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Lipid Metabolism During Bacterial Growth, Sporulation, and Germination: an Obligate Nutritional Requirement in *Bacillus thuringiensis* for Compounds That Stimulate Fatty Acid Synthesis

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The regulation of fatty acid biosynthesis by compounds that are required for growth of *Bacillus thuringiensis* was investigated using an in vivo assay developed to measure fatty acid synthesis in germinating spores. A minimal glucose-ammonium-salts medium does not support growth even though previous radiorespirometric studies have shown *B. thuringiensis* to possess intact tricarboxylic acid and Embden-Meyerhof-Parnas pathways. Abundant growth does occur, however, when this medium is supplemented with either glutamate, aspartate, citrate, thiosulfate, cystine, or ethylenediaminetetraacetic acid. Cells held under nongrowing conditions incorporate acetate into fatty acids; fatty acid synthesis is stimulated by the compounds that permit growth. These alternate nutritional requirements are not manifestations of a vitamin or trace metal deficiency and do not reflect a chelation phenomenon. These results indicate a direct correlation between the capacity of these compounds to promote growth and to stimulate formation of fatty acids.

The crystal-forming bacterium *Bacillus thuringiensis* is an economically important microbial insecticide (27). We have been studying the nutritional requirements of this microorganism in an effort to delineate the control mechanisms regulating parasporal crystal production. We have already shown that all 12 serotypes of *B. thuringiensis* exhibit an auxotrophic requirement for either glutamate, aspartate, or citrate when grown on a glucose-salts medium designated BM (23). Neither succinate nor fumarate were able to replace these compounds.

We have sought to clarify the physiological function of glutamate, aspartate, and citrate because subsequent radiorespirometric studies clearly demonstrated that this requirement was not the result of defects in either glucose metabolism or the tricarboxylic acid cycle (25), nor was it the manifestation of an unfulfilled vitamin requirement (23). In the present paper we have found that the requirement for aspartate, glutamate, and citrate can be bypassed if BM is instead supplemented with either cystine, thiosulfate, or ethylenediaminetetraacetic acid (EDTA). The physiological function that these diverse compounds have in common is not obvious. However, it is known that citrate (17, 35) and EDTA (26) are positive regulators of fatty acid synthesis and that cystine may control membrane synthesis (33). Consequently, we suggest that *B. thuringiensis* requires a stimulator of fatty acid synthesis in order to grow in a defined medium. The observed nutritional requirements illustrate members of a class of such compounds.

To test this idea we have developed an in vivo assay which measures incorporation of [^{14}C]acetate into purified fatty acids under a variety of nutritional conditions. The ideal system in which to develop such an assay was found in our recent study (24) of germinating *B. thuringiensis* spores, wherein extensive incorporation of acetate into fatty acids was detected 20 min after the onset of germination. In the present paper we have used this system to study the nutritional control of fatty acid synthesis during the third half-hour of spore outgrowth. The BM defined medium containing chloramphenicol and [^{14}C]acetate was supplemented in turn by those compounds previously found to permit growth. The formation of additional fatty acid synthesizing capacity was precluded by the chloramphenicol. (Presented in part at the 75th annual meeting of the American Society for Microbiology, New York, 27 April to 2 May 1975.)

MATERIALS AND METHODS

Organisms and cultural conditions. The organisms and culture conditions employed have been described previously (23). The 18 strains of *B. thuringiensis* used in our nutritional studies are deposited in the Agricultural Research Service Culture Collection, Peoria, Ill. These strains include the 12 recognized serotypes (27). *B. thuringiensis* NRRL B-4027 was chosen for those experiments determining the extent of fatty acid synthesis resulting from incorporation of radioactive acetate. This niacin-requiring strain was originally obtained from A. A. Yousten and is the same strain used in our previous studies of lipid synthesis (3, 24). Its flagellar antigen designation is galleriae. Spores of strain 4027 were prepared as previously described (24).

Our unsupplemented basal medium (BM) is a weakly buffered glucose-salts medium containing ample magnesium. It consists of: 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, 0.08 g of $CaCl_2$, 0.005 g of $ZnSO_4 \cdot 7H_2O$, 0.005 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of K_2HPO_4, 0.0005 g of $FeSO_4 \cdot 7H_2O$, 2.0 g of $(NH_4)_2SO_4$, and 1.0 g of glucose per liter of distilled water (pH adjusted to 7.4). BM is the G medium of Nakata and Halvorson (21) except that the yeast extract is omitted. All chemicals were reagent grade. The glucose and phosphate were autoclaved separately and added aseptically before inoculation. Identical results were obtained with Na_2HPO_4/KH_2PO_4 as with K_2HPO_4.

B. thuringiensis does not grow in an unsupplemented BM medium. The following procedure was used to screen the effectiveness of numerous supplemental compounds. Spores from stock slants were germinated in BM + 0.1% vitamin-free Casamino Acids. After growth, 0.1 ml of culture was transferred to 10 ml of BM + 0.2% glutamate (KOH neutralized), allowed to grow, and 0.04 ml of this culture was used as inoculum. Supplemental test cultures (10 ml) were grown at 28 C in 25-ml Erlenmeyer flasks and aerated by rotary agitation at 250 rpm. Growth was determined by the presence of visible turbidity. All vitamins were filter sterilized.

Germination and outgrowth. The techniques used duplicate those described by Nickerson et al. (24). Spores of strain B-4027 were heat-activated before germination at a concentration of 120 mg/ml by homogenizing in a Potter-Elvehjem hand homogenizer for 2 min, heat shocking for 30 min at 80 C, and homogenizing an additional 2 min. The spores were germinated at a concentration of 14 mg/ml in a modified GYS medium [0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, and 0.05% K_2HPO_4 (pH 7.3)] (25) to which the germinating agents L-alanine (100 μg/ml), adenosine (500 μg/ml), and EDTA (25 μg/ml) were added. Germination and outgrowth were allowed to proceed at 28 C for 1 h. Aeration was by rotary agitation at 150 rpm.

In vivo assay for fatty acid synthesis. One hour after the start of germination the cells of strain B-4027 were harvested by centrifugation at 4 C and the pellet was suspended in 60 ml of BM plus niacin (4 μg/ml). The cells were equally distributed among 50-ml Erlenmeyer flasks containing 25 ml of BM supplemented with niacin (4 μg/ml), chloramphenicol (200 μg/ml), [U-^{14}C]acetate (1.66 μCi per 2 μg/ml), and the designated test nutrient. Final spore concentration was 160 mg/flask. Acetate concentrations far greater than those used here have previously been shown not to permit growth when added to BM (23). The cells were incubated in this medium at 28 C with rotary agitation at 200 rpm. After 30 min the cells were harvested by centrifugation at 4 C and their fatty acids were purified as the methyl esters.

Preparation of fatty acid methyl esters. The procedure used was that of Bulla et al. (2). The cell pellet was extracted for 72 h in 40 ml of $CHCl_3$-CH_3OH (2:1 vol/vol), hereafter CM. All glassware were CH_3OH-washed. The cells were filtered through CH_3OH presoaked Whatman no. 1 paper (12.5 cm) and allowed to drain for 15 min. The filtrate was evaporated under N_2, dissolved in 10 ml of CM, and allowed to sit for 15 min. This solution was filtered through a 13-mm Swinney syringe holder fitted with a CH_3OH presoaked S & S 740-E filter and washed with 5 ml of CM. The filtrates were combined, evaporated under N_2, and taken up in 0.5 ml of ether. The methyl esters were formed by adding 1.0 ml of 0.5 N KOH in CH_3OH. After 20 min the solution was neutralized with 1.0 N HCl and overlaid with 1.0 ml of hexane. The solution was swirled gently and the hexane layer was decanted into a vial. This extraction was repeated three times and the combined hexane fractions were dried under N_2. The residue was suspended in 0.5 ml of hexane and 5-μl samples were taken for determination of the radioactivity present.

Chemicals. Desferal (desferioxamine) was obtained from the Ciba Pharmaceutical Co., Summit, N. J. The [U-^{14}C]acetate was purchased from International Chemical and Nuclear Corp., Irvine, Calif.

RESULTS

Nutritional requirements for growth. The nutritional studies reported here confirm our previous finding that *B. thuringiensis* will not grow in the weakly buffered glucose-salts medium BM (23). We reported that all 12 serotypes of *B. thuringiensis* will grow and sporulate when supplemented with 0.2% of either glutamate, aspartate, or citrate. We have now found that growth will also occur if our glucose-salts medium is instead supplemented with 0.09 mM EDTA, 0.01 mM cystine, or 4.5 mM sodium thiosulfate. The minimal concentrations of EDTA and thiosulfate that would support growth are indicated in Tables 1 and 2, respectively. These additions allowed all serotypes to grow, but in no case was sporulation as consistent as when the glucose-salts medium was supplemented with glutamate, aspartate, or citrate. This limitation was especially true for the lower substrate levels reported in Tables 1 and 2. Laue and MacDonald (15) described

TABLE 1. *Minimal concentration of EDTA sufficient to permit growth*[a]

Concn (mM)[b]	*B. thuringiensis* var.		
	alesti	*dendro-limus*	*galleriae*
0.3	++	++	++
0.09	++	++	++
0.03	−	−	−
0.009	−	−	−

[a] Inoculum consists of 0.04 ml of cells grown in BM plus 0.2% glutamate. Symbols: ++, optical density > 0.6; −, no growth.

[b] BM plus sufficient 3 mM EDTA to give the indicated concentration in a final volume of 10 ml.

TABLE 2. *Minimal concentration of sodium thiosulfate sufficient to permit growth*[a]

Concn (mM)[b]	*B. thuringiensis* var.		
	alesti	*dendro-limus*	*galleriae*
90.9	++	++	++
45.5	++	++	++
22.7	++	++	++
9.09	++	++	++
4.55	++	++	++
0.91	−	+	−
0.45	−	−	−

[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 909 mM $Na_2S_2O_3 \cdot 5H_2O$ to give the indicated concentration in a final volume of 10 ml.

a cystine-requiring strain of *Staphylococcus aureus* that had the interesting property of being able to grow equally as well on L-djenkolic acid, the analog of L-cystine with a methylene group inserted between the two sulfur atoms. We found that *B. thuringiensis* var. *entomocidus* was not able to substitute either L-djenkolic acid, cysteic acid, cystamine, or D-methionine for L-cystine. However, L-methionine (0.20 mM) was able to replace L-cystine in var. *entomocidus* and presumably will work as well in the other serotypes.

In the process of further defining the nutritional requirements of *B. thuringiensis* we have resolved the controversy of whether a glucose-salts medium is sufficient for growth (23, 27, 31). Singer et al. (31) reported that it was sufficient; but, it is now apparent that they observed growth only because their salts contained 0.0016% ferric ammonium citrate and 0.01% (0.3 mM) EDTA.

Nutritional requirements for fatty acid synthesis. The compounds presently found to permit growth when added to the glucose-salts medium BM are listed in Table 3. The physiological function these compounds have in common is not obvious. We have considered many hypotheses regarding why the BM medium is deficient. These are also listed in Table 3. Only after each of these hypotheses had proven negative did we consider the possibility that *B. thuringiensis* had an obligate nutritional requirement for compounds able to stimulate fatty acid synthesis.

A method of testing this hypothesis was developed during our recent studies of lipid synthesis in germinating spores (24) wherein we found that acetate was incorporated extensively into purified fatty acids 20 min after the onset of spore germination. We have developed an in vivo assay for fatty acid synthesis that measures how much acetate was incorporated into purified fatty acids during the third half-hour of spore outgrowth under a variety of nutritional conditions. The cells were harvested after 1 h of outgrowth and resuspended in the glucose-salts medium BM containing uniformly ^{14}C-labeled acetate and those compounds previously found to permit growth. Chloramphenicol (200 μg/ml) was included to prevent the formation of new fatty acid synthesizing machinery.

The data in Table 4 demonstrate that there is a direct correlation between those compounds that stimulate fatty acid synthesis and those previously found to permit growth in defined media. Table 4 shows that an energy source

TABLE 3. *Nutritional control of growth in B. thuringiensis*

Approximate lowest effective concentration of compounds that permit growth when added to the glucose-salts medium BM	Possible nutritional defects that have been tested and eliminated
Glutamate 1 mM (23)	Vitamin deficiency (23)
Aspartate 0.6 mM (23)	Trace metal deficiency
Citrate 0.4 mM (23)	Polyamine deficiency
EDTA 0.09 mM	Insufficient CO_2 pressure
Thiosulfate 4.5 mM	Chelation phenomena
Cystine 0.01 mM	Insufficient buffering capacity
Methionine 0.20 mM	Insufficient osmotic strength
	Defective glucose metabolism (25)
	Defective tricarboxylic acid cycle (25)

TABLE 4. *Nutritional control of fatty acid synthesis in B. thuringiensis*

BM supplement[a]	Counts/min in fatty acid esters $\times 10^{-3}$	Nanograms of acetate in fatty acid esters per milligram of spores[b]
Distilled water	363	1.36
Cystine (0.18 mM)	481	1.80
Thiosulfate (80.6 mM)	465	1.74
EDTA (0.30 mM)	425	1.59
Glutamate (13.6 mM)	1,060	3.97
Citrate (10.4 mM)	412	1.54
Succinate (17 mM)	315	1.18
Phenylalanine (12.1 mM)	370	1.38
Glucose omitted	52	0.19
Organic acids (78 mM)[c]	0	0.00
Distilled water; chloramphenicol omitted	359	1.35
Citrate (10.4 mM); chloramphenicol omitted	405	1.52

[a] Final concentrations in 25 ml of total volume.

[b] Average of duplicate experiments.

[c] Isobutyrate, isovalerate, isocaproate (1:1:1 vol/vol).

must be supplied for fatty acid synthesis to occur. There was very little acetate incorporation in the absence of glucose. As an additional control, no radioactivity was incorporated in the presence of the three branched-chain organic acids when the cells underwent immediate lysis. The known growth-permitting compounds cystine, EDTA, thiosulfate, citrate, and glutamate were all found to stimulate fatty acid synthesis; glutamate was the most effective. Glutamate has long been recognized as a crucial nutrient for growth and sporulation in the bacilli (4, 20). Succinate does not permit growth of *B. thuringiensis* (23) and, indeed, does not stimulate fatty acid synthesis either. Phenylalanine was chosen as an additional representative of the many unrelated compounds known not to effect growth in BM. It too did not stimulate fatty acid synthesis. Because the fatty acids were purified before the extent of acetate incorporation was determined, we are confident that our assay measures fatty acid synthesis only. The high background observed in the distilled water controls was undoubtedly because no effort was made to starve the cells or deplete their pool sizes before assay. In the interests of synchronous germination and to duplicate the conditions of our previous studies of lipid synthesis (24) we used a yeast extract-containing medium during the first hour of germination and outgrowth.

Chelation. Glutamate, aspartate, and citrate are effective chelators and EDTA is generally regarded solely as a chelating agent. As expected, radiorespirometric experiments using radioactive EDTA revealed that *B. thuringiensis* does not metabolize EDTA to CO_2. Several microbial systems are known to exhibit a nutritional requirement for chelating agents (5, 6, 22, 28, 29, 36); however, we do not believe it is the common ability to chelate that distinguishes those compounds listed in Table 3. The following modifications of our glucose-salts basal medium did not allow growth: (i) deletion of Cu(II), Zn(II), or Ca(II); (ii) a 100-fold increase in the $FeSO_4 \cdot 7H_2O$ concentration; (iii) use of ferric salts instead of ferrous salts; (iv) use of filter-sterilized salts rather than autoclaved salts; (v) addition of 0.01 or 0.001 M tris(hydroxymethyl)aminomethane buffer; (vi) addition of (5 μg/ml) desferal (desferioxamine); (vii) addition of either 8-hydroxyquinoline (0.1 to 2.0 μg/ml) or salicylic acid (0.4 to 8.0 μg/ml). These modifications stressed possible defects in iron metabolism because of the relationship between iron and the siderochrome growth factors. None of these chelation-related modifications allowed *B. thuringiensis* var. *entomocidus* to grow. In addition, we have previously showed that succinate is incapable of stimulating growth (23) although it is as good a chelator as glutamate or citrate (7) and uptake studies using radioactive succinate demonstrated that the succinate does have access to the cell interior (23). Chelating strength does not appear to be important. The concentration of EDTA required for growth of *B. thuringiensis* (Table 1) is roughly equivalent to the lowest concentrations of glutamate or aspartate that can be employed (23) even though EDTA is at least a 10^8-fold better chelator for most metal ions (7). Salicylic acid, 8-hydroxyquinoline, and desferioxamine, a naturally occurring trihydroxamic acid, were not effective even though their chelating abilities are similar to that of EDTA.

DISCUSSION

We have demonstrated that glutamate, aspartate, citrate, EDTA, cystine, and thiosulfate form a class of compounds that permit growth of *B. thuringiensis* and that stimulate fatty acid synthesis in the presence of chloramphenicol. Compounds such as succinate and phenylalanine that did not permit growth likewise did not stimulate fatty acid synthesis.

Demain (8) found that *B. subtilis* required glutamate and either EDTA or cysteine. Significantly, he also demonstrated that cysteine was not functioning as a reducing agent and that EDTA was not removing toxic metal ions. Evidence that the class of growth-stimulating compounds we have defined is not restricted to the bacilli is derived partly from Starr's nutritional study of the genus *Xanthomonas* (32). He examined 113 strains of *Xanthomonas* and found that they would not grow well on a simple glucose-ammonium-salts medium. However, abundant growth resulted from the addition of either 6.8 mM glutamate or 1.33 mM methionine. So far we have been unable to demonstrate that these nutritional requirements are manifestations of an unfulfilled vitamin requirement, or that they result from osmotic or chelation phenomena. An apparent unifying physiological function would be to stimulate fatty acid and membrane synthesis. Kaneshiro et al. (12) interpreted a nutritional requirement for aspartate in a pantothenate auxotroph of *Agrobacterium tumefaciens* in an identical fashion.

Several lines of evidence support such a hypothesis. Acetyl coenzyme A carboxylase (EC 6.4.1.2) catalyzes the rate-governing step in fatty acid synthesis. This enzyme is well known to be allosterically regulated by citrate and isocitrate (17, 18, 34, 35). Significantly, the citrate concentrations found necessary for growth of *B. thuringiensis* (23) approximate those required to stimulate acetyl coenzyme A carboxylase in vitro. Both Matsuhashi et al. (18) and Martin and Vagelos (17) found that 0.3 to 3.0 mM citrate stimulated acetyl coenzyme A carboxylase half-maximally. In addition, the fatty acid synthetase complex has been reported to be under positive regulation by EDTA (26) and the work of Toennies (33) and Shockman (30) on unbalanced cell wall synthesis in *Streptococcus faecalis* indicates that cystine may regulate membrane synthesis. Synthesis of lipid-containing membrane was stimulated in the presence of cystine (0.17 mM) or methionine (0.07 mM), whereas the cells underwent autolysis in the absence of these amino acids. Toennies (33) postulated that cystine and methionine are the primary regulators of membrane and cell wall synthesis.

The conclusion that the compounds listed in Table 3 represent a class of compounds that enable *B. thuringiensis* to grow because they stimulate fatty acid synthesis is strengthened by the elimination of other reasonable hypotheses. Other ideas that we have tested and have found inadequate to explain our nutritional data are listed in Table 3. The inability of unsupplemented BM to support growth cannot be due to either inadequate buffering capacity or insufficient osmotic strength because addition of 0.2% succinate, fumarate, acetate, or pyruvate should then work equally as well as 0.2% citrate; they do not (23). Likewise, the addition of either 0.5 M sucrose or 0.5 M KCl was ineffective. Additional evidence that BM does not suffer from insufficient buffering capacity arises from the constancy of the pH in the absence of growth after inoculation.

Our glucose-salts medium could be deficient due to the absence of certain polyamines. Indeed, Kihara and Snell (13) showed that strepogenin's growth stimulatory activity was probably due to contamination by the polyamines spermine and spermidine. However, we found that *B. thuringiensis* var. *dendrolimus* did not respond to supplementary putrescine, spermine, or spermidine (10^{-3} to 10^{-4} M).

A requirement for four-carbon intermediates may result from a defect in the anaplerotic biotin-containing enzyme pyruvate carboxylase (EC 6.4.1.1) or from an unfulfilled biotin requirement (14). However, *B. thuringiensis* var. *entomocidus* did not grow when supplemented with biotin alone, even though biotin was added over a wide range of concentrations (1 to 500 ng/ml) at both neutrality and pH 4. Addition of biotin at an acid pH was tried because Moat and Lichstein (19) found that for *Lactobacillus arabinosus* addition of exogenous biotin was only effective at or below this pH. A defective pyruvate carboxylase could also result in a requirement for CO_2 pressures greater than atmospheric. Many instances are known in which bacteria require substantially elevated CO_2 pressures to grow (10). Accordingly, we attempted to grow *B. thuringiensis* vars. *alesti*, *dendrolimus*, and *galleriae* on unsupplemented BM in an atmosphere consisting of 5% CO_2 and 95% air. The results were consistently negative. In addition, var. *entomocidus* did not grow when BM was modified by the inclusion of 0.1 M bicarbonate. This latter modification was attempted because Brady and Gurin (1) had reported that fatty acid synthesis often could proceed in a bicarbonate buffer but not in a phosphate buffer.

Alternatively, the glucose-salts medium BM could be deficient in a requisite trace metal. Foster (9) has stressed that such a deficiency may be the actual cause of an observed requirement for relatively large amounts of substrates such as glutamic acid. Our basal medium contains adequate Mg(II), Ca(II), Mn(II), Cu(II),

Zn(II), and Fe(II); in addition, we supplemented it with the trace elements recommended by Hutner (11). Addition of Na, Mo, V, Co, B, Ni, Cr, and Sn did not allow the *B. thuringiensis* serotypes *alesti*, *dendrolimus*, or *galleriae* to grow. This result is not surprising in view of Lee and Weinberg's observation that the metal ion requirements in *B. megaterium* are greater for sporulation than for growth alone (16). We have no evidence that the nutritional requirements observed in Table 3 arise from an unfulfilled vitamin or trace metal requirement.

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