Isolation and phenotypic characterization of methanol oxidation mutants of the restricted facultative methylotroph *Methylophaga marina*

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Methanol oxidation in *Methylophaga marina* appears to be organized in a way similar to that in other Gram-negative methylotrophs, involving a quinoprotein methanol dehydrogenase (MDH; EC 1.1.99.8) and two soluble cytochromes. The MDH is composed of two different subunits, most probably arranged in an $\alpha_2\beta_2$ structure. The two cytochromes are of the *c*-type and differ in size (molecular mass 19.5 and 12.5 kDa) and isoelectric point (pI 4.6 and 9.2). The one with the lowest isoelectric point, commonly designated as cytochrome *c_L*, is able to oxidize reduced MDH. Taking advantage of the ability of *M. marina*, a restricted facultative methylotroph, to utilize fructose as a growth substrate, mutants impaired in methanol utilization were isolated after application of optimal concentrations of ethylmethane sulphonate. Three classes of methanol oxidation mutants were obtained. Class I mutants were affected in a global regulation of the synthesis of both apo-methanol-dehydrogenase and cytochrome *c_L* as well as PQQ (pyrroloquinoline quinone). Class II mutants did not produce an active MDH, but instead a comparable amount of a 65 kDa protein was found in the cell-free extract upon SDS-PAGE. This mutant protein was purified and compared to wild-type MDH. It was located in the periplasm, but unlike MDH it was composed of only two identical large subunits. Each of these subunits was able to bind one molecule of PQQ. An antiserum raised against wild-type MDH did not react with the mutant protein. Conversely, an antiserum raised against mutant protein weakly cross-reacted with wild-type MDH, suggesting that the presence of the β -subunit in MDH dramatically changes its immunochemical behaviour. Three of the class II mutants did not produce PQQ. In the presence of PQQ, partial revertants able to grow on methanol medium were obtained (class III). Class III mutants produced a stable apo-MDH consisting of α - and β -subunits and showing a normal reaction with the anti-MDH serum. Although this apo-MDH can bind PQQ, enzymic activity was not restored *in vitro*. This suggests that, in addition to apo-MDH and PQQ, other factors are required for the assembly of an enzymically active MDH.

Introduction

Methylophaga marina is a Gram-negative methylotroph that is able to utilize two *C*₁ compounds, methanol and methylamine; the formaldehyde resulting from their oxidation is assimilated by the ribulose monophosphate (RuMP) pathway (Janvier *et al.*, 1985).

With the exception of *Acetobacter methanolicus* (Uhlir *et al.*, 1986), all known Gram-negative methanol-utilizing bacteria are obligate methylotrophs, growing only on methanol and sometimes methylamine. Originally described under various names, they are now

grouped under two generic designations: *Methylobacillus* (Urakami & Komagata, 1986) and *Methylophilus* (Jenkins *et al.*, 1987). These two genera and *Methylophaga* unambiguously belong to distinct taxonomic groups as evidenced by their different G + C content and by the absence of hybridization of their respective DNAs (Janvier *et al.*, 1985). A further distinguishing property of *Methylophaga marina* is its ability to use efficiently a single multi-carbon compound, fructose, as a growth substrate.

In all the Gram-negative bacteria studied so far, methanol is oxidized by a periplasmic quinoprotein methanol dehydrogenase (MDH) consisting of two identical, large α -subunits (reviewed by de Vries *et al.*, 1990; Anthony, 1992). Two additional, small β -subunits have been discovered recently in the MDH from

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Abbreviations: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; RuMP, ribulose monophosphate.

Methylobacterium extorquens AM1 (Nunn *et al.*, 1989) and *Methylobacillus glycogenes* (Adachi *et al.*, 1990b). Since this β -subunit is only faintly stained in SDS-PAGE gels, it is conceivable that its presence in MDH has been overlooked in most cases. A gene (*moxI*) coding for a similar subunit has been found in *Paracoccus denitrificans* (van Spanning *et al.*, 1991) and in the present paper it is reported that MDH from *Methylophaga marina* also contains a β -subunit, suggesting that the $\alpha_2\beta_2$ structure is a general feature of quinoprotein MDH. All MDHs contain the cofactor pyrroloquinoline quinone (PQQ) and donate their electrons to a special type of cytochrome *c*, cytochrome *c_L* (Dijkstra *et al.*, 1989; Nunn & Anthony, 1988).

Presently, most of our knowledge of the genetics of methanol oxidation comes from studies with *P. denitrificans*, a methanol-utilizing facultative autotroph (Harms *et al.*, 1985; Harms & van Spanning, 1991; van Spanning *et al.*, 1991) and with two species of Gram-negative facultative methylotrophs using the serine cycle of formaldehyde assimilation: *Methylobacterium organophilum* (Allen & Hanson, 1985; Machlin *et al.*, 1988; Mazodier *et al.*, 1988; Biville *et al.*, 1989) and *Methylobacterium extorquens* strain AM1 (Tatra & Goodwin, 1985; Nunn & Lidstrom, 1986a, b). In strain AM1, a minimum of 17 *mox* genes involved in methanol oxidation have been postulated (Lidstrom, 1990). The function of some of these genes is still unclear. Well established is the role of a cluster of four genes (*moxF, J, G, I*), which transcribe successively the α -subunit of MDH (*moxF*), a 30 kDa protein of unknown function (*moxJ*), the cytochrome *c_L* (*moxG*) and finally the β -subunit of MDH (*moxI*) (Anderson and Lidstrom, 1988; Nunn *et al.*, 1989). An identical gene organization is found in *P. denitrificans* (van Spanning *et al.*, 1991). In *Methylobacterium organophilum* six *pqq* genes involved in PQQ biosynthesis have been identified (Biville *et al.*, 1989). Several *mox* genes of *Methylobacterium extorquens* AM1 that encode the biosynthesis and utilization of PQQ (Lidstrom, 1990) show similarity to the *pqq* genes of *Methylobacterium organophilum* (M. Lidstrom & F. Biville, personal communication).

In *Methylobacterium* and *Paracoccus*, the isolation of mutants unable to utilize methanol (or other *C₁* compounds) is facilitated by the facultative methylotrophy of these organisms. The obligate methylotrophy of most of the strains containing the RuMP pathway constitutes a difficulty in the isolation of mutants affected in their methylotrophic metabolism, since mutations in catabolic genes are necessarily lethal. One possibility to circumvent this difficulty is the isolation of conditionally lethal mutants [see Kletsova *et al.* (1986) and Dawson & Goodwin (1990)]. Another approach used by the latter authors was specific selection on a

methylamine-containing medium of mutants of *Methylophilus methylotrophus* impaired in the initial step of the oxidation of methanol to formaldehyde. This selection eliminates the mutants affected in the formaldehyde assimilation pathway, which is common to both methanol and methylamine metabolism. In this work we took advantage of the ability of *Methylophaga marina* to utilize fructose as a growth substrate to isolate mutants affected specifically in methanol utilization. The characteristics of these mutants regarding MDH, cytochrome *c_L* and PQQ were analysed.

Methods

Bacterial strain and cultivation. *Methylophaga marina* strain 42, a spontaneous streptomycin-resistant mutant of strain 92b, previously described, was cultivated in minimal ASW (artificial sea-water) medium supplemented with methanol (0.3%, v/v), fructose (0.4%, w/v) or methylamine (0.2%) (Janvier *et al.*, 1985). The concentration of magnesium in ASW was lowered to 0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O l}^{-1}$ (instead of 2 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O} + 3 \text{ g MgCl}_2 \cdot 5 \text{H}_2\text{O l}^{-1}$) for growth with penicillin and cycloserine during the enrichment procedure. Antibiotics were added as filter-sterilized solutions. Final concentrations per ml were 20 μg for rifamycin (Merck), 2 mg for cycloserine and 10^4 I.U. for penicillin G (Rhône-Poulenc).

Biochemical and immunological methods. The methods used for growth of bacteria, preparation of crude extracts, preparation of antisera and immuno-diffusion in gels (Ouchterlony plates) have been described previously (Janvier *et al.*, 1985; Janvier & Gasser, 1987). Activities of methanol dehydrogenase (EC 1.1.99.8), methylglutamate dehydrogenase (EC 1.4.99.5), hexulose phosphate synthase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were determined as previously described (Janvier *et al.*, 1985). NAD^+ -dependent formaldehyde dehydrogenase (EC 1.2.1.1) was assayed by the method of Stirling & Dalton (1978), formate dehydrogenase (EC 1.2.1.2) by the method of Johnson & Quayle (1964), 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) by the method of Meloch *et al.* (1966) and oxoglutarate dehydrogenase (EC 1.2.4.2) by the method of Reed & Murkherjee (1969). Assay of PQQ was performed as reported previously (Biville *et al.*, 1988). SDS-PAGE and PAGE at pH 4.5 without SDS were performed as described by Laemmli (1970) and by Reisfeed *et al.* (1962), respectively. Molecular mass markers were SDS-PAGE standards (low range) from Bio-Rad. Electrophoretic transfer of protein from polyacrylamide gels (without SDS) onto nitrocellulose sheets and immunoblots was done by the method of Towbin (1979). Antisera raised against MDH or the 4206 mutant protein were diluted at 1/500 before use.

Purification of MDH and related mutant proteins. Cells (40 g wet wt) of *M. marina* 42 grown in ASW-methanol medium or of mutants 4206 and 42061 grown with fructose as carbon source were suspended in 50 ml 0.05 M-potassium phosphate buffer, pH 7. After addition of 10 mg DNAase I, the suspension was passed twice through a French pressure cell at 30 MPa and cell debris was removed by centrifugation at 49000 g for 30 min. The protein precipitating between 45 and 80% saturation ammonium sulphate was dissolved in a minimum volume of 10 mM-MOPS buffer, pH 7, and subjected to hydrophobic interaction chromatography on a phenyl-Sepharose HP column (10 $\times$ 2.6 cm, Pharmacia) with a linear gradient of ammonium sulphate in 10 mM-MOPS (1.5–0 M in 60 min at a flow rate of 2.0 ml min^{-1}). This step was included in the purification procedure for MDH since it has been

shown that it is essential to obtain preparations that are not subject to autoreduction (Frank & Duine, 1990). Fractions (4 ml) containing either MDH activity or the mutant protein (eluting at the same position as MDH, i.e. between 128 and 136 ml) were pooled and dialysed overnight against 2 litres 10 mM-CHES buffer, pH 8, and chromatographed on a DEAE Superformance Fractogel 650(S) column (15 × 2.6 cm; Merck) with a linear gradient of 0–1 M-NaCl in 10 mM-CHES, pH 8, over 60 min at 2.0 ml min⁻¹. The eluate was monitored at 280 nm. In the case of MDH and 42061 mutant protein, the identity of the protein eluting in the main peak was established by testing for enzymic activity (MDH) and cross-reaction with anti-MDH serum, respectively. Fractions (4 ml) containing the main protein peak were pooled, concentrated to 2 ml with a Centricon 30 kDa ultrafiltration membrane and chromatographed (injection volume 0.5 ml) on a Superose-12 gel-filtration column (30 × 1 cm; Pharmacia) in 0.1 M-sodium phosphate buffer, pH 6.5, at a flow rate of 0.5 ml min⁻¹. The eluate was monitored at 280 nm and the material eluting in the main peak was stored at –20 °C.

Determination of the subunit structure of MDH and mutant proteins. The proteins were denatured by dissolving 36 mg urea per 0.1 ml of protein solution followed by heating at 100 °C for 2 min. After centrifugation (10 min, 49000 g), the samples were chromatographed on a Superose-12 gel-filtration column (Pharmacia) using 0.1 M-sodium phosphate, 0.1 M-NaCl, 6 M-urea, pH 6.5, as the eluant at a flow rate of 0.5 ml min⁻¹.

Binding of PQQ to the mutant proteins. Protein purified from mutants 4206 and 42061 (3–6 nmol in 200 µl 10 mM-MOPS buffer, pH 7.0) was incubated for 10 min with PQQ (20 nmol in water). The effect of Ca²⁺ ions on binding was investigated by adding CaCl₂ (200 nmol in water) prior to addition of PQQ. The mixtures were analysed by gel-filtration on a Superose-12 10/30 column (Pharmacia) in 100 mM-sodium phosphate buffer, pH 7.0. The eluate was monitored with a Waters 991 photodiode array detector and the peak of the protein was collected.

Analysis of soluble c-type cytochromes. Methanol- or fructose-grown cells (1.5–2 g wet wt) suspended in 5 ml 50 mM-sodium phosphate buffer, pH 7, were passed twice through a French pressure cell at 110 MPa. The resulting extract was centrifuged for 2 h at 100000 g. The supernatant was dialysed against 10 mM-Tris/HCl buffer, pH 7.2, and loaded onto a short DEAE-trisacryl column (23 cm × 23 mm) equilibrated with the same buffer. The column was washed with Tris/HCl buffer and the proteins retained by DEAE were eluted by a 0–0.2 M-NaCl gradient in 10 mM-Tris/HCl, pH 7.2. Pink-coloured fractions from the void volume (cytochrome c_H) of this column and from the gradient eluate (cytochrome c_L) were pooled, dialysed and concentrated in an Amicon cell through a YM30 membrane before SDS-PAGE (12.5%, w/v, acrylamide). After electrophoresis, the gels were soaked in 12.5% (w/v) trichloroacetic acid (TCA) (30 min) and rinsed in distilled water (30 min). Cytochrome bands were then revealed by their peroxidase activities using the method of Thomas *et al.* (1976) modified as follows. Gels were soaked in 0.25 M-sodium acetate buffer, pH 5, containing 2% (w/v) sodium perchlorate and 1.9 mM-*N,N,N',N'*-tetramethylbenzidine. After 2 h, hydrogen peroxide was added (final concentration 30 mM). Deep blue bands appeared in about 15 min. Absorption spectra of purified cytochromes were recorded at room temperature in a Beckman DU40 spectrophotometer and their isoelectric points estimated using the method of Peltre *et al.* (1982). The purified cytochromes were also used as markers in SDS-PAGE to identify cytochromes present in crude extracts.

Reaction of reduced MDH with cytochrome c. The purified MDH (in the semiquinone form) and cytochrome c fractions were dialysed overnight against 10 mM-MOPS buffer, pH 7.0. Cytochrome c was diluted to approximately 1 µM [assuming a specific absorption coefficient, A₂₈₀ (1 mg ml⁻¹) = 2] and the spectrum of 1 ml of this

solution was recorded. Since the cytochrome preparations are generally reduced after purification, a slightly less than stoichiometric amount of potassium ferricyanide was added. Before adding a tenfold excess of MDH, the A₅₅₀ was followed for 5 min to judge the extent of autoreduction.

Localization of MDH and of the 4206 mutant protein. *M. marina* 42 and the mutant 4206 were grown in 10 ml ASW-fructose medium up to OD₆₀₀ = 0.9. Bacterial cells were centrifuged at room temperature, washed twice in 0.1 M-Tris/HCl buffer, pH 7.4, and resuspended in 2 ml 20% (w/v) sucrose containing 4 mM-EDTA in order to be subjected to osmotic shock, essentially according to the method of Brockman & Heppel (1968) but modified as follows. After 20 min in the sucrose/EDTA solution, protoplasts were centrifuged at room temperature and the pellet rapidly dispersed in 2 ml cold 0.5 mM-MgCl₂. This suspension was kept for 10 min at 0 °C and then centrifuged. The pellet was the (cytoplasm + membrane) fraction. The supernatant (periplasm fraction) was centrifuged a second time to eliminate remaining cells and debris. Proteins of the periplasm fraction were precipitated with a cold, saturated solution of TCA in water (i.e. 25 g TCA in 11.3 ml of distilled water). The resulting pellet was washed with 95% ethanol and resuspended in the sample buffer used for PAGE. The (cytoplasm + membrane) fraction was directly resuspended in sample buffer before electrophoresis.

Mutagenesis and mutant selection. *M. marina* 42 grown in ASW-methanol medium was harvested in mid-exponential phase and resuspended at OD₆₀₀ = 0.8 either in ASW for ethylmethane sulphonate (EMS) treatment or in 50 mM-Tris/maleate buffer, pH 6, for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Both mutagens were used at different concentrations. EMS was pure methanesulphonic acid ethyl ester (*d* = 1.17) from Sigma. After 30 min incubation at 30 °C with shaking, the suspensions were centrifuged, washed twice in ASW, diluted for plate count (percentage survivors) and inoculated into fresh ASW-fructose medium. After overnight incubation at 30 °C with shaking, the number of rifamycin-resistant mutants in the population was determined by plating dilutions onto ASW-fructose agar with or without the antibiotic. For enrichment of mutants unable to utilize methanol, cells were diluted at OD₆₀₀ = 0.1 in ASW-methanol medium containing a low Mg²⁺ concentration. At OD₆₀₀ = 0.3, in the early exponential phase, penicillin (10⁴ I.U. ml⁻¹) and cycloserine (2 mg ml⁻¹) were added. After a further 2 h incubation, cultures were diluted and spread onto ASW-fructose medium so as to obtain 100–200 colonies per plate. Well-grown colonies obtained after 4–5 d at 30 °C were replica-plated onto methanol- and methylamine-ASW media for detection of clones impaired in the utilization of these compounds.

Results

Phenotypic characterization of Methylophaga marina 42 in relation to oxidation of C₁ compounds

M. marina 42 grew fairly rapidly in ASW medium supplemented with methanol (μ = 0.86 h⁻¹), methylamine (μ = 0.25 h⁻¹) or fructose (μ = 0.14 h⁻¹). Growth in methylamine medium (solid or liquid) was slow and poor when the inoculum was of methanol- or fructose-grown cells. Only a second transfer in methylamine medium produced a satisfactory culture with the growth rate indicated above. This poor culture after a first transfer to methylamine medium prevented the use of this substrate in genetic selection experiments.

Table 1. *Specific activities of the main enzymes involved in C₁ metabolism in Methylophaga marina 42*

Specific activities are expressed as nmol disappearing substrate or appearing product min⁻¹ (mg protein)⁻¹. ND, Not determined.

	Growth substrate		
	Methanol	Methylamine	Fructose
Methanol dehydrogenase	728	800	810
Methylamine dehydrogenase	ND	0	ND
Methyl-L-glutamate dehydrogenase	0	23	0
Formaldehyde dehydrogenase (NAD ⁺)	11	87	0
Formate dehydrogenase (NAD ⁺)	61	315	14
Hexulose phosphate synthase	3400	4370	2600
Glucose-6-phosphate dehydrogenase (NAD ⁺)	261	322	256
(NADP ⁺)	250	ND	ND
6-Phosphogluconate dehydrogenase (NAD ⁺)	8	0	0
(NADP ⁺)	37	42	40
2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (6-Phosphogluconate dehydrase)	474	86	149
Oxoglutarate dehydrogenase	0	ND	23

Crude extracts of *M. marina* 42 contain two soluble *c*-type cytochromes which could be separated on DEAE-trisacryl at pH 7.2. According to the nomenclature proposed by O'Keefe & Anthony (1980), cytochrome *c_H* was not retained by DEAE, and cytochrome *c_L* was eluted from the column by 0.2 M-NaCl. After further purification, the apparent molecular mass estimated after SDS-PAGE was 19500 Da for cytochrome *c_L* and 12500 Da for cytochrome *c_H*. Their pI values were 4.6 and 9.2, respectively. Only cytochrome *c_L* was reduced by MDH. These data, together with their absorption spectrum (not shown), indicate that the two cytochromes *c* of *M. marina* are similar to those already described in methylotrophic bacteria that use the RuMP pathway (Cross & Anthony, 1980) and other methylotrophs (Dijkstra *et al.*, 1988).

The main enzymic activities related to C₁ catabolism of *M. marina* are reported in Table 1. A report on the qualitative detection of some of these enzymes for taxonomic purposes has already been published (Janvier *et al.*, 1985). *M. marina* has a constitutive MDH and dissimilates methylamine through an inducible methylglutamate dehydrogenase. Consequently, as in *Methylobacterium organophilum* (Mazodier *et al.*, 1988), MDH appears to be the only catabolic quinoprotein in *M. marina*. The amounts of PQQ detected in crude extracts of methanol- or fructose-grown cells (Table 2) were comparable to those found in other methylotrophs (Mazodier *et al.*, 1988). However, no PQQ was detected in the culture supernatants.

The enzymic activities of formaldehyde and formate dehydrogenases were inducible and were higher in methylamine-grown cells than in methanol-grown cells.

Other enzymic activities reported in Table 1 show that *M. marina* uses the RuMP pathway of formaldehyde assimilation. A high hexulose phosphate synthase activity was present and the Entner-Doudoroff pathway of hexose phosphate dissimilation was operative as evidenced by the high specific activity of KDPG aldolase. As usual in methylotrophs, the Krebs' cycle is not used for catabolism (Anthony, 1986). However oxoglutarate dehydrogenase activity was found in extracts of fructose-grown cells. The pathway of fructose catabolism in *M. marina* is not known.

Optimal conditions for mutagenesis

The survival curve obtained with different EMS concentrations and the corresponding efficiency of mutagenesis estimated from the number of rifamycin-resistant mutants in the bacterial population are reported in Fig. 1. The initial decrease in survival was accompanied by an increase in the number of mutants up to a hundred times for a survival of 5% with an EMS concentration of 7.5 µl ml⁻¹. At higher EMS concentrations, a sharper decline of survival was observed with a large fall in the number of mutants. Variations in these results were recorded with different EMS batches but the most efficient EMS concentrations were always lower than 10 µl ml⁻¹ under the experimental conditions employed. *M. marina* was apparently very sensitive to EMS as compared to *E. coli*, for which the efficient concentrations recommended are 15–50 µl ml⁻¹ (Miller, 1972).

Similar results were obtained with nitrosoguanidine (results not shown). The most efficient concentration was 100 µg ml⁻¹ leading to 6% survival and a maximal

Table 2. Phenotypic characterization of *Methylophaga marina* 42 and of mutants unable to utilize methanol

In all cases, bacteria were grown on ASW medium with fructose as growth substrate. (w), weak reaction; ND, not determined.

α -MDH or mutant protein detected by:†							
Frequency of revertants	MDH activity*	SDS-PAGE	ICR with		PQQ concentration in crude extracts‡	Cytochrome c_L	
			(a)	(b)			
<i>M. marina</i> 42	—	810	+	+	+(w)	2:1	+
<i>Methanol oxidation mutants</i>							
Class I							
4236	10^{-7} – 10^{-8}	<10	+(w)	+(w)	ND	0.05	+(w)
4252	10^{-7} – 10^{-8}	30	+(w)	+(w)	ND	<0.02	+(w)
4296	10^{-7} – 10^{-8}	<10	+(w)	+(w)	ND	0.06	+(w)
4217	< 10^{-9}	<10	—	—	ND	<0.02	—
Class II							
4203	10^{-6} – 10^{-7}	<10	+	—	+	0.75	—
4226	10^{-6} – 10^{-7}	<10	+	—	+	2:1	—
4273	10^{-6} – 10^{-7}	<10	+	—	+	0.37	—
4206	< 10^{-9}	<10	+	—	+	0.06	—
4263	< 10^{-9}	<10	+	—	+	0.15	—
6042	< 10^{-9}	<10	+	—	+	0.14	—
Class III							
42061	10^{-4} – 10^{-6}	<10	+	+	+(w)	0.06	+
42631	10^{-4} – 10^{-6}	<10	+	+	ND	0.15	+
60421	10^{-4} – 10^{-6}	<10	+	+	ND	0.14	+

* nmol dichlorophenol indophenol reduced min^{-1} (mg protein) $^{-1}$.

† The β -subunit was too small to be detected by SDS-PAGE in the gel used. Immunological cross-reactions, as determined by immunoblotting, were with antisera raised against wild-type MDH (a) or against the mutant protein purified from mutant 4206 (b). In Ouchterlony plates, similar results were obtained with serum (a) but serum (b) failed to show any cross-reactions against crude extracts of strain 42 or class III mutants.

‡ nmol PQQ (mg protein) $^{-1}$.

increase of 100 times the initial number of rifamycin-resistant mutants. This concentration is similar to that employed with *E. coli* (Adelberg *et al.*, 1965; Miller, 1972).

The mutants described below were obtained from independent mutagenesis experiments with EMS, except mutant 4296 which was obtained after nitrosoguanidine treatment.

Mutants impaired in methanol oxidation

The mutants described in this work were affected only in methanol utilization with a corresponding defect in methanol oxidation. Their growth in methylamine medium was not modified. When grown on fructose, the enzymic activities in crude extracts of the mutants were similar to those reported in Table 1 for wild-type cells grown on fructose, except for MDH activities. Hexulose phosphate synthase activity was not changed. Table 2 presents the different classes of mutants arranged according to the characteristics of three relevant factors of methanol oxidation: MDH, cytochrome c_L and PQQ.

Class I mutants are characterized by a sharp decrease or a complete disappearance of all three of these factors.

Mutants 4236, 4252 and 4296 contain a low amount of MDH as evidenced by the thin protein band corresponding to this enzyme on SDS-PAGE (Fig. 2), the weak cross-reaction in Ouchterlony plates or on Western blots (not shown), and in some cases a low MDH activity (Table 2). Cytochrome c_L is present in low amounts and PQQ barely detectable. Cells of these mutants displayed very slow growth on methanol medium, yielding distinct colonies, 0.2–0.3 mm in diameter after 2 weeks of incubation, whereas wild-type cells produced 2 mm colonies in 3 d. Mutants 4236, 4252 and 4296 reverted in a single step to the wild-type phenotype, i.e. growth in methanol medium as rapid and abundant as in the wild-type with concomitant recovery of all the enzymic characteristics of normal methanol oxidation (results not shown).

Mutant 4217 contains no detectable amounts of MDH, cytochrome c_L and PQQ, yet a slow growth was observed on methanol plates as with other class I mutants. As a control, no residual growth of class I mutants was observed on ASW plates without carbon source. Mutant 4217 did not revert spontaneously to the wild-type even on a methanol medium plate supplemented with PQQ. This suggests that it is not affected by the same type of mutation found in some class II mutants (see below).

Fig. 1. Survival curve (■) of *Methylophaga marina* 42 with EMS and corresponding effect on the number of rifamycin-resistant mutants in the population (●).

Fig. 2. SDS-PAGE of crude extracts after Coomassie blue staining. Lane 1, molecular mass markers; 2, methanol dehydrogenase (α -subunit); 3 and 4, *Methylophaga marina* 42 grown with methanol or fructose, respectively; 5–10, representatives of different classes of mutants (see Table 2).

Mutants of class II are all affected in the synthesis of an active MDH and of cytochrome c_L . A strong protein band was observed on SDS-PAGE (Fig. 2) at a position corresponding to that of the α -subunit of MDH (65 kDa). However, no cross-reaction was observed between an anti-MDH serum and this protein in Ouchterlony plates (Fig. 3) or upon immunoblotting (see Fig. 5).

Fig. 3. Double diffusion in agarose gel (Ouchterlony) of an anti-MDH serum (centre well) and of crude extracts of: 1, *Methylophaga marina* 42 grown in fructose medium; 2, mutant 42061 grown in fructose medium; 3, mutant 42061 grown in methanol supplemented with PQQ; 4, 5 and 6, mutants 4203, 4206 and 4263 grown in fructose medium. The central well contained 30 μl of anti-serum and each lateral well 200 μg of crude extract protein.

Mutants 4203, 4226 and 4273 have a normal or a lowered but still present PQQ content. From these mutants, revertants with normal methanol oxidation ability were easily obtained, recovering in a single step an active MDH yielding the immunological cross-reactions of the original strain 42 and normal amounts of cytochrome c_L . Spontaneous revertants to the wild-type phenotype were not obtained from the three other mutants of class II, 4206, 4263 and 6042, in which the amount of PQQ found in crude extracts was close to the lower limit that the assay method could detect. They were, in fact, affected by a second mutation regarding PQQ biosynthesis: partial revertants 42061, 42631 and 60421 (class III) were obtained at a frequency of about 10^{-7} by spreading mutants 4206, 4263 or 6042 onto methanol medium plates supplemented with PQQ. These partial revertants recovered in a single step cytochrome c_L and a functional MDH. This MDH produced a normal precipitin line in Ouchterlony plates, not only as active holoenzyme in crude extracts of cells grown in methanol medium supplemented with PQQ but also as inactive apoenzyme in extracts of fructose-grown cells (Fig. 3). Therefore mutants of class III are affected only in PQQ biosynthesis and this confirms the lack of PQQ in the class II mutants 4206, 4263 and 6042.

Purification and subunit structure of MDH and related proteins present in mutants of classes II and III

In a typical purification of MDH, 137 mg of pure enzyme was obtained from 40 g of wet cells, which is comparable

Fig. 4. HPLC gel-filtration in 0.1 M-sodium phosphate, 0.1 M-NaCl, 6 M-urea, pH 6.5, of denatured MDH (a) and mutant 4206 protein before (b) and after (c) recombination with PQQ. The proteins eluted as single peaks under non-denaturing conditions. The chromatograms have been scaled to approximately the same size to facilitate comparison.

to the result reported previously, i.e. 62 mg from 20 g wet cells (Janvier & Gasser, 1987). The purification procedure leads to electrophoretically pure preparations of MDH and mutant 4206 protein, while mutant 42061 protein was slightly contaminated by a higher molecular mass protein (results not shown). As judged from the A_{280} of the purified solution, the final yield of the 4206 and 42061 proteins was 14% and 26%, respectively, that of MDH.

In addition to a protein band at 65 kDa, a faint band with a molecular mass of 6.5 kDa was observed when wild-type MDH (which elutes as a single band on a Superose-12 gel-filtration column) was subjected to SDS-PAGE, indicating that MDH from *M. marina* also contains a small β -subunit as was previously shown for the MDH from *Methylobacterium extorquens* AM1 (Nunn *et al.*, 1989). This was confirmed by gel-filtration on Superose-12 in 6 M-urea (Fig. 4a).

SDS-PAGE of crude extracts (Fig. 2) revealed that mutants of class II synthesize a 65 kDa polypeptide in amounts comparable to and co-migrating with the α -subunit of wild-type MDH. From one of the mutants of class II (4206), a protein could be isolated that eluted from the Superose-12 gel-filtration column at the same position as MDH (elution volume 12.4 ml indicating a molecular mass of about 130 kDa), and with an A_{280} (1 mg ml⁻¹) value close to that of wild-type MDH (2.03 vs 2.04) (van Iersel *et al.*, 1985). In contrast, gel-filtration

Fig. 5. PAGE without SDS at pH 4.5. The gel was stained with Coomassie blue (a); the corresponding immunoblots were incubated with (b) an antiserum raised against purified 4206 mutant protein and (c) an anti-MDH serum. Lane 1 was charged with pure MDH, lanes 2 and 3 with crude extracts of cells of *Methylophaga marina* 42 grown in methanol or fructose medium respectively, and lanes 4 with the purified 4206 mutant protein.

of the purified native protein under denaturing conditions showed that it does not contain a β -subunit. A further difference between the two proteins was noted during chromatography on DEAE-Fractogel, the mutant protein eluting at 0.5 M-NaCl vs 0.3 M for the wild-type protein. Apparently, the mutant 4206 protein has a lower isoelectric point. This was confirmed by the different migration behaviour of the native proteins upon electrophoresis in polyacrylamide gels without SDS at pH 4.5 (Fig. 5). At pH 8.0 the mutant 4206 protein did not enter the gel (result not shown).

The gel-filtration chromatogram in 6 M-urea of the purified protein (reactive with anti-wild-type MDH serum) from mutant 42061, one of the revertants of mutant 4206, was similar to that of MDH (Fig. 4a), except for the absence of PQQ, indicating that mutants of class III have recovered the ability to synthesize the small β -subunit. This was also reflected by the almost identical retention behaviour of the protein on DEAE-Fractogel compared to that of wild-type MDH.

Binding of PQQ to the 4206 and 42061 mutant proteins

The spectra of the mutant proteins before and after incubation with PQQ, taken during elution from the gel-filtration column, are shown in Fig. 6. Although the global shape of the spectra of the two proteins is the same, the absorbance maximum of the 42061 spectrum is shifted 25 nm to the red. The latter spectrum resembles that of one of the oxidized forms of MDH (Frank *et al.*,

Fig. 6. Binding of PQQ by 4206 and 42061 mutant proteins. Absorption spectra before (---) and after (—) incubation with PQQ and chromatography on Superose-12. (a) 4206 protein; (b) 42061 protein. The absorbance above 300 nm of the reconstituted proteins is also shown magnified by a factor 30.

1989). Neither binding of PQQ, nor the shape of the resulting spectra were affected by the presence or absence of Ca^{2+} in the reconstitution medium, and the same applies to the lack of enzymic activity exhibited by the proteins upon binding of PQQ.

Immunological relations between MDH, the mutant protein of class II, and the apo-MDH of class III

Table 2 summarizes the results obtained with anti-MDH and anti-protein-4206 sera. Mutant proteins of class II did not react against the anti-MDH serum whatever the method used: precipitation in Ouchterlony plates (Fig. 3) or immunoblot after PAGE with or without SDS (Fig. 5). These mutant proteins gave normal, strong cross-reactions as expected against an anti-protein-4206 serum (Fig. 5). However, the latter serum also gave a cross-reaction with native MDH, but clearly visible only after PAGE without SDS (Fig. 5). A very weak cross-reaction was also given by the α -subunit of MDH by immunoblotting after SDS-PAGE. Ouchterlony plates did not reveal this cross-reaction. Immunoblotting with serum anti-4206-protein also showed the presence of a small amount of 4206 protein in the cell-free extract of fructose-grown wild-type cells (Fig. 5).

With respect to the anti-MDH serum, the PQQ-requiring mutants from class III produced immunologically reactive proteins upon growth in fructose medium (Fig. 3), even though MDH activity could not be detected in crude extracts. Hence, the lack of immunological response of the mutant proteins of class II with the anti-MDH serum does not seem to be related to the absence of PQQ.

Fig. 7. SDS-PAGE of (a) the (cytoplasmic + membrane) fractions and (b) the periplasmic fractions of *Methylophaga marina* 42 and the mutant 4206. The left-hand lane contained crude extract of *M. marina* 42 and the right-hand lane contained molecular mass markers. The large spots of MDH and of the 4206 mutant protein are visible only in the periplasmic fractions.

Localization of MDH and of mutant protein of class II mutants

MDHs are periplasmic proteins (Alefounder & Ferguson, 1981; Anthony, 1986). A change of structure in the

mutant protein related to a defect in MDH translocation to the periplasm was explored by analysing membrane and cytoplasmic fractions. Fig. 7 shows that wild-type MDH of *M. marina* 42 is located in the periplasm and the same applies to the mutant protein from 4206 and 4203 (not shown).

Discussion

Methanol oxidation in *Methylophaga marina* appears to be organized in a way comparable to that seen in most other Gram-negative methylotrophs. Native MDH (130 kDa) in this organism is composed of two different types of subunits, a large α -subunit (65 kDa) and a small β -subunit (6.5 kDa) suggesting an $\alpha_2\beta_2$ structure for the enzyme and PQQ as cofactor. In addition two soluble cytochromes *c* are present, one of which, cytochrome c_L , is reactive with MDH.

In the four mutants of class I, the levels of MDH, cytochrome c_L and PQQ are strongly lowered or even undetectable (mutant 4217). Except for the latter mutant, which did not revert even in the presence of PQQ, the frequencies of reversion to the wild-type phenotype of methanol utilization indicate that they are single-step reversions resulting in normal levels of MDH, cytochrome c_L and PQQ. Thus these three factors of methanol oxidation are placed under the control of a common regulatory gene. This is also suggested by the presence in mutants 4236, 4252 and 4296 of small amounts of MDH and of cytochrome c_L in accordance with a slow residual growth observed on methanol medium plates. Numerous regulatory genes involved in MDH and cytochrome c_L have been described (Nunn & Lidstrom, 1986b; Lidstrom, 1990) but most often their phenotypic description does not include the results of PQQ assay and for this reason it is difficult to compare them with class I mutants of *M. marina*. For example, mutants of *Methylobacterium extorquens* affected in *moxB*, *moxC* or *moxH*, which synthesize only minute amounts of both MDH and cytochrome c_L (Lidstrom *et al.*, 1987), might be similar to class I mutants of *Methylophaga marina*. An alternative hypothesis regarding the nature of class I mutants would be the presence of a polar mutation affecting an operon encoding α - and β -subunits of MDH and cytochrome c_L as in other methylotrophs but containing also a regulatory gene involved in PQQ synthesis or its regulation. With this hypothesis, the presently unknown function of *moxJ* might be clarified with the help of PQQ assays. In *Methylophilus methylotrophus*, a Tn5 insertion in *moxJ* prevented the synthesis of both the α -subunit of MDH encoded by *moxF* located upstream of *moxJ* and cytochrome c_L encoded by *moxG* located downstream,

leading to the conclusion that *moxJ* is a positive activator of the MDH-cytochrome c_L operon (Lee *et al.*, 1991). Unfortunately, no data are available about the PQQ content of these *moxJ* mutants that could allow a clear comparison.

Mutants of class II do not contain an active MDH nor cytochrome c_L but they all produce large amounts of a dimeric protein, located in the periplasm like MDH and with a molecular mass close to that of MDH, but containing only two subunits of molecular mass 65 kDa, identical to the α -subunit of MDH. Apparently mutants of class II cannot synthesize the β -subunit of MDH and its absence in the mutant protein might be responsible for its lower isoelectric point, since it is known that the β -subunit occurring in MDH from *Methylobacterium extorquens* AM1 contains a large number of lysine residues (Nunn *et al.*, 1989). The purified mutant protein of 4206 is capable of binding PQQ and upon association the absorption maxima of PQQ at 327 and 475 nm are shifted to 365 and 520 nm respectively. The shape of the resulting spectrum and the magnitude of the wavelength shifts is comparable to those observed when apoquinoprotein glucose dehydrogenase is reconstituted with PQQ (Dokter *et al.*, 1986). This indicates a strong interaction between PQQ and the protein. However, the resulting spectrum does not resemble any of the known spectra of MDH, including the one resulting from the *in vitro* reconstitution of apo-MDH (see below). Apparently, the absence of the β -subunits markedly affects the interaction between PQQ and MDH.

Surprisingly, immunological data only partially support the identity of the α -subunit of MDH and the mutant protein. The compared cross-reactivities of MDH and 4206 protein against their respective anti-sera indicate appreciable structural differences in the two proteins. This might be explained by supposing that the presence of the β -subunits in wild-type MDH induces significant conformational changes in the α -subunits leading to the formation of largely different epitopes, in agreement with the difference in interaction with PQQ. In that case, the presence of a small amount of 4206 protein in cell-free extracts of wild-type cells (Fig. 5) would mean that some part of the α -subunits do not combine with β -subunits, due either to a relative excess of α - over β -subunits or to an inefficiency in assembly. Alternatively, the mutant protein might represent a differently processed α -subunit.

The mutants of class II, 4203, 4226 and 4273, produce PQQ and revert spontaneously to the wild-type phenotype of methanol utilization with the concomitant reappearance of normal amounts of MDH and cytochrome c_L . The other mutants of class II that do not produce PQQ, 4206, 4263 and 6042, revert similarly on methanol medium plates supplemented with PQQ to

class III mutants which are affected only in PQQ synthesis. The lack of PQQ synthesis in mutants 4206, 4263 and 6042 therefore seems to be due to an independent second mutation. Alternatively MDH, cytochrome c_L and PQQ might also be controlled by successive suppressor mutations. As in the case of class I, discussed above, the simultaneous recovery of MDH and cytochrome c_L in a single reversion step suggests a co-ordinated synthesis of these factors of methanol oxidation. This can be explained by the presence of a positive regulation gene controlling the synthesis of both MDH and cytochrome c_L , but the well-established model present in the serine pathway methylotrophs, of a *moxF,J,G,I* operon encoding the two subunits of MDH and cytochrome c_L , seems a more likely hypothesis for the organization of these genes in *Methylophaga marina*.

Mutants of class III 42061, 42631 and 60421 produce cytochrome c_L and an apo-MDH that cross-reacts normally with an anti-MDH serum. This apo-MDH, containing α - and β -subunits, is reconstituted to a functional holoenzyme when PQQ is incorporated in the culture medium. In contrast, when PQQ is added to purified apo-MDH, PQQ is bound to the enzyme, but enzymic activity is not restored. The spectrum observed upon association of PQQ (Fig. 6b) closely resembles that of a transient intermediate occurring during the oxidation of MDH (Frank *et al.*, 1989) and which has been identified as the MDH_{ox}-methanol complex, with methanol added to the C(5)-carbonyl of PQQ. This implies that either the *in vitro* reconstituted holo-MDH is still capable of binding methanol, but is unable to oxidize it, or that apo-MDH preferentially binds hydrated PQQ, which has a spectrum similar to that of the methanol adduct of PQQ (Dekker *et al.*, 1982). In both cases, this may be caused by a defective assembly of PQQ and apo-MDH, indicating that one or more external factors are involved in the assembly process as has been suggested previously (Nunn & Lidstrom, 1986b). It has been reported that MDH from *Methylobacillus glycogenes* contains Ca²⁺ which is essential for enzymic activity (Adachi *et al.*, 1990a). However, since the resulting spectrum and lack of enzymic activity were not affected by prior addition of Ca²⁺, it seems that Ca²⁺ is not the only factor.

Interestingly, the PQQ⁻ mutants of class III prove that the apo-MDH of *M. marina* is a stable protein, even in the absence of PQQ. By contrast, in all the PQQ⁻ mutants of another methylotroph, *Methylobacterium organophilum*, apo-MDH is at best barely detectable, either because it is unstable in the absence of PQQ or because it is hardly synthesized in the absence of its cofactor (Mazodier *et al.*, 1988; Biville *et al.*, 1989).

Work is in progress to clarify the precise relationship between the 4206 mutant protein and the wild-type

MDH protein and to characterize possible factors involved in the correct assembly *in vivo* of the 42061 mutant protein.

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