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Physiology of Sporeforming Bacteria Associated with Insects: Minimal Nutritional Requirements for Growth, Sporulation, and Parasporal Crystal Formation of *Bacillus thuringiensis*.

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A defined medium is described in which 18 strains of *Bacillus thuringiensis* representing the 12 established serotypes grow, sporulate, and produce a parasporal crystal. This minimal medium contains glucose and salts supplemented with either aspartate, glutamate, or citrate. These organic acids are required and cannot be replaced by vitamin mixtures or succinate even though succinate is taken up at a rate similar to that of aspartate, glutamate, and citrate.

Bacillus thuringiensis, a bacterium found in natural association with lepidopterous insects, differs from most other sporeformers by synthesizing a discrete parasporal protein crystal in addition to the endospore. The importance of this crystal to the physiology of *B. thuringiensis* is obvious when one considers that it often constitutes 30% of the sporangium dry weight (16). Delafield et al. (7) postulated that the parasporal crystal arises from an unregulated superproduction of spore coat protein.

The parasporal crystal is toxic to insects and, consequently, *B. thuringiensis* has become an economically important microbial insecticide (16). Use of the organism as such has considerably outpaced our understanding of the cause of parasporal crystal formation, the chemical nature of crystal structure, the mechanism of toxicity, and the control of crystal formation vis-à-vis sporulation. Particularly, there is a lack of information on the nutrition and physiology of *B. thuringiensis* relating to growth, sporulation, and crystal formation.

In this communication and the accompanying report (15), we present results of comparative nutritional studies with 18 strains of *B. thuringiensis*. These strains include the 12 recognized serotypes and esterase types (5, 6, 11, 12). Serotype denotes the presence of a specific flagellar H antigen, whereas the esterase type refers to an electrophoretic variant. To our knowledge, there are no reports comparing nutritional requirements of the 12 serotypes. Furthermore, most work reported on *B. thuringiensis* has been done in undefined media containing yeast extract. Use of such media can interfere with proper interpretation of physiological and biochemical analyses, as well as preclude entirely genetic techniques to pinpoint various control mechanisms. We describe here a minimal medium that supports abundant growth and sporulation of all strains of *B. thuringiensis* tested. Each serotype exhibits an auxotrophy that can be satisfied by either glutamate, aspartate, or citrate.

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MATERIALS AND METHODS

Organisms and cultural conditions. The 18 strains of *B. thuringiensis* used in this investigation, which were kindly supplied by T. A. Angus, Insect Pathology Research Institute, Canadian Forestry Service, Sault Ste. Marie, Ontario, and H. de Barjac, Service de lutte bacteriologique, Institut Pasteur, Paris, are listed in Table 1. All strains are now deposited in the Agricultural Research Service Culture Collection, Peoria, Ill. Stock cultures were maintained on TGY agar slants (10). Our unsupplemented basal medium (BM) consisted of: $MgSO_4 \cdot 7H_2O$, 0.3 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2$, 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; K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 2.0 g; and 1.0 g of glucose per liter of distilled water (pH adjusted to 7.4). BM is essentially the G medium devised by Nakata and Halvorson (14) for *B. cereus*, except that yeast extract is omitted. All chemicals used were reagent grade. The glucose and phosphate were autoclaved sepa-

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rately and added aseptically before inoculation; all vitamins were filter-sterilized. Cultures (10 ml) were grown at 28 C in 25-ml Erlenmeyer flasks and aerated by rotary agitation at 250 rpm. Inocula were prepared as previously described (2) by using BM plus glucose and glutamate.

Radioisotope uptake studies. *B. thuringiensis* var. *entomocidus* (B-4046) was grown on BM plus 0.2% glutamate. Vegetative cells were harvested and washed twice in BM plus 0.02% chloramphenicol. These cells were suspended in the same medium at a final concentration of 1.0 mg (dry weight)/ml and equilibrated for 2 min at 28 C. Radioactive substrate (1 μCi) and carrier substrate were added to bring the final volume to 5 ml. Samples (1 ml) were removed at 2, 5, 10, and 15 min, filtered on pre-wetted 25-mm membrane filters (0.45 μ) (Millipore Corp.), and washed twice with 5-ml portions of BM plus 0.02% chloramphenicol. The filters were transferred to vials containing 10 ml of scintillation fluid (20) and were counted in a liquid scintillation spectrometer. Zero time radioactivity, determined in the absence of cells for each substrate, was subtracted from the resulting values. [2,3-^{14}C]succinic acid, [3,4-^{14}C]glutamic acid, [1,5-^{14}C]citric acid, and [U-^{14}C]aspartic acid were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

It should be emphasized at the outset that *B. thuringiensis* does not grow in minimal glucose-salts media. Unsupplemented BM did not support growth of any of the 18 strains listed in Table 1. However, all strains grew and sporulated when BM was supplemented with either citrate, aspartate, or glutamate (Table 2). Crystal formation always accompanied sporulation, and these crystals were toxic to lepidopterous insects (H. T. Dulmage, personal communication). Each strain grew well when BM was supplemented with either citrate, aspartate, or glutamate at a concentration of 0.2%. This substrate concentration is greater than that normally required to fulfill other amino acid auxotrophic requirements. Guirard and Snell (9) concluded that non-bacilli require amino acids at a concentration of about 0.001%; however, the bacilli may require abnormally large amounts of glutamate in chemically defined media. The defined medium devised by Nakata (13) for *B. cereus* contained 0.184% (12.5 mM) glutamate, and Buono et al. (3) reported that 1.0% (68 mM) glutamate is required for sporulation of *B. cereus*.

If an intact tricarboxylic acid cycle is present, the carbons of exogenously supplied aspartate and glutamate should equilibrate with those of citrate and the other intermediates of the tricarboxylic acid cycle. Table 2 reveals that when BM was supplemented with either citrate, malate, fumarate, succinate, or oxalacetate, only citrate promoted growth of all strains at levels comparable to those observed when aspartate or glutamate was added. A few strains were able to grow on malate, but none were able to grow on fumarate, succinate, or oxalacetate. The inability of succinate to support growth was suprising, because we had previously shown by radiorespirometry (1) that *B. thuringiensis* var. *berliner* grown in a yeast extract medium metabolized radioactive succinate at concentrations (0.003%) far lower than those insufficient to support growth (Table 2).

We sought to determine the minimal concentrations of citrate, aspartate, and glutamate that would support growth. Flasks containing BM plus decreasing concentrations of the three substrates were inoculated with *B. thuringiensis* var. *entomocidus* and var. *alesti* (Table 3). No growth occurred at substrate concentrations below 0.4 mM. Microscope examination revealed that the growth that did occur at substrate concentrations below 1 mM often resulted in swollen vegetative cells and aberrant sporulation. For this reason we routinely used BM plus 0.2% (13.6 mM) glutamate to prepare all inocula.

Impaired amino acid uptake is the most

TABLE 1. *Established serotypes[a] of B. thuringiensis*

ARS culture collection no.[b]	Variety name	H antigen serotype	Esterase type
B-4039	*berliner*	1	berliner
B-4040	*finitimus*	2	finitimus
B-4041	*alesti*	3a	alesti
B-4055	*kurstaki*	3a3b	ND[c]
B-4042	*sotto*	4a4b	sotto
B-4043	*dendrolimus*	4a4b	dendrolimus
B-4044	*kenyae*	4a4c	kenyae
B-4045	*galleriae*	5a5b	galleriae
B-4056	*canadensis*	5a5c	ND
B-4046	*entomocidus*	6	entomocidus
B-4047	*entomocidus-limassol*	6	entomocidus
B-4057	*subtoxicus*	6	entomocidus
B-4048	*aizawai*	7	galleriae
B-4049	*morrisoni*	8	morrisoni
B-4050	*tolworthi*	9	tolworthi
B-4058	*darmstadiensis*	10	ND
B-4059	*toumanoffi*	11	toumanoffi
B-4060	*thompsoni*	12	thompsoni

[a] Based on classification according to de Barjac and Bonnefoi (5).

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

[c] ND, not determined.

TABLE 2. *Nutritional requirements of B. thuringiensis*[a]

Variety name	BM + 0.2% KOH neutralized			Glucose-free BM + 0.2% KOH neutralized		Vitamin requirement
	Glutamate, aspartate, or citrate	Malate	Fumarate, succinate, or oxalacetate	Glutamate	Citrate	
berliner	+	+	−	+	+	None
finitimus	+	−	−	+	−	None
alesti	+	−	−	+	−	None
kurstaki	+	+	−	−	−	None
sotto	+	−	−	+	+	None
dendrolimus	+	−	−	+	+	None
kenyae	+	+	−	+	+	None
galleriae	+	−	−	+	−	None
canadensis	+	−	−	+	+	None
entomocidus	+	+	−	+	+	None
entomocidus-limassol	+	−	−	+	−	None
subtoxicus	+	+	−	ND[b]	ND	Riboflavin
aizawai	+	+	−	+	+	None
morrisoni	+	−	−	+	+	None
tolworthi	+	−	−	+	+	None
darmstadiensis	+	−	−	+	+	None
toumanoffi	+	−	−	+	+	None
thompsoni	+	−	−	+	+	None

[a] Presence or absence of growth as determined by turbidity is indicated by (+) and (−); sporulation and crystal formation were observed through phase-contrast optics of a light microscope.
[b] ND, not determined.

TABLE 3. *Minimal concentrations of glutamate, aspartate, and citrate sufficient to support growth*[a]

Concentration (mM)[b]	*B. thuringiensis* var. *entomocidus*			*B. thuringiensis* var. *alesti*		
	Glutamate[c]	Aspartate[c]	Citrate[c]	Glutamate	Aspartate	Citrate
6	++	++	++	++	++	++
5	++	++	++	+	++	++
4	++	++	++	+	++	++
3	++	++	++	+	++	++
2	++	++	++	−	++	++
1	++	++	++	−	++	++
0.8	++	++	++	−	+	++
0.6	++	++	++	−	−	++
0.4	−	+	++	−	−	+
0.2	−	−	−	−	−	−
0.1	−	−	−	−	−	−

[a] Inoculum consisted of 0.04 ml of cells grown in BM plus 0.2% glutamate. Symbols: + +, optical density >0.6; +, optical density ≥0.1; and −, no growth.
[b] BM plus sufficient 10 mM substrate to give the indicated concentration in a final volume of 10 ml.
[c] Aspartate and glutamate were KOH-neutralized; citrate was present as the trisodium salt.

obvious explanation for the minimal aspartate and glutamate concentrations that support growth (Table 3); these concentrations are well above those necessary for strictly metabolic purposes if the substrates had unrestricted access to the cell interior. Also, because Ghei and Kay (8) reported that the dicarboxylic acid transport system in *B. subtilis* is less efficient for succinate and fumarate, we suspected that the inability of succinate to promote growth (Table 2) was also due, in part, to a transport problem. Accordingly, we conducted uptake studies (Fig. 1A–D) using radioactive aspartate, citrate, glutamate, and succinate. All four substrates were taken up by *B. thuringiensis*, but only the citrate and succinate pools showed saturation during the time periods employed. The pool sizes observed are in excellent agreement with those reported by Ghei and Kay (8) for *B. subtilis*. Figure 1A demonstrates that succinate does indeed have access to the cell interior, and we had to search for another reason for its inability to promote growth.

We considered the possibility that succinate might be toxic to *B. thuringiensis* when present at concentrations as high as 0.2%. Succinate could have an adverse regulatory effect only evident when present in abnormally large amounts (17 mM). Unique regulatory phenomena have been frequently observed in *B. thuringiensis* (4, 16). This possibility was eliminated by the following observations: (i) BM plus only 0.02% succinate did not support growth; (ii) when *B. thuringiensis* var.

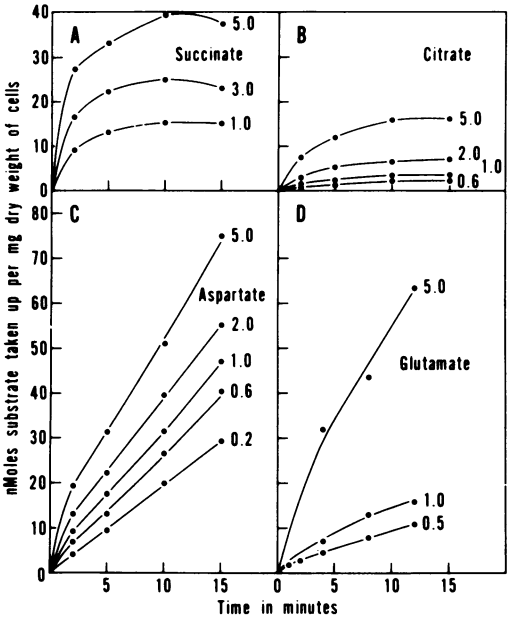

FIG. 1. *Kinetics of substrate uptake by B. thuringiensis var. entomocidus. Substrate concentrations shown in mM: (A) [2,3-^{14}C]succinate; (B) [1,5-^{14}C]citrate; (C) [U-^{14}C]aspartate; (D) [3,4-^{14}C]glutamate. Final cell concentration was 1.0 mg (dry weight)/ml for A, B, and C, and 0.1 mg (dry weight)/ml for D.*

entomocidus was grown at a wide variety of succinate-glutamate ratios, all glutamate concentrations that allowed growth in the absence of succinate also allowed growth in the presence of up to a ninefold excess of succinate; (iii) similarly, succinate did not prevent the citrate-stimulated growth.

The inadequacy of unsupplemented BM cannot be attributed to an unfulfilled vitamin requirement. Agar plates made with BM plus 0.2% glutamate allowed var. *alesti* and var. *entomocidus* to form colonies. Moreover, strains were routinely germinated and grown in BM plus 0.1% vitamin-free Casamino Acids plus 10^{-4} M tryptophan. Seventeen of the 18 strains grew well through four serial transfers in this vitamin-free medium. We did find (Table 2) that var. *subtoxicus* (B-4057) required riboflavin. Supplementation of BM with a mixture of several vitamins elicited no growth of the remaining 17 strains. Specifically, biotin (1 to 500 ng/ml), lipoic acid (1 to 2 ng/ml), thiamine (0.01 to 4.0 μg/ml), pantothenate (0.2 to 10 μg/ml), folic acid, vitamin B_{12} (100 to 200 ng/ml), nicotinic acid (0.1 to 1.5 μg/ml), nicotinamide (100 to 200 ng/ml), riboflavin (0.1 to 2.0 μg/ml), and pyridox (-al, -amine, and -ine; 0.04 to 2.0 μg/ml) were without effect.

Glucose uptake can be selectively inhibited under certain nutritional conditions. Romano and Kornberg (17) found that growth of *Aspergillus nidulans* on an acetate-containing medium inhibited glucose uptake, whereas sucrose uptake and catabolism remained unaffected. With this in mind, we modified BM by eliminating the glucose and replacing it with either sucrose, lactose, or mannitol. These modified BM also did not allow growth. Further, when *B. thuringiensis* was grown on BM plus aspartate or glutamate, glucose was taken up and catabolized via the Embden-Meyerhof-Parnas pathway (15), the same as it would be in yeast extract-grown cells. The presence of glucose is not, however, essential in most cases. Table 2 shows that most strains were capable of growing when glutamate or citrate were added to a glucose-free BM; the absence of glucose generally resulted in defective sporulation.

DISCUSSION

We have developed an appropriate chemically defined medium suitable for various biochemical, genetic, and physiological studies of *B. thuringiensis*. All of the strains not requiring vitamins grow and sporulate in our BM with the addition of 0.2% citrate, aspartate, or glutamate. This medium is a significant improvement over the two defined media previously reported (4, 18). That of Singer et al. (18) is basically a glucose-salts medium, whereas that of Conner and Hansen (4) contains citrate as the sole carbon and energy source. Neither medium is adequate; each allows only minimal growth in just a few strains. In addition, sporulation is very poor in those strains that do exhibit growth. The contention that *B. thuringiensis* var. *berliner* grows in glucose-salts media (16, 18, 19) is misleading because the "salts" used in those media included 0.0016% ferric ammonium citrate, 0.005% zinc acetate, and 0.01% ethylenediaminetetraacetic acid. The citrate and ethylenediaminetetraacetic acid may have been responsible for the slight growth observed by Singer et al. (18).

The inability of succinate to promote growth when added to BM remains unclear. The succinate is not toxic, and uptake studies demonstrate that it does have access to the cell interior. We are currently investigating the possibility that succinate cannot be further metabolized in BM. This phenomenon is suggested by the absence of a continued increase in succinate uptake with time (Fig. 1A), unlike

that occurring with aspartate (Fig. 1C). In addition, radiorespirometry using radioactive succinate (K. W. Nickerson and L. A. Bulla, unpublished data) shows that cells grown in BM plus 0.2% glutamate do not oxidize succinate to carbon dioxide, whereas under identical conditions yeast extract-grown cells do (1). Citrate is able to promote growth in the lowest concentrations of those substrates tested (Table 3) and yet is taken up least by the cells (Fig. 1B). This observation suggests that citrate may be the essential component. Aspartate and glutamate work just as well because they can be converted to citrate, whereas this conversion is unavailable to succinate.

Attention must be paid to the pitfalls encountered when conducting nutritional studies on sporeformers. A medium may be sufficient to support vegetative growth and yet not be sufficient for germination and outgrowth of spores. BM plus 0.2% glutamate gave excellent growth in every case, and yet the timing of spore germination in this medium was erratic. The vitamin-free Casamino Acids starter culture represented a compromise in that the medium had to be rich enough to promote prompt spore germination and yet defined enough to allow rapid detection of vitamin and other nutritional requirements on subsequent transfer. It is important to examine each inoculum microscopically to determine whether the cells are still vegetative. An inoculum committed to sporulation would test a medium's ability to support sporulation, germination, and outgrowth, in addition to vegetative growth. It is much harder to define nutritional requirements for sporulation because a medium is no longer chemically defined after vegetative growth has been completed. A distinction between the ability to sporulate and the ability to form stable spores requires frequent microscope observation. Dormant spores are stable and may be observed any time after growth has been completed. However, spores unable to maintain dormancy may lyse following premature germination and outgrowth. Absence of or reduction in the number of cellular forms can mean either that the cells lysed without ever sporulating or that defective spores were formed. The media described in this report produce stable dormant spores.

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