A periplasmic, \(\alpha\)-type carbonic anhydrase from \textit{Rhodopseudomonas palustris} is essential for bicarbonate uptake

László G. Puskás,† Masayuki Inui, Kenneth Zahn and Hideaki Yukawa

Intact cells of the purple non-sulfur bacterium \textit{Rhodopseudomonas palustris} growing anaerobically, but not aerobically, contain carbonic anhydrase (CA) activity. The native enzyme was purified \(>2000\)-fold to apparent homogeneity and found to be a dimer with an estimated molecular mass of \(54\) kDa and a subunit molecular mass of \(27\) kDa. The CA gene (\(acaP\)) was cloned and its sequence revealed that it was homologous to \(\alpha\)-type CAs. The upstream region of \(acaP\) was fused to the \(lacZ\) gene and \(\beta\)-galactosidase activity was measured under different growth conditions. Acetazolamide inhibited purified CA with an \(IC_{50}\) in the range of \(10^{-8}\) M, and in the culture media concentrations as low as \(30\) \(\mu\)M inhibited phototrophic growth under anaerobic, light conditions when bicarbonate was used. An \(acaP::\text{Kan}^r\) mutant strain was constructed by insertion of a kanamycin-resistance cassette and showed a growth pattern similar to wild-type cells grown in the presence of CA inhibitor. \(CO_2\) gas supplied as an inorganic carbon source reversed the effect of mutation or acetazolamide. CA activity measurements, fusion and Western blot experiments confirmed that CA is expressed under different anaerobic conditions independently of bicarbonate or \(CO_2\) and that there is no expression under aerobic conditions.

Keywords: carbonic anhydrase, inorganic carbon uptake, \textit{Rhodopseudomonas palustris}, periplasmic enzyme

INTRODUCTION

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing metalloenzyme catalysing the reversible hydration of \(CO_2\). CA is important in biological systems because the uncatalysed interconversion between \(CO_2\) and \(HCO_3^-\) is slow around neutral pH. Its high efficiency catalysis is fundamental to many biological processes, such as photosynthesis, respiration, pH homeostasis and ion transport (Badger & Price, 1992; Tashian, 1989; Vandenberg et al., 1996). Among these gene families, significant sequence homologies cannot be recognized. CA has been described in photosynthetic organisms, including higher plants, micro- and macroalgae, and cyanobacteria (Burnell et al., 1990; Fukuzawa et al., 1992; Hatch & Burnell, 1990; Kimpel et al., 1983; Price et al., 1992). Some photosynthetic organisms have multiple types of CA enzyme found in different cellular locations that serve different functions in the photosynthetic process. In cyanobacteria and algae, different types of CA have been found. The role of extracellular CA is to improve the efficiency of inorganic carbon (\(C_i\)) transport and it is located in the periplasm or in the cell membrane (Price et al., 1992). The function of the intracellular CA in these organisms is to convert \(HCO_3^-\) to \(CO_2\) for fixation by ribulose-bisphosphate carboxylase-oxygenase (RubisCo) (McKay et al., 1993; Rawat & Moroney, 1995). In plants, intracellular CA enhances the rate of \(CO_2\) to \(HCO_3^-\) conversion, for fixation by phosphoenolpyruvate carboxylase (Hatch & Burnell, 1990). CA has been proposed to be involved in \(C_i\) utilization in

†Present address: Biological Research Center, Hungarian Academy of Sciences, DNA-Chip Laboratory, Szeged, PO Box 521, H-6701, Hungary.

Abbreviations: AZ, acetazolamide; CA, carbonic anhydrase; \(C_i\), inorganic carbon; PNSB, purple non-sulfur bacterium; RubisCo, ribulose-bisphosphate carboxylase-oxygenase.

The GenBank accession number for the sequence reported in this paper is AB022175.
most anaerobic bacteria and archaea (Alber & Ferry, 1994; Kaplan et al., 1991). CA function in processes other than C₆ utilization has also been verified in several bacteria. In Escherichia coli the cyaT gene, encoding CA, is part of the cyanate degradation operon. Its physiological role is to prevent depletion of cellular HCO₃⁻ during cyanate decomposition due to loss of CO₂ (Guilloton et al., 1992). In aceticogenic bacteria the function of CA might be to participate in increasing the intracellular CO₂ concentration and to regulate intracellular pH during acetate production (Braus-Stromeyer et al., 1997).

Purple non-sulfur bacteria (PNSB) can grow either heterotrophically under aerobic conditions or photoheterotrophically under anaerobic light conditions using biocatalysts for bio-remediation has been suggested (Harwood & Gibson, 1988). Recently, the use of PNSB for waste treatment and subsequent fixation of CO₂ released during industrial processes was proposed (Brenner et al., 1998). Little information exists with regard to the mechanism of CO₂ utilization in these bacteria, and their C₆ uptake mechanism is unexplored. Because CA is known to play an important role in C₆ transport and in photosynthetic processes in other organisms, in the present study we intended to verify the presence and the possible function of this enzyme in PNSB. Intracellular, but not periplasmic, CA has been detected in Rhodospirillum rubrum (Gill et al., 1984); however, the biological function of this enzyme was not investigated.

Here we report the identification of a periplasmic, α-type CA from Rhodopsseudomonas palustris. This is the only type found in animals, but it can also be found in the periplasm and the thylakoid membranes of the eukaryotic unicellular green alga Chlamydomonas reinhardtii (Coleman et al., 1984; Karlsson et al., 1998) and in the eubacterium Neisseria gonorrhoeae (Chirica et al., 1997). In the PNSB Rhodobacter sphaeroides, Rhodobacter capsulatus and Rps. palustris α- and γ-type, but not α-type, CAs have been detected by immuno-blotting with polyclonal antisera and based on sequence searches of the incomplete genome sequences (Smith et al., 1999). We describe the cloning of CA gene, designated acAP, encoding CA protein (AcA) in Rps. palustris. 

**METHODS**

**Cultivation of bacteria.** Rps. palustris No. 7, Rba. sphaeroides and Rsp. rubrum were cultivated at 30 °C in a minimal medium (Fujii et al., 1983) containing 5 mM 1-propanol and 20 mM NaHCO₃ (pH 7.5) under anaerobic conditions using a light incubator when the activity of the strains were compared, when the effect of different concentrations of acetazolamide (AZ; Sigma) was determined and for purification of CA from Rps. palustris No. 7. When comparing CA activities using different organic compounds, the same minimal media was used but instead of 1-propanol, 2 mM hexanoic acid or butyramine was added. When comparing CA activities in different C₆ concentrations, 1, 5, 10 and 20 mM sodium bicarbonate and 2 mM 1-propanol was used in minimal media (pH 7.5). When the effect of CO₂ was studied instead of NaHCO₃, CO₂ was bubbled into the media (3% CO₂ in argon). Photoautotrophic growth was studied by growing cells on agar plates anaerobically using CO₂ and H₂ gas. Comparison of growth of mutant and wild-type cells was also performed on Petri plates set in a small chamber and filled with CO₂ with or without H₂ at the concentration mentioned above. Aerobic cultivation was performed in van Niel medium (van Niel, 1944) containing 5 mM 1-propanol on a rotary shaker (150 r.p.m.) with or without 20 mM sodium bicarbonate. Fermentative growth of cells was carried out anaerobically in the dark using van Niel minimal medium with 4 mM pyruvate on agar plates.

**Sample preparation and enzyme assays.** Cells were collected by centrifugation, washed and suspended in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM zinc sulfate. For measurement of CA of intact cells, 200 ml of this suspension (15 OD₅₅₀ U ml⁻¹) was used directly. Cells were treated to obtain the periplasmic and cytoplasmic fractions as previously published (Brancher et al., 1989; Tai & Kaplan, 1985). For total-cell CA activity and for purification of CA from Rps. palustris No. 7, cell suspension (0.4 g wet weight ml⁻¹) was passed through a French pressure cell (20000 p.s.i.; 138 MPa) in the presence of DNase I. The cell debris was centrifuged at 16000 r.p.m. for 20 min at 4 °C and the supernatant was used. CA activity was measured from the pH decrease after the addition of 1 ml CO₂-saturated water to 20 mM Veronal buffer (pH 8.5) (Wako Chemicals) containing the test material in a total volume of 2.8 ml. Units of enzyme activity (U) were calculated using the equation U = (tₐ − t₀)/t, where t and t₀ represent the time for a pH change from 8.5 to 7.0 with and without sample (Wilbur & Anderson, 1948). Protein concentrations were determined colorimetrically by the method of Bradford (1976) using Bio-Rad dye reagents and standards. β-Galactosidase activity was determined by measuring the initial rate of hydrolysis of ONPG (Malamy & Horecker, 1964). Cytochrome c₅₅ levels were determined by measuring the difference in the A₅₅₀ between the fully reduced and oxidized forms (Brancher et al., 1989).

**Enzyme purification and N-terminal amino acid sequence determination.** Cells from 21 total culture volume were collected and disrupted with a French press. Centrifugation of the cell extract was then performed for 20 min at 16000 g. About 90% of the total CA activity was obtained in the supernatant fraction. Ammonium sulfate was added to the supernatant (15 g 100 ml⁻¹) and the mixture stirred for 1 h at 4 °C. After centrifugation for 20 min at 16000 g, the same amount of ammonium sulfate was added and the extract treated as above. The precipitate was collected by centrifugation, dissolved in sodium phosphate buffer (50 mM, pH 7.2 containing 1 mM zinc sulfate) and stored on ice for 2 h. The mixture was loaded onto a p-aminomethylbenzene-sulfonamide-agarose (Sigma) (3 ml bed volume) column equilibrated with the same buffer. The column was washed with buffer (20 ml), buffer containing 0.7 M NaCl (30 ml) and 0.3 M sodium azide in the same buffer (30 ml). CA activity was eluted with 0.6 M sodium azide. The eluate was concentrated using a 50 kDa cut-off Amicon column. The concentrated eluate was diluted with phosphate buffer, reconcentrated with the same column and rediluted. The diluted enzyme solution was loaded onto Q-Sepharose (Pharmacia) and the flow-through was collected. This step was repeated using a cation-exchange column, CM-Sepharose (Pharmacia). Under various conditions...
pH conditions CA activity would not bind to anion or cation exchange resins. Although CA could not bind to these columns, collections of the flow-through served to purify CA an additional 12-fold. Ammonium sulfate was added to 1 M and the sample was loaded onto a Phenyl-Sepharose (Pharmacia) column equilibrated with phosphate buffer (50 mM, pH 7.2) containing 1 M ammonium sulfate. After a 50 ml washing step (in the same buffer as used in equilibration), the activity was eluted with 1-6 M ammonium sulfate solution. The fractions containing CA activities were dialysed against phosphate buffer and concentrated as described in the affinity chromatography step.

After purification, 8 µg CA enzyme was transferred onto a nitrocellulose membrane (Millipore) and the N-terminal sequence of the protein was determined commercially by the automated Edman degradation procedure (Takara).

**Immunoblot analysis of Rps. palustris CA.** The soluble cell fractions were analysed by immunoblotting for CA content after separating the proteins on polyacrylamide gels under denaturing (using SDS) or native conditions. Proteins were electrophoretically transferred to nitrocellulose (Millipore). Immunoblotting was performed with mouse antiserum against purified CA and goat anti-mouse antibody–alkaline phosphates conjugate as a detection method, as described previously (Blake et al., 1984).

**Cloning and nucleotide sequence determination of acap.** Based on the amino acid sequence of the N terminus of CA from *Rps. palustris* (AEGAYHW) the following degenerate primer was synthesized: acap1, 5’-GC(GA)(AG)(GG)(GT)-GC(CG)TGGGG-3’. The second primer for PCR was designed according to the highly conserved amino acid motif GSLTTTP among α-type CAs: acap2, 5’-GGGGGG(GG)-GT(CG)GT(CG)AG(GA)(CG)(GG)-CC-3’. PCR was carried out in a total volume of 100 µl with 10 ng *Rps. palustris* genomic DNA, 0.2 mM dNTPs, 2% DMSO and 1 × Taq polymerase buffer with MgCl2 and 4 U Taq DNA polymerase (Takara) for 30 cycles at temperatures of 95 °C for denaturation (1 min), 57 °C for annealing (1 min) and 72 °C for extension (1 min). After agarose gel electrophoresis of the PCR mixture, the desired fragment (516 nt long) was purified with a PCR purification kit (Pharmacia) and cloned into the GS-digest (Bgl II–Hind III) digested with the 225th nt position. The recombinant fragment was amplified with two specific primers complementary to regions flanking the genomic NaeI sites, which also contained BamHI sites at their 5’ ends. The resulting fragment was digested with BamHI and cloned into the pGEM-7 vector that had been digested with BglII. Procedures used to obtain the *Rps. palustris* double crossover acap::kanR insertion mutant strain were as described by Inui et al. (1999).

**Construction of acap–lacZ fusion vector.** The coding region of the lacZ gene was excised from the plasmid pMC1871 (Pharmacia) with PstI/BamHI and cloned into the pBluescript SK (II) multicloning site resulting in the plasmid pBlac. A 500 bp upstream region of acap (from the first Ala codon of the mature CA enzyme) was amplified with primers having EcoRI and PstI sites, cut with the same enzymes and cloned into pBlac upstream of the lacZ gene. The EcoRI–BamHI fragment was cut out and cloned into the corresponding sites of pMG103 to produce plasmid pMLaca. pMLaca was electroporated into *Rps. palustris* cells or transformed into *E. coli* cells and the transformants were selected with kanamycin and used in β-galactosidase fusion studies.

**DNA sequencing.** DNA sequencing was performed using Thermo Sequenase dye-terminators and samples were run on an ABI 373 fluorescent sequencer. All plasmid inserts produced by PCR in this study were confirmed by sequencing.

**Materials.** All chemicals were purchased from Wako Chemicals unless otherwise mentioned. Goat anti-mouse antibody–alkaline phosphates conjugate was purchased from Boehringer Mannheim Biochemicals. Restriction endonucleases were obtained from Takara Biochemicals and were used according to the manufacturer’s specifications.

**RESULTS**

**CA activity in Rps. palustris cells**

Protein preparations of *Rps. palustris* cells cultivated under anaerobic conditions exhibited total CA activities from total cell protein [2.95–3.20 U (mg protein)−1], similar to those of other CA-containing bacteria (Alber & Ferry, 1994; Braus-Stromeyer et al., 1997), whereas cells grown aerobically showed little or no activity (<0.1 U) (Table 1). Intact cells showed CA activity.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaerobic conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Photoheterotrophic light (minimal medium)</td>
<td></td>
</tr>
<tr>
<td>Ethanol + bicarbonate</td>
<td>3:05</td>
</tr>
<tr>
<td>1-Propionate + bicarbonate</td>
<td>3:20</td>
</tr>
<tr>
<td>1-Butylamine + bicarbonate</td>
<td>3:02</td>
</tr>
<tr>
<td>Ethanol + CO2</td>
<td>3:08</td>
</tr>
<tr>
<td>Photosynthetic light (minimal medium)</td>
<td></td>
</tr>
<tr>
<td>CO2 + H2</td>
<td>2:98</td>
</tr>
<tr>
<td>Fermentative dark (rich medium)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2:95</td>
</tr>
<tr>
<td><strong>Aerobic conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Heterotrophic dark (rich medium)</td>
<td></td>
</tr>
<tr>
<td>Ethanol + bicarbonate</td>
<td>0:05</td>
</tr>
<tr>
<td>Ethanol−bicarbonate</td>
<td>0:07</td>
</tr>
<tr>
<td>1-Butylamine</td>
<td>0:03</td>
</tr>
<tr>
<td>Ethanol (minimal medium)</td>
<td>0:07</td>
</tr>
<tr>
<td>Pyruvate (rich medium)</td>
<td>0:08</td>
</tr>
</tbody>
</table>

Activities of cell extracts cultivated under different growth conditions and substrates. Activity is expressed as U (mg protein)−1.
**Table 2.** Purification of CA from *Rps. palustris*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell extract</td>
<td>100</td>
<td>1575</td>
<td>4798</td>
<td>3·05</td>
<td>100</td>
<td>1·0</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>98</td>
<td>1160</td>
<td>3980</td>
<td>3·43</td>
<td>83</td>
<td>1·1</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>80</td>
<td>710</td>
<td>3360</td>
<td>4·73</td>
<td>70</td>
<td>1·6</td>
</tr>
<tr>
<td>Affinity column</td>
<td>15</td>
<td>1·5</td>
<td>2845</td>
<td>1896·7</td>
<td>59</td>
<td>621·9</td>
</tr>
<tr>
<td>Ion-exchange column</td>
<td>20</td>
<td>1·03</td>
<td>2342</td>
<td>2276·7</td>
<td>49</td>
<td>746·5</td>
</tr>
<tr>
<td>Phenyl-Sepharose column</td>
<td>15</td>
<td>0·29</td>
<td>1818</td>
<td>6269·0</td>
<td>38</td>
<td>2055·4</td>
</tr>
</tbody>
</table>

[0·6 U (OD\(_{600}\) U bacteria\(^{-1}\)] due to periplasmic or membrane-bound CA enzyme. Activity was measured from cells that were cultivated under different anaerobic conditions: fermentative dark conditions using pyruvate as carbon source, photoheterotrophic light conditions using different organic compounds and CO\(_2\) or bicarbonate as the C\(_i\) source, photoautotrophic light condition using H\(_2\) and CO\(_2\). Cells that were cultivated under aerobic heterotrophic conditions with or without bicarbonate in the media showed no activity. This result did not depend on the applied organic carbon source (Table 1).

**Enzyme purification**

Under a variety of conditions, CA did not bind to cation- or anion-exchange columns. However, these steps were useful to remove certain contaminants. Major purification was achieved with affinity chromatography. Strong binding of the enzyme to the p-aminomethylbenzenesulfonamide-agarose column was observed and this is consistent with the high affinity of CA for AZ. The enzyme was purified more than 2000-fold, with an overall recovery of 38\% (Table 2). The specific activity of the purified enzyme (6269 U mg\(^{-1}\)) is comparable to that of commercially available human erythrocyte isozyme II (7870 U mg\(^{-1}\)).

**Properties of the purified enzyme**

The enzyme appeared as a single band of about 27 kDa on a 4–20\% gradient SDS polyacrylamide gel after gel electrophoresis and Coomassie brilliant blue staining (Fig. 1a). On a native gel (acrylamide gradient 4–20\%) CA also gave a single band (Fig. 1b). To examine the presence of CA enzyme in cells cultivated under different conditions and for localization studies, mouse polyclonal antisera were raised against the purified CA from *Rps. palustris*. As shown in Fig. 2, the antibodies detected a polypeptide of the same size as CA in a Western blot analysis and could detect very small amounts of purified CA protein. When antiserum was used against whole-cell extracts, no other protein showed reactivity, thus it seems that the antiserum is specific to CA from *Rps. palustris*.

The migration of the purified enzyme was analysed on a series of denaturing and native gels containing different polyacrylamide concentrations and the apparent size of the enzyme was determined by the method of Ferguson (1964). Under denaturing conditions the estimated molecular mass of CA is about 27 kDa while under native conditions the molecular mass is approximately 54 kDa, suggesting that the native enzyme is a dimer. The NH\(_2\) terminus of the purified CA protein was determined by the automated Edman degradation pro-
using marker enzymes with known localizations. We chose cytochrome $c_1$ as the periplasmic, and $\beta$-galactosidase as the cytoplasmic marker. Fractionation and measurements of marker enzyme activities were performed as published elsewhere (Brandner et al., 1989; Tai & Kaplan, 1985). In the cells grown under photosynthetic conditions, cytochrome $c_1$ and CA were found only in the periplasmic fraction (997 pmol Cyt $c_1$ in the periplasm, and 173 pmol Cyt $c$ in the cytoplasmic fraction), while $\beta$-galactosidase activity appeared only in the cytoplasmic fraction (260 Miller U in the cytoplasm and 38 Miller U in the periplasmic fraction). Since wild-type Rps. palustris No. 7 strain does not show $\beta$-galactosidase activity, we constructed a plasmid which contains the E. coli lacZ gene using an Rps. palustris shuttle vector (Inui et al., 2000). To examine the localization of CA by another method we used immunoblot analysis. We used specific antisera against the purified enzyme and checked the protein fractions from the periplasm and the cytosol. We were able to confirm the periplasmic location of CA as observed with enzymic assays (data not shown).

**Cloning and sequence data comparison of CA gene, acaP**

Based on the sequence of the first 12 NH$_2$-terminal amino acid residues of the purified CA and from studies of inhibitor specificity, we assumed that Rps. palustris CA belongs to the $\alpha$ class. Two degenerate primers were designed for PCR based on the first NH$_2$-terminal 8 aa and on one internal conserved sequence region present in all $\alpha$-CAs (Hewett-Emmett & Tashian, 1996) (see Methods). PCR was used to amplify a DNA fragment of 516 bp which was cloned and sequenced. Nucleotide sequence similarity comparisons showed that this fragment had significant homology to other $\alpha$-CAs in the database. To identify an E. coli clone having the full-length Rps. palustris CA gene, PCR amplification was used to screen a NaeI genomic library from Rps. palustris in pBluescript SK II. A 1443 bp NaeI fragment was found to contain the full-length CA gene. From the DNA sequence and the NH$_2$-terminal amino acid determination we assume that there is a 25 aa signal sequence which is responsible for the periplasmic export of the enzyme. A positively charged part in the amino terminus of the possible signal motif and a following hydrophobic core are recognized characteristics of bacterial signal peptides (von Heijne, 1984). The 18 residue hydrophobic region ends in the $+1$, $−1$ AXA motif characteristic of the recognition site of signal peptidase (Perlman & Halvorson, 1983). The protein is thus predicted to be made as a 27,302 Da precursor which is cleaved between Ala-25 and Ala-26 to produce a mature 230 residue protein of 24772 Da. The putative initiation codon (ATG) is preceded by a possible ribosome-binding site sequence (GGAGA), 5 nt upstream. Downstream from the ORF is a possible rho-independent transcription terminator. The nucleotide sequence and the ORF, designated acaP, encodes the 255 aa Rps. palustris CA. The deduced amino acid

**Cellular localization of CA**

Rps. palustris is a Gram-negative bacterium and contains two cellular compartments, the cytoplasm and the periplasm. CA activity was found only in the periplasmic space (the cytoplasmic fraction exhibited 0.49 U, while the periplasmic fraction showed 11.5 U). The purity of the periplasmic extract was evaluated by

**Fig. 2.** Immunoblot analysis of protein extracts from Rps. palustris cultivated under different conditions. Lane 1 contains purified CA as a control. Under photoautotrophic (lane 2) and photoheterotrophic (lanes 3 and 4) conditions, cells were cultivated in minimal media at 35 °C in the light using bicarbonate or CO$_2$ as the C$_1$ source as indicated. Under fermentative conditions (lane 5) cells were grown in the dark in an anaerobic chamber using pyruvate in rich media at 35 °C. When cells were cultivated under heterotrophic conditions in the presence of oxygen (lanes 6, 7 and 8), the same media, and organic and inorganic carbon compounds were used as in the case of the corresponding photoheterotrophic conditions (lanes 3, 4 and 5). Aerobic growth in the dark was at 35 °C with shaking. Blotting was performed with mouse antiserum against purified CA and goat anti-mouse antibody–alkaline phosphatase conjugate as a detection method.
sequence of acaP was aligned with the sequences of CA from *Anabaena*, *Synechococcus*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *N. gonorrhoeae* and human CA isozymes I and II. *Rps. palustris* CA showed the highest homology to bacterial CAs (35.7–45.7% identities). Comparing *Rps. palustris* CA with the two human CA isozymes I and II, the identities were 33% and 34.6%, respectively. The highest identity was found with the β-Proteobacterium *Neisseria* (45.7%) and the γ-Proteobacteria *Erwinia* (45.7%) and *Klebsiella* (42.9%).

**Regulation of acaP expression**

CA activity was measured and was the same when cells were grown under different anaerobic conditions: photoheterotrophic light, photoautotrophic light or fermentative dark conditions. In the presence of oxygen, CA activity could not be detected using the media listed in Table 1. The presence of a CA band as demonstrated by immunoblot analysis under each condition (Fig. 2) was in agreement with the activity data. The enzyme was detected under different anaerobic conditions. No difference was detected in the amount of CA enzyme in cells grown in low and high concentrations of bicarbonate under different anaerobic conditions (data not shown).

To investigate the transcriptional regulation of acaP, an acaP-lacZ fusion was constructed. The non-coding, upstream region with the proposed translation initiation signals of acaP was fused translationally in-frame to the first codon of lacZ. In *E. coli* the β-galactosidase activity remained unchanged when cells were shifted from aerobic to anaerobic conditions, while a dramatic change was observed in the case of *Rps. palustris*, where activity was more than 20-fold higher when cells were incubated in the absence of oxygen (0.9 compared to 19.7 Miller U). Expression of β-galactosidase occurred within several hours and cells plated in the presence of X-Gal became blue when examined on LB plates containing the indicator. In *E. coli* the expression of acaP gene seemed to be constitutive compared to expression from the native lac promoter, and the strength of the acaP promoter was approximately 13-fold weaker than the lac promoter both in *E. coli* and in *Rps. palustris*. The immunoblotting and β-galactosidase fusion studies suggest that CA expression is regulated at the transcriptional level.

**Inhibition of phototrophic growth by CA inhibitors**

Since AZ was an effective in vitro inhibitor of CA, we studied the effect of AZ on the growth of *Rps. palustris* under anaerobic light conditions. Preliminary studies showed that CA activity could be completely inhibited in intact cells using 0.2 mM AZ. Lower concentrations of AZ inhibited photosynthetic growth in a concentration-dependent manner when bicarbonate was used as the C source (Fig. 3a). Even higher concentrations of AZ than those had no effect on aerobic growth (data not shown). These results are in good agreement with the observation that extracts from cells harvested under aerobic conditions exhibited no CA activity. Cells showed CA activity when grown under photoautotrophic and fermentative conditions. When CO₂ was used instead of bicarbonate under photoheterotrophic conditions, AZ had no inhibitory effect on growth (data not shown).
Insertion mutation of acaP and characterization of the mutant strain

To obtain more information regarding the physiological role of CA, we made a mutant strain of *Rps. palustris* by insertion of a kanamycin-resistance cassette into the coding region of *acaP*. The single-crossover mutants showed resistance to another antibiotic, gentamicin, while strains that displayed only kanamycin resistance were double-crossover mutants and these were selected for further analysis. The homologous recombination event was confirmed by PCR and Southern blotting (data not shown). The mutant strain showed a similar growth pattern to wild-type cells cultivated in the presence of 60 μM AZ in minimal medium containing bicarbonate as the C source (Fig. 3a). Phototrophic growth was inhibited for several days when bicarbonate was used. The very slow growth can be explained by the slow conversion of bicarbonate to CO₂ without catalysis at close-to-neutral pH in liquid media. Using CO₂ with different organic compounds or with H₂, no difference in growth was observed between the wild-type and the mutant cells using solid media in small anaerobic chambers (data not shown).

Effect of CO₂ and pH on acaP mutant and wild-type cells

When cell growth was arrested by AZ under phototrophic conditions using bicarbonate, the inhibitory effect could be diminished by bubbling CO₂ gas into the media (data not shown). An *acaP* mutant strain could also grow when the media was supplemented with CO₂ (Fig. 3b). The same effect could be seen when the pH of the media was lowered with buffers. Although wild-type cells grew more slowly in lower pH (pH 5–6) media, the media was lowered with buffers. Although wild-type cells grew more slowly in lower pH (pH 5–6) media, the media was lowered with buffers. Although wild-type cells grew more slowly in lower pH (pH 5–6) media, the media was lowered with buffers. Although wild-type cells grew more slowly in lower pH (pH 5–6) media, the media was lowered with buffers.

**DISCUSSION**

One report on CA from the PNSB has so far appeared where a cytoplasmic CA from *Rsp. rubrum* was purified and some features of the enzyme were studied (Gill et al., 1984). The physiological role of this enzyme was not studied, nor was a periplasmic location established. In this report, CA from *Rps. palustris* is studied in detail. We have found that intact cells exhibit CA activity when cultivated under photosynthetic growth conditions. Enzyme activity could be localized mainly to the periplasmic space and this was confirmed by using cytochrome c₅₅ and β-galactosidase enzymes as control markers localized to the periplasm and cytoplasm, respectively. CA activity was unchanged when cells were grown under different anaerobic conditions: photoheterotrophic light, photoautotrophic light or fermentative dark conditions. Under photoheterotrophic conditions, the levels of CA are not regulated in response to the growth substrate, and enzymic activities are similar in alcohol, organic acid or alkylamine-grown cells. No correlation was established between CA activity and the concentration of C₅ in the media. Little [<0.1 U (mg protein)⁻¹] or no activity was detected when cells were cultured under aerobic conditions.

**CA protein structural relationships**

Cloning of the *acaP* gene and determination of the amino acid sequence demonstrates that *Rps. palustris* CA is homologous to x-type CAs. Stronger homology and other distinctive features characteristic of bacterial CAs of the same family were recognized, which suggest a common molecular structure and also a role in the localization of the enzyme. The NH₂ terminus of all bacterial CAs contains a possible signal sequence and the AXA signal peptide recognition motif can be seen in all of the cases except in the *Klebsiella* enzyme. Four distinct deletions in the sequence can be seen when compared to human CA isozymes I and II (data not shown). Three of them are located in the regions that connect β strands D, E and F, which are central characteristics of the dominating structural element, the 10-stranded β sheet of all x-CAs (Eriksson et al., 1988). A fourth gap is located close to the C terminus and might result in a shorter corner loop which is longer in the human isoenzymes molecular periphery region (Eriksson et al., 1988). Thirty-six amino acids are found to be highly conserved among all x-CAs. Three histidines (His-94, His-96, His-119), which are three zinc ligands in the enzyme, as well as other conserved amino acids (Gln-92, Glu-117, Ala/Val-121, Val-143) are unchanged. Interestingly, there are two positions where all bacterial CAs have cysteine residues (Cys-53 and Cys-200; *Rps. palustris* numbering) while human isozymes have alanine and leucine in those positions. In the case of the newly discovered x-type CA from the thylakoid membrane of *C. reinhardtii*, cysteines are also found in these positions (Karlsson et al., 1998); this may suggest a close evolutionary relationship to bacterial CAs.

**Localization and expression of CA**

CA and RubisCo are associated in the carboxysomes of cyanobacteria and possibly in the pyrenoids of algae (Price et al., 1992). The carboxysomes are postulated to contain a specialized CA at the active site to provide CO₂. It is also possible that intracellular CA aids in the conversion of CO₂ to HCO⁻₃ for fixation by phosphoenolpyruvate carboxylase (Hatch & Burnell, 1990). Both biochemical pathways are found in photosynthetic bacteria (Buchanan et al., 1967; Inui et al., 1997; Tabita, 1995) although carboxysomes are not found in PNSB and our results suggest that CA enzyme is not associated with RubisCo, because the main CA activity was found in the periplasmic fraction. Periplasmic localization and the presence of the enzyme only under anaerobic conditions suggests that CA participates in C₅ utilization in this bacterium. We also tested two other PNSB, *Rba. sphaeroides* and *Rsp. rubrum*, and found the same phenomenon (data not shown). This suggests that an x-type CA exists in these PNSB as well. We cannot exclude the presence of other
intracellular accumulation systems for CO₂ because when different CO₂ concentrations were used under photosynthetic conditions, the expression and activity of the CA studied here did not change. Other CO₂ transport proteins probably act under photosynthetic conditions, either in cooperation with CA or independently. These aspects of the uptake mechanism await clarification.

Anaerobic induction and transcriptional regulation of the acaP gene is suggested by the analysis of the acaP::lacZ fusion. A 20-fold change in β-galactosidase activity was observed in the case of Rps. palustris, when cells were incubated anaerobically. Induction occurred within several hours and was also detected on plates where the cells became blue in the presence of X-Gal. In E. coli, the β-galactosidase activity remained unchanged when cells were shifted from aerobic to anaerobic conditions, suggesting that the anaerobic regulation of CA expression is specific for Rps. palustris. In E. coli, β-galactosidase expression from the acaP–lacZ fusion gene was much weaker than in the case of the full length lacZ gene with its own promoter. These results suggest that the promoter of acaP is quite weak and may be regulated at the transcriptional level by oxygen via an oxygen-responsive transcriptional regulatory system as found in other bacteria (Monson et al., 1995; Unden & Schirawski, 1997). However, FixL-like and Fnr-like recognition sequences found in other oxygen regulatory systems could not be discerned. It is also possible that the expression of CA is controlled by a global regulatory system, like the one recognized to control aerobic and anaerobic CO₂ metabolism in Rps. rubrum and Rba. sphaeroides. In these bacteria the RegA/RegB system was found to regulate the aerobic and anaerobic expression of genes encoding enzymes of primary and alternative CO₂ fixation and integrates photosynthesis and nitrogen fixation (Qian & Tabita, 1996; Joshi & Tabita, 1996). However, a RegA-like consensus recognition motif (Du et al., 1998) was not found in the sequence of acaP, nor upstream of the coding region, which suggests that a different regulatory system is involved.

Physiological role of CA

In PNSB, CA has an important role under anaerobic growth conditions when bicarbonate is the CO₂ source. Growth inhibition could be detected by inhibiting the extracellular CA of Rps. palustris by the specific CA inhibitor AZ, and a growth defect was observed in the acaP mutant. Growth of wild-type cells in the presence of AZ or the acaP mutant was slowed by several days but this inhibitory effect could be largely reversed using CO₂, or partially reversed when the pH of the media was lowered and the non-enzymic conversion of bicarbonate to CO₂ is much faster. From these results we assume that the cell membrane is a barrier to the bicarbonate ion, but that CO₂ can penetrate by diffusion into the cytoplasm. The function of CA in Rps. palustris, and probably in other PNSB, is to accelerate the bicarbonate–CO₂ conversion in the periplasmic space. This process might be analogous to CA function in cyanobacteria and algae; however, intact cells of cyanobacteria exhibit very low CA activity and inactivation of the cyanobacterial ecaA gene, which encodes extracellular CA, did not give a dramatic change in photosynthetic growth (Soltes-Rak et al., 1997). Although there are slight differences in periplasmic CA activities, CA seems to function universally in CO₂ utilization in photosynthetic microorganisms.

We believe that this work contributes to a basic understanding of the key reactions in CO₂ utilization and of the nature of anaerobic/aerobic gene regulation in Rps. palustris. We also hope that this knowledge will prove useful in efforts to improve CO₂ fixation and photosynthetic ability in this species for a variety of biotechnological applications.

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