The Role of Manganese in Growth and Sporulation of *Bacillus subtilis*

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Phosphoglycerate phosphomutase of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus megaterium* required Mn^{2+} as cofactor, whereas the wheat germ and rabbit liver enzymes did not. In the absence of Mn^{2+} , *B. subtilis* did not sporulate in normal sporulation media but it did sporulate if the proper ratio of glucose or glycerol and malate was used. Decoyinine, an inhibitor of guanosine monophosphate synthesis, induced sporulation in the presence of excess glucose and malate to the same extent with and without Mn^{2+} . Apparently, phosphoglycerate phosphomutase is the only strictly Mn^{2+} -requiring enzyme needed for optimal sporulation in normal sporulation media.

INTRODUCTION

Manganese ions are essential for endospore formation in bacilli (Charney *et al.*, 1951). In the absence of Mn^{2+} , rapidly metabolizable carbohydrates, such as glucose and glycerol, are not completely metabolized by *Bacillus subtilis* and the bacteria accumulate 3-phosphoglyceric acid (3-PGA) (Oh & Freese, 1976). This results from the strict Mn^{2+} requirement of phosphoglycerate phosphomutase (EC 5.4.2.1; PGA-mutase) demonstrated both in crude bacterial extracts (Oh & Freese, 1976) and in pure PGA-mutase of *B. subtilis* (Watabe & Freese, 1979). Whether, in addition to PGA-mutase, some other reaction essential for sporulation specifically requires Mn^{2+} is so far unknown. This paper demonstrates that sporulation of *B. subtilis* can be restored in the absence of Mn^{2+} by the proper supply and balance of carbon compounds entering the metabolic pathways above and below the PGA-mutase reaction. It is also possible to induce sporulation in a medium containing excess of both glucose and malate by adding Decoyinine (Mitani *et al.*, 1977), an inhibitor of guanosine monophosphate synthetase; when Decoyinine is added while the culture has a sufficiently low cell titre (i.e. has not accumulated too much 3-PGA), the same extent of sporulation is observed in the presence and the absence of Mn^{2+} .

METHODS

Bacteria and growth. The principal strain used was 60015 (*metC trpC*), a transformable derivative of *B. subtilis* 168. *Bacillus megaterium* 80013 (ATCC 19243) and *Bacillus cereus* 80001 were used for comparison of Mn^{2+} requirements.

Nutrient sporulation medium (NSMP) contained nutrient broth (Difco) at 8 g l⁻¹ (autoclaved) and sterile additions of K_2HPO_4/KH_2PO_4 (10 mM-P_i, pH 6.5), 1 mM-MgCl₂, 0.7 mM-CaCl₂, 50 μ M-MnCl₂, 1 μ M-FeCl₃, 25 μ g L-tryptophan ml⁻¹ and 10 μ g L-methionine ml⁻¹. For some experiments MnCl₂ was omitted (NSMP-Mn²⁺).

Synthetic growth medium contained KH_2PO_4/K_2HPO_4 (10 mM-P_i, pH 7.0), 10 mM-(NH₄)₂SO₄, 5 μ M-FeCl₃, 1 μ M-ZnCl₂ and Mg²⁺, Ca²⁺, Mn²⁺, tryptophan and methionine as in NSMP, with glucose, glycerol or potassium L-malate (adjusted to pH 7.0 with KOH) as stated.

Cultures were grown overnight on plates of tryptose blood agar base (Difco; 33 g l⁻¹) and inoculated into medium to give an A_{600} of 0.05 to 0.1. The flasks, containing medium that comprised less than 20 % of their volume, were shaken at 120 strokes min⁻¹ at 37 °C. Growth was followed turbidimetrically at 600 nm. The viable cell count was determined by diluting samples in KH₂PO₄/K₂HPO₄ (100 mM-P_i, pH 7.0) containing 1 mM-MgCl₂ and plating on tryptose blood agar plates. The dilution tubes were then heated for 15 min at 75 °C, and samples were plated on tryptose blood agar to determine the number of heat-resistant colony-forming units (spores). The plates were incubated for 16 h at 37 °C before colonies were counted.

For sporulation, a synthetic sporulation medium was used which contained KH₂PO₄/K₂HPO₄ (5 mM-P_i, pH 7.0), 10 mM-(NH₄)₂SO₄, 100 mM-3-(*N*-morpholino)propanesulphonic acid (MOPS; adjusted to pH 7.0 with KOH), 20 mM-sodium glutamate (pH 7.0), 2 mM-MgCl₂, 0.7 mM-CaCl₂, 50 µM-MnCl₂, 5 µM-FeCl₃, 1 µM-ZnCl₂, 2 µM-thiamin, 50 µg L-tryptophan ml⁻¹, 25 µg L-methionine ml⁻¹, 100 mM-glucose and 100 mM-malate (adjusted to pH 7.0 with KOH). Where stated, Mn²⁺ was omitted from the medium.

Mn²⁺ assay. Mn²⁺ was determined by atomic absorption in a graphite furnace using a manganese lamp at 403 nm (Perkin Elmer). Media without added Mn²⁺ usually contained less than 80 nM-Mn²⁺. If the Mn²⁺ concentration was higher than this, the medium was discarded.

3-Phosphoglycerate (PGA) accumulation. Strain 60015 was grown in NSMP containing 25 mM-glycerol and ³²P_i (0.3 µCi µmol⁻¹). Bacteria from a 5 ml sample were collected on a membrane filter (pore size 0.45 µm), the filter was immediately transferred to a small Petri plate containing 0.5 ml ice-cold 0.5 M-formic acid and left on ice for 30 min. After centrifugation of this extract, 5 µl of the supernatant were chromatographed on a thin-layer plate containing polyethylene-impregnated cellulose as previously described (Oh & Freese, 1976).

Induction of sporulation. Strain 60015 was grown in synthetic sporulation medium to an A_{600} of 0.2. The culture was rapidly filtered through a membrane and the bacteria were resuspended in fresh prewarmed medium to give an A_{600} of 0.005. This culture was shaken at 37 °C and, at intervals, 20 ml portions were filtered and the bacteria were resuspended in fresh prewarmed medium to give the same A_{600} (as at the time of withdrawal). Three 5 ml samples of each portion were added to three prewarmed (25 mm diam.) tubes, one containing Decoyinine (150 µg ml⁻¹), another containing Decoyinine and MnCl₂ (50 µM), and the third containing no addition. The tubes were rapidly shaken (120 strokes min⁻¹) and growth was followed turbidimetrically by taking 0.5 ml samples, if necessary diluting them in phosphate/Mg²⁺ buffer so that the A_{600} was less than 0.8. The titres of viable cells and heat-resistant spores were determined 10 h after transfer of the cells to the tubes. Decoyinine was a kind gift from Dr G. B. Whitfield of the Upjohn Co., Kalamazoo, Michigan, U.S.A.

Preparation of cell extracts for PGA-mutase assay.

(i) **Bacilli.** *Bacillus megaterium* was grown in NSMP to an A_{600} of 1.0, centrifuged (4 °C, 12000 g), washed twice with 10 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES; adjusted to pH 7.6 with KOH) containing 2 mM-mercaptoethanol and 0.5 mM-MnCl₂, suspended at an A_{600} of 30 in similar buffer, and treated with lysozyme (150 µg ml⁻¹) for 20 min at 37 °C. The lysed preparation was centrifuged (4 °C, 35000 g) for 20 min. Ammonium sulphate was added to the supernatant to give 61 % saturation at 4 °C. After 30 min, the precipitate was collected and dissolved in 5 ml HEPES/mercaptoethanol/MnCl₂ buffer, and 1 ml portions were dialysed overnight with constant stirring against 500 ml of either HEPES/mercaptoethanol, or similar buffer containing 10 mM-EDTA and 0.5 mM-MnCl₂, or HEPES/mercaptoethanol containing 0.5 mM-MnCl₂; each buffer was changed twice. The PGA-mutase activity of the dialysed material was assayed by following the oxidation of NADH at 340 nm. The assay mixture, in 1 ml, contained 50 mM-K⁺, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer (pH 7.6), 5 mM-MgCl₂, 50 mM-KCl, 1 mM-MnCl₂, 1 mM-ADP, 0.15 mM-NADH, 1.5 units enolase, 1.5 units pyruvate kinase, 2.0 units lactate dehydrogenase and 5 mM-3-PGA. Protein concentrations were determined according to Lowry *et al.* (1951). The PGA-mutase from *B. cereus* was isolated and assayed according to the same procedure.

(ii) **Wheat germ.** Wheat germs (5 g) were suspended in 25 ml ice-cold HEPES/mercaptoethanol/MnCl₂ buffer, ground in a mortar and homogenized for 30 s. The homogenate was centrifuged at 35000 g for 20 min and, to the supernatant, (NH₄)₂SO₄ was added to give 61 % saturation in the cold. The precipitate was resuspended and portions were dialysed and then assayed as described for the *B. megaterium* enzyme.

(iii) **Rabbit liver.** Fresh rabbit liver (5 g) was minced using scissors in ice-cold HEPES/mercaptoethanol/MnCl₂ buffer containing 0.25 M-sucrose. The cells were broken in a homogenizer, the homogenate was centrifuged at 600 g for 5 min and the supernatant was recentrifuged at 35000 g for 15 min. A 61 % (NH₄)₂SO₄ fraction of the supernatant was prepared and processed as described for the *B. megaterium* enzyme. The PGA-mutase was assayed in the presence of 4 mM-2,3-diphosphoglycerate.

Fig. 1. Growth of strain 60015 in minimal medium containing different carbohydrates with (●) or without (○) $50 \mu M$ - $MnCl_2$. (a) Minimal medium with 25 mM-glucose; (b) minimal medium with 50 mM-malate (adjusted to pH 7.0 with KOH); (c) minimal medium with both glucose and malate.

RESULTS

In synthetic medium with malate as sole carbon source, strain 60015 grew at the same rate in the presence and absence of Mn^{2+} (Fig. 1*b*). With glucose as sole carbon source, the bacteria grew faster with Mn^{2+} (doubling time 135 min) than without it (doubling time 235 min) (Fig. 1*a*). With glucose and malate together, the initial growth rate was the same with and without Mn^{2+} , up to an A_{600} of 0.4, but subsequently the growth rate without Mn^{2+} steadily decreased (Fig. 1*c*); this suggested that a growth inhibitor (3-PGA) was accumulating in the Mn^{2+} -deficient culture, as had previously been observed in Mn^{2+} -deficient nutrient sporulation medium (NSMP- Mn^{2+}) containing glucose (Oh & Freese, 1976).

In NSMP- Mn^{2+} without any additional carbon source, the bacteria sporulated reasonably well. Addition of malate (10 mM) did not significantly affect the extent of sporulation (Fig. 2). However, glycerol (≥ 1 mM) or glucose (> 2 mM) significantly reduced sporulation (Fig. 2). In this case the spore titre increased with the concentration of added Mn^{2+} , reaching a maximum of 5×10^8 to 7×10^8 spores ml^{-1} (0.62 to 0.87 spores per viable cell) at $1 \mu M$ - Mn^{2+} , or higher if the medium contained 5 mM-glucose or glycerol at the beginning (results not shown). The adverse effect of glucose or glycerol could also be counteracted by malate. For example, in NSMP- Mn^{2+} containing 2.5 mM-glycerol (or glucose) the spore titre increased with the malate concentration to a maximum at 15 mM (Fig. 3*a*). Conversely, if the initial malate concentration was 10 mM the spore titre decreased with increasing concentration of glucose (Fig. 3*b*) or glycerol (not shown).

The presence of glucose or glycerol resulted in the accumulation of 3-PGA (Fig. 4) causing the arrest of growth at the low A_{600} value of 1. When malate was added to such a culture, growth resumed and the intracellular concentration of 3-PGA decreased, and then increased later when a new plateau of growth was reached (Fig. 4).

Fig. 2. Effect of carbohydrates on sporulation in the absence of Mn^{2+} . Strain 60015 was grown in NSMP- Mn^{2+} containing either glucose (○, ●) or glycerol (△, ▲) or potassium malate (adjusted to pH 7.0 with KOH) (□, ■). The viable bacterial titre (○, △, □) and spore titre (●, ▲, ■) were determined after 18 h.

Fig. 3. Restoration of sporulation by malate and suppression by glucose in the absence of Mn^{2+} . Strain 60015 was grown in NSMP- Mn^{2+} containing (a) 2.5 mM-glycerol and different amounts of malate (2 to 20 mM) or (b) 10 mM-potassium malate (adjusted to pH 7.0 with KOH) and different amounts of glucose (1 to 5 mM). The total viable bacterial titre (○) and the spore titre (●) were determined after 18 h.

Fig. 4. Inhibition of growth in the absence of Mn^{2+} by 3-PGA accumulation and restoration by malate. Strain 60015 was grown in NSMP— Mn^{2+} containing 25 mM-glycerol and $^{32}P_1$ ($0.3 \mu Ci \mu mol^{-1}$). At the time indicated by the arrow, 50 mM-potassium malate was added. At different times, the bacteria from 5 ml samples were collected on membrane filters (pore size $0.45 \mu m$) and extracted with ice-cold 0.5 M-formic acid. Portions ($5 \mu l$) of the extracts were chromatographed and 3-PGA was determined as described in Methods. ●, A_{600} of culture without malate addition; ○, A_{600} after malate addition. ▲, 3-PGA concentration in culture without malate addition; △, 3-PGA concentration after malate addition.

Induction of sporulation by Decoyinine in the absence of Mn^{2+}

The above results suggested that Mn^{2+} was needed for sporulation mainly because it had to activate PGA-mutase thereby preventing the accumulation of the inhibitory 3-PGA and assuring the supply of carbon compounds in both parts of the metabolic pathways interconnected by PGA-mutase. If that were correct, it should be possible to induce sporulation in the presence of excess glucose and malate by Decoyinine, irrespective of the presence or absence of Mn^{2+} . To be successful, Decoyinine would probably have to be added before too much 3-PGA had accumulated. This experiment was performed in synthetic sporulation medium in which significant growth inhibition due to deficiency of Mn^{2+} was observed when the A_{600} increased beyond 0.6 (Fig. 5a). When Decoyinine ($150 \mu g ml^{-1}$) was added to such cultures and sporulation was measured 10 h later, excellent sporulation was observed for cultures having A_{600} values of 0.3 to 0.5 whereas much lower spore titres (and long cell filaments) were obtained when Decoyinine was added to cultures with A_{600} values above 0.7 (Fig. 5b). In contrast, the cultures containing Mn^{2+} produced good sporulation for a wider A_{600} range as they did not accumulate the inhibitory 3-PGA.

Mn^{2+} requirement of the PGA-mutase of other organisms

Ammonium sulphate precipitates of cell-free extracts, assayed after dialysis against a buffer containing Mn^{2+} or Mn^{2+} and EDTA, demonstrated that the PGA-mutases of *B. megaterium* and *B. cereus* also required Mn^{2+} whereas that of wheat germ did not and that of rabbit liver required 2,3-diphosphoglycerate (Table 1). On dialysis in the absence of Mn^{2+}

Fig. 5. Induction of sporulation by Decoyinine in the presence or absence of Mn^{2+} . Strain 60015 was grown in the presence (—) or absence (---) of Mn^{2+} in synthetic sporulation medium. Decoyinine ($150 \mu g \text{ ml}^{-1}$) was added at different times, and the viable bacterial titre and spore titre were determined 10 h after the addition of drug. (a) A_{600} in the presence (○) and absence (△) of Mn^{2+} . (b) Spore titre (●) and viable bacterial titre (○) in the absence of Mn^{2+} ; spore titre (▲) and viable bacterial titre (△) in the presence of Mn^{2+} .

Table 1. *Effect of Mn^{2+} on phosphoglycerate phosphomutase activity*

PGA-mutase was isolated and assayed as described in Methods. The 61 % $(NH_4)_2SO_4$ fraction was divided into three portions and dialysed against 10 mM-HEPES pH 7.6 containing 2 mM- β -mercaptoethanol and either (i) 0.5 mM- $MnCl_2$ (+ Mn^{2+}), (ii) no addition (− Mn^{2+}) or (iii) both 0.5 mM- $MnCl_2$ and 10 mM-EDTA (+ Mn^{2+} + EDTA). Enzyme activities were determined as μmol 3-PGA converted $\text{min}^{-1} (\text{mg protein})^{-1}$, and the results show the percentage of activity remaining after dialysis against the various HEPES/mercaptoethanol buffers.

Source of PGA-mutase	Activity remaining (%) after dialysis		
	+ Mn^{2+}	− Mn^{2+}	+ Mn^{2+} + EDTA
<i>B. megaterium</i>	96	6	18
<i>B. cereus</i>	102	9	17
Wheat germ	98	84	127
Rabbit liver*	106	108	111

* The enzyme isolated from rabbit liver was assayed in the presence of 4 mM-2,3-diphosphoglycerate.

or in the presence of Mn^{2+} and EDTA, the PGA-mutase activities of *Bacillus* extracts were irreversibly lost whereas those of wheat germ and rabbit liver were not. Thus the Mn^{2+} requirement of PGA-mutase seems to be characteristic of the genus *Bacillus*.

DISCUSSION

The metabolic processes leading to endospore formation depend on the establishment of a proper balance between the concentrations of intracellular metabolites. If this balance is

disturbed, either by inhibition of an enzyme or by mutation, sporulation remains suppressed (Freese *et al.*, 1974). Such an imbalance is produced by deficiency of Mn^{2+} in bacilli where it results in an inactive PGA-mutase. In *B. subtilis*, the physiological effect of this deficiency is similar to that of a mutation in phosphoglycerate kinase. In both cases the overall metabolic pathways are divided into two parts, and the ratio of carbon compounds entering each part determines whether the cell can differentiate, or whether it accumulates a compound which suppresses sporulation (Freese *et al.*, 1972; Oh & Freese, 1976). The ability of the tricarboxylic acid cycle to convert carbon compounds into CO_2 allows the efficient elimination of compounds (such as malate) entering this portion of the metabolic pathway system. In the absence of Mn^{2+} , the proper ratio of malate to glucose allows complete consumption of glucose towards the end of growth, without excess accumulation of 3-PGA, and thus allows initiation of sporulation which can be completed because just sufficient (glucose-derived) intermediates are present to enable the required synthesis of membrane and peptidoglycans.

Sporulation can be induced in the presence of excess carbohydrates by Decoyinine, irrespective of the presence or absence of Mn^{2+} . Decoyinine has only to be added before 3-PGA has reached a concentration at which it suppresses sporulation. The induction of sporulation demonstrates that in the presence of Decoyinine, where certain metabolic reactions (tricarboxylic acid cycle, gluconeogenesis, etc.) are no longer needed for sporulation (Freese *et al.*, 1977), no Mn^{2+} -requiring reaction is needed for sporulation. This indicates that under normal sporulation conditions (without Decoyinine) the only reactions which specifically require Mn^{2+} are those which are not needed in the presence of Decoyinine. Only two such reactions are known. One is PGA-mutase and the other is fructose-1,6-bisphosphatase, the strict Mn^{2+} requirement of which has been demonstrated in *B. licheniformis* (Opheim & Bernlohr, 1975) and in *B. subtilis* (Y. Fujita & E. Freese, unpublished results). A mutant lacking all fructose-1,6-bisphosphatase activity has been isolated in our laboratory; in nutrient sporulation medium, this strain can sporulate as well as the parent strain (unpublished results). Akrigg & Mandelstam (1978) and Akrigg (1978) have recently reported the formation (after stage II of sporulation) of an extracellular Mn^{2+} -stimulated deoxyribonuclease which requires 10 mM- Mn^{2+} for optimal activity and has 15 % of this activity if Mn^{2+} is replaced by Mg^{2+} . This enzyme is either not needed for sporulation in the presence of Decoyinine or its Mg^{2+} activation suffices. Thus it appears likely that the strict Mn^{2+} requirement observed for *Bacillus* sporulation results exclusively from the Mn^{2+} requirement of PGA-mutase.

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