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Regulation of Synthesis of Pyruvate Carboxylase in the Photosynthetic Bacterium *Rhodobacter capsulatus*

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The synthesis of pyruvate carboxylase (PC) was studied by using quantitative immunoblot analysis with an antibody raised against PC purified from *Rhodobacter capsulatus* and was found to vary 20-fold depending on the growth conditions. The PC content was high in cells grown on pyruvate or on carbon substrates metabolized via pyruvate (lactate, D-malate, glucose, or fructose) and low in cells grown on tricarboxylic acid (TCA) cycle intermediates or substrates metabolized without intermediate formation of pyruvate (acetate or glutamate). Under dark aerobic growth conditions with lactate as a carbon source, the PC content was approximately twofold higher than that found under light anaerobic growth conditions. The results of incubation experiments demonstrate that PC synthesis is induced by pyruvate and repressed by TCA cycle intermediates, with negative control dominating over positive control. The content of PC in *R. capsulatus* cells was also directly related to the growth rate in continuous cultures. The analysis of intracellular levels of pyruvate and TCA cycle intermediates in cells grown under different conditions demonstrated that the content of PC is directly proportional to the ratio between pyruvate and C_4 dicarboxylates. These results suggest that the regulation of PC synthesis by oxygen and its direct correlation with growth rate may reflect effects on the balance of intracellular pyruvate and C_4 dicarboxylates. Thus, this important enzyme is potentially regulated both allosterically and at the level of synthesis.

Pyruvate carboxylase (PC) (EC 6.4.1.1), a biotin-containing enzyme, plays an important anaplerotic function in replenishing intermediates of the tricarboxylic acid (TCA) cycle by catalyzing the ATP-dependent carboxylation of pyruvate to form oxaloacetate (for a review, see reference 1). The enzyme is highly conserved and is found in a wide variety of organisms, including bacteria, fungi, animals, and plants (39, 46). PC from most sources is a tetramer of four identical subunits with molecular masses of 110 to 130 kDa. Native (tetrameric) PC has a molecular mass of 440 to 520 kDa and is allosterically activated by acetyl coenzyme A (acetyl-CoA) (1, 45). PC from *Pseudomonas citronellolis* consists of two different subunits, one of 65 kDa (α), which contains biotin, and one of 54 kDa (β). The native protein has a molecular mass of 454 to 530 kDa, which is consistent with an $\alpha_4\beta_4$ structure (8). Preliminary studies of PC from *Azotobacter vinelandii* indicate that this enzyme is quite similar to the *Pseudomonas* enzyme (33).

Bacteria have evolved a number of strategies for regulating the activities of enzymes that play key roles in carbon or nitrogen metabolism. In the long term, levels of potential enzyme activity are controlled by varying the synthesis of the enzyme to appropriately match particular metabolic conditions. Overlaying this level of control is the possibility of the short-term modulation of enzyme activity, either by interaction with allosteric effectors or through activation and inactivation via covalent modification. Key enzymes, such as glutamine synthetase in the enteric bacteria, may be subject to all three means of control. The short-term regulation of PC activity by allosteric activation and/or inhibition has been well documented (for a review, see reference 13). For instance, the enzyme is inhibited by L-aspartate and oxaloacetate and is activated by monovalent cations and acetyl-CoA. However, little is known about the regulation of the synthesis of PC, which is apparently a constitutive protein. Changes in levels of PC activity have been observed in many organisms. In yeasts and *Bacillus stearothermophilus* the synthesis of PC is apparently controlled at the level of holoenzyme formation by a limiting supply of biotin (7). The level of PC activity in *P. citronellolis* and *A. vinelandii* is dependent on the carbon source used for cell growth (33, 37). Enzyme activity was highest in extracts from cells grown on lactate and virtually absent in cells grown on malate and succinate. Based on these results, it has been proposed that PC synthesis is induced by pyruvate or some related metabolite (33, 37).

The photosynthetic bacterium *Rhodobacter capsulatus* is metabolically versatile, being able to grow both in the light (anaerobically) and in the dark (aerobically or anaerobically) with either organic compounds or CO_2 as a carbon source (36, 43). PC activity in *R. capsulatus* has been previously shown to be much lower in extracts from malate- or acetate-grown cells than in extracts from lactate-grown cells (42). *R. capsulatus* mutants which lack PC activity are unable to utilize pyruvate or substrates metabolized via pyruvate as the sole carbon source for growth (42, 44). A PC-overexpressing strain of *R. capsulatus* has been described and used for enzyme purification and partial enzymatic characterization (24). In this study we have investigated the regulation of PC synthesis in *R. capsulatus* on the protein level by using quantitative immunoblot analysis. The results clearly establish that in *R. capsulatus* the synthesis of PC is highly regulated in response to growth conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. capsulatus* SB1003 was grown photoheterotrophically on either a complex medium (YPS [0.3% yeast extract, 0.3% peptone, 2 mM $CaCl_2$, 2 mM $MgCl_2$]) or a minimal medium (modified RCV, containing previously described mineral salts [40], 10 mg $FeSO_4 \cdot 7H_2O \cdot liter^{-1}$, 19.1 mM phosphate buffer [twice the normal concentration], and, unless otherwise noted, 30 mM NH_4^+ as a nitrogen source). Throughout this paper, minimal media are indicated as C (carbon source)-RCV, including the changes to RCV noted above. For the large-scale purification of PC, cells were grown in 80-liter

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batches on lactate (30 mM DL-lactate)-RCV, collected by flow centrifugation, and stored in liquid nitrogen. Various growth conditions were used for the study of the regulation of PC synthesis. For anaerobic growth in the light (in screw-capped tubes filled to capacity), growth was with different carbon sources (30 mM final concentration; acetate-RCV was supplemented with 10 mM $NaHCO_3$ [42]) or under iron limitation (lactate-RCV, with iron omitted, was prepared with deionized water with a conductivity of $<10^{-7}$ S). Cultures in the late exponential growth phase which had been grown on lactate-RCV medium were used as inocula (5%, vol/vol). Tubes were incubated at 30°C in a Biotronette Mark III environmental chamber (Labline Instruments) equipped with three 150-W incandescent lamps. Culture growth was followed by measuring the A_{660}. For aerobic growth in the dark, 1-liter flasks containing 0.4 liters of lactate-RCV were wrapped with aluminium foil and incubated for 1 day with agitation at 30°C. For anaerobic growth in the dark, screw-capped glass tubes, wrapped with aluminium foil, were filled completely with RCV base medium containing 11 mM fructose and 80 mM dimethyl sulfoxide (no lactate) and placed in an argon-sparged anaerobic jar. Cultivation was at 30°C for 3 days.

Continuous cultivation of *R. capsulatus* was performed by using a photobioreactor specially designed for the continuous cultivation of photosynthetic microorganisms (38). Cultures were grown under photoheterotrophic anaerobic conditions with lactate (45 mM) and $(NH_4)_2SO_4$ (10 mM for turbidostat conditions and 2 mM for chemostat conditions) as carbon and nitrogen sources, respectively. All assays were performed under steady-state conditions after at least five culture doublings.

Preparation of cell extracts. Extracts were routinely made from cells grown to mid-exponential phase. Cells were harvested by centrifugation (18,000 × *g*, 20 min, 4°C) and resuspended in 0.1 M Tris-HCl buffer (pH 7.4). Cell suspensions were disrupted by sonication (six times for 20 s each at maximal power on ice) with a Biosonik III sonicator (Brownwill Scientific, Rochester, N.Y.). The crude extracts were clarified by centrifugation (100,000 × *g*, 1 h, 4°C), and the supernatant was retained for determination of enzyme activity.

Purification of PC. Cells (~300 g, wet weight) were thawed in 600 ml of disruption buffer (0.1 M Tris-HCl [pH 7.4], 0.2 mM dithiothreitol, 1 mM $MgCl_2$), sonicated eight times (1-min periods, 4°C, maximal power) with a Biosonik III sonicator, and centrifuged (100,000 × *g*, 1 h, 4°C). The supernatant, diluted with an equal volume of buffer A (50 mM Tris-HCl [pH 7.4], 0.2 mM dithiothreitol, 1 mM $MgCl_2$), was passed through a DEAE-cellulose column (DE_{52}, 20 by 2.5 cm) previously equilibrated with buffer A. After washing with this buffer, PC activity was eluted with an exponential NaCl gradient (50 ml of buffer A and 200 ml of 0.2 M NaCl in buffer A). The PC fraction from the DEAE-column was loaded onto a Reactive Red 120-agarose column (Sigma; 26 by 1.5 cm) previously equilibrated with buffer A. PC activity was then eluted with buffer A containing 0.4 M NaCl. The main PC fraction was applied directly to a hydroxylapatite column (Bio-Gel HTP; Bio-Rad; 17 by 1 cm) equilibrated with 1 mM K-phosphate in buffer B (buffer A containing 0.1 M NaCl and 10% glycerol). The column was washed with 3 bed volumes of this buffer, and then PC activity was eluted by increasing the K-phosphate concentration to 50 mM. PC-containing fractions were subsequently loaded onto an Ultrogel AcA-34 column (90 by 2.5 cm) equilibrated and developed with buffer B. Concentrated (by ultrafiltration with an XM-50 membrane) PC fractions from the previous step were chromatographed on a Varian 5000 liquid chromatograph equipped with a Protein-Pak DEAE SPW column (Waters) equilibrated with buffer A. The column was developed (1 ml/min) with a linear NaCl gradient (0 to 0.25 M) in buffer A over 35 min.

While the present work was in progress, another PC purification procedure, which appears to be more efficacious, was published (24). However, that procedure used a PC-overproducing strain, which, although the enzymatic properties of the purified enzyme should be the same, affects the number and types of purification steps used. Purification of PC from the wild-type strain necessitates multiple purification steps. (For example, Modak and Kelly [24] used ammonium sulfate precipitation in addition to dye-ligand chromatography, whereas we used hydroxyapatite, gel filtration, and anion-exchange chromatography.) Moreover, dye-ligand affinity chromatography cannot be used until an appreciable degree of purification has been achieved; otherwise, the columns quickly become contaminated due to their lipophilic character and lose capacity. Additionally, the purification procedure described by Modak and Kelly (24) requires the relatively expensive use of acetyl-CoA for the final purification step and necessitates its removal for subsequent enzymatic studies.

Spectrophotometric assay for PC activity. PC activity was assayed by coupling the production of oxaloacetate to the reduction of 5,5-dithiobis-(2′-nitrobenzoic acid) (DTNB), using citrate synthase, as described by Payne and Morris (30). The reaction mixture comprised (in a final volume of 1 ml) 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM Na-bicarbonate, 5 mM ATP, 5 mM Na-pyruvate, 0.1 mM acetyl-CoA, 0.25 mM DTNB, and 2 U of citrate synthase (Boehringer). The rate of increase in the A_{412} (E = 13.6 $mM^{-1} \cdot cm^{-1}$) at room temperature was measured, and the reaction rate was calculated from the difference between the rates of DTNB reduction in the presence and absence of ATP (1 U = 1 μmol of DTNB reduced/min).

Molecular mass determinations. Analytical gel filtration–high-performance liquid chromatography (HPLC) was performed with a Bio-Silect SEC-400-5 column (7.8 by 300 mm; Bio-Rad) with a Varian 5000 HPLC system. The eluent was 50 mM Tris-HCl (pH 7.4)–0.1 M NaCl, and the flow rate was 1 ml/min. Molecular mass markers used were thyroglobulin (670,000 Da), *R. capsulatus* nitrogenase MoFe protein (230,000 Da), immunoglobulin G (158,000 Da), ovalbumin (44,000 Da), and myoglobin (17,000 Da). Subunit size and protein purity were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% total acrylamide) by the method of Laemmli and Favre (16) with reference proteins (high-molecular weight standards; Pharmacia). Protein bands were visualized by staining with Coomassie blue R-250.

N-terminal amino acid sequencing. The N-terminal amino acid sequence of PC was determined by electroblotting homogenous protein onto a polyvinylidene difluoride membrane, staining the membrane with Coomassie blue R-250, destaining with 50% methanol, and subjecting the band to automated Edman degradation. N-terminal sequencing was performed by the Sheldon Biotechnology Center, McGill University, Montreal, Canada.

Determination of organic acids. *R. capsulatus* cultures (150 ml) were grown as indicated and fixed by the addition of H_2SO_4 (50 mM final concentration) before centrifugation (18,000 × *g*, 20 min, 4°C). Analysis of concentrated supernatants showed that no organic acids were extracted during this method of cell fixation. Cell pellets were resuspended in cold 70% ethanol, sonicated (six times for 20 s each), heated for 2 min in a boiling water bath, chilled on ice, and clarified by centrifugation (20,000 × *g*, 30 min, 4°C). Supernatants were lyophilyzed, dissolved in 2 to 3 ml of 5 mM H_2SO_4, centrifuged for 10 min in an Eppendorf 5415 centrifuge (maximum speed), and filtered through 0.45-μm-pore-size nylon syringe filters. The levels of organic acids in cell samples were determined by HPLC on a Varian 5000 liquid chromatograph equipped with an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad). The column was equilibrated and developed with 5 mM H_2SO_4 (with detection at 210 nm).

Quantitative immunoblot analysis of PC in *R. capsulatus* cells. *R. capsulatus* cells were grown under different conditions (as described above) to mid-exponential growth phase, and whole-cell extracts were prepared by mixing culture aliquots with an equal volume of Laemmli sample buffer followed by 5 min of incubation in boiling water. Samples were separated by SDS-PAGE (7.5% total acrylamide) (16) and electroblotted onto nylon membranes. Western immunoblotting with rabbit anti-PC serum (1:2,000 dilution) was carried out essentially as described earlier (47). For PC quantitation, the nylon membranes were developed with Amersham ECL chemiluminescence detection reagents (according to the manufacturer's protocol), the X-ray films were scanned, and the bands corresponding to PC were quantitated with a Molecular Dynamics personal densitometer. The PC content was calculated by using a standard curve established with a homogenous preparation of *R. capsulatus* PC, which was linear over a range of at least from 0.5 to 20 ng of PC. Antiserum against *R. capsulatus* PC was obtained by subcutaneous injections of homogenous preparations in rabbits (9). Protein concentrations were determined by the Bradford method (6) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Properties of purified PC. *R. capsulatus* PC purified from the wild-type strain as described in Materials and Methods had a specific activity of 18.7 ± 4 μmol of DTNB reduced/min · mg of protein and gave a single protein band on SDS-PAGE with an apparent molecular mass of 128 ± 4 kDa. This probably represents a more accurate molecular mass than the previously determined 140 kDa (24), since we used molecular mass markers that bracketed PC, whereas in the previous measurement (24), PC exceeded the range of markers used. The molecular mass of native *R. capsulatus* PC was determined by HPLC-gel filtration to be 500 ± 20 kDa (not shown). Since SDS-PAGE analysis of the isolated enzyme gave a single band of 128 kDa, this suggests that *R. capsulatus* PC is composed of four apparently identical subunits. Thus, *R. capsulatus* PC has a molecular organization similar to that of eukaryotic enzymes. A homogenous sample of *R. capsulatus* PC, purified as described in Materials and Methods, was subjected to SDS-PAGE, and the N-terminal amino acid sequence of the PC polypeptide was determined by Edman degradation after transfer to a polyvinylidene difluoride membrane. The sequence was MFEKILVA NRGEIAIRVLRAANEL, which shows a high degree of similarity with the N-terminal regions of PCs from yeast and animals (18, 20, 22, 35, 41, 48). The sequence is almost identical to that previously determined for *R. capsulatus* (24), with the only difference being in the seventh residue (underlined); it was Val in the sequence presented here and Met according to the data of Modak and Kelly (24). It should be noted that there is Val at the analogous position in the N-terminal regions of all

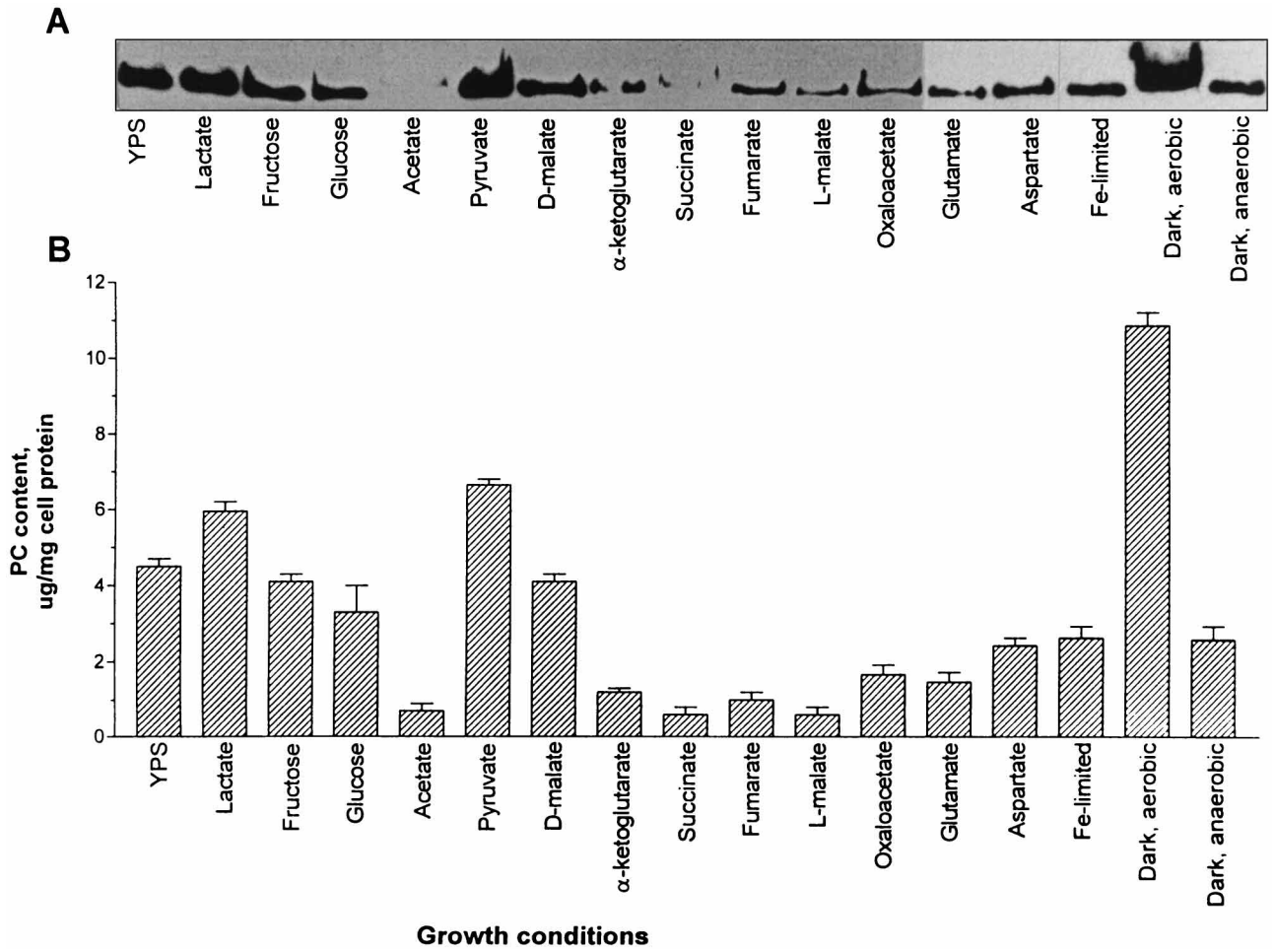

FIG. 1. PC content of *R. capsulatus* cells grown under various conditions (for experimental details, see Materials and Methods). Aliquots (100 to 500 μl) were withdrawn from cultures grown under the indicated conditions, SDS-treated samples were prepared, and Western immunoblot analysis with anti-PC antiserum was performed as described in Materials and Methods. Immunoblots were scanned, and PC contents were calculated by using a standard curve. (A) Western immunoblot of PC. (B) Content of PC calculated from the scan of the immunoblot. The data represent averages from at least four independent determinations, with standard deviations indicated by error bars.

other PCs whose sequences are known at this time (18, 20, 22, 35, 41, 48). Despite these minor differences, we believe that the protein isolated here is identical to that previously isolated (24). In support of this, Western blot analysis indicates that the enzyme that was purified and subsequently used for antibody production is immunologically identical to that previously described (not shown). A detailed enzymatic analysis of this protein has recently been presented by others (24).

Effect of growth conditions on PC synthesis and activity. *R. capsulatus* can use a number of organic substrates as a carbon source, including sugars, organic acids, and fatty acids (36, 40). Western immunoblot analysis of *R. capsulatus* cells with a polyclonal antiserum directed against *R. capsulatus* PC revealed the presence of this protein under all tested growth conditions (Fig. 1A). Scanning densitometry of immunoblots showed that the content of PC in the cells varied over a 20-fold range (Fig. 1B). In lactate-grown cells the content of this enzyme was about 0.5% of total cell protein. The data presented in Fig. 1 clearly show that the quantity of PC in *R. capsulatus* cells is greatly dependent on the type of carbon source used. Cultures grown on pyruvate or on carbon substrates metabolized via pyruvate, e.g., lactate, D-malate, glucose, or fructose, showed a higher content of PC than cells grown on substrates which are metabolized without the intermediate formation of pyruvate, e.g., acetate, α-ketoglutarate, succinate, L-malate, oxaloacetate, glutamate, or aspartate. Acetate, succinate, and L-malate gave a stronger suppression of PC synthesis than oxaloacetate, aspartate, α-ketoglutarate, and glutamate (Fig. 1). The relatively high PC content of *R. capsulatus* cells grown on YPS medium suggests that under this condition, this bacterium uses as a carbon source amino acids which are degraded mainly to pyruvate (alanine, cysteine, glycine, serine, threonine, and tryptophan). These results suggest that in *R. capsulatus* the synthesis of PC is induced by the presence of pyruvate or some closely related metabolite.

To determine if the pattern of PC protein regulation was reflected in the level of PC activity, extracts were prepared from cells showing low or high contents of PC, and the specific PC activity was determined. Similarly to the results obtained with immunoblot analysis, *R. capsulatus* cells grown on acetate, fumarate, or succinate showed significantly lower specific PC activity than cultures grown on lactate or pyruvate (Fig. 2). Therefore, in general, the PC activity of *R. capsulatus* cells correlates with the level of PC protein present. Interestingly, while the difference in the levels of PC protein between cells with high and low PC contents was 10- to 20-fold, the differ-

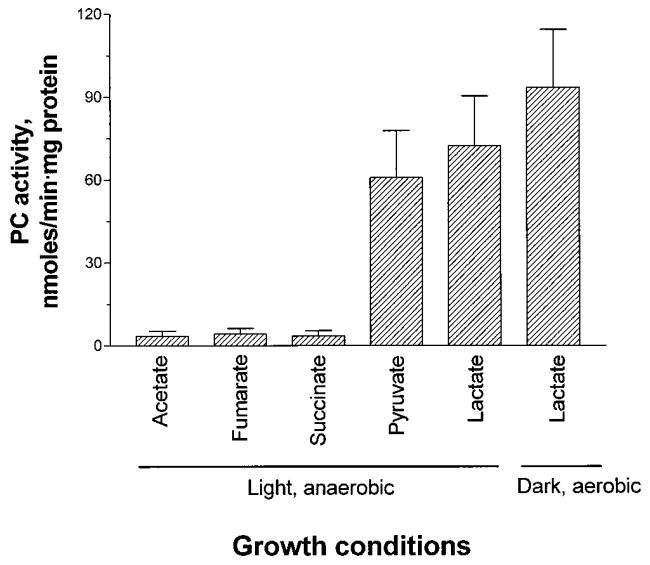

FIG. 2. PC activities in extracts of *R. capsulatus* cells grown under different conditions. Cells were grown as indicated, and extracts were prepared and assayed for PC activity as described in Materials and Methods. Results are averages from at least four independent determinations, with standard deviations indicated by error bars.

ence in PC activity between these cultures was somewhat higher (20- to 30-fold). Similarly, it has been previously shown that the level of PC activity was much lower (sometimes undetectable) in extracts from malate- or acetate-grown cultures than that in extracts from lactate-grown cells (42). Aerobically grown cells of *R. sphaeroides* had a higher PC activity than photoheterotrophically grown cultures (30). Another interesting observation based on the comparison of the data shown in Fig. 1 and 2 is that the PC activity in extracts from lactate- or pyruvate-grown cells was reasonably close (60 to 100%) to that calculated from the content of PC protein and the specific activity of the purified enzyme. However, the observed PC activity in extracts from acetate-, fumarate-, or succinate-grown cells constituted less than 10% of potential PC activity. Low and variable PC activity in cells of *R. capsulatus* and *R. sphaeroides* grown with these carbon sources has also been reported by other investigators (30, 42). There are several possible explanations for this effect. For one thing, PC in these cells could be more labile. We have already observed that purified PC is sensitive to dilution (results not shown). This could also reflect a secondary system of control of PC activity, for example, inhibition by covalent modification. Understanding these differences requires further analysis on the molecular level. At any rate, these results demonstrate that immunoblot analysis of the level of PC protein in *R. capsulatus* cells gives a better reflection of the regulation of its synthesis than determination of PC activity in cell extracts.

Effect of incubation with organic compounds. To further investigate the regulation of PC synthesis, pyruvate-limited cultures of *R. capsulatus* were incubated with different carbon sources, and their PC contents were determined after 4 and 18 h (Fig. 3). Control cultures (no additions) showed that PC was being turned over, with a 50% decrease in light-incubated cultures and a 80% decrease in dark-incubated cultures after 18 h. Fourfold-higher levels of PC were observed in dark-incubated cultures to which pyruvate had been added. Likewise, somewhat higher levels were found in light-incubated cultures when pyruvate was present. Addition of fumarate, succinate, oxaloacetate, or L-malate to light-incubated cultures

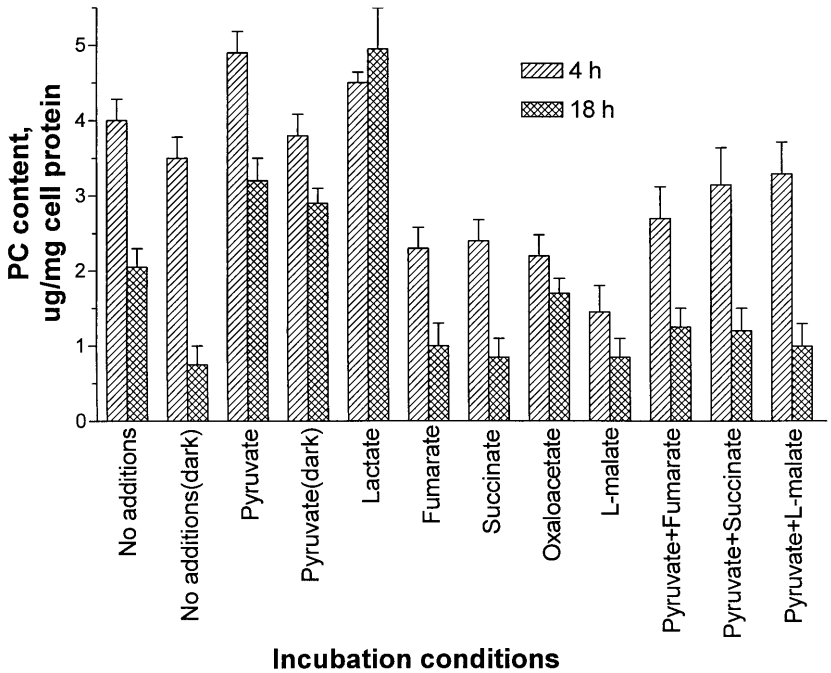

FIG. 3. Effect of different incubation conditions on PC content. Cells were grown overnight on RCV medium containing a limiting (5 mM) concentration of pyruvate (A_{660} = 1.0; early stationary phase of growth). Aliquots (4 ml) of the culture were transferred anaerobically via a syringe to sterile serum vials (preflushed with argon) containing different substrates as indicated (30 mM final concentration). The vials were incubated at 30°C in the light or in darkness (wrapped in aluminium foil). Aliquots (200 μl) of culture were withdrawn from the vials after 4 and 18 h of incubation for the determination of culture density (A_{660}) and PC content by quantitative immunoblot analysis as described in Materials and Methods. Results are averages from at least four independent determinations, with standard deviations indicated by error bars.

resulted in a greatly decreased level of PC (over that of the control), suggesting that PC turnover was increased under these conditions. This was confirmed in separate experiments with chloramphenicol (data not shown). The addition of lactate caused the greatest accumulation of PC, which is presumably due to the conversion of lactate to pyruvate by lactate dehydrogenase. One possible reason for lactate having a greater effect than pyruvate is discussed below. The previous growth data (Fig. 1) suggested that PC synthesis was stimulated by pyruvate. It was not known if the low level of synthesis in the presence of TCA cycle intermediates was merely due to the absence of pyruvate or reflected an inhibitory effect of these compounds on PC synthesis. When pyruvate-limited cultures were incubated in the presence of pyruvate and fumarate, succinate, or L-malate, low levels of PC, approximately those seen in the absence of pyruvate, were found (Fig. 3). This indicates an additional level of negative control by TCA cycle intermediates.

PC synthesis is induced by pyruvate and repressed by TCA cycle intermediates. Since the addition of pyruvate or lactate significantly increased the level of PC in pyruvate-limited cultures, we examined how the addition of these substrates affected the PC content of cultures grown under different conditions. The addition of pyruvate induced a rapid increase in the level of PC in cultures grown with limiting concentrations of fumarate (Fig. 4A) or other TCA cycle intermediates (data not shown). However, the stimulating effect of pyruvate was significantly reduced in cells grown with an excess of fumarate (Fig. 4A) or other TCA cycle intermediates. Analogous results were obtained with lactate (Fig. 4B), but in contrast to pyruvate, lactate addition induced PC synthesis only after a 30-min lag period, which may reflect the time required for the accumulation of sufficient pyruvate. In both cases, the increase in PC content correlated with an increase in the PC activity of cell extracts (data not shown) and was completely suppressed by the simultaneous addition of chloramphenicol (Fig. 4).

The decrease in PC content after incubation of *R. capsulatus* cells with C_4 dicarboxylic acids (Fig. 3) and the reduced induction of PC in cells grown with an excess of fumarate (Fig. 4) again suggest that the synthesis of PC is also negatively controlled by TCA cycle intermediates in *R. capsulatus*. Indeed, these substrates act similarly to chloramphenicol in that their simultaneous addition with pyruvate completely suppresses PC induction (Fig. 5). Oxaloacetate was only somewhat inhibitory, while the addition of acetate did not prevent the induction of PC synthesis by pyruvate (Fig. 5). These results indicate that in *R. capsulatus* the negative control of PC synthesis by TCA cycle intermediates supersedes the positive control by pyruvate. In fumarate-limited *R. capsulatus*, the addition of 0.2 to 5.0 mM pyruvate increased the PC content almost twofold. Higher pyruvate concentrations (8 mM and greater) caused an increase in the levels of PC which was proportional to the amount of pyruvate added (not shown). Thus, it appears that pyruvate in concentrations as low as 0.2 mM is sufficient for the induction of PC synthesis in fumarate-limited cells. Full induction of PC at low concentrations of pyruvate (0.2 to 5.0 mM) is presumably inhibited by the endogenous pool of C_4 dicarboxylates. The effect of this endogenous pool is gradually overcome by pyruvate at concentrations of 8 mM and higher. Addition of 1 to 2 mM fumarate causes a 50% inhibition of the PC synthesis induced by 50 mM pyruvate, while lower fumarate concentrations inhibit PC induction by 20 mM pyruvate (data not shown). These results suggest that the synthesis of PC in *R. capsulatus* is dependent on the intracellular ratio of pyruvate and C_4 dicarboxylates. This may explain why lactate causes a stronger stimulation than pyruvate during long-term incubations (Fig. 3). The content of PC reaches a maximum 2 h after pyruvate addition and then decreases significantly (two- to threefold), possibly due to an increase in the level of TCA cycle intermediates (results not shown). Lactate-induced PC synthesis showed a lag (Fig. 4B), and there was not a significant decrease after the maximum was reached. One hypothesis is that assimilation of exogenous lactate produces intracellular pyruvate at a concentration which is sufficient to induce PC synthesis, while the level of TCA cycle intermediates remains too low to have a negative effect. Although succinate, fumarate, and L-malate were found to be the most potent inhibitors of PC synthesis, it has not yet been possible to identify the

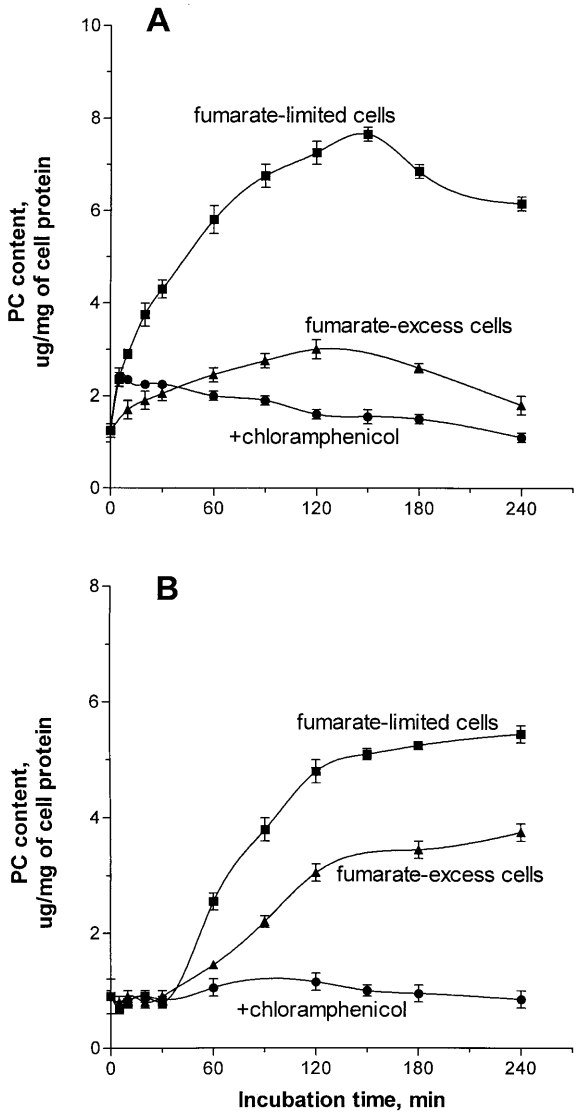

FIG. 4. Induction of PC synthesis by pyruvate (A) or lactate (B). Cells were grown overnight on RCV medium containing a limiting (5 mM; A_{660} = 1.2) or excess (30 mM; A_{660} = 5.0) fumarate concentration. At time zero, anaerobic solutions of pyruvate (50 mM final concentration) or lactate (30 mM final concentration) were added, followed by incubation at 30°C in the light. The effect of chloramphenicol (50 μg/ml, added simultaneously with the carbon substrate) was checked with fumarate-limited cells. At the times indicated, 200-μl aliquots of culture were withdrawn for the determination of culture density (A_{660}) and PC content by immunoblot analysis as described in Materials and Methods. Each point is the average of four independent measurements, with standard deviations indicated by error bars.

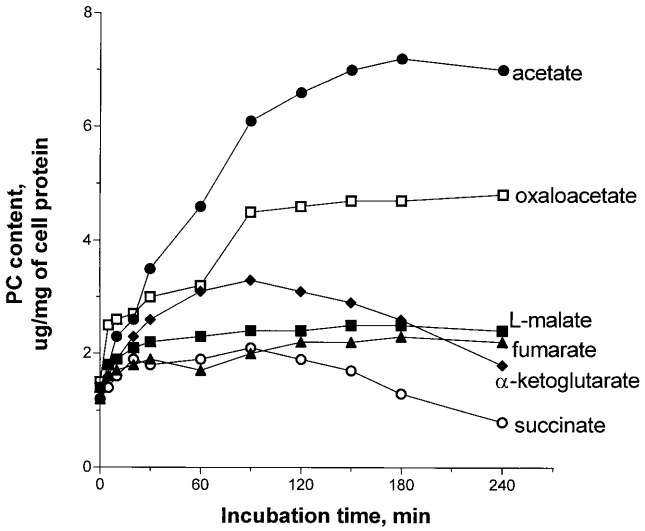

FIG. 5. Effect of different carbon substrates on pyruvate-induced PC synthesis in *R. capsulatus* cells. Cultures were grown overnight on RCV medium containing a limiting (5 mM) fumarate concentration (A_{660} = 1.2). At time zero, an anaerobic mixture of pyruvate (50 mM final concentration) and the indicated carbon substrate (30 mM final concentration) was added to the cultures, which were incubated under light anaerobic conditions (30°C). At the times indicated, 200-μl aliquots of culture were withdrawn for the determination of culture density (A_{660}) and PC content by immunoblot analysis as described in Materials and Methods. Each point represents an average from at least three replicate assays.

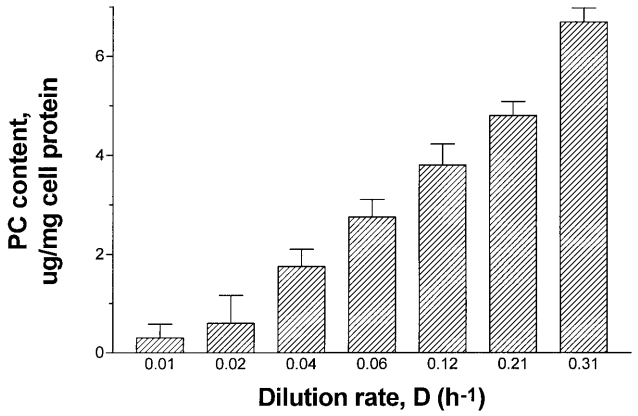

FIG. 6. PC content of *R. capsulatus* cells grown in continuous culture with lactate as a carbon source at different dilution rates (D) under turbidostat conditions (D = 0.31 h^{-1}) or under chemostat conditions with ammonium limitation (D = 0.01 to 0.21 h^{-1}). After the culture had reached steady state at a given dilution rate, aliquots were removed for quantitative immunoblot analysis of PC content as described in Materials and Methods. Results are averages from at least four independent determinations, with standard deviations indicated by error bars.

metabolite responsible for this repression. Since succinate, fumarate, and L-malate have a high degree of structural similarity (they share a common transport system encoded by *dct* locus [11, 34]), it is possible that all three compounds are effectors in the regulation of PC synthesis. Pyruvate or a closely related metabolite such as acetyl-CoA may be responsible for the induction of PC synthesis. However, the PC levels of acetate-grown cultures were very low (Fig. 1), suggesting that pyruvate, rather than acetyl-CoA, is the physiological inducer of PC synthesis in *R. capsulatus*.

Maximal levels of PC were observed in *R. capsulatus* cells grown under dark aerobic conditions with lactate as a carbon source (Fig. 1). This apparently reflects an indirect effect of O_2 on PC synthesis, since 2 h of incubation of fumarate-limited cells under aerobic conditions did not cause an increase in the cellular content of PC. In *R. capsulatus*, the operation of the TCA cycle is increased under dark aerobic conditions (2, 3). Therefore, the elevated level of PC in aerobically grown cells may be due to the lower level of TCA cycle intermediates under these conditions.

Effect of growth rate and growth phase on PC synthesis. The levels of PC in *R. capsulatus* cells grown under iron limitation were significantly reduced (Fig. 1). This appears to be an indirect effect, since no iron was detected in purified PC. A low PC content was also found in cultures grown under dark anaerobic conditions (Fig. 1). Since *R. capsulatus* cells had low growth rates under both conditions, we suspected that there was a correlation between growth rate and PC content. We chose continuous culture methodology to examine this relationship, since this method allows one to make physiological and biochemical measurements under steady-state (balanced growth) conditions at different growth rates. In preliminary experiments with lactate-grown batch cultures, we observed that nitrogen (ammonium) limitation did not affect the content of PC (data not shown). Thus, the regulatory systems important in responding to the cellular nitrogen status do not appear to play a role in regulating PC synthesis. Therefore, we determined PC levels in cells grown in continuous culture with lactate as a carbon source under turbidostat conditions (maximal growth rate) or under chemostat conditions with ammonium limitation (growth rate = dilution rate). We chose ammonium limitation instead of lactate limitation since carbon limitation, in addition to controlling culture growth, could very well affect the intracellular balance of pyruvate and C_4 dicarboxylates. Quantitative immunoblot analysis shows that the PC content was maximal in turbidostat-grown cultures and that it was directly proportional to the growth rate in chemostat cultures (Fig. 6). Similarly, shifts from turbidostat to chemostat conditions and vice versa resulted in the decrease or increase, respectively, of cellular levels of PC (data not shown).

In lactate-grown batch cultures of *R. capsulatus*, the level of PC also varied during the growth cycle, reaching a maximum during the early exponential phase of growth and then progressively declining (Fig. 7). Similarly, the highest PC specific activity was observed in extracts prepared from exponentially grown cells of *A. vinelandii* and *P. citronellolis* (33, 37). It can be suggested that this growth phase dependence of the levels of PC is also based on the growth rate control of PC synthesis. This is supported by the observation that the maximal content of PC in lactate-grown batch cultures (exponential growth phase) (Fig. 7) is close to that in a turbidostat culture (Fig. 6) (D = 0.31 h^{-1}). The higher level of PC found for the stationary phase of batch cultures compared to the low-growth-rate chemostat cultures may be due to a relatively low rate of PC turnover. Thus, in *R. capsulatus* the synthesis of PC appears to be under growth rate control. In *Escherichia coli* the synthesis of another biotin-containing enzyme, acetyl-CoA carboxylase, has been found to be directly correlated with growth rate (19), while the levels of several enzymes of TCA cycle varied inversely with growth rate (27–29). Relatively little is known about the mechanism responsible for growth rate control, but the increased level of PC in faster-growing *R. capsulatus* cells may reflect the reduced level of TCA cycle intermediates under these conditions.

Intracellular levels of pyruvate and TCA cycle intermediates under different growth conditions. The results presented here

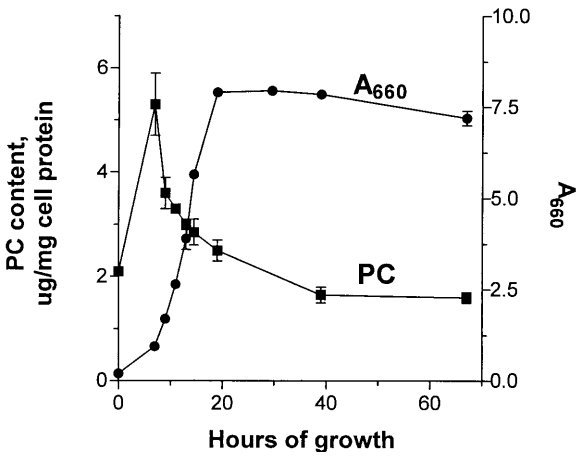

FIG. 7. PC content of *R. capsulatus* cultures during batch growth under light anaerobic conditions with lactate as a carbon source. Cells were grown on RCV medium containing 30 mM lactate. At the times indicated, 200-μl aliquots were withdrawn for the determination of culture density (A_{660}) and PC content as described in Materials and Methods. Results are averages from at least four independent determinations, with standard deviations indicated by error bars.

suggest that PC synthesis in *R. capsulatus* responds to the intracellular ratio between pyruvate and C_4 dicarboxylates. To examine this directly in more detail, we determined the contents of some organic acids in cells grown under different conditions (Table 1). It can be seen that the content of individual C_4 dicarboxylates (especially fumarate) varied over a 300- to 1,000-fold range, while the variation in pyruvate levels was much less dramatic (5-fold variation). This corresponds, using an internal water space of 5 μl per mg of protein (10), to 0.14 to 0.7 mM pyruvate and 0.02 to 6.12 mM L-malate. Previous studies with glucose-grown cultures of *E. coli* have shown the same magnitudes of concentrations of pyruvate and malate (21). Succinate levels in butyrate-grown cells of *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, and *R. capsulatus* that are similar to those found here for acetate-grown cells (Table 1) have been reported (17).

The data presented in Table 1 show that the PC content is directly proportional to the level of pyruvate and inversely proportional to the levels of succinate, fumarate, and L-malate. Incubation with any of these C_4 dicarboxylates has a similar effect on PC synthesis (Fig. 3 and 5). Therefore, for purposes of discussion, we will relate the synthesis of PC to the ratio between the level of pyruvate and the sum of the levels of succinate, fumarate, and L-malate (P/C_4 ratio). Thus, the low PC content of cells grown on acetate, glutamate, α-ketoglutarate, succinate, fumarate, or L-malate correlates well with the low P/C_4 ratio (0.01 to 0.09) found with these cultures. Conversely, lactate-grown cells (exponential growth phase, light anaerobic conditions) have a much higher P/C_4 ratio (2.24) and a significantly higher content of PC. Aerobically grown cultures showed increased levels of pyruvate, citrate, and α-ketoglutarate (to be expected due to increased activity of the TCA cycle under aerobic growth conditions) and correspondingly higher levels of PC (Table 1; Fig. 1). In the stationary phase of growth, lactate-grown cells have a decreased pool of pyruvate and increased contents of fumarate and L-malate. At the same time, PC levels are decreased. Thus, in general, these results demonstrate that under different growth conditions, cellular PC levels are directly correlated with the P/C_4 ratio. Moreover, regulation of PC synthesis by oxygen and growth rate may be due to the effects of these factors on the P/C_4 ratio.

Conclusion. Taken together, the results reported here show that in *R. capsulatus* the synthesis of PC is (i) induced by pyruvate, (ii) repressed by TCA cycle intermediates, (iii) stimulated by aerobiosis, and (iv) directly correlated with growth rate. The synthesis of this enzyme appears to be controlled by both positive (pyruvate) and negative (C_4 dicarboxylates) effectors. The necessity for a dual regulation of PC synthesis with the dominance of negative control is understandable in terms of the metabolic versatility of *R. capsulatus*. Positive control (induction by pyruvate) is essential, since there is no necessity for PC synthesis in the presence of carbon substrates which can be assimilated without the intermediate formation of pyruvate (C_4 dicarboxylates, many amino acids, acetate, butyrate, and propionate). Negative control (repression by TCA cycle intermediates) is essential, since this anaplerotic pathway does not require PC activity (synthesis) when the concentration of C_4 dicarboxylates is high (even if pyruvate is abundant). The presence of two apparently antagonistic regulatory systems controlling the synthesis of PC ensures that the content of PC is commensurate with metabolic demand. Regulation of PC synthesis seems to respond to the balance between the intracellular pools of pyruvate and C_4 dicarboxylates (P/C_4 ratio). Thus, in *R. capsulatus*, PC is potentially subject to two levels of control; allosteric regulation of enzyme activity, as previously demonstrated (24), and, as we show here, regulation of protein synthesis. Previously, ADP, L-asparate, α-ketoglutarate, and acetyl-CoA have been identified as potential allosteric effec-

TABLE 1. Contents of different organic acids in *R. capsulatus* cells grown under various conditions[a]

Carbon source and growth conditions	μmol/mg of cell protein (mean ± SD)						P/C_4[c]
	Pyruvate	Citrate	α-Ketoglutarate	Succinate	Fumarate	L-Malate	
Acetate, light anaerobic	0.71 ± 0.05	1.79 ± 0.15	1.77 ± 0.22	1.47 ± 0.2	1.02 ± 0.32	11.02 ± 2.0	0.05
Glutamate, light anaerobic	3.02 ± 0.57	5.34 ± 1.24	7.1 ± 2.1	44.4 ± 5.0	0.97 ± 0.29	5.2 ± 0.5	0.06
α-Ketoglutarate, light anaerobic	1.63 ± 0.13	<0.2	246.7 ± 13.6	42.2 ± 4.9	1.80 ± 0.14	3.41 ± 0.33	0.04
Succinate, light anaerobic	3.78 ± 0.3	12.27 ± 0.65	3.75 ± 0.1	266 ± 7.6	12.08 ± 0.3	30.59 ± 6.1	0.01
Fumarate, light anaerobic	2.1 ± 0.1	7.34 ± 0.1	5.04 ± 0.1	<0.2	21.43 ± 1.0	36.79 ± 7.3	0.04
L-Malate, light anaerobic	4.17 ± 0.4	4.63 ± 0.8	4.55 ± 0.5	35.85 ± 6.5	3.17 ± 0.17	7.63 ± 1.3	0.09
Lactate, light anaerobic	3.42 ± 0.72	3.47 ± 0.57	2.41 ± 0.59	<0.2	0.01 ± 0.01	1.33 ± 0.23	2.24
Lactate, dark aerobic	4.7 ± 0.28	13.43 ± 0.83	8.76 ± 0.44	<0.2	0.05 ± 0.01	<0.1	13.43
Lactate, light anaerobic (old cells)[b]	1.15 ± 0.03	6.54 ± 0.4	2.76 ± 0.03	<0.2	0.13 ± 0.02	9.48 ± 0.7	0.12

[a] Samples were prepared from duplicate, independently grown cultures and assayed as described in Materials and Methods.
[b] Late stationary growth phase (48 h of growth).
[c] Ratio of the level of pyruvate to the sum of the levels of succinate, fumarate, and L-malate. The calculated ratio represents a minimum value for those conditions under which the concentration of an organic acid was below the limit of detection.

tors. Additionally, PC may be subject to some type of control via covalent modification. Unfortunately, since there is no available direct method of measuring PC activity in situ, it is presently impossible to assess the relative importance of these potential regulatory mechanisms in the control of this metabolically important enzyme.

In *R. capsulatus* the synthesis of isocitrate lyase, the key enzyme of the glyoxylate shunt, is suppressed by TCA cycle intermediates (malate and succinate) and stimulated by acetate or butyrate (5, 15, 26). Interestingly, pyruvate prevents the induction of isocitrate lyase synthesis by acetate (5), which implies that the PC-dependent anaplerotic pathway is dominant over the glyoxylate shunt. Similarly, the activity and/or synthesis of propionyl-CoA carboxylase, which is responsible for the assimilation of propionate, was repressed in *Rhodospirillum rubrum* cells grown on a malate-containing medium (14). Therefore, repression by TCA cycle intermediates and induction by carbon substrates may represent general mechanisms of regulation of the biosynthesis of enzymes involved in anaplerotic pathways in *R. capsulatus* and possibly in other bacteria as well.

In *E. coli*, the expression of L-lactate dehydrogenase and a number of enzymes of the TCA cycle (citrate synthase, succinate dehydrogenase, and malate dehydrogenase) is elevated under aerobic conditions and also varies depending on the type of carbon substrate and the growth rate (12, 27–29). The synthesis of these enzymes, as well as those involved in the glyoxylate shunt, is under the control of several global transcriptional regulators (Fnr, ArcA, FadR, IclR, and FruR) which sense the intracellular levels of some cellular metabolites (4, 12, 22, 23, 27, 29, 32). Our results suggest that a similar regulatory mechanism(s) which monitors the ratio of intracellular concentrations of pyruvate and C_4 dicarboxylates may be involved in the control of PC synthesis in *R. capsulatus*. Recently, RegB-RegA, a global two-component regulatory system, was identified in *R. capsulatus* and *R. sphaeroides* and shown to be involved in the control of carbon (CO_2) metabolism and photosystem biosynthesis (25, 31). However, this regulatory system does not appear to be involved in the regulation of PC synthesis, since a *regB* mutant showed wild-type levels of PC protein when grown under light anaerobic conditions (47a). Obviously, further work is required to identify the molecular mechanisms of transcriptional regulation controlling the synthesis of PC in *R. capsulatus.*

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