# *Bacillus megaterium* – from simple soil bacterium to industrial protein production host

Patricia S. Vary[1], Rebekka Biedendieck[2], Tobias Fuerch[3], Friedhelm Meinhardt[4], Manfred Rohde[5], Wolf – Dieter Deckwer[3], Dieter Jahn[2]*

[1]Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA; [2]Institute of Microbiology, Technical University Braunschweig, Spielmannstrasse 7, 38106 Braunschweig, Germany; [3]Institute of Biochemical Engineering (TU-BCE), Technical University Braunschweig/Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany; [4]Institute for Molecular Microbiology and Biotechnology, Westfaelische Wilhelms-University Muenster, Corrensstrasse 3, 48149 Muenster, Germany; [5]Department of Microbial Pathogenesis, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany.

*Corresponding author. Tel.: +49 (0) 531-391-5801; fax: +49 (0) 531-391-5854

*E-mail address*: d.jahn@tu-bs.de (D. Jahn)

URL: http://www.tu-braunschweig.de/ifm

**Abstract**

*Bacillus megaterium* has been industrially employed for over 50 years since it possesses some very useful and unusual enzymes, and a high capacity for the production of exoenzymes. It is also a desirable cloning host for the production of intact proteins since it does not possess external alkaline proteases and can stably maintain a variety of plasmid vectors. Genetic tools for this species include transducing phages and several hundred mutants covering the processes of biosynthesis, catabolism, division, sporulation, germination, antibiotic resistance, and recombination. The seven plasmids of *B. megaterium* strain QM B1551 contain several unusual metabolic genes that may be useful in bioremediation. Recently, several recombinant shuttle vectors carrying different strong inducible promoters and various combinations of affinity tags for simple protein purification have been constructed. Leader sequences mediated export of affinity tagged proteins into the growth medium was made possible. These plasmids are commercially available. For a broader application of *B. megaterium* in industry, sporulation and protease deficient as well as UV-sensitive mutants were constructed. The genome sequence of two different strains, plasmidless DSM319 and QM B1551 carrying seven natural plasmids, is now available. These sequences allow for a systems biotechnology optimization of the production host *B. megaterium*.

Altogether, a “toolbox” of hundreds of genetically characterized strains, genetic methods, vectors, hosts, and genomic sequences make *B. megaterium* an ideal organism for industrial, environmental, and experimental applications.

**Keywords**

*Bacillus megaterium*, high level production, biosafety, metabolic flux analysis, genome sequencing

## Introduction

In 1884, De Bary named *B. megaterium* “big beast” because of its large size with a volume approximately 100 times that of *Escherichia coli* (**Fig. 1**) (De Bary 1884). It has been ideal for studies of cell structure, protein localization, sporulation, and membranes (Hrafnsdottir et al. 1997; McCool and Cannon 2001). Lysogeny was discovered by Lwoff and Gutman using strain 899 and phage T (Lwoff and Gutmann 1950).

*B. megaterium* is a Gram-positive, mainly aerobic spore-forming bacterium found in widely diverse habitats from soil to seawater, sediment, rice paddies, honey, fish, and dried food. It can grow in simple media on over 62 carbon sources out of 95 tested including all tricarboxylic acid cycle intermediates, formate, and acetate. This has made it an ideal industrial organism for over 50 years. Among its products are proteins like penicillin acylase (Martin et al. 1995; Suga et al. 1990), used to make synthetic penicillins, various amylases (Hebeda et al. 1988; Metz et al. 1988; Takasaki 1989; Vihinen and Mantsala 1989) which are of interest in starch modification in the baking industry, glucose dehydrogenase (Kittsteiner-Eberle et al. 1989; Nagao et al. 1992), used for regeneration of the cofactor NADPH in biochemical reactions and glucose blood tests (**Tab. 1**). Further, it is used for the production of pyruvate, vitamin $B_{12}$ (Raux et al. 1998; Wolf and Brey 1986), fungicidal toxins, and oxetanocin, a viral inhibitor active on HIV (Human immunodeficiency virus), hepatitis B virus, and herpes simplex corneal ulcers (Morita et al. 1999; Shimada et al. 1986; Shiota et al. 1996; Tseng et al. 1991). Many of its industrial applications have been reviewed in detail (Vary 1992; Vary 1994). Some of the most interesting proteins produced by *B. megaterium* are the family of P-450 cytochrome monooxygenases (He and Fulco 1991). These have been of great interest since they have considerable similarity to eukaryotic P-450 important in many disease conditions.

**Genetics of *Bacillus megaterium***

The *B. megaterium* strain QM B1551 was used for the isolation of the transducing phage MP13 (Vary 1979; Vary et al. 1982). In this process, 41 further bacteriophages were characterized that are specific for this strain (Vary and Halsey 1980). Over the next several years, hundreds of mutants were isolated and characterized for auxotrophy, recombination, division, sporulation, germination, antibiotic resistance, UV sensitivity, and neutral protease (Vary 1994). Surprisingly, the *Bacillus subtilis* map did not serve well in transductional mapping. However, the construction of a rough map covering approximately 80 % of the chromosome by the early 80's was managed (Vary 1993; Vary and Muse 1993). Protoplast transformation conditions were established in the laboratories of Carlton and Alfoldi (Brown and Carlton 1980; Vorobjeva et al. 1980). Although protoplast transformation requires experimental experience and is less effective compared to standard *E. coli* transformation procedures, the ability to transform *B. megaterium* immediately led to the cloning of several genes, the study of its plasmids, and its use as a very efficient host for gene expression. The transposon Tn*917* was introduced into strain QM B1551 and showed that it inserted randomly (Bohall and Vary 1986). The transposons constructions allowing *lacZ* fusion genetics in *B. subtilis* (Camilli et al. 1990; Youngman et al. 1989) also work well in *B. megaterium*.

**Natural Plasmids of *Bacillus megaterium***

Most strains of *B. megaterium* carry multiple plasmids, usually more than four. However, the well characterized strain QM B1551 has seven resident plasmids ranging from a size of 5.4 to 165 kb representing over 11 % of cellular DNA (Kieselburg et al. 1984). The two smallest ones replicate by the rolling circle mechanism whereas the other five are theta replication plasmids (Stevenson et al. 1998). *B. megaterium* strain 216 studied by Carlton (Carlton and Helinski 1969) has ten plasmids. QM B1551 was randomly cured of plasmids so that the role of plasmid genes could be determined.

Very few phenotypic differences were determined for the various cured strains. However, one originated strain, PV361, was cured of all seven plasmids (Sussman et al. 1988). Surprisingly, its growth under laboratory conditions was close to wild type QM B1551. The strain has proven to be a good host for gene expression. It has been used for many years to produce the antigen for HIV diagnostic kits (Ginsburgh et al. 1989).

To get an idea about the additional genetical material and its relevance for the organism, the DNA sequence of the plasmids of QM B1551 has been determined over the last few years. The plasmids are carrying genes encoding enzymes like dehydrogenases, amylases, a P-450 cytochrome, monooxygenases, carboxylases, a styrene monoxygenase, biotin carboxylases, permeases, transposases, and integrases. Furthermore, genes encoding proteins for heavy metal export, many possible transport proteins, acyl carriers, sigma factors, and sterols were found. Redox, mobilization, and transfer genes, as well as sporulation and germination genes were identified (Kunnimalaiyaan et al. 2001; Kunnimalaiyaan and Vary 2002; Scholle et al. 2003). Moreover, there are genes very similar to some found on the virulence plasmids of *Bacillus anthracis* and at least one complete rRNA operon with 18 tRNA genes (Kunnimalaiyaan et al. 2001). Interestingly, the five theta plasmids have unusually similar Rep proteins (82 – 95 % identity at the amino acid sequence level), as do the iterons they must bind to. Normally, such identity precludes compatibility. The replicons are found in several strains of *B. megaterium*, but not in other *Bacillus* species tested (Rosso and Vary 2005). Moreover, no proteins in the databases are similar to the proteins encoded in the replicon region. Hence, the theta replicons appear to be unique. The two rolling circle plasmids (5.4 and 9.1 kb) are not similar to each other, but their replicons show similarities to sequences of plasmids known from *Bacillus thuringiensis* and *B. anthracis* (Scholle et al. 2003). In QM B1551, the plasmids are very stable and several have been followed through 100 generations with no selection or loss.

Overall, the genes found on the plasmids suggest that much exchange has been made with other Gram-positive bacteria. Many of the genes show no phenotype on laboratory media after their knock-out. Hence, they must be necessary for the success of *B. megaterium* in diverse habitats. Further, many of the genes are of unknown function, or they can only be assigned as "an ABC transporter" or a "kinase", but cannot be assigned to a specific metabolic pathway. Encoded proteins may be candidates for unusual reactions and pathways yet to be discovered.

### *Bacillus megaterium* as Expression Host for Recombinant Genes

*B. megaterium* does not have alkaline proteases which advanced the recovery of recombinant proteins and has the ability to stably maintain plasmids. Hence, the plasmidless derivatives of *B. megaterium*, specifically PV361 (Sussman et al. 1988) and DSM319 (Stahl and Esser 1983), have proven to be excellent production hosts for intact foreign proteins.

### *Bacillus megaterium Vector Systems for Recombinant Protein Production*

Rygus and Hillen (1991) isolated the xylose-inducible promoter $P_{xylA}$ with the gene for the regulatory repressor protein XylR from the genome of *B. megaterium*. In the absence of xylose, XylR binds to an operator sequence located in $P_{xylA}$ preventing transcription of the *xyl* operon (Gärtner et al. 1988). In the presence of xylose in the cell, the sugar is bound to the repressor protein introducing a conformational change to the protein which leads to a release of XylR from the promoter. Now, the RNA polymerase efficiently recognizes the promoter and gene expression is induced approximately 350-fold. An additional level of regulation of the original *xyl* operon is mediated by glucose via the catabolite regulatory protein CcpA (Gärtner et al. 1988). Based on this promoter, Rygus and Hillen (1991) developed a free replicating plasmid-borne system for the xylose-inducible overproduction of recombinant proteins. The designed vector, called pWH1520,

was successfully employed for the recombinant intracellular production of prokaryotic and eukaryotic proteins including *E. coli* β-galactosidase, *B. megaterium* glucose dehydrogenase, *Acinetobacter calcoaceticus* mutarotase, human urokinase-like plasminogen activator (Rygus and Hillen 1991), and *Clostridium difficile* toxin A with a high relative molecular mass of 308,000 (Burger et al. 2003).

In further studies, the promoter region of pWH1520 was modified and optimized for recombinant gene expression (Malten et al. 2005a). The resulting vector pMM1520 allows for the simple cloning of target genes by the use of 15 different DNA restriction enzyme cleavage sites located in a newly designed multiple cloning site (**Fig. 2A**). Furthermore, inactivation of the binding site for the catabolite regulatory protein CcpA made promoter utilization independent of the presence of glucose in the growth medium. To further optimize the overall gene expression system, a derivative of the plasmidless *B. megaterium* strain DSM319, named WH323 (**Tab. 3**), was constructed (Rygus and Hillen 1992). This strain is deficient in the xylose isomerase gene *xylA* and consequently unable to consume the inducer xylose as carbon source.

**Plasmids for the Production and Purification of Intracellular Recombinant Proteins**

Based on this xylose-inducible protein production system, novel expression vectors for the intracellular production of recombinant affinity tagged proteins were developed (**Fig. 2B**) (Biedendieck et al. 2007b). Using these vectors, proteins of interest N- or C-terminally fused to a $His_6$- or Strep II-tag can be produced (Lichty et al. 2005). Testing this newly developed plasmid system with the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Tsien 1998) as a model protein yielded up to 17.9 mg of soluble recombinant GFP per liter shaking flask cell culture (**Tab. 2**). A one-column purification step resulted in up to 9 mg purified GFP per liter cell culture for the differentially tagged GFP forms (**Tab. 2**). The vectors encode protease cleavage sites for

Factor Xa (Jenny et al. 2003) or the tobacco etch virus (TEV) protease (Kapust et al. 2002) which allow for the removal of the affinity tags. Hence, untagged pure GFP was obtained after protease digestion and a single second purification step in which the cleaved tag and the protease were removed (**Fig. 3**) (Biedendieck et al. 2007b).

In high cell density cultivations of *B. megaterium* without recombinant protein production, final biomass concentrations of up to 80 g per liter were achieved (Hollmann and Deckwer 2004; Malten et al. 2005a). For up-scaling the recombinant production process, GFP production in *B. megaterium* was analyzed after a glucose limited fed batch cultivation (Biedendieck et al. 2007b). This process yielded 52 g cell dry weight (CDW) per liter and up to 274 mg GFP per liter culture corresponding to 5.2 mg GFP per g CDW. Flow cytometric analysis allowed for the detection of the recombinantly produced GFP at the single cell level during the fermentation process indicating that the recombinant protein production hardly influenced the viability of the cell population (Biedendieck et al. 2007b). The xylose-inducible vector system of *B. megaterium* was also successfully used for the high yield biotransformation of fructose into D-mannitol. For this purpose, *Leuconostoc pseudomesenteroides* ATCC12291 mannitol dehydrogenase, converting D-fructose into D-mannitol, and *Mycobacterium vaccae* N10 formate dehydrogenase, which is necessary for regeneration of NADH reduction equivalents, were recombinantly synthesized and the resting recombinant *B. megaterium* strain produced large amounts of mannitol (personal communication). Here, the capability of *B. megaterium* to tolerate high amounts of formate was utilized (personal communication). Interestingly, high amounts of formate dehydrogenase were only obtained after complete codon adaptation to the *B. megaterium* codon usage via the use of a completely synthetic gene.

**Plasmids for the Production and Purification of Extracellular Recombinant Proteins**

In order to maximize the capacity of *B. megaterium* for the secretion of exoenzymes in high amounts into the environment, a series of vectors employing the signal peptides (SP) of the *B. megaterium* extracellular esterase LipA (Malten et al. 2006) and penicillin G acylase (PGA) (Biedendieck et al. 2007a) was developed for the recombinant production of extracellular proteins. Levansucrase from *Lactobacillus reuteri* strain 121 (van Hijum et al. 2001; van Hijum et al. 2004) was secreted best using the leader peptide of *B. megaterium* LipA (Biedendieck et al. 2007a). More than 4 mg enzyme per liter culture of this large enzyme ($M_r$ = 110,000) were found as the predominant protein in the growth medium of *B. megaterium* in shaking flask cultivations (Malten et al. 2006). Homologous penicillin G acylase (PGA) from *B. megaterium* strain ATCC14945 was secreted up to 40 mg per liter shake flask culture (Yang et al. 2006). A hydrolase of *Thermobifida fusca* (TFH) (Kleeberg et al. 2005), which possesses unique hydrolytic properties as it can act as esterase, lipase, cutinase, and is also able to cleave polyesters, yielded up to 2.6 mg per liter culture (Yang et al. 2007). Both proteins were directed by the signal peptide of LipA into the growth medium. An upscale of the recombinant PGA secretion process to a 2 liter bioreactor batch cultivation resulted in 28.5 mg recombinant PGA per liter (Yang et al. 2006) and 16.1 mg recombinant TFH per liter (Yang et al. 2007), respectively. The secretion vector system was extended by the addition of DNA sequences encoding $His_6$- and StrepII-tags (Lichty et al. 2005) (**Fig. 3C**). However, the stepwise introduction of affinity tags decreased the amount of produced and exported recombinant protein (Malten et al. 2006; Ruiz et al. 2002). A one-step affinity chromatography purification of $His_6$-tagged TFH from the growth medium resulted in 8.3 mg protein per liter (Yang et al. 2007).

Loss of secreted enzymes due to proteolytic degradation was negligible since the organism displays low extracellular proteolytic activities (Millet et al. 1969). However, in order to further improve the

stability of secreted proteins the *nprM* gene encoding the major protease (neutral metalloprotease) was isolated, characterized, and completely deleted (Meinhardt et al. 1994; Wittchen and Meinhardt 1995). The resulting *B. megaterium* strain MS941 (**Tab. 3**) proved to be useful for efficient secretion of heterologous proteins like a dextransucrase (DsrS) from *Leuconostoc mesenteroides*, a *L. reuteri* levansucrase (Lev), the *B. megaterium* penicillin G amidase (PGA), and a *T. fusca* hydrolase (TFH) (Biedendieck et al. 2007a; Malten et al. 2006; Malten et al. 2005a; Yang et al. 2006; Yang et al. 2007). Concomitant expression of the gene encoding the major signal-peptidase SipM of *B. megaterium* strain DSM319 additionally enhanced recombinant protein secretion (Biedendieck et al. 2007a; Malten et al. 2005b; Nahrstedt et al. 2004).

**Strain development in *Bacillus megaterium* with respect to enzyme production and biosafety**

For cloning purposes, a number of strains based on *B. megaterium* strain DSM319 with deletions in several genes were constructed (**Tab. 3**). Among these are strains lacking β-galactosidase (Rygus et al. 1991; Strey et al. 1999) and amylase activity (Lee et al. 2001). For industrial applications, the availability of strains being genetically debilitated when released into the environment is desirable. Different genetic approaches to obtain biological containment were followed, eventually giving rise to mutants which have lost their ability to form viable endospores (Wittchen et al. 1998). An alternative approach for obtaining biologically contained strains aimed at generating strains with a reduced capacity to repair DNA damage. Although non-sporulating, auxotrophic, and protease deficient mutants could easily be developed, the creation of a mutant lacking homologous recombination (*recA*-mutants), which renders the bacterium UV-sensitive, failed (Nahrstedt et al. 2005). Uniquely, *B. megaterium* was found to possess two *recA*-genes, *recA1* and *recA2*, one of which even turned out to be essential for survival (Nahrstedt et al. 2005). The *recA1* gene was knocked out (strain MS991; **Fig. 4**), but all attempts failed to destroy the *recA2* gene. Since

homologous recombination is also quite often required as a tool for strain development, an alternative strategy was followed. Instead of the inactivation of the homologous recombination system, the genes *uvrA* and *uvrB* encoding the nucleotide excision repair were directly knocked out (strains MS011, MS022, and MS033; **Fig. 4**) (Nahrstedt and Meinhardt 2004).

**Dynamic Studies of Recombinant Protein Production in *Bacillus megaterium* Using Metabolic Flux Analysis**

In the field of "White Biotechnology" knowledge about microbial metabolism is of remarkable importance. "White Biotechnology" stands for the biotechnological production of chemical bulk products and fine chemicals. Rational process development and optimization are strongly dependent on accurate data on the regulation and the dynamic processes involved. Thus, in the last ten years metabolic flux analysis has been developed as a major tool in the field of metabolic engineering (Sauer 2004; Stephanopoulos 1999; Wiechert 2002). In the meantime, the metabolism of various organisms has been characterized which finally led to optimized production strains (Christiansen et al. 2002; Dauner et al. 2001; Gombert et al. 2001; Kiefer et al. 2004). Consequently, dynamic studies of the central and amino acid metabolism of *B. megaterium* will facilitate the process of recombinant protein production (**Fig. 5**). Undesired limitations of the *Bacillus* system are recognized and subsequently targeted by the application of genetic engineering. Carbon labelling experiments using stable isotopes ($^{13}C$) for metabolic flux analysis in *B. megaterium* were carried out in continuous cultures. The experimental set up is shown in **figure 6**. When metabolic steady-state conditions were achieved, the feed medium, containing naturally labelled glucose as carbon source, was changed to an identical medium comprising a mixture of naturally and $^{13}C$-enriched glucose (Fürch et al. 2006; Fürch et al. 2007).

In initial experiments, the metabolic fluxes of carbon limited growth at a dilution rate (D) of 0.11 $h^{-1}$ was compared to not limited growth of *B. megaterium* MS941 at a D of 0.426 $h^{-1}$. Faster growing *B. megaterium* revealed a remarkable reversible activity of the enzymes in the pentose phosphate pathway, namely transaldolase and -ketolase. At the interface of glycolysis and tricarboxylic acid cycle, a significant contribution of an anaplerotic reaction catalyzed by pyruvate carboxylase leading to an increased oxaloacetate pool was determined. In connection with the detected activity of the malic enzyme, the presence of a so called futile cycle around the pyruvate node with a reaction series of pyruvate, oxaloactetate, malate, and pyruvate can be assumed. Interestingly, the gluconeogenic reaction catalyzed by phosphoenolpyruvate carboxykinase was proven not to be active in all cases tested (Fürch et al. 2006; Fürch et al. 2007).

The analysis of the transients during the various carbon labelling experiment revealed new information about the dynamic behaviour of the *B. megaterium* central metabolism. Although only a few fluxes of the central carbon metabolism and of the amino acid biosynthesis were determined, the identified time constants for the single amino acids revealed significant differences (Fürch et al. 2006). This time constant describes the average cellular existence of an amino acid at a certain growth rate. For instance, the amino acids aspartate (6.4 h) and glutamate (4.5 h) exhibited relatively small time constants, whereas the time constants of the aromatic amino acids, *e.g.* phenylalanine (11.4 h) and tyrosine (12.3 h), were found remarkably higher. The acidic amino acids are derived from the tricarboxylic acid cycle. Therefore, it was assumed that due to the cyclic character of the pathway stationary conditions were achieved noticeably faster than for intermediates derived from glycolysis. They contribute to the biosynthesis of the aromatic amino acids.

Next, *B. megaterium* recombinantly producing and exporting a *T. fusca* hydrolase (TFH) was investigated. Continuous cultivations were carried out in a chemostat using either glucose or

pyruvate as sole carbon source. A remarkable increase of produced TFH was detected for the pyruvate-dependent cultivation compared to glucose-dependent growth. Estimation of intracellular carbon fluxes through the central metabolism for both growth conditions using $^{13}C$-labelled substrates revealed noticeable changes of the fluxes through the tricarboxylic acid cycle, the pentose phosphate pathway, and around the pyruvate node when protein production was induced. With pyruvate as sole carbon source, the observed alterations of the fluxes yielded an increased production of ATP and NADPH, both required for the anabolism. Additionally, the analysis of the corresponding secretome revealed significantly reduced amounts of extracellular proteases in the medium compared to glucose-limited cultivations. Thus, pyruvate-dependent chemostat cultivation was identified as a favourable condition for recombinant protein production and secretion using *B. megaterium* as production host (personal communication).

**From Genome Sequencing to Systems Biotechnology**

At present, the genome of two *B. megaterium* strains, QM B1551 and DSM319, have been sequenced and are now in closure and annotation (Sun et al. 2006). The laboratory of Patricia S. Vary (Northern Illinois University, De Kalb, IL, USA) with The Institute for Genomic Research (TIGR, Rockville, MD, USA) and the laboratory of Dieter Jahn (Technical University Braunschweig, Germany) with the company GATC (Konstanz, Germany) are collaborating to complete both sequences.

Knowledge of the sequence will enhance the usefulness of this efficient expression host. Further, total DNA of strain QM B1551 is sequenced so that the sequence of all seven plasmids is available now. This will be one of the first multiple plasmid arrays within one cell to be completely sequenced, a “plasmid genome”. Our knowledge of especially the large Gram-positive plasmids should be greatly expanded.

Finally, the completed genomes give access to whole genome DNA arrays and high through-put proteomics. In combination with the determination of hundreds of metabolites in a metabolomics approach, all necessary information for an integrated systems biotechnology approach are now at hand. Using the integrative database/bioinformatics tool platform "Megabac" (www.prodoric.de/megabac), the generation of first models for the cellular processes in *B. megaterium* during recombinant protein production will be next (Bunk et al. 2006; Choi et al. 2007; Grote et al. 2005; Hiller et al. 2006; Hiller et al. 2004; Hiller et al. 2003; Munch et al. 2003; Scheer et al. 2006; Schomburg et al. 2002).

## Conclusion

The versatility of *B. megaterium* spans its habitats, its unusual enzymes, its industrial record, its secretion capacity, its cloning host capabilities, and its many plasmids. Now, we are on our way to a systems biotechnology approach to further systematically enhance biotechnological potential of this interesting bacterium. *B. megaterium* is an organism rich in potential and usefulness in the years to come. In 1994, Patricia S. Vary wrote (Vary 1994) "Prime time for *Bacillus megaterium*" – perhaps a little naïve, a little soon – but now it is becoming true.

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**TABLES**

**Table 1** Industrial products of *B. megaterium* and their applications (modified after Vary, 1994 (Vary 1994))

| **Product/Use** | **Comments** | **References** |
|---|---|---|
| α-amylases | Can replace pullulanases | (Takasaki 1989; Vihinen and Mantsala 1989) |
| β-amylases | Bread industry | (Hebeda et al. 1988; Metz et al. 1988) |
| Chitosanasen | Yeast cell wall analysis | (Pelletier and Sygusch 1990) |
| Glucose dehydrogenase | Generator of NADH, immobilization, biosensors | (Kittsteiner-Eberle et al. 1989; Nagao et al. 1992) |
| Neutral protease | Leather industry | (Kühn and Fortnagel 1993; Meinhardt et al. 1994; Millet et al. 1969) |
| Oxetanocin production | Inhibits HIV, hepatitis B virus, cytomegalovirus, herpes virus | (Kohlbrenner et al. 1990; Morita et al. 1999; Shimada et al. 1986; Shiota et al. 1996; Tseng et al. 1991) |
| Penicillin amidase | Construction of synthetic penicillins | (Martin et al. 1995; Suga et al. 1990) |
| Toxic waste cleanup | Herbicides, C-P bond lysis | (Quinn et al. 1989) |
| Vitamin $B_{12}$ production | Aerobic and anaerobic Vitamin $B_{12}$ producer | (Raux et al. 1998; Wolf and Brey 1986) |

**Table 2** Production and purification of GFP. Different fusion forms of GFP were produced in *B. megaterium* WH323. Purification was performed using affinity chromatography. CDW: cell dry weight.

| | **Protein produced** | | **Protein purified** | |
|---|---|---|---|---|
| | [$mg/l_{cell\ culture}$] | [$mg/g_{CDW}$] | [$mg/l_{cell\ culture}$] | [$mg/g_{CDW}$] |
| GFP-His | 9.6 | 6.8 | 2.4 | 1.5 |
| His-TEV-GFP | 17.9 | 14.0 | 5.0 | 3.0 |
| Strep-TEV-GFP | 13.2 | 10.5 | 9.0 | 6.0 |
| Strep-Xa-GFP | 8.4 | 6.3 | 6.0 | 4.0 |
| GFP-Strep | 16.0 | 11.2 | 6.0 | 4.0 |
| GFP-Strep/HCDC | 274 | 5.2 | | |

**Table 3** Selection of mutants generated in *B. megaterium* strain DSM319. Tn*917* insertion mutations in sporulation genes *spoIIA* (strains PV517 and PV518) and *spoIIAC*, coding for sigma factor F, (strains PV519), and a useful *lac* negative mutant (strain PV586) are also available in plasmidless derivatives of QM B1551 (Tao et al. 1992; Tao and Vary 1991). Analysis of *rec*, *uvr* and *npr* mutants in QM B1551 has also been reported (English and Vary 1986; Vary 1994).

| **Strain** | **Mutations** | **Reference** |
|---|---|---|
| *spo*3::Tn917 | *spo*$^-$ | (Meinhardt et al. 1994) |
| MS941 | Δ*nprM* | (Wittchen and Meinhardt 1995) |
| MS942 | Δ*nprM*, Δ*leuC* | (Wittchen et al. 1998) |
| MS943 | Δ*nprM*, Δ*leuC*, Δ*spoIV* | (Wittchen et al. 1998) |
| MS944 | Δ*nprM*, Δ*leuC*, Δ*spoIV*, Δ*recA* | (Wittchen 1995) |
| MS961 | Δ*leuC::nprM* | unpublished |
| MS970 | *bgaR::bgl* | (Strey et al. 1999) |
| MS971 | Δ*bgaR* | (Strey et al. 1999) |
| MS972 | *ORF2::bgl* | (Strey et al. 1999) |
| MS981 | *bgaM::nprM* | (Strey et al. 1999) |
| MS982 | Δ*bamM* | (Lee et al. 2001) |
| MS983 | Δ*nprM*, Δ*leuC*, Δ*bgaR/bgaM* | unpublished |
| MS991 | Δ*recA1* | (Nahrstedt et al. 2005) |
| MS001 | Δ*nprM*, Δ*barM/bamM* | unpublished |
| MS011 | Δ*uvrA::bgl* | (Nahrstedt and Meinhardt 2004) |
| MS021 | Δ*bgaR/bgaM* | (Schmidt et al. 2005) |
| MS022 | Δ*uvrBA::cat* | (Nahrstedt and Meinhardt 2004) |
| MS023 | Δ*bgaR/bgaM, leuC::"empty"* | (Schmidt et al. 2005) |
| MS024 | Δ*bgaR/bgaM, leuC::"xylA"-promoter testcartridge* | (Schmidt et al. 2005) |
| MS031 | Δ*bgaR/bgaM, leuC::"recA1"-promoter testcartridge* | (Nahrstedt et al. 2005) |
| MS032 | Δ*bgaR/bgaM, leuC::"recA2"-promoter testcartridge* | (Nahrstedt et al. 2005) |
| MS033 | Δ*uvrB* | (Nahrstedt and Meinhardt 2004) |
| WH320 | *lacZ*$^-$ | (Rygus et al. 1991) |
| WH323 | *xylA1-spoVG-lacZ* | (Rygus and Hillen 1992) |

**FIGURES**

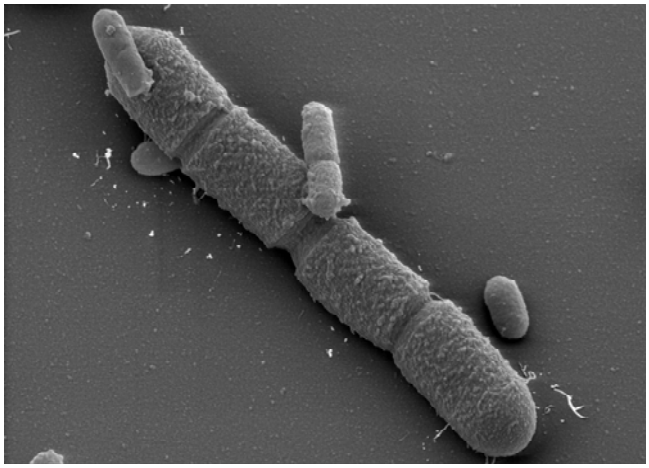


**Fig. 1 Electron microscope image of *Bacillus megaterium* and *Escherichia coli* vegetative cells.** *B. megaterium* cells grow up to a volume of more than 60 µm$^3$ (2.5 x 2.5 x 10). Compared to the *E. coli* volume of 0.5 µm$^3$ (0.5 x 0.5 x 2), *B. megaterium* has at least an up to 100-fold higher 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 x15,000; white bar: 2 µm.

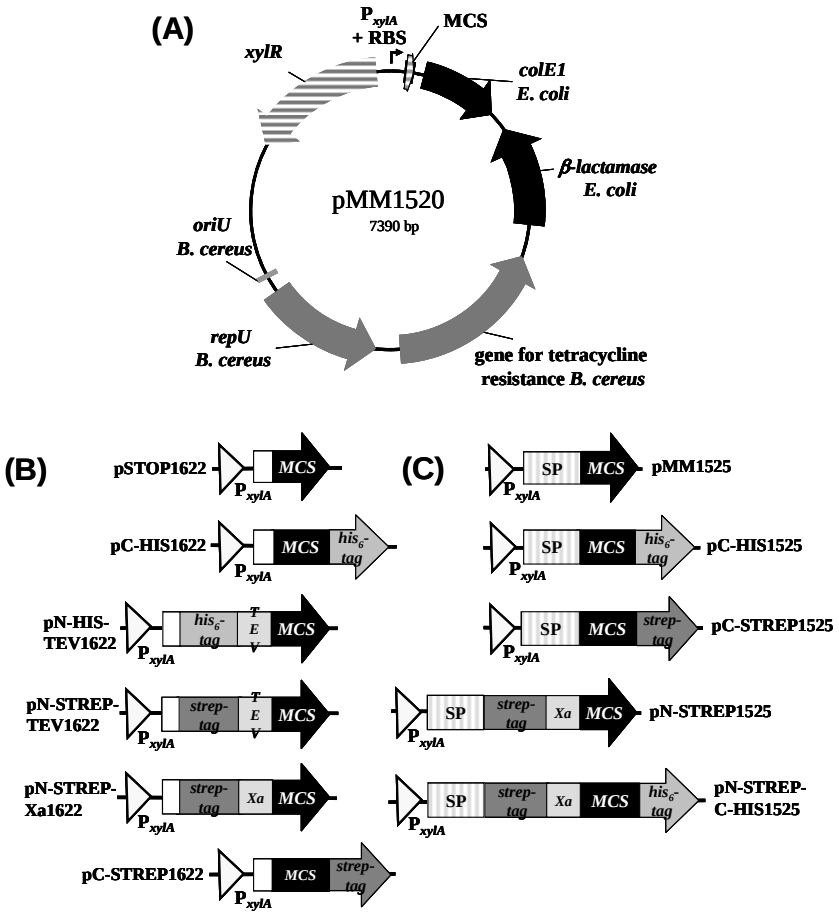



**Fig. 2 Expression plasmids for recombinant production of tagged proteins in *B. megaterium*.** **(A)** Structure of the *B. megaterium* expression vector pMM1520 (Malten et al. 2005a). Elements for xylose-inducible recombinant gene expression in *B. megaterium* are $P_{xylA}$ and the gene encoding the xylose repressor (*xylR*). Elements for plasmid replication in *Bacillus* are the origin of replication *oriU*, *repU*, and the tetracycline resistance gene. Elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the *β-lactamase* gene. **(B)** Scheme of expression plasmids for intracellular protein production (Biedendieck et al. 2007b). **(C)** Scheme of expression plasmids for extracellular protein production (Biedendieck et al. 2007a; Malten et al. 2006). $P_{xylA}$: promoter of *xylA*; TEV: tobacco etch virus protease cleavage site; Xa: Factor Xa protease cleavage site.

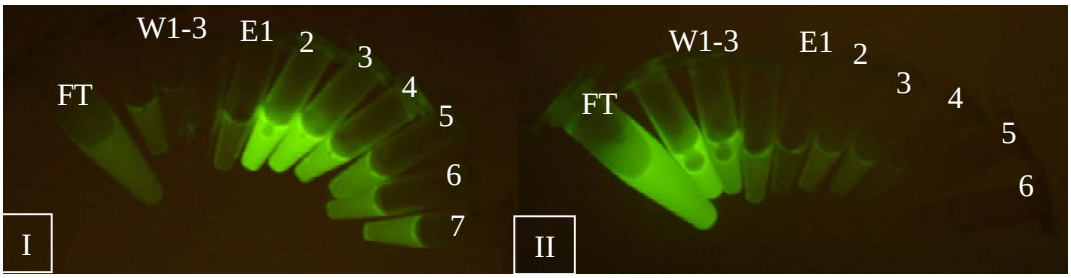



**Fig. 3 Purification of recombinant GFP produced in *B. megaterium* WH323 plasmid strains (Biedendieck et al. 2007b).** After the first affinity chromatography purification, His-TEV-GFP was incubated with or without TEV protease. Thereafter, the total volume of each reaction was loaded onto a Ni-NTA affinity column. **(I)** Without protease addition, the His-TEV-GFP binds to the affinity material. After washing (W1-3), His-TEV-GFP was eluted from the affinity material (E1-7). **(II)** After protease addition, GFP does not bind to the Ni-NTA affinity column (FT + W1-3). The elution fractions (E1-3) show less or no (E4-6) GFP.

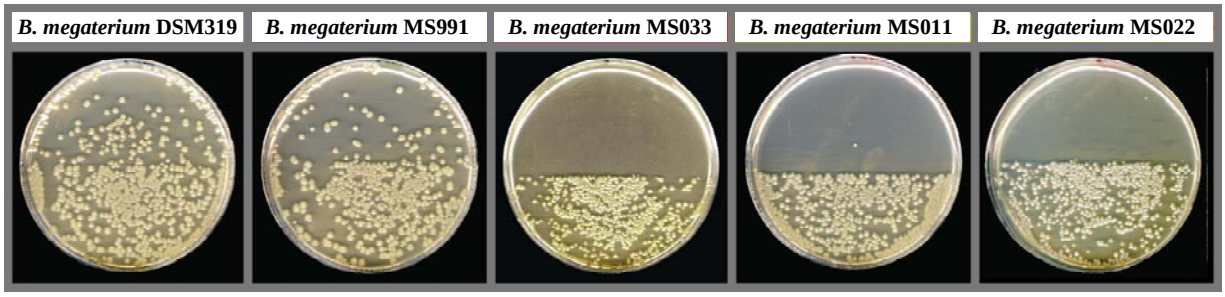
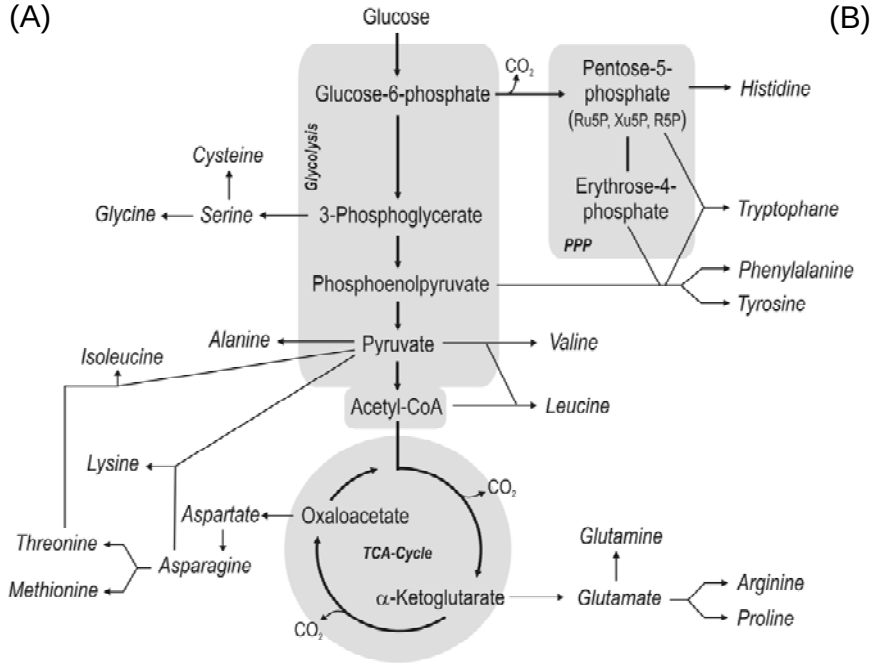



**Fig. 4 Analysis of UV sensitivity.** *B. megaterium* mutant strains were grown until the med-exponential growth phase and then spread in appropriate dilutions on LB plates for irradiation with increasing UV doses. For rapid testing, half of the plates were covered prior to irradiation (100 J/m$^2$). Plates shown after incubation overnight in the dark. *B. megaterium* strains DSM319: wild type, MS991: Δ*recA1*, MS033: Δ*uvr*B, MS011: Δ*uvr*A::*bgl*, MS022: Δ*uvr*BA::*cat*.

(A)




(B)

| Amino acid | Time constant τ [h] |
|---|---|
| alanine | 9.1 ± 0.3 |
| arginine | 8.6 ± 0.4 |
| aspartate | 6.4 ± 0.3 |
| glutamate | 4.5 ± 0.2 |
| glycine | 13.2 ± 1.1 |
| histidine | 9.7 ± 0.7 |
| isoleucine | 11.0 ± 0.3 |
| leucine | 11.6 ± 0.4 |
| lysine | 8.9 ± 0.3 |
| phenylalanine | 11.4 ± 0.3 |
| proline | 12.4 ± 0.3 |
| serine | 6.7 ± 0.1 |
| threonine | 7.4 ± 0.2 |
| tyrosine | 12.3 ± 0.6 |
| valine | 9.7 ± 0.2 |

**Fig. 5 Amino acid metabolism in *B. megaterium*. (A)** Schematic illustration of the biosynthesis of the 20 proteinogenic amino acids starting from their precursor molecules of the glycolysis, the pentose phosphate pathway and the TCA cycle. **(B)** Overview of the time constants τ of the single amino acids.

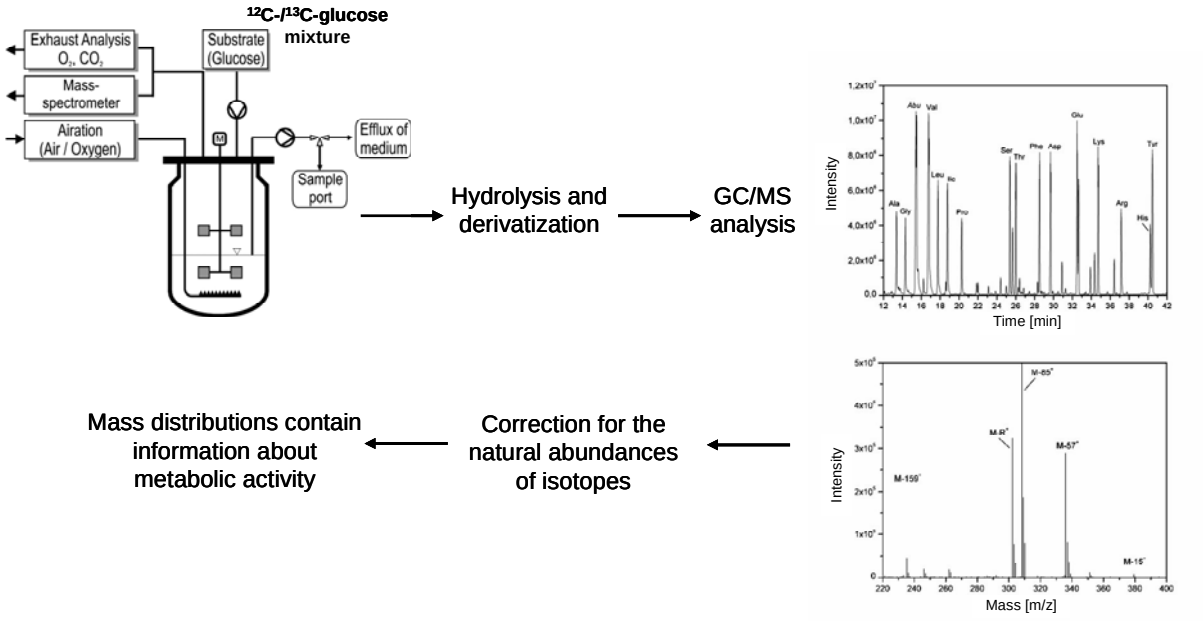



**Fig. 6 Experimental set up.** Schematic illustration of the experimental set up for dynamic studies of amino acids exchange using the metabolic flux analysis of *B. megaterium*.