Phenotypic characterization of *c*-type-cytochrome-deficient mutants of *Methylobacterium extorquens* AM1 and identification of two chromosomal regions essential for the production of *c*-type cytochromes

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(Received 21 July 1992; revised 28 September 1992; accepted 5 October 1992)

Four mutants of *Methylobacterium extorquens* AM1 which were deficient in the production of soluble *c*-type cytochromes have been isolated and characterized. They were unable to grow on methanol, ethanol or methylamine and *c*-type cytochromes were not detected in cell extracts by spectroscopy or in haem-stained SDS-polyacrylamide gels. Western blotting demonstrated that the mutants did not contain either the apo- or the holo-forms of cytochrome *c*_L, although trace amounts of holo-cytochrome *c*_H were detected. The mutants had only trace levels of methylamine dehydrogenase activity, suggesting a role for at least one of the *c*-type cytochromes in the production of the active form of this enzyme. Two regions of the *M. extorquens* AM1 chromosome which encode genes required for the synthesis of *c*-type cytochromes were identified and we have shown that, although the four mutants were phenotypically identical, they represent at least three complementation groups.

Introduction

Methylobacterium extorquens AM1 can grow not only on one-carbon compounds such as methanol and methylamine, but also on a variety of compounds containing more than one carbon atom, including ethanol and succinate. Methanol and ethanol are oxidized by methanol dehydrogenase, a periplasmic pyrroloquinoline-quinone-dependent enzyme (Anthony, 1988); electrons are then transferred to an unusual periplasmic *c*-type cytochrome, designated cytochrome *c*_L (Beardmore-Gray *et al.*, 1983). The oxidation of methylamine is catalysed by methylamine dehydrogenase, another periplasmic protein, and it has recently been shown that the prosthetic group of this enzyme is tryptophan tryptophylquinone (McIntire *et al.*, 1991). A blue copper protein, amicyanin, is normally the primary electron acceptor of methylamine dehydrogenase (see Anthony, 1992).

M. extorquens AM1 produces at least two other soluble *c* cytochromes in addition to cytochrome *c*_L. One is a typical Class I *c*-type cytochrome, designated cytochrome *c*_H; it transfers electrons from cytochrome *c*_L to the terminal oxidase and is also involved in electron

transfer during growth on methylamine (Anthony, 1992). The other, cytochrome *c*₅₅₃, is produced in trace amounts in wild-type cells, but is overexpressed in some mutants which are defective in methanol oxidation (Mox mutants); its function is unknown (Day *et al.*, 1990).

Anthony (1975) described a mutant of *M. extorquens* AM1 (PCT76) which did not synthesize any *c*-type cytochromes and was unable to grow on methanol, ethanol or methylamine; growth on formate, succinate and other compounds containing more than one carbon atom was not impaired, indicating that none of the *c*-type cytochromes is necessary for utilization of these substrates. These results suggested that one or more *c*-type cytochromes is essential for growth on methanol and ethanol. However, the role of such cytochromes during growth on methylamine could not be deduced from this work since PCT76 also lacked methylamine dehydrogenase activity and may have contained two independent mutations.

Periplasmic *c*-type cytochromes such as those described in *M. extorquens* AM1 must undergo several post-translational processing events, including removal of a signal sequence during transport to the periplasm and attachment of haem to the polypeptide. By analogy with mitochondrial *c*-type cytochromes this is likely to be catalysed by a haem lyase (Page & Ferguson, 1989, 1990) but it is unclear whether this enzyme acts before or after translocation to the periplasm. The further elucidation of

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the steps involved in *c*-type cytochrome biosynthesis in bacteria is being aided by the isolation and characterization of mutants which are deficient in *c*-type cytochromes (Kranz, 1989; Page & Ferguson, 1989, 1990; Biel & Biel, 1990; Ramseier *et al.*, 1991) and in this paper we describe four such mutants of *M. extorquens* AM1. They were phenotypically similar to mutant PCT76, being defective in the production of both methylamine dehydrogenase and the soluble *c*-type cytochromes, and fell into three complementation groups.

Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. Mutant PCT76 was not available for comparison.

Table 1. *Bacterial strains and plasmids*

Strain/ cosmid	Relevant properties	Source/ reference
<i>(a) Bacteria</i>		
<i>M. extorquens</i> AM1		
Wild-type		Peel & Quayle (1961)
FO13	Deficient in <i>c</i> -type cytochromes and methylamine dehydrogenase	This study
FO24		
FO35		
FO43		
PG1	MoxA mutant	Tatra & Goodwin (1983)
MoxB, C, D, E, F, G, H, K, L mutants		Nunn & Lidstrom (1986a)
<i>E. coli</i>		
HB101		Boyer & Roulland-Dussoix (1969)
S17-1	Contains the <i>tra</i> region of RP4 integrated in the chromosome	Simon <i>et al.</i> (1983)
<i>(b) Cosmids</i>		
pVK100	Inc P1 Tc ^r Km ^r cos	Knauf & Nester (1982)
PRK2013	Km ^r mobilizing plasmid, ColE1 replicon	Figurski & Helinski (1979)
Pooled clone bank of <i>M. extorquens</i> AM1 DNA in pVK100		Fulton <i>et al.</i> (1984)
pHIN-AB, pHIN-CD, pHIN-E, pHIN-FG, pHIN-H	Cosmids complementing Mox mutants	Nunn & Lidstrom (1986a)
pFO13	Complements FO13 and FO35	
pFO24	Complements FO24 and FO43	
pFO13-1, pFO13-3, pFO13-5	Subclones of pFO13	
pFO24-1, pFO24-2	Subclones of pFO24	
		This study

Growth and maintenance of bacteria. *M. extorquens* AM1 was grown on MacLennan's medium as described by Tatra & Goodwin (1983). *Escherichia coli* was grown in L broth (Sambrook *et al.*, 1989). Antibiotics were added to the medium when appropriate, at the following concentrations: oxytetracycline hydrochloride, 20 µg ml⁻¹ for *E. coli* and 15 µg ml⁻¹ for *M. extorquens* AM1; kanamycin sulphate, 50 µg ml⁻¹ for *E. coli* and 100 µg ml⁻¹ for *M. extorquens* AM1.

Mutagenesis and isolation of revertants. Mutants were isolated using ethyl methanesulphonate as described by Biville *et al.* (1989). Spontaneous revertants were never isolated, but after treatment of the mutants with ethyl methanesulphonate, cells with a wild-type phenotype (Tables 2 and 3) were obtained at a frequency of approximately 10⁻⁷.

Whole cell oxidation studies. These were done as described by Dunstan *et al.* (1972). Assays were repeated using at least two independent cultures and were reproducible to within 10%.

Preparation of cell extracts and assay of methanol and methylamine dehydrogenases. These procedures were done as described by Tatra & Goodwin (1985). All assays were repeated using at least two independent cultures and were reproducible to within 5%.

Cytochrome spectra. The presence of cytochromes in soluble and membrane fractions was determined by the method of O'Keeffe & Anthony (1980).

SDS-PAGE and haem staining. SDS-PAGE was done as described by Lee *et al.* (1991), except that soluble extracts rather than whole cells were used when indicated. The method of Thomas *et al.* (1976) was used for haem staining, under conditions in which only *c*-type cytochromes retain their haem stain.

Production of antibodies to holo- and apo-forms of *c* cytochromes. Cytochromes *c_H* and *c_L* were purified as described by O'Keeffe & Anthony (1980) except that metal chelate affinity chromatography was used instead of preparative isoelectric focusing at the final stage in the purification of cytochrome *c_L*. Pooled samples of cytochrome *c_L* collected from the second gel-filtration column were dialysed against 0.1 M-sodium phosphate (pH 8.0) and loaded at a flow rate of 5 ml h⁻¹ onto a column (1 × 8 cm) of chelating Sepharose FF (Pharmacia); this had been equilibrated with the same buffer after loading with Cu²⁺. The column was washed with 15 ml 0.1 M-sodium phosphate (pH 8.0), then eluted with a gradient of decreasing pH formed by mixing 0.1 M-sodium acetate (pH 4.0) into 0.1 M-sodium phosphate (pH 8.0). The total elution volume was 100 ml. Cytochrome *c_L* eluted at approximately pH 6.1. Both purified cytochromes were homogeneous as judged by SDS-PAGE.

The cytochromes were converted to their apo-forms as described by Page & Ferguson (1990). Each of the apo-forms was coupled to bovine serum albumin (BSA) using the cross-linking procedure of Hennig & Neupert (1983) and the resulting conjugates were injected into male New Zealand White rabbits. Primary inoculations containing 200 µg cytochrome plus Freund's complete adjuvant were followed after a 4-week interval by injection of 50 µg cytochrome plus Freund's incomplete adjuvant. The appearance of antibodies was monitored by immunoblotting using apo-cytochrome *c_H* or *c_L* immobilized on nitrocellulose strips.

The antisera obtained contained antibodies which reacted with BSA. These were removed as follows. BSA was immobilized on cyanogen-bromide-activated Sepharose 4B and 0.5 ml of the gel containing 10 mg BSA was equilibrated with phosphate-buffered saline and then used to absorb 0.5 ml of antiserum. This treatment removed the anti-BSA activity as judged by Ouchterlony double diffusion (Ouchterlony & Nilsson, 1978).

The antiserum raised against apo-cytochrome c_L reacted with the holo-form of this cytochrome, but not with either form of cytochrome c_H . Similarly, the antiserum raised against apo-cytochrome c_H reacted with the apo- and holo- forms of this cytochrome but not with either form of cytochrome c_L .

Cytochrome c_{553} was purified as described by Day *et al.* (1990). We did not attempt to raise antibodies to apo-cytochrome c_{553} because the strong tendency of this form of the protein to aggregate would have complicated any analysis of its synthesis.

Western blotting. This was done as described by Page & Ferguson (1990), using the immuno-absorbed antisera at 1/100 dilution. Prior to electrophoresis, all samples were solubilized in the presence of 2-mercaptoethanol; if this was omitted the relevant apocytochromes reacted more strongly than the holo-cytochromes after Western blotting. The inclusion of 2-mercaptoethanol also eliminated the formation of a putative dimer of apocytochrome c_H .

HPLC. This was done as described by Page & Ferguson (1989, 1990), except that a TSKG600SW column was used; antibody was used to detect when cytochrome c_H was eluted and the retention times of the apo- and holo- forms of this cytochrome were established using purified samples.

Mating procedures and DNA manipulations. The pooled genomic library of *M. extorquens* AM1 DNA constructed by Fulton *et al.* (1984) was used to complement the mutants. This library was made by digesting chromosomal DNA with *Hind*III, isolating fragments of more than 15 kbp and ligating them into the broad-host-range mobilizable cosmid vector pVK100. The recombinant cosmids were packaged into lambda procapsids and used to infect *E. coli* HB101. About 10000 transductants were obtained, and random screening indicated that they contained inserts with an average size of 20 kbp. Recombinant cosmids from this pooled library were mobilized into mutants of *M. extorquens* AM1 using a three-way cross as described by Fulton *et al.* (1984) and modified by Stone & Goodwin (1989). Complemented mutants were selected on medium containing methanol plus tetracycline. The complemented *M. extorquens* AM1 mutants were purified and then the recombinant cosmids were mobilized into *E. coli* S17-1. The ability of each cosmid to complement the appropriate mutant was confirmed by further crosses.

Cosmid DNA was isolated from *E. coli* by the method of Birnboim & Doly (1979) as modified by Sambrook *et al.* (1989). DNA manipulations and agarose gel electrophoresis were done as described by Sambrook *et al.* (1989).

Enzymes for restriction digestion and DNA manipulation were obtained from Sigma and used according to the manufacturers' instructions.

Results

Isolation and growth properties of mutants

Mutagenesis of wild-type cells with ethyl methane-sulphonate, followed by selection on medium containing succinate plus allyl alcohol, resulted in the isolation of 24 allyl-alcohol-resistant mutants. This paper describes the four isolates (FO13, FO24, FO35 and FO43) which were unable to grow on methanol, ethanol or methylamine; growth of these mutants on other substrates was not impaired. The remaining 20 isolates were able to grow on methylamine but not on methanol or ethanol. Preliminary studies indicated that they did not have a general defect in *c*-type cytochrome biosynthesis, but

Fig. 1. Visible absorption spectra of soluble cell extracts of wild-type *M. extorquens* AM1 and mutant FO13. Bacteria were grown on medium containing succinate plus methanol plus methylamine. Samples, which contained 6–8 mg protein ml⁻¹, were reduced by addition of a crystal of dithionite and the spectrum was measured against a buffer reference. (a) Wild-type; (b) mutant FO13 complemented with pFO13; (c) mutant FO13.

were methanol oxidation mutants similar to those described by Nunn & Lidstrom (1968*a, b*).

Biochemical characterization of the mutant strains

Wild-type and mutant strains were grown on medium containing methanol plus methylamine plus succinate, since it has previously been shown that the enzymes involved in C-1 oxidation are induced under these growth conditions (Dunstan *et al.*, 1972).

The ability of whole cells of the mutant and wild-type strains to oxidize a variety of substrates was compared (Table 2). Methanol, ethanol and methylamine were oxidized by the mutants at less than 10% of the rate observed in wild-type cells. Other substrates were oxidized by mutant and wild-type cells at similar rates, apart from formate, which was oxidized by the mutants at about 25% of the rate observed with the wild-type.

The failure of our mutants to oxidize methanol and ethanol cannot be explained by a lack of methanol dehydrogenase activity, although the specific activities of this enzyme in extracts of the mutants were only 30–60% of the values measured in the wild-type extracts (Table 3). In contrast, only trace amounts of methylamine dehydrogenase activity were observed in the mutant strains.

Soluble *c*-type cytochromes were readily demonstrated in extracts of wild-type cells but the typical absorption peaks due to *c*-type cytochromes were not evident in extracts of the mutants, and this could account for their inability to oxidize methanol and ethanol. Fig. 1 shows the spectrum obtained after reducing soluble extracts of

Table 2. *Oxidation of C-1 compounds, ethanol and succinate by whole cells of wild-type and mutant strains*

Bacteria were grown on medium containing succinate, methanol and methylamine as carbon sources. Values are the means of at least two independent determinations. The endogenous rate of oxygen uptake was approximately $0.08 \mu\text{mol O}_2 \text{ used (mg dry wt}^{-1}) \text{ h}^{-1}$ and was subtracted from the values shown above.

Strain	Rate of oxidation [$\mu\text{mol O}_2 \text{ used (mg dry wt}^{-1}) \text{ h}^{-1}$] of:					
	Methanol	Ethanol	Methylamine	Formaldehyde	Formate	Succinate
Wild-type	1.3	1.4	2.3	1.1	1.2	1.8
FO13	0.08	0.05	0.03	1.1	0.3	2.1
FO24	0.08	0.08	0.1	1.2	0.4	2.6
FO35	0.07	0.07	0.1	1.5	0.3	2.0
FO43	0.05	0.05	0.05	0.7	0.4	2.1
FO13(pFO13)	1.1	1.1	1.1	0.9	0.6	1.4
FO24(pFO24)	1.3	1.0	1.2	1.0	1.0	1.0
FO35(pFO13)	0.9	1.1	0.9	1.0	0.9	1.3
FO43(pFO24)	1.2	0.9	1.2	1.1	0.7	0.9
FO13 revertant	0.9	1.1	1.3	0.9	0.8	1.3
FO24 revertant	1.1	1.1	1.1	1.1	0.9	1.0
FO35 revertant	1.1	1.1	1.3	1.1	0.8	1.5
FO43 revertant	1.2	1.1	1.2	1.1	0.7	1.4

Table 3. *Specific activities of methanol and methylamine dehydrogenases in extracts of wild-type and mutant cells*

Bacteria were grown on medium containing succinate, methanol and methylamine as carbon sources. Enzyme activities were assayed using phenazine ethosulphate as the electron acceptor. Values are means of at least two independent determinations.

Strain	Specific activity [$\mu\text{mol (mg protein)}^{-1} \text{ h}^{-1}$] of:	
	Methanol dehydrogenase	Methylamine dehydrogenase
Wild-type	17.0	6.0
FO13	5.1	0.06
FO24	5.8	0.12
FO35	7.5	0.09
FO43	9.8	0.05
FO13(pFO13)	16.0	3.4
FO24(pFO24)	11.0	3.1
FO35(pFO13)	10.5	3.8
FO43(pFO24)	14.1	3.0
FO13 revertant	15.3	3.7
FO24 revertant	15.5	3.7
FO35 revertant	16.1	3.9
FO43 revertant	13.8	3.8

mutant FO13 with dithionite, measured against a buffer reference. Similar results were obtained using extracts of the other mutants, and cytochrome *c* was not observed in reduced-minus-oxidized difference spectra of any of the mutant extracts. In contrast, the reduced-minus-oxidized difference spectra of membrane preparations of the wild-type and mutant strains were similar (data not shown), *a* and *b* cytochromes being apparently normal in the

mutants. There have not been any detailed studies of membrane-bound *c*-type cytochromes of *M. extorquens* AM1, but they are difficult to detect and at least 90% of the *c*-type cytochromes are soluble when this organism is grown methylotrophically (Anthony, 1975).

The absence of normal levels of *c*-type cytochromes in the mutants was confirmed by gel electrophoresis and Western blotting. There was no obvious difference in the protein profiles of wild-type and mutant extracts after staining SDS-polyacrylamide gels with Coomassie blue (data not shown). However, haem staining of similar gels demonstrated the presence of cytochromes c_H , c_L and c_{553} in extracts of the wild-type but not of the mutants (Fig. 2).

Western blotting showed that the antiserum raised against the apo-form of cytochrome c_H also recognized the holo-cytochrome, both in the purified form and in extracts of wild-type cells (Fig. 3*a*, lanes 9–11). Trace amounts of cytochrome c_H were detected in the mutant extracts (Fig. 3*a*, lanes 5–8) and further analysis using HPLC gel-filtration established that all four mutants contained trace amounts of the holo- but not the apo-form of cytochrome c_H ; this was present at too low a level to be detected on the SDS-polyacrylamide gels by haem staining. There was no evidence for the accumulation of a putative precursor of the cytochrome which contained an N-terminal signal sequence.

The antiserum raised against cytochrome c_L also recognized both the holo- and the apo-forms of this protein (Fig. 3*b*, lanes 9–11). No traces of either form of cytochrome c_L were detected in the mutant extracts by

Fig. 2. SDS-polyacrylamide gel showing haem staining of proteins in cell extracts of wild-type and mutant strains. Bacteria were grown on medium containing succinate plus methanol plus methylamine. Each lane contained approximately 0.25 mg protein. Lanes: 1, FO43(pFO24); 2, FO35(pFO13); 3, FO24(pFO24); 4, FO13(pFO13); 5, FO43; 6, FO35; 7, FO24; 8, FO13; 9, wild-type. The bands that stained for haem were identified on the basis of the known relative mobilities of these cytochromes on SDS-PAGE.

Western blotting and there was no evidence of a putative precursor of the apocytochrome (Fig. 3*b*, lanes 5–8).

Both mutant and wild-type extracts contained a polypeptide of about 17 kDa which cross-reacted with the antiserum raised against cytochrome c_L . In another methylotroph (*Methylophilus methylotrophus*), it has been shown that cytochrome c_L degrades to a 17 kDa

haem-containing polypeptide (Cross & Anthony, 1980). However, the 17 kDa polypeptide shown in Fig. 3 was not evident on haem staining (Fig. 2), indicating that it is unlikely that it derived from limited proteolysis of cytochrome c_L . It was not detected by SDS-PAGE in the purified cytochrome preparations used to raise the antibody, but was presumably a minor impurity which was highly antigenic.

Complementation of mutant strains

pFO13, a recombinant cosmid of about 17 kbp that complemented FO13 and FO35, was isolated from the *Hind*III genomic library of *M. extorquens* AM1 DNA constructed by Fulton *et al.* (1984). A second recombinant cosmid, pFO24, complemented FO24 and FO43. The complemented mutants grew on methanol and methylamine; furthermore, both cytochromes c_H and c_L were detected in oxidized-minus-reduced difference spectra, in soluble extracts reduced with dithionite (Fig. 1 shows the spectrum for FO13), after haem staining of SDS-polyacrylamide gels (Fig. 2) and by Western blotting (Fig. 3). Whole cells of the complemented mutants oxidized methylamine and extracts contained methylamine dehydrogenase activity, at about 50% of the level in the wild-type (Tables 2 and 3). None of the mutants was complemented by clones carrying genes previously shown to be involved in methanol oxidation (*mox* genes); the clones tested contained the gene which complements MoxG mutants (which are defective in the structural gene encoding cytochrome c_L), as well as

Fig. 3. Western blotting of extracts of wild-type and mutant strains. Antisera were raised against (a) apocytochrome c_H ; (b) apocytochrome c_L . Each lane contained approximately 15 μ g protein (a). Lanes: 1, FO43(pFO24); 2, FO35(pFO13); 3, FO24(pFO24); 4, FO13(pFO13); 5, FO43; 6, FO35; 7, FO24; 8, FO13; 9, wild-type; 10, holocytochrome c_H (a) or holocytochrome c_L (b); 11, apocytochrome c_H (a) or apocytochrome c_L (b).

Fig. 4. Restriction maps of cosmids which complement cytochrome-*c*-deficient mutants of *M. extorquens* AM1. (i) Restriction map of cosmid pFO13 and its subclones; (ii) restriction map of cosmid pFO24 and its subclones.

genes which complement three mutants (MoxB, Cou-1 and Cou-6) defective in the production of methanol dehydrogenase and cytochrome *c_L* (Nunn & Lidstrom, 1986*a, b*; Lee *et al.*, 1991). The two clones isolated here did not complement any of the previously described Mox mutants of *M. extorquens* AM1 which are listed in Table 1.

Three subclones of cosmid pFO13 were generated and consideration of their restriction maps indicated that the gene(s) complementing FO13 and FO35 lies within a region of about 7 kbp (Fig. 4). Subclones of pFO24 were also constructed; one of these complemented FO24 but not FO43, indicating that the mutations in these two strains are located in different genes. The gene complementing FO24 lies within an 8.3 kbp region which is

adjacent to a 2 kbp region required for complementation of FO43 (Fig. 4).

Discussion

At present little is known of the details of *c*-type cytochrome biosynthesis in bacteria and it is likely that it will be necessary to study this process in several different organisms in order to gain an overall picture of the pathways involved. To date, selection of bacterial mutants which are unable to produce any *c*-type cytochromes has mainly made use of the Nadi test (Marrs & Gest, 1973; Willison & John, 1979; Biel & Biel, 1990), since a positive result is thought to depend on the presence of a *c*-type cytochrome, together with an

oxidase which reacts with such cytochromes. However, mutants with other phenotypes, for example deficient in the cytochrome oxidase (Willison *et al.*, 1981) are also selected by this method; furthermore, the Nadi test is difficult to use with pigmented organisms such as *M. extorquens* AM1. In this paper, we describe the use of a different selection procedure, which is based on the method originally devised by Nunn & Lidstrom (1986*a*) to isolate Mox mutants of *M. extorquens* AM1. In this organism, *c*-type cytochromes are essential for growth of *M. extorquens* AM1 on methanol but not on succinate; thus mutants deficient in these cytochromes can grow on succinate in the presence of allyl alcohol, whereas wild-type cells are killed following the oxidation of this suicide substrate by the methanol oxidase system. Further application of this method should result in the isolation of more classes of mutants deficient in *c*-type cytochromes, which can easily be distinguished from Mox mutants by their inability to grow on methylamine. We have also tried to use this selection procedure to isolate *c*-type-cytochrome-deficient mutants of another facultative methylotroph, *Paracoccus denitrificans*, which has been used as a model organism to study the biosynthesis of these cytochromes, but have been unsuccessful. This is probably because the selection procedure relies on the ability of *M. extorquens* AM1 to express methanol dehydrogenase when grown on medium containing alternative carbon sources such as succinate (Dunstan *et al.*, 1972); in contrast, in *P. denitrificans* expression of this enzyme is repressed to low levels by multi-carbon compounds (de Vries *et al.*, 1988).

All of the four mutants that we investigated were defective in the production of *c*-type cytochromes and this accounts for their inability to oxidize methanol and ethanol. Neither the mature form nor the apo-form of cytochrome c_L , which is the primary electron acceptor of methanol dehydrogenase, was present in extracts of the mutants; however, trace amounts of holo-cytochrome c_H were detected by Western blotting and HPLC. This low level of synthesis is probably due to the mutations being slightly 'leaky', as is often the case with point mutations. The mutants fell into three complementation groups and the genes complementing two of these groups are linked. This indicates that in *M. extorquens* AM1 there are at least three gene products which are essential for the production of all the soluble *c*-type cytochromes, but are not required for synthesis of the *a* or *b* cytochromes.

There have been recent reports describing *c*-type-cytochrome-deficient mutants of *Bradyrhizobium japonicum* (Ramseier *et al.*, 1991), *P. denitrificans* (Page & Ferguson, 1989, 1990) and *Rhodobacter capsulatus* (Kranz, 1989; Biel & Biel, 1990). In the case of *P. denitrificans*, a mutant lacking all the holo-forms of the *c*-type cytochromes contained the apo-forms of at least

two of these cytochromes in the periplasm, indicating that in this organism haem attachment may occur in the periplasm. In contrast, mutants of *B. japonicum* which lacked all soluble and membrane-bound haem-staining *c*-type cytochromes also lacked the apo-form of cytochrome c_1 ; the presence of the apo-forms of other *c*-type cytochromes was not investigated. However, the failure to detect apo-cytochromes in the *B. japonicum* mutants and in our *M. extorquens* AM1 mutants does not necessarily mean that these mutants are unable to synthesize precursor forms of the *c*-type cytochromes. It is more likely that the apocytochromes are synthesized and that they are unstable, as is the case with the apo-iso-1-cytochrome *c* of *Saccharomyces cerevisiae* (Dumont *et al.*, 1990).

Genetic analysis has indicated that in *B. japonicum* at least six clustered genes are required for *c*-type cytochrome biosynthesis (Ramseier *et al.*, 1991); in *R. capsulatus* six genes involved in this process have been identified also (Beckman *et al.*, 1992), but they are found at two different loci (*hel* and *ccl*). Future work will determine if the two regions of the *M. extorquens* AM1 chromosome that we have isolated contain genes corresponding to any of those described in *B. japonicum* or *R. capsulatus*.

The elucidation of the exact nature of the defects in our mutants also requires further work. However, it is unlikely that there is a defect in transcription of the structural genes encoding the *c*-type cytochromes since *moxG*, which encodes cytochrome c_L , is thought to be part of an operon which includes the genes encoding the α - and β -subunits of methanol dehydrogenase (Anderson *et al.*, 1990), and this enzyme is active in our mutants.

Since our mutants synthesize normal amounts of *a*- and *b*-type cytochromes they cannot be defective in haem synthesis. If, as is the case in mitochondria, the haem moiety must be reduced before it is inserted into the apocytochrome *c* (Nicholson & Neupert, 1989) one of our mutants may be unable to catalyse this step. Other explanations for the phenotypes of our mutants include defects in a postulated haem lyase, in the process of translocation of precursors (including haem) into the periplasm, or in a molecular chaperone specifically required for translocation and/or the correct assembly of the *c*-type cytochromes.

An unexpected property of our *M. extorquens* AM1 cytochrome-*c*-deficient mutants is their lack of methylamine dehydrogenase activity. When the first mutant of this type (PCT76) was isolated it was unclear whether or not it had one mutation which affected the production of both the *c*-type cytochromes and methylamine dehydrogenase, or whether this phenotype was due to two independent mutations (Anthony, 1975). Surprisingly, the four cytochrome-*c*-deficient mutants we

have isolated also lacked methylamine dehydrogenase activity. All four mutants reverted to a wild-type phenotype; furthermore, they fell into three complementation groups, and in all cases the presence of complementing cosmids restored the ability to produce both *c*-type cytochromes and methylamine dehydrogenase. It is thus very unlikely that the pleiotropic phenotypes of these mutants are each due to two independent mutations. The alternative explanation is that synthesis of methylamine dehydrogenase is in some way dependent on the production of one or more of the *c*-type cytochromes. Cytochrome c_L is unlikely to be important in this respect since it is not involved in methylamine oxidation and mutants which lack this cytochrome (but still produce cytochrome c_H) grow normally on methylamine (Nunn & Lidstrom, 1986b). On the other hand, cytochrome c_H is thought to be involved in electron transfer during the oxidation of both methanol and methylamine (Anthony, 1992). Further work is required to determine whether it has a role in methylamine dehydrogenase synthesis in *M. extorquens* AM1, but mutants defective in the equivalent cytochrome in *P. denitrificans* (cytochrome c_{550}) have wild-type levels of methylamine dehydrogenase (van Spanning *et al.*, 1991). It is, perhaps, more likely that either cytochrome c_{553} or an intermediate in the production of the *c*-type cytochromes plays a key role in the processing or regulation of methylamine dehydrogenase synthesis, and it is clearly important to investigate this unexpected aspect of methylamine metabolism in *M. extorquens* AM1 in more detail.

M.D.P. and S.J.F. were supported by SERC grant GR/F/48746.

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