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Role of the Fused Corrinoid/Methyl Transfer Protein CmtA during CO-Dependent Growth of *Methanosarcina acetivorans*

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The genome of *Methanosarcina acetivorans* encodes three homologs, initially annotated as hypothetical fused corrinoid/methyl transfer proteins, which are highly elevated in CO-grown cells versus cells grown with alternate substrates. Based only on phenotypic analyses of deletion mutants, it was previously concluded that the homologs are strictly dimethylsulfide:coenzyme M (CoM) methyltransferases not involved in the metabolism of CO (E. Oelgeschlager and M. Rother, Mol. Microbiol. 72:1260–1272, 2009). The homolog encoded by MA4383 (here designated CmtA) was reexamined via biochemical characterization of the protein overproduced in *Escherichia coli*. Purified CmtA reconstituted with methylcob(III)alamin contained a molar ratio of cobalt to protein of 1.0 ± 0.2. The UV-visible spectrum was typical of methylated corrinoid-containing proteins, with absorbance maxima at 370 and 420 nm and a band of broad absorbance between 450 and 600 nm with maxima at 525, 490, and 550 nm. CmtA reconstituted with aquocobalamin showed methyl-tetrahydromethanopterin:CoM (CH_3-THMPT:HS-CoM) methyltransferase activity (0.31 μmol/min/mg) with apparent K_m values of 135 μM for CH_3-THMPT and 277 μM for HS-CoM. The ratio of CH_3-THMPT:HS-CoM methyltransferase activity in the soluble versus membrane cellular fractions was 15-fold greater in CO-grown versus methanol-grown cells. A mutant strain deleted for the CmtA gene showed lower growth rates and final yields when cultured with growth-limiting partial pressures of CO, demonstrating a role for CmtA during growth with this substrate. The results establish that CmtA is a soluble CH_3-THSPT:HS-CoM methyltransferase postulated to supplement the membrane-bound CH_3-THMPT:HS-CoM methyltransferase during CO-dependent growth of *M. acetivorans*. Thus, we propose that the name of the enzyme encoded by MA4384 be CmtA (for cytoplasmic methyltransferase).

Most methane-producing species (methanogens) presently characterized obtain energy for growth by the CO_2 reduction pathway (Table 1, reactions 1 to 3), in which CO_2 is reduced stepwise with pairs of electrons derived from the oxidation of H_2 (7). Although the oxidation of CO can serve as a source of electrons in the CO_2 reduction pathway (Table 1, reactions 4 to 6), only three species have been investigated. Growth of *Methanothermobacter thermautotrophicus* (basonym, *Methanobacterium thermoautotrophicum* strain ΔH) with CO is extremely poor, with a rate only 1% of that of H_2 (6). Although it was previously shown that *Methanosarcina barkeri* grows more rapidly (24), it was concluded that this species is not well evolved for growth with CO based on a poor doubling time (65 h) compared to growth with acetate (48 h) or methanol (12 h). The pathway in *M. barkeri* (Table 1, reactions 7 to 10) begins with the oxidation of CO to H_2 followed by reduction of CO_2 to methane with electrons derived from the oxidation of H_2 (24). Conversely, the doubling time (20 h) for CO-dependent growth of *Methanosarcina acetivorans* is triple that of *M. barkeri* (28). *M. acetivorans* is incapable of metabolizing H_2 (12, 35), and H_2 is not detected during growth with CO (28), suggesting novel features of the CO_2 reduction pathway in converting CO to CH_4. Indeed, quantitative global proteomic profiling coupled with molecular and biochemical analyses of *M. acetivorans* grown with CO versus acetate or methanol revealed an H_2-independent CO_2 reduction pathway in which electron transfer reactions deviate substantially from that of *M. barkeri* and other H_2-oxidizing, CO_2-reducing species (18). Furthermore, *M. acetivorans* also produces acetate, formate, and dimethylsulfide (DMS) during CO-dependent growth (18, 23, 28), the only reported products other than CH_4 for any methanogenic species.

An important step common to both *M. barkeri* and *M. acetivorans* CO_2 reduction pathways is transfer of the methyl group from tetrahydrosarcinapterin (THSPT) to coenzyme M (HS-CoM), which is catalyzed by the membrane-bound N^5-CH_3-THSPT:HS-CoM methyltransferase complex (MtrA-H). The MtrA-H complex couples the exergonic reaction to generation of a sodium gradient (with the concentration high outside) (10) with the potential to drive ATP synthesis via the Na^+/H^+ ATP synthase (32). The CH_3-S-CoM so produced is reductively demethylated to CH_4 in the final step of the pathway. Quantitative proteomic profiling supported by quantitative reverse transcription-PCR (RT-PCR) revealed levels of MtrA-H subunits with a 9-fold mean lower abundance in CO- versus acetate-grown *M. acetivorans*, a result indicating downregulation of the complex in response to growth with CO (18). The analyses also revealed 50-fold elevated levels of proteins encoded by loci MA0859, MA4384, and MA4558 in cells grown with CO versus methanol or acetate (18). These proteins were first named fused corrinoid/methyltransferases (FCMT) based solely on sequence identity to methyltransferases, which transfer the methyl groups of methylamines and methanol to HS-CoM in pathways converting these growth substrates to CH_4 (8). The amino-terminal domains of the FCMT homologs each contain a corrinoid-binding motif (GDVHDIGKNLV) with the conserved active-site histidine (underlined), while the C-terminal methyltransferase domains have a conserved $HXCX_nC$

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TABLE 1 Reactions and free energy yields of the carbon dioxide reduction pathway

No.	Reaction	(kJ/mol)
1	$4H_2 \rightarrow 8e^- + 8H^+$	
2	$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$	
3 (Sum of no. 1 and 2)	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	−131
4	$4CO + 4H_2O \rightarrow 8e^- + 8H^+ + 4CO_2$	
5	$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$	
6 (Sum of no. 4 and 5)	$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	−210.4
7	$4CO + 4H_2O \rightarrow 4H_2 + 4CO_2$	
8	$4H_2 \rightarrow 8e^- + 8H^+$	
9	$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$	
10 (Sum of no. 7, 8, and 9)	$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	−210.4
11	$2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 2H_2S + H^+$	−49.4

motif found in several methyltransferases that binds zinc, which is required for deprotonation of HS-CoM in the catalytic mechanism (9, 17, 30). Thus, it is proposed that the FCMT homologs transfer the methyl groups of unknown substrates to HS-CoM. The reduction of MtrA-H levels, concomitant with elevated abundance of FCMT homologs in CO- versus methanol- or acetate-grown *M. acetivorans*, led to the proposal that the homologs function as cytoplasmic CH_3-THSPT:HS-CoM methyltransferases which supplement activity of the membrane-bound sodium-pumping MtrA-H (18). However, *M. acetivorans* mutants, for which combinations of MA0859, MA4384, and MA4558 were deleted or disrupted, fail to produce DMS or utilize it for methanogenesis or growth (25). Furthermore, the growth phenotypes of the mutants cultured with growth-saturating CO levels are not significantly different from wild-type *M. acetivorans* (25). Based on these data alone, it was concluded that MA0859, MA4384, and MA4558 function exclusively in the pathway of methanogenesis from DMS, encoding DMS:HS-CoM methyltransferases that were designated MtsD, MtsF, and MtsH (methyltransferases specific for methylsulfides) (25). Remarkably, there are no reports of biochemical characterizations of any FCMT homolog validating this conclusion or investigation of the previously hypothesized CH_3-THSPT:HS-CoM methyltransferase activity.

Here, we present a reexamination of the CO-dependent growth characteristics for an MA4384 deletion mutant strain of *M. acetivorans* and an initial biochemical investigation of the heterologously produced FCMT homolog (CmtA) encoded by MA4384. The results support the previously proposed role of cytoplasmic CH_3-THSPT:HS-CoM methyltransferase for CmtA and FCMT homologs which supplement the membrane-bound CH_3-THMPT:HS-CoM methyltransferase during CO-limited growth of *M. acetivorans*. Thus, we propose changing the name of the enzyme encoded by MA4384 to CmtA (cytoplasmic methyltransferase).

MATERIALS AND METHODS

Strains, plasmids, and chemicals. *M. acetivorans* strain C2A (DSM 804) was from laboratory stocks, strain WWM1 (Δ*hpt*) was a gift from W. W. Metcalf (University of Illinois, Urbana-Champaign), and the MA4384 deletion mutant strain DmtsF(26) (here referred to as Δ*cmtA*) was a gift from M. Rother (Goethe-Universität, Frankfurt, Germany). *Escherichia coli* strain Rosetta DE3 (pLacI) and the pET22b expression vector were from Novagen (Madison, WI). Tetrahydromethanopterin (THMPT) was a gift from R. K. Thauer (Max Planck Institute for Terrestrial Microbiology, Marburg, Germany). Preparation of CH_3-THMPT from THMPT was performed as published previously (2). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical or molecular biology grade.

Preparation of cell extracts and isolation of soluble and membrane fractions. *M. acetivorans* strains were grown in high-salt medium at 37°C with 125 mM methanol or 1.0 atm of CO as previously described (18, 19). The adaptation of the wild-type and mutant strains of *M. acetivorans* to CO was performed as described previously (25). All steps requiring transfer of suspensions and solutions were performed under strictly anaerobic conditions in an inert-atmosphere glove bag (Coy Laboratory Products, Ann Arbor, MI), except where indicated, and at 4°C. All buffers were degassed with N_2 to remove oxygen. Protein was determined by the method of Bradford (5).

Cells were harvested by centrifugation, and the cell paste was stored at −80°C. Thawed cells were resuspended in 50 mM morpholinepropanesulfonic acid (MOPS)-KOH buffer (pH 7) containing 1.5 mM phenylmethylsulfonyl fluoride and DNase I (20 μg/ml) at 2 g (wet weight) per 7 ml. Cells were disrupted by two passes through a French pressure cell at 6.9×10^3 kPa. Cell extract was obtained by centrifugation of the lysate at 1,200 × *g* for 12 min to pellet cell debris and unbroken cells. The membrane and soluble cellular fractions were separated by centrifugation in a discontinuous sucrose gradient as previously described (39, 41), with the following exceptions. All manipulations were performed in dim light. Cell extract (1 ml) was layered onto a gradient comprised of 70, 30, and 20% sucrose (1:2:1 ml) in 50 mM MOPS-KOH buffer (pH 7.5) and centrifuged at 150,000 × *g* for 2 h in a Beckman SW55 Ti swinging-bucket rotor. The membrane fraction was harvested from a tight band located at the interface of the 70 and 30% sucrose layers. The soluble protein fraction was harvested from above the 20% sucrose layer. All fractions were stored at 4°C in sealed brown bottles under a N_2 atmosphere and assayed immediately.

Heterologous overproduction, purification, and reconstitution of CmtA. The MA4384 gene sequence, obtained from the Comprehensive Microbial Resource website (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), was amplified by PCR from genomic DNA of *M. acetivorans* strain C2A (DSM 804) (35) with an NdeI site at the 5′ end and an XhoI site at the 3′ end introduced into the primers (sequences of the primers are available on request). The PCR product was ligated into the pET22b expression vector to construct the recombinant plasmid pET-*MA4384*, with which *E. coli* Rosetta (DE3) cells were transformed. A stop codon (TAG) was introduced immediately after the MA4384 sequence to block expression of the His tag present in the pET22b vector to prevent possible interference with binding of corrinoid cofactors and zinc to the protein. The transformed cells were cultured at 37°C in Luria-Bertani broth (pH 7.0) containing 100 μg ampicillin/ml and 35 μg chloramphenicol/ml (final concentrations). Expression was induced by addition of isopropyl thio-β-D-galactopyranoside (1 mM final concentration) at an optical density at 600 nm (OD_{600}) of 0.6, and the culture was incubated at 16°C for 14 h. Cells were harvested by centrifugation. Approximately 10 g (wet weight) of cells was suspended in 30 ml of 50 mM MOPS-KOH buffer (pH 7.0) containing 1.5 mM phenylmethylsulfonyl fluoride and 20 μg DNase I/ml (final concentration). Cells were disrupted by two passages through a French pressure cell at 6.9×10^3 kPa. The lysate was centrifuged at 4°C for 12 min at 1,200 × *g*. The supernatant solution containing cell extract was centrifuged for 30 min at 75,000 × *g* to pellet inclusion bodies containing CmtA and cell debris. The pellet was washed three times with 150 mM NaCl to remove remaining cell debris from inclusion bodies. The inclusion bodies were solubilized by resuspension in 5 ml of 50 mM anaerobic MOPS-KOH buffer (pH 7.0) containing 7 M urea and 10 mM dithiothreitol (DTT). From this stage onwards, all steps were performed

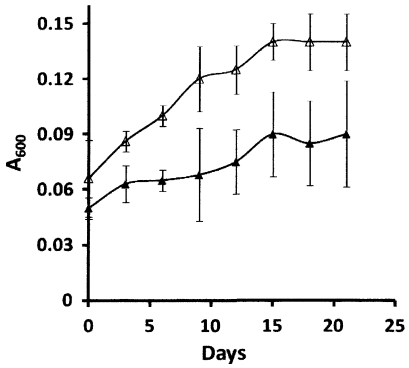

FIG 1 Growth of *Methanosarcina acetivorans* with a limiting partial pressure of CO. Growth was with 1.01×10^4 Pa (0.1 atm) of CO in the headspace. The inoculum was CO-grown cells. Symbols: Δ, wild-type strain WWM1; ▲, mutant strain deleted for MA4384 [DmtsF(26)].

in dim light under strictly anaerobic conditions in an inert-atmosphere glove bag.

The concentration of urea in the solution (6.5 ml) containing solubilized CmtA was reduced stepwise as follows. The solution was mixed with 153.5 ml of 50 mM MOPS-KOH buffer (pH 7.0) containing 10 mM DTT. This solution was concentrated to 60 ml using a Vivacell (Sartorius Stedim Biotech GmbH, Gottingen, Germany) pressure-operated system fitted with a 50,000-molecular-weight cutoff membrane. Buffer (102.5 ml) was added to the concentrated solution, followed again by concentration to 75 ml. Finally, buffer (75 ml) was added to this solution, followed by concentration to 1.5 ml. SDS-PAGE revealed CmtA was greater than 95% pure.

The purified protein was reconstituted with either methylcob(III)alamin or aquocobalamin by following published procedures for the MtaC corrinoid-containing protein from *Methanosarcina barkeri* (29) with the following modifications. The cobalamins (300 μM final concentration) and $ZnCl_2$ (75 μM final concentration) were added to the solubilized protein and incubated at 21°C for 2 h with gentle agitation in the dark. Unbound cofactors and zinc were removed by passage through a PD-10 gel filtration column (GE Healthcare, Buckinghamshire, United Kingdom). Protein in the pass-through fraction was concentrated by filtration to 1.0 ml. The cobalt content was determined by inductively coupled plasma emission spectroscopy at the Center for Isotope Studies, University of Georgia, Athens, GA.

Methyltransferase assay. Methylation of HS-CoM was assayed as described previously for the membrane-bound CH_3-THMPT:HS-CoM methyltransferase of *Methanosarcina mazei* (basonym, *Methanosarcina* strain Gö1) (1, 3) by measuring the disappearance of HS-CoM based on thiol analysis using Ellman's reagent and an extinction coefficient for the 2-nitro-5-thiobenzoate dianion of 13,600 M^{-1} cm^{-1} (13). When activity in extracts was assayed, a final concentration of 5 mM potassium–2-bromoethanesulfonate was included to inhibit CH_3-SCoM reductase activity. When DMS was the methyl donor, the methylthiol produced was removed prior to the detection of HS-CoM as follows. Protein was precipitated by the addition of cold acetone (−20°C) to 10 μl of assay mix and centrifuged. Methylthiol was removed from the supernatant solution by spinning in a Speed Vac for 20 min. Controls with a range of methylthiol (0.5 to 20 mM) added to assay mixtures confirmed complete removal.

The methylcob(III)alamin:HS-CoM methyltransferase gel activity stain was performed as previously described (36). Development of activity was dependent on both methylcob(III)alamin and HS-CoM.

RESULTS

Role in CO-dependent growth. All previous investigations (4, 25) of the FCMT homologs were conducted with wild-type and mutant strains of *M. acetivorans* grown with a CO partial pressure of 1.5×10^5 Pa (1.5 atm), which is not growth limiting, and it is considerably greater than concentrations likely to occur in the environment. The FCMT homolog encoded by MA4384 (CmtA) is reported to be upregulated to the highest level among the three homologs (18) and was the primary focus of previous investigations (25). Thus, growth of the wild type versus the MA4384 deletion mutant Δ*cmtA* [DmtsF(26)] strain was undertaken with a growth-limiting partial pressure of 1.01×10^4 Pa (0.1 atm) CO (Fig. 1). Growth of the Δ*cmtA* strain was significantly less in terms of both doubling times (30 versus 13 days) and final yield (A_{600}, 0.09 versus 0.14) than that of the wild type. No growth was observed in the absence of CO for either strain. The results indicate that CmtA is required for optimal growth when cultured with a growth-limiting partial pressure of CO.

Distribution of CH_3-THMPT:HS-CoM methyltransferase activity in the soluble and membrane fractions of CO- versus methanol-grown *M. acetivorans*. Previous quantitative proteomic analysis of *M. acetivorans* revealed a decrease in the level of several subunits belonging to the membrane-bound CH_3-THSPT:HS-CoM methyltransferase (MtrA-H) complex and a substantially larger 50-fold increase in the FCMT homolog encoded by MA0859 in CO- versus methanol-grown cells (18). With translational fusions, it was also shown that CO-grown *M. acetivorans* contains at least 320-fold higher levels of CmtA than methanol-grown cells (4). If CmtA (MA4384) and the homolog encoded by MA0859 are soluble CH_3-THSPT:HS-CoM methyltransferases as previously proposed (18), the ratio of activity in the soluble versus membrane fraction should be greater in CO-grown cells than in methanol-grown cells. The membrane and soluble fractions were cleanly isolated by discontinuous sucrose concentration gradients and assayed with THMPT, a structural and functional analog of THSPT (20, 40), from the methanogen *Methanothermobacter marburgensis*. The activity ratio in methanol-grown *M. acetivorans* was 0.16 (Table 2), similar to the ratio of 0.13 reported for methanol-grown *M. mazei* (3), whereas the ratio in CO-grown *M. acetivorans* was nearly 15-fold greater (Table 2). Furthermore, no transmembrane-spanning regions were detected in the sequences

TABLE 2 Methyl-THMPT:HSCoM methyltransferase activity in the soluble and membrane fractions of CO- or methanol-grown *Methanosarcina acetivorans*

	Total protein (mg)		Total activity[a] (U)		Sp act (U/mg)		Total activity (%)	
Fraction	CH_3OH	CO	CH_3OH	CO	CH_3OH	CO	CH_3OH	CO
Extract	50.0	50.0	6.15 ± 0.45	10.9 ± 1.1	0.12 ± 0.02	0.22 ± 0.03	100	100
Soluble	37.5	41.4	0.79 ± 0.15	7.5 ± 0.8	0.02 ± 0.005	0.18 ± 0.02	12.7	69.0
Membrane	4.5	3.5	4.77 ± 0.43	3.2 ± 0.2	1.06 ± 0.06	0.92 ± 0.08	77.5	29.0

[a] The 100-μl assay was performed at 37°C in 50 mM MOPS-KOH (pH 7.0) containing 100 μg CmtA reconstituted with aquocobalamin, 1 mM Ti(III)-citrate, 5 mM methyl viologen, 10 mM $MgCl_2$, 10 mM ATP, 5 mM methyl donor, 5 mM HS-CoM, and 5 mM potassium 2-bromoethanesulfonate. Unit (U) indicates μmol CH_3-S-CoM/min.

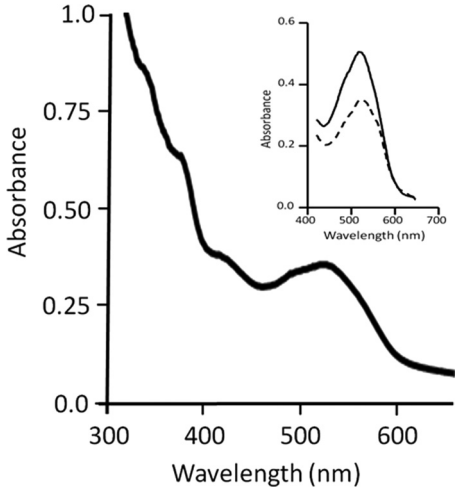

FIG 2 UV-visible spectrum of CmtA. The protein (4.5 mg/ml) was reconstituted with methylcob(III)alamin. The inset shows results before (solid line) and after (dashed line) the addition of 5 mM HS-CoM (final concentration).

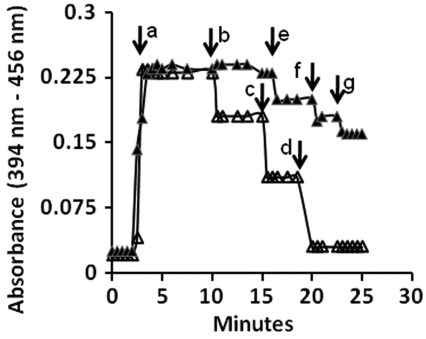

FIG 3 Reduction of cobamide bound to CmtA and methylation with CH_3-THMPT or DMS. The reaction mixture (80-μl final volume) contained 10 μM (final concentration) CmtA reconstituted with aquocobalamin in 50 mM MOPS-KOH buffer (pH 7.0). Reactions were performed at 21°C. At the time indicated by the arrow labeled a, 2.5 μl of 32 mM Ti(III)-citrate (1.0 mM final concentration) was added to reduce aquocobalamin. Additions of methyl donors were made at time points indicated by the arrows: b and e, 1.0 nmol each of CH_3-THMPT and DMS, respectively; c and d, 2.0 nmol of CH_3-THMPT; f and g, 2.0 nmol of DMS. Absorbencies were corrected for dilution when adding reagents. Symbols: Δ, change in absorbance on addition of CH_3-THMPT; ▲, change in absorbance on addition of DMS.

deduced from MA0859, MA4384, or MA4558 evaluated with the TMPRED program (15). These results are consistent with the previously proposed role of a soluble CH_3-THSPT:HS-CoM methyltransferase for the protein encoded by MA0859 and homologs MA4384 and MA4558 (18).

Methyl-THMPT:HS-CoM methyltransferase activity of CmtA. The biochemical characterization of any FCMT homolog has yet to be reported. Thus, the product of MA4384 (CmtA) was overproduced in *E. coli* and purified to determine the enzymatic activities. The protein was solubilized from inclusion bodies and reconstituted with methylcob(III)alamin. SDS-PAGE of the purified protein showed a single band corresponding to the predicted monomeric molecular mass of 69 kDa (see Fig. S1 in the supplemental material). Preparations were pink in color, which is typical of corrinoid-containing proteins. The UV-visible spectrum (Fig. 2) showed absorbance maxima at 370 and 420 nm with additional broad absorbance between 450 and 600 nm, containing maxima at 525 nm and shoulders at 490 and 550 nm, which is characteristic of methylated corrinoid-containing proteins of methane-producing species (14, 22, 36). Metal analysis of 6 preparations revealed 1.0 ± 0.2 mol of cobalt per mol of protein, indicating the presence of approximately one corrinoid cofactor bound to each CmtA molecule.

Nondenaturing gels stained positive for methylcob(III)alamin:HS-CoM methyltransferase activity with the nonphysiological substrate methylcob(III)alamin (see Fig. S1 in the supplemental material). Furthermore, incubation of methylcob(III)alamin-reconstituted CmtA with HS-CoM resulted in decreased absorbance at 525 nm (Fig. 2, inset), indicating loss of the corrinoid-containing methyl group to HS-CoM. These results indicated the potential for CH_3-THSPT:HS-CoM or DMS:HS-CoM methyltransferase activity. Methyltransferase activity was further investigated by first addressing if CmtA reconstituted with aquocobalamin was competent to accept methyl groups from CH_3-THMPT or DMS (Fig. 3). The reduced Co(I) cobamide form of the cofactor was first generated by reaction with 1 mM Ti(III)-citrate as revealed by the rapid development of absorbance at 394 nm (Fig. 3). Each successive addition of methyl group donor to the reduced enzyme decreased the absorbance at 394 nm relative to that at 456 nm, indicating the generation of base-off Co(III)-methyl cobamide that was observed previously for corrinoid-containing methyl transfer enzymes from species of the genus *Methanosarcina* (11, 16). These results suggest a mechanism in which the methyl group of CH_3-THMPT is transferred to the corrinoid cofactor and subsequently transferred to HS-CoM. The transfer of methyl groups from the addition of 1 nmol CH_3-THMPT was highly efficient, whereas transfer from the addition of 2 nmol of DMS was substantially less efficient.

Figure 4 shows the CH_3-THMPT:HS-CoM methyltransferase activity of CmtA reconstituted with aquocobalamin. The specific activity with saturating concentrations of substrates was 0.31

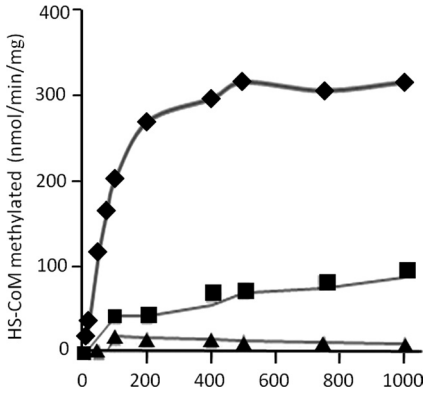

FIG 4 Time course of methyl group transfer from CH_3-THMPT, CH_3-THF, or DMS to HS-CoM catalyzed by CmtA. The reaction was performed at 37°C in 50 mM MOPS-KOH buffer (pH 7.0) containing 100 μg of aquocobalamin-reconstituted CmtA, 1.0 mM Ti(III)-citrate, 5 mM methyl viologen, 10 mM $MgCl_2$, 10 mM ATP, 5 mM methyl donor, and 5 mM HS-CoM in a final volume of 100 μl. Methylation of HS-CoM was determined by measuring the disappearance of HS-CoM as described in Materials and Methods. When DMS was the methyl donor, samples were processed for removal of methylthiol before determining HS-CoM as described in Materials and Methods. Symbols: ◆, CH_3-THMPT; ■, CH_3-THF; ▲, DMS.

μmol/min/mg with CH_3-THMPT and 0.18 μmol/min/mg with CH_3-THF under the conditions tested. The ability of CH_3-THF to substitute for CH_3-THMPT is consistent with that reported for the membrane-bound CH_3-THMPT:HS-CoM methyltransferase (MtrA-H) of *M. mazei* (2). Low levels of DMS:HS-CoM methyltransferase activity were observed early in the time course, although they were not sustained (Fig. 4). The enzyme displayed normal Michaelis-Menten kinetics, revealing apparent K_m values of 135 μM for CH_3-THMPT and 277 μM for HS-CoM. These results support a physiological role for CmtA in catalyzing a soluble CH_3-THSPT:HS-CoM methyltransferase activity.

Sequence analysis. A BLASTP (http://blast.ncbi.nlm.nih.gov) search of the nonredundant databases was performed with the query sequence deduced from MA4384. The first five retrieved sequences were with full-length coverage (99%) and 54 to 57% identity to sequences deduced from FCMT homologs MA0859 and MA4558 and proteins annotated as methyltransferase cognate corrinoid proteins (see Fig. S2 in the supplemental material) from the methanogens *Methanohalobium evestigatum* (basonym, *Methanohalobium evestigatus*), *Methanohalophilus mahii*, and *Methanosalsum zhilinae* (basonym, *Methanohalophilus zhilinae*). The remaining 45 retrieved sequences were from diverse corrinoid-containing proteins distributed among diverse species belonging to the domains *Bacteria* and *Archaea*, having less than 35% identity and 30% coverage confined to the first 220 N-terminal residues of CmtA. The results confirm the previously reported sequence identity of the N-terminal domain to corrinoid-containing proteins (8). Since many enzymes with diverse functions contain corrinoid cofactors, the C-terminal domain of CmtA most likely determines the specific enzyme activity. Thus, the only candidate homologs of CmtA are those retrieved sequences annotated as methyltransferase corrinoid cognate proteins with identity to both the N- and C-terminal domains of CmtA. The putative homologs are from species which convert the methyl groups of methylamines or methanol to methane, and all except *M. evestigatum* are reported to metabolize DMS (21, 26, 27, 34, 42). However, none of these species were examined for the ability to grow with CO except *M. acetivorans* (18, 28), precluding assignment of the putative methyltransferase corrinoid cognate proteins to a specific pathway of methanogenesis. Nonetheless, the BLASTP search results indicate that FCMT homologs are limited to only a few methanogenic species, suggesting a novel physiological function consistent with the results documenting CH_3-THSPT:HS-CoM methyltransferase activity of CmtA.

It was previously reported that *M. acetivorans* FCMT homologs function exclusively as DMS:HS-CoM methyltransferases based only on growth phenotypes of mutant strains in which genes encoding FCMT homologs were deleted or disrupted (25). Thus, the deduced sequence of MA4384 was aligned with the two-component DMS:HS-CoM methyltransferase of *M. barkeri* (see Fig. S3 in the supplemental material) comprised of two nonidentical subunits, MtsA and MtsB, for which the enzyme mechanism is well documented (37, 38). The MtsA subunit binds DMS, transferring a methyl group to the corrinoid-containing MtsB subunit, which donates the methyl group back to the MtsA subunit, where HS-CoM is bound and methylated. MtsB aligned most favorably with 31% identity to N-terminal residues 10 to 191 from the deduced sequence of MA4384, a result consistent with the N-terminal domain binding the corrinoid cofactor. MtsA aligned most favorably with C-terminal residues 270 to 620 from the deduced sequence of MA4384, although with only 19% identity. Nonetheless, the deduced sequences of MA4384 and homologs of MA4384 (see Fig. S2) contain a motif in MtsA ($HXCX_nC$) which binds zinc, which is important for deprotonation of HS-CoM and further supports a role for the C-terminal domain of CmtA in methylation of HS-CoM. Although the low sequence identity with MtsA does not necessarily rule out DMS:HS-CoM methyltransferase activity of CmtA, the results suggest a novel function consistent with the results documenting CH_3-THSPT:HS-CoM methyltransferase activity.

DISCUSSION

The results presented here establish CH_3-THMPT:HS-CoM methyltransferase activity of CmtA, the FCMT homolog encoded by MA4384 in *M. acetivorans*. The previously reported increased levels of FCMT homologs (18) and expression of the encoding genes correlate positively with increased CH_3-THMPT:HS-CoM methyltransferase activity in the soluble protein fraction of CO- versus methanol-grown *M. acetivorans* reported here, a result that is consistent with CH_3-THMPT:HS-CoM methyltransferase activity of CmtA. The identity of CmtA to homologs encoded by MA0859 (51.7%) and MA4558 (53.7%) suggest that they also catalyze the same reaction, although biochemical analyses are necessary to draw conclusions. The results are in contrast to the previous conclusion that FCMT homologs only function in the metabolism of methylsulfides, which leads to the naming of the FCMT homologs MtsD, MtsF, and MtsH (for methyltransferases specific for methylsulfides) encoded by MA0859, MA4384, and MA4558 (25). Based on the results presented here, we propose renaming MtsF as CmtA (cytoplasmic methyltransferase).

Furthermore, the results support a role for CmtA in CO-dependent growth of *M. acetivorans*. Compared to the parental strain, the growth rate and final yield were significantly less for the mutant strain deleted for the gene (MA4384) encoding CmtA when cultured with a growth-limiting partial pressure of CO. This result is in contrast to growth of the same mutant in a pressurized atmosphere of 100% CO, which was previously reported to have no growth defects relative to the parental strain (25). Clearly, CO-limited growth parameters best approximate those expected in the environment from which *M. acetivorans* was isolated (35). The methyltransferase activity of the purified CmtA (0.31 μmol/min/mg), which was assayed with CH_3-THMPT, was found to be substantially lower than that reported (7.0 μmol/min/mg) for the membrane-bound CH_3-THSPT:HS-CoM methyltransferase (MtrA-H) purified from acetate-grown *M. mazei* and assayed with CH_3-THMPT (20). Less than optimal activity of CmtA could result from substitution of the commercially available cobalamins (dimethylbenzimidazolylcobamide) for reconstitution of CmtA in place of 5-hydroxybenzimidazolylcobamide, which is present in species of the genus *Methanosarcina* (31). Regardless, a relatively low level of activity for CmtA is consistent with the ≥50-fold increase in levels of CmtA and FCMT homologs reported for CO- versus methanol- or acetate-grown cells to achieve the greater ratio of soluble versus membrane-bound CH_3-THMPT:HS-CoM methyltransferase activities in CO-grown cells compared to methanol-grown cells reported here. Nevertheless, the results unequivocally establish the CH_3-THMPT:HS-CoM methyltransferase activity of CmtA.

Overall, the results support the previously postulated role for CmtA and homologs in supplementing the sodium-pumping

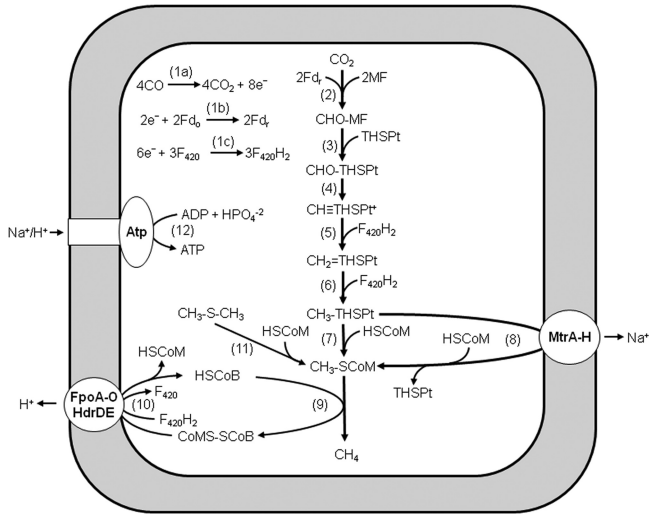

FIG 5 Pathway proposed for the conversion of CO to CH_4 by *Methanosarcina acetivorans*. Fd_o, oxidized ferredoxin; Fd_r, reduced ferredoxin; F_{420}, coenzyme F_{420}; MF, methanofuran; THSPt, tetrahydrosarcinapterin; HSCoM, coenzyme M; HSCoB, coenzyme B; FpoA-O, $F_{420}H_2$ dehydrogenase complex; HdrDE, heterodisulfide reductase; MtrA-H, CH_3-THSPt:HS-CoM methyltransferase; and Atp, Na^+/H^+-dependent ATP synthase.

CH_3-THSPT:HS-CoM methyltransferase complex MtrA-H during CO-dependent growth of *M. acetivorans* (18). This role for CmtA is indicated by defective growth of the mutant deleted for the encoding gene when cultured with a growth-limiting partial pressure of CO. It is conceivable that with low partial pressures of CO, the lower available free energy limits reactions coupled to the energy-requiring generation of ion gradients that drive ATP synthesis. Only two reactions in the CO-dependent CO_2-reducing pathway of *M. acetivorans* generate ion gradients (Fig. 5) (18), the sodium-pumping CH_3-THSPT:HS-CoM methyltransferase (MtrA-H) (Fig. 5, reaction 8) and the proton-pumping FpoA-O complex, which donates electrons to the heterodisulfide reductase (HdrDE), catalyzing the final reductive step (Fig. 5, reaction 10). The soluble CmtA and homologs potentially provide a mechanism for bypassing MtrA-H (Fig. 5, reaction 7), allowing growth at otherwise prohibitively low CO concentrations and equipping the cell to accommodate fluctuations in the CO concentrations that are encountered in the environment, thereby maximizing the thermodynamic efficiency for optimal ATP synthesis and growth by partitioning methyl transfer through CmtA and MtrA-H. Under laboratory conditions where cells are routinely cultured with greater than 1.0 atm of CO, CmtA and homologs would be dispensable, explaining why no growth defect was previously reported for *M. acetivorans* mutants cultured with 1.5 atm CO (25).

It is interesting that genes encoding *M. acetivorans* FCMT homologs are not present in the genomes of phylogenetically related *M. barkeri* or *M. mazei*. In contrast to *M. acetivorans* (12, 35), *M. barkeri* and *M. mazei* utilize H_2 as a reductant in the CO_2 reduction pathway, which is derived from the metabolism of fermentative and syntrophic species and is significantly more abundant than CO in the environment. Thus, *M. barkeri* or *M. mazei* is not strictly dependent on low concentrations of CO for growth, although conversion of CO to H_2 could supplement abundant H_2 already available from fermentative and syntrophic species in the environment. Indeed, *M. barkeri* grows with 0.5 atm of CO by first converting it to H_2 (24). Growth of *M. barkeri* or *M. mazei* at lower partial pressures of CO as a sole energy source, as demonstrated here for *M. acetivorans*, has not been reported.

Mutants of *M. acetivorans* with deleted or disrupted genes encoding FCMT homologs, including CmtA, are defective in growth and methanogenesis with DMS, leading to the previous conclusion that FCMT homologs function exclusively in the pathway for conversion of DMS to CH_4 (Table 1, reaction 11) (25). In the proposed pathway, FCMT homologs catalyze transfer of the methyl groups of DMS to HS-CoM (Fig. 5, reaction 11). Three molecules of CH_3-S-CoM are reductively demethylated with three electron pairs, producing CH_4 (Fig. 5, reaction 9). The methyl group from one molecule of CH_3-S-CoM is transferred to THSPT for oxidation to CO_2 by reversal of reactions 2 to 6, which supply the three electron pairs for reductive demethylation of CH_3-S-CoM. Transfer of methyl groups from CH_3-S-CoM to THSPT is accomplished by the reversal of reaction 8 (Fig. 5) ($\Delta G^{\circ\prime} = 30$ kJ/mol), which is catalyzed by MtrA-H and driven by a sodium gradient. The results presented here neither support nor preclude the proposed role of FCMT homologs in the pathway. Although it is substantially less robust than CH_3-THMPT, DMS served as a methyl donor to CmtA, predicting DMS:HS-CoM methyltransferase activity based on the demonstrated competency of CmtA to methylate HS-CoM. The weaker methylation with DMS and unsustained activity is explained in part by the unfavorable thermodynamics ($\Delta G^{\circ\prime} = 17.9$ kJ/mol) predicted from the equilibrium constant for the DMS:cob(I)alamin methyltransferase reaction mediated by the MtsA component of the DMS:HSCoM methyltransferase from *M. barkeri* (38). It is also possible that the methylthiol product inhibits the enzyme. Thus, no firm conclusions can be drawn from these negative results regarding the potential for DMS:HSCoM methyltransferase activity of CmtA. However, although the thermodynamics for conversion of DMS to CH_4 (33) predict good growth (Table 1, reaction 11), DMS-dependent growth of wild-type *M. acetivorans* is exceedingly poor, with final optical densities of ~0.1 and maximum doubling times of 60 h (25). The poor growth suggests DMS is not a preferred substrate for *M. acetivorans*. The poor growth with DMS can be explained by the most plausible mechanism for CmtA. The mechanism is analogous to the three- and two-component methyltransferase systems of methanogens, wherein a subunit transfers the substrate methyl group to a corrinoid-containing subunit, which then donates the methyl group to the subunit, which binds and methylates HS-CoM. In the mechanism postulated for CH_3-THMPT:HS-CoM methyltransferase activity of CmtA, the C-terminal domain binds CH_3-THSPT and transfers the methyl group to the corrinoid cofactor of the N-terminal domain, which then returns the methyl group to the C-terminal domain, where HS-CoM is bound and methylated, producing CH_3-S-CoM. The robust CH_3-THMPT:HS-CoM methyltransferase activity demonstrated for CmtA suggests that FCMT homologs evolved with this as the primary function. The proposed mechanism for the predicted DMS:HS-CoM methyltransferase activity is similar, with the possible exception that DMS is a fortuitous methyl donor that could explain poor growth with DMS (25).

Although the reported doubling times are not significantly different between the wild-type and Δ*cmtA* [DmtsF(26)] strains of *M. acetivorans* cultured with trimethylamine, it is also reported that levels of a *uidA* translational fusion to *cmtA* [*mtsF*(26)] in-

crease severalfold in wild-type *M. acetivorans* cultured with monomethylamine, dimethylamine, or trimethylamine versus CO, which leads to the conclusion that CmtA [MtsF(26)] is involved in the metabolism of methylotrophic substrates via an undetermined mechanism (4). Clearly, further research is needed to resolve the role of CmtA in the metabolism of methylotrophic substrates.

Conclusions. The previous conclusion that FCMT homologs only catalyze DMS:HS-CoM methyltransferase activity was based solely on the analysis of mutants with no biochemical characterization of enzyme activity. The results reported here document the CH_3-THMPT:HS-CoM methyltransferase activity of CmtA, the FCMT homolog encoded by the MA4384 gene. Thus, we propose renaming MA4384 with the designation CmtA (cytoplasmic methyl transferase), for which the methyl donor is not specified. The results also support a role for CmtA in the CO-dependent pathway of methanogenesis when cultured with growth-limiting partial pressures of CO. Finally, the results call for further biochemical investigations to determine the function of other FCMT homologs in *M. acetivorans* and other methanogenic species reported here.

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