Bacillus Megaterium
Protein Expression System

Product Information and Instructions
January 2000

Mo Bi Tec
MOLECULAR BIOLOGISCHE TECHNOLOGIE

Bacillus megaterium

"- *one of the most efficient expression systems described in any organism so far!* "

(Rygus and Hillen, 1991)

Content

B.meg. 3/2000

A new alternative to *E. coli* : stable protein expression with high yield - suited not only for industrial scale.

MoBiTec offers this new expression system as an easy-to-handle kit with *E. coli/ B. megaterium* shuttle vector pWH1520 and *B. megaterium* protoplasts ready for transformation.

1. Introduction

1.1. General features of *Bacillus megaterium*

Being first described over 100 years ago, *Bacillus 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 has, thus, 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; 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.

Several *B. megaterium* proteins are of importance. A family of P-450 cytochrome 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. In the MoBiTec system with pWH1520 as cloning vector, the above mentioned xylose operon is used as regulatory element. The pWH1520 vector map is shown in Fig. 1, page 5.

The DNA sequence is available for download on our internet web page http://www.mobitec.de.

Over 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 and Hillen, 1991). In addition, there are no endotoxins found in the cell wall.

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 application examples below). 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 protein expression, high yield
- xylose operon: tightly regulated and efficiently inducible by xylose (up to 350-fold)
- polylinker downstream of promoter allows versatile cloning
- 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
- endotoxins are not found in the cell wall
- suited for industrial large scale protein production
- all *Bacillus subtilis* vectors are compatible

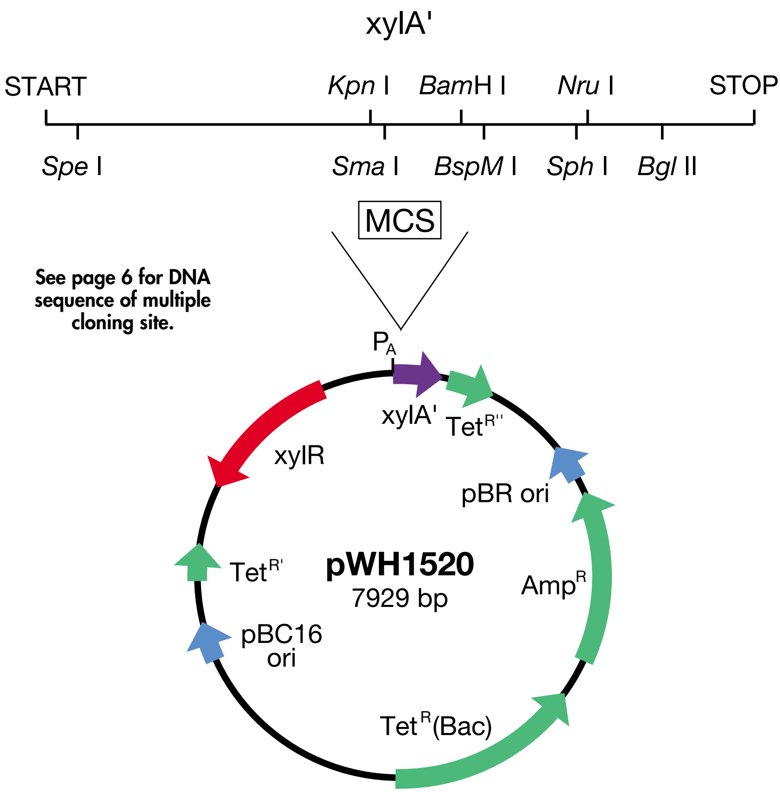

Fig. 1
Map of pWH1520. *Shuttle vector for* E. coli/B. megaterium. *Tet*^{R} *(Bac), tetracycline resistance* Bacillus; *Tet*$^{R'}$, *Tet*$^{R''}$, *tetracycline resistance, interrupted; Amp*^{R}, *ampicillin resistance; XylR, xylose-dependent repressor; XylA', xylose isomerase, gene incomplete;* P_A, *xylA promoter; MCS, multiple cloning site; pBC16 ori,* Bacillus *origin of replication; pBR, ColE1 origin of replication. The complete DNA sequence is available upon request.*

The complete DNA sequence is available for download on our internet web page http://www.mobitec.de.

```
START XylA
bp#9          Spe I
ATG GTC CAA ACT AGT ACT AAT AAA ATT AAT CAT TTT GAA AGC GCA AAC AAA GTT TTA TAC
Met val gln thr ser thr asn lys ile asn his phe glu ser ala asn lys val leu tyr

GAA GGT AAA GAT TCT AAA AAT CCT TTA GCT TTT AAA TAC TAT AAC CCT GAA GAA GTA GTA
glu gly lys asp ser lys asn pro leu ala phe lys tyr tyr asn pro glu glu val val

GGC GGT AAA ACG ATG AAA GAT CAG CTG CGT TTT TCT GTT GCT TAC TGG CAC CAG TTT ACA
gly gly lys thr met lys asp gln leu arg phe ser val ala tyr trp his gln phe thr

                                              Sma I
                                        Kpn I       BamH I                BspM I
GCA GAT GGT ACG GAT CAA TTC GAG CTC GGT ACC CGG GGA TCC TCT AGA GTC GAC CTG CAG
ala asp gly thr asp gln phe glu leu gly thr arg gly ser ser arg val asp leu gln

 Sph I          Nru I         Bgl II
GCA TGC AAG CTT TCG CGA GCT CGA GAT CTA GAT ATC GAT GAA TTG ATC CGA CGC GAG GCT
ala cys lys leu ser arg ala arg asp leu asp ile asp glu leu ile arg arg glu ala

GGA TGG CCT TCC CCA TTA TGA TTC TTC TCG CTT CCG GCG GCA
gly trp pro ser pro leu  *  phe phe ser leu pro ala ala
                        STOP                          bp#350
```

Fig. 2 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).

3. Application Examples

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

- the catabolite control protein ccpA -Ref. 2-
- xylose repressor (XylR) -Ref. 1-
- trehalose repressor (TreR) -Ref. 1-
- heat stable protein (HPr) from PTS (phosphotransferase sugar transport system)
- mutarotase (Mro) -Ref. 1-
- glucose dehydrogenase (Gdh) -Ref. 1-
- β-galactosidase
- human single-chain urokinase-like plasminogen activator (rscuPA)
- cellulase

Protein yield:

Protein yields vary depending on the protein expressed. Rygus and 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.

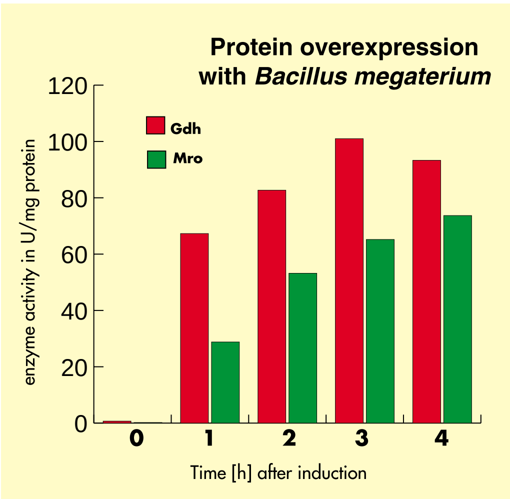

Fig. 2
Time dependence of induced expression of the enzymes Gdh (glucose dehydrogenase) and Mro (mutarotase) in Bacillus megaterium. *Enzymatic activity given in U/mg protein.*

4. Using pWH1520 for heterologous gene expression in *Bacillus megaterium*

Plasmid pWH1520 contains the strong *xylA* promoter originating from *Bacillus megaterium*. Transcription from this promoter is xylose inducible. The most convenient cloning sites for insertion of DNA fragments carrying heterologous genes are located in the reading frame of *xylA* (see sequence). 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 *xylA*. Whether the resulting transcriptional fusion leads to expression of the gene of interest, which is independent from *xylA* expression, depends on the location of the created *xylA* stop codon with respect to the start codon of the gene of interest. If these are close together, translational coupling may occur, in which the ribosomes translating the *xylA* reading frame would terminate at its stop codon, creating a locally high concentration of ribosomes, so that translation initiation at the new start codon would be more efficient compared to a construct in which the *xylA* translation terminates farther away from the start codon.

On the other hand, if the *xylA* reading frame continues for a long distance into the reading frame of the gene of interest, the ribosomes translating the created *xylA* 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 *xylA* reading frame, and any fusion thereof 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 *xylA* reading frame on pWH1520 to create a translational or protein fusion. This will result in expression of a chimeric protein consisting of up to 90 amino acids specified by the *xylA* encoding sequence, followed by the sequence encoded by the gene of interest.

5. Protocols

5.1. Cloning the DNA fragment of interest

The *E. coli/B. megaterium* shuttle vector pWH1520 is supplied as lyophilized DNA (5 µg). 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 into pWH1520, the vector 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
- pWH1520 and derivatives can be selected for by adding 5 to 10 mg/l tetracycline to the growth medium
- protoplast transformation and conditions for overproduction are described separately

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 well with *Bacillus megaterium.*

5.3. Transformation of *B. megaterium* protoplasts

For protein expression the vector pWH1520 with the insert coding for the protein of interest is transformed into *B. megaterium.*

B. megaterium cannot be naturally transformed. Below you find a standard protocol for protoplast transformation, which is a modification of the method from Puyet *et al.* (1987).

MoBiTec conveniently provides protoplasts of *B. megaterium*, which are ready for transformation. MoBiTec produces the 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: We have of course made this test for each protoplast lot during our quality control as well.

2. Positive control: protoplasts transformed with pWH1520 (without insert)

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

Essential buffers are listed in chapter 6.

Transformation:

- in one 12 ml tube for each transformation combine 500 µl of protoplast suspension and 0.5 to 1 µg of DNA (in SMMP, see chapter 6)
- add 1.5 ml of PEG-P, incubate 2 minutes at room temperature (RT)
- add 5 ml SMMP, mix by inverting the tube three times
- harvest cells by gentle centrifugation (in e.g. a Heraeus Biofuge/Minifuge at 3000 rpm for 10 minutes at RT), pour off supernatant immediately after centrifugation, do not check for a pellet - most of the time there will be none visible and the pellet may be fragile
- add 500 µl SMMP
- incubate at 37°C for 90 minutes with gentle shaking or rolling of tubes (max. 100 rpm)

- prepare 2.5 ml aliquots of CR5-top agar in sterile tubes in a waterbath (max. 43°C)
- 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
- incubate at 37°C over night - expect colonies of varying diameter 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)
- streak on fresh plates within two days

5.4. Protein production

The strong, tightly 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. The multiple cloning site downstream of the promoter allows versatile cloning of genes under its transcriptional control.

Relevant restriction sites are indicated in the vector map in Fig. 1, page 5.

<u>I. Test protein expression</u>

- 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
- withdraw sample as control before induction
- induce the xylose promoter by addition of 0.5 % xylose
- incubate at 37°C
- withdraw samples every 30 to 60 minutes until an OD_{600} of 1.5 is reached (i.e. the cells enter the stationary phase)
- centrifuge each sample to harvest cells
- resuspend cells in sonication buffer to a final concentration of 0.01 OD/ml
- sonicate 3 times in short bursts (20 seconds) at 50 W; allow sample to cool for 20 seconds between each burst
- centrifuge lysate to separate the insoluble fraction (pellet) from the soluble fraction (supernatant)

- dilute the insoluble fraction in sonication buffer to a final concentration of 0.02 OD/ml
- 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)
- determine enzymatic activities with the appropriate assays
- make Western blot, if appropriate antibodies are available

II. Scale up protein production

- grow larger culture and induce as indicated above
- harvest cells at point of maximal protein overproduction, as determined by the test experiment

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

Antibiotics

Ampicillin	100 µg/ml final concentration (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

ad 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)

<u>CR5 topagar for 500 ml: components a)-c)</u>

component a)

51.5 g	sucrose
3.25 g	MOPS
0.33 g	NaOH

ad 250 ml

adjust to pH 7.3 with NaOH and autoclave for 12 minutes

component b)

2 g	agar
0.1 g	casamino acids
5 g	yeast extract

ad 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 (*see next page)
25 ml	12 % proline (w/v; sterilize by filtration)
25 ml	20 % glucose (w/v; sterilize by filtration)

Aliquot in sterilized containers - contaminates easily.

B.meg. 3/2000

***CR5 salts 8x stock:**

1.25 g K_2SO4

50 g $MgCl_2$ x 6 H_2O

0.25 g KH_2PO4

11 g $CaCl_2$ x 2 H_2O

ad 625 ml H_2O

autoclave for 20 minutes

Adjust to 42°C - 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 g/l
MOPS	6.5 g/l
NaOH	0.66 g/l

adjust to pH 7.3 and

autoclave for 12 minutes

component b)

agar	4 g/l
casamino acids	0.2 g/l
yeast extract	10 g/l

autoclave for 20 minutes

component c)

K_2SO_4	0.25 g/l
$MgCl_2$ x 6 H_2O	10 g/l
glucose	10 g/l
proline	6 g/l
KH_2PO_4	0.05 g/l
$CaCl_2$	2.2 g/l

sterilize glucose and proline by

filtration; autoclave other

components for 20 minutes

7. References

1. *Rygus T. and Hillen, W., Inducible high-level expression of heterologous genes in* Bacillus megaterium *using the regulatory elements of the xylose-utilization operon, Appl. Microbiol. Biotechnol. (1991), 35, 594-599.*

2. *Rygus, T.* et al.*, Molecular cloning, structure, promoters and regulatory elements for transcription of* Bacillus megaterium *encoded regulon for xylose utilization. Arch. Microbiol. (1991) 155, 535-542.*

3. *Hueck, C.P.* et al.*, Cloning expression and functional analyses of the catabolite control protein ccpA from* Bacillus megaterium, *Mol. Microbiol. (1995) 16(5), 855-864.*

4. *Vary, P., Prime time for* Bacillus megaterium*, Microbiology (1994) 140, 1001-1013.*

5. *Sexana, A.* et al.*, Microorganisms capable of metabolizing the herbicide metolachlor. Appl. Environ. Microbiol. (1987) 53, 390-396.*

6. *Selvanayagam, M. and Vijaya, J., Degradation of persistent insecticides by aquatic bacteria as sole source of carbon. J. Environ. Biol. (1989) 10, 399-407.*

7. *Meinhardt, F.* et al.*, Highly efficient expression of homologous and heterologous genes in* Bacillus megaterium*. Appl. Microbiol. Biotechnol. (1989) 30, 343-350.*

8. *Puyet, A.* et al.*, A simple medium for rapid regeneration of* Bacillus subtilis *protoplasts transformed with plasmid DNA. FEMS Microbiol. Lett. (1987) 40, 1-5.*

9. *Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (1970) 227, 680-685*

<u>Potential industrial and diagnostical applications:</u>

10. *Vary, P. S. (1992) Development of genetic engineering in* Bacillus megaterium*: an example of the versatility and potential of industrially important bacilli, Biology of bacilli: Applications to Industry, pp.251-310. Ed. by Doi and McGloughlin, Butterworths-Heinemann.*

11. *Ginsburgh* et al.*, (1989) Sporulation promoter spoVG controlled expression of PP42 gene of HIV-1 in* Bacillus megaterium*, Abstr. International Conf. on AIDS, Montreal.*

<u>Further:</u>

12. *Sambrook, J.* et al.*, eds. Molecular Cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).*

8. Order Information

order #	description
BMEG01	<u>*Bacillus megaterium* kit</u> • 5 µg pWH1520; lyophilized DNA (*E. coli/B. megaterium* shuttle vector) • 5 x 500 µl *B. megaterium* protoplasts ready for transformation (strain WH320; Rygus, T. *et al.*,1991) Material is sufficient for 4 transformations plus control experiment. A detailed handbook is provided.
BMEG02	<u>Protoplasts only</u> 5 x 500 µl *B. megaterium* protoplasts ready for transformation (strain WH320; Rygus, T. *et al.*,1991) Material is sufficient for 4 transformations plus control experiment. A detailed handbook is provided.
BMEG03	<u>Vector only</u> • 5 µg pWH1520; lyophilized DNA (*E. coli/B. megaterium* shuttle vector) A detailed handbook is provided.

B.meg. 3/2000