The Metabolism of Propane in *Rhodococcus rhodochrous* PNKb1

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A bacterium that utilized propane as a sole carbon source was isolated from soil and identified as a strain of *Rhodococcus rhodochrous*. Of the *n*-alkanes tested (C_1 – C_8) it grew only on propane, and it was not capable of growth on alkenes. The organism grew on most of the potential intermediates of propane metabolism and simultaneous adaptation studies showed that it could oxidize both terminal and sub-terminal intermediates. Assays of enzyme activities in cell-free extracts revealed elevated levels of enzymes of both terminal and sub-terminal pathways in propane-grown cells. The initial propane-specific oxygenase activity was measured by its ability to co-oxidize propene to epoxyp propane. This oxygenase system was investigated in terms of its inhibitor profile and was compared with *n*-alkane oxygenase systems described in the literature. On the basis of the comparison, the oxygenase appears to be of a type not previously reported.

INTRODUCTION

The ability of micro-organisms to utilize propane as a sole carbon source is well documented (Perry, 1980). Such organisms belong mainly to the *Corynebacterium*–*Mycobacterium*–*Nocardia* complex, a loosely defined group containing other genera such as *Rhodococcus*, *Brevibacterium* and *Arthrobacter* (Klug & Markovetz, 1971; Perry, 1980). The understanding of the physiology and biochemistry of this group of organisms, particularly regarding their metabolism of gaseous alkanes, is somewhat limited. There appears to be a general opinion that propane is metabolized principally by a sub-terminal oxidation pathway via acetone (Fig. 1) (Perry, 1968, 1980). Much of the evidence to support this opinion is based on growth substrate specificities, product excretion and simultaneous adaptation studies, all techniques that should be interpreted with caution (Dagley & Chapman, 1971). Recently Stephens & Dalton (1986) reassessed much of the data and concluded that terminal oxidation of propane (Fig. 1) may be more significant than was previously supposed. Other recent reports (Babu & Brown, 1984; MacMichael & Brown, 1987) have also suggested that terminal oxidation of propane may be occurring in at least some gaseous alkane utilizers.

Measurements of enzyme activities within cell-free extracts of alkane-grown organisms are lacking in many of these studies (but see Taylor *et al.*, 1980; Van Ginkel *et al.*, 1987) and investigation of the initial oxygenase in cell-free systems has only recently been attempted (Patel *et al.*, 1983).

This paper reports on the isolation and investigation of a *Rhodococcus* sp. that grows on propane, with reference to propane-specific enzyme activities in cell-free extracts and the nature of the propane oxygenase enzyme system.

METHODS

Isolation of R. rhodochrous PNKb1. Soil and water samples were obtained from both environments known to have been exposed to *n*-alkanes and environments apparently having no such exposure. Samples were suspended in 10 ml of sterile mineral salts (MS) medium (Whittenbury *et al.*, 1970). Suspensions were allowed to clear slightly and 100 μ l of supernatant was used to inoculate 50 ml of MS medium, in a 250 ml Quickfit flask, to which 0.5 g NH_4Cl l^{-1} , 0.5 g KNO_3 l^{-1} and a vitamin supplement (Gest *et al.*, 1983) had been added (ANMSV medium). The flask was sealed with a 'Suba seal' and gassed with propane to give 50% (v/v) propane in air, and then incubated at

30 °C on an orbital shaker until significant turbidity developed. Serial dilutions of the culture were spread onto ANMSV agar plates and single colonies then streaked onto ANMSV agar plates. Plates were incubated at 30 °C in a 50% (v/v) propane/air atmosphere. Purity was checked by several passages through liquid medium and serial dilutions onto ANMSV plates followed by rigorous microscopic examination.

A large number of different propane-utilizing bacteria were also obtained using a continuous enrichment procedure in a one-litre fermenter vessel sparged with a 50% (v/v) propane in air gas mixture at 120 ml min⁻¹. Temperature (30 °C), pH (6.8) and agitation were controlled and dilution rates of between 0.014 h⁻¹ and 0.04 h⁻¹ were employed.

Growth studies. Growth of *R. rhodochrous* PNKb1 was routinely achieved using AMS medium (MS medium plus 1 g NH₄Cl l⁻¹) in 250 ml Quickfit flasks. These flasks were sealed with 'Suba seals' and 50 ml of air replaced with 50 ml of gaseous substrate (alkane, alkene or alkyne). Volatile liquid substrates were supplied at 0.1 or 0.05% (v/v) into similar sealed flasks and solid substrates were supplied at 0.1% (w/v). Where large volumes of alkane-grown cells were required, growth was batchwise in an LH500 series fermenter of 1.7 l working volume, fed with propane (50%, v/v, in air) at 60 ml min⁻¹, stirred at 800 r.p.m. and operated at constant pH (6.8) and temperature (30 °C).

Measurement of cell density. Cell density was routinely measured as OD₅₄₀ using a Pye Unicam SP1800 spectrophotometer. Dry weights (established using a filtration/drying method: Gerhardt, 1981) were estimated from a standard curve of OD₅₄₀ versus dry weight.

Preparation of cell suspensions. Cultures from flasks or fermenters were centrifuged at 20000 g for 10 min at 4 °C. Cell pellets were washed in 30 ml of ice-cold buffer (20 mM-Tris/HCl, pH 6.8) and centrifuged as before. The cell pellets were then resuspended in the same buffer to an OD₅₄₀ of between 20 and 40 (4.6 and 11.6 mg dry weight cells ml⁻¹).

Preparation of cell-free extracts. Cells, prepared as above, were disrupted by three passages through a precooled French pressure cell operated at 138 MPa. Disrupted cells were then centrifuged at 30000 g for 10 min at 4 °C to remove any unbroken cells and membrane debris.

Oxygen electrode assays. The ability of *R. rhodochrous* PNKb1 to oxidize a variety of substrates was tested by measuring the stimulation of oxygen uptake on addition of the substrate to cell suspensions contained in a Clark-type oxygen electrode. Assays were done at 30 °C in 2.9 ml of 20 mM-phosphate buffer, pH 6.8. Air-saturated buffer was placed in the reaction chamber, the plunger inserted and the system allowed to equilibrate. Cell suspension (50 µl) was then added by syringe and the endogenous rate of oxygen uptake was measured. Substrate (50 µl) was then injected and any stimulation in oxygen uptake was recorded. Rates of oxygen uptake were corrected for endogenous rate and expressed as nmol O₂ min⁻¹ (mg dry weight of cells)⁻¹. The dissolved oxygen concentration of air-saturated buffer was calculated using the method of Robinson & Cooper (1970).

Gaseous substrates were prepared by degassing 5 ml distilled water under vacuum then passing the contents of a football bladder inflated with the gas through the water. Liquid alkanes and other relatively insoluble substrates were prepared as saturated solutions. The final concentrations of these substrates were calculated using the data of McAuliffe (1966). Substrates readily soluble in water were prepared as 2 mM solutions giving a final concentration in the assay of 33.3 µM.

Enzyme assays. (a) *Alcohol/aldehyde dehydrogenase* (EC 1.1.1.-). This was measured spectrophotometrically as an NAD⁺-linked activity using a Pye Unicam SP1800 spectrophotometer at 340 nm. Assays were done in a total volume of 1 ml. The cuvette contained, in 20 mM-Tris/NaOH, pH 10.0, 0.2 µmol NAD⁺ and 0.1–1.0 mg of protein extract (enough to give a linear rate for more than 5 min). Cuvettes were allowed to equilibrate for 1 min at 30 °C before the reaction was initiated by the addition of 10 µmol alcohol or aldehyde.

(b) *Propionyl-CoA synthetase* (EC 6.2.1.-). This was measured by the determination of propanoate-dependent sulphhydryl CoA disappearance using the method of Grunert & Phillips (1951).

(c) *Ketone oxygenases*. Acetone and acetol oxygenase activities were measured by substrate-dependent stimulation of oxygen uptake in a Clark-type oxygen electrode using the method of Hartmans & de Bont (1986).

(d) *Acetol dehydrogenase* (EC 1.1.1.-). This was measured spectrophotometrically using an NAD(P)⁺-linked assay as described by Taylor *et al.* (1980).

(e) *Isocitrate lyase* (EC 4.1.3.1). The methods used were those of Dixon & Kornberg (1959), and Reeves *et al.* (1971).

(f) *Propane oxygenase*. Assays were performed in 2 ml gas chromatography (GC) vials in a total volume of 0.25 ml. Sufficient 20 mM-Tris/HCl, pH 6.8, to give a final assay volume of 0.25 ml was placed in a GC vial along with 1–5 mg of protein from a cell-free extract. The vial was sealed and preincubated at 30 °C for 30 s in a shaking water bath (250 r.p.m.). NADH (1 µmol) was added and 0.9 ml of air was removed and replaced with 0.9 ml of propene. The vial was returned to the water bath and 5 µl samples were removed every 5 min and analysed on a Pye Unicam gas chromatograph fitted with a 1.5 m × 2.3 mm glass column packed with Poropak Q. The column was run isothermally at 180 °C with nitrogen (30 ml min⁻¹) as carrier gas. Output from the gas chromatograph was linked to a reporting integrator (Hewlett Packard) that had been calibrated with standard solutions. Rates were expressed as nmol 1,2-epoxypropane formed min⁻¹ (mg protein)⁻¹. Whole-cell oxygenase activity was measured as above (omitting NADH) but using 12 µl cell suspension plus 238 µl buffer.

Inhibitor studies. Potential inhibitors were made up as 100 mM solutions except CO and C₂H₂, which were made up as saturated solutions having concentrations of 1 mM and 44 mM, respectively (Stirling, 1978). They were added to vials, to give a final concentration as indicated in Table 3, before addition of the substrate. Control vials lacked inhibitor.

For all whole-cell oxidation studies and preparation of cell-free extracts for use in enzyme assay work, *R. rhodochrous* PNKb1 was harvested at culture cell densities (OD₅₄₀) of 0.7, i.e. during the late exponential phase of growth. All enzyme activities reported are mean values after at least three determinations.

Polyacrylamide gel electrophoresis. The method was based on that of O'Farrell (1975) and used a discontinuous buffer system as described by Laemmli (1970). The resolving gel was prepared in 3.0 M-Tris/HCl, pH 8.8, and the stacking gel in 0.5 M-Tris/HCl, pH 6.8. The running buffer was Tris/glycine (25 mM-Tris base, 129 mM-glycine). Gels were formed from 10 to 30% (w/v) exponential gradients of acrylamide. Electrophoresis was done at a constant current of 15 mA. The stacking gel, resolving gel and running buffer contained sodium dodecyl sulphate (SDS) at 0.1% (w/v) and 2-mercaptoethanol at 1 mM. Cell-free extracts for SDS-PAGE were boiled for 5 min in sample buffer (125 mM-Tris/HCl, pH 6.8, sucrose 10%, w/v, and SDS 4%, w/v). Prior to loading, samples were mixed with bromophenol blue tracking buffer (10 µl, 0.1%, w/v, bromophenol blue per 0.5 ml sample). Gels were stained for 5 h in 0.1% (w/v) Coomassie Blue R in 10% (v/v) acetic acid and 40% (v/v) methanol. They were destained in the same solvent for 2–4 h.

Chemicals. Gases were of the purest grade available (greater than 99%) obtained from British Oxygen Co., Matheson Gases and Cambrian Chemicals. All other compounds, inhibitors, substrates, media components, etc. were obtained from Sigma, Aldrich, BDH and Fisons Scientific Apparatus.

RESULTS

Isolation and characterization of the propane-utilizing strain

Over 80 strains of Gram-positive propane-utilizing bacteria were isolated using both batch and continuous enrichment techniques. All of the organisms isolated had morphologies consistent with their being members of the *Corynebacterium*–*Mycobacterium*–*Nocardia* complex. Organisms were isolated from terrestrial and aquatic environments with and without evidence of *n*-alkane contamination. Strains showed varying degrees of specificity towards ethane, propane and butane as growth substrates, some growing on all three, others only on two or one.

One of these strains, designated PNKb1, was studied in detail since it grew rapidly on propane and, unlike many other isolates, did not flocculate in liquid culture. Colonies on nutrient agar and propane/AMS agar plates were pink in colour and showed a mycelial morphology when viewed under low magnification ($\times 30$). The organisms were non-motile, Gram-positive rods that were catalase-positive, non-sporeforming and showed oxidative metabolism of glucose. On entering the stationary phase of growth, cells underwent a rod to coccus transition. These characteristics suggested that the strain was a *Rhodococcus* or *Nocardia* sp. A more complete identification was performed by NCIMB Ltd (Aberdeen, UK), who assigned PNKb1 to the species *Rhodococcus rhodochrous*.

Of the *n*-alkanes tested (i.e. methane to octane) *R. rhodochrous* PNKb1 grew only on propane. The unsaturated hydrocarbons ethene, propene and butene were not growth substrates but ethyne and propyne were (see de Bont & Peck, 1980). Other growth substrates included acetate, pyruvate, succinate, glucose and nutrient broth. Batch culture doubling-times of *R. rhodochrous* PNKb1 on propane and pyruvate were 8 and 3 h, respectively.

Growth on potential intermediates of propane metabolism

R. rhodochrous PNKb1 was tested for the ability to grow on the potential intermediates of propane metabolism (see Fig. 1). Rapid growth occurred on propan-1-ol, propanal, propanoate, propan-2-ol, propanone (acetone) and hydroxypropanone (acetol). Propanal and propanone appeared to be toxic at 0.1% (v/v) and were supplied at 0.05% (v/v). Methylacetate, methanol and methylglyoxal (pyruvaldehyde) did not support growth at 0.1 or 0.05% (v/v) but were not toxic at 0.05% (v/v) in the presence of propane. Evidently *R. rhodochrous* PNKb1 can metabolize the potential products of either terminal or sub-terminal oxidation of propane.

Fig. 1. Possible pathways of propane metabolism. (a) Terminal oxidation via propanoate; (b) sub-terminal oxidation via acetol and hydroxymethylacetate; (c) sub-terminal oxidation via pyruvate; (d) sub-terminal oxidation via methyl acetate.

Oxidation of potential intermediates of propane metabolism by R. rhodochrous PNKb1

Substrate-dependent oxygen uptake was determined for propane-, propan-1-ol-, propan-2-ol- and pyruvate-grown cells (Table 1). It was evident that propane oxidation was inducible only by growth on propane (of the four growth substrates tested). The ability of pyruvate-grown cells to oxidize all the potential intermediates of propane metabolism tested suggested that the enzymes required for their metabolism are constitutively expressed. However, there appeared to be an increase in the levels of activity after growth on either the alcohols or the alkane. The low level of propanoate oxidation and the inability to oxidize methylacetate by propane-grown cells may be due to the lack of an uptake system for these substrates. In total these results suggest that *R. rhodochrous* PNKb1 has the metabolic potential to further metabolize both terminal and subterminal oxidation products of propane.

Table 1. Ability of *R. rhodochrous* PNKb1 to oxidize potential intermediates of propane metabolism after batch growth on propane, propan-1-ol, propan-2-ol or pyruvateRates are expressed as nmol oxygen consumed min⁻¹ (mg dry weight cells)⁻¹.

Assay substrate	Growth substrate:			
	Propane	Propan-1-ol	Propan-2-ol	Pyruvate
Propane	21.0	0	0	0
Propan-1-ol	24.6	31.3	27.1	9.2
Propanal	26.3	45.8	33.1	10.2
Propanoate	6.9	43.8	8.5	2.7
Propan-2-ol	35.2	45.8	44.8	4.6
Propanone	41.9	50.0	20.6	5.6
Acetol	12.1	50.0	38.8	33.3
Methylacetate	0	0	0	ND
Methanol	0	0	0	ND
Methylglyoxal	0	ND	ND	ND
Acetate	27.7	22.9	15.8	ND
Pyruvate	6.0	4.8	0	22.9
Succinate	5.8	4.4	0	ND

ND, Not determined.

Table 2. Specific activities of enzymes in cell-free extracts of *R. rhodochrous* PNKb1 batch-grown on propane or pyruvate

Enzyme activity	Growth substrate:	
	Propane	Pyruvate
Propan-1-ol dehydrogenase*	40	33
Propanal dehydrogenase*	14	3
Propionyl-CoA synthetase†	26	6
Propan-2-ol dehydrogenase*	185	49
Acetone oxygenase‡	0	0
Acetol oxygenase‡	27	1
Acetol dehydrogenase*	0	0

* Activity measured as nmol NAD⁺ reduced min⁻¹ (mg protein)⁻¹.† Activity measured as nmol CoA-SH utilized min⁻¹ (mg protein)⁻¹.‡ Activity measured as nmol O₂ consumed min⁻¹ (mg protein)⁻¹.*Enzyme activities in cell-free extracts*

Specific activities of key enzymes of propane oxidation in cell-free extracts of *R. rhodochrous* PNKb1 were measured after growth on propane or pyruvate (Table 2). NAD⁺-linked propan-1-ol dehydrogenase activity showed little increase in propane-grown cells compared to pyruvate-grown cells. The other enzymes of the terminal oxidation pathway, propanal dehydrogenase and propionyl-CoA synthetase, showed a fivefold increase in levels of activity in propane-grown cells compared to pyruvate-grown cells. NAD⁺-linked propan-2-ol dehydrogenase activity showed a fourfold increase in propane-grown cells, and acetol oxygenase activity, which was virtually absent from pyruvate-grown cells, was present in propane-grown cells. The absence of acetol dehydrogenase activity and the presence of acetol oxygenase activity would seem to rule out conversion of acetol to pyruvate, at least in cells grown under these conditions. The absence of a measurable acetone oxygenase activity is perplexing but has been noted before in several acetone utilizers (Taylor *et al.*, 1980). More recently, Platen & Schink (1987) and Bonnet-Smits *et al.* (1988) have reported that carboxylation of acetone to acetoacetate can occur in a mixed anaerobic culture and in *Thiosphaera*, respectively. Therefore, such a mechanism, which thus far

Fig. 2. SDS-PAGE of cell-free extracts of *R. rhodochrous* PNKb1 grown on various potential intermediates of propane metabolism. Growth substrates were: 2, pyruvate; 3, succinate; 4, acetol; 5, acetone; 6, propan-2-ol; 7, propanoate; 8, propanal; 9, propan-1-ol; 10, propane. Track 1 contained molecular mass markers. Each track contained 50 μ g of protein. Arrows indicate major polypeptides specific to propane-grown cells.

has only been demonstrated in organisms capable of anaerobic growth, although unlikely, cannot be completely ruled out for the obligate aerobe *R. rhodochrous* PNKb1.

Isocitrate lyase activity appeared to be induced in propane-grown cells, suggesting a possible role for acetate in propane metabolism. The activity in propane-grown cells was 12 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, compared to zero in pyruvate-grown cells and 78 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ in acetate-grown cells.

Under all growth conditions tested there was no evidence for any dye-linked alcohol dehydrogenase activity in cell-free extracts or in particulate fractions remaining after preparation of protein extracts. It should also be noted that the specific activities of all enzymes assayed for in cell-free extracts (see Table 2) did not vary significantly throughout the growth cycle of *R. rhodochrous* PNKb1 and cell-free extracts from cells harvested during early and mid-exponential growth had similar levels of enzyme activities to those shown in Table 2.

The nature of the propane oxygenase enzyme

Oxygenase activity was shown to be induced only during growth on propane. It appeared to have a narrow substrate range and only oxidized the short-chain unbranched *n*-alkanes ethane, propane and butane at rates of 3.8, 18.1 and 9.2 $\text{nmol oxygen consumed min}^{-1} (\text{mg dry weight cells})^{-1}$, respectively. Methane, isobutane, pentane, hexane and octane were not oxidized. SDS-PAGE of cell-free extracts of cells grown on various intermediates of propane metabolism showed the presence of three major polypeptide bands, of apparent molecular masses 69, 59 and 57 kDa, specific to only propane-grown cells (Fig. 2). These are likely to be components of the propane oxygenase system as they only appeared during induction of propane oxidation (see below).

Despite exhaustive efforts, it was not possible to measure propane-stimulated oxygen uptake or the build-up of potential intermediates of propane metabolism with cell-free extracts.

Table 3. *Effect of potential inhibitors on the formation of 1,2-epoxypropane from propene by cell-free extracts of propane-grown R. rhodochrous PNKb1*100% activity is 10.5 nmol 1,2-epoxypropane formed min⁻¹ (mg protein)⁻¹.

Inhibitor	Concentration (mM)	Relative activity (%)
None	—	100
Azide	1	70
	5	52
KCN	1	51
	5	39
	10	29
Na ₂ EDTA	1	100
	5	75
	10	70
8-Hydroxyquinoline	10	100
<i>p</i> -Hydroxymecuribenzoate	10	100
2-Mercaptoethanol	1	100
	5	90
	10	70
Carbon monoxide	0.5	100
Acetylene	7.5	100

However, it was possible to measure the formation of 1,2-epoxypropane from propene, the epoxide apparently not being metabolized further. In whole-cell assays, addition of increasing amounts of propane to the assay system inhibited the formation of the epoxide, suggesting that propane and propene were competing for the same active site. NADH and oxygen appeared to be required for activity, suggesting a monooxygenase-type reaction (Colby *et al.*, 1975). Oxygenase activity, as measured by epoxide formation, was found to be induced upon switching a chemostat culture growing on propan-1-ol to growth on propane concomitantly with the appearance of the propane-specific bands on SDS-PAGE (data not shown).

Using the formation of epoxide as a measure of oxygenase activity, the effects of various inhibitors on this activity were assayed (Table 3). The activity was relatively unaffected by the chelating agents EDTA and 8-hydroxyquinoline, or by the reducing effects of *p*-hydroxymecuribenzoate and 2-mercaptoethanol. Oxygenase activity was totally unaffected by the presence of carbon monoxide and acetylene but was susceptible to inhibition by cyanide. Using whole-cell suspensions an identical inhibitor profile was obtained when measuring either propane-stimulated oxygen uptake in an oxygen electrode or epoxypropane formation, suggesting that epoxide formation is indeed catalysed by the propane oxygenase.

DISCUSSION

The ubiquitous nature of propane-oxidizing bacteria was demonstrated by the ease with which organisms were isolated from a variety of different terrestrial and aquatic environments. The dominance of Gram-positive organisms in both batch and continuous enrichments has been noted before (Perry, 1980; Stephens & Dalton, 1987). *R. rhodochrous* was shown to grow on propane by Babu & Brown (1984) and MacMichael & Brown (1987) but the specificity of *R. rhodochrous* PNKb1 for propane alone, out of the short-chain alkanes, is unusual although not unique (Perry, 1968). The inability of alkane-utilizing species to grow on alkenes has also been frequently noted but the ability to utilize alkynes is much less well documented (Kanner & Bartha, 1982; de Bont & Peck, 1980).

The ability of *R. rhodochrous* PNKb1 to grow on most of the potential intermediates of propane metabolism would suggest that it had the metabolic capability to utilize either the terminal or the sub-terminal pathway of propane metabolism. Previous studies (e.g. Stephens &

Dalton, 1986) have isolated strains of *Arthrobacter* with an inability to utilize specific intermediates, making the relative importance of each pathway clearer. Likewise, the oxidation of potential intermediates of propane metabolism by *R. rhodochrous* PNKb1 would suggest that the organism had the ability to use either pathway.

Elevated levels of enzyme activities in both the terminal and sub-terminal pathways in propane-grown cells would suggest that both play a role in metabolizing propane. The inability to measure acetone oxygenase activity could be due to the enzyme being extremely labile (as was the propane oxygenase) or due to a failure to achieve optimal conditions for the assay, as may have been the case with Taylor *et al.* (1980). The ability of the organism to grow on and oxidize acetone must indicate the requirement of an acetone-metabolizing enzyme, and the other enzyme activities present would suggest an acetone oxygenase as a likely candidate. The presence of (albeit low) levels of isocitrate lyase in propane-grown cells might suggest a role for acetate in propane metabolism, but it need not arise as an end-product of sub-terminal oxidation. Metabolism of propanoate via malonic semialdehyde could also result in cells being isocitrate lyase positive (Dagley & Nicholson, 1970).

Assuming that the propane oxygenase enzyme was capable of producing a mixture of terminal and sub-terminal products, as indeed previously characterized alkane monooxygenase systems have been found to do (Dalton, 1980; Patel *et al.*, 1983*a*), then the ability of the organism to assimilate both terminal and sub-terminal products could be an advantage and may explain the ability of *R. rhodochrous* PNKb1 to do so and why such organisms are predominant in enrichment cultures on propane.

A partial purification of the propane oxygenase would answer the question of the relative carbon flow along each pathway. Attempts to purify the oxygenase from this organism were thwarted by the difficulty encountered in forming a cell-free extract (due to the extreme mechanical resistance of the cell wall) and the extreme lability of the oxygenase activity, which was not lessened despite the use of numerous stabilization agents including dithiothreitol, sodium thioglycollate and phenylmethylsulphonyl fluoride. Cells of *R. rhodochrous* PNKb1 appeared to be resistant to enzymic and chemical pretreatments to degrade their walls. Three passages through a French pressure cell at 200 MPa were required to release cell protein and the oxygenase activity in the resulting extract had a half-life of 150 min. The inability to measure a build-up of the products of propane oxidation prevented an estimation of the relative proportions of terminal and sub-terminal oxidation products. However, propene was epoxidized by propane oxygenase to form 1,2-epoxypropane, the build-up of which gave a good measure of oxygenase activity. The rates measured using this assay system compared favourably with the only previous report of such measurements (Patel *et al.*, 1983*b*).

The effect of various potential inhibitors on this activity allowed it to be compared and contrasted with those alkane oxygenase systems most studied to date, i.e. methane monooxygenase (Dalton, 1980), octane monooxygenase from a *Corynebacterium* sp. (Cardini & Jurtshuk, 1970) and octane monooxygenase from a *Pseudomonas* sp. (McKenna & Coon, 1970). Of particular interest was the lack of inhibition of the *R. rhodochrous* PNKb1 propane oxygenase by carbon monoxide, indicating the lack of involvement of P₄₅₀-type cytochromes. This was also confirmed by the absence of a typical CO-reduced P₄₅₀ spectrum (unpublished data). This shows that the propane oxygenase system in *R. rhodochrous* PNKb1 is significantly different from that in *Corynebacterium* sp. 7E1C, the only alkane oxygenase system from a Gram-positive organism studied in any detail to date (Cardini & Jurtshuk, 1970), which appeared to be P₄₅₀ cytochrome linked. Lack of inhibition by the chelating agents 8-hydroxyquinoline and EDTA contrasts the propane oxygenase from *R. rhodochrous* PNKb1 with that found in *Pseudomonas oleovorans*, which was highly susceptible to inhibition by 8-hydroxyquinoline (McKenna & Coon, 1970). This result would suggest that any metal centres in the propane oxygenase of *R. rhodochrous* PNKb1 are tightly bound within the protein. Lack of inhibition by ethyne, which is a potent inhibitor of the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) shows that the enzyme in *R. rhodochrous* PNKb1 is unlike that found in the methanotrophs.

On the basis of product excretion by resting cell suspensions of *R. rhodochrous* ATCC 21198,

Babu & Brown (1984) suggested that the propane oxygenase from this organism was a novel intramolecular oxygenase producing mainly propan-1-ol. *R. rhodochrous* PNKb1 differs from *R. rhodochrous* ATCC 21198 in that it did not oxidize isobutane or excrete propan-1-ol under any conditions. The similarity between these two oxygenase systems therefore remains unclear.

In conclusion, the relative importance of terminal and sub-terminal oxidation of propane in *R. rhodochrous* PNKb1 remains uncertain. However, a cell-free assay system has been developed and future work will allow the stabilization and purification of what appears to be a novel oxygenase system, thus answering the question of the relative importance of each pathway of propane oxidation in this organism. Establishment of growth of this *Rhodochrous* sp. on the potential intermediates of propane metabolism will also aid future genetic analysis of the pathways of propane metabolism.

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REFERENCES

- BABU, J. P. & BROWN, L. R. (1984). A new type of oxygenase involved in the metabolism of propane and isobutane. *Applied and Environmental Microbiology* **48**, 260–264.
- BONNET-SMITS, E. M., ROBERTSON, L. A., VAN DIJKEN, J. P., SENIOR, E. & KUENEN, J. G. (1988). Carbon dioxide as the initial step in the metabolism of acetone by *Thiosphaera pantotropha*. *Journal of General Microbiology* **134**, 2281–2289.
- DE BONT, J. A. M. & PECK, M. W. (1980). Metabolism of acetylene by *Rhodococcus* A1. *Archives of Microbiology* **127**, 99–104.
- CARDINI, G. & JURTSCHUK, P. (1970). The enzymatic hydroxylation of n-octane by *Corynebacterium* sp. strain 7EIC. *Journal of Biological Chemistry* **245**, 2789–2796.
- COLBY, J., DALTON, H. & WHITTENBURY, R. (1975). An improved assay for bacterial methane monooxygenase: some properties of the enzyme from *Methylobacterium methanica*. *Biochemical Journal* **151**, 459–462.
- DAGLEY, S. & CHAPMAN, P. J. (1971). Evaluation of methods used to determine metabolic pathways. *Methods in Microbiology* **6A**, 217–269.
- DAGLEY, S. & NICHOLSON, D. E. (1970). *An Introduction to Metabolic Pathways*. Oxford: Blackwell Scientific Publications.
- DALTON, H. (1980). Oxidation of hydrocarbons by methane monooxygenases from a variety of microbes. *Advances in Applied Microbiology* **26**, 71–87.
- DIXON, G. H. & KORNBERG, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochemical Journal* **72**, 3–33.
- GERHARDT, P. (1981). Diluents and biomass measurement. In *Manual of Methods for General Bacteriology*, pp. 504–507. Edited by P. Gerhardt, R. Murray, R. Costilow, E. Nester, W. Wood, N. Krieg & G. Phillips. Washington, DC: American Society for Microbiology.
- GEST, H., DITS, M. W. & FAVINGER, J. L. (1983). Characterisation of *Rhodopseudomonas sphaeroides*. *FEMS Microbiology Letters* **17**, 321–325.
- GRUNERT, R. R. & PHILLIPS, P. H. (1951). A modification of the nitroprusside method of analysis for glutathione. *Archives of Biochemistry & Biophysics* **30**, 217–225.
- HARTMANS, S. & DE BONT, J. A. M. (1986). Acetol monooxygenase from *Mycobacterium* Pyl cleaves acetol into acetate and formaldehyde. *FEMS Microbiology Letters* **36**, 155–158.
- KANNER, D. & BARTHA, R. (1982). Metabolism of acetylene by *Nocardia rhodochrous*. *Journal of Bacteriology* **150**, 989–992.
- KLUG, M. J. & MARKOVETZ, A. J. (1971). Utilization of aliphatic hydrocarbons by microorganisms. *Advances in Microbial Physiology* **5**, 1–43.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MACMICHAEL, G. & BROWN, L. (1987). Role of carbon dioxide in catabolism of propane by '*Nocardia paraffinicum*' (*Rhodococcus rhodochrous*). *Applied and Environmental Microbiology* **53**, 65–69.
- MCAULIFFE, C. (1966). Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin and aromatic hydrocarbons. *Journal of Physical Chemistry* **70**, 1267–1275.
- MCKENNA, E. J. & COON, M. J. (1970). Enzymatic ω -oxidation IV. Purification and properties of the ω -hydroxylase of *Pseudomonas oleovorans*. *Journal of Biological Chemistry* **245**, 3882–3889.
- O'FARRELL, P. H. (1975). High-resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007–4021.
- PATEL, R. N., HOU, C. T., LASKIN, A. I., FELIX, A. & DERELANKO, P. (1983a). Oxidation of alkanes by organisms grown on C₂–C₄ alkanes. *Journal of Applied Biochemistry* **5**, 107–120.
- PATEL, R. N., HOU, C. T., LASKIN, A. I., FELIX, A. & DERELANKO, P. (1983b). Epoxidation of n-alkenes by organisms grown on gaseous alkanes. *Journal of Applied Biochemistry* **5**, 121–131.
- PERRY, J. J. (1968). Substrate specificity in hydrocarbon utilizing organisms. *Antonie van Leeuwenhoek* **34**, 27–36.
- PERRY, J. J. (1980). Propane utilization by microorganisms. *Advances in Applied Microbiology* **26**, 89–115.
- PLATEN, H. & SCHINK, B. (1987). Methanogenic degradation of acetone by an enrichment culture. *Archives of Microbiology* **149**, 136–141.
- REEVES, H. C., RABIN, R., WEGENER, W. S. & AJL, S. J. (1971). Assays of enzymes of the tricarboxylic acid and glyoxalate cycles. *Methods in Microbiology* **6A**, 452–455.

- ROBINSON, J. & COOPER, R. M. (1970). Method of determining oxygen concentration in biological media, suitable for calibration of the oxygen electrode. *Analytical Biochemistry* **33**, 390–399.
- STEPHENS, G. M. & DALTON, H. (1986). The role of terminal and subterminal oxidation pathways in propane metabolism by bacteria. *Journal of General Microbiology* **132**, 2453–2462.
- STEPHENS, G. M. & DALTON, H. (1987). Is toxin production by coryneform bacteria linked to their ability to utilize hydrocarbons? *Trends in Biotechnology* **5**, 5–7.
- STIRLING, D. I. (1978). *Oxidation of carbon compounds by Methylococcus capsulatus*. PhD thesis, University of Warwick, UK.
- TAYLOR, D. G., TRUDGILL, P. W., CRIPPS, R. E. & HARRIS, P. R. (1980). The microbial metabolism of acetone. *Journal of General Microbiology* **118**, 159–170.
- VAN GINKEL, C. G., WELTON, H. G., HARTMANS, S. & DE BONT, J. A. M. (1987). Metabolism of trans-2-butene and butane in *Nocardia* TB1. *Journal of General Microbiology* **133**, 1713–1720.
- WHITTENBURY, R., PHILLIPS, K. C. & WILKINSON, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology* **61**, 205–218.