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Soluble Cytochromes from the Marine Methanotroph *Methylomonas* sp. Strain A4

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Soluble *c*-type cytochromes are central to metabolism of C_1 compounds in methylotrophic bacteria. In order to characterize the role of *c*-type cytochromes in methane-utilizing bacteria (methanotrophs), we have purified four different cytochromes, cytochromes *c*-554, *c*-553, *c*-552, and *c*-551, from the marine methanotroph *Methylomonas* sp. strain A4. The two major species, cytochromes *c*-554 and *c*-552, were monoheme cytochromes and accounted for 57 and 26%, respectively, of the soluble *c*-heme. The approximate molecular masses were 8,500 daltons (Da) (cytochrome *c*-554) and 14,000 Da (cytochrome *c*-552), and the isoelectric points were pH 6.4 and 4.7, respectively. Two possible diheme *c*-type cytochromes were also isolated in lesser amounts from *Methylomonas* sp. strain A4, cytochromes *c*-551 and *c*-553. These were 16,500 and 34,000 Da, respectively, and had isoelectric points at pH 4.75 and 4.8, respectively. Cytochrome *c*-551 accounted for 9% of the soluble *c*-heme, and cytochrome *c*-553 accounted for 8%. All four cytochromes differed in their oxidized versus reduced absorption maxima and their extinction coefficients. In addition, cytochromes *c*-554, *c*-552, and *c*-551 were shown to have different electron paramagnetic spectra and N-terminal amino acid sequences. None of the cytochromes showed significant activity with purified methanol dehydrogenase in vitro, but our data suggested that cytochrome *c*-552 is probably the in vivo electron acceptor for the methanol dehydrogenase.

Methylomonas sp. strain A4 is a type I marine methanotroph that utilizes methane or methanol as its sole energy and carbon source (20). This methanotroph is similar to freshwater *Methylomonas* strains except for its NaCl requirement, its rapid growth rate (generation time, 3.5 h), and the presence of both the serine and ribulose monophosphate pathways for carbon assimilation. Freshwater methanotrophs oxidize methane to carbon dioxide by a series of two electron steps, with methanol, formaldehyde, and formate as intermediates (6). Soluble *c*-type cytochromes have been shown or proposed to be directly involved as either an electron acceptor or electron donor in each step of the methane oxidation pathway except the last (formate oxidation) (7, 29).

Consistent with the importance of cytochromes *c* in metabolism of C_1 compounds, several freshwater methanol utilizers have been shown to contain at least two different soluble *c*-type cytochromes (7, 14). *Methylobacterium extorquens* AM1 (10), *Hyphomicrobium* sp. strain X (14), and *Methylophilus methylotrophus* (27) have two major monoheme cytochromes *c*, one, usually in lesser abundance, of approximately 17 to 20 kDa with a low isoelectric point (often termed cytochrome c_L) and a second, usually in higher abundance, of approximately 8 to 15 kDa with a higher isoelectric point (often termed cytochrome c_H). These cytochromes are thought to mediate electron flow from the enzyme involved in the oxidation of methanol to formaldehyde, methanol dehydrogenase (MeDH), in the order MeDH — cytochrome c_L — cytochrome c_H — terminal oxidase (7). *M. extorquens* has recently been shown to contain a third, minor monoheme cytochrome *c*, cytochrome *c*-553 (11). Cytochrome *c*-553 is 23 kDa, with a low isoelectric point, and its function is unknown, although it does not appear to be involved in methanol metabolism. *M. methylotrophus* has been shown to contain two other cytochromes *c* of unknown function (27, 28). These are approximately 20 and 15 kDa, with isoelectric points of 4 and 8.7. The latter, termed cytochrome *c*″, bound CO and showed a redox-linked change of spin state (27). The classes of *c*-type cytochromes isolated from the methanol utilizer *Methylomonas* sp. strain J (25) and the autotrophic methanol utilizer *Paracoccus denitrificans* (1, 3) show some differences from the others noted above, since the isoelectric points of these cytochromes were all below pH 7.0, and the sizes and abundances were also more variable.

In contrast to the work noted above, little is known about cytochromes *c* in methane utilizers. One study involved the freshwater type X strain *Methylococcus capsulatus* Bath (2). Four soluble *c*-type cytochromes were reported, including one major cytochrome, cytochrome *c*-555, which was sequenced (molecular mass, 11 kDa), and three uncharacterized *c*-type cytochromes (2). The functions of these cytochromes are not known. We are interested in defining the role of cytochromes *c* in *Methylomonas* sp. strain A4, and this report describes the first step in this goal, the identification and isolation of four cytochromes *c*, two of which may be diheme types. The purification procedures and the characteristics of these four cytochromes are presented.

MATERIALS AND METHODS

Culture conditions. *Methylomonas* sp. strain A4 was grown in a nitrate mineral salts medium (NMS) (30) plus 1.5% (wt/vol) NaCl at 37°C under an atmosphere of 20% methane and 80% (vol/vol) air. Cells were grown in a 10-liter fermentor and harvested by centrifugation at 13,200 × *g* at 4°C. Cells were resuspended in NMS medium containing 1.5% NaCl and centrifuged at 13,200 × *g* for 15 min at 4°C. Approximately 2.5 g of cell protein (10 to 15 g, wet weight)

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was obtained per 10 liters. Cells were stored at −20°C until 110 liters of cells were harvested.

Electrophoresis. Three different sodium dodecyl sulfate-polyacrylamide gel electrophoresis methods were performed. Method I utilized the procedure of Laemmli (19) on 12 to 20% gels. Methods II and III were performed by the procedures of Anderson et al. (4) and Merle and Kadenbach (23) on 18 or 20% gels.

Isoelectric focusing in tube gels was carried out by the procedure of O'Farrell (25) with 2% ampholyte pH 3.5 to 10.0 (Bio-Rad Laboratories, Richmond, Calif.) or 2.0% ampholyte pH 4.0 to 6.0. Preparative flat-bed (10 by 20 cm) isoelectric focusing with 4% Ultrodex (LKB, Bromma, Sweden) and 2% ampholyte (pH 4.0 to 6.0, pH 3.5 to 10.0, pH 5.0 to 8.0, or a combination of two of the above) was performed on a 2117 Multiphore II electrophoresis unit (LKB).

Densitometric scanning of slab gels was performed with an LKB densitometer (2202 Ultroscan) equipped with an LKB model 2220 recording integrator model 2220.

Protein and heme determination. The concentration of heme *c* was measured by the pyridine ferrohemochrome method (10) with the $\Delta\epsilon$ (550 nm) of 29.1 cm^{-1} mM^{-1}. The type of heme was determined by the acid acetone method (16). Protein concentration was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard during the initial purification steps and equine cytochrome *c* (Sigma Chemical Co., type IV) as a standard during later steps and on the final samples.

Amino acid analysis and peptide sequence. Amino acid analysis was carried out with either a Dionex D-300 amino acid peptide analyzer with a Dionex DC-5A column (0.33 by 14 cm) for cytochrome *c*-551 and cytochrome *c*-552 or with an Applied Biosystems 420A derivatizer coupled to an Applied Biosystems 130A separation system for cytochrome *c*-553 and cytochrome *c*-554. Samples were hydrolyzed in 6 N HCl for 33 h at 110°C under an N_2 atmosphere. For cysteine determination, dimethyl sulfoxide was added to a separate sample prior to acid hydrolysis (5). To allow calculation of molecular mass per heme *c*, total amino acid content and heme *c* were measured on the same portion of cytochrome.

The N-terminal amino acid sequence of cytochromes *c*-554, *c*-552, and *c*-551 was analyzed by a gas phase peptide sequenator (Applied Biosystems 477A).

Reduction by methanol dehydrogenase. Reduction of ferricytochromes *c*-554, *c*-553, *c*-552, and *c*-551 by methanol dehydrogenase from *Methylomonas* sp. strain A4 was monitored at 554 nm for cytochromes *c*-554 and *c*-553 and at 552 nm for cytochromes *c*-552 and *c*-551 on a Hewlett-Packard 8452A photodiode-array spectrophotometer. The reaction mixture contained 50 mM buffer, 50 mM NH_4Cl, 10 to 20 mM ferricytochrome *c*, 0.5 mM methanol dehydrogenase, 50 mM methanol, and, when added, 0.5 mM phenazine methosulfate. The buffers used were acetate (pH 3.5, 4.5, and 5.5), sodium phosphate (pH 7.0), Tris hydrochloride (Tris-HCl, pH 8.0 and 9.0), and Bistris (pH 10.0). Methanol dehydrogenase was purified to homogeneity by a standard protocol (7), which will be described elsewhere (DiSpirito et al., unpublished data).

Spectroscopy. Room temperature spectroscopy was performed with either an Aminco DW2, DW-2A spectrophotometer in the split-beam mode or a Cary/Varian 219 spectrophotometer. Low-temperature (77 K) spectra were recorded with a 2-mm light path low-temperature attachment for an Aminco DW2.

Electron paramagnetic resonance spectra were recorded at x-band on a Varian E-109 spectrophotometer equipped with an Oxford ESR-10 liquid helium cryostat. Temperature *g*-value calibrations and spin quantitations were achieved as described previously (21).

RESULTS

Purification of cytochrome *c*-554. All isolation steps were performed at 0 to 4°C. Freeze-thawed cells were suspended in 50 mM Tris-HCl–100 mM KCl, pH 7.5, buffer (buffer A) and passed through a French pressure cell three times at 20,000 lb/in^2. The cell slurry was centrifuged at 13,200 × *g* for 30 min to remove unlysed cells and cell debris. The supernatant was centrifuged at 155,000 × *g* for 90 min. The supernatant (S_{144}) was brought to 30% saturation with solid ammonium sulfate, stirred for 3 h, and centrifuged at 13,200 × *g* for 15 min. The pellet was discarded, and the concentration of ammonium sulfate in the supernatant was raised to 60% saturation. The solution was then stirred for 3 h and centrifuged at 13,200 × *g* for 15 min, and the pellet was suspended in a minimal volume of 10 mM Tris-HCl (pH 8.5) buffer (buffer B) (30 to 60% ammonium sulfate fraction). The concentration of ammonium sulfate in the supernatant was raised to 80% saturation, and the solution was stirred and centrifuged as before. The resulting precipitate from the 60 to 80% ammonium sulfate fractionation step was suspended in a minimal volume of buffer B. The proteins precipitating in 30 to 60% and 60 to 80% ammonium sulfate and the supernatant obtained from the above centrifugation were dialyzed for 18 h against three changes of buffer B. The supernatant from the above centrifugations (soluble 80% ammonium sulfate fraction) was also dialyzed for 24 h against four changes of buffer B.

The three ammonium sulfate fractions were concentrated with a stirred cell (Diaflow, Amicon Corp.) with a YM10 filter. Cytochrome *c*-554 was observed in all three of the ammonium sulfate fractions. The major portion (75.6%) of cytochrome *c*-554 was observed in the soluble 80% ammonium sulfate fraction. The cytochrome in this fraction was purified in one step by separation on a preparative isoelectric focusing bed (10 by 20 cm) containing 4% Ultrodex, 1% ampholyte pH 4.0 to 6.0, and 1% ampholyte pH 3.5 to 10.0. Cytochrome *c*-554 migrated as two major bands focusing at pH 6.4 and at pH 5.65. The two bands were apparently the result of the reduction of a fraction of the oxidized sample by the ampholytes, as they had identical N-terminal amino acid sequences and amino acid compositions (see below). The isoelectric focusing points of the oxidized and reduced cytochromes *c*-554 were at pH 6.4 and 5.6, respectively. Both bands were eluted from the Ultrodex with 50 mM sodium potassium phosphate (NaKPi) buffer, pH 7.3 (buffer C). Cytochrome *c*-554 was dialyzed against three changes of buffer C and concentrated with an Amicon Centricon (Diaflow Corp., Amicon) YM10 filter.

Approximately 12% of cytochrome *c*-554 was observed in the 60 to 80% ammonium sulfate fraction. This fraction was separated on a preparative isoelectric focusing bed as described above. The major red band focusing at pH 6.4 was eluted with buffer A and concentrated with a stirred cell (YM10 filter). The sample was then loaded on a Sephadex G-50 column (2.5 by 47 cm) equilibrated with buffer A, and the eluting heme *c*-containing fraction was concentrated with a Centricon YM10 filter.

Cytochrome *c*-554 was also observed in the 30 to 60% ammonium sulfate fraction. This fraction was dialyzed for 8

TABLE 1. Purification of cytochromes c-554, c-553, and c-552 from *Methylomonas* sp. strain A4

Cytochrome	Prepn	Purification step[b]	Protein		Heme c[a]		
			Total (mg)	Yield (%)	Total (μmol)	Yield (%)	Concn (nmol/mg of protein)
All		Cell extract	25,220	100.0	42.9	100.0	1.7
		Soluble S_{144}	15,700	62.2	37.2	86.7	2.4
		Sol. 80% amm. sulfate	1,775	7.0	20.8	48.5	11.7
		60–80% amm. sulfate	2,200	8.7	10.3	24.0	4.7
		30–60% amm. sulfate	6,725	26.7	4.6	10.7	0.7
c-554	I	Sol. 80% amm. sulfate					
		Isoelectric focusing	73.50	0.29	9.7	22.6	132
	II	60–80% amm. sulfate					
		Isoelectric focusing	14.0	0.06	1.8	4.2	129
		Sephadex G-50	11.9	0.05	1.6	3.7	134
	III	30–60% amm. sulfate					
		DEAE-cellulose (CP3)	470.0	1.90	3.4	7.9	7.2
		Isoelectric focusing	12.6	0.05	1.7	4.0	135
	I+II+III		98.0	0.39	13.0	30.3	133
c-553		60–80% amm. sulfate					
		Isoelectric focusing	57.5	0.23	2.0	4.7	3.48
		DEAE-Sepharose CL-6B	37.9	0.15	1.7	4.0	44.9
c-552		60–80% amm. sulfate					
		Isoelectric focusing	103.3	0.41	6.5	15.2	62.9
c-551		30–60% amm. sulfate					
		Isoelectric focusing	23.6	0.09	2.1	4.9	89
		Sephadex G-75	20.4	0.08	2.0	4.7	98

[a] The heme c concentration was measured by using the pyridine ferrohemochrome method (10) with the Δε (550 nm) of 29.1 cm^{-1} mM^{-1}.
[b] Sol., Soluble; amm., ammonium.

h against three changes of buffer B and loaded on a DEAE-cellulose column (2.5 by 34 cm) equilibrated with the same buffer. The column was washed with approximately 400 ml of column buffer, and a red band (CP3) slowly eluted with 50 mM Tris-HCl (pH 8.0) buffer. The red band was concentrated with a stirred cell (YM10 filter) and applied to a preparative isoelectric focusing bed containing 4% Ultrodex, 1% ampholyte pH 5.0 to 8.0, and 1% ampholyte pH 3.5 to 5.0. The sample was focused for 17 h at 500 V, and cytochrome c-554 focused as two bands at pH 6.4 and at pH 5.6. The samples were eluted from the Ultrodex and concentrated as described above.

Purification of cytochrome c-551. The third major red band observed in the isoelectric focusing bed of the CP3 fraction was cytochrome c-551. This fraction was eluted from the Ultrodex and concentrated with a Centricon as described above. The sample was loaded on a Sephadex G-75 column (2.5 by 47 cm) equilibrated with buffer A. The red fraction that eluted from the Sephadex G-75 column was concentrated with a Centricon (YM10 filter).

Purification of cytochrome c-552 and cytochrome c-553. The two red bands migrating at pH 4.9 and pH 4.7 in the isoelectric focusing bed of the 60 to 80% ammonium sulfate fraction were cytochrome c-553 and cytochrome c-552, respectively. The two cytochromes were eluted from the Ultrodex, dialyzed for 20 h against three changes of buffer B, and concentrated with a stirred cell (YM10 filter). Cytochrome c-553 was loaded on a DEAE-Sepharose CL-6B column (1.25 by 25 cm) (Pharmacia P-L Biochemicals) equilibrated with the dialysis buffer. The red band slowly eluted from the column with the dialysis buffer, and the sample was concentrated with a Centricon (YM10 filter). Cytochrome c-552 was loaded on a DEAE-Sepharose column (1.0 by 32 cm) equilibrated with buffer B. The column was washed with approximately 100 ml of column buffer (100 ml of 25 mM Tris-HCl [pH 8.0] buffer), and the sample was eluted with 50 mM Tris-HCl (pH 8.0) buffer.

Purification of the four soluble cytochromes is summarized in Table 1. Each cytochrome was purified a minimum of three times, and Table 1 lists a representative purification scheme.

Properties of soluble cytochromes. The properties of the four soluble cytochromes from *Methylomonas* sp. strain A4 are listed in Tables 2 and 3.

Molecular mass. The molecular mass of cytochrome c-554 was determined to be 8,500 ± 600 Da on a Sephadex G-50 column (2.5 by 64 cm). The reference proteins were aprotinin (M_r 6,500), horse heart cytochrome c (M_r 12,400), lysozyme (M_r 14,300), *Nitrosomonas europaea* cytochrome c-554 (M_r 25,000), and carbonic anhydrase (M_r 29,000). In sodium dodecyl sulfate (SDS)-polyacrylamide gels as performed by the Laemmli method (19), cytochrome c-554 migrated as a single band corresponding to a molecular mass of 4,000 ± 900 Da (Fig. 1, lanes A and B). In SDS-polyacrylamide gels containing 8 M urea (4) or 3.6 M urea plus 13% glycerol (23), the cytochrome migrated as single bands corresponding to a molecular mass of 4,200 ± 400 or 4,100 ± 600 Da, respectively (data not shown). However, the molecular mass determined per heme from the amino acid composition was similar to that determined by gel filtration (Table 3), and the sequence for the first 49 amino acids showed a molecular mass greater than 6,000 Da (data not shown). Therefore, it appears that this cytochrome runs anomalously on denaturing gels, and the true molecular mass is approximately 8,500 Da.

The molecular mass of cytochrome c-552 was 14,000 ±

TABLE 2. Properties of the soluble cytochromes from *Methylomonas* sp. strain A4

Property	Value for cytochrome:			
	c-554	*c*-553	*c*-552	*c*-551
Soluble *c*-heme[a] (%)	57.3	7.5	26.4	8.8
Heme *c* concn (nmol/nmol of cytochrome)	1.1	1.6	1.1	1.6
Mass (Da)				
SDS-PAGE	4,000[b]	34,000	14,000	16,500
Column	8,500	41,000	14,000	17,500
Isoelectric point	5.6[c], 6.4[d]	4.9	4.7	4.8
Extinction coefficient (α-band; cm^{-1} mM^{-1})	21.3	25.0	24.0	28.7
Absorption maxima (nm)				
Oxidized	413	406	408	410
Reduced				
γ-band	418	419	417	416
β-band	524	524	522	523
α-band	554	553	552	551
Purity index ($A_{280}/A_{\alpha red}$)	0.8	1.2	1.4	1.7
Binds CO		+	−	−
EPR (*g* values)	3.15, 2.19, 1.32	5.39, 3.316, 2.88, 2.78, 2.35, 2.0, 1.55	6.2, 3.42, 2.1	6.05, 2.98, 2.28

[a] Percentage based on 100% of the total soluble cytochrome recovered, with the assumption that losses during purification were equal.
[b] This cytochrome apparently runs anomalously on denaturing polyacrylamide gels.
[c] Oxidized form.
[d] Reduced form.

800 Da as determined by gel filtration on a Sephadex G-50 column (2.5 by 64 cm). The molecular mass standards were the same as those used for cytochrome *c*-554. SDS-polyacrylamide gel electrophoresis of the purified sample revealed a major band with a molecular mass of 14,000 ± 600 Da and a few minor bands with higher molecular masses. Densitometric scanning of SDS-polyacrylamide gels stained with Coomassie brilliant blue G showed the major band to constitute 91% of the protein. Under certain gel conditions, the major band at 14,000 Da was revealed as a doublet (Fig. 1, lane C). N-terminal amino acid sequencing of these preparations generated only one sequence, suggesting that this doublet represents two forms of the same protein. Two forms of cytochrome c_L from *M. methylotrophus* have been reported that differ slightly in apparent size (10).

TABLE 3. Amino acid composition of *Methylomonas* sp. strain A4 soluble cytochromes[a]

Amino acid residue	No. of residues/ heme *c*		No. of residues/ 2 heme *c*	
	c-554	*c*-552	*c*-553	*c*-551
Asx	5	14	34	15
Gly	4	11	30	2
Glx	9	16	30	16
Ser	2	4	6	10
His	1	4	7	3
Arg	4	1	13	3
Thr	3	11	24	9
Ala	4	10	42	19
Pro	2	5	20	9
Tyr	2	4	10	4
Val	3	3	15	3
Met	2	3	4	1
Ile	4	3	13	5
Leu	4	9	28	10
Phe	3	5	13	4
Lys	4	11	13	9
Cys	2	2	ND[b]	4

[a] The minimum molecular masses were based on the heme *c* concentration, assuming 1 heme *c* per cytochrome *c*-554 or *c*-552 and 2 hemes per cytochrome *c*-553 or *c*-551. Tryptophan was not determined. The minimum molecular masses were calculated to be 7,200, 13,700, 34,900, and 14,800 Da for *c*-554, *c*-552, *c*-553, and *c*-551, respectively.
[b] ND, Not determined.

Cytochrome *c*-553 and cytochrome *c*-551 were electrophoretically homogeneous on SDS-polyacrylamide gels, yielding single bands with molecular masses of 34,000 ± 400 Da for cytochrome *c*-553 (Fig. 1, lane E) and 16,500 ± 400 Da for cytochrome *c*-551 (Fig. 1, lane D). The molecular mass of cytochrome *c*-553 was determined to be 41,000 ± 1,300 Da and cytochrome *c*-551 to be 17,500 ± 1,400 Da by gel filtration with a Sephadex G-75 column (2.5 by 51 cm). The reference proteins were horse heart cytochrome *c*, *N. europaea* cytochrome *c*-554, ovalbumin (M_r 45,000), and bovine serum albumin (M_r 67,000).

Based on the heme *c* and amino acid composition, the minimum molecular masses of cytochromes *c*-554, *c*-553, *c*-552, and *c*-551 were estimated at 7,200, 34,900, 13,700, and 14,800 Da, respectively (Table 3). These values are similar to the molecular masses derived from gel data, with the exception of cytochrome *c*-554. The amino acid composition was

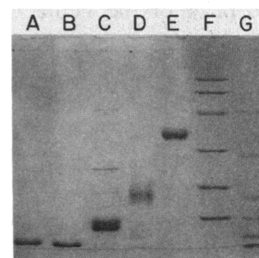

FIG. 1. SDS-polyacrylamide slab gel electrophoresis of cytochromes *c*-554 (oxidized) (A), *c*-554 (reduced) (B), *c*-552 (C), *c*-551 (D), *c*-553 (E), and molecular mass standards (F and G). The standards correspond to molecular masses of 92,000, 66,200, 45,000, 31,000, 21,500, and 14,400 Da for lane F and 43,000, 25,700, 14,300, 6,200, and 3,000 Da for lane G.

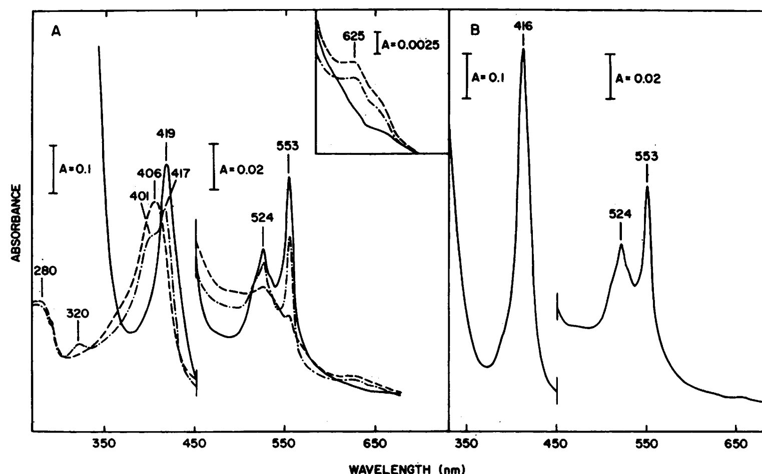

FIG. 2. Absorption spectra of cytochrome c-553; 115 μg of protein per ml, in 50 mM NaKPi buffer (pH 7.3). (A) Absorption of the native cytochrome (- · · -), after oxidation with ferricyanide (--), and following reduction with dithionite (——). (B) Absorption spectrum of the dithionite-reduced plus CO cytochrome c-553 (115 μg of protein per ml).

determined twice for cytochromes c-551, c-552, and c-553 and three times for cytochrome c-554.

Isoelectric points. In isoelectric focusing gels containing 5 M urea, 0.2% Nonidet P-40, and 2% carrier ampholyte (pH 3.5 to 10.0) (25), cytochrome c-554 migrated as two bands with isoelectric points at 6.4 and 5.3 and in preparative isoelectric focusing beds at pH 6.4 and 5.6. Approximately 57% migrated at pH 6.4 and 43% at pH 5.6. The amino acid composition and N-terminal amino acid sequence (see below) of the cytochrome c-554 migrating at pH 6.4 and that migrating at pH 5.6 showed that the two cytochromes were identical. Cytochromes c-553, c-552, and c-551 all migrated as single bands with isoelectric points at pH 4.7, 4.6, and 5.45, respectively, in gels containing 5 M urea and 0.2% Nonidet P-40 (25) and at pH 4.85, 4.7, and 4.75, respectively, in preparative isoelectric focusing beds.

Heme. The heme groups of the four soluble cytochromes were not extractable with acid acetone (16), and the pyridine ferrohemeochromogen absorption spectrum showed maxima at 414, 520, and 550 nm ± 1 nm. Thus, all four cytochromes may be concluded to be c-types. From the absorbance at 550 nm of the pyridine ferrohemeochromogen spectra, the number of heme groups per cytochrome was calculated to be 1.1, 1.6, 1.1, and 1.6 for cytochromes c-554, c-553, c-552, and c-551, respectively, assuming the molecular mass from the SDS-polyacrylamide gels, except for c-554, in which column molecular mass was used, and assuming the extinction coefficient at the α-peak of the heme c to be 29.1 cm^{-1} mM^{-1}.

Absorption spectra. The absorption maxima for oxidized and reduced cytochromes c-554, c-553, c-552, and c-551 are noted in Table 2. At low temperature (77 K), the absorption maxima for cytochromes c-554, c-552, and c-551 shifted 2 to 3 nm towards the blue and did not show a split α-peak.

The optical spectra of cytochrome c-553 were more complex and are shown in Fig. 2. The absorption spectrum of the cytochrome as isolated showed maxima at 320, 401, 417, 525, 553, and 625 nm, at 406 and 635 nm in the ferricyanide oxidized form, and at 419, 524, and 553 nm in the dithionite reduced form (Fig. 2A). The absorption bands at 625 nm in the resting and ferricyanide spectra are typical of high-spin hemoproteins (16) (see electron paramagnetic resonance [EPR] spectra, Fig. 4). Addition of CO to the dithionite reduced cytochrome resulted in a shift of the soret band to 416nm, an increased absorbance at this wavelength, and decreased alpha and beta bands (Fig. 2B). Cytochrome c-553 was the only cytochrome isolated from *Methylomonas* sp. A4 that bound carbon monoxide. Addition of CO had no effect on either the oxidized or the dithionite reduced spectra of cytochromes c-554, c-552, or c-551 at pH 4.3, 6.0, 7.3, 8.5, or 10.0.

EPR spectrum. The EPR spectrum of cytochrome c-554 recorded at 10K showed typical signals for a low-spin (S = 1/2) c-type heme (g = 3.15, 2.19, 1.32) (Fig. 3, top; Table 2).

The EPR spectrum of the diheme cytochrome c-551 was more complex, showing the presence of both high-spin (S = 5/2; g = 6.05) and low-spin (S = 1/2; g = 2.98, 2.28) hemes (Fig. 3, bottom). Spin quantitation of the EPR active high-spin fraction suggests that it accounted for less than 5% of the total heme in the sample. Thus, it is likely that two hemes are present and both are low spin. The second heme would give rise to the broad resonance on the low-field side of the g = 2.98 resonance arising from both hemes.

The monoheme cytochrome c-552 also showed signals characteristic of high-spin (g = 6.2) and low-spin (g = 3.42, 2.1) heme (Fig. 4A). Again, quantitation of the g = 6.2 signal suggests that the high-spin fraction accounted for less than 5% of the heme in the sample. Thus, the majority of the heme was low spin, in accord with the optical results. The resonance from the low-spin heme at g = 3.34 was relatively broad and occurred at a somewhat lower field than usually observed for c-type hemes. In other monoheme proteins exhibiting similar EPR spectral characteristics, the spectral width has been attributed to multiple ionization states of the heme ligands or of the amino acids in the vicinity of the heme (9). The signals near g = 4.3 observed in the EPR spectra of those proteins are often observed in biological samples and are due to adventitiously bound high-spin iron in a rhombic environment.

The EPR spectrum of cytochrome c-553 was more complex, again suggesting the presence of two hemes (Fig. 4B). Approximately 10% of the heme was in a high-spin state, with resonance positions at g = 5.39 and g = 1.997. Three types of low-spin heme were also present. One showed g values at 2.88, 2.35, and 1.55, one had a g value at 2.78, and the third had a g value at 3.32. It is possible that ionization

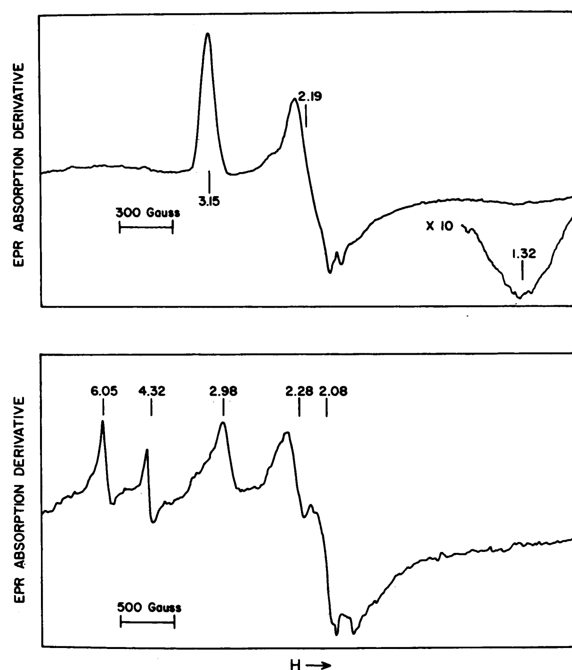

FIG. 3. EPR spectra of cytochrome *c*-554 (top) and cytochrome *c*-551 (bottom) in 50 mM NaKPi (pH 7.3) buffer at 10 K. Instrumental conditions were the following: microwave frequency, 9.22 GHz; modulation amplitude, 10 G; modulation frequency, 100 kHz; microwave power, 0.2 mW.

of an axial ligand accounts for some of the heterogeneity. However, the $g = 3.32$ and $g = 2.78$ almost certainly have a different axial heme ligand.

Amino acid sequence. The data presented above suggest that the four cytochromes identified are in fact different

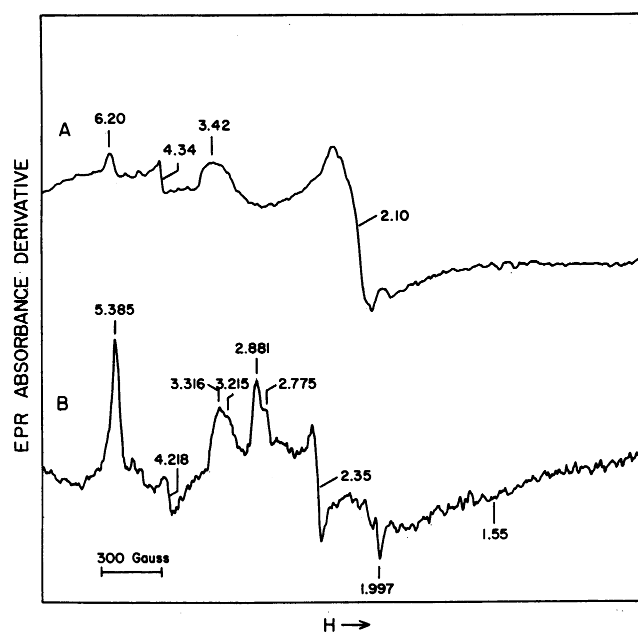

FIG. 4. EPR spectra of cytochrome *c*-552 (A) and cytochrome *c*-553 (B) in 50 mM NaKPi (pH 7.3) buffer at 10 K. Instrumental conditions were the same as in Fig. 3.

species. In order to confirm this, N-terminal amino acid sequencing was carried out for cytochromes *c*-554 (oxidized and reduced forms), *c*-552, and *c*-551. Cytochrome *c*-553 was not obtained in sufficient quantity for this work. Sequencing was repeated twice for cytochromes *c*-552 and *c*-551 and four times for cytochrome *c*-554, twice for each form. The first 17 amino acids for cytochromes *c*-554 and *c*-551 and the first 37 amino acids for cytochrome *c*-552 are shown in Table 4, and these were clearly different for cytochromes *c*-554, *c*-552, and *c*-551. The only question in the sequence data reported in Table 4 was at position 16 of *c*-551. In one sequence run, a weak signal coincident with cysteine was also present. The sequences for the two forms of cytochrome *c*-554 were identical. Further sequence data were also obtained for cytochrome *c*-554 (49 amino acids total; data not shown but available upon request).

Concentration of soluble cytochromes in *Methylomonas* sp. strain A4. A recovery of 53% of the initial soluble *c*-heme was obtained during the purification of the four soluble cytochromes from *Methylomonas* sp. strain A4. Correcting for the loss of *c*-heme during the purification procedures, the heme concentrations of cytochrome *c*-554, *c*-553, *c*-552, and *c*-551 were approximately 974, 128, 449, and 150 pmol of heme *c* per mg of cell protein, respectively. The relative concentrations were calculated under the assumption that the loss of each cytochrome was proportional during purification. The total concentration of soluble *c*-type cytochromes was similar in *M. methylotrophus* (10) and *M. extorquens* AM1 (27) as well as the related ammonia-oxidizing bacterium *N. europaea* (15).

Reduction of cytochromes by MeDH. In methanol-utilizing methylotrophs, the in vitro reduction of soluble cytochromes *c* by purified MeDH is consistently low, usually at less than a percent of the whole-cell activities (7, 13). Therefore, it is difficult to assess from in vitro assays whether a particular cytochrome *c* is coupled to MeDH in vivo. However, since this had not yet been tested in a methanotroph, we purified MeDH from *Methylomonas* sp. strain A4 to determine the rate of reduction of the four different cytochromes *c* isolated in this study. In the absence of an artificial electron acceptor (phenazine methosulfate), none of the four cytochromes was reduced at a significant rate. The highest activity (3.2 nmol of cytochrome *c*-554 reduced per min per nmol of MeDH) was observed with cytochrome *c*-554, but this was still only a few percent of the total whole-cell rate. In the presence of phenazine methosulfate, higher rates (107 nmol of cytochrome *c*-554 reduced per min per nmol of MeDH) were observed, suggesting the lack of an intermediary, as proposed for cytochrome c_H for *Hyphomicrobium* sp. strain X (13). Combinations of cytochromes did not result in increased rates of reduction over the single cytochrome rates. The pH optimum for these low rates of reduction appeared to be 9.0, which is the pH optimum for the in vitro assay of MeDH with artificial electron acceptors. This is in contrast to the results for *Hyphomicrobium* sp. strain X, in which the pH optimum for the reduction of cytochrome c_L by MeDH was 7.0 (13). In addition, in the system for methanol-utilizing methylotrophs, significant autoreduction activity occurs (reduction of cytochrome *c* by MeDH in the absence of methanol [7, 8, 27]), but this was not the case for *Methylomonas* sp. strain A4. No reduction of any cytochromes was observed in the absence of methanol.

DISCUSSION

The results presented here show that four distinct *c*-type cytochromes are present in *Methylomonas* sp. strain A4,

TABLE 4. N-terminal amino acid sequences of cytochromes *c*-551, *c*-552 and *c*-554 from *Methylomonas* A4 and cytochrome c_L from *M. extorquens* AM1 (24)[a]

Cytochrome	Sequence
c-551	Ala-Asp-Ile-Ala-Ala-Gly-Glu-Gln-Lys-Ala-Glu-Thr-Cys-Ser-Gly-Phe[b]-His
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
c-554[c]	Gln-Glu-Asp-Ile-Lys-Val-Gly-Gln-Lys-Ile-Tyr-Asp-Arg-Ala-Phe-Gly-Arg
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
c-552	Glu-Ile-Thr-Leu-His-Thr-Ala-Ile-**Thr-Gly-Glu**-Val-**Leu-Asp**-Met-**Ser**-Val-Ala-Pro-
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
c_L	Gly-Val-Val-Phe-Arg-Asn-Thr-Val-**Thr-Gly-Glu**-Ala-**Leu-Asp**-Val-**Ser**-Gln-Gly-Lys-
	9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
c-552	Cys-**Gly-Gly**-Asn-----**Thr**-Glu-**Ala**-Phe-**Lys**-Gln-**Phe**-Met-**Glu**-Leu-Thr-Gly-**Asn**-Pro
	20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
c_L	Glu-**Gly-Gly**-Arg-Asp-**Thr**-Pro-**Ala**-Val-**Lys**-Lys-**Phe**-Leu-**Glu**-Thr-Gly-Glu-**Asn**-Leu
	28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46

[a] Bold-faced residues indicate conservation between *c*-552 and c_L.
[b] A weak signal coincident with cysteine was also present.
[c] Both forms showed identical sequences.

and, as in other methylotrophs, two monoheme cytochromes represented the major *c*-type species. These monoheme cytochromes from *Methylomonas* sp. strain A4 show both similarities to and differences from *c*-type cytochromes from other methylotrophic bacteria, but in general follow the patterns for the major monoheme cytochromes *c* found in other methylotrophs. The smaller, more abundant species with the higher isoelectric point (cytochrome *c*-554) is probably equivalent to the cytochrome c_H in methanol utilizers, and the larger, less abundant species with the lower isoelectric point is probably equivalent to cytochrome c_L. This suggestion is further supported by a comparison of N-terminal amino acid sequences. No significant similarity was observed between these sequences and cytochrome sequences deposited in GenBank. However, 37.5% similarity was observed between the N-terminal sequences of cytochrome c_L from *M. extorquens* AM1 and cytochrome *c*-552 (Table 4), further supporting the hypothesis that these are equivalent. No sequence data have been published for cytochrome c_H, but no significant similarities were observed between the sequence for cytochrome *c*-554 and that for other published cytochrome *c* sequences from methylotrophs (2, 3, 24, 28).

The EPR spectra and heme content (1.6 heme per cytochrome) suggest that cytochromes *c*-551 and *c*-553 are probably diheme cytochromes. If correct, these would be the first diheme cytochromes reported from a methylotrophic bacterium. Cytochrome *c*-553 also differed from the three other cytochromes isolated from *Methylomonas* sp. strain A4 and from most of the cytochromes from other methylotrophic bacteria in the rate and extent of CO binding. Historically, the reaction of *c*-type cytochromes from methylotrophic bacteria with CO has led to the classification of these cytochromes as cytochrome c_{CO}. However, with the exception of cytochrome *c*-553 from *M. extorquens* AM1, the CO binding in these cytochromes is only observed after a long incubation period (30 min or more) and the binding is often incomplete. In the case of cytochrome *c*-553 from *Methylomonas* sp. strain A4, the binding of CO is rapid (less than 30 s) and complete. No significant sequence similarity was observed for either of these cytochromes to sequences in GenBank or of other known cytochromes from methylotrophs (2, 3, 24, 28).

The functions of the soluble cytochromes in *Methylomonas* sp. strain A4 are still not defined, although, as noted above, cytochrome *c*-552 is a likely candidate for the MeDH-associated cytochrome. However, none of these cytochromes showed significant reduction by MeDH in the absence of phenazine methosulfate. Low reaction rates (i.e., 0.06 to 14.2 nmol of cytochrome *c* reduced per min per nmol of MeDH) with purified MeDH and cytochromes have been observed in all the methylotrophic bacteria studied (8, 12, 13), and so these negative data are not meaningful concerning in vivo electron acceptors. Further biochemical and genetic studies are needed to determine the functions of these cytochromes and their potential role in C_1 metabolism in *Methylomonas* spp. The N-terminal amino acid sequences reported here will be useful for generating probes to clone the genes and construct mutants by reverse genetics.

The cellular locations of the four *c*-type cytochromes from *Methylomonas* sp. strain A4 have not yet been determined. Since none of the N-terminal amino acid sequences started with a methionine, it is likely that the cytochromes are transported with leader sequences to the periplasmic space, where the leader sequences are removed, as is the case with other soluble *c*-type cytochromes (1, 17, 18, 31). Future studies will address the cellular location and function of these cytochromes.

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LITERATURE CITED

1. **Alefounder, P. R., and S. J. Ferguson.** 1981. A periplasmic location for methanol dehydrogenase from *Paracoccus denitrificans*: implications for proton pumping by cytochrome aa_3. Biochem. Biophys. Res. Commun. **98**:778–784.
2. **Ambler, R. P., H. Dalton, T. E. Meyer, R. G. Bartsch, and M. D. Kamen.** 1986. The amino acid sequence of cytochrome *c*-555 from the methane-oxidizing bacterium *Methylococcus capsulatus*. Biochem. J. **233**:333–337.
3. **Ambler, R. P., T. E. Meyer, P. A. Trudinger, and M. D. Kamen.** 1985. The amino acid sequences of cytochromes *c*-554 (547) from the chemolithotroph *Thiobacillus neapolitanus*. Biochem. J. **227**:1009–1013.
4. **Anderson, B. L., R. W. Berry, and A. Telser.** 1983. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis system that separates polypeptides and protein in the molecular weight range of 25,000 to 90,000. Anal. Biochem. **132**:365–375.
5. **Anderson, K. K., J. D. Lipscomb, M. Valentine, E. Munck, and A. B. Hooper.** 1986. Tetraheme cytochrome *c*-554 from *Nitrosomonas europaea*. J. Biol. Chem. **261**:1126–1138.
6. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, Inc., New York.
7. **Anthony, C.** 1986. Bacterial oxidation of methane and methanol. Adv. Microb. Physiol. **27**:113–209.
8. **Beardmore-Gray, M., D. T. O'Keeffe, and C. Anthony.** 1983. The methanol:cytochrome *c* oxidoreductase activity of methanotrophs. J. Gen. Microbiol. **129**:923–933.
9. **Brautigan, D. L., B. A. Feinberg, B. M. Hoffman, E. Margoliash, J. Peisach, and W. E. Blumberg.** 1977. Multiple low spin form of the cytochrome *c* ferrihemochrome. J. Biol. Chem. **252**:574–582.
10. **Cross, A. R., and C. Anthony.** 1980. The electron transport chains of the obligate methylotroph *Methylophilus methylotrophus*. Biochem. J. **192**:429–439.
11. **Day, D. J., D. N. Nunn, and C. Anthony.** 1990. Characterization of a novel *c*-type cytochrome in a *moxD* mutant of *Methylobacterium extorquens* AM1. J. Gen. Microbiol. **136**:181–188.
12. **Dijkstra, M., J. Frank, and J. A. Duine.** 1988. Methanol oxidation under physiological conditions using methanol dehydrogenase and a factor isolated from *Hyphomicrobium* X. FEBS Lett. **227**:198–202.
13. **Dijkstra, M., J. Frank, and J. A. Duine.** 1989. Studies on electron transfer from methanol dehydrogenase to cytochrome c_L, both purified from *Hyphomicrobium* X. Biochem. J. **257**:87–94.
14. **Dijkstra, M., J. Frank, J. E. van Wielink, and J. D. Duine.** 1988. The soluble cytochromes *c* of methanol-grown *Hyphomicrobium* X. Biochem. J. **251**:467–474.
15. **DiSpirito, A. A., L. R. Taaffe, and A. B. Hooper.** 1985. Localization and concentration of hydroxylamine oxidoreductase and cytochromes *c*-552, *c*-554, c_M-553, c_M-552, and *a* in *Nitrosomonas europaea*. Biochim. Biophys. Acta **806**:320–330.
16. **Fuhrhop, J.-H.** 1975. Laboratory methods in porphyrin and metalloporphyrin research. Elsevier Scientific Publishers, New York.
17. **Hooper, A. B., and A. A. DiSpirito.** 1985. In bacteria which grow on simple reductants, generation of a proton gradient involves extracytoplasmic oxidation of substrate. Microbiol. Rev. **49**:140–157.
18. **Kasprzak, A. A., and D. J. Steenkamp.** 1984. Localization of the major dehydrogenases in two methylotrophs by radiochemical labeling. J. Bacteriol. **156**:348–353.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lidstrom, M. E.** 1988. Isolation and characterization of marine methanotrophs. Antonie van Leewenhoek J. Microbiol. Serol. **54**:189–199.
21. **Lipscomb, J. D.** 1980. Electron paramagnetic resonance detectable states of chtochrome P-450_{CAM}. Biochemistry **19**:3590–3599.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Merle, P., and B. Kadenbach.** 1980. The subunit composition of mammalian cytochrome *c* oxidase. Eur. J. Biochem. **105**:499–507.
24. **Nunn, D. N., and C. Anthony.** 1988. The nucleotide sequence and deduced amino acid sequence of the genes for cytochrome c_L of *Methylobacterium* AM1. Nucleic Acids Res. **16**:7722–7723.
25. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
26. **Ohata, S., and J. Tobari.** 1981. Two cytochromes *c* from *Methylomonas* J. J. Biochem. **90**:215–224.
27. **O'Keeffe, D. T., and C. Anthony.** 1980. The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1. Biochem. J. **192**:411–419.
28. **Santos, H., and D. L. Turner.** 1988. Characterization and NMR studies of a novel cytochrome *c* isolated from *Methylophilus methylotrophus* which shows a redox-linked change of spin state. Biochim. Biophys. Acta **954**:277–286.
29. **Tonge, G. M., D. E. F. Harrison, and I. J. Higgins.** 1977. Purification and properties of the methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3b. Biochem. J. **161**:333–344.
30. **Whittenbury, R., and H. Dalton.** 1981. The methylotrophic bacteria, p. 894–902. *In* M. P. Starr, H. G. Stolp, A. Truper, A. Belows, and H. G. Schlegel (ed.), The prokaryotes, vol. I. Springer-Verlag, Berlin.
31. **Wood, P. M.** 1983. Why do *c*-type cytochromes exist? FEBS Lett. **164**:223–226.