

Characterization of a novel soluble *c*-type cytochrome in a *moxD* mutant of *Methylobacterium extorquens* AM1

DARREN J. DAY, DAVID N. NUNN and CHRISTOPHER ANTHONY*

Biochemistry Department, University of Southampton, Southampton SO9 3TU, UK

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Methylobacterium extorquens AM1 contains a novel *c*-type cytochrome, called cytochrome *c*-553, previously thought to be a precursor of the electron acceptor (cytochrome *c*_L) for methanol dehydrogenase. Its amino acid composition and serological characteristics show that it has no structural relationship to cytochrome *c*_L. It usually comprises less than 5% of the total *c*-type cytochromes. In a *moxD* mutant, which contains neither methanol dehydrogenase nor cytochrome *c*_L, it comprises 30% of the soluble cytochrome and it has been purified and characterized from that mutant. Cytochrome *c*-553 is large (*M*_r 23000), acidic and monohaem, with a redox potential of 194 mV. It reacts rapidly and completely with CO but is not autooxidizable. It is not autoreducible, and it is not an electron acceptor from methanol dehydrogenase or methylamine dehydrogenase, nor an important electron donor to the oxidase. It is able to accept electrons from cytochrome *c*_L and to donate electrons to cytochrome *c*_H. It is present in the soluble fraction (presumably periplasmic) and membrane fraction of wild-type bacteria during growth on a wide range of growth substrates, but its function in these bacteria or in the *moxD* mutant has not been determined.

Introduction

Methylobacterium extorquens AM1 is a pink facultative methylotroph, previously known as *Pseudomonas* AM1 or *Methylobacterium* AM1 (Anthony, 1982). It was recently classified and renamed, and shown to be similar to other pink facultative methylotrophs such as *M. organophilum* and *M. zatmanii* (Green & Bousefield, 1982; Green *et al.*, 1988). It oxidizes methanol by way of the periplasmic quinoprotein methanol dehydrogenase (MDH) which interacts directly with a specific cytochrome *c* (called cytochrome *c*_L). This then transfers electrons to a typical small cytochrome *c* (called cytochrome *c*_H) which is the substrate for the membrane-bound oxidase cytochrome *aa*₃ (for reviews see Anthony, 1982, 1986, 1988). In the first extensive study of the genes involved in methanol oxidation, 10 gene functions were described in *M. extorquens* (Nunn & Lindstrom, 1986*a*, *b*), including the structural gene for cytochrome *c*_L (*moxG*) which has been recently sequenced. The deduced protein structure showed that cytochrome *c*_L constitutes a completely novel class of *c*-type cytochrome (Nunn & Anthony, 1988). It was also shown that it has a typical N-terminal signal peptide. A second mutation affecting the *c*-type cytochromes is at the *moxD* locus; this mutation

leads to production of an altered cytochrome *c* which, it has been suggested (Nunn & Lidstrom, 1986*b*), might represent cytochrome *c*_L with its signal peptide still attached.

The present paper investigates this possibility and concludes that the cytochrome observed in the *moxD* mutant is not related to cytochrome *c*_L, but that it is a novel cytochrome which is always present in wild-type *M. extorquens* but which usually comprises less than 5% of the soluble cytochrome *c*. In *moxD* mutants it is produced in high concentrations, in the absence of either the normal cytochrome *c*_L or MDH.

Methods

Bacterial strains, growth and disruption of bacteria. *Methylobacterium extorquens* (NCIB 9133), previously known as *Pseudomonas* AM1 or *Methylobacterium* AM1 (Green *et al.*, 1988), was grown at 30 °C on the medium of MacLennan *et al.* (1971) as described by O'Keeffe & Anthony (1980*b*), with methanol (0.5%) as carbon source unless otherwise stated. The *moxD* mutant (UV9), described by Nunn & Lidstrom (1988*a*, *b*), was grown on the same medium as the wild-type strain except that the carbon source was 0.5% methylamine. It was grown in a 20 litre fermentor, harvested at the end of the exponential phase by centrifugation, and washed and resuspended in 20 mM-Tris/HCl (pH 8.0). A suspension of 120 g wet wt in 900 ml was disrupted by sonication in 50 ml batches using an MSE Soniprep ultrasonic disintegrator at full power for 6 min (in cycles of 30 s on and

Abbreviation: MDH, methanol dehydrogenase.

30 s off) with cooling in an ice/salt bath. Cell debris and unbroken cells were removed by centrifugation for 10 min at 10000 *g*. The supernatant liquid was decanted and centrifuged to remove membranes at 120000 *g* for 90 min to provide crude soluble extract. The membranes were washed once in 20 mM-MOPS/NaOH buffer (pH 7.0) and resuspended in the same buffer.

Purification of cytochrome *c*-553. The crude soluble extract was applied to a column of DEAE-Sephacel (Pharmacia) (45 mm diameter, 75 mm length) equilibrated in 20 mM-Tris/HCl buffer (pH 8.0). The column was then washed with 5 column vols of the same buffer at a flow-rate of 3 ml min⁻¹. This eluted the cytochrome *c_H*. Cytochrome *c*-553 was eluted with 250 mM-NaCl in the same Tris buffer, the salt removed by dialysis against the same buffer and the cytochrome applied to a column (95 mm × 25 mm diameter) of DEAE-Sepharose (Fast-Flow; Pharmacia). The column was washed with 100 mM-NaCl and the cytochrome eluted with 150 mM-NaCl in the same Tris buffer. It was taken to 45% saturation with ammonium sulphate at 4 °C and the precipitate removed by centrifugation (3000 *g* for 15 min). The supernatant liquid, containing the cytochrome, was applied to a column of phenyl Superose (Pharmacia HR 5/5 column) equilibrated with 1.8 M-ammonium sulphate in 20 mM-MOPS buffer (final pH 7.0). The column was washed with 1 M-ammonium sulphate in the same buffer before eluting the cytochrome with 0.8 M-ammonium sulphate in the same buffer. Final purification was achieved by gel-filtration on a Superose-12 column (Pharmacia) equilibrated with 50 mM-Tris/HCl containing 100 mM-NaCl (pH 8.0) at a flow rate of 0.5 ml min⁻¹. For estimation of relative molecular mass by gel-filtration the same column was used with the following standards (*M_r* values in parentheses): apoferritin (443000); ovalbumin (44000); haemoglobin (64000); myoglobin (17000); horse heart cytochrome *c* (12400); and vitamin B₁₂ (1350).

Determination of proportions of cytochromes *c*-553, *c_H* and *c_L*. Crude soluble extracts (100 μl containing 1 mg protein) were applied to a Pharmacia Mono-Q column (1 ml) equilibrated with 20 mM-Tris/HCl buffer (pH 8.0). The column was washed with 5 ml of the same buffer in order to wash-out the cytochrome *c_H* and cytochromes *c*-553 or *c_L* were eluted with a linear gradient (0–180 mM-NaCl; total vol. 12 ml). The acidic cytochromes eluted between 100 and 120 mM-NaCl. The concentration of cytochromes was determined using the absorption coefficients of the cytochromes (Table 1, and O'Keeffe & Anthony, 1980*a*).

Protein and haem determination. Protein was measured as described by Smith *et al.* (1988) using the bicinchoninic acid method with horse heart cytochrome *c* as standard. Haem was determined as the pyridine haemochrome as described by Fuhrhop & Smith (1975).

Spectrophotometry. Absorption spectra were recorded using a Shimadzu UV-3000. A few grains of dithionite or ascorbic acid were added to reduce the samples and a few grains of ferricyanide or ammonium persulphate to oxidize the samples. All spectra were run at 100 nm min⁻¹ (spectral bandwidth 1 nm). For spectra in the near-infrared region the sum of 16 accumulated spectra (0.1 nm sampling pitch) was recorded.

Redox potentials. These were measured by the method of O'Keeffe & Anthony (1980*a*) except that the concentration of cytochrome *c*-553 was 1.7 μM and the following mediators were used: diaminodurol (75 μM), hydroquinone (50 μM), pyocyanin (7.5 μM) and potassium ferricyanide (75 μM). All solutions were in 20 mM-MOPS buffer (pH 7.0) containing 100 mM-KCl.

Antisera preparation and Western blotting. Purified cytochrome *c*-553 or cytochrome *c_L* (400 μg) was suspended in Freund's complete adjuvant and used to immunize two New Zealand White rabbits; two

boosters of cytochrome (100 μg each) were given at 1 month intervals. Blood was collected (from the ear) and an IgG fraction prepared from the serum by ammonium sulphate fractionation as described by Hudson & Hay (1980). After SDS-PAGE, Western blotting was done essentially as described by Gershoni & Palade (1983); the electrophoretic transfer buffer was 25 mM-Tris/192 mM-glycine (pH 8.3) containing 20% (w/v) methanol. Blocking was done with a 5% (w/v) solution of non-fat dried milk in Tris-buffered saline (50 mM-Tris/HCl containing 100 mM-NaCl). Bound antibodies were detected by horse-radish peroxidase conjugated to rabbit anti-IgG.

Preparation of proteins from *Methylophilus methylotrophus*, organism 4025, *Paracoccus denitrificans* and *Acetobacter methanolicus*. Methods for growth and preparation of cell fractions and purified proteins were exactly as described previously: *M. methylotrophus* (Cross & Anthony, 1980); organism 4025 (Lawton & Anthony, 1985); *P. denitrificans* (Beardmore-Gray *et al.*, 1983); *A. methanolicus* (Elliott & Anthony, 1988).

SDS-PAGE. The SDS-Tris/glycine pH 8.3 system (Laemmli, 1970) was used and protein bands were stained with Coomassie Brilliant Blue R250, as described by Weber & Osborn (1975). Final acrylamide concentrations (w/v) of 10% (for whole cells and membranes) or 15% (for soluble fractions) were used. When whole bacteria were used they were boiled for 10 min in disruption buffer and insoluble debris removed by centrifugation. Gels were stained for haem proteins with 3,3',5,5'-tetramethylbenzidine (TMBZ) by the method of Thomas *et al.* (1976), and haem *c* was detected specifically as described by Goodhew *et al.* (1986). Samples that were to be haem-stained were mixed with disruption buffer lacking mercaptoethanol because heating in its presence caused loss of haem iron and a decrease in staining intensity. *M_r* values and proportions of each protein in electrophoresed samples were determined as described by Elliott & Anthony (1988).

Autoreduction and reaction with dehydrogenases. Cytochrome was reduced with ascorbic acid or oxidized with ferricyanide, the excess being removed by gel-filtration on a PD-10 column (Pharmacia). Oxidation or reduction reactions were determined by monitoring changes in absorption of the α absorption maximum of each cytochrome. Autoreduction in the presence or absence of methanol dehydrogenase (MDH), and reduction of cytochrome *c* by MDH was determined as described previously (O'Keeffe & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1983). The MDH was prepared from *M. extorquens* as described by Nunn *et al.* (1989); cytochrome *c_H* was prepared as described by O'Keeffe & Anthony (1980*a*) and cytochrome *c_L* was prepared as described by Nunn & Anthony (1988). The concentration of MDH was about 0.3 μM, which was sufficient to reduce rapidly cytochrome *c_L* (30 μM). Reactions were in 400 μl cuvettes in 20 mM-MOPS buffer (pH 7.0) containing 5 mM-methanol and 13 mM-ammonium chloride. A similar assay was used for testing reaction with methylamine dehydrogenase. The reaction was in 1 ml cuvettes in 20 mM-MOPS buffer (pH 7.0) containing 25 mM-methylamine, 26 μM-cytochrome *c*-553 and about 0.3 μM-methylamine dehydrogenase, prepared and assayed as previously described (Lawton & Anthony, 1985); its specific activity was 3.5 μmol min⁻¹ (mg protein)⁻¹ in the standard dye-linked assay.

Oxidation of cytochromes by membranes. This was measured spectrophotometrically using 10 μl of membrane suspension (0.2 mg ml⁻¹) in 20 mM-MOPS, pH 7.0, in a 400 μl cuvette. Cytochromes were reduced by a grain of ascorbate followed by rapid desalting by gel-filtration on a Pharmacia PD10 column. A range of cytochrome *c* concentrations was used so that values for *V* (maximal velocity) could be determined.

Results

Replacement of cytochrome *c_L* by cytochrome *c-553* in the *moxD* mutant (UV9)

The *moxD* mutant (UV9), grown on methylamine, contained no cytochrome *c_L*, as shown by Western blots of SDS extracts of whole bacteria, and of membrane and soluble fractions after breakage by sonication. The spectrum of the membrane fraction of mutant UV9 was slightly different from that in wild-type bacteria (Fig. 1). The proportions of cytochromes *a*, *b* and *c* were similar to those in wild-type bacteria but the α absorption peak of cytochrome *c* was slightly higher, suggesting that the cytochrome *c_L* observed in membranes of wild-type bacteria might be replaced by cytochrome *c-553* in the mutant. This was confirmed by Western blot analysis of SDS extracts of membranes using antibodies to cytochromes *c_L* and *c-553*. It is not possible to say whether or not this implies any function for these membrane-bound cytochromes in *M. extorquens*. The soluble fraction of mutant UV9 (which, of course, contains the periplasm) had two *c*-type cytochromes which could be separated by ion-exchange chromatography on DEAE-Sephacel; one had a high isoelectric point (not adsorbing to DEAE-Sephacel) and was indistinguishable from cytochrome *c_H*; the other adsorbed to the column, suggesting a low isoelectric point. This acidic cytochrome had an α peak

at 553 nm instead of 550 nm as expected for cytochrome *c_L*, and hence is called cytochrome *c-553* in the rest of this paper. The amount of cytochrome *c_H* in soluble fractions was very similar to that in wild-type bacteria and the amount of cytochrome *c-553* was similar to that of cytochrome *c_L* in wild-type bacteria. Because of the presence of large amounts of cytochrome *c-553*, the absorbance maximum of crude soluble fractions of mutant UV9 was at 552 nm rather than 550 nm as in wild-type bacteria.

In order to characterize further the novel cytochrome *c-553* it was purified and characterized from the *moxD* mutant (UV9).

Purification and characterization of cytochrome *c-553*

Purification of cytochrome *c-553*. In order to compare it with cytochrome *c_L* from wild-type bacteria, the cytochrome *c-553* was purified from mutant UV9 as described in Methods. The yield was about 15% and the purification achieved was about 25-fold, based on mols cytochrome *c-553* (g soluble protein)⁻¹ (calculated by assuming that this cytochrome constituted 30% of the total soluble *c*-type cytochrome). The cytochrome *c-553* was judged to be pure on the basis of its behaviour on SDS-PAGE (a single band when stained with Coomassie Blue and when haem-stained); it was the only protein

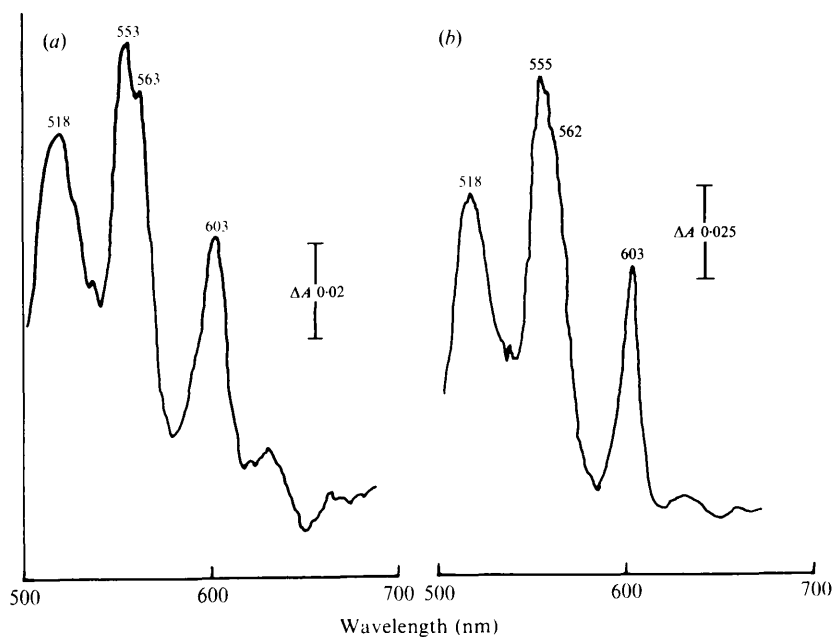


Fig. 1. Reduced-minus-oxidized difference spectra of membranes from wild-type and mutant bacteria at 77 K. Membranes, in 25 mM-HEPES/NaOH buffer (pH 7.5) containing 40% (w/v) sucrose were reduced with dithionite and oxidized with ferricyanide. (a) Wild-type bacteria (11.6 mg membrane protein ml⁻¹); (b) mutant UV9 (7.0 mg ml⁻¹). The light path was 1 mm, the bandwidth was 1 nm and the scan speed was 100 nm min⁻¹.

present as judged by its behaviour (single peak) on gel-filtration on Superose-12, and on ion-exchange chromatography on Pharmacia Mono-Q. On the basis of the purification achieved, the cytochrome comprised about 4% of the soluble protein of the bacteria. This is similar to the proportion calculated for the cytochrome c_L in wild-type bacteria.

M_r , isoelectric point and midpoint redox potential of cytochrome c -553. The M_r was 23000 as determined by SDS-PAGE with M_r standards. This was the same as that estimated by gel-filtration on a calibrated Superose-12 column (Pharmacia). The isoelectric point was judged to be low because of its behaviour on ion-exchange chromatography (binding to anion-exchange materials but not to cation-exchange materials). Cytochrome c -553 titrated as a single electron carrier with a midpoint redox potential at pH 7.0 of 194 mV. Using the same equipment and method it was confirmed that the midpoint redox potential of cytochrome c_L was 256 mV as shown previously by O'Keeffe & Anthony (1980a).

Amino acid composition of cytochrome c -553. The amino acid composition of the cytochrome was determined as described previously (Beardmore-Gray *et al.*, 1982). It is presented with the values for cytochrome c_L (from Beardmore-Gray *et al.*, 1983) given for comparison in parentheses (ND, not determined): Asx, 23 (24); Thr, 4 (14); Ser 1 (7); Glx, 20 (26); Pro, 8 (13); Gly, 19 (23); Ala, 24 (14); Cys, ND (3); Val, 8 (5); Met, 3 (3); Ile, 4 (6); Leu, 11 (15); Tyr, 10 (6); Phe, 5 (5); His, 8 (7); Lys, 17 (13); Arg, 6 (4); Trp, ND (2). It is obvious from these values that cytochrome c -553 is not a derivative of cytochrome c_L (or vice versa). It was not possible to determine the N-terminal amino acid sequence of cytochrome c -553 because it was shown to be blocked.

Absorption spectra of cytochrome c -553. Cytochrome c -553 was in the oxidized state when eluted from DEAE-Sephacel; in this it differs from cytochromes c_H and c_L which are usually almost completely reduced when isolated. Reaction with alkaline pyridine gave a typical haem c spectrum, with 1.06 mol haem (mol cytochrome) $^{-1}$ (assuming $M_r = 23000$). This demonstrates that the cytochrome is a monohaem cytochrome c and this is confirmed by its spectrum which is that of a typical low-spin c -type cytochrome (Fig. 2). The absorption band in the near-infrared region (about 695 nm) shown by the cytochrome when oxidized by air, ferricyanide, ammonium persulphate or hydrogen peroxide (Fig. 2) is characteristic of c -cytochromes having methionine as their 6th ligand (Pettigrew & Moore, 1987). The absorption peaks of cytochrome c_L and cytochrome c -553 (Fig. 3) are clearly distinguishable, being 550 nm, 520 nm and 417 nm for cytochrome c_L , and 553 nm, 523–524 nm and 419 nm for cytochrome c -553.

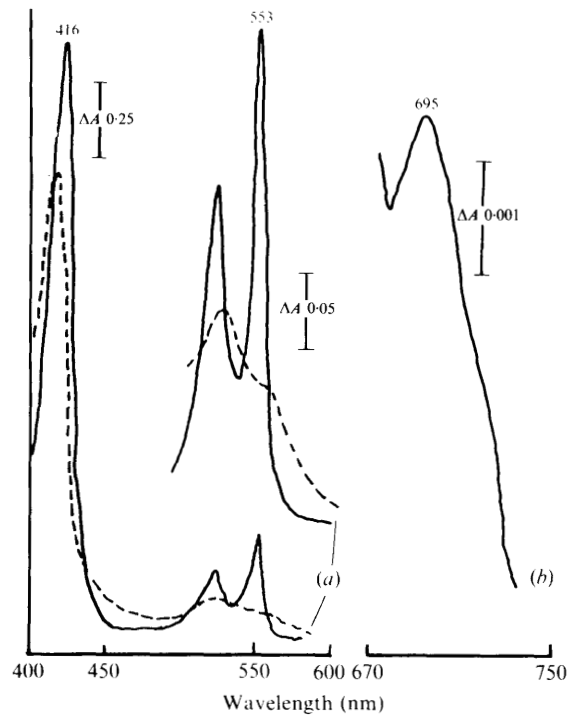


Fig. 2. Absorption spectra of cytochrome c -553. (a) Cytochrome ($9 \mu\text{M}$, 20 mM-HEPES buffer, pH 7.5) was reduced by addition of a few grains of dithionite, or oxidized by leaving in air at 2°C for 24 h. (b) Oxidized cytochrome in the near-infrared region (accumulated spectrum as described in Methods).

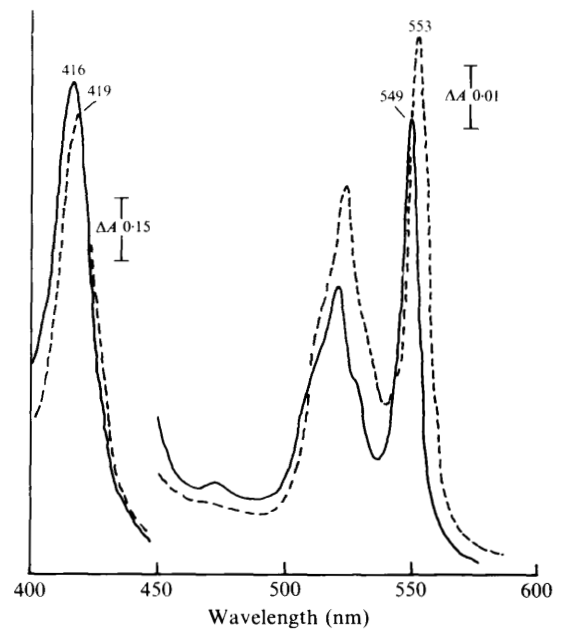


Fig. 3. Comparison of cytochromes c -553 and c_L . The cytochromes were reduced with dithionite. —, Cytochrome c_L ($4.9 \mu\text{M}$); ---, cytochrome c -553 ($6.9 \mu\text{M}$).

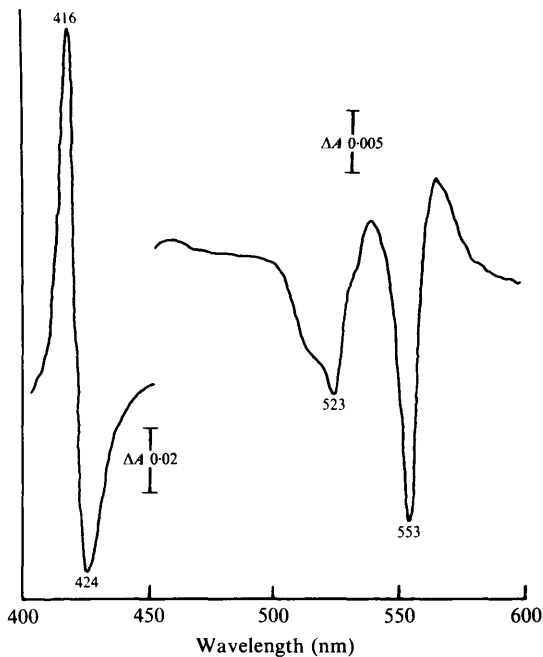


Fig. 4. The (reduced plus CO)-minus-reduced difference spectrum of cytochrome *c*-553. Cytochrome (2.3 μM in 20 mM-HEPES buffer, pH 7.5) was reduced with a few grains of dithionite and CO was bubbled through a sample as a fine stream for 30 s; its spectrum was recorded and CO passed through for a further 60 s and the spectrum recorded again.

Reaction with carbon monoxide and autoxidizability of cytochrome *c*-553. The spectrum shown in Fig. 4 shows that cytochrome *c*-553 reacted rapidly with CO. When CO was bubbled through a solution for 30 s the spectrum indicated 81% binding to CO, assuming the usual extinction coefficient for CO-reactive *c*-type cytochromes (Wood, 1984). After a further 60 s of bubbling the reaction was complete (this was after a total exposure time of about 4 min, including the time for running the spectra). This rapid and complete reaction with CO is markedly different from the reaction of cytochrome *c*_L of *M. extorquens*, which is well-known as a CO-reactive cytochrome and which has a slow partial reaction with 36% reaction after 5 min and 72% reaction after 30 min (O'Keeffe & Anthony, 1980a).

When freshly reduced cytochrome was exposed to air it was slowly oxidized, the time taken for half-oxidation being about 2 h.

Electron transfer reactions of cytochrome *c*-553

Autoreduction and reaction with methanol dehydrogenase and methylamine dehydrogenase. Key characteristics of cytochrome *c*_L are that it is autoreduced at high pH and that it is the electron acceptor for MDH. In the absence of methanol, addition of MDH to cytochrome *c*_L leads to

its reduction either by stimulation of autoreduction or by transfer of electrons from endogenous reductant on the MDH (O'Keeffe & Anthony, 1980a; Beardmore-Gray *et al.*, 1983). By contrast with cytochrome *c*_L, cytochrome *c*-553 was not autoreduced (at pH 9.0) and it showed no reaction with pure MDH from *M. extorquens* in the presence or absence of methanol. Possible reaction was assessed at pH 7 or pH 9 in the presence or absence of ammonia as activator, either with cytochrome *c*-553 as sole potential electron acceptor or with a second cytochrome *c* as terminal acceptor, and in the presence or absence of air. The amounts of cytochrome and dehydrogenase used were such that a rate of 1% of that observed with MDH and cytochrome *c*_L would have been readily measured.

No reaction occurred in a similar system in which MDH was replaced by methylamine dehydrogenase.

Oxidation of cytochrome *c*-553 by the oxidase in membranes of *M. extorquens*. It has been shown previously that *M. extorquens* contains a single major oxidase, cytochrome *aa*₃, which has been purified and characterized (Fukumori *et al.*, 1985; Fukumori & Yamanaka, 1987). In the present work membranes prepared from wild-type bacteria catalysed the rapid, azide-sensitive oxidation of cytochrome *c*_H and horse heart cytochrome *c* [49 and 28 nmol cytochrome oxidized min^{-1} (mg membrane protein)⁻¹ respectively]. By contrast, the rate of oxidation of cytochrome *c*-553 was only 1.5 $\text{nmol min}^{-1} \text{mg}^{-1}$, a value similar to that measured with cytochrome *c*_L (1.7 $\text{nmol min}^{-1} \text{mg}^{-1}$). Cytochrome *c*-553 had no effect on the rate of oxidation of cytochrome *c*_H by the oxidase.

The K_m values for oxidation of all cytochromes tested were very similar to each other (about 7 μM) and similar to the K_m values measured for the oxidation of cytochrome *c*_H and *c*_L by the pure oxidase from *M. extorquens* (Fukumori *et al.*, 1985; Fukumori & Yamanaka, 1987). The oxidation rates (and K_m values) for the oxidation of cytochromes by membranes prepared from the mutant UV9 were almost identical to those measured with membranes from wild-type bacteria. All these results are consistent with the properties of the purified oxidase and indicate that the novel cytochrome *c*-553 does not function as an important electron donor to the oxidase, a function previously concluded to be that of cytochrome *c*_H (Fukumori *et al.*, 1985; Fukumori & Yamanaka, 1987; Froud & Anthony, 1984).

Electron transfer between cytochromes *c*-553, *c*_H and *c*_L. Using the membrane oxidase system described above, it was shown that the rate of oxidation of cytochrome *c*-553 (31 μM) was increased 15-fold by addition of 0.6 μM cytochrome *c*_H, and the cytochrome *c*-553 became

completely oxidized. This confirms that rapid electron transfer can occur between cytochrome *c*-553 and cytochrome c_H , the usual electron donor to the oxidase.

Using cytochrome *c*-553 as terminal electron acceptor in the MDH/cytochrome c_L system it was shown that rapid electron transfer could occur between cytochrome c_L and cytochrome *c*-553. When cytochrome *c*-553 was present in 8-fold excess over cytochrome c_L it became completely reduced by MDH although, as shown above, it was not itself an electron acceptor from MDH. The rate measured with both cytochromes together was the same as that when cytochrome c_L was the only cytochrome present.

Serological characterization and distribution of the cytochrome

Using Western blot analysis it was shown that antiserum raised to pure cytochrome *c*-553 reacted with the cytochrome but with no other cytochrome tested. These included the following: horse heart cytochrome *c*, cytochromes c_H and c_L of *M. extorquens*, and cytochromes c_L from *Methylophilus methylotrophus* and organism 4025 (obligate methylotrophs), *Acetobacter methanolicus* (an acidophile) and *Paracoccus denitrificans* (a facultative autotroph). No cross-reaction was observed when using SDS extracts of whole cells, membranes or soluble fractions of any of these organisms (except for *M. extorquens*).

Antiserum raised to pure cytochrome c_L of *M. extorquens* failed to react with cytochromes *c*-553 or c_H of this organism or with cytochromes c_L from *M. methylotrophus*, organism 4025 or *P. denitrificans*. There was a weak reaction with the pure cytochrome c_L from *A. methanolicus*.

Western blot analysis was used to show that protein reacting with antiserum specific to cytochrome *c*-553 was present in large amounts in SDS-solubilized extracts of whole cells of mutant UV9, from which cytochrome *c*-553 was isolated, but in very much lower amounts (estimated to be less than 5% of that in the mutant) in extracts of wild-type *M. extorquens* after growth on methanol, methylamine, ethanol, propanediol, pyruvate, lactate, succinate and nutrient broth. There was no indication from these experiments that the cytochrome was induced to higher levels on any of these substrates; hence no suggestion of any specific function has arisen from this work.

Discussion

The results described above have demonstrated that the previous explanation of the *moxD* mutation (Nunn &

Lidstrom, 1986*b*) was incorrect. It was suggested that this mutant might be lacking a processing function that explained three aspects of its phenotype: the lack of MDH, lack of normal cytochrome c_L and the presence of a slightly larger, acidic cytochrome *c*. It was proposed that this cytochrome might be cytochrome c_L with its signal peptide still attached. That this is not the case is demonstrated by the lack of serological relationship between the two cytochromes, and the markedly different amino acid composition. The other differences in properties, listed in Table 1, confirm the separate identity of the two cytochromes. This conclusion is supported by the genetic evidence: cytochrome *c*-553 is still synthesized in a mutant in which the gene for cytochrome c_L has been completely deleted (data not shown).

The conclusion that cytochrome *c*-553 is a completely different cytochrome raises the question of its nature and its function. It is a typical cytochrome *c* in being a soluble (presumably periplasmic), monohaem cytochrome, having a high midpoint redox potential and methionine as its 6th ligand to the haem iron. It is unusual in its large size, its rapid and complete reaction with CO and its low rate of oxidation by the cytochrome aa_3 in membranes of *M. extorquens*.

The function of this cytochrome remains obscure. It is neither an electron acceptor for MDH or methylamine dehydrogenase nor an effective electron donor to the oxidase. It is present on all growth substrates but only at low concentrations.

The nature of the *moxD* mutation is very difficult to comprehend. An obvious possibility is that it is a regulatory mutant which inhibits expression of the operon coding for MDH and its electron acceptor, cytochrome c_L . Why this mutation should lead to 'overproduction' of what is usually a very minor cytochrome of no known function remains, however, completely obscure. The possibility that cytochrome *c*-553 is induced to high levels in order to replace a function normally carried out by cytochrome c_L , but not specifically involved in methanol oxidation, cannot be discounted.

It should be noted that cytochrome *c*-553 of *M. extorquens* bears only a superficial resemblance to *c*-type cytochromes having absorption maxima above 552 nm that have been isolated from other methylotrophs. These include cytochrome *c* (IV) of *Hyphomicrobium* ZV580 which is, however, almost certainly the electron acceptor for MDH (cited by Dijkstra *et al.*, 1988). More similar is cytochrome *c*-553i from *P. denitrificans*, which is, however, also involved in methanol oxidation and is also much larger than the cytochrome *c*-553 described in the present paper (Husain & Davidson, 1986; Gray *et al.*, 1986). Cytochrome *c*-553 of *M. extorquens* also bears no

Table 1. Physical properties of cytochrome *c*-553 and cytochrome *c_L*

Data for cytochrome *c_L* are taken from O'Keeffe & Anthony (1980*a, b*) and Beardmore-Gray *et al.* (1982). The proportion of cytochrome *c_H* in both wild-type bacteria and mutant UV9 was about 70% of the total soluble cytochrome.

Property	Cytochrome <i>c</i> -553	Cytochrome <i>c_L</i>
Relative proportion in crude extracts		
Wild-type	< 5%	28%
Mutant UV9	30%	0
<i>M_r</i>	23000	20900
Haem groups per mol	1	1
Midpoint redox potential (pH 7.0)	+194 mV	+256 mV
Absorption maxima		
Ferrocytochrome	553 nm, 419 nm	549 nm, 416 nm
Ferricytochrome	414 nm, 695 nm	410 nm, 695 nm
Absorption coefficients (mm ⁻¹ cm ⁻¹)		
Ferrocytochrome (α and Soret)	25.3, 153	26, 163
Ferricytochrome (695 nm)	0.58	0.35
Ratio of Soret/α absorption in ferrocytochrome	6.05	6.25
Autoreduction at high pH	None	Fast
Electron acceptor for MDH	No	Yes
Autoxidizability	Very slow	Slow
Oxidation by membrane oxidase	Very slow	Very slow
Reaction with CO (% binding)	Fast (100%)	Slow (72%)

resemblance to unusual *c*-type cytochromes having no known function that have been isolated from other methylotrophs; these include cytochrome *c*-555 (a small basic protein) from *Methylococcus capsulatus* (Ambler *et al.*, 1986), and cytochrome *c''* from *Methylophilus methylotrophus*, which is exceptional in being a high-spin cytochrome when in the reduced state but low-spin in its oxidized state (Santos & Turner, 1988).

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