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# Role of Glutamate in the Sporogenesis of *Bacillus cereus*

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*Bacillus cereus* T, sporulating in a chemically defined medium under optimum conditions, requires substrate quantities of glutamate during the first 4 h of sporogenesis. Seventy percent of the glutamate utilized was catabolized to $CO_2$ during this period, with the remaining glutamate carbon assimilated into various spore constituents, principally protein and nucleic acid. The importance of glutamate as the primary source of reducing potential and energy for early stages of spore formation was investigated. Although the relative efficiency at which tricarboxylic acid cycle intermediates substituted for glutamate was suggestive of oxidation via the tricarboxylic acid cycle, only partial inhibition of glutamate oxidation by fluoroacetate was observed.

Nakata (29) described a replacement culture procedure that employs the active culture technique and a chemically defined sporulation (CDS) medium. This procedure permits the study of sequential events during sporulation of *Bacillus cereus* T in the absence of growth. CDS medium contains a mineral salts mixture, methionine, and acetate, lactate, and L-glutamate as carbon and energy sources. The medium is buffered at pH 6.4 throughout spore formation. During sporogenesis, the acetate and lactate serve as sources of carbon via synthesis and degradation of poly-β-hydroxybutyrate (PHB) (30). A substantial amount of glutamate was essential for complete sporulation to take place in the CDS medium (29), but the function of this amino acid was not determined.

Of the amino acids influencing growth and sporulation of bacilli, glutamate has received the most attention. Its role in providing carbon and nitrogen precursors for spore biogenesis (3-5, 13, 27, 41), in the regulation of tricarboxylic acid cycle enzymes (14, 15), and in the oxidation of glucose during growth and sporulation (11, 21, 25) has been reported. Glutamate influences ultraviolet resistance, dipicolinic acid content, and heat resistance of spores of *B. cereus* (20). The oxidation of glutamate to $CO_2$ by *Bacillus* species (3, 5, 15) and the utilization of endogenous glutamate oxidation in providing the major portion of the energy initially required for sporogenesis have not been described in detail.

Since acetate and lactate supply carbon for spore constituents of *B. cereus* T during sporulation in CDS medium, the metabolism of exogenously supplied glutamate was investigated. The results presented here indicate that its primary role is that of a source of reducing potential and energy.

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## MATERIALS AND METHODS

**Organism, media, and cultural conditions.** *B. cereus* T was grown in G medium, employing the active culture technique as described earlier (28). When cells were committed to sporulation ($T_0$), as evidenced by the characteristic rise in pH of the medium, they were routinely harvested by centrifugation, quickly washed in sterile 0.1 M potassium phosphate buffer (pH 6.4) at room temperature, and immediately placed in a volume of CDS medium equivalent to the volume of G medium culture harvested (replacement procedure). The preparation of CDS medium was described elsewhere (29).

Sporulating cultures were incubated at 30°C on a rotary shaker set at 300 rpm. Cytological changes during sporulation were followed microscopically by observing smears stained with gentian violet. Turbidimetric measurements were made with a Klett-Summerson photoelectric colorimeter equipped with a blue filter (400-465 nm). Dry weight measurements were made on suspensions dried overnight at 105°C. Although spores were still encased in sporangia, sporulation was essentially complete by 11 to 12 h after placement into CDS medium.

For $CO_2$ evolution experiments, cultures were connected to an aeration system placed on the shaker. This system consisted of a sterile cotton filter trap, a sterile water trap to humidify incoming air, and the replacement culture flask. Two $CO_2$ traps, each containing a predetermined volume of 1.0 N NaOH (and equipped with sintered-glass spargers), were connected in series to the culture flask and finally to a portable vacuum pump. A slight continuous vacuum was applied to the system during the incubation period to provide a constant supply of sterile moist air to the culture and to remove the $CO_2$ produced by the culture.

**Radioisotope experiments.** A 4-μCi amount of L-

[U-$^{14}$C]glutamic acid (200 mCi/mmol) was added per 100 ml of medium. Immediately after placement of sporulating cells into CDS or G medium containing the radioisotope, and at intervals thereafter, 1.0-ml culture samples were removed and diluted 10-fold in distilled water, and duplicate 2.0-ml portions of these dilutions were filtered rapidly through membrane filters (0.45 μm; Millipore Corp.). The filters retaining the cells were glued to planchets, dried, and measured for radioactivity. Duplicate 1.0-ml portions of the filtrates were plated in ringed planchets, dried, and counted to measure radioactivity residual in the supernatant. Similarly, 10-fold dilutions were prepared from the alkali traps, and 1.0-ml portions were plated to determine the radioactivity trapped as $[^{14}C]CO_2$. The $CO_2$ traps were changed at intervals, and the corresponding radioactivities were summed. Samples from cultures in labeled growth medium were treated similarly. Suitable controls were prepared for each type of sample, and all measurements were corrected for self-absorption. All radioactivity measurements were made with an automatic Nuclear-Chicago gas flow detection system for a 10-min period.

**Fractionation of spores.** Spores produced in the presence of [$^{14}$C]glutamate were fractionated 32 h after replacement by the procedure of Park and Hancock (33). Duplicate 1.0-ml portions of each fraction were plated and dried overnight at 105°C for dry weight determinations. For radioactivity measurements, 1.0-ml volumes of 10-fold dilutions of the residue suspensions were filtered in duplicate, and the filters were dried and counted.

**Cell-free extracts.** Cell-free extracts were prepared from a 20-fold-concentrated suspension of cells. All steps were done at 4°C. Cell suspensions (200 ml) from either G medium or CDS medium cultures were harvested, washed once in 0.1 M phosphate buffer (pH 7.0), suspended in 10 ml of the buffer, and passed through a French pressure cell at 20,000 lb/in$^2$. Cell debris was removed by centrifugation at 17,000 × *g* for 10 min. The supernatants were subsequently centrifuged for 1.5 h at 200,000 × *g* in a preparative ultracentrifuge (International Equipment Co., model B-60). Extracts were stored in an ice bath and used within 12 h.

**Enzyme assays.** The reaction mixtures and procedure for the assay of glutamic-oxaloacetate transaminase (EC 2.6.1.1) and glutamic-pyruvic transaminase (EC 2.6.1.2) were performed as described in Sigma technical bulletin no. 505 (Sigma Chemical Co., St. Louis, Mo.), except that reagent volumes were reduced twofold. Rate measurements were determined by stopping the reactions at regular time intervals with acidic 2,4-dinitrophenylhydrazine and measuring the keto acid hydrazone at 505 nm.

Phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.32) was measured by a modification of the method of Vennesland (42). A unit reaction mixture contained (at 35°C): 0.5 ml of 0.12 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.6), 0.1 ml of 0.15 M reduced glutathione, 0.1 ml 0.02 M adenosine 5′-triphosphate, 0.1 ml of 0.02 M $MnCl_2$, 0.45 ml of distilled water, and 0.1 ml of cell extract (1 to 2 mg of protein). The reaction was started by the addition of 0.15 ml of freshly prepared 0.03 M oxaloacetate. The final volume was 1.5 ml. A blank containing all components, except cell extract, was used to monitor the rate of nonenzymatic decomposition of oxaloacetate. The standard curve for oxaloacetate was prepared from reaction mixtures containing 0.0 to 0.3 μmol of freshly prepared oxaloacetate, except that cell extracts were first boiled for 5 min. The organic acid produced by the reaction was determined chromatographically by the method of Fortnagel and Freese (9).

Citrate synthase (EC 4.1.3.7) was measured by the procedure of Srere et al. (39). The activity of fumarase (EC 4.2.1.2) was determined by the methods of Massey (26) and Racker (35). Malate dehydrogenase (EC 1.1.1.37) was assayed by the procedure of Ochoa (32). The methods of Strecker (40) and West et al. (43) were employed to assay L-glutamate dehydrogenase enzymes (EC 1.4.1.4 and EC 1.4.1.2).

Both glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed by methods described by Doi et al. (7) and Goldman and Blumenthal (12).

Fructose-1,6-diphosphatase (EC 3.1.3.11) was measured by a modification of the method of Pontremoli (34). The final volume of the reaction mixture was 3.0 ml and included 0.1 ml of 0.1 M reduced glutathione.

Protein concentrations were estimated by the method of Lowry et al. (24). The specific activities of all enzymes measured were expressed as micromoles of substrate converted per minute per milligram of protein.

All isotopes used in this study were purchased from New England Nuclear Corp.

## RESULTS

**Glutamate requirement.** L-Glutamate is required in substrate quantity during sporulation of *B. cereus* T in the CDS medium (Table 1). In

TABLE 1. *Glutamate requirement for early stages of sporulation of B. cereus T*[a]

| Time of transfer[b] (h) | Dry wt (mg/ml of culture) | Viable count[c] (cells × $10^8$/ml) | Spore count[d] (cells × $10^8$/ml) | Spores (% of control value) |
|---|---|---|---|---|
| Control | 0.82 | 7.6 | 7.2 | 100 |
| 0 | 0.38 | 3.7 | 1.2 | 17 |
| 2 | 0.47 | 7.4 | 4.8 | 67 |
| 4 | 0.70 | 7.4 | 7.1 | 99 |
| 6 | 0.77 | 7.4 | 7.0 | 98 |

[a] All values were determined after 26 h of sporulation at 30°C.

[b] Time interval between placement of cells into complete CDS medium at $T_0$ and transfer to CDS medium minus glutamate.

[c] Culture samples were diluted in 0.1% peptone-water, and duplicate platings were prepared by using Trypticase soy agar (BBL) or plate count agar (Difco).

[d] Spores are defined as cells that survived 80°C for 20 min prior to diluting and plating.

the absence of this amino acid, about 50% of the cells routinely lysed and 10 to 20% eventually sporulated. The latter figures are probably high since nutrients are released during cell lysis. If cells were deprived of glutamate 2 h after placement into CDS medium, lysis was negligible and the percentage of the population forming spores increased to 60 to 70%. The presence of glutamate during the first 4 h was adequate for both survival and almost complete sporulation, indicating that the glutamate requirement for sporogenesis under these conditions was satisfied. In all cell cultures, there were no significant differences in the time spore structures first appeared, about 9 h after placement into CDS medium. Spore formation was approximately 95% complete by h 11 in the control culture.

**Oxidation of glutamate.** The distribution of radioactivity added to CDS medium as L-[U-$^{14}$C]glutamic acid was monitored at various time intervals (Fig. 1A). By h 6, about 80% of the glutamate utilized was oxidized to [$^{14}$C]$CO_2$, and about 20% was associated with the sporulating cells. The amount incorporated into mature spores (24 h) was 10 to 15%. Chemical fractionation analysis (33) showed that, of the radioactivity incorporated, 75 to 80% remained in the nucleic acid and protein fractions. Stained preparations of cultures and chemical analysis of cells for PHB (30) show that the accumulation of this polymer coincides with the period of maximum glutamate oxidation.

**Tricarboxylic acid cycle intermediate substitution for glutamate.** Although earlier results from this laboratory indicated that acetate was not significantly oxidized to $CO_2$ during the first 4 h of sporulation in the sporulation medium (30), it seemed likely that glutamate oxidation occurred via the tricarboxylic acid cycle or some part of it. Various tricarboxylic acid cycle intermediates were tested to determine the extent to which each could substitute for glutamate in producing heat-resistant spores, as compared with the normal CDS culture containing glutamate. The most effective was $\alpha$-ketoglutarate; fumarate, malate, citrate, and succinate were successively less effective, and finally oxaloacetate and isocitrate were least effective in replacing glutamate for sporulation (Table 2). Buono et al. (5) reported that several tricarboxylic acid cycle intermediates replaced glutamate during sporulation of a different strain of *B. cereus* if $(NH_4)_2SO_4$ was present in the medium employed. Both G and CDS media used in this investigation contain $(NH_4)_2SO_4$. Despite the instability of oxaloacetate and permeability differences among intermediates tested, it appeared from these data that the tricarboxylic acid cycle or a part of it was involved in the oxidation of glutamate.

**Effect of fluoroacetate on glutamate oxidation.** The addition of 0.01 M fluoroacetate to CDS medium cultures containing uniformly labeled glutamate revealed that glutamate oxidation may not be entirely dependent upon the complete tricarboxylic acid cycle. This concentration of fluoroacetate completely inhibited acetate metabolism, PHB synthesis, and spore formation. Of the glutamate that was utilized, about 80% was oxidized to [$^{14}$C]$CO_2$ by h 6 (in the presence or absence of fluoroacetate), but the rates were lower in the presence of fluoroacetate (Fig. 1B). Moreover, in noninhibited cultures an appreciable amount of radioactivity was associated with the hot trichloroacetic acid-soluble portion of fractionated mature spores. Further analysis of this fraction by the method of Smith and Salmon (38) showed that the pentose residue of ribonucleic acid contained the

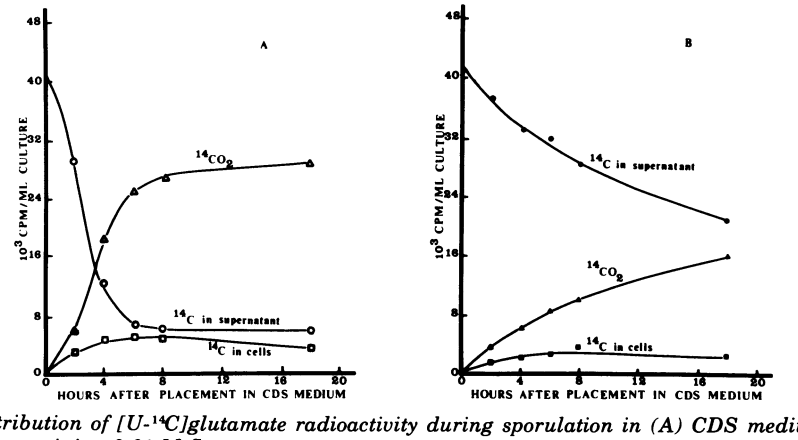


FIG. 1. *Distribution of [U-$^{14}$C]glutamate radioactivity during sporulation in (A) CDS medium and (B) CDS medium containing 0.01 M fluoroacetate.*

TABLE 2. *Substitution of glutamate with tricarboxylic acid cycle intermediates*[a]

| Substitute intermediate[b] | Spores[c] (% of maximum value) |
|---|---|
| CDS control | 100 |
| Alpha-ketoglutarate | 89 |
| Fumarate | 80 |
| Malate | 79 |
| Citrate | 66 |
| Succinate | 65 |
| Oxaloacetate | 54 |
| Isocitrate | 49 |
| Neither glutamate nor intermediate | 22 |

[a] All measurements were made after 27 h of incubation at 30°C.

[b] Initial concentration of intermediates was 0.2% (wt/vol), except for isocitrate, which was 0.3%.

[c] Cells surviving 20 min at 80°C.

highest percentage of the incorporated radioactivity.

These data suggested that glutamate oxidation occurred partially by the tricarboxylic acid cycle (glutamate to oxaloacetate) and partially by the pentose phosphate pathway via gluconeogenesis. Experimental analysis showed that the PHB synthesized by cells sporulating in glutamate-labeled media contained only 1.0 to 2.0% of the glutamate carbon available (unpublished data), suggesting that only a small quantity of glutamate-derived PEP is metabolized to acetyl coenzyme A. Hence, attempts were made to demonstrate the presence of certain enzymes essential for the oxidation of glutamate during early stages of sporulation.

**Demonstration of enzymes essential for the oxidation of glutamate.** Extracts were prepared at various time intervals during spore formation, and the specific activities of these enzymes were determined (Fig. 2). Our attempts and those of others (19, 25) to detect nicotinamide adenine dinucleotide phosphate-dependent L-glutamic dehydrogenase activities in *B. cereus* failed, but both glutamic-oxaloacetic and glutamic-pyruvic transaminases occur throughout sporulation (Fig. 2B). These could be responsible for $\alpha$-ketoglutarate formation from glutamate, as suggested by Buono et al. (5). Glutamate may enter the tricarboxylic acid cycle as succinate by the $\gamma$-aminobutyrate pathway, analogous to the mechanism in *B. thuringiensis* (1).

Very little fluctuation in activities was observed during sporulation for the transaminases and fructose-1,6-diphosphatase. However, the specific activities of fumarase, malate dehydrogenase, PEP carboxykinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase all increased rapidly with the onset of sporulation and glutamate oxidation. In the time in which the rate of glutamate oxidation was maximum, the activities of malate dehydrogenase and PEP carboxykinase increased approximately three- and fourfold, respectively, in contrast to smaller increases in the other enzymes measured. After 2 h into sporulation, the specific activity of citrate synthase was $6.0 \times 10^{-3}$ $\mu$mol/min per mg of protein and was the least active of the tricarboxylic acid cycle enzymes tested.

**Energy metabolism and enzyme activities in cells in G medium.** The possibility that glutamate oxidation does not occur during vegetative growth or that it is not unique to the CDS medium was tested. In G medium cultures (supplemented with 0.18% [wt/vol]glutamate) glutamate oxidation is minimal during vegetative growth, but rapidly increases with the commencement of sporulation (Fig. 3). Also, several of the selected enzymes were examined in extracts prepared from cells growing and sporulating in G medium with and without added glutamate. In either instance, the specific activities of malate dehydrogenase, PEP carboxykinase, and 6-phosphogluconate dehydrogenase increased as sporulation began, and no significant differences in specific activities were noted between these curves and the ones obtained from CDS culture extracts (Fig. 2).

The extent of oxidation of vegetative cell material that might contribute to the production of energy during sporulation was also estimated by labeling G medium with either D-[U-$^{14}$C]glucose (1 to 5 mCi/mmol) or L-U-$^{14}$C-amino acid mixture (10 mCi/mmol) or both. When sporulation began, the labeled cells were placed in unlabeled CDS medium and monitored for [$^{14}$C]$CO_2$ release during sporogenesis. Only 10 to 15% of the $^{14}$C label associated with vegetative cell material was oxidized to [$^{14}$C]$CO_2$ after 8 h of incubation. A greater proportion of the [$^{14}$C]$CO_2$ was evolved during the later stages of sporulation, thereby indicating that cell turnover is not a major source of energy early in sporulation.

## DISCUSSION

The presence of L-glutamate, at a concentration of 1.8 mg/ml, was essential to permit maximum yields of heat-resistant spores (29). This investigation revealed that during the first 4 h of sporogenesis, nearly 70% of the available glutamate was utilized. As much as 80% of the glutamate taken up from the medium could be recovered as $CO_2$ in the initial 4-h period,

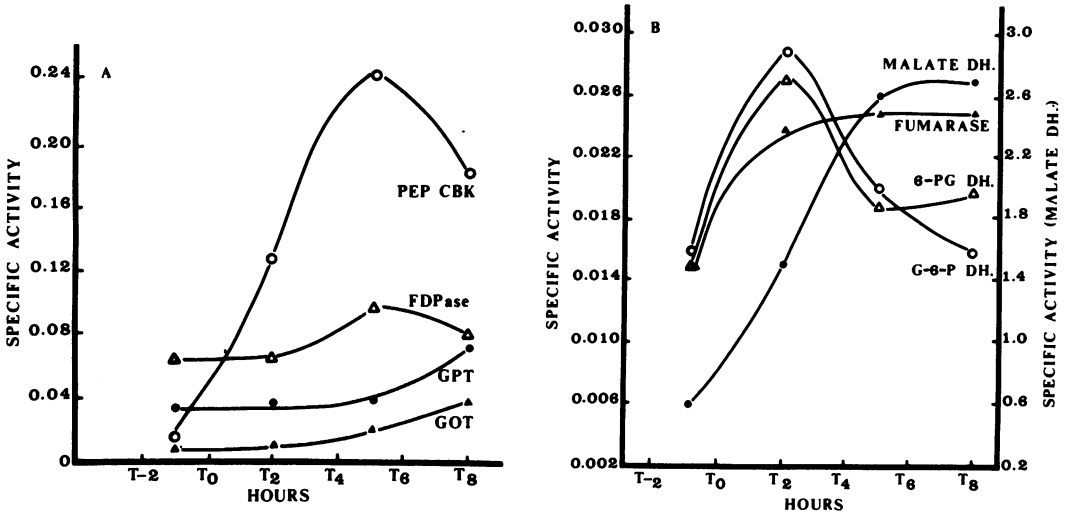


FIG. 2. *Specific activity profiles of (A) PEP carboxykinase (PEP CBK), fructose-1,6-diphosphatase (FDPase), glutamic-pyruvic transaminase (GPT), and glutamic-oxaloacetate transaminase (GOT) and (B) fumarase, malate dehydrogenase (malate DH), glucose-6-phosphate dehydrogenase (G-6-P DH), and 6-phosphogluconate dehydrogenase (6-PG DH) prior to and during sporogenesis. $T_0$ indicates the time cells were placed in CDS medium. Data for $T_{-1}$ were obtained with extracts prepared from G medium cultures 1 h prior to replacement.*

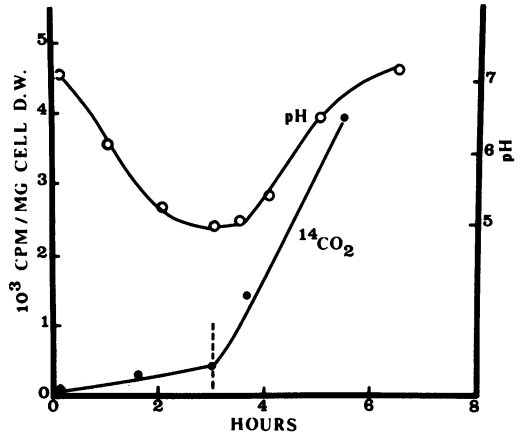


FIG. 3. *Oxidation of glutamate to $CO_2$ in G medium. Dashed line indicates the time sporulation began as evidenced by the rise in pH.*

whereas only 15 to 20% was associated with the cells.

In the sporulation conditions employed, the catabolism of glutamate appears to have the overall function of providing energy for spore formation and, to a lesser extent, of providing precursors for spore synthesis. The latter does not conflict with the findings of Kennedy et al. (20), who observed glutamate to be involved in many physiological processes during sporulation and to influence a number of spore properties. Bernlohr (2) suggested that endogenous amino acids may serve as the primary source of energy and of anabolic precursors during sporulation of *B. licheniformis* and later showed glutamate to constitute a high percentage of the amino acid pool of that organism (4).

The implication of glutamate as the major source of energy seems to conflict with reports from other laboratories in which oxidation of acetate via the tricarboxylic acid cycle is assigned this function, not only in *B. cereus* T but also in other bacilli as well (10, 16–18). This difference lies basically in the substrate specified as the source of energy and cultural conditions employed.

All the date from this laboratory suggest that in the first 4 h sporulating cells of *B. cereus* T couple the energy and reduced coenzyme derived from glutamate catabolism to the assimilation of a large portion of the exogenous acetate into PHB. The divergence in the metabolic fates of glutamate and acetate is supported by the detection of only a small percentage of labeled glutamate carbon in PHB. The results here are consistent with our earlier findings on the role of acetate during sporulation of *B. cereus* T (30).

We have presented data that indicate that in early sporulation *B. cereus* T may not utilize the entire tricarboxylic acid cycle for glutamate catabolism. It has been reported before (9, 18) that sporulation in aerobic *Bacillus* spp. required the tricarboxylic acid cycle. The fluoroacetate data coupled with detection of signifi-

cant quantities of glutamate radioactivity in the pentose of ribonucleic acid suggested a catabolic sequence involving a portion of the tricarboxylic acid cycle, gluconeogenesis, and the pentose phosphate pathway. Increases in pertinent enzyme activities during sporulation provide additional support. A similar pathway was described for *B. subtilis* (10). Singh (37) found that a complete tricarboxylic acid cycle might not be necessary for spore formation by *B. megaterium* 735, and sporulation without derepression of the normal tricarboxylic acid cycle seems to occur in *B. thuringiensis* (31).

Malate dehydrogenase and PEP carboxykinase have pronounced activity at the time the rate of glutamate oxidation is the greatest. PEP carboxykinase, biologically active only in the direction of PEP formation (23), would be necessary for the gluconeogenic metabolism of glutamate. That PEP carboxykinase is associated with sporulation in *B. cereus* T was reported previously (J. F. Charba, Diss. Abstr. Int. B, 30:3007, 1970). Diesterhaft and Freese (6) later reported that this enzyme is needed for sporulation in *B. subtilis*.

The cellular events governing simultaneous glutamate catabolism and acetate assimilation during sporulation in CDS medium are yet unknown. Modulation of tricarboxylic acid cycle enzymes by adenosine 5'-triphosphate concentration and energy charge may be partly responsible. It has been shown that adenosine 5'-triphosphate synthesis reaches a peak early in *B. subtilis* sporulation (22). Other evidence indicates that L-glutamine functions in the repression of sporulation in *B. megaterium* when both ammonia and glucose are present in the medium (8, 36). When cells of *B. cereus* T are transferred to CDS medium at $T_0$, the high availability of ammonia and glutamate (a combination possibly analogous to ammonia and glucose before $T_0$) may promote a temporary repression of one or more sporulation-specific synthetic processes by a mechanism similar to the one described for *B. megaterium*.

The conclusion that glutamate (rather than acetate) is the primary energy source during beginning stages of spore formation was noted earlier (J. F. Charba and H. M. Nakata, Bacteriol. Proc., p. 16, 1966) but not emphasized. It was also pointed out before that acetate did not appear to have this function for *B. cereus* T sporulating in CDS medium (30). The requirement for a relatively high concentration of glutamate is viewed as fulfilling mainly a catabolic function, that of yielding reducing potential and energy for the assimilation of acetate into PHB (consumed in later stages of sporulation) and other early syntheses.

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## LITERATURE CITED

1. **Aronson, J. N., J. F. Doerner, E. W. Akers, D. P. Borris, and M. Mani.** 1975. γ-Aminobutyric acid pathway of glutamate metabolism by *Bacillus thuringiensis*, p. 404–410. *In* P. Gerhardt, H. L. Sadoff, and R. N. Costilow (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
2. **Bernlohr, R. W.** 1964. Postlogarithmic phase metabolism of sporulating microorganisms. I. Protease of *Bacillus licheniformis*. J. Biol. Chem. 239:538–543.
3. **Bernlohr, R. W.** 1965. Role of amino acids in sporulation, p. 75–87. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
4. **Bernlohr, R. W.** 1967. Changes in amino acid permeation during sporulation. J. Bacteriol. 93:1031–1044.
5. **Buono, F., R. Testa, and D. G. Lundgren.** 1966. Physiology of growth and sporulation in *Bacillus cereus*. I. Effect of glutamic and other amino acids. J. Bacteriol. 91:2291–2299.
6. **Diesterhaft, M. D., and E. Freese.** 1973. Role of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme during growth and sporulation of *Bacillus subtilis*. J. Biol. Chem. 248:6062–6070.
7. **Doi, R., H. Halvorson, and B. Church.** 1959. Intermediate metabolism of aerobic spores. III. The mechanism of glucose and hexose phosphate oxidation in extracts of *Bacillus cereus* spores. J. Bacteriol. 77:43–54.
8. **Elmerich, C., and J.-P. Aubert.** 1975. Involvement of glutamine synthetase and the purine nucleotide pathway in repression of bacterial sporulation, p. 385–390. *In* P. Gerhardt, H. L. Sadoff, and R. N. Costilow (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
9. **Fortnagel, P., and E. Freese.** 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. 95:1431–1438.
10. **Freese, E., and P. Fortnagel.** 1967. Analysis of sporulation mutants. I. Response of uracil incorporation to carbon sources, and other mutant properties. J. Bacteriol. 94:1957–1969.
11. **Gary, N. D., and R. C. Bard.** 1952. Effect of nutrition on the growth and metabolism of *Bacillus subtilis*. J. Bacteriol. 64:501–512.
12. **Goldman, M., and H. J. Blumenthal.** 1964. Pathways of glucose catabolism in *Bacillus cereus*. J. Bacteriol. 87:377–386.
13. **Grelet, N.** 1955. Nutrition azotee et sporulation de *Bacillus cereus* var. *mycoides*. Ann. Inst. Pasteur Paris 88:60–75.
14. **Hanson, R. S., J. Blicharska, M. Arnaud, and J. Szulmajster.** 1964. Observations on the regulation of the synthesis of the tricarboxylic acid cycle enzymes in *Bacillus subtilis*, Marburg. Biochem. Biophys. Res. Commun. 17:690–695.
15. **Hanson, R. S., and D. P. Cox.** 1967. Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. J. Bacteriol. 93:1777–1787.
16. **Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson.** 1961. Citrate formation in *B. cereus* strain T. Biochem. Biophys. Res. Commun. 5:457–460.
17. **Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson.** 1963. Biochemistry of sporulation. I. Metabolism of acetate by vegetative and sporulating cells. J. Bacteriol. 85:451–460.

18. **Hanson, R. S., V. R. Srinivasan, and H. O. Halvorson.** 1963. Biochemistry of sporulation. II. Enzymatic changes during sporulation of *Bacillus cereus*. J. Bacteriol. 86:45–50.
19. **Hong, M. M., S. C. Shen, and A. E. Braunstein.** 1959. Distribution of L-alanine dehydrogenase and L-glutamate dehydrogenase in *Bacilli*. Biochim. Biophys. Acta 36:288–289.
20. **Kennedy, R. S., F. J. Malveaux, and J. J. Cooney.** 1971. Effects of glutamic acid on sporulation of *Bacillus cereus* and on spore properties. Can. J. Microbiol. 17:511–519.
21. **Keynan, A., H. S. Stracker, and H. Waelsch.** 1954. Glutamine, glutamic acid, and glycolysis in *Bacillus subtilis*. J. Biol. Chem. 211:883–890.
22. **Klofat, W., G. Picciolo, E. W. Chapelle, and E. Freese.** 1969. Production of adenosine triphosphate in normal cells and sporulation mutants of *Bacillus subtilis*. J. Biol. Chem. 244:3270–3276.
23. **Lehninger, A. L.** 1975. Biochemistry, 2nd ed. Worth Publishers, Inc., New York.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
25. **McCormick, N. G., and H. O. Halvorson.** 1964. Purification and properties of L-alanine dehydrogenase from vegetative cells of *Bacillus cereus*. J. Bacteriol. 87:68–74.
26. **Massey, V.** 1952. The crystallization of fumarase. Biochem. J. 51:490–494.
27. **Millet, J., and J. P. Aubert.** 1960. Le metabolism de l'acide glutamique au cours de la sporulation chez *Bacillus megaterium*. Ann. Inst. Pasteur Paris 98:282–290.
28. **Nakata, H. M.** 1963. Effect of pH on intermediates produced during growth and sporulation of *Bacillus cereus*. J. Bacteriol. 86:577–581.
29. **Nakata, H. M.** 1964. Organic nutrients required for growth and sporulation of *Bacillus cereus*. J. Bacteriol. 88:1522–1524.
30. **Nakata, H. M.** 1966. Role of acetate in sporogenesis of *Bacillus cereus*. J. Bacteriol. 91:784–788.
31. **Nickerson, K. W., J. DePinto, and L. A. Bulla, Jr.** 1974. Sporulation of *Bacillus thuringiensis* without concurrent derepression of the tricarboxylic acid cycle. J. Bacteriol. 117:321–323.
32. **Ochoa, S.** 1955. Malic dehydrogenase from pig heart, p. 735–739. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
33. **Park, J. T., and R. Hanocock.** 1960. A fractionation procedure for studies of the synthesis of cell wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. 22:249–258.
34. **Pontremoli, S.** 1966. Fructose-1,6-diphosphatase, p. 625–631. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 9. Academic Press Inc., New York.
35. **Racker, E.** 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta 4:211–214.
36. **Reysset, G., and J.-P. Aubert.** 1975. Relationship between sporulation and mutations impairing glutamine synthetase in *Bacillus megaterium*. Biochem. Biophys. Res. Commun. 65:1237–1241.
37. **Singh, R. M. M.** 1970. Role of tricarboxylic acid cycle in bacterial sporulation. Biochem. Biophys. Res. Commun. 39:651–654.
38. **Smith, R. C., and W. D. Salmon.** 1965. Effect of ethionine on the ribonucleic acid, deoxyribonucleic acid, and protein content of *Escherichia coli*. J. Bacteriol. 89:687–692.
39. **Srere, P. A., H. Braxil, and L. Gonen.** 1963. The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. Acta Chem. Scand. 17:S-129–S-134.
40. **Strecker, H. J.** 1955. L-glutamic dehydrogenase from liver, p. 220–225. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
41. **Szulmajster, J., and R. S. Hanson.** 1965. Physiological control of sporulation in *Bacillus subtilis*, p. 162–173. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
42. **Vennesland, B.** 1962. Conversion of phosphoenolpyruvate to oxaloacetate, p. 619–620. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
43. **West, D. J., R. W. Tuveson, and R. W. Barratt.** 1967. Allosteric effects in nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Neurospora*. J. Biol. Chem. 242:2134–2138.