Evidence for the presence of the reductive pentose phosphate cycle in a filamentous anoxygenic photosynthetic bacterium, *Oscillochloris trichoides* strain DG-6

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Studies on autotrophic CO₂ fixation by the filamentous anoxygenic photosynthetic bacterium *Oscillochloris trichoides* strain DG-6 demonstrated that, unlike other green bacteria, this organism metabolized CO₂ via the reductive pentose phosphate cycle. Both key enzymes of this cycle – ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoribulokinase – were detected in cell extracts. The main product of ribulose 1,5-bisphosphate-dependent CO₂ fixation was 3-phosphoglyceric acid. KCN, which is known to be a competitive inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase, completely inhibited the CO₂ assimilation by whole cells as well as by cell extracts of *O. trichoides*. The ¹³C/¹²C carbon isotope fractionation during photoautotrophic growth of *O. trichoides* was –19.7‰, which is close to that obtained for autotrophic organisms that use ribulose-1,5-bisphosphate carboxylase as the primary carboxylation enzyme. Cell extracts of *O. trichoides* contained all the enzymes of the tricarboxylic acid cycle except 2-oxoglutarate dehydrogenase. No activity of isocitrate lyase, a key enzyme of the glyoxylate shunt, was found in cell extracts of *O. trichoides* DG-6.

Keywords: green bacteria, *Oscillochloris trichoides*, CO₂ fixation pathway, ribulose-1,5-bisphosphate carboxylase/oxygenase, tricarboxylic acid cycle

INTRODUCTION

Oscillochloris trichoides DG-6 belongs to the filamentous anoxygenic photosynthetic bacteria (Pierson & Castenholz, 1995). As far as we know, this strain of *Oscillochloris* is the only one that has been isolated in pure culture (Keppen *et al.*, 1993, 1994). Morphologically it is similar to *Chloroflexus aurantiacus*. *O. trichoides* DG-6, however, unlike *Chloroflexus aurantiacus*, produces gas vesicles, is mesophilic and grows only in the light under anaerobic conditions. The results of DNA–DNA hybridization and nucleotide sequencing of 5S rRNA indicate the absence of a close phylogenetic relationship between this species, *Chloroflexus aurantiacus* and the green sulfur bacterium *Chlorobium vibrioforme* (Keppen *et al.*, 1994). *O. trichoides* is a photolithoautotroph that uses hydrogen or sulfide for

CO₂ fixation. The pathway of CO₂ fixation is not known. The ability to use organic substrates is very restricted. Acetate and pyruvate were the only substrates that stimulated the growth of *O. trichoides* DG-6 on medium that contained sulfide and bicarbonate (Keppen *et al.*, 1994). In this communication, we present evidence for operation of the reductive pentose phosphate cycle in *O. trichoides* strain DG-6.

METHODS

Bacterial strains and growth conditions. *O. trichoides* strain DG-6 (DM MSU 327) was grown in the medium described earlier (Keppen *et al.*, 1994). The medium contained Na₂S·9H₂O (0.05%, w/v), NaHCO₃ (0.1%, w/v) and sodium acetate (0.1%, w/v) as growth substrates. Carbon isotope fractionation was determined in cells grown photoautotrophically in medium without acetate. The concentration of bicarbonate was 0.5% (w/v). The cultures were grown under anaerobic conditions (28 °C, 2000 lx) in completely filled 500 ml bottles with magnetic stirring.

Abbreviations: PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate.

Chlorobium vibrioforme strain 8327 was grown photoautotrophically in completely filled bottles (28 °C, 2000 lx) on the medium described by Larsen (1952) with NaHCO_3 (0.5%), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.1%) and sodium thiosulfate (0.2%, w/v).

Thiocapsa roseopersicina strain BBS (DM MSU 317) was grown photoautotrophically in completely filled bottles (28 °C, 2000 lx) on modified Pfennig medium (Bogorov, 1974) with NaHCO_3 (0.5%), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.1%), sodium thiosulfate (0.2%) and NaCl (2%, w/v).

Chloroflexus aurantiacus strain B-3 (DM MSU 322) was grown photoautotrophically on modified DGN medium (Keppen *et al.*, 1994) in which sulfide was replaced by hydrogen as the electron source and the final concentration of NaHCO_3 was 0.5% (w/v). The cells were grown at 55 °C (2000 lx) in screw-capped bottles approximately one-third full of medium and the remaining volume filled with H_2 gas.

Whole-cell labelling studies. Cultures from the late exponential-growth phase were harvested, washed twice with mineral growth medium lacking Na_2S and NaHCO_3 and resuspended in the same medium at a density of 0.1–0.3 mg protein ml^{-1} . Experiments for the assimilation of $^{14}\text{CO}_2$ by cells of phototrophic bacteria were carried out in medical syringes at a light intensity of 3000 lx. After 30 min preincubation with NaHCO_3 (5 mM), Na_2S (500 mg l^{-1}) (or H_2) and inhibitors (if necessary), the reaction was started by the addition of $\text{NaH}^{14}\text{CO}_3$ (0.04 MBq), and stopped at fixed time intervals by the filtration of 1 ml cell suspension through 0.45 μm nitrocellulose filters. Filters were counted in an LKB RacBeta model 1127 liquid scintillation counter.

Preparation of cell extracts and enzyme assays. Cells were collected and washed as described above and finally resuspended in TMD buffer (50 mM Tris/HCl, 5 mM MgCl_2 , 5 mM DTT, pH 7.8) at a density of 5–10 mg protein ml^{-1} . Unless otherwise indicated, cell extracts were prepared by sonication at 22 kHz, 2 min, 4 °C. Debris was removed by centrifugation of the extract at 45 000 g for 30 min (4 °C) and the supernatant (cell extract) was used for enzymic studies.

Enzyme activities were assayed immediately after preparation of cell extracts. All enzyme assays were carried out at room temperature under aerobic conditions and were repeated using at least two independent cultures.

Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was determined as the ribulose 1,5-bisphosphate-dependent fixation of $\text{NaH}^{14}\text{CO}_3$ into acid-stable products using a modification of the procedure described by Schauder *et al.* (1987). The assay was performed in Eppendorf tubes. The reaction mixture (0.5 ml) contained: TMD buffer, $\text{NaH}^{14}\text{CO}_3$ (15 mM, 0.04 MBq) and cell extract (0.5–1.0 mg protein). When needed, KCN (1 mM) was added. After preincubation for 5 min the reaction was started by the addition of ribulose 1,5-bisphosphate (1.0 mM). The reaction was stopped after 5, 10 or 15 min by the addition of 100 μl 12 M formic acid. ^{14}C fixed during the incubation period was determined from samples dried at 80 °C and counted by liquid scintillation.

Phosphoribulokinase (EC 2.7.1.19) was assayed by two methods. The first involved coupling ribulose 5-phosphate conversion to ribulose 1,5-bisphosphate with the ribulose-1,5-bisphosphate carboxylase reaction. The reaction conditions were as with ribulose 1,5-bisphosphate carboxylase, except that ribulose 1,5-bisphosphate was replaced by ribulose 5-phosphate (1.5 mM). The reaction was started by the addition of ribulose 5-phosphate. The second method followed the appearance of ADP (Hart & Gibson, 1975). The reaction mixture contained 50 mM Tris/HCl pH 7.5, 5 mM ATP, 15 mM MgCl_2 , 1 mM ribulose 5-phosphate, 0.4 mM NADH,

2.5 mM phosphoenolpyruvate (PEP), 5 units pyruvate kinase (type II, from rabbit muscle), 5 units lactate dehydrogenase (type XVII, from bovine heart), 5 mM DTT and cell extract (0.03–0.05 mg protein ml^{-1}).

PEP carboxylase (EC 4.1.1.31), PEP carboxykinase (EC 4.1.1.32) and PEP carboxytransphosphorylase (EC 4.1.1.38) were assayed radiochemically by determining PEP-dependent CO_2 fixation. The assay mixture contained: 50 mM Tris/HCl pH 7.5, 6 mM PEP, 15 mM (0.04 MBq) $\text{NaH}^{14}\text{CO}_3$, 0.6 mM NADH, 4 mM MnCl_2 , 5 mM DTT and cell extract (0.5–1.5 mg protein ml^{-1}). For PEP carboxykinase and PEP carboxytransphosphorylase determination, the above mixture was supplemented with 5 mM ADP and 2 mM K_2HPO_4 , respectively.

Pyruvate carboxylase (EC 6.4.1.1) was assayed radiochemically by determining pyruvate-dependent CO_2 fixation. The reaction mixture contained: TMD buffer, 80 mM pyruvate, 15 mM (0.04 MBq) $\text{NaH}^{14}\text{CO}_3$, 5 mM ATP and cell extract (2.5–3.0 mg protein ml^{-1}).

Acetyl-CoA synthetase (EC 6.2.1.1) was measured according to Berg (1956). Citrate synthase (EC 4.1.3.7) was assayed using 5,5'-dithiobis-2-nitrobenzoate as described by Srere (1969). Isocitrate dehydrogenase (EC 1.1.1.42) was determined by following the reduction of NADP with isocitrate according to Cleland *et al.* (1969). Aconitase (EC 4.2.1.3) was assayed in a manner similar to the assay for isocitrate dehydrogenase, but isocitrate was substituted with citrate. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was determined by following the reduction of NAD with 2-oxoglutarate as described by Reed & Mukherjee (1969). Succinate dehydrogenase (EC 1.3.99.1) was assayed by following the reduction of triphenyltetrazolium chloride at 485 nm as described by Holo & Sirevag (1986). Fumarate hydratase (EC 4.2.1.2) was determined photometrically by following fumarate disappearance at 240 nm as described by Brandis-Heep *et al.* (1983). Malate dehydrogenase (EC 1.1.1.37) was assayed by following NADH oxidation by oxaloacetate according to Yoshida (1969). Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) were measured as described Dixon & Kornberg (1959).

For the measurement of pyruvate- and 2-oxoglutarate synthase reactions, cells were resuspended in TMD buffer with 1 mM EDTA. Frozen cell suspensions were broken by passing through a cooled X-press. Debris was removed by centrifugation of the extract at 45 000 g for 15 min (4 °C). The supernatant was used for enzymic studies immediately. Pyruvate synthase (EC 1.2.7.1) was demonstrated as pyruvate synthesis and as pyruvate- $^{14}\text{CO}_2$ exchange. The pyruvate synthesis reaction mixture (in a final volume of 1 ml) consisted of: TMD buffer, 0.5 mM EDTA, 2 mM methyl viologen, 1 mM acetyl-CoA, 2 mM serine, 10 mM $\text{NaH}^{14}\text{CO}_3$ (0.04 MBq) and cell extract (0.5–1.0 mg protein). Acetyl-CoA was omitted in the control. The exchange reaction mixture (in a final volume of 1 ml) consisted of: TMD buffer, 0.5 mM EDTA, 0.1 mM acetyl-CoA, 10 mM sodium pyruvate, 10 mM (0.04 MBq) $\text{NaH}^{14}\text{CO}_3$ and cell extract (0.5–1.0 mg protein). Both assays were conducted anaerobically under hydrogen. 2-Oxoglutarate synthase (EC 1.2.7.3) was assayed in a 2-oxoglutarate- $^{14}\text{CO}_2$ exchange reaction in similar manner to the assay for pyruvate synthase, except that pyruvate was substituted with 2-oxoglutarate and acetyl-CoA was substituted with succinyl-CoA.

Paper chromatography. After termination of the ribulose-1,5-bisphosphate carboxylase reaction by the addition of ethanol (1 ml) and removal of denatured protein by centrifugation, the products of the reaction were examined by ascending two-

dimensional chromatographic procedures using Whatman no. 1 filter paper (12 × 12 cm) according to established procedures (Bandurski & Axelrod, 1951; Wood, 1968). Following addition of about 3500 c.p.m. of the reaction mixture to the paper, it was developed first in an acid solvent (methanol/88% formic acid/water; 80:15:5, by vol.) and then in the basic solvent (methanol/ammonium hydroxide/water; 60:10:30, by vol.). Another method used for the determination of the reaction products was the method described by Bassham & Calvin (1957). The solvent used in the first dimension was a neutral solvent (phenol/water; 72:28, w/w) and the second solvent was an acid one (n-butanol/propionic acid/water; 100:50:70, by vol.). 3-Phosphoglycerate (3-PGA) was visualized by using phosphomolybdate as the indicator and labelled compounds were located by autoradiography. The verification of 3-PGA on chromatograms was established by co-chromatographic procedures.

Carbon isotope measurements. Dry biomass samples were combusted to CO₂ in sealed Pyrex tubes. Approximately 5 mg dried sample and 0.5 g copper oxide wire were placed into a preheated Pyrex tube (length 20 cm, outer diameter 8 mm) sealed at one end. Sample tubes were then attached to a vacuum line, evacuated to 2.67 Pa and sealed with a torch. The sealed tubes were placed in a muffle furnace at 550 °C for 48 h.

Gases from the combusted tubes were released into a sampling vacuum line. The CO₂ was cryogenically purified and transferred into tubes (2 ml volume). The tubes were broken in a device connected with an inlet system to the mass spectrometer.

The CO₂ was precipitated as BaCO₃ following addition of the saturated solution of Ba(OH)₂ to the culture medium. Organic admixtures were removed by ignition in the oxygen atmosphere at 550 °C. Carbonate was converted to CO₂ by the addition of H₃PO₄ after the evacuation of the atmospheric gases from the reaction vessel.

Isotopic abundance was determined using a dual-inlet, dual-collector mass spectrometer CH-7 (Varian). The $\delta^{13}\text{C}$ value is presented as per mille deviation from the PDB standard (Craig, 1957):

$$\delta^{13}\text{C} = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right] \times 10^3 \text{‰}$$

Protein was measured according to the Lowry method, using bovine serum albumin as standard.

Materials. Enzymes and biochemicals were purchased from Sigma. NaH¹⁴CO₃ was obtained from Amersham. Other materials were from standard commercial sources.

RESULTS AND DISCUSSION

O. trichoides DG-6 is capable of photoautotrophic assimilation of bicarbonate using sulfide as an electron donor. The addition of cyanide, a competitive inhibitor of ribulose-1,5-bisphosphate carboxylase (Takabe & Akazawa, 1977), leads to complete inhibition of ¹⁴CO₂ assimilation in *O. trichoides* DG-6 cells (Table 1). Analogous data were obtained for *T. roseopersicina*, which uses the Calvin cycle for CO₂ assimilation. By contrast, CO₂ fixation by whole cells of *Chlorobium* and *Chloroflexus*, which do not use the reductive pentose phosphate cycle for autotrophic CO₂ fixation, was not affected by KCN (or cyanide had a subtle effect

Table 1. Effect of KCN on bicarbonate assimilation by whole cells of phototrophic bacteria

The rate of bicarbonate fixation in the absence of electron donor (sulfide or hydrogen) was negligible (<10%). Measurements were done in triplicate and values were within ±25% of each other.

Organism	Rate of bicarbonate fixation [nmol min ⁻¹ (mg protein) ⁻¹]	
	Sulfide	Sulfide + KCN*
<i>Oscillochloris trichoides</i>	27.3	0.7
<i>Thiocapsa roseopersicina</i> †	76.0	3.6
<i>Chloroflexus aurantiacus</i> †	7.1	4.3
<i>Chlorobium vibrioforme</i> ‡	246.2	229.0

* The concentration of KCN was 1 mM.

† Sulfide was replaced by hydrogen as the electron source.

‡ The concentration of Na₂S was 1 g l⁻¹.

Table 2. Activity of enzymes involved in CO₂ assimilation in *Oscillochloris trichoides*

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
Ribulose-1,5-bisphosphate carboxylase	33.0
Phosphoribulokinase*	48.7
PEP carboxylase	1.5
PEP carboxykinase	8.0
PEP carboxytransphosphorylase	12.5
Pyruvate carboxylase	13.0
Pyruvate synthase	
Pyruvate synthesis	2.3
Pyruvate- ¹⁴ CO ₂ exchange	2.8
2-Oxoglutarate synthase†	2.6

* This activity was obtained by following the appearance of ADP. When measured by the method which involved coupling ribulose 5-phosphate conversion to ribulose 1,5-bisphosphate with the ribulose-1,5-bisphosphate carboxylase reaction, lower activity was obtained [11 nmol min⁻¹ (mg protein)⁻¹].

† This enzyme was measured as 2-oxoglutarate-¹⁴CO₂ exchange.

on CO₂ fixation) (Table 1). These data indicate that a functional reductive pentose phosphate cycle is present in *O. trichoides* DG-6. Activity of ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase, the key enzymes of this cycle, was detected in cell extracts of *O. trichoides* DG-6 (Table 2) and the observed activity of these enzymes is sufficient to support the observed growth rate of *O. trichoides* DG-6 under autotrophic conditions ($\mu = 0.045 \text{ h}^{-1}$).

The main product of ¹⁴C-bicarbonate assimilation in the presence of ribulose 1,5-bisphosphate by cell extracts of

Fig. 1. Radioactive products formed from $\text{NaH}^{14}\text{CO}_3$ in the presence of ribulose 1,5-bisphosphate and cell extracts of *O. trichoides*. The basic solvent (methanol/ammonium hydroxide/water) was used in the second dimension (arrow 1) and the acid solvent (methanol/formic acid/water) was used in the first dimension (arrow 2). The assay and chromatography analysis were performed as described in Methods.

O. trichoides DG-6 was identified by paper chromatography: in the systems used [methanol/formic acid/water and methanol/ammonium hydroxide/water (see Fig. 1), and phenol/water and n-butanol/propionic acid/water (data not shown)], a labelled product comigrated with authentic 3-PGA. Like ^{14}C -bicarbonate fixation by whole cells, carboxylation of ribulose 1,5-

bisphosphate in cell extracts of *O. trichoides* DG-6, as well as in cell extracts of *T. roseopersicina*, was repressed in the presence of cyanide (Fig. 2). These data support the conclusion that CO_2 fixation in *O. trichoides* DG-6 under photoautotrophic conditions is carried out via the reductive pentose phosphate cycle. This was confirmed by the analysis of the results of $^{13}\text{C}/^{12}\text{C}$ stable isotope fractionation. It was shown earlier (Sirevag *et al.*, 1977; Quandt *et al.*, 1977) that photosynthetic bacteria which incorporate CO_2 by ribulose-1,5-bisphosphate carboxylase fractionate ^{13}C isotope to a much larger degree than bacteria that use other carboxylation reactions. The results of our isotope studies (Table 3) are consistent with the conclusion that the photosynthetic bacterium *O. trichoides* DG-6, like *T. roseopersicina*, fixes CO_2 via the reductive pentose phosphate cycle. A higher level of ^{13}C isotope fractionation ($\Delta^{13}\text{C}$) was observed in *O. trichoides* DG-6 than in *Chloroflexus aurantiacus* and *Chlorobium vibrioforme*, which use different pathways for CO_2 utilization. Thus, taken together, the biochemical data and fractionation studies indicate that *O. trichoides* DG-6 fixes CO_2 via the reductive pentose phosphate cycle.

A number of enzymes catalysing carboxylation of pyruvate and PEP were also determined (Table 2). The function of these enzymes in *O. trichoides* DG-6 may be the utilization of PEP synthesized in the course of bicarbonate fixation via the reductive pentose phosphate cycle. Oxaloacetate and malate, formed as a result of these carboxylation reactions, are metabolized via a truncated tricarboxylic acid cycle. All enzymes of this cycle except 2-oxoglutarate dehydrogenase were de-

Fig. 2. Activity of ribulose-1,5-bisphosphate carboxylase in cell extracts of *O. trichoides* (4 mg protein) (a) or *T. roseopersicina* (2.7 mg protein) (b) in the presence of KCN. ■, Complete assay mixture; ▲, complete assay mixture + KCN; ◆, complete assay mixture without ribulose 1,5-bisphosphate.

Table 3. $^{13}\text{C}/^{12}\text{C}$ -carbon isotope fractionation by different phototrophic bacteria

Organism	$\delta^{13}\text{C}_{\text{cells}}$ (‰)*	$\delta^{13}\text{C}_{\text{medium}}$ (‰)†	$\Delta^{13}\text{C}$ (‰)‡
<i>Oscillochloris trichoides</i>	-28.2	-8.5	-19.7
<i>Thiocapsa roseopersicina</i>	-29.8	-8.2	-21.6
<i>Chloroflexus aurantiacus</i>	-22.5	-10.0	-12.5
<i>Chlorobium vibrioforme</i>	-20.5	-9.3	-11.2

* $\delta^{13}\text{C}_{\text{cells}}$, carbon isotope contents of CO_2 in the cell biomass.

† $\delta^{13}\text{C}_{\text{medium}}$, carbon isotope contents of the culture medium.

‡ $\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{cells}} - \delta^{13}\text{C}_{\text{medium}}$.

Table 4. Activity of enzymes of the tricarboxylic acid cycle in *Oscillochloris trichoides*

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
Acetyl-CoA synthetase	78.2
Citrate synthase	11.6
Aconitase	63.8
Isocitrate dehydrogenase	34.3
2-Oxoglutarate dehydrogenase	<0.1
Succinate dehydrogenase	11.2
Fumarate hydratase	200.9
Malate dehydrogenase	173.3
Isocitrate lyase	<0.1
Malate synthase	15.2

tected (Table 4). No activity of isocitrate lyase, a key enzyme of the glyoxylate shunt, was found in cell extracts of *O. trichoides* DG-6. The absence of 2-oxoglutarate dehydrogenase and isocitrate lyase explains the inability of this bacterium to maintain photoheterotrophic growth with organic substrates as the electron donor (Keppen *et al.*, 1994). As an additional carbon source, acetate might be involved in metabolism via a citrate synthase reaction leading to synthesis of 2-oxoglutarate. Assimilation of acetate and pyruvate is also feasible by the following sequence of reactions:

The possibility of such reactions is provided by the presence of pyruvate- and 2-oxoglutarate synthases in *O. trichoides* DG-6 (Table 2). In this respect *O. trichoides* DG-6 resembles the purple sulfur bacterium *T. roseopersicina* strain BBS which is an obligate autotroph that is able to assimilate a limited number of organic substrates in the presence of CO_2 (Kondratieva *et al.*, 1981).

According to available data, three autotrophic CO_2 fixation pathways have been found among green bacteria so far: the reductive citric acid cycle in green sulfur bacteria (Evans *et al.*, 1966), the 3-hydroxypropionate cycle in *Chloroflexus aurantiacus* OK-70 (Holo, 1989; Strauss & Fuchs, 1993) and the reductive dicarboxylic acid cycle in *Chloroflexus aurantiacus* B-3 (Ivanovsky *et al.*, 1993). This communication provides evidence that in some green bacteria, i.e. *O. trichoides* DG-6, the reductive pentose phosphate cycle is operating as the mechanism of CO_2 fixation.

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