The Archaeon *Methanosarcina acetivorans* Contains a Protein Disulfide Reductase with an Iron-Sulfur Cluster^{∇†}

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Methanosarcina acetivorans, a strictly anaerobic methane-producing species belonging to the domain *Archaea*, contains a gene cluster annotated with homologs encoding oxidative stress proteins. One of the genes (MA3736) is annotated as a gene encoding an uncharacterized carboxymuconolactone decarboxylase, an enzyme required for aerobic growth with aromatic compounds by species in the domain *Bacteria*. Methane-producing species are not known to utilize aromatic compounds, suggesting that MA3736 is incorrectly annotated. The product of MA3736, overproduced in *Escherichia coli*, had protein disulfide reductase activity dependent on a C₆₇XXC₇₀ motif not found in carboxymuconolactone decarboxylase. We propose that MA3736 be renamed *mdrA* (*methanosarcina* disulfide reductase). Further, unlike carboxymuconolactone decarboxylase, MdrA contained an Fe-S cluster. Binding of the Fe-S cluster was dependent on essential cysteines C₆₇ and C₇₀, while cysteines C₃₉ and C₁₀₇ were not required. Loss of the Fe-S cluster resulted in conversion of MdrA from an inactive hexamer to a trimer with protein disulfide reductase activity. The data suggest that MdrA is the prototype of a previously unrecognized protein disulfide reductase family which contains an intermolecular Fe-S cluster that controls oligomerization as a mechanism to regulate protein disulfide reductase activity.

The oxidative stress defense mechanisms utilized by prokaryotes of the domain *Bacteria* are well understood (61). Considerably less is known about these mechanisms in members of the domain *Archaea*, including the strictly anaerobic methane-producing archaea (methanoarchaea). It has been documented that *Methanosarcina* and *Methanobrevibacter* species are aerotolerant (34, 38). *Methanosarcina barkeri* survives exposure to air and resumes growth immediately after a return to anaerobiosis (20, 67), suggesting that it mounts a substantial defense against oxidative stress. An iron superoxide dismutase and catalase have been characterized from *M. barkeri* (7, 58). Recently, an iron-sulfur flavoprotein (Isf) from *Methanosarcina thermophila* was shown to reduce O₂ and H₂O₂ to water (13). The sequenced genomes of *Methanosarcina* species (14, 22) contain homologs of genes encoding superoxide reductase and rubrerythrin, proteins unique to anaerobes that reduce superoxide and hydrogen peroxide, respectively, and have been characterized from other strict anaerobes (12, 25, 31, 46, 64). The genome annotations also include homologs of genes encoding flavoprotein A (FprA), which reduces O₂ to water (56).

RC-I_{MRE50} is an uncultured methanoarchaeon closely related to *Methanosarcina* species and is a representative of the rice cluster I (RC-I) methanoarchaea, which are the predominant methanoarchaea in the rice rhizosphere (11, 16). The RC-I_{MRE50} group is the primary contributor to methane emissions from rice fields, which are estimated to contribute 10 to 25% of the global methane emissions to the atmosphere (17).

The recent sequencing of the RC-I_{MRE50} genome revealed genes encoding homologs of antioxidant enzymes, including superoxide dismutase, superoxide reductase, catalase, rubrerythrin, FprA, and peroxiredoxins. Thus, it has been suggested that aerotolerance is a key component of the competitive superiority of RC-I_{MRE50}, allowing survival during transient oxic conditions associated with life in the rhizosphere (17). The genome of *Methanosarcina acetivorans*, a marine methanoarchaeon phylogenetically related to RC-I_{MRE50} (16), also contains homologs of genes encoding antioxidant enzymes similar to those found in RC-I_{MRE50} (17, 22), suggesting that *M. acetivorans* can also survive transient oxic conditions found in the kelp bed sediment from which it was isolated (60). To date, attempts to obtain RC-I organisms in pure culture have not been successful. *M. acetivorans* has a robust genetic system (49, 66), making this organism an attractive model for studying the specific function of the annotated antioxidant genes and for discovering additional genes important for aerotolerance of *Methanosarcina* and related species, including RC-I_{MRE50}.

Here we show that the genome of *M. acetivorans* contains a 10-gene transcriptional unit annotated with homologs of genes encoding superoxide reductase, FprA, and Isf. MA3736 in the cotranscribed gene cluster is annotated as a gene encoding carboxymuconolactone decarboxylase (CMD), an enzyme essential in aerobic species in the domain *Bacteria* utilizing aromatic compounds as growth substrates (18, 52). Methanogens are strictly anaerobic, and none are known to metabolize aromatic compounds for growth (68), suggesting that MA3736 is annotated incorrectly. We overproduced the MA3736 product in *Escherichia coli* and found that the purified product had protein disulfide reductase activity dependent on a CXXC motif typical of protein disulfide reductases. Unexpectedly, the MA3736 product was found to contain an Fe-S cluster(s) with binding also dependent on the CXXC motif. Loss of the Fe-S

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cluster(s) was necessary for protein disulfide reductase activity. We propose that MA3736 is distinct from CMD and should be renamed *mdrA* (*methanosarcina* disulfide reductase).

MATERIALS AND METHODS

RT-PCR analysis. Sequence information for *M. acetivorans*, *Methanosarcina mazei*, and *M. barkeri* was obtained from The Institute for Genomic Research (<http://www.tigr.org>), and sequence information for *Methanococcoides burtonii* was obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Total RNA was isolated from methanol-grown *M. acetivorans*, and reverse transcription (RT)-PCR analysis of the gene cluster containing MA4664 and MA3734 to MA3743 (designated the MA4664/MA3734-MA3743 cluster) was performed as described previously (43). The primer sequences used are listed in Table S1 in the supplemental material.

Cloning, expression, and purification of MdrA. The gene encoding MdrA was amplified from *M. acetivorans* genomic DNA by PCR. The PCR-amplified DNA fragment was cloned into the pTYB12 vector from an IMPACT T7 kit (New England Biolabs), generating plasmid pDJL200. pDJL200 contains the chitin-binding domain (CBD)-intein-MdrA fusion.

The CBD-intein-MdrA fusion was overproduced in *E. coli* Rosetta (DE3)(pLacI) cells transformed with pDJL200. Cells were grown in Terrific broth at 37°C with shaking at 250 rpm until an optical density at 600 nm of 0.5 to 0.7 was reached, at which time the growth temperature was adjusted to 16°C. After 30 min the culture was induced with 500 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) and then harvested by centrifugation 16 h after induction. All subsequent purification procedures were performed anaerobically using an anaerobic chamber (Coy Laboratory Products) containing an atmosphere of 95% N₂ and 5% H₂. Approximately 15 g (wet weight) of cells was suspended in 20 ml of 50 mM HEPES (pH 7.5) containing 300 mM NaCl and 2 mM benzimidazole. The cells were lysed by two passages through a French pressure cell at 138 MPa. The lysate was centrifuged at 74,000 $\times$ g for 30 min at 4°C. The supernatant solution containing the CBD-intein-MdrA fusion protein was filtered (pore size, 0.45 μ m) and applied at a flow rate of 0.5 ml/min to a column containing 20 ml of chitin bead resin (New England Biolabs). The column was then washed with 200 ml of 50 mM HEPES (pH 7.5) containing 300 mM NaCl and 1% Triton X-100 at a flow rate of 2 ml/min. MdrA was cleaved from the CBD by flushing the column with 60 ml of 50 mM HEPES (pH 7.5) containing 300 mM NaCl and 40 mM dithiothreitol (DTT), followed by incubation of the column for 16 h at room temperature. MdrA was then eluted from the column with 60 ml of 50 mM HEPES (pH 7.5) containing 300 mM NaCl. The elute was concentrated to 2.5 ml using a Vivacell concentrator with a 10,000-molecular-weight cutoff under a nitrogen flow inside the anaerobic chamber. The concentrated protein was desalted with 3.5 ml of 50 mM HEPES (pH 7.5) containing 300 mM NaCl using a PD-10 column (Amersham Biosciences). The purity of MdrA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. MdrA purified using this method contained one additional histidine residue in the N terminus.

MdrA variants were generated by site-directed mutagenesis with primers listed in Table S1 in the supplemental material, using a QuickChange site-directed mutagenesis kit (Stratagene). Each variant protein was purified as described above for wild-type MdrA.

Protein concentrations were determined by the method of Bradford (6), using bovine serum albumin as a standard.

Enzyme assays. The protein disulfide reductase activity of MdrA was determined using the turbidimetric assay for insulin disulfide reduction described by Holmgren (30). For determination of DTT-dependent activity, the assay mixture contained 0.4 ml (final volume) of 100 mM potassium phosphate (pH 7.0), 0.13 mM insulin, 1 mM EDTA, and 0 to 10 μ M MdrA. The reaction was initiated by addition of 0.33 mM DTT and was performed at 21°C. The absorbance at 650 nm was plotted against time. Assays were done in an anaerobic chamber (Coy). Activity was expressed as the ratio of the slope of a linear part of the turbidity curve to the lag time (reported as $\Delta A_{650}/\text{min}^2$, 10^{-5}), as described previously (48, 57). The lipamide-dependent insulin disulfide reduction activity of MdrA was assayed with an assay similar to the DTT-dependent assays using NADH, lipamide, and bovine lipamide dehydrogenase (8, 30). The typical assay was performed anaerobically, and the assay mixture contained 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 0.13 mM bovine insulin, 0.4 U of lipamide dehydrogenase, 50 μ M lipamide, and 0 to 10 μ M MdrA. The reaction was initiated by addition of 0.5 mM NADH, and turbidity was monitored at 650 nm.

Characterization of chromophore content. The iron and acid-labile sulfide content of MdrA was determined as previously described (4, 65). UV-visible

spectra of MdrA and variants were recorded with a Beckman DU-7400 spectrophotometer inside an anaerobic chamber (Coy). The putative Fe-S cluster was removed by anaerobic incubation of MdrA with dithionite and 20 mM EDTA in 50 mM HEPES (pH 7.5) containing 300 mM NaCl for 2 h at 25°C. The protein was then desalted with a PD-10 column equilibrated with 50 mM HEPES (pH 7.5) containing 300 mM NaCl. The resulting form of MdrA is referred to as apo-MdrA below.

Size exclusion chromatography. Estimates of the native molecular masses of MdrA and variants of MdrA were based on elution from a Sephacryl Hiprep S-200 gel filtration fast protein liquid chromatography column (Amersham Biosciences) using an AKTA explorer (Pharmacia Biotech). The column was calibrated with the following proteins having known molecular masses: β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The buffer used was 50 mM HEPES (pH 7.5) containing 150 mM NaCl and 10 mM DTT to provide reducing conditions. A flow rate of 0.5 ml min⁻¹ was used. Samples containing 0.5 to 0.6 mM protein were loaded onto the column. To determine the effect of EDTA on the oligomeric state of wild-type MdrA and cysteine variants of MdrA, proteins were incubated with 10 mM EDTA under anaerobic conditions at 25°C for 30 min prior to injection onto the column with 10 mM EDTA in the elution buffer.

Construction of a phylogenetic tree. Database searches and alignments were carried out using BLAST and CLUSTALX. The output was edited with the Alignment Editor of MEGA (v3.1) (37). A phylogenetic tree was constructed with the MEGA package using the neighbor-joining method, including 500 bootstrap replicates. The accession numbers for all protein sequences used for the phylogenetic analysis are as follows: *M. acetivorans* MA3736, gi: 19917805; *M. mazei* Goe1 MM0631, gi: 20905023; uncultured RC-I methanogenic archaeon RCIX2594, gi: 110622368; *Thermus thermophilus* HB8 TTHA0727, gi: 55772109; *Rhodococcus* sp. strain RHA1 RHA1_ro11235, gi: 110825601; *Mycobacterium tuberculosis* H37Rv Rv1767, gi: 2131035; *Thermotoga maritima* MSB8 TM1620, gi: 15644368; *Rhodospirillum rubrum* BisB18 RPC_4301, gi: 90107787; *Lactobacillus sakei* 23K LSA1776, gi: 78611031; *Thermoanaerobacter tengcongensis* MB4(T) TTE0299, gi: 20515286; *R. palustris* BisB18 RPC444, gi: 90107930; *Legionella pneumophila* Philadelphia 1 lpg2349, gi: 52629670; *Streptomyces coelicolor* A3(2) SCO5031, gi: 9967658; *M. tuberculosis* H37Rv Rv2429, gi: 1666155; *Caulobacter crescentus* CB15 CC_3698, gi: 13425462; *Myxococcus xanthus* DK 1622 MXAN_1563, gi: 108465278; *Brucella abortus* 9-941 BruAb2_0523, gi: 62197643; *Corynebacterium diphtheriae* NCTC13129 DIP1419, gi: 38200266; *Ralstonia eutropha* JMP134 Reut_A1364, gi: 72118471; *Nocardia farcinica* IFM10152 nfa37900, gi: 54017268; *Cytophaga hutchinsonii* ATCC 33406 CHU_3759, gi: 110282806; *Acinetobacter* sp. strain ADP1 ACIAD1710, gi: 49530840; *Methanobacterium thermoautotrophicum* delta H MTH234, gi: 2621282; *M. acetivorans* C2A MA0409, gi: 19914189; *Sulfolobus acidocaldarius* DSM 639 Saci_1814, gi: 68568191; *M. tuberculosis* H37Rv Rv0771, gi: 1550649; *Rhodococcus* sp. strain RHA1 RHA1_ro11338, gi: 110817878; *Pseudomonas putida* KT2440 PP_1381, gi: 24982843; *Burkholderia xenovorans* LB400 Bxe_B0647, gi: 91692108; *S. coelicolor* A3(2) SCO6339, gi: 3367745; *R. palustris* CGA009 RPA4740, gi: 39651658; and *Shewanella oneidensis* MR-1 SO_0083, gi: 24345456.

RESULTS

Analysis of the MA4664/MA3734-MA3743 gene cluster. Similar to other *Methanosarcina* spp. (34), *M. acetivorans* can withstand prolonged exposure to atmospheric levels of O₂ and resume growth once anaerobiosis is restored (data not shown), suggesting that this organism contains enzymes for protection from and/or repair of damage caused by reactive O₂ species. Indeed, the MA4664/MA3734-MA3743 gene cluster (Fig. 1) contains homologs of genes encoding oxidative stress proteins that have been characterized from other strict anaerobes. This gene arrangement is similar to that of gene clusters in other sequenced *Methanosarcina* and related species (Fig. 1), suggesting that the gene products have an important function in these organisms. However, the original annotation of MA3739 appears to be incorrect, as the first 53 amino acids of the gene product are missing compared to the gene products of MM0633 and Mbur2376 (see Fig. S1 in the supplemental material). We propose that MA3739 starts at a codon that is

FIG. 1. Organization of the *M. acetivorans* MA4664/MA3734-MA3743 gene cluster and comparison to gene clusters in other *Methanosarcina* species. (A) The MA4664/MA3734-MA3743 gene organization shown in line a is the original annotation, and that shown in line b is the proposed annotation. MA4664/MA3734-MA3743 is compared to the following gene clusters from other sequenced methanogens: *M. mazei* Go1 MM0629 to MM0638, *M. burtonii* DSM 6242 Mbur2373 to Mbur2380, and *M. barkeri* strain Fusaro Mbar_A2452 to Mbar_A2454 and Mbar_A0252 to Mbar_A0250. The arrows indicate the gene direction and relative size and spacing. Homologous genes are indicated by the same pattern and are centered on MA3736 (indicated by the solid arrow). Genes indicated by asterisks in the *M. mazei* and *M. barkeri* gene clusters were missed in the original annotation and encode desulfiredoxin (Dx) homologs similar to the MA4664 product. Mbur2378 and Mbur2379 encode homologs of flavodoxin and rubrerythrin, respectively. The genes in *M. barkeri* are not contiguous, as indicated by slashes. (B) RT-PCR analysis of the MA4664/MA3734-MA3743 gene cluster in *M. acetivorans*. Predicted RT-PCR products are indicated in panel A by lines under the genes and are labeled with roman numerals. Predicted RT-PCR product sizes are indicated in parentheses. The roman numerals above the gel lanes correspond to the predicted RT-PCR products. For lane IV' the reaction was performed without reverse transcriptase.

within MA3738 previously annotated as divergently transcribed from MA3739, suggesting that MA3738 is not a functional open reading frame (Fig. 1). RT-PCR analysis of each intergenic region in the MA4664/MA3734-MA3743 gene cluster (data not shown) and across several genes (Fig. 1B) indicated that the genes are cotranscribed and further suggested that MA3738 is not a functional gene. Furthermore, the products of most of the genes (MA3735, MA3736, MA3737, MA3740, MA3741, MA3742, and MA3743) were detected at similar levels in CO₂-, acetate-, and methanol-grown cells by global proteomic analyses (41, 42), consistent with a physiological function for the encoded proteins.

In the MA4664/MA3734-MA3743 transcriptional unit, three of the gene products are annotated as proteins that directly reduce reactive O₂ species. MA3737 is annotated as a gene encoding a class II superoxide reductase (see Fig. S2 in the supplemental material) (3). MA3740 is annotated as a gene encoding a homolog of Isf (see Fig. S3 in the supplemental material), and MA3743 is annotated as a gene encoding FprA (see Fig. S4 in the supplemental material); both of these gene products reduce O₂ to H₂O (13, 56). In addition, MA4664 is annotated as a gene encoding a homolog of desulfiredoxin (see Fig. S5 in the supplemental material), the physiological electron donor to the class II superoxide reductase of *Desul-*

fovibrio gigas (3). A role for the remaining gene products in response to oxidative stress has not been documented.

In contrast to the annotation of other genes in the MA4664/MA3734-MA3743 transcriptional unit that could function in oxidative stress, MA3736 is annotated as a gene encoding an uncharacterized CMD homolog. CMD is an essential enzyme in aerobic species in the domain *Bacteria* that utilize aromatic compounds as growth substrates (18, 52). Methanogens are strictly anaerobic, and none are known to metabolize aromatic compounds for growth (68), suggesting that MA3736 is annotated incorrectly, which prompted an investigation of the physiological function of the protein previously shown to be present in CO₂-, acetate-, and methanol-grown cells of *M. acetivorans* (41, 42).

Purification of the MA3736 product and initial characterization. Unlike characterized CMD proteins, the deduced sequences of the MA3736 product and homologs (Fig. 1) contain a CXXC motif within a domain that has sequence identity (~30%) to the active site domain of the prototypical AhpD protein from *M. tuberculosis* (Fig. 2). Although AhpD has alkylperoxide reductase activity, it functions primarily as a disulfide reductase, reducing the active site disulfide of AhpC, a peroxiredoxin (8, 28, 36). AhpD and AhpC are key components of the oxidative stress response in *M. tuberculosis* (8, 26).

FIG. 2. Alignment of amino acid sequences of MdrA homologs and AhpD from *M. tuberculosis*. Identical amino acid residues are indicated by asterisks. The active site cysteines of AhpD that are conserved in the MdrA homologs (C_{67} and C_{70} in MdrA) are indicated by solid arrowheads, and additional conserved cysteines (C_{39} and C_{107} in MdrA) not found in AhpD are indicated by open arrowheads. Sequences were aligned using CLUSTAL W. MdrA, *M. acetivorans* C2A; MM0631, *M. mazei* Go1; Mbar_A2454, *M. barkeri* strain Fusaro; Mbur2375, *M. burtonii* DSM 6242; AhpD, *M. tuberculosis*.

Thus, MA3736 was heterologously expressed, and the protein was anaerobically purified to test for AhpD-like activities. The protein was judged homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, which also indicated that the subunit molecular mass was consistent with the calculated value, 12.9 kDa (data not shown). The purified MA3736 product was assayed for alkylperoxide reductase activity using DTT or a reducing system comprised of NADH, lipoamide, and lipoamide dehydrogenase, as previously described for AhpD (28, 36). No activity was detected under anaerobic conditions (data not shown), suggesting that the protein does not function as an alkylperoxide reductase. However, the MA3736 product exhibited both DTT- and lipoamide-dependent protein disulfide reductase activity as measured by the insulin turbidimetric assay (30) under anaerobic conditions (Fig. 3). No protein disulfide reductase activity was detected when the product was assayed aerobically. The DTT-dependent protein disulfide reductase activity of MdrA was approximately 20% of that measured for thioredoxin from *E. coli* (data not shown). Lipoamide-dependent activity was dependent on all three assay components (data not shown), suggesting that lipoamide directly reduces the oxidized MA3736 product, similar to AhpD (8). This is the first enzymatic activity determined for a product of genes annotated as genes encoding putative CMD enzymes with a CXXC motif. We propose that MA3736 encodes a protein distinct from CMD and should be renamed *mdrA* (methanosarcina disulfide reductase).

It is unclear what protein(s) or cofactor(s) functions as an in vivo electron donor to MdrA. Reduced coenzyme F_{420} , a universal electron carrier in methanogens, was ineffective as a direct electron donor (data not shown). NADPH-dependent thioredoxin reductase from *E. coli* also could not supply electrons to support MdrA protein disulfide reductase activity (data not shown). Reduction of AhpD in vivo is linked to metabolic enzymes of the tricarboxylic acid cycle in *M. tuberculosis* (8). Dihydrolipoamide succinyltransferase (SucB), a lipoamide-containing protein, is a reducing partner of AhpD. SucB

is subsequently reduced by lipoamide dehydrogenase via NADH in vivo (8, 36). To examine the specificity of MdrA for the AhpD reducing partners, we assayed MdrA for disulfide reductase activity with the 5,5'-dithiobis(2-nitrobenzoic acid) assay developed by Bryk et al. (8), using purified SucB and lipoamide dehydrogenase from *M. tuberculosis*. MdrA could not substitute for AhpD in this assay (data not shown), suggesting that there are differences in the specificities of AhpD and MdrA for redox partners.

Analysis of MdrA cysteine variants. MdrA contains two additional conserved cysteine residues independent of the $C_{67}XXC_{70}$ motif; one is located in the N terminus (C_{39}), while the second (C_{107}) is located in the C terminus (Fig. 2). Protein disulfide reductases, including AhpD, thioredoxin, and glutaredoxin, possess redox-active cysteine residues within a CXXC motif (29). However, the redox-active cysteine residues in AhpC-like peroxiredoxins are located on opposite ends of the protein (15, 53), similar to the locations of C_{39} and C_{107} in MdrA. To determine which MdrA cysteines are functionally important for protein disulfide reductase activity, cysteine-to-serine variants, including single variants ($C_{39}S$, $C_{67}S$, $C_{70}S$, and $C_{107}S$) and double variants ($C_{39}S/C_{107}S$ and $C_{67}S/C_{70}S$), were generated.

All of the MdrA variants were expressed and purified at levels similar to that of the wild type (data not shown). The $C_{39}S$, $C_{107}S$, and $C_{39}S/C_{107}S$ variants retained wild-type levels of activity in the DTT- and lipoamide-dependent assays (Fig. 4). However, the $C_{67}S$ and $C_{70}S$ single variants exhibited only 3 to 9% of the wild-type MdrA activity in both assays (Fig. 4). In addition, the $C_{67}S/C_{70}S$ double variant had no detectable activity in either assay (Fig. 4). These results indicate that C_{67} and C_{70} are required for protein disulfide reductase activity, consistent with a requirement for the CXXC motif in other characterized protein disulfide reductases (29).

Detection of an Fe-S cluster in MdrA. Unexpectedly, wild-type MdrA and the $C_{39}S/C_{107}S$ variant were red-brown in color

FIG. 3. Protein disulfide reductase activity of MdrA as determined by the insulin turbidimetric method. (A) DTT-dependent protein disulfide reductase activity of MdrA. The assay was carried out by addition of 0.33 mM DTT in 100 mM potassium phosphate (pH 7.0) containing 0.13 mM bovine insulin in the absence of MdrA ($\diamond$) and in the presence of different concentrations of MdrA, including 2.5 μ M ($\blacksquare$), 5 μ M ($\blacktriangle$), 7.5 μ M ($\square$), and 10 μ M ($\diamond$). (B) Lipoamide-dependent protein disulfide reductase activity of MdrA. The assay was carried out by addition of 0.5 mM NADH in 100 mM potassium phosphate (pH 7.0) containing 0.13 mM bovine insulin, 0.05 mM lipoamide, and 0.4 U bovine lipoamide dehydrogenase in the absence of MdrA ($\diamond$) and in the presence of different concentrations of MdrA, including 2.5 μ M ($\blacksquare$), 5 μ M ($\blacktriangle$), 7.5 μ M ($\diamond$), and 10 μ M ($\square$). The insets show the linear dependence of the activity on the MdrA concentration.

(Fig. 5), and iron and acid-labile sulfide were detected in both proteins (Table 1). The UV-visible spectrum of the $C_{39}S/C_{107}S$ variant had absorbance peaks at 335, 412, 460, and 520 nm (Fig. 5). Similar spectral features were observed for the wild type, although the overall absorption was less (Fig. 5). These results suggest that MdrA contains an Fe-S cluster having an undetermined composition. The ratio of iron or acid-labile sulfide per monomer was less than unity for the wild type and the variant, which suggests either that the proteins do not

FIG. 4. Protein disulfide reductase activities of wild-type MdrA and cysteine variants. (A) DTT-dependent activity. (B) Lipoamide-dependent activity. Assays were performed as described in Materials and Methods. The values are reported as $\Delta A_{650}/\text{min}^2 \cdot 10^{-5}$. WT, wild type.

contain a full complement of the Fe-S cluster(s) or that the cluster(s) is bound to more than one monomer. A double cysteine-to-alanine variant ($C_{39}A/C_{107}A$) was also red-brown in color and had a UV-visible absorption spectrum similar to that of the $C_{39}S/C_{107}S$ variant (data not shown). These results suggest that residues at positions 39 and 107 are not essential for Fe-S cluster binding. However, the $C_{67}S/C_{70}S$ variant was colorless and lacked spectral features of the wild type and the $C_{39}S/C_{107}S$ variant (Fig. 5). Further, the levels of iron and acid-labile sulfide were below the limits of detection in the

FIG. 5. UV-visible spectra of wild-type MdrA and variants. Line a, wild-type MdrA (400 μ M); line b, $C_{39}S/C_{107}S$ (200 μ M); line c, $C_{67}S/C_{70}S$ (400 μ M). The inset shows vials containing the protein solutions.

TABLE 1. Analysis of iron and acid-labile sulfide in wild-type MdrA and cysteine variants

Protein	Amt of iron (nmol/ nmol of MdrA monomer)	Amt of sulfide (nmol/ nmol of MdrA monomer)
MdrA ^a	0.37 ± 0.05	0.15 ± 0.02
C ₃₉ S/C ₁₀₇ S	0.44 ± 0.06	0.23 ± 0.02
C ₆₇ S/C ₇₀ S	BDL ^b	BDL
apo-MdrA ^c	BDL	BDL

^a As-purified MdrA.^b BDL, below the detection limit (0.01 nmol).^c As-purified MdrA pretreated with EDTA.

C₆₇S/C₇₀S variant (Table 1). Single-cysteine variants (C₆₇S and C₇₀S) were also colorless and lacked spectral features of the wild type and the C₃₉S/C₁₀₇S variant (data not shown). These results suggest not only that the active site cysteines function in protein disulfide reduction but also that both of these residues play a role in ligation of the Fe-S cluster(s).

Effect of the Fe-S cluster on protein disulfide reductase activity and the oligomeric state of MdrA. As residues Cys₆₇ and Cys₇₀ appear to be necessary for protein disulfide reductase activity and binding of an Fe-S cluster, the effect of the presence or absence of the Fe-S cluster on protein disulfide reductase activity was determined. The presence of EDTA in the assay mixture was necessary for activity with purified MdrA, unless MdrA was pretreated with EDTA (Table 2), in which case iron or acid-labile sulfide was undetectable (apo-MdrA) (Table 1). These results suggest that loss of the Fe-S cluster(s) is required for protein disulfide reductase activity.

Four cysteines typically coordinate Fe-S clusters. However, only Cys₆₇ and Cys₇₀ appear to be required for Fe-S cluster binding in MdrA, suggesting that the cluster has ligands other than cysteine or that MdrA contains an intermolecular cluster coordinated by cysteines from more than one monomer. Indeed, the disulfide oxidoreductase glutaredoxin 2 (Grx2) from humans contains an intermolecular bridging [2Fe-2S] cluster that has been shown to regulate disulfide reductase activity (44). Thus, the effect of loss of the Fe-S cluster on the oligomeric state of wild-type MdrA and the cysteine variants was determined by size exclusion chromatography (Fig. 6). The elution profile of as-purified wild-type MdrA was consistent with the profile of a hexamer (Fig. 6A). The C₃₉S/C₁₀₇S variant elution profile was similar to that of the wild type, which was also consistent with the profile of a hexamer (Fig. 6C). However, the C₆₇S/C₇₀S variant migrated as a trimer (Fig. 6B).

TABLE 2. Comparison of as-purified MdrA and apo-MdrA protein disulfide reductase activities

Protein	DTT-dependent activity (U/mg) ^a		Lipoamide-dependent activity (U/mg) ^a	
	With EDTA	Without EDTA	With EDTA	Without EDTA
MdrA	95 ± 11	BDL ^b	58 ± 6	BDL
apo-MdrA	80 ± 10	72 ± 15	50 ± 4	52 ± 9

^a Assays were performed as described in Materials and Methods with 10 μM protein with or without 1 mM EDTA in the assay mixture. The values are reported as ΔA₄₅₀/min², 10⁻⁵.

^b BDL, below the detection limit.

FIG. 6. Effect of EDTA on the oligomeric state of MdrA and cysteine variants as analyzed by size exclusion chromatography. (A) Elution profiles of wild-type MdrA as purified (black line) and with EDTA (light gray line) and of apo-MdrA (dark gray line). (B) Elution profiles of the C₆₇S/C₇₀S variant as purified (black line) and with EDTA (gray line). (C) Elution profiles of the C₃₉S/C₁₀₇S variant as purified (black line) and with EDTA (gray line). Dashed vertical line a indicates the volume corresponding to the hexameric form of MdrA, and dashed vertical line b indicates the volume corresponding to the trimeric form of MdrA. Hexameric and trimeric volumes were calculated based on a standard curve generated with molecular mass standards (data not shown).

Inclusion of EDTA in the buffers used in size exclusion chromatography of as-purified wild-type MdrA resulted in a mixture of smaller oligomers of MdrA, including a trimer (Fig. 6A). A similar elution profile was obtained for MdrA pretreated with EDTA (apo-MdrA) even though EDTA was not included in the buffers (Fig. 6A and Table 1). The C₃₉S/C₁₀₇S variation had a similar effect, as the protein migrated primarily as a trimer when it was eluted in the presence of EDTA (Fig. 6C). However, the C₆₇S/C₇₀S variant continued to migrate as a trimer when it was eluted in the presence of EDTA (Fig. 6B). These results demonstrate the importance of Cys₆₇ and Cys₇₀ in modulating the oligomeric state of MdrA. Thus, Cys₆₇ and Cys₇₀ may coordinate an intermolecular bridging Fe-S cluster(s) between trimers to form a hexamer, and loss of the Fe-S cluster converts the enzyme to a trimer that is active.

Phylogenetic analyses. The finding that MdrA is a protein disulfide reductase prompted an investigation of the databases to determine the extent to which proteins encoded by genes annotated as CMD genes contain the CXXC motif and have the potential to be MdrA-like protein disulfide reductases. A BLAST search of all nonredundant databases was performed with the protein sequence of MdrA as the query. A survey of the returned sequences revealed 189 putative proteins that contained a CXXC motif and had between 22 and 84% identity

FIG. 7. Phylogenetic tree of selected CMD-, MdrA-, and AhpD-related sequences. The phylogenetic tree was constructed using the neighbor-joining method. The scale bar indicates the average number of amino acid substitutions per site. Prototypical functionally analyzed CMD and AhpD, as well as MdrA, are in bold type. Cluster I contains AhpD-related proteins, and cluster II contains MdrA-related proteins. Cluster I and II proteins contain a CXXC motif, with the exception of TTHA0727 from *T. thermophilus*, which contains an SXXC motif (indicated by an asterisk). Cluster III contains prototypical CMD-related proteins that do not contain a CXXC motif.

to MdrA, suggesting that putative homologs are widespread. The analysis was further extended to understand the relatedness of MdrA and putative homologs to prototypical CMD and AhpD. A BLAST search with prototypical CMD (PcaC from *Acinetobacter* sp. strain ADP1 [24]) as the query revealed 212 putative proteins without a CXXC motif that had $\geq 24\%$ identity to PcaC. A BLAST search with prototypical AhpD from *M. tuberculosis* (28) as a query revealed 113 putative proteins with a CXXC motif that had $\geq 26\%$ identity to AhpD. To elucidate the phylogeny of MdrA and CXXC-containing and non-CXXC-containing putative CMD and AhpD proteins, 32 sequences were selected from the first 50 sequences retrieved from each BLAST search. The selections were based on previously characterized proteins and uncharacterized proteins from physiologically and phylogenetically diverse organisms. These sequences were aligned, and a phylogenetic tree was constructed (Fig. 7). The non-CXXC-containing sequences from both *Bacteria* and *Archaea* group together (cluster III),

including the prototypical CMD (PcaC) from *Acinetobacter* sp. strain ADP1. The CXXC-containing sequences display a dichotomy. Cluster II contains MdrA and various sequences from *Bacteria* and *Archaea*, whereas cluster I contains AhpD from *M. tuberculosis* (8), *S. coelicolor* (27), *L. pneumophila* (39) and sequences from other *Bacteria*. The phylogenetic analyses indicate that MdrA is distinct from both prototypical CMD and AhpD, suggesting that MdrA is the prototype of a new family. The phylogenetic analyses further suggest that there is wide distribution of CMD-related, MdrA-related, and AhpD-related enzymes among diverse prokaryotes. Two non-CXXC-containing proteins from methanogens, encoded by MTH234 from *M. thermoautotrophicum* and by MA0409 from *M. acetivorans*, group in cluster III with prototypical CMD (Fig. 7). Methanogens are strictly anaerobic, and none are known to metabolize aromatic compounds, indicating that MTH234 and MA0409 most likely do not function as CMD or as MdrA but may have an unknown function.

DISCUSSION

A major challenge in the postgenomic era is avoiding the perpetuation of incorrect annotations. Resolution of this growing problem rests on biochemical and molecular biology experimental approaches for validation of questionable annotations, as discussed recently (63). The resolution of incorrect annotations often leads to discovery of function and protein families, as is the case reported here for MA3736 (encoding MdrA) from *M. acetivorans*. MA3736 was originally annotated as a gene encoding an uncharacterized CMD homolog, but the results presented here support the conclusion that MdrA is a protein disulfide reductase with the potential to function in the oxidative stress response of *M. acetivorans* and related species, including RC-I_{MRE50}.

A role for CMD in the physiology of *M. acetivorans* is highly improbable as methanogens are strictly anaerobic and none are known to metabolize aromatic compounds for growth (68). Therefore, although MdrA shares some sequence identity (<30%) with CMD enzymes, such as PcaC from *Acinetobacter* sp. strain ADP1 (24), MdrA most likely does not function as previously annotated. Instead, MdrA was shown to contain an Fe-S cluster and to have protein disulfide reductase activity dependent on a CXXC motif that is not found in characterized CMD proteins (18, 45, 52) but is essential in other characterized protein disulfide reductases, including AhpD (8, 29, 36). Further, phylogenetic analyses indicate that MdrA is distinct from both CMD and AhpD, suggesting that MdrA is the prototype of a new family.

The active site domain of MdrA and AhpD also has identity to that of sestrins (9), proteins that play a role in peroxide signaling pathways in higher eukaryotic organisms, including humans. Analogous to AhpD, sestrin 2 catalyzes the reduction of a peroxiredoxin. However, sestrins contain only the proximal cysteine of the essential CXXC motif of AhpD and MdrA. Sestrins are not disulfide reductases but instead function as cysteine sulfinyl reductases, reducing overoxidized peroxiredoxins to modulate peroxide signaling and antioxidant defense (9). Therefore, MdrA and homologs found in ancient methanarchaea may provide an evolutionary link not only to the structurally related proteins AhpD and CMD but also to sestrins.

The data presented here suggest that the CXXC-containing domain is important for oligomerization of MdrA and control of activity. Purified wild-type MdrA and the C₃₉S/C₁₀₇S variant are hexamers, while the C₆₇S/C₇₀S variant is a trimer. Oligomerization of MdrA also appears to be dependent on Fe-S cluster binding. Although additional characterization to identify the type of Fe-S cluster was beyond the scope of this study, the UV-visible spectrum and extrapolation of the amount of iron (nanomoles per nanomole of hexamer: 2.22 ± 0.30 for the wild type and 2.64 ± 0.36 for the C₃₉S/C₁₀₇S variant) are consistent with the hypothesis that wild-type MdrA and the C₃₉S/C₁₀₇S variant contain one [2Fe-2S] cluster per hexamer, while the Fe-S cluster is absent in the trimeric C₆₇S/C₇₀S variant. In addition, the protein disulfide reductase activity of wild-type MdrA was dependent on loss of the cluster, and addition of EDTA to wild-type MdrA and the C₃₉S/C₁₀₇S variant resulted in a change from a hexamer to primarily a trimer. Taken together, these results suggest that oligomeriza-

tion of MdrA trimers is Fe-S cluster dependent and that Cys₆₇ and Cys₇₀ are important for Fe-S cluster binding.

The first disulfide reductase shown to contain a regulatory Fe-S cluster, [2Fe-2S], is Grx2 (44). Recently, a poplar glutaredoxin (Grx-C1) was also shown to contain a subunit-bridging [2Fe-2S] cluster (19, 55). The [2Fe-2S] cluster in Grx2 and Grx-C1 is coordinated by the N-terminal active site cysteine of two monomers and two noncovalently bound molecules of glutathione (5, 55). Dimeric holo-Grx2 and holo-Grx-C1 are inactive as disulfide oxidoreductases, similar to hexameric, [Fe-S]-containing MdrA. Loss of the [2Fe-2S] cluster results in activation of Grx2 and Grx-C1. In MdrA, the active site cysteines (Cys₆₇ and Cys₇₀) also appear to be necessary for Fe-S cluster binding, suggesting functional similarity to Grx2 and Grx-C1. Grx2 also contains two additional cysteine residues that are outside the active site cysteines and are postulated to play a structural role (5, 32). It is unclear what role, if any, the two additional cysteine residues (Cys₃₉ and Cys₁₀₇) play in MdrA. However, most CXXC-containing CMD homologs do not contain the additional cysteine residues found in the *Methanoscarchina*-related MdrA homologs.

Recently, WhiB4/Rv3681c from *M. tuberculosis* was shown to have protein disulfide reductase activity and to contain a labile Fe-S cluster hypothesized to regulate protein disulfide reductase activity (2). WhiB homologs have been shown to be important for survival and for the response to oxidative stress (23, 35). WhiB4 and MdrA share no overall sequence identity, as confirmed by the inability to align the amino acid sequences (62), indicating that WhiB and MdrA are members of distinct protein disulfide reductase families. WhiB proteins have four conserved cysteines, two of which are in a CXXC motif (59), similar to MdrA, suggesting that WhiB and MdrA may be functionally similar protein disulfide reductases. However, all four cysteines are important for coordinating an intramolecular Fe-S cluster in WhiB, while only the CXXC motif appears to be necessary for coordinating an intermolecular Fe-S cluster in MdrA. The Fe-S cluster(s) in MdrA may also serve as a sensor of oxidative stress, similar to the [2Fe-2S] cluster in Grx2 and the Fe-S cluster in WhiB. Thus, it appears that at least three distinct protein disulfide reductase families that employ an Fe-S cluster as a mechanism to regulate activity have evolved.

The gene encoding MdrA (MA3736) was shown to reside in a transcriptional unit with several putative oxidative stress genes, consistent with a role for MdrA in the oxidative stress response of *M. acetivorans*. MdrA (encoded by MA3736) and the products of most of the other genes (MA3735, MA3737, MA3740, MA3741, MA3742, and MA3743) were detected at similar levels in CO-, acetate-, and methanol-grown cells by global proteomic analyses (41, 42), consistent with a physiological function for these proteins. Conservation of the gene organization in other methanogen species also supports the hypothesis that these genes have a physiological role. Further sequence analysis suggested potential functions for two of the remaining gene products. MA3742 is annotated as a gene encoding a conserved hypothetical protein, which contains a conserved di-iron-binding motif (see Fig. S6 in the supplemental material), similar to bacterioferritin and rubrerythrin, which function in iron storage/detoxification and in reduction of hydrogen peroxide to water, respectively (10, 21, 51). MA3739

encodes a protein with five CXXCH heme-binding motifs (see Fig. S1 in the supplemental material), which suggests that this protein is a multiheme cytochrome *c*.

One potential function that can be postulated for MdrA is the repair of proteins in which disulfide bonds are formed by oxidation during exposure to O₂. An intriguing alternative hypothesis is that MdrA functions in Fe-S cluster assembly or delivery, a process which is relatively unknown in methanoarchaea. Indeed, the genome of *M. acetivorans* does not encode complete homologs of known Fe-S cluster biosynthesis proteins (e.g., NifU, Nfu, and IscA) (33, 40). Further, the properties of MdrA are consistent with those of proteins known to function in Fe-S cluster assembly, such as the CXXC-containing Fe-S cluster scaffold SyNifU/Nfu, which also binds a bridging [2Fe-2S] cluster (40, 50), and other disulfide reductases (glutaredoxins) (1, 54). Thus, MdrA may also function in repair of Fe-S cluster proteins damaged during oxidative stress.

The genome of *M. acetivorans* contains six additional genes annotated as genes encoding CMD homologs with CXXC motifs, which is similar to the number found in other *Methanosarcina*-related species. Although the genes encoding these homologs are not clustered with genes encoding oxidative stress proteins, the results are consistent with the hypothesis that the homologs have a function similar to that of MdrA. The multiple MdrA homologs found in *Methanosarcina*-related species, including RC-I_{MRE50} (17), suggest that these proteins are physiologically important components of methanoarchaea that significantly contribute to global methane emissions and may further suggest a broader function, such as Fe-S cluster assembly or delivery. Thus, it is important to note that methanoarchaea appear to contain the greatest number of Fe-S proteins, as estimated by the abundance of the CX₂CX₂CX₃C motif in proteins encoded in methanoarchaeon genomes (47). Further, it is estimated that of the methanoarchaea, *Methanosarcina* species contain the highest number of Fe-S proteins, which may reflect their metabolic diversity and large genome size. That *Methanosarcina* species contain the highest number of putative Fe-S proteins may also reflect a need for a high number of Fe-S cluster assembly and delivery proteins, consistent with the multiple copies of MdrA functioning in Fe-S cluster assembly or delivery. MdrA may function in repair of Fe-S cluster proteins damaged during oxidative stress, and homologs could function in general Fe-S cluster biosynthesis. We are currently investigating the ability of MdrA and homologs to function in Fe-S cluster assembly or delivery.

Conclusions. Although the *mdrA* gene was originally annotated as a gene encoding an uncharacterized CMD homolog, the results presented here support the hypothesis that MdrA is a protein disulfide reductase. The report of the protein disulfide reductase activity of MdrA is the first report of an enzymatic activity for CXXC-containing putative CMD homologs, and the findings suggest that MdrA is the prototype of a family. MdrA was also shown to contain an Fe-S cluster(s) with the potential to play a regulatory role in protein disulfide reductase activity or to additionally function in Fe-S cluster assembly or delivery. The activity of MdrA and the organization of *mdrA* in a transcriptional unit with oxidative stress genes are consistent with a role in the oxidative stress response of *M. acetivorans*.

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