JOURNAL OF BACTERIOLOGY, Sept. 1991, p. 5901–5908
0021-9193/91/185901-08$02.00/0

Vol. 173, No. 18

Genetic Organization of Methylamine Utilization Genes from *Methylobacterium extorquens* AM1

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Received 15 February 1991/Accepted 25 June 1991

An isolated 5.2-kb fragment of *Methylobacterium extorquens* AM1 DNA was found to contain a gene cluster involved in methylamine utilization. Analysis of polypeptides synthesized in an *Escherichia coli* T7 expression system showed that five genes were present. Two of the genes encoded the large and small subunits of methylamine dehydrogenase, and a third encoded amicyanin, the presumed electron acceptor for methylamine dehydrogenase, but the function of the other two genes is not known. The order on the 5.2-kb fragment was found to be large-subunit gene, the two genes of unknown function, small-subunit gene, amicyanin gene. The gene for azurin, another possible electron acceptor in methylamine oxidation, does not appear to be present within this cluster of methylamine utilization genes.

Gram-negative bacteria of diverse systematic groups are able to utilize methylamine as a carbon, energy, and nitrogen source. These groups include facultative autotrophs such as *Paracoccus denitrificans* (25) and *Thiobacillus versutus* A2 (23); facultative methylotrophs such as pink-pigmented *Methylobacterium* (formerly "*Pseudomonas*") spp. (6, 10, 18, 19, 37), *Hyphomicrobium* spp. (36, 45, 56), and nonpigmented "*Pseudomonas*" spp. (7, 24); restricted facultative methylotrophs such as *Methylophilus* spp. (11, 12, 15, 29); and obligate methylotrophs such as *Methylobacillus* spp. (22, 30, 41) and "*Methylomonas*" spp. (42, 44). The majority of these strains use methylamine dehydrogenase (MADH; EC 1.4.99.3) for the initial oxidation of methylamine, although some oxidize methylamine via *N*-methylglutamate dehydrogenase (EC 1.5.99.5), including some *Methylobacterium* strains (8–10), all *Hyphomicrobium* strains (45, 56), and all nonpigmented "*Pseudomonas*" strains (5, 7, 24, 32).

Methylobacterium extorquens AM1 utilizes methylamine via MADH (18). The enzyme consists of two large (40-kDa) and two small (14-kDa) subunits (51), with a cofactor covalently bound to the small subunit. MADHs from other methylotrophic bacteria have a similar structure (23, 27, 42, 51), except the MADH from *Methylobacillus flagellatum* KT, which is a heterodimer (30). Amino acid (28) and nucleotide (14) sequences of the small subunit of MADH from *M. extorquens* AM1 have been published. Recent evidence has shown that the MADH cofactor has a unique structure, having two tryptophans within the polypeptide chain that are modified and covalently linked (14). A novel structure has been proposed for this cofactor (43).

The copper-containing protein amicyanin is proposed to be the natural electron acceptor for MADH (17, 25, 34, 35, 54, 55, 57, 58). Amicyanin is thought to transfer electrons to a soluble periplasmic *c*-type cytochrome, but some uncertainty exists concerning the identity of the in vivo acceptor. In the case of *M. extorquens* AM1, both cytochrome c_L and c_H are electron acceptors in vitro (4, 18, 51) but mutants lacking cytochrome c_L grow normally on methylamine (48), suggesting that this cytochrome is not required for in vivo methylamine utilization. Fukumori and Yamanaka (20) have shown that MADH from *M. extorquens* AM1 may directly pass electrons to cytochrome c_H in vitro in the absence of amicyanin and cytochrome c_L, but the significance of this pathway in vivo is unknown. In *Paracoccus denitrificans*, special methylamine-induced *c*-type cytochromes have been reported (26). In *Methylophilus* sp. strain W3A1 and *Methylophilus methylotrophus*, amicyanin has not been found and a *c*-type cytochrome is apparently the direct in vivo electron acceptor for MADH (11, 12).

Another copper-containing protein, azurin, has been found in *M. extorquens* AM1. It accepts electrons in vitro from amicyanin, cytochrome c_L, or cytochrome c_H and transfers them to a terminal oxidase (54). However, it is induced only in the presence of high concentrations of copper in the growth medium. Azurin is also induced by high copper concentrations in *M. flagellatum* KT (17), "*Methylomonas*" strain J (54), and *P. denitrificans* (40).

Although the first step of methylamine oxidation has been well studied biochemically, little is known about the organization of genes involved in methylamine metabolism. Recently, we reported the cloning and sequencing of the genes for the small subunit of MADH (14) and amicyanin from *M. extorquens* AM1 (14a). In this paper, we show that the genes for the MADH small and large subunits and for amicyanin are closely clustered with two other genes of unknown function.

MATERIALS AND METHODS

Bacterial strains. 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. (38). Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added at 40 μg/ml. *M. extorquens* AM1 was grown in the minimal medium described previously (21). *M. flagellatum* KT was grown in M9 medium (47).

Purification of MADH. MADHs from *M. extorquens* AM1 and *M. flagellatum* KT were purified as described previously (30). Rabbit antibodies against MADH from *M. flagellatum*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Methylotrophic bacteria		
Methylobacterium extorquens AM1	Wild type, Rf^r	49
Methylobacillus flagellatum KT	Wild type	31
E. coli strains		
DH5α	F^- *recA1* Δ(*lacZYA-argF*)U169 *hsdR17 thi-1 gyrA66 supE44 endA1 relA1* φ80d*lac*Δ(*lacZ*)M15	Bethesda Research Laboratories
GM2163	F^- *ara-14 leuB6 tonA31 lacY1 tsx-78 supE44 galK2 galT22 hisG4 rpsL136 xyl-5 mtl-1 thi-1 dam-13*::Tn*9 dcm-6 hsdR2 mcrB*	New England Biolabs
Plasmids		
pUC18, pUC19	Ap^r *lacZ'*	Pharmacia
pTZ19TT	Ap^r Φ(*cat-lacZ'*)	2
pGP1-2	Km^r *c*I856(Ts) p_L:polT7	52
pAYC139	Tc^r IncP1 *mob*$^+$; plasmid from cosmid library, containing *mau* genes	A. Chistoserdov
pAYC141	Tc^r IncP1 *mob*$^+$; plasmid from cosmid library that hybridizes weakly to *mauA* probe; contains no overlap with pAYC139 or pAYC142	This study
pAYC142	Ap^r; 7.3-kb *Bam*HI insert of *M. extorquens* AM1 chromosome, containing *mau* genes in pUC7	14
pAYC147	Ap^r *mauB*(E)DAC Φ(*cat-lacZ'*)	This study
pAYC147.1	Ap^r *mauB'*1 Φ(*cat-lacZ'*)	This study
pAYC147.2	Ap^r *mauB'*2 Φ(*cat-lacZ'*)	This study
pAYC148	Ap^r *mauDAC* Φ(*cat-lacZ'*)	This study
pAYC152	Ap^r *mauAC* Φ(*cat-lacZ'*)	This study
pAYC152.1	Ap^r *mauA* Φ(*cat-lacZ'*)	This study
pAYC152.2	Ap^r *mauA* Φ(*cat-lacZ'*)	This study
pAYC152.3	Ap^r *mauA* Φ(*cat-lacZ'*)	This study
pAYC152.5	Ap^r *mauC* Φ(*cat-lacZ'*)	This study
pAYC153	Ap^r *mauD'1* Φ(*cat-lacZ'*)	This study
pAYC153.1	Ap^r *mauE* Φ(*cat-lacZ'*)	This study
pAYC156	Ap^r *mauB(E)DAC* Φ(*cat-lacZ'*)	This study
pAYC156.1	Ap^r *mauB(E'1)* Φ(*cat-lacZ'*)	This study

KT were received from the laboratory of V. L. Yurin (VNII Genetica, Moscow, USSR).

β-Galactosidase assays. β-Galactosidase was assayed as described by Miller (47). The *lac* promoter was activated by supplementing liquid medium with IPTG.

DNA-DNA hybridizations. DNA-DNA hybridizations were carried out in dried agarose gels as described by Meinkoth and Wahl (46). Hybridizations were carried out at 45°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Washing was done at 42°C in 0.5 × SSC.

T7 expression system. For T7 expression of proteins from cloned genes, the protocol of Tabor and Richardson (52) was used. Plasmid pTZ19TT was used as the T7 expression vector (2).

SDS gel electrophoresis. The protocol of Laemmli (33) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting. Proteins were transferred from gels to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad, Richmond, Calif.). Subsequent incubation with anti-MADH and anti-cytochrome *c*-553 antibodies and detection with the Immun-Blot Alkaline Phosphatase Assay kit were done in accordance with the protocols of the manufacturer (Bio-Rad). Antibody to *M. extorquens* AM1 cytochrome *c*-553 was generously provided by C. Anthony (University of Southampton, Southampton, United Kingdom).

DNA manipulations. Plasmid isolation, *E. coli* strain transformation, restriction endonuclease digestion, ligation, blunting of ends with Klenow fragment and/or T4 DNA polymerase, and polynucleotide kinase reactions were carried out as described by Maniatis et al. (38). Chromosomal DNA of *M. extorquens* was isolated by the procedure of Marmur (39).

Oligonucleotide probe synthesis. The oligonucleotide probe 5'-CACTAYATGATGGGNATGGT-3' was synthesized on the basis of the known amino acid sequence of azurin (HYMMGMV) (1) by the Caltech Microchemical Facility.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method (3, 50) with Sequenase and Taquenase sequencing kits according to instructions of the manufacturer (U.S. Biochemical Corp., Cleveland, Ohio).

Computer analysis of DNA sequences. Computer analysis was carried out by using the PCGENE program (Genofit SA, Geneva, Switzerland) and searches for hairpin structures were in accordance with the method of Tinoco et al. (53).

Nucleotide sequence accession number. The GenBank accession number for sequences used in this study is M57963.

RESULTS

Expression of polypeptides. In a previous paper (14), we reported the cloning of a 5.2-kb *Bam*HI-*Hin*dIII fragment that was shown to contain the genes for the small subunit of MADH (*mauA*) of *M. extorquens* AM1 (14) and amicyanin (14a). This fragment was cloned into the T7 expression vector pTZ19TT in the orientation allowing transcription of both genes (the MADH gene and the amicyanin gene) from T7 and *lac* promoters (plasmid pAYC147, Fig. 1). Expression of this plasmid in *E. coli* gave a number of polypeptide bands on SDS-PAGE. For precise mapping of the genes

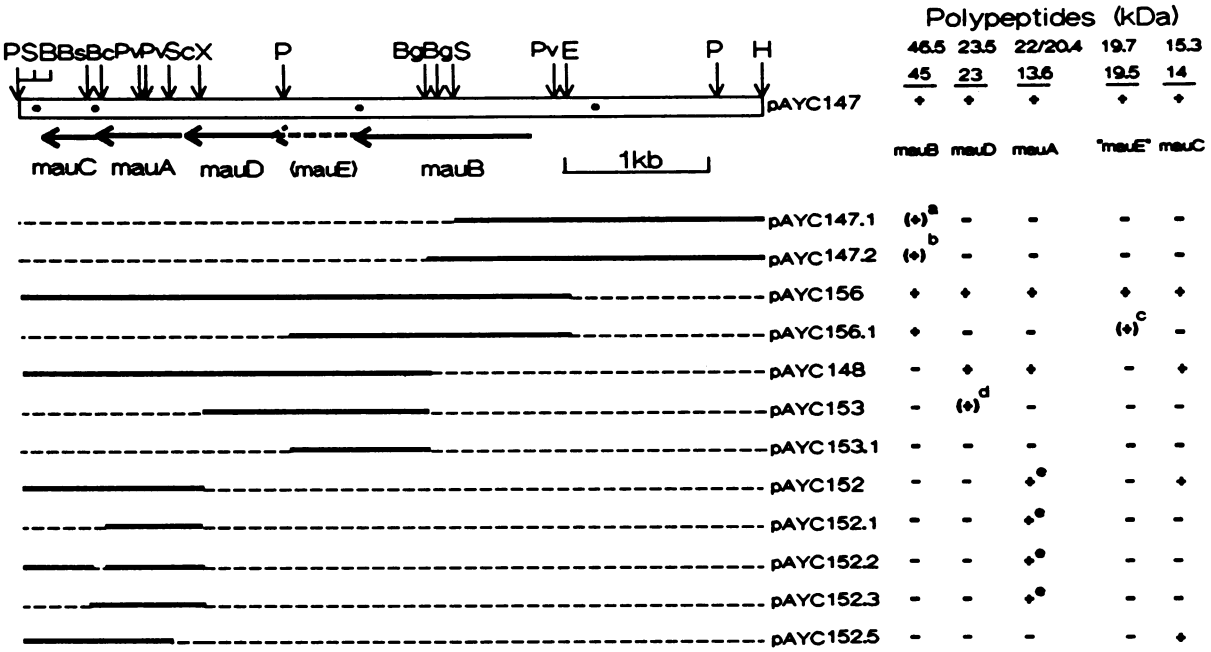

FIG. 1. The genetic map of the 5.2-kb insertion in plasmid pAYC147 and its deletion derivatives. *, the assumed position of the terminators; *mauA*, the gene of the small subunit of MADH; *mauB*, the gene of the large subunit of MADH; *mauC*, the amicyanin gene; *mauD*, the gene for the 23.5- and 23-kDa polypeptides (all solid arrows); *mauE*, proposed gene for the 19.7- and 19.5-kDa polypeptides (dashed arrow). Restriction sites: P, *Pst*I; S, *Sal*I; B, *Bam*HI; Bs, *Bss*HII; Bc, *Bcl*I; Pv, *Pvu*II; Sc, *Sac*II; X, *Xho*I; Bg, *Bgl*II; E, *Eco*RV; H, *Hin*dIII. Not all *Bss*HII, *Bcl*I, and *Sac*II sites are shown. Dashed lines denote deleted regions of the 5.2-kb fragment; solid lines denote retained regions of the 5.2-kb fragment. + or − in chart on the right designates the presence or lack of expression of the polypeptides shown above each column by the plasmid shown in the same row. Parentheses designate the presence of smaller polypeptides, which may be due to truncated forms of the gene noted. Footnotes: a, *mauB* (20.8- and 20.0-kDa polypeptides observed); b, *mauB* (29.2- and 28.5-kDa polypeptides observed); c, *mauE* (18.2- and 17.6-kDa polypeptides observed); d, *mauD* (21.8- and 21.2-kDa polypeptides observed); e, the 13.6-kDa band is very weak. P_{T7}, P_{lac} and the corresponding arrows designate T7 and *lac* promoters. Arrows indicate the direction of transcription.

encoding these polypeptides, plasmid pAYC147 was subcloned further, as shown in Fig. 2. The maps of the resulting plasmids are shown in Fig. 1. All listed plasmids were expressed in the T7 system, and the results for each gene are described separately in the next sections.

Identification and mapping of the first gene, encoding the MADH large subunit. Polypeptides with apparent molecular masses of 46.5 and 45 kDa were encoded by three plasmids: the original plasmid pAYC147 and two subclones, pAYC156 and pAYC156.1 (Fig. 1 and 3). The 2-kb insert in pAYC156.1

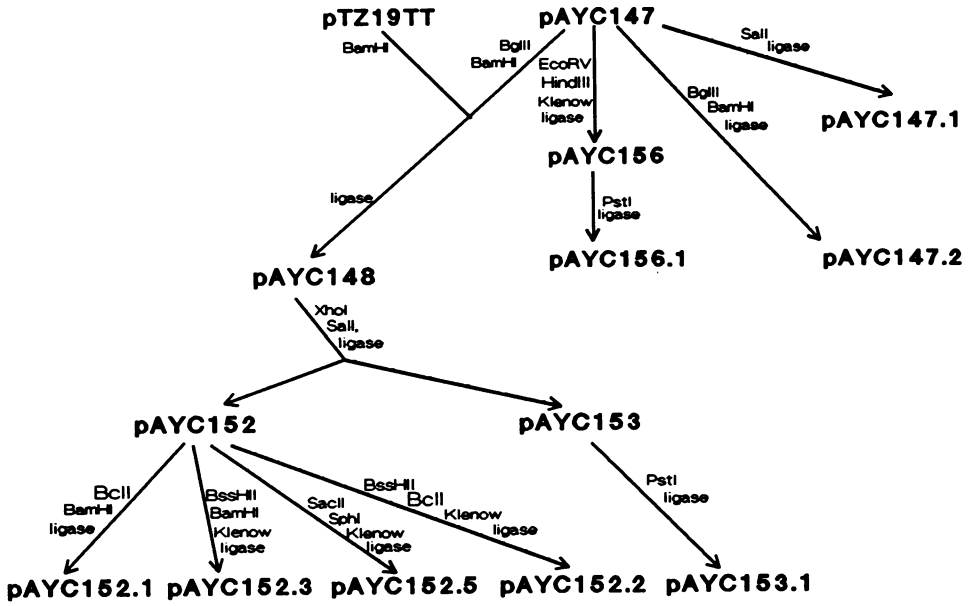

FIG. 2. Scheme of subcloning of plasmid pAYC147.

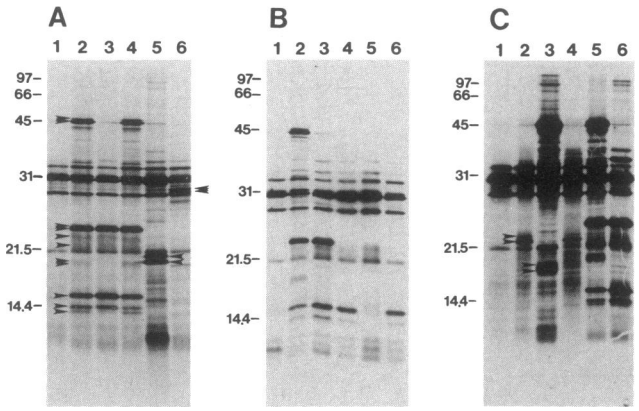

FIG. 3. Autoradiogram of polypeptides expressed by the T7 expression system in *E. coli* strains containing (A) plasmids pTZ19TT (lane 1), pAYC147 (lane 2), pAYC148 (lane 3), pAYC156 (lane 4), pAYC147.1 (lane 5), pAYC147.2 (lane 6); (B) plasmids pTZ19TT (lane 1), pAYC147 (lane 2), pAYC148 (lane 3), pAYC152 (lane 4), pAYC152.2 (lane 5), pAYC152.5 (lane 6); (C) plasmids pTZ19TT (lane 1), pAYC153 (lanes 2 and 4), pAYC156.1 (lane 3), pAYC156 (lane 5), pAYC148 (lane 6). Arrows denote polypeptides listed in Fig. 1, and molecular weight standards (in thousands) are noted on the left.

is not large enough to encode both polypeptides, suggesting that these represented two forms of the same gene product. The MADH large subunit is known to have a molecular mass of about 40 kDa (51), and so an available antibody against MADH from *M. flagellatum* KT was used to determine whether these polypeptides were forms of the MADH large subunit. As shown in Fig. 4, lane 3, both the 46.5- and 45-kDa polypeptides bound this antibody. As positive controls, a preparation of purified MADH from *M. extorquens* AM1 (lane 5) and crude extracts from *M. extorquens* AM1 grown on two different substrates known to induce MADH (lanes 6 and 7) and *M. flagellatum* KT grown on methylamine (lane 1) are shown. This antibody binds the MADH large subunits from both strains but does not detect the MADH small subunit from *M. extorquens* AM1. Antibodies also bind a smaller polypeptide (42 kDa) in the purified preparation from *M. extorquens* AM1 (Fig. 4, lane 5). This may be a specific breakdown product of the MADH large subunit. A polypeptide of similar size which faintly binds antibody (Fig. 4, lane 3) is synthesized in the T7 expression system (Fig. 3). A number of other smaller polypeptides which could be other breakdown products that do not bind antibody are also present in expression extracts containing the MADH large subunit. A negative control with a plasmid lacking the large-subunit gene, the pAYC148 expression extract, showed no antibody binding to polypeptides in the size class of 40 to 45 kDa. The gene for the large subunit was therefore localized to the 2-kb *Pst*I-*Eco*RV insertion fragment of plasmid pAYC156.1. Two constructs, pAYC147.1 and pAYC147.2, contained inserts truncated before the 3′ end of the pAYC156.1 insert, and expression of these two plasmids generated pairs of polypeptides of 20.8 and 20 kDa (Fig. 3, lane 5A) and 29.2 and 28.5 kDa (Fig. 3, lane 6A), respectively, that were not observed in other expression extracts. These polypeptides may represent truncated large-subunit polypeptide(s), but if so, they did not bind antibody (data not shown). The difference between the molecular masses of the 29.2- and 20.8-kDa polypeptides (8.4 kDa) corresponds well to the difference between the sizes of the DNA insertions in plasmid pAYC147.1 and pAYC147.2 (0.25 kb).

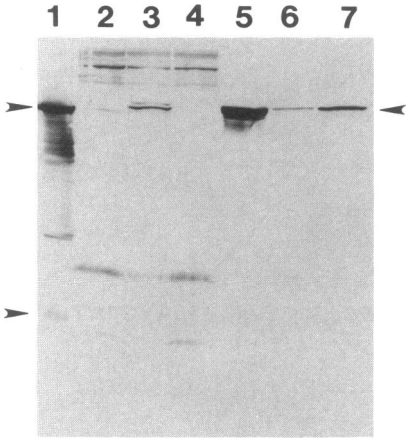

FIG. 4. Immunoblot of authentic MADH and polypeptides synthesized in the T7 expression system after SDS-PAGE, by using antisera prepared against MADH from *M. flagellatum* KT. Lanes: 1, crude extract of *M. flagellatum* KT; 2, polypeptides expressed in strain with pTZ19TT; 3, polypeptides expressed in strain with pAYC147; 4, polypeptides expressed in strain with pAYC148; 5, preparation of purified MADH from *M. extorquens* AM1; 6, crude extract of propylamine-grown cells of *M. extorquens* AM1; 7, crude extract of methylamine-grown cells of *M. extorquens* AM1. Arrowheads denote full-size MADH large and small subunits.

Mapping of the second gene. Plasmids pAYC147 and pAYC156 direct the synthesis of another polypeptide pair with apparent molecular masses of 19.7 and 19.5 kDa (Fig. 1 and 3). Although these polypeptides are similar in size, they are resolved in gels exposed for short time periods (Fig. 3A, lane 4 and Fig. 3C, lane 5). In most cases, expression was low but reproducible. These polypeptides are not synthesized by the other plasmids tested, suggesting that they are the products of a gene located immediately downstream of the large-subunit gene. This suggestion is supported by the observation that a smaller polypeptide pair (of 18.2 and 17.6 kDa) is synthesized from pAYC156.1, which carries an insert truncated by the 3′ end with respect to pAYC156. These polypeptides could represent truncated products of the second gene, since the full-size large-subunit polypeptides are also present in extracts containing this construct. In this case, the two polypeptides are clearly resolved (Fig. 3C, lane 3), providing further evidence that the full-sized polypeptides are expressed as a pair. The second gene should also be present in plasmids pAYC148 and pAYC153, and the truncated gene should be present in pAYC153.1. However, the 19.7- and 19.5-kDa polypeptides are not present in extracts containing pAYC148 and pAYC153, and the 18.2- and 17.6-kDa polypeptides are not expressed from pAYC153.1. The reason for this is not known, but it is clear that the products of the second gene are observed only with constructions that include the upstream large-subunit gene.

Mapping of the third gene. The synthesis of another pair of polypeptides with apparent molecular masses of 23.5 and 23 kDa is directed by plasmids pAYC147, pAYC148, and pAYC156 (Fig. 1 and 3), suggesting the presence of a third gene within the smaller of the *Pst*I-*Pst*I fragments shown in Fig. 1. *Xho*I-*Sal*I subcloning of the insertion from plasmid pAYC148 resulted in the creation of two plasmids, pAYC152 and pAYC153. Plasmid pAYC152 did not direct synthesis of

these polypeptides (Fig. 3B, lane 4), while plasmid pAYC153 encoded only one polypeptide pair of 21.8 and 21.2 kDa (Fig. 3C, lanes 2 and 4). These polypeptides may be truncated products of the third gene. Although the 23.5- and 23-kDa polypeptides are difficult to resolve, the apparent truncated pair is clearly resolved (Fig. 3C, lanes 2 and 4). A portion of an open reading frame corresponding to a peptide of approximately 2 kDa has been found upstream of the MADH small-subunit gene (13). Assuming this open reading frame encodes a portion of *mauD*, the *Xho*I site would truncate the polypeptide by about 2 kDa, consistent with the sizes of the observed polypeptides.

Identification of the fourth gene, encoding the MADH small subunit. As reported previously (14), the structural part of the gene for the small subunit of MADH (*mauA*) has been localized by sequencing within a 0.57-kb *Sac*II-*Bcl*I fragment in the orientation shown in Fig. 1. Synthesis of a pair of bands with apparent molecular masses of 22 and 20.4 kDa is directed by plasmids pAYC147, pAYC148, pAYC152, and pAYC156, with the larger polypeptide predominating (Fig. 3C, lane 5). Synthesis of the same polypeptides was also directed by plasmids pAYC152.1, pAYC152.3 (data not shown), and pAYC152.2 (Fig. 3B, lane 5), which have the *Sac*II-*Bcl*II region and the upstream region (coding for the putative leader of the small subunit of MADH) intact. Deletion of the *Pst*I-*Sac*II fragment from plasmid pAYC152, resulting in plasmid pAYC152.5, led to loss of the ability to synthesize these bands (Fig. 3B, lane 6). Since the gene for the small subunit of MADH can encode polypeptides of 20 or 18.5 kDa, two polypeptides of about this size were expected, depending on which of the two possible start codons is used (13). The deletion in pAYC152.5 deprives the small-subunit gene of both possible start sites and leads as a result to loss of expression of these polypeptides. Both polypeptides are premature forms of the small subunit, which has a molecular mass of 13 kDa, according to the sequencing data (14). However, the mature polypeptide of the MADH small subunit is apparently not expressed well in *E. coli*. A polypeptide with an apparent molecular mass of 13.6 kDa was observed in extracts from strains containing plasmids with the entire cluster of methylamine metabolism genes (pAYC147 and pAYC156), but it was seen only faintly in strains containing pAYC148, pAYC152, pAYC152.1, pAYC152.2, and pAYC152.3 (Fig. 3). As expected, this polypeptide was not synthesized in strains with plasmid pAYC152.5, which carries an insert that starts within *mauA*. It is not possible to identify the 13.6-kDa polypeptide conclusively, since the available MADH antibody does not bind this polypeptide (Fig. 4).

Identification of the fifth gene, encoding amicyanin. In the T7 expression system, polypeptides with apparent molecular masses of 15.3 and 14 kDa were expressed from all plasmids containing the extreme 3′ end of the insert in pAYC147, including pAYC156, pAYC148, pAYC152, and pAYC152.5 (Fig. 3). The sequence of this region (the *Bcl*I-*Bam*HI fragment from plasmid pAYC152) has shown that it contains the amicyanin gene (14a). This gene has only one possible start site and encodes 12.7-kDa premature and 10.7-kDa mature amicyanin proteins, suggesting that the polypeptides synthesized in the T7 expression system correspond to the premature and mature forms of amicyanin. This is confirmed by the sequence, which shows that in pAYC152.5, only the amicyanin reading frame exists intact. Any deletion disturbing the amicyanin reading frame (such as seen with plasmids pAYC152.1, pAYC152.2, and pAYC152.3) prevents the synthesis of these two polypeptides (see Fig. 3B, lane 5, for results with plasmid pAYC152.2).

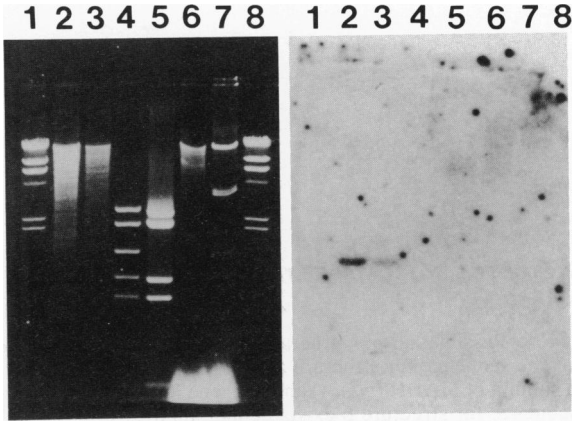

FIG. 5. Agarose electrophoresis (left) and autoradiogram (right) after hybridization with the probe for azurin. Lanes 2 and 3, 1 and 0.3 μg, respectively, of *M. extorquens* AM1 chromosomal DNA digested with *Bam*HI; lane 4, pAYC147 digested with *Pvu*II; lane 5, pAYC156 digested with *Pvu*II; lane 6, pAYC139 digested with *Bam*HI; lane 7, pAYC141 digested with *Bam*HI; lanes 1 and 8, lambda DNA digested with *Hin*dIII (sizes [in kilobases], 23, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56).

Probing for the azurin gene. Azurin, a blue copper protein of 13 kDa (1), has been proposed as a possible electron acceptor in methylamine oxidation in *M. extorquens* AM1 (54). To determine whether the gene for azurin is linked with the other genes of methylamine metabolism, an oligonucleotide was synthesized on the basis of the known amino acid sequence of azurin (see Materials and Methods) and used as a probe. *Bam*HI digests of *M. extorquens* AM1 chromosomal DNA as well as the plasmids pAYC147, pAYC156, pAYC139, and pAYC141 were probed with the oligonucleotide. The oligonucleotide did not hybridize with any of the plasmids, but did hybridize with a 1.3-kb *Bam*HI fragment of the *M. extorquens* AM1 chromosome (Fig. 5).

Immunoblotting with anti-cytochrome *c*-553. Data from the literature suggest that a cytochrome *c* may be involved in accepting electrons from amicyanin (20, 26), but in *M. extorquens* AM1, no amicyanin-specific cytochrome has been identified. However, a cytochrome *c* of unknown function, cytochrome *c*-553, has been identified in *M. extorquens* AM1 (16) and is therefore a possible candidate for this role. We were interested in determining whether the genes of unknown function in the methylamine utilization cluster described here encode cytochrome *c*-553, which is approximately 23 kDa in molecular mass. However, antibody to cytochrome *c*-553 did not show detectable binding to any of the expressed polypeptides, although it did bind to a polypeptide of approximately 23 kDa in extracts of both methanol- and methylamine-grown *M. extorquens* AM1 cells (data not shown).

Terminators for *E. coli* in the 5.4-kb *M. extorquens* AM1 chromosomal fragment. Sequence data for the MADH small-subunit and amicyanin genes revealed the presence of two putative hairpin structures, one located behind the MADH small-subunit gene and the other located behind the amicyanin gene (Fig. 6). The calculated free energy values for these structures (ΔG [25.5°C]) are −15.2 and −32.2 kcal, respectively. In order to assess these and other possible structures in this gene cluster, several constructs were tested

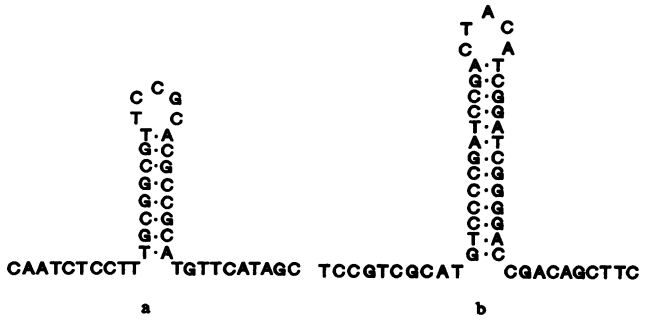

FIG. 6. Possible hairpin structures from DNA of *M. extorquens* AM1. The sequence for structure a exists between the gene for the small subunit of MADH (*mauA*) and the gene for amicyanin (*mauC*); the sequence for structure b exists behind *mauC*.

for terminator activity in *E. coli*. Plasmid pTZ19TT can be used for the estimation of terminator activity within a fragment of DNA cloned in the multiple-cloning site, since it has translation terminators in all three reading frames between the multiple-cloning site and *lacZ*. β-Galactosidase activity in strains containing pTZ19TT derivatives is expressed from the *lac* promoter and is absent only in the cases in which transcriptional termination occurs within the cloned fragment. Table 2 shows the β-galactosidase activities in strains containing pTZ19TT or different pAYC147 derivatives. Termination occurs in plasmid pAYC152, which contains both putative hairpin structures. Deletion of both hairpin structures (plasmid pAYC152.1) relieves the termination effect, whereas deletion of either one of them results in intermediate β-galactosidase levels (as seen with plasmids pAYC152.2 and pAYC152.3). At least two other transcriptional terminators exist in the 5.2-kb *Bam*HI-*Hin*dIII fragment. One is located upstream of the large subunit (plasmid pAYC147.1), and another is in the 0.95-kb *Bgl*I-*Pst*I fragment of plasmid pAYC153.1. The approximate location of terminatorlike structures is shown on the genetic map (Fig. 1).

DISCUSSION

The results presented here show that three genes encoding known or proposed methylamine utilization functions are closely clustered with two genes of unknown function in *M. extorquens* AM1. All of these genes are transcribed in the same direction. Recently, we proposed to designate genes of methylamine metabolism *mau*, for methylamine utilization, and named the gene for the small subunit of MADH *mauA*. Continuing this policy, we propose also to designate the genes described here as *mau*.

TABLE 2. Activities of β-galactosidase expressed by different plasmids in *E. coli* DH5α in the presence of IPTG

Plasmid	Activity (nmol min^{-1} mg of $protein^{-1}$)	% of activity[a]
None	0.016	<0.2
pTZ19TT	10.6	100
pAYC147	0.028	0.26
pAYC148	0.053	0.5
pAYC147.1	0.25	2.3
pAYC153	0.42	4.0
pAYC153.1	0.39	3.7
pAYC152	0.23	2.0
pAYC152.1	6.33	59.7
pAYC152.2	1.39	13.0
pAYC152.3	1.04	9.8

[a] Activity in pTZ19TT is used for the 100% level.

The first gene in the cluster encodes the MADH large subunit, and we propose to designate it *mauB*. Two major polypeptides were expressed from *mauB* in *E. coli*, both of which bound antibody for the MADH large subunit and corresponded in size to the premature and mature forms of this secreted polypeptide. Several smaller polypeptides were observed that were always associated with the presence of intact *mauB* and were presumed to be breakdown products of the large subunit. The T7 expression data concerning full-size and truncated polypeptides suggest that *mauB* begins approximately 550 bp upstream of the right *Sal*I site shown in Fig. 1 and continues 450 bp downstream of the left *Bgl*II site. Immediately downstream of *mauB* is a second gene, which we tentatively propose to designate "*mauE*". This gene directs the synthesis of a pair of polypeptides in *E. coli* but only in constructions in which *mauB* is intact. These polypeptides are not breakdown products of MauB, since one construction (pAYC156.1) that contained the complete *mauB* sequence and expressed MauB well did not produce them. It is possible that expression of *mauE* is coupled to expression of *mauB* or that the *mauE* gene product is unstable in the absence of MauB. It is unlikely that the polypeptides observed in the expression extracts are artifacts, since an apparently truncated pair of polypeptides was expressed from pAYC156.1, which contains an insert truncated at the 3′ end with respect to the inserts that expressed the full-length polypeptides. This is consistent with mapping data showing that a space that is about the right size (0.55 kb) to encode a polypeptide of 20 kDa exists between *mauB* and the third gene (both of which have been mapped precisely). The function of *mauE* is unknown. It does not encode azurin, and it does not appear to encode cytochrome *c*-553, both possible electron acceptors in methylamine utilization. We cannot be certain that this gene functions in methylamine utilization, but this seems likely, since it is situated within a group of four other *mau* genes. For convenience, we propose this tentative designation as a *mau* gene, until the function of this gene can be determined. The expression data suggest that two forms of the gene product were synthesized in *E. coli*, but the origin of these polypeptides is not known.

The third gene is located immediately downstream of *mauE*, and we propose to designate it *mauD*. The function of this gene, like that of *mauE*, is unknown. It does not encode azurin or cytochrome *c*-553. However, sequence data have shown that an open reading frame which must correspond to *mauD* exists immediately upstream of the small-subunit gene (13). We have also constructed a mutant that contains an insertion in this open reading frame that does not disrupt downstream functions and that exhibits a methylamine-negative phenotype (unpublished data). Therefore, this unknown gene does appear to be involved in methylamine utilization. As is true for the first and second genes, *mauD* also encodes a pair of polypeptides in *E. coli*, but the origin of these is unknown. The sequence data and T7 expression results suggest that *mauD* starts near the *Pst*I site shown in Fig. 1 and extends 80 bp downstream of the *Xho*I site.

The fourth gene is *mauA*, encoding the MADH small subunit. Three forms of this polypeptide were observed in *E. coli*. Two of these corresponded in size to polypeptides that would result from use of the two different start sites that have been identified from the DNA sequence (13). The presence of both polypeptides suggests that *E. coli* translates

both, although the larger polypeptide is the most abundant. The first start site has a better ribosome-binding sequence, and the second start site is covered by an inverted repeat sequence (13), so it seems likely that *E. coli* would produce the large form preferentially. However, it is also possible that the different amounts are observed because of different stabilities of the two forms. The third polypeptide expressed from *mauA* is the correct size (13.6 kDa) to be the mature form. However, this was observed only in constructions containing the entire gene cluster. In other constructions, the two larger polypeptides were observed but the smaller polypeptide was present only at trace levels. The reason for this difference is not known. The 13.6-kDa polypeptide is not a breakdown product of MauB because it was not present in extracts expressing pAYC156.1, which contains the intact *mauB* but no *mauA*. Therefore, it seems likely that it is the mature form of the MADH small subunit. It is not surprising that this polypeptide might be poorly processed in *E. coli*, since it contains an unusual leader sequence (13).

The fifth gene identified encodes amicyanin, and we propose to designate it *mauC*. It is located 7 bp downstream of *mauA* (14a). Two polypeptides which correspond in size to the premature and mature forms of amicyanin were expressed in *E. coli* from this gene. Sequence data predict an amicyanin signal sequence that has features typical of *E. coli* signal sequences (14a), suggesting that amicyanin should be secreted and processed by *E. coli*. Sequence data have been recently reported showing that the amicyanin gene in *P. denitrificans* is also located immediately downstream of the MADH small-subunit gene of this organism (58). An amicyanin mutant that was shown to be incapable of growth on methylamine was constructed, demonstrating that amicyanin is required for growth of this organism on methylamine. It is not yet known whether *P. denitrificans* contains other *mau* genes clustered with the amicyanin and MADH small-subunit gene. However, a third open reading frame of unknown function was identified downstream of the amicyanin gene. Sequence data are not available for this downstream region in *M. extorquens* AM1, but it is possible that more methylamine utilization genes are present in this cluster.

The existence of four terminators functioning in *E. coli* raises the question of whether this gene cluster is organized as an operon or is expressed as separate transcripts. Although it is not known whether the terminators that function in *E. coli* are functional in *M. extorquens* AM1, the position of the structures that bracket *mauC* and the suggested position of the terminator downstream of *mauB* argue that these terminators may have some function in gene expression in *M. extorquens* AM1. Future work will address transcription and transcriptional regulation of this gene cluster.

ACKNOWLEDGMENTS

This work was supported by a grant from the NIH (GM 36296).

We thank Chris Anthony for supplying antibody to cytochrome *c*-553.

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