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Pyruvate Carboxylase from *Rhizobium etli*: Mutant Characterization, Nucleotide Sequence, and Physiological Role

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Pyruvate carboxylase (PYC), a biotin-dependent enzyme which catalyzes the conversion of pyruvate to oxaloacetate, was hypothesized to play an important anaplerotic role in the growth of *Rhizobium etli* during serial subcultivation in minimal media containing succinate (S. Encarnación, M. Dunn, K. Willms, and J. Mora, J. Bacteriol. 177:3058–3066, 1995). *R. etli* and *R. tropici pyc*::Tn*5*-mob mutants were selected for their inability to grow in minimal medium with pyruvate as a sole carbon source. During serial subcultivation in minimal medium containing 30 mM succinate, the *R. etli* parent and *pyc* mutant strains exhibited similar decreases in growth rate with each subculture. Supplementation of the medium with biotin prevented the growth decrease of the parent but not the mutant strain, indicating that PYC was necessary for the growth of *R. etli* under these conditions. The *R. tropici pyc* mutant grew normally in subcultures regardless of biotin supplementation. The symbiotic phenotypes of the *pyc* mutants from both species were similar to those of the parent strains. The *R. etli pyc* was cloned, sequenced, and found to encode a 126-kDa protein of 1,154 amino acids. The deduced amino acid sequence is highly homologous to other PYC sequences, and the catalytic domains involved in carboxylation, pyruvate binding, and biotinylation are conserved. The sequence and biochemical data show that the *R. etli* PYC is a member of the α_4 homotetrameric, acetyl coenzyme A-activated class of PYCs.

The biotin-dependent enzyme pyruvate carboxylase (PYC) (pyruvate:CO_2 ligase [ADP forming] [EC 6.4.1.1]) plays an important anaplerotic role in many organisms by catalyzing the carboxylation of pyruvate to form oxaloacetate (OAA), which is used to replenish tricarboxylic acid cycle intermediates used for energy generation and biosynthesis (for reviews, see references 4 and 54). In bacteria, anaplerotic CO_2 fixation is critical for the maintenance of the OAA pool during growth on compounds that are utilized via pyruvate (1, 28). Although PYC fulfills this function in many prokaryotes (1, 13, 46, 50, 68), enteric bacteria as well as some other groups convert phosphoenolpyruvate (PEP) to OAA in the reaction catalyzed by PEP carboxylase (PPC) (10, 37). These two pathways for OAA synthesis are not mutually exclusive, since some bacteria produce both PYC and PPC (18, 28, 38, 45, 55).

In eukaryotes and some prokaryotes (e.g., a thermophilic *Bacillus* strain [33] and *Rhodobacter capsulatus* [39]), the PYC holoenzyme is an α_4 homotetramer composed of 113- to 130-kDa subunits (4). Each subunit polypeptide contains three domains that work in concert to catalyze the overall reaction, which occurs as the sum of the two half-reactions shown below, where ENZ represents the enzyme.

$$\mathrm{MgATP} + \mathrm{HCO_3^-} + \mathrm{ENZ\text{-}biotin} \xrightleftharpoons{\mathrm{Mg^{2+}},\ \text{acetyl-CoA}} \mathrm{MgADP} + \mathrm{P_i} + \mathrm{ENZ\text{-}biotin\text{-}CO_2^-} \quad (1)$$

$$\mathrm{ENZ\text{-}biotin\text{-}CO_2^-} + \text{pyruvate} \rightleftharpoons \mathrm{ENZ\text{-}biotin} + \mathrm{OAA} \quad (2)$$

In reaction 1, the ATP-dependent biotin carboxylase domain carboxylates a biotin prosthetic group linked to a specific lysine residue in the biotin carboxyl carrier protein (BCCP) domain. Acetyl coenzyme A (acetyl-CoA) activates reaction 1 by increasing the rate of bicarbonate-dependent ATP cleavage. In reaction 2, the BCCP domain donates the CO_2 to pyruvate in a reaction catalyzed by the transcarboxylase domain (4).

The PYC holoenzymes from *Pseudomonas citronellolis* and *Azotobacter vinelandii* are exceptional in having an $\alpha_4\beta_4$ structure in which the α subunit (65 kDa) contains the catalytic domains (12, 26, 55). In contrast to the α_4 PYCs, all of which require or are activated by acetyl-CoA (33, 39, 42), the $\alpha_4\beta_4$ PYCs are fully active in the absence of this effector (12, 56). Although bacterial PYCs from both structural classes have been studied at the biochemical and physiological levels (12, 13, 26, 38, 39, 49, 55, 60), little genetic characterization has been done and only one nucleotide sequence for a putative prokaryotic PYC has been obtained (57).

Previous results from this laboratory led us to hypothesize that PYC plays an important role in determining if strains of *Rhizobium etli* and *R. tropici* display an aerobic or fermentative metabolism in vitro (17, 18). We showed that *R. etli* CE3 develops a fermentative metabolism, with a concomitant decrease in growth rate, during serial subcultivation in minimal medium (MM) containing succinate as a sole carbon source. The fermentative metabolism was prevented when the medium was supplemented with biotin or thiamine. The presence of either of these vitamins increased the activities of several carbon-metabolic enzymes, most notably PYC and pyruvate and oxoglutarate dehydrogenases, during subcultivation. Although the activities of all three enzymes were markedly increased with either supplement, biotin stimulated PYC to a greater

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
R. etli strains		
CE3	Derivative of wild-type strain CFN42, Sm^r Nal^r Suc^+ Glc^+ Pyr^+	7
12-53	CE3 *pyc*::Tn*5*-mob, Sm^r Nal^r Km^r Suc^+ $Glc^{\pm}$ Pyr^-	This study
R. tropici strains		
CFN299	Wild-type strain, Sp^r Nal^r Suc^+ Glc^+ Pyr^+	36
2-176	CFN299 *pyc*::Tn*5*-mob, Sp^r Nal^r Km^r Suc^+ $Glc^{\pm}$ Pyr^-	This study
E. coli strains		
S17-1	294 *recA*; mobilizing strain for Tn*5*	56
DH5α	*recA1 lacU169* ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories
HB101	F^- *recA13*; host for *R. etli* pLAFR1 genomic library	Laboratory stock
Plasmids		
pLAFR1	Broad-host-range cloning vector, Tc^r	21
pSUP5011	pBR233 Tn*5*-mob, Ap^r Cm^r Km^r	56
pRK2013	Helper plasmid, Km^r	20
pBluescript II SK (+)	Cloning vector, Ap^r	Stratagene
pHPD5g	pBluescript containing the Tn*5*-mob insertion and flanking regions from strain 12-53	This study
pDG1	pBluescript with an *Eco*RI fragment containing the Tn*5*-mob insertion from strain 12-53	This study
pDG1-2	*Eco*RI-*Hin*dIII subclone of pDG1	This study
p2-176	pBluescript with an *Eco*RI fragment containing the Tn*5*-mob insertion from strain 2-176	This study
p2-1	*Eco*RI-*Hin*dIII subclone of p2-176	This study
pPC	pLAFR1 harboring 15.2 kb of *R. etli* genomic DNA containing *pyc*; Tc^r	This study
pPC1	*Eco*RI deletion subclone of pPC containing *R. etli pyc*	This study

[a] Suc, succinate; Glc, glucose; Pyr, pyruvate; +, normal growth; ±, reduced growth; −, no growth.

degree than it did the dehydrogenases, whereas thiamine had the opposite effect. In contrast to *R. etli*, *R. tropici* CFN299 maintained high levels of these activities during subcultivation in unsupplemented MM and did not develop a fermentative metabolism (18). We chose to further study the effect of biotin supplementation on the growth of *R. etli* and to test our hypothesis that PYC was the major site at which biotin acts in promoting an aerobic metabolism in this species (15, 18). A corollary to this hypothesis is that *R. tropici* might maintain its growth in unsupplemented medium because it does not lose PYC activity under these conditions. Our approach, as reported here, was to inactivate the *pyc* gene in *R. etli* and *R. tropici* and characterize the free-living and symbiotic phenotypes of the mutants. To begin a comparative genetic and biochemical analysis of the PYCs produced by these species, we cloned and sequenced the *R. etli pyc* and also report some characteristics of the enzyme.

(Preliminary reports of part of this work were presented previously [15, 17].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Rhizobia were grown either in PY rich medium (8) or in MM (5) containing a carbon source and filter-sterilized biotin as indicated for each experiment. To prepare MM plates, carbon sources were added to a final concentration of 20 mM and media were solidified with 1.5% purified agar (Merck, Darmstadt, Germany). For the growth of *Rhizobium* strains in batch cultures, cells were prepared and media were inoculated and incubated as described previously (18). To culture cells in the presence of radiolabeled biotin, MM-succinate (10 mM) containing 1 ng of unlabeled biotin plus 0.05 μCi (0.28 ng) d-[8,9-^{3}H]biotin per ml was inoculated and incubated for 18 h as described previously (18) except that 1 liter of medium in a 2.8-liter flask was used. Strains of *Escherichia coli* were grown in LB medium (51). Cultures of *Rhizobium* spp. or *E. coli* were supplemented with antibiotics at the following concentrations (in micrograms per milliliter): spectinomycin, 200; streptomycin, 200; nalidixic acid, 20; tetracycline, 10; kanamycin, 30; and carbenicillin, 100.

Mutagenesis of *Rhizobium* spp. and selection of *pyc* mutants. Cells were mutagenized by a standard biparental mating with *E. coli* S17-1/pSUP5011 as the donor strain. Clones containing Tn*5*-mob insertions were selected by spreading dilutions of 24-h matings onto PY medium containing spectinomycin or streptomycin (for strains CFN299 or CE3, respectively), nalidixic acid, and kanamycin. After incubation for 3 days at 30°C, colonies were patched onto MM containing succinate or pyruvate as a sole carbon source. Clones able to grow on succinate but unable to grow on pyruvate were selected and further characterized.

Isolation of the *R. etli pyc* gene. A genomic DNA bank (Table 1) prepared from *R. etli* CE3 was mobilized to the *R. tropici pyc* mutant 2-176 (Table 1), using pRK2013 as a helper strain. Mating mixtures were incubated on PY medium for 24 h at 30°C, resuspended in 0.85% NaCl, and spread onto MM with glucose or pyruvate as a sole carbon source and containing spectinomycin, nalidixic acid, and tetracycline. One colony appearing on a MM-glucose plate after 3 days of incubation was purified by restreaking and used for further studies. The complementing plasmid isolated from this clone was designated pPC (Table 1).

DNA manipulations. Routine DNA manipulations were performed by standard techniques (51). Southern blot hybridizations to detect transposon insertions were performed with ^{32}P-labeled Tn*5*-mob prepared by using a Megaprime kit (Amersham International) and were performed as described previously (51). Indigenous plasmids from the *pyc* mutants were isolated and electrophoretically separated by the Eckhardt procedure (16). The DNA fragment containing the Tn*5*-mob insertion in *R. tropici* 2-176 (Table 1) was isolated by completely digesting genomic DNA with *Eco*RI, ligating the fragments into pBluescript, and selecting carbenicillin- and kanamycin-resistant transformants of *E. coli* DH5α. The plasmid obtained was designated p2-176. The Tn*5*-mob insertion from *R. etli* 12-53 was cloned by a similar strategy except that partial *Eco*RI digests of the genomic DNA were used so that the insertion and several contiguous *Eco*RI fragments were cloned. This plasmid was designated pHPD5g. The *Eco*RI fragment containing the insertion was subcloned from pHPD5g and designated pDG1 (Table 1). DNA flanking either end of the Tn*5*-mob insertions in p2-176 and pDG1 were subcloned as *Hin*dIII-*Eco*RI fragments (where the *Hin*dIII site is within the transposon and the *Eco*RI site is within the genomic DNA) to obtain plasmids pDG1-2 and p2-1, respectively (Table 1). The nucleotide sequence of 100 bp near the Tn*5*-mob insertion subcloned from *R. tropici* 2-176 (plasmid p2-1) was determined by using an *Eco*RI primer and was 91% identical to a segment of the cloned *R. etli pyc* sequence. By correlating this information with the distance of the sequenced portion from the Tn*5*-mob insertion in p2-1, the probable site of the insertion was determined. This approximation is based on the assumption that the *R. tropici pyc* is similar in size (see Discussion) and sequence to the *R. etli pyc*. The Tn*5*-mob insertion site in *R. etli* 12-53 was localized by sequencing the DNA from pDG1-2 with a primer specific for the *Xho*I site located near the border of the transposon. The DNA sequencing described above was performed by the Sanger dideoxy method (53). Double-stranded sequencing of the *R. etli pyc* was performed by Medigene GmbH, Munich, Germany, using an Applied Biosystems (Foster City, Calif.) 373A DNA sequencing system. Nucleotide and protein sequence homology searches were made by using the BLAST program (2) via the National Institute for Biotech-

TABLE 2. Effect of culture carbon source and biotin supplementation on PYC activity in *R. etli* CE3[a]

Carbon source	Culture biotin concn (μg/ml)	PYC activity (nmol/min/mg of protein)
Succinate	0	6.3 ± 0.5 (1.0)
	0.00005	5.2 ± 1.5 (0.83)
	0.00025	24.2 ± 6.5 (3.8)
	0.05	28.2 ± 4.7 (4.5)
	0.5	27.3 ± 2.8 (4.3)
	1.0	26.5 ± 2.5 (4.2)
Glucose	0	10.5 ± 1.6 (1.0)
	1.0	28.9 ± 2.3 (2.8)
Pyruvate	0	16.9 ± 6.3 (1.0)
	1.0	42.4 ± 2.5 (2.5)

[a] Cells were grown for 16 h in MM containing the indicated carbon source at 10 mM with or without biotin supplementation as indicated. Activities are the means ± standard errors for values obtained in two to three separate experiments. For each carbon source, values in parentheses are normalized to the activity of cells cultured without added biotin, set at unity.

nology Information server. Multiple protein sequence alignments were made with Clustal W version 1.5 (63) at the Baylor College of Medicine-Human Genome Center server.

Enzyme and protein assays. For enzyme activity screening, cells grown for 3 days on PY plates containing the appropriate antibiotics were washed twice in sterile PY and used to inoculate 100 ml of PY medium to an initial A_{540} of 0.05. Cultures were incubated at 30°C for 16 h with shaking at 200 rpm. The late-log-phase cells were harvested by centrifugation (9,800 × *g*, 10 min) and washed twice in 20 mM potassium phosphate buffer (pH 7.2) containing 0.85% NaCl. The cells were resuspended in 5 ml of cell lysis buffer (20 mM potassium phosphate [pH 7.2], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.75% glycerol) and lysed by sonication as described previously (18). To prepare protein extracts from cells grown in the presence of [^{3}H]biotin or for the partial purification of PYC, cells were harvested and washed as described above, resuspended in 10 ml of cell lysis buffer per g (fresh weight), and sonicated in 10 1-min bursts using a MSE sonicator (Dalon Scientific Ltd., London, United Kingdom) with a 1-cm probe at 75% of maximum power, with cooling of the sample to 5°C between bursts. Cell-free supernatants were obtained by centrifugation at 90,000 × *g* for 1.5 h at 4°C. Enzyme assays were performed as described previously (18) except that PYC was assayed in the presence of 20 μM acetyl-CoA. Protein contents of culture biomass and cell lysates were determined as described previously (18).

Partial purification of PYC from *R. etli*. Batch cultures of strain CE3/pPC1 (Table 1) were grown for 16 h in a total of 3 liters of MM-pyruvate (30 mM) containing biotin (1 μg/ml) and tetracycline. Cell extracts were prepared as described above, and proteins were precipitated at 4°C by the addition of solid $(NH_4)_2SO_4$. Following equilibration for 20 min at each final salt concentration, precipitates were collected by centrifugation at 10,000 × *g* for 10 min. The precipitate from the 40 to 60% of saturation fraction contained the majority of the PYC activity and was stored overnight at −80°C. The protein was redissolved in a small volume of equilibration buffer (EQB; 20 mM potassium phosphate [pH 8.0], 5 mM $MgSO_4$, 20 mM KCl, 5% glycerol, 1 mM dithiothreitol, 1 mM EDTA) and desalted on a Sephadex G-25 column equilibrated in EQB. The desalted sample was chromatographed on a DEAE-cellulose column (1 by 14 cm) equilibrated in EQB and eluted at 1.5 ml/min. Following the elution of nonbinding proteins, the column was eluted with a linear 20 to 250 mM gradient of KCl in EQB over 134 min. Fractions containing PYC activity were pooled and precipitated with $(NH_4)_2SO_4$ to 60% saturation. The preparation was centrifuged and the pellet was stored as described above. The protein pellet was redissolved in a small volume of 1 mM NaCl (unbuffered) and desalted on a G-25 column equilibrated in the same buffer. The desalted preparation was loaded onto a column (0.7 by 8 cm) of Bio-Gel HTP hydroxylapatite (Bio-Rad Corporation, Hercules, Calif.) equilibrated in the same buffer. The column was washed with about 2 column volumes of 1 mM NaCl, and bound proteins were eluted with a 50-ml linear gradient of 0 to 0.3 M potassium phosphate at 0.5 ml/min. Active fractions were pooled, precipitated, and stored as described above. Before use in enzyme assays, the precipitate was redissolved in a minimal amount of cell lysis buffer and dialyzed against the same buffer. All purification steps were carried out at 4°C.

Detection of biotin-containing proteins by Western blotting (immunoblotting) and [^{3}H]biotin labeling. Proteins in cell extracts were separated in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (23) and electroblotted onto nitrocellulose for 1 h at 300 mA as described by Towbin et al. (65). Biotin-containing proteins on the blots were detected by using a Sigma-Blot protein detection kit (Sigma Chemical Corp., St. Louis, Mo.) as instructed by the manufacturer but omitting the protein biotinylation step so that only proteins naturally containing biotin would bind the streptavidin-horseradish peroxidase conjugate. Blots were imaged with a pdi Desktop scanner and Diversity One software (pdi Inc., Huntington Station, N.Y.). Protein bands were manually contoured prior to quantitation. Radioactivity in biotin-containing proteins prepared from cultures grown in the presence of [^{3}H]biotin was quantitated by excising the bands, dissolving them in 5 ml of scintillation fluid (9), and counting in a scintillation counter. Portions of membrane excised from areas in the lanes not containing biotinylated proteins were included as background checks. Specific radioactivity was calculated by dividing the counts per minute obtained for a band by its contour quantity (units of optical density [OD] multiplied by area [square millimeters]) obtained by scanning densitometry.

Nodulation and nitrogen fixation assays. Seeds of *Phaseolus vulgaris* cv. Negro jamapa were surface disinfected and germinated as described previously (8). Cultures of the *pyc* mutants and their respective parent strains were grown to saturation overnight in PY media containing the appropriate antibiotics (Table 1), washed once in 0.85% NaCl, and resuspended to an OD at 540 nm of 0.05. One milliliter of this suspension was used to inoculate 3-day-old seedlings, and plants were maintained aseptically under greenhouse conditions. At 12, 15, 18, 25, 32, 40, and 55 days postinoculation, plants were harvested and analyzed for nodule number and dry weight, plant dry weight, and acetylene reduction activity as described previously (11). Seed yields were determined at 116 days postinoculation. Nodule microsymbiont occupancy was determined at all time points by plating nodule homogenates on PY medium with or without the antibiotics (Table 1) required to differentiate each strain.

Nucleotide sequence accession number. The nucleotide sequence data presented in this report are available under GenBank accession number U51439.

RESULTS

Physiological and biochemical characterization of the PYC from *R. etli* CE3. Because PYC activity in strain CE3 is significantly affected by growth conditions (17, 18), we examined the effects of culture carbon source and biotin supplementation on the production of PYC in this strain (Table 2). In cells cultured in MM without added biotin, PYC activities were 2.7-fold higher in pyruvate than in succinate, while glucose-grown cells had an intermediate level of activity. Supplementation of the media with 1 μg of biotin per ml increased PYC activities, relative to unsupplemented media, 4.2-fold in succinate, 2.8-fold in glucose, and 2.5-fold in pyruvate. In cells grown in MM-succinate, a biotin concentration of 0.25 ng/ml was sufficient to induce PYC activity to near-maximal levels (Table 2).

For use as a standard on Western blots, PYC was partially purified from *R. etli* CE3 by ion-exchange chromatography (14). On Western blots developed to detect biotinylated proteins, this preparation gave an intense band which migrated with an apparent molecular weight (MW) of 120,000 (Fig. 1). In blots of extracts prepared from cells grown in MM-succinate

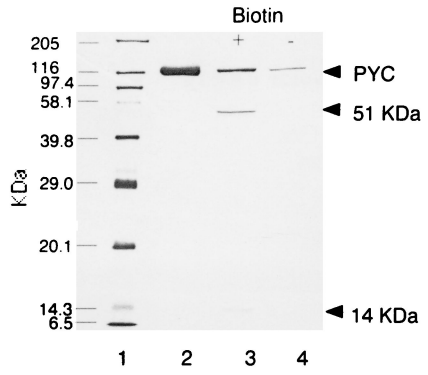

FIG. 1. Effect of culture medium biotin supplementation on the production of biotin-containing proteins in *R. etli* CE3. Biotin-containing proteins were detected in the Western blot as described in Materials and Methods. Lanes: 1, biotinylated protein standards with molecular masses as indicated; 2, partially purified *R. etli* PYC; 3, extract from cells cultured in MM-succinate (10 mM) containing 1 μg of biotin per ml; 4, extract from cells cultured in MM-succinate (10 mM) without added biotin. Lanes 3 and 4 were each loaded with 110 μg of total protein.

TABLE 3. Effect of assay reaction mixture composition on the activity of PYC from *R. etli*

Reaction mixture[a]	Activity (mU)[b]	Relative rate
Standard	43.22	1.00
Standard − pyruvate	0.48	0.01
Standard − ATP	1.05	0.02
Standard − HCO_3^-	1.84	0.04
Standard − $MgCl_2$	NA[c]	0.00
Standard − acetyl-CoA	1.72	0.04
Standard + 2 μM acetyl-CoA	2.75	0.06
Standard + 5 μM acetyl-CoA	14.59	0.34
Standard + 20 μM acetyl-CoA[d]	44.49	1.03
Standard + 50 μM acetyl-CoA	43.33	1.00
Standard + 200 μM acetyl-CoA	44.25	1.02
Standard + 1 mM L-Asp	34.73	0.80
Standard + 5 mM L-Asp	4.22	0.10
Standard + 10 mM L-Asp	2.16	0.05
Standard + 10 mM 2-OG	45.00	1.04
Standard + 10 mM L-Glu	45.72	1.06

[a] The standard reaction mixture contained (per milliliter) *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), 100 mM; $MgCl_2$, 10 mM; ATP, 2.5 mM; $NaHCO_3$, 20 mM; NADH, 0.2 mM; acetyl-CoA, 20 μM; malate dehydrogenase; 0.5 U; and pyruvate, 10 mM. Substrates and amino or organic acid stocks used in the reactions were prepared in 50 mM HEPES buffer (pH 7.0).

[b] One unit of activity catalyzes the reduction of 1 nmol of NADH per min. The specific activity of the enzyme preparation used in all assays was 2,470 nmol/min/mg of protein.

[c] NA, no activity detected.

[d] Note that this reaction mixture is identical to that used in the standard assay.

without added biotin, PYC accounted for greater than 99% of the total biotin-containing proteins detected; a 51-kDa protein (see below) comprised the remainder of the biotinylated protein in the sample (Fig. 1). In extracts obtained from cells cultured in the presence of biotin, the quantity of PYC protein increased 5.3-fold (Fig. 1), in fairly close agreement with the 4.2-fold increase in enzymatic activity observed under the same conditions (Table 2). Two additional biotin-containing proteins with MWs of 51,000 and 14,000 were induced to easily detectable levels when cells were grown in biotin-supplemented medium (Fig. 1). By scanning densitometry, PYC and the 51- and 14-kDa proteins accounted for 79, 19.4, and 1.6%, respectively, of the total biotinylated protein in the sample.

When strain CE3 was grown in MM-succinate supplemented with [^{3}H] biotin, the quantity of biotin in the media was reduced 93% after 18 h of growth. On Western blots prepared from these cultures, specific radioactivities for PYC and the 51- and 14-kDa bands were in the range of 800 to 6,000 cpm/OD × mm^2, while background radioactivity in sample blanks, prepared as described in Materials and Methods, were less than 1% of these values.

Holo-PYC was overproduced when strain CE3 containing plasmid-borne copies of the *R. etli pyc* (pPC1; Table 1) was grown in biotin-containing media. The enzyme was purified 12-fold to a specific activity of 2,470 nmol/min/mg of protein. The activity of the enzyme was substantially dependent on the presence of substrates (pyruvate, MgATP, and bicarbonate) in the reaction mixture. In the absence of acetyl-CoA, the enzyme retained 4% of its activity relative to that measured in the standard assay (Table 3). From these data, we calculate that the acetyl-CoA concentration necessary for half-maximal activation of the enzyme is 8.8 μM. The enzyme was inhibited 50% by 2.8 mM L-aspartate but was not inhibited by 2-oxoglutarate or L-glutamate (Table 3). When CE3/pPC1 was grown in MM-succinate lacking biotin, virtually all of the PYC produced was present in the apo form (14).

Genetic and phenotypic characterization of the *Rhizobium pyc* mutants. Putative *pyc* mutants were selected from populations of Tn*5*-mob-containing clones on the basis of their inability to grow on solid MM-pyruvate. Enzyme assays showed that two clones (of 607 screened) with this phenotype isolated from *R. tropici* CFN299 were both PYC negative, and one, strain 2-176 (Table 1), was used in further studies. For *R. etli* CE3, 6,500 clones were screened and 3 having a pyruvate-negative phenotype were isolated. Of these three clones, only strain 12-53 (Table 1) lacked PYC activity.

Hybridization of a Tn*5*-mob probe against *Eco*RI fragments of genomic DNA prepared from the *pyc* mutants and the parent strains showed that each mutant contained a single Tn*5*-mob insertion, and no hybridization was observed against DNA isolated from the parent strains. The hybridizing band for mutant 12-53 was approximately 8.5 kb, while that for mutant 2-176 was 8.0 kb. By correlating the size of the hybridizing fragment from *R. etli* 12-53 with the restriction enzyme map of pHPD5g (Table 1) (47), we found that the DNA surrounding the insertion had the same restriction sites as the cloned *R. etli pyc* (47) and that the insertion site was in a fragment corresponding to the 0.86-kb *Eco*RI fragment of pPC (see Fig. 6a). When indigenous plasmids from the *pyc* mutants were separated on Eckhardt gels and hybridized with Tn*5*-mob, no hybridization was observed. As a positive control, plasmids isolated from an *R. etli* mutant with a Tn*5*-mob insertion in the b plasmid (61) were run in the same gel and gave a strong hybridization signal (19).

The Tn*5*-mob-containing DNA fragments cloned from both mutants were analyzed by partial sequencing. The insertion site in *R. tropici* 2-176 was approximated as described in Materials and Methods and is estimated to occur approximately midway in the *pyc* open reading frame (15). The insertion site in *R. etli* 12-53 is very near the 5′ end of the gene (following nucleotide 117) and is in the same *Eco*RI fragment predicted by hybridization and restriction enzyme mapping.

Growth curves for the mutant and parent strains in MM-pyruvate (10 mM) show that the mutants of either species were unable to grow on this carbon source except when it was supplemented with 1 mM L-aspartate (Fig. 2A). In MM-glucose, the *R. etli* and *R. tropici pyc* mutants grew slowly and gave

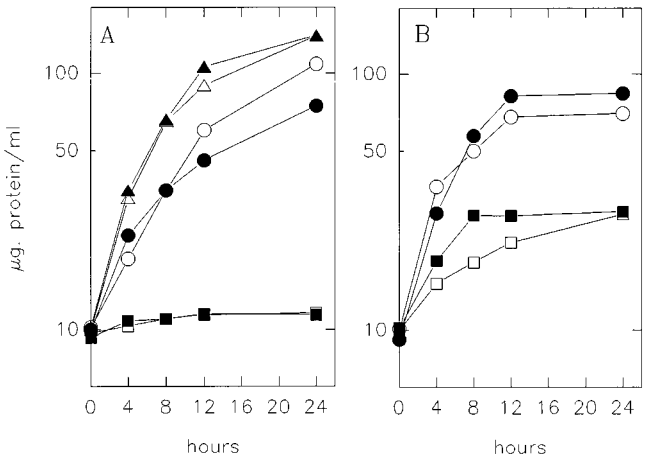

FIG. 2. Growth of *Rhizobium* wild-type and *pyc* mutant strains in MM-pyruvate (10 mM) with and without 1 mM L-aspartate (A) and in MM-glucose (10 mM) (B). Strains tested were *R. etli* CE3 (closed circles), *R. tropici* CFN299 (open circles), *R. etli pyc* mutant 12-53 (open boxes), and *R. tropici pyc* mutant 2-176 (closed boxes). Symbols in panel A for growth in pyruvate-containing aspartate: closed triangles, 12-53; open triangles, 2-176.

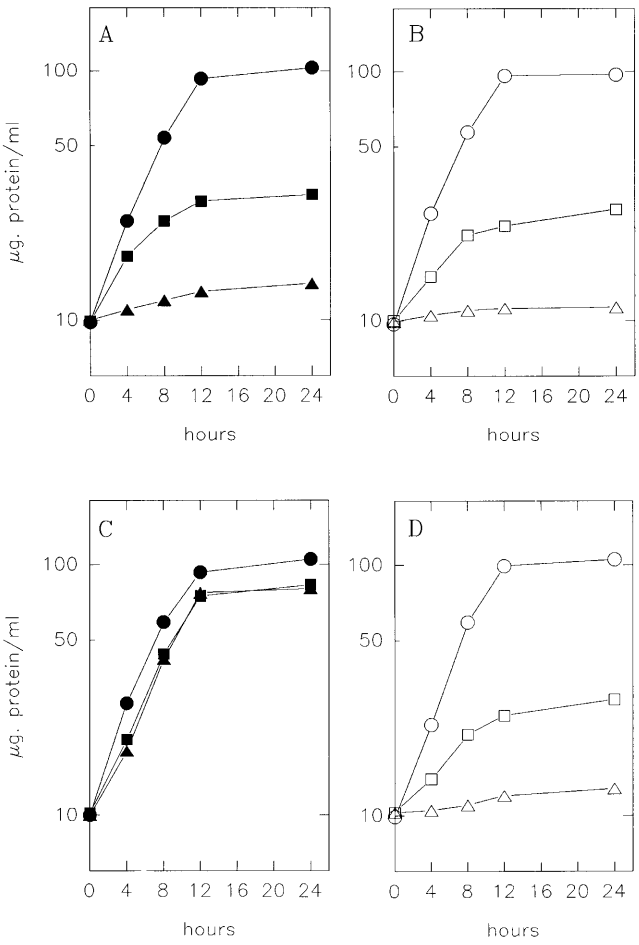

FIG. 3. Growth of *R. etli* parent strain CE3 (closed symbols) and *pyc* mutant 12-53 (open symbols) during subcultivation in MM containing 30 mM succinate with or without 1 μg of biotin per ml. (A) Strain CE3 without biotin; (B) strain 12-53 without biotin; (C) strain CE3 with biotin; (D) strain 12-53 with biotin. Symbols: circles, first subculture; squares, second subculture; triangles, third subculture.

final cell yields of 41 and 34% in comparison with the respective parent strains (Fig. 2B). Supplementation of the pyruvate or glucose minimal medium with biotin (1 μg/ml) or thiamine (10 μg/ml) did not allow growth of the mutants (19).

During serial subcultivation in MM containing 10 or 30 mM succinate without added biotin, the *R. tropici* parent strain

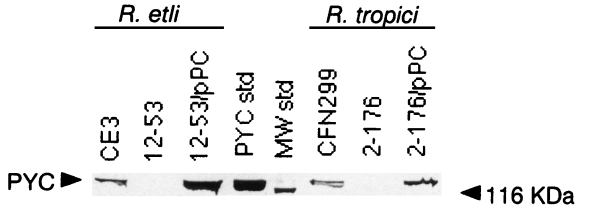

FIG. 4. Western blot analysis of PYC produced by the *Rhizobium pyc* mutants and complemented strains. Cells were grown in biotin-supplemented media as indicated in Materials and Methods. The positions of PYC and the 116-kDa protein standard are indicated. Sample lanes were loaded with 12.4 μg of total protein.

and *pyc* mutant showed no differences in growth rate or final cell yield (19). In MM containing 10 (19) or 30 mM (Fig. 3A and B) succinate without added biotin, *R. etli pyc* mutant 12-53 decreased its growth rate in a manner similar to that of the parent strain. In biotin-supplemented MM containing 10 mM succinate, both the mutant and the parent strain grew well, with the mutant having only a slightly lower final cell yield in the third subculture (19). Under these conditions, thiamine supplementation of the MM also allowed good growth of strain 12-53 in subcultures (19). A physiological effect of the *pyc* mutation in *R. etli* was much more apparent during subculturing in MM containing 30 mM succinate, in which case addition of biotin to the medium allowed continuous growth of the parent strain (Fig. 3C) but not of the mutant (Fig. 3D). The effect of thiamine supplementation on the growth of the mutant has not been determined under these conditions.

Enzyme activities in the *pyc* mutants. Preliminary PYC activity assays of cell extracts detected a low to intermediate level of apparent PYC activity in preparations from both *pyc* mutants. We therefore assayed PYC in assay reaction mixtures with and without ATP in order to differentiate true, ATP-dependent PYC activity from nonspecific background activity (Table 4). The apparent PYC activity detected in the mutant strains, in contrast to that of the parent strains, was not significantly decreased when ATP was excluded from the assays of cell lysates prepared from cells grown in PY or MM-succinate (Table 4), indicating that this apparent PYC activity is actually due to an ATP-independent enzyme and not to PYC. The lack of detectable, biotinylated PYC protein on Western blots of proteins prepared from the mutants (Fig. 4) supports this conclusion. The activities of several additional carbon-metabolic enzymes were assayed and did not differ significantly in the mutants and their respective parent strains (Table 5).

TABLE 4. PYC specific activities in *Rhizobium* wild-type and *pyc* mutant strains

Strain	Mean (nmol/min/mg of protein) ± SE[a]					
	PY (rich) medium			MM-succinate (10 mM) + biotin (1 μg/ml)		
	Assay + ATP	Assay − ATP	ATP-dependent activity	Assay + ATP	Assay − ATP	ATP-dependent activity
R. etli						
CE3	43.5 ± 1.7	13.2 ± 0.8	30.3	28.4 ± 2.0	4.5 ± 0.5	23.9
12-53	9.9 ± 1.6	13.0 ± 1.6	NA[b]	0.95 ± 0.95	3.1 ± 3.4	NA
R. tropici						
CFN299	57.1 ± 1.3	22.0 ± 1.6	35.1	55.2 ± 1.3	15.6 ± 2.0	39.6
2-176	9.8 ± 0.7	7.3 ± 0.9	2.5	11.8 ± 2.6	13.0 ± 0.5	NA

[a] Assay + ATP is the standard PYC assay (see Table 3); assay − ATP lacked only ATP. ATP-dependent activity is the difference between these two values.
[b] NA, no activity detected.

TABLE 5. Activities of selected carbon-metabolic enzymes in the parent and *pyc* mutant strains[a]

Enzyme	Activity (nmol/min/mg of protein)			
	R. etli		*R. tropici*	
	CE3	12-53	CFN299	2-176
PDH	28 ± 8	24 ± 2	21 ± 5	20 ± 3
ODH	25 ± 6	20 ± 1	54 ± 13	58 ± 9
MDH	2,142 ± 645	2,421 ± 861	3,518 ± 631	4,058 ± 845
PYK	190 ± 14	170 ± 22	130 ± 5	164 ± 20
PCK	130 ± 11	122 ± 13	161 ± 16	168 ± 28
PPC	52 ± 15	44 ± 20	54 ± 20	30 ± 2
ME (NAD^+)	152 ± 21	129 ± 24	103 ± 5	75 ± 8
ME ($NADP^+$)	88 ± 43	77 ± 24	97 ± 7	86 ± 3

[a] Duplicate PY cultures were harvested at 16 h, and lysates prepared from each were assayed in duplicate. Results are the means ± standard errors from two separate experiments. Abbreviations: PDH, pyruvate dehydrogenase (EC 1.2.2.2); ODH, oxoglutarate dehydrogenase (EC1.2.4.2); MDH, malate dehydrogenase (EC 1.1.1.37); PYK, pyruvate kinase (EC 2.7.1.40); PCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.49); PPC, phosphoenolpyruvate carboxylase (EC 4.1.1.31); ME, malic enzyme (assayed with NAD^+ [EC 1.1.1.39] or $NADP^+$ [EC 1.1.1.40] as indicated).

Cloning and characterization of the PYC gene from *R. etli*. Plasmid pPC (Table 1) was isolated from an *R. etli* genomic DNA bank on the basis of its ability to restore the growth phenotype of *R. tropici* 2-176. As expected, the presence of pPC in either *pyc* mutant 2-176 or *pyc* mutant 12-53 restored the normal growth phenotype in MM-pyruvate (Fig. 5). In MM-succinate (10 mM) supplemented with biotin, the level of ATP-dependent PYC activity produced by the complemented *R. tropici* mutant was 84 U/mg of protein, or about twofold higher than that of the parent strain (Table 4). When pPC was introduced into the *R. etli* mutant, the ATP-dependent PYC activity was 182 U/mg of protein, a 7.6-fold increase over that produced by the parent strain (Table 4). In Western blots of cell extracts prepared from the complemented mutants, the PYC band, absent in the mutants, was restored (Fig. 4). Densitometric quantitation of the PYC protein produced by the complemented mutants relative to the parent strains (Fig. 4) revealed 3.2- and 6.6-fold increases for strains 2-176/pPC and 12-53/pPC, respectively. These values are in fairly close agreement with the increase in PYC activity determined in the enzyme assays.

The growth phenotypes and PYC activities of the *R. tropici* mutant containing subclones of pPC are shown in Fig. 6a. Partial nucleotide sequencing of *Eco*RI fragments derived from subclone pPC1 indicated that the putative PYC-coding region was located in the 3′ half of the fragment, and subsequent sequencing of this region localized the *pyc* open reading frame as shown in Fig. 6b. The 3,462-bp sequence of the *R. etli pyc* is predicted to encode a protein of 1,154 amino acids with an MW of 126,008, which is similar to the subunit MW of 120,000 estimated by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1). The putative initiation codon (TTG) is preceded by a ribosomal binding site sequence (GGAGG [25]) 9 nucleotides upstream.

Nucleotide sequence similarity comparisons showed that the *R. etli pyc* had significant homology only to *pyc* or other biotin carboxylase sequences in the databases (14). The deduced amino acid sequence of the *R. etli pyc* is 44% identical to that of the putative *Mycobacterium tuberculosis* PYC and 47% identical to sequences of both yeast and human PYCs (Fig. 7). The N terminus is 77% identical to the first 26 amino acid residues of the *R. capsulatus* PYC recently determined by amino acid sequencing (39) (Fig. 7). The *R. etli* PYC sequence (1,154 amino acids) is intermediate in length between those of yeast and human PYCs (both containing 1,178 amino acids including the leader sequence) and the putative *M. tuberculosis* (1,124 residues) PYC (Fig. 7 and 8).

A comparison of substrate-binding-site motifs in the *R. etli* sequence (Fig. 7) with those found in related proteins is shown schematically in Fig. 8. In the amino-terminal segment of the *R. etli* PYC, the hexapeptide GGGGRG matches the GGGG (R/K)G sequence found in all biotin-containing enzymes (67) and is believed to function in ATP binding (22, 48) (Fig. 7 and 8). A variant of this motif also occurs in carbamylphosphate synthetase, which shares some mechanistic properties with PYC (4) but does not require a biotin cofactor (29) (Fig. 8). A Cys residue (Cys-237 [Fig. 7]) is conserved 62 residues downstream of the ultimate Gly residue in this site and is believed to be involved in the CO_2 fixation reaction catalyzed by biotin-dependent carboxylases (32) (Fig. 7 and 8). A second region proposed to be involved in ATP binding is present in carbamylphosphate synthetase and biotin-dependent carboxylases (34) and is also conserved in the *R. etli* sequence (Fig. 7). In the intermediate region of the protein, a putative pyruvate-binding motif, FLTEDPWER, has significant homology with those found in the transcarboxylase domains of *Mycobacterium*, yeast, and human PYCs (Fig. 7) as well as in the *Propionibacterium shermanii* 5s subunit of transcarboxylase (Fig. 8). Tryptophan fluorescence studies with transcarboxylase indicate that the Trp residue present in this motif is involved in pyruvate binding (30). In the carboxy-terminal segment of the enzyme, a putative biotin-binding site (AMKM) is identical to those found in the other PYCs (Fig. 7) as well as the BCCP domains of other biotin-dependent enzymes (Fig. 8). Beginning 29 residues upstream of the biotinyl-lysine in all of the PYC sequences (Fig. 7) and in the α subunit of propionyl-CoA carboxylase (Fig. 8), a PX(P/A) sequence occurs; this sequence is proposed to be involved in the recognition and biotinylation of the biotin carboxylases (32).

Symbiotic phenotypes of the *pyc* mutants in bean plants. At all time points assayed, the symbiotic phenotypes of the *R. etli* and *R. tropici pyc* strains were statistically indistinguishable from those of the parent strains by the criteria of nitrogen

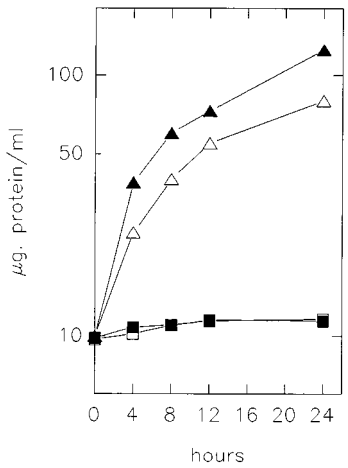

FIG. 5. Phenotypic complementation of the *Rhizobium pyc* mutants for growth in MM-pyruvate (10 mM) when complemented with the cloned *pyc* gene (pPC) from *R. etli*. Strains tested were *R. etli pyc* mutant 12-53 (open boxes), 12-53/pPC (open triangles), *R. tropici pyc* mutant 2-176 (closed boxes), and 2-176/pPC (closed triangles). Growth curves for the mutant strains are reproduced from Fig. 2A.

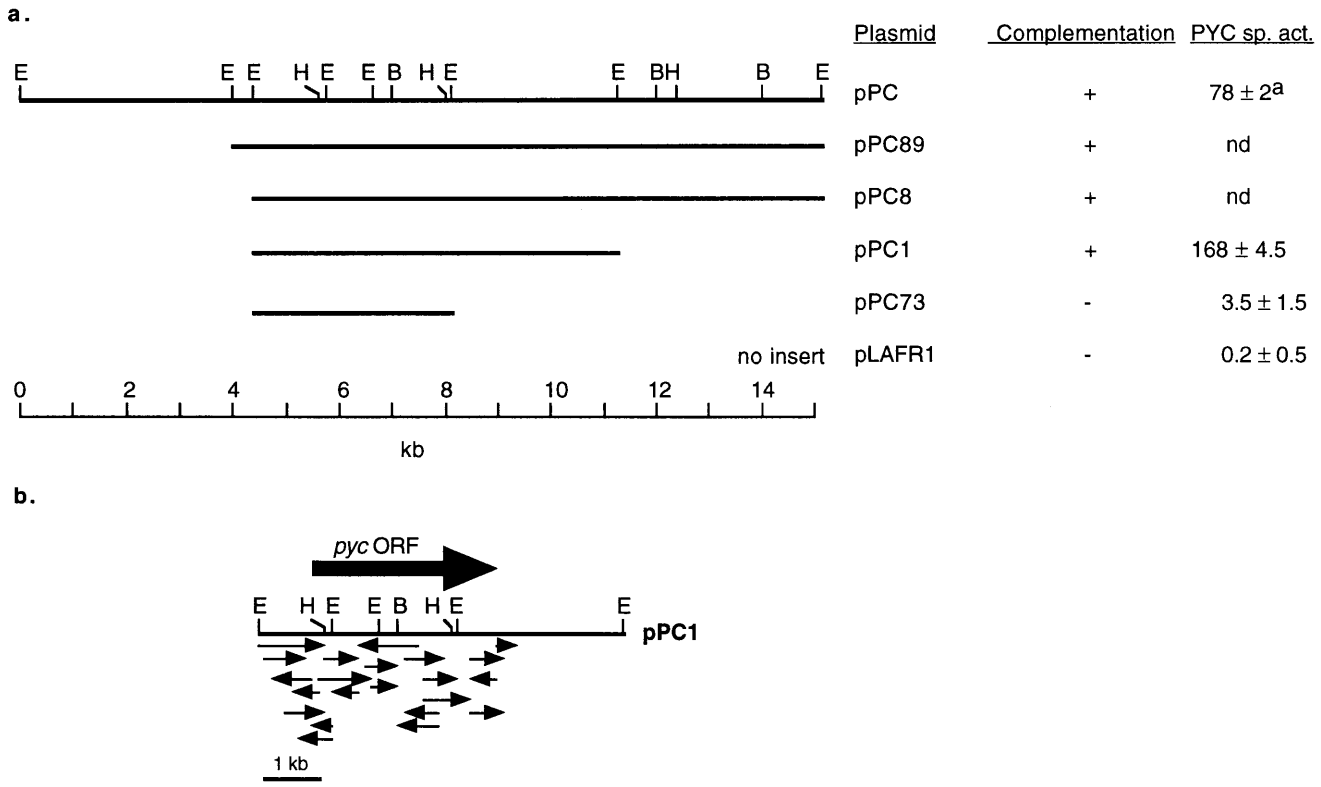

FIG. 6. (a) Subcloning of the *R. etli pyc* gene. Subclones were generated by deleting *Eco*RI fragments from plasmid pPC (Table 1). Complementation refers to the restoration of the wild-type growth phenotype in MM-pyruvate. PYC specific activities (nanomoles per minute per milligram of protein) were determined in 12-h PY (for pPC) or MM-succinate plus biotin (all others) cultures with cell extracts from strain 2-176 containing the indicated plasmid. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; nd, not detected. (b) Sequencing strategy and the location of the *R. etli* pyc open reading frame (ORF) in pPC1.

fixation activity, number of nodules formed, and nodule and plant dry weights. Final seed yields of plants inoculated with the mutant or parent strains were indistinguishable (40). Microsymbionts isolated from nodules at all time points retained the expected antibiotic resistances.

DISCUSSION

Biotin stimulates the growth of a variety of fast-growing *Rhizobium* species under laboratory conditions (19, 59, 66), and the enzyme activities affected by this vitamin have been investigated in *R. etli* and *R. tropici* (18). We now extend these observations by showing that in biotin-supplemented MM, *R. etli* CE3 produces three biotinylated polypeptides, the 120-kDa PYC subunit and the 51- and 14-kDa proteins, neither of which we have identified (Fig. 1). The unidentified biotin proteins were also produced by *pyc* mutant strain 12-53 (14), indicating that they are not degradation products of PYC. Extracts of *R. tropici* contained biotinylated proteins with sizes very similar to those produced by *R. etli* (14), and because the *pyc* mutant 2-176 lacked only a 120-kDa protein produced by the wild-type strain (Fig. 4), this protein probably represents the PYC subunit in this species. Thus, it appears that the PYCs of *R. etli* and *R. tropici* are similar in size. The results of our hybridization studies indicate that the *pyc* genes in these species are not borne on any of their indigenous plasmids but are instead chromosomal.

When strain CE3 was grown in MM-succinate without added biotin, PYC was easily detected in Western blots but at a level of less than 20% of that produced in biotin-containing cultures (Fig. 1). Our data show that strain CE3 can effectively take up exogenously supplied biotin from the medium and incorporate it into PYC and the other biotin-containing proteins. However, under some conditions in which biotin is limiting, this strain appears to produce insufficient biotin to fully biotinylate PYC (Fig. 1) (19). It is important to note several lines of evidence suggesting that *R. etli* CE3 is not a biotin auxotroph. (i) The growth arrest observed when strain CE3 is subcultured in MM without added biotin occurs only after two successive subcultures (18, 19), whereas biotin auxotrophs show no or very little growth when inoculated into medium lacking the vitamin (24, 59). (ii) *Rhizobium meliloti* 1021, like *R. etli* CE3, decreases its growth rate in successive MM subcultures in the absence of biotin (18, 19). Nevertheless, strain 1021 is a biotin prototroph from which genetically defined biotin auxotrophs have been isolated. These auxotrophs do not grow in minimal media lacking biotin (59). (iii) The activity of acetyl-CoA carboxylase, a biotin-requiring enzyme, is maintained in *R. etli* during subculturing in MM lacking biotin (18), suggesting that sufficient biotin is being produced to maintain the enzyme in its active (biotinylated) form. (iv) Pimelic acid (10 μg/ml), the earliest known precursor in the biotin biosynthetic pathway (24), can substitute for biotin in allowing strain CE3 to grow in subcultures (14). This finding suggests that *R. etli* is capable of synthesizing biotin de novo. Collectively, these data indicate that *R. etli* CE3 is a biotin prototroph which regulates biotin synthesis (or the efficiency of its ligation to biotin-requiring carboxylases) during growth on succinate. We note parenthetically that thiamine, which can substitute for

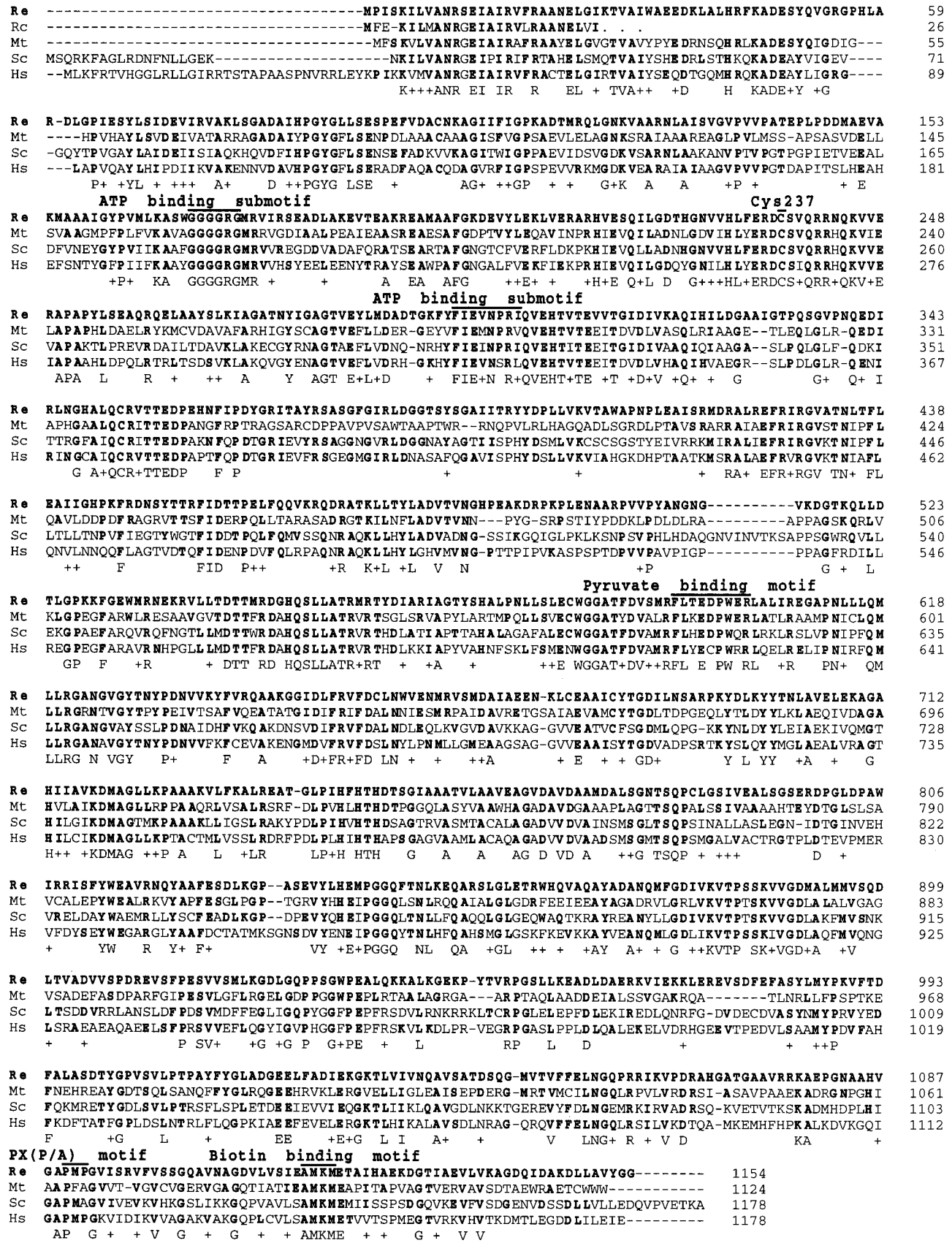

FIG. 7. Comparison of the deduced amino acid sequence of the *R. etli* (Re) PYC with the N-terminal 26 amino acid residues of the *R. capsulatus* (Rc) PYC and the entire deduced amino acid sequences of the *M. tuberculosis* (Mt), *Saccharomyces cerevisiae* (Sc), and *Homo sapiens* (Hs) PYCs. Amino acids identical to those in the Re PYC are in boldface, and residues conserved in all sequences are listed below the alignments, with plus signs indicating conservative amino acid substitutions. Sequence motifs discussed in the text are overbarred. Dashes indicate gaps introduced into the sequences to maximize alignment. Sequences were taken from the references cited in Fig. 8 except for the *R. capsulatus* (39) and *S. cerevisiae* (34) PYCs.

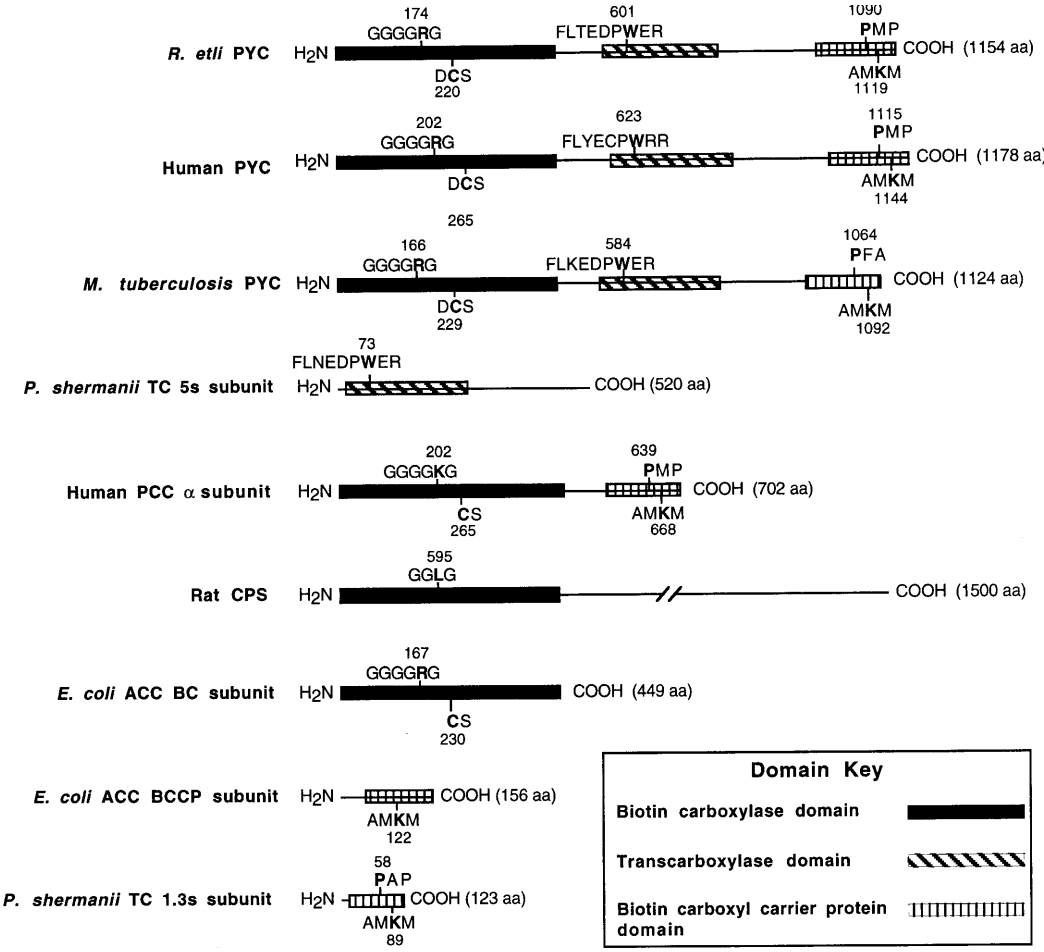

FIG. 8. Schematic representation of the deduced domain structure of the *R. etli* PYC based on sequence homologies to other proteins containing defined sequence motifs. The functional domains are indicated in the key, and some of their associated amino acid (aa) sequence motifs are shown in the diagrams. The extent of the shading representing each of the various motifs was determined by homology comparisons of the following proteins with the *R. etli* PYC: transcarboxylase (TC) domain, *P. shermanii* TC 5s subunit; biotin carboxylase (BC) and BCCP domains, *E. coli* acetyl-CoA carboxylase (ACC) BC and BCCP subunits, respectively. Amino acid residues corresponding to the numbering in the sequence motifs are in boldface. Sequences were taken from the references indicated: human PYC (67); *M. tuberculosis* PYC (57); *P. shermanii* TC 5s subunit (64); human propionyl-CoA carboxylase (PCC) α subunit (31); rat carbamylphosphate synthase (CPS) (44); *E. coli* ACC BC subunit (32); *E. coli* ACC BCCP subunit (41); and *P. shermanii* TC 1.3s subunit (35).

biotin in maintaining an aerobic metabolism in *R. etli*, is a required cofactor for the biotin synthase of *E. coli* (6).

The biochemical data presented show that the *R. etli* PYC is composed of subunits of approximately 120 kDa, and analysis of the enzyme by gel chromatography indicate that the native enzyme is approximately 400 kDa (15). These data are consistent with the enzyme having an α_4 structure. This conclusion is supported by the finding that the partially purified PYC (Table 3) has regulatory properties similar to those of many α_4 PYCs (4, 54). The relatively small variation in *R. etli* PYC activity during growth on different carbon sources (Table 2) is similar to that observed with the allosterically regulated α_4 PYCs produced by *Bacillus subtilis* (13) and *B. licheniformis* (49). In contrast, the $\alpha_4\beta_4$ PYCs from *A. vinelandii* (55) and *P. citronellolis* (62) are highly inducible and are not subject to regulation by effectors. The simultaneous presence of the α_4 PYC and PPC (Table 5) in *R. etli* is worthy of note. Although an allosterically regulated PPC occurs in many bacterial species which produce the $\alpha_4\beta_4$ form of PYC (12, 28, 45, 55), PPC has been shown to be absent in species producing the α_4 subclass of PYC (1, 13, 46, 50, 68). This finding raises the question of how PYC and PPC are regulated in *R. etli*, since many bacterial PPCs are allosterically regulated in a manner very similar to that of the α_4 PYCs (42, 55).

Under what growth conditions do *R. etli* and *R. tropici* utilize PYC? When grown on glucose or other carbon sources utilized via the C3 compounds pyruvate or PEP, bacteria anaplerotically produce C_4 compounds by using CO_2-fixing enzymes (PYC or PPC) or the glyoxylate shunt (1). For example, the growth of *Bacillus megaterium* on C_6 or C_3 compounds requires PYC unless a source of C_4 compounds (e.g., aspartate) is available or unless the glyoxylate cycle is functioning (1). The *R. etli* and *R. tropici pyc* and parent strains grew equally well in rich medium (14, 19) and to similar final cell densities during the first subculture in MM-succinate (Fig. 3) (15, 19). This finding is consistent with the belief that PYC activity is not required for growth on a C_4 carbon source (MM-succinate) or in medium in which a precursor thereof (in PY) is present (1, 13). The mutant strains of both species grew to some extent in glucose (Fig. 2B), a substrate which can be catabolized to PEP in *Rhizobium* spp. (50). The PEP produced from glucose could be converted to OAA in the reaction catalyzed by PPC, thereby

allowing some growth of the mutants. In contrast, *pyc* mutants of *B. subtilis* do not grow on glucose probably because PPC is not produced by this species (13). The *Rhizobium* mutants failed to grow on pyruvate (Fig. 2A) as demonstrated for *pyc* mutants isolated from other species (39, 46, 68). Our failure to detect PEP synthase, or pyruvate or orthophosphate dikinase (either of which can convert pyruvate to PEP [14]), in *R. etli* and *R. tropici* indicates that OAA synthesis from pyruvate (via PEP) cannot occur and prevents the growth of the mutants on this carbon source. We believe that the *Rhizobium* mutants are able to grow in pyruvate medium supplemented with aspartate (Fig. 2A) because OAA is produced by the action of aspartate aminotransferase (54), for which there is considerable activity in *R. etli* and *R. tropici* (14). The production of OAA catalyzed by malate dehydrogenase appears to be possible in the *pyc* mutants (Table 5), although it has been proposed that during growth on C_3 or C_6 substrates, this reaction produces insufficient OAA for both energy generation (via the tricarboxylic acid cycle) and biosynthesis. Consequently, the anaplerotic activity of PYC is necessary for growth under these conditions (13). Finally, the production of C_4 metabolites via the glyoxylate shunt is unlikely to occur in the *pyc* mutants, since the enzymes required for this pathway in *R. etli* CE3 (14) and *R. tropici* CFN299 (27) are present at significant levels only during growth in acetate-containing medias, as previously documented for other bacterial systems (43).

Our hypothesis that PYC is the enzyme upon which biotin acts in preventing the fermentative metabolism in *R. etli* was based on results of experiments using MM containing 10 mM succinate (18). Under these conditions, biotin promoted almost equally good growth of the *pyc* mutant and parent strains in subcultures, suggesting that a biotin-dependent system, distinct from PYC, is the site for biotin action in the mutant. We do not know, however, if this alternative biotin-dependent system is also the site at which biotin acts in the parent strain, or whether it merely compensates for a lack of PYC in the mutant. PYC does appear to have a clear role in allowing *R. etli* (but not *R. tropici*) to grow continuously in serial subcultures in media containing 30 mM succinate (Fig. 3). This conclusion is based on the fact that biotin supplementation was not able to restore the growth of the *R. etli* mutant during subculturing, indicating that PYC is responsible for the growth-maintaining effect of biotin under these conditions. Although a requirement for PYC during growth on succinate appears to be inconsistent with the generally accepted metabolic role for this enzyme (see above), we speculate that growth in higher concentrations of succinate might cause a greater flux, via malic enzyme, of succinate-derived carbon to pyruvate rather than to OAA (via malate dehydrogenase). If this in fact does occur, PYC could be essential in generating sufficient OAA for biosynthetic and energy-generating purposes. This hypothesis remains to be tested.

The deduced amino acid sequence of the *R. etli pyc* has significant similarity to the PYC sequences from a diverse group of organisms (Fig. 7) and contains a biotin carboxylase domain in its N-terminal region, a BCCP domain in its C-terminal region, and a transcarboxylase domain, with a binding site specific for pyruvate, in its central region (Fig. 8). The subunit MW of the protein as determined by SDS-PAGE or as predicted from the deduced amino acid sequence of the cloned gene is similar to those of eukaryotic α_4 PYCs (4). This finding extends the observation that there is a strong evolutionary conservation among biotin-containing carboxylases (29, 52) and a prokaryotic PYC. Our sequence comparison results also indicate that the putative *M. tuberculosis pyc* (57) does indeed encode an α_4 PYC.

Putative *pyc* mutants of *Rhizobium leguminosarum* bv. trifolii (50) and *R. leguminosarum* bv. viciae (3) were reported to be symbiotically proficient on clover and pea plants, respectively. The findings from our in planta experiments are consistent with these data and support the generally accepted proposal that the major carbon sources used by bacteroids are dicarboxylic acids such as malate or succinate (58). Interestingly, by using very low inoculum densities, the importance of biotin in the infective capability of *R. meliloti* was recently demonstrated, although the biochemical basis for this effect was not determined (59). Because the activity of PYC in *R. etli* is greatly affected by biotin, it would be of interest to determine if our mutant strain is altered in competitiveness or growth in the bean rhizosphere.

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