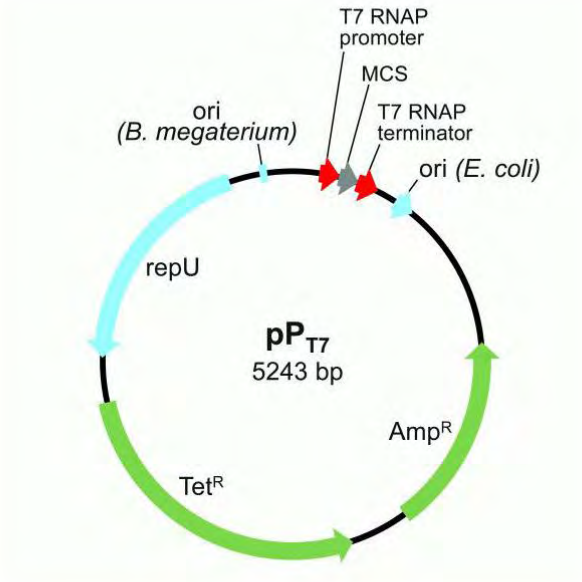


Fig. 3 Map of pP_{T7}: replicon derived from *B. cereus* (RepU); *B. megaterium* origin of replication (ori *B. megaterium*); T7 RNA polymerase promoter and terminator; multiple cloning site (MCS); *E. coli* origin of replication (ori *E. coli*); resistances for ampicillin (Amp^R) and tetracycline (Tet^R).

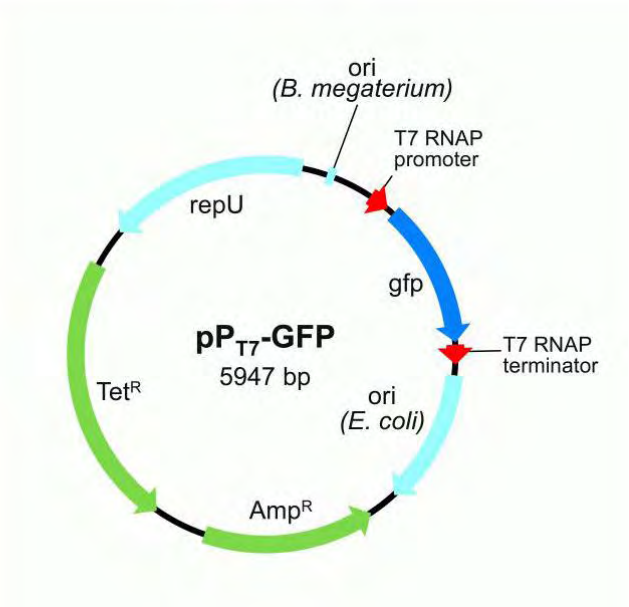


Fig. 4 Map of pP_{T7}-GFP: replicon derived from *B. cereus* (RepU); *B. megaterium* origin of replication (ori *B. megaterium*); T7 RNA polymerase promoter and terminator; GFP (green fluorescent protein) gene sequence (gfp); *E. coli* origin of replication (ori *E. coli*); resistances for ampicillin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

Fig. 5 Incomplete sequence of pP_{T7}. T7 RNAP promoter and terminator are underlined. The Multiple cloning site (MCS) is marked with a red bar and unique restriction sites are marked with a red rectangle. The stop codon (TAAT) is illustrated by a dashed line.

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8. Order Information, Shipping and Storage

order#	description	amount
BMEGT702	*Bacillus megaterium* protoplasts, strain MS941, pretransformed with pT7-RNAP	5x500 µL
BMEGT701	*Bacillus megaterium* high yield T7 gene expression kit, includes pretransformed protoplasts BMEGT702(5x500µl), pP_{T7} cloning vector and pP_{T7}-GFP control vector (vectors lyophilized, 10 µg each)	1 Kit
BMEGT710	*Bacillus megaterium* pP_{T7} cloning vector, lyophilized	10 µg
BMEG50	*Bacillus megaterium* protoplasts, strain MS941	5 x 500 µl

shipped at RT, protoplasts and kit shipped on dry ice
store lyophilized vectors at 4 °C, reconstituted vectors at -20 °C, protoplasts at -70 °C
Vectors are *E. coli / B. megaterium* shuttle vectors

9. Contact and Support

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