Commitment to Sporulation in *Bacillus megaterium* and Uptake of Specific Compounds

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SUMMARY

Commitment of *Bacillus megaterium* cells to continue the sporulation process was tested at different times during the developmental period with respect to either addition of different carbon sources (sugars or amino acids) or dilution into media containing these. Organisms grown in minimal medium containing sucrose as sole carbon source were committed earliest with respect to aspartic or glutamic acid as sole carbon source, later with respect to fructose, glucose, glycerol or sucrose, and latest with respect to nutrient medium supplemented with casein hydrolysate. Addition of both aspartate and a carbohydrate resulted in later commitment than addition of either compound alone. The initial uptake rates of aspartate, glutamate, glucose and sucrose increased toward the end of growth in complex medium (but not in minimal medium for glucose and sucrose) and then decreased during the developmental period.

INTRODUCTION

In a suitable medium, members of the genus *Bacillus* start the process of sporulation at some time after the end of exponential growth, when all rapidly metabolizable carbon sources have been used up. Early sporulation development can be aborted and growth can be resumed if the culture is diluted into fresh medium (Fitz-James, 1965; Frehel & Ryter, 1969; Hardwick & Foster, 1952) or if it is supplemented with glucose or casein hydrolysate (Freese, Klofat & Galliers, 1970; Grelet, 1951; Sterlini & Mandelstam, 1969). Cells restart development after they have again used up all rapidly metabolizable carbon sources. However, at some time after its onset, development can no longer be interrupted, i.e. the cells are committed to continue the sporulation process. Reports that commitment occurs at different stages of development in different *Bacillus* species have appeared, but different conditions were used for this test (Buono, Testa & Lundgren, 1966; Greene & Slepecky, 1972; Schaeffer, 1969). Commitment of *B. megaterium* seemingly occurred at the same stage of development irrespective of the medium to which the cells were exposed (Greene & Slepecky, 1972; Hendry & Fitz-James, 1974), whereas commitment of *B. subtilis* occurred much earlier with respect to addition of glucose than with respect to either dilution into nutrient medium or addition of casein hydrolysate (Freese *et al.*, 1970; Frehel & Ryter, 1969). Under certain conditions, inability to resume growth seems to appear earlier than commitment to continue sporulation (Kretschmer, 1972).

The mechanism by which cells become committed is poorly understood, except for commitment with respect to glucose in *B. subtilis* (Freese *et al.*, 1970). In this organism the activity of the glucose-phosphoenolpyruvate transferase system, which is necessary for

the uptake of glucose (Roseman, 1969), declines significantly after the end of exponential growth.

METHODS

Organism and culture methods. *Bacillus megaterium* ATCC19213 was used. Minimal sucrose medium (MS plus 0.1 % sucrose) was that of Slepecky & Foster (1959). Minimal salts medium (MS) was the same, but without sucrose.

Spores harvested from MS plus sucrose were washed four times with distilled water and stored in distilled water at -20°C . To obtain an inoculum, spores were heat-shocked at 75°C for 10 to 15 min, streaked on Difco nutrient agar plates and incubated for 24 h at 30°C . A loop of these organisms was inoculated into half-strength Difco nutrient broth (4 g l^{-1}), and incubated with shaking (New Brunswick reciprocating water-bath shaker, 200 to 250 rev./min) for 24 h at 30°C . Portions (0.75 and 1.5 ml) of these cultures were inoculated into two 1 l flasks each containing 200 ml MS plus sucrose; this ensured that at least one of the cultures had an E_{600} of about 0.2 (Gilford spectrophotometer, model 220) after 15 h.

Nutrient sporulation medium (Fortnagel & Freese, 1968) was supplemented with 0.5 % vitamin-free casein hydrolysate (NSMC). Phosphate-buffered nutrient broth contained Difco nutrient broth (8 g l^{-1}) and 100 mM-potassium phosphate pH 6.5.

Commitment. Commitment to sporulation was measured following either fivefold dilution of bacilli into the medium to be tested, or addition of 5 ml portions of the culture to pre-warmed tubes ($25 \times 150\text{ mm}$) containing 10 mg test compound in as little water as possible, except where otherwise noted (aspartic and glutamic acid were neutralized with KOH); sucrose was used at 1 mg ml^{-1} . Incubation was continued with shaking at 30°C . The time of commitment with respect to a given carbon source was the same irrespective of which technique was used. The treated portions, and a portion of the original culture, were frozen 9 to 10 h after the end of exponential growth (T_9 to T_{10}), a time which was sufficient for complete development of phase-bright spores. After thawing, phase-bright spore titres were determined in a Petroff-Hauser chamber, counting a minimum of 25 squares in each of two slides. The ratio of the phase-bright spore titre of a treated sample multiplied by the dilution factor (five) to the phase-bright spore titre of an untreated sample gave a measure of the fraction of bacilli that were committed with respect to the particular medium change. Untreated cultures always sporulated at a frequency above 85 %.

Uptake studies. A portion (2 ml) of the culture was centrifuged for 1 min in an Eppendorf Microfuge (Brinkman Instruments, Westbury, New York, U.S.A.), and the bacilli were washed with and resuspended in the same volume of warm (30°C) MS. The centrifuge was kept at room temperature to avoid cold-shock effects on permeability (Henneberry & Freese, 1973). Uptake experiments were initiated by adding the uniformly ^{14}C -labelled compound ($20\text{ }\mu\text{l}$) to give an isotope concentration of 200 nCi ml^{-1} . In cases specially noted, the labelled compound was added directly to unwashed samples of the culture, or chloramphenicol (crystalline; Sigma) was added to the samples to give a concentration of $100\text{ }\mu\text{g ml}^{-1}$. The final concentrations of the compounds were sucrose 2.92 mM, D-glucose 5.55 mM, L-glutamate 0.5 mM, and L-aspartate 0.2 mM; all radioactive compounds were from New England Nuclear, Boston, Massachusetts, U.S.A.

As rapidly as possible (all within 5 min), five samples (0.2 ml each) were removed, filtered (Millipore, 25 mm diam., $0.45\text{ }\mu\text{m}$ pore size) and washed twice with 5 ml MS (30°C). The filters were placed into vials with 10 ml scintillation fluid [containing (per litre): 758 ml toluene; 200 ml Triton X-100; and 42 ml Liquifluor, from New England Nuclear]

Table 1. *Effect of groups of amino acids on the suppression of sporulation*

Cells were grown in MS plus sucrose to T_1 or T_5 and diluted fivefold in MS containing the stated additions. The titre of phase-bright spores is expressed as a percentage of that of the control culture, which had 2.42×10^8 phase-bright spores/ml, at T_{10} . The groups contained amino acids at a final concentration of 2 mg ml⁻¹ each, except for tyrosine which was used as a saturated solution.

Addition to MS	Percentage of phase-bright spores at T_{10} when compounds were added at:	
	T_1	T_5
Group I	< 0.8	< 0.8
Group II	< 0.8	104
Group III	103	94
Group IV	99	103
Group V	< 0.8	112
Casein hydrolysate (2 %)	< 0.8	89
Sucrose (0.1 %)	< 0.8	109

Group I: L-arginine, L-cysteine, L-lysine, L-methionine. Group II: L-glutamic acid, L-isoleucine, L-leucine, L-valine. Group III: L-phenylalanine, L-tryptophan, L-tyrosine. Group IV: L-histidine, L-proline, L-threonine. Group V: L-alanine, L-aspartic acid, glycine, L-serine.

and were counted in a Packard Tri-Carb liquid scintillation spectrometer. Initial rates of uptake were calculated.

For thin-layer chromatography after 1, 2 or 3 min of glutamate or aspartate uptake, 1 ml of the bacillus suspension was washed twice with 5 ml MS (30 °C) on a Millipore filter, and the filters were extracted with 3 ml water at 80 °C for 1 h. The extract was freeze-dried, the residue dissolved in 50 µl water, and 2 µl portions were chromatographed on MN300 cellulose thin-layer plates (250 µm thick; Analtech) using, as solvent, phenol (liquefied; Fisher)/water/formic acid (75:25:1, by vol.). Dried plates were exposed for 5 days to no-screen X-ray film (Kodak) and then developed with RP X-OMAT developer (Kodak) for 1 min.

RESULTS

Identification of amino acids which suppress sporulation

Bacillus megaterium was grown in MS plus sucrose. At different times during the developmental period, portions of the culture were exposed to media changes. Commitment to continue sporulation was measured by the ratio of phase-bright spores in the treated cultures to those in untreated cultures. Once the bacilli contained phase-bright forespores, they did not lose this property for at least 4 h. Under our conditions, committed bacilli also formed heat-resistant spores because the same titre of phase-bright and heat-resistant spores was observed at T_{11} when cultures were diluted into fresh MS plus sucrose, MS plus aspartate or NSMC. Commitment occurred earlier with respect to sucrose than with respect to casein hydrolysate or a rich medium (NSMC). To determine which of the amino acids in casein hydrolysate suppressed sporulation, groups of three or four amino acids were tested for their effect at T_1 and T_5 , i.e. at times before and after the period of commitment with respect to casein hydrolysate (Table 1). All possible combinations of amino acids from the three groups which suppressed sporulation were tested. With the exception of glycine, each amino acid had, by itself, the same effect on sporulation as when it was tested with other compounds in its group. Only the effect of single amino acids is shown in Table 2. Cysteine suppressed sporulation at T_1 and T_5 . Methionine also suppressed sporulation at T_1 and less effectively

Table 2. *Effect of amino acids on the suppression of sporulation*

Cells were grown in MS plus sucrose to T_1 or T_5 and diluted fivefold in MS containing the stated addition. The titre of phase-bright spores is expressed as a percentage of that of the appropriate control culture at T_{10} . Absolute numbers of phase-bright spores/ml are given for each control culture.

Addition to MS	Percentage of phase-bright spores at T_{10} when compounds were added at:	
	T_1	T_5
Group I		
Lysine	101	91
Methionine	0.75	9.7
Cysteine	< 0.18	< 0.18
Arginine	100	97
Sucrose (0.1 %)	< 0.18	93
None (control culture)	2.14×10^8 phase-bright spores/ml	
Group III		
Leucine	89	100
Isoleucine	107	118
Valine	83	97
Glutamic acid	< 0.17	102
Sucrose (0.1 %)	< 0.17	106
None (control culture)	2.29×10^8 phase-bright spores/ml	
Group V		
Alanine	113	113
Glycine	< 0.09	< 0.09
Glycine + alanine	113	102
Serine	94	93
Aspartic acid	< 0.09	108
Sucrose (0.1 %)	< 0.09	106
None (control culture)	2.12×10^8 phase-bright spores/ml	

(90 %) at T_5 . The effects of cysteine and methionine were not further investigated because these compounds did not restore growth. They allowed the formation of phase-dark forespores which are known to contain a cortex (Greene *et al.*, 1971), but they inhibited the development of phase-bright spores, apparently by interfering with coat assembly (Aronson & Fitz-James, 1968; Vinter, 1957). Glutamic acid and aspartic acid suppressed sporulation at T_1 but not at T_5 . Glycine suppressed sporulation at both T_1 and T_5 . Separate experiments demonstrated that glycine alone inhibited development of phase-bright spores when added at any time prior to the appearance of these spores. Whereas the addition of glutamate or aspartate at T_1 caused the resumption of growth, the addition of glycine did not. Alanine completely reversed the inhibitory effect of glycine (Table 2) but not the suppression of sporulation by aspartate or glutamate. Glycine (1 mg ml^{-1}) also reduced the rate of growth in MS plus sucrose by about 50 %, an effect which was again counteracted by alanine. Thus glycine seemed to act by inhibiting cell metabolism rather than by reversing a development already started. The only two compounds that suppressed sporulation at T_1 but not at T_5 , aspartate and glutamate, were also the only amino acids that could serve as sole carbon source yielding moderately rapid growth rates (doubling times of less than 3 h; Table 3).

Distinct timing of commitment with respect to different carbon sources

Commitment occurred earliest with respect to aspartate and glutamate followed by fructose, glucose, glycerol and sucrose, and latest with respect to the rich NSMC medium (Fig. 1). Since the definition of T_0 as the 'end of exponential growth' was not very accurate

Fig. 1. Time of commitment. (a) At different times during growth and development of *B. megaterium* in MS plus sucrose, samples were diluted fivefold into MS containing aspartate (∇), glutamate ($\blacktriangle$), or sucrose ($\square$), or into NSMC ($\triangle$). A fifth sample ($\blacksquare$) was added to a tube containing sucrose at a final concentration of 0.5%. E_{600} ($\circ$). A control culture had no change of medium. Phase-bright spores were counted at T_{10} and expressed as a percentage of the number found in the control culture: this ratio represents the percentage of bacilli committed at the time of dilution. The time of commitment with respect to addition of glucose (not shown) was the same as that for sucrose.

(b) Samples were diluted into MS containing (1 mg ml⁻¹) aspartate (∇), sucrose ($\square$), or both sucrose and aspartic acid ($\triangle$), or into NSMC ($\blacksquare$). E_{600} ($\circ$). Combinations of glutamate and sucrose, glucose, or glycerol also produced later commitment than either compound alone.

The points represent the average of two counts; bars represent the range.

Table 3. Growth of *B. megaterium* in the presence of different carbon sources capable of suppressing sporulation

Medium	Doubling time (h)
NSMC	0.75
MS + sucrose	1.2
MS + glucose	1.3
MS + glutamate	2.0
MS + aspartate	2.4

and the culture conditions of different experiments may have altered slightly, the T_n values of commitment varied. Differences in commitment times were therefore established by transfer of cells from the same culture.

The higher the growth rate supported by a medium (Table 3), the later were cells committed with respect to that particular medium (Fig. 1). The combination of sucrose and aspartate (or of glutamate and any one of glucose, glycerol, or sucrose, not shown) showed a later commitment than either compound alone (Fig. 1b). However, the latest commitment was observed with respect to the rich NSMC medium.

As observed by phase-contrast microscopy, spores developing in the presence of an added carbon source were always larger than spores of the control culture. This was true even when the bacilli were exposed to a compound after the forespore had been completely engulfed,

as established by electron microscopy. Most non-committed cells apparently resumed growth under our experimental conditions. This was determined by extrapolating the later portion of the growth curve of the diluted culture back to the time of dilution, and it also followed from the lengthening lag in the E_{600} increase observed after the dilution of cultures containing an increasing proportion of committed cells.

Commitment and uptake of specific compounds

Commitment with respect to glucose addition has been correlated in *B. subtilis* to a reduced transport of glucose (Freese *et al.*, 1970). In *B. megaterium* a 20-fold reduction in the initial rate of [U- 14 C]sucrose uptake occurred during the same time interval during which cells became committed with respect to sucrose (Fig. 2). Experiments with D-[U- 14 C]glucose gave the same patterns.

The changes of L-[U- 14 C]glutamate uptake during growth were more complex. Early in growth, the uptake rates were low (Fig. 3). Toward the end of exponential growth, they increased about threefold, reaching a maximum near T_1 , and subsequently declined while the cells became committed with respect to glutamate. The pattern of aspartic acid uptake (Fig. 4) was similar.

The amount and pattern of glutamate uptake in the presence of chloramphenicol were essentially the same as in its absence. Most glutamate was not incorporated into polymers, because 20 min after glutamate addition (in the absence of chloramphenicol) only 7.3 % of the total glutamate taken up was incorporated into trichloroacetic acid-insoluble material. A similar observation was made in the case of aspartate. Chromatographic analysis showed that at 1, 2, or 3 min after addition of the compound, most of the radioactivity that had entered the bacilli was still in the aspartate or glutamate form. Therefore, measurements of the initial rate of uptake reflect the rate of transport rather than that of metabolism, so that the three- to fivefold decrease in the uptake rate during the developmental period probably indicates a decrease in the rate of transport.

Glutamate uptake in the absence of manganese

Bacillus megaterium does not sporulate in phosphate-buffered nutrient broth without added Mn^{2+} ; in 24 h only 5 % of the bacilli reach stages II or III of sporulation (Cooney, 1972). If the nutrient medium contains $10\ \mu M$ - Mn^{2+} , more than 90 % of the bacilli develop phase-bright spores by T_{10} . Figure 5 indicates that in the presence of manganese, glutamate uptake showed a similar increase and decline during growth and sporulation to that shown in MS plus sucrose. Without added manganese, however, the rate of glutamate uptake did not change significantly, and no spore forms were visible by phase-contrast microscopy after 24 to 48 h.

In phosphate-buffered half-strength nutrient broth containing 0.2 % sucrose, extra $CaCl_2$ ($90\ \mu M$) was needed to allow development of phase-brightness in all cells, owing to the high final E_{600} . In the absence of added Mn^{2+} , the culture grew to a lower E_{600} and no spore forms were observed in the phase-contrast microscope after 3 days. In the presence of Mn^{2+} , complete sporulation (>90 %) was observed at T_{12} . The uptake of sucrose changed in the presence of manganese but not in its absence (Fig. 6), in a similar way to the uptake of glutamate.

Fig. 2. Commitment with respect to sucrose and rate of sucrose uptake. *Bacillus megaterium* was grown in MS plus sucrose. At different times, a sample was diluted fivefold into MS plus sucrose to determine commitment (Δ), expressed as in Fig. 1, and another sample was washed and its initial rate of sucrose uptake ($\square$) was measured as described in Methods. E_{600} ($\circ$).

Fig. 3

Fig. 4

Fig. 3. Commitment with respect to glutamate and rate of glutamate uptake. *Bacillus megaterium* was grown in MS plus sucrose. At different times, a sample was diluted fivefold into MS plus glutamate to determine commitment (Δ), expressed as in Fig. 1. [^{14}C]Glutamic acid uptake ($\square$) was measured directly by adding [^{14}C]glutamate to samples of the culture. E_{600} ($\circ$).

Fig. 4. Commitment with respect to aspartate and rate of aspartate uptake. *Bacillus megaterium* was grown in MS plus sucrose. At different times, a sample was fivefold diluted into MS plus aspartate to determine commitment (Δ), expressed as in Fig. 1. [^{14}C]Aspartate uptake ($\square$) was measured as in Fig. 3. E_{600} ($\circ$).

Fig. 5

Fig. 6

Fig. 5. Glutamate uptake in the presence and absence of Mn^{2+} . *Bacillus megaterium* was grown in phosphate-buffered nutrient broth with (closed symbols) and without (open symbols) $10 \mu\text{M}$ - MnCl_2 . At different times, samples were taken, washed, and $[^{14}\text{C}]$ glutamate uptake was measured (■, □). E_{600} (●, ○). The growth temperature was 37°C ; similar results were obtained at 30°C .

Fig. 6. Sucrose uptake in nutrient medium in the presence and absence of Mn^{2+} . *Bacillus megaterium* was grown in phosphate-buffered half-strength nutrient broth containing 0.2% sucrose and $90 \mu\text{M}$ - CaCl_2 with (closed symbols) and without (open symbols) added Mn^{2+} ($10 \mu\text{M}$). At different times, samples were taken, washed, and $[^{14}\text{C}]$ sucrose uptake was measured (■, □). E_{600} (●, ○).

DISCUSSION

Commitment of *B. megaterium* occurs at different times during the developmental period with respect to different carbon sources (Fig. 1). Even after bacilli are committed with respect to a given carbon source, they are not completely unaffected by it, because larger spores are produced in its presence than in its absence. Different spore sizes have previously been reported when media were used that contained different carbon sources from the beginning of growth (Hitchins, Greene & Slepecky, 1972). Glucose, added after commitment, also increased the sporulation frequency of *B. subtilis* (Freese *et al.*, 1970). These observations demonstrate that small intracellular concentrations of a carbon source, which can apparently enter bacilli after they are committed, do not suppress sporulation.

In our view, sporulation development involves a competition between prespore membrane growth and bacillus growth and division. If no rapidly metabolizable carbon source is available, the prespore membrane increasingly engulfs the small prespore compartment; after engulfment is completed, the forespore develops into a mature spore. If all metabolites needed for growth are suddenly made available to the inside of the cell, synthesis of murein fills the prespore septum while synthesis of cytoplasmic material increases the size of both cell compartments and allows the resumption of growth and, later, cell division. When few compounds are added to the medium after T_0 , a small amount of a rapidly metabolizable carbon source inside of the cell can slightly increase the rate of cell metabolism without aborting sporulation: thus it produces larger spores. But when too much of one or more carbon sources can enter the cell and be metabolized, the concentration of the metabolites favouring growth and division can increase beyond a critical value. The mother (Frehel & Ryter, 1969) or both cell compartments (Fitz-James, 1965; Fitz-James & Young, 1969) can then resume growth and further division, depending on the species and the medium used.

We believe that during the course of development, bacilli become committed with respect to a given carbon source when the rate of transport or cellular metabolism of that carbon source has sufficiently decreased. When only one compound is added, the intracellular concentration of it, or of its metabolites, may no longer increase to the level needed to abort sporulation. Even increasing the concentration in the medium to 100 mM does not help.

The resistance to sucrose and glucose develops during the same time interval during which the uptake of these compounds decreases 20-fold (Fig. 2); this decrease may explain the corresponding commitment. The uptake rate of aspartate and glutamate also decreases (by a factor three to five) while commitment with respect to these compounds develops (Figs 3 and 4). But the spread in time is so large that the uptake rate has decreased only 20 % by the time most cells are committed with respect to aspartate or glutamate. Some other (intracellular) change may therefore be necessary to cause this commitment.

Cells grow fastest in a rich medium (NSMC) and commitment with respect to this medium occurs latest (Fig. 1). Whereas each component of this medium alone does not suppress sporulation at such a late stage, the combination of all components apparently provides enough intracellular compounds to prevent sporulation. The finding that aspartate (or glutamate) and a carbohydrate together show a later commitment time than either compound alone agrees with this interpretation (Fig. 1*b*). The combination of different compounds may be more effective because most compounds are transported by different transport carriers, so that their combination can provide a higher intracellular concentration of metabolizable carbon. In addition, the activity of some intracellular enzyme(s), required for the interconversion of different metabolites, may decrease during development. In that case, two carbon sources would allow later commitment than either one alone, if they supply the two metabolic areas separated from each other by the postulated enzyme(s) of decreased activity.

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