Isolation and characterization of sporulation *lacZ* fusion mutants of *Bacillus megaterium*

YI-PING TAO and PATRICIA S. VARY*

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

(Received 2 July 1990; revised 9 November 1990; accepted 12 December 1990)

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^r) 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

Abbreviation: DPA, dipicolinic acid.

Table 1. *B. megaterium* strains used in this study

Strain	Genotype*	Source or reference
QM B1551	Wild-type	J.C. Vary†
PV3	<i>hisH21</i>	Callahan <i>et al.</i> (1983)
PV195	<i>trpB3 pheA2</i>	This study
PV415	<i>p⁻⁷ lac-3</i>	This study
PV447	<i>p⁻⁷ lac-3 lac-6</i>	This study
PV469	<i>p⁻⁷ lac-3 lac-6/pTV53 tet Tn917-lacZ-cat</i>	This study
PV497	<i>p⁻⁷ lac-3 lac-6 spo-54::Tn917-lacZ-cat</i>	This study
PV503	<i>spo-54::Tn917-lacZ-cat</i>	This study
JV59	<i>trpE4</i>	J. C. Vary
JV62	<i>trpE7 rpsL3</i>	J. C. Vary
JV109	<i>rib-2</i>	J. C. Vary
PV517	<i>spo-56::Tn917-lacZ-cat</i>	This study
PV518	<i>spo-57::Tn917-lacZ-cat</i>	This study
PV519	<i>spo-58::Tn917-lacZ-cat</i>	This study

* Genotype abbreviations: *cat*, chloramphenicol resistance; Tn917, transposon Tn917; MLS^r, 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.

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 inactivation (Bohall & Vary, 1986), but to our knowledge, no sporulation mutants have been genetically characterized 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* mutations 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 spo0A12*), IS65 (*metC3 tal-1 spoIVF88*) and IS66 (*metC3 rpo-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^r) 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 (MLS^r) (Youngman *et al.*, 1985a). Luria-Bertani (LB) medium (Levine, 1957) was used in transposition experiments, and SNB medium (English & Vary, 1986) was used for routine growth, sporulation, and preparation of phage lysates. Antibiotic concentrations were 10 µg tetracycline (Tc) ml⁻¹, 0.15 µg erythromycin (Em) ml⁻¹ for induction, 5 µg erythromycin ml⁻¹ and 250 µg lincomycin (MLS) ml⁻¹ for transposon selection, and 3.5 µg chloramphenicol (Cm) ml⁻¹ for selection of fusions. Cultures were grown at 30 °C with shaking unless otherwise indicated.

Plasmid isolation, transformation, transduction and mutagenesis. Plasmid DNA was isolated by the method of Kawamura *et al.* (1985). For transformations, the PEG-mediated protoplast method of Von

Tersch & Carlton (1983) was used except that cells were first regenerated overnight on nonselective RHAF 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 $\text{KH}_2\text{PO}_4 \text{ l}^{-1}$ 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 (Bohall & Vary, 1986). After 36–48 h incubation, the 47 °C plates were replica plated to SNB-MLS plates containing 60 $\mu\text{g X-Gal ml}^{-1}$, 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^s), and to SNB-MLS-Tc plates to test for plasmid curing (Tc^c). The $\text{Cm}^s \text{ Tc}^c$ 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 Hirsch & 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 0.8 ml Z buffer instead of 1 ml. Dipicolinic acid (DPA) was assayed as described by Janssen *et al.* (1958) 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 Celler '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* PY332, 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 MLS^r and visualization of the plasmid by agarose gel electrophoresis (data not shown).

Isolation of insertion spo mutants

Transposition experiments were done using PV469 as described in Methods. The average transposition frequency was 8.9×10^{-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^s 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^c). These colonies were tested for Cm^s during exponential growth, then were screened by phase microscopy for inability to sporulate. The *spo* mutants represented about 15% of the deep-blue Cm^s 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 5×10^{-6} in the Lac⁻ strain, but was 1×10^{-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 1×10^{-2} , 6×10^{-7} and 8×10^{-7} , respectively.

Fig. 1. Electron micrographs of PV503 and PV519. (a) PV503 showing most cells blocked before septum formation; (b) PV503 with early aborted septa (arrows) occurring in about 1% of the cells. (c) PV519 showing block at stage II at completion of septum formation and presence of aberrant septa. Bars, 1 μ m.

Transduction of the *spo* fusion mutants.

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 MLS^r 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 MLS^r 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

Fig. 2. Measurement of β -galactosidase activity and DPA of wild-type and PV503. Filled symbols represent the OD_{660} 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.

expressed in relation to DPA synthesis and appearance of refractile spores, *spo-54::Tn917-lacZ-cat* and QM B1551 were grown in SNB as shown in Fig. 2. Deviation from exponential growth was designated as t_0 . It can be seen that, although QM B1551 grows on minimal-lactose plates and is blue on X-Gal plates, it expressed only about 5 units of β -galactosidase activity. The net enzyme activity of the *spo* fusion mutant began to increase at $t_{1.5}-t_2$. The production of DPA could be detected in the wild-type at $t_{5.5}-t_6$, when less than 1% intracellular refractile forespores were observed. DPA synthesis in the fusion mutant could not be detected. The time of expression of β -galactosidase in all four mutants is shown in Fig. 3.

Enzyme assays

Synthesis of several enzymes such as alkaline phosphatase and glucose dehydrogenase begins during specific stages of sporulation in *B. subtilis* (Waites *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 t_3-t_4 ; glucose dehydrogenase at t_4 . Mutant *spo-54* produced little or no alkaline phosphatase (Fig. 4b) and only 19% of the glucose dehydrogenase of wild-type (Fig.

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*.

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

Fig. 4. Production of alkaline phosphatase (AP) and glucose dehydrogenase (GDH) in the wild-type and in fusion mutant PV503. Filled symbols in (a) and (b) represent the OD₆₆₀ of QM B1551 (■) and PV503 (▲). Open symbols represent production of glucose dehydrogenase (a) and alkaline phosphatase (b) by QM B1551 (□) and PV503 (△) (see Methods for details).

Table 2. Assay of glucose dehydrogenase at *t*₆, alkaline phosphatase at *t*₅ and DPA after 24 h incubation, of wild-type and mutants

Strain	GDH*	AP†	DPA‡
QM B1551	17.26	3.51	120.9
PV503 (<i>spo-54</i>)	3.31	0.30	11.4
PV517 (<i>spo-56</i>)	2.34	0.28	9.0
PV518 (<i>spo-57</i>)	1.16	0.16	7.9
PV519 (<i>spo-58</i>)	1.68	0.13	10.4

* Glucose dehydrogenase, nmol min⁻¹.

† Alkaline phosphatase, nmol min⁻¹ per OD₆₆₀ unit.

‡ Dipicolinic acid, µg (ml culture)⁻¹.

was tested by overlaying an antibiotic-sensitive *B. subtilis* *spo0A* 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 *spo0A* growth (data not shown). Since *B. megaterium* might produce an antibiotic not active on the *B. subtilis* *spo0A* 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* *spo0A*, *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 metalloprotease 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 MLS^r 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 *NotI* fragment as *trp* and *hisH* by using pulse gel electrophoresis and probing *NotI* 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

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

Donor	Recipient	Cotransduction*	Cotransduction frequency (%)
PV497 (<i>spo-54</i>)	PV195 (<i>trpB3</i>)	MLS ^r /Trp ⁺ 19/716 (3)	2.7
	JV59 (<i>trpE4</i>)	MLS ^r /Trp ⁺ 3/345 (1)	0.9
PV517 (<i>spo-56</i>)	PV195	MLS ^r /Trp ⁺ 6/235 (2)	2.6
PV518 (<i>spo-57</i>)	PV195	MLS ^r /Trp ⁺ 4/304 (2)	1.3
PV519 (<i>spo-58</i>)	PV195	MLS ^r /Trp ⁺ 3/245 (2)	1.2
PV503 (<i>spo-54</i>)	PV3 (<i>hisH21</i>)	MLS ^r /His ⁺ 2/99 (1)	2.0
PV497 (<i>spo-54</i>)	PV3	MLS ^r /His ⁺ 2/398 (2)	0.5
PV517 (<i>spo-56</i>)	PV3	MLS ^r /His ⁺ 5/237 (2)	2.1
PV518 (<i>spo-57</i>)	PV3	MLS ^r /His ⁺ 0/262 (2)	<0.4
PV519 (<i>spo-58</i>)	PV3	MLS ^r /His ⁺ 1/210 (2)	0.5
JV62 (<i>trpE7</i>)	JV109 (<i>rib-2</i>)	Trp ⁻ /Rib ⁺ 22/805 (4)	2.7
PV497 (<i>spo-54</i>)	JV109	MLS ^r /Rib ⁺ 0/704 (2)	<0.1
PV517 (<i>spo-56</i>)	JV109	MLS ^r /Rib ⁺ 0/177 (1)	<0.6
PV518 (<i>spo-57</i>)	JV109	MLS ^r /Rib ⁺ 0/189 (1)	<0.5
PV519 (<i>spo-58</i>)	JV109	MLS ^r /Rib ⁺ 0/177 (1)	<0.6

* 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 t_0 . The mutant carrying the *spo-57* fusion expressed the enzyme weakly, starting at $t_{1.5}$ – t_2 , 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 t_2 , glucose dehydrogenase at t_3 and DPA between t_4 and t_5 (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 t_3 – t_4 , but all four mutants failed to produce this enzyme. Chatelain (1975) had previously reported production of alkaline phosphatase in *B. megaterium* strain KM at t_0 , 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 t_6 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 t_4 . In agreement with our results, Chatelain (1975) also reported detection of glucose dehydrogenase between t_3 and t_5 . DPA was detectable at $t_{5.5}$ 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 t_4 – t_5 , 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 t_3 .

Mutations in some *spo0* 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 t_0 and t_1 , 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 *spoI* or *spoII*. 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-hisH* 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 were 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-hisH* 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 β -galactosidase production reported for *B. subtilis* sporulation genes has been low. It is fairly certain that mutations *spoI54* and *spoII58* 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 *spo58* exhibiting very strong expression) and at least 1–2 h apart. In *B. subtilis*, the *spo0A* locus is at 218°. There are two *spoII* mutations near the *trp-hisH* 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 & Mandelstam, 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 *ssp* 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*.

In summary, the results demonstrate that the plasmid pTV53 carrying Tn917-*lacZ-cat* can be used as a fusion probe to identify *spo* mutants, map the *spo* loci and study the expression of their promoters in *B. megaterium*.

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

We are grateful to James Vary for providing the JV mutants, and to Philip Youngman for providing pTV53 and his continued advice and support. We thank Dian Molsen for assistance in electron microscopy, and Freyja Altepetter for typing the manuscript.

This work was supported by grant DMB 8607259 from the National Science Foundation.

References

- BACH, M. L. & GILVARG, C. (1966). Biosynthesis of dipicolinic acid in sporulating *Bacillus megaterium*. *Journal of Biological Chemistry* **241**, 4563–4566.
- BOHALL, N. A. & VARY, P. S. (1986). Transposition of Tn917 in *Bacillus megaterium*. *Journal of Bacteriology* **167**, 716–718.
- BRONNER, F. & FREUND, T. S. (1972). Calcium accumulation during sporulation of *Bacillus megaterium*. In *Spores V*, pp. 187–190. Edited by H. O. Halvorson, R. Hanson & L. L. Campbell. Washington, DC: American Society for Microbiology.
- CALLAHAN, J. P., CRAWFORD, I. P., HESS, G. F. & VARY, P. S. (1983). Cotransductional mapping of the *trp-his* region of *Bacillus megaterium*. *Journal of Bacteriology* **154**, 1455–1458.
- CHALOUPKA, J., SEVERIN, A. I., SASTRY, K. J., KUČEROVÁ, H. & STRNADOVÁ, M. (1982). Differences in the regulation of exocellular proteinase synthesis during growth and sporogenesis of *Bacillus megaterium*. *Canadian Journal of Microbiology* **28**, 1214–1218.
- CHATELAIN, S. (1975). Variations d'activités enzymatiques chez *Bacillus megaterium* dans différentes conditions de sporulation. *Comptes Rendus de l'Académie des Sciences, D* **281**, 1529–1532.
- CHATELAIN, S. & FARGETTE, F. (1976). Variations d'activités enzymatiques chez un mutant asporogène de *Bacillus megaterium* pour différentes carences nutritionnelles. *Comptes Rendus de l'Académie des Sciences, D* **283**, 1563–1566.
- CRAFTS-LIGHTY, A. & ELLAR, D. J. (1980). The structure of the outer membrane in dormant and germinated spores of *Bacillus megaterium*. *Journal of Applied Bacteriology* **48**, 135–145.
- DEUTSCHER, M. P., CHAMBER, P. & KORNBERG, A. (1968). Biochemical studies of bacterial sporulation and germination. XI. Protein-synthesizing systems from vegetative cells and spores of *Bacillus megaterium*. *Journal of Biological Chemistry* **243**, 5117–5125.
- ELLAR, D. J. & POSGATE, J. A. (1974). Characterization of forespores isolated from *Bacillus megaterium* at different stages of development into mature spores. In *Spore Research*, pp. 21–40. Edited by A. N. Barker, G. W. Gould & J. Wolf. New York: Academic Press.
- ELLAR, D. J., LUNDGREN, D. G. & SLEPECKY, R. A. (1967). Fine structure of *Bacillus megaterium* during synchronous growth. *Journal of Bacteriology* **94**, 1189–1205.
- ELLAR, D. J., EATON, M. W., HOGARTH, C., WILKINSON, B. J., DEANS, J. & LA NAUZE, J. (1975). Comparative biochemistry and function of forespore and mother-cell compartments during sporulation of *Bacillus megaterium* cells. In *Spores VI*, pp. 425–433. Edited by P. Gerhardt, R. N. Costilow & H. L. Sadoff. Washington, DC: American Society for Microbiology.
- ENGLISH, J. D. & VARY, P. S. (1986). Isolation of recombination defective and UV sensitive mutants of *Bacillus megaterium*. *Journal of Bacteriology* **165**, 155–160.
- ERRINGTON, J. & MANDELSTAM, J. (1986). Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2967–2976.
- FLISS, E. R. & SETLOW, P. (1985). Genes for *Bacillus megaterium* small, acid-soluble spore proteins: nucleotide sequence of two genes and their expression during sporulation. *Gene* **35**, 151–157.
- FLISS, E. R., CONNORS, M. J., LOSHON, C. A., CURIEL-QUESADA, E., SETLOW, B. & SETLOW, P. (1985). Small, acid-soluble spore proteins

- of *Bacillus*: products of a sporulation-specific, multigene family. In *Molecular Biology of Microbial Differentiation*, pp. 60–66. Edited by J. Hoch & P. Setlow. Washington, DC: American Society for Microbiology.
- GREENE, R. A., HOLT, S. C., LEADBETTER, E. R. & SLEPECKY, R. A. (1971). Correlation of light and electron microscopic observations of sporulation in *Bacillus megaterium*. In *Spore Research*, pp. 161–180. Edited by A. N. Barker, G. W. Gould & J. Wolf. London: Academic Press.
- HILL, S. H. A. (1983). *spoVH* and *spoVJ* – new sporulation loci in *Bacillus subtilis* 168. *Journal of General Microbiology* **129**, 293–302.
- HIRSCH, J. G. & FEDORKO, M. F. (1968). Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and 'post-fixation' in uranyl acetate. *Journal of Cell Biology* **38**, 615–627.
- HOGARTH, C. & ELLAR, D. J. (1978). Calcium accumulation during sporulation of *Bacillus megaterium* KM. *Biochemical Journal* **176**, 197–203.
- HULETT, F. M. & JENSEN, K. (1988). Critical roles of *spo0A* and *spo0H* in vegetative alkaline phosphatase production in *Bacillus subtilis*. *Journal of Bacteriology* **170**, 3765–3768.
- IMAGAWA, M., OKU, Y., EL-BELBASI, H. I., TEROAKA, M., NISHIHARA, T. & KONDO, M. (1985). Synthesis and deposition of spore coat proteins during sporulation of *Bacillus megaterium*. *Microbiology and Immunology* **29**, 1151–1162.
- JANOFF, A. S., COUGHLIN, R. T., RACINE, F. M., MCGROARTY, E. J. & VARY, J. C. (1979). Use of electron spin resonance to study *Bacillus megaterium* spore membranes. *Biochemical and Biophysical Research Communications* **89**, 565–570.
- JANSSEN, F. W., LUND, A. J. & ANDERSON, L. E. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**, 26–27.
- KAWAMURA, F., WANG, L.-F. & DOI, R. H. (1985). Catabolite-resistant sporulation (*crsA*) mutations in the *Bacillus subtilis* RNA polymerase σ^{43} gene (*rpoD*) can suppress and be suppressed by mutations in *spoO* genes. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8124–8128.
- KEMPER, J. (1974). Gene order and cotransduction in the *leu-ara-fol-pyrA* region of the *Salmonella typhimurium* linkage map. *Journal of Bacteriology* **117**, 94–97.
- KOSHIKAWA, T., BEAMAN, T. C., PANKRATZ, H. S., NAKASHIO, S., CORNER, T. R. & GERHARDT, P. (1984). Resistance, germination, and permeability correlates of *Bacillus megaterium* spores successfully divested of integument layers. *Journal of Bacteriology* **159**, 624–632.
- KRETSCHMER, S. & FIELDER, G. (1974). Septation sporogener und asporogener *Bacillus megaterium*-Zellen während des Übergangs zu Stickstoff- und Kohlenstoff-hunger. *Zeitschrift für Allgemeine Mikrobiologie* **14**, 303–312.
- LA NAUZE, J. M., ELLAR, D. J., DENTON, G. & POSGATE, J. A. (1974). Some properties of forespores isolated from *Bacillus megaterium*. In *Spore Research*, pp. 41–46. Edited by A. N. Barker, G. W. Gould & J. Wolf. New York: Academic Press.
- LANDMAN, O. E. (1957). Properties and induction of β -galactosidase in *Bacillus megaterium*. *Biochimica et Biophysica Acta* **23**, 558–569.
- LEVINE, M. (1957). Mutations in the temperate phage P22 and lysogeny in *Salmonella*. *Virology* **3**, 22–41.
- LOSICK, R., YOUNGMAN, P. & PIGGOT, P. J. (1986). Genetics of endospore formation in *Bacillus subtilis*. *Annual Review of Genetics* **20**, 625–669.
- MANDELSTAM, J. & ERRINGTON, J. (1987). Dependent sequences of gene expression controlling spore formation in *Bacillus subtilis*. *Microbiological Sciences* **4**, 238–244.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- MILLET, J. & AUBERT, J.-P. (1969). Étude de la mégateriopeptidase, protéase exocellulaire de *Bacillus megaterium*. III. Biosynthèse et rôle physiologique. *Annales de l'Institut Pasteur* **117**, 461–473.
- OTA, A. (1980). Calcium uptake by *Bacillus megaterium* mutant strains unable to sporulate. *International Journal of Biochemistry* **11**, 363–367.
- PIGGOT, P. J. & HOCH, J. A. (1985). Revised genetic linkage map of *Bacillus subtilis*. *Microbiological Reviews* **49**, 158–179.
- PRIEST, F. G. (1977). Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Reviews* **41**, 711–753.
- ROTMAN, Y. & FIELDS, M. L. (1968). A modified reagent for dipicolinic acid analysis. *Analytical Biochemistry* **22**, 168–172.
- SADOFF, H. L., BACH, J. A. & KOOLS, J. W. (1965). Significance of multiple forms of glucose dehydrogenase in relation to its heat resistance. In *Spores III*, pp. 97–109. Edited by L. L. Campbell & H. O. Halvorson. Ann Arbor, Michigan: American Society for Microbiology.
- SANDMAN, K., LOSICK, R. & YOUNGMAN, P. (1987). Genetic analysis of *Bacillus subtilis* *spo* mutations generated by Tn917-mediated insertional mutagenesis. *Genetics* **117**, 603–617.
- SCANDELLA, C. J. & KORNBERG, A. (1969). Biochemical studies of bacterial sporulation and germination. XV. Fatty acids in growth, sporulation and germination of *Bacillus megaterium*. *Journal of Bacteriology* **98**, 82–86.
- SETLOW, P. (1974). Polyamine levels during growth, sporulation, and spore germination of *Bacillus megaterium*. *Journal of Bacteriology* **117**, 1171–1177.
- SETLOW, P. (1975). Protease and peptidase activities in growing and sporulating cells and dormant spores of *Bacillus megaterium*. *Journal of Bacteriology* **122**, 642–649.
- SINGH, R. P. & SETLOW, P. (1979). Regulation of phosphoglycerate phosphomutase in developing forespores and dormant and germinated spores of *Bacillus megaterium* by the level of free manganous ions. *Journal of Bacteriology* **139**, 889–898.
- SUSSMAN, M. D., VARY, P. S., HARTMAN, C. & SETLOW, P. (1988). Integration and mapping of *Bacillus megaterium* genes which code for small, acid soluble spore proteins and their protease. *Journal of Bacteriology* **170**, 4942–4945.
- SZULMAJSTER, J. & HANSON, R. (1965). Physiological control of sporulation in *Bacillus subtilis*. In *Spores III*, pp. 162–173. Edited by L. L. Campbell & H. O. Halvorson. Ann Arbor, Michigan: American Society for Microbiology.
- TODD, J. A. & ELLAR, D. J. (1982). Alteration in the penicillin-binding profile of *Bacillus megaterium* during sporulation. *Nature, London* **300**, 640–643.
- TOMICH, P. K., AN, F. Y. & CLEWELL, D. B. (1980). Properties of erythromycin-inducible transposon Tn917 in *Streptococcus faecalis*. *Journal of Bacteriology* **141**, 1366–1374.
- VARY, P. S. (1979). Transduction in *Bacillus megaterium*. *Biochemical and Biophysical Research Communications* **88**, 1119–1124.
- VARY, P. S. & TAO, Y.-P. (1988). Development of genetic methods in *Bacillus megaterium*. In *Genetics and Biotechnology of Bacilli*, vol. 2, pp. 403–407. Edited by A. T. Ganesan & J. A. Hoch. New York: Academic Press.
- VON TERSCH, M. A. & CARLTON, B. C. (1983). Megacinogenic plasmids of *Bacillus megaterium*. *Journal of Bacteriology* **155**, 872–877.
- WAITES, W. M., KAY, D., DAWES, I. W., WOOD, D. A., WARREN, S. C. & MANDELSTAM, J. (1970). Sporulation in *Bacillus subtilis*. Correlation of biochemical events with morphological changes in asporogenous mutants. *Biochemical Journal* **118**, 667–676.
- WILKINSON, B. J. & ELLAR, D. J. (1975). Morphogenesis of the membrane bound electron-transport system in sporulating *Bacillus megaterium* KM. *European Journal of Biochemistry* **55**, 131–139.
- YAMAMOTO, T. & BALASSA, G. (1969). Biochemical genetics of bacterial sporulation. II. Membrane development during sporulation of *B. subtilis* and its mutants. *Molecular and General Genetics* **106**, 1–13.
- YOUNGMAN, P., PERKINS, J. B. & SANDMAN, K. (1985a). Use of Tn917-mediated transcriptional gene fusions to *lacZ* and *cat-86* for the identification and study of *spo* genes in *Bacillus subtilis*. In *Molecular Biology of Microbial Differentiation*, pp. 47–54. Edited by J. A. Hoch & P. Setlow. Washington, DC: American Society for Microbiology.
- YOUNGMAN, P., ZUBER, P., PERKINS, J. B., SANDMAN, K., IGO, M. & LOSICK, R. (1985b). New ways to study developmental genes in spore-forming bacteria. *Science* **228**, 285–291.
- YOUNGMAN, P., POTH, H., GREEN, B., YORK, K., OLMEDE, G. & SMITH, K. (1989). Methods for genetic manipulation, cloning and functional analysis of sporulation genes in *Bacillus subtilis*. In *Regulation of Prokaryotes Development, Structural and Functional Analysis of Bacterial Sporulation and Germination*, pp. 65–88. Edited by I. Smith, R. A. Slepecky & P. Setlow. Washington, DC: American Society for Microbiology.