Genetic Analysis of the Methanol- and Methylamine-Specific Methyltransferase 2 Genes of *Methanosarcina acetivorans* C2A[∇]

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The entry of methanol into the methylotrophic pathway of methanogenesis is mediated by the concerted effort of two methyltransferases, namely, methyltransferase 1 (MT1) and methyltransferase 2 (MT2). The *mtaA1*, *mtaA2*, and *mtbA* genes of *Methanosarcina acetivorans* C2A encode putative methanol- or methylamine-specific MT2 enzymes. To address the *in vivo* roles of these genes in growth and methanogenesis from known substrates, we constructed and characterized mutants with deletions of each of these genes. The *mtaA1* gene is required for growth on methanol, whereas *mtaA2* was dispensable. However, the *mtaA2* mutant had a reduced rate of methane production from methanol. Surprisingly, deletion of *mtaA1* in combination with deletions of the genes encoding three methanol-specific MT1 isozymes led to lack of growth on acetate, suggesting that MT1 and MT2 enzymes might play an important role during growth on this substrate. The *mtbA* gene was required for dimethylamine and monomethylamine (MMA) utilization and was important, but not required, for trimethylamine utilization. Analysis of reporter gene fusions revealed that both *mtaA1* and *mtbA* were expressed on all methanogenic substrates tested. However, *mtaA1* expression was induced on methanol, while *mtbA* expression was down-regulated on MMA and acetate. *mtaA2* was expressed at very low levels on all substrates. The *mtaA1* transcript had a large 5' untranslated region (UTR) (275 bp), while the 5' UTR of the *mtbA* transcript was only 28 bp long.

Methanogenesis, the biological formation of methane (CH₄), is carried out by a unique group of microorganisms from the domain *Archaea* known as methanogens. These organisms convert a limited number of small carbon-containing compounds to CH₄, conserving energy for growth in the process. The substrates used by methanogens include H₂-CO₂, acetate, and a variety of one-carbon compounds (C₁ compounds) that are disproportionated into CO₂ and CH₄ via the methylotrophic methanogenic pathways (11, 41). Methylotrophic methanogens are found exclusively among members of the *Methanosarcinales*, including the three sequenced species *Methanosarcina barkeri*, *Methanosarcina mazei*, and *Methanosarcina acetivorans*. Although *Methanospira* species (members of the *Methanobacteriales*) are also able to utilize methanol, they do so via a distinct methanogenic pathway that requires hydrogen as a cosubstrate (47).

Detailed biochemical characterization of methylotrophic methanogenesis has demonstrated that the methyl group from C₁ compounds enters the methanogenic pathway at the level of methyl-coenzyme M (CH₃-CoM) (reviewed in reference 23). This is mediated by the concerted action of two methyltransferases called methyltransferase 1 (MT1) and MT2. MT1 consists of two protein components; the first component is a methyltransferase [encoded by the genes *mtaB* for methanol, *mttB* for trimethylamine [TMA], *mtbB* for dimethylamine [DMA], and *mtmB* for monomethylamine [MMA]] that catalyzes the

transfer of the methyl group from the methylated substrate to a second protein component, a cognate corrinoid protein (encoded by the genes *mtaC* for methanol, *mttC* for TMA, *mtbC* for DMA, and *mtmC* for MMA). The methylated corrinoid protein then becomes the substrate for the MT2 methyltransferase, which transfers the methyl group to CoM.

A variety of *in vitro* biochemical studies in *M. barkeri* have shown that the MT1 enzyme systems are exquisitely specific with respect to their substrates. Thus, discrete MT1 enzymes for the activation of methanol, MMA, DMA, and TMA have been purified and biochemically characterized (7, 14, 15, 43). This substrate specificity is reflected in the amino acid sequences of the MT1 proteins. Although the corrinoid proteins are similar, there is no significant homology between the methyltransferase proteins for any of the MT1 enzymes. Interestingly, however, there are multiple, highly homologous MT1 enzymes for each of the known C₁ substrates in *Methanosarcina* spp. Thus, there are three methanol-specific (MtaCB1, -2, and -3), two TMA-specific (MttCB1 and -2), three DMA-specific (MtbCB1, -2, and -3), and two MMA-specific (MtmCB1 and -2) MT1 isozymes (10, 17, 26).

In *M. barkeri* Fusaro two different MT2 isozymes have been described, one that predominates in methanol-grown cells (MT2-M) and another that predominates in acetate-grown cells (MT2-A); however, both proteins are present in methanol- and acetate-grown cells (19). Later, MT2-M was renamed MtaA while MT2-A was renamed MtbA in this organism (21). These MT2 isozymes are also substrate specific but not to the same degree as the MT1 components. Accordingly, MtaA is capable of transferring the methyl group from the methanol-specific corrinoid protein (MtaC) to CoM *in vitro*, whereas MtbA catalyzes the analogous transfer from the MMA-, DMA-, and TMA-specific corrinoid proteins *in vitro*. Interest-

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TABLE 1. Plasmids used in the study

Plasmid	Description and/or construction	Reference or source
pSL1180	Ap ^r cloning vector; pUC replicon	5
pJK3	<i>pac</i> cassette source	28
pJK41	Ap ^r Pm ^r cloning vector; R6K replicon	27
pMP44	Vector used to construct up- and down-region cassettes to delete genes from the <i>M. acetivorans</i> C2A chromosome using the markerless exchange method	33
pMP47	HindIII/KpnI-digested up-mtaA1 PCR product (with primers 5-upmtaA and 3-upmtaA) cloned into HindIII/KpnI-digested pSL1180	This study
pMP48	KpnI/BstBI-digested dn-mtaA1 PCR product (with primers 5-dnmtaA and 3-dnmtaA) cloned into KpnI/BstBI-digested pSL1180	This study
pMP54	Replacement of 166-bp HindIII/KpnI fragment of pMP48 with 1,027-bp HindIII/KpnI fragment of pMP47	This study
pMP64	AflII/NotI-digested up-mtbA PCR product (with primers AflII-upmtbA and NotI-upmtbA) cloned into AflII/NotI-digested pMP44	This study
pMP65	NotI/KpnI-digested dn-mtbA PCR product (with primers NotI-dnmtbA and KpnI-dnmtbA) cloned into NotI/KpnI-digested pMP64	This study
pMP68	Replacement of 52-bp NruI/AvrII fragment of pMP44 with 1,874-bp SmaI/NruI Δ <i>mtaA1</i> fragment of pMP54	This study
pMP70	SpeI/SstI-digested up-mtaA2 PCR product (with primers SpeI-upA2 and SacI-upA2) and SstI/NotI-digested dn-mtaA2 PCR product (with primers SacI-dnA2 and NotI-dnA2) cloned into SstI/NotI-digested pMP44	This study
pAMG82	Vector for construction of promoter fusions to the <i>uidA</i> gene of <i>E. coli</i>	20
pAB46	AscI-digested 1,000-bp upstream region of <i>mtaA1</i> PCR product (using primers mtaA1rev and mtaA1for) was treated with T4 kinase and cloned into Ecl136II/AscI-digested pAMG82	This study
pAB47	AscI-digested 1,000-bp upstream region of <i>mtaA1</i> PCR product (using primers mtaA1rev2 and mtaA1for) was treated with T4 kinase and cloned into Ecl136II/AscI-digested pAMG82	This study
pAB48	AscI-digested 1,000-bp upstream region of <i>mtaA2</i> PCR product (using primers mtaA2rev and mtaA2for) was treated with T4 kinase and cloned into Ecl136II/AscI-digested pAMG82	This study
pAB49	AscI-digested 1,000-bp upstream region of <i>mtbA</i> PCR product (using primers mtbArev and mtbAfor) was treated with T4 kinase and cloned into Ecl136II/AscI-digested pAMG82	This study

ingly, biochemical studies demonstrate that MtaA can also act as the MT2 enzyme for TMA, but not for DMA and MMA, in *M. barkeri* (6, 14–16, 45).

Regulation of the *mtaA* and *mtbA* genes in *M. barkeri* is consistent with their biochemical function, i.e., that MtaA is the methanol-specific MT2 while MtbA is the methylamine-specific MT2. Qualitative expression levels determined using Northern blot analysis revealed that the *mtaA* transcript predominates in methanol-grown cells, whereas transcription of *mtbA* is most abundant during growth on TMA and H₂-CO₂. Nevertheless, these mRNA studies and the biochemical studies described above indicate that both genes are expressed on multiple substrates (19, 21). Thus, it seems quite possible that these proteins might play as-yet-unknown metabolic roles during growth on these substrates. Interestingly, two *mtaA* genes, designated *mtaA1* and *mtaA2*, are present in each of the sequenced *Methanosarcina* genomes. Akin to the methanol-specific *mtaCB1*, *mtaCB2*, and *mtaCB3* operons, these two genes might be differentially regulated and/or encode isozymes with different functions (4). Importantly, it should be noted that there are numerous other MT2 proteins encoded in the *Methanosarcina* genomes. For example, *M. acetivorans* has 10 MT2 homologs in addition to the *mtaA1*, *mtaA2*, and *mtbA* genes (17). Whether these play a role in methanol or methylamine metabolism or in the metabolism of other substrates has yet to be experimentally addressed. Thus, numerous questions regarding the in vivo function of MT2 enzymes remain to be answered.

Here, we report the first use of genetic methods to understand the in vivo role of the *mtaA* and *mtbA* genes of *M.*

acetivorans C2A. Our data reveal a novel and broader role for these enzymes than previously suspected.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Standard conditions were used for growth of *Escherichia coli* strains (44). DH5 α λ *pir* (30) was used as the host for all *pir*-dependent replicons. DH10B (Stratagene, La Jolla, CA) was used for all other plasmid replicons. *M. acetivorans* C2A (DSM 2834) (37) was from laboratory stocks. *Methanosarcina* was grown in single-cell morphology (38) at 37°C in high-salt (HS) broth medium containing either 125 mM methanol, 50 mM trimethylamine, 50 mM dimethylamine, 50 mM monomethylamine, or 120 mM acetate. Growth of *M. acetivorans* on medium solidified with 1.5% agar was as described previously (2). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic glove box. Solid-medium plates were incubated in an intrachamber anaerobic incubator as described previously (29). Puromycin (CalBiochem, San Diego, CA) was added from sterile, anaerobic stocks at a final concentration of 2 μ g/ml for selection of *Methanosarcina* strains carrying the puromycin transacetylase gene cassette (*pac*) (18, 28). The purine analog 8-aza-2,6-diaminopurine (Sigma, St. Louis, MO) was added from sterile, anaerobic stocks at a final concentration of 20 μ g/ml for selection against the *hpt* gene.

DNA methods. Standard methods were used throughout for isolation and manipulation of plasmid DNA from *E. coli* (1). Genomic DNA from *M. acetivorans* was isolated as described previously (32). DNA hybridizations were performed using the DIG System (Roche, Mannheim, Germany). MagnaGraph Nylon transfer membranes were from Micron Separations Inc. The DNA sequence was determined from double-stranded templates at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

Transformation. *E. coli* strains were transformed by electroporation using an *E. coli* Gene Pulser (Bio-Rad, Hercules, CA) as recommended. Liposome-mediated transformation was used for *Methanosarcina* species as described previously (28).

Plasmid constructions. Standard methods were used for construction of all plasmids. The plasmid constructions and primers used are described in Tables 1

TABLE 2. Primers used in the study

Primer	Sequence ^a	Added site(s)
5-upmtaA	GGGGGGAAGCTTCTCGAGAGCCAGGATGTCCTTCACC	HindIII, XhoI
3-upmtaA	GGGGGGGGTACCCATATGGGTCATACC	KpnI, NdeI
5-dnmtaA	GGGGGGGGTACCAGATCTTGAAAACGCATAAAAAACCC	KpnI, BglII
3-dnmtaA	GGGGGGTTTGAACCTAGGTAACAGCGCAGGTTCTCTCC	BstBI, AvrII
SpeI-upA2	GCCGCCACTAGTTTCGAGGATAGTTTACGTTACGA	SpeI
SacI-upA2	GGCGCCGAGCTCCATATGACTCATCTTCAATCTTCCCCAAAT	SstI, NdeI
SacI-dnA2	GCCGCCGAGCTCGGATCCTGAAGAACCTGAAAAATAGAAA	SstI, BamHI
NotI-dnA2	GCCGCCGCGGCCGCTCCGAGACTCTTGCATACGA	NotI
AflII-upmtbA	CCGCCGCTTAAGAAAAATCTACTCCCAAACTA	AflII
NotI-upmtbA	CCGCCGCGCGCCGCACATGTTATAATCCCTCTCTAAGTTCTA	NotI, BspLU11I
NotI-dnmtbA	CCGCCGCGCGCCGCAGATCTGTAAACAAGTACTGAGTAATGC	NotI, BglII
KpnI-dnmtbA	CCGCCGGGTACCATTTATCAAAAAATCTCTTGG	KpnI
A1L	GCACTATCTGCCCCCATTTA	None
A1R	AAGCTGCCCTTATGGGTTTT	None
A2L	TCGTTGCCCAAACAATGATA	None
A2R	GCAGTTCTTTCTCCCTGTGC	None
mtbA up	TAAATTGTGCACGGAACCTGC	None
mtbA down	CTTCCGGAATAATTTGAGAGC	None
mtaA1for	GGCGCGCCTCCGTCCCTGAAGACTTTTG	AscI
mtaA1rev	TTTTTTACTTTTATCTTAGTACATATATTTTTATTGC	None
mtaA1rev2	GGTCATACCTTTTTTACTTTTATCTTAGTACATATATTTTTATTG	None
mtaA2for	GGCGCGCCAATATCTGAAAGGACATTCTAAATCC	AscI
mtaA2rev	CAATCTTCCCCAAATATTCAATTAC	None
mtbAfor	GGCGCGCCACCATACCGCCCAAGTATTT	AscI
mtbArev	TAATCCCTCTCTAAGTTCTATAGACAGC	None

^a Added restriction sites are underlined.

and 2, respectively. pJK41 and all derivatives are nonreplicating in *Methanoscarchina*.

Construction of *mtaA* and *mtbA* deletion mutants. The markerless deletion method (33) was used to create deletion mutants of the *mtaA1*, *mtaA2*, and *mtbA* genes in the Δhpt (WWM1) background of *M. acetivorans* (Table 3). The plasmids pMP68, pMP70, and pMP65 were used to delete *mtaA1*, *mtaA2*, and *mtbA*, respectively. The $\Delta mtaA1 \Delta mtaA2$ mutant was constructed from the $\Delta mtaA2$

(WWM27) mutant by deleting the *mtaA1* gene. The $\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta mtaA1$ (WWM346) and $\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta mtaA2$ (WWM28) deletion mutants were constructed using the previously constructed mutant $\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3$ (WWM15) that is incapable of growth on methanol (32). The $\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta mtaA1 \Delta mtaA2$ (WWM147) deletion mutant was constructed by using the $\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta mtaA2$ (WWM28) background and subsequently deleting *mtaA1*. The *mtaA1*, *mtaA2*, and *mtbA* deletions were isolated on TMA while the *mtbA* deletion was isolated on methanol. These substrates were selected as we expected the *mtaA* genes to be dispensable for growth on TMA and the *mtbA* gene to be dispensable for growth on methanol.

Determination of growth characteristics. To determine the growth parameters of *M. acetivorans mtaA* deletion mutants on TMA, DMA, acetate, and methanol, mid-exponential phase, TMA-grown cells (optical density at 600 nm [OD₆₀₀] of 0.4 to 0.5) were collected anaerobically by centrifugation (for 10 min at 5,000 × g), washed once with 5 ml of HS medium, and resuspended in 10 ml of HS medium. For MMA, cells were adapted once from TMA to MMA, and growth parameters were then determined on this substrate as described above. Ten-milliliter cultures of HS medium containing 50 mM trimethylamine, 125 mM methanol, 50 mM dimethylamine, 50 mM monomethylamine, or 120 mM acetate were inoculated with 0.3 ml of washed cells (in triplicate) and incubated at 37°C. Cell growth was monitored at 600 nm in a Bausch and Lomb Spectronic 21. The growth parameters of the *mtbA* deletion mutant were determined in the same way as above except that mid-exponential methanol-grown cells were used. Lag time was defined as the time required to achieve a half-maximal OD₆₀₀ value.

Rate of methane production. A 250-ml methanol- or TMA-grown culture (OD₆₀₀ of 0.4 to 0.5) was pelleted anaerobically by centrifugation (10 min at 5,000 × g), washed with an equal volume of HS medium, and resuspended in HS medium supplemented with puromycin at a concentration of 1 × 10⁹ cells/ml as determined by visible count using a Petroff-Hausser counting chamber. A total of 2 × 10⁹ cells (2 ml of resuspended cells per tube) were aliquoted into Balch tubes on ice, and the headspace was exchanged for 250 kPa N₂-CO₂ (80%/20%). The reaction was started by the addition of 500 μmol of methanol for methanol-grown cells or 500 μmol of TMA for TMA-grown cells. Samples (50 μl or 100 μl) of headspace gas were removed every 8 to 10 min and analyzed on a Hewlett Packard 5890 Series II gas chromatograph using an 80- to 120-mesh Carboxpack B column (Supelco, Bellefonte, PA). To determine protein concentration, 1 ml of the resuspended cells was centrifuged, and the resulting pellet was lysed by resuspending it in 100 μl of double-distilled H₂O with 1 μg/ml of RNase and DNase. Protein concentration was determined by the method of Bradford using

TABLE 3. *M. acetivorans* C2A strains used

Strain ^a	Genotype or description	Source or reference
C2A	Wild type (DSM 2834)	37
WWM1	Δhpt	33
WWM13	$\Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta hpt$	32
WWM21	$\Delta mtaA1 \Delta hpt$	This study
WWM27	$\Delta mtaA2 \Delta hpt$	This study
WWM28	$\Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta hpt$	This study
WWM33	$\Delta mtaA1 \Delta mtaA2 \Delta hpt$	This study
WWM40	$\Delta mtaA1 \Delta hpt$	This study
WWM147	$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta hpt$	This study
WWM346	$\Delta mtaA1 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3 \Delta hpt$	This study
WWM82	$\Delta hpt::\phi C31 \text{ int-attP}$	20
WWM347	$\Delta hpt::\phi C31 \text{ int-attR PmtaA1-uidA}$ (annotated start codon)-attL	This study
WWM348	$\Delta hpt::\phi C31 \text{ int-attR PmtaA1-uidA}$ (alternate start codon)-attL	This study
WWM349	$\Delta hpt::\phi C31 \text{ int-attR PmtaA2-uidA}$ (annotated start codon)-attL	This study
WWM350	$\Delta hpt::\phi C31 \text{ int-attR PmtbA-uidA-attL}$	This study

^a All strains are derivatives of *M. acetivorans* WWM1 and were constructed by markerless exchange as described in Materials and Methods. Plasmids used for introduction of the *mtaA1*, *mtaA2*, and *mtbA* alleles were pMP68, pMP70, and pMP65, respectively. The construction of WWM15 has been previously published (32) and was used to construct WWM28, WWM147, and WWM346.

TABLE 4. Primers used for 5' RLM-RACE

Primer	Purpose	Sequence
5' RACEouter	5' RLM- RACE, sequencing	GCTGATGGCGATGAATGAACACTG
5' RACEinner	Sequencing	CATCAAAGCCAGCAAACGCAGTGTTCGGATCCGCG
mtaA1raceinner	Sequencing	TCTGCAGGAGGTCTGCAGGCACGGCTGC
mtaA1raceouter	5' RLM- RACE, sequencing	TCTGTAGTCTGGGCTGGAGGTACTG
mtaA1rev2	Sequencing	GGTCATACCTTTTACTTTTATCTTACGTACATATTTTTATTG
mtaA2raceinner	Sequencing	TCCCCAGCAAATTATCGGGCATTTCAG
mtaA2raceouter	5' RLM- RACE, sequencing	TCTGCAGCCGCGGCTCAAGTTCGGT
mtaA2rev	Sequencing	CAATCTTCCCCAAATATTCAATTAC
mtbAinner	5' RLM- RACE, sequencing	ATCTTGATAGCTTCAAGGATTAAC
mtbAouter	Sequencing	GCCGGAACGATCTCCTCGCCTGTGAAAAG

the Pierce protein assay kit following the manufacturer's guidelines. Specific activity was calculated from a CH_4 standard curve and reported as milliunits (mU) is calculated as $\text{nmol of CH}_4 \text{ produced min}^{-1} \text{ mg}^{-1}$ of protein).

Integrating promoter fusions on the *M. acetivorans* chromosome. All plasmids constructed in either pAMG82 or pJK200 were integrated on the *M. acetivorans* chromosome using site-specific recombination between the $\phi\text{C31 attB}$ site on the plasmid with the $\phi\text{C31 attP}$ site on the chromosome as described previously (20).

Extract preparation and β -glucuronidase assay. The preparation of cell extracts and the β -glucuronidase assay method were as previously described (34).

Determination of transcription start site. Transcription start sites were determined using 5' RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) as previously described (3). The primers used for amplification are listed in Table 4. The products were then treated with ExoSAP-IT (USB, Cleveland, OH) to remove primers as per the manufacturer's guidelines. The PCR products were sequenced at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

RESULTS

In silico analysis of the *mtaA* and *mtbA* genes in *M. acetivorans*, *M. mazei*, and *M. barkeri*. The proximity of the *mtaA* and *mtbA* genes to the *mtaCB2* and *mtmCB1* operons, respectively, supports their proposed in vivo roles in methanol and methylamine metabolism. In all sequenced *Methanosarcina* genomes, the *mtaA1* gene is located about 16 kbp upstream of *mtaCB2* whereas *mtaA2* is directly downstream of *mtaCB3*. The *mtbA* gene is located upstream of the genes encoding an MMA MT1 (*mtmB* and *mtmC*) (Fig. 1 shows these loci in *M. acetivorans*).

The annotated *mtaA1*, *mtaA2*, and *mtbA* genes of *M. acetivorans* are 1,029 bp, 1,020 bp, and 1,020 bp in length, respec-

tively, and are predicted to encode MT2 methyltransferase proteins of 342 amino acids (aa), 339 aa, and 339 aa, respectively. All three annotated genes have ATG start codons; however, alignment of these genes with those from the other sequenced *Methanosarcina* genomes shows that the start codons of the *mtaA1* genes from *M. acetivorans* and *M. mazei* are not equivalent to the experimentally determined start codon for the *M. barkeri mtaA1* gene. Furthermore, the annotated *M. acetivorans* and *M. mazei* genes lack potential ribosome-binding sites (RBS), which can be found upstream of the annotated *M. barkeri* translation start (Fig. 2). Because a homologous ATG codon and RBS are present within the coding sequences of the *M. acetivorans* and *M. mazei mtaA1* genes, we favor this internal start codon and have used the shorter coding sequence for all of the following analyses. This assignment is supported by the gene fusion data reported below.

All *Methanosarcina* MtaA1 and MtaA2 proteins share 88% to 76% identity (Table 5), with most differences being conservative amino acid changes. For comparison, the MtbA and MtaA share only 35 to 37% identity. Biochemical analyses of the MtaA and MtbA proteins have shown that both coordinate zinc, potentially by virtue of a HXCX_nC motif (where n is 74 or 76) (24, 36) that is conserved in the MtaA1, MtaA2, and MtbA proteins from all three *Methanosarcina* spp. (data not shown).

Phylogenetic analyses of the MtaA1, MtaA2, and MtbA proteins from *M. acetivorans*, *M. barkeri*, and *M. mazei* (Fig. 3) show that, with one exception, each of the isozymes forms a monophyletic group, suggesting that they evolved prior to separation of the three *Methanosarcina* spp. However, the MtaA1 protein from *M. barkeri* clusters with the MtaA2 proteins, consistent with a gene conversion event in which the *M. barkeri* Fusaro *mtaA1* gene was replaced by a copy of the *mtaA2* gene. If true, this suggests that MtaA1 and MtaA2 subfamilies are functionally equivalent.

Construction and phenotypic characterization of *mtaA* and *mtbA* deletion mutants. Mutants carrying deletions of the *mtaA1*, *mtaA2*, and *mtbA* genes were constructed as previously described using the markerless method of gene deletion (33). Various deletion combinations were also constructed, including ones in which the *mtaA* mutations were combined with previously constructed strains lacking the three methanol specific MT1 operons: *mtaCB1*, *mtaCB2*, and *mtaCB3* (32) (Table 3).

The resulting deletion mutants were tested for their ability to grow on five methanogenic substrates: methanol, TMA, DMA, MMA, and acetate (Table 6). The *mtaA1* single dele-

FIG. 1. Physical maps of the *mtaA* genes in *M. acetivorans*. A 20-kbp DNA region surrounding *mtaA1* (A), *mtaA2* (B), and the *mtbA* gene *mtaA2* (C) is shown. *mtaA1*, *mtaA2*, and *mtbA* are shown as aquamarine arrows; *mtaCB2* and *mtaCB3* are shown as red arrows; and *mtaCB2* and *mtaCB3* are shown as blue arrows. Other open reading frames are shown as gray arrows.

FIG. 2. Sequence alignment of the upstream regions of *Methanosarcina mtaA1*, *mtaA1*, and *mtbA* genes. A sequence of approximately 60 bp of DNA upstream of the predicted start site of the *M. acetivorans* (Ma), *M. barkeri* (Mb), and *M. mazei* (Mm) *mtaA1* gene (A), *mtaA2* gene (B), and *mtbA* gene (C) was compared using CLUSTALW (42). The predicted start codon for each gene is shown in cyan. The putative RBS is shown in yellow. (A) Conserved bases are shown in red. The annotated start site is underlined. (B) Conserved bases are shown in green. (C) Bases conserved in all three *Methanosarcina* spp. are shown in blue while those conserved in *M. acetivorans* and *M. mazei* are shown in red.

tion mutant was unable to grow on methanol, indicating that *mtaA1* is the sole functional methanol-specific MT2 enzyme and that the presence of *mtaA2* does not compensate for lack of *mtaA1*. Consistent with this result, the *mtaA2* single deletion mutant showed similar generation times, lag times, and growth yields to the parental strain on methanol, and, thus, *mtaA2* is not required for growth on this substrate. The Δ *mtaA1* Δ *mtaA2* mutant did not grow on methanol, which is also consistent with the growth phenotypes of the single deletion mutants. Interestingly, some cultures of the Δ *mtaA1* and Δ *mtaA1* Δ *mtaA2* mutants acquired the ability to utilize methanol after extended incubation. When these cultures were readapted to growth on TMA and subsequently inoculated into methanol medium, they showed a substantially shorter lag time than they exhibited during the first transfer from TMA to methanol (data not shown). Thus, it is likely that these cultures had acquired suppressor mutations allowing them to use methanol.

We previously showed that a mutant lacking the operons encoding all three methanol-specific MT1 enzymes (*mtaCB1*, *mtaCB2*, and *mtaCB3*) does not grow on methanol (32). Therefore, the inability of the Δ *mtaA1* Δ *mtaCB1* Δ *mtaCB2* Δ *mtaCB3* mutant to grow on methanol was not surprising; however, we were surprised to observe that this mutant failed to grow on acetate, whereas the Δ *mtaCB1* Δ *mtaCB2* Δ *mtaCB3* mutant does not have any observable growth defect on acetate (32). Relative to this mutant, the Δ *mtaA2* Δ *mtaCB1* Δ *mtaCB2*

Δ *mtaCB3* mutant did grow on acetate, though with a slightly longer generation time. Thus, analogous to the methanol growth phenotype, the *mtaA2* gene does not appear to be required for growth on acetate. Results with the deletion mutant lacking all five methanol-specific MT1 and MT2 enzymes are consistent with this interpretation. All the *mtaA* mutants tested in this study showed a modest but reproducible increase in generation time and lag time on TMA, DMA, and MMA compared to the parental strain, the significance of which is not clear (Table 6).

The Δ *mtbA* mutant is incapable of growth on DMA and MMA, and, thus, MtbA is probably the sole MT2 enzyme for use of these substrates. The Δ *mtbA* strain is able to grow on TMA; however, the generation time of the mutant was threefold longer, the lag phase when switching from methanol to TMA was fivefold longer, and the growth yield was only half relative to the parent strain. Interestingly, this growth yield is similar to that achieved by the wild-type strain on MMA, suggesting that only a single methyl group resulting from the demethylation of TMA to DMA is being channeled into the methylotrophic pathway in this mutant (i.e., the data suggest that the product DMA is not further catabolized in this mutant). Growth of the Δ *mtbA* mutant is unlikely to be due to suppressor mutations because this mutant retained the characteristic lag time, slow growth rate, and low yield after being switched from TMA to methanol and back to TMA (data not

TABLE 5. Percent amino acid identity of *M. acetivorans*, *M. mazei*, and *M. barkeri* MtaA1, MtaA2, and MtbA proteins^a

Protein	% Amino acid identity								
	MtaA1 _{Ma}	MtaA1 _{Mm}	MtaA1 _{Mb}	MtaA2 _{Ma}	MtaA2 _{Mm}	MtaA2 _{Mb}	MtbA _{Ma}	MtbA _{Mm}	MtbA _{Mb}
MtaA1 _{Ma}		88	82	82	80	78	38	38	37
MtaA1 _{Mm}			87	79	77	76	36	36	35
MtaA1 _{Mb}				76	77	76	36	35	36
MtaA2 _{Ma}					83	81	38	39	38
MtaA2 _{Mm}						86	35	35	35
MtaA2 _{Mb}							37	38	37
MtbA _{Ma}								90	87
MtbA _{Mm}									87
MtbA _{Mb}									

^a CLUSTALW was used for calculations (42). The following subscripts were used to identify the proteins: Ma, *M. acetivorans*; Mm, *M. mazei*; and Mb, *M. barkeri*.

FIG. 3. Phylogeny of *Methanosarcina* MtaA and MtbA proteins. Unrooted neighbor-joining tree generated by the DrawTree program (<http://workbench.sdsc.edu>) (13, 22, 42) for the MtaA1 (red), MtaA2 (green), and MtbA (blue) proteins from *M. acetivorans* (Ma), *M. mazei* (Mm), and *M. barkeri* (Mb) are shown. Note that in all cases the individual isozymes in the three *Methanosarcina* spp. are more similar to each other than they are to other isozymes found in the same organism. The *M. barkeri* MtaA1 protein is an exception as it is more similar to the MtaA2 isozymes from the three *Methanosarcina* spp.

shown). Thus, MtbA is very important, but not essential, for growth on TMA. The $\Delta mtaA$ mutant had only slight growth defects on methanol or acetate.

Methanogenesis from various substrates in *mtaA* and *mtbA* deletion mutants. Because growth can be affected by a variety of factors, we also measured the rate of methane production by mutant and wild-type cell suspensions (Table 7). We were unable to measure the rate of methane production by the $\Delta mtaA1$, the $\Delta mtaA1 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3$, the $\Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3$, and the $\Delta mtaA1 \Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2 \Delta mtaCB3$ mutants on methanol as these strains do not grow on this substrate. We attempted to circumvent this problem by measuring methane production from methanol in TMA-grown cells but were unsuccessful. This is likely due to a lack of MT1 expression under these growth conditions (4). Interestingly, the rate of methane production from methanol by resting cell suspensions in methanol-grown $\Delta mtaA2$ cells was nearly twofold slower than wild-type. Thus, despite the lack of observable growth phenotypes in this mutant, MtaA2 clearly plays a role in methanol metabolism. The $\Delta mtaA$ mutant had no significant effect on methane production from methanol, and neither the $\Delta mtaA1$ nor the $\Delta mtaA2$ mutation affected the ability of TMA-grown cells to produce methane from TMA. In contrast, the $\Delta mtaA$ mutant displayed a 50-fold reduction in growth rate, relative to wild-type, on TMA. Therefore, the slow growth of the $\Delta mtaA$ mutant is probably due to the slow rate of substrate catabolism.

Identification of the TSS of *mtaA1* and *mtbA*. We determined the transcription start site (TSS) of *mtaA1* and *mtbA* (Fig. 4) using 5' RACE as described previously. The *mtaA1* TSS is a G residue 275 bp upstream of the putative start codon. Twenty-four base pairs upstream of the TSS lies a putative TATA-box adjacent to a purine rich element representing a potential transcription factor B recognition element (BRE). These elements are conserved in all three *Methanosarcina* spp. In sharp contrast, the *mtbA* TSS is a G residue only 28 bp upstream of the putative start codon. Twenty-eight base pairs upstream of that lies a putative TATA-box next to a poorly recognizable potential BRE. Interestingly, the *mtbA* TSS was not conserved in *M. barkeri* Fusaro. We were unable to deter-

TABLE 6. Growth of $\Delta mtaA$ and *mtbA* mutants in various methanogenic substrates^a

Strain genotype	Substrate generation time (h)				Substrate lag time (h)				Substrate growth yield (maximum OD ₆₀₀)				
	Methanol	TMA	DMA	MMA	Acetate	Methanol	TMA	DMA	MMA	DMA	TMA	MMA	Acetate
$\Delta mtaA$	7.2 ± 0.2	6.5 ± 0.3	15.3 ± 0.6	11.7 ± 1.1	50.8 ± 2.9	29 ± 2	20 ± 1	44 ± 1	28 ± 1	0.51 ± 0.03	0.82 ± 0.01	0.39 ± 0.02	0.42 ± 0.1
$\Delta mtaA1 \Delta mtaA2$	NG	8.5 ± 0.4	24.9 ± 3.0	12.8 ± 0.3	55.6 ± 0.8	NG	27 ± 2	53 ± 5	30 ± 2	0.85 ± 0.02	0.85 ± 0.02	0.38 ± 0.02	0.44 ± 0.1
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB1$	6.5 ± 0.6	8.8 ± 0.6	32.8 ± 0.9	21.6 ± 0.9	55.7 ± 3.1	18 ± 2	29 ± 1	68 ± 14	52 ± 3	0.50 ± 0.02	0.83 ± 0.02	0.39 ± 0.0	0.42 ± 0.0
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB2$	NG	9.7 ± 0.9	28.5 ± 0.7	25.7 ± 0.6	57.1 ± 1.7	NG	34 ± 2	67 ± 2	36 ± 1	0.51 ± 0.02	0.84 ± 0.04	0.39 ± 0.01	0.44 ± 0.0
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB3$	NG	8.7 ± 0.7	27.9 ± 0.3	30.2 ± 0.4 ^b	72.7 ± 3.3	NG	25 ± 3	71 ± 3	134 ± 4 ^b	0.52 ± 0.01	0.85 ± 0.00	0.36 ± 0.01 ^b	0.4 ± 0.1
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2$	NG	8.3 ± 0.1	25.1 ± 0.5	13.9 ± 0.4	NG	NG	33 ± 1	64 ± 2	31 ± 2	0.46 ± 0.04	0.89 ± 0.01	0.38 ± 0.02	NG
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB3$	NG	10.5 ± 0.9	26.6 ± 3.8	29.6 ± 0.6	85.2 ± 2.4	NG	40 ± 2	76 ± 2	21 ± 1	0.51 ± 0.01	0.84 ± 0.01	0.38 ± 0.02	0.4 ± 0.1
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB1 \Delta mtaCB2$	NG	8.4 ± 0.9	25.1 ± 0.5	17.3 ± 1.1	NG	NG	28 ± 3	64 ± 5	38 ± 2	0.44 ± 0.02	0.84 ± 0.02	0.36 ± 0.01	NG
$\Delta mtaA1 \Delta mtaA2 \Delta mtaCB3$	NG	9.5 ± 1.4	21.2 ± 0.5	NG	46.5 ± 1.1	29 ± 2	107 ± 5	NG	109 ± 5	0.72 ± 0.01	0.42 ± 0.01	NG	0.40 ± 0.02

^a Growth was measured as indicated in Materials and Methods. Lag time represents the time required to reach one-half of the maximum OD value. Values represent the average and standard deviations of triplicate measurements. NG, no growth was seen for at least 3 months.

^b Growth parameters for this strain on MMA were determined during a TMA-to-MMA substrate switch growth experiment.

TABLE 7. Rate of methane production of the *mtaA* and *mtbA* single deletion mutants

Strain genotype	Specific activity (mU/mg of protein) ^a	
	Methanol	TMA
<i>Δhpt</i>	161 ± 2	24 ± 3
<i>ΔmtaA1 Δhpt</i>	NG ^b	21 ± 2
<i>ΔmtaA2 Δhpt</i>	78 ± 4	21 ± 2
<i>ΔmtbA Δhpt</i>	197 ± 7	0.4 ± 0.04

^a Activity was determined as nmol of CH₄ produced min⁻¹ mg⁻¹ protein. Values represent the average and standard deviations of four independent measurements.

^b NG, no growth occurs on this substrate.

mine the TSS of *mtaA2*, possibly due to the extremely low levels of expression of this gene (see below).

Expression analysis of *mtaA1*, *mtaA2*, and *mtbA* genes. To gain further insight into their in vivo functions we examined the substrate-dependent regulation of the *mtaA1*, *mtaA2*, and *mtbA* genes using reporter gene fusions. Accordingly, translational gene fusions were constructed such that a 1-kbp region immediately upstream of each gene including the start codon was fused to the *uidA* gene, which encodes the easily assayable

enzyme β-glucuronidase (33). Based on the mapped TSSs for each gene (Fig. 4), we believe that this region should include all necessary regulatory elements required for controlled gene expression, although it remains formally possible that distally located elements or elements within the coding sequence could also be required for expression. These translational fusions were integrated into the chromosome in single copies as described previously (20) and assayed for β-glucuronidase activity after growth on methanol, TMA, DMA, MMA, and acetate (Table 8).

Each of the three gene fusions was expressed on all substrates tested. Expression of the *mtaA1* fusion was strongly induced by methanol (10-fold relative to MMA) but remained at relatively high levels on TMA, DMA, and acetate (only two- to threefold lower than methanol). The *mtbA* fusion was expressed at equally high levels on methanol, TMA, and DMA, with two- to threefold lower expression on MMA and 20-fold lower expression on acetate. These observations are in contrast to those made by previous workers in *M. barkeri* using Northern blot analysis and immunochemical approaches (19, 21). These differences might be species specific and/or might be a reflection of the different techniques used by us and these

A

Ma <i>mtaA1</i>	GAGATTTATTAAAAAAGGCAAGAAAAATTCCTGACAATTATTTTATAATTGATA	107
Mm <i>mtaA1</i>	GTGATTTATCAAAAAAGAGGTAGAAAAAATTCCTGGCAATTAGCTTTTATAATTGCTA	112
Mb <i>mtaA1</i>	CTCGTTTATTAAAAATATGATAAAAAATATTCTTGACAAATAGCTTTTATAAGTTGTTA	120
▼		
Ma <i>mtaA1</i>	ACTAAAAAATATCCGCATCCCATCCCAATTGAGGGACTTGAGAATTGTTTTACGGCCA	167
Mm <i>mtaA1</i>	ACTAAAAAATGTCCGCATTCTGACTCAACAGAGGGGTTTCGAGAATTGTTTTACTTTGA	172
Mb <i>mtaA1</i>	ACTAAAAAATGCCCGTACTCTAATGTATCAAGGAGCCTGAGAATTATTTTTAGTGCCC	180
Ma <i>mtaA1</i>	ATAATTCTCATTCCTGCTCATATGAGACAAAAAGGTGATACAGAAGGCACATCTGCCCC	227
Mm <i>mtaA1</i>	TTATTCTCATTCCTCGCTCAGGCAGAGA--AAAAAGATGATTGAGAG---CTATCTATTCC	226
Mb <i>mtaA1</i>	TGGTTTCTATCTCTGCTCGGGCGAGGTGGAAAGGGAGATTCAAAAT---TTATTT---TT	233
Ma <i>mtaA1</i>	CATTTACCGTAAAACTACAGTTTTTTAATATGTTTGTAA--CACCGGATACTCTAGTCT	286
Mm <i>mtaA1</i>	CGTTTATCAAAAGAGACCTTAATTTTTTAATATGCTTTTAA--CTCTGGATACTCTAGTCT	285
Mb <i>mtaA1</i>	GAGTTTTCAGGTGAACCTAAGATTTTAACTGTGTTTCAAACTCTGGATTTTCTATTCC	293
Ma <i>mtaA1</i>	GGAGTATGAAACATTTTGGATTGAAAGGTCTATA---TAATATTGTCAAGTAGACAATA	343
Mm <i>mtaA1</i>	GGAGTGTGAAACATTTTGGATTGAAAGGTCTATA---TAATATTGTCAAGTAGACAATA	342
Mb <i>mtaA1</i>	GGGGCGTGAACATTTTGGATTGAAAGGTCTGAAACAATAATATTGTCAAGTAGGCAATA	353
Ma <i>mtaA1</i>	AAATTGCAATAAAAAATATGTACTAAGATAAAAGTAAAAAA--GGTATGACCGATATG	400
Mm <i>mtaA1</i>	AAATTGTAATAAAAAATAATGTACAAGGATAAAAGTAAAAAAAGGTATGACCGATATG	400
Mb <i>mtaA1</i>	AAATT-TAATAAAAAATAAT-----AGAGATAAAATAAAAA--GGTATGAC---TATG	400

B

Ma <i>mtbA</i>	TAATAAGAGCTTTAATTAAGGAATAATAATACATATTTATTAA--TAGTATGGGTTTTTG	58
Mm <i>mtbA</i>	TAATAAAATCTTTAATTAAGGAATAATAATACATATTTATTAA--TAGTATGGGTTTTTG	58
Mb <i>mtbA</i>	TAACATAATTTTAAATTAAGGGTATAAATACATATTTATTAAAAAGTATGTATTTTTC	60
▼		
Ma <i>mtbA</i>	A-ATCTAGGGCTGTCTATAGAACTTAGAGAGGATTATAGATG	100
Mm <i>mtbA</i>	A-AATAGGGCTGTCTATAGAACTTAGAGAGGATTATAGATG	100
Mb <i>mtbA</i>	ATAATCCTACACCTTATACAAATAAGAGAGGATTATAGATG	103

FIG. 4. The TSS of *mtaA1* and *mtbA* in *M. acetivorans* determined by 5' RLM-RACE. The 5' mRNA leader region upstream of the putative start codon (cyan and underlined bases) for the three genes along with the experimentally determined TSS (arrow) and the putative TATA box (black bracket) and RBS (orange text) are shown. Panel A shows this region for the *mtaA1* promoter; the red letters represent bases conserved in all three *Methanosarcina* spp., namely *M. acetivorans* (Ma), *M. mazei* (Mm), and *M. barkeri* (Mb). Panel B shows the 5' mRNA leader region for the *mtbA* promoter; the blue letters represent bases conserved in all three *Methanosarcina* spp. while the red letters represent bases conserved between *M. acetivorans* and *M. mazei*.

TABLE 8. β -Glucuronidase activities of *uidA* translational fusions to *mtaA1*, *mtaA2* and *mtbA* in cells grown on various methanogenic substrates

Fusion	Activity on the indicated substrate (mU/mg of protein) ^a				
	Methanol	TMA	DMA	MMA	Acetate
<i>mtaA1</i> (annotated)	8 $\pm$ 0.3	6 $\pm$ 0.3	10 $\pm$ 1.8	7 $\pm$ 0.7	1.8 $\pm$ 0.5
<i>mtaA1</i> (alternate)	157 $\pm$ 10	42 $\pm$ 3	65 $\pm$ 9	13 $\pm$ 3	68 $\pm$ 11
<i>mtaA2</i>	8 $\pm$ 0.2	1 $\pm$ 0.1	2 $\pm$ 0.1	1 $\pm$ 0.5	2 $\pm$ 0.9
<i>mtbA</i>	418 $\pm$ 37	381 $\pm$ 36	460 $\pm$ 39	164 $\pm$ 38	22 $\pm$ 2

^a Activity was determined as nmol of β -glucuronidase produced min⁻¹ mg⁻¹ of protein. Values represent the average and standard error of nine independent measurements. The limit of detection for this assay is 0.1 mU/mg of protein.

groups to assess expression. The *mtaA2* gene was expressed at very low levels on all methanogenic substrates tested but did show an eightfold increase during growth on methanol. We also observed that the translational fusion constructed using the annotated start codon for the *mtaA1* gene was expressed at very low levels on all substrates, consistent with the annotation's being incorrect, as suggested above.

DISCUSSION

The data presented here are consistent with the idea that *mtaA1* and *mtbA* play primary roles in methanol and methylamine metabolism, respectively. The observation that *mtaA1* is necessary for growth on methanol, in combination with its proven *in vitro* activity in *M. barkeri* (43), demonstrates that MtaA1 is a methanol-specific MT2. Furthermore, it shows that no other MT2 enzyme, including the 82% identical homolog MtaA2, can substitute for MtaA1 during growth of *M. acetivorans* on methanol. Similarly, our data indicate that MtbA is the sole MT2 enzyme used for DMA and MMA metabolism. The proximity of these genes to other genes known to be involved in the metabolism of methanol and methylamines supports these primary functional assignments. Nevertheless, our data also clearly indicate that the various MT2 enzymes play additional roles in the metabolism of nonmethylotrophic substrates and that cross-reactivity between substrates occurs.

An interesting observation that came out of this study is the requirement of MtaCB and MtaA1 for growth on acetate, a substrate for which there is no known or apparent role for the MT1/MT2 methyltransferase pathway. The nature of the MT1/MT2 requirement for growth on acetate remains mysterious at this time but is supported by the relatively high-level expression of *mtaA1* on acetate. MtaA1 expression is not simply constitutive but 10-fold lower on MMA than on methanol and, therefore, clearly regulated. Thus, the expression of *mtaA1* on acetate is likely a reflection of an important role during growth on this nonmethylotrophic substrate. These data indicate that there is a required flow of methyl groups from acetate through the methanol-specific MT1/MT2 pathway during growth on acetate. Interestingly, the data presented here suggest that this phenomenon may be a general function of MT1/MT2 systems. Other MT1 and MT2 enzymes can take the place of MtaCB and MtaA1 in performing this function as both the Δ *mtaCB1* Δ *mtaCB2* Δ *mtaCB3* mutant and the Δ *mtaA1* mutant can grow

on acetate. Accordingly, it appears that in the Δ *mtaA1* mutant another MT2 enzyme acts to transfer methyl groups either to or from MtaCB, and in the Δ *mtaCB* mutant another MT1 enzyme acts to transfer methyl groups to or from MtaA. It should be noted that in this study the Δ *mtaCB* mutants lacked all three isozymes (*mtaCB1*, *mtaCB2*, and *mtaCB3*). Therefore, it is possible that only one of the *mtaCB* operons in conjunction with *mtaA1* is responsible for this phenotype. In this regard it is interesting that both *mtaCB2* and *mtaCB3* are specifically induced on acetate compared to TMA (4). The possibility that other MT1/MT2 enzymes may be involved is supported by numerous studies showing their expression during growth on acetate. These include genes encoding MtaCB2, MtaCB3 as mentioned above, MtaA, MtbA, MtmC, and MtsB (4, 6, 9, 16, 19, 25, 31, 40, 46).

Two results demonstrate cross-reactivity between MT2 enzymes. First, the Δ *mtbA* mutant retains the ability to grow on TMA, albeit poorly. Thus, MtbA is not the sole MT2 that can be used for growth on TMA, although it is clearly the predominant one. These genetic data support previous immunochemical experiments showing that MtbA-depleted extracts retain some ability to produce methane from TMA (16). The data also suggest that another MT2 enzyme is capable of transferring the methyl group from methylated MttC to CoM. Although we did not directly test this idea, it is highly likely, based on *in vitro* biochemistry (15), that the MT2 enzyme responsible for this activity is MtaA1 though it is quite possible that another MT2 enzyme might be responsible for this activity. Second, we observed that strains carrying the Δ *mtaA1* mutation, alone or in combination with the Δ *mtaA2* mutation, are capable of acquiring suppressor mutations that allow them to utilize methanol. Deletion mutants lacking the genes encoding the three methanol-specific MT1 isozymes (*mtaCB1*, *mtaCB2*, and *mtaCB3*), either alone or in conjunction with Δ *mtaA1* or Δ *mtaA1* Δ *mtaA2*, did not acquire suppressor mutations allowing growth on methanol. Therefore, the genes encoding the MT1 isozymes are needed for this suppression, which most likely occurs by activating or modifying another MT2 gene. The existence of 11 additional MT2 genes (17) suggests likely candidates for the locus modified by the suppressor mutation. Surprisingly, *mtaA2* is not a candidate, given that suppressors arise with similar frequency in both the Δ *mtaA1* and Δ *mtaA1* Δ *mtaA2* strains.

Our data do not provide any clear indications of the function of MtaA2. Unlike *mtaA1*, *mtaA2* is neither necessary nor sufficient for growth on methanol. This was surprising, given the proximity of *mtaA2* to the *mtaCB3* operon, which we have previously shown encodes a methanol-specific MT1 isozyme (32). However, MtaA2 does appear to contribute to methanogenesis from methanol because this deletion mutant had a slower rate of methane production from methanol. Methane production from TMA was not affected, indicating that this is a substrate-specific effect. The *mtaA2* gene was expressed at very low levels on all methanogenic substrates tested, although it is up-regulated eightfold on methanol. These observations are in accordance with proteomic and microarray studies done on methanol-grown *M. thermophila* and *M. acetivorans* (12, 25). Interestingly, phylogenetic analysis shows that the biochemically characterized methanol-specific MT2 enzyme in *M. barkeri* Fusaro is an MtaA2 protein but in an identical genomic

context as the *mtaA1* genes of *M. acetivorans* and *M. mazei*. Thus, it seems probable that the MtaA2 proteins from all the other *Methanosarcina* species are capable of activity with the methanol-specific MT1 enzymes.

Finally, we along with other workers have observed large 5' untranslated regions (UTRs) for a number of methanogenic genes (3, 8, 35, 39). This was the case for the *mtaA1* gene as well, which has a large 5' UTR (275 nucleotides). The significance of these long leader sequences is not completely understood, but deletion analysis shows that these regions can play an important role in regulating expression (3). The 5' UTR for the *M. acetivorans mtbA* transcript is very short (28 nucleotides) and is completely conserved in *M. mazei*, along with appropriately spaced putative TATA-box and BREs immediately upstream. While the putative TATA-box and BRE are conserved in *M. barkeri* Fusaro, the TSS is not. Although *M. barkeri* Fusaro is reported to grow on methylamines, the strain maintained in our laboratory, which was also the source of DNA used for genome sequencing, does not grow on TMA and DMA (data not shown) (26). The lack of conservation in the *mtbA* promoter therefore reflects a potential inactivating mutation that might explain this phenotype.

The genetic experiments presented in this study confirmed the implications of biochemical studies and underscored the importance of the MT2 methyltransferases in methanogenesis. This study also raised new questions, the answers to some of which we are seeking presently in our laboratory. These include the role of MT1/MT2 systems during growth on non-methylotrophic substrates, the nature of the suppressor mutations that arise in the Δ *mtaA1* backgrounds, and the potential regulatory proteins that might affect expression of these genes. We would also like to determine the MT2 enzyme(s) that substitute for *mtbA* in TMA utilization and also determine the potential differences in the interactions of MtaA1 and MtbA with the various MT1 isozymes specific for methanol and TMA.

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