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Sporulation of *Bacillus thuringiensis* Without Concurrent Derepression of the Tricarboxylic Acid Cycle

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Bacillus thuringiensis sporulates in a glucose-glutamate medium without concurrent derepression of the tricarboxylic acid cycle. Glutamate appears to regulate tricarboxylic acid cycle activity as well as to influence spore heat resistance and production of dipicolinic acid.

Bacterial sporulation is a model system for studying cellular differentiation. However, biochemical and genetic characterization of spore formation has been plagued by difficulty in distinguishing between causal necessity and fortuitous coincidence of physiological events (7). Some events implicated as requisite for sporulation, because of their temporal relationship to morphological development or because of their absence in certain mutants unable to sporulate, may not be involved in spore formation at all. One such physiological phenomenon in aerobic sporeformers is the appearance of tricarboxylic acid cycle activity during postlogarithmic growth and sporogenesis.

Yousten and Hanson (13) provided the first evidence that the tricarboxylic acid cycle may not be an absolute requirement for sporulation. They showed that mutants of *Bacillus subtilis* with lesions in the tricarboxylic acid cycle could be made to sporulate by appropriate manipulation of the culture media. In this communication, we provide direct evidence that a fully operational tricarboxylic acid cycle is not necessarily required for sporulation.

We developed a chemically defined growth medium in which *B. thuringiensis* var. *entomocidus* NRRL B-4046 has sporulated repeatedly without an active tricarboxylic acid cycle. This medium consists of (per liter of distilled water): glucose, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; $ZnSO_4 \cdot 7H_2O$, 0.005 g; $CuSO_4 \cdot 5H_2O$, 0.005 g; $FeSO_4 \cdot 7H_2O$, 0.0005 g; and glutamate, 0.16 g (pH adjusted to 7.3 with KOH). The glutamate concentration is critical. This medium contains 0.016% glutamate and is designated LG (low glutamate), in contrast to our high glutamate (HG) medium which contains 0.2% glutamate. No growth occurs in the absence of glutamate. Refractile spores were formed in both the LG medium (Fig. 1) and the HG medium, but whereas the final pH of the HG medium was above 8.0, the pH of the LG medium dropped to about 4.5 and remained there throughout sporulation.

A final pH of 4.5 in the LG medium is presumptive evidence that the tricarboxylic acid cycle was not activated. This conclusion was confirmed by comparative radiorespirometric studies of sporulating cells from HG and LG media. Figure 2 shows the pattern of $^{14}CO_2$ released from specifically labeled glutamate and acetate. Sporulating cells grown in the HG medium actively metabolized glutamate; $^{14}CO_2$ release in the order C2 = C5 > C3,4 is consistent with glutamate oxidation via an intact tricarboxylic acid cycle. In contrast, sporulating cells grown in LG medium did not oxidize either labeled glutamate or labeled acetate. Virtually no $^{14}CO_2$ was released from either substrate. These results provide strong evidence that a functional tricarboxylic acid cycle was absent during sporulation in the LG medium because: (i) glutamate was incorporated into the sporulating cells during radiorespirometry; e.g., 20% of the glutamic acid-*3,4*-^{14}C was incorporated into the cells, whereas only 0.23% was oxidized to CO_2; (ii) separate uptake experiments showed that the concentration of carrier glutamate used in the radiorespirometry (0.02% in all experiments) was sufficient to ensure entry of the labeled glutamate into the cells; and (iii) by identical techniques, an active tricarboxylic acid cycle was demon-

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strated in sporulating cells grown in HG medium.

Table 1 compares the properties of spores produced in HG and LG media. The LG-grown spores formed in the absence of a functional tricarboxylic acid cycle were: (i) refractile; (ii) frequently of normal *B. thuringiensis* spore morphology (see Fig. 1); (iii) octanol resistant (6); (iv) resistant to 62 C for 1 h; and (v) not stained by fat (4) and metachromatic granule (2) specific stains. The spores differed from those produced in HG media because they were: (i) sensitive to heat shock (30 min) at 80 C; (ii) reduced in dipicolinic acid (8) content; and (iii) of decreased density. These properties are similar to those of the dipicolinic acid-deficient spores produced by dipicolinic acidless mutants (12) and by cells grown in the presence of ethyl oxamate (6), picolinamide (11), nicotinamide (9), or excess phenylalanine (5). Our work indicates that glutamate, either directly or indirectly, regulates dipicolinic acid production and heat resistance. Lack of a functional tricarboxylic acid cycle may be important to

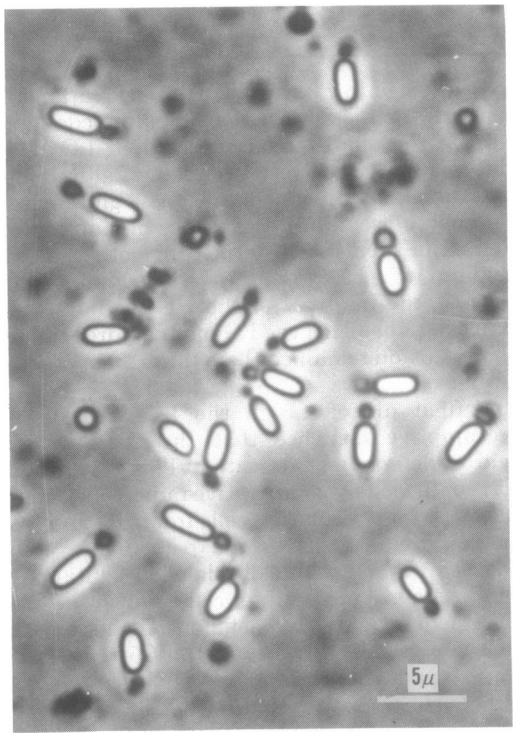

FIG. 1. *Phase contrast micrograph of B. thuringiensis spores formed in low glutamate medium.*

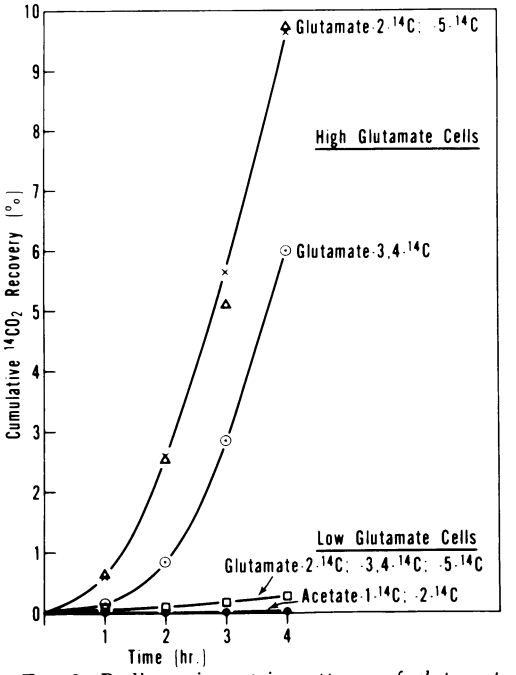

FIG. 2. *Radiorespirometric patterns of glutamate and acetate utilization by B. thuringiensis var. entomocidus. Sporulating cells grown in high and low glutamate media were assayed by the procedures of Bulla et al. (3). Glutamate-2-14C; -3,4-14C; and -5-14C (0.02% glutamate) at pH 7. Acetate-1-14C; and -2-14C (0.003% acetate) at pH 4.6.*

TABLE 1. *Characteristics of spores formed in media of differing glutamate concentrations*

Glutamate concn	Properties								
	Final pH of sporulation medium	Total viable count (per ml)	Octanol-resistant (6) count (per ml)	Heat-stable spores (%)		Dipicolinic acid content (μg/10⁸ spores)[a]	Spore density[b] (g/ml)	Tricarboxylic acid cycle during sporulation	Glucose utilized during growth (%)
				80 C for 0.5 h	62 C for 1 h				
High	~8	1.8×10^8	1.9×10^7	100	100	6.7	1.30	Present	95.1
Low	4.5–4.8	2.8×10^8	2.8×10^7	<0.01	90–95	≤3 0[c]	1.27	Absent	92.3

[a] Spores defined as octanol resistant counts.
[b] Determined by Renografin density gradient centrifugation (12).
[c] Highest of five determinations. Other values 2 to 2.5 μg/10⁸ spores.

dipicolinic acid synthesis because aspartate is a precursor of dipicolinic acid.

Our data establish that the tricarboxylic acid cycle is inoperative during sporulation of *B. thuringiensis* var. *entomocidus* in LG medium; the presence or absence of individual tricarboxylic acid cycle enzymes was not established. The phenomenon of sporulation at low pH occurred also in *B. thuringiensis* var. *alesti* NRRL B-4041, the only other organism we tested.

High amino acid concentrations may induce enzymes for the catabolic degradation of that amino acid, and glutamate decarboxylase (L-glutamate l-carboxy-lyase EC 4.1.1.15) activity in *B. thuringiensis* can be high (1). J. N. Aronson (personal communication) has suggested that *B. thuringiensis* possesses a modified tricarboxylic acid cycle in which alpha-ketoglutarate dehydrogenase is absent. Instead, alpha-ketoglutarate is converted to glutamate and then to succinate via gamma-aminobutyric acid. We feel that high concentrations of glutamate may be necessary to induce glutamate decarboxylase or another enzyme of the gamma-aminobutyric acid pathway. This theory explains the high concentrations of glutamate required for sporulation in a great number of different organisms (7). Our radiorespirometric data (see also ref. 3) would not distinguish such a modified tricarboxylic acid cycle from one containing alpha-ketoglutarate dehydrogenase. At present, we are conducting enzyme assays to test these ideas.

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