Isolation and characterization of sporulation lacZ fusion mutants of Bacillus megaterium

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A derivative of Bacillus megaterium QM B1551 cured of all seven resident plasmids was mutated to Lac-. Transposon Tn917 carrying lacZ and cat was then used to isolate four spo::lacZ-cat fusion mutants by screening for colonies expressing the fusion in stationary phase. The sporulation frequencies of the mutants ranged from $10^{-7}$ to $10^{-2}$. Macrolide, lincosamide and streptogramin B resistance (MLS') of the mutants cotransduced 100%, with the sporulation defect and all four mutations mapped near trp by transduction in the order: trp-hisH-spo. All four mutants isolated were defective early in sporulation since β-galactosidase could be detected between zero and 2 h after the end of exponential growth. Electron microscopy of two of the mutants expressing the enzyme at t₁-t₂ revealed a defect prior to or just at the beginning of septum formation in one, and after completion of septum formation in the other. Little or no synthesis of dipicolinic acid, glucose dehydrogenase or alkaline phosphatase was detected in the mutants, but each was neutral protease positive. These results show that the mutations were in at least two early genes expressed before glucose dehydrogenase production. This study represents the first genetic characterization of sporulation mutants in B. megaterium and also demonstrates that gene fusion technology can be used in this species.

Introduction

Sporulation in Bacillus is a complex cell differentiation event involving over 50 operons (Losick et al., 1986; Piggot & Hoch, 1985). The genes that are specifically required for endospore formation are called spo genes and, when mutated, arrest development at one of six morphologically defined stages (stage 0, II, III, etc.) without significantly affecting the vegetative phase of growth. The genes are designated as spo0, spoII, spoIII, etc. Several laboratories have used the presence or absence of sporulation-specific enzymes and compounds synthesized at defined stages in sporulation to help identify the stages at which those mutants are blocked (Losick et al., 1986; Sandman et al., 1987). The sporulation genes have been studied most extensively in Bacillus subtilis and many of them have been cloned (see review by Mandelstam & Errington, 1987).

Because Bacillus megaterium sporulates more efficiently than most Bacillus spp. (Millet & Aubert, 1969), it has been the species of choice among biochemists for the biochemical characterization of the sporulation process. For example, the spore has been examined for its protein-synthesizing systems (Deutscher et al., 1968), for several electron-transport enzymes (Crafts-Lighty & Ellar, 1980; Wilkinson & Ellar, 1975) and for its membrane characteristics by electron spin resonance (Janoff et al., 1979). Sporulating cells have been tested for several enzymes (Chatelain, 1975), fatty acids (Scandella & Kornberg, 1969), polyamines (Setlow, 1974), proteases (Chaloupka et al., 1982; Setlow, 1975), electron transport (Wilkinson & Ellar, 1975), calcium accumulation (Bronner & Freund, 1972; Hogarth & Ellar, 1978), dipicolinic acid (DPA) (Bach & Gilvarg, 1966), coat protein synthesis (Imagawa et al., 1985) and penicillin-binding proteins (Todd & Ellar, 1982). Several small acid-soluble proteins (SASPs) and their specific protease have also been isolated, cloned and characterized by Setlow and co-workers. These SASPs have been shown to interact with DNA and to provide an important source of amino acids during germination and outgrowth (Fliss et al., 1985; Fliss & Setlow, 1985). However, it has been difficult to progress very far in the analysis of sporulation in B. megaterium without methods of genetic exchange.

In the last few years, our laboratory has developed genetic systems for B. megaterium and has mapped over 45 genes into linkage groups covering approximately...
70% of the chromosome (English & Vary, 1986; Sussman et al., 1988; Vary, 1979; Vary & Tao, 1988; S. L. Palm & P. S. Vary, unpublished). Although the sporulation process has been studied extensively in this species (Chatelain, 1975; Ellar et al., 1967, 1975; Ellar & Posgate, 1974; Greene et al., 1971), only a few spo mutants have been reported. Several DPA mutants that could not sporulate were analysed for their accumulation of calcium by Ota (1980), and spo mutants were analysed for nutritional defects by Chatelain & Fargette (1976) and Kretschmer & Fielder (1974). Coatless mutants have been isolated by Koshikawa et al. (1984) and Imagawa et al. (1985). We have also previously reported the isolation of several sporulation-defective mutants by insertional mutagenesis in this species.

In B. subtilis, a genetic tool that has greatly facilitated the genetic analysis of sporulation has been the transposon Tn917 originally derived from Streptococcus faecalis (Tomich et al., 1980). This transposon has been modified by Youngman and coworkers in a series of constructions to generate derivatives that can be used for insertion mutagenesis, for constructing gene fusions to lacZ and/or cat (chloramphenicol acetyltransferase), and for cloning (Youngman et al., 1985a, b, 1989). Sandman et al. (1987) have demonstrated the usefulness of Tn917 in studying sporulation by isolating and characterizing 21 new spo mutants in B. subtilis, among which nine represented new loci. Bohall & Vary (1986) introduced Tn917 into B. megaterium and found that it transposed randomly without hotspots. Of the mutants isolated, approximately 1% were spo mutants. The use of transposition should now facilitate progress toward isolating and characterizing sporulation mutants in B. megaterium. This paper reports the construction of a Lac- strain and the use of lacZ fusion technology to isolate and characterize four spo::lacZ-cat fusion mutants.

### Methods

**Strains, plasmids and media.** All B. megaterium strains were derived from strain QM B1551 (ATCC 12872) and are listed in Table 1. B. subtilis mutants IS9 (trpC2 pheA1 spoA12), IS65 (metC3 tal-1 spoIV F88) and IS66 (metC3 spo-B2 spoVC285) were obtained from the Bacillus Genetic Stock Center, Ohio State University, USA. Strain PY332 (trpC2 thrA5), carrying pTV53, was kindly provided by Dr Philip Youngman (Department of Microbiology, University of Pennsylvania School of Medicine, USA). Plasmid pTV53 carries resistance to tetracycline (Tc') and is temperature sensitive for replication (Youngman et al., 1985a). It also carries transposon Tn917 into which promoterless tandem lacZ and cat genes, preceded by a Bacillus ribosome-binding site, have been inserted without interference with transposition or resistance to macrolides, lincosamides and streptogramin B; p-7, cured of all seven resident plasmids; tet, tetracycline resistance; lacZ, promoterless gene of β-galactosidase; lac, lactose; spo, sporulation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM B1551</td>
<td>Wild-type</td>
<td>J.C. Vary†</td>
</tr>
<tr>
<td>PV3</td>
<td>hisH2I</td>
<td>Callahan et al. (1983)</td>
</tr>
<tr>
<td>PV195</td>
<td>trpB3 pheA2</td>
<td>This study</td>
</tr>
<tr>
<td>PV415</td>
<td>p-7 lac-3</td>
<td>This study</td>
</tr>
<tr>
<td>PV447</td>
<td>p-7 lac-3 lac-6</td>
<td>This study</td>
</tr>
<tr>
<td>PV469</td>
<td>p-7 lac-3 lac-6/pTV53 tet Tn917-lacZ-cat</td>
<td>This study</td>
</tr>
<tr>
<td>PV497</td>
<td>p-7 lac-3 lac-6 spo-54::Tn917-lacZ-cat</td>
<td>This study</td>
</tr>
<tr>
<td>PV503</td>
<td>spo-54::Tn917-lacZ-cat</td>
<td>This study</td>
</tr>
<tr>
<td>JV99</td>
<td>trpE4</td>
<td>J. C. Vary</td>
</tr>
<tr>
<td>JV62</td>
<td>trpE7 rpsL3</td>
<td>J. C. Vary</td>
</tr>
<tr>
<td>JV109</td>
<td>rib-2</td>
<td>J. C. Vary</td>
</tr>
<tr>
<td>PV517</td>
<td>spo-56::Tn917-lacZ-cat</td>
<td>This study</td>
</tr>
<tr>
<td>PV518</td>
<td>spo-57::Tn917-lacZ-cat</td>
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<tr>
<td>PV519</td>
<td>spo-58::Tn917-lacZ-cat</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Genotype abbreviations: cat, chloramphenicol resistance; Tn917, transposon Tn917; MLS', resistant to macrolides, lincosamides and streptogramin B; p-7, cured of all seven resident plasmids; tet, tetracycline resistance; lacZ, promoterless gene of β-galactosidase; lac, lactose; spo, sporulation.

† J. C. Vary, University of Illinois School of Medicine at Chicago.
dehydrogenase was assayed during growth and stationary phase by the method of Sadoff et al. (1986). One unit of enzyme was defined as the amount necessary to convert 1 nmol substrate min$^{-1}$.

Antibiotic production in B. megaterium was assayed by the method of Hill (1983) except that cells were first regenerative overnight on nonselective RHA plates, then replicated to SNB plates containing the selective antibiotic(s). After 12-24 h further incubation, transformants could be observed. Media, procedures for preparing lysates, and transductions with phage MP13 have been described previously (English & Vary, 1986) except that 6 g KH$_2$PO$_4$$L^{-1}$ was also added to the minimal glucose medium. Transductants appearing on minimal plates were picked to minimal medium with inducing Em. After 24-48 h incubation, colonies were replica plated to minimal medium and LB-MLS medium to determine cotransduction frequency. Mutagenesis by nitrosoguanidine and by UV irradiation have been described previously (English & Vary, 1986).

Selection of transposon insertions. To isolate transposition mutants, a plate method using a single incubation at 47°C was used as follows. Strain PV469 carrying pTV53 was streaked on an LB-Tc-Em plate and was incubated overnight. A loopful of the culture was inoculated into 3 ml LB-MLS-Tc broth with aeration. When the OD$_{660}$ reached 0.5-0.6, cells were diluted and spread onto LB-MLS plates and incubated at 30°C and 47°C so that transposition frequencies could be calculated as previously described (Bohali & Vary, 1986). After 36-48 h incubation, the 47°C plates were replica plated to SNB-MLS plates containing 60 µg X-Gal ml$^{-1}$, and were allowed to sporulate for 3 d at 30°C. Deep-blue colonies were picked to SNB-MLS plates, incubated overnight, then replica plated to fresh SNB-MLS plates. After 8-12 h incubation, colonies were replicated to SNB-MLS plates plus or minus Cm to test Cm sensitivity (Cm$^{-}$), and to SNB-MLS-Tc plates to test for plasmid curing (Tc$^{-}$). The Cm$^{-}$/Tc$^{-}$ colonies were purified, incubated for 3 d and examined microscopically for absence of refractive spores. Sporulation frequencies were tested by growing cultures with shaking at 30°C for 24 h, diluting and plating both heated (80°C, 10 min) and unheated cultures and calculating no. of viable colonies (heated)/no. of viable colonies (unheated) relative to the wild-type control.

Electron microscopy. Cultures were incubated with shaking for 24 h and harvested. The method of Hirsh & Fedorko (1968) was used to simultaneously fix the cells in 2.5% (v/v) glutaraldehyde and 2% (w/v) osmium tetroxide and to embed them in a Mollenhauer-Epon Araldite Resin mixture. The cells were sectioned with a Sorval 'Porter Blum' ultramicrotome, stained with 2% (w/v) uranyl acetate and counterstained with 0.2% (w/v) lead citrate. The preparations were viewed with an Hitachi HS-9 transmission electron microscope operated at 75 kV.

Enzyme and antibiotic assays. Cells were prepared and assayed for β-galactosidase by the method of Miller (1972) as modified by Errington & Mandelstam (1986) except that the cell pellet was resuspended in 0.6 ml Z buffer instead of 1 ml. Dipicolinic acid (DPA) was assayed as described by Janssen et al. (1998) and modified by Rotman & Fields (1968). Protease was assayed on SNB plates containing 1% (w/v) skim milk. Following cell growth in SNB plus 5 mM-potassium phosphate (pH 7.0), to inhibit possible vegetative alkaline phosphatase, alkaline phosphatase was assayed by the method of Hulett & Jensen (1988). One unit of alkaline phosphatase was defined as the amount of enzyme necessary to hydrolyse 1 nmol p-nitrophenyl phosphate min$^{-1}$. The specific activity was expressed as units per OD$_{660}$ unit. Glucose dehydrogenase was assayed during growth and stationary phase by the method of Sadoff et al. (1965) except that NADH was used as a standard. One unit of enzyme was defined as the amount necessary to convert 1 nmol substrate min$^{-1}$. The cells were collected at various times, spun down, quick-frozen in dry ice/ethanol, and stored at $-70°C$ until assayed. The enzyme was released by sonicating cells using the method of Hill (1983) except that 40 ml sample was used and sonication was applied 15 times (30 s each time) at 38 W with a microtip probe on a Vibra Cell ‘VC 250’ ultrasonic processor. Antibiotic production in B. megaterium was assayed by the method of Sandman et al. (1987) using B. subtilis spo0A and three of the B. megaterium early spo mutants as sensitive overlay strains.

Results

Isolation of a Lac$^{-}$ strain and transformation of pTV53

Strain QM B1551 has been cured of all seven plasmids to yield strain PV361 (Vary & Tao, 1988). To isolate a Lac$^{-}$ derivative, PV361 was then mutagenized by nitrosoguanidine and five colonies were found that could not grow on minimal lactose, yet were still blue on X-Gal. One of these colonies, PV415, was then spread on X-Gal plates, exposed to UV light and screened for white colonies. Out of several hundred colonies screened, four white colonies were isolated and purified. One of them was designated as PV447. Plasmid pTV53, isolated from B. subtilis PS332, was then used to transform B. megaterium PV447 protoplasts with selection for tetracycline-resistance. A pTV53-containing transformant was purified and designated as PV469. The presence of the plasmid was verified by erythromycin induction, selection for MLSR and visualization of the plasmid by agarose gel electrophoresis (data not shown).

Isolation of insertion mutants spo

Transposition experiments were done using PV469 as described in Methods. The average transposition frequency was $8.9 \times 10^{-4}$. In Tn917-lacZ-cat, a promoterless lacZ gene and a promoterless cat gene are arranged as a tandem pair without a transcriptional terminator between them. Thus, insertions that activate the transposon-borne lacZ-cat genes late in cell growth would give blue colonies on X-Gal plates, but would be Cm$^{+}$ during exponential growth (Youngman et al., 1985a). In each transposition experiment when deep-blue colonies were picked, about 80% were found to be cured of the plasmid (Tc$^{-}$). These colonies were tested for Cm$^{+}$ during exponential growth, then were screened by phase microscopy for inability to sporulate. The spo mutants represented about 15% of the deep-blue Cm$^{+}$ colonies.

Mutants spo-56 : Tn917-lacZ-cat and spo-57 : Tn917-lacZ-cat were from the same transposition experiment, but were retained because of differences in expression of β-galactosidase. The sporulation frequency of spo-54 : Tn917-lacZ-cat was $5 \times 10^{-6}$ in the Lac$^{-}$ strain, but was $1 \times 10^{-3}$ in the wild-type strain. The frequencies of sporulation (after transfer to the wild-type strain) for spo-56 : Tn917-lacZ-cat, spo-57 : Tn917-lacZ-cat and spo-58 : Tn917-lacZ-cat were $1 \times 10^{-2}$, $6 \times 10^{-7}$ and $8 \times 10^{-7}$, respectively.
During preliminary tests, it was shown that the Lac− strain PV447 sporulated more slowly than the wild-type (data not shown), so that the timed expression of a sporulation gene could not be precisely studied in this background. Therefore, all four mutations were transduced into wild-type QM B1551 selecting for MLSr to generate isogenic strains. Phage and cells were plated on SNB with inducing Em and incubated overnight. The confluent growth was replica plated to SNB-MLS plates. Colonies appeared after 48 h only on the test plates, not on controls to which no phage was added. Transductants grew on minimal medium without glucose and sodium citrate, but with 0.2% lactate and 25 mM-glutamate, and so were not TCA cycle mutants (Szulmajster & Hanson, 1965). Cotransduction of each of the spo mutations with MLSr was 100%.

Electron microscopy

When the mutant containing spo-54::Tn917-lacZ-cat (PV503) was viewed by electron microscopy, it was found to be blocked at the onset of septum formation (see Fig. 1a). Rare cells (<1%) were observed that had slight indentations of septa (Fig. 1b). Mutant spo-58::Tn917-lacZ-cat (PV519), in contrast, was able to complete septum formation, although some aberrant septa were also observed (see Fig. 1c). It is now designated as a late spoII mutant.

β-Galactosidase and DPA production

Since transcription of the promoterless lacZ gene on Tn917 should be under the control of the spo promoters, spo gene expression could be monitored by the assay of β-galactosidase activity. To determine when a spo gene was
B. megaterium spo insertion mutants

Fig. 2. Measurement of β-galactosidase activity and DPA of wild-type and PV503. Filled symbols represent the OD₆₆₀ of QM B1551 (■) and PV503 (▲). Open symbols represent the levels of DPA in QM B1551 (□) and PV503 (△). Dashed lines represent the β-galactosidase activities of QM B1551 (□—□) and of PV503 minus that of QM B1551 (△—△). The time when intracellular refractile spores of QM B1551 are first observed (<1%) is shown at A.

Fig. 3. Comparison of expression of β-galactosidase in all four spo::Tn917-lacZ-cat mutants. ■, QM B1551; ○, spo-54; △, spo-56; ▲, spo-57; □, spo-58.

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Enzyme assays

Synthesis of several enzymes such as alkaline phosphatase and glucose dehydrogenase begins during specific stages of sporulation in B. subtilis (Waines et al., 1970) and their synthesis has been used to help delineate mutants blocked at specific stages (Mandelstam & Errington, 1987; Sandman et al., 1987). Expression of these enzymes was tested in wild-type B. megaterium and compared to one of the mutants as shown in Fig. 4. Alkaline phosphatase was detectable in the wild-type at t₃–t₄, glucose dehydrogenase at t₄. Mutant spo-54 produced little or no alkaline phosphatase (Fig. 4b) and only 19% of the glucose dehydrogenase of wild-type (Fig. 4a). The data in Table 2 show that none of the four mutants produced significant amounts of either enzyme.

Assays for antibiotic and protease production and motility

Mutations in most spo0 genes in B. subtilis also prevent motility and production of extracellular proteases and antibiotics (Losick et al., 1986; Sandman et al., 1987). These effects were also tested in B. megaterium wild-type and the early sporulation mutants. Antibiotic production
Table 2. Assay of glucose dehydrogenase at $t_5$, alkaline phosphatase at $t_5$ and DPA after 24 h incubation, of wild-type and mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>GDH*</th>
<th>AP†</th>
<th>DPA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM B1551</td>
<td>17.26</td>
<td>3.51</td>
<td>120.9</td>
</tr>
<tr>
<td>PV503 (spo-54)</td>
<td>3.3</td>
<td>0.30</td>
<td>11.4</td>
</tr>
<tr>
<td>PV517 (spo-56)</td>
<td>2.34</td>
<td>0.28</td>
<td>9.0</td>
</tr>
<tr>
<td>PV519 (spo-57)</td>
<td>1.68</td>
<td>0.13</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Glucose dehydrogenase, nmol min$^{-1}$.
† Alkaline phosphatase, nmol min$^{-1}$ per OD$_{600}$ unit.
‡ Dipicolinic acid, µg (ml culture)$^{-1}$.

Table 2 was tested by overlaying an antibiotic-sensitive *B. subtilis* spoOA strain onto *B. megaterium* wild-type, PV503, and positive controls of *B. subtilis* spoIVF and spoVC. Only the late spo mutants of *B. subtilis* inhibited spoOA growth (data not shown). Since *B. megaterium* might produce an antibiotic not active on the spoOA mutant, all four mutants which expressed β-galactosidase very early were used as the sensitive overlay strains over *B. megaterium* wild-type and *B. subtilis* spoOA, spoIVF and spoVC strains. Again, the *B. megaterium* mutants were inhibited only by the *B. subtilis* spoIVF and spoVC strains.

When the wild-type strain and the fusion mutants were tested for protease production, all were found to be positive. The extracellular protease of QM B1551 is completely inhibited by EDTA (data not shown) and therefore is probably the single neutral, or metallo-protease reported for other strains of *B. megaterium* (Chaloupka et al., 1982; Millet & Aubert, 1969; Priest, 1977). Indeed, we have isolated a mutant that produces no detectable extracellular proteases on milk plates (D. A. Lach & P. S. Vary, unpublished data). When the supernatants of cultures of wild-type and spo mutants produced at different stages of growth were dropped on SNB + 1% skim milk plates, a zone of clearing was seen from $t_1$ to $t_6$ in both wild-type and fusion mutants.

Motility was measured by stabbing wild-type and PV503 into SNB containing 0.25% agar, and also by microscopic observation. There was only weak motility in all strains tested, with no evident differences.

Mapping of the insertional mutations

Since a transposon insertion gives the mutated gene an easily selectable marker, it was possible to map the spo loci by screening for MLSR cotransductants. A mapping strain kit of 12 strains was used to test for cotransduction (Vary & Tao, 1988). As shown in Table 3 and Fig. 5, all four of the mutant loci could be mapped in the trp-his region of the chromosome. As none were linked to rib, they probably lie on the rib-distal side of hisH. All four mutant loci have also been detected on the same 339 kb NorI fragment as trp and hisH by using pulse gel electrophoresis and probing NorI digests of mutants and wild-type with an internal fragment of Tn917 (W. B. Muse & P. S. Vary, unpublished data).

Discussion

In this study, we report the first isolation and characterization of spo::lacZ fusion mutants in *B. megaterium* using the Tn917-lacZ-cat transposon. Tn917-lacZ-cat transposed efficiently and randomly at a slightly higher frequency than the unmodified transposon. The extent of curing of the plasmid by elevated temperature could also be monitored as in *B. subtilis* (Youngman et al., 1985a) by the intensity of the blue colour of colonies on X-Gal.
B. megaterium spo insertion mutants

Fig. 5. The genetic linkages of the spo mutations with various loci. Linkages are expressed as 100% minus the percentage cotransduction. The hisH trp cotransduction data are from Callahan et al. (1983). Numbers in parentheses are values obtained by the formula of Kemper (1974) and correlate more closely to actual physical distances.

Table 3. Mapping of spo : : Tn917-lacZ-cat mutations

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Cotransduction*</th>
<th>Cotransduction frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV497 (spo-54)</td>
<td>PV195 (trpB3)</td>
<td>MLSr/Trp* 19/716 (3)</td>
<td>2.7</td>
</tr>
<tr>
<td>PV517 (spo-56)</td>
<td>JVS9 (trpE4)</td>
<td>MLSr/Trp* 3/345 (1)</td>
<td>0.9</td>
</tr>
<tr>
<td>PV518 (spo-57)</td>
<td>PV195</td>
<td>MLSr/Trp* 6/235 (2)</td>
<td>2.6</td>
</tr>
<tr>
<td>PV519 (spo-58)</td>
<td>PV195</td>
<td>MLSr/Trp* 4/304 (2)</td>
<td>1.3</td>
</tr>
<tr>
<td>PV503 (spo-54)</td>
<td>PV3 (hisH21)</td>
<td>MLSr/His* 2/99 (1)</td>
<td>2.0</td>
</tr>
<tr>
<td>PV497 (spo-54)</td>
<td>PV3</td>
<td>MLSr/His* 2/398 (2)</td>
<td>0.5</td>
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<tr>
<td>PV517 (spo-56)</td>
<td>PV3</td>
<td>MLSr/His* 5/237 (2)</td>
<td>2.1</td>
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<td>MLSr/His* 0/262 (2)</td>
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<td>MLSr/His* 1/210 (2)</td>
<td>&lt;0.5</td>
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<tr>
<td>JV62 (trpE7)</td>
<td>JV109 (rib-2)</td>
<td>Trp*/Rib* 22/805 (4)</td>
<td>2.7</td>
</tr>
<tr>
<td>PV497 (spo-54)</td>
<td>JV109</td>
<td>MLSr/Rib* 0/704 (2)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PV517 (spo-56)</td>
<td>JV109</td>
<td>MLSr/Rib* 0/177 (1)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PV518 (spo-57)</td>
<td>JV109</td>
<td>MLSr/Rib* 0/177 (1)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PV519 (spo-58)</td>
<td>JV109</td>
<td>MLSr/Rib* 0/177 (1)</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* Cotransduction frequencies are reported as no. of unselected/no. of selected colonies. Numbers in parentheses denote the number of experiments.

plates. Since the plasmidless derivative of QM B1551 was blue on X-Gal plates, it was necessary to isolate a Lac- strain, which required a two-step mutagenesis. Whether both mutations were in the same gene is not known. Landman (1957) reported that wild-type B. megaterium, unlike B. subtilis, contains a β-galactosidase that is induced similarly to that of Escherichia coli, but is more unstable. By using the Lac- strain to isolate spo : : lacZ-cat fusions, it was demonstrated that the transposon fusion method worked well in B. megaterium. The transposon not only facilitated the isolation of spo mutants, but also provided a simple way to study the kinetics of gene expression in the mutants by monitoring β-galactosidase activity. The fusion mutations were then transferred to a completely prototrophic background to test for the onset of gene expression in isogenic strains. The 100% cotransduction of MLS resistance with the Spo- phenotype and the correlation between the microscopically determined stage and the onset of expression of the lacZ gene in spo-54 strongly suggests that the transposon was within, or very closely linked to the spo gene. By electron microscopy, spo-54 was shown to be either a spo0 or a spo1 mutant since some cells were observed with slightly indented asymmetric septa. Yamamoto & Balassa (1969) described a similar mutant, CA15, in B. subtilis. However, their mutant showed a mixture of cells with no septa, abortive septa (spikes) as well as completed septa. They designated it as an early
**spoII** mutant. Chatelain & Fargette (1976) briefly described a mutant of *B. megaterium* KM that is very similar to *spo-54* and they designated it as a *spoI* mutant. *SpoI* mutants have not been isolated in *B. subtilis*, so the designation has been dropped. However, now at least two mutants have been isolated in two different backgrounds of *B. megaterium* (QM B1551 and KM) that seem to be blocked just before or at the start of septation. These mutants may be very useful in determining what gene product(s) is necessary for the initiation or elongation of asymmetric septation. Mutant *spo-58*, in contrast, was shown to complete septum formation and so is blocked at stage II. Mutants carrying *spo-56* and *spo-58* fusions expressed the β-galactosidase at or just before *t₀*. The mutant carrying the *spo-57* fusion expressed the enzyme weakly, starting at *t₁*, and may be similar to *spo-54*. However, the electron microscopy and β-galactosidase assays have revealed that mutations in at least two genes have probably occurred among the four mutants characterized.

Various enzymes and compounds that are expressed at specific times during sporulation in *B. subtilis* (Waites et al., 1970) were tested in *B. megaterium* QM B1551 to establish the kinetics of expression in this strain and to determine whether they might be used to help distinguish mutants blocked at specific stages during our characterization of the fusion mutants. In *B. subtilis*, alkaline phosphatase is expressed at *t₂*, glucose dehydrogenase at *t₃* and DPA between *t₄* and *t₅* (Waites et al., 1970). Production of alkaline phosphatase can be used to delineate some *spoII* mutants (Sandman et al., 1987) if care is taken to grow cells in the presence of a high phosphate concentration in order to inhibit the vegetative alkaline phosphatase produced at the beginning of stationary phase (Hulett & Jensen, 1988). In SNB broth plus 5 mM-phosphate, *B. megaterium* wild-type did not begin to produce alkaline phosphatase until *t₃*—*t₄*, but all four mutants failed to produce this enzyme. Chatelain (1975) had previously reported production of alkaline phosphatase in *B. megaterium* strain KM at *t₀*, but the experiment was done in medium containing no inorganic phosphate, so he may have measured the vegetative enzyme. Interestingly, he observed no enzyme activity even at *t₀* in high phosphate. Our results suggest that alkaline phosphatase is produced 1–2 h later in *B. megaterium* than in *B. subtilis* and cannot be used to delineate earlier *spo* mutants. Glucose dehydrogenase, on the other hand, is produced in both species at about *t₄*. In agreement with our results, Chatelain (1975) also reported detection of glucose dehydrogenase between *t₃* and *t₅*. DPA was detectable at *t₃*—*t₅* in the whole cells used in this study, consistent with the results of Singh & Setlow (1979), who could detect DPA in isolated forespores at about *t₄*—*t₅*, and La Nauze et al. (1974), who reported that DPA was produced during stage IV. The inability of the *spo* fusion mutants to produce alkaline phosphatase, glucose dehydrogenase or DPA confirmed that each mutant was blocked at a stage prior to their expression, i.e. before *t₃*.

Mutations in some *spo* genes in *B. subtilis* cause pleiotropic effects such as inhibition of production of antibiotic and proteases, and the development of motility and competency (Kawamura et al., 1985; Losick et al., 1986; Sandman et al., 1987). It was found during our study that both the mutant and wild-type strains of *B. megaterium* produced comparable levels of neutral protease. Millet & Aubert (1969) reported that production of neutral protease in strain KM begins at the end of exponential growth in complex medium, but throughout vegetative growth in minimal medium. We also found that when grown in SNB, QM B1551 began to produce protease between *t₀* and *t₁*, as has also been observed by Setlow (1974) for QM B1551. No correlation of protease production with sporulation was found by Chatelain (1975), Chatelain & Fargette (1976) or Millet & Aubert (1969) and no other extracellular protease has been reported for this species. The observation that all four *spo* mutants were protease positive was, therefore, consistent with previously reported results.

Other parameters considered were antibiotic production, competency and motility. No transformation competency has been detected in wild-type *B. megaterium* (P. S. Vary, unpublished data) and motility was poor, so these parameters could not be used to characterize mutants. Moreover, production of an antibiotic could not be demonstrated, although some strains of *B. megaterium* produce them. Either *B. megaterium* QM B1551 does not produce an antibiotic, or the early *spo* mutant strains we used were not sensitive to it. However, even wild-type *B. megaterium* was sensitive to the antibiotic produced by *B. subtilis*, while the antibiotic-sensitive *spo0A* mutant of *B. subtilis* exhibited no sensitivity to *B. megaterium*. However, these results do not completely rule out possible production of an antibiotic that does not affect *B. subtilis*.

Thus, we have shown that neutral protease production, competency or antibiotic production cannot be used to distinguish early sporulation mutants in *B. megaterium*. In contrast, glucose dehydrogenase and alkaline phosphatase production may in the future be useful in distinguishing some *spoIII* from *spoIV* mutants, and DPA production may differentiate some *spoIV* from *spoV* mutants. The production of these compounds during sporulation in QM B1551 permits the assay of new sporulation mutants.

Based upon the data presented, the fusion mutant *spo-54* has been tentatively designated as a *spoI* mutant, and the *spo-58* mutant as *spoII*. Mutants *spo-56* and *spo-57* are
expressed between $t_0$ and $t_1$, and may be $spol$ or $spolI$. The similarity of timing of the production of glucose dehydrogenase, DPA and intracellular refractile phase-bright spores in $B. megaterium$ and in $B. subtilis$ also suggests that our designation of $t_0$ is probably accurate to within 30 min.

The mutant loci were mapped using a recently constructed $B. megaterium$ mapping kit (Vary & Tao, 1988), and were all found to be in the $trp$-$his$H region previously mapped by Callahan et al. (1983) and recently extended (Vary & Tao, 1988; P. S. Vary & S. L. Palm, unpublished). The four mutants isolated were all in the same region and were all early $spo$ mutations. The two-factor crosses could not order the four mutations. Unfortunately, a marker to the 'right' of the $spo$ genes close enough for cotransduction is not yet available. Why only early genes in the same region is isolated is not known. Possibilities are that there was a selection bias in the intensity of expression (deep-blue colonies) or perhaps there is a cluster of early $spo$ genes near $trp$-$his$H in $B. megaterium$. Indeed, the low frequency of sporulation mutants recovered (15%) may also reflect a bias introduced by picking only deep-blue colonies since expression of early sporulation genes may have accumulated more of the 5-bromo-4-chloroindigo product of X-Gal. Alternatively, only genes with very strong promoters may have been selected and may be those in the $trp$-$his$ region. Much of the $\beta$-galactosidase production reported for $B. subtilis$ sporulation genes has been low. It is fairly certain that mutations $spoI54$ and $spoI158$ are in different early genes based upon both the timing of gene expression and electron microscopy. All four mutations expressed at consistently different intensities (with $spo$-I exhibited very strong expression) and at least 1-2 h apart. In $B. subtilis$, the $spoA$ locus is at 218°. There are two $spoII$ mutations near the $trp$-$his$H region, $spoIIA$ and $spoIIM$ at 211° (Losick et al., 1986; Sandman et al., 1987). The $spoIIA$ locus is expressed at about $t_1$ in $B. subtilis$ and is composed of three genes (Errington & Mendelstam, 1986). To our knowledge, the time of expression of $spoIIM$ has not been reported. Since mapping of $spo$ genes has just begun in $B. megaterium$, it is not known how the positions of $spo$ genes on the chromosome will correlate with positions on $B. subtilis$. However, we have recently found that $spol$ genes homologous to those in $B. subtilis$ are more conserved in position than surrounding genes (Sussman et al., 1988). Whether that conservation of position will hold true with other $spo$ genes should be of interest in understanding the evolution of the sporulation process in $Bacillus$

Moreover, the assay of several sporulation compounds and enzymes in both mutant and wild-type strains has helped establish parameters for the characterization of future $spo$ mutants in this species.

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