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Physiology of Sporeforming Bacteria Associated with Insects: Radiorespirometric Survey of Carbohydrate Metabolism In the 12 Serotypes of *Bacillus thuringiensis*

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Radiorespirometry was used to compare the primary pathways of glucose catabolism in 18 strains of *Bacillus thuringiensis* representing the 12 established serotypes. Every strain utilizes the Embden-Meyerhof-Parnas pathway almost exclusively; pentose-phosphate pathway participation is minor. The Embden-Meyerhof-Parnas pathway predominates regardless of whether the cells were grown in a minimal medium or one containing yeast extract. The results indicate that the absolute requirement for citrate and related compounds is not a result of defective citrate or glucose transport and metabolism.

In an attempt to define the metabolic characteristics of bacteria with insecticidal properties, we previously compared *Bacillus thuringiensis*, *B. alvei*, *B. lentimorbus*, and *B. popilliae* with regard to their catabolism of glucose (3) and oxidation of certain tricarboxylic acid cycle intermediates (2). These studies were necessarily restricted to single isolates of each species. In the present investigation, we examined the metabolic homogeneity of *B. thuringiensis* as a group by using radiorespirometry to compare the primary pathways of carbohydrate catabolism in 18 natural isolates. The percent participation of the Embden-Meyerhof-Parnas (EMP) and pentose-phosphate (PP) pathways was calculated for each strain.

Extensive taxonomic studies have been conducted on the numerous isolates of *B. thuringiensis* (5, 13). A classification system dependent on the flagellar H antigen has been devised, and 12 serotypes are now recognized (6). The extent of the differences and similarities among these serotypes is of interest. We have already observed that the serotypes do not differ significantly with regard to their nutritional requirements (10). Results presented in this communication demonstrate that the 18 strains of *B. thuringiensis* also form a cohesive unit when compared metabolically.

Apparently, *B. thuringiensis* is closely related to *Bacillus cereus* (13). Whether this relatedness derives from a single evolutionary divergence to crystal-forming ability is not known. Norris (12) showed that *B. thuringiensis* and *B. cereus* possess phage in common, whereas Somerville and Jones (15) used nucleic acid hybridization to compare these two groups of bacilli. Our metabolic data also reflect a close relationship between *B. thuringiensis* and *B. cereus*.

In a separate series of experiments, we employed *B. thuringiensis* var. *entomocidus* as a test organism to study glucose metabolism in the defined minimal media described in the previous paper (10). Particularly, we found that when grown in a glucose-salts medium *B. thuringiensis* exhibits an absolute requirement for either citrate, aspartate, or glutamate; in contrast, a citrate-salts medium (4) was reported to support growth in the absence of glucose. Because of this apparent paradox, we considered the possibility that either glucose uptake or metabolism was defective in our defined media (10). For example, Romano and Kornberg (14) found that glucose uptake was selectively inhibited when *Aspergillus nidulans* was grown on an acetate-containing medium. Alternatively, glucose catabolism could be defective if our defined media were deficient in thiamine, lipoic acid, or another cofactor required for glycolysis. However, when glucose radiorespirometry was performed on *B. thuringiensis* cells grown in the defined media (10), the results differed only slightly from those obtained when the cells were grown in a medium containing yeast extract. *B.*

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thuringiensis does not require citrate because of any defect in glucose uptake or metabolism.

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MATERIALS AND METHODS

Organisms and cultural conditions. The strains of *B. thuringiensis* listed in Table 1 were obtained from the Agricultural Research Service Culture Collection, Peoria, Ill. Vegetative cells suitable for radiorespirometry were grown in GYS medium, which consisted of 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, and 0.05% K_2HPO_4 (pH 7.3). Aseptic addition of 0.1% glucose, 0.02% $MgSO_4$, 0.008% $CaCl_2 \cdot 2H_2O$, and 0.005% $MnSO_4 \cdot H_2O$ followed autoclaving. A uniform spore inoculum (10) was put into 500 ml of GYS medium contained in a Fernbach flask; the culture was aerated by rotary agitation at 200 rpm. Cells were harvested after 4 h of growth at 28 C.

In addition, vegetative cells of *B. thuringiensis* var. *entomocidus* were cultured in the glucose-glutamate-salts and glucose-aspartate-salts media described by Nickerson and Bulla (10). Such cells were washed twice in 0.003 M phosphate-salts medium after harvesting.

Radiorespirometry. The procedures used were modified from those of Bulla et al. (2, 3). Respirometer flasks containing about 60 mg of a vegetative cell suspension were incubated at 28 C in a modified Gilson differential respirometer. The cells were acclimated 30 min in 30 ml of glucose-free growth medium to reduce endogenous metabolism. Carrier glucose (1 mg) was added to each flask, and after an additional 20 min of incubation 0.25 μCi of radioactive glucose was added. D-Glucose specifically labeled at C1, C3,4, and C6 was purchased from New England Nuclear Corp., Boston, Mass. The flasks were shaken while air was passed through at a rate of 60 cm^3/min. Respired $^{14}CO_2$ was trapped in 10 ml of a mixture of absolute ethanol and monoethanolamine (2:1, vol/vol) that was removed and replenished at half-hour intervals. The trapping solutions then were adjusted to 15 ml with absolute ethanol, and a 5-ml portion was mixed with 10 ml of toluene containing 6 mg of 2,5-diphenyloxazole per ml and 0.1 mg of 1,4-bis-[2]-(5-phenyloxazolyl)benzene per ml. The mixtures were placed in scintillation vials, and a liquid scintillation spectrometer was used to measure radioactivity.

At the end of each experiment, the cells were separated by centrifugation at 4 C, homogenized in NCS solubilizer (Amersham/Searle Corp., Des Plaines, Ill.) for 36 h at 45 C, and counted in a scintillation fluid containing: toluene/2,5-diphenyloxazole/1,4-bis-[2]-(5-phenyloxazoly) benzene:Triton X-100:ethanol (8:4:3; vol/vol/vol). This scintillation mixture was also used to count 1-ml samples of the cell-free supernatant following centrifugation.

Estimation of pathway participation. A simplification of the procedure of Wang (16) was used to estimate participation of the EMP and PP pathways. The following equation shows how the fraction of glucose catabolized via the PP pathway (G_p) was estimated from the yields of $^{14}CO_2$ recovered from D-[1-^{14}C]glucose (G_1) and D-[6-^{14}C]glucose (G_6):

TABLE 1. *Glucose catabolism in B. thuringiensis*

ARS culture collection no.[a]	Variety name	H antigen serotype	Recovery as $^{14}CO_2$ (%)[b]			Pathway participation (%)	
			C-1	C-3,4	C-6	EMP	PP
B-4039	*berliner*	1	5.2	60.9	1.7	96.5	3.5
B-4040	*finitimus*	2	3.6	75.5	4.3	100	0
B-4041	*alesti*	3a	2.6	57.7	3.1	100	0
B-4055	*kurstaki*	3a3b	5.9	32.8	2.3	96.4	3.6
B-4042	*sotto*	4a4b	3.2	55.4	0.6	97.6	2.4
B-4043	*dendrolimus*	4a4b	9.5	58.4	2.5	93	7
B-4044	*kenyae*	4a4c	3.4	45.2	2.9	99.5	0.5
B-4045	*galleriae*	5a5b	3.1	42.2	2.5	99.4	0.6
B-4056	*canadensis*	5a5c	1.9	43.4	2.4	100	0
B-4046	*entomocidus*	6	1.2	43.4	3.1	100	0
B-4047	*entomocidus-limassol*	6	3.4	70.2	2.2	98.8	1.2
B-4057	*subtoxicus*	6	2.5	82.1	3.2	100	0
B-4048	*aizawai*	7	6.3	44.9	5.2	98.9	1.1
B-4049	*morrisoni*	8	1.8	65.5	2.4	100	0
B-4050	*tolworthi*	9	3.6	37.0	2.5	98.9	1.1
B-4058	*darmstadiensis*	10	1.1	83.8	4.4	100	0
B-4059	*toumanoffi*	11	1.9	52.8	2.8	100	0
B-4060	*thompsoni*	12	2.5	64.1	2.4	100	0

[a] ARS, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

[b] Averages of triplicate experiments.

$$G_p = \frac{G_1 - G_6}{1 - G_6}$$

The fraction of glucose catabolized via the EMP pathway (G_E) was calculated by difference: $G_E = 1 - G_p$. The $^{14}CO_2$ yields from specifically labeled D-glucose were calculated as follows: (i) interval $^{14}CO_2$ recoveries as counts per minute were summed (most experiments entailed three half-hour intervals); (ii) interval recoveries were multiplied by three because only 5 ml of trapping solution was counted; (iii) total counts per minute were converted to disintegrations per minute by dividing by the overall counting efficiency (approximately 85%); (iv) the percent yield of $^{14}CO_2$ then was derived by dividing the total disintegrations per minute by 555,000 (the number of disintegrations per minute in 0.25 μCi of radioactive glucose used in these experiments) and multiplying by 100.

This simplified procedure is valid because of the tremendously high yield of $^{14}CO_2$ from D-[3,4-^{14}C]glucose (Table 1) and because tricarboxylic acid cycle activity is minimal during vegetative growth of *B. thuringiensis* in GYS medium (2, 3). A similar phenomenon of diminished tricarboxylic acid cycle activity is true for several other bacilli (1, 7, 8, 9). If the tricarboxylic acid cycle were active, increased $^{14}CO_2$ yields from D-[1-^{14}C]glucose and D-[6-^{14}C]glucose would hinder estimation of G_p by this method (1).

RESULTS AND DISCUSSION

Eighteen strains of *B. thuringiensis*, including the 12 recognized serotypes (6), have been surveyed with regard to their pathways of glucose catabolism. The $^{14}CO_2$ recovery and pathway participation percentages are listed in Table 1. The high yields of $^{14}CO_2$ from D-[3,4-^{14}C]glucose indicate that the EMP pathway is predominant in every strain tested. With one exception (*B. thuringiensis* var. *dendrolimus*), 96 to 100% of the glucose is catabolized via the EMP pathway. As such, *B. thuringiensis* represents a cohesive group by the criterion of metabolic homogeneity. The observed small differences in PP pathway participation are as great within a single serotype as between serotypes.

In addition to substantiating the unity of *B. thuringiensis* as a group, the uniform dominance of the EMP pathway points out the relatedness of *B. thuringiensis* to *B. cereus*. Previous radiorespirometric studies demonstrated that, whereas 98% of glucose catabolism is via the EMP pathway in *B. cereus* (8), only 65% is routed that way in *B. subtilis* (7, 17).

Figures 1 and 2 show the aerobic radiorespirometric patterns of glucose utilization by *B. thuringiensis* var. *entomocidus* cells grown in glucose-glutamate-salts and glucose-aspartate-salts media, respectively. The rapid appearance of $^{14}CO_2$ from D-[3,4-^{14}C]glucose is indicative of a functional EMP pathway and, more specifically, demonstrates pyruvate dehydrogenase

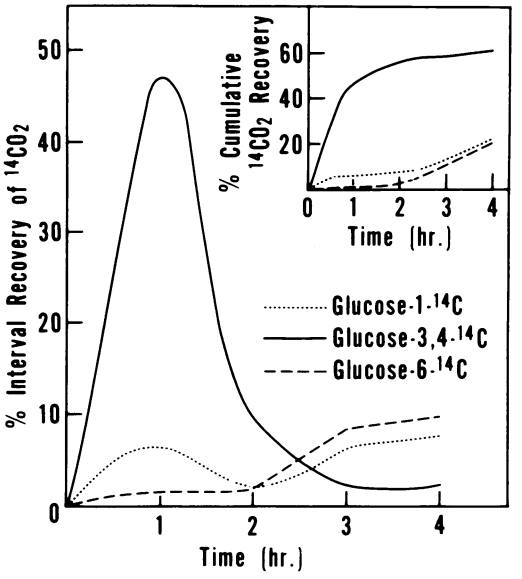

FIG. 1. *Aerobic radiorespirometric patterns of glucose utilization by vegetative cells of B. thuringiensis var. entomocidus grown in a glucose-glutamate-salts medium (10). C_1, $C_{3,4}$, and C_6 designate specifically labeled carbon atoms. One-half milligram of specifically labeled substrate (0.25 μCi) was added to each reaction flask. Final volume per flask was 30 ml.*

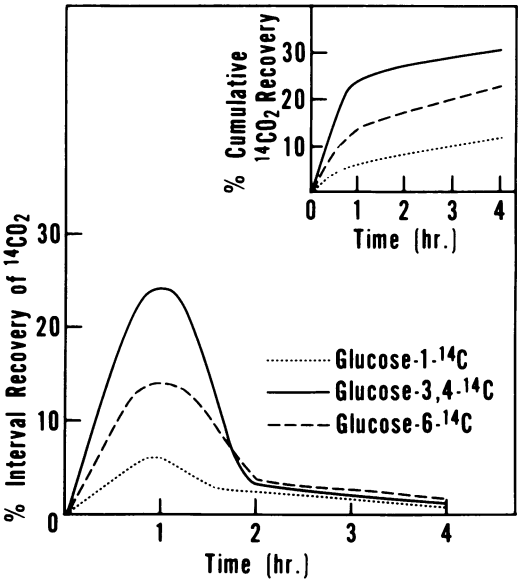

FIG. 2. *Aerobic radiorespirometric patterns of glucose utilization by vegetative cells of B. thuringiensis var. entomocidus grown in a glucose-aspartate-salts medium (10). C_1, $C_{3,4}$, and C_6 designate specifically labeled carbon atoms. One-half milligram of specifically labeled substrate (0.25 μCi) was added to each reaction flask. Final volume per flask was 30 ml.*

(pyruvate:lipoate oxidoreductase, EC 1.2.4.1) activity. The high yield of $^{14}CO_2$ from D-[6-^{14}C]glucose (Fig. 2) indicates that *B. thuringiensis* var. *entomocidus* possesses an intact tricarboxylic acid cycle (1) when grown in the glucose-aspartate-salts medium. This observation confirms previous radiorespirometric work with radioactive glutamate (2, 3) indicating the intactness of the tricarboxylic acid cycle. Taken together, these results demonstrate that the absolute requirement shown by *B. thuringiensis* for citrate or a related compound (10) is not caused by a defect in either citrate or glucose metabolism or in glucose uptake. We have shown in the accompanying paper (10) that the requirement for citrate cannot be replaced by biotin, thiamine, lipoic acid, or any other vitamin tested.

It is known that in other bacilli glucose and glutamate are required together to repress enzymes of the tricarboxylic acid cycle (9). Our results are consistent with this view because tricarboxylic acid cycle activity (based on $^{14}CO_2$ evolution from [6-^{14}C]glucose during the first 2 h of incubation) is absent in vegetative cells grown in either glucose-glutamate-salts (Fig. 1) or GYS media, but is present in those cells grown in the glucose-aspartate-salts medium (Fig. 2). We have no explanation for the higher $^{14}CO_2$ yield from D-[6-^{14}C]glucose than from D-[1-^{14}C]glucose in Fig. 2, except that D-glucose may be rapidly converted to uridine 5'-diphosphate glucose and subsequently to uridine 5'-diphosphate-galacturonic acid, which in turn undergoes oxidative decarboxylation to give the observed order of $^{14}CO_2$ evolution. However, because the tricarboxylic acid cycle is inactive in the glucose-glutamate-salts-grown cells we can calculate from the data in Fig. 1 that the percent contribution of the PP pathway is 5%. This slight PP activity probably supports biosynthetic needs. Table 1 shows that glucose catabolism was 100% via the EMP pathway when the cells were grown in GYS medium, wherein biosynthetic needs are minimal.

Though enzyme assays demonstrate the simultaneous presence in cell-free extracts of the EMP and PP pathways (L. A. Bulla et al., unpublished data), we have used radiorespirometry to ascertain that the EMP pathway is the dominant energy-generating system in all strains of *B. thuringiensis* tested.

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