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A sucrose-inducible promoter system for the intra- and extracellular protein production in *Bacillus megaterium*

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Abstract

A sucrose-inducible promoter system (P_{sacB}) from *Bacillus megaterium* was identified using a secretome approach. It was successfully employed for the extracellular production of the homologous levansucrase SacB (4252.4 U l^{-1}) and the heterologous green fluorescent protein GFP (7.9 mg g_{CDW}^{-1}). Mutational analysis of *B. megaterium* P_{sacB} allowed the identification of important promoter elements. The sucrose-inducible promoter provides a useful alternative to the established xylose-inducible promoter system (P_{xylA}) for recombinant gene expression in *B. megaterium*.

Keywords: *Bacillus megaterium*; Sucrose-inducible promoter; Recombinant protein production

Bacillus megaterium is used in industry and academia since many years for the production of several biotechnologically relevant vitamins and proteins. It is characterized by its large size (2.5 μm × 2.5 μm × 10 μm) which accounts for its 100-fold higher volume compared to the often employed production host *Escherichia coli* (Fig. 1). In contrast to Gram negative bacteria like *E. coli*, *B. megaterium* does not produce toxins associated with the outer membrane. The utilisation of a wide variety of carbon sources allows for its growth on low cost substances (Vary, 1994). In contrast to *Bacillus subtilis, B. megaterium* does not possess alkaline proteases and is known for the stable replication and maintenance of plasmids (Kim, 2003; Vary, 1992; von Tersch and Robbins, 1990). A further advantage is its ability to secrete proteins directly into the growth medium (Priest, 1977). Due to the Gram positive nature of the bacterium, an outer membrane hampering protein export is missing.

In 1991, Rygus and Hillen identified the xylose-inducible promoter P_{xylA} in the genome of *B. megaterium*. In the presence of xylose, the expression of the xylose isomerase gene *xylA* under control of P_{xylA} was found to be 200-fold induced (Rygus et al., 1991). Using this promoter system localized on free replicating plasmids for the overexpression of recombinant genes in *B. megaterium* yielded an induction of up to 350-fold while adding xylose to the growth medium. This system was successfully used for the intra- and extracellular production of various heterologous prokaryotic and eukaryotic proteins (Biedendieck et al., 2007a; Biedendieck et al., 2007b; Jordan et al., 2007; Malten et al., 2005; Malten et al., 2006; Rygus and Hillen, 1991; Yang et al., 2006).

1. Identification of the sucrose-inducible promoter of *B. megaterium* via secretome analysis

For strong recombinant gene expression in the host organism *E. coli* various different well characterized promoter systems like the P_{lac}, the P_{tetA}, the P_{ara}, or the T7 phage RNA polymerase promoter are available (reviewed in Terpe, 2006). For a broader application of the bacterial host *B. megaterium*, alternative promoter systems in addition to the established P_{xylA} are demanded. The availability of essential parts of the *B. megaterium* strain DSM319 genome sequence significantly

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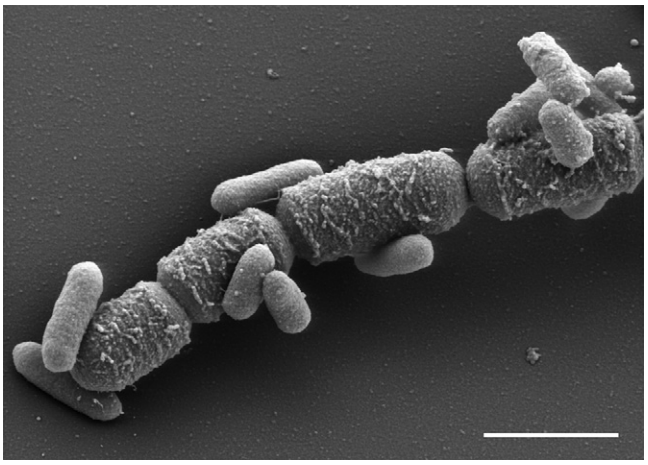

Fig. 1. Electron microscope image of *Bacillus megaterium* and *Escherichia coli* vegetative cells. *B. megaterium* and *E. coli* cells were aerobically cultivated separately in LB medium at 37 °C. They were grown until reaching the stationary phase. The two cultures were mixed in the ration of 1:1. *B. megaterium* cells grow up to a volume of more than 60 μm^3 ($2.5 \times 2.5 \times 10$). Compared to the *E. coli* volume of 0.5 μm^3 ($0.5 \times 0.5 \times 2$), *B. megaterium* has at least an up to 100-fold higher volume. Aldehyde-fixed bacteria were dehydrated with a graded series of acetone, critical-point-dried with liquid CO_2, and sputter-coated with gold. Samples were examined in a field emission scanning electron microscope (FESEM) Zeiss DSM982 Gemini at an acceleration voltage of 5 kV using the Everhart-Thronley SE-detector and the SE-Inlens-detector in a 50:50 ratio. Magnification ×15,000; white bar: 2 μm.

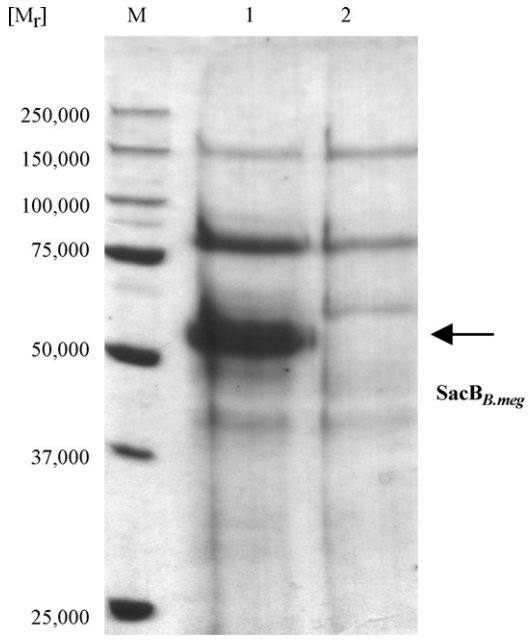

Fig. 2. Sucrose-dependent secretome of *B. megaterium*. *B. megaterium* DSM319 was cultivated aerobically in 100 ml of Luria–Bertani (LB) medium at 37 °C with (lane 1) or without (lane 2) the addition of 5 g l^{-1} sucrose (Sambrook and Russell, 2001). Six hours after cultivation start, 1.5 ml of cell-free growth medium were desalted using a PD-10 column (GE Healthcare; Uppsala; Sweden) and subsequently lyophilized. The precipitated proteins were analyzed via 10% SDS–PAGE and stained with Coomassie Brilliant Blue G250. Lane M shows Precision Plus Protein Standard (Bio-Rad; Munich; Germany).

simplified this task (Sun et al., 2006). In the presence of 5 g l^{-1} sucrose in Luria–Bertani (LB) medium, *B. megaterium* strongly secreted a protein with a molecular weight of 52,000 which was found absent in the absence of sucrose (Sambrook and Russell, 2001) (Fig. 2). The secreted protein was excised from an SDS–PAGE gel and identified via matrix assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF/MS) as a levansucrase with over 70% identity at the amino acid sequence level to levansucrases SacB from *B. subtilis* and *Bacillus amyloliquefaciens*. Therefore, the *B. megaterium* protein was also named levansucrase SacB. Interestingly, strong SacB secretion significantly reduced the growth of *B. megaterium*. Only half of the cell densities were achieved compared to the cultivation without 5 g l^{-1} sucrose (data not shown). This behaviour is common for bacterial recombinant strains producing and secreting high amounts of proteins (Glick, 1995). The addition of less than 5 g l^{-1} sucrose resulted in higher final cell densities but significantly reduced SacB secretion. Sucrose concentration above 5 g l^{-1} did not show an effect on growth behaviour and SacB export (data not shown).

2. P_{sacB}-dependent SacB production and export using a free replicating plasmid

The promoter region P_{sacB} followed by the *sacB* gene was cloned into *B. megaterium* vector pMM1522 (Malten et al., 2006). The corresponding 1,452 bp DNA fragment for *B. megaterium sacB* gene and 630 bp of its upstream region were amplified from genomic *B. megaterium* DNA using PCR and the primers PsacB_for630 (TATCA*CTTAAG*CTGATTCCAGCCG TGAAGG) and PRBEc1_rev (TATCA*GGATCC*GCTATTG CAAAGCGCTCAGTC) (restriction sites are shown in *italic* letters.). The 2,082 bp PCR fragment was digested with *Aat*II and *Bam*HI and cloned into similarly cut pMM1522. Protoplasted *B. megaterium* strain MS941 was transformed with this novel vector named pRBBm69 (encoding P_{sacB}-SacB) as outlined before (Barg et al., 2005; Wittchen and Meinhardt, 1995). Strain MS941 carrying the target geneless vector pMM1522 served as control. The efficiency of sucrose-inducible P_{sacB}-dependent multi copy *sacB* expression was compared with single copy chromosomal *sacB* expression. After adding 5 g l^{-1} sucrose to the LB growth medium, the enzymatic activity of levansucrase in the growth medium was determined using the dinitrosalicylic acid (D.N.S.) activity test (pH 5.4; $T = 37$ °C) as outlined before (Biedendieck et al., 2007b). Within the first 3 h, the volumetric activity of levansucrase strongly increased 373-fold from 2.4 to its maximal value of 896.1 U l^{-1} for *B. megaterium* carrying P_{sacB}-*sacB* as single copy in the genome (Fig. 3A). Due to the reduced growth in the presence of sucrose, the activity concerning the cell dry weight (CDW) increased 478-fold (0.53 and 259.7 U $g_{CDW}{}^{-1}$, respectively). An increase to 4252.4 U l^{-1} (4169 U $g_{CDW}{}^{-1}$) was observed for *B. megaterium* carrying the vector pRBBm69 (encoding P_{sacB}-SacB) encoded multi copy version of *sacB* (Fig. 3A). The induction of multi copy plasmid vector encoded P_{sacB}-SacB by sucrose addition was less intensive due to the already present SacB activity in the not induced state (77.6 U l^{-1} corresponding to 4169 U $g_{CDW}{}^{-1}$). To avoid the latter undesirable effect, the repression of promoter