Organization of methylamine utilization genes (*mau*) in '*Methylobacillus flagellatum*' KT and analysis of *mau* mutants

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The organization of genes involved in utilization of methylamine (*mau* genes) was studied in the obligate methylotroph '*Methylobacillus flagellatum*' KT. Nine open reading frames were identified as corresponding to the genes *mauFBEDAGLMN*. In addition, an open reading frame (*orf-1*) encoding a polypeptide with unknown function was identified upstream of the *mau* gene cluster. Subclones of the '*M. flagellatum*' KT gene cluster were used for complementation of a series of chemically induced *mau* mutants of '*M. flagellatum*' KT. Mutants in *mauF*, *mauB*, *mauE/D*, *mauA*, *mauG*, *mauL* and *mauM* were identified. Two mutants (*mau-18* and *mau-19*) were not complemented by the known *mau* genes. Since none of the chemically induced mutants studied had a defect in *orf-1* or *mauN*, insertion mutants in these genes were constructed. Phenotypically the mutants fell into three groups. The *mauF*, *mauB*, *mauE/D*, *mauA*, *mauG*, *mauL* and *mauM* mutants do not grow on methylamine as a source of carbon and lack methylamine dehydrogenase activity, but they synthesize both the large and the small subunit polypeptides albeit at different ratios. The *mau-18* and *mau-19* mutants do not grow on methylamine as a source of carbon, and lack both methylamine dehydrogenase activity and the methylamine dehydrogenase subunits. The *orf-1* and *mauN* mutants grow on methylamine as a source of carbon and synthesize wild-type levels of methylamine dehydrogenase. It has been shown earlier that the product of the *mauM* gene is not required for synthesis of active methylamine dehydrogenase in *Methylobacterium extorquens* AM1 and *Paracoccus denitrificans*. However, *MauM* is required for synthesis of functional methylamine dehydrogenase in '*M. flagellatum*'.

Keywords: '*Methylobacillus flagellatum*', methylamine dehydrogenase, *mau* mutants, tryptophan tryptophylquinone

INTRODUCTION

Methylamine dehydrogenase (MADH) is a primary enzyme for the utilization of methylamine as a source of carbon in many methylotrophic bacteria. These bacteria include the facultative autotrophs *Paracoccus*

denitrificans (Husain & Davidson, 1987) and *Thiobacillus versutus* (Haywood *et al.*, 1982), the facultative methylotroph *Methylobacterium extorquens* AM1 (Eady & Large, 1968) belonging to the α subgroup of proteobacteria, the restricted facultative methylotroph *Methylophilus methylotrophus* W3A1 (Kenny & McIntire, 1983) and the obligate methylotrophs '*Methylobacillus flagellatum*' KT (Kiriukhin *et al.*, 1990), *Methylomonas* sp. J. (Matsumoto *et al.*, 1980) and Organism 4025 (Lawton & Anthony, 1985) belonging to the β subgroup of proteobacteria, and *Methylophaga* spp. (Janvier *et al.*, 1985), which have been recently assigned to the γ subgroup of the proteobacteria.

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Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.

The GenBank accession numbers for the *mau* gene cluster sequences from '*M. flagellatum*' KT are L37426, L37427, L37428, L37429, L37433, L37434 and L37435.

The MADH enzymes from diverse physiological groups of methylotrophs have been well studied and characterized biochemically (Eady & Large, 1968; Haywood *et al.*, 1982; Husain & Davidson, 1987; Kenny & McIntire, 1983; Kiriukhin *et al.*, 1990; Lawton & Anthony, 1985; Matsumoto *et al.*, 1980) and have been shown to be similar. For all cases studied, MADH is a periplasmic protein consisting of two small and two large subunits. Each small subunit has a covalently bound prosthetic group called tryptophan tryptophylquinone (TTQ), synthesized from two tryptophan residues belonging to the small-subunit polypeptide chain (McIntire *et al.*, 1991). The MADHs from restricted facultative methylotrophic bacteria belonging to the genus *Methylophilus* (Burton *et al.*, 1983; Chandrasekar & Klapper, 1986) use a *c*-type cytochrome as an electron acceptor, whereas MADHs from other methylotrophs use blue copper proteins – cupredoxins (Anthony & Jones, 1987; Dinarieva & Netrusov, 1989; Van Houwelingen *et al.*, 1985; Husain & Davidson, 1985; Lawson & Anthony, 1985; Tobari, 1984; Tobari & Harada, 1981). Organism 4025 and '*Methylobacillus flagellatum*' KT can grow slowly on methylamine in a medium depleted of copper, and no cupredoxins can be detected (Dinarieva & Netrusov, 1989; Lawton & Anthony, 1985). An unknown cytochrome was suspected to function under such growth conditions, since the well-characterized cytochromes c_L and c_H in Organism 4025 do not accept electrons from MADH *in vitro* (Auton & Anthony, 1989).

All or some of the genes responsible for MADH synthesis (*mau* genes) have been cloned from *Methylobacterium extorquens* AM1 (Chistoserdov *et al.*, 1990, 1994a), *P. denitrificans* (Van Spanning *et al.*, 1990; Chistoserdov *et al.*, 1992; Van der Palen *et al.*, 1995), *T. versutus* (Ubbink *et al.*, 1991; Huitema *et al.*, 1993), '*Methylobacillus flagellatum*' KT (Gak *et al.*, 1995) and *Methylophilus methylotrophus* W3A1 (Chistoserdov *et al.*, 1994b). The *mau* gene clusters from *T. versutus*, *P. denitrificans* and *M. extorquens* AM1 contain genes for amicyanin (*mauC*) and a polypeptide with unknown function (*mauJ*) between *mauA* (encoding the MADH small-subunit polypeptide) and *mauG* (encoding a putative peroxidase) (Chistoserdov *et al.*, 1994a; Van Spanning *et al.*, 1990; Ubbink *et al.*, 1991). In the *M. methylotrophus* W3A1-NS and '*M. flagellatum*' KT chromosome *mauC* and *mauJ* are missing, and *mauG* immediately follows *mauA* (Chistoserdov *et al.*, 1994b; Gak *et al.*, 1995). A distinctive feature of the *mau* gene cluster from '*M. flagellatum*' KT is that the cluster is followed by the gene for azurin (Gak *et al.*, 1995). The present article addresses the question of the organization of the *mau* gene cluster from '*M. flagellatum*' KT and describes the analysis of a set of chemically induced and insertional *mau* mutants.

METHODS

Bacterial strains and plasmids and growth conditions. The bacterial strains and plasmids used in this work are shown in Table 1. All *Escherichia coli* strains were grown in

Luria–Bertani medium in the presence of appropriate antibiotics as described by Maniatis *et al.* (1982), except that the concentration of chloramphenicol for pAYC63 derivatives was 0.01 mg ml⁻¹. IPTG and X-Gal were added at 0.04 mg ml⁻¹. The '*Methylobacillus flagellatum*' KT strains were grown in the minimal medium described by Fulton *et al.* (1984), except that it was used in double strength and its pH was adjusted to 7.2–7.3 with sodium hydroxide. The nitrogen-free medium used was the minimal medium with sodium sulfate (0.2 g l⁻¹) substituted for ammonium sulfate and ammonium molybdate omitted. The concentrations of tetracycline (Tc), ampicillin (Ap), rifamycin, chloramphenicol (Cm) and kanamycin (Km) for growing the *M. methylotrophus* KT strains were 0.001, 1, 0.1, 0.15 and 0.15 mg ml⁻¹ respectively. Methanol (2%, v/v, unless indicated differently in the text) and methylamine hydrochloride (0.4%, w/v) were used as carbon sources. Ethylamine, ethanolamine, propylamine, dimethylamine and trimethylamine hydrochlorides in 0.2% concentration were assessed as nitrogen sources.

DNA–DNA hybridizations. DNA–DNA hybridizations were carried out in dried agarose gels in accordance with the procedure described by Meinkoth & Wahl (1984). The temperature of hybridizations (6 × SSC, 0.1% SDS) and washes (0.5 × SSC, 0.1% SDS) was 68 °C. 1 × SSC is 0.15 M sodium chloride and 0.015 M sodium citrate solution, pH 7.0.

DNA manipulations. Plasmid isolation, *E. coli* strain transformation, preparative isolation of the DNA fragments from agarose gels, restriction endonuclease digestion, ligation, and blunting of ends with Klenow fragment or T4 DNA polymerase were carried out as described by Maniatis *et al.* (1982). Random primer labelling of DNA fragments was done as suggested by the manufacturer (Boehringer Mannheim). Chromosomal DNA of the '*M. flagellatum*' KT strains was isolated in accordance with the procedure of Marmur (1961).

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method on both strands in the UCLA DNA Sequenator Core Facility on an Applied Biosystems sequenator. Plasmid pAYC63 (Cm^R; Chistoserdov *et al.*, 1992) was used as a vector for subcloning and sequencing in addition to pUC19 (Ap^R).

Matings. Bi- and triparental matings were done as described by Fulton *et al.* (1984). pRK2013 was used as a helper plasmid in triparental matings.

Isolation of chemically induced mutants of '*M. flagellatum*' KT. The 1-methyl-3-nitro-1-nitrosoguanidine mutagenesis procedure used in this research was as described by Miller (1972) with modifications for '*M. flagellatum*' KT (Kletsova *et al.*, 1988). Carbenicillin (1 mg ml⁻¹) and D-cycloserine (0.3 mg ml⁻¹) were used for enrichment of mutants as described by Miller (1972), except that incubation time with antibiotics was increased to 24 h.

Construction of insertional *mau* strains. Insertion *mau* mutants were constructed by homologous recombination as described previously (Ruvkun & Ausubel, 1981). The Km^R cassette from pUC4K was used as a selective inactivating marker and plasmids pAYC61 (Chistoserdov *et al.*, 1994a) and pSUP2021 (Simon *et al.*, 1983) were used as suicide vectors.

MADH assay. '*M. flagellatum*' KT cells were broken by the following osmotic shock procedure. Cells from 50 ml cultures were collected by centrifugation, suspended in 1.5 ml of 25% (w/v) sucrose solution in 50 mM Tris/HCl buffer pH 7.5, and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference*
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d(<i>lac</i> Δ Z)M15 <i>hsdR17 supE44 thi-1 gyrA96 endA1 recA1 relA?</i> Δ (<i>lacZYA-argF</i>)U169	New England Bio-Labs
S17-1	F ⁻ Δ <i>proAB thi-1 recA56</i> RP4-2(Tc ^R : :Mu Km ^R : :Tn7 (Tp ^R Sm ^R)) integrated into chromosome	Simon <i>et al.</i> (1983)
' <i>M. flagellatum</i> ' KT		
KT- <i>rif</i>	Wild-type, <i>rif-1</i>	Govorukhina <i>et al.</i> (1987)
MFK-51	<i>met-1 rif-2</i>	Kletsova <i>et al.</i> (1988)
KT-2.1	<i>mauA1 met-1 rif-2</i>	
KT-2.9	<i>mau-19 met-1 rif-2</i>	
KT-2.18	<i>mau-18 met-1 rif-2</i>	
KT-3	<i>mauB1 met-1 rif-2</i>	
KT-5	<i>mauG1 met-1 rif-2</i>	
KT-8	<i>mauF1 met-1 rif-2</i>	
KT-10	<i>mau-10 met-1 rif-2</i>	
KT-13	<i>mauM1 met-1 rif-2</i>	
KT-24	<i>mau-24 met-1 rif-2</i>	
KT-25	<i>mauG2 met-1 rif-2</i>	
KT-26	<i>mau-26 met-1 rif-2</i>	
KT-32	<i>mau-32 met-1 rif-2</i>	
KT-56	<i>mauL1 met-1 rif-2</i>	
KT-61	<i>mauL2 met-1 rif-2</i>	
KT-78	<i>mauG3 rif-1</i>	
KT-79	<i>mauB2 met-1 rif-2</i>	
KT-85	<i>mauL3 met-1 rif-2</i>	
KT-86	<i>mauF2 met-1 rif-2</i>	
80-2	<i>mauN1</i> : :Km ^R <i>rif-1</i>	
305c-1	<i>azu</i> : :Km ^R <i>rif-1</i>	
306a-1	<i>orf-1</i> : :Km ^R <i>rif-1</i>	
Plasmids		
pRK310	Tc ^R <i>lacZ'</i> <i>mob</i> ⁺ IncP	Ditta <i>et al.</i> (1985)
pRK2013	Km ^R <i>tra</i> ⁺ IncColE1	Ditta <i>et al.</i> (1985)
pUC19	Ap ^R <i>lacZ'</i>	New England Bio-Labs
pAYC61	Ap ^R Tc ^R <i>mob</i> ⁺ IncColE1	Chistoserdov <i>et al.</i> (1994a)
pAYC63	Cm ^R <i>lacZ'</i>	Chistoserdov <i>et al.</i> (1992)
pSUP202	Ap ^R Cm ^R Tc ^R <i>mob</i> ⁺ IncColE1	Simon <i>et al.</i> (1983)
pUC4K	Ap ^R Km ^R	Pharmacia
pGak1	Ap ^R <i>mauD'</i> AGLMN <i>azu</i>	
pGak2	Tc ^R <i>orf-1 mauFBEDAGLM'</i> <i>mob</i> ⁺	
pGak4	Tc ^R <i>mauD'</i> AGLMN <i>azu mob</i> ⁺	
pGak6	Tc ^R <i>mauD'</i> AG' <i>mob</i> ⁺	
pGak7	Tc ^R <i>mauD'</i> AGL'N' <i>azu mob</i> ⁺	
pGak11	Tc ^R <i>orf-1 mauFB'</i> <i>mob</i> ⁺	
pGak60	Tc ^R <i>mauM'</i> N <i>azu'</i> <i>mob</i> ⁺	
pGakS	Tc ^R <i>mauB'</i> EDAG' <i>mob</i> ⁺	
pGakR	Tc ^R <i>mauB'</i> E <i>mob</i> ⁺	

* Where no source or reference is given, the strain or plasmid was derived in this study.

washed twice using 1 ml of the same buffer. A 0.48 ml sample of the cell suspension was withdrawn and was supplemented with 0.5 ml lysozyme solution in the same sucrose buffer (0.2 mg ml⁻¹) and 0.02 ml 0.5 M EDTA to form protoplasts.

After incubation at 37 °C for 15 min 0.2 ml protoplast suspension was diluted with 0.8 ml cold distilled water, causing lysis. Cell debris was pelleted by centrifugation for 30 min in a refrigerated Eppendorf centrifuge (14000 r.p.m.).

MADH in periplasmic extracts was measured spectrophotometrically in accordance with Eady & Large (1968). Protein concentrations and the precise concentration of lysozyme in the lysozyme solutions were measured as described by Whitaker & Granum (1980). The lysozyme concentration was subtracted to obtain concentrations of cellular proteins in extracts.

Computer analysis of DNA sequences. Computer analysis was carried out by using PCGENE (Genofit) and GCG (University of Wisconsin Genetic Computer Group).

Electrophoresis of proteins. The Laemmli system was used for SDS-PAGE (Laemmli, 1970). Separations were done in 13% (w/v) or 15% (w/v) gels. Protein standards for SDS-PAGE were from Bio-Rad.

Transfer of proteins onto membranes. Proteins were transferred onto nitrocellulose membranes for immunoblot experiments and quinone-specific staining using the standard protocol provided by the manufacturer of the 'Trans-blot cell' apparatus (Bio-Rad), using a 4 h transfer time.

Immunoblot experiments. The 'i Assay Kit' (Bio-Rad) was used to detect the MADH large-subunit polypeptide and the 'Amplified Alkaline Phosphatase i Kit' (Bio-Rad) was used to detect the MADH small-subunit polypeptide. The kits were used in accordance with the manufacturer's recommendations.

Quinone-specific staining. This was done as described by Paz *et al.* (1991).

RESULTS

Organization of the *mau* gene cluster

Cloning of the *mau* gene cluster from '*Methylobacillus flagellatum*' KT was reported in our earlier work (Gak *et al.*, 1995). This *mau* gene cluster was cloned as two overlapping fragments of chromosomal DNA: an 8.5 kb *Pst*I fragment and a 4.5 kb *Hind*III fragment (Fig. 1). Since complete or partial nucleotide sequences of several *mau* gene clusters were available (Chistoserdov *et al.*, 1994a, b), partial sequencing was used to identify open reading frames sharing substantial similarity to known *mau* genes and thus to define the *mau* gene cluster organization. The sequencing strategy was based on the assumption that the *mau* gene cluster from '*M. flagellatum*' KT might resemble that of *Methylophilus methylotrophus* W3A1. Nine open reading frames (*mau*FBEDAGLMN) transcribed in the same direction were identified (Fig. 1) that show substantial similarity (33 to 95% identity) to their counterparts from *M. methylotrophus* W3A1. All *mau* genes have strong ribosome-binding sites (at least four bases complementary to the 3' end sequence of 16S rRNA), with the strongest, AGGAGGAAA, in front of *mau*B.

Areas adjacent to the *mau* gene cluster were also sequenced. One open reading frame was found (*orf*-1 in Fig. 1) which is transcribed in the same direction as the *mau* genes and is located 450 bp upstream of *mau*F. It is predicted to encode a putative periplasmic polypeptide of 200 amino acids (21706 Da). A search of the GenBank database did not reveal any gene or protein with considerable similarity to *orf*-1 or its corresponding polypeptide.

Several potential hairpin structures were identified in the sequenced region (Fig. 1). A hairpin structure with predicted energies of production of $-20.6 \text{ kcal mol}^{-1}$ ($-86.1 \text{ kJ mol}^{-1}$) is located between *mau*N and *azu*. This hairpin structure closely resembles a ρ -independent terminator of *E. coli*. Four possible hairpin structures are located between *orf*-1 and *mau*F. They all have relatively low free energies of production (-9.4 to $-11.8 \text{ kcal mol}^{-1}$; -39.3 to $-49.3 \text{ kJ mol}^{-1}$) and they do not resemble ρ -independent terminators of *E. coli*.

Generation of chemically induced *mau* mutants

In order to further analyse the *mau* system, a number of *Mau*⁻ mutants of '*M. flagellatum*' KT were generated using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (Kletsova *et al.*, 1988). All attempts to generate *mau* mutants by chemical treatment were unsuccessful until carbenicillin and D-cycloserine in combination were used for enrichment. The frequency of occurrence of *mau* mutants after such the enrichment was 5×10^{-8} . Of 62 mutants initially isolated as growing on methanol but not on methylamine, only 18 were found to inherit the *Mau*⁻ phenotype stably, with reversion frequencies less than 10^{-8} , and they were used for further work (Table 1).

Complementation analysis of *mau* mutants

To identify mutants containing lesions in the known *mau* genes, the 8.5 kb *Pst*I and 4.5 kb *Hind*III fragments were subcloned into the broad-host-range vector pRK310 such that the *mau* genes were transcribed from the *lac* promoter. The plasmids used were designed to distinguish between mutants in each *mau* gene, and included pGak2 (*orf*-1 *mau*FBEDAGLMN') containing the entire 8.5 kb *Pst*I fragment, pGak4 (*mau*D'AGLMN *azu*) containing the entire 4.5 kb *Hind*III fragment, pGak6 (*mau*D'AG') containing a *Hind*III-*Nsi*I subfragment of pGak1, pGak7 (*mau*D'AGL'N' *azu*), the result of an *Eco*RV-*Pml*I deletion in pGak4, pGak11 (*orf*-1 *mau*FB') containing a *Pst*I-*Bam*HI subfragment of pGak2, pGak60 (*mau*M'N *azu*) containing a *Pst*I-*Hinc*II subfragment of pGak1, pGakS (*mau*B'EDAG') containing a *Hinc*II-*Sma*I subfragment of the 8.5 kb *Pst*I fragment, and pGakR (*mau*B'E) containing a *Hinc*II-*Eco*RV subfragment of the 8.5 kb fragment (Fig. 1). These plasmids were introduced into each mutant in triparental matings with selection for Tc^R. One hundred transconjugants from each mating were checked for the ability to grow on methanol. In all the cases, transconjugants showed a uniform phenotype (all *Mau*⁻ or all *Mau*⁺). The colonies of transconjugants selected after the transfer of pGakR into KT-10, KT-24, KT-26 and KT-32 were tiny and we were not able to restreak them. Therefore, we cannot identify precisely the nature of the lesion in these mutants; they are either *mau*E or *mau*D. The plasmids used would also not distinguish between mutants in *orf*-1 and *mau*E, but in this case *orf*-1 mutants were shown to be *Mau*⁺ (see below).

Fig. 1. Physical and genetic maps of the *mau* gene cluster from '*M. flagellatum*' KT. (a) Restriction map of the 8.5 kb *Pst*I fragment in pGak2. (b) Restriction map of the 4.5 kb *Hind*III fragment in pGak1. Restriction sites with hash signs (#) are not unique on these fragments; not all sites are shown. Arrows above the maps indicate sites of the Km-resistance gene insertions and corresponding mutant names. (c) Genetic map of the *mau* gene cluster. Sites of predicted hairpin structures are denoted by triangles; open boxes denote open reading frames encoding putative periplasmic polypeptides; hatched boxes denote open reading frames encoding putative membrane-bound polypeptides. Boxes whose edges are dashed represent unsequenced portions of *mau* genes. The single dashed line represents unsequenced areas or areas sequenced from one strand. All genes shown are transcribed left to right except *azu*, which is transcribed right to left. (d) The inserts in the plasmids (pGak2, pGak4, pGak6, pGak7, pGak11, pGak60, pGakS, pGakR) used for complementation analysis.

Complementation analysis allowed us to identify the nature of *mau* mutations in all of the mutants (Table 2) except for two, *mau-18* and *mau-19*. These two mutants were not complemented by the known *mau* genes, and apparently they have impairments in unknown genes. No *mauN* or *orf-1* mutants were found. Of the 16 mutants identified one each was defective in *mauA* and *mauM*, two each in *mauB* and *mauF*, three each in *mauG* and *mauL*, and four in *mauD/mauE*.

Generation of insertion mutations in the *mauN* gene and *orf-1*

In order to obtain information about the function of *mauN* and *orf-1*, insertion mutants were generated in these two genes by homologous recombination between

the chromosome and plasmids containing insertions in the genes. The Km^R cassette from pUC4K was inserted in the genes at the sites noted in Fig. 1. The insertions of the Km^R cassette in which the *aph* gene is transcribed in the same direction as the inactivated gene (*mauN* or *orf-1*) were selected for further studies. The constructs with mutagenized genes were recloned into the suicide vector pAYC61 (*orf-1*) or pSUP2021 (*mauN*) and introduced into the '*M. flagellatum*' chromosome by conjugation and selection on plates with methanol and Km. Approximately 90–95 % of the transconjugants analysed were Km^RAp^STc^S (or Km^RAp^SCm^S for pSUP202), the phenotypes indicative of double crossover recombination.

Chromosomal DNAs of these mutants were hybridized with pUC4K and the corresponding DNA fragment

Table 2. Characteristics of chemically induced *Mau*⁻ mutants of '*M. flagellatum*' KT

None of the *Mau*⁻ mutants had detectable MADH activity. The MADH activity of the wild-type was 50 nmol min⁻¹ (mg protein)⁻¹.

Mutant (initial mutation designation)	Complementation by pGak plasmids*								Presence of subunits†		Quinone staining†	Nature of mutation
	pGak2	pGak4	pGak6	pGak7	pGak11	pGak60	pGakS	pGakR				
									Large	Small		
Wild-type									++	++	++	
KT-2.1 (<i>mau-11</i>)	+	+	+	+					++	+	++	<i>mauA1</i>
KT-2.9 (<i>mau-19</i>)	-	-	-	-	-				-	-	-	Unknown
KT-2.18 (<i>mau-18</i>)	-	-	-	-	-				-	-	-	Unknown
KT-3 (<i>mau-3</i>)	+	-	-	-	-		-	-	++	+	++	<i>mauB1</i>
KT-5 (<i>mau-5</i>)	+	+	-	+					++	++	++	<i>mauG1</i>
KT-8 (<i>mau-8</i>)	+	-	-	-	+				++	++	++	<i>mauF1</i>
KT-10 (<i>mau-10</i>)	+	-	-	-	-		+		++	+	++	<i>mauD</i> or <i>mauE</i>
KT-13 (<i>mau-13</i>)	-	+	-	-		-			++	++	++	<i>mauM1</i>
KT-24 (<i>mau-24</i>)	+	-	-	-	-		+		++	+	++	<i>mauD</i> or <i>mauE</i>
KT-25 (<i>mau-25</i>)	+	+	-	+					++	++	++	<i>mauG2</i>
KT-26 (<i>mau-26</i>)	+	-	-	-	-		+		++	+	++	<i>mauD</i> or <i>mauE</i>
KT-32 (<i>mau-32</i>)	+	-	-	-	-		+		++	+	++	<i>mauD</i> or <i>mauE</i>
KT-56 (<i>mau-56</i>)	+	+	-	-					++	++	++	<i>mauL1</i>
KT-61 (<i>mau-61</i>)	+	+	-	-					++	++	++	<i>mauL2</i>
KT-78 (<i>mau-78</i>)	+	+	-	+					++	++	++	<i>mauG3</i>
KT-79 (<i>mau-79</i>)	+	-	-	-	-		-	-	++	++	++	<i>mauB2</i>
KT-85 (<i>mau-85</i>)	+	+	-	-					++	++	++	<i>mauL3</i>
KT-86 (<i>mau-85</i>)	+	-	-	-	+				+	+	++	<i>mauF2</i>

* +, Complementation; -, absence of complementation.

† ++, Property is the same as wild-type; +, considerable decrease compared to the wild-type; -, absence of property.

which was used in generating the mutant. In all cases a 1.4 kb increase in the mutagenized fragments was observed, confirming the presence of only the Km^R cassette (data not shown).

All of the insertion mutants were able to grow on methylamine. This fact may explain why no chemically induced mutants were identified for these two genes. Mutants 306a-1 (*orf-1*) and 80-2 (*mauN*) were indistinguishable in their properties from the wild-type strain and therefore they were not used for further studies.

Phenotypic analysis of *mau* mutants

'*M. flagellatum*' KT can grow only on methylamine or methanol as sources of carbon (Govorukhina *et al.*, 1987). To determine which amines this bacterium can utilize as sources of nitrogen, growth of the wild-type '*M. flagellatum*' KT was assessed in nitrogen-free medium containing methanol as a source of carbon to which each of the amines listed in Methods were added. Ethylamine, ethanolamine, dimethylamine and n-propylamine, but not trimethylamine, served as nitrogen sources for '*M. flagellatum*' KT.

The chemically induced *mau* mutants were checked for their ability to utilize the amines listed above as

sources of carbon and/or nitrogen. All mutants were able to use methylamine, ethylamine, ethanolamine, dimethylamine and n-propylamine as sources of nitrogen, but not trimethylamine. The growth rates of the mutants with the amines as sources of nitrogen were essentially identical to that of the wild-type.

To further characterize the mutant phenotypes, the following properties were studied: the presence of MADH activity; the presence of large and small subunits in immunoblot experiments; and the presence of quinone in the small-subunit polypeptide as assessed using a quinone-specific stain.

It is known for several methylotrophic bacteria that the presence of methanol in the growth medium substantially represses MADH activity even if the natural inducer of MADH, methylamine, is also present. The concentration of methanol (2%) normally used for growing '*M. flagellatum*' KT totally represses MADH synthesis in the presence of 0.4% methylamine. At 0.5% methanol in the presence of 0.4% methylamine, partial derepression of MADH can be achieved (Table 2), and these concentrations of growth substrate and the inducer were used to measure MADH activities in the mutants and wild-type.

All *Mau*⁻ mutants showed no detectable MADH ac-

tivity, while the Mau⁺ insertion strains had activities virtually identical to that of the wild-type strain (data not shown). To determine whether the large- and small-subunit polypeptides are synthesized in the Mau⁻ mutants, immunoblot experiments with antibodies specific for the MADH large or small subunits were performed. The large- and small-subunit polypeptides were detected in extracts of all strains except *mau-18* and *mau-19*, which lacked both subunits. The ratio between the large and small subunits differed from strain to strain. In the *mauA* (KT2.1) and *mauE/D* (KT10, KT24, KT26, KT32) strains the amounts of the small-subunit polypeptide were decreased relative to the amounts of the large subunit polypeptide. In one mutant (KT86) both the large and small subunit polypeptides were present at levels lower than that of the wild-type strain, but the ratio between the large and small polypeptides for KT86 was the same as for the wild-type. A redox-cycling staining procedure has been developed by Paz *et al.* (1991) for specific staining of quinoproteins; it allows the specific staining of the MADH small-subunit polypeptide after transfer to a membrane from a denaturing polyacrylamide gel. A set of polypeptides was stained by this procedure in extracts from all strains, including a polypeptide with molecular mass around 14 kDa, which corresponds to the MADH small subunit. The only two exceptions were *mau-18* and *mau-19*, which lacked the 14 kDa polypeptide.

DISCUSSION

The organization of the *mau* gene cluster in 'Methylobacillus flagellatum' KT is very similar to that in *Methylophilus methylotrophus* W3A1-NS. Not only is the gene order the same, but the partial sequences determined for each 'M. flagellatum' KT *mau* gene also shows high similarity to the analogous *M. methylotrophus* W3A1-NS *mau* genes. Mutants of 'M. flagellatum' KT that were defective in each of the *mau* genes also showed phenotypes consistent with the proposed function of each gene and resembling phenotypes described for analogous *mau* mutants of other micro-organisms (Chistoserdov *et al.*, 1994a). Thus, *mauN* mutants of both 'M. flagellatum' KT and *Methylobacterium extorquens* AM1 can grow on methylamine. *mauD*, *mauE* and *mauF* mutants of 'M. flagellatum' KT and *M. extorquens* AM1 also appeared somewhat similar. These mutants of 'M. flagellatum' KT produce considerably decreased amounts of the small subunit polypeptide compared to the wild-type whereas *mauD*, *mauE* and *mauF* mutants of *M. extorquens* AM1 produce amounts of the small subunit polypeptide which are decreased to undetectable levels. However, two 'M. flagellatum' KT mutants were isolated that were not complemented by the DNA fragments that cover the entire *mau* gene cluster, and these mutants had pleiotropic phenotypes. Since the Mau system is positively regulated by the presence of amines and is negatively regulated by methanol, it is

possible that these mutants are defective in a regulatory function that is not encoded by known *mau* genes. A positive LysR-type regulator of *mau* genes in *P. denitrificans* has been described recently by Van Spanning *et al.* (1995) and its gene was called *mauR*. Properties of 'M. flagellatum' KT-2.9 and KT2.18 appear to be similar to the *mauR* mutant described by Van Spanning *et al.* (1995). Further work will be necessary to assess the role of the gene or genes defective in these mutants. It is also important that the *mauM* gene product was shown to be required for MADH activity, since other *mauM* mutants, isolated in *M. extorquens* AM1 and *P. denitrificans*, had normal MADH activity (Chistoserdov *et al.*, 1994a; Van der Palen *et al.*, 1995). It is possible that this function is redundant in *M. extorquens* AM1, *P. denitrificans* but present in only one form in 'M. flagellatum' KT.

Another important difference was also found in properties of *mauG* mutants of *Methylobacterium extorquens* AM1, *P. denitrificans* and 'Methylobacillus flagellatum'. *MauG* is a putative *c*-haem-containing peroxidase believed to be involved in TTQ biosynthesis (Lidstrom & Chistoserdov, 1993). Earlier data of Page & Ferguson (1993) unambiguously proved that mutants of *M. extorquens* and *P. denitrificans* deficient in *c*-type cytochrome biogenesis synthesize the methylamine dehydrogenase polypeptides but cannot assemble TTQ. A *mauG* mutant of *P. denitrificans* was not able to synthesize a quinone moiety (Van der Palen *et al.*, 1995). A *mauG* mutant of *M. extorquens* AM1 was able to synthesize a quinone moiety in MADH (Chistoserdov *et al.*, 1994a) albeit in only about 10% of all MADH molecules, and the enzyme has a low residual MADH activity (G. N. Marchenko & A. Y. Chistoserdov, unpublished). *mauG* mutants of 'M. flagellatum' were also able to synthesize a quinone moiety in MADH, although it was difficult to estimate quantitatively the intensities of quinone staining in wild-type and *mauG* strains. The possible explanation of the observed discrepancies could be that in 'M. flagellatum' KT and *M. extorquens* AM1, but not in *P. denitrificans*, an isoenzyme exists which is able to carry out the function of *MauG* but much less efficiently.

The ability of *mau* mutants of 'M. flagellatum' KT to utilize amines as sources of nitrogen indicates that MADH is not the only amine-oxidizing system in this bacterium. Particularly interesting is that 'M. flagellatum' KT has a system which allows it to use dimethylamine as a source of nitrogen but not as a source of carbon. Two enzymes are known that are able to oxidize dimethylamine: dimethylamine monooxygenase (Burton *et al.*, 1983) and dimethylamine dehydrogenase (Meiberg & Harder, 1979). Both enzymes produce methylamine as a product of dimethylamine oxidation. Methylamine would allow 'M. flagellatum' KT to grow on dimethylamine if it had either of these two enzymes. Apparently, 'M. flagellatum' KT has a novel dimethylamine oxidation system, which oxidizes dimethylamine directly to ammonia without conservation of energy.

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REFERENCES

- Anthony, C. & Jones, C. W. (1987). Energy metabolism of aerobic, methylotrophic bacteria. In *Microbial Growth on C₁ Compounds*, pp. 195–202. Edited by H. W. Van Verseveld & J. A. Duine. Groningen: University of Groningen.
- Auton, K. A. & Anthony, C. (1989). The role of cytochromes and blue copper proteins in growth of an obligate methylotroph on methanol and methylamine. *J Gen Microbiol* 135, 1923–1931.
- Burton, S. M., Byrom, D., Carver, M., Jones, G. D. D. & Jones, C. W. (1983). The oxidation of methylated amines by methylotrophic bacterium *Methylophilus methylotrophus*. *FEMS Microbiol Lett* 17, 185–190.
- Chandrasekar, R. & Klapper, M. H. (1986). Methylamine dehydrogenase and cytochrome *c*₅₅₂ from the bacterium W3A1. *J Biol Chem* 261, 3616–3619.
- Chistoserdov, A. Y., Tsygankov, Y. D. & Lidstrom, M. E. (1990). Cloning and sequencing of the structural gene for the small subunit of methylamine dehydrogenase from *Methylobacterium extorquens* AM1: evidence for two tryptophan residues involved in the active center. *Biochem Biophys Res Commun* 172, 211–216.
- Chistoserdov, A. Y., Boyd, J., Mathews, F. S. & Lidstrom, M. E. (1992). The genetic organization of the *mau* gene cluster of the facultative autotroph *Paracoccus denitrificans*. *Biochem Biophys Res Commun* 184, 1226–1234.
- Chistoserdov, A. Y., Chistoserdova, L. V., McIntire, W. S. & Lidstrom, M. E. (1994a). The genetic organization of the *mau* gene cluster in *Methylobacterium extorquens* AM1: complete nucleotide sequence and generation and characteristics of *mau* mutants. *J Bacteriol* 176, 4052–4065.
- Chistoserdov, A. Y., McIntire, W. S., Mathews, F. S. & Lidstrom, M. E. (1994b). Organization of the methylamine utilization (*mau*) genes in *Methylophilus methylotrophus* W3A1-NS. *J Bacteriol* 176, 4073–4080.
- Dinariyeva, T. & Netrusov, A. (1989). Cupredoxins of obligate methylotroph. *FEBS Lett* 259, 47–49.
- Ditt, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X. W., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985). Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* 13, 149–153.
- Eady, R. R. & Large, P. J. (1968). Purification and properties of an amine dehydrogenase from *Pseudomonas* AM1 and its role in growth on methylamine. *Biochem J* 106, 245–255.
- Fulton, G. L., Nunn, D. N. & Lidstrom, M. E. (1984). Molecular cloning of a malyd coenzyme A lyase gene from *Pseudomonas* sp. strain AM1, a facultative methylotroph. *J Bacteriol* 160, 718–723.
- Gak, E. R., Chistoserdov, A. Y. & Lidstrom, M. E. (1995). Cloning, sequencing, and mutation of a gene for azurin in '*Methylobacillus flagellatum*' KT. *J Bacteriol* 177, 4575–4578.
- Govorukhina, N. I., Kletsova, L. V., Tsygankov, Yu. D., Trotsenko, Yu. A. & Netrusov, A. I. (1987). Characteristics of a new obligate methylotroph. *Mikrobiologiya* 56, 673–677 (in Russian).
- Haywood, G. W., Janschke, N. S., Large, P. J. & Wallis, J. M. (1982). Properties and subunit structure of methylamine dehydrogenase from *Thiobacillus* A2 and *Methylophilus methylotrophus*. *FEMS Microbiol Lett* 15, 79–82.
- Huitema, F., Van Beeumen, J., Van Driessche, G., Duine, J. A. & Canters, G. W. (1993). Cloning and sequencing of the gene coding for the large subunit of methylamine dehydrogenase from *Thiobacillus versutus*. *J Bacteriol* 175, 6254–6259.
- Husain, M. & Davidson, V. L. (1985). An inducible periplasmic blue copper protein from *Paracoccus denitrificans*: purification, properties, and physiological role. *J Biol Chem* 260, 14626–14629.
- Husain, M. & Davidson, V. L. (1987). Purification and properties of methylamine dehydrogenase from *Paracoccus denitrificans*. *J Bacteriol* 169, 1712–1717.
- Janvier, M., Frehel, C., Grimont, F. & Gasser, F. (1985). *Methylophaga marina* gen. nov., sp. nov. and *Methylophaga thalassica* sp. nov., marine methylotrophs. *Int J Syst Bacteriol* 35, 131–139.
- Kenny, W. C. & McIntire, W. (1983). Characterization of methylamine dehydrogenase from bacterium W3A1: interaction with reductant and amino-containing compounds. *Biochemistry* 22, 3858–3868.
- Kiriukhin, M. Y., Chistoserdov, A. Y. & Tsygankov, Y. D. (1990). Methylamine dehydrogenase from '*Methylobacillus flagellatum*'. *Methods Enzymol* 188, 247–256.
- Kletsova, L. V., Chibisova, E. S. & Tsygankov, Y. D. (1988). Mutants of obligate methylotroph '*Methylobacillus flagellatum*' KT defective in genes of the ribulose monophosphate cycle of formaldehyde fixation. *Arch Microbiol* 149, 441–449.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lawton, S. A. & Anthony, C. (1985). The role of copper proteins in the oxidation of methylamine by an obligate methylotroph. *Biochem J* 228, 719–725.
- Lidstrom, M. E. & Chistoserdov, A. Y. (1993). Molecular biology and genetics of methylamine dehydrogenase. In *Microbial Growth on C₁ Compounds*, pp. 381–400. Edited by J. C. Murrell & D. P. Kelly. Andover: Intercept.
- Maniatis, T., Fritsch, E. T. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208–218.
- Matsumoto, T., Shirai, S., Ishii, Y. & Tobari, J. (1980). Methylamine dehydrogenase of *Methylomonas* J and *Pseudomonas* AM1. Hybridization of light and heavy subunits and some properties of an isolated hybrid enzymes. *J Biochem* 88, 1097–1102.
- McIntire, W. S., Wemmer, D. E., Chistoserdov, A. Y. & Lidstrom, M. E. (1991). A new cofactor in a prokaryotic enzyme: tryptophan tryptophylquinone as the redox prosthetic group in methylamine dehydrogenase. *Science* 252, 817–824.
- Meiberg, J. B. & Harder, W. (1979). Dimethylamine dehydrogenase from *Hyphomicrobium* X: purification and some properties of a new enzyme that oxidizes secondary amines. *J Gen Microbiol* 115, 49–58.
- Meinkoth, J. & Wahl, G. (1984). Hybridization of nucleic acids immobilized on a solid support. *Anal Biochem* 138, 267–284.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Page, M. D. & Ferguson, S. J. (1993). Mutants of *Methylobacterium extorquens* and *Paracoccus denitrificans* deficient in *c*-type cytochrome biogenesis synthesize the methylamine-dehydrogenase polypeptides but cannot assemble the tryptophan-tryptophanylquinone group. *Eur J Biochem* 218, 711–718.
- Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M. & Gallop, P. M.

- (1991). Specific detection of quinoproteins by redox-cycling staining. *J Biol Chem* **266**, 689–692.
- Ruvkun, G. B. & Ausubel, J. R. (1981). A general method for site-directed mutagenesis in prokaryotes. *Nature* **289**, 85–88.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad-host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**, 784–791.
- Tobari, J. (1984). Blue copper proteins in electron transport in methylotrophic bacteria. In *Microbial Growth on C₁ Compounds*, pp. 106–112. Edited by R. L. Crawford & R. S. Hanson. Washington, DC: American Society for Microbiology.
- Tobari, J. & Harada, Y. (1981). Amicyanin: an electron acceptor of methylamine dehydrogenase. *Biochem Biophys Res Commun* **101**, 502–508.
- Ubbink, M., van Kleef, M. A. G., Kleinjan, D.-J., Hoitink, C. W. G., Huitema, F., Beintema, J. J., Duine, J. A. & Canters, G. W. (1991). Cloning, sequencing and expression studies of the genes encoding amicyanin and the β -subunit of methylamine dehydrogenase from *Thiobacillus versutus*. *Eur J Biochem* **202**, 1003–1012.
- Van der Palen, C. J. N. M., Slotboom, D. J., Jongejan, L., Reijnders, W. N. M., Harms, N., Duine, J. A. & Van Spanning, R. J. M. (1995). Mutational analysis of *mau* genes involved in methylamine metabolism in *Paracoccus denitrificans*. *Eur J Biochem* **230**, 860–871.
- Van Houwelingen, T., Canters, G. W., Stobbelaar, G., Duine, J. A., Frank, J., Jzn. & Tsugita, A. (1985). Isolation and characterization of a blue copper protein from *Thiobacillus versutus*. *Eur J Biochem* **153**, 75–80.
- Van Spanning, R. J. M., Wansell, C. W., Reijnders, W. N. M., Oltmann, L. F. & Stouthamer, A. H. (1990). Mutagenesis of the gene encoding amicyanin of *Paracoccus denitrificans* and the resultant effect on methylamine oxidation. *FEBS Lett* **275**, 217–220.
- Van Spanning, R. J. M., van der Palen, C. J. N. M., Slotboom, D.-J., Reijnders, W. N. M., Stouthamer, A. H. & Duine, J. A. (1995). Expression of the *mau* genes involved in methylamine metabolism in *Paracoccus denitrificans* is under control of a LysR-type transcriptional activator. *Eur J Biochem* **226**, 201–210.
- Whitaker, J. R. & Granum, P. E. (1980). An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal Biochem* **109**, 156–159.

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