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Structural and Functional Analysis of the Phosphoenolpyruvate Carboxylase Gene from the Purple Nonsulfur Bacterium *Rhodopseudomonas palustris* No. 7

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The *ppc* gene, encoding phosphoenolpyruvate carboxylase (PEPC), from *Rhodopseudomonas palustris* No. 7 was cloned and sequenced. Primer extension analysis identified a transcriptional start site 42 bp upstream of the *ppc* initiation codon. An *R. palustris* No. 7 PEPC-deficient strain showed a slower doubling time compared with the wild-type strain either anaerobically in the light or aerobically in the dark, when pyruvate was used as a carbon source.

Purple nonsulfur bacteria are able to utilize a wide range of organic compounds as photosynthetic electron donors and carbon sources. When these compounds are metabolized under photoanaerobic conditions, the intracellular redox balance is maintained by CO_2 reduction, hydrogen formation, and synthesis of intracellular reserve materials (21). Ribulose bisphosphate carboxylase-oxygenase (RubisCO) plays a major role in CO_2 fixation in photosynthetic bacteria. However, the presence of at least one other independent CO_2 fixation pathway in these purple nonsulfur bacteria was recently suggested, since *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* are capable of photolithoautotrophic growth in the absence of RubisCO (26). This prompted us to perform a more-precise analysis of CO_2 fixation mechanisms in the purple nonsulfur bacterium *Rhodopseudomonas palustris* No. 7 through genetic and biochemical characterization of several kinds of carboxylases. This strain was isolated from 1-propanol enrichment cultures under anaerobic light conditions (3), and morphology and physiological studies indicate that it is a species of *R. palustris* (3, 17).

Phosphoenolpyruvate (PEP) carboxylase (PEPC) (EC 4.1.1.31) is an enzyme involved in carbon metabolism that catalyzes the fixation of CO_2 to PEP in the presence of HCO_3^- to yield oxaloacetate and inorganic phosphate. Known PEPCs are tetramers with a subunit molecular mass of approximately 100 kDa. The reaction driven by PEPC plays an anaplerotic role in supplying oxaloacetate to the citric acid cycle, thus ensuring a continuous replenishment of C_4-dicarboxylic acid, which is necessary for nitrogen assimilation and amino acid biosynthesis (1, 10).

We report here the cloning, sequencing, and transcriptional analysis of the *ppc* gene from the purple nonsulfur bacterium *R. palustris* No. 7 and also analyze the physiological role of PEPC in this strain by constructing a *ppc* gene disruptant.

Cloning and sequencing of the *R. palustris* No. 7 *ppc* gene. The *R. palustris* No. 7 PEPC protein, which was purified by the protocol described by Sadaie et al. (18), was kindly provided by T. Fujii. The NH_2-terminal amino acid sequence of the purified PEPC protein was determined with a Protein Sequencer 473A (Applied Biosystems/Perkin-Elmer, Foster City, Calif.) to be SSLNLSAGPEPVSERPDDAA. Based on this result and on the observation that amino acid sequences of known PEPC proteins share a highly conserved domain [W(M/I/V)G(G/S)D(R/H)DGNP], primer 1 (5′-YTIAAYYTIWSIGCIGGIC CIGARCCIGT-3′ [where I = inosine, R = A or G, Y = C or T, S = G or C, and W = A or T]) and primer 2 (5′-GGRT TICCRTCIYKRTCISIICCIAYCCA-3′ [where K = G or T]) were synthesized to amplify a portion of the *ppc* gene from *R. palustris* No. 7. Primers 1 and 2 were used in a PCR with *R. palustris* No. 7 chromosomal DNA as a template and produced a 0.85-kb PCR product which was subsequently cloned into the pGEM-T vector. The DNA sequence of the insert revealed homology to a part of all known *ppc* gene products. A λFixII library (Stratagene, La Jolla, Calif.) of *R. palustris* No. 7 chromosomal DNA was constructed as per the manufacturer's instructions, and the 0.85-kb α-^{32}P-labelled PCR fragment was used as a probe to screen this library. Screening of 10^4 plaques yielded four positive clones. Southern blotting of *Eco*RI-digested *R. palustris* No. 7 chromosomal DNA demonstrated that a 6.8-kb *Eco*RI fragment hybridized with the same labelled PCR fragment DNA and that each of the recombinant λ phages contained the same fragment (data not shown). The 6.8-kb *Eco*RI fragment was cloned into plasmid pUC118 and sequenced by the dideoxy chain termination method as described by Sanger et al. (19) with a 373A DNA sequencer (Applied Biosystems/Perkin-Elmer). Nucleotide sequences on both strands were determined. The coding region corresponding to the *R. palustris* No. 7 *ppc* gene consists of an open reading frame (ORF) comprising 2,811 nucleotides, corresponding to 936 amino acids. A methionine codon and codons immediately downstream correspond to the NH_2-terminal sequence determined previously for the purified protein (see above). Preceding the start codon of the *ppc* gene there is a G+A-rich putative ribosome binding site (Fig. 1A). The deduced amino acid sequence is 33.7, 44.9, and 35.3% identical to those of the *ppc* gene products from *Escherichia coli* (4), *Corynebacterium glutamicum* (13), and a *Thermus* sp. (12), respectively.

Amino acid residues His^{138} and Arg^{587} of *E. coli* PEPC, which have been described as functionally essential from chemical modification data, amino acid alignments, and site-directed mutagenesis (7, 23) and which are strictly conserved in all PEPC enzymes analyzed to date, are also found in the *R. palustris* No. 7 enzyme (data not shown).

The functionality of the *R. palustris* No. 7 *ppc* gene was

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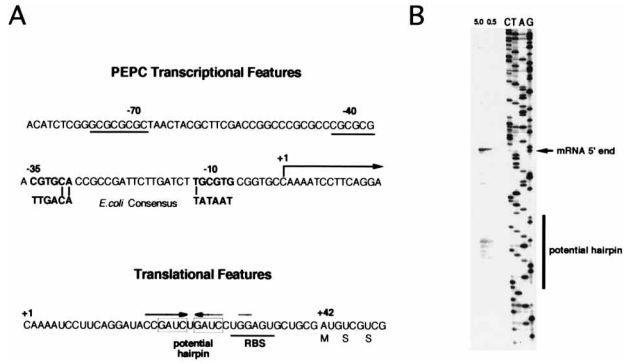

FIG. 1. Transcriptional analysis of the *ppc* gene. (A) Sequence features around the start point of transcription. Top, DNA sequence around and including the *ppc* mRNA 5′ end. Boldface underlining, two potential Z-DNA forming regions at approximately −70 and −40 (8). Boldface letters, regions at the canonical promoter distances of −35 and −10 (the *E. coli* sigma 70 recognition consensus sequence is indicated below) (2). Vertical bars, conserved bases in the two sequences. Lower portion, sequence of the first 50 bases of the *ppc* mRNA. Underlining, the putative ribosome binding site (RBS); rightward and leftward arrows, a region of dyad symmetry possibly involving the ribosome binding site; +42, the first base of the start codon. The single-letter amino acid code for the first three codons of the PEPC protein is presented below the sequence. (B) Sequencing gel analysis of primer extension (primer 5′ end at 633). High-resolution electrophoresis of 5′-labelled cDNA products is aligned with a sequencing ladder generated from DNA from the same region with the same primer. CTAG, dideoxy sequencing marker lanes; lanes 0.5 and 5.0, cDNA synthesis with a 10-fold dilution of the RNA sample; arrow, primary mRNA end point; potential hairpin, region of dyad symmetry in which significant cDNA termination is seen.

tested by complementation of the *E. coli ppc* mutant 342-167 (5). When the *R. palustris* No. 7 PEPC coding region was placed under the control of the *trc* promoter in the expression vector pTrc99A (Pharmacia), which carries a copy of the *lacI*^{q} gene, the *E. coli ppc* mutant 342-167 grew only on minimal medium containing glucose and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). No growth was observed in the absence of inducer or with inducer and vector alone.

Analysis of transcription. Primer extension with primers at positions 633 and 610, which are close to the translational start, gives clear and consistent results and identifies a 5′ end unique to the *ppc* mRNA. The result with the primer at position 633 is shown in Fig. 1B.

The major mRNA start point was seen at a C residue +42 bases upstream of the translation initiation codon. Another, weaker reverse transcriptase stop was seen at +24, which is coincident with the site of a predicted hairpin and may reflect cDNA termination either due to difficulty crossing this site or due to cleavage at this hairpin. No strong differences in primer extension with RNA from phototrophically or heterotrophically grown cells or with prolonged heat shock under either of these conditions (data not shown) were observed.

Primer extension suggests that regulatory sequences constituting the *ppc* promoter are likely to reside close to the structural gene. However, DNA elements at the canonical promoter distance (−35 and −10) show no resemblance to the sigma 70 promoter (or other) consensus derived from *E. coli* (2) (Fig. 1A). Examination of the DNA sequence upstream of this region also fails to detect putative promoters based on sequence homology searches. If regions responsible for promoting transcription lie at −35 and closer, these reflect the GC richness (65%) of *R. palustris* No. 7 DNA and, therefore, constitute an atypical promoter. The role of sequences at −35 and further upstream is currently being studied with gene fusions and by deletional analysis.

Direct repeats in the 41-nucleotide leader RNA have the potential to form secondary structure around the ribosome binding site, and strong primer extension stops in this region (Fig. 1B) suggest that this structure exists. This result could suggest a role for mRNA secondary structure involving ribosome binding site accessibility in translational control of *ppc* mRNA.

TABLE 1. Specific activities of anaplerotic enzymes in cell extracts of wild-type and PEPC-deficient strains of *R. palustris* No. 7

R. palustris No. 7	Sp act (nmol of product/mg of protein/min)[a]		
	PEPC	PEPCK	PC
Wild type	119 (±10)	17 (±2)	<2
ppc::Km	<2	16 (±2)	<2

[a] Values are means ± standard deviations from at least three independent determinations. PEPC, PEPCK, and PC activities were determined spectrophotometrically at 30°C by a modified malate dehydrogenase (EC 1.1.1.37)-coupled assay (18).

Comparative growth experiments of a PEPC-deficient *R. palustris* No. 7 strain and alternative anaplerotic enzyme activities. To determine the role of PEPC in *R. palustris* No. 7 carbon metabolism, we constructed a PEPC-deficient mutant by in vitro gene disruption using a kanamycin resistance cassette. The inactivated copy of the *ppc* gene was introduced into *R. palustris* No. 7 by conjugation as described by Herrero et al. (6) with a gentamicin derivative of the suicide vector pGP704 (11), which can replicate only in a strain that produces the R6K-specified π protein and, therefore, not in *R. palustris* No. 7. Of 300 Km^r transconjugants, 9 clones that had lost the vector mediating gentamicin resistance were identified. Integration of the kanamycin cassette via a double-crossover event was confirmed by Southern hybridization experiments (data not shown).

The PEPC-specific activities in cell extracts of one recombinant strain and the parental strain were determined (Table 1). The recombinant strain showed no detectable PEPC activity, indicating that the *ppc* gene was inactivated.

We compared growth of the *ppc* mutant strain with that of wild-type *R. palustris* No. 7 in synthetic minimal medium in which yeast extract was removed from the basal salt medium (3) under both aerobic dark and anaerobic light conditions with various carbon sources (Fig. 2). The *ppc* mutant differs from the parental strain in exhibiting a slower doubling time either anaerobically in the light or aerobically in the dark with pyruvate (Fig. 2) or lactate (data not shown) as a carbon source. When grown aerobically in the dark with pyruvate as a carbon source, the *ppc* strain shows a threefold increase in its doubling time (25 h) compared to that for the wild-type strain (8 h). Normal growth is restored by supplementing the medium with 0.05% malate (Fig. 2B). When other carbon sources such as succinate, malate, acetate, or ethanol were used, the mutant showed no significant difference from the wild-type strain. Therefore, PEPC has an important function as an anaplerotic enzyme of the Krebs cycle in *R. palustris* No. 7 under both aerobic and photosynthetic conditions.

The residual growth of the *ppc* mutant derivative seen in comparative growth experiments with pyruvate (or lactate) as the carbon source indicated that *R. palustris* No. 7 may possess anaplerotic enzymes other than PEPC. Southern hybridization shows that only one *ppc* gene is present in *R. palustris* No. 7 chromosomal DNA (data not shown). We checked alternative anaplerotic enzyme activities by measuring pyruvate carboxylase (PC) and PEP carboxykinase (PEPCK) specific activities in both the wild-type and *ppc* mutant strains (Table 1).

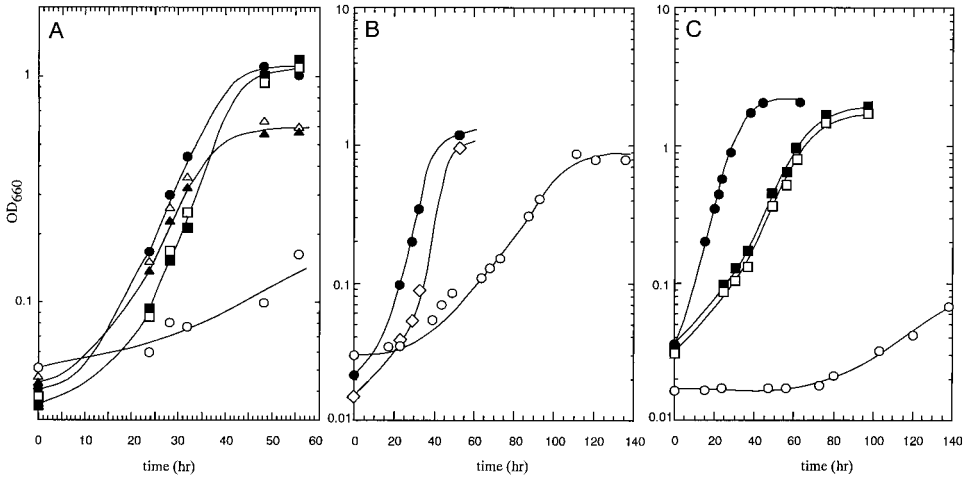

FIG. 2. Growth of *R. palustris* No. 7 strains. Wild-type (closed symbols) and *ppc*-deficient (open symbols) strains were grown in minimal medium with pyruvate (circles), ethanol (squares), succinate (triangles), or pyruvate plus 0.05% malate (diamonds) as the carbon source. (A and B) Growth under aerobic dark conditions, (C) growth under anaerobic light conditions. OD_{660}, optical density at 660 nm.

Whereas PC activity was undetectable under our experimental conditions, both strains showed the same PEPCK activity (about 15 to 20 nmol/mg of protein/min). This latter activity could, therefore, contribute to the growth of the *ppc* mutant and could indicate that *R. palustris* No. 7 PEPCK plays a role analogous to that of PEPCK of *Alcaligenes eutrophus*, in which it has been reported to be the only anaplerotic enzyme (20). However, in most bacterial strains, PEPCK is a gluconeogenic enzyme which converts oxaloacetate to PEP and CO_2 (24). In fact, it was reported that PEPCK in vivo must function to produce PEP in *R. sphaeroides* (14).

The anaplerotic reaction shows great variety in various bacterial strains. An *E. coli* PEPC-deficient mutant could not grow on minimal medium containing glucose as the sole carbon source (5, 25), indicating that PEPC is the essential anaplerotic enzyme in this organism. In *C. glutamicum*, PEPC is dispensable as an anaplerotic enzyme for growth on glucose and for lysine production, suggesting the presence of other anaplerotic enzymes (15, 16). Possible candidates are PC and/or PEPCK, although PC activity was detectable in only one strain of this organism and PEPCK is generally thought to function in gluconeogenesis (16).

The purple nonsulfur bacteria *R. sphaeroides* and *R. capsulatus* contain no PEPC activity. Instead, PC, which converts pyruvate to oxaloacetate, is found at significant levels in both phototropically and chemotrophically grown cells. Furthermore, PC-defective mutants of both species were unable to grow on pyruvate or other compounds metabolized through pyruvate (22), suggesting that PC plays an important role in supplying C_4-dicarboxylic acids during growth on carbon sources which yield only PEP and/or pyruvate. Although it has been reported that *R. rubrum* contains both PEPC and PC enzymes (9), the role of each of these enzymes in carbon metabolism is still unclear. *R. palustris* No. 7, which also belongs to the purple nonsulfur bacteria, shows a significant level of PEPC activity, suggesting that this strain employs a different anaplerotic mechanism.

Nucleotide sequence accession number. The sequence data reported here have been deposited in the DDBJ/EMBL/GenBank database under accession no. D89668.

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