Prime time for Bacillus megaterium

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Keywords: Bacillus megaterium, sporulation, germination, cloning host, genetics

Bacillus megaterium: general features and taxonomic position

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, inter-species 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
found in linkage, inversion of the *leu-ile* region and presence of both *cytB* and *cytC* 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 *cbl* genes involved in haem metabolism (leading to vitamin B<sub>12</sub> 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 _spollA_ 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 (Rybus & 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 _10^4_ transformants (µg 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. Shank 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 (µg 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 conjunctively 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_ (Guffants 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 promotorless lacZ and _cat_ genes have been used to select auxotrophic, sporulation and germination mutants; to study, via the fusions produced, the regulation of _spol_ and _spoII_ genes; and to clone the _spaII_A 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 _spE_ genes has also been integrated, allowing their mapping (Sussman et al., 1988). Rybus & 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 1 1% 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 (Carillo-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.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
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<td>KM†</td>
<td>70</td>
<td>ATCC 19213†</td>
<td>240</td>
<td>M1286§</td>
<td>5.4</td>
<td>DSM 90§</td>
<td>8.9</td>
<td></td>
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<tr>
<td>pVY109 165</td>
<td>216†</td>
<td>pBM109 166</td>
<td>70</td>
<td>pBM114 91</td>
<td>240</td>
<td>M1365§</td>
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<td>pBM109 139</td>
<td>5.4</td>
<td>pBM113 44</td>
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<td>DSM 337§</td>
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<tr>
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<td>3.6</td>
<td>pBM108 112</td>
<td>3.6</td>
<td>pBM112 26</td>
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<td>DSM 339§</td>
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<td>pBM107 91</td>
<td>2.0</td>
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† 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 daA* 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 (Khoury *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 $\sigma^F$, has been isolated and sequenced and has a promoter homologous to the B. subtilis $\sigma^H$ consensus sequence (Tao & Vary, 1992). The downstream spoVA operon has a $\sigma^P$ promoter consensus. The spoOH gene (for $\sigma^H$) of B. megaterium as well as several $\sigma^H$ promoters have been cloned (Naumann et al., 1990). The spoIIGB gene (for $\sigma^K$) lacking the first five codons has also been cloned (B. Diederich, personal communication), and P. Setlow has shown that the spo genes and grp gene of B. megaterium are transcribed by a RNA polymerase $\sigma^K$ 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 of 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 spoIIVA 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 beijerinckii* 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, glucose, KBr, leucine or proline, unlike strains carrying this observation. A germination gene(s) for this gene in other bacilli. A germination gene from of Qh4 B1551 (Carrillo-Martinez et al., 1993) 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 process 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 xyllose operon (Ryguš & 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 monoxygenases. 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

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<th>Product/use</th>
<th>Comments</th>
<th>References</th>
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<td>Inhibitor of alkaline phosphatase</td>
<td>Aoyagi et al. (1989)</td>
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<tr>
<td>α-Amylases</td>
<td>Can replace pullulanases</td>
<td>Takasaki (1989); Vinhinen &amp; Matsala (1989)</td>
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<tr>
<td>β-Amylases</td>
<td>Bread industry</td>
<td>Metz et al. (1988); Hebeda et al. (1988)</td>
</tr>
<tr>
<td>Chitosanases</td>
<td>Yeast cell wall analysis</td>
<td>Pelletier &amp; Sygusch (1990)</td>
</tr>
<tr>
<td>Fungicidal toxins</td>
<td>For Rhizoctonia, one isolated</td>
<td>Liu &amp; Sinclair (1992); Bhattacharyya &amp; Pukayasta (1989)</td>
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<tr>
<td>Glucose dehydrogenase</td>
<td>Generator of NADH, immobilization, biosensors</td>
<td>Nagao et al. (1992); Kittsteiner-Eberle et al. (1989)</td>
</tr>
<tr>
<td>Glutamate production</td>
<td></td>
<td>1987 Patent JP62048393</td>
</tr>
<tr>
<td>Modification of steroids</td>
<td>4-α-Glucanotransferase A</td>
<td>1989 Patent DD266592</td>
</tr>
<tr>
<td>Oxetanocin production</td>
<td>Inhibits HIV, hepatitis B, cytomegalovirus, herpes</td>
<td>Kohlbrenner et al. (1990); Tseng et al. (1992)</td>
</tr>
<tr>
<td>Phosphate solubilization</td>
<td>Phosphate fertilizer</td>
<td>Vary (1992)</td>
</tr>
<tr>
<td>Penicillin amidase</td>
<td>Construction of synthetic penicillins</td>
<td>Suga et al. (1990)</td>
</tr>
<tr>
<td>Sensitivity testing</td>
<td>Heat, sterilization, antimicrobials</td>
<td>Vary (1992)</td>
</tr>
<tr>
<td>Toxic waste cleanup</td>
<td>Herbicides, C-P bonds</td>
<td>Quinn et al. (1989); Saxena et al. (1987); Selvanayagam &amp; Vijaya (1989)</td>
</tr>
<tr>
<td>Vitamin B₁₂ production</td>
<td>Only aerobic producer</td>
<td>Wolf &amp; Brey (1986); Robin et al. (1990)</td>
</tr>
<tr>
<td>Expression host</td>
<td>Secretes, processes, produces intact proteins</td>
<td>Ahn et al. (1993); Rygus &amp; Hillen (1991); Von Tersch &amp; Robbins (1990); Shivikumar et al. (1989)</td>
</tr>
</tbody>
</table>

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 (Kuhna & 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 glucoamyloses. 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-1-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 (Kohlbrener *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 (Kohlbrener *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 Bhattacharya & 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 pesticide compounds tested. The ability of *B. megaterium* to degrade persistent insecticides such as metachlor, Baytex 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 cry1A 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*, gdh from *B. megaterium*, mnr (mutarotase from Acinetobacter) and human gene puk (a urokinase-like plasmidogen 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*.

The author would like to thank David Stevenson for critical reading of this manuscript and to apologize to all those researchers whose work on *B. megaterium* made this review possible, but who could not be included because of space limitations.

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