Electron Transport in the Pathway of Acetate Conversion to Methane in the Marine Archaeon *Methanosarcina acetivorans*†

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A liquid chromatography–hybrid linear ion trap–Fourier transform ion cyclotron resonance mass spectrometry approach was used to determine the differential abundance of proteins in acetate-grown cells compared to that of proteins in methanol-grown cells of the marine isolate *Methanosarcina acetivorans* metabolically labeled with ¹⁴N versus ¹⁵N. The 246 differentially abundant proteins in *M. acetivorans* were compared with the previously reported 240 differentially expressed genes of the freshwater isolate *Methanosarcina mazei* determined by transcriptional profiling of acetate-grown cells compared to methanol-grown cells. Profound differences were revealed for proteins involved in electron transport and energy conservation. Compared to methanol-grown cells, acetate-grown *M. acetivorans* synthesized greater amounts of subunits encoded in an eight-gene transcriptional unit homologous to operons encoding the ion-translocating Rnf electron transport complex previously characterized from the *Bacteria* domain. Combined with sequence and physiological analyses, these results suggest that *M. acetivorans* replaces the H₂-evolving Ech hydrogenase complex of freshwater *Methanosarcina* species with the Rnf complex, which generates a transmembrane ion gradient for ATP synthesis. Compared to methanol-grown cells, acetate-grown *M. acetivorans* synthesized a greater abundance of proteins encoded in a seven-gene transcriptional unit annotated for the Mrp complex previously reported to function as a sodium/proton antiporter in the *Bacteria* domain. The differences reported here between *M. acetivorans* and *M. mazei* can be attributed to an adaptation of *M. acetivorans* to the marine environment.

The anaerobic conversion of biomass to methane is essential to the global carbon cycle. Most of the methane is produced by a two-step process in which complex organic matter is fermented to acetate that is further converted to methane and carbon dioxide. The same process is also key to the conversion of renewable plant biomass to methane (biomethanation) as an alternative energy source. The production of renewable energy crops for biomethanation is particularly advantageous for reducing CO₂ emissions to the atmosphere, as the CO₂ produced by burning methane is recycled through plant photosynthesis. An understanding of the pathway for acetate conversion to methane is paramount to the development of process parameters for control and optimization of large-scale biomethanation of renewable biomass. Fundamental to this understanding is the identification of novel proteins essential to the pathway for methane formation from acetate. Transcriptional profiling of the freshwater species *Methanosarcina mazei* (17) has identified genes up-regulated in response to growth with acetate compared to growth with methanol, suggesting roles for the

encoded proteins that are specific to the pathway for acetate conversion to methane. The results show that the pathway in *M. mazei* is similar to that previously proposed for other freshwater *Methanosarcina* species (8–10, 29). A recent proteomic analysis of the marine species *Methanosarcina acetivorans* that utilized two-dimensional gel electrophoresis and matrix-assisted laser desorption–time of flight tandem mass spectrometry (MS) (2-D gel/MS) identified 34 proteins with greater abundance in acetate-grown cells than in methanol-grown cells (25, 26). Here, we report a deeper analysis using liquid chromatography (LC)–hybrid linear ion trap–Fourier transform ion cyclotron resonance (FTICR) MS to identify differentially abundant proteins from acetate-grown cells versus methanol-grown cells metabolically labeled with ¹⁴N versus ¹⁵N, respectively. The results provide a more detailed understanding of the acetate pathway in this marine species, revealing profound differences in the pathway for acetate conversion to methane by *M. acetivorans* compared to those of *M. mazei* and other freshwater *Methanosarcina* species.

MATERIALS AND METHODS

Cell growth. *M. acetivorans* acetate-grown cells were cultured as previously described (25). Methanol-grown cells were cultured as previously described (25), except that ¹⁴NH₄Cl was substituted with ¹⁵NH₄Cl (98%) (Sigma, St. Louis, MO). Cells from both cultures were harvested in the mid-exponential phase of growth at optical densities at 600 nm of 0.8 and 0.6 for acetate and methanol cultures, respectively, as previously described (25).

Protein extraction, SDS-polyacrylamide gel electrophoresis (PAGE) fractionation, and in-gel digestion. The cell pellet from about 40 ml of culture was resuspended in 100 μl of 10 mM Tris-HCl containing 5 mM MgCl₂ and 100 U DNase (Roche, Indianapolis, IN) and incubated on ice for 20 min. This treat-

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ment was followed by the addition of 900 μ l of 8 M urea containing 0.05% sodium dodecyl sulfate (SDS) and vortexing for 3 min. The whole-cell lysate was cleared by centrifugation at $13,000 \times g$ for 20 min at 4°C. The concentrations of whole-cell protein extracts, determined by the Bradford assay (Bio-Rad, Hercules, CA), from acetate- and methanol-grown cells were 5.8 and 3.5 mg/ml respectively.

Whole-cell extracts of acetate- and methanol-grown cells were combined to generate a 1:1 (wt/wt) mixture of the ^{14}N - and ^{15}N -labeled proteins. An aliquot containing 40 μ g of the mixture was diluted to 45 μ l with SDS-PAGE sample buffer consisting of 2% (wt/vol) SDS, 25% (vol/vol) glycerol, 100 mM dithiothreitol, 0.01% bromophenol blue, and 62.5 mM Tris-HCl (pH 7). The sample was resolved in a precast 12-well 10.5 to 14% linear gradient Criterion Tris-HCl gel (Bio-Rad, Hercules, CA) developed at 160 V for 50 min. The gel was stained with silver as previously described (32). The lanes were cut into 10 fractions, each of which contained roughly the same total density as estimated by visual inspection aided with a translucent illuminator. Each fraction was separately minced into $\sim 1\text{-mm}^3$ cubes and subjected to washing, in-gel digestion, and peptide extraction steps as described previously (36), except that the volume of solution added for each step was adjusted to accommodate the volume of gel pieces for each SDS-PAGE fraction. Sequencing-grade trypsin (Promega, Madison, WI) was used as the digestion enzyme. The collected peptide extract solution for each fraction (~ 1.2 ml) was concentrated to a $\sim 30\text{-}\mu$ l final volume in an SPD1010 SpeedVac system (Thermo Savant, Holbrook, NY) at 45°C in a 1.5-ml microcentrifuge tube.

Protein identification and abundance ratio determination. Proteins were identified from the peptide extract of each one-dimensional SDS-PAGE gel fraction using a shotgun proteomics strategy similar to that previously described (42). Approximately 10 μ l of peptide extract solution was loaded onto a 100- μ m by 15-cm column packed with MagicC18 5- μ m particles (Michrom BioResources, Auburn, CA) followed by a 75-min linear gradient of 2% to 35% (vol/vol) acetonitrile in 0.1% formic acid using a 300-nl/min flow rate. A hybrid linear ion trap-FTICR instrument (LTQ FT MS; Thermo Electron, San Jose, CA) was used for the analysis. In one data acquisition cycle, a single high-resolution MS Fourier transform (FT) scan with accumulation of up to 2×10^6 ions was followed by the acquisition of tandem MS spectra using the linear ion trap (accumulation of 3×10^4 ions) for up to seven of the most intense ions with the dynamic exclusion set to 1 min. A single data acquisition cycle was completed in approximately 3.5 s. Each one-dimensional SDS-PAGE fraction was analyzed twice. The acquired data were searched against the NCBI database of *M. acetivorans* C2A in two separate Sequest searches, one corresponding to ^{14}N and the other corresponding to ^{15}N labeling. The precursor ion mass tolerance was set to ± 1.4 Da, and trypsin was designated as the proteolytic enzyme with up to two missed cleavages. In order to minimize the rate of false-negative identification, peptides identified with cross-correlation (Xcorr) values greater than 1.5 (1+), 2.0 (2+), and 2.5 (3+) in either ^{14}N or ^{15}N searches in two replicate LC/hybrid linear ion trap-FTICR MS runs were initially selected. Only peptides with the precursor mass tolerance of ± 10 ppm were then accepted as correct identifications. In about 15% of peptide identifications, the second isotope was initially assigned as the precursor ion, resulting in a 1-Da mass shift. In these cases, the shift was corrected for the monoisotopic peak before applying the 10-ppm precursor mass accuracy restriction. The relative abundance of the identified peptides was then calculated using a laboratory-developed program (2) that determines ratios of chromatographic peak areas of the isotopically labeled peptide pairs. For successful quantitation, a coeluting $^{14}\text{N}^{15}\text{N}$ pair of peptides with at least one peptide identified using the database search criteria specified above and the mass difference between the peptides corresponding to the number of nitrogen atoms in the peptide sequence had to be found. In the next step, the relative abundance ratios of proteins were calculated by averaging identified peptide abundance ratios. Importantly, the combination of precursor mass tolerance (± 10 ppm), Sequest Xcorr values, and the presence of a pair of coeluting peaks provided highly confident protein identifications.

Cytochrome analysis. Cell membrane and cytoplasmic fractions were separated in a manner similar to that described previously (21). Cells were resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing DNase I (10 μ g/ml) and broken by passing them twice through a French pressure cell at 1.38×10^8 Pa. Cell debris was separated by centrifugation at $10,000 \times g$ for 20 min at 4°C. The resulting cell extract was separated into membrane and cytoplasmic fractions by centrifugation at $100,000 \times g$ for 60 min at 4°C. The supernatant solution was removed, and the pellet containing membranes was washed once with the above-described buffer and resuspended in the same buffer. Heme staining of proteins separated by SDS-PAGE was performed using *o*-dianisidine (Sigma) as previously described (35). The 55-kDa band revealed by heme staining of the membrane fraction from acetate-grown cells was excised and subjected to in-gel

digestion and peptide extraction as described above. The peptides were analyzed by LC/hybrid linear ion trap-FTICR MS. Reduced-minus-oxidized-difference spectra of membrane fractions were obtained at room temperature as previously described (22) by using a Beckman DU-7400 spectrophotometer. Samples were air oxidized and then reduced with a few grains of dithionite.

RT-PCR. Reverse transcriptase (RT)-PCR was performed as described previously (24), with modifications. Total RNA was isolated from acetate- or methanol-grown *M. acetivorans* cells by using an RNeasy Total RNA Mini kit (QIAGEN). Purified RNA was treated twice with RNase-free DNase I (QIAGEN) and once with RQ1 DNase (Promega) to remove contaminating DNA. RT-PCR was carried out with an Access RT-PCR kit (Promega) using 50 to 100 ng of purified total RNA (primer sequences and primer pairs used are listed in Tables S1 and S2, respectively, in the supplemental material). Control PCRs were performed without the addition of RT with four different primer sets to confirm the complete removal of DNA.

RESULTS AND DISCUSSION

Protein identification. Previous 2-D gel/MS analyses of the marine isolate *M. acetivorans* identified a total of 412 gene products (25). The determination of spot intensities indicated that there were 34 gene products that were differentially abundant between acetate- and methanol-grown cells (26). To obtain a deeper qualitative and quantitative analysis, we utilized LC/hybrid linear ion trap-FTICR MS to analyze acetate-grown cells and methanol-grown cells metabolically labeled with ^{14}N and ^{15}N , respectively. A total of 1,081 proteins were detected (unpublished data), representing 21% of the 4,524 genes reported in the genome (11). Of the 1,081 proteins detected, 246 were found to have ≥ 3 -fold differential abundance between acetate- and methanol-grown cells as determined with two or more peptide pairs. These 246 proteins were considered candidates for roles specific to growth or methane formation from either methanol or acetate. The recent transcriptional profiling of the freshwater isolate *M. mazei* (17) detected 3,371 expressed genes, 240 of which showed ≥ 2.5 -fold differential expression between acetate- and methanol-grown cells.

Of the 246 differentially abundant proteins determined for *M. acetivorans*, 60 were encoded by homologs of genes ≥ 2.5 -fold differentially expressed in *M. mazei*. Of these 60 proteins from *M. acetivorans*, the patterns of differential abundance for 57 proteins were in agreement with the transcriptional profile of *M. mazei* (17). Furthermore, the methods reported here for *M. acetivorans* identified 20 differentially abundant proteins previously determined by 2-D gel/MS (26) for which the differential abundance patterns of all 20 proteins were in agreement with the 2-D gel/MS analyses. These results are a robust validation of the experimental approaches used in this study. Here, we focus on select proteins with high differential abundance that have identified two enzyme complexes proposed to function in electron transport in acetate-grown *M. acetivorans*.

The pathway for conversion of acetate to methane in *M. acetivorans*. Transcriptional profiling of acetate-grown compared methanol-grown *M. mazei* cells (17) showed that the pathway of acetate conversion to methane is similar to that previously determined for other freshwater *Methanosarcina* species (7, 9, 12). However, *M. acetivorans* was isolated from marine sediments (38) where it functions to convert acetate to methane during the anaerobic decomposition of giant kelp (39). Thus, comparison of the transcriptome of the freshwater isolate *M. mazei* with the proteome of *M. acetivorans* is valuable for identifying essential core enzymes in the acetate path-

FIG. 1. Pathway proposed for the conversion of acetate to methane by *M. acetivorans*. Ack, acetate kinase; Pta, phosphotransacetylase; CoA-SH, coenzyme A; THMPT, tetrahydromethanopterin; Fd_L, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; Cdh, CO dehydrogenase/acetyl-CoA synthase; CoM-SH, coenzyme M; Mtr, methyl-THMPT:CoM-SH methyltransferase; CoB-SH, coenzyme B; Cam, carbonic anhydrase; Ma-Rnf, *M. acetivorans* Rnf; MP, methanophenazine; Hdr-DE, heterodisulfide reductase; Mrp, multiple resistance/pH regulation Na⁺/H⁺ antiporter; Atp, H⁺-transporting ATP synthase. Carbon transfer reactions are catalyzed by the enzymes shown in blue (see text). Electron transfer reactions are catalyzed by enzymes shown in green.

way and assessing metabolic flexibility in response to the environment. The advanced experimental approach utilized in this study has identified several proteins not identified in the previous 2-D gel/MS analysis (25, 26), leading to a more detailed understanding of the pathway as shown in Fig. 1. The overall pathway can be divided into two parts. The first part involves conversion of the methyl group of acetate to methane, referred to as “one-carbon” reactions catalyzed by the enzymes shown in blue in Fig. 1. Part two of the pathway involves electron transfer reactions catalyzed by enzymes shown in green in Fig. 1.

(i) **One-carbon reactions leading to methane.** The results shown in Table 1 are consistent with the previously proposed roles for acetate kinase, phosphotransacetylase, CO dehydrogenase/acetyl coenzyme A (CoA) synthase, methyl-tetrahydromethanopterin (THMPT):coenzyme M (CoM) methyltransferase, and methyl-CoM methylreductase supported by 2-D gel/MS analyses (25, 26). However, the advanced methods used in this study further showed that carbonic anhydrase (Cam; MA2536) was more abundant in acetate-grown *M. acetivorans* cells, in agreement with the up-regulation of the gene encoding the cognate enzyme in *M. maezi* (17). Involvement of this enzyme in the conversion of acetate to methane was first reported for *Methanosarcina thermophila* (1) and was previously proposed to facilitate the removal of CO₂ from the cell by converting it to bicarbonate outside the cell (Fig. 1).

All of the enzymes proposed to catalyze conversion of the methyl group of acetate to methane by *M. acetivorans* (Fig. 1)

have been identified in other freshwater *Methanosarcina* species (7, 10, 12) and more recently by transcriptional profiling of the freshwater isolate *M. mazei* (17). Thus, these enzymes and reactions can be considered to be core to the pathway for acetate conversion to methane by freshwater and marine *Methanosarcina* species. However, the results presented in the next section suggest dramatic differences in electron transport reactions in the acetate pathway of *M. acetivorans* compared to those in freshwater *Methanosarcina* species.

(ii) Electron transport and energy conservation. The greater abundance of Cdh in acetate-grown cells than in methanol-grown cells reported previously (25, 26) and confirmed here suggests that the first electron transport step proposed for *M. acetivorans* is the same as those for *M. mazei* and other freshwater *Methanosarcina* species, where Cdh oxidizes the carbonyl group of acetyl-CoA and donates electrons to ferredoxin (40, 41). However, as detailed below in the following sections, electron transport from the reduced ferredoxin to CoM-S-S-CoB proposed here for *M. acetivorans* (Fig. 1) differs substantially from that of freshwater species. In freshwater species, reduced ferredoxin is proposed to donate electrons to the membrane-bound Ech hydrogenase that produces H₂ and pumps protons outside the membrane (15, 28, 29). The H₂ is oxidized by a different hydrogenase that donates electrons to methanophenazine in the membrane. Finally, the reduced methanophenazine donates electrons to the heterodisulfide reductase which reduces CoM-S-S-CoB, accompanied by the extrusion of protons. However, the genome of *M. acetivorans* does not

TABLE 1. Differential abundance of proteins with predicted roles in the pathway for conversion of acetate to methane by *M. acetivorans*

Locus	Annotation ^c	No. of peptides ^d	Abundance ratio ^e (AC/ME) $\pm$ SD
MA0269 ^a	Methyl-THMPT:CoM methyltransferase (MtrH), subunit H	7	3.2 $\pm$ 0.9
MA0270 ^a	Methyl-THMPT:CoM methyltransferase (MtrG), subunit G	2	3.4 $\pm$ 1.1
MA0272 ^a	Methyl-THMPT:CoM methyltransferase (MtrA), subunit A	6	3.3 $\pm$ 1.2
MA0273	Methyl-THMPT:CoM methyltransferase (MtrB), subunit B	2	5.1 $\pm$ 0.6
MA0274 ^a	Methyl-THMPT:CoM methyltransferase (MtrC), subunit C	2	3.2 $\pm$ 0.8
MA0275	Methyl-THMPT:CoM methyltransferase (MtrD), subunit D	3	4.7 $\pm$ 1.2
MA0659	Na ⁺ -transporting NADH:ubiquinone oxidoreductase (Nqr), subunit 1	4	11.2 $\pm$ 3.5
MA0661	Na ⁺ -transporting NADH:ubiquinone oxidoreductase (Nqr), subunit 3	3	12.5 $\pm$ 2.8
MA0664	Na ⁺ -transporting NADH:ubiquinone oxidoreductase (Nqr), subunit 6	3	9.7 $\pm$ 2.1
MA0687 ^a	Heterodisulfide reductase (HdrE), subunit E	2	1.2 $\pm$ 0.6
MA0688 ^a	Heterodisulfide reductase (HdrD), subunit D	3	1.9 $\pm$ 0.7
MA1014	CO dehydrogenase/acetyl-CoA synthase (CdhB), subunit beta	7	26 $\pm$ 12
MA1015	CO dehydrogenase/acetyl-CoA synthase (CdhE), subunit epsilon	5	10.5 $\pm$ 3.8
MA1016	CO dehydrogenase/acetyl-CoA synthase (CdhA), subunit alpha	7	27 $\pm$ 11
MA2536	Carbonic anhydrase (Cam)	2	9.4 $\pm$ 2.5
MA3606 ^b	Acetate kinase (Ack)	10	50 $\pm$ 25
MA3607 ^b	Phosphotransacetylase (Pta)	11	18 $\pm$ 8
MA3860	CO dehydrogenase/acetyl-CoA synthase (CdhA), subunit alpha	3	250 $\pm$ 110
MA3861	CO dehydrogenase/acetyl-CoA synthase (CdhE), subunit epsilon	4	260 $\pm$ 90
MA3862	CO dehydrogenase/acetyl-CoA synthase (CdhB), subunit beta	4	160 $\pm$ 85
MA4152 ^a	H ⁺ -transporting ATP synthase (AtpH), subunit H	2	3.5 $\pm$ 1.2
MA4153	H ⁺ -transporting ATP synthase (AtpI), subunit I	8	9.8 $\pm$ 5.5
MA4154	H ⁺ -transporting ATP synthase (AtpC), subunit C	2	13 $\pm$ 4.1
MA4155	H ⁺ -transporting ATP synthase (AtpE), subunit E	3	7.5 $\pm$ 2.3
MA4156	H ⁺ -transporting ATP synthase (AtpC), subunit C	9	9.5 $\pm$ 3.6
MA4157	H ⁺ -transporting ATP synthase (AtpF), subunit F	3	7.6 $\pm$ 2.7
MA4158	H ⁺ -transporting ATP synthase (AtpA), subunit A	14	8.1 $\pm$ 3.4
MA4159	H ⁺ -transporting ATP synthase (AtpB), subunit B	11	7.1 $\pm$ 3.2
MA4160	H ⁺ -transporting ATP synthase (AtpD), subunit D	4	9.7 $\pm$ 3.1
MA4547 ^a	Methyl coenzyme M reductase (McrG), subunit gamma	9	3.2 $\pm$ 0.9
MA4550 ^a	Methyl coenzyme M reductase (McrB), subunit beta	8	3.5 $\pm$ 1.3
MA4567	Multiple-resistance/pH regulation Na ⁺ /H ⁺ antiporter (MrpF), subunit F	4	40 $\pm$ 21
MA4568	Multiple-resistance/pH regulation Na ⁺ /H ⁺ antiporter (MrpE), subunit E	3	31 $\pm$ 17

^a Proteins not statistically ≥ 3 -fold differentially abundant (see text).^b Proteins detected by 2-D gel/MS (26).^c Annotations are the primary annotations listed at <http://www.tigr.org>.^d The number of unique identified peptides used for quantitation.^e AC, acetate-grown cells; ME, methanol-grown cells. Relative abundance was calculated by averaging duplicate runs of abundance ratios of acetate-grown versus methanol-grown *M. acetivorans* cells (see Materials and Methods).

contain genes encoding a functional Ech hydrogenase (11, 18), suggesting alternative electron transport components involved in the transfer of electrons to CoM-S-S-CoB.

Previous 2-D gel/MS analyses indicated that the product of MA0659 is present in acetate-grown *M. acetivorans* cells; however, the abundance relative to methanol-grown cells was not determined, which precluded proposing a role for this product in the conversion of either substrate to methane (25). Nonetheless, it was hypothesized that MA0659 encodes one of six subunits of a complex encoded by the gene cluster MA0659-0664 with the potential to replace the ion-pumping function of Ech hydrogenase. Here, we show that the products of MA0659, MA0661, and MA0664 are severalfold more abundant in acetate-grown cells than in methanol-grown cells of *M. acetivorans* (Table 1), supporting a role in the acetate pathway. Furthermore, RT-PCR analysis showed that MA0658 and MA0665 are cotranscribed with MA0659-664 (Fig. 2). Finally, deletion of the cotranscribed genes yields a mutant of *M. acetivorans* that is unable to grow with acetate (P. C. W. Metcalf, University of Illinois). These results strongly suggest that MA0658-0665 encodes an eight-subunit complex that functions during growth of *M. acetivorans* on acetate. Notably, the genome of *M. mazei* does not contain genes homologous to MA0658-0665 (18),

further supporting a role for the complex that is specific to *M. acetivorans*.

Although MA0659-0664 is annotated as encoding subunits of the sodium-translocating NADH:ubiquinone oxidoreductase (Nqr), a search of the databases revealed that the amino acid sequences have greater identity to subunits of the related Rnf (*Rhodobacter* nitrogen fixation) complex, and the greatest identity is with the *mf* gene cluster annotated for *Clostridium tetani* (Fig. 3A). The Rnf complex was first described in *Rhodobacter capsulatus* (34), where it is proposed to encode a six-subunit (20) membrane-bound complex (RnfABCDGE) oxidizing NADH and reducing a ferredoxin which then donates electrons to nitrogenase (23, 34). It is further proposed that the thermodynamically unfavorable reverse electron transport is driven by an electrochemical gradient (27, 33). It has previously been suggested (23) that the Rnf complex is a new energy-coupling family that may also function to generate an electrochemical gradient for ATP synthesis, consistent with the proposed function in *M. acetivorans*. For example, it was previously proposed that the Rnf complex of *C. tetani*, encoded by the gene cluster shown in Fig. 3, oxidizes ferredoxin and reduces NAD coupled to the extrusion of sodium to outside the cell membrane (5).

The subunits of the Rnf complex from *R. capsulatus* (Rc-Rnf)

FIG. 2. Transcriptional mapping of *M. acetivorans* gene clusters by RT-PCR. (A) MA4566-MA4572. (B) MA0658-MA0665. Arrows represent genes and direction of transcription. Predicted RT-PCR products are represented by lines under the genes and are labeled with letters. Predicted RT-PCR product sizes are shown in parentheses. Letters above the gel lanes correspond to predicted RT-PCR products. RT-PCR was performed with total RNA from acetate-grown *M. acetivorans* cells.

have been previously characterized (20, 23), predicting functions for subunits of the *M. acetivorans* Rnf complex (Ma-Rnf) encoded by MA0658-0665 (Fig. 4). The products of MA0660/MA0662/MA0663 share sequence identity and conservation of hydrophobic segments (not shown) with RnfD/RnfE/RnfA previously proposed to comprise the ion channel of the Rc-Rnf complex. Thus, the products of MA0660/MA0662/MA0663 are hypothesized to reside in a membrane-bound subcomplex that functions as an ion channel for the translocation of either

protons or sodium outside the membrane (Fig. 4), generating an ion gradient that drives ATP synthesis (Fig. 1). The amino acid sequences of MA0664/MA0659/MA0661 share identity with RnfB/RnfC/RnfG (Fig. 5), which were previously proposed to reside in an electron transfer subcomplex in *R. capsulatus*, suggesting an analogous function for the products of MA0664/MA0659/MA0661. The amino acid sequence of MA0664 has identity to that of RnfB (Fig. 5A), which was previously shown

FIG. 3. Gene organization and sequence comparisons of *M. acetivorans* genes with *mrf* and *mrp* genes from the Bacteria domain. (A) Comparison of the *M. acetivorans* MA0658-0665 transcription unit with the *C. tetani* *mrfCDGEAB* cluster (5). (B) Comparison of the *M. acetivorans* MA4566-4572 transcription unit with the *B. subtilis* *mrpABCDEFG* operon (19). The numbers in parentheses are percent identities between the proteins encoded by the genes as indicated by the arrows.

FIG. 4. Topology and function predicted for the MA0658-0665 gene products of *M. acetivorans*. Fd_r, reduced ferredoxin; Fd_o, oxidized ferredoxin; Cyt c, cytochrome c; MP, methanophenazine.

FIG. 5. Comparison of amino acid sequences deduced from MA0658-0665 with sequences of the Rnf subunits from *Rhodobacter capsulatus* (21). Identical residues are indicated asterisks. Cysteine motifs are in reverse contrast in panels A and B. The threonine postulated to covalently bind FMN is indicated by the arrow below the sequence in panel C. Numbers on the right indicate the amino acid positions. Truncated sequences are indicated by +. Sequences were aligned with ClustalW.

to contain at least a [2Fe-2S] cluster and was postulated to interact with ferredoxin. Thus, the MA0664 product is hypothesized to interact with ferredoxin (Fig. 4). This proposal is supported by the amino acid sequence of MA0664, which contains four cysteine motifs capable of ligating four [4Fe-4S] clusters, three of which are conserved with RnfB (Fig. 5A). These results are consistent with a role for the product of

MA0664 forming an iron-sulfur electron-conducting "wire" originating with the iron-sulfur clusters of ferredoxin. The product of MA0659 has identity with RnfC (Fig. 5B), which was previously proposed to oxidize NADH and to harbor two [4Fe-4S] clusters in addition to flavin mononucleotide (FMN), although the amino acid sequence of MA0659 has no consensus FMN-binding motif. The amino acid sequence of MA0659

FIG. 6. Analysis of *c*-type cytochromes in *M. acetivorans*. (A) SDS-PAGE-separated and heme-stained proteins from whole-cell lysates (35 μ g) of acetate-grown (AC) and methanol-grown (ME) cells. (B) SDS-PAGE-separated and heme-stained proteins (18 μ g) from the cytoplasmic (AC-CF) and membrane (AC-MF) fractions of acetate-grown cells. (C) Reduced-minus-oxidized-difference spectrum of the membrane fractions of acetate- and methanol-grown cells. Each membrane suspension contained 2 mg/ml of protein. Arrows indicate the approximate molecular mass of each band determined after staining with Coomassie brilliant blue R.

also has two cysteine motifs consistent with ligation of two [4Fe-4S] clusters (Fig. 5B). Thus, the product of MA0659 is postulated to extend the iron-sulfur "wire" mediating electron transfer between ferredoxin and MA0661 (Fig. 4). The MA0661 product shares identity with the RnfG subunit (Fig. 5C), which extends to a motif (ETPGLGX₃₇₋₄₃GAT) that is also conserved with the NqrC subunit of *Vibrio* species (23) in which the C-terminal threonine of the motif covalently binds FMN (3, 14, 30). Therefore, the product of MA0661 is hypothesized to contain FMN, accepting electrons from the product of MA0659 (Fig. 4).

Although the annotation for MA0658 (18) is a predicted protein, closer inspection indicates five CXXCH motifs and one CXXXCH motif consistent with a multiheme *c*-type cytochrome that covalently binds hemes with a histidine axial ligand. SDS-PAGE of whole-cell lysates identified two protein bands in acetate- and methanol-grown cells that stained positive for heme, one approximately 25 kDa and the other 55 kDa (Fig. 6A). The product of MA0658 has a calculated molecular mass of 55 kDa, and the heme-stained 55-kDa protein was more abundant in acetate-grown cells than in methanol-grown cells, consistent with the greater abundance of other products encoded by the cotranscribed MA0658-0665 gene cluster. These results indicate that the heme-stained 55-kDa protein is encoded by MA0658 and contains covalently bound heme predicted from the amino acid sequence. SDS-PAGE and heme staining of the cytoplasmic and membrane fractions of acetate-

grown cells (Fig. 6B) indicated that the product of MA0658 is membrane associated. LC/hybrid linear ion trap-FTICR MS analysis of the 55-kDa protein band identified two peptides (Y₁₂₁GLYDFDAR₁₂₉ and S₄₈₁IADVTDKPRPVEVETEPAL₅₀₀) that are unique to the product of MA0658. Furthermore, the reduced-minus-oxidized-difference spectrum indicated the prevalence of a *c*₅₅₂-type cytochrome in the membrane fraction of acetate-grown cells (Fig. 6C). These results indicate that the product of MA0658 is a membrane-bound cytochrome *c*. Thus, it is hypothesized that cytochrome *c* mediates electron transfer from the product of MA0661 to methanophenazine (Fig. 4) during growth with acetate.

The amino acid sequence of MA0665 has no identity to any protein with a known function (18). However, the product of MA0665 belongs to a family (UPF0132) of small integral membrane proteins identified in all methane-producing *Archaea* for which the genome has been sequenced (18). Thus, although the function cannot be predicted, the product of MA0665 is postulated to associate with the membrane (Fig. 4).

As discussed above, the results presented here indicate that the terminal step in electron transport for acetate-grown *M. acetivorans* is the reduction of the heterodisulfide CoM-S-S-CoB (Fig. 1). The products of MA0687 and MA0688, annotated to encode the two-subunit heterodisulfide reductase (HdrDE type) (18), were found in acetate-grown cells (Table 1), indicating synthesis of the enzyme. Furthermore, RT-PCR analyses (Fig. 2) showed cotranscription of MA0687-0688 in acetate-grown cells. The HdrDE type functions to reduce CoM-S-S-CoB in the pathway for methanol conversion to methane in all *Methanosarcina* species previously investigated (6). Thus, assuming that the HdrDE type also functions in the methanol pathway for *M. acetivorans*, the relative abundance found in acetate-grown cells compared to that found in methanol-grown cells (Table 1) suggests that the HdrDE type is a major contributor to the heterodisulfide reductase activity in acetate-grown cells. The HdrDE type has been characterized from acetate-grown *M. thermophila* (37) and *Methanosarcina barkeri* (16). In these freshwater species, the HdrDE type is associated with a hydrogenase (Vho) proposed to oxidize H₂ and reduce methanophenazine which donates electrons to HdrDE. However, in the absence of an Ech hydrogenase in *M. acetivorans*, it is hypothesized that methanophenazine mediates electron transfer directly from the Ma-Rnf complex to HdrDE without the participation of H₂ and hydrogenase (Fig. 1).

The proposed electron transport scheme in the pathway of acetate conversion to methane by *M. acetivorans* (Fig. 1 and 4) is consistent with the absence of a functional Ech hydrogenase encoded in the genome (18) and with previous biochemical characterizations indicating that H₂ is not an intermediate for this species (31). Furthermore, a recent genetic analysis has concluded that *M. acetivorans* utilizes an unknown energy-conserving electron transport scheme fundamentally different from that of freshwater *Methanosarcina* species in that it does not involve H₂ (13). The noninvolvement of H₂ as an obligatory electron carrier is compatible with the marine environment from which *M. acetivorans* was isolated (38). In the marine environment, sulfate-reducing species outcompete methane-producing species for H₂ (44). Unless the evolved H₂ was channeled internally by an unknown mechanism, a significant amount could be lost to sulfate-reducing species. Thus, *M. acetivorans*

may have evolved an electron transport scheme not involving H_2 , as opposed to freshwater species. It is also tempting to postulate that the Ma-Rnf complex pumps sodium to generate a sodium gradient that directly or indirectly drives ATP synthesis, consistent with the similarity of Ma-Rnf to the sodium-pumping Nqr complex of marine species (4, 43). Indeed, a sodium-pumping role has previously been proposed (5) for the homologous Rnf complex of *C. tetani* (Fig. 3).

The genome of *M. acetivorans* is annotated with a seven-gene cluster (MA4566-72) encoding an Mrp (multiple-resistance/pH regulation) complex for which the products of two genes (MA4567 and MA4568) were substantially more abundant in acetate-grown cells, suggesting a role for this Mrp complex during growth with acetate. RT-PCR analysis showed expression of the MA4566-72 genes in a transcriptional unit (Fig. 2). Homologs of genes encoding the Mrp complex are found in diverse species from the *Bacteria* domain, including *Bacillus subtilis* (Fig. 3B). This is the first evidence reported for synthesis of an Mrp complex in the *Archaea* domain. A function for Mrp in *B. subtilis* is that of a secondary sodium/proton antiporter (19); thus, a possible function for the Mrp of *M. acetivorans* is to convert sodium gradients to a proton gradient that drives ATP synthesis by the archaeal proton-translocating A_1A_0 -type ATP synthase (Fig. 1) abundant in acetate-grown cells (Table 1). It was previously noted (19) that subunits of the Mrp complex from the *Bacteria* domain have high identity with several subunits of proton-translocating NADH dehydrogenases and the formate hydrogenlyase system from *Escherichia coli*, and it was hypothesized that the Mrp complex could possibly function as a primary ion extrusion complex energized by electron transport. The genome of *M. mazei* is not annotated to encode the Mrp complex (18), consistent with a role for the complex specific to *M. acetivorans* and the marine environment. Clearly, the results presented here invite investigation of these potential functions.

Conclusions. The first global comparison of regulated gene expression and protein synthesis between methanoarchaeal species has suggested major differences in the pathway of acetate conversion to methane between freshwater and marine species. The results suggest an adaptation of *M. acetivorans* to the marine environment that utilizes two multisubunit protein complexes, also found in the *Bacteria* domain, for electron transport and energy conservation.

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