Transcriptional Profiling of Methyltransferase Genes during Growth of *Methanosarcina mazei* on Trimethylamine[†]

Christian Krätzer,¹ Paul Carini,² Raymond Hovey,³ and Uwe Deppenmeier^{1*}

Institut für Mikrobiologie und Biotechnologie, University of Bonn, 53115 Bonn, Germany¹; Department of Microbiology, Oregon State University, Corvallis, Oregon 97331²; and Department of Biological Sciences, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53211³

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The genomic expression patterns of *Methanosarcina mazei* growing with trimethylamine were measured in comparison to those of cells grown with methanol. We identified a total of 72 genes with either an increased level (49 genes) or a decreased level (23 genes) of mRNA during growth on trimethylamine with methanol-grown cells as the control. Major differences in transcript levels were observed for the *mta*, *mtb*, *mtt*, and *mtm* genes, which encode enzymes involved in methane formation from methanol and trimethylamine, respectively. Other differences in mRNA abundance were found for genes encoding enzymes involved in isopentenyl pyrophosphate synthesis and in the formation of aromatic amino acids, as well as a number of proteins with unknown functions. The results were verified by in-depth analysis of methyltransferase genes using specific primers for real-time quantitative reverse transcription-PCR (RT-PCR). The monitored transcript levels of genes encoding corrinoid proteins involved in methyl group transfer from methylated C₁ compounds (*mtaC*, *mtbC*, *mttC*, and *mtmC*) indicated increased amounts of mRNA from the *mtaBC1*, *mtaBC2*, and *mtaBC3* operons in methanol-grown cells, whereas mRNA of the *mtb1-mtt1* operon was found in high concentrations during trimethylamine consumption. The genes of the *mtb1-mtt1* operon encode methyltransferases that are responsible for sequential demethylation of trimethylamine. The analysis of product formation of trimethylamine-grown cells at different optical densities revealed that large amounts of dimethylamine and monomethylamine were excreted into the medium. The intermediate compounds were consumed only in the very late exponential growth phase. RT-PCR analysis of key genes involved in methanogenesis led to the conclusion that *M. mazei* is able to adapt to changing trimethylamine concentrations and the consumption of intermediate compounds. Hence, we assume that the organism possesses a regulatory network for optimal substrate utilization.

Methanogenic archaea are responsible for the biological formation of methane. An understanding of the biological processes leading to CH₄ formation is of major interest since methane is an important greenhouse gas that directly contributes to climate change and global warming. The substrates used by methanogens include H₂-CO₂, acetate, methanol, and a variety of methylamines. These last substrates are disproportionated into CO₂ and CH₄ by methylotrophic methanogens that are found exclusively among members of the *Methanosarcinales*, including the three sequenced species *Methanosarcina barkeri*, *Methanosarcina mazei*, and *Methanosarcina acetivorans* (10, 17, 27). These species are metabolically versatile methanogens and may have acquired new pathways for energy generation from acetate and methylamines, along with additional genes for environmental adaptation, by lateral gene transfer. 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) (11). These processes are catalyzed by the concerted action of distinct methyltransferases (see Fig. 1) called methyltransferase 1 (MT1) and MT2 and a

corrinoid protein (15, 16). MT1 proteins are substrate specific and are encoded by the genes *mtaB* for methanol, *mttB* for trimethylamine (TMA), *mtbB* for dimethylamine (DMA), and *mtmB* for monomethylamine (MMA) (8, 9, 32, 33). The corresponding enzymes (see Fig. 1) catalyze the transfer of the methyl group from the methylated substrate to their appropriate corrinoid proteins, which are encoded by the genes *mtaC* for methanol, *mttC* for TMA, *mtbC* for DMA, and *mtmC* for MMA (30). From the methylated corrinoid protein, the methyl group is transferred to coenzyme M (HS-CoM) (14, 18) by the catalytic activity of the MT2 methyltransferases (MtaA1, MtaA2, and MtbA). Methyl-CoM is reduced to methane with the help of coenzyme B (HS-CoB). The resulting heterodisulfide, CoM-S-S-CoB, is finally reduced to HS-CoM and HS-CoB by a membrane-bound electron transport system. The electrons derive from methyl group oxidation forming CO₂ (11). *M. barkeri* and *M. mazei* can also use molecular hydrogen for the reduction of the heterodisulfide (23, 29).

The methyltransferases initiating methanogenesis from TMA, DMA, and MMA, as well as some of the permeases for substrate uptake, possess a novel residue, pyrrolysine (see Fig. 1), which is encoded by a single amber (UAG) codon (24, 28). Interestingly, multiple highly homologous MT1 enzymes and corrinoid proteins for the degradation of TMA (two copies, MttBC-1 and MttBC-2), DMA (three copies, MtbBC1, -2, and -3), MMA (two copies, MtmBC1 and -2), and methanol (three copies, MtaBC1, -2, and -3) have been identified in *Methanosarcina* spp. (10, 17, 27). Genetic analysis of the three metha-

* Corresponding author. Mailing address: Institut für Mikrobiologie und Biotechnologie, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany. Phone: 49-228-735590. Fax: 49-228-737576. E-mail: udeppen@uni-bonn.de.

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nol-specific methyltransferase 1 operons (*mtaBC1*, *mtaBC2*, and *mtaBC3*) in *M. acetivorans* demonstrated that each of them has a discrete function during growth on methanol, which is reflected by differential gene regulation (3, 32). In contrast, relatively little is known about the regulation of the methylamine-utilizing methyltransferase isoenzymes. Here we report on the mRNA abundance of the above-mentioned methyltransferases and corrinoid proteins from *M. mazei* during growth on TMA or methanol by genome-wide transcriptional profiling, real-time RT-PCR, and culture parameter analysis.

MATERIALS AND METHODS

Culture conditions. *M. mazei* G61 (DSM 7222) was grown at 37°C in complex medium under an atmosphere of N₂-CO₂ (80:20). The complex medium was prepared as described by Hippe et al. (19). Prior to inoculation, either methanol (150 mM) or TMA (50 mM) was added as the substrate and the medium was reduced using Na₂S (0.3 mg/ml). Cysteine was not added because it interfered with TMA detection. Cells were harvested from cultures that had been inoculated at low density and had maintained a constant growth rate for at least six generations. During growth, the pH value remained between 6.6 and 6.9.

Genome-wide transcriptional profiling. The monitoring of genomic transcription levels of *M. mazei* provided data commonly referred to as gene expression patterns (35). It is important to keep in mind that the term gene expression in microarray hybridization indicates mRNA abundance, which is the result of the rate of transcription initiation and transcription extension, efficiency of termination, and mRNA degradation. For genome-wide expression profiling, total RNA was extracted from three independent cultures grown on TMA and methanol, respectively. Each RNA pair was subjected to two independent labeling reactions, including the dye swap experiment, resulting in six experimental data sets in total. As each open reading frame was spotted in duplicate on each microarray slide, 12 data points of mRNA abundance were generated for each open reading frame. For each labeling reaction, total RNA was isolated from 50-ml cultures. The cells were centrifuged under anaerobic conditions for 5 min at 4°C, and the pellets were resuspended in 5 ml Tri reagent (Sigma Aldrich, Schnelldorf, Germany). After freezing (−80°C) and thawing, 1 ml of 1-bromo-3-chloro propane was added and the cell lysate was centrifuged again (4°C, 10 min). Total RNA was precipitated with isopropanol and was pelleted by centrifugation. After a wash with 75% ethanol, the pellet was dried and directly resuspended in DNase buffer. RNase-free DNase was added and incubated at 37°C for 2 h. A second purification of the RNA preparations was performed using an RNA cleanup kit (Zymo Research, Orange, CA). The RNA quality was checked by agarose gel electrophoresis and the 260-nm/280-nm absorption ratio. Randomly primed cDNA synthesis of total RNA from *M. mazei* was used to prepare fluorescently labeled cDNA probes for array hybridization according to the protocol of the CyScribe First Strand cDNA labeling kit (GE Healthcare, Munich, Germany). Labeled cDNA was purified and DNA arrays were generated as described previously (20). DNA array hybridization, scanning, and normalization were done according to the method of Hovey et al. (20) using the software program GenPixPro 3.0 (Axon Instruments, Union City, CA). Afterwards, the data were computationally filtered using the “R” value, which is defined by the signal-minus-background value divided by the background standard deviation (21). For statistical analysis, we used the program SAM (Significance Analysis of Microarrays) (37).

Real-time qRT-PCR. mRNA quantification was done by real-time quantitative reverse transcription-PCR (qRT-PCR) with total RNA as a template. The RNA preparations were obtained from 50-ml cultures as described above. Transcript levels of methanol and TMA cultures were determined from at least three different cultures. For the analysis of the relationship between optical density and substrate consumption/product formation, seven TMA-grown cultures were harvested (optical density [OD] of 0.5 to 2.2) and the total RNA of each culture was used for the real-time qRT-PCR experiments to determine the level of mRNA abundance. For labeling and quantification, the iCycler (Bio-Rad, Munich, Germany) and the Quantitect SYBR green reverse transcription-PCR (RT-PCR) kit (Qiagen, Hilden, Germany) were used. The composition of the PCRs was according to the manufacturer's manual.

To overcome the problem of cross-hybridization between highly homologous methyltransferase-encoding genes, we designed gene-specific primers for in-depth RT-PCR experiments. First, homologous genes were aligned using the program ClustalW (36) and regions showing maximal differences were chosen for primer construction (see Table S1 in the supplemental material). Second, we

checked annealing of each primer pair and primer combination under real-time PCR conditions using chromosomal DNA or plasmids containing the corresponding gene inserts as templates. As evident from data in Table S2 in the supplemental material, only combinations of specific forward and reverse primers designed for each gene were efficient in real-time quantitative PCR. When primer combinations of different homologous genes were applied, the differences in the threshold cycle (*C_T*) values were at least sevenfold in comparison to gene specific primers, indicating that there was no significant cross-annealing between homologous genes.

The primers had been generated with the Primer3 software program (<http://frodo.wi.mit.edu/>). These primers amplified DNA fragments between 100 and 250 bp, which is optimal for real-time qRT-PCR. The *C_T* values for the initial target concentration in each reaction were determined using the iCycler software. Control reactions without reverse transcriptase were performed to verify the absence of genomic DNA. The mean concentration of RNA derived from the gene encoding glyceraldehyde dehydrogenase (*gap*) was used as a standard for the real-time qRT-PCR experiments because it is considered to be a stable housekeeping gene in *M. mazei*. Indeed, there was no evidence of different mRNA content for *gap* by genome-wide expression profiling using cells grown under different conditions (acetate versus methanol) (20), high and low salt (31), or low and high nitrogen content (39). The relative change in mRNA abundance was recorded as the ratio of normalized target concentrations ($\Delta\Delta C_T$). Also, the gene MM2536, encoding the 50S ribosomal protein L21e, was used as an internal standard. The $\Delta\Delta C_T$ values obtained using this standard were almost identical to the $\Delta\Delta C_T$ values of mRNA abundance calculated from *gap* (not shown). In addition, the specificity of each reaction was monitored by determination of the product melting temperature. To address the question of whether there were differences in target and reference gene amplification efficiency, we repeated the experiments using a second primer pair for the *mtb*, *mtt*, and *mtm* genes. The annealing positions were different from those of the first primer set. The $\Delta\Delta C_T$ values obtained using the second primer pair were almost identical to the data from the first set of gene-specific primers, indicating similar reverse transcriptase efficiencies and amplification rates for the target DNA by PCR (not shown).

Determination of TMA, DMA, and MMA. One-milliliter samples from TMA-grown cultures were taken at different time points (ODs). Cells were separated from the culture medium by paraffin oil centrifugation. Two hundred microliters of the paraffin oil was added to a 2-ml Eppendorf tube, and the culture broth was carefully loaded on top of the oil. After 2 min of centrifugation (15,000 × *g*), the cell supernatant was used for methylamine determination. Standard curves were prepared for all compounds from 0 to 50 mM concentrations using complex medium for dilution. TMA was analyzed by the utilization of the Folin-Ciocalteu phenol reagent (22). To 50 μ l of cell supernatant, 783 μ l H₂O, 125 μ l Na₂CO₃ (20% [wt/vol]), and 42 μ l Folin-Ciocalteu phenol reagent were added. The mixture was allowed to stand at room temperature for 30 min, and the absorbance was read at 745 nm. Concentrations of up to 100 mM DMA or MMA did not interfere with the TMA detection method. DMA was quantified by the copper-dithiocarbamate procedure (13). Fifty microliters of cell supernatant was diluted in 500 μ l H₂O, followed by the addition of 50 μ l of an ammonium acetate (20 g), CuSO₄ (0.2 g), NaOH (10 g), and NH₄OH (30 ml) solution (final volume, 100 ml). After the addition of 500 μ l carbon disulfide in benzene (5% [vol/vol]) and 50 μ l acetic acid (30% [vol/vol]), the solution was mixed and centrifuged for 1 min. The absorbance of the upper layer at 430 nm was measured photometrically. MMA in the supernatant was reacted with acetylacetone and formaldehyde in pH 5.6 acetate buffer to form *N*-methyl-2,6-dimethyl-3,5-diacetyl-1,4-dihydropyridine, which could be detected photometrically at 410 nm (40). Control experiments indicated that the TMA, DMA, and MMA test systems were all specific for their respective substrates and interference did not occur.

Information about the genome of *M. mazei* is available at GenBank, National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), TIGR (<http://www.tigr.org/tigr-scripts/CMR2/GenomeTabs.spl?database=ntmm01#1>), and KEGG (<http://www.genome.jp/kegg/>).

Information on primer sequences and specificity checks for primers used in real-time RT-PCR is listed in Tables S1 and S2 in the supplemental material, respectively.

Microarray data accession number. Information concerning DNA array design and original array results can be accessed in the Array Express database (<http://www.ebi.ac.uk/microarray-as/ae/>) under accession number E-MEXP-2108.

RESULTS AND DISCUSSION

Transcript profiling using DNA microarrays and real-time qRT-PCR. In this study, the genomic expression profiles of *M.*

mazei strain Gö1 (DSM 7222) growing in complex medium with methanol or TMA as the carbon and energy source were determined. For this purpose, three cultures grown on methanol and TMA were harvested independently in the mid-exponential phase (OD at 600 nm [OD₆₀₀] = 0.6 to 0.9), and the corresponding total RNA of each culture was used to generate fluorescent-dye-labeled probes for transcriptional profiling. The genome of *M. mazei* contains 3,371 genes (10), of which 3,242 were represented on the DNA array chip used for the experiments. After normalization and initial filtering, 1,612 genes showed transcript levels above the threshold (signal minus background/background standard deviation ratio of >2) in at least 6 out of 12 data sets. The statistical software package SAM (37) was applied to identify those genes with different transcript levels using a minimal change of 2.25-fold. From this selection, a total of 72 genes were identified with either an increased level (49 genes) or a decreased level (23 genes) of mRNA during growth on TMA with methanol-grown cells as the control (Table 1).

Methanol and TMA are both metabolized by the methylotrophic pathway of methanogenesis. Hence, we did not observe differences in mRNA abundances of genes encoding the enzymes of the central part of this pathway or enzymes involved in the energy-conserving systems of *M. mazei*. However, the utilization of methanol and TMA differs in the transfer of methyl moieties to CoM (HS-CoM), leading to the formation of methyl-S-CoM. In accordance, several sets of those genes showed different transcript patterns under the culture conditions tested (Table 1). Major differences in RNA abundance were observed for the *mta*, *mtm*, *mtb*, and *mtt* genes, involved in methyl group transfer from methanol or TMA. Biochemical evidence indicates that methanol catabolism in *Methanosarcina* species requires the concerted effort of the methyltransferase MtaB, a corrinoid-containing methyl-accepting protein (MtaC), and the methyltransferase MtaA (18, 34, 38). As previously mentioned, *Methanosarcina* species possess three homologous operons encoding methanol-specific MtaB and corrinoid proteins, *mtaBC1*, *mtaBC2*, and *mtaBC3* (Fig. 1). All these operons revealed elevated mRNA concentrations in methanol-grown *M. mazei* (Table 1). Most prominent was the operon *mtaBC2*, with a 200-fold increase in comparison to results with TMA-grown cells, followed by *mtaBC1*, with an elevation up to 14-fold, and *mtaBC3*, with a ratio of 4 (methanol/TMA).

However, a comprehensive investigation of mRNA levels is not possible with this technique because highly homologous genes could cause cross-hybridization of the corresponding cDNAs, and the interaction of labeled cDNA probes with different spots (representing the *mta*, *mtt*, *mtb*, and *mtm* genes) was highly possible (Fig. 1). To overcome this problem, we designed gene-specific primers (for details, see Table S1 in the supplemental material) for in-depth qRT-PCR experiments using the *gap* housekeeping gene for comparison and RNA preparations from cells harvested in mid-exponential phase (OD₆₀₀ = 0.6 to 0.9). The corrinoid protein-encoding gene *mtaC2* and the gene *mtaR* (MM1075), encoding a putative regulatory protein, showed 200- to 500-fold-higher mRNA concentrations in methanol-grown cells than in TMA cells (Table 1). Similar results were obtained for *mtaC1*, representing the *mtaBC1* operon (a 45-fold increase in methanol-grown

cells), and *mtaC3*, representing the *mtaBC3* operon (a 14-fold increase in methanol-grown cells). In summary, the results are comparable to those of genome-wide expression profiling. However, the degree-of-change values were higher because of the greater dynamic range in real-time PCR. These findings are in accordance with proteome analysis and with results obtained from reporter gene fusions and deletion mutants of *Methanosarcina* species (3, 5, 12, 25, 26, 32). It was found that the genes *mtaB2* and *mtaC2* from *M. acetivorans* are among the most highly regulated genes known in the *Archaea* (3). Results for *M. acetivorans* deletion mutants lacking the three *mtaBC* operons in all possible combinations indicated that each operon encodes a methanol-utilizing MtaB/corrinoid protein pair (32). Furthermore, regulatory proteins were identified that either activate or inactivate gene expression of *mtaBC* operons (5). In summary, the results suggest that the regulation of the *mtaBC* genes is universal among *Methanosarcina* species.

Transcriptional profiling experiments showed increased mRNA levels of the *mttBC1* and *mtbBC1* genes in TMA-grown cells, reaching ratios up to 40-fold those in methanol-grown cells (Table 1). These genes (MM1687 to MM1694) encode TMA- and DMA-dependent methyltransferases involved in degradation of the corresponding substrates (Fig. 1). The results are in accordance with findings from real-time qRT-PCR. In contrast to results with methanol-grown cells, the genes *mtbC1* and *mttC1* showed a 100- to 200-fold-higher RNA abundance in TMA cells (Table 1). Furthermore, the gene *mttP1* (MM1691), which encodes a putative permease for transport of TMA into the cell, showed a higher transcript level during growth on TMA. The predicted TMA permease MttP1 is similar to CAN1, a cationic amino acid permease from *Candida albicans* (1). It was shown that in *M. barkeri*, the genes *mtbC1*, *mttB1*, *mttC1*, *mttP1*, *mtbB1*, and *mttC1* form an operon (30). This might also be true for *M. mazei*, since the transcript levels of *mttC1* (MM1690) and *mtbC1* (MM1687) were essentially the same under all growth conditions tested, indicating that the genes are cotranscribed.

Other methyltransferase genes involved in TMA degradation (*mtmBC1*, *mtmBC2*, *mttBC2*, *mtbBC2*, and *mtbB3*) also showed different levels of RNA in genome-wide transcript profiling experiments but only by factors between 2 and 10 (TMA/methanol). Real-time qRT-PCR revealed a moderate elevation of mRNA abundance for *mtmC1* with TMA as a substrate. The gene *mtmC1* probably forms an operon structure with *mtmB1* and a gene encoding a putative methylamine permease. Evidence for this presumption was found in *M. barkeri*, where separate transcripts of 2.4 and 4.7 kb were detected, both of which carried the *mtmBC* genes. The larger transcript also encoded *mtmP*, which is homologous to the APC family of cationic amine permeases (9). MtmB1 and MtmC1 form one of the MMA-dependent methyltransferases (8). Other methyltransferase genes encoding isoenzymes of MttBC1 (*mttBC2*) and MtbBC1 (*mtbBC2* and *mtbBC3*) were transcribed at very low levels with the substrate methanol or TMA, and differences in mRNA abundance in real-time RT-PCR were not observed (Table 1). These results are in contrast to the microarray data and indicate that cross-hybridization of homologous cDNA fragments occurred during genome-wide expression profiling (Table 1). Transcripts of *mtmBC2* were almost undetectable, revealing C_T values near the chromo-

TABLE 1. Different transcript levels for *M. mazei* during growth on TMA versus that on methanol, identified by genome-wide expression profiling and real-time RT-PCR

Gene annotation	Gene no.	Score (<i>d</i>) ^{a,d,e}	Fold change ^{b,d,e}	Putative operon	Real-time RT-PCR result ^c
Methanol corrinoid protein MtaC3	MM0174			0174–0175	0.06
Methanol:corrinoid methyltransferase MtaB3	MM0175	−8.00	0.25	0174–0175	
Hypothetical protein	MM0312	−5.83	0.36	0311–0312	
Hypothetical protein	MM0408	−5.66	0.34	Single gene	
Hypothetical protein	MM0479	−2.39	0.37	Single gene	0.006
Hypothetical protein	MM0924	−2.21	0.28	Single gene	
Hypothetical protein	MM1071	−6.04	0.42	Single gene	
Methanol corrinoid protein MtaC2	MM1073	−12.09	0.005	1073–1075	
Methanol:corrinoid methyltransferase MtaB2	MM1074	−20.80	0.006	1073–1075	0.002
Putative regulatory protein	MM1075	−9.20	0.07	1073–1075	
Hypothetical protein	MM1112	−9.03	0.15	Single gene	
2-Dehydro-3-desoxyphosphoheptanote aldolase	MM1271	−8.76	0.18	1271–1275	
3-Dehydroquinate synthase	MM1272	−5.86	0.24	1271–1275	0.15
3-Dehydroquinate dehydratase	MM1273	−9.15	0.22	1271–1275	
Shikimate 5-dehydrogenase	MM1274	−5.05	0.30	1271–1275	
Prephenate dehydrogenase	MM1275	−6.22	0.33	1271–1275	
Methanol:corrinoid methyltransferase MtaB1	MM1647	−3.44	0.16	1647–1648	0.02
Methanol corrinoid protein MtaC1	MM1648	−2.94	0.07	1647–1648	
Hypothetical protein	MM1761	−7.13	0.35	1760–1764	
Mevalonate kinase	MM1762	−5.78	0.32	1760–1764	
Hypothetical protein	MM1977	−9.19	0.38	1976–1977	0.91
Hypothetical protein	MM2882	−8.95	0.23	2882–2884	
Hypothetical protein	MM2933	−7.15	0.30	Single gene	
Hypothetical protein	MM3197	−7.40	0.28	Single gene	
Hypothetical protein	MM0011	7.34	2.38	Single gene	
Cobyrinic acid synthase CbiP	MM0093	6.61	2.28	Single gene	
Phosphate acetyltransferase	MM0496	5.05	2.25	0495–0496	
Hypothetical protein	MM0583	4.89	3.83	Single gene	
2-Isopropylmalate synthase	MM0671	15.78	3.04	Single gene	4.57
Hypothetical protein	MM0772	10.75	3.53	Single gene	
Hypothetical protein	MM0869	8.65	3.96	0869–0872	
Beta-ketoacyl synthase/thiolase	MM0870	7.92	5.86	0869–0872	
Hydroxymethylglutaryl-CoA synthase	MM0871	5.65	5.27	0869–0872	4.12
Putative transcriptional regulator	MM0872	8.56	5.10	0869–0872	
Thiamine biosynthesis protein ThiC	MM1025	6.22	3.34	Single gene	
2-Isopropylmalate synthase	MM1284	8.73	5.26	1284–1287	
Hypothetical protein	MM1304	7.87	2.94	Single gene	15.9
Formylmethanofuran H ₄ MPT formyltransferase	MM1321	6.37	2.25	Single gene	
Monomethylamine:corrinoid methyltransferase MtmB1	MM1436 ^f	5.74	2.26	1436–1438	
Monomethylamine corrinoid protein MtmC1	MM1438 ^f	6.58	3.42	1436–1438	
Methylcobalamin:coenzyme M methyltransferase MtbA2	MM1439	6.33	3.46	Single gene	1.9
Hypothetical protein	MM1488	4.99	2.93	1487–1488	
Cobalamin biosynthesis protein CobN	MM1601	4.73	3.19	1601–1602	
Cobalamin biosynthesis protein CobN	MM1602	9.66	5.40	1601–1602	
Hypothetical protein	MM1612	9.97	6.15	Single gene	188.7
Dimethylamine corrinoid protein MtbC1	MM1687 ^g	14.22	32.89	1687–1694	
Trimethylamine:corrinoid methyltransferase MttB1 (N-terminal domain)	MM1688 ^h	12.23	40.59	1687–1694	
Trimethylamine:corrinoid methyltransferase MttB1 (C-terminal domain)	MM1689 ^h	15.33	22.30	1687–1694	
Trimethylamine corrinoid protein MttC1	MM1690 ^h	13.43	16.76	1687–1694	150.1
Trimethylamine permease MttP1	MM1691	8.04	4.93	1687–1694	95.0
Conserved protein	MM1692	5.77	2.90	1687–1694	0.88
Dimethylamine:corrinoid methyltransferase MtbB1	MM1693 ^g	7.58	3.11	1687–1694	
Dimethylamine:corrinoid methyltransferase MtbB1 (C-terminal domain)	MM1694 ^g	12.58	15.21	1687–1694	
Catalase	MM1950	6.17	4.31	Single gene	
Hypothetical protein	MM1951	4.60	12.89	Single gene	0.52
Alkyl sulfatase	MM1982	6.56	4.39	Single gene	
Hypothetical protein	MM1988	8.55	18.36	Single gene	
Trimethylamine corrinoid protein MttC2	MM2047 ^h	7.66	2.86	2047–2052	
Trimethylamine:corrinoid methyltransferase MttB2	MM2049 ^h	5.82	3.45	2047–2052	0.94
Dimethylamine:corrinoid methyltransferase MtbB2	MM2051 ^g	11.01	3.51	2047–2052	
Dimethylamine corrinoid protein MtbC2	MM2052 ^g			2047–2052	
Hypothetical protein	MM2338	3.23	2.33	Single gene	
Cobalt transport ATP-binding protein CbiO	MM2387	2.67	2.32	2386–2388	0.73
Anthranilate synthase component I	MM2818	5.16	2.51	2817–2823	
Tryptophan synthase, alpha chain	MM2821	2.31	2.39	2817–2823	
Tryptophan synthase subunit beta	MM2822	2.28	4.51	2817–2823	
Hypothetical protein	MM2843	7.30	2.89	Single gene	0.94
Dimethylamine corrinoid protein MtbC3	MM2961 ^g			2961–2963	
Dimethylamine:corrinoid methyltransferase MtbB3	MM2962 ^g	7.32	6.33	2961–2963	
Dimethylamine:corrinoid methyltransferase MtbB3 (C-terminal domain)	MM2963 ^g	8.40	11.54	2961–2963	
Hypothetical protein	MM3011	7.22	2.52	Single gene	0.73
Hypothetical protein	MM3108	9.76	3.32	Single gene	
Monomethylamine corrinoid protein MtmC2	MM3334 ^f	7.96	2.60	3334–3336	
Monomethylamine:corrinoid methyltransferase MtmB2	MM3335 ^f	6.77	2.50	3334–3336	
Monomethylamine:corrinoid methyltransferase MtmB2 (C-terminal domain)	MM3336 ^f	3.94	2.31	3334–3336	

^a Data obtained from the program SAM (TMA- versus methanol-grown cells). The score *d* is calculated on the basis of the change in gene expression relative to the standard deviation of repeated measurements for that gene (for details, see reference 37).

^b Transcript level ratios (TMA- versus methanol-grown cells).

^c Data obtained from real-time RT-PCR (transcript ratios of TMA- versus methanol-grown cells).

^d The gene *mtaC3* was not represented on the chip.

^e The genes *mtbC2* and *mtbC3* showed transcript levels below the threshold (signal minus background/background standard deviation ratio of >2) in more than six data sets. Hence, they failed the filtering standard for the microarray experiments.

^f Gene belongs to the highly homologous *mtmBC* gene clusters.

^g Gene belongs to the highly homologous *mtbBC* gene clusters.

^h Gene belongs to the highly homologous *mttBC* gene clusters.

FIG. 1. (A) Gene clusters encoding enzymes involved in methyl group transfer from methylated C₁ compounds. The gene numbers of the genes are as follows: *mtaA1*, MM1070; *mtaC2*, MM1073; *mtaB2*, MM1074; *mtaR* (= putative regulatory gene), MM1075; *mtaB1*, MM1647; *mtaC1*, MM1648; *mtaC3*, MM0174; *mtaB3*, MM0175; *mtaA2*, MM0176; *mtbC1*, MM1687; *mttB1*, MM1688/MM1689; *mttC1*, MM1690; *mttP1*, MM1691/MM1692; *mtbB1*, MM1693/MM1694; *mttP2*, MM2045/MM2046; *mttC2*, MM2047; *mttB2*, MM2048/MM2049; *mtbB2*, MM2050/2051; *mtbC2*, MM2052; *mtbC3*, MM2961; *mtbB3*, MM2962/MM2963; *mtbP*, MM2964; *mtmC2*, MM3334; *mtmB2*, MM3335; *mtmP*, MM1434/MM1435; *mtmB1*, MM1436/MM1437; *mtmC1*, MM1438; *mtbA*, MM1439. Black horizontal arrows show the orientation and length of genes; boxes indicate operons or predicted operons; small vertical arrows indicate genes containing a pyrrolysine codon; dashed lines indicate that the genes are in close proximity on the chromosome. (B) Schematic view of the functions of MT1, corrinoid protein, and MT2.

somal DNA background (not shown). This result is in accordance to findings by Veit et al. (39), who showed that the *mtmBC2* operon is induced only during nitrogen starvation.

One of the methyltransferase genes, *mtbA* (MM1439), involved in methyl transfer from the corrinoid protein to HS-CoM (Fig. 1), also showed a change in transcript levels (Table 1). It was shown that MtbA catalyzes the analogous transfer from the MMA-, DMA-, and TMA-specific corrinoid proteins in vitro (14). The analysis of *M. acetivorans* deletion mutants revealed that the *mtbA* gene was required for DMA and MMA utilization and was important for TMA degradation (4). A second set of MT2 enzymes, referred to as MtaA1 and MtaA2, act on methanol together with the MtaBC proteins (Fig. 1). However, biochemical studies demonstrate that MtaA can also function as the MT2 enzyme for TMA utilization (14). Analysis of reporter gene fusions revealed that both *mtaA1* and *mtbA* were expressed on all methanogenic substrates tested. Additionally, *mtaA1* expression was induced on methanol (4). Our DNA array studies and real-time qRT-PCR results did not reveal differences in mRNA abundances of *mtaA1* or *mtaA2* in *M. mazei* (not shown). However, the *mtaA1* transcript content was high under both growth conditions (not shown).

Other differences in mRNA abundance concerned genes encoding enzymes involved in biosynthetic pathways and genes

encoding hypothetical proteins (Table 1). Most prominent in transcriptome and real-time qRT-PCR analysis of methanol-grown cells was the gene cluster MM1271-MM1275, encoding proteins for the synthesis of shikimate from erythrose-4-phosphate and phosphoenolpyruvate from prephenate (*tyrA*) (Table 1). These intermediates are precursors for the biosynthesis of the aromatic amino acids tyrosine and phenylalanine. Interestingly, microarray experiments also showed increased transcript levels for these enzymes during growth on acetate compared to that on methanol (20). Hence, there is obviously a hierarchical pattern of mRNA abundance, with increasing amounts of *aroBDEF* transcripts from cells grown on TMA, methanol, and acetate. The representative gene MM1272, encoding the 3-dehydroquinate synthase, was used to validate the differences in mRNA abundance of genes involved in aromatic amino acid biosynthesis identified by microarray analysis. Real-time qRT-PCR was performed, and in agreement with the microarray data, the transcript level of MM1272 was elevated in methanol-grown cells by a factor of 7 (Table 1). In contrast, the gene MM1762, predicted to encode a mevalonate kinase, showed different mRNA concentrations in the genome-wide expression profiles, but this result could not be confirmed by RT-PCR (Table 1).

TMA cells had elevated transcript levels for genes encoding proteins predicted to be involved in isopentenyl pyrophosphate synthesis (MM0869 to MM0872; Table 1). This result was validated by real-time qRT-PCR showing that the transcript levels of MM0870 and MM0872 were about five times higher in TMA-grown cells than in cells grown on methanol (Table 1). The closest homologue of MM0869 is a predicted nucleic-acid-binding protein containing a Zn ribbon from *Methanopyrus kandleri*; thus, the corresponding gene product could function as a regulatory protein. MM0870 encodes an acetyl-coenzyme A acetyltransferase, which is the first enzyme in the pathway for the synthesis of mevalonate (6). The second enzyme of this pathway is MM0871, which functions as 3-hydroxy-3-methylglutaryl-coenzyme A synthase. MM0872 contains a helix-turn-helix domain and could be a transcriptional regulator. The aforementioned proteins catalyze the first steps in mevalonate synthesis for the production of ether lipids. What would be the advantage of increasing the rate of synthesis for precursors of ether lipids? The substrate TMA is a weak base (pK_a of ~9.8) that exists mostly in the protonated form at the medium pH of 6.6 to 6.9. The internal pH value of *M. mazei* is not known, but the close relative *M. barkeri* has a cellular pH of 7 to 7.5 (2), which is slightly more alkaline than the medium. Hence, it is tempting to speculate that TMA is actively taken up by the cells in the protonated form by specific permeases (MttP) and is partly deprotonated in the cytoplasm. The deprotonated weak base is highly membrane permeable, and the diffusion would lead to a constant drop in intracellular pH. Indeed, it has been shown that TMA strongly depolarized membrane potential and intracellular pH in *Xenopus laevis* oocytes (7). Hence, the need for an increased biosynthesis of ether lipids in *M. mazei* might reflect an adaptation mechanism of the organism for improved membrane stability to keep TMA within the cell. Other genes with elevated mRNA abundance in TMA cells encode proteins involved in tryptophan formation (MM2818, MM2821, and MM2822) and the biosynthesis of

FIG. 2. TMA consumption and DMA/MMA formation during growth on TMA. Trimethylamine (■), dimethylamine (□), and monomethylamine (●) concentrations in the culture supernatant are shown. Inset: sequential demethylation of trimethylamine.

cofactors (e.g., MM0011, MM1025, MM1601, and MM1602). Two genes encoding proteins with unknown function (MM1951 and MM1988) showed transcript ratios greater than 10. There were a few other genes showing a slight increase in transcript levels during growth on TMA (Table 1). The specific function of the corresponding proteins in TMA metabolism is unknown.

Dependence of mRNA abundance on OD and product formation in TMA-grown *M. mazei*. A detailed analysis of transcript levels in TMA cells is possible only with the exact knowledge of substrate consumption and product formation. TMA contains three methyl groups, which are sequentially transferred to HS-CoM in the course of methane formation. Hence, there are two possible intermediates, DMA and MMA (Fig. 2, inset), and the question arose of whether these intermediates are accumulated or whether TMA is immediately converted to CH_4 , CO_2 , and NH_3 .

To answer the question, we performed experiments to analyze growth parameters and product formation in *M. mazei* with TMA as a substrate. Samples were taken from growing cultures at different time points to determine the optical density and the concentration of methylamines in the culture supernatants (Fig. 2). The TMA concentration decreased continuously until all TMA was consumed when the cultures reached an OD of about 2.0 (doubling time for all cultures tested = 7.4 ± 0.4 h; maximal OD = ~ 2.4). Interestingly, there were significant concentrations of DMA in the culture supernatants, reaching concentrations up to 7.5 mM. Cultures with ODs of about 0.3 started to excrete DMA, and the concentration of this intermediate stayed almost constant until the OD reached a value of about 1.7. In the late exponential phase, DMA was consumed, and the concentration dropped to zero when the cell cultures reached an OD of about 2 (Fig. 2). Most surprising was the fact that large amounts of MMA were secreted in the course of growth. The concentration of MMA in the culture supernatant constantly increased up to 35 mM and started to drop in the very late exponential growth phase (OD₆₀₀ ≥ 2).

This phenomenon was even more evident when the formation of MMA was compared to the consumption of TMA.

FIG. 3. mRNA abundance of genes in TMA cells at different growth phases. ΔC_T values were calculated using the relative amount of *gap* transcripts as a standard ($C_{T, \text{gene}} - C_{T, \text{gap}}$). Ratios were calculated from ΔC_T values by using the function $2^{-\Delta C_T}$. Light-gray bars, expression levels of genes in cells harvested in the early to mid-exponential phase (OD = 0.5 to 1.7). Dark gray bars, expression levels of genes in cells harvested in the late exponential phase (OD ≥ 2).

Two-thirds of consumed TMA was excreted into the medium as MMA when the cells were in the exponential phase (OD₆₀₀ = 0.4 to 2.0). An immediate complete degradation of TMA within the cell would result in an enormous release of ammonia and could lead to high intracellular concentrations of NH_4^+ , which could have a toxic effect on the cell metabolism. It is tempting to speculate that MMA is excreted to avoid ammonia-induced toxicity. When all other substrates are depleted in the medium, MMA is slowly taken up and metabolized (Fig. 2).

With the knowledge of growth parameters and product/intermediate formation, qRT-PCR experiments were performed using RNA preparations from *M. mazei* cultures harvested at different ODs. The results indicated two major transcript patterns when the cells were grown on TMA (Fig. 3). RNA prepared from cultures with ODs from 0.5 to 1.7 (early to late exponential phase) showed large amounts of *mttC1* (MM1690) and *mtbC1* (MM1687) transcripts, which are part of the operon MM1687-MM1693. The second transcript pattern was detected in cultures harvested in the very late exponential phase (OD ≥ 2). Under this condition there was a high mRNA content of the *mtbBC3* operon (MM2961-MM2964) and the *mtmBC1* operon (MM1434-MM1438). Interestingly, both operons contain genes encoding substrate-specific permeases, MtbP and MtmP, for the transport of DMA and MMA, respectively. The expression of *mttBC1* and *mtbBC1* was almost completely repressed (Fig. 3). It became evident from the results of the growth parameters and real-time qRT-PCR how *M. mazei* adapts to changing TMA concentrations. In the first phase, TMA is taken up from the medium by the permease MttP1. The methyltransferase MttBC1 demethylates the substrate and forms DMA, part of which is excreted. The DMA-

dependent methyltransferase MtbBC1 converts intracellular DMA into MMA, which is then excreted in large amounts. Consequently, the concentrations of RNA molecules carrying the information for production of MMA-degrading enzymes are only moderate (*mtmBC1*) or even very low (*mtmBC2*).

When TMA was completely consumed, the cells switched their metabolism and highly expressed the *mtb3* operon (MM2961-MM2964) containing *mtbP*, which is most probably a permease-encoding gene for DMA transport. This switch explains the disappearance of DMA in the medium, which is taken up and converted into MMA. Furthermore, there was an increase in the *mtm1* operon transcript level containing *mtmP* (MM1436-MM1438). We predict that the permease MtmP transports MMA into the cells, where it is slowly metabolized to CH₄, CO₂, and NH₃. Obviously, the *mtb1-mtm1* operon (MM1687-MM1694) is downregulated, because in the very late exponential growth phase, there is no need for TMA-utilizing proteins. In summary, there is strong evidence that *M. mazei* possesses a sophisticated regulatory mechanism for the utilization of methylamines. Evidence supporting this assumption is also found in *M. barkeri*, where highly homologous DNA sequences upstream of the promoter regions of the *mtb1-mtm1* operon, the *mtmCBP* operon, and *mtbA* were found, indicating binding motifs for transcriptional regulators (30). We conclude that *M. mazei* is able to adapt to changing TMA concentrations and regulates the degradation of intermediary compounds. Hence, we believe that the organism possesses a highly elaborate mechanism of gene regulation for optimal substrate utilization.

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