**REVIEW
ARTICLE****Prime time for *Bacillus megaterium***

Patricia S. Vary

Tel: +1 815 753 7801. Fax: +1 815 753 0461. e-mail (Internet): T80PSV1@MVS.CSO.NIU.EDU

Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115, USA

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Bacillus megaterium has fascinated microbiologists since it was first described over 100 years ago. It is interesting especially because of its physiology, unusual and useful enzymes and products, and wide range of ecological habitats. It is also capable of sporulation, a simple cell differentiation cycle that serves as a model system for understanding gene regulation during temporal and morphological development. Moreover, the large size of its vegetative cells and spores (the source of its name) make it especially amenable to morphological analysis. Although it is generally considered a soil organism, it is found in diverse environments from rice paddies to dried food, seawater, sediments, fish, normal flora, and even in bee honey. Strains are often isolated on unusual substrates (such as herbicides) in the company of pseudomonads and actinomycetes.

B. megaterium has economic importance because of its commercially important enzymes such as penicillin amidase and steroid hydrolases. It is the major aerobic producer of vitamin B₁₂ and is one of the organisms involved in fish spoilage. An extensive review emphasizing its commercial applications has recently been published (Vary, 1992); major research areas and commercial applications are summarized in the illustration that appears on the cover of this issue of *Microbiology* [140(5)]. During the 1980s, genetic techniques of transduction, plasmid transformation, protoplast fusion and transposition became developed enough in *B. megaterium* to apply them to the study of many of its metabolic and developmental functions. Moreover, it is increasingly used as a host to produce foreign genes since it has been found to express, secrete and process foreign proteins without degradation.

In order to understand the species, it is necessary to be aware of the great diversity in the taxonomy of *Bacillus*, and within *B. megaterium* (Claus & Fritze, 1989; Priest, 1993). As can be seen from Fig. 1, it is within the *B. subtilis* group, but much more distantly related to *B. subtilis* than *B. licheniformis*, *B. cereus*, *B. anthracis* or *B. pumilus* by 16S rRNA sequence analysis (Ash *et al.*, 1991; Priest, 1993).

Major research strains of *B. megaterium* include QM B1551, KM, 216, DSM 319, ATTC 10778 and ATTC 19213. Strains QM B1551, 216 and IWG3, as well as the plasmidless strains PV361, DSM 319 and VT1660 are well-known industrially. Because of such diversity, interspecies and intraspecies comparisons can help to reveal essential genes, protein active sites and critical regulatory regions. Are the genes of the genus interchangeable? Even within *B. megaterium*, are there significant differences in physiology, differentiation and plasmids? Some intriguing answers to these questions are fuelling a renewed interest in *B. megaterium*. It occupies a unique position, somewhat removed from the genetically well-characterized *B. subtilis*, yet with better genetic and physiological characterization than most of the other bacilli. It can therefore serve to test some assumptions and observations in regulation, differentiation and physiology of Gram-positive bacteria. The purpose of this Short Review is to introduce *B. megaterium*, emphasizing recent advances in its genetics, cell structure, sporulation and germination, its broad physiological capabilities, and its expanding use as a cloning host.

Genetics***Transductional mapping***

The characterization of many physiological processes in cells of *B. megaterium* has been ongoing, yet an understanding of such areas was hampered for many years because of a lack of genetic methods. Phage MP13, a generalized transducing phage (Vary *et al.*, 1982), has made possible the mapping of over 50 loci spanning approximately 75–80% of the probable chromosome (Vary & Muse, 1993), and a mapping strain kit is available (Vary & Tao, 1988). Strains carrying multiple markers, including auxotrophy, antibiotic resistance, germination, sporulation, division, recombination and DNA repair have now been characterized. The genes for leucine, tryptophan and pyrimidine biosynthesis have been characterized in strain QM B1551 in detail, as have some genes for sporulation, germination, ribosomal proteins and division (Callahan *et al.*, 1983; Garbe *et al.*, 1984; Lach *et al.*, 1990; Vary & Muse, 1993). When the maps of *B. subtilis* and *B. megaterium* are compared, differences are

Fig. 1. Phylogenetic tree of some members of the genus *Bacillus* based on 16S rRNA sequence analysis (Ash *et al.*, 1991). Modified from Priest (1993). Reproduced with permission. Evolutionary distance between two species is the sum of the total branch lengths between them.

found in linkage, inversion of the *leu-ilv* region and presence of both *cysB* and *cysC* in the ribosomal protein region, to name a few. Interestingly, the position of the spore specific SASP (*ssp*) genes is more conserved than the surrounding auxotrophic loci (Sussman *et al.*, 1988). Wolf & Brey (1986) used MP13 to show that the *cob* and *chl*

genes involved in haem metabolism (leading to vitamin B₁₂ synthesis) are clustered in strain ATCC 10778. Although they did not locate these on the chromosome, two distinct *hem* loci have been mapped (Callahan *et al.*, 1983; Sussman *et al.*, 1988). The *B. megaterium* chromosome map is now complete enough that most gaps

remaining can be spanned by the cotransductional range of MP13. Progress has also been made in generating a physical map with *NotI* and *SfiI* restriction enzymes and pulse gel electrophoresis (Vary & Muse, 1993).

The dramatic increase in cloning of *B. megaterium* genes in the last 5 years has further contributed to the determination of physical linkages between genes and our understanding of gene regulation. For example, W. S. A. Brusilow and co-workers cloned the genes for the ATP synthetase subunits and found all of them to be contiguous (Brusilow *et al.*, 1989; Hawthorne & Brusilow, 1988). A cluster of sporulation genes including the *spoIIA* and *spoI'A* operons and an upstream penicillin binding protein have also been cloned and shown to be contiguous (Tao & Vary, 1992). W. Hillen and co-workers have cloned and sequenced the genes of the xylose operon of *B. megaterium*, which can be induced by xylose over 200-fold (Rygus & Hillen, 1992). A summary of all the known genes that have been mapped or cloned through mid-1992 has recently been tabulated (Vary, 1993). At least 40 genes have now been cloned and 36 sequences reported in the literature.

Transformation

Plasmid DNA can be transferred by protoplast transformation between strains of *B. megaterium*, other *Bacillus* spp., and *Streptococcus* and *Staphylococcus*. *B. megaterium*, like most species of *Bacillus* (including most industrial strains) cannot be naturally transformed, but the laboratories of L. Alföldi and B. C. Carlton developed polyethylene-glycol-mediated protoplast transformation (Von Tersch & Carlton, 1983; Vorobjeva *et al.*, 1980), and many recombinant plasmids have been introduced (Von Tersch & Robbins, 1990). All the Gram-positive vectors function well in *B. megaterium*, and there is excellent segregational and structural stability of both natural and recombinant plasmids in strains QM B1551, 216 and DSM 319 (Kieselburg *et al.*, 1984; Meinhardt *et al.*, 1989; Von Tersch & Robbins, 1990). Frequencies of 10^6 transformants ($\mu\text{g DNA}^{-1}$) allow direct cloning into *B. megaterium* (Meinhardt *et al.*, 1989). Such protoplast transformation has facilitated the use of transposons to develop the genetics of *B. megaterium* and for its emergence as an effective cloning host for foreign DNA. However, the protoplast fusion method is cumbersome and is sensitive to detergents in DNA or glassware, so other techniques are being developed. Shark *et al.* (1991) have reported biolistic transformation of *B. megaterium* (the first example in a prokaryote), transforming several plasmids at a frequency of $> 10^4$ transformants ($\mu\text{g DNA}^{-1}$). There are a few reports of very low frequencies of transformation by electroporation but so far optimal conditions have not been found. Of potential use is pLS20, a plasmid from *B. subtilis* (natto) that Koehler & Thorne (1987) showed can transfer conjugatively among several species of *Bacillus* including *B. megaterium*. In addition, conjugative transposon Tn925 can transfer itself and recombinant plasmids from *B. subtilis* to *B. firmus* (Guffanti *et al.*, 1991). It would seem reasonable to expect that it should also transfer into *B. megaterium*.

Integrative genetics

Transposons and plasmids lacking Gram-positive origins of replication have been successfully integrated in *B. megaterium*. Transposon Tn917 from *Streptococcus faecalis* and many engineered derivatives constructed by J. B. Perkins, R. Losick and P. Youngman (Youngman *et al.*, 1989) have been extremely useful in advancing the genetics of *B. megaterium*. It has been used to generate auxotrophic, sporulation and germination mutants. For example, Tn917 derivatives with promoterless *lacZ* and *cat* genes have been used to select auxotrophic, sporulation and germination mutants; to study, via the fusions produced, the regulation of *spoI* and *spoII* genes; and to clone the *spoIIA* operon (Tao & Vary, 1992). DNA from transpositions has been successfully cloned from *B. megaterium* into *E. coli* (Stevenson *et al.*, 1993) using a modified Tn917 containing pBR322. An integrative plasmid carrying fragments of cloned *ssp* genes has also been integrated, allowing their mapping (Sussman *et al.*, 1988). Rygus & Hillen (1992) constructed a vector for *B. megaterium* that integrates using homology with a cloned *xyl* gene to generate gene conversions.

Analysis of indigenous plasmids

Surveys involving a total of 18 strains of *B. megaterium* showed that almost all strains had at least four plasmids (N. A. Bohall & P. S. Vary, unpublished results; Stahl & Esser, 1983). Table 1 compares the plasmid arrays of a few strains of *B. megaterium*. It is evident that there are specific size categories that are present. Unfortunately, it has not been determined whether, say, the 70 kb plasmid of QM B1551 is homologous to the 70 kb plasmid of KM or the 76 kb plasmid of 216. Why is QM B1551 (as well as other strains) stably carrying over 11 % of its cellular DNA as plasmid DNA, even under laboratory conditions with no obvious selection? A completely plasmidless derivative of QM B1551 (which usually contains seven plasmids) still sporulates and grows normally on a variety of carbon sources. *B. megaterium* produces several megacins, or bacteriocins (Rostas *et al.*, 1980; Von Tersch & Carlton, 1984). When 400 randomly cured strains were tested for several possible phenotypes only two differences were found (Weiland, 1985): many strains were either megacin negative or defective for germination. It is known that megacin genes are present on the 47 kb plasmid of strain 216 and the 44 kb plasmid of ATCC 19213 and show limited homology by hybridization (Von Tersch & Carlton, 1984). QM B1551 also has a plasmid-borne megacin, but it is similar to MegCx (Kieselburg *et al.*, 1984). The germination gene was recently located on the 165 kb plasmid (Stevenson & Vary, 1993). Indeed, another germination gene, SASPG, has now been located on the 106 kb plasmid (Carrillo-Martinez & Setlow, 1993; Stevenson & Vary, 1993). It is, of course, possible that other plasmid genes are necessary for growth in environments not found in the laboratory. In an interesting twist to the plasmid analysis, plasmidless strains PV361 and its Lac⁻ derivative PV447, from QM B1551 (Vary & Tao, 1988), VT1660 from ATCC 19213 (Von Tersch & Carlton, 1984), AF421 (Rostas *et al.*, 1980) and DSM 319

Table 1. Plasmids of *B. megaterium*

Sizes are in kb.

QM B1551*	216†	KM‡	ATCC 19213†	ATTC 10778¶	M1286¶	M1365¶	DSM 90¶	DSM 337¶	DSM 339¶
pVY109 165	pBM110 166	70	pBM114 91	24.0	19.5	9.1	26.0	6.2	8.9
pVY170 106	pBM109 139	5.4	pBM113 44	8.5		8.6	8.9	5.6	4.2
pVY146 70	pBM108 112	3.6	pBM112 26	7.4		3.9	7.3		
pVY132 49	pBM107 91	2.0	pBM111 9.7	5.2		2.1	4.2		
pVY113 20	pBM106 76								
pVY105 8.1	pBM105 50								
pVY103 5.4	pBM309 47								
	pBM103 24								
	pBM102 9.4								
	pBM101 6.1								

* Kieselburg *et al.* (1984).

† Von Tersch & Carlton (1983).

‡ Weickert (1982).

¶ Stahl & Esser (1982) (technique used could not detect plasmids > 26 kb).

(Stahl & Esser, 1983) are proving to be ideal cloning hosts for foreign DNA.

Cell structure

B. megaterium is one of the few *Bacillus* strains that has a cell width greater than 1 µm. This large size and its ability to take up diaminopimelic acid has been exploited in several morphological studies. It has been used effectively to study cell wall synthesis as well as membrane and spore structure as reviewed recently (Archibald *et al.*, 1993). The kinetics of the growing vegetative cell has been determined by electron microscopy with labelled diaminopimelic acid in elegant experiments by Frehel & Ryter (1979). Teichuronic acids in addition to teichoic acids are present and are transferred from uridine nucleotides to a unique lipid carrier for assembly. A new model for assembly of *B. megaterium* peptidoglycan into a cylindrical wall by a monomer addition process proposed by Gally *et al.* (1991) is also applicable to Gram-negative bacteria.

The membranes of both vegetative cells and spores have been analysed. For example, a unique mutant resistant to oxidative uncouplers has recently been shown to have increased amounts of unsaturated fatty acids in its phospholipids (Clejan *et al.*, 1988). Changes in lipid composition of membranes following growth on different substrates have been documented by the use of fluorescent probes, nuclear magnetic resonance and circular dichroism (Janoff, 1979; Nikolopoulou & Vary, 1987). Mitchell & Vary (1989) have further detected 15 membrane proteins that interact with guanine nucleotides, four of which appear during sporulation. Penicillin binding proteins (PBPs) involved in the metabolism of peptidoglycan should be required for spore development as well as vegetative growth, and indeed, two sporulation-specific

PBPs, PBP 3e and PBP 5a, both of which were involved in cortex maturation have been found in *B. megaterium* strain KM (Todd & Ellar, 1983). Even though homologues for several other genes have been detected, Buchanan & Gustafson (1991) could find no homologue between *B. megaterium* ATCC 14581 DNA and the *B. subtilis* *dacA* gene, which codes for the major PBP 5 protein in *B. subtilis*. In contrast, such homologues have been detected in several other species, once again indicating the diversity of the genus.

By far the most extensive morphological studies using *B. megaterium* have been to study its large, distinctive spore structure. This is best illustrated by the electron-microscopic sequential analysis of QM B1551 spores during microcycle sporogenesis (spore to primary cell to spore again without intervening division) made several years ago by Freer & Levinson (1967). The sequence of events and compartmentalization of spore coat protein synthesis in the sporangium can be seen in their electron micrographs, events only recently shown by molecular techniques. The spore of QM B1551 is walnut-shaped and has a very distinct electron-dense outer layer, several complex, fairly amorphous inner spore coat layers, and a thick cortex (Fig. 2A). During germination, the spore opens along the equatorial ridge. Spores are less heat resistant than several other *Bacillus* spores, which seems to be a reflection of its more heat sensitive growth as a vegetative cell (Khouri *et al.*, 1987). T. Nishihara and co-workers have shown that the three most abundant coat proteins of QM B1551, of mol. mass 44 kDa, 25 kDa and 22 kDa, are absent from the spore of an outer-coatless mutant (even though the 22 kDa protein accumulates in the forespore of the mutant 6 h after sporulation starts) (Takubo *et al.*, 1992). They have found that coatless mutants aggregate in salt, which should allow many more mutants to be

isolated. Since there has been considerable progress in characterizing the genes of *B. subtilis* coat proteins and their regulation, it would be very useful to correlate these with the morphological and biochemical analysis in *B. megaterium*.

Sporulation

There has been a long-term effort in *Bacillus* to study sporulation for two principal reasons. First, to better understand the process in order to circumvent the devastating effects of spore-mediated diseases such as food poisoning, anthrax, botulism and tetanus. Second, to use *Bacillus* cell differentiation as a model system to understand cell development. Sporulation proceeds through seven morphological stages (0, II–VII) culminating in the lysis of the mother cell and release of a mature spore approximately 7–8 h later. Mutants blocked during the process have defined the stages (*spo0*, *spoII*, etc.). Many of the seminal biochemical studies on sporulation were done using *B. megaterium* because of its large size, its ability to sporulate very efficiently on diverse media and to germinate synchronously on a wide range of germinants (Setlow & Kornberg, 1969; Vary & Kornberg, 1970). Much progress has been made since in understanding the regulation and temporal expression of sporulation genes. There are over 150 genes involved in sporulation and germination that have been mapped in *B. subtilis*, and many loci have been cloned (Hoch, 1993).

Even limited comparison of the two species *B. subtilis* and *B. megaterium* is increasing our understanding of regulation, and it now becomes possible to distinguish some of the essential processes of sporulation from individual variations. For example, several Tn917 *lacZ* fusion sporulation mutants have been isolated in *B. megaterium*, and one mutant was blocked very early in septum formation, showing slight indentations where the septa should form (Tao & Vary, 1991). Two such mutants have now been described in *B. megaterium* but no similar mutants have been reported among the great number of *spo* mutants described in *B. subtilis*. Temporal synthesis of glucose dehydrogenase, proteases, calcium and dipicolinic acid accumulation, antibiotic production and motility have been well established in both species (Vary, 1992), but neither antibiotic production nor alkaline protease have been detected during sporulation, and neither motility nor protease production correlated with sporulation in *B. megaterium*, in contrast to *B. subtilis*. Alkaline phosphatase is also synthesized 2–3 h earlier. Yet there are important similarities. The cascade of sigma factors that helps regulate the temporal expression of the developmental genes (Stragier & Losick, 1990) is present as shown by the following evidence. The *spoIIAC* gene, coding for σ^F , has been isolated and sequenced and has a promoter homologous to the *B. subtilis* σ^H consensus sequence (Tao & Vary, 1992). The downstream *spoVA* operon has a σ^F promoter consensus. The *spo0H* gene (for σ^H) of *B. megaterium* as well as several σ^H promoters have been cloned (Naumann *et al.*, 1990). The *spoIIGB* gene

(for σ^B) lacking the first five codons has also been cloned (B. Diederich, personal communication), and P. Setlow has shown that the *ssp* genes and *grp* gene of *B. megaterium* are transcribed by a RNA polymerase σ^G holoenzyme (Setlow, 1988). There are some differences in sigma factor expression, however, including the absence of DNA rearrangement of the *sigK* gene of *B. megaterium* (P. S. Vary, unpublished data). Still another sigma factor may be active during sporulation in *B. megaterium* since Strnadova *et al.* (1991) have detected an increase in over 10 heat shock proteins during sporulation.

The *spoIIA* operon of *B. megaterium* codes for three proteins of almost identical size to those from *B. subtilis* with 78–92% amino acid homology (Tao & Vary, 1992). The genes are in the same order as those of *B. subtilis* and *B. licheniformis*, but while *B. megaterium* expresses two transcripts, like *B. subtilis*, these were expressed 1–3 h earlier in *B. megaterium*, perhaps one reason for faster sporulation in this species. Considerable gene overlap for translational coupling, more than is found in the *spoIIA* operon of *B. subtilis* was observed. In addition, the promoter region of the *spoIVVA* operon contained regions that were conserved across all three species of *Bacillus*, but were not found in other sequenced genes, so that they would not have been detected by sequence comparisons within one species (Moldover *et al.*, 1991; Tao & Vary, 1992). The promoter region of *spoIIA* was also found to contain a 'spoOA' box, similar to *B. subtilis* and a *B. megaterium* gene homologous to *spoOA* of *B. subtilis* has recently been cloned (Brown *et al.*, 1992). Greater homology was found when the *spoVG* gene from *B. megaterium* was cloned and sequenced (Hudspeth & Vary, 1992). The protein encoded has 98% amino acid homology with *spoVG* from *B. subtilis*. It is therefore clear that the sporulation genes have been conserved among the bacilli and that comparisons of genes from different species can help distinguish some of the important factors essential for the prokaryotic cell differentiation process (regulation of *spoIIA*) from individual species strategies (rearrangement of the *sigK* gene).

Two types of germination in *B. megaterium*

While the study of sporulation has been possible because of a sophisticated network of genetic and molecular techniques, mutants and observable morphological changes, it has been more difficult to understand germination. Germination is very rapid and not as easily followed sequentially since early events occur in a dormant, resistant spore with few morphological landmarks. Mutants must also be conditional or they are lethal, locked in perpetual dormancy (since they cannot easily be kept from sporulating). What is very intriguing is that this spore, one of the most resistant cells known in nature, responds to only a few compounds that must find their way through several layers of spore coat, an outer membrane and a cortex to a receptor(s) located on the inner membrane. Once accomplished, the effects are dramatic and rapid, utilizing enzymes and other com-

Fig. 2. Comparison of the spore structure of *B. megaterium* strains QM B1551 (A) and KM (B). Electron micrograph A, P. S. Vary; B, from Aronson & Fitz-James (1976); reproduced with the permission of the American Society for Microbiology. Abbreviations: OC, outer coat; CP, cross patch material in strain KM (and probable equivalent in QM B1551); Cx, cortex; Ex, exosporium; UC, undercoat; GCW, germinal cell wall; IM, inner membrane; OM, outer membrane.

pounds already present in the spore. The rest of germination is a poorly understood sequence of biosynthetic events, called outgrowth, that result in the release from its spore-shell of a new, actively metabolizing cell within 90 min. Since *B. megaterium* spores germinate synchronously after heat activation, they have been very useful in studying the first critical events. The sequence of events in germination are: commitment, increased heat sensitivity, swelling, dipicolinic acid and hexosamine release, decrease in absorbance at 660 nm, onset of metabolism, net ATP generation (end of germination at 5–6 min and beginning of outgrowth), RNA and DNA synthesis, protein synthesis, and finally, cell emergence and division (Foster & Johnstone, 1990; Levinson & Hyatt, 1966).

If one compares the scattered data on germinants and spores in different strains, it is evident that there are at least two distinct types of *B. megaterium* spores and these have very different germination requirements. As shown in Fig. 2, the spores of strains KM and QM B1551 are morphologically distinct. QM B1551 has a thick, outer spore coat and exosporium while KM (as well as strain ATCC 19213) has a simpler coat, no exosporium and no outer coat. Likewise, germination seems to be very different in KM and QM B1551. A germination cortex lytic enzyme has been purified from strain KM that hydrolyses the cortex during germination (Foster & Johnstone, 1990). Moreover, Foster and Johnstone have detected this enzyme in *B. cereus* T, *B. subtilis* and *Clostridium bifermentans* M, but not in QM B1551. Foerster & Foster (1966) analysed germination of 21 strains of *B. megaterium* and 25 other strains of 13 species of *Bacillus* on a variety of germinants. Their data show QM B1551 germinates on almost all substrates tested. Strains KM and ATCC 19213, in contrast, germinate only on alanine, or alanine plus inosine. It thus seems clear that there are at minimum two kinds of *B. megaterium* as determined not only by spore morphology, but by response to germinants as well: those similar to *B. subtilis* that germinate on alanine, and those, similar to QM B1551, that respond to a wider range of germinants.

Recent evidence from our laboratory may help to explain this observation. A germination gene(s) (*gerP*) was discovered on the largest (165 kb) plasmid (Stevenson & Vary, 1993). Among randomly cured strains, only strains lacking the 165 kb plasmid were unable to germinate on glucose, KBr, leucine or proline, unlike strains carrying the plasmid. All could still germinate on rich medium. Neither KM nor ATCC 19213 have a comparable plasmid, at least in size (Table 1). One more germination gene is also plasmid-borne in QM B1551. The minor unique SASPG gene, mentioned above, is on the 106 kb plasmid of QM B1551 (Carrillo-Martinez & Setlow, 1993; Stevenson *et al.*, 1993), explaining the failure to find homologues for this gene in other bacilli. A germination gene from QM B1551 has been cloned by transforming a gene library of QM B1551 into ATCC 19213, and it changed the germination requirement from alanine to a mixture of glucose and KNO₃ (Tani *et al.*, 1990), but it has not been localized to either chromosome or plasmid.

Are there multiple receptors for germination in QM B1551, and if so, where are they located? It is now well established that triggering occurs at the inner spore membrane. Rossignol & Vary (1979) labelled a 10·2 kDa inner membrane protein in QM B1551, probably part of the proline receptor, with the analogue L-proline chloromethyl ketone. They also isolated a mutant that could not germinate on proline, but could germinate on all the other triggers, suggesting that there are at least two receptors for the single germinants. There is strong evidence that the earliest change is a change in the fluidity of the membrane (Janoff *et al.*, 1979), and ion channels may play a role in the germination process (Mitchell *et al.*, 1986). Of three outer coatless mutants isolated, two were Ger⁻ but one was Ger⁺, suggesting that the outer coat is not essential for germination (Takubo *et al.*, 1992). New tools are now available to dissect some of the events in *B. megaterium* with inhibitors of intermediate germination stages such as HgCl₂ and CdCl₂ (Foster & Johnstone, 1990; Rossignol & Vary, 1977). In addition, W. M. Waites and co-workers have followed the germination progress of strain KM using a bioluminescence reporter gene (Hall *et al.*, 1991) and were able to study the effect of rifampicin, nisin and chloramphenicol on germination.

The role of several unique spore compounds called small acid soluble proteins, or SASP, has been studied by P. Setlow and co-workers in *B. megaterium*, and recently reviewed (Setlow, 1988). Nine SASP genes (*ssp*) from *B. megaterium* have been cloned (Curiel-Quesada, 1983; Fliss *et al.*, 1985; Fliss & Setlow, 1984a, b). These proteins, which make up approximately 20% of the total spore protein, have a role in heat and UV resistance and in supplying amino acids early in germination. They are hydrolysed very rapidly during germination by a specific protease, called Gpr or germination protease. Most of the *ssp* genes are highly conserved (with the exception of the unique minor SASPG plasmid gene and the major γ gene) and the α/β major *ssp* genes have been cloned from several spore formers by homology with the *B. megaterium* genes. This is one of the first examples of a multigene family in prokaryotes (Setlow, 1993).

Physiology and commercial products

B. megaterium has the ability to grow on many carbon sources including waste from the meat industry and corn syrups as well as a wide range of sugars; it has been found in petrochemical effluents and can oxidize thiosulfate compounds (Priest *et al.*, 1988; Vary, 1992). Only a few examples of the extensive physiological studies on *B. megaterium* can be mentioned in this review. Currently, one of the best characterized carbon source operons is the xylose operon (Ryguis & Hillen, 1992). The biochemical pathways of many amino acids have also been studied in depth. Some of the most interesting proteins of *B. megaterium* are a family of P-450 cytochrome monooxygenases. These have been of great interest since they have considerable similarity to eukaryotic P-450 important in many disease conditions (He & Fulco, 1991). A. J. Fulco and co-workers have characterized and se-

Table 2. Examples of recent industrial uses of *B. megaterium*

Product/use	Comments	References
Alphostatin production	Inhibitor of alkaline phosphatase	Aoyagi <i>et al.</i> (1989)
α -Amylases	Can replace pullulanases	Takasaki (1989); Vinhinen & Matsala (1989)
β -Amylases	Bread industry	Metz <i>et al.</i> (1988); Hebeda <i>et al.</i> (1988)
Chitosanases	Yeast cell wall analysis	Pelletier & Sygusch (1990)
Fungicidal toxins	For <i>Rhizoctonia</i> , one isolated	Liu & Sinclair (1992); Bhattacharyya & Pukayastha (1989)
Glucose dehydrogenase	Generator of NADH, immobilization, biosensors	Nagao <i>et al.</i> (1992); Kittsteiner-Eberle <i>et al.</i> (1989)
Glutamate production		1987 Patent JP62048393
Modification of steroids	4- α -Glucanotransferase A	1989 Patent DD266592
Oxetanocin production	Inhibits HIV, hepatitis B, cytomegalovirus, herpes	Kohlbrenner <i>et al.</i> (1990); Tseng <i>et al.</i> (1992)
Phosphate solubilization	Phosphate fertilizer	Vary (1992)
Penicillin amidase	Construction of synthetic penicillins	Suga <i>et al.</i> (1990)
Sensitivity testing	Heat, sterilization, antimicrobials	Vary (1992)
Toxic waste cleanup	Herbicides, C-P bonds	Quinn <i>et al.</i> (1989); Saxena <i>et al.</i> (1987); Selvanayagam & Vijaya (1989)
Vitamin B ₁₂ production	Only aerobic producer	Wolf & Brey (1986); Robin <i>et al.</i> (1990)
Expression host	Secretes, processes, produces intact proteins	Ahn <i>et al.</i> (1993); Rygus & Hillen (1991); Von Tersch & Robbins (1990); Shivikumar <i>et al.</i> (1989)

quenced these interesting enzymes (Wen *et al.*, 1989). They have described one, P-450_{BM-3} that is induced by barbiturates and is catalytically self-sufficient, requiring only NADPH and oxygen to catalyse the hydroxylation of long-chain fatty acids. It is drawing considerable interest as a model system for the understanding of the eukaryotic enzymes (Lie *et al.*, 1991).

Several enzymes are known to be secreted by *B. megaterium*, including α -amylase, β -amylase, penicillin amidase, neutral protease, β -glucanase, megacins (the phospholipase MegA), glucanotransferase and chitosanases (Vary, 1992). The species also produces unusual enzymes such as an epoxide hydrase, an isomerase for maleylpyruvate, a β -glucanase used as a probe for structural analysis of yeast cell walls, and the enzyme alphostatin, which inhibits calf alkaline phosphatase. The neutral, or metalloprotease (Npr) first characterized by Millet *et al.* (1969), has been studied extensively by J. Chaloupka and co-workers who have recently characterized three intracellular proteases (Moravcova & Chaloupka, 1990). The extracellular calcium-dependent protease has now been cloned also (Kuhn & Fortnagle, 1993). Two mutants in QM B1551 have also been described that are *npr* negative (Vary & Tao, 1988). One of these produces no observable protease even when culture supernatant was concentrated 20-fold and dropped on casein plates (C. Hansen, unpublished results).

B. megaterium enzymes and products have been used in industrial applications for several years and are also effective in immobilized systems, making industrial processes even more efficient. Table 2 lists some of the commercial applications and products of *B. megaterium* and a few are discussed below.

Amylases

Several amylases are produced. The advantage of these amylases is that they have unusual cleavage capabilities. The bread industry *B. megaterium* amylase (BMA) converts branched saccharides to a form that is easily hydrolysed by glucoamylases. The gene has been cloned, sequenced and characterized (Hebeda *et al.*, 1988; Metz *et al.*, 1988) and has no homology to other amylases. A unique α -amylase that generates maltose subunits from starch has also been described (Takasaki, 1989). Unfortunately, it is difficult from the literature to determine just how many amylases the species produces since there has been little comparison. *B. megaterium* produces at least two amylases that are distinct and commercially important.

Glucose dehydrogenase

Laboratories in both Japan and Germany have described multiple glucose dehydrogenase (*gdh*) genes from species of *B. megaterium*. In fact, four *gdh* genes have now been cloned and sequenced from one strain, IAM1030 (Nagao *et al.*, 1992) and at least two have been found in each of several other strains, coding for three different proteins. The genes appear to be regulated differently. Meinhardt *et al.* (1989) used the promoter of one of the *gdh* genes to construct an efficient expression vector in *B. megaterium*. GDH stability has also been enhanced by mutation (Nagao *et al.*, 1989). It is easily immobilized and is often used both to generate NADH in industrial processes and as a biosensor (Kittsteiner-Eberle *et al.*, 1989). Honorat *et al.* (1990) reported that enzyme activity of both L-alanine and L-valine dehydrogenases can be purified and coupled

with a purified glucose dehydrogenase from the same strain to synthesize valine and alanine very efficiently in permeabilized cells of *B. megaterium*.

Penicillin amidase

Another important enzyme is penicillin acylase, or amidase, which is used to cleave the side-chain of penicillins to generate new synthetic antibiotics. The gene was cloned directly into a *B. megaterium* production strain generating a very high producing strain (McCullough, 1983). Recently, Suga *et al.* (1990) developed an immobilization process to increase the efficiency of the enzyme and Kang *et al.* (1991) cloned the gene into *B. subtilis* and *E. coli* with a 20-fold increase in yield.

Vitamin B₁₂

B. megaterium is the major aerobic source for vitamin B₁₂, or cobalamin, which is synthesized from the haem biosynthetic pathway. Several *chl* and *cob* mutants in the specific cobalamin pathway have been isolated and the genes cloned (Brey *et al.*, 1986; Wolf & Brey, 1986). Additionally, Robin *et al.* (1991) have sequenced the key enzyme in the pathway, S-adenosyl-L-methionine:uroporphyrinogen-III methyltransferase. This gene has 43.5% amino acid identity with the enzyme from another major B₁₂ producer, *Pseudomonas denitrificans*.

Oxetanocin and other antimicrobial agents

A few antibiotics such as emimycin are produced by *B. megaterium* (Vary, 1992), but by far the most exciting is a unique analogue antibiotic, oxetanocin, that has been shown to be effective against a number of important pathogenic viruses, even ones that do not produce thymidine kinase (Kohlbrenner *et al.*, 1990). N. Shimada and co-workers were among the first to characterize its action and tested several derivatives (Shimada *et al.*, 1990). It is very potent in inhibiting herpes (HSV-1), hepatitis B and cytomegalovirus (Kohlbrenner *et al.*, 1990), and the recent discovery that it is also effective against HIV (Tseng *et al.*, 1991) is stimulating considerable interest. *B. megaterium* also has fungicidal properties. A fungicidal toxin has been isolated from *B. megaterium* by Bhattacharyya & Pukayastha (1989). Moreover, *B. megaterium* is not only stably maintained in the rhizosphere of soybeans, but causes both a decrease in the fungal infectivity of *Rhizoctonia solani* Kuhn, which causes root decay, and an increase in the growth of soybeans (Liu & Sinclair, 1992).

Bioremediation

B. megaterium is found in unusual and sometimes toxic environments and may have potential as a detoxifying agent. Quinn *et al.* (1989) described a carbon-phosphorylase in both *Pseudomonas* and *B. megaterium* that cleaves C-P bonds so that both species were able to degrade 14 of 15 C-P herbicide compounds tested. The ability of *B. megaterium* to degrade persistent insecticides such as

metachlor, Baytext and Paris green, and utilize them as carbon sources has also been documented (Saxena *et al.*, 1987; Selvanayagam & Vijaya, 1989).

B. megaterium as an expression host

Within the last few years, several laboratories have shown that *B. megaterium* has many advantages as a host for expression of foreign DNA. First, it has all the advantages of *Bacillus*, that is, it secretes proteins readily, has no endotoxin in its cell wall in contrast to the Gram-negatives, and is industrially proven as an organism that can give excellent yields on inexpensive substrates. Second, it has none of the alkaline proteases that have plagued *Bacillus* cloning efforts. Laboratories in the US, Korea, UK and Germany have documented excellent expression of foreign proteins with no degradation (Ahmad *et al.*, 1989; Kim *et al.*, 1991; Meinhardt *et al.*, 1989; Rygus & Hillen, 1991; Shivakumar *et al.*, 1989; Von Tersch & Robbins, 1990). In addition, Ahn *et al.* (1993) have described not only secretion, but efficient processing of *B. subtilis* glucanase in *B. megaterium*. A third major advantage of cloning in *B. megaterium* is the structural and segregational stability of recombinant plasmids. Even its own glucose dehydrogenase genes cloned into *B. megaterium* were stable through 3 weeks of daily subculturing without selective pressure (Meinhardt *et al.*, 1989). Many of the expression studies have used the insecticidal toxin crystal genes of *B. thuringiensis*. Two *cryIA* genes carried on high and low copy number plasmids were expressed much better and gave far superior protein stability (no degradation) in *B. megaterium* when compared to *B. subtilis* (Shivakumar *et al.*, 1989). In addition, *B. megaterium* sporulated normally with intact crystals evident in the cells. No protein was produced in *B. subtilis* from the high copy plasmid, and it was produced more slowly with extensive degradation from the low copy plasmid. In another comparative study, Von Tersch & Robbins (1990) cloned random fragments of *B. thuringiensis* DNA into *B. megaterium*, *B. subtilis* and *E. coli* and found that *B. megaterium* had the lowest transformation frequency, but was comparable to *E. coli* in structural stability. The average size of inserts was much larger in *E. coli* and *B. megaterium* than in *B. subtilis*. Segregational stability was better in *E. coli*, but the observed instability in both bacilli was shown to be because of the pBR322 sequences in the shuttle vector.

Several expression systems developed for *B. megaterium* hosts have been reviewed recently (Vary, 1992). Since that review, a strong, tightly controlled expression system using the promoter and repressor gene of the *B. megaterium* xylose operon has been developed by W. Hillen and co-workers (Rygus & Hillen, 1991). Four genes – *lacZ* from *E. coli*, *gdb* from *B. megaterium*, *mro* (mutarotase from *Acinetobacter*) and human gene *puk* (a urokinase-like plasminogen activator) were induced 130–350-fold by 0.5% xylose with no proteolysis. This ability to efficiently express foreign genes has been successfully transferred to industrial production. For example, the plasmidless strain

PV361 is now used for the commercial production of HIV coat protein genes for diagnostic tests for AIDS (Ginsburgh *et al.*, 1989).

Summary

It is evident that *B. megaterium* is an intriguing organism because of its biochemical versatility, its wide distribution ecologically, its ability to undergo sporulation, and its usefulness as an industrial production strain and expression host. With the progress in genetics and the availability of molecular tools such as new transposons, vectors and efficient transformation, an understanding of some of the organization and regulation of many genes is increasing rapidly. Such recent discoveries as the ability of oxetanocin to combat some medically significant, recalcitrant viruses further demonstrates that there is much to be learned and much to benefit from continued study of *B. megaterium*.

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