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# Genetic Organization of the *mau* Gene Cluster in *Methylobacterium extorquens* AM1: Complete Nucleotide Sequence and Generation and Characteristics of *mau* Mutants

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**The nucleotide sequence of the methylamine utilization (*mau*) gene region from *Methylobacterium extorquens* AM1 was determined. Open reading frames for 11 genes (*mauFBEDACJGLMN*) were found, all transcribed in the same orientation. The *mauB*, *mauA*, and *mauC* genes encode the periplasmic methylamine dehydrogenase (MADH) large and small subunit polypeptides and amicyanin, respectively. The products of *mauD*, *mauG*, *mauL*, and *mauM* were also predicted to be periplasmic. The products of *mauF*, *mauE*, and *mauN* were predicted to be membrane associated. The *mauJ* product is the only polypeptide encoded by the *mau* gene cluster which is predicted to be cytoplasmic. Computer analysis showed that the MauG polypeptide contains two putative heme binding sites and that the MauM and MauN polypeptides have four and two FeS cluster signatures, respectively. Mutants generated by insertions in *mauF*, *mauB*, *mauE*, *mauD*, *mauA*, *mauG*, and *mauL* were not able to grow on methylamine or any other primary amine as carbon sources, while a mutant generated from an insertion in *mauC* was not able to utilize methylamine as a source of carbon but utilized $C_2$ to $C_4$ *n*-alkylamines as carbon sources. Insertion mutations in *mauJ*, *mauM*, and *mauN* did not impair the ability of the mutants to utilize primary *n*-alkylamines as carbon sources. All *mau* mutants were able to utilize methylamine as a nitrogen source, implying the existence of an alternative (methyl)amine oxidation system, and a low activity of *N*-methylglutamate dehydrogenase was detected. The *mauD*, *mauE*, and *mauF* mutants were found to lack the MADH small subunit polypeptide and have a decreased amount of the MADH large subunit polypeptide. In the *mauG* and *mauL* mutants, the MADH large and small subunit polypeptides were present at wild-type levels, although the MADHs in these strains were not functional. In addition, MauG has sequence similarity to cytochrome *c* peroxidase from *Pseudomonas* sp. The *mauA*, *mauD*, and *mauE* genes from *Paracoccus denitrificans* and the *mauD* and *mauG* genes from *Methylophilus methylotrophus* W3A1 were able to complement corresponding mutants of *M. extorquens* AM1, confirming their functional equivalence. Comparison of amino acid sequences of polypeptides encoded by *mau* genes from *M. extorquens* AM1, *P. denitrificans*, and *Thiobacillus versutus* shows that they have considerable similarity.**

Several species of methylotrophic bacteria are able to utilize methylamine as a sole source of carbon and energy, and they usually are also able to use methylamine as well as other primary amines as a nitrogen source (39). Three different systems for oxidation of primary amines are known. These are (methyl)amine dehydrogenase, found in some gram-negative methylotrophs and facultative autotrophs (17, 27, 63), amine oxidase, found in gram-positive methylotrophs (41), eukaryotes (27, 46), and members of the family *Enterobacteriaceae* (52), and indirect methylamine oxidation via *N*-methylglutamate dehydrogenase, found in the remaining gram-negative methylotrophs (2, 29, 43).

The methylamine dehydrogenases (MADH) from diverse physiological groups of methylotrophs are well studied and characterized biochemically (17, 27, 33, 35, 37, 40), and they are similar in organization. For all studied cases, MADHs are periplasmic proteins consisting of two small and two large subunits (17, 27, 33, 35, 37, 40). Each small subunit has a covalently bound prosthetic group synthesized from two tryptophans belonging to the small subunit polypeptide chain. The prosthetic group is called tryptophan tryptophylquinone (TTQ) (47). MADHs can be divided into two groups based on the electron acceptors that they use. The MADHs from restricted facultative methylotrophic bacteria belonging to the genus *Methylophilus* (4, 5) use a *c*-type cytochrome as an electron acceptor, whereas all other MADHs use blue copper proteins called amicyanins (13, 32, 40, 60, 63). Several genes responsible for the synthesis of MADH in *Methylobacterium extorquens* AM1 (the *mau* genes) have been cloned recently (11); in addition, the MADH large and small subunit gene and the amicyanin gene from *Paracoccus denitrificans* (6, 64) and from *Thiobacillus versutus* have been cloned (30, 62). In *M. extorquens* AM1, the genes for the small subunit and amicyanin were identified by direct sequencing of the corresponding area of the cloned DNA fragment (10, 12). The gene for the large subunit along with two genes for polypeptides with unknown functions were also mapped by using a T7 expression system (11). The order of the genes has been shown to be *mauBE DAC*. *mauB* encodes the MADH large subunit polypeptide, *mauE* and *mauD* encode polypeptides with unknown functions, *mauA* encodes the MADH small subunit polypeptide, and *mauC* encodes amicyanin.

To understand the organization of the *mau* gene cluster,

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| *E. coli* DH5α | F⁻ φ80d(*lacΔZ*)*M15 hsdR17 supE44 thi-1 gyrA96 endA1 recA1 relA*? Δ(*lacZYA-argF*)*U169* | New England Biolabs |
| *M. extorquens* AM1 | | |
| AM1-*rif* | Wild type, *rif-1* | 20 |
| UV10 | *moxG1 rif-1* | 50 |
| UV9 | *moxD1 rif-1* | 50 |
| 196c-7 | *mau*⁺::Km$^r$1 *rif-1* | This study |
| *Pvu*IIKmM | *mauA1*::Km$^r$ *rif-1* | This study |
| *Bss*HIIKm2 | *mauC1*::Km$^r$ *rif-1* | This study |
| 2*Bcl*IKm8 | *mau*⁺::*Km*$^r$2 *rif-1* | This study |
| 194a-6 | *mauB1*::Km$^r$ *rif-1* | This study |
| 195c-7 | *mauD1*::Km$^r$ *rif-1* | This study |
| *Rsr*II-4 | *mauJ1*::Km$^r$ *rif-1* | This study |
| 261c-14 | *mauJ2*::Km$^r$ *rif-1* | This study |
| 257c-10 | *mauE1*::Km$^r$ *rif-1* | This study |
| 260c-9 | *mauF1*::Km$^r$ *rif-1* | This study |
| 262c-6 | *mauN1*::Km$^r$ *rif-1* | This study |
| 263c-12 | *mauG1*::Km$^r$ *rif-1* | This study |
| 264c-1 | *mauL1*::Km$^r$ *rif-1* | This study |
| 265a-2 | *mauM1*::Km$^r$ *rif-1* | This study |
| 262cG | *moxG1 mauN1*::Km$^r$ *rif-1* | This study |
| 262cD1 | *moxD1 mauN1*::Km$^r$ *rif-1* | This study |
| 252aG | *moxG1 mauM2*::Km$^r$ *rif-1* | This study |
| 252aD1 | *moxD1 mauM2*::Km$^r$ *rif-1* | This study |
| Plasmids | | |
| pRK310 | Tc$^r$ *lacZ' mob*⁺ IncP | 15 |
| pRK2013 | Km$^r$ *tra*⁺ IncColE1 | 15 |
| pUC19 | Ap$^r$ *lacZ'* | New England Biolabs |
| pAYC61 | Ap$^r$ Tc$^r$ *mob*⁺ IncColE1 | This study |
| pAYC63 | Cm$^r$ *lacZ'* | 6 |
| pAYC139 | Tc$^r$ *mauFBEDACJGLKMN mob*⁺ | 10 |
| pAYC163a | Tc$^r$ *mauFBEDACJ' mob*⁺ | 6 |
| pAYC163b | Tc$^r$ *mauFBEDACJ' mob*⁺ | 6 |
| pAYC208 | Tc$^r$ *mauFBEDAGLMN*(?) *mob*⁺ | 9 |
| pAYC267a | Tc$^r$ $P_{mau}$:*cat mob*⁺ | This study |
| pAYC267b | Tc$^r$ $P_{orf}$:*cat mob*⁺ | This study |

sequencing of the cluster was conducted. Several new open reading frames as well as the open reading frames for *mauBE DAC* were found to constitute the cluster, and a promoter was identified upstream of this cluster. Mutants in each known *mau* gene of *M. extorquens* AM1 were generated. The properties of the mutants and of the products encoded by *mau* genes are described in this report.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this work are described 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. (44), except that the concentration of chloramphenicol for pAYC63 derivatives was 0.01 mg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added at 0.04 mg/ml. The *M. extorquens* AM1 strains were grown in the minimal medium described previously (20) except that the microelement solution did not include $(NH_4)_2MoO_4$. The nitrogen-free medium used was the minimal medium in which sodium sulfate (0.2 g/liter) substitutes for ammonium sulfate. The concentrations of tetracycline, ampicillin, rifamycin, and kanamycin for growing the *M. extorquens* strains were 0.01, 0.1, 0.05, and 0.15 mg/ml, respectively. Methanol (1% [vol/vol]) was used as a carbon source. Methylamine hydrochloride (0.5% [wt/vol]), ethylamine hydrochloride (0.5% [wt/vol]), propylamine (0.5% [vol/vol], neutralized with HCl), butylamine (0.5% [vol/vol], neutralized with HCl), amylamine (0.5% [vol/vol], neutralized with HCl), hexylamine (0.5% [vol/vol], neutralized with HCl), isopropylamine (0.5% [vol/vol], neutralized with HCl), phenylethylamine hydrochloride (0.5% [wt/vol]), benzylamine hydrochloride (0.5% [wt/vol]), dimethylamine hydrochloride (0.5% [wt/vol]), and trimethylamine hydrochloride (0.5% [wt/vol]) were used as nitrogen and/or carbon sources. These amines were purchased from Aldrich (Milwaukee, Wis.).

Growth rates of various *M. extorquens* AM1 strains on amines as sources of carbon and/or nitrogen were estimated by determining the time required for single colonies to reach a size of 1.0 mm. The wild type produces colonies of the indicated size on medium with methylamine as a source of carbon or nitrogen or both in 5 days. As much as 1 month was required for some mutant strains to develop colonies 1 mm in diameter (see Results).

**DNA-DNA hybridizations.** DNA-DNA hybridizations were carried out in dried agarose gels in accordance with the procedure described by Meinkoth and Wahl (48). The temperature of hybridizations (6× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0], 0.1% sodium dodecyl sulfate [SDS]) and washes (0.5× SSC, 0.1% SDS) was 68°C.

**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. (44). Random primer labeling of DNA fragments was conducted as suggested by the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). Chromosomal DNA of the *M. extorquens* AM1 strains was isolated in accordance with the procedure of Marmur (45).

**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$^r$) (6) was used as a vector for subcloning and sequencing in addition to pUC19 (Ap$^r$).

**Matings.** Bi- and triparental matings were conducted as described previously (20). Plasmid pRK2013 was used as a helper plasmid in triparental matings.

**Construction of *mau* strains.** Insertion *mau* mutants were constructed by homologous recombination as described previously (56). The Km$^r$ cassette from plasmid pUC4K was used as a selective inactivating marker, and plasmid pAYC61 was used as a suicide vector. pAYC61 was constructed from pUC18 in two steps. First, the *Bam*HI *ori*T-bearing fragment from plasmid pSUP5011 (58) was inserted into the unique *Bam*HI site of pUC18. In the resulting plasmid, the *Eco*RI-*Alw*NI fragment carrying *ori*V was superseded with the *Eco*RI-*Alw*NI fragment from pAT153 (44). Thus, the vector pAYC61 has Ap$^r$ and Tc$^r$ markers and unique sites for enzymes *Pvu*II, *Pst*I, *Xba*I, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI, *Aat*II, *Ssp*I, *Sca*I, *Alw*NI and *Bal*I (Fig. 1).

**Induction of *mau* genes in the *mau* mutants.** Methanol at normal growth substrate levels represses synthesis of MADH. Therefore, in order to induce *mau* genes in mutants, the minimal medium was supplemented with 0.1% methanol and

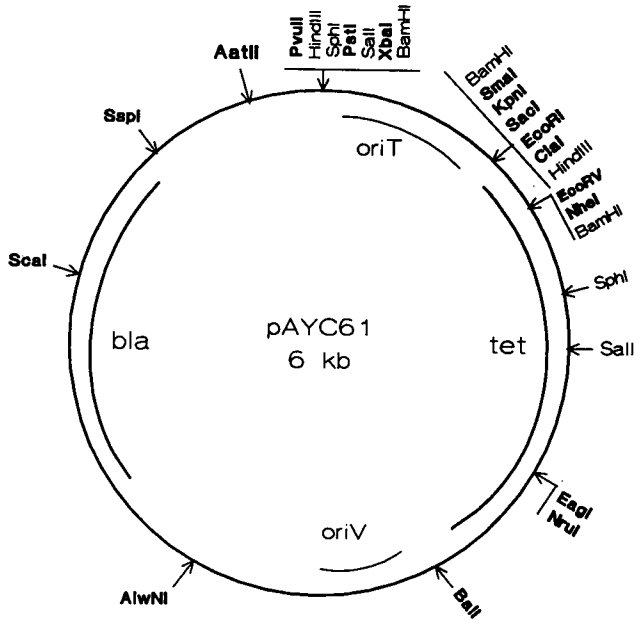


FIG. 1. Physical map of pAYC61. Unique restriction enzyme sites are shown in boldface. bla, ampicillin resistance gene; tet, tetracycline resistance gene; oriT, origin of transfer; oriV, origin of replication.

0.4% methylamine. MADH is fully derepressed under such growth conditions.

**Enzyme assays.** *M. extorquens* AM1 cells were broken open for all biochemical analyses in 10 mM potassium phosphate buffer ($KP_i$; pH 7.0), using a French Press (SLM-AMINCO, Urbana, Ill.) at 20,000 lb/in$^2$. MADH activity was measured as described earlier (17). Amine oxidase and *N*-methylglutamate dehydrogenase were measured as described previously (references 28 and 29, respectively). Oxygen-resistant dye-linked and NAD(P)-dependent formaldehyde and formate dehydrogenase activities were measured as described elsewhere (34). Activities of all enzymes are expressed in micromoles per minute per milligram of protein. Protein concentrations were determined by published methods (66).

**Isoelectrofocusing.** Isoelectrofocusing was performed with PhastGel isoelectric focusing gels of appropriate pH range and the PhastSystem in accordance with recommendations of the manufacturer (Pharmacia, Piscataway, N.J.). MADHs were visualized by specific staining. The reaction mixture for activity staining of MADHs contained 100 mM $KP_i$ (pH 6.8), 10 mM methylamine hydrochloride, 0.5 mM phenazine methosulfate (PMS), and 1 mM nitroblue tetrazolium.

**MADH isolation, subunit separation, and generation of antibodies.** MADH was isolated by combining steps from published procedures (3, 57, 61). Five hundred grams of *M. extorquens* AM1 cell paste was suspended to a volume of 1,700 ml with 10 mM $KP_i$ (pH 7.0). The suspension was passed twice through a Gaulin press at 11,000 lb/in$^2$. Cell debris was removed by centrifugation at 45,000 × *g* for 15 min. The resulting solution was applied to and eluted from a DEAE-Trisacryl M column (5 by 55 cm; Sepracor, Marlborough, Mass.), using a 4-liter, 10 to 250 mM KCl gradient in 10 mM $KP_i$ (pH 7.0). The fractions containing MADH were pooled and concentrated. This material was chromatographed on an Ultrogel AcA34 sieving column (2.5 by 110 cm; $M_r$ range, 20,000 to 350,000; IBF Biotechnics). Final chromatography on a hydroxylapatite column (2.5 by 50 cm; Bio-Gel HPT; Bio-Rad, Richmond, Calif.) with a 0 to 250 mM $KP_i$ (pH 7.0) gradient provided pure enzyme. The subunits were separated as described earlier (57). Rabbits were immunized by using a standard procedure (24).

**Computer analysis of DNA sequences.** Computer analysis was carried out by using PCGENE (Genofit SA, Geneva, Switzerland). Algorithms used in this program package were those of Tinoco et al. (59) for hairpin structure searches and of Rao and Argos (54) and Eisenberg et al. (18) for searches of membrane-associated or membrane-spanning helices.

**Electrophoresis of proteins.** The Laemmli system was used to carry out SDS-gel electrophoresis (38). Separations were done in 13% (wt/vol) or 15% (wt/vol) gels. Protein standards for SDS-gel electrophoresis 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 for native gels.

**Immunoblot experiments.** The i Assay Kit (Bio-Rad) was used for detection of the MADH large subunit polypeptide, and the Amplified Alkaline Phosphatase i Kit (Bio-Rad) was used for detection of the MADH small subunit polypeptide. The kits were used in accordance with the recommendations of manufacturer.

**Quinone-specific staining.** The procedure was conducted as described previously (53).

**Nucleotide sequence accession number.** The GenBank accession number for the *mau* gene cluster from *M. extorquens* AM1 is L26406.

## RESULTS

**Nucleotide sequence of the *mau* gene cluster from *M. extorquens* AM1.** We reported previously (11) that the 5.2-kb *Hin*dIII-*Bam*HI fragment of the *M. extorquens* AM1 chromosome bearing the *mau* genes directs synthesis of several polypeptides in a T7 expression system. Coding areas for the polypeptides were mapped inside this fragment. Three of them were ascribed to the MADH large and small subunits and amicyanin. The genes for the small subunit (*mauA*) and amicyanin (*mauC*) were sequenced (10, 12). Sequence data were not available for the MADH large subunit gene (*mauB*) or polypeptide or for two other genes coding for the polypeptides with unknown function (*mauE* and *mauD*). The 5.2-kb fragment is a part of a larger 9.4-kb *Hin*dIII fragment found in a clone library of the *M. extorquens* AM1 chromosome (10) (Fig. 2). The 9.4-kb fragment was sequenced, and the sequencing data are shown in Fig. 3.

In total, 12 open reading frames were found in this fragment, including the open reading frames for the MADH small subunit polypeptide and amicyanin. Eleven open reading frames (*mauFBEDACJGLMN*) are transcribed in the direction coinciding with the direction of the transcription of the MADH small subunit and amicyanin. One open reading frame (*orf*-1 in Fig. 2) is transcribed in the opposite direction. A 497-bp gap without apparent polypeptide coding exists between the translation starts of *orf*-1 and *mauF*. No open reading frame was found in the 120-bp region downstream of *mauN*. A long open reading frame adjacent to a *Eco*RV site was found in the area where *mauB* (the MADH large subunit gene) had been mapped (11). It is predicted to encode a 411-amino-acid-long periplasmic polypeptide with a molecular mass of 44,635 Da, which corresponds to the known molecular mass of the MADH large subunit polypeptide (42 kDa [57]). The open reading

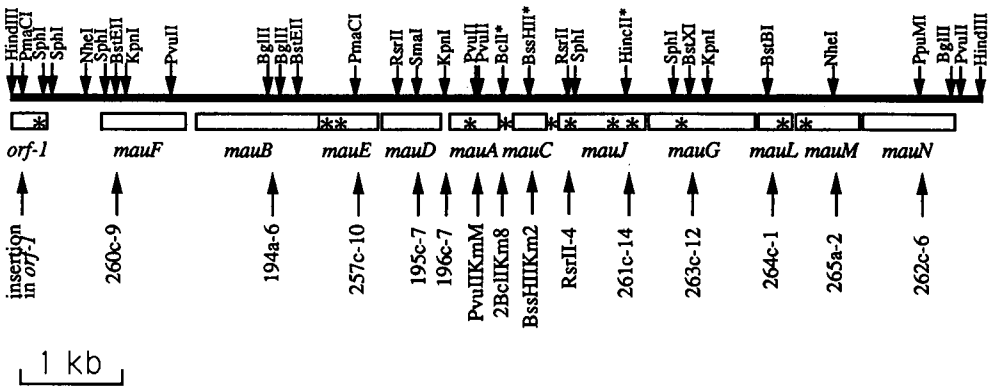


FIG. 2. Physical and genetic map of the 9,441-bp *Hin*dIII fragment containing the *mau* operon of *M. extorquens* AM1. Restriction sites with asterisks are not unique on this fragment. *, sites of hairpin structures. Arrows at the bottom indicate sites of the kanamycin resistance gene insertions and corresponding mutant names.

frame downstream of *mauB* corresponding to *mauE* is predicted to encode polypeptides of 187 (19,521 Da), 186 (19,422 Da), and 184 (19,178 Da) amino acids, depending on which of the potential start codons is used. T7 expression data have shown that translation of the *mauE* gene products (polypeptides of 19.5 and 19.7 kDa) is coupled to translation of *mauB* (11). This indicates that the polypeptides starting with the Val (187 amino acids) and the first Met (186 amino acids) codons are likely to be the initiator triplets responsible for the presence of these two polypeptides in the T7 system. The third potential start is a Met codon inside the *mauE* open reading frame (Fig. 3), which would result in synthesis of a polypeptide of 10,000 Da (100 amino acids). A polypeptide of such mass was not observed in the T7 expression system. This start site probably does not function in vivo, since it was not found in the corresponding positions of the highly similar *mauE* genes from *P. denitrificans* (6) and *Methylophilus methylotrophus* W3A1 (9). The *mauE* open reading frame is followed by an open reading frame coding for polypeptides 205 and 203 amino acids long (22,223 and 22,000 Da), depending on which of two Met codons serves as the initiator. It coincides in frame with the previously described partial open reading frame for *mauD* (8).

We identified several additional open reading frames which had not been previously mapped in T7 expression experiments. The *mauF* open reading frame located upstream of *mauB* is predicted to encode a 285-amino-acid-long polypeptide of 29,464 Da. The synthesis of the MauF polypeptide was not detected in the T7 expression experiments although *mauF* is intact on the plasmid (pAYC147) used in those studies (11). Five open reading frames (designated *mauJGLMN*) were identified in the area downstream of *mauC*, the amicyanin gene. They encode polypeptides 295 (32,682 Da), 353 (38,145 Da), 114 (12,771 Da), 220 (23,308 Da), and 287 (30,683 Da) amino acids long, respectively.

An interesting feature of the *mau* cluster from *M. extorquens* AM1 is the presence of a number of hairpin structures in intergenic spaces as well as inside genes. The hairpin structures with predicted energies of production higher than −14 kcal (ca. −58.6 kJ)mol are shown in Fig. 2 and 3. Two hairpin structures with the highest energies of production are located between *mauA* and *mauC* and between *mauC* and *mauJ*.

**Computer analysis of the amino acid sequences of the polypeptides encoded by the *mau* cluster.** Analysis of the amino acid sequences of the polypeptides encoded by the *mau* cluster allowed predictions about their location in the cell compartments. The MauB, MauA, and MauC polypeptides are known to be periplasmic, and as expected, the gene sequences predict leader sequences for these polypeptides (Fig. 4). The MauG, MauM, and MauL polypeptides are also predicted to be periplasmic, since they have putative leader sequences (Fig. 4). The MauD polypeptide is also probably periplasmic, although it has an unusual leader sequence lacking positively charged amino acids (Fig. 4). Such leader sequences provide transport to the *E. coli* periplasm but with much lower rates than regular leader sequences (21). In addition, MauD is predicted to have a lipoprotein signal peptidase recognition site rather than that for signal peptidase I.

A search of the GenBank sequence banks did not reveal any gene or protein with considerable similarity to *mauF*, *mauE*, *mauD*, *mauJ*, or *mauL* or their corresponding polypeptides. However, MauG has 29% identity with the amino acid sequence of cytochrome *c* peroxidase from *Pseudomonas* sp. (55). The highest identity can be observed near the first heme binding site (Fig. 5). The MauM and MauN polypeptides show similarity to a number of proteins having iron-sulfur clusters, including ferredoxins. The highest identity for MauM was to the ferredoxin from *Methanosarcina barkeri* (25%) (26), and for MauN it was to the ferredoxin I from *Desulfovibrio desulfuricans* (37%; Fig. 5) (22). The MauM and MauN polypeptides are predicted to have four and two [4Fe-4S] cluster signatures, respectively.

Two independent programs in the PCGENE package showed the presence of hydrophobic transmembrane (54) or membrane-associated (18) helices in the MauF, MauE, and MauN polypeptides. Each of them is predicted to have four transmembrane helices.

The only polypeptide encoded by the *mau* cluster which is predicted to be cytoplasmic is MauJ. No substantial similarity was identified for MauJ to any polypeptide in the GenBank sequences. *mauJ* has at least two possible in frame starts. One would generate a polypeptide of 295 amino acids, and another would generate a polypeptide of 236 amino acids. Comparison of the MauJ polypeptide sequence from *M. extorquens* AM1 with that from *T. versutus* indicates that the first start is most likely to be used for initiation of translation in vivo, since the second possible start is absent from the MauJ sequence from *T. versutus* (62).

**Generation of insertion mutations in the *mau* genes and *orf*-1.** To obtain information on the function of the *mau* genes identified by sequencing, mutants that were defective in each

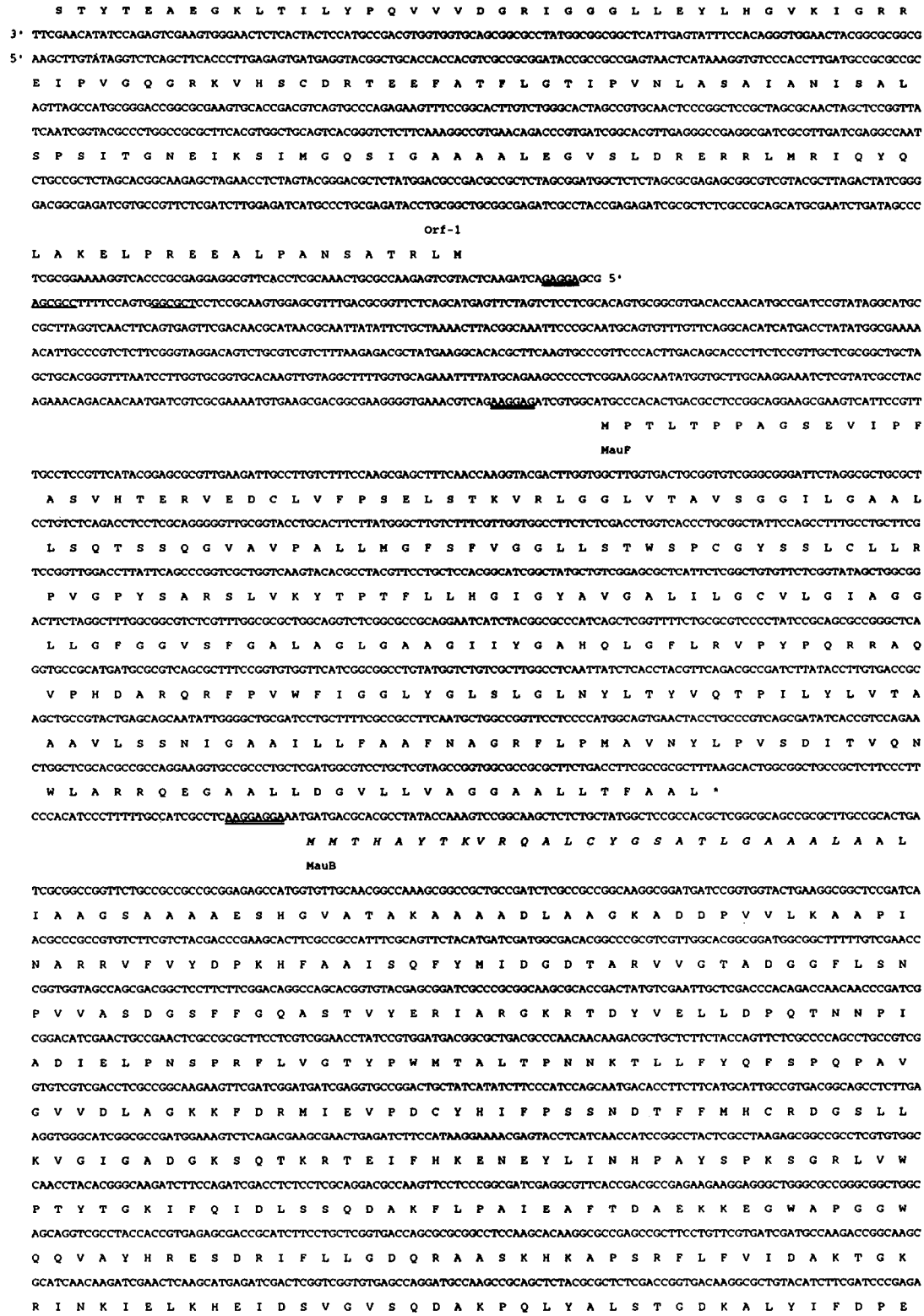


FIG. 3. Nucleotide sequence of the 9,441-bp *Hin*dIII fragment of the *M. extorquens* AM1 chromosome containing the *mau* operon. Putative Shine-Dalgarno sequences are double underlined, hairpin structures are underlined, and putative leader sequences are italicized. In boldface are shown the following: in MauA, the two tryptophans participating in the synthesis of the TTQ cofactor; in MauC, the putative heme *c* binding sites; and in MauM and MauN, the putative iron-sulfur cluster signatures.

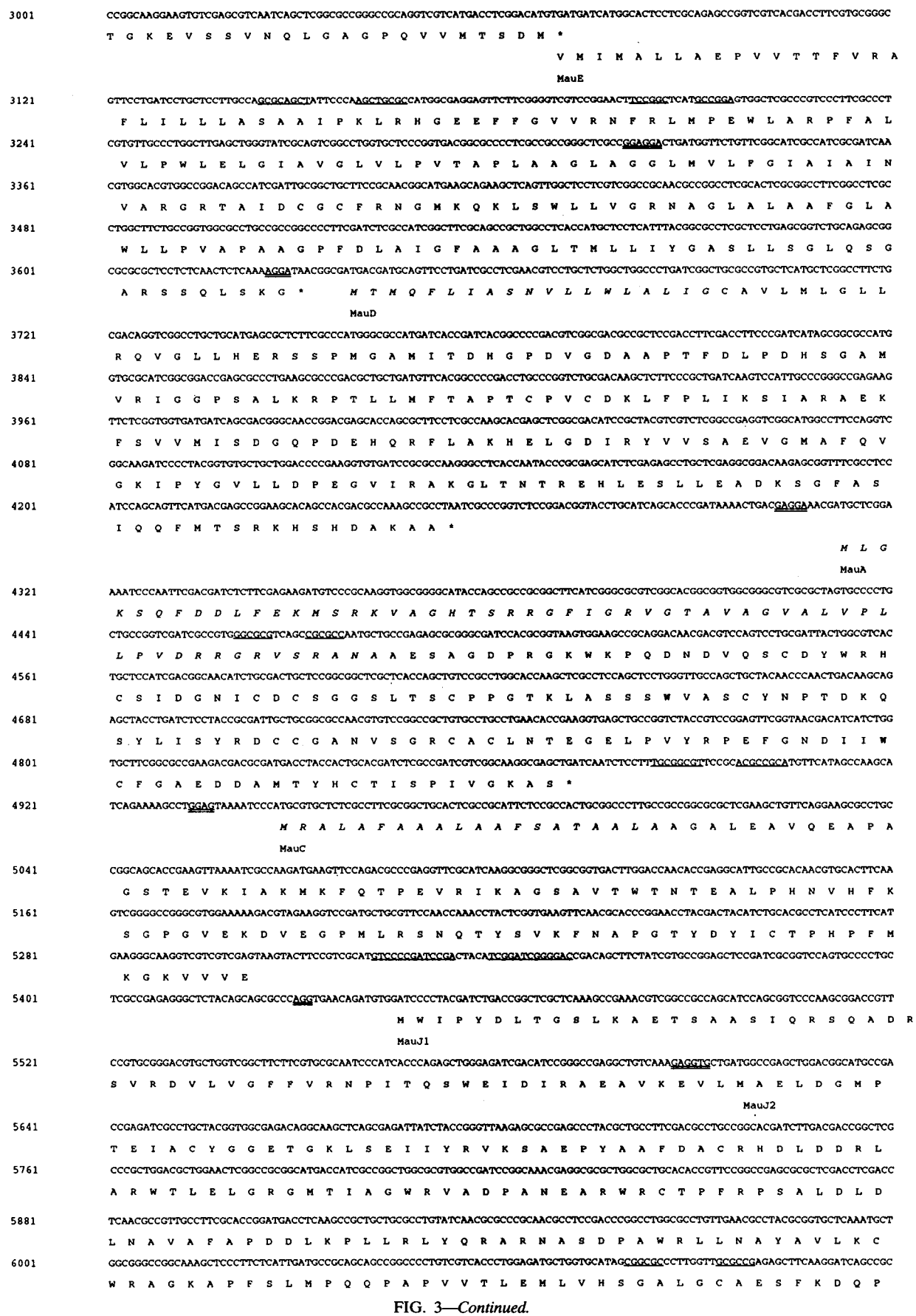


FIG. 3—*Continued.*

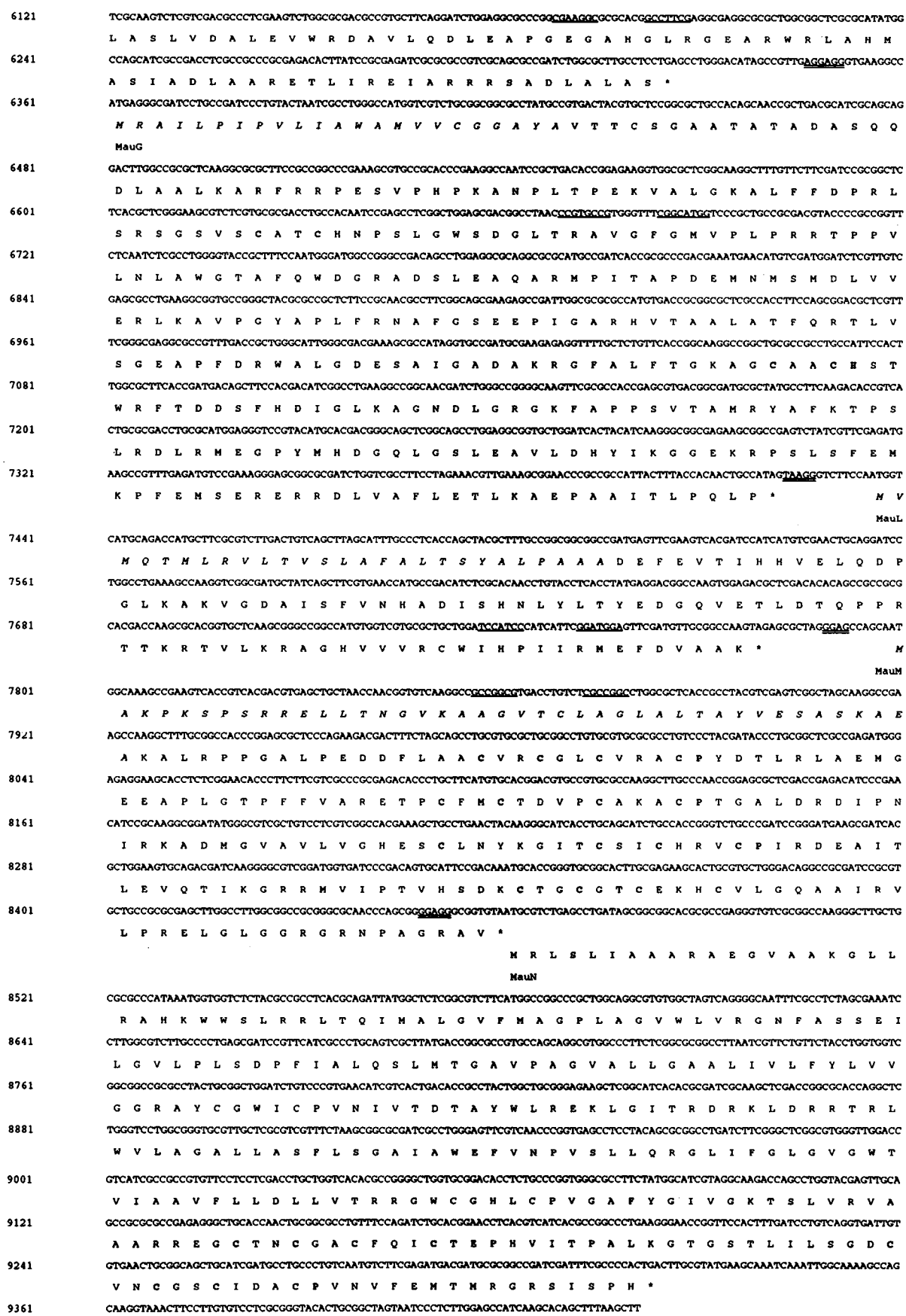


FIG. 3—*Continued.*

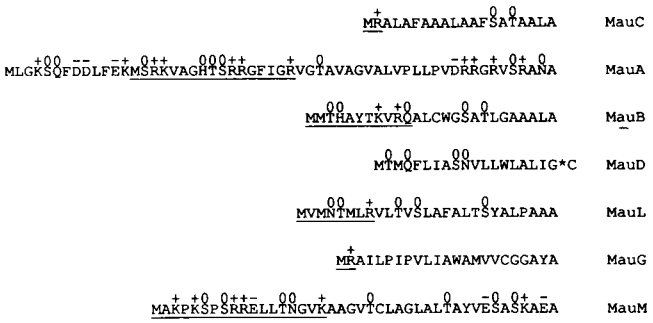


FIG. 4. Putative leader sequences of Mau polypeptides. Positively charged N-terminal sequences proposed to interact with the phospholipid layer are underlined. *, cleavage site in the putative lipoprotein; +, −, and 0, positively charged, negatively charged, and polar amino acids.

gene were generated. Insertion mutations in each *mau* gene and in *orf*-1 were generated by homologous recombination between the chromosome and plasmids containing insertions 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 an inactivated gene (*mau* or *orf*-1) were selected for further studies. Two insertions in different sites inside genes were generated for *mauJ*. The insertion in *mauE* was generated so as to impair all possible polypeptides encoded by this gene. The constructs with mutagenized genes were recloned into the suicide vector pAYC61 and introduced into the *M. extorquens* AM1 chromosome by conjugation and selection on plates with methanol and kanamycin. To assess whether the Km$^r$ cassette has a polar effect on downstream genes, insertions between *mauA* and *mauC* and between *mauD* and *mauA* were obtained. In these cases also, the insertions selected contained the *aph* gene transcribed in the same direction as the surrounding *mau* genes. With the exception of *orf*-1, all insertion mutants studied were Km$^r$ Tc$^s$, the phenotype indicative of double-crossover recombination.

Chromosomal DNAs of these mutants were hybridized with radioactively labeled plasmid pUC4K and the corresponding DNA fragment which was used in generating the mutant. In all cases, a 1.4-kb increase in the mutagenized fragments was observed, indicating the presence of only the Km$^r$ cassette (data not shown).

All attempts to generate a double-crossover recombinant Km$^r$ insertion for *orf*-1 were unsuccessful. Of 500 Km$^r$ transconjugants selected on medium with methanol as a source of carbon and 1,000 Km$^r$ transconjugants selected on medium with succinate as a source of carbon, all were simultaneously Tc$^r$. Sequence data suggest that *orf*-1 is the first open reading frame of another gene cluster (7). Therefore, the inability to obtain null mutants implies that the *orf*-1 gene product or products of genes downstream of *orf*-1 are vital for growth of *M. extorquens* AM1 cells on both succinate and methanol. The mutants obtained apparently contained an insertion of the entire suicide plasmid. This single-crossover recombinational event generates a complete copy of the mutated gene and does not have a polar effect on downstream genes (7).

**Phenotypic analysis of *mau* mutants.** *M. extorquens* AM1 can grow on methylamine, ethylamine, *n*-propylamine, and *n*-butylamine, but not grow on *n*-amylamine, dimethylamine, trimethylamine, or isopropylamine, as sources of carbon (17). In addition, we found that *M. extorquens* AM1 cannot utilize phenylethylamine and benzylamine as sources of carbon, and

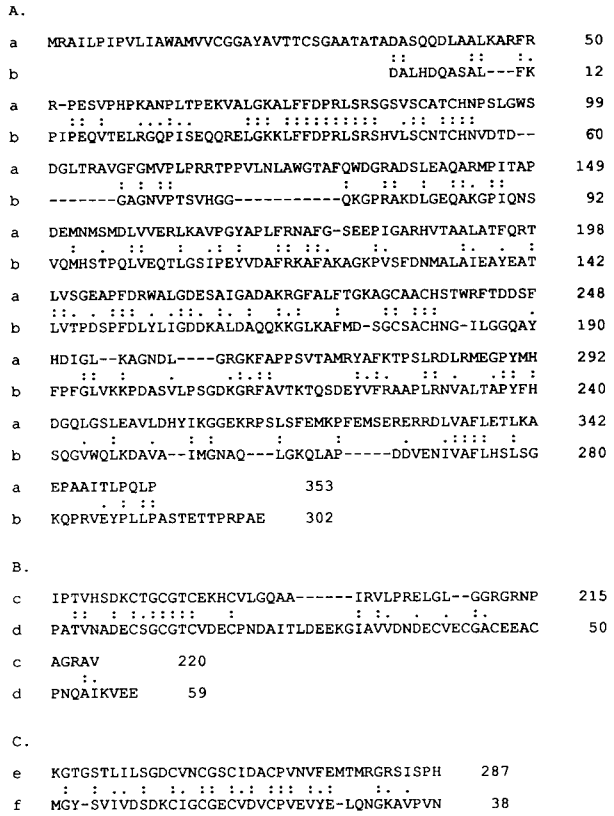


FIG. 5. (A) Alignment of the deduced amino acid sequence of MauG (a) with the sequence of cytochrome *c* peroxidase (b) from *Pseudomonas* sp. (54). (B) Alignment of a fragment (amino acids 174 to 220) of MauM (c) with ferredoxin (d) from *M. barkeri* (25). (C) Alignment of a fragment (amino acids 248 to 287) of MauN (e) with a fragment (amino acids 1 to 38) of ferredoxin II (f) from *D. desulfuricans* (21). Identical residues are indicated by double dots; conserved substitutions are shown by single dots.

*n*-hexylamine is highly toxic for this bacterium. To determine which alkylamines this bacterium can utilize as sources of nitrogen, growth of the wild-type strain *M. extorquens* AM1 was assessed in nitrogen-free medium containing methanol as a source of carbon to which each of the amines listed above had been added. Only the $C_1$ to $C_5$ *n*-alkylamines served as nitrogen sources for *M. extorquens* AM1.

The insertion mutants in the *mau* cluster were checked for the ability to utilize *n*-alkylamines as sources of carbon and/or nitrogen (Table 2). All mutants were uniformly able to use methylamine, ethylamine, and *n*-propylamine, but not *n*-butyl- or *n*-amylamine, as sources of nitrogen; therefore, only data for growth on methylamine and ethylamine are shown in Table 2. All strains able to grow on ethylamine as a source of carbon were also able to utilize *n*-propyl- and *n*-butylamines as sources of carbon.

The mutants fall into three phenotypic groups. Group 1 consists of mutants which grow on the same amines as carbon and nitrogen sources as the wild type with growth rates similar to that of the wild type. This group includes the two *mauJ* mutants and the *mauM* and *mauN* mutants. Although these mutants grow normally on amines, since these genes are closely clustered with the other *mau* genes, we will designate them as *mau* throughout this report. In addition, normal growth on amines was observed for mutants with insertions of the Km$^r$

TABLE 2. Properties of the wild-type strain and the insertion *mau* mutants of *M. extorquens* AM1

| Strain | Property[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ability to utilize methylamine | | Ability to utilize ethylamine | | MADH activity[b] (nmol/min/mg of protein) | Large subunit | Small subunit | Quinone staining, 14 kDa[c] | Formaldehyde dehydrogenase activity[b] (nmol/min/mg of protein) | | Formate dehydrogenase activity[b] (nmol/min/mg of protein) | |
| | As carbon | As nitrogen | As carbon | As nitrogen | | | | | NAD | DCPIP[d] | NAD | DCPIP |
| Wild type | +++ | +++ | +++ | +++ | 110 | +++ | +++ | +++ | 8 | 6 | 30 | 13 |
| *Pvu*IIKmM (*mauA*) | − | + | − | + | 0 | + | − | − | 8 | 10 | 50 | 33 |
| 2*Bcl*IKm8 (*mauA/mauC*)[e] | +++ | +++ | +++ | +++ | 60 | +++ | +++ | +++ | 6 | 6 | 10 | 12 |
| *Bss*HIIKm2 (*mauC*) | − | + | + | ++ | 30 | ++ | ++ | +++ | 2 | 8 | 35 | 20 |
| 194a-6 (*mauB*) | − | + | − | + | 0 | − | − | − | 8 | 12 | 19 | 24 |
| 195c-7 (*mauD*) | − | + | − | + | 0 | ++ | − | − | 10 | 2 | 32 | 24 |
| 257c-10 (*mauE*) | − | + | − | + | 0 | ++ | − | − | 5 | 3 | 33 | 25 |
| 260c-9 (*mauF*) | − | + | − | + | 0 | ++ | − | − | 7 | 2 | 37 | 30 |
| 196c-7 (*mauD/mauA*)[e] | +++ | +++ | +++ | +++ | 40 | +++ | +++ | +++ | 6 | 5 | 10 | 18 |
| *Rsr*II-4 (*mauJ*) | +++ | +++ | +++ | +++ | 35 | +++ | +++ | +++ | 18 | 7 | 21 | 17 |
| 261c-14 (*mauJ*) | +++ | +++ | +++ | +++ | 75 | +++ | +++ | +++ | 6 | 5 | 33 | 14 |
| 263c-12 (*mauG*) | − | + | − | + | 0 | +++ | +++ | +++ | 7 | 4 | 38 | 30 |
| 264c-1 (*mauL*) | − | + | − | + | 0 | +++ | +++ | +++ | 8 | 8 | 41 | 32 |
| 265a-2 (*mauM*) | +++ | +++ | +++ | +++ | 105 | +++ | +++ | +++ | 4 | 3 | 46 | 16 |
| 262c-6 (*mauN*) | +++ | +++ | +++ | +++ | 76 | +++ | +++ | +++ | 3 | 2 | 50 | 15 |

[a] +++, same as wild type; ++, slight decrease compared with wild type; +, considerable decrease compared with wild type; −, absence.
[b] Results are averages of three independent measurements, which agree ± 15%. 0, not detectable.
[c] 14 kDa is the small subunit polypeptide observed in denaturing gels.
[d] DCPIP, dichlorophenol indolphenol.
[e] Mutation is located between the two genes.

cassette between *mauD* and *mauA* and between *mauA* and *mauC*. This result is an indication that the Km$^r$ cassette does not have a polar effect on downstream genes. Group 2 consists of the amicyanin mutant (*mauC*). It cannot grow on methylamine as a source of carbon but readily utilizes $C_2$ to $C_4$ *n*-alkylamines as sources of carbon, with growth rates 2.5- to 3-fold lower than that of the wild type. The growth rates of the *mauC* mutant on $C_1$ to $C_5$ *n*-alkylamines as sources of nitrogen were also 2- to 2.5-fold lower than that of the wild type. Group 3 consists of mutants that were unable to grow on any *n*-alkylamines as carbon sources. This group includes the *mauF*, *mauB*, *mauE*, *mauD*, *mauA*, *mauG*, and *mauL* mutants. The growth rates of all mutants within this group on amines as sources of nitrogen were approximately fivefold lower than that of the wild type.

The prediction of iron-sulfur clusters in the MauM and MauN polypeptides suggests that these polypeptides might participate in a redox process such as electron transfer from amicyanin. If this is so, mutants in *mauM* and *mauN* might grow normally on amines as a result of an alternative electron transport route. The cytochrome $c_L$ has been implicated as an alternative electron acceptor for MADH, since it is known that methanol metabolism genes, including the $c_L$ cytochrome gene (*moxG*), are induced by methylamine (50), and in in vitro experiments, cytochrome $c_L$ accelerated transfer of electrons from MADH in the presence of amicyanin (19). To rule out such a possibility, *moxG mauM* and *moxG mauN* double mutants were constructed by using the same double-crossover recombination procedure (56) with the Km$^r$ cassette from pUC4K and the *M. extorquens moxG* mutant UV10 as the recipient. *moxD mauN* and *moxD mauM* strains were also constructed using the *moxD* mutant UV9 to serve as additional negative controls (*moxD* mutants are devoid of cytochrome $c_L$; instead, they have a high level of cytochrome $c_{553}$). All four double mutants were able to grow on methylamine as a source of carbon with growth rates comparable to that of the wild type.

**Activities of enzymes involved in the oxidation of amines in the *mau* mutants.** Activities of several enzymes involved or potentially involved in methylamine oxidation in *M. extorquens* AM1 were measured in an attempt to identify functions of polypeptides encoded by the *mau* gene cluster. This enzyme list includes not only MADH but also NAD(P)-dependent and dye-linked formaldehyde and formate dehydrogenases. It has been shown (36) that in *Hyphomicrobium* strain ZV580, the induction of two different formaldehyde dehydrogenases depends on the carbon source. Growth on methylamine results in induction of a specific formaldehyde dehydrogenase in this strain (36). Thus, it is feasible that a defect in a methylamine pathway-specific formaldehyde or formate dehydrogenase could impair the ability to grow on methylamine (and possibly other amines) as a carbon source. However, the results of screening the NAD(P)-dependent and dye-linked formate and formaldehyde dehydrogenase activities in the mutants and the wild type (Table 2) show that all have similar levels of these enzymes. These results indicate that the Mau$^-$ phenotype of the mutants is not connected with a lesion in a formate or formaldehyde dehydrogenase.

Mau$^-$ mutants except the *mauC* mutant showed no detectable MADH activity in crude extracts (Table 2). In addition, no MADH activities were found in these mutant extracts after isoelectrofocusing separation and specific staining for MADH activity (data not shown). In vitro, the *mauC* mutant had MADH activity of 30 μmol/min/mg of protein.

**Immunological analyses of the *mau* mutants.** To determine whether the large and small subunit polypeptides are synthesized in the insertion mutants, immunoblot experiments with antibodies specific for the MADH large or small subunits were

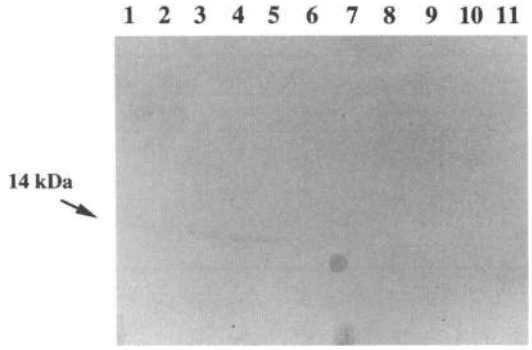


FIG. 6. Immunoblot of crude extracts of mutant and wild-type *M. extorquens* AM1 after electrophoresis on an SDS–15% polyacrylamide gel, using antisera prepared against the small subunit of MADH from *M. extorquens* AM1. Lanes: 1, wild type grown on methylamine (positive control); 2, *Bss*HIIKm2 (MauC); 3, 262c-12 (MauG); 4, 264c-1 (MauL); 5, 196c-7 (MauD/A); 6, 260c-9 (MauF); 7, 194a-6 (MauB); 8, *Pvu*IIKmM (MauA); 9, 195c-7 (MauD); 10, 257c-10 (MauE); 11, wild type grown on methanol (negative control). Mutants were grown on methanol in the presence of inducing concentrations of methylamine as described in Materials and Methods. About 10 μg of protein per lane was loaded. Arrow denotes a weak band of 14 kDa corresponding to the small subunit of MADH.

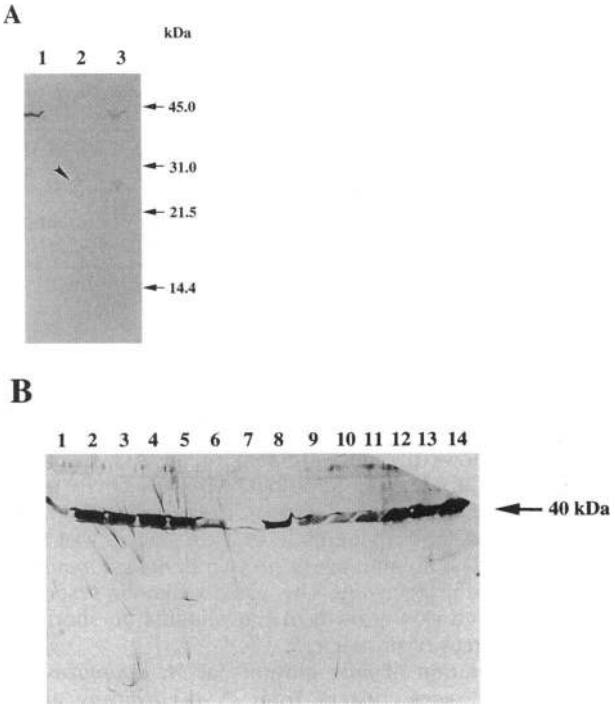


FIG. 7. Immunoblot of crude extracts from wild-type *M. extorquens* AM1 and the mutant in the gene encoding the large subunit of MADH after electrophoresis on an SDS–13% polyacrylamide gel, using antisera prepared against the large subunit of MADH from *M. extorquens* AM1. (A) Lanes: 1, wild type grown on methylamine; 2, 194a-6 (MauB); 3, a mutant resistant to both kanamycin and tetracycline carrying a copy of the suicide vector pAYC61 inserted in the chromosome. It contains one whole and one truncated copy of *mauB*. Molecular masses of standard proteins are shown on the right. The arrowhead denotes a polypeptide with an approximate molecular mass of 28 kDa corresponding to the truncated form of MADH. (B) Lanes: 1, 260c-9 (MauF); 2, 262c-6 (MauN); 3, 263c-12 (MauG); 4, 264c-1 (MauL); 5, 2*Bcl*IKm8 (MauA/C); 6 and 11, *Pvu*IIKmM (MauA); 7, wild type grown on methanol (negative control); 8, 261c-14 (MauJ); 9, 195c-7 (MauD); 10, 257c-10 (MauE); 12, *Rsr*II-4 (MauJ); 13, *Bss*HIIKm2 (MauC); 14, wild type grown on methylamine (positive control). The arrow denotes the position of the large subunit. Mutants were grown on methanol in the presence of inducing concentrations of methylamine as described in Materials and Methods. About 10 μg of protein was loaded per lane.

conducted. The data obtained after immunoblot experiments of mutant extracts separated in denaturing gels are shown in Fig. 6 and 7 and are summarized in Table 2. Immunoblot detection of the MADH small subunit was poor (Fig. 6), possibly because of the high degree of cross-linking found in the small subunit polypeptide, which has six disulfide bonds in addition to the TTQ cross-link (47). The small subunit polypeptide was detected in extracts of the strains with the wild-type phenotype when they were grown in the presence of methylamine and in extracts of the *mauC*, *mauG*, and *mauL* mutant strains. The small subunit polypeptide was not detected in *mauF*, *mauB*, *mauE*, *mauD*, and *mauA* mutants and in strains grown under uninduced conditions. It is not clear from the experimental data whether the small subunit is not detected in these mutants because it is synthesized at a level which is below the sensitivity of the method or because of its total absence. The large subunit polypeptide was found to be truncated in the *mauB* mutant (Fig. 7A). The mutants with the wild-type phenotype and the *mauC*, *mauG*, and *mauL* mutants synthesize wild-type levels of the MADH large subunit polypeptide under induced conditions (Fig. 7B). All other mutants synthesized decreased levels of the large subunit polypeptide, although extremely low amounts of the MADH large subunit polypeptide were detected in the *mauF* mutant.

**The presence of quinoproteins in *mau* mutants.** A redox cycling staining procedure was developed recently by Paz et al. (53) for specific staining of quinoproteins. It was shown that this procedure allowed the specific staining of the MADH small subunit polypeptide after it was transferred to a membrane from a denaturing polyacrylamide gel. With this procedure, extracts from mutants with the wild-type phenotype and the *mauC*, *mauG*, and *mauL* mutants grown under inducing conditions gave staining of a 14-kDa band (Table 2). This band was absent in lanes loaded with the crude extracts from other mutants and the uninduced wild type. An additional band with molecular mass of around 16 kDa was observed in all lanes after redox straining for quinoproteins, and it may represent a second quinoprotein with a covalently bound quinone moiety. The presence of this band was not dependent on induction by methylamine, and therefore it appears not to pertain to methylamine metabolism.

**The presence of alternative amine oxidation activities.** The ability of Mau$^-$ mutants to utilize *n*-alkylamines as nitrogen sources suggests the presence of an alternative amine oxidation system. Three different (methyl)amine oxidation systems are currently known to be able to metabolize methylamine: methylamine dehydrogenase, *N*-methylglutamate dehydrogenase, and amine oxidase (AO). To identify whether any of these systems is responsible for the ability of Mau$^-$ mutants to grow on methylamine as a source of nitrogen, we tested for the activities of these enzymes. The possibility of the existence of a second methylamine dehydrogenase cannot be ruled out, and therefore MADH activity was also included. Cultures of the *mauA*, *mauB*, and *mauF* mutants were grown in nitrogen-free minimal medium supplemented with methylamine as a source of nitrogen, and enzyme activities were measured in extracts.

TABLE 3. Complementation of the *M. extorquens* AM1 *mau* mutants by plasmids with the *mau* genes from *P. denitrificans* and *M. methylotrophus* W3A1

| Plasmid | Organism | Genes harbored | Complementation[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mauF* | *mauB* | *mauE* | *mauD* | *mauA* | *mauC* | *mauG* | *mauL* |
| pAYC139 | *M. extorquens* AM1 | *mauFBEDACJGLMN* | + | + | + | + | + | + | + | + |
| pAYC163a | *P. denitrificans* | *mauFBEDACJ'* | − | − | + | + | + | − | − | − |
| pAYC163b | *P. denitrificans* | *mauFBEDACJ'* (opposite orientation) | − | − | + | + | + | − | − | − |
| pAYC208 | *M. methylotrophus* W3A1 | *mauFBEDAGLMN(?)O* | − | − | − | + | − | − | + | − |

[a] +, complementation observed; −, complementation not observed.

Two methods of measuring of AO activity were used. One allows measurement of an AO specific for aromatic and long-chain amines which can oxidize methylamine (16), and the second allows the measurement of an AO specific for methylamine (28). Neither additional MADH activity nor any AO activity was found in the extracts; instead, very low activity of dye-linked *N*-methylglutamate dehydrogenase was found: 0.2, 0.3, and 2.2 nmol/min/mg of protein in *mauA*, *mauB*, and *mauF* mutants, respectively. This activity may be responsible for the observed slow growth of the mutants on short-chain amines as sources of nitrogen.

**Complementation of *mau* mutants of *M. extorquens* AM1 with the *mau* gene clusters from *P. denitrificans* and *M. methylotrophus* W3A1.** In addition to the *mau* gene cluster from *M. extorquens* AM1, two other *mau* gene clusters were used for complementation experiments. A 6.3-kb *Hin*dIII fragment from the *P. denitrificans* chromosome has been cloned previously (6) and contains *mauFBEDAC* and a part of *mauJ*. This fragment was cloned in the broad-host-range vector pRK310 in both orientations (plasmids pAYC163a and pAYC163b). The cloning of the *mau* gene cluster from *M. methylotrophus* W3A1 is described in the accompanying report (9), and the DNA fragment used for complementation contains the *mauFBEDAGLMN* genes in pRK310. Only the orientation in which the insert with the *mau* gene cluster cannot be transcribed from $P_{lac}$ was obtained in *E. coli* (plasmid pAYC208). pAYC208, pAYC163a, and pAYC163b were transconjugated into all $Mau^-$ mutants of *M. extorquens* AM1 with selection for $Tc^r$, and then 100 transconjugants from each were checked for the ability to grow on methylamine as a source of carbon (Table 3). In all cases in which complementation was observed, all 100 colonies tested were able to grow on methylamine, suggesting that in each case the complete functionally similar gene was present. If complementation required recombination, the frequency of $Mau^+$ transconjugants would be expected to drop. Although *P. denitrificans* is fairly closely related to *M. extorquens* AM1 (they both belong to the α subgroup of proteobacteria [23]), the *mauF*, *mauB*, and *mauC* mutations of *M. extorquens* AM1 were not complemented by either pAYC163a or pAYC163b. This finding is especially surprising in the case of amicyanins (*mauC*), since these structurally and functionally related electron acceptors were expected to be interchangeable. However, the *mauD*, *mauE*, and *mauA* mutations were complemented with both pAYC163a and pAYC163b. *M. methylotrophus* W3A1 is a member of the β subgroup of proteobacteria (23) and therefore is not closely related to *M. extorquens* AM1. However, the *mau* genes from the former are able to complement the *mauD* and *mauG* mutants of the latter.

**Regulation of MADH in the MauJ mutants.** Since MauJ is the only cytoplasmic polypeptide found so far in the *mau* gene cluster and is not required for growth on methylamine, it is logical to suspect that it might be involved in regulation. To determine whether MauJ is involved in negative regulation (repression) of MADH synthesis, both *mauJ1* and *mauJ2* mutant strains along with the wild type were grown in medium supplemented with either methylamine, methanol, or succinate, and MADH activity was measured in all crude extracts. The wild-type and the *mauJ* mutants had similar levels of MADH activity when they were grown on methylamine and no MADH activity when they were grown on methanol or succinate. Thus, if MauJ is involved in some kind of regulation, it does not drastically affect MADH synthesis under the growth conditions tested.

## DISCUSSION

**Suggested functions of the Mau polypeptides.** MADH is a unique quinoprotein in which the prosthetic group, TTQ, is apparently synthesized posttranslationally from the MADH small subunit polypeptide chain. We have shown here that MADH is responsible in *M. extorquens* AM1 not only for consumption of methylamine as a source of carbon but also for utilization of other *n*-alkylamines as carbon sources. It is expected that the MADH system should include not only genes encoding structural components of MADH and its electron acceptor but also genes for prosthetic group synthesis and genes responsible for regulation and coordination of the expression of the first two groups of genes. Taking into consideration the presence of an unusual leader sequence in the MauA polypeptide and its inability to provide transport into the periplasm of *E. coli* (8), specific functions involved in the MauA polypeptide transport into the periplasm should also exist. The synthesis of TTQ has been proposed to include two reactions, oxidation (hydroxylation) of the first Trp into tryptophylquinone and cross-linking of tryptophylquinone and the second tryptophan (42). These processes might occur in different cell compartments (in the cytoplasm, within the inner membrane, or in the periplasm), and they might occur sequentially or simultaneously. However, it is unlikely that cross-linking occurs prior to transport, because of the difficulties of transporting a looped polypeptide. Analyses of the primary structure of the polypeptides encoded by the *mau* genes as well as properties of the $Mau^-$ mutants can shed some light on the function of the corresponding polypeptides in the process of MADH synthesis and assembly.

The functions of MauB, MauA, and MauC are clear. They are structural components encoding the MADH large subunit, MADH small subunit, and amicyanin, respectively. The small subunit polypeptide contains an unusual leader sequence structure which is conserved in all four known MADHs and may be involved in TTQ synthesis (6, 9, 10, 62).

The *mauA* mutation of *M. extorquens* AM1 can be complemented by the highly similar *mauA* from *P. denitrificans*. Amicyanin (MauC) seems to be required as an acceptor of

electrons from MADH for growth of *M. extorquens* AM1 on methylamine as a source of carbon, as has been shown for *P. denitrificans* (64). However, the *mauC* mutant grew normally on $C_2$ to $C_4$ *n*-alkylamines. This finding suggests that alternative electron carriers can serve as direct electron acceptors from MADH in vivo, but they probably do not function in proton translocation. Proton translocation at this step is important for bacteria to grow on methylamine (1). However, during growth on multicarbon compounds, energy can be acquired from subsequent oxidation of the substrate (1), and so it is not necessary that energy be generated in the first step. Unexpectedly, the *mauC* mutation of *M. extorquens* AM1 was not complemented with the *mauC* gene from *P. denitrificans*, probably because of either difficulties of its expression in a heterologous host or incompatibility with the electron transfer system of *M. extorquens* AM1.

The MauE and MauD polypeptides seem likely to be involved either directly or indirectly in the stability of the MADH small subunit. The mature form of MauA was not detected in MauE and MauD mutants in immunoblot experiments and in quinone staining experiments. The MauE polypeptide is structurally and functionally conserved in methylotrophs belonging to the α subgroup of proteobacteria, and *mauE* from *P. denitrificans* complements the *mauE* mutation of *M. extorquens* AM1.

MauD is more unusual, since its proposed leader sequence contains no positively charged amino acids. Several cases of such leader sequences are known; they result in slow transport of polypeptides into the periplasm of *E. coli*, which still occurs probably as a result of the N-terminal amino group of a premature polypeptide (21). The presence of a putative lipoprotein leader sequence in MauD seems to be irrelevant to the function carried out, since MauD from *M. methylotrophus* W3A1 has a normal leader sequence which also does not bear positively charged amino acids. Our complementation data (Table 3) show that MauD from *M. methylotrophus* W3A1 can function in *M. extorquens* AM1.

The MauF polypeptide might be involved in regulation or transport from the phenotype of the *mauF* mutant. It has no detectable MauA and a low amount of MauB, and it is predicted to be a membrane polypeptide. Our results show that it is unlikely that MauF is involved in regulation. First, the sequence of MauF does not resemble that of any known transmembrane regulator (25). Second, a *mauFp-cat* transcriptional fusion provides the same level of $Cm^r$ in the *mauF* mutant as in the wild type (7, 49).

The MauJ polypeptide seems to be nonessential for MADH synthesis and activity. Its position inside the *mau* cluster suggests that it is involved in the Mau system somehow, perhaps in a function redundant in *M. extorquens* AM1. *mauJ* is linked to *mauC*, and both are absent in the genome of *M. methylotrophus* W3A1, which does not have cupredoxins such as amicyanin or azurin (9).

The MauG and MauL polypeptides appear to be involved in the synthesis of TTQ in MauA. The *mauG* and *mauL* mutants contain the MADH small and large subunits; the MADH small subunit polypeptide stains positively for quinones, but the MADH does not have activity. Therefore, it is likely that MauG and MauL are involved in the cross-linking reaction of TTQ biosynthesis. MauG is predicted to be a diheme cytochrome *c* which has similarity to cytochrome *c* peroxidase from *Pseudomonas* sp. A peroxidase could mediate cross-linking of tryptophylquinone and tryptophan, since peroxidases are known to cross-link indole groups (14, 31). The fact that a *c*-type cytochrome is required for synthesis of active MADH was observed by Oozeer et al. (51). MauG from *M. methylotrophus* W3A1 can complement the *mauG* mutation of *M. extorquens* AM1. The function of MauL is apparently not as conserved, and the identity between MauLs from these two species is only 31%.

The functions of MauM and MauN in MADH synthesis are unclear. These polypeptides are not required for generation of active MADH, and yet their position in the *mau* cluster suggests that they are involved somehow. *mauM* is present in the *mau* gene cluster from *M. methylotrophus* W3A1 and shows 55% identity with *mauM* of *M. extorquens* AM1, indicating that at least MauM carries out some important functions in the *mau* system. The sequence analysis suggests that MauM and MauN participate in redox processes, but our data suggest that they probably do not participate in electron transfer between amicyanin and cytochrome $c_H$.

**An alternative system of amine oxidation in *M. extorquens* AM1.** Mutants devoid of MADH activity grow slowly on methylamine, ethylamine, and *n*-propylamine as sources of nitrogen, suggesting that an alternative amine oxidation system must be present. We were unable to detect activities of AO or an alternative MADH in *M. extorquens* AM1. However, low activities of dye-linked *N*-methylglutamate dehydrogenase were found in all *M. extorquens* AM1 strains tested growing with methylamine as a source of nitrogen, and this may explain the slow growth observed. It has been reported that *M. extorquens* AM1 does not produce intermediates of the *N*-methylglutamate dehydrogenase reaction (65). However, it is possible that they were not detectable at such low concentrations.

## ACKNOWLEDGMENTS

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