Genetic Basis for Metabolism of Methylated Sulfur Compounds in *Methanosarcina* Species

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ABSTRACT

***Methanosarcina acetivorans* uses a variety of methylated sulfur compounds as carbon and energy sources. Previous studies implicated the *mtsD*, *mtsF*, and *mtsH* genes in catabolism of dimethylsulfide, but the genes required for use of other methylsulfides have yet to be established. Here, we show that a four-gene locus, designated *mtpCAP-msrH*, is specifically required for growth on methylmercaptopropionate (MMPA). The *mtpC*, *mtpA*, and *mtpP* genes encode a putative corrinoid protein, a coenzyme M (CoM) methyltransferase, and a major facilitator superfamily (MFS) transporter, respectively, while *msrH* encodes a putative transcriptional regulator. Mutants lacking *mtpC* or *mtpA* display a severe growth defect in MMPA medium but are unimpaired during growth on other substrates. The *mtpCAP* genes comprise a transcriptional unit that is highly and specifically upregulated during growth on MMPA, whereas *msrH* is monocistronic and constitutively expressed. Mutants lacking *msrH* fail to transcribe *mtpCAP* and grow poorly in MMPA medium, consistent with the assignment of its product as a transcriptional activator. The *mtpCAP-msrH* locus is conserved in numerous marine methanogens, including eight *Methanosarcina* species that we showed are capable of growth on MMPA. Mutants lacking the *mtsD*, *mtsF*, and *mtsH* genes display a 30% reduction in growth yield when grown on MMPA, suggesting that these genes play an auxiliary role in MMPA catabolism. A quadruple Δ*mtpCAP* Δ*mtsD* Δ*mtsF* Δ*mtsH* mutant strain was incapable of growth on MMPA. Reanalysis of *mtsD*, *mtsF*, and *mtsH* mutants suggests that the preferred substrate for MtsD is dimethylsulfide, while the preferred substrate for MtsF is methanethiol.**

IMPORTANCE

Methylated sulfur compounds play pivotal roles in the global sulfur and carbon cycles and contribute to global temperature homeostasis. Although the degradation of these molecules by aerobic bacteria has been well studied, relatively little is known regarding their fate in anaerobic ecosystems. In this study, we identify the genetic basis for metabolism of methylmercaptopropionate, dimethylsulfide, and methanethiol by strictly anaerobic methanogens of the genus *Methanosarcina*. These data will aid the development of predictive sulfur cycle models and enable molecular ecological approaches for the study of methylated sulfur metabolism in anaerobic ecosystems.

Methylated sulfur compounds (generically known as methylsulfides), including methylmercaptopropionate (MMPA), dimethylsulfide (DMS), and methanethiol (MeSH), are abundant in many ecosystems and play pivotal roles in the global sulfur and carbon cycles (1, 2). The predominate source of methylsulfides in marine systems is believed to be dimethylsulfoniopropionate (DMSP), a compatible solute whose synthesis by marine algae is estimated to account for 1 to 10% of global primary production (3). DMSP is degraded via two distinct microbial metabolic processes: the cleavage pathway and the demethylation pathway (1). The majority of DMSP (50 to 90%) is degraded via the demethylation pathway, producing MMPA, which is subsequently converted to 3-mercaptopropionate or MeSH (4, 5). MMPA and MeSH are often assimilated by marine microorganisms, thus retaining sulfur in the marine microbial food web (5). The cleavage pathway, on the other hand, generates volatile DMS, which is the primary contributor to the ocean-atmosphere sulfur flux (6) and a precursor to aerosol particles that facilitate cloud condensation, thus contributing to the global temperature homeostasis (7).

While the aerobic catabolism of DMSP and its methylsulfide by-products has been studied extensively (1, 2), considerably less is known regarding the fate of these compounds in anaerobic ecosystems. In anoxic marine sediments, DMSP is ultimately converted to methane; however, pure cultures of methanogenic archaea are not known to use DMSP (8). Instead, it is likely that anaerobic bacteria convert DMSP into MMPA and DMS, which are then used by methanogens. This conclusion is based on the observations that DMS is produced via the cleavage pathway in certain clostridia (9) and that both sulfate-reducing and acetogenic bacteria produce MMPA via the demethylation pathway (10–12). Significantly, it was noted that MMPA was not further metabolized by these anaerobic bacteria (11). However, methylsulfides, including DMS, MMPA, and MeSH, are known to be substrates for the growth of methanogenic archaea (8, 13, 14). Several members of the order *Methanosarcinales* have been reported to grow on DMS (13), while *Methanosarcina* sp. strain

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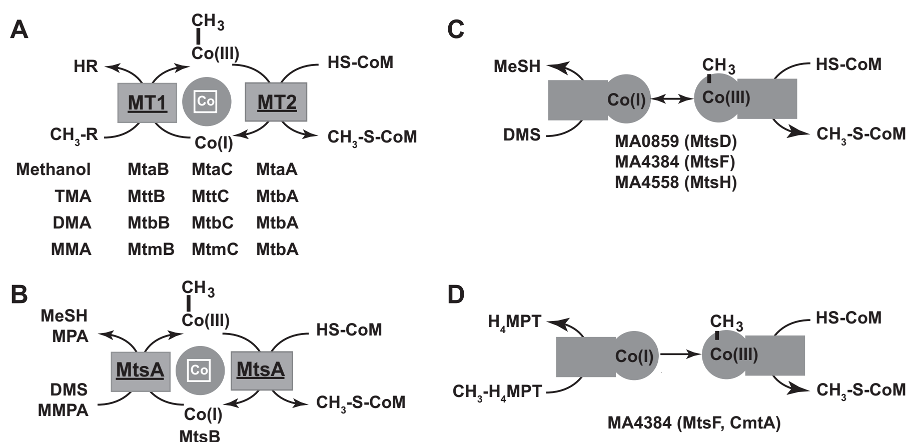

FIG 1 *Methanosarcina* enzymes involved in C-1 metabolism. (A) Schematic of the three-subunit, substrate-specific MT1/MT2 system for activation of methanol and methylamines found in all *Methanosarcina* species. The designations for specific MT1/MT2 enzymes and their cognate substrates are shown. (B) Schematic of the biochemically characterized two-subunit methylsulfide MT1/MT2 system from *M. barkeri*. (C) Schematic of the putative function of a single-subunit DMS MT1/MT2 system, based on genetic analysis of the *mtsD*, *mtsF*, and *mtsH* genes of *M. acetivorans* (19). (D) Schematic of the putative function of an H_4MPT:CoM methyltransferase system encoded by the *mtsD*, *mtsF*, and *mtsH* genes of *M. acetivorans*, based on biochemical analysis of the MtsF (CmtA) protein (21).

MTP4 (14), *Methanosarcina siciliae* strain HI350, and *Methanolobus taylorii* strain GS-16 have been shown to grow on MeSH (15). Methanogenic catabolism of MMPA is less common, with *Methanosarcina* sp. strain MTP4, *Methanosarcina acetivorans* strain C2A, and *Methanosarcina siciliae* strain T4/M being the only pure cultures known to use this compound (8). Despite numerous reports demonstrating the use of methylsulfides by methanogens, mechanistic studies of methylsulfide metabolism have only been carried out in *Methanosarcina barkeri* and *M. acetivorans*.

In methylotrophic methanogens, the methyl moiety from C-1 compounds like methanol, methylamines, and methylsulfides is channeled into methanogenesis via transfer from the substrate to coenzyme M (CoM) (reviewed in reference 16). This C-1 activation reaction is catalyzed by a series of substrate-specific CoM methyltransferases. Typically, these enzymes consist of a two-subunit methyltransferase 1 (MT1) component that transfers the methyl group from the substrate to a corrinoid carrier protein and a single-subunit methyltransferase 2 (MT2), which then transfers the methyl group from the corrinoid protein to CoM (Fig. 1). A variation on this theme was discovered during the characterization of a 480-kDa corrinoid protein purified from acetate-grown *Methanosarcina barkeri* strain MS (17, 18). This two-subunit enzyme complex, comprised of the MtsA and MtsB proteins, is capable of methylating CoM using either DMS or MMPA as the substrate. It is believed that MtsA catalyzes the methyl transfer reaction from DMS to the corrinoid subunit, MtsB, as well as the subsequent transfer from methyl-MtsB to the CoM (17). Nevertheless, *M. barkeri* MS does not utilize either DMS or MMPA as a sole growth substrate (8, 18), and thus, the biological function of this interesting enzyme has yet to be established. A family of single-subunit CoM methyltransferases, encoded by the *MA0859*, *MA4384*, and *MA4558* loci of *M. acetivorans*, has also been characterized. These proteins are comprised of an N-terminal corrinoid domain and a C-terminal MT2 domain. Genetic studies showed that these proteins are both required and sufficient for the synthesis and catabolism of DMS, leading to their designation as MtsD, MtsF, and MtsH, respectively (19, 20). It was suggested that these proteins catalyze a DMS:CoM methyltransferase reaction analogous to that catalyzed by the MtsA/B system of *M. barkeri* (19). However, biochemical characterization of MA4384 (MtsF) showed that the enzyme is capable of catalyzing the transfer of methyl groups from methyl-tetrahydromethanopterin (H_4MPT) to CoM. DMS also served as a methyl donor, but at lower rates, prompting these authors to rename the protein CmtA (21). Thus, although the *MA0859*, *MA4384*, and *MA4558* loci are clearly involved in DMS metabolism, the mechanism remains obscure.

Analysis of the *M. acetivorans* genome reveals numerous genes that appear to encode MT1/MT2 homologs, including 10 MT1 methyltransferase subunits, 15 MT1 corrinoid subunits, and 13 MT2 proteins (22). While most of these can be confidently assigned to known substrates, several remain unassigned. Among the known substrates used by *M. acetivorans*, only MMPA and MeSH have yet to be characterized at a genetic level. Thus, we suspected that some of the unassigned MT1/MT2 homologs might be involved in the use of these substrates. Here, we show that the *MA4164* to *MA4166* locus (which we designated the *mtpCAP* operon) is specifically required for MMPA metabolism and that its expression is positively regulated by MA4167 (MsrH) when MMPA is present. We also reanalyzed the phenotypes of Δ*mtsD*, Δ*mtsF*, and Δ*mtsH* mutants, showing that these genes contribute to the efficient metabolism of MMPA and that MtsD is the primary enzyme for DMS metabolism, while MtsF is the primary enzyme for MeSH metabolism. MtsH, however, appears to accept both substrates.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *Methanosarcina* strains used in the study are described in Table 1. The *Methanosarcina* strains were grown in single-cell morphology (23) at 37°C using high-salt (HS) broth containing one or more of the following substrates: 125 mM methanol, 50 mM trimethylamine (TMA), 50 mM dimethylamine (DMA), 50 mM monomethylamine (MMA), 120 mM acetate (24), 20 mM MMPA, 20 mM DMS, or 20 mM MeSH. Growth on medium solidified with 1.5% agar was as described previously (25). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic incubator as described previously (26). Puromycin (CalBiochem, San Diego, CA) was added from sterile, anaerobic stocks at a final concentration of 2

TABLE 1 Strains used in the study[a]

Strain	Genotype or description	Source or reference
M. acetivorans C2A	Wild type	DSM 2834
WWM82	Δ*hpt*::(P*mcrB*-ϕC31 *int-attP*)	31
WWM829	Δ*hpt* Δ*mtpCAP*	This study
WWM830	Δ*hpt* Δ*mtpC*	This study
WWM831	Δ*hpt* Δ*mtpA*	This study
WWM832	Δ*hpt* Δ*mtpP*	This study
WWM833	Δ*hpt* Δ*msrH*	This study
WWM898	Δ*hpt*::pFH009 Δ*mtpC*	This study
WWM899	Δ*hpt*::pFH010 Δ*mtpA*	This study
WWM900	Δ*hpt*::pFH013A Δ*msrH*	This study
WWM901	Δ*hpt*::pFH012 Δ*mtpCAP*	This study
WWM810	Δ*hpt* Δ*mtsD*::*frt*	19
WWM811	Δ*hpt* Δ*mtsF*::*frt*	19
WWM812	Δ*hpt* Δ*mtsH*::*frt*	19
WWM813	Δ*hpt* Δ*mtsD*::*frt* Δ*mtsF*::*frt*	19
WWM814	Δ*hpt* Δ*mtsD*::*frt* Δ*mtsH*::*frt*	19
WWM815	Δ*hpt* Δ*mtsF*::*frt* Δ*mtsH*::*frt*	19
WWM816	Δ*hpt* Δ*mtsD*::*frt* Δ*mtsF*::*frt* Δ*mtsH*::*frt*	19
WWM897	Δ*hpt* Δ*mtsD*::*frt* Δ*mtsF*::*frt* Δ*mtsH*::*frt* Δ*mtpCAP*	This study
M. siciliae C2J	Wild type	Laboratory stock from K. Sowers
M. siciliae T4/M	Wild type	DSM 3028
M. siciliae HI350	Wild type	DSM 6564
Methanosarcina sp. Naples 100	Wild type	DSM 8689
Methanosarcina sp. WH1	Wild type	DSM 4659
Methanosarcina sp. MTP4	Wild type	DSM 6636
Methanosarcina sp. WWM596	Wild type	Laboratory stock from K. Sowers

[a] Plasmids and primers used for strain constructions are described in Tables S1 and S2 in the supplemental material.

μg/ml for selection of *Methanosarcina* strains carrying the puromycin transacetylase gene (*pac*) (27, 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 μg/ml for selection against the *hpt* gene. MMPA was prepared by alkaline hydrolysis of methyl-3-methylmercaptopropionate (Sigma-Aldrich, St. Louis, MO) (29) or purchased directly from Tokyo Chemical Industry Co. (Japan).

Construction, verification, and complementation of mutant strains. Plasmids used for creating the *mtpC*, *mtpA*, *mtpP*, *msrH*, and *mtpCAP* mutants were constructed as described in Table S1 in the supplemental material. Individual gene deletions removed most of the coding sequence, leaving behind an in-frame fusion peptide consisting of the first and last 10 amino acids, with the goal of creating nonpolar mutations. The mutated alleles were recombined onto the chromosome using the markerless genetic exchange method as described previously (30). All mutations were verified by PCR and by DNA hybridization experiments, using pFH002 as the probe against restriction endonuclease-digested genomic DNA isolated from each mutant (see Fig. S1). Plasmids used for complementation were constructed using pJK027A, which, in the strain background used, expresses the genes in question from the strong, constitutive p*mcrB-tetO1* promoter (31). Each complementation plasmid was integrated in single copy into the chromosome of its respective mutant via a site-specific recombination method described previously (31).

DNA methods. The plasmid constructions and primers used are described in Tables S1 and S2 in the supplemental material. Standard methods were used throughout for isolation and manipulation of plasmid DNA from *Escherichia coli* (32). Genomic DNA from *M. acetivorans* was isolated as described previously (33). DNA hybridizations were performed using the DIG (digoxigenin) System (Roche, Mannheim, Germany) as recommended by the manufacturer, using MagnaGraph Nylon transfer membranes (Micron Separations, Inc., Westborough, MA). DNA sequencing was performed at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois.

Phenotypic characterization. Growth rates were determined by monitoring the optical densities of three or more independent cultures at 600 nm using a Bausch and Lomb Spectronic 21.

Determination of metabolites. DMS, MeSH, and methane were quantified using a Hewlett-Packard gas chromatograph (5890 series II) equipped with a stainless steel 80/120 Carbopack B column (Supelco; Sigma-Aldrich, St. Louis, MO) and a flame ionization detector. The column, injector, and detector were at 130°C, and the nitrogen flow rate was 22.5 ml/min. Under these conditions, methane, MeSH, and DMS were eluted with retention times of 0.9, 1.9, and 4.0 min, respectively. The partitioning coefficients of DMS (11.2) and MeSH (7.8) between liquid and gas phases were determined using at least six replicates in stoppered Balch tubes containing the desired substrate in 10 ml HS medium. The measured partitioning coefficients did not vary significantly over the concentrations examined, as previously observed (8, 18, 34).

RNA-seq analysis. *M. acetivorans* C2A strains were adapted to different growth media for at least 30 generations prior to RNA isolation. The total RNA was isolated from early exponential-phase cultures using TRIzol (Invitrogen, Carlsbad, CA) and Zymo Direct-zol RNA miniprep kits (Zymo Research, Irvine, CA). The 16S and 23S rRNAs were subtracted using biotin-labeled 16S and 23S RNA probes as previously described (35). Briefly, 16S and 23S rRNA genes were amplified from *M. acetivorans* strain C2A with T7 promoter-appended primers (see Table S2 in the supplemental material) to allow *in vitro* synthesis of biotinylated antisense rRNA probes, which were then hybridized to total RNA prior to removal using streptavidin-coated magnetic beads. Construction and sequencing of libraries on the Illumina HiSeq2000 was performed at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. Briefly, 50 nanograms of rRNA-depleted mRNA was converted to indexed high-throughput RNA sequencing (RNA-seq) libraries with the ScriptSeq version 2 RNA-seq library preparation kit (Epicentre Biotechnologies, Madison, WI). The libraries were pooled in equimolar concentrations and quantified by

quantitative PCR (qPCR) with the Illumina-compatible KAPA library quantification kit (Kapa Biosystems, Woburn, MA). The pooled libraries were sequenced for 101 cycles, plus 7 cycles for the index read, on a HiSeq2000 using TruSeq SBS version 3 reagents. The fastq files were generated and demultiplexed with Casava 1.8.2 (Illumina, San Diego, CA). Further bioinformatics processing was performed using the CLC Genomics Workbench (version 7.0; CLCbio, Aarhus, Denmark). fastq files were imported as high-throughput data and trimmed for quality (quality, 0.001; ambiguous, 2: discard, min 30, max 100) and to remove adapter sequences. After trimming, the remaining reads that mapped to stable RNAs (16S, 23S, and 5S rRNA and all tRNAs) were removed (similarity, 0.9; length, 0.85; mismatch, 2; insertion, 3; deletion, 3; global alignment and stand-alone read). The remaining reads were then mapped to the genome for RNA-seq analyses (mapping parameters, 0.9; length, 0.85; mismatch, 2; insertion, 3; deletion, 3; maximum number of hits for a read, 10). The mapped reads were normalized using the default settings, and differentially expressed genes identified using empirical analysis of differential gene expression (EDGE test) (36). Genes showing fold expression changes greater than 4 or less than −4 with *P* values of <0.01 were considered to be differentially expressed.

Microarray data accession number. The raw and processed RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE64349.

RESULTS

***In silico* analysis of the *M. acetivorans mtpCAP-msrH* locus.** The results of bioinformatics analyses suggested that the *MA4164* to *MA4167* locus, which we designated *mtpCAP-msrH*, is involved in the metabolism of an unknown C-1 compound. The MtpC protein (MA4164) shares 30 to 50% identity with the corrinoid protein subunits of numerous characterized *Methanosarcina* methyltransferase I (MT1) enzymes, being most closely related to the putative DMS methyltransferase MtsD, whereas MtpA (MA4165) is homologous (25% to 31% identity) to MT2 methyltransferase proteins, being most closely related to MtsA from *M. barkeri* (see Fig. S2 in the supplemental material). The MtpP (MA4166) protein is a member of the major facilitator superfamily (MFS) and is likely to be a transporter for the cognate substrate. Lastly, as noted by Reichlen et al. (37), MsrH (MA4167) is a member of the methanol-specific regulator (Msr) family, known to be involved in the transcriptional activation of numerous MT1-/MT2-encoding genes in *Methanosarcina* (20, 38).

Construction and characterization of *mtpCAP-msrH* mutants. To address the *in vivo* function of the *mtpCAP-msrH* locus, we constructed Δ*mtpCAP*, Δ*mtpC*, Δ*mtpA*, Δ*mtpP*, and Δ*msrH* mutants and examined their growth on various substrates (Fig. 2; see also Table S3 in the supplemental material). The mutants were indistinguishable from the parent when grown on methanol, acetate, methylamines, DMS, or MeSH. The parental strain and each of the mutants also transiently accumulate MeSH when grown on DMS and DMS when grown on MeSH (see Fig. S3 and S4). Therefore, the *mtpCAP-msrH* locus is not required for the catabolism of methanol, acetate, methylamines, DMS, or MeSH, nor is it involved in the synthesis of DMS or MeSH. In contrast, the Δ*mtpCAP*, Δ*mtpC*, Δ*mtpA*, and Δ*msrH* mutants were severely impaired in their ability to utilize MMPA as a growth substrate, having ca. one-third of the cell yield, measured by optical density, and lag phases over 200 h longer than that of the parental strain. The Δ*mtpC* and Δ*mtpA* phenotypes were alleviated upon complementation with the respective genes; however, the defect of the Δ*msrH* mutant was not recovered, possibly due to inappropriate levels of MsrH caused by the strong promoter used in the complementation plasmid (31). The Δ*mtpP* strain showed no observable defect during growth on MMPA.

MMPA metabolism in diverse *Methanosarcina* species. Analysis of 30 sequenced *Methanosarcina* genomes revealed 10 strains that contain homologs of the *mtpCAP-msrH* locus (see Fig. S5 in the supplemental material). Seven of eight strains examined grew on MMPA, including *M. acetivorans*, *M. siciliae* C2J, *M. siciliae* HI350, *Methanosarcina* sp. strain WH1, *Methanosarcina* sp. strain WWM596, *Methanosarcina* sp. strain Naples 100, and *Methanosarcina siciliae* T4/M. The two *M. lacustris* strains were not tested due to their very poor growth in our standard medium. Surprisingly, *Methanosarcina* sp. MTP4, which had previously been reported to grow on MMPA (8), did not use this substrate in our experiments. We note that the *Methanosarcina* sp. MTP4 genome carries a duplication of the *mtpCAP-msrH* locus that is not found in any of the other strains, raising the possibility that rearrangement of this genomic region, which may have occurred during serial transfer following its original isolation, altered the ability to use MMPA. This result notwithstanding, these data provide strong, correlative support for the role of the *mtpCAP-msrH* locus in MMPA catabolism.

Reassessment of the roles of *mtsD*, *mtsF*, and *mtsH* in methylsulfide metabolism. Based on the observation that MMPA is a substrate for *M. barkeri* MtsA/B, we suspected that the related *M. acetivorans* MtsD, MtsF, and MtsH proteins might be responsible for the residual MMPA-dependent growth of the *mtpCAP* mutants. To test this, we obtained the previously described (19) *mts* mutants and examined their growth on MMPA (Fig. 2 and Table 2). None of the single or double *mts* mutants differed significantly from the parental strain with respect to growth rate on MMPA; however, when all three genes were deleted, the growth yield was ca. one-third lower than that of the parental strain (Fig. 2). Thus, the MtsD, MtsF, and MtsH proteins play a role in MMPA metabolism in otherwise wild-type cells. The quadruple mutant lacking all three *mts* genes and the *mtpCAP* operon showed no growth in MMPA medium.

We also reexamined the phenotypes of the *mts* mutants during growth on DMS and MeSH (Table 2). Contrary to previously published results (19), we found that strains lacking *mtsD* were incapable of growth on DMS, regardless of the presence or absence of *mtsF* and *mtsH*, whereas strains lacking *mtsF* failed to grow on MeSH, regardless of the presence or absence of *mtsD* and *mtsH*. The *mtsH* gene was not required for growth on either MeSH or DMS; however, the generation times of strains lacking this gene were substantially longer on both substrates, with the exception of the Δ*mtsDH* mutant, which grew significantly faster than the wild-type on MeSH. Interestingly, unlike the parental strain, the Δ*mtsDH* mutant did not produce DMS during growth on MeSH (see Fig. S4 in the supplemental material), suggesting that the higher growth rate may be due to more efficient catabolism of the substrate. It should be noted that our DMS results differ from those obtained by Oelgeschlager et al. (19), who showed that any one of the *mts* genes was sufficient to allow growth on DMS. Although lower levels of DMS were used in the previous study, we obtained similar results at both 5 mM and 20 mM DMA (see Fig. S6). Thus, we believe subtle differences in medium preparation or precultivation conditions may be responsible for the discrepancy.

Transcriptional profiling of *M. acetivorans* grown on methylsulfides. Transcriptional profiling of *M. acetivorans* via RNA-seq provided additional support for the substrate specificities as-

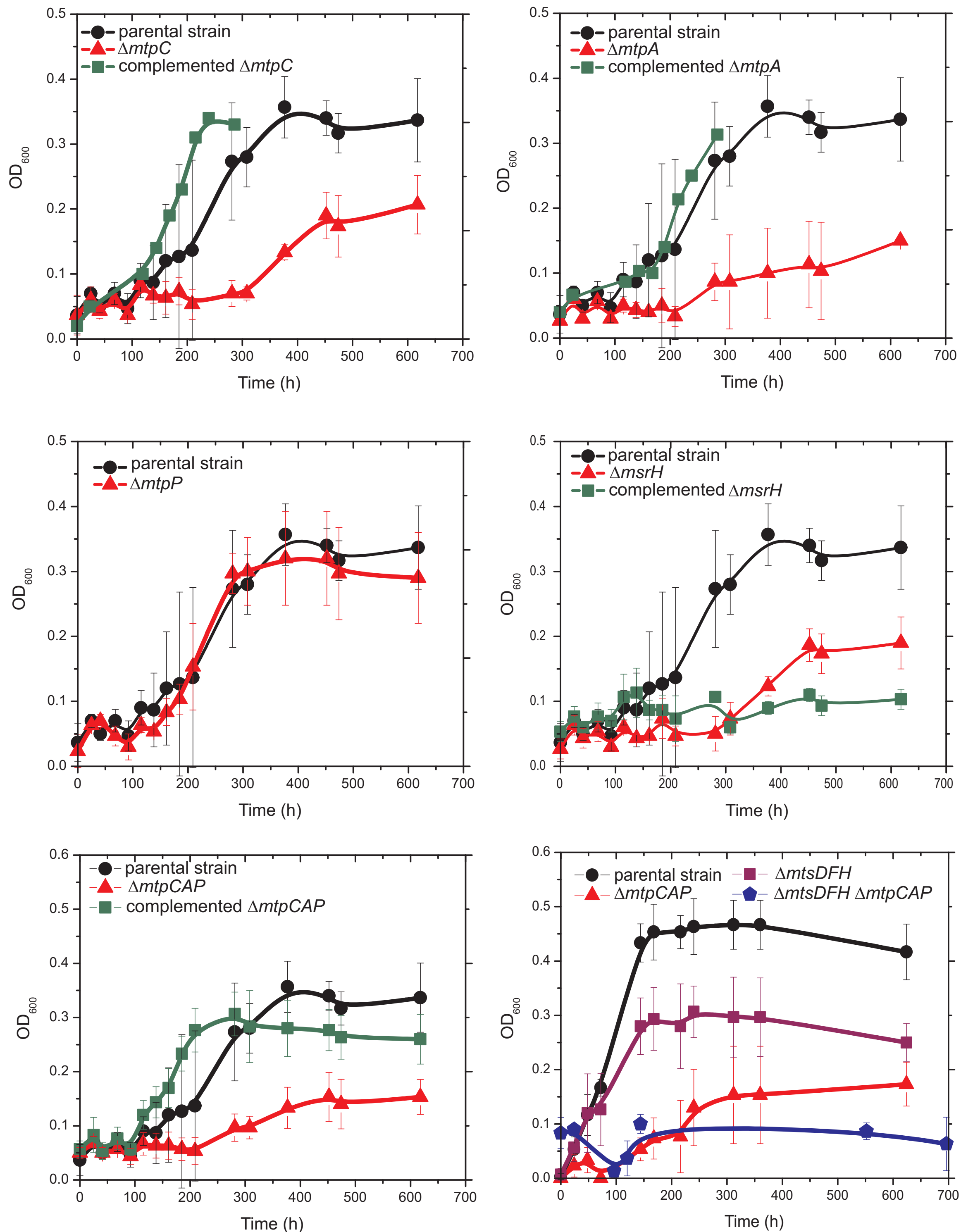

FIG 2 Growth of *M. acetivorans* strains in MMPA medium. The indicated mutants were grown in HS medium with 20 mM MMPA. The strains used were WWM82 (parental strain), WWM830 (Δ*mtpC*), WWM898 (complemented Δ*mtpC*), WWM831 (Δ*mtpA*), WWM899 (complemented Δ*mtpA*), WWM832 (Δ*mtpP*), WWM833 (Δ*msrH*), WWM900 (complemented Δ*msrH*), WWM829 (Δ*mtpCAP*), WWM901 (complemented Δ*mtpCAP*), WWM816 (Δ*mtsDFH*), and WWM897 (Δ*mtpCAP* Δ*mtsDFH*). Error bars represent standard deviations of the results from triplicate cultures. OD_{600}, optical density at 600 nm.

signed to the various methyltransferase genes based on the mutant phenotypes. Accordingly, the *mtpCAP* locus is highly and specifically upregulated during growth on MMPA relative to its transcription during growth on methanol, MeSH, or DMS (Table 3; see also Table S3 in the supplemental material). Moreover, during growth on MMPA, *mtpA*, *mtpC*, and *mtpP* are among the most abundant transcripts in the cell, but their expression is low during growth on the other substrates. The *mtsD*, *mtsF*, and *mtsH* genes

TABLE 2 Generation times of *mtsD*, *mtsF*, and *mtsH* mutants on methylsulfides

Strain genotype	Avg generation time ± SD (h) on[a]: MMPA	DMS	MeSH
C2A WT	44.7 ± 1.9	20.7 ± 3.6	44.8 ± 5.7
Δ*mtsD*	35.3 ± 7.5	No growth	61.3 ± 0.8
Δ*mtsF*	39.6 ± 7.5	31.6 ± 6.6	No growth
Δ*mtsH*	40.4 ± 2.8	71.0 ± 5.1	187.3
Δ*mtsDF*	38.1 ± 5.2	No growth	No growth
Δ*mtsDH*	40.4 ± 6.2	No growth	28.1 ± 4.1
Δ*mtsFH*	45.3 ± 13.2	86.1 ± 8.4	No growth
Δ*mtsDFH*	51.0 ± 4.8	No growth	No growth

[a] Growth was measured as indicated in Materials and Methods. Values represent the results from three replicates, with the exception of the Δ*mtsH* strain on MeSH, whose growth was measured once. MMPA, methylmercaptopropionate; DMS, dimethylsulfide; MeSH, methanethiol.

are also upregulated during growth on MMPA and DMS relative to their transcription in methanol-grown cells, consistent with these genes having a role during growth on both substrates. However, only *mtsF* is upregulated during growth on MeSH (and only relative to its transcription during growth on methanol), consistent with the observation that *mtsF* mutants fail to grow on this substrate. It should be noted that MeSH is an intermediate of DMS catabolism, explaining the induction of *mtsF* during growth on DMS.

Several additional classes of genes showed significant transcriptional regulation during growth on methylsulfides. The *MA0849* locus, which encodes a protein with homology to the methylamine methyltransferase-activating protein RamA (39), is strongly upregulated on MMPA, DMS, and MeSH, suggesting a specific role in activation of methylsulfide methyltransferases. The *mtaCB3* operon was also highly expressed during growth on all methylsulfides. Although the results of genetic experiments strongly support the idea that this operon encodes a methanol-specific MT1 enzyme, it is only expressed during growth on energetically poor substrates, such as acetate and MMA (40). Our data now add MMPA, DMS, and MeSH to the list of substrates that induce *mtaCB3*. Another group of highly regulated genes encompasses ones previously shown to be controlled by the global regulator MreA (37), including *mreA* itself, as well as the *cdh-2* operon and the *ack*, *pta*, and *mreD* genes. Interestingly, the putative *mreA* regulon genes were only upregulated on MMPA. A third group involves genes with putative roles in sulfur metabolism. These include a homolog of the sulfur transfer protein ThiS (41), encoded by *MA3300*, the protein disulfide reductase MdrA (42), encoded by *MA3736*, and a putative sulfite reductase, encoded by *MA0685* (43). Lastly, we observed that the protein encoded by *MA0803* was strongly and specifically upregulated during growth on DMS. This protein has a C-terminal domain that is related to the Msr family transcriptional regulators but has a significantly different N-terminal DNA binding domain, suggesting that it is the founding member of a new family of archaeal transcriptional regulators involved in DMS-mediated regulation.

TABLE 3 Relative abundances of mRNAs of wild-type *M. acetivorans* C2A on different substrates[a]

Locus	Gene or function	Rank on indicated substrate[b]: MMPA	DMS	MeSH	MeOH	Fold change for[c]: MeOH vs MMPA	DMS vs MMPA	MeSH vs MMPA	MeOH vs DMS	MeSH vs DMS	MeOH vs MeSH
MA4164	*mtpC*	7	1,568	1,574	2,015	−173	−145	−149	NS	NS	NS
MA4165	*mtpA*	4	1,659	1,121	736	−153	−188	−161	NS	NS	NS
MA4166	*mtpP*	21	3,310	3,733	2,995	−57	−65	−82	NS	NS	NS
MA4167	*msrH*	1,534	2,053	1,155	938	NS	NS	NS	NS	NS	NS
MA1617	*mtaC3*	8	251	92	2,688	−80	−12	−9	−7	NS	−9
MA1616	*mtaB3*	13	289	144	2,826	−58	−10	−9	−6	NS	−7
MA0849	*ramS*	34	235	114	3,144	−37	−5	−4	−7	NS	−8
MA3860	*cdhA2*	24	277	1,275	1,009	−24	−11	−25	NS	NS	NS
MA3861	*cdhB2*	16	136	608	333	−21	−13	−22	NS	NS	NS
MA3862	*cdhC2*	17	348	788	880	−29	−11	−24	−3	NS	NS
MA3863	*cooC2*	9	159	113	106	−22	−8	−11	−3	NS	NS
MA3864	*cdhD2*	32	386	628	971	−21	−7	−15	−3	NS	NS
MA3865	*cdhE2*	22	467	939	1,576	−30	−11	−22	−3	NS	NS
MA3300	*thiS* homolog	97	308	2,774	4,084	−20	NS	−12	−9	−5	NS
MA0859	*mtsD*	70	41	366	2,001	−16	NS	NS	−18	NS	NS
MA4384	*mtsF*	44	16	4	1,333	−20	4	NS	−73	NS	−62
MA4558	*mtsH*	192	60	695	2,047	−8	NS	NS	−16	−9	NS
MA3302	*mreA*	25	424	1,428	3,577	−42	−6	−22	−7	NS	NS
MA3130	*mreD*	14	48	30	123	−18	NS	NS	−9	NS	−4
MA0685	Sulfite reductase	37	166	53	572	−16	NS	NS	−5	NS	−6
MA3736	*mdrA*	23	125	59	227	−15	−4	−4	−4	NS	−4
MA3607	*pta*	35	266	335	229	−11	−6	−9	NS	NS	NS
MA3606	*ack*	39	178	288	256	−11	−3	−8	−4	NS	NS
MA0803	Putative regulator	2,777	1	3,233	2,548	NS	7,277	NS	−6,037	−7,783	NS
MA0269	*mtrH*	287	146	119	148	NS	NS	NS	−3	NS	NS
MA0276	*mtrE*	195	102	101	93	NS	NS	NS	−3	NS	NS

[a] The full RNA-seq data set is presented in Table S4 in the supplemental material.
[b] Rank based on the normalized reads per kilobase per million mapped reads (RPKM values) for all genes in the genome, sorted from high to low, is presented.
[c] Statistically significant changes ($P < 0.01$) based on the EDGE test are shown. NS, not statistically significant ($P \geq 0.01$).

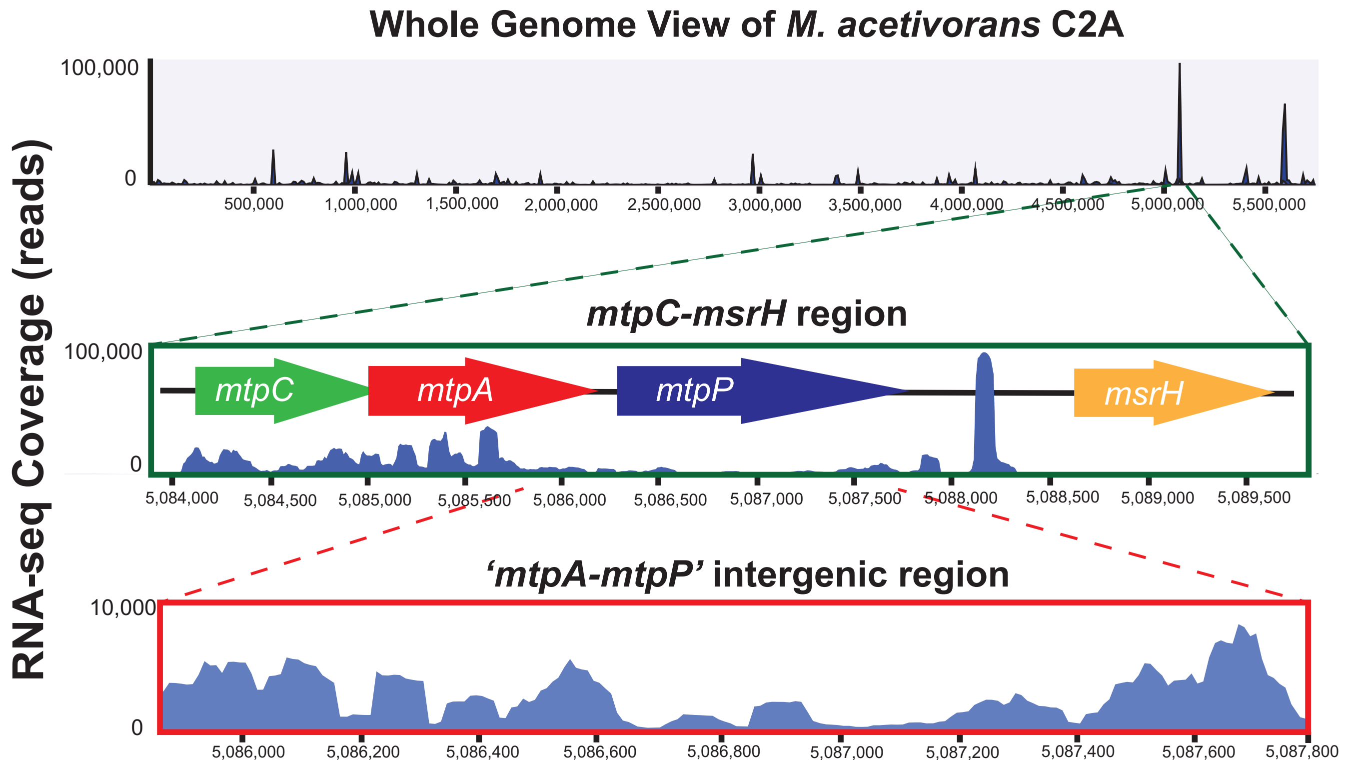

FIG 3 RNA-seq read coverage of *M. acetivorans* grown on MMPA. (Top) mRNA read coverage of the entire chromosome; (middle) mRNA read coverage of the *mtpCAP-msrH* locus; (bottom) mRNA read coverage of the *mtpA-mtpP* intergenic region. Note that the coverage between genes never drops below the coverage within the genes, suggesting that *mtpCAP* are cotranscribed.

The RNA-seq data also support the notion that *mtpCAP* comprises an operon transcriptionally regulated by MsrH. Thus, continuous mapping of mRNA reads was observed across the entire operon (Fig. 3). The *msrH* gene is not cotranscribed with *mtpCAP* but instead comprises a monocistronic transcript that is constitutively expressed at low levels on all growth media (Table 3). To investigate the idea that *msrH* encodes a transcriptional regulator of *mtpCAP*, we used RNA-seq to compare the levels of mRNA abundance between the Δ*msrH* strain and the parental strain. Because the Δ*msrH* mutant does not grow on MMPA as the sole substrate, we grew both strains on 5 mM TMA supplemented with 20 mM MMPA. Under these conditions, *mtpC*, *mtpA*, and *mtpP* were the only genes that were differentially expressed between the two strains (Fig. 4). Thus, *msrH* appears to be a highly specific, MMPA-dependent activator of *mtpCAP*.

DISCUSSION

The data presented here suggest that the products of the *mtpCAP* operon are the primary means for catabolism of MMPA in *Methanosarcina* species. These genes are strongly and specifically induced by MMPA and required for wild-type growth rates and yields on this substrate. Based on our analyses, it seems likely that MMPA is catabolized via a two-component MT1/MT2 system comprised of the methyltransferase MtpA and the corrinoid protein MtpC (Fig. 5). By analogy to the biochemically characterized MtsA/B system of *M. barkeri* (17), we suggest that MtpA will catalyze both the methyl transfer from MMPA to MtpC and the subsequent methyl transfer from methyl-MtpC to CoM. The highly similar chemical structures of MMPA and methyl-CoM strengthen the idea that these molecules could be substrates/products for the same enzyme. Although we found that MtpP is not required for growth on MMPA under laboratory conditions, the transporter is probably required in nature. Based on the pKa of MMPA (4.7), the ratio of protonated to unprotonated MMPA in our medium (pH 6.8) is ca. 1:100. Thus, because weak acids diffuse freely across the membrane, the transporter is probably not required when substrate concentrations are high and the pH is near neutrality. Moreover, at the marine pH of 8.3 (44), the ratio of protonated to unprotonated MMPA will be >10-fold lower than in our medium. Under these conditions, the putative transporter MtpP will almost certainly be required. The universal con-

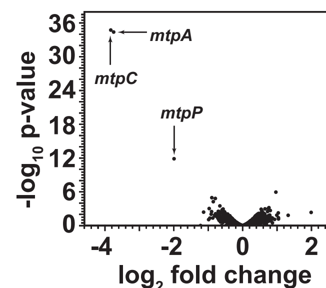

FIG 4 Relative mRNA abundances of the Δ*msrH* mutant versus the parental strain during growth on TMA plus MMPA. Volcano plot shows the fold differences in transcript abundance between WWM833 (Δ*msrH*) and WWM82 (parental strain), based on the EDGE test plotted against statistical significance. Only the three genes indicated show significant differences between the two strains.

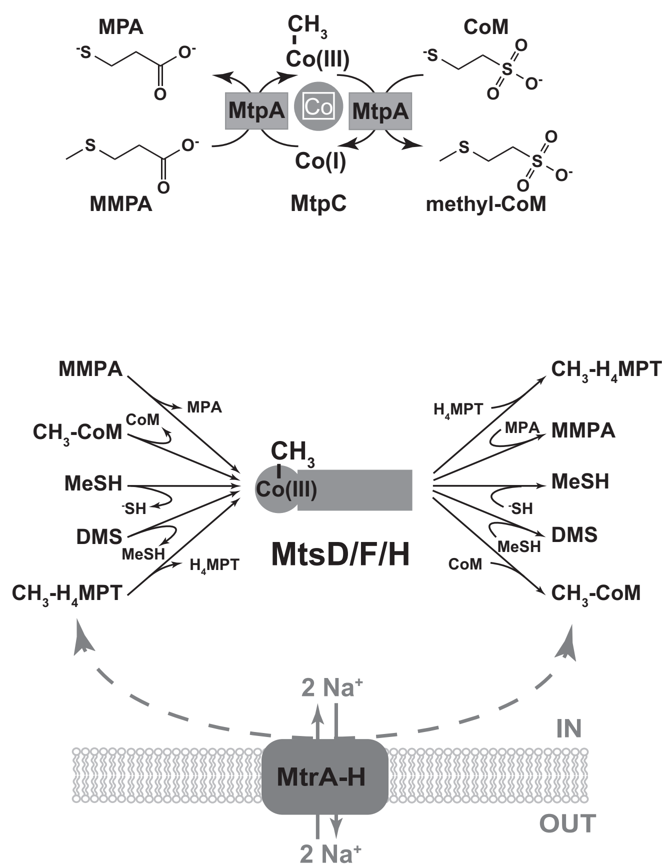

FIG 5 Putative roles for MtpCA and MtsD/-F/-H in methylsulfide metabolism. (Top) Schematic of a proposed bifunctional MT1/MT2 enzyme, in which MtpA catalyzes transfer of the methyl group from MMPA to the corrinoid protein MtpC and subsequent methyl transfer from methyl-MtpC to CoM. (Bottom) Schematic of a proposal for broad substrate specificity in the MtsD/-F/-H proteins. These putative multifunctional enzymes would allow bypass of the normal energy-conserving/-dependent Mtr H_4MPT:CoM methyltransferase while providing a mechanism to introduce methyl groups from methylsulfides into both the reductive and the oxidative branch of the methylotrophic pathway of methanogenesis. Note that the direction of methyl transfer between CH_3-H_4MPT and CH_3-CoM determines whether it is exergonic or endergonic and, thus, whether sodium ions are extruded or consumed.

servation of the *mtpP* gene in all methanogens that contain homologs of *mtpCA* supports this conclusion.

The observation that the *mtpCAP* mutant showed partial growth on MMPA indicates the presence of an alternate, albeit inefficient pathway for catabolism of this compound by *M. acetivorans*. If MtpA/MtpC is indeed the primary MT1/MT2 system for this substrate, then the observed residual growth of the *mtpCAP* mutant must depend on an alternate activating system to generate methyl-CoM from MMPA. The loss of residual growth upon deletion of the three *mts* genes suggests that MtsF, MtsD, or MtsH acting as single-subunit MT1/MT2 enzymes for MMPA can provide this function. Our genetic data, along with the previously published results of Oelgeschlager et al. (19), show that the Mts proteins also use MeSH and DMS as substrates, with different substrate preferences for individual isozymes. Biochemical evidence for the use of multiple methylsulfide substrates has previously been demonstrated for the MtsA/B system of *M. barkeri*, which uses both DMS and MMPA as substrates (17).

Intriguingly, we observed that the Δ*mtsDFH* mutant reached a lower cell density on MMPA than did otherwise wild-type cells, suggesting that the proteins encoded by these genes play an additional role beyond substrate activation. While this phenotype is more difficult to explain, it may be related to the CoM:H_4MPT methyltransferase activity of this family of proteins (21). In reporting this novel biochemical activity, Vepachedu and Ferry (21) suggested that MtsF, and probably the homologous MtsD and MtsH proteins, would act to bypass the membrane-bound, energy-conserving Mtr CoM:H_4MPT methyltransferase, providing an advantage during growth on low-energy substrates, such as carbon monoxide. Assuming that both the genetic and biochemical data are correct, the Mts family of proteins would have an exceptionally broad substrate range, including methylated and unmethylated sulfides and H_4MPT (Fig. 5). The ability to recognize numerous sulfide and methyl-sulfide substrates in addition to H_4MPT and methyl-H_4MPT would explain all available data. These multifunctional enzymes would allow bypass of the ion-pumping Mtr H_4MPT:CoM methyltransferase, as well as provide a mechanism to introduce methyl groups from methylsulfides into both the reductive and oxidative branches of the methylotrophic methanogenesis pathway. Thus, the energetic advantage of bypassing Mtr would apply to growth on any of these substrates. Accordingly, the Δ*mtsDFH* mutants would lack this energetic advantage and would achieve a lower cell density, as seen in our data. Moreover, the broad specificity of Mts isozymes could also explain the synthesis of DMS and MeSH during growth on CO (19, 45), because the methylated corrinoid cofactor can donate the methyl moiety to sulfide or MeSH in a reaction that is thermodynamically equivalent to transfer to CoM. There is, however, one major caveat to this model. The possession of CoM:H_4MPT methyltransferase activity by both Mts and Mtr introduces the possibility of a futile cycle that would deplete the ion gradient across the cell membrane, essentially preventing energy conservation. If this model, shown in Fig. 5, is correct, then the cell must possess a mechanism to prevent this from happening.

Given the dramatic differences between the aceticlastic and methylotrophic pathways for methanogenesis (reviewed in references 16 and 46), we were surprised to note the striking similarity between the transcriptional profiles of MMPA- and acetate-grown cells. This shared phenotype may involve the global regulator MreA, although the intricacies for this regulation remain murky at this point. Based on a combination of the transcriptomic profiles of acetate- versus methanol-grown cells and those of *mreA* mutants, Reichlen et al. (37) proposed that MreA mediates repression of methylotrophic and activation of aceticlastic genes. Our data are consistent with this interpretation but suggest that the regulation is considerably more complex. Thus, the aceticlastic genes encoding acetate kinase (*ack*), phosphotransacetylase (*pta*), and acetyl coenzyme A (acetyl-CoA) synthase (*cdh*-2) are highly upregulated on both MMPA and acetate, as is the *mreA* gene itself. However, deletion of *mreA* leads to increases of ca. 40- to 130-fold in *mtpCAP* expression while concomitantly reducing the expression of *pta*, *ack*, and *cdh* by 5-fold (37). This effect is likely to be indirect, probably mediated via *msrH*, which is required for *mtpCAP* expression and is significantly upregulated in the *mreA* mutant. The question then remains, what is the common regulatory signal that is sensed during growth on MMPA and acetate? The simplest explanation is that MMPA catabolism generates acetate; however, it has previously been shown that growth on MMPA

results in nearly stoichiometric conversion of the substrate to mercaptopropionate (8). Nonetheless, it is conceivable that some mercaptopropionate is metabolized to acetate, triggering an aceticlastic transcriptional response. Alternatively, mercaptopropionate may be sufficiently similar to acetate to trigger this response without catabolism. We do not favor this idea because *mreA* mutants recapitulate the regulatory phenotype in the absence of MMPA. Further clues come from the observation that *mtpCAP*, along with *mtsD*, *mtsF*, and *mtsH*, are also highly upregulated upon deletion of the alternate heterodisulfide reductase encoded by *hdrABC* (47). Previous studies suggested that loss of HdrABC slows the regeneration of the free coenzymes M and B (CoM and CoB), which are needed for the terminal step in methanogenesis. Loss of HdrABC also slows the regeneration of oxidized ferredoxin, which is the central electron carrier in aceticlastic methanogenesis. Interestingly, *hdrABC* expression is highly downregulated during growth on acetate (47). Taken together, these data suggest a highly intertwined and complex mechanism for the regulation of energy-conserving metabolism in *Methanosarcina* that may involve direct sensing of intermediates in the methanogenic pathway.

Finally, our results shed additional light on the fate of methylated sulfur compounds in marine environments. The aerobic flux of DMSP into either the cleavage pathway to generate DMS or the demethylation pathway to produce MMPA has attracted considerable attention due to the significant consequences for climate regulation (48). Our data establishing the genetic basis for both DMS and MMPA metabolism in marine methanogens will empower molecular microbial ecology approaches to assess the fate of methylsulfides in anaerobic marine ecosystems as well.

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