Physiology of sporeforming bacteria associated with insects. V. Tricarboxylic acid cycle activity and adenosine triphosphate levels in *Bacillus popilliae* and *Bacillus thuringiensis*

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Six tricarboxylic acid cycle enzymes were assayed in cell-free extracts of *Bacillus popilliae* and *B. thuringiensis* at various times during the early and late stationary phases of growth. In *B. popilliae*, citrate synthase and isocitric dehydrogenase were present at very low levels at all times. After completion of exponential growth, adenosine triphosphate (ATP) levels in *B. popilliae* cells fell over 100-fold and the cells failed to sporulate. Supplementation with glucose allowed reestablishment of high ATP levels but did not allow sporulation. Resuspension of postexponential cells of *B. popilliae* in glucose-supplemented spent broth prepared from a sporogenaous strain of *B. subtilis* did not allow sporulation. This technique had previously been successful in allowing sporulation of tricarboxylic acid cycle mutants of *B. subtilis*. In contrast, *B. thuringiensis* tricarboxylic acid cycle enzymes increased in activity after completion of exponential growth, ATP levels remained high, and most cells in the population underwent sporulation.

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**Introduction**

The best evidence that tricarboxylic acid cycle activity is necessary for normal sporulation of bacilli has been obtained from work with *Bacillus subtilis*. Many mutants of this organism, which have defects in the various tricarboxylic acid cycle enzymes, are available and all of these sporulate at frequencies less than the parent strain (3, 5). Defects in the cycle render the mutants deficient in adenosine triphosphate (ATP) after exponential growth and may inhibit depletion of one or more inhibitors of sporulation from the medium (5, 7, 11). Mutants lacking isocitric dehydrogenase or aconitase were made to sporulate at nearly normal frequencies by re-suspension of postexponential phase cells in spent broth from the parent strain along with a compound such as glucose that could be metabolized to provide ATP (11). In *B. thuringiensis*...
the specific activity of aconitase increased markedly at the completion of exponential growth and inhibitors of tricarboxylic acid cycle activity inhibited sporulation (12). The inability of _B. popilliae_ to oxidize acetate and succinate in manometric and radiorespirometric studies indicated that this microorganism may lack a functional tricarboxylic acid cycle (1, 10). In the radiorespirometric experiments, C-1 was the only carbon of pyruvate and glutamate that was decarboxylated. McKay et al. (9) found that acetate oxidation was dependent upon the strain and medium used. Very little work has been done on the relationship between tricarboxylic acid cycle activity and sporulation in other bacilli.

*Bacillus thuringiensis* and _B. popilliae_ are finding increasing use as biological insecticides. With both it is necessary to achieve good sporulation to produce the optimum level of insecticidal activity. In _B. thuringiensis_ insecticidal activity is associated with production of a toxic parasporal protein (6-endotoxin) and, in _B. popilliae_, the spore itself is the infectious agent. It was therefore of interest to examine the tricarboxylic acid cycle enzymes and ATP levels in each of these organisms. This was particularly true in the case of _B. popilliae_ since the failure to achieve large-scale in vitro sporulation has retarded the use of the bacterium for insecticidal purposes.

### Materials and Methods

#### Organisms and Cultural Conditions

*Bacillus popilliae* Northern Regional Research Laboratory (NRRL) H-2309 was obtained from the Agricultural Research Service (ARS) culture collection of the Northern Regional Research Center, Peoria, Illinois. It was maintained by weekly transfer on slants of MD agar (1.5% yeast extract, 0.6% K$_3$HPO$_4$, 0.2% glucose, and 2.0% agar). _Bacillus thuringiensis_ var. galleriae and _B. subtilis_ Marburg 168 were obtained from the culture collections of the Biology Department of Virginia Polytechnic Institute and State University and the Bacteriology Department of the University of Wisconsin, respectively.

Cells of _B. popilliae_ and _B. thuringiensis_ for enzyme assays, ATP assays, and resuspension experiments were grown in MD broth. The broth was prepared by autoclaving concentrated solutions of each of the ingredients separately and combining them when cool to the final concentration. Espon狙ial cells grown in 10 ml of MD broth at 30 °C on a rotary shaker were used as inoculum (5% v/v) for larger volumes. Large flasks containing 10% of their capacity of MD medium plus cells were shaken by rotary agitation at 30 °C. When samples were removed for ATP assay, vigorous agitation was maintained until the moment the samples were withdrawn.

In experiments testing the effect of nutritional supplements on ATP pools, 9.9 ml of broth from a shaker flasks was added to a 125-ml flask containing 0.1 ml of the supplement. The final concentration of each supplement was 0.01 M. The supplemented flasks were then shaken at 30 °C along with an unsupplemented flasks and samples were taken at specific intervals for ATP analysis.

#### Viable Cell Counts

Total viable cell counts were performed by dilution in 0.2% yeast extract and subsequent plating on MD agar. Spore counts were obtained by heating a 1-ml sample for 12 min at 80 °C. Dilutions and plating were the same as those done for total counts.

#### Enzyme Assays

The following enzymes were prepared and assayed as previously described (3): citrate synthase [citrate oxidase-lyase (CoA acetylating), EC. 4.1.3.7]; aconitase [citrate (isocitrate) hydro-lyase, EC. 4.2.1.3]; succinate dehydrogenase (NADP) [malate-isocitrate : NADP oxido-reductase, EC. 1.1.1.42]; succinate dehydrogenase [succinate : (acceptor) oxido-reductase, EC. 1.3.99.1]; fumarase (l-malate hydro-lyase, EC. 4.2.1.2); and malate dehydrogenase (l-malate : NAD oxido-reductase, EC. 1.1.1.37). α-Ketoglutarate dehydrogenase activity is not reported because of low activity of the enzyme and interference by NADH oxidase. Protein was determined by the method of Lowry et al. (8).

#### ATP Assay

ATP was extracted from cells by adding 0.1 ml of culture to 0.9 ml of a mixture of 90% dimethyl sulfoxide (DMSO) - 10% 0.01 M morpholinepropanesulfonic acid (MOPS) buffer pH 7.1. The mixture was stirred vigorously, allowed to stand at room temperature for 5 min, diluted by addition of 3.0 ml of 0.01 M MOPS buffer (pH 7.1), and frozen until assayed. The assay was carried out by adding 0.2 ml of extract to 0.2 ml of a 1:1 dilution of DuPont ATP assay reagent (rehydratable mixture of MOPS buffer, MgSO$_4$, lactofen, and luciferase) purchased from the DuPont Co., Wilmington, Delaware. The resultant light energy output was measured in a JRB photometer (JRB, Inc., La Jolla, Calif.) and the ATP in the samples was determined from a standard curve prepared with ATP dissolved in the DMSO-MOPS extraction solvent.

#### Resuspension Experiments

Ten milliliters of cells was removed from a _B. popilliae_ culture at the times indicated and the cells were sedimented by centrifugation (10 000 g for 10 min) at 25 °C. The resulting cell pellets were suspended in 20 ml of sterile spent broth (see below) and 10 ml of this suspension was transferred to each of two 125-ml flasks. To each of these flasks was then added 0.1 ml of a sterile glucose solution (200 mg/ml). One of the pair of flasks also received 1.0 ml of 1.0 M Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5. The flasks containing resuspended cells were incubated at 30 °C on a rotary shaker. Spent broth was prepared by growing _B. subtilis_ Marburg 168 in MD broth or nutrient sporulation medium (7) 2 h past the end of exponential growth (t$_f$). The spent medium freed of _B. subtilis_ cells by centrifugation was autoclaved.
Results

Tricarboxylic Acid Cycle Enzyme Activity and ATP Levels in B. thuringiensis

The specific activities of the six tricarboxylic acid cycle enzymes assayed in B. thuringiensis are reported in Table 1. Exponential growth by B. thuringiensis in MD broth was completed after 3 h although the absorbance (not shown) continued to increase slowly for several hours. Enzymes of the first half of the cycle showed marked increases upon completion of growth as would be expected if their synthesis was regulated by catabolite repression. Sporulation of most of the population had occurred by 21 h. Levels of ATP in growing and sporulating cells are reported in Table 2. An increase in ATP occurred during sporogenesis whereas there was a small decline in B. subtilis (5) and a much larger decrease in B. popilliae. Otherwise, the general patterns of tricarboxylic acid cycle enzyme and ATP levels were not greatly different from those in sporulating strains of B. subtilis.

Tricarboxylic Acid Cycle Enzyme Activity and ATP Levels in B. popilliae

There is some evidence that B. popilliae lacks a functional tricarboxylic acid cycle (1, 9, 10). We have assayed individual enzymes of the pathway and their specific activities are reported in Table 3. Exponential growth of the culture was complete at about 12–13 h. At this time, citrate synthase activity was not detectable and isocitric dehydrogenase activity was very low. In other bacilli (6), derepression of the tricarboxylic acid cycle enzymes parallels the completion of exponential growth. However, in B. popilliae there was very little increase of enzyme activity at 15 and 17 h, the times at which growth was stationary. Though all enzymes were detected during the stationary phase, it is unlikely that the tricarboxylic acid cycle was functioning effectively. Enzymes of the second half of the cycle were present in B. popilliae at reasonably high levels though succinic dehydrogenase was lower than in B. subtilis (3). The mutants of B. subtilis, which lack a functional tricarboxylic acid cycle, undergo a marked drop in ATP content after exhausting glucose from the medium and completing exponential growth (5, 7, 11). A similar phenomenon was observed in B. popilliae (Table 4). The ATP content of the culture de-
creased over 100-fold between 13 and 26 h of growth. It was during this period that the cells would normally be expected to begin the series of events leading to spore formation. Interestingly, the decline in ATP level occurred several hours before decrease in the number of viable cells. Neither refractile nor heat-stable spores were formed in this culture.

Supplementation and Resuspension Cultivation of *B. popillae*

The levels of ATP in tricarboxylic acid cycle mutants of *B. subtilis* could be increased by supplementing the medium with compounds metabolizable by pathways other than the tricarboxylic acid cycle or by enzymes beyond the block in the cycle (11). However, normal sporulation was not restored by this technique. To determine whether a similar situation was true for *B. popillae*, ATP levels were determined in exponential cultures grown to peak populations. After ATP levels had decreased, aliquots of the cultures were transferred from the main flask to smaller separate flasks, each of which contained a solution of only one compound (final concentration, 0.01 M). All flasks then were sampled and assayed periodically for ATP. Plate counts showed that the number of viable cells in the main flask remained at about 1.1 × 10⁹/ml during the course of the experiment. The data in Fig. 1 reveal responses in ATP synthesis to metabolism of various compounds. Glucose metabolism elicited an immediate burst of ATP synthesis that decreased only slightly during the next 7 h. Pyruvate also was metabolized along with a sudden increase in ATP formation although there was no prolonged synthesis as was found with glucose. Acetate, citrate, glutamate, and succinate evoked little or no such response.

Permeability of *B. popillae* to these compounds has been demonstrated before (1). Final pH of the culture supplemented with glucose was 6.0; final pH of all others was 6.65. No refractile or heat-resistant spores were present 24 h after supplementation with the various compounds.

Mutants of *B. subtilis* that are blocked in the first half of the tricarboxylic acid cycle can be caused to sporulate in spent broth when provided with an energy source (11). This technique was applied to *B. popillae* using spent broth from a sporulating strain of *B. subtilis*. Both MD broth and nutrient sporulation medium (nutrient broth supplemented with Mg²⁺, Mn²⁺, Ca²⁺, and Fe²⁺) were used. Cells harvested from the main growth flask at 13, 16, 19, and 22 h were resuspended and monitored by phase-contrast microscopy to observe spore formation. One of each pair of the flasks containing resuspended cells was buffered to a pH of 7.5 with Tris-HCl. The pH dropped to 5.5-6.0 in those flasks containing only resuspended cells and glucose supplement. No phase-dark or refractile spores were detected in any of the re-suspension cultures. Neither were any heat-stable forms present after 24 h of re-suspension cultivation.

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**TABLE 4**

Growth and ATP content of *B. popillae*<sup>a</sup>  

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Colony-forming units per ml</th>
<th>µM ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3 × 10⁷</td>
<td>N.D.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>4.9 × 10⁷</td>
<td>8.4</td>
</tr>
<tr>
<td>13</td>
<td>1.0 × 10⁹</td>
<td>5.6</td>
</tr>
<tr>
<td>21</td>
<td>1.0 × 10⁹</td>
<td>0.16</td>
</tr>
<tr>
<td>26</td>
<td>1.0 × 10⁹</td>
<td>0.03</td>
</tr>
<tr>
<td>31</td>
<td>1.3 × 10⁹</td>
<td>0.01</td>
</tr>
<tr>
<td>37</td>
<td>1.4 × 10⁹</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells were grown in MD broth at 30°C on a rotary shaker.  
<sup>b</sup>This indicates that the level of ATP was not determined.
Discussion

During exponential growth of *B. popilliae*, the ATP pool of the cells is kept at a high level by metabolism of glucose via the Embden-Myerhof-Parnas and pentose phosphate pathways (2, 10). In sporogenic bacilli, energy for syntheses associated with spore formation is provided by metabolism of intermediate metabolites via the tricarboxylic acid cycle. The specific activities of citrate synthase and isocitric dehydrogenase were extremely low in *B. popilliae*. Mutants of *B. subtilis* with levels of citrate synthase and isocitric dehydrogenase comparable to those found in *B. popilliae* are unable to synthesize α-ketoglutaric acid and are therefore auxotrophic for glutamic acid (3). We believe that these activities reflect low levels of the enzymes in postexponential cells and are inadequate to allow the tricarboxylic acid cycle to function efficiently to maintain ATP at the same levels found in vegetative cells. The decline of ATP in *B. popilliae* began at least 13 h before decrease in the number of viable cells. Apparently, maximum ATP levels are not necessary to maintain cell viability. Chapman et al. (4) provided evidence that cell viability is maintained above an energy-charge value of 0.5. The energy charge (ATP + 1/2 ADP)/(ATP + ADP + AMP) of *B. popilliae* was not determined in these experiments, but it is reasonable to assume that the cellular adenosine monophosphate and adenosine diphosphate pools could have shifted during the decline in ATP level to maintain an energy charge above 0.5, at least temporarily.

The response in ATP synthesis to glucose supplementation was expected because the Embden-Myerhof-Parnas pathway functions during exponential growth of the organism; decline in ATP was probably due to exhaustion of glucose from the medium. That a pyruvate dehydrogenase system operates normally was indicated by the ability of cells to decarboxylate pyruvate (1) and by the burst of ATP synthesis observed in response to supplementation with pyruvate (see Fig. 1). *Bacillus popilliae* failed to oxidize acetate or succinate in radiospirometric studies (1) and ATP production was not stimulated when these compounds were added as supplements. Synthesis of ATP in response to citrate was minimal and it was somewhat surprising to observe any net increase in ATP because of the low levels of isocitric dehydrogenase. Possibly, isocitric dehydrogenase is present at higher levels in vivo and deteriorates rapidly when cells are lysed for enzyme analysis. Supplementation with glutamate elicited very little increase in ATP synthesis probably because of incomplete oxidation of this compound (2). The failure of resuspension cultivation to promote sporulation of *B. popilliae* indicates one or more fundamental difference between *B. popilliae* and *B. subtilis* oligosporogenesis. It is impossible from the present study to decide what those differences may be.

In the *B. subtilis* system a sporogenic parent existed to allow selection of a suitable medium. In the case of *B. popilliae* the spent media tested may have lacked factors critical to the sporogenic response. Although glucose supplementation allowed ATP synthesis for at least 7 h, adequate for sporulation in *B. subtilis*, prolonged ATP synthesis may be necessary in *B. popilliae*. The time required for a single *B. popilliae* cell to sporulate once it has completed growth is unknown. Thin sections of resuspended cells would be useful to determine if any progress had taken place toward sporulation.

*Bacillus subtilis* mutants blocked in the second half of the tricarboxylic acid cycle (at succinic dehydrogenase or fumarase) did not sporulate in the resuspension culture system (11). The level of succinic dehydrogenase in *B. popilliae* was low and succinate was not metabolized to yield ATP. It is possible that this enzyme, though detectable in vitro, was not functioning adequately in vivo to perform some required function and this prevented sporulation.

In contrast to the low tricarboxylic acid cycle enzyme activities and decreasing ATP levels found in *B. popilliae*, *B. thuringiensis* exhibited a pattern not unlike that found in *B. subtilis*. During sporogenesis, the tricarboxylic acid cycle enzymes were present and ATP was produced at a rate sufficient to provide adequate amounts for biosynthetic purposes.