Dependence of *Bacillus stearothermophilus* Spore Germination on Nutrient Depletion and Manganese

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Spores of *Bacillus stearothermophilus* NCTC 10003 germinated without delay in glucose/glutamate chemically defined medium at 60 °C. The nature of the nutrient limitation inducing sporulation affected the ability of spores to germinate under the conditions studied. Spores produced after glucose depletion of the medium (carbon-depleted spores) germinated faster and to a greater extent than did spores produced after sulphate depletion of the medium (sulphur-depleted spores). Sub-lethal heat treatment (80 °C, 10 min) prior to germination enhanced dormancy. L-Alanine (0.2 mg ml⁻¹) did not reverse this effect, nor did it enhance the germination of sulphur-depleted spores. The rate and extent of germination for both kinds of spores was proportional to the intrasporal manganese concentration. In contrast, extrasporal manganese exerted an inhibitory effect and it was not required for germination. We conclude that the nature of the nutrient depletion and the level of intrasporal manganese distinctly affect the extent and rate of spore germination.

INTRODUCTION

Conditions for bacterial spore germination have been reviewed by Gould (1969), and overt morphological and biochemical changes accompanying germination have been characterized (Levinson & Hyatt, 1966; Dring & Gould, 1971; Setlow & Primus, 1975; Watabe & Kondo, 1975; Scott *et al.*, 1978). Since the discovery of the specific requirement for manganese in sporulation (Charney *et al.*, 1951) and the manganese stimulatory effect on germination (Levinson & Sevag, 1952), the role of divalent metal ions in the cell cycle of *Bacillus* species has been intensely studied. However, previous studies on the influence of manganese on germination did not distinguish the intrasporal Mn²⁺ from that in the germination media (Levinson & Sevag, 1952; Gould, 1969). Recent experimental evidence has provided strong indications that mobilization of spore manganese plays a critical regulatory role in germination (Singh & Setlow, 1978; 1979; Vasantha & Freese, 1979). If this is true, then the level of intrasporal manganese may be more important in spore germination than that of extrasporal manganese added to germination media.

Furthermore, comparatively little is known concerning how nutrient depletion and other sporulation conditions affect germination. It has been reported that depletion of a particular nutrient by cells growing in a chemically defined medium results in spores of specific characteristics (Brown & Hodges, 1974). Therefore, in order to explore the germination mechanism, the history of the spores under study should be carefully traced. Appropriate manipulation of the sporulation medium by nutrient depletion in combination with adjustment of divalent metal ion concentrations may yield spores of specified characteristics offering a clearer insight into spore germination.

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In this paper, we describe the effects of nutrient depletion during sporulation on germination of *Bacillus stearothermophilus* spores and how these effects vary with the intra- and extraspore concentrations of manganese.

METHODS

Spore preparation. *Bacillus stearothermophilus* NCTC 10003 cells growing exponentially in 25 ml of a chemically defined medium (CDM) were used as an inoculum after 6 h incubation at 60 °C. The CDM contained 17.6 mM- Na_2HPO_4 , 2.4 mM-L-glutamate, 7.3 mM- KH_2PO_4 , 7.5 mM-D-glucose, 9.35 mM- NH_4Cl , 10 μM - FeCl_3 , 10 μM - MnCl_2 , 0.5 mM- MgCl_2 , 0.1 mM- CaCl_2 and 0.1 mM- Na_2SO_4 (pH 7); this medium was found to support good growth of this strain (Cheung *et al.*, 1982). The inoculum cells were washed twice with pre-warmed medium containing all nutrients in the sporulation medium except the nutrient intended to limit cell growth. They were then added to 500 ml chemically defined medium such that depletion of either the carbon source (glucose) or the sulphur source (Na_2SO_4) limited exponential growth and led to spore formation. When preparing nutrient-depleted spores with different intraspore manganese levels, the manganese concentration in the medium was varied as stated below, keeping all other nutrient concentrations constant. Spores were harvested by centrifugation after 60 h incubation at 60 °C with aeration at 800 ml min⁻¹. They were then washed 10 times in cold sterile deionized distilled water, resuspended ($1-5 \times 10^8$ spores ml⁻¹) in phosphate-buffered saline (5 mM, pH 7.2) and stored at 4 °C. In each batch, 1-4% of spores appeared non-refractile under the phase-contrast microscope after more than 2 months storage.

Assay of divalent metal ions. Spore contents of Mn^{2+} , Mg^{2+} , Ca^{2+} and Fe^{2+} were measured using a Unicam SP90 atomic absorption spectrophotometer. Spores were washed twice with deionized distilled water, dried in a desiccator and dissolved in 1 M-HCl by autoclaving for 1 h. The samples were then diluted in water and assayed in duplicate, taking three readings for each measurement. Contaminating levels of Mn^{2+} in the medium were always less than 10^{-7} M as determined by a Perkin-Elmer 560 flameless atomic absorption spectrophotometer.

Determination of dipicolinic acid. The dipicolinic acid content of spores was measured polarographically in acidified samples using method 1 of Porter *et al.* (1967).

Heat-shock treatment. Spores washed twice and resuspended in appropriate amounts of sterile deionized distilled water were heated for 10 min at 80 °C. After heat-shock, the spores were immediately transferred to pre-warmed CDM for an assessment of germination. Survival of the heat-shocked spores was measured by plating serially diluted spore suspensions in triplicate on 0.5% (w/v) glucose/1% (w/v) tryptone agar. Plates were incubated at 60 °C for 24 h and colonies were then counted.

Spore germination. Nutrient-depleted spores suspended in phosphate-buffered saline (5 mM, pH 7.2) and kept at 4 °C were resuspended in pre-warmed CDM which induced germination. Germination was measured either by monitoring the decrease in turbidity at 470 nm (A_{470}) of appropriately diluted spore suspensions and/or by counting the number of phase-dark spores in a haemocytometer counting chamber (Hawksley & Son) under a phase-contrast microscope. The initial A_{470} was always between 0.8 and 1.0. Spores were induced to germinate with or without prior heat-shock by shaking the suspension at 60 °C. The A_{470} was measured at specified time intervals and a standard curve for the conversion of low percentage transmittance to true turbidity was used (Lawrence & Maier, 1977). Confirmation of germination was obtained by observing changes in refractility using a phase-contrast microscope. In the method of phase-dark spore counting, a sample of the germinating spore suspension was immediately mixed with an equal quantity of 5 M-HCl in a test tube in order to prevent further germination. A few drops of the mixture were later placed on counting chambers and counted. Germination rates were estimated by measuring the initial slope of the germination curves.

RESULTS

Spore composition and properties

We examined the spore contents of divalent cations and dipicolinic acid in relation to the initial Mn^{2+} concentration in the medium with sulphur or carbon source depletion limiting growth and causing sporulation. Spore contents of Ca^{2+} , Fe^{2+} , Mg^{2+} and dipicolinic acid appeared to vary slightly and randomly with respect to the Mn^{2+} concentration in the medium (Table 1). The intraspore concentration of Mn^{2+} was proportional to the initial concentration of Mn^{2+} in the medium for both carbon- and sulphur-depleted spores. Both kinds of spores were, on average, equally capable of giving rise to colonies after 24 h incubation on plates. Approximately 66-90% of spores were viable.

Effect of nutrient depletion and heat shock on germination

Spores of *B. stearothermophilus* were induced to germinate by resuspending them in CDM at 60 °C. When approximately 25% of the phase-bright spores had become phase-dark, the A_{470}

Table 1. *Dipicolinic acid (DPA) and divalent metal ion contents of B. stearothermophilus spores harvested from media containing various concentrations of Mn²⁺*DPA and metal ion contents are expressed as μg per 10^7 spores.

Medium Mn^{2+} concn (μM)	Sulphur-depleted spores					Carbon-depleted spores				
	DPA	Ca^{2+}	Fe^{2+}	Mg^{2+}	Mn^{2+}	DPA	Ca^{2+}	Fe^{2+}	Mg^{2+}	Mn^{2+}
2.1	1.29	0.32	0.091	0.038	0.035	1.15	0.32	0.055	0.025	0.027
5.0	1.59	0.44	0.084	0.056	0.039	—	—	—	—	—
10.0	1.89	0.57	0.165	0.053	0.067	1.53	0.49	0.251	0.052	0.067
62.5	2.06	0.64	0.169	0.061	0.147	1.57	0.48	0.179	0.032	0.083
100.0	1.74	0.59	0.107	0.052	0.158	1.59	0.48	0.223	0.048	0.186

Fig. 1

Fig. 2

Fig. 1. Correlation between germination and decrease in A_{470} . Carbon-depleted spores containing 186 ng Mn^{2+} per 10^7 spores were induced to germinate as described in Methods. Every 5 min a sample of the spore suspension was removed. Its A_{470} was measured and expressed as a percentage of the initial A_{470} . The phase-dark spores in this sample were then counted and their number expressed as a percentage of the total number of spores present.

Fig. 2. Germination of nutrient-depleted spores with or without heat shock. Carbon-depleted (a) and sulphur-depleted (b) spores were induced to germinate with (□, ○) or without (■, ●) heat shock as described in Methods. The extent of germination was assessed at 5 min intervals by measuring the decrease in A_{470} of the suspension. The arrow in (b) indicates the time when L-alanine (0.2 mg ml^{-1}) was added to the germination medium. Carbon-depleted spores contained 186 ng Mn^{2+} per 10^7 spores and sulphur-depleted spores contained 158 ng Mn^{2+} per 10^7 spores.

had decreased by 10%. This relationship departed from linearity when the decrease in A_{470} was more than 20% (Fig. 1). All spores examined germinated without the apparent delay commonly observed with spores of *Bacillus cereus* T (Gould, 1969). Although the exact time courses varied slightly from experiment to experiment, the extent and rate of germination was reproducible and was always higher for carbon-depleted spores than for sulphur-depleted spores (Figs 2, 3 and 4). These observations suggest that sulphur-depleted spores were more dormant, although colony counts indicated that approximately similar percentages of spores were viable after long periods of incubation.

Fig. 3. Effect of extrasporal Mn^{2+} on the germination of nutrient-depleted spores. Carbon-depleted (a) and sulphur-depleted (b) spores were induced to germinate in CDM containing 2.1 (●), 10 (◐), 62.5 (●) and 100 (○) μM - Mn^{2+} , as described in Methods. Germination was assessed by measuring the fall in A_{470} . Intraspore Mn^{2+} levels were the same as in Fig. 2.

Spores of some *Bacillus* species are activated to germinate by heat shock (Gould, 1969; Srivastava & Fitz-James, 1981). Figure 2 depicts the germination of heat-shocked carbon- and sulphur-depleted spores compared with controls. In both cases spores became more dormant after the heat-shock treatment. The majority of control spores germinated in 60 min while the heat-shocked spores remained dormant for more than 6 h and then the A_{470} increased rather than decreased. The increase in A_{470} was associated with outgrowth of some spores followed by the first cell division (as observed microscopically). Plating on glucose/tryptone agar indicated that only 13% of both kinds of spores were inactivated by heat-shock treatment before germination. In contrast, 62% of sulphur- and 53% of carbon-depleted spores were inactivated by incubation at 100 °C for 30 min (data not shown). Therefore, sub-lethal heat treatment of this strain was not only ineffective in activating germination but actually enhanced dormancy. In CDM, spores were most probably triggered to germinate by glucose. In order to assess whether an additional triggering compound would accelerate germination, L-alanine (0.2 mg ml⁻¹) was added to the medium (Fig. 2b, arrow). In repeated experiments A_{470} remained unchanged indicating that L-alanine was unable to trigger germination of sulphur-depleted spores. Carbon-depleted spores were not treated with L-alanine since they germinated extensively without it. We conclude from these data that CDM permits germination of *B. stearothermophilus* spores and that the nature of the nutrient limitation inducing sporulation affects their ability to germinate.

Effect of extra- and intraspore manganese on spore germination

We wanted to investigate whether the germination kinetics were influenced by extra- and intraspore manganese levels. Nutrient-depleted spores containing different amounts of manganese were suspended in media containing increasing concentrations of Mn^{2+} and were examined for germination. Spores germinated similarly in the absence and presence of 2.1 μM - Mn^{2+} . The total numbers of germinated sulphur-depleted spores at 60 min, as well as their germination rates, decreased slightly as the Mn^{2+} concentration in the germination medium was increased (Fig. 3b). The germination of carbon-depleted spores was similarly affected by extrasporal

Fig. 4. Germination time course of nutrient-depleted spores as a function of intrasporal manganese concentration. Carbon-depleted spores (a) containing 67 (○), 83 (◐) and 186 (●) ng Mn^{2+} per 10^7 spores and sulphur-depleted spores (b) containing 35 (□), 67 (◐), 147 (◑) and 158 (■) ng Mn^{2+} per 10^7 spores were induced to germinate as described in Methods. Germination was assessed by measuring the decrease in A_{470} of samples withdrawn at 5 min intervals. Chemically defined germination medium contained $10 \mu\text{M-Mn}^{2+}$.

manganese except that it proceeded faster and to a greater extent than that of sulphur-depleted spores (Fig. 3a). Germination of spores containing lower levels of manganese was less inhibited by the Mn^{2+} concentration in the germination medium when compared to spores with high manganese content (data not shown). Intrasporal manganese concentration appeared to have a larger impact on the rate and extent of germination than did manganese present in the germination medium (Fig. 4). The rate and extent of germination of both sulphur- and carbon-depleted spores was proportional to the intrasporal manganese concentration. Approximately two to three times as many carbon-depleted spores germinated in 60 min as compared to sulphur-depleted spores containing comparable amounts of Mn^{2+} . The germination of carbon-depleted spores showed a greater dependence on intrasporal Mn^{2+} than did germination of sulphur-depleted spores (Fig. 4).

DISCUSSION

The time course of germination of *B. stearotheophilus* spores varied with the nature of the nutrient limiting vegetative growth and inducing sporulation in the chemically defined medium. Sulphur-depleted spores appeared to be more dormant; they germinated more slowly and to a lesser extent than carbon-depleted spores. These observations are consistent with the findings of Morris & Hansen (1981) who have recently reported that thiol groups in the spore coat of *B. cereus* became increasingly exposed during germination and that their covalent modification inhibited outgrowth. Similarly, an increase in the thiol content of coat proteins extracted from both isolated coats and whole cells accompanies augmentation of germination by heat shock in *B. cereus* (Srivastava & Fitz-James, 1981). The structure of the spore coat proteins determines their hydrolysis rates as well as the interaction with germination-triggering compounds. Both processes contribute to the overall germination rate. Sulphate in the sporulation medium was the only sulphur source for the synthesis of cysteine and other sulphur-containing compounds. Polypeptides rich in half-cysteine cause the spore coat to be a high sulphur-containing keratin-

like structure (Aronson & Fitz-James, 1976) and this may be modified in sulphur-depleted spores.

Spores of *Bacillus* species require different triggering compounds and may need heat shock to initiate germination (Foerster & Foster, 1966; Gould, 1969). Some spores can be triggered to germinate by glucose (Vary, 1978), others by L-alanine (Scott *et al.*, 1978) and L-proline (Rossignol & Vary, 1979). Our results indicate that spores of *B. stearothermophilus* NCTC 10003 require glucose for germination. Exposure of *B. stearothermophilus* spores to 80 °C for 10 min failed to activate their germination system and enhanced dormancy. This effect could not be reversed by L-alanine or D-glucose. Heat-induced spore dormancy in two other *B. stearothermophilus* strains has also been observed, when spores were heated in water at 80, 90 and 100 °C (Finley & Fields, 1962). The reasons for this phenomenon remain unknown.

Manganese levels in carbon- and sulphur-depleted spores of *B. stearothermophilus* were directly proportional to the initial concentrations of this ion in the sporulation medium. Similar results were reported for *B. megaterium* (Slepecky & Foster, 1959; Levinson & Hyatt, 1966) and *B. fastidiosus* (Aoki & Slepecky, 1973). Both the rate and extent of germination increased as the manganese concentration in the spores increased. This effect was observed regardless of the nutrient depletion, although it was less pronounced in sulphur-depleted spores probably due to their high dormancy. Free Mn^{2+} serves as a preferred cofactor for 3-phosphoglycerate mutase (Oh & Freese, 1976), the enzyme which provides energy for germination (Singh & Setlow, 1979). It is possible that spores with higher intrasporal manganese concentrations have higher amounts of free Mn^{2+} released from bound forms at the earliest stage of germination; this would increase energy production and accelerate germination. However, our results do not exclude other possibly unidentified changes which may have altered the extent and rate of germination. In contrast to intrasporal manganese, exogenous manganese exerted a smaller and inhibitory effect on germination. Manganese in the medium also inhibits germination of spores of *B. megaterium* (Levinson & Hyatt, 1966) and *B. fastidiosus* (Aoki & Slepecky, 1973). The effect was smaller because exogenous Mn^{2+} may reach the spore protoplast after intrasporal ions have already exerted their influence on germination. During the early stages of germination, transport systems are neither activated nor synthesized (Scott *et al.*, 1978). The effect was inhibitory possibly due to manganese uptake utilizing some of the metabolic energy (Eisenstadt *et al.*, 1973; Silver *et al.*, 1975; Stahl, 1978) needed for germination or because of a manganese inhibition of macromolecular synthesis as occurs in vegetative cells (Eisenstadt *et al.*, 1973). However, further experimental evidence is necessary to investigate these possibilities.

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