Assay and properties of acetone carboxylase, a novel enzyme involved in acetone-dependent growth and CO₂ fixation in *Rhodobacter capsulatus* and other photosynthetic and denitrifying bacteria

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The photosynthetic bacterium *Rhodobacter capsulatus* is able to grow, in the presence of carbon dioxide, under anaerobic (photosynthetic) conditions with the solvents acetone or butanone as carbon source. The carboxylation of acetone to form acetoacetate is the most likely initial step in acetone metabolism. This paper describes an assay for acetone carboxylation, in which fixation of radiolabelled carbon dioxide from NaH¹⁴CO₃ is measured in the presence of acetone, ATP, magnesium ions and acetyl-CoA. Acetone carboxylase activity was specifically induced by growth of *R. capsulatus* on acetone or butanone and was associated with a high-molecular-mass protein complex containing two major polypeptides, of 70 and 85 kDa. Partial purification of the activity was achieved by FPLC ion-exchange chromatography, which confirmed that the 70 and 85 kDa proteins were subunits of the enzyme and suggested that at least one additional protein (60 kDa) may be associated with carboxylase activity. N-terminal sequences of the two major subunits were not significantly similar to any other carboxylases in the databases and neither contained covalently bound biotin, indicating that the enzyme represents a novel type of carboxylase. Acetone carboxylase activity was also demonstrated in cell-free extracts of acetone-grown *Rhodomicrobium vannielii* and the denitrifying bacterium *Thiosphaera pantotropha*.

Keywords: acetone, carboxylase, *Rhodobacter*, *Rhodomicrobium*, *Thiosphaera*

INTRODUCTION

Acetone and other ketones become available for bacterial metabolism from both natural and man-made sources. Several bacteria, for example *Clostridium acetobutylicum*, produce acetone as a product of their fermentative metabolism (Gottschalk, 1986) and ketones are also produced and used in industrial processes, notably in the production of perspex and as organic solvents. Since the initial discovery that the purple photosynthetic bacterium *Rhodocyclus gelatinosus* was capable of utilizing acetone as a substrate for growth (Seigel, 1950), subsequent studies

have demonstrated that several different physiological groups of bacteria are capable of growing on ketones aerobically (Taylor *et al.*, 1980; Sluis *et al.*, 1996), anaerobically (Platen & Schink, 1989; Platen *et al.*, 1990) or under both conditions (Bonnet-Smits *et al.*, 1988). Given the known metabolic diversity of the *Rhodospirillaceae*, it would be expected that some other members of this group would utilize acetone as a carbon source. Madigan (1990) studied a range of purple non-sulphur bacteria, and found that some strains of *Rhodobacter capsulatus* and *Rhodomicrobium vannielii* could indeed grow phototrophically on acetone. Preliminary experiments also suggested that *R. capsulatus* was capable of growth on acetone under microaerobic (but not fully aerobic) conditions.

Anaerobic growth on ketones has been studied mainly in

Abbreviations: PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate.

denitrifying bacteria, and several observations have led to the conclusion that an initial carboxylation of acetone is the first step in its anaerobic metabolism. Firstly, anaerobic growth on acetone in all bacteria so far studied is strictly dependent on CO₂, usually added in the form of bicarbonate. Secondly, labelling studies (Bonnet-Smits *et al.*, 1988; Platen & Schink, 1989) have shown that ¹³CO₂ or ¹⁴CO₂ was incorporated mainly into the C-1 atom of the polyhydroxybutyrate (PHB) monomer, consistent with an initial carboxylation of acetone to acetoacetate. This reaction is, however, endergonic [$\Delta G^{\circ} = +17.1 \text{ kJ (mol acetone)}^{-1}$; Thauer *et al.*, 1977; Platen & Schink, 1989, 1990], so the actual mechanism of carboxylation must be more complex, involving activation of acetone before carboxylation, possibly by phosphorylation (Platen & Schink, 1989, Lack & Fuchs, 1992, 1994).

Until very recently, attempts to assay acetone carboxylase activity directly have failed. Previous studies with the denitrifying strain BunN (Platen & Schink, 1990) have demonstrated an acetoacetate decarboxylase activity which was induced in acetone-grown cells. The activity was low [$4\text{--}8 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] but was shown to be dependent on both ADP and magnesium ions. Subsequent work (Janssen & Schink, 1995), using dialysed cell free extracts taken from the same strain, demonstrated ¹⁴CO₂ incorporation into acetoacetate in an isotope-exchange reaction which may be a partial reaction of the acetone carboxylase, catalysed by an acetoacetyl-enzyme-ADP complex which is a postulated intermediate of the net carboxylation reaction. More recently, Sluis *et al.* (1996) have reported the direct assay of CO₂ and ATP-dependent acetone degradation activity in cell-free extracts of the obligate aerobe *Xanthobacter* strain Py2.

In this study, an assay for acetone-dependent ¹⁴CO₂ fixation has been developed and has been used to characterize the acetone carboxylase activity of *Rhodobacter capsulatus* in cell-free extracts and in a partially purified form. The results indicate that this enzyme is a novel type of biotin-independent carboxylase.

METHODS

Bacterial strains, growth media and culture conditions. *Rhodobacter capsulatus* strain PAS100 (Taylor *et al.*, 1983) and *Rhodospirillum rubrum* strain RB4 (Leyland & Kelly, 1991) were routinely grown and maintained photoheterotrophically (anaerobic/light) in RCV minimal salts medium (Weaver *et al.*, 1975) with the modifications of Hillmer & Gest (1977). For routine subculturing, RCV medium contained 30 mM DL-lactate as carbon source, plus 0.05% (w/v) yeast extract. For growth experiments, acetone (25 mM), butanone (25 mM) or sodium pyruvate (50 mM) was added to minimal RCV medium after autoclaving. For growth on ketones, sodium ascorbate (0.1%) and sodium bicarbonate (final concentration 50 mM) were also added to the autoclaved medium, from filter-sterilized stock solutions. Cultures were routinely grown in 500 ml medical flat bottles filled almost to capacity with medium and incubated at 30 °C at a distance of 20 cm from a 60 W tungsten bulb. *Thiosphaera pantotropha*

LMD82.1 (Robertson & Kuenen, 1983) was grown at 30 °C in the dark in RCV-acetone with the addition of sodium nitrate (final concentration 50 mM) as electron acceptor. In all cases, growth was monitored by aseptically removing samples and measuring optical density at 710 nm.

¹H-NMR analysis of culture supernatants. Samples (1 ml) from cultures grown on acetone were centrifuged at 13800 g for 3 min to remove cells, and the supernatant was stored at -20 °C. ¹H-NMR spectra at 500 MHz were obtained at 30 °C with a presaturation pulse for water suppression using a Bruker AMX-500 spectrometer. Typical acquisition parameters were: sweep width of 12500 Hz, pulse width of 8 µs (90°), 16384 data points, with an acquisition time of 655 ms and a relaxation delay of 1 s. Spectra were obtained in 5 mm (outer diameter) NMR tubes with 10% (v/v) D₂O added for the field frequency lock, without sample spinning. Known concentrations of sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate (TSP) were also added to the samples as a 0 p.p.m. reference and for quantification. Spectra were processed and displayed using the software package FELIX (Biosym Technologies).

HPLC analysis of polyhydroxyalkanoates. PHA polymer samples were isolated from lyophilized cells, by 24 h incubation in chloroform. The PHA sample was then decomposed to its constituent acids by acid hydrolysis with 55% (w/v) perchloric acid (80 °C for 60 min). This was subsequently neutralized with an appropriate quantity of NaOH, determined by titration with methyl red indicator. A 20 µl sample of the neutralized acid products was then filtered through a 0.45 µm Acrodisc HT and separated by HPLC on an Aminex HPLC-87H (300 mm × 7.8 mm i.d.) column, using a Waters model 510 HPLC pump. Sulphuric acid (8 mM) was used as the mobile phase, which also served to continuously regenerate the column during its operation. PHA monomers were detected using a Waters model 481 UV detector, and the retention times were compared to samples of known PHA monomers.

Preparation of cell-free extracts. Cells were harvested during the late exponential phase of growth by centrifugation (12000 g, 4 °C, 20 min). The cells were then washed in 10 mM potassium phosphate buffer (pH 7.5) by resuspension, pelleted by centrifugation and finally resuspended in phosphate buffer to the required cell density. Cell-free extracts were prepared by ultrasonication, using an LKB sonicator (5 × 30 s bursts). Debris was removed by centrifugation of the extract at 13800 g for 10 min, and the supernatant (cell-free extract) stored on ice and used within 8 h. Cell-free extracts for use in the acetone carboxylase assay were routinely dialysed with stirring at 4 °C against 1.5 litres 10 mM phosphate buffer, pH 7.2, to remove residual ketone. Cell-free extracts were dialysed for 1 h, with a change of buffer every 15 min. Removal of cytoplasmic membranes from cell-free extracts was accomplished by ultracentrifugation at 150000 g at 4 °C for 2 h. The supernatant was removed and used for partial purification of acetone carboxylase activity.

Spectrophotometric enzyme assays

All assays were carried out at 30 °C in quartz cuvettes (1 cm pathlength) in a total volume of 1 ml. Changes in absorbance were measured using a PYE-Unicam SP1800 double-beam recording spectrophotometer coupled to a Servoscribe chart recorder.

Acetoacetate reductase and acetoacetyl-CoA reductase. Acetoacetate or acetoacetyl-CoA reduction was assayed spectro-

photometrically by following the initial rate of substrate-dependent NAD(P)H oxidation at 340 nm. The reaction mixture contained: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; NADH or NADPH, 1 mM; lithium acetoacetate, 10 mM, or acetoacetyl-CoA, 1 mM; cell-free extract, 0.1–0.5 mg protein. In assays where NADH oxidation was monitored, rotenone (0.1 mM) was also added to the reaction mixture. The reaction was started by the addition of lithium acetoacetate or acetoacetyl-CoA. The initial linear decrease in absorbance was used to calculate the enzyme activity [absorption coefficient for NAD(P)H at 340 nm: $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$].

3-Hydroxybutyrate and 3-hydroxybutyryl-CoA dehydrogenase. 3-Hydroxybutyrate or 3-hydroxybutyryl-CoA reduction was assayed by following the initial rate of substrate-dependent NAD(P) reduction at 340 nm. The reaction mixture contained: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; NAD or NADP, 1 mM; DL-3-hydroxybutyrate, 10 mM, or 3-hydroxybutyryl-CoA, 0.1 mM; cell-free extract, 0.1–0.5 mg protein. Rotenone (0.1 mM) was also added to assays in which NAD reduction was measured. The reaction was started by the addition of DL-3-hydroxybutyrate or 3-hydroxybutyryl-CoA. The initial linear increase in absorbance was used to calculate the enzyme activity.

Acetoacetate:succinyl-CoA/acetyl-CoA transferase. These enzymes catalyse the formation of acetoacetyl-CoA from acetoacetate and succinyl-CoA and/or acetyl-CoA as CoA donor. The initial rate of acetoacetyl-CoA formation was measured directly at 303 nm. The reaction mixture contained: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; lithium acetoacetate, 10 mM; succinyl-CoA or acetyl-CoA, 0.1 mM; cell free extract, 0.1–0.5 mg protein. The reaction was started by the addition of the acyl-CoA. The initial linear increase in absorbance was used to calculate the enzyme activity (absorption coefficient for acetoacetyl-CoA at 303 nm: $14 \text{ mM}^{-1} \text{ cm}^{-1}$). The assay was also carried out in the reverse direction (i.e. as acetoacetyl-CoA:succinate/acetate CoA-transferase). Here, the initial rate of acetoacetyl-CoA disappearance was measured directly at 303 nm. The reaction mixture for the assay contained the following: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; acetoacetyl-CoA, 0.1 mM; sodium succinate or sodium acetate, 10 mM; cell-free extract, 0.1–0.5 mg protein. The reaction was started by the addition of the organic acid. In both the forward and reverse directions, the measured activities were corrected for the deacylation of acetoacetyl-CoA, which was catalysed at a low rate [a few $\text{nmol min}^{-1} (\text{mg protein})^{-1}$] by the cell-free extracts.

3-Ketothiolase. Thiolase catalyses the CoA-dependent cleavage of acetoacetyl-CoA into two moles of acetyl-CoA. The reaction was assayed by following the initial rate of acetoacetyl-CoA cleavage at 303 nm. The reaction mixture contained: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; CoASH, 10 mM; acetoacetyl-CoA, 0.1 mM; cell-free extract, 0.1–0.5 mg protein. The reaction was started by the addition of the CoA. The initial linear decrease in absorbance was used to calculate the enzyme activity.

Isocitrate lyase. The assay method used was a modification of that described by Reeves *et al.* (1971). The assay mixture contained the following: Tris/HCl pH 7.5, 50 mM; MgCl_2 , 10 mM; L-cysteine, 0.3 mM; phenylhydrazine.HCl, 3.3 mM; sodium isocitrate, 3 mM; cell-free extract, 0.1–0.5 mg protein. The reaction was started by the addition of isocitrate. After a short lag period the absorbance at 324 nm increased in a linear fashion due to the formation of glyoxylate phenylhydrazone (absorption coefficient at 324 nm: $17 \text{ mM}^{-1} \text{ cm}^{-1}$).

Acetone carboxylation assay. The standard reaction mixture contained: potassium phosphate buffer, pH 7.2, 10 mM; MgCl_2 , 1 mM; acetone, 1 mM; ATP, 1 mM; CoA, acetyl-CoA or other acyl-CoA, 100 μM ; cell-free extract, 0.2–5.0 mg protein; $\text{NaH}^{14}\text{CO}_3$ ($0.37 \text{ GBq mmol}^{-1}$), 1 mM, in a final volume of 0.25 ml. The assay mixture was preincubated at 30 °C for 5 min prior to the reaction being initiated by the addition of the cell extract, which had also been preincubated separately at the same temperature. The complete assay mixture was then incubated at 30 °C for various time periods, after which 50 μl 12 M trichloroacetic acid was added, followed by incubation at 80 °C for 45 min; this was found to be sufficient to remove any unfixed labelled $\text{NaH}^{14}\text{CO}_3$. Aliquots were then placed in a scintillation vial containing 3 ml scintillation fluid (Beckman Safe-Fluor) and their radioactivity measured in a Beckman LS1801 scintillation counter. For time-course experiments, the reaction mixture had a larger total volume, and 50 μl samples were removed at intervals, transferred to screw-capped Eppendorf tubes containing 10 μl 12 M trichloroacetic acid, sealed, and stored on ice until the total incubation time was completed. Then, all of the tubes were opened and incubated at 80 °C for 45 min. The samples were then scintillation counted. Results are reported as d.p.m. ^{14}C fixed during the incubation period (1 pmol $\text{NaH}^{14}\text{CO}_3$ fixed corresponds to 2.2×10^4 d.p.m.).

Separation of ^{14}C labelled compounds by thin-layer chromatography. Samples for TLC analysis were prepared using the above acetone carboxylase assay, except that higher specific activity $\text{NaH}^{14}\text{CO}_3$ ($1.85 \text{ GBq mmol}^{-1}$) was used. In addition, after the addition of trichloroacetic acid, the samples were incubated on ice for 10 min, omitting the normal heating step, and centrifuged at 14000 g for 5 min. The deproteinized supernatant was spotted onto 250 mm Merck silica gel 60 plates (containing fluorescent indicator F_{254}). The solvent system was ethanol/ammonia/water (80:4:16, by vol.). Plates were developed at 4 °C in this solvent system until the solvent front approached the upper edge of the plate. The position of authentic acetoacetate (lithium salt) was determined by exposure of the plates to UV light. To determine the position of the ^{14}C -containing compounds, the TLC plate was exposed to X-ray film (hyperfilm β -max, Amersham) for 1–2 d.

Localization of acetone carboxylation activity. Cell-free extracts obtained from *R. capsulatus* were fractionated on a 10–60 % (w/v) sucrose gradient prepared in 10 mM phosphate buffer (pH 7.2), which was then centrifuged at 150000 g for 20 h at 4 °C. The gradient was fractionated into 0.5 ml fractions. Each fraction was assayed for acetone carboxylase activity, absorbance at 280 nm (protein) and 850 nm (bacteriochlorophyll), and the sucrose concentrations were determined by refractometry.

Polyacrylamide gel electrophoresis. SDS-PAGE gels (10%, w/v, acrylamide) were prepared and run according to standard methods (Laemmli, 1970), with samples boiled for 2 min in SDS-sample buffer (Laemmli, 1970) before application to the gel. The following molecular mass standards (Pharmacia) were used: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α -lactalbumin (14.4 kDa). Native gel electrophoresis on 5% (w/v) polyacrylamide gels was carried out by essentially the same method, but with SDS omitted from the stacking and resolving gels, running buffer and sample buffer, and with samples being unheated before application to the gel. For the analysis of acetone carboxylase activity associated with protein bands on native gels, the standard Tris/glycine buffer

was replaced with 0.1 M potassium phosphate buffer pH 7.2 in the resolving gel and running buffer. In this case, resolving gels were cast without a stacking gel and samples prepared in 20 mM potassium phosphate buffer pH 7.2 containing 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue. The gels were run at 30 mA constant current in a cold-room maintained at 4 °C. All types of gel were routinely stained with Coomassie brilliant blue. Where increased sensitivity was required, the silver staining method of Wray *et al.* (1981) was used.

Detection of biotinylated proteins by Western blotting. SDS-PAGE, Western blotting and subsequent detection of proteins on the blots containing covalently bound biotin was carried out exactly as described by Modak & Kelly (1995).

Partial purification of acetone carboxylase activity. A membrane-free cell extract was prepared as described above. An aliquot (2 ml) was then injected onto a Mono-Q HR 5/5 column pre-equilibrated with 10 mM phosphate buffer, pH 7.2. The column was eluted (1 ml min⁻¹; 1 ml fractions) with a 0–1 M KCl gradient (0 M KCl for 4 min, 0–1 M KCl for 46 min). The protein content of each of the fractions was monitored by measuring their absorbance at 280 nm. Individual fractions were assayed for acetone carboxylase activity as described above. The active fractions were then pooled and either used immediately, if enzyme activity was required, or stored at –20 °C.

Determination of protein concentration. Protein concentration was determined using the Lowry method with bovine serum albumin as standard.

RESULTS

Phototrophic growth of *Rhodobacter capsulatus* on ketones

It was demonstrated by Madigan (1990) that the B10 strain of *R. capsulatus* was capable of growth on acetone. Strain PAS100 (Taylor *et al.*, 1983), used in this study, is derived from strain B10 and was found to grow on butanone in addition to acetone, but at a slower rate (turbidity doubling time of 15 h on butanone compared with 7 h on acetone). However, no growth was evident on any of several higher ketones tested (pentanone, hexanone, 2-methyl-3-pentanone, acetophenone). In the case of acetone and butanone, growth was dependent on the addition of CO₂ (as sodium bicarbonate) to the medium. The apparent molar growth yield on acetone was determined from an experiment in which the residual acetone concentration after growth had ceased was determined by ¹H-NMR spectroscopy of the culture supernatant. A value of 30 g (dry weight) cells (mol acetone)⁻¹ was obtained.

PHA formation and enzyme profile of ketone-grown cells

Cells grown on acetone and subsequently incubated in N-free RCV-acetone medium deposited significant amounts of PHA (up to about 30% of the dry weight), which HPLC analysis revealed to be exclusively composed of PHB, with no PHV detected. Cells grown on butanone and subsequently incubated in N-free RCV-butanone medium did incorporate small amounts of

PHV (0.8% w/w) into their PHA, but the PHA content of the cells was less than that of acetone-grown cells (about 18% of the dry weight).

Significant PHA formation during growth on acetone has been observed previously (Madigan, 1990) and is consistent with the operation of pathways for the incorporation into PHA of the acetoacetate or acetoacetyl-CoA resulting from acetone carboxylation. Assays for the major enzymes associated with PHA synthesis and degradation were therefore carried out (Table 1). An acetoacetate:succinyl-CoA transferase activity was detected which could form the key intermediate acetoacetyl-CoA as a precursor for both biosynthesis and PHA formation. Its activity was greatest when assayed in the reverse direction (i.e. in the direction of acetoacetate formation). No activity was detected with acetyl-CoA as CoA donor. Thiolase and isocitrate lyase were present at significant specific activity in ketone-grown cells, allowing acetyl-CoA to be formed from acetoacetyl-CoA and assimilated via the citric acid cycle for biosynthetic purposes. The PHA biosynthetic enzyme acetoacetyl-CoA reductase (i.e. the acetoacetyl-CoA reducing activity of 3-hydroxybutyryl-CoA dehydrogenase) was significantly induced during growth on acetone and was most active with NADPH as coenzyme. Conversely, the PHA degradative enzyme 3-hydroxybutyrate dehydrogenase was NAD specific, but when assayed in the reductive direction a significant NADPH-specific acetoacetate reductase activity was also present. In general terms, the specific activities of all of the enzymes detected were considerably higher in ketone-grown cells compared to cells grown on pyruvate (Table 1).

Development of an assay for acetone carboxylase activity: requirements for acetone-dependent CO₂ fixation in cell-free extracts

An assay was developed for acetone carboxylase by including various combinations of possible substrates and cofactors in an assay system designed to detect acetone-dependent ¹⁴CO₂ fixation from NaH¹⁴CO₃. Table 2 shows that significant acetone-dependent fixation of ¹⁴CO₂ into acid-stable products was obtained in a system containing phosphate buffer, ATP, CoA (or acetyl-CoA; see below), magnesium ions and cell-free extract. However, it was consistently found that the use of undialysed cell-free extracts resulted in a much lower level of acetone-dependent ¹⁴CO₂ fixation compared to that seen when dialysed extracts were employed (Table 2). Dialysis presumably removes residual acetone from the extracts. Removing ATP from the dialysed or undialysed reaction mixtures resulted in a large decrease in the ¹⁴CO₂ fixed in the assay. Removing magnesium ions from the assays also decreased the radiolabel fixed, but to a lesser degree. The addition of CoA was clearly needed to obtain maximal acetone-dependent CO₂ fixation. Table 2 also shows that the addition of avidin to the assay resulted in only a small inhibition of activity, indicating that biotin is unlikely to be necessary

Table 1. Representative specific activities of enzymes associated with PHA synthesis and degradation in cell-free extracts of *R. capsulatus* grown on pyruvate, acetone or butanone

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹] with growth substrate:		
	Acetone	Butanone	Pyruvate
Acetoacetate reductase			
+ NADH	60	82	53
+ NADPH	16	23	9
3-Hydroxybutyrate dehydrogenase			
+ NAD	178	163	12
+ NADP	< 1	< 1	< 1
Acetoacetate:succinyl-CoA transferase	8	8	< 1
Acetoacetate:acetyl-CoA transferase	< 1	< 1	< 1
Acetoacetyl-CoA:succinate CoA-transferase	275	165	102
Acetoacetyl-CoA:acetate CoA-transferase	< 1	< 1	< 1
Acetoacetyl-CoA reductase			
+ NADH	23	25	12
+ NADPH	165	50	23
Hydroxybutyryl-CoA dehydrogenase			
+ NAD	6	< 1	< 1
+ NADP	50	16	2
3-Ketothiolase	178	270	51
Isocitrate lyase	20	10	< 1

for carboxylase activity. In contrast to acetone, butanone was unable to act as a substrate in the assay. Finally, activity was completely dependent on both labelled bicarbonate and cell-free extract.

Representative time-courses of activity in the complete system are illustrated in Fig. 1(a, b). Although the inclusion of CoA in the assay clearly stimulated acetone-dependent ¹⁴CO₂ fixation (Fig. 1a, Table 1), it was subsequently found that acetyl-CoA gave a more reproducible and larger degree of stimulation (Fig. 1b). Acetyl-CoA was therefore routinely used in activity measurements. In either case, acetone-dependent ¹⁴CO₂ fixation increased for at least 20 min before reaching a plateau, although linear initial rates were not always obtained. Fig. 1(c) illustrates the pH profile of ¹⁴CO₂ fixation in the presence and absence of acetone. A clearly defined optimum of pH 7.2 characterized acetone carboxylase activity, while the counts incorporated in the absence of added acetone increased only at alkaline pH values. These experiments were carried out using potassium phosphate buffers adjusted to the required pH value, as it was noted that in the presence of Tris/HCl, a strong inhibition of acetone-dependent ¹⁴CO₂ fixation occurred (data not shown).

Growth on ketones induces acetone carboxylase activity

The ability of cell-free extracts derived from either acetone- or pyruvate-grown cells to fix ¹⁴CO₂ in an acetone-dependent manner is illustrated by the time-courses shown in Fig. 1(d), where the difference in d.p.m. fixed in the presence and absence of acetone has been plotted. It is apparent that pyruvate-grown cells contain negligible acetone carboxylase activity but that this is strongly induced by growth on acetone. In separate experiments, it was found that butanone-grown cells also showed acetone-dependent ¹⁴CO₂ fixation but, in keeping with the data in Table 2 for acetone-grown cells, butanone carboxylase activity could not be demonstrated (data not shown).

Acetone carboxylase activity in other bacteria

The purple photosynthetic bacterium *Rhodomicrobium vannielii* and the denitrifying bacterium *Thiosphaera pantotrophica* have previously been shown to be capable of growth on acetone (Madigan, 1990; Bonnet-Smits *et al.*, 1988). As shown in Table 3, acetone-dependent ¹⁴CO₂ fixation was detected in cell-free extracts of both

Table 2. Reaction requirements and effect of dialysis on acetone-dependent fixation of $\text{NaH}^{14}\text{CO}_3$ by cell-free extracts of acetone-grown *R. capsulatus*

Assay mixture	$^{14}\text{CO}_2$ fixed after 2 h incubation			
	Undialysed cell-free extract*		Dialysed cell-free extract*	
	D.p.m.	% of complete	D.p.m.	% of complete
Complete†	41913	100	67150	100
Minus acetone	34449	82	17226	26
Minus ATP	6562	16	3339	5
Minus CoA	18988	45	26392	39
Minus acetone and ATP	6450	15	2943	4
Minus acetone and CoA	11796	28	9844	15
Minus acetone, ATP and CoA	2857	7	959	1
Complete plus 2 U avidin	NA		54350	81
Complete minus Mg^{2+}	NA		45000	67
Complete minus acetone, plus butanone	NA		9483	14
Complete minus $\text{NaH}^{14}\text{CO}_3$	NA		15	< 0.1
Complete minus cell-free extract	NA		59	< 0.1

NA, Not assayed.

* 0.3 mg protein per assay.

† The complete assay system consisted of: 10 mM potassium phosphate buffer, pH 7.5, 1 mM MgCl_2 , 1 mM acetone, 1 mM ATP, 0.1 mM CoA, cell-free extract, 1 mM $\text{NaH}^{14}\text{CO}_3$ (0.37 GBq mmol^{-1}) in a total volume of 250 μl . **Fig. 1.** Characteristics of acetone-dependent $^{14}\text{CO}_2$ fixation catalysed by cell-free extracts of *R. capsulatus*, using the standard assay (Methods). (a) Time-course in the presence (●) or absence (○) of acetone with 0.1 mM CoA included in the assay (containing 3.7 mg cell-free extract protein). (b) Time-course in the presence (●) or absence (○) of acetone with 0.1 mM acetyl-CoA included in the assay (containing 3.7 mg cell-free extract protein). (c) pH profile of carboxylase activity in the presence (●) or absence (○) of acetone. Samples were taken after 45 min incubation of 4 mg cell-free extract protein in the standard assay (containing acetyl-CoA) buffered to the appropriate pH value with 10 mM potassium phosphate buffer. (d) Time-course of acetone carboxylase activity (i.e. d.p.m. $^{14}\text{CO}_2$ fixed in the presence of acetone minus d.p.m. $^{14}\text{CO}_2$ fixed in the absence of acetone) with cell-free extract (0.7 mg protein) derived from acetone-grown cells (▲) or pyruvate-grown cells (■).

organisms after growth on acetone, indicating the presence of similar enzyme activities. In the case of *T. pantotropha*, a similar pattern of dependence on ATP

and acetyl-CoA as observed with *R. capsulatus* (Table 2) was seen, but this was less apparent with the *Rm. vannielii* extract.

Table 3. Acetone carboxylation activity catalysed by dialysed cell-free extracts of acetone-grown photosynthetic and denitrifying bacteria

Assay mixture	$^{14}\text{CO}_2$ fixed after 2 h incubation					
	<i>R. capsulatus</i> (0.7 mg protein)		<i>Rm. vannielii</i> (0.6 mg protein)		<i>T. pantotropha</i> (1.1 mg protein)	
	D.p.m.	% of complete	D.p.m.	% of complete	D.p.m.	% of complete
Complete*	157 576	100	47 859	100	48 701	100
Minus acetone	38 901	25	29 057	61	14 573	30
Minus ATP	NA		33 264	70	7 554	16
Minus acetyl-CoA	NA		45 472	95	15 452	32
Minus acetyl-CoA and acetone	NA		17 046	36	5 108	11

NA, Not assayed.

* The complete assay system consisted of: 10 mM potassium phosphate buffer, pH 7.5, 1 mM MgCl_2 , 1 mM acetone, 1 mM ATP, 0.1 mM acetyl-CoA, 1 mM $\text{NaH}^{14}\text{CO}_3$ (0.37 GBq mmol^{-1}) and cell-free extract in a total volume of 250 μl .

Fig. 2. Sucrose-gradient localization of acetone carboxylase activity. (a) Cell-free extract of strain PAS100 was loaded onto a discontinuous 5–60% (w/v) sucrose gradient. After ultracentrifugation and fractionation, the total protein content of the fractions was assessed by absorbance at 280 nm (●), the location of the cytoplasmic-membrane-containing fractions by absorbance of bacteriochlorophyll at 850 nm (○) and acetone-dependent carboxylase activity (i.e. d.p.m. in the presence of acetone minus d.p.m. in the absence of acetone) was measured using the standard assay with acetyl-CoA (■). In (b) the total $^{14}\text{CO}_2$ fixation activity of fractions incubated with acetone (▲) and without acetone (△) is plotted, to show the peak of acetone-independent activity associated with the membrane fraction.

Cellular localization of acetone carboxylase activity in *R. capsulatus*

Differential centrifugation of cell-free extracts to give 'cytoplasmic' (supernatant) and 'membrane' (pellet) fractions was initially used to determine whether acetone carboxylase activity was 'soluble' or membrane-associated. $^{14}\text{CO}_2$ fixation activity was in fact found in both fractions but was only acetone dependent in the 'cytoplasmic' fraction. This was examined in more detail by sucrose-gradient centrifugation of cell-free extracts (Fig. 2). The broad peak in the absorbance at 280 nm towards the top (least dense) part of the gradient represents the majority of the soluble proteins that are largely cytoplasmic in origin, while the second large protein peak is the prominent red-pigmented cytoplasmic membrane band (containing bacteriochlorophyll-binding light-harvesting proteins absorbing at 850 nm) centred at a density of 1.16 g cm^{-3} (Fig. 2a). Two peaks of $^{14}\text{CO}_2$ fixation occurred in the gradient (Fig. 2b). The first was centred at a density of about 1.09 g cm^{-3} , well separated from the membrane fraction, and was largely, although not completely, acetone dependent. The second peak of $^{14}\text{CO}_2$ fixation was not acetone dependent and was partially associated with the membrane fraction. The difference in d.p.m. $^{14}\text{CO}_2$ fixed in the presence and absence of acetone is plotted separately in Fig. 2(a), which more clearly illustrates the acetone-dependent nature of the activity in the first peak.

Analysis of the products of acetone carboxylation by thin-layer chromatography

To examine labelled products resulting from $^{14}\text{CO}_2$ fixation, samples from an acetone carboxylase assay were deproteinized and transferred to TLC plates, which

Fig. 3. Identification of products of acetone carboxylation. Cell-free extract of acetone-grown cells of *R. capsulatus* (3 mg protein) was incubated for 2 h in the standard assay mixture (with 1 mM $\text{NaH}^{14}\text{CO}_3$ of specific activity $1.85 \text{ GBq mmol}^{-1}$). After deproteinizing, samples ($10 \mu\text{l}$) of the supernatants were applied to a silica gel TLC plate, which was run as described in Methods. The resulting autoradiogram is shown. Track 1, complete assay system (plus acetone); track 2, minus acetone; track 3, minus ATP; track 4, minus acetone and ATP; track 5, minus acetyl-CoA; track 6, minus acetyl-CoA and acetone. The position of authentic lithium acetoacetate is shown by the arrow labelled 'A'.

were developed in a variety of solvent systems. The most effective solvent system was found to be ethanol/ammonia/water. On the representative autoradiogram shown in Fig. 3, a labelled product (R_F 0.56) which co-migrated with authentic acetoacetate (R_F 0.6) was formed in significant amounts only in the complete assay system containing acetone. In the absence of added acetone, small amounts of this product were still formed, presumably from traces of acetone still remaining in the cell-free extract even after dialysis. At least one other labelled product (R_F 0.2) was formed in the complete assay system in the presence or absence of added acetone, but was clearly dependent on the inclusion of acetyl-CoA in the assay. The chromatographic behaviour of this compound is consistent with its being a CoA derivative: acetoacetyl-CoA and acetyl-CoA had R_F values of 0.05–0.2 in the solvent system used. Removal of ATP from the reaction mixtures gave no visible radioactive products.

Identification and partial purification of proteins associated with acetone carboxylase activity

Growth of *R. capsulatus* on either acetone or butanone consistently resulted in the specific overproduction of two polypeptides, of about 70 and 85 kDa (Fig. 4a). On native (non-denaturing) gels (Fig. 4b) a prominent band was present only in extracts derived from ketone-grown,

Fig. 4. Identification of proteins associated with acetone carboxylase activity in *R. capsulatus*. (a) SDS-PAGE protein profile of cell-free extracts derived from acetone-grown cells (lane 2), pyruvate-grown cells (lane 3) and butanone-grown cells (lane 4). In each case, $150 \mu\text{g}$ total protein was loaded onto the gel, which was subsequently stained with Coomassie blue. Lane 1 contains molecular mass markers. (b) Native PAGE protein profile (Coomassie blue staining) of cell-free extracts derived from butanone-grown cells (lane 1), pyruvate-grown cells (lane 2) and acetone-grown cells (lane 3). (c) A gel slice containing the high-molecular-mass band indicated from lane 3 in (b) was excised, boiled in SDS-sample buffer and the supernatant applied (lanes 2 and 3) to an SDS-PAGE gel which was subsequently silver-stained. Lane 1 contains molecular mass markers.

Fig. 5. (a) Partial purification of acetone carboxylase activity by anion-exchange FPLC. A membrane-free cell extract of *R. capsulatus* was loaded onto a MonoQ column and proteins eluted with a 0–1 M KCl gradient (dotted line). The absorbance profile at 280 nm was measured continuously (solid line) and $^{14}\text{CO}_2$ fixation from $\text{NaH}^{14}\text{CO}_3$ measured in collected fractions by the standard assay in the presence (●) and absence (○) of acetone. Highest activity was observed in fraction 17 (0.25 M KCl). (b) SDS-PAGE analysis of acetone carboxylase purification. Lane 1, cell-free extract from acetone-grown *R. capsulatus*. Lane 2, membrane-free cell-extract (150 000 g supernatant) prior to anion-exchange. Lanes 3–6, equal volumes (50 μl) of fractions 16–19 respectively from the anion-exchange FPLC in (a). Lane 7, molecular mass markers.

but not pyruvate-grown, cells, and this contained both the 70 and 85 kDa polypeptides in approximately equal ratios (Fig. 4c). The gel in Fig. 4(c) was silver-stained to increase sensitivity and several other minor proteins are visible. Although these may be contaminants, the possibility that one or more of these proteins are derived from the native complex cannot be excluded. In order to determine if the prominent band observed on native gels was actually associated with acetone carboxylase activity, a phosphate-buffered native gel was run, the band was excised and the eluted protein was assayed as described above. Significant $^{14}\text{CO}_2$ fixation was observed in the presence of acetone (105 455 d.p.m. ^{14}C fixed after

2 h incubation), while in the absence of acetone the incorporation was much lower (14 677 d.p.m. ^{14}C fixed after 2 h incubation). Taken together, these data are consistent with the 70 and 85 kDa proteins being subunits of the acetone-carboxylating enzyme.

Initial attempts to purify the acetone carboxylase activity, using a variety of techniques, were unsuccessful. A major problem was that the enzyme easily lost activity. The most successful method was partial purification by FPLC ion-exchange chromatography of undialysed cell-free extracts from which the cytoplasmic membrane fraction had first been removed (Fig. 5a). This con-

Table 4. Component assay for partially purified acetone carboxylase

Assay mixture	$^{14}\text{CO}_2$ fixed after 2 h incubation			
	Plus acetone		Minus acetone	
	D.p.m.	% of complete	D.p.m.	% of complete
Complete*	133 313	100	36 428	100
Minus ATP	1742	1	969	3
Minus acetyl-CoA	4343	3	4343	12
Minus Mg^{2+}	33 910	25	16 864	46
Minus acetyl-CoA, plus acetoacetyl-CoA	198 911	150	23 528	65
Minus acetyl-CoA, plus acetoacetyl-CoA and 2 U avidin	191 965	144	22 119	61
Minus acetyl-CoA and ATP, plus acetoacetyl-CoA	1701	1	851	2

* The complete assay system consisted of: 10 mM potassium phosphate buffer, pH 7.5, 1 mM MgCl_2 , 1 mM acetone, 1 mM ATP, 0.1 mM acetyl-CoA (or acetoacetyl-CoA where indicated), partially pure enzyme preparation (0.5 mg protein), 1 mM $\text{NaH}^{14}\text{CO}_3$ ($1.85 \text{ GBq mmol}^{-1}$) in a total volume of 250 μl .

sistently resulted in a clear peak of acetone-dependent CO_2 fixation eluting from the column, corresponding to a KCl concentration of approximately 0.25 M. However, all attempts at further purification of active fractions from the MonoQ column resulted in complete loss of activity.

The polypeptide composition of the MonoQ active fractions is shown in Fig. 5(b). The 70 and 85 kDa polypeptides associated with growth on acetone (see Fig. 4) are clearly visible in fractions 16 and 17. However, although fraction 16 contained the largest amounts of these polypeptides, it had negligible acetone carboxylase activity compared to fraction 17 (see Fig. 5a). Fraction 17 (but not 16) also contained a protein of about 60 kDa, and both fractions contained small amounts of a 35 kDa protein.

The acetone carboxylase activity in cell-free extracts had previously been shown to be essentially insensitive to avidin (Table 2), but in order to prove that the enzyme contained no biotin, an SDS-PAGE gel containing the active fraction (17) from a MonoQ partial purification was blotted onto nitrocellulose and probed for biotin using a chemiluminescence detection procedure after incubation with an avidin conjugate. None of the polypeptides in the active fraction reacted with the conjugate, while a control sample of purified pyruvate carboxylase from *R. capsulatus* (Modak & Kelly, 1995) reacted strongly (data not shown).

Properties of the partially purified enzyme

The reaction requirements of the partially purified enzyme were examined (Table 4). Acetone-dependent

CO_2 fixation required both ATP and acetyl-CoA and was clearly stimulated by magnesium ions. However, in contrast to the activity measured in cell-free extracts, replacing acetyl-CoA with CoA was completely ineffective. It seems likely that limited synthesis of acetyl-CoA from CoA is possible in crude extracts, which does not occur with the partially purified preparation. Interestingly, acetoacetyl-CoA could substitute for acetyl-CoA (Table 4). However, this is almost certainly explained by the presence of some contaminating thiolase activity in the acetone carboxylase fractions from the ion-exchange column, which would allow acetyl-CoA to be formed.

A distinctly non-linear relationship was observed between the initial rate of acetone carboxylase activity and the amount of protein added to the assay mixture. Below about 0.1 mg protein per assay, acetone-dependent CO_2 fixation was undetectable, but it appeared optimal at about 0.4–0.5 mg protein per assay. However, above this concentration, the rate decreased. For acetone, sodium bicarbonate and acetyl-CoA, saturation-type kinetics were observed and apparent K_m values of 30 μM , 750 μM and 50 μM , respectively, were determined. An ATP concentration of 0.5–1.0 mM resulted in the highest initial rates, but higher concentrations were inhibitory. This effect may be due to chelation of magnesium ions and was not investigated further.

The N-terminal sequences of the 70 and 85 kDa subunits were obtained by automated Edman degradation of blotted proteins separated by SDS-PAGE. The N-terminal sequence obtained for the 70 kDa polypeptide was LDREKTRSVXV and for the 85 kDa polypeptide,

LNAPLVIRGDV. Although limited in length, database searches failed to reveal any significant similarity of these sequences with those of known carboxylases or other proteins concerned with CO₂ metabolism.

DISCUSSION

An assay for acetone carboxylation in cell-free extracts of *R. capsulatus* was developed in this study. Acetone-dependent fixation of ¹⁴CO₂ into acid-stable products required the inclusion of ATP and, for maximal activity, CoA or acetyl-CoA in addition to magnesium ions. Consistent activity could also only be obtained by the use of dialysed extracts, which presumably effected the removal of residual acetone. Although *R. capsulatus* only appears to grow well under anaerobic conditions on acetone (Madigan, 1990), no special precautions had to be taken here to exclude oxygen from the assay in order to obtain activity, suggesting that the lack of aerobic growth on this ketone is not due to an inherent oxygen sensitivity of the enzyme. Compelling evidence for the carboxylation of acetone to acetoacetate has been obtained in previous studies (Bonnet-Smits *et al.*, 1988; Platen & Schink, 1989, 1990; Janssen & Schink, 1995; Sluis *et al.*, 1996). TLC analysis revealed that the major product formed in the assay described here was a polar compound which co-chromatographed with authentic acetoacetate. The assay protocol included a heating step (necessary to remove unfixed ¹⁴CO₂), and was successful despite the fact that acetoacetate is a relatively unstable compound. However, because non-linear initial rates were often obtained in crude extracts and even with the partially purified preparation, those initial rates that were measurable did not increase linearly with protein concentration (see below), specific activities of acetone carboxylation were highly variable and were estimated to be only of the order of a few hundred pmol min⁻¹ (mg protein)⁻¹ at most. This is similar to the range of values obtained by Janssen & Schink (1995) in their isotope exchange assay with the denitrifying strain BunN. Given a doubling time of 7 h on acetone and an apparent molar growth yield of 30 g cells (mol acetone)⁻¹, it can be calculated that an acetone carboxylation rate of about 110 nmol min⁻¹ (mg protein)⁻¹ would be required to support the growth of *R. capsulatus* under the conditions used in this study. A similar discrepancy was noted in the work of Janssen & Schink (1995) and indicates that the assay is not optimal and/or that the enzyme loses most of its activity upon cell-breakage (or both).

Interestingly, butanone could not substitute for acetone in assays carried out with extracts derived from acetone- or butanone-grown cells. It is possible that the product of butanone carboxylation is not stable under the assay conditions used, or that the acetone carboxylase is specific for acetone and that a separate butanone carboxylase exists which may have different requirements. Nevertheless, growth on butanone induced the synthesis of acetone carboxylase, as judged by activity measurements and PAGE profiles, and so the possibility

that butanone is first converted to acetone before carboxylation should be considered.

A requirement for ATP in the acetone carboxylase assay is not surprising, given the fact that direct carboxylation of acetone to acetoacetate is not thermodynamically favourable. Sluis *et al.* (1996) also reported ATP dependence of acetone degradation in *Xanthobacter*. Platen & Schink (1990) and Lack & Fuchs (1992, 1994) have proposed, by analogy with the carboxylation of phenol to 4-hydroxybenzoate via phenylphosphate, that activation of acetone for carboxylation may occur by phosphorylation of the *enol* tautomer to give phosphoenolacetone. This could be subsequently dephosphorylated to give an enzyme-bound carbanion which could be carboxylated directly in a thermodynamically favourable reaction to produce acetoacetate. Although our results could be consistent with such a mechanism, the instability and commercial unavailability of phosphoenolacetone precluded its use in our *in vitro* carboxylation assay to test this hypothesis directly.

The involvement of acetyl-CoA is more complex. One plausible explanation, at least in the case of cell-free extracts, is that the CoA moiety is transferred to the acetoacetate formed in the carboxylase reaction to give acetoacetyl-CoA, which shifts the equilibrium of the net carboxylation reaction in favour of acetoacetate formation, hence giving a higher rate of acetone-dependent ¹⁴CO₂ fixation. In this case, both acetoacetate and acetoacetyl-CoA would be radiolabelled products, with the amounts formed depending on the relative activities of the carboxylase and transferase. This is consistent with the results of the TLC analysis, but requires the activity of an acetoacetate:acetyl-CoA transferase activity as a 'coupling enzyme', which could not be detected (Table 1). In addition, succinyl-CoA did not stimulate acetone carboxylation activity and conversion of acetoacetate to the more stable 3-hydroxybutyrate by including NADH and excess commercial 3-hydroxybutyrate dehydrogenase in the carboxylation assay also failed to result in increased ¹⁴CO₂ fixed (unpublished observations). Perhaps acetyl-CoA has a direct role as an activator of the carboxylase, or it could take part in the reaction mechanism itself.

Partial purification of acetone carboxylase activity was achieved by ion-exchange chromatography. This, combined with PAGE analysis, showed that the two abundant cell proteins specifically induced by growth on acetone were subunits of the enzyme but the limited N-terminal sequences obtained gave no indication of similarity to known proteins involved in CO₂ metabolism. Bonnet-Smits *et al.* (1988) previously noted the acetone-dependent overproduction of two high-molecular-mass proteins in *T. pantotropha* by HPLC analysis, and SDS-PAGE of extracts of this bacterium has revealed abundant 70 and 85 kDa proteins as in *R. capsulatus* (A. Day & D. J. Kelly, unpublished observations). The purified enzyme from *R. capsulatus* showed the same requirements as observed in cell-free

extracts, with a clearer dependence on acetyl-CoA. It is known that acetoacetate can undergo non-specific decarboxylation in the presence of high concentrations of proteins such as bovine serum albumin (Platen & Schink, 1990) and this is a possible explanation for the decline in rate as the amount of protein in the assay was increased above an 'optimum' value.

The assay developed here is clearly applicable to other bacteria, as demonstrated by the detection of activity in *Rm. vannielii* and *T. pantotropha*, and should allow additional properties of acetone carboxylase to be elucidated. In particular, purification to homogeneity will be required to unequivocally establish the reaction mechanism and thus determine if similarities to phenol carboxylase exist, as proposed by Platen & Schink (1990) and Lack & Fuchs (1992, 1994). Our results do suggest that the enzyme is of a novel type, although confirmation of this will probably have to await the cloning of the genes encoding its subunits.

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REFERENCES

- Bonnet-Smits, E. M., Robertson, L. A., Van Dijken, J. P., Senior, E. & Kuenen, J. G. (1988). Carbon dioxide fixation as the initial step in the metabolism of acetone by *Thiosphaera pantotropha*. *J Gen Microbiol* **134**, 2281–2289.
- Gottschalk, G. (1986). *Bacterial Metabolism*, 2nd edn. Berlin: Springer-Verlag.
- Hillmer, P. & Gest, H. (1977). H₂ metabolism in the photosynthetic bacterium *Rhodospseudomonas capsulata*: H₂ production by growing cultures. *J Bacteriol* **129**, 724–731.
- Janssen, P. H. & Schink, B. (1995). ¹⁴CO₂ exchange with acetoacetate catalysed by dialysed cell-free extracts of the bacterial strain BunN grown with acetone and nitrate. *Eur J Biochem* **228**, 677–682.
- Lack, A. & Fuchs, G. (1992). Carboxylation of phenylphosphate by phenol carboxylase, an enzyme system of anaerobic phenol metabolism. *J Bacteriol* **174**, 3629–3636.
- Lack, A. & Fuchs, G. (1994). Evidence that phenol phosphorylation to phenylphosphate is the first step in anaerobic phenol metabolism in a denitrifying *Pseudomonas* sp. *Arch Microbiol* **161**, 132–139.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Leyland, M. L. & Kelly, D. J. (1991). Purification and characterisation of a monomeric isocitrate dehydrogenase with dual coenzyme specificity from the photosynthetic bacterium *Rhodospirillum rubrum*. *Eur J Biochem* **202**, 85–93.
- Madigan, M. T. (1990). Photocatabolism of acetone by nonsulfur purple bacteria. *FEMS Microbiol Lett* **71**, 281–286.
- Modak, H. & Kelly, D. J. (1995). Acetyl-CoA dependent pyruvate carboxylase from the photosynthetic bacterium *Rhodobacter capsulatus*: rapid and efficient purification using dye-ligand affinity chromatography. *Microbiology* **141**, 2619–2628.
- Platen, H. & Schink, B. (1989). Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. *J Gen Microbiol* **135**, 883–891.
- Platen, H. & Schink, B. (1990). Enzymes involved in anaerobic degradation of acetone by a denitrifying bacterium. *Bio-degradation* **1**, 243–251.
- Platen, H., Temmes, A. & Schink, B. (1990). Anaerobic degradation of acetone by *Desulfococcus biacutus* spec. nov. *Arch Microbiol* **154**, 355–361.
- Reeves, H. C., Rabin, R., Wegener, W. S. & Aji, S. J. (1971). Assays of enzymes of the tricarboxylic acid and glyoxylate cycles. *Methods Microbiol* **6A**, 437–439.
- Robertson, L. A. & Kuenen, J. G. (1983). *Thiosphaera pantotropha* gen. nov., sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. *J Gen Microbiol* **129**, 2847–2855.
- Siegel, J. M. (1950). The metabolism of acetone by the photosynthetic bacterium *Rhodospseudomonas gelatinosa*. *J Bacteriol* **60**, 595–606.
- Sluis, M. K., Small, F. J., Allen, J. R. & Ensign, S. A. (1996). Involvement of an ATP-dependent carboxylase in a CO₂-dependent pathway of acetone metabolism by *Xanthobacter* strain Py2. *J Bacteriol* **178**, 4020–4026.
- Taylor, D. G., Trudgill, P. W., Cripps, R. E. & Harris, P. R. (1980). The microbial metabolism of acetone. *J Gen Microbiol* **118**, 159–170.
- Taylor, D. P., Cohen, S. N., Clark, N. G. & Marrs, B. L. (1983). Alignment of genetic and restriction maps of the photosynthetic region of the *Rhodospseudomonas capsulata* chromosome by a conjugation mediated marker-rescue technique. *J Bacteriol* **154**, 580–590.
- Thauer, R. K., Jungermann, K. & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**, 100–180.
- Weaver, P. F., Wall, J. D. & Gest, H. (1975). Characterisation of *Rhodospseudomonas capsulata*. *Arch Microbiol* **105**, 207–216.
- Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118**, 197–203.

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