Characterization and Regulation of Pyruvate Carboxylase of Bacillus licheniformis

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Cell-free extracts of Bacillus licheniformis were found to contain pyruvate carboxylase which catalyzes the reaction between pyruvate and bicarbonate to yield oxalacetate in the presence of adenosine triphosphate (ATP), acetylcoenzyme A (CoA), and manganese. The plot between the reaction velocity of the carboxylation by the partially purified pyruvate carboxylase (25-fold) and the concentration of pyruvate, bicarbonate, manganese, and ATP did not indicate a pronounced deviation from the Michaelis-Menten hyperbola. The enzyme was inhibited by avidin and aspartate. Biotin partially protected the enzyme from avidin inhibition, whereas the amount of inhibition by aspartate was dependent on the concentration of acetyl-CoA present. The intracellular concentration of acetyl-CoA did not vary significantly enough to allow control of the enzyme by this method. Extracts of 4-hr postexponential-phase cells of B. licheniformis were also found to contain phosphoenolpyruvate carboxykinase, which appears to be under catabolite repression control. It is suggested that the endogenous induction of this enzyme is the determining factor allowing the shift to gluconeogenesis from glycolysis during sporulation of glucose-grown cells.

Sporulation is initiated in Bacillus licheniformis, as it is in many Bacillus species, by decreasing the growth rate of the organism (12, 23, 29), which usually occurs when one or more nutrients become limiting (3). Thus, when B. licheniformis is grown on a medium composed of minimal salts plus limiting glucose, sporulation is initiated at the cessation of exponential growth, which is concomitant with the disappearance of vegetative cells (17).

B. licheniformis, like B. subtilis, does not store poly-β-hydroxybutyrate nor any detectable amounts of polysaccharide and cannot sporulate endotrophically (34, 43). B. subtilis requires acetate and glycine or serine for sporulation (37). In B. licheniformis, the energy and carbon compounds necessary for the synthesis of the spore structural components are derived from the oxidation products of glucose released into the medium during growth, from endogenous metabolites resulting from protein turnover, and from compounds released from lysed vegetative cells (26).

The synthesis of phosphoenolpyruvate (PEP) from pyruvate, lactate, alanine, and other precursors of pyruvate, a crucial step in gluconeogenesis, is accomplished in microbes either by PEP synthetase (11), pyruvate-phosphate dikinase (4), or the combined activities of pyruvate carboxylase [pyruvate: carbon dioxide ligase (adenosine diphosphate), EC 6.4.1.1] and PEP carboxykinase (31). The apparent metabolic shift by B. licheniformis from glycolysis during growth to gluconeogenesis during sporulation involves important control mechanisms affecting these enzymes (41).

Pyruvate carboxylase has been found to occur in B. coagulans (10), Clostridium kluyveri (J. R. Stern, Fed. Proc. 22:355, 1963), Saccharomyces cerevisiae (27), Pseudomonas citronellolis (32), Micrococcus lysodeikticus (21), Aspergillus niger (6), Rhodopseudomonas spheroides (24), Arthrobacter globiformis (7), and Chromatium species (13) as well as in avian liver (30) and mammalian liver (33).

The widespread distribution of this enzyme reflects a diversity of its function in both microorganisms and vertebrates since this enzyme has been shown to have an anaplerotic role (16) as well as a role in gluconeogenesis (31) and glycogenesis (2). Some microorganisms, however, do not have a pyruvate carboxylase. These organisms carboxylate PEP instead of pyruvate (14).

In the present work, we have demonstrated that extracts of B. licheniformis contain pyruvate-phosphate dikinase. The role of this enzyme in the metabolic shift from glycolysis to gluconeogenesis during sporulation has been investigated. The enzyme was shown to be present in extracts of 4-hr postexponential-phase cells of B. licheniformis (34, 43). B. subtilis requires acetate and glycine or serine for sporulation (37). In B. licheniformis, the energy and carbon compounds necessary for the synthesis of the spore structural components are derived from the oxidation products of glucose released into the medium during growth, from endogenous metabolites resulting from protein turnover, and from compounds released from lysed vegetative cells (26).

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vate carboxylase. Some of the characteristics of the partially purified pyruvate carboxylase are presented, and the possible significance of the reaction catalyzed by this enzyme both by itself and in series with PEP carboxykinase in growing and sporulating cells is discussed.

**MATERIALS AND METHODS**

**Growth of bacteria.** All experiments were performed with cultures of *Bacillus licheniformis* A-5 that were started by adding approximately 10⁵ spores per ml to 50 ml of glucose minimal salts medium composed of: 64 mM K₂HPO₄, KH₂PO₄ (pH, 7.2), 0.8 mM MgSO₄, 0.02 mM MnCl₂, 0.5 mM CaCl₂, 15 mM NH₄Cl, and 20 mM glucose. The solutions containing glucose, NH₄Cl, MnCl₂, MgSO₄ and CaCl₂ were sterilized by membrane filtration and added to sterile phosphate buffer after the pH had been adjusted.

When the culture reached an optical density (OD) of 0.7 to 1.0, generally after 16 hr of shaking at 37 C, it was transferred to Fernbach flasks (2.8 liter) containing 1 liter of prewarmed (37 C) medium. Cultures were incubated aerobically at 37 C on a rotary shaker. When larger volumes of culture were required, a New Brunswick Scientific Co. fermentor was used. One liter of cells (OD 0.7) was transferred to a fermentor containing 10 liters of sterile, prewarmed medium. Growth was monitored by periodic turbidity determinations in a Klett-Summerson colorimeter (no. 54 filter). The turbidity was converted to units of absorbancy by use of a standard curve.

**Preparation of cell extracts.** Cells were suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5 [5 g (wet weight) per 10 ml], containing 1.0 M sucrose, 0.1 M KCl, 5 mM MnSO₄, 0.5 mM disodium adenosine-5′-triphosphate (ATP), 0.1 mM dithiothreitol, and 0.1 mM ethylenediaimidetetracetate (EDTA). (This solution was found to give maximum stability to the enzyme at 0 C.) The cells were disrupted by passing them through a French pressure cell at 18,000 psi. The mixture was centrifuged at 105,000 × g for 90 min. The supernatant solution obtained was used for determining the enzyme level in the cells or for further purification of the enzyme. The protein concentration was determined either by the biuret method (19) or the method of Lowry et al. (20).

**Assay of pyruvate carboxylase.** The activity of pyruvate carboxylase was determined in the following ways.

(i) **¹⁴C-bicarbonate fixation assay.** The reaction mixture (1.0 ml) contained (in micromoles): Tris-hydrochloride (pH 7.5), 100; MnCl₂, 5; sodium pyruvate, 10; ATP, 5; NaH¹⁴CO₃, 10 (approximately 10⁴ counts per min per micromole); acetyl-coenzyme A (CoA), 0.5; KCl, 5; reduced nicotinamide adenine dinucleotide (NADH), 2.5; and malic dehydrogenase, 5 units. The reaction was initiated by the addition of cell extract; after 10 min of incubation at 30 C, the reaction was terminated by the addition of 0.1 ml of 10% trichloroacetic acid. CO₂ was bubbled through the reaction mixture for 5 min, and the solution was centrifuged at 5,000 × g for 5 min. Duplicate samples (0.1 ml) of the supernatant solution were transferred to scintillation counting vials and 10 ml of scintillation fluid was added. Acid-stable ¹⁴C activity was determined by using a Beckman model LS100 liquid scintillation spectrometer.

(ii) **Spectrophotometric assay.** The reaction mixture and conditions for this assay were modified from those described above for the NaH¹⁴CO₃ fixation assay to include unlabeled bicarbonate and less NADH (0.15 micromole). Since it was determined that acetyl-CoA is necessary for pyruvate carboxylase activity, it was possible to determine the activity of the enzyme in crude dialyzed extracts. The initial velocity of NADH oxidation (without acetyl-CoA) at 30 C was followed for 2 min at 340 nm in a Beckman DU2 spectrophotometer after initiating the reaction with extract. Acetyl-CoA was then added, and NADH oxidation was again followed for 2 min. The difference was due to pyruvate carboxylase, as it was dependent on the presence of pyruvate and the observed reaction rate was the same as that seen with the ¹⁴C-bicarbonate assay coupled either to malic dehydrogenase or to glutamate-oxalacetate transaminase (GOT) as described below. There was no significant NADH oxidation when acetyl-CoA alone was added to the reaction mixture.

(iii) **¹⁴C-bicarbonate assay coupled to GOT.** In this reaction mixture, GOT (10 units) and glutamate (20 micromoles) were used in place of malic dehydrogenase and NADH in the ¹⁴C-bicarbonate assay described above. The ¹⁴C-aspartate formed was estimated by passing the acidified reaction mixture through an updated Beckman-Spinco amino acid analyzer, model 120B (25) which was coupled to a Beckman LS100 scintillation counter with a flow-through cuvette containing anthracene crystals.

**Assay of PEP carboxykinase.** Cell-free extracts of *B. licheniformis* used for assaying PEP carboxykinase were prepared by suspending the cells [5 g (wet weight) per 10 ml] in 0.1 M imidazole buffer (Cl⁻), pH 7.0, and passing the preparation through a French pressure cell. The suspension was then centrifuged at 105,000 × g for 90 min. The supernatant solution obtained was dialyzed for 2 hr against a 100-fold volume of the same buffer.

The activity of PEP carboxykinase was determined by the ¹⁴C-bicarbonate fixation assay (18). The reaction mixture contained (in micromoles in a final volume of 1.0 ml): imidazole buffer (Cl⁻), pH 7.0; 100; KH₂CO₃, 50 (2 × 10⁴ counts per min per micromole); cyclohexylammonium PEP, 5; sodium adenosine-5′-diphosphate (ADP), 4; MnCl₂, 1.0; reduced glutathione, 2.0; NADH 2.5; malate dehydrogenase, 8 units; and 0.1 ml of crude extracts (1.0 to 1.5 mg of protein). The reaction was terminated after 10 min at 30 C with 0.1 ml of 10% trichloroacetic acid. The procedures for the assay of the product, oxaloacetate, during the reaction were similar to those for pyruvate carboxylase.

**Partial purification of pyruvate carboxylase.** Unless otherwise noted all operations were performed at 0 to 4 C. Extracts of *B. licheniformis* cells that had been grown to an OD of 1.0 on glucose-minimal salts medium [approximately 5 g (wet weight)] were prepared as described above. The rup-
tured cell suspension was centrifuged at 105,000 × g for 2 hr in a Spinco model L preparative ultracentrifuge, and the supernatant solution, which had a specific activity of 0.04 (units/mg of protein) and a pH of 7.5, was collected. Solid (NH₄)₂SO₄ was added to this solution, with constant stirring, to 65% saturation. After 10 min of stirring, the precipitated protein was removed by centrifugation (105,000 × g for 20 min), the supernatant fraction was collected, and additional (NH₄)₂SO₄ was added until 70% saturation was reached. Stirring was continued for 10 min, and then the resulting suspension was centrifuged (105,000 × g for 30 min) and the supernatant solution was discarded. The sediments protein was dissolved in the original buffer with a resulting specific activity of 0.14. After the pH was adjusted to 6.8 with 0.1 m HCl, (NH₄)₂SO₄ was added to the resulting solution to 55% saturation. After stirring for 10 min, the suspended protein was removed by centrifugation (105,000 × g for 20 min), and the resulting supernatant solution was brought to 60% saturation with solid (NH₄)₂SO₄. The precipitated protein was removed by centrifugation (105,000 × g for 20 min), and the supernatant solution was discarded.

The following step was performed at room temperature and completed within 30 min. The precipitated protein was dissolved in 5 ml of 0.05 M Tris-hydrochloride buffer (pH 7.0) and had a specific activity of 0.32. A 2.5-ml portion was added to a diethylaminoethyl (DEAE)-cellulose column (3.5 by 20 cm) which had been equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.0). The column was quickly washed with 50 ml of this buffer followed by 50 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4) and finally 50 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4) containing 0.1 M KCl. The first 20 ml of solution eluting with the KCl buffer was collected, (NH₄)₂SO₄ was added to 60% saturation, and the solution was immediately centrifuged (50,000 × g for 10 min). The supernatant solution was discarded, and the precipitated protein was dissolved in the original buffer; the resulting specific activity was 1.0. The specific activity decreased 50% in 2 days at 0 C or 1 week at 20 C.

**Assay of citric acid cycle compounds.** Reaction mixtures containing 14C-labeled citric acid cycle compounds were deproteinized by adding 0.1 ml of 10% perchloric acid followed by centrifugation at 5,000 × g for 10 min. The supernatant solutions were neutralized with 1 m KHCO₃ and left overnight at 0 C. The solutions were then centrifuged at 10,000 × g for 10 min, and the supernatant solutions were concentrated under N₂ at 40 C. The resulting concentrated solutions were applied to cellulose plates (MN-polygram Cel 300; Macherey-Nagel and Co., Duren). The plates were subjected to one-dimensional development with a solvent system composed of ether-formic acid-water, [10:2:1 (v/v)] for 1 hour. After drying for at least 2 hr, the radioactive acids were counted on the plates using a radiochromatogram scanner (model RSC-363, Atomic Accessories, Inc.).

**Assay of CoA and acetyl-CoA.** CoA and acetyl-CoA were determined in cells of _B. licheniformis_ by the method of Allred and Guy (1) as modified by Gilber A. Leveille, University of Illinois (personal communication). Samples of culture (10.0 ml) were added to 1 ml of 60% perchloric acid at 0 C and mixed as rapidly as possible. After 5 min, the solution was neutralized (pH 6.5) by adding 3 ml K₂CO₃ containing 0.5 m triethanolamine, and 1.0 ml of 0.1 m dithiothreitol solution was added. After 10 min, the solution was centrifuged at 50,000 × g for 20 min, and the supernatant solution was analyzed for CoA and acetyl-CoA immediately by using a Turner model 111 fluorometer (40).

The results obtained from the above procedure were within 15% of those obtained with an assay utilizing α-ketoglutarate dehydrogenase as outlined by Tubbs and Garland (40). The α-ketoglutarate dehydrogenase was prepared from pig heart by the procedure of Sanadi et al. (28). Cell extracts prepared as above were taken to dryness overnight in a desiccator containing P₂O₅. The dry residue was taken up in 1.0 ml of water and centrifuged at 20,000 × g for 10 min. The solution was analyzed for CoA and acetyl-CoA fluorometrically (40).

To determine how much CoA and acetyl-CoA were present in the medium, 1.0 ml of a growing culture taken at different times during the growth phase was filtered through a membrane filter (24-mm diameter, 0.2-μm pore size) into 0.1 ml of 60% perchloric acid (0 C) and analyzed for these compounds as mentioned above. No CoA or acetyl-CoA could be found in the culture filtrate.

**Glucose assay.** Glucose concentration was determined by the glucose oxidase (Glucostat) method (Worthington Biochemical Corp., Freehold, N.J.).

**Chemicals.** Nicotinamide adenine dinucleotide (NAD-oxidized), NADH, acetyl-CoA, CoA, dithiothreitol, citrate synthetase, phosphotransacetylase, ATP, ADP, sodium cytidine-5'-diphosphate (CDP), sodium inosine-5'-diphosphate (IDP), sodium guanosine-5'-diphosphate, (GDP), and reduced glutathione were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Enzyme grade (NH₄)₂SO₄, was purchased from Mann Research Laboratories, New York, N.Y. 1C-sodium bicarbonate was obtained from Calbi- ochem., Los Angeles, Calif. Sodium pyruvate, the tricyclohexyl-ammonium salt of PEP, GTP, malate, and glyceral were obtained from Sigma Chemical Corp., St. Louis, Mo. Acetyl phosphate was prepared by the method of Stadtman (36). Cellex D (DEAE-cellulose) was obtained from Bio-Rad Laboratories, Richmond, Calif. All other reagents and chemicals used were of reagent grade.

**RESULTS**

**Requirements of 14CO₂ fixation in cell-free extracts.** The requirements for the pyruvate-dependent fixation of carbon dioxide into acid-stable products by cell extracts of _B. licheniformis_ were determined as an indication of the enzyme (or enzymes) present in this organism catalyzing this reaction (Table 1).

The fixation of carbon dioxide by extracts of exponential-phase cells of _B. licheniformis_ was dependent on the presence of ATP, the diva-
TABLE 1. Pyruvate carboxylase activity in dialyzed cell-free extracts

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Specific activity (μmoles/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.025</td>
</tr>
<tr>
<td>ATP omitted</td>
<td>0</td>
</tr>
<tr>
<td>Pyruvate omitted</td>
<td>0.004</td>
</tr>
<tr>
<td>Mn2+ or Mg2+ omitted</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA omitted</td>
<td>0.02</td>
</tr>
<tr>
<td>KCl omitted</td>
<td>0.03</td>
</tr>
<tr>
<td>Complete + avidin (0.5 unit)</td>
<td>0</td>
</tr>
<tr>
<td>ATP and pyruvate omitted; phosphoenolpyruvate (10 mM) added</td>
<td>0.005</td>
</tr>
<tr>
<td>ATP added; NADH or NADPH (2.5 mM added)</td>
<td>0</td>
</tr>
</tbody>
</table>

*14C-bicarbonate fixation assay.

lent cations magnesium or manganese, pyruvate, and acetyl-CoA. Biotin and potassium chloride stimulated the fixation of carbon dioxide marginally. Extracts of cells, in the presence of the above compounds, fixed carbon dioxide into trichloroacetic acid-insoluble products at a rate which varied from 0.02 to 0.03 μmole of carbon dioxide per min per mg of protein. The presence of 0.5 unit of avidin completely inhibited the reaction.

When PEP was substituted for pyruvate and ATP, carbon dioxide was not incorporated into acid-stable products. Fixation of carbon dioxide in the presence of pyruvate and NADH or nicotinamide adenine dinucleotide phosphate, reactions catalyzed by the malic enzyme, could not be found in B. licheniformis.

Stabilization of pyruvate carboxylase. The carbon dioxide-fixing ability of cell-free extracts of B. licheniformis suspended in 0.1 M Tris-hydrochloride (pH 7.4) was quickly lost when the extract was stored at 0 C or room temperature (Fig. 1). It was necessary to stabilize this activity for further studies on the nature of the reaction to characterize the enzyme responsible. The temperature as well as the components present affected the stability of the carbon dioxide-fixing ability of the cell extracts. At 0 C in 0.1 M Tris-hydrochloride (pH 7.4), 50% of the activity present in the cell extracts was lost in 2 hr; 50% of the activity was lost in 4 hr at 30 C; and 50% was lost in 15 min at 45 C (Fig. 1). Of the various components tried, those listed in Fig. 1 added to 0.1 M Tris-hydrochloride buffer gave maximum stability to the enzyme at 0 C. Acetyl-CoA could replace ATP and sucrose with the same results: approximately 20% of the activity was lost in 10 hr. This same stability could be achieved at 30 C without the addition of sucrose but with the other components. The stability was not significantly influenced by pH between 6.5 and 7.5; however, below pH 6.0, there was a rapid loss of activity by the crude extracts.

Products of carbon dioxide fixation. Although the requirements of the carbon dioxide fixation reaction indicated pyruvate carboxylase as the enzyme responsible for this reaction, it was necessary to identify oxalacetate as the primary product to conclude that this was the enzyme functioning in B. licheniformis.

In the 14CO2-fixing assay coupled to malic dehydrogenase, radioactivity fixed by crude extracts of B. licheniformis appeared in several citric acid cycle intermediates (Fig. 2), including citrate and fumarate as well as malate. When the partially purified enzyme catalyzed the reaction, the primary product detected by this method was malate (Fig. 2). To further determine that oxalacetate was the main product of the reaction, the reaction mixture was coupled to GOT instead of malic dehydrogenase. The specific activity of the recovered aspartate agreed with that of malate and was within 95% of the specific activity of the carbon dioxide used in the reaction.

Characterization of partially purified pyruvate carboxylase. The apparent kinetic constants of the partially purified enzyme were
determined by constructing reciprocal substrate versus velocity plots. The apparent $K_m$ values of the various components used in the spectrophotometric assay are listed in Table 2. When the $K_m$ values for Mn$^{2+}$ ions and ATP were determined, these two reactants were omitted from the buffer.

The effect of avidin on the activity of pyruvate carboxylase was determined since biotin has been reported to be a cofactor for many carboxylases and since avidin is a specific inhibitor of biotin (44). Avidin inhibits the pyruvate carboxylase of *B. licheniformis*, and this inhibition is partially reversed by biotin (Fig. 3).

Aspartate has been reported to be a potent inhibitor of the pyruvate carboxylases from bakers' yeast (9) and *B. coagulans* (38) and also the PEP carboxylase from *Escherichia coli* (35). This amino acid also inhibits the enzyme from *B. licheniformis* (Fig. 4). The inhibition is dependent on the concentration of acetyl-CoA present and independent of the concentration of Mn$^{2+}$, ATP, or HCO$_3^-$.  

**Levels of pyruvate carboxylase and PEP carboxykinase in growing cultures.** To determine whether the maximal catalytic capacity of pyruvate carboxylase was dependent on the age of the culture, the levels of pyruvate carboxylase in growing cultures of *B. licheniformis* were determined. In addition, assays were performed for PEP carboxykinase activity and the glucose concentration in the culture fluid. The PEP carboxykinase activity in extracts was dependent on PEP, ADP, and Mn$^{2+}$ (Table 3).

The levels of pyruvate carboxylase in cells of *B. licheniformis* are not dependent on the carbon source used to grow *B. licheniformis*.

![Graph](http://jb.asm.org/)

**Table 2. Apparent $K_m$ values for the partially purified pyruvate carboxylase from *Bacillus licheniformis*^a^**

<table>
<thead>
<tr>
<th>Component</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO$_3$</td>
<td>$2.4 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>$4.1 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>ATP</td>
<td>$1.5 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>$2.3 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>$8.6 \times 10^{-4}$ M</td>
</tr>
</tbody>
</table>

*a* Pyruvate carboxylase with a specific activity of 0.96 (μmoles of CO$_2$ fixed per min per mg of protein) was used in the spectrophotometric assay.

![Radiochromatograms](http://jb.asm.org/)
partate in the VOL. 109, 1972 to added acetyl-CoA

ADP omitted

omitted

Phosphoenolpyruvate omitted

Mn** omitted

ADP omitted

IDP or GDP added at 4 mM

0.7

FIG. 4. Inhibition of pyruvate carboxylase by aspartate in the presence of acetyl-CoA. Aspartate and acetyl-CoA at the indicated concentrations were added to the reaction mixture for the spectrophotometric assay. The reaction was initiated by adding the partially purified pyruvate carboxylase (0.96 unit/mg).

**TABLE 3. Requirements of the phosphoenolpyruvate carboxykinase from cell-free extracts of postexponential-phase cultures**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Activity (nmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2.0</td>
</tr>
<tr>
<td>ADP omitted</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphoenolpyruvate omitted</td>
<td>0.2</td>
</tr>
<tr>
<td>Mn** omitted</td>
<td>0.3</td>
</tr>
<tr>
<td>ADP omitted</td>
<td></td>
</tr>
<tr>
<td>IDP or GDP added at 4 mM</td>
<td></td>
</tr>
</tbody>
</table>

(Table 4). In growing cultures of B. licheniformis, the specific activity of pyruvate carboxylase increased approximately twofold during the log phase of growth (Fig. 5). There was a decrease in activity after the end of growth corresponding to the depletion of glucose in the medium. At this time, there was a sharp increase in PEP carboxykinase activity, which reached its maximum activity 3 to 5 hr after growth, at which time the activity of PEP carboxykinase was 2 nmoles per min per mg of protein. This enzyme, which catalyzes the reaction oxaloacetate + ATP = PEP + CO₂ + ADP, was assayed by measuring the PEP-dependent fixation of carbon dioxide. The nucleotides GDP and IDP were ineffective in this assay.

**Intracellular level of CoA and acetyl-CoA.** Since the specific requirement for acti-

_**TABLE 4. Levels of pyruvate carboxylase in cells grown on different carbon sources**_

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Specific activity (μmoles/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.04</td>
</tr>
<tr>
<td>Malate</td>
<td>0.03</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.04</td>
</tr>
<tr>
<td>Peptone-yeast extract</td>
<td>0.03</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Cell extracts of B. licheniformis grown in media containing 20 mM concentrations of the above carbon sources were prepared as described in the text. Pyruvate carboxylase was assayed by the ¹⁴CO₂ method.

FIG. 5. Growth of B. licheniformis on glucose-minimal salts medium (○) and the relative activities of pyruvate carboxylase (△; 100% = 0.04 μmole per min per mg of protein) and phosphoenolpyruvate (PEP) carboxykinase (▲; 100% = 2.0 nmoles per min per mg of protein). Dialyzed 105,000 x g supernatant fractions were prepared from extracts of cells from 100-ml volumes of culture harvested at the indicated times. Pyruvate carboxylase and PEP carboxykinase were determined by the ¹⁴CO₂ methods described in the text.

viation by acetyl-CoA shown by pyruvate carboxylase may be of considerable importance in connection with the proposed key role of this enzyme in the regulation of gluconeogenesis, as suggested by Utter et al. (44), the intracellular level of acetyl-CoA was determined (Fig. 6). When B. licheniformis was grown in a glucose-minimal salts medium, the concentration of total CoA (defined as CoA and acetyl-CoA) increased from 0.7 to 1.0 n mole per mg of bacterial protein during growth. This increase in total CoA was due to acetyl-CoA since this compound increased from 0.52 to 0.85 n mole per mg during this growth phase. After the end of growth, total CoA started to decline and fell to 0.4 n mole per mg at 6 hr after the end of
exponential growth; however, acetyl-CoA fell to 0.075 nmole at this time.

**DISCUSSION**

The pyruvate carboxylase in extracts of *B. licheniformis* catalyzes the reaction between pyruvate and bicarbonate to yield oxalacetate in the presence of ATP, acetyl-CoA, and Mn$^{2+}$. KCl and biotin stimulated the fixation of carbon dioxide marginally, whereas avidin, a specific inhibitor of biotin, inhibited the reaction. The latter observation would indicate that the pyruvate carboxylase of *B. licheniformis* is a biotin-requiring enzyme. The enzyme is cold-labile and markedly unstable in 0.1 M Tris-hydrochloride buffer. It was necessary to add sucrose as well as various other components to this buffer to achieve maximum stability at 0 C.

The plot between the reaction velocity of the carboxylation and the concentration of pyruvate, bicarbonate, Mn$^{2+}$, and ATP did not indicate a pronounced deviation from the Michaelis-Menten hyperbola. Taylor (38) reported that the pyruvate carboxylase isolated from sheep liver demonstrated a negative-type cooperativity in the binding of pyruvate to this enzyme. Scrutton (30), however, questions whether negative cooperative interaction between pyruvate sites is the only explanation for the observed deviations from Michaelis-Menten behavior. Seufert et al. (33), in kinetic analysis of the pyruvate carboxylase from rat liver, found that the enzyme exhibits negative cooperativity with respect to binding of pyruvate only at suboptimal levels of acetyl-CoA.

In its absolute dependence upon acetyl-CoA, the pyruvate carboxylase of *B. licheniformis* resembles more closely the enzyme from avian liver (15) than those from microbial sources which either show activity in the absence of acetyl-CoA but are stimulated in its presence (8) or are unaffected (12). However, in contrast to the enzyme from avian liver, the enzyme from *B. licheniformis* is inhibited by aspartate. Aspartate also inhibits the pyruvate carboxylase from yeast (9), and the PEP carboxylase of the *Enterobacteriaceae* (22). Scrutton (30) suggested that failure to observe a similar effect for pyruvate carboxylase from avian liver may be related to a decrease in the relative importance of the anaplerotic role in this tissue. The competitive regulation by aspartate and acetyl-CoA of the enzyme in this organism has also been observed for the pyruvate carboxylase of yeast (9) and *B. coagulans* (10) as well as the PEP-carboxylase in *E. coli* (35). The reversal of the inhibition of aspartate by acetyl-CoA may have physiological importance in these organisms since this provides a mechanism for regulating carbon entering the tricarboxylic acid cycle via oxalacetate in the presence of high intracellular concentrations of acetyl-CoA while maintaining an aspartate pool for protein synthesis (5).

The reaction catalyzed by this enzyme in Bacillus species is apparently obligatory for the maintenance of the tricarboxylic acid cycle during growth on glucose (38). In addition to this anaplerotic function, the synthesis of some spore-specific compounds from lactate, alanine, and other precursors of pyruvate requires the combined action of pyruvate carboxylase and PEP carboxykinase. The conversion of pyruvate to PEP is a crucial step in gluconeogenesis. The results indicate that one of the important control mechanisms involved in the metabolic shift from glycolysis during growth to gluconeogenesis sporulation is the derepression of PEP carboxykinase. The levels of pyruvate carboxylase do not vary significantly in cells throughout growth and sporulation. Although the concentration of acetyl-CoA remains fairly constant throughout growth and the early stages of development, calculations show that the concentration of acetyl-CoA decreases from an intracellular concentration of 100 μM in 3-hr postexponential-phase cells to 20 μM in 6.5-hr postexponential-phase cells. The fact that the latter value is fourfold lower than the $K_m$ of acetyl-CoA suggests that the acetyl-CoA concentration could limit the synthesis of spore-specific compounds from pyruvate or precursors of this compound by the end of 5 hr postexponential-phase growth. This
conclusion is tentative, however, since the activation of rat liver pyruvate carboxylase by acetyl-CoA is dependent on pH, the concentrations of HCO$_3^-$, MgATP$^+$, free magnesium, and, in turn, by calcium as an antagonist of magnesium (35). Furthermore, it is possible that the three carbon compounds used for synthesis by the developing cell have been converted to the four-carbon substrates of PEP carboxykinase by the time the concentration of acetyl-CoA could influence the reaction rate of pyruvate carboxylase. Thus we believe that the determining factor allowing the shift to gluconeogenesis during the first few hours of sporulation of glucose-grown cells is the appearance in these cells of PEP carboxykinase, an enzyme that appears to be under catabolite repression control.

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LITERATURE CITED


