METABOLISM OF ONE CARBON COMPOUNDS: CYTOCHROMES OF METHANE- AND METHANOL-UTILISING BACTERIA

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1. Introduction

There have been many reports on the enzymology and intermediary metabolism of methane- and methanol-utilising microorganisms [1,2]. However, there is little information concerning the energy metabolism and respiratory pathways involved. Cytochrome spectra of intact organisms have recently been described for two methane utilisers, *Methylomonas albus* and *Methylosinus trichosporium* [3] and Anthony [4] has briefly described studies of the cytochromes of *Pseudomonas* AM1, using both intact organisms and extracts.

The present paper reports comparative spectrophotometric studies of the cytochromes in extracts of three methanol utilisers (*Pseudomonas* AM1, *Pseudomonas extorquens* and *Hyphomicrobium*) and a representative of each of the major groups [5,6] of methane utilisers, *Pseudomonas methanica* (contains Type I membranes and utilises a pentose pathway for carbon incorporation) and *Methylosinus trichosporium* (contains Type II membranes and utilises the serine pathway for carbon incorporation).

2. Materials and methods

Hyphomicrobium sp. and *Pseudomonas* AM1 were grown on methanol (1%, v/v) as sole carbon and energy source [7,8]. *Pseudomonas extorquens* NCIB 9399 was grown on methanol (1% v/v) or succinate (0.6% w/v) as sole carbon and energy sources both in batch and continuous culture at 30°C on a minimal medium containing in 1 litre at pH 7.0, Na_2HPO_4, 3 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 3g; $MgSO_4.7H_2O$, 0.10 g; $FeCl_3$, 16.7 mg; $CaCl_2.2H_2O$, 0.6 mg; $ZnSO_4.7H_2O$, 0.18 mg; $CuSO_4.5H_2O$, 0.16 mg; $MnSO_4.4H_2O$, 0.5 mg; $CoCl_2.6H_2O$, 0.18 mg; $Na_2MoO_4.2H_2O$, 0.3 mg.

Methylosinus trichosporium OB3b and *Pseudomonas methanica* were grown on methane, supplied as a methane:air mixture (1:1, v/v), as sole source of carbon and energy [3,9].

After harvesting, organisms were disrupted by ultrasonication (3 min, MSE type 150 W sonicator) and the resulting extract centrifuged (12 000 *g* for 10 min). The supernatant fraction was further centrifuged (150 000 *g* for 90 min) yielding particulate and supernatant fractions. The particulate fraction was washed and resuspended in 10 mM potassium phosphate buffer, pH 7.0.

Cytochrome spectra at room temperature and 77°K and pyridine haemochromes were measured using a Hitachi Perkin–Elmer 356 spectrophotometer [10]. Protein concentration was determined by a modified biuret method [11].

3. Results

3.1. *Cytochromes of P. extorquens*

Fig. 1 shows room temperature reduced *minus* oxidised difference spectra of the particulate and supernatant fractions of *P. extorquens*. The supernatant fraction contains a *c*-type cytochrome with

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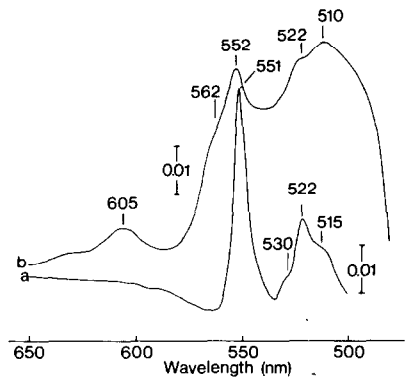

Fig. 1. $Na_2S_2O_4$ – reduced *minus* $K_3Fe(CN)_6$ – oxidised difference spectra of the supernatant (a) and particulate (b) fractions of *P. extorquens*. The light path was 1 cm and the temperature 21°C. Absorbance scale indicated by numbers in vertical bars.

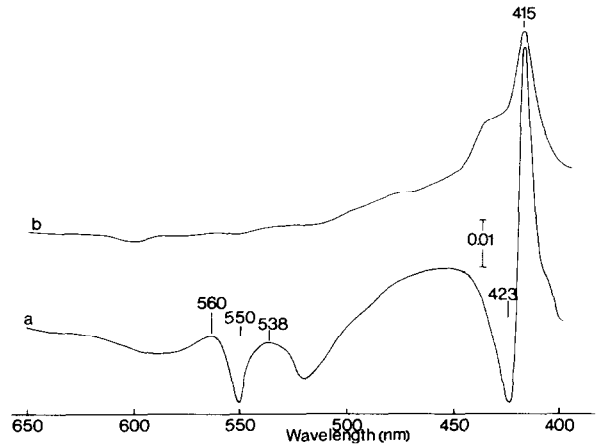

Fig. 2. $Na_2S_2O_4$ – reduced *plus* CO *minus* $Na_2S_2O_4$ – reduced difference spectra of the supernatant (a) and particulate (b) fractions of *P. extorquens*. CO was bubbled for 30 sec through one cuvette followed by a 10 min incubation before recording the spectra at room temperature. Absorbance scale indicated by numbers in vertical bars.

peaks at 551 nm and 522 nm (Soret peak at 418 nm, not shown) but no other cytochromes. The particulate fraction contains cytochromes aa_3 (605 nm), *b* (562, 530 nm) and *c* (552, 522 nm). The Soret peak is at 430 nm due to cytochromes *b* and *c* and has a shoulder at 441 nm due to cytochromes aa_3 (not shown). Note the upwards displacement of the cytochrome *c* α-peak relative to that of the cytochrome *b* (fig. 1). This unusual effect is seen in all the organisms used in this study.

Pyridine haemochrome reduced *minus* oxidised difference spectra of the residue from acid–acetone extractions [10] of the supernatant fraction of *P. extorquens* have an α-peak at 551 nm, confirming that the cytochrome of the supernatant is of the *c*-type. No pyridine haemochrome derivatives can be detected in acid–acetone extracts of the supernatant, i.e. there is no protohaem (cytochrome *b*) present.

Fig. 2 shows room temperature reduced *plus* CO *minus* reduced difference spectra of the particulate and supernatant fractions of *P. extorquens*. The supernatant fraction has peaks at 561, 538 and 415 nm and troughs at 550 and 423 nm. This spectrum is typical of CO-binding *c*-type cytochromes [10]. It is also similar to that of cytochrome *o*, but shifted 2–10 nm towards the blue region [12,13]. Cytochrome *o* is a protohaem-containing cytochrome [14] but reduced *minus* oxidised difference spectra and pyridine haemochrome difference spectra of both residue and extract from acid–acetone extraction of the supernatant fraction of *P. extorquens* indicate that it contains a *c*-type cytochrome and not a protohaem-containing one Therefore the peaks must be due to CO-binding by the *c*-type cytochrome. A similar pattern of peaks and troughs occurs in the particulate fraction CO-spectra (fig. 2). The intensity of the spectrum is, however, much lower, indicating that only a small proportion of the CO-binding *c*-type cytochrome is present in the particles. In addition, a shoulder at 430 nm occurs on the Soret peak of athe particles; this is due to cytochrome a_3.

Low temperature (77°K) reduced *minus* oxidised or reduced *plus* CO *minus* reduced difference spectra of the particulate and supernatant fractions of *P. extorquens* result in improved definition of the absorbance peaks and a shift of 2–3 nm towards the blue region, but no resolution of extra peaks or shoulders.

3.2. *Cytochromes of other methane- and methanol-utilising bacteria*

A survey of other methane and methanol oxidisers indicates that in each case there is a similar cytochrome composition to that of *P. extorquens* (table 1). The particulate fractions contain cytochromes aa_3, *b* and *c*, of which cytochrome a_3 and notably some

Table 1
Distribution of cytochromes in cell-free extracts of methane- and methanol-utilising bacteria

Organism	Concentration in fraction shown (pmoles (mg of protein)$^{-1}$)								
	Particulate				Supernatant				Supernatant
	aa_3	b	c	c_{CO}	c	c_{CO}	aa_3	b	$\frac{c_{CO}}{c}$ (%)
P. extorquens	74	327	123	55	2263	1344	+	–	59.2
Hyphomicrobium	++	285	173	73	1263	763	+	–	61.1
P. AMI	++	236	122	68	946	750	++	–	79.2
P. methanica	++	129	238	117	583	372	++	–	63.9
M. trichosporium	++	215	136	104	897	590	++	–	65.9

+, ++ = Detected but concentration too low to measure accurately.
The concentrations of the total *b* and *c*-type cytochromes were determined from the heights of their α-peaks in room temperature $Na_2S_2O_4$ reduced *minus* oxidised difference spectra using the extinction coefficients given by Jones and Redfearn [15]. The total concentrations of the CO-binding *c*-type cytochrome were determined from the Soret peak in room temperature $Na_2S_2O_4$ reduced *plus* CO *minus* reduced difference spectra, as described by Bartsch [16].

of the *c*-type cytochrome bind CO. The supernatant fractions all contain substantial quantities of a *c*-type cytochrome which is able to bind CO. The proportion of the total cytochrome *c* that binds CO varies between the bacteria from 59.2–79.2% (table 1). This may indicate that two *c*-type cytochromes are present, only one of which binds CO, but it is more likely that the results represent incomplete binding due to the low binding rate [10,17].

3.3. *Effect of growth substrate on cytochrome complement of P. extorquens*

Fig. 3 shows cytochrome spectra of *P. extorquens* particulate fraction after growth on succinate. Spectra of the supernatant fraction show the presence of the *c*-type cytochrome only (not shown). Determinations of cytochrome content were made (table 2) on preparations of organisms from five cultures, independently grown on methanol (three continuous and two batch cultures). These results are compared with those from analogous preparations after growth on succinate (two continuous and one batch culture). Growth conditions in continuous culture were closely similar when either methanol or succinate was the growth substrate and the cultures were grown under conditions of oxygen limitation. The differences in cytochrome complement after growth on those substrates in batch culture were quantitatively similar to those obtained after growth in continuous culture. Growth on succinate resulted in an average 8.4- and 3.4-fold increase in particulate fraction cytochromes aa_3 and *b* respectively together with an average 35% reduction of cytochrome *c* in the supernatant compared to methanol grown cells. There is no change in

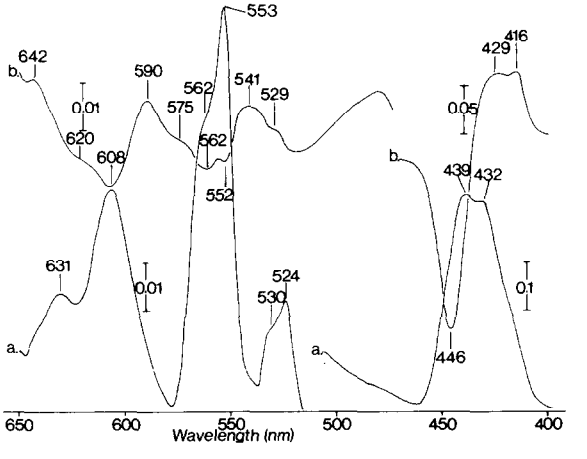

Fig. 3. (a) $Na_2S_2O_4$ – reduced *minus* H_2O_2 – oxidised difference spectra of particulate fraction of succinate-grown *P. extorquens*. (b) $Na_2S_2O_4$ – reduced *plus* CO *minus* $Na_2S_2O_4$ – reduced difference spectra of the particulate fraction of succinate-grown *P. extorquens*. Absorbance scale indicated by numbers in vertical bars.

Table 2
Comparison of the cytochrome complements of *P. extorquens* after growth on succinate or methanol

Growth Substrate	Cytochrome concentration (pmoles (mg of protein)$^{-1}$)				
	Particulate fraction				Supernatant fraction
	aa_3	a_2	b	c	c
Methanol	74 ± 2.0	–	327 ± 3.2	123 ± 3.3	1344 ± 2.8
Succinate	623 ± 3.5	++	1102 ± 3.8	125 ± 2.5	864 ± 2.6

– = not detectable, ++ = present but not quantitated.
Values represent the arithmetical mean of duplicate determinations on five- and three-independent preparations of methanol- and succinate grown organism respectively. In each case the reproducibility is indicated by the standard error of the mean. Growth conditions were as follows:- culture vol, 1.5 l in a magnetically-stirred culture vessel (capacity, 2 l.), aeration rate, 2.0 l min^{-1}, other conditions as described in Materials and methods. Batch cultures were harvested during exponential growth (mean generation time, 2.5–3.0 h) when the cell density was approx. 2.0 g. dry wt. l^{-1}. In continuous cultures the dilution rate was 0.09–0.10 hr^{-1}.

particulate fraction cytochrome *c* concentration, but a small amount of cytochrome a_2 (α-peak at 631 nm) is now seen. Reduced *plus* CO *minus* reduced difference spectra of the supernatant fraction of succinate grown cells are similar to those of the methanol grown cells with peaks due to the CO-binding cytochrome *c* only (not shown). CO spectra of the particulate fraction of succinate grown cells (fig. 3) contain peaks due to CO-binding cytochrome *c* (416, 541 nm and trough at 552 nm), cytochrome a_3 (429, 590 nm) and cytochrome a_2 (642 nm, inflexion at 620 nm).

4. Discussion

The particulate fractions of several methane- and methanol-utilising bacteria have all been found to contain only low concentrations of cytochromes *b*, *c* and aa_3 after growth on methane or methanol; the quantities found are low for aerobically-grown bacteria. Of great interest is the presence of extremely high concentrations of a soluble CO-binding *c*-type cytochrome in all the methylotrophs studied.

Anthony [4] has previously described briefly the purification of a *c*-type cytochrome from *P.* AM1, which presumably corresponds to the soluble cytochrome that we have observed, although CO-binding was not reported. Ribbons and coworkers [2] have likewise noted the presence of a 412 nm absorbing component in CO-spectra of the obligate 'methylotroph', *Methylococcus capsulatus;* it is most likely due to the Soret peak of the CO-binding *c*-type cytochrome. Davey and Mitton [3] have also noted a peak at 416 nm in CO-spectra of intact organisms of *M. albus* and *M. trichosporium* which they assumed to be due to cytochrome *o*, although from the peak intensity compared to the relative protohaem and cytochrome *c* contents of *M. trichosporium* [3] it is probably due to the CO-binding *c*-type cytochrome that we have observed.

CO-binding is not usually associated with *c*-type cytochromes and it may indicate an oxidase or oxygenase function for this cytochrome in 'methylotrophic' bacteria. Recent studies in this laboratory have shown that two other heterotrophic bacteria, *Beneckea natriegens* and *Chromobacterium violaceum* also contain soluble CO-binding *c*-type cytochromes. Action spectra in intact organisms of *Beneckea natriegens* indicate that the CO-binding *c*-type cytochrome in this bacterium has an oxidase function [18,19].

These heterotrophs contain higher concentrations of membrane-bound cytochromes than the methane utilisers and methanol-grown methanol-utilisers. This is also true of succinate-grown *P. extorquens* and in this case the gross difference in cytochrome complement between methanol- and succinate-grown organisms supports the hypothesis that the CO-bind-

ing *c*-type cytochrome may have some special function in the metabolism of methane and methanol.

On the basis of preliminary studies of the cytochromes of the obligate 'methylotroph', *Methylococcus capsulatus*, and the fact that low NADH oxidase activities have been found in several obligate 'methylotrophs' [20], Ribbons and coworkers [2] suggested that obligate 'methylotrophy' may be due to possession of electron transport and energy transduction mechanisms that are different from those of common heterotrophs. The results described in the present paper show a remarkable similarity between the cytochrome complements of obligate 'methylotrophs' and methanol-grown heterotrophic methanol utilisers. However *P. extorquens* grown heterotrophically on succinate contains massively greater concentrations of membrane bound cytochromes compared to cells grown 'methylotrophically' on methanol. This infers a radically different requirement for respiration and energy transduction between heterotrophically-grown and 'methylotrophically'-grown methylotrophs, rather than a distinction between obligate 'methylotrophs' grown on methane and obligate and facultative 'methylotrophs' grown on methanol or other substrates.

The possibility that the soluble CO-binding *c*-type cytochrome found in such large quantities in these bacteria may have an oxidase or oxygenase function in methane and/or methanol oxidation is supported by the very recent work of Ferenci [21] on CO stimulated respiration in *P. methanica* and *M. trichosporium*, published after completion of these studies.

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