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γ-Aminobutyric Acid Pathway and Modified Tricarboxylic Acid Cycle Activity During Growth and Sporulation of *Bacillus thuringiensis*

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Enzymatic analyses of *Bacillus thuringiensis* extracts suggest that a modified Krebs tricarboxylic acid cycle (without α-ketoglutarate dehydrogenase) can operate during sporulation in conjunction with the glyoxylic acid cycle and the γ-aminobutyric acid pathway.

Bacillus species are generally thought to require tricarboxylic acid cycle activity for successful sporulation, possibly in order to maintain necessary adenosine 5'-triphosphate levels and to remove some accumulated suppressor substance (29). *Bacillus thuringiensis* exhibits a somewhat typical metabolic pattern in that several tricarboxylic acid cycle enzymes appear at the onset of sporulation (37). However, studies in our laboratory have revealed the presence of an ancillary pathway in *B. thuringiensis* which allows for glutamate and α-ketoglutarate catabolism via the γ-aminobutyric acid pathway. This obviates the necessity for α-ketoglutarate dehydrogenase, which activity we have been unsuccessful in demonstrating by several procedures. In this communication we provide evidence for the operation of a modified tricarboxylic acid cycle along with the glyoxylic acid cycle and the γ-aminobutyric acid pathway. Specific activity data is also given for enzymes that catalyze the formation of glutamate, aspartate, alanine, and reduced pyridine nucleotides.

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B. thuringiensis var. *berliner* was used in this study and was grown at 30 C on a salts medium (33) supplemented with 0.1% glucose, 0.033% Casamino Acids (Difco), and trace metals as described previously (3). Maximal culture absorbance at 600 nm was reached after 10 h following a steady decrease in the pH and the glucose concentration. Culture samples were centrifuged for 20 min in a Sorvall RC2-B refrigerated centrifuge at 12,100 × *g*, and the cell pellets were analyzed for protein by the procedure of Lowry et al. (21). Supernatant fluids were analyzed for residual glucose by the Glucostat procedure (Worthington Biochemical Corp., Freehold, N.J.). The maximal culture absorbance was reached when the glucose had been completely depleted from the medium, at which time the pH suddenly rose; in typical cultures of *Bacillus* species this would be interpreted to result from tricarboxylic acid cycle activity (16). Cultures were sampled during four stages representative of the developmental pattern: mid-exponential phase (4 to 6 h), late exponential phase (9 to 10 h), early sporulation (11 to 13 h), and late sporulation (15 to 16 h).

Appropriate dilutions of sporulated cultures were heated at 70 C for 15 min, and portions were plated on nutrient agar. Colonies that developed after 24 h of incubation were counted as spores. This medium usually yielded 8.0×10^7 spores/ml, which was greater than 90% sporulation by microscopic examination.

The cells were washed once in cold water and then suspended in the appropriate buffer as determined by the enzyme to be assayed. Cells were disrupted by sonication in an aluminum cell with a Branson Sonifier model S-125. To minimize heating, the cell was kept in an ice water bath, and sonication was accomplished in seven 30-s bursts with a 30-s delay between each burst. The cell debris was removed by centrifugation at 12,100 × *g* for 20 min. Fractions of the resulting supernatant fluid were analyzed or quick-frozen in a dry ice-acetone bath and stored at −20 C for later analysis.

Bacterial extracts were assayed for enzyme activities by standard spectrophotometric procedures; references are cited in Table 1. Results of several experiments are summarized in Table 1. Early enzymes of the tricarboxylic acid cycle (Table 1, c and d) exhibited increased specific

TABLE 1. *Specific activities of various enzymes during growth and sporulation of B. thuringiensis*

Enzyme[a]	Sp act (nmol/min/mg of protein)			
	Mid-log phase extracts	Late-log phase extracts	Early sporulation extracts	Late sporulation extracts
a. Fructose diphosphate aldolase, EC 4.1.2.13 (13)	2.5	1.8	1.2	0.0
b. Glyceraldehyde phosphate dehydrogenase, EC 1.1.1.12 (14)	12.2	6.8	8.7	0.0
c. Aconitate hydratase, EC 4.2.1.3 (2)	25.0	36.2	50.0	42.8
d. Isocitrate dehydrogenase (NADP), EC 1.1.1.42 (24)	NA[b]	15.7	35.0	NA
e. Fumarate hydratase, EC 4.2.1.2 (27)	26.5	21.5	18.5	9.7
f. Malate dehydrogenase, EC 1.1.1.37	1.2×10^2	1.2×10^2	1.2×10^2	0.7×10^2
g. Malate dehydrogenase (decarboxylating), (NADP), EC 1.1.1.40 (25)	1.2×10	1.2×10	2.0×10	1.3×10^2
h. Isocitrate lyase, EC 4.1.3.1 (22)	0.1	0.1	0.8	3.1
i. Malate synthase, EC 4.1.3.2 (22)	0.8	0.7	8.5	6.5
j. Glutamate dehydrogenase, EC 1.4.1.2 (28, 32)	1.2	1.9	3.1	0.1
k. Alanine dehydrogenase, EC 1.4.1.1 (34)	9.0	9.0	11.5	0.7
l. L-Aspartate aminotransferase (GOT), EC 2.6.1.1 (5, 11)	20.0	19.6	17.0	16.0
m. L-Alanine aminotransferase (GPT), EC 2.6.1.2 (30)	NA	1.0	1.2	1.9
n. Glutamate decarboxylase, EC 4.1.1.15 (1, 20)	4.1	2.9	9.0	5.1
o. Aminobutyrate aminotransferase, EC 2.6.1.19 (17)	8.3	5.7	1.5	1.0
p. Succinate-semialdehyde dehydrogenase [NAD(P)$^+$], EC 1.2.1.16 (8, 17)	24.3	11.7	6.2	5.0
q. (+)-Citramalate lyase,	0.2	0.8	1.3	1.2
(−)-citramalate lyase (7)	0.3	0.3	0.7	0.7
r. Methylaspartate ammonia lyase, EC 4.3.1.2 (7)	0.2	0.3	0.2	1.0
s. "Methylaspartate mutase," EC 5.4.99.1 (7)	0.3	0.5	1.4	1.6

[a] NADP, Nicotinamide adenine dinucleotide phosphate; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase.

[b] NA, Not assayed.

activity (about twofold) during early sporulation, an observation similar to that reported for the closely related *B. thuringiensis* var. *galleriae* (37). Related anaplerotic enzymes (Table 1, g, h, and i) also showed increases at sporulation.

Extracts (0.2 ml) of sonically disrupted cells were prepared for amino acid analysis by adjusting with 1.0 N HCl to pH 2.2, diluting to 0.9 ml with citrate buffer (0.2 M, pH 2.2), and then deproteinizing by centrifugation through a Centriflo ultrafiltration membrane (Amicon Corp., Lexington, Mass.). Samples were applied to the long column of a Beckman-Spinco model 120C amino acid analyzer. Alanine and glutamic acid accounted for greater than 60% of the free amino acids, as was reported by Bernlohr (6) for three *Bacillus* species and by Young and Fitz-James for a closely related strain of *B. thuringiensis* var. *alesti* (35). Their concentrations are interrelated through transaminases (Table 1, l and m). During sporulation some glutamate is most likely made via glutamate dehydrogenase activity (Table 1, j), possibly supplemented by the citramalate pathway (Table 1, q, r, and s) (7).

Glutamate catabolism would be expected to proceed via the activity of glutamic acid dehydrogenase, α-ketoglutarate dehydrogenase, and the remainder of the tricarboxylic acid cycle. We have unsuccessfully assayed for α-ketoglutarate dehydrogenase activity in these extracts by three different procedures (12, 18, 19). Extracts from cells grown in a phosphate buffer medium (4) in which 0.2% glutamic acid served as the only nitrogen source did possess α-ketoglutarate dehydrogenase activity; however, the activity was not evident until the protein had first been precipitated at 80% ammonium sulfate saturation, which presumably removed some endogenous inhibitor. Therefore, it seems that derepression of all of the tricarboxylic acid cycle enzymes may not be necessary for sporulation of *B. thuringiensis*. A similar conclusion was reached for *B. thuringiensis* var. *entomocidus* grown in a medium containing approximately two times the amount of glutamate contained in our medium (23). The γ-aminobutyric

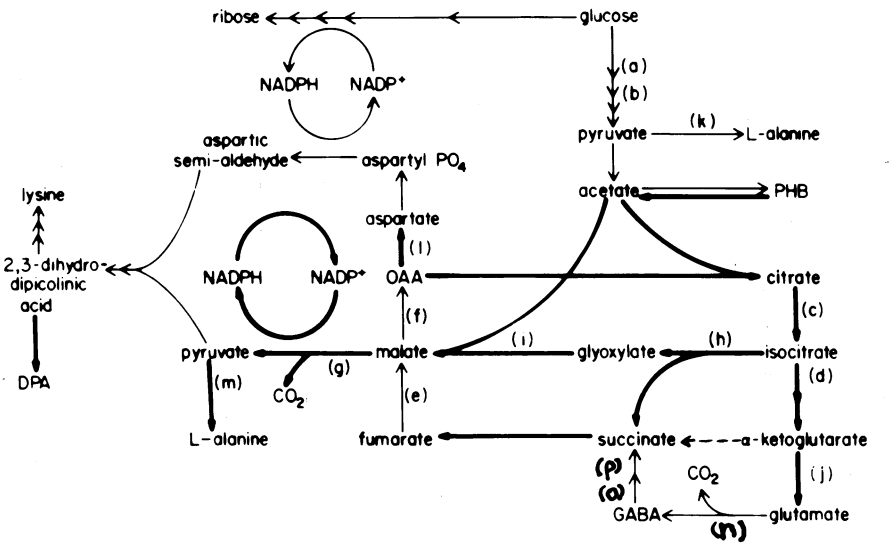

FIG. 1. *Summary of some metabolic pathways utilized by B. thuringiensis during sporulation (heavy arrows). Those pathways thought to be important during growth or during both growth and sporulation are shown by light arrows. Letters in parentheses refer to enzyme assays tabulated in Table 1. Enzymes q, r, and s from Table 1 have been left out for clarity and because of uncertainty of their importance for this organism.*

acid pathway (Table 1, enzymes n, o, and p) would allow circumvention of the α-ketoglutarate dehydrogenase step. This pathway has not previously been implicated in growth and/or sporulation of *Bacillus* species, although certain mutant strains of *Bacillus megaterium* have been shown (15) to require γ-aminobutyric acid for germination.

The utilization by *B. thuringiensis* of the ancillary pathways shown in this work would not be distinguishable by standard radiorespirometry experiments from the operation of the normal tricarboxylic acid cycle (9). Buono et al. (10) showed the importance of glutamate for the sporulation of *Bacillus cereus*, but they also were unable to demonstrate α-ketoglutarate dehydrogenase activity by three different assay procedures. Apparent lack of α-ketoglutarate dehydrogenase activity is not conclusive evidence against an intact tricarboxylic acid, especially since some *Bacillus* species do appear to have an intact tricarboxylic acid cycle (29). However, the γ-aminobutyric acid modification proposed in this communication would obviate the absolute necessity for the α-ketoglutarate dehydrogenase step, and with little energy loss to the organism. If peak enzyme activity occurs in *B. thuringiensis* over a very short time span, as was shown by Ohné (26) for *Bacillus subtilis*, then detection of the α-ketoglutarate dehydrogenase would be more difficult. As shown by the work of Ohné (26), and as indicated by this study with *B. thuringiensis*, high medium glutamate enhances cellular α-ketoglutarate dehydrogenase activity. Studies with other *Bacillus* species, however, have also cast doubt on the essentiality of the tricarboxylic acid cycle for sporulation under certain conditions (31, 36).

Figure 1 summarizes the interactions of the enzymatic reactions discussed in this communication.

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