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Understanding Intracellular Redox Regulation in Methanogens: Characterization of the Components and Targets of the NADPH-dependent Thioredoxin System from Methanosarcina acetivorans

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Understanding Intracellular Redox Regulation in Methanogens: Characterization of the Components and Targets of the NADPH-dependent Thioredoxin System from *Methanosarcina acetivorans*.

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Abstract

All cells have a reduced intracellular environment. In the presence of oxygen, the non-specific oxidation of intracellular components leads to the production of reactive oxygen species (ROS) within cells leading to oxidative stress. During oxidative stress labile cofactors (e.g. Fe-S clusters) are lost and deleterious disulfide bonds are formed within proteins. Intracellular redox maintenance systems are used to direct reducing equivalents towards the repair of oxidatively-damaged proteins. The thioredoxin system is the ubiquitous intracellular redox system, found in virtually all species. The canonical thioredoxin system is comprised of a NADPH-dependent thioredoxin reductase (TrxR) that functions to reduced thioredoxin (Trx). Although the thioredoxin system is well understood in many bacteria and eukaryotes, it is far less understood in archaea, in particular strictly anaerobic methane-producing archaea (methanogens). Methanogens are the only organisms capable of methane production. Biologically produced methane is essential for the global carbon cycle, but is also a byproduct of agriculture and farming of ruminants thus exacerbating the extent of anthropogenic climate change. The ability of methanogens to produce methane requires a large number of oxygen-sensitive metalloenzymes. However, methanogens can survive oxygen exposure, indicating that they possess intracellular redox maintenance systems. Methanogens use the deazaflavin F_{420} and the Fe-S cluster protein ferredoxin as primary electron carriers, instead of NADPH. Results presented here reveal that *Methanosarcina acetivorans*, and likely the majority of methanogens, use NADPH-dependent thioredoxin systems. NADPH is produced through the oxidation of the primary electron carriers F_{420} and ferredoxin. *M. acetivorans* contains multiple Trx homologs (MaTrx1-7) that serve alternative purposes within *M. acetivorans*. In particular, MaTrx3 and MaTrx6 are membraned associated where they likely function in the oxidation/reduction of extracellular proteins. MaTrx7 is the primary intracellular Trx, as it is the only MaTrx reduced

by MaTrxR, and it is capable of reducing several hundred *M. acetivorans* proteins. Enzyme assays reveal that *M. acetivorans* can produce NADPH in the presence of oxygen, supporting a role for the NADPH system in response to oxidative stress. Overall, these results provide insight into the roles of a thioredoxin system in *M. acetivorans*, which may lead to methods to control methane production in methanogens.

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Dedication

Acquisition of a doctoral degree is no short or easy task, and those who supported me along the way are due credit. Thank you, family and friends, for your advice, education, and support along the way. In particular, thanks to my maternal grandparents Jimmy and Judi Conway for the financial support and constant reminders to complete the task after accepting an additional full-time job near the end of my degree. Jimmy and his father W.R. Conway both spoke frequently about the importance of achieving the highest form of education possible, and while both passed away during the course of obtaining this degree both will be remembered for their love and support. To my paternal grandparents Robert and Charlotte McCarver, thank you for constantly reminding me that both of you hold degrees, one a doctorate of practical theology and the other a RN, and for your financial assistance. After nine and a half years I can finally say my degree was harder, at least in my opinion. To my uncle Heath McCarver, thank you for being a father to me after moving from home to begin school nearly a decade ago. Your advice and discussions helped me to look past the immediate hurdles and onto the goal of finishing. To my friend John Gomez, thank you for showing me throughout our friendship that advanced education is for anyone who applies themselves. To my fiancé and future wife Melissa Welch, thank you for dealing with my mood swings after long hours of seemingly wasted time in lab and for supporting my numerous hobbies that helped to maintain my sanity. I look forward to many years of scuba diving and a lower stress lifestyle with you now that my degrees are complete.

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Chapter II - McCarver, A. C., Lessner F. H., Soroeta J. M., Lessner D. J. *Methanosarcina acetivorans* Utilizes a Single NADPH-Dependent Thioredoxin System and Contains Additional Thioredoxin Homologues with Distinct Functions. *Microbiology-Sgm*, vol. 163, no. 1, 2017, pp. 62-74. (http://mic.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.000406)

Chapter IV - Sheehan R. C., McCarver A. C., Isom C. E., Karr E. A., Lessner D. J. The *Methanosarcina acetivorans* Thioredoxin System Activates DNA Binding of the Redox-sensitive Transcriptional Regulator MsvR. *Journal of Industrial Microbiology & Biotechnology*. June 2015;42(6):965-969. (https://link.springer.com/article/10.1007/s10295-015-1592-y)

Introduction

Growth of an organism is dependent upon the ability to consume a substrate (catabolism) in order to conserve the necessary energy to synthesize macromolecules (anabolism). In order for catabolic and anabolic reactions to occur within the cell the cell must maintain a reduced intracellular environment, or the flow of electrons (from the most electronegative species to the most electropositive species) will occur independent of metabolism. For this reason, cells have evolved to maintain tight regulation on the redox state of the cell through the use of systems and compounds dedicated to serve as antioxidants. Change in the intracellular redox state inside the cell must be rapidly sensed by intracellular markers before oxidative damage becomes irreversible. Common markers for sensing redox state include having reduced cysteine residues among intracellular proteins and a larger proportion of reduced electron donor pools over their oxidized counterparts. Nearly all organisms reduce the majority of disulfides among intracellular proteins via the thiol redox maintenance systems such as glutaredoxin and thioredoxin systems.

Intracellular thiol redox maintenance systems

Many cells have evolved multiple redox maintenance systems like the thioredoxin and glutaredoxin systems with functional redundancy to guarantee redox homeostasis. However, growing evidence suggests the thioredoxin system may be ubiquitous to anaerobes and aerobes alike, while the Grx system appears more often in aerobic species suggesting that the thioredoxin system plays an important thiol redox maintenance role in all forms of life. The canonical thioredoxin system is comprised of a thioredoxin reductase that utilizes NADPH as the electron donor to reduce two internal cysteine residues that in turn can reduce the disulfide bond formed between two cysteines in the thioredoxin (Trx) active site motif WCGPC (Fig. 1). The thioredoxin system often has a single thioredoxin reductase (TrxR), and to date only two types of

TrxR have been observed, a low molecular weight (L-TrxR) comprised of ~35 kDa subunits and a high molecular weight (H-TrxR) comprised of ~55 kDa subunits [1]. Typically, the H-TrxR is found among higher eukaryotes, while the L-TrxR is found in archaea, bacteria, and eukaryotes. Whether L-TrxR or H-TrxR the mechanism of each is the same, where each subunit binds NADPH and oxidizes it to then move the electrons through the flavin adenine dinucleotide (FAD) molecule that is associated with each subunit (Fig. 1). The reduced FAD molecule can then pass the pair of electrons to the internal cysteine residues within the active site motif (CXXC) of its respective subunit. The reduced pair of cysteines in TrxR are then capable of reducing the cysteines within the active site of Trx (WCGPC or CXXC), and then reduced Trx can in turn reduce a disulfide among its target proteins (Fig. 1) [2]. Trx is a small (~12 kDa) protein with active site cysteine residues exposed on the protein's surface for quick access to target protein disulfides. Due to the exposed nature of the active site cysteine residues Trx is capable of reducing a multitude of proteins ranging from biosynthetic enzymes such as ribonucleotide reductase to reducing inter- and intramolecular disulfide bonds generated in proteins as result of redox reaction or unwanted oxidation. In order to provide additional specificity, some organisms contain multiple Trxs with each having a subset of target proteins rather than a single Trx being responsible for the entire redox regulated proteome. This additional layer of specificity allows the cell to sense the oxidation state of various biological functions simultaneously, while also providing rapid reduction by decreasing the amount of proteins necessary for one Trx to reduce. Thus, in many organisms the thioredoxin system serves a critical role in redox maintenance. Trx-like proteins are also utilized at the membrane of periplasm of some species where they are responsible for reducing disulfide bonds in the more oxidizing environment outside the cytoplasm. The most common example of Trx-like proteins

functioning at the membrane or periplasm of some species is in cytochrome *c* maturation where binding of heme must be controlled to prevent environmental metals from coordinating with the cytochromes, so cytochromes are oxidized outside the cytoplasm and reduced just before heme insertion by Trx-like proteins.

Among organisms using NADPH-dependent thioredoxin systems, $NADP^+$ is often reduced by the pentose phosphate pathway of metabolism, and thus linking the thioredoxin system to anabolic pathways in the cell. As mentioned previously, many of key biosynthesis enzymes are reduced by Trx. For example, ribonucleotide reductase, which utilizes ribonucleotides to form deoxyribonucleotides needed for DNA synthesis, is reduced by the NADPH-dependent thioredoxin system. Synthesis of ribonucleotides begins at ribose 5-phosphate, a product of the pentose phosphate pathway. Therefore, if central anabolism is to occur then there must be sufficient carbon for the pentose phosphate pathway to produce the NADPH needed by the thioredoxin system to catalytically reduce key biosynthesis enzymes to keep anabolic pathways functioning. Oxidation of anabolic enzymes or depletion of NADPH serves as an indicator for the cell that conditions are not suitable for anabolic growth, and resources are allocated to restore and repair the cell. Alternative mechanisms for obtaining NADPH include, but are not limited to, oxidizing alternative electron donors such as ferredoxin to reduce $NADP^+$, and oxidizing carbon storage molecules such as glucose. Numerous prokaryotes utilize a NADPH-dependent glyceraldehyde -3-phosphate dehydrogenase (GAPDH) as a primary source of NADPH production in the absence of a fully functioning pentose phosphate pathway [3]. By having additional electron donors like ferredoxin and NADH, the cell can dedicate and regulate the functionality of a given metabolic pathway. NADH, for instance, is used for catabolic reactions and energy conservation in *E. coli*, and thus reducing equivalents

needed for catabolic reactions and energy conservation are not being competed for those needed in anabolic reactions. Limiting the competition for reducing equivalents also allows the cell to sense the current state of a given metabolic pathway, and adjust metabolism as needed.

Other thiol redox maintenance systems similar to the NADPH-dependent thioredoxin system exist, for instance, photosynthetic organisms utilize the ferredoxin:thioredoxin reductase (FTR) to reduce Trx(s) that are specific for FTR. Electrons from ferredoxin serve to reduce the ferredoxin-dependent thioredoxin system in photosynthetic organisms because the primary electron donor pool generated during photosynthesis is reduced ferredoxin. Organisms using ferredoxin as an electron carrier other than phototrophs often utilize a ferredoxin-dependent thioredoxin system as well. The ferredoxin-dependent thioredoxin system is comprised of similar components to that of the NADPH-dependent thioredoxin system where a TrxR reduces Trx except TrxR oxidizes ferredoxin instead of NADPH. Although the ferredoxin-dependent thioredoxin system functions similarly to the NADPH-dependent thioredoxin system, the major difference is between the reductases TrxR and FTR. FTR does not utilize the prosthetic group FAD to move electrons from the electron donor source to the internal cysteine residues, rather FTR contains an FeS cluster similar to ferredoxin. By utilizing ferredoxin and FeS clusters to mediate electron transfer the ferredoxin-dependent thioredoxin system is coupled to phototrophic growth since reduced ferredoxin is only generated during phototrophic growth.

An additional widely used thiol redox maintenance system exists, the glutaredoxin system. Like the thioredoxin system, the glutaredoxin system utilizes NADPH as the electron donor to reduce glutathione reductase (GrxR). The glutaredoxin system is comprised of GrxR that receives electrons from NADPH to reduce an internal FAD and ultimately glutathione, a small dithiol containing peptide, which in turn reduces the small protein (~12 kDa) glutaredoxin

(Grx) (Fig. 2). Reduced Grx can reduce a multitude of proteins, and is generally capable of reducing more intracellular protein than Trx [4]. In addition to Grx being capable of reducing more proteins than Trx, Grx systems are often found in aerobic species and not in strictly anaerobic species, while Trx is more ubiquitous among anaerobes and aerobes alike. In aerobes exploiting the use of glutaredoxin systems alongside thioredoxin systems, the two systems share target proteins and have a few unique target proteins. Additionally, the glutaredoxin system functions with an extra step that is not utilized by the thioredoxin system in which reduced glutathione is produced. Reduced glutathione is a small redox active dithiol peptide that is capable of reducing some disulfides and can also serve as a redox indicator inside the cell. When the levels of reduced glutathione decrease the intracellular redox state becomes more electropositive and the cell responds by generating more reducing equivalents needed for redox maintenance. The ability of glutaredoxin systems to function as a more general antioxidant in the cell and their predominance in aerobes suggest that the glutaredoxin system may have evolved to provide further redox maintenance needed when growing in the presence of oxygen.

Methanogens and methanogenesis

Methanogens are prokaryotes belonging to the domain *Archaea*, and all species of methanogens are obligate anaerobes with habitats ranging from fresh and salt water sediments to the intestinal microflora of ruminants and humans. Methanogens are responsible for all biologically produced methane, and are capable of growth using CO_2 as a carbon source and H_2 as an energy source or acetate or small methylated compounds (ie methanol and trimethylamine) as both carbon and energy sources (Fig. 3). Due to their unique metabolic pathway, methanogens are critical to the global carbon cycle and are also important players in global climate change (Fig. 4). Methanogens are estimated to be one of the earliest organisms to have evolved on earth

[5], thus providing a window into how early life metabolic pathways may have functioned. There are four classes of methanogens: Methanopyri, Methanococci, Methanobacteria, and Methanomicrobia. Organisms of the class Methanomicrobia are considered to be the most recently evolved methanogens exhibiting growth on some or all of the methanogenesis substrates (Fig. 3). Growth on acetate accounts for nearly two-thirds of biologically produced methane today. However, only some methanogens of the class Methanomicrobia, in particular members of the order Methanosarcinales, are capable of acetate metabolism, indicating that these recently evolved methanogens are the most influential in the global carbon cycle and climate change.

Geological evidence dates the origin of life likely occurred 3.4 ± 0.3 Gyr ago and the earliest signs of methane production approximately 3.5 Gyr ago [5], predating oxygenic photosynthesis. The production of methane from CO_2 is thought to have increased early earth's temperature thus allowing for the evolution of oxygenic phototrophs and eventually gave rise to aerobes that can utilize oxygen during respiration. Methanogens in the classes Methanopyri and Methanococci more closely resemble the ancestral methanogens that inhabited early earth, likely because many of them still inhabit harsh anaerobic environments similar to early earth. Other methanogens inhabit areas that are exposed to oxygen much more frequently, such as the hind guts of ruminants, than that of methanococci species which typically live near hydrothermal vents deep in the ocean. Collectively methanogens are capable of representing how early earth's strict anaerobes adapted to rapid climate change leading to a more oxygenated atmosphere. As larger quantities of oxygen were introduced into the atmosphere of earth the number of oxidative stress events encountered by anaerobes increased, and thus necessitated evolving methods in which strict anaerobes could sense and repair oxidative damage. Methanogens belonging to the class Methanomicrobia are some of the most aerotolerant methanogens surviving oxygen

exposure for up to several hours or in some cases even capable of producing methane in the presence of oxygen [6, 7], suggesting that higher order methanogens evolved mechanisms to cope with oxygen exposure.

As discussed previously, all methanogens utilize methanogenesis for growth and methanogenesis can only function under strict anaerobic conditions even if some species of methanogens can tolerate increased oxygen exposure. Oxygen sensitivity of methanogenesis enzymes is attributed to many of these enzymes utilizing reduced cysteines and FeS clusters for catalytic activity (Fig. 3). Additionally, one of the two primary electron carriers in methanogens, ferredoxin, also binds a FeS cluster for function and can only be reduced by oxygen sensitive methanogenesis enzymes. For example, methanogens capable of growth on acetate utilize carbon monoxide dehydrogenase/acetyl-CoA synthase (Cdh/Acs) to oxidize acetate and generate reduced ferredoxin, but Cdh/Acs is a multi FeS cluster binding protein incapable of functioning in the presence of oxygen (Fig. 3). Thus, methanogens capable of surviving oxygen exposure must have oxygen sensing and repair mechanisms that can restore key methanogenesis enzyme functionality. In particular, methanogens with increased aerotolerance would need mechanisms to actively reduce oxygen, the reactive oxygen species generated by oxygen exposure, and mechanisms for repairing damaged proteins. However, many of the reactive oxygen species rapidly oxidize/damage DNA, proteins, lipids, RNA, and cofactors/coenzymes like the previously discussed FeS clusters that are incorporated in the majority of the key metabolic enzymes within methanogens. One type of reactive oxygen species that is readily formed during oxidative stress is hydrogen peroxide, which readily reacts with thiol compounds/containing proteins to generate water and oxidized compounds/proteins. Proteins containing FeS clusters utilize reduced cysteine residues (thiols) to coordinate FeS clusters, and thus methanogen

physiology by in large functions on the basis of thiols. Oxygen exposure for methanogens would lead to thiol oxidation and would undoubtedly necessitate mechanisms for thiol reduction if the organism is to survive.

Thiol Redox Systems in Methanogens

Systems responsible for detoxifying oxygen and the resulting reactive oxygen species have been examined in some methanogens, thiol redox maintenance systems have not been examined as extensively. Thiol redox maintenance systems like the thioredoxin system could be performing oxidation sensing, signaling, and repair roles in methanogens much like the role of the system in many other organisms. Moreover, the demand for thiol redox systems in methanogens is likely to be much higher since methanogens are predicted to utilize the largest number of FeS cluster proteins that are very oxygen sensitive and are dependent upon reduced cysteine residues within proteins, which could only be achieved by thiol redox systems or thiol based antioxidants. Methanogens use the coenzymes B and M, which are thiol containing, during methanogenesis and may be using them to facilitate thiol reduction in some circumstances. However, it is unlikely that coenzyme B and M are the primary thiol redox maintenance system given their critical role in central metabolism. Additionally, the more evolved methanogens capable of growth on multiple substrates exhibit additional metabolic constraints, for instance, *Methanosarcina* species when growing on acetate require an increase in the number of FeS cluster containing proteins and consequently more FeS clusters must be generated and inserted into these proteins. Each step of FeS cluster biosynthesis utilizes reduced thiols in proteins to facilitate the generation of components and the transfer of complete FeS cluster between carriers and target proteins. The increased demand for FeS cluster proteins during growth on acetate by

Methanosarcina species supports a need for additional or robust thiol redox systems capable of extensive maintenance.

While metabolic pathways and energy conservation mechanisms have been examined, little work has addressed redox maintenance systems in methanogens despite the apparent demand for thiol redox systems in metabolism and energy conservation. The work in thiol redox maintenance systems of methanogens focused largely on individual components of the system such as thioredoxin [8-10]. At the beginning of the work presented in this dissertation it was unclear which thiol redox maintenance systems were incorporated into methanogen physiology, as homologs of predicted NADPH-dependent TrxRs, ferredoxin-dependent TrxRs, and Grxs were all found in the genomes of various methanogens. In particular, canonical thiol redox maintenance systems utilize NADPH as the electron donor, however, methanogens utilize the electron carriers F_{420} and ferredoxin, suggesting methanogens might be utilizing a thiol redox maintenance system capable of oxidizing reduced F_{420} or ferredoxin. Glutaredoxin systems were omitted as a candidate thiol redox system in methanogens due to the lack of the ability to synthesize glutathione in all methanogens [11-13], and thus leaving the candidate F_{420} and ferredoxin thiol redox systems as key players in the redox maintenance of methanogens with the possibility of NADPH-dependent thiol redox systems being utilized. Indeed recent work examined the FTR encoded in *Methanosarcina acetivorans* (named ferredoxin:disulfide reductase FDR) and determined that the FDR did oxidized ferredoxin but the redox partner to FDR in *M. acetivorans* was not elucidated [14]. Recent work in *Methanosarcina barkerii* with FDR indicated that FDR reduces a thioredoxin-like protein called NrdH which is specific for reducing ribonucleotide reductase (NrdD) [15], suggesting that methanogens might be using a

ferredoxin dependent thioredoxin system in a similar fashion to that of the NADPH dependent thioredoxin system performs in many other organisms.

The electron donor F_{420}, however, has not been observed as a direct donor to a thioredoxin-like system until recently during the work presented in this dissertation. The novel F_{420}-dependent TrxR was identified in the more ancestral methanogen *Methanocaldococcus jannaschii* where it is specific for Trx1 [16]. Having a F_{420}-dependent thioredoxin system directly ties the system into methanogen physiology, and thus allows for rapid thiol redox maintenance within the cell. However, there are pitfalls to F_{420} and ferredoxin dependent systems such as the thiol redox maintenance system is utilizing the primary electron donors for methanogenesis causing competition between metabolism/energy conservation and redox maintenance.

Early studies of methanogens and methanogenesis led many researchers to hold the opinion that methanogens do not utilize pyridine dinucleotides like NADPH since methanogenesis enzymes utilize F_{420} and ferredoxin [17, 18]. However later work indicated that metabolically diverse methanogens (capable of growth on methylated compounds or acetate) contained NADPH dependent alcohol dehydrogenases, and thus posed the question of how $NADP^+$ was being reduced [17]. Further examination found that methanogens with NADPH-dependent alcohol dehydrogenases possessed the ability to oxidize $F_{420}H_2$ in order to reduce $NADP^+$ through the use of the enzyme F_{420}-dependent NADP reductase [17]. The ability to use reducing equivalents produced during methanogenesis to reduce $NADP^+$ directly ties NADPH-dependent pathways into methanogenesis, suggesting that methanogens have the capability to support NADPH-dependent thiol redox systems. However, the ability to reduce $NADP^+$ from primary electron carriers generated during methanogenesis still limits the functionality of the

NADPH-dependent thioredoxin system to anaerobic growth with some activity immediately after oxidative stress using residual NADPH. If a thiol redox system is providing increased oxidative stress tolerance in any methanogen through rapid repair post-oxidation then there must be a source of electrons that are insensitive to oxygen. Similar to other prokaryotes studied to date, methanogens utilizing a NADPH-dependent thioredoxin system may have carbon storage molecules that can be oxidized when primary metabolism ceases (methanogenesis after oxidative stress) such as glycolysis pathways.

Experimental Sections Included in this Dissertation

One type of thiol redox maintenance system was examined in methanogens during the work encompassed in this dissertation, the canonical NADPH-dependent thioredoxin system. The genome of model methanogen *M. acetivorans* encodes one homolog of a predicted NADPH-dependent TrxR and seven predicted Trxs. This dissertation addresses the questions of 1) does *M. acetivorans* contain a functional NADPH-dependent thioredoxin system, 2) if the system can be integrated in the physiology of the methanogen (i.e. obtain electrons from methanogenesis), 3) if the complete NADPH-dependent thioredoxin system can serve as a general thiol redox maintenance system to oxidatively damaged proteins, 4) can $NADP^+$ be reduced post oxidative stress by an endogenous source, and 5) does the NADPH-dependent thioredoxin system play a redox signaling role in *M. acetivorans* similar to other prokaryotes.

Chapter I- Initial characterization of the TrxR in *M. acetivorans* (MaTrxR) and three of the putative Trxs (MaTrxs). Recombinant MaTrxR was purified and the activity assessed *in vitro*. Spectrophotometric studies indicated that MaTrxR purified from *E. coli* could readily be reconstituted with FAD, the cofactor bound to all NADPH-dependent TrxRs, suggesting that

MaTrxR also binds FAD and that overexpression in *E. coli* led to an enzyme product without the full complement of FAD. Additionally, F_{420}, NADH, and NADPH were examined as potential electron donors to MaTrxR and indeed MaTrxR is NADPH-dependent. Three of the seven MaTrxs were examined for disulfide reductase activity, MaTrx2, 6, and 7, and all three-exhibited robust disulfide reductase activity consistent with each functioning as Trxs. Insulin disulfide reductase and Trx-dependent oxidized glutathione reduction assays were performed using NADH/NADPH with MaTrxR and either MaTrx2, 6, or 7 to address which candidates are likely *in vivo* targets to MaTrxR. Of the three MaTrxs only MaTrx7 was observed to be reduced by MaTrxR, and thus the first complete NADPH-dependent thioredoxin system was characterized in a methanogen. Due to the large number of Trxs in *M. acetivorans* and methanogens as a whole (~150 Trx homologs in ~45 methanogen genomes), additional work was done to categorize the Trxs in methanogens by analyzing them on the basis of sequence similarity. The activity and reducing partner to a given Trx and its respective group or type in methanogens can be applied the Trx type as a whole to provide the initial probable function. This chapter was published and is available online.

Chapter II- Further characterization of the remaining Trxs in *M. acetivorans* was performed, and again MaTrx7 remained the sole reducing partner to MaTrxR. Sources of NADPH production were examined since the primary electron carriers in methanogenesis are F_{420} and ferredoxin. Using a supply of reduced F_{420} ($F_{420}H_2$) to *M. acetivorans* cell lysate, rapid oxidation of $F_{420}H_2$ was observed when $NADP^+$ was present, supporting that NADPH could be generated using $F_{420}H_2$. Similarly, ferredoxin was assessed as an electron donor to generate NADPH, but was done indirectly through the activity of Cdh/Acs which produces reduced ferredoxin through the oxidation of carbon monoxide. Carbon monoxide was supplied to *M.*

acetivorans cell lysate along with $NADP^+$ and compared to the activity of lysate supplied nitrogen instead. Production of NADPH was higher in carbon monoxide containing lysates as opposed to nitrogen, and thus collectively supporting that $F_{420}H_2$ and ferredoxin could be utilized to generate NADPH through the use of F_{420}-dependent $NADP^+$ reductase (Fno) and ferredoxin $NADP^+$ reductase (Fnr) like activities. Additional work indicated that nearly all methanogens possess predicted NADPH-dependent TrxRs and Fno/Fnr indicating that most methanogens have evolved to utilize NADPH-dependent thioredoxin systems. Further work examined possible roles for the remaining MaTrxs that are not reducing partners to MaTrxR. MaTrx3 and MaTrx6 were shown to be localized to the membrane of *M. acetivorans* where they may play a role in cytochrome c maturation. MaTrx3 and MaTrx1 appear to have disulfide oxidase activity (generate disulfide bonds). The MaTrx3/6 pair may perform a role at the membrane similar to cytochrome maturation systems in other prokaryotes, where the cytochrome is oxidized (MaTrx3) to prevent improper metal incorporation and then reduced (MaTrx6) just before heme insertion. This chapter was published and is available online.

Chapter III- The ability of the MaTrxR-MaTrx7 system to serve as a broad thiol redox maintenance system to *M. acetivorans* was addressed. A MaTrx7 mutant was generated that could not completely reduce its substrates and anchored to a resin. Air oxidized *M. acetivorans* cell lysate was incubated with the reduced resin and an alkylated resin (negative control) to mimic post oxidative stress exposure, and the resulting proteins identified via mass spectrometry. Nearly 700 proteins were identified, supporting a role for the MaTrxR-MaTrx7 system as a broad thiol redox maintenance system. Additionally, many enzymes that have activities dependent upon Trx in other studies were identified as targets to MaTrx7, and thus it is likely that many *in vivo* targets were identified as well. The ability of *M. acetivorans* to produce

NADPH without additional electron donor was addressed both before and after aerobic exposure to determine if the MaTrxR-MaTrx7 system could function after oxidative stress. NADPH was produced at the same rate aerobically as anaerobically and in relatively large quantities, supporting that some source of oxygen insensitive electrons and the enzymes needed to utilize it exist in *M. acetivorans.* This work will be published at a future date. During this work Santiago-Martinez *et al* in 2016 released a study indicating that *M. acetivorans* contained oxygen insensitive glycolysis enzymes and intermediates that could be used to generate relatively large quantities of NADPH. It is likely that during methanogenesis glycogen is stored and can be oxidized in the event of oxidative stress to serve as an electron donor for oxidative repair. Collectively this work supports that the MaTrxR-MaTrx7 system can serve as a broad thiol redox maintenance system post-oxidative stress.

Chapter IV- This work examined one of the identified proteins from chapter III *in vivo.* The transcriptional regulator MsvR binds to DNA when internal thiols are reduced to repress transcription. In lieu of strong, non-physiologically relevant reductants, such as dithiothreitol, the MaTrxR-MaTrx7 system was used to determine if the system could reduce MsvR as observed in chapter III while simultaneously exhibiting a mode of redox sensing and transcriptional regulation in *M. acetivorans*. The work presented supports that the MaTrxR-MaTrx7 system can serve as a redox partner to MsvR. The work performed was a part of a previous graduate student at the University of Arkansas Fayetteville thesis, and was performed as a collaborative project among Ryan C. Sheehan and Addison C. McCarver. This chapter has been published and is available online.

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Figures

Figure 1

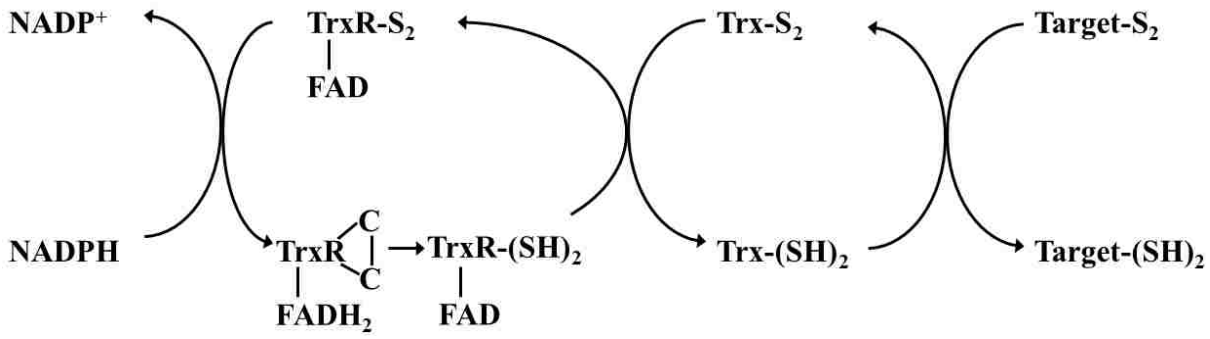

Figure 1. NADPH-dependent thioredoxin system where NADPH is oxidized by thioredoxin reductase (TrxR) to reduce the bound FAD group and ultimately the internal cysteine residues. The dithiols in TrxR can then reduce oxidized thioredoxin (Trx), and thus allowing Trx to reduce a wide array of target proteins. Figure adapted from Holmgren *et al* 2010.

Figure 2

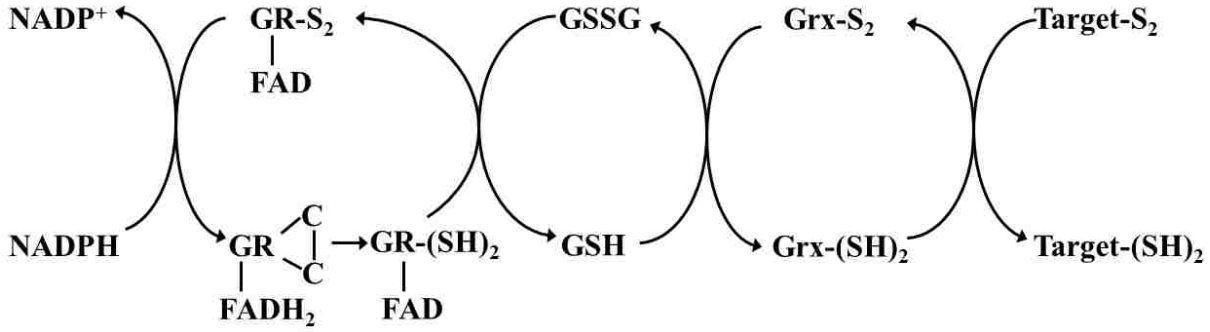

Figure 2. The glutaredoxin system components where NADPH is oxidized by glutathione reductase (GR) to reduce bound FAD and ultimately internal cysteine residues. Thiols in GR can then reduce oxidized glutathione (GSSG), and reduced glutathione (GSH) can reduce glutaredoxin (Grx). Reduced Grx redudces a wide array of target proteins.

Figure 3

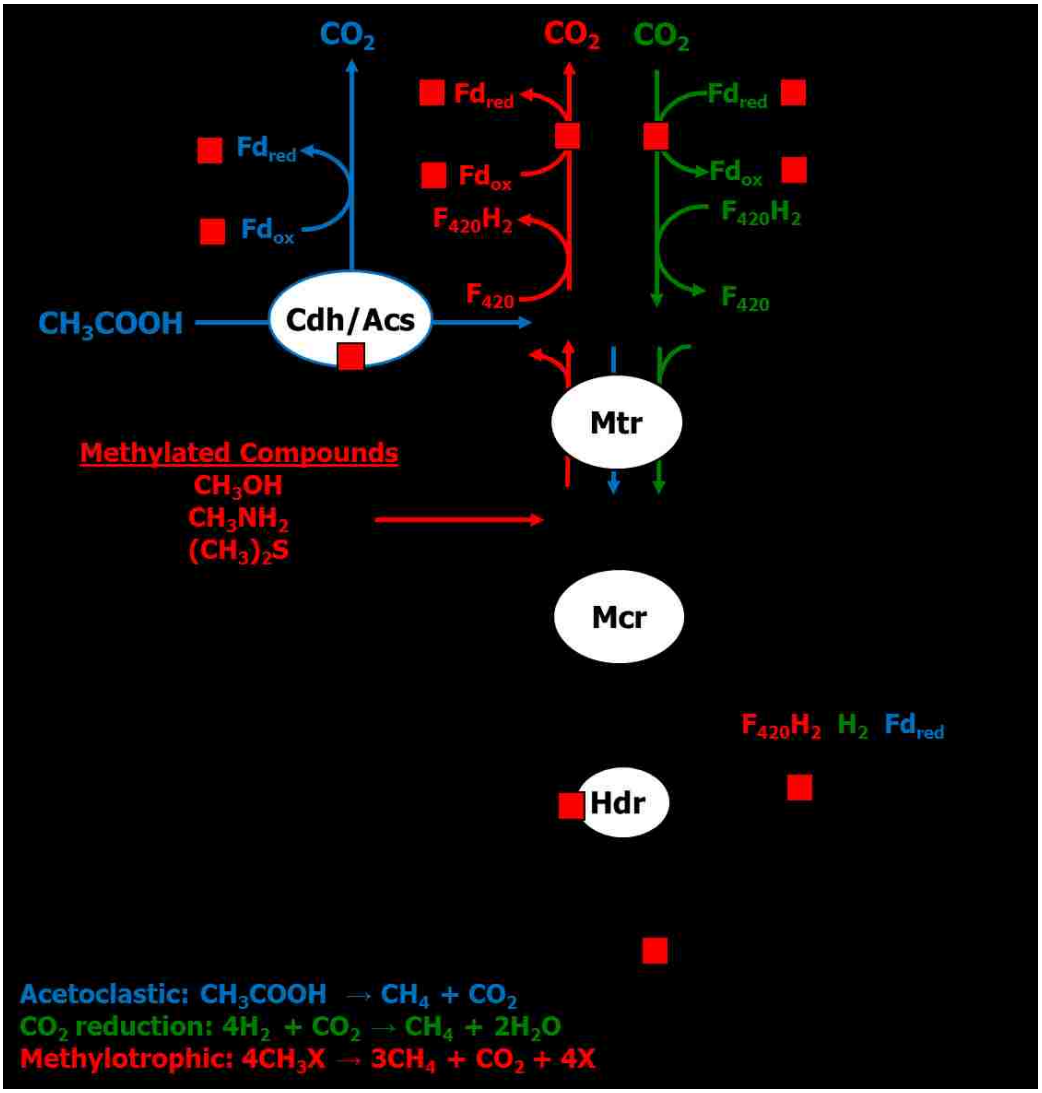

Figure 3. Simplified diagram of the three methanogenic pathways: Acetoclastic, CO_2, and methylotrophic pathways. Presence of FeS clusters in proteins are indicated by red boxes. Abreviations: Cdh/Acs, carbon monoxide dehydrogenase/acetyl-CoA synthase; Mtr, methyl-H_4MPT coenzyme M methyltransferase; Mcr, methyl-coenzyme M reductase; Hdr, CoM-S-S-CoB heterodisulfide reductase; CoM and CoB, reduced coenzymes M and B.

Figure 4

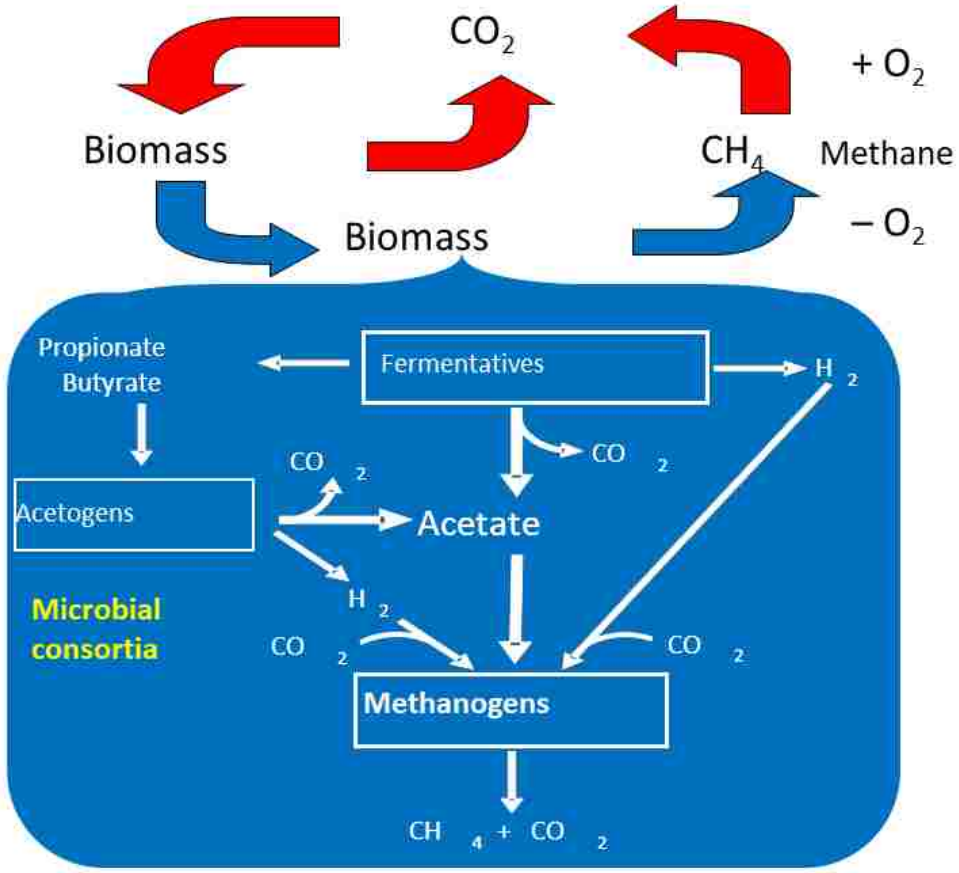

Figure 4. Sources of methanogen growth substrates indicating the role of methanogens in global carbon cycles. During anaerobic fermentation carbon dioxide (CO_2), hydrogen (H_2), acetate, and methylated compounds accumulate. Gasses like CO_2 and H_2 can reenter the atmosphere or alternatively be used by methanogens to produce methane. The solutes acetate and methylated compounds must be consumed by methanogens to produce methane for the carbon to reenter the atmosphere and complete the carbon cycle.

Chapter I

Molecular Characterization of the Thioredoxin System from *Methanosarcina acetivorans*

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Abstract

The thioredoxin system, composed of thioredoxin reductase (TrxR) and thioredoxin (Trx), is widely distributed in nature, where it serves key roles in electron transfer and in defense against oxidative stress. Although recent evidence reveals Trx homologues are almost universally present among the methane-producing archaea (methanogens), a complete thioredoxin system has not been characterized from any methanogen. We examined the phylogeny of Trx homologues among methanogens and characterized the thioredoxin system from *Methanosarcina acetivorans*. Phylogenetic analysis of Trx homologues from methanogens revealed eight clades, with one clade containing Trxs broadly distributed among methanogens. The *Methanococci* and *Methanobacteria* each contain one additional Trx from another clade, respectively, whereas the *Methanomicrobia* contain an additional five distinct Trxs. *M. acetivorans*, a member of the *Methanomicrobia,* contains a single TrxR (MaTrxR) and seven Trx homologues (MaTrx1-7), with representatives from five of the methanogen Trx clades. Purified recombinant MaTrxR had DTNB reductase and oxidase activities. The apparent K_m value for NADPH was 115-fold lower than the apparent K_m value for NADH, consistent with NADPH as the physiological electron donor to MaTrxR. Purified recombinant MaTrx2, MaTrx6, and MaTrx7 exhibited DTT- and lipoamide-dependent insulin disulfide reductase activities. However, only MaTrx7, which is encoded adjacent to MaTrxR, could serve as a redox partner to MaTrxR. These results reveal that *M. acetivorans* harbors at least three functional and distinct Trxs, and a complete thioredoxin system composed of NADPH, MaTrxR, and at least MaTrx7. This is the first characterization of a complete thioredoxin system from a methanogen, which provides a foundation to understand the system in methanogens.

Introduction

Thiol-disulfide exchange reactions are universal among all living cells. The most ubiquitous is the thioredoxin system, composed of thioredoxin (Trx) and the partner enzyme thioredoxin reductase (TrxR). TrxR and Trx are found in species from all three domains of life and the thioredoxin system is well characterized in species from the *Bacteria* and *Eukarya* domains, including humans [1]. The thioredoxin system plays a primary role in cellular redox maintenance and reduces disulfides in certain proteins. The two basic functions of the system are to supply electrons to biosynthetic enzymes, including ribonucleotide reductase, methionine sulfoxide reductase, and sulfate reductases, and to reduce inter- and intramolecular disulfides in oxidized proteins. TrxR specifically catalyzes the reduction of the disulfide in oxidized Trx using metabolism-derived NADPH as a source of reducing equivalents. The thioredoxin system also serves a critical role in protection from oxidative stress in many organisms [2]. Trx can reduce deleterious disulfide bonds in oxidatively-damaged proteins and also serve as a reducing partner to peroxiredoxins, which scavenge hydrogen peroxide. In bacteria, plants, and mammals the thioredoxin system plays a role in the regulation of gene expression and cell signaling [3]. The thioredoxin system is also important to the survival of pathogens [4]. Despite the ubiquitous importance of Trx, the properties and role(s) of the thioredoxin system in species from the domain *Archaea* is far less understood.

TrxR is a member of the dimeric flavoprotein family of pyridine nucleotide disulfide oxidoreductases, which includes lipoamide dehydrogenase, glutathione reductase, and mercuric reductase. Each TrxR subunit contains a FAD molecule and a redox-active disulfide, but two distinct types are currently known, a low molecular weight (L-TrxR) type comprised of ~ 35 kDa subunits and a high molecular weight (H-TrxR) type comprised of ~55 kDa subunits [5]. Both

types of TrxR possess a NADPH-binding site and obtain reducing equivalents from NADPH. H-TrxR is found primarily in higher eukaryotes and the protozoan malaria parasite, while L-TrxR is found in archaea, bacteria, and eukaryotes. Trxs are small proteins (~12 kDa) that contain a CXXC motif, whereby the two active site cysteines are separated by two amino acid residues. The canonical Trx active site motif is WCGPC, which is present in well-characterized Trxs from *Escherichia coli* and yeast [1]. Many organisms possess multiple Trxs, which can have distinct or overlapping activities and specificities. For example, *E. coli* and yeast contain two and three Trxs, respectively [6]. However, plants contain numerous Trxs which function in all compartments of plant cells [7].

Complete NADPH-dependent thioredoxin systems have been characterized from three archaea, *Sulfolobus solfataricus*, *Aeropyrum pernix* K1, and *Pyrococcus horikoshii* [8-10]. All three species are hyperthermophiles, with *P. horikoshii* being the only anaerobe. However, the target proteins of each system and the importance of the system to the metabolism and oxidative stress response of each archaeon is largely unknown. The methane-producing archaea (methanogens) are strict anaerobes and are the only organisms capable of biological methane production. There are currently four Classes of methanogens, the *Methanopyri*, *Methanococci*, *Methanobacteria*, and *Methanomicrobia* [11]. Species within the *Methanopyri*, *Methanococci*, and *Methanobacteria* are only capable of producing methane by the reduction of CO_2. However, members of the *Methanosarcinales*, within the *Methanomicrobia*, are more metabolically diverse, capable of methanogenesis with methylated compounds and acetate. Moreover, only species of the *Methanosarcinales* possess cytochromes and are capable of producing methane from acetate, which is estimated to account for two-thirds of all biologically-produced methane [11]. Recent evidence revealed the presence of Trx homologues within all methanogens, except

the single member of the *Methanopyrales* [12]. Thus, Trx likely serves a fundamental role in methanogens. Members of the *Methanomicrobia* are predicted to contain approximately twice as many Trxs as the *Methanococci* and *Methanobacteria* (~4 vs 2), which is likely a result of the metabolic diversity and larger genomes of the *Methanomicrobia*. The majority of species within the *Methanosarcinales* contain >5 Trx homologues [12]. A few Trxs have been characterized from methanogens, including *Methanocaldococcus jannaschii* and *Methanothermobacter thermautotrophicus* [13-15]. Recent evidence revealed Trx in *M. jannaschii* targets fundamental processes, including proteins involved in methanogenesis [12]. However, a complete thioredoxin system, in particular, a NADPH-dependent TrxR, has yet to be characterized from a methanogen. Moreover, none of the components of the thioredoxin system from a member of the *Methanosarcinales* have been characterized. We are particularly interested in deciphering the role of the thioredoxin system in the *Methanosarcinales*, using *Methanosarcina acetivorans* as a model system. We report here that *M. acetivorans* contains seven Trx homologues and a single TrxR homologue. Purification and characterization studies reveal that *M. acetivorans* contains at least three functional Trxs and a complete NADPH-dependent thioredoxin system.

Materials and methods

Phylogenetic analysis. 123 Trx amino acid sequences were obtained from GenBank using their accession numbers provided by Sustani *et al* [12] and an additional 17 Trx amino acid sequences were included (see **Table S1**). The 140 Trx amino acid sequences were aligned using MUSCLE [16], and columns in the alignment containing a fraction of gaps of 0.6 or greater were omitted using trimAl [17]. The trimmed alignment file was inputted into RAxML 7.3.1 [18] where a rapid bootstrap analysis was performed using 1,000 bootstrap replicates, 1,070,065 parsimony random seeds, and 3,535,411 rapid bootstrap random seeds. The best scoring maximum

likelihood (ML) tree was obtained and bootstrap values greater than 50% were included on the nodes within the tree (**Fig. S1**). The resulting tree file from RAxML was pruned to 50 taxa using PAUP, and nodes with >50% support were reported (**Fig. 1**).

Cloning of *M. acetivorans* thioredoxin system genes. The genes encoding MaTrxR, MaTrx2, MaTrx6, MaTrx6ΔSp (deleted of signal peptide amino acids 1-30), and MaTrx7 (see Table 1 for gene designations) were PCR amplified using chromosomal DNA from *M. acetivorans* C2A as a template. All forward and reverse primers contained the restriction enzyme sites *Nde*I and *Bam*HI respectively. Purified PCR products and the pET28a plasmid were digested with *Nde*1 and *Bam*H1 for 16 hr at 37 °C. Digested PCR products and vector were ligated using T4 DNA ligase for 16 hr at 16 °C. *Escherichia coli* DH5α cells were transformed with the ligation reactions and cells containing plasmid were selected on LB agar containing 100 µg/mL kanamycin. Plasmids containing *matrxR*, *matrx2*, *matrx6*, *matrx6ΔSp* and *matrx7* were verified by DNA sequencing and named pDL335, pDL331, pDL333, pDL332, and pDL336 respectively.

Purification of recombinant proteins. Proteins were expressed in *E. coli* Rosetta DE3 (pLacI) transformed with pDL335, pDL331, pDL332, pDL333, or pDL336. Each *E. coli* expression strain was grown in LB medium containing kanamycin (50 µg/mL) and chloramphenicol (17 µg/mL) at 37°C to an OD_{600} of 0.5-0.7. Protein expression was induced with 500 µM isopropyl-β-D-thiogalactopyranoside and cultures were incubated at 25°C for 16 hr. Cells were harvested by centrifugation and stored at -80 °C.

For the purification of MaTrxs, cell pellets (2-4 g) were resuspended in 25-30 mL of buffer A (20 mM Tris-HCl, 500 mM NaCl pH 8.0) containing a few crystals of DNaseI and benzamidine. Cells were lysed by three passes in a French pressure cell at a minimum of 100

MPa. Cell lysate was centrifuged at 41,000 x *g* for 35 min at 4 °C. The supernatant containing the expressed protein was filtered (pore size, 0.45 µm) and loaded by gravity flow onto a column containing 5 mL of Ni^{2+}-agarose resin (Genscript). The column was then washed with 25 ml of buffer A three separate times with the second wash containing 10 mM imidazole. The column was then incubated in Buffer A containing 50 U of thrombin at 25 °C for 16 hr. Thrombin-cleaved protein was eluted from the column by the addition of 10 mL of buffer A. The eluate was passed through a 1 mL benzamidine column (GE Healthcare) to remove thrombin. The flowthrough was concentrated using a Vivacell concentrator (Sartorius) with a 5,000-Dalton molecular weight cutoff under nitrogen flow. The concentrated protein was desalted into buffer B (50 mM Tris-HCl, 150 mM NaCl pH 7.2) using a PD-10 column (GE Healthcare). The desalted protein was stored at -80 °C.

For the purification of MaTrxR, cell lysate was prepared as described above, except that 10% glycerol was added to buffer A. The supernatant containing the expressed protein was filtered (pore size, 0.45 µm) and loaded by gravity flow onto a column containing 5 mL of Ni^{2+}-agarose resin (Genscript). The column was washed with 25 mL of buffer A two separate times with the second wash containing 10 mM imidazole. Total bound protein was eluted from the column by two steps, first the addition of 10 mL of buffer A containing 75 mM imidazole, second by the addition of 10 mL of buffer A containing 150 mM imidazole. The eluates were combined and concentrated using a Vivacell concentrator with a 10,000-Dalton MW cutoff under nitrogen flow. The concentrated protein was desalted into buffer C (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol pH 7.2) using a PD-10 column and stored at -80 °C.

Reconstitution of MaTrxR with FAD was carried out by incubation of purified MaTrxR in buffer C containing 1 mM dithiothreitol and a 10 molar excess of FAD at 25 °C for 1 hr. The

protein was desalted into buffer C using a NAP-5 column (GE Healthcare). Incorporation of FAD into MaTrxR was monitored by UV-visible spectroscopy and quantified based on the ratio of A_{280}/A_{460}.

Thioredoxin reductase activity assays. The ability of NADH and NADPH to reduce the FAD within MaTrxR was monitored by UV-visible spectroscopy before and after incubation of MaTrxR in buffer B with a >10-fold molar excess of either NADH or NADPH within an anaerobic chamber (Coy Laboratories). Reduction of DTNB by purified MaTrxR was monitored by the increase in absorbance at 412 nm using either NADPH or NADH as electron donors. The assays were performed anaerobically in buffer B containing 0.5 µM MaTrxR and 1 mM DTNB. The reactions were initiated by the addition of NADPH (1-20 µM) or NADH (5-2000 µM). The concentration of TNB produced was calculated using ε_{412} =14,150 M^{-1} cm^{-1} [19]. The apparent kinetic constants were determined by nonlinear regression of Michaelis-Menten plots using Microsoft Excel with the XL_kinetics add-in. Measured activities in all assays were corrected for by subtracting the rates of control reactions without MaTrxR. Three independent assays were performed at each NADPH or NADH concentration.

NADH and NADPH oxidase activity of MaTrxR was measured spectrophotometrically by the decrease in absorbance at 340 nm in the presence of oxygen. Reactions contained 160 µM NADH or NADPH in buffer B. The reactions containing NADH as the electron donor contained 1 µM MaTrxR, while the NADPH-dependent reactions contained 100 nM MaTrxR. Oxidase activity of MaTrxR with each reductant was calculated using ε_{340} =6,220 M^{-1} cm^{-1}.

The ability of MaTrxR to use $F_{420}H_2$ as an electron donor was examined with DTNB reduction assays. F_{420} purified from *Mycobacterium smegmatis* was provided as gift from Lacy

Daniels (Texas A&M University, Kingsville). F_{420} was chemically reduced to $F_{420}H_2$ using sodium borohydride as previously described [20]. Assays were performed anaerobically in buffer A containing 0.5 µM MaTrxR and 1 mM DTNB, and were initiated by the addition of 50 µM $F_{420}H_2$.

Thioredoxin activity assays: Thioredoxin activity was determined by the turbidimeteric insulin reduction assay using DTT, lipoamide, glutathione, or coenzyme M as potential electron donors as described [21]. The standard assay mixture contained 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 130 µM insulin, and up to 11 µM Trx. Standard assays contained 330 µM DTT, 660 µM glutathione or 660 µM coenzyme M, whereas lipoamide-dependent assays contained 50 µM lipoamide, 0.4 units of bovine lipoamide dehydrogenase (Sigma-Aldrich), and 500 µM NADH. Reactions were initiated by the addition of either reductant. An increase in the absorbance at 650 nm was monitored every 0.5 min. Activity was expressed as the ratio of the slope of a linear part of the turbidity curve to the lag time (reported as $\Delta A650/min^2$, 10^{-3}), as described previously [22]. *E. coli* Trx1 (Sigma-Aldrich) was assayed for comparison.

MaTrxR-MaTrx interaction assays. MaTrxR activity with thioredoxin substrates was assayed using the turbidimetric insulin reduction assay. The assays were performed anaerobically in 400 µL containing 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 130 µM insulin, 0.5 µM MaTrxR, and 5 or 10 µM Trx. Reactions were initiated by the addition of either NADH (1 mM) or NADPH (350 µM). An increase in the absorbance at 650 nm was monitored every 0.5 min.

MaTrxR-MaTrx7 kinetic parameters were obtained with assays that used oxidized glutathione as a substrate for thioredoxin as described [23]. The assays were performed

anaerobically in buffer B containing 0.5 µM MaTrxR, 1 mM oxidized glutathione (Sigma Aldrich), and increasing amounts of MaTrx7. The reactions were initiated by the addition of NADPH (100 µM). Activity was monitored by the decrease in absorbance at 340 nm by the oxidation of NADPH (ε_{340}=6,220 M^{-1} cm^{-1}). The apparent kinetic constants were determined by nonlinear regression of Michaelis-Menten plots using Microsoft Excel with the XL_kinetics add-in.

Results

Phylogenetic analysis of methanogen thioredoxins. Recent analysis of the sequenced genomes of methanogens identified 123 Trx homologues [12]. Using *E. coli* Trx1 (EcTrx1) and *M. jannaschii* Trx1 (MjTrx1) as BLAST queries, we found seven Trx homologues encoded in the genome of *M. acetivorans* C2A, which is two more than previously reported [12]. Because of this discrepancy we further searched the genomes of methanogens for additional Trxs, finding another 18 (**Table S1**). On average one additional Trx homologue was found specifically in some members of the *Methanomicrobia.* However, four additional Trx homologue were found in the genome of *Methanosarcina barkeri* str. Fusaro, bringing the total to 9 Trx, the most predicted in any methanogen. All of the sequenced *Methanosarcina* species contain at least seven Trx homologues (**Table S1**).

Phylogenetic analysis of the methanogen Trx homologues revealed a relationship between Trxs at the Class and Order levels. **Figure 1** is a simplified version of the complete phylogenetic tree (**Fig. S1**). Based on the phylogeny, we identified at least 8 clades (A-H), recognizing that some of these groupings have more support than others. Clade A contains the largest number of Trxs, including sequences from the *Methanococci*, *Methanobacteria*, and *Methanomicrobia*. *M. jannaschii* Trx2, which was shown to have limited Trx activity [12], is a

member of clade A. Clade A also contains MTH895 from *Methanothermobacter thermautotrophicus* ΔH for which the structure has been determined [14]. Clades B and C only include Trxs from the *Methanobacteria* and *Methanococci*, respectively, indicating Trxs within these clades are distinct from other Trxs found in the *Methanomicrobia*. *M. jannaschii* Trx1 (MjTrx1), which has Trx activity and was shown to target fundamental processes [12, 15], is found in clade C. Clades D through H contain Trxs that are restricted to members of the *Methanomicrobia*. All seven Trxs of the well-supported clade D are restricted to the Order *Methanosarcinales* and are encoded by a gene that is directly upstream of the gene encoding the putative TrxR in each species. This gene location indicates these Trxs likely serve as a substrate for the corresponding TrxR. Clade E contains Trxs that have a predicted N-terminal signal peptide, indicating these Trxs are likely extracellular. Interestingly, many of the genes encoding clade E Trxs are directly upstream of a gene encoding a homolog of CcdA, which functions in transferring electrons to extracellular ResA, a Trx-like protein. CcdA/ResA are components of cytochrome *c* biogenesis system II [24]. Thus, clade E Trxs may play a role in cytochrome *c* maturation or in the general reduction of disulfides in extracellular proteins. All clade F Trxs contain the consensus Trx active site motif (WCGPC) and are not located near genes that hint at a particular function or location. Clade G Trxs are distributed within the *Methanomicrobia*, but are not present in the genomes of *Methanosarcina* species. However, Trxs within clade H are primarily restricted to members of the order *Methanosarcinales*. Overall, it appears that the majority of methanogens contain a clade A Trx, but methanogens within the *Methanomicrobia* have acquired at least five different Trxs that are distinct from the additional Trxs found in the *Methanococci* and *Methanobacteria*.

***Methanosarcina acetivorans* thioredoxin homologues**. We have named the seven Trx homologues in *M. acetivorans* C2A MaTrx1-7 based on gene annotation number (**Table 1**). Overall, the sequence identity between the seven MaTrxs is <40%, with the exception of MaTrx4 and MaTrx5 (~48%) and MaTrx3 and MaTrx6 (~70%). *M. acetivorans* contains Trx homologues from five of the eight identified Trx clades based on phylogeny (**Fig. 1 and Table 1**), with MaTrx4/5 and MaTrx3/6 of the same clade. Each MaTrx contains an active site CXXC-motif (**Fig. S2**) and has 30-40% overall sequence identity to EcTrx1. Of the seven MaTrxs, only MaTrx2 and MaTrx6 have the conventional WCGPC active site motif (**Fig. S2**). MaTrx1 contains a CPYC motif, typical of glutaredoxins [1]. The genes encoding MaTrx1 and MaTrx2 are likely monocistronic. MaTrx3 and MaTrx6 contain a putative N-terminal signal peptide, including a lipobox (**Fig. S2**) [25], indicating each is likely targeted across the membrane and function extracellularly. The gene encoding MaTrx6 is adjacent to *ccdA* encoding a membrane protein predicted to function in cytochrome *c* maturation [24]. The gene encoding MaTrx3 is downstream of *ma3703* encoding a predicted cell surface protein. MaTrx4 and MaTrx5 are the smallest MaTrxs (**Table 1**) and have the same active site sequence (**Fig. S2**). The gene encoding MaTrx4 may be co-transcribed with *ma3937* and *ma3939*, each encoding a hypothetical protein. The gene encoding MaTrx5 is likely in an operon, adjacent to *maTrx4*, which includes hypothetical proteins and a universal stress protein. MaTrx7 is encoded by a gene directly upstream of *ma1368*, encoding the only predicted TrxR in *M. acetivorans*. Four (MaTrx1, MaTrx2, MaTrx6, and MaTrx7) of the MaTrxs were detected in previous proteomic analyses [26-28], consistent with each having cellular function.

Conserved TrxR in the *Methanosarcinaceae.* A BLAST with the EcTrxR amino acid sequence revealed the majority of methanogens contain at least one protein with homology to EcTrxR, including conservation of the coenzyme-binding and active site residues (data not shown). Therefore, the majority of methanogens may contain a complete thioredoxin system, composed of a L-TrxR and at least one Trx. Interestingly, *Methanopyrus kandleri* AV19, which does not contain an apparent Trx [12], encodes a putative TrxR (MK1561) that contains conserved coenzyme-binding and active-site residues. The TrxR in *M. kandleri* may be linked to proteins other than Trx. The TrxR in seven of the sequenced species of the *Methanosarcinaceae* (listed in **Fig. S3**) is encoded downstream of a clade D Trx. The *Methanosarcinaceae* TrxRs share >50% sequence identity to each other and >35% sequence identity to EcTrxR. Moreover, the FAD-binding, NAD(P)H-binding, and active site cysteine residues are all conserved in the *Methanosarcinaceae* TrxRs, including the only TrxR from *M. acetivorans* (**Fig. S3 and Table 1**). These results indicate that the *Methanosarcinaceae* have at least one NAD(P)H-dependent TrxR, which likely serves as the reducing partner to at least the clade D Trx in each species.

Purification and biochemical properties of recombinant MaTrxR. To examine the catalytic properties of MaTrxR, His-tagged recombinant MaTrxR was purified to homogeneity as revealed by SDS-PAGE (**Fig. 2**). Purified MaTrxR was slightly yellow indicative of the presence of flavin. The visible absorption spectrum of purified MaTrxR revealed absorbance maxima at 380 and 460 nm (**Fig. 3A**), typical for flavoproteins [29]. However, as-purified MaTrxR yielded an A_{280}/A_{460} ratio of 13.0, higher than the ratio observed for other TrxRs, including EcTrxR [29], indicating recombinant MaTrxR may not have full incorporation of FAD. To determine if as-purified MaTrxR was specific for FAD and had full incorporation, the protein was incubated

with excess FAD in the presence of DTT and subsequently re-purified. FAD-reconstituted MaTrxR had a visible spectrum with a substantial increase in absorbance at 380 and 460 nm (**Fig. 3A**), resulting in an A_{280}/A_{460} ratio of 3.3 consistent with full incorporation of FAD. FAD-reconstituted MaTrxR was used for all subsequent analyses.

The majority of TrxRs are reduced by NADPH and NADH, but have a strong preference for NADPH [5]. Anaerobic incubation of MaTrxR with excess NADPH or NADH resulted in rapid reduction of the bound FAD, as revealed by the decrease in absorbance at 460 nm (**Fig. 3**). Exposure of both NADH- or NADPH-reduced MaTrxR to oxygen resulted in a rapid oxidation of the bound FAD and restoration of the absorbance maxima at 380 and 460 nm (data not shown). Similar to TrxR from *S. solfataricus* [30], MaTrxR exhibited NADPH- and NADH-dependent oxidase activity (**Table 2**). Although, the majority of L-TrxRs are incapable of direct reduction of DTNB, unlike H-TrxRs, L-TrxRs characterized from some archaea and bacteria have been shown to catalyze the direct reduction of DTNB [9, 31, 32]. MaTrxR also possesses DTNB-reductase activity with both NADPH and NADH (**Table 2**), similar to L-TrxRs from other archaea [9, 31].

To examine coenzyme specificity of MaTrxR, the DTNB reduction assay was used to determine kinetic parameters with either NADPH or NADH as the electron donor. The apparent K_m value for NAPDH was 6.3 ± 0.5 µM, with a catalytic efficiency of 6.2 (μM^{-1} min^{-1}), which was approximately 100 times higher than the value obtained with NADH (**Table 3**). The apparent K_m value for NADPH is similar to those from other TrxRs, including EcTrxR [33]. DTNB reduction activity of MaTrxR with $F_{420}H_2$ as the electron donor was below the detection limit (data not shown). These results are consistent with MaTrxR as a NADPH-dependent TrxR, similar to TrxRs from bacteria, other archaea, and eukaryotes.

Purification and biochemical properties of MaTrx2, MaTrx6, and MaTrx7. MaTrx2, MaTrx6, and MaTrx7 were chosen for initial biochemical characterization, because all three proteins have been detected in the proteome of *M. acetivorans* [27, 28] and MaTrx2 and MaTrx6 each contain the consensus Trx active site (WCGPC) (**Fig. S2**). Although, MaTrx7 lacks the consensus Trx active site, it is linked to MaTrxR on the chromosome of *M. acetivorans*, indicating MaTrxR may be specific for MaTrx7. MaTrx2, MaTrx6, and MaTrx7 were expressed in *E. coli* with a thrombin-cleavable His-tag. His-tagged MaTrx2 and MaTrx7 were found in the soluble (cytoplasmic) fraction of *E. coli*, whereas full length MaTrx6 was found in the insoluble (membrane) fraction (data not shown), consistent with the predicted location of each MaTrx (**Table 1**). However, expression of MaTrx6 deleted of the putative signal peptide **(Fig. S2)**, designated MaTrx6Δsp, resulted in MaTrx6 being found in the soluble fraction of *E. coli* lysate (data not shown). This result suggests *E. coli* recognizes full-length MaTrx6 as a membrane-associated protein, consistent with MaTrx6 containing a signal peptide. MaTrx2, MaTrx6Δsp, and MaTrx7, each with the His-tag removed, were purified to homogeneity as revealed by SDS-PAGE (**Fig. 2**).

MaTrx2, MaTrx6Δsp, and MaTrx7 were examined for disulfide reductase activity using the insulin reduction assay, with DTT, lipoamide, glutathione, or coenzyme M as the source of reducing equivalents [21]. All three purified MaTrxs exhibited both DTT- and lipoamide-dependent insulin reduction activity (**Fig. 4**), but no activity was observed with glutathione or coenzyme M (data not shown), typical for Trxs. However, despite both MaTrx2 and MaTrx6Δsp possessing the consensus Trx active site motif, the insulin reduction activity of MaTrx2 was 8-18 fold lower than the activities determined for MaTrx6Δsp (**Fig. 4, insets**). The insulin reduction activity of MaTrx6Δsp was also approximately 2-fold higher than the activity determined for

MaTrx7. The DTT-dependent insulin reduction activity of EcTrx1, assayed under the same experimental conditions, was 785 (ΔA650/min^2 × 10^{-3})/mg, similar to the activity obtained for MaTrx7, but 2-fold lower than MaTrx6Δsp (**Fig. 4**). These results reveal that MaTrx2, MaTrx6, and MaTrx7 are capable of reducing disulfides in proteins and therefore have the capacity to function as *in vivo* disulfide reductases. Also, MaTrx6 has the highest disulfide reductase activity of the MaTrxs examined, which could be related to MaTrx6 likely being an extracellular protein.

Specificity of MaTrxR for MaTrxs. The ability of MaTrxR to serve as a direct electron donor to MaTrx2, MaTrx6Δsp, and MaTrx7 was examined. Initial assays examining MaTrxR-dependent NADPH or NADH oxidation in the presence of each oxidized MaTrx as an electron acceptor indicated MaTrxR is specific for MaTrx7 (data not shown). The ability of MaTrxR to form a complete thioredoxin system with MaTrx2, MaTrx6Δsp, or MaTrx7 was tested using the insulin reduction assay. Of the three MaTrxs, only MaTrx7 catalyzed the reduction of insulin when incubated with MaTrxR and either NADPH or NADH at a concentration above the apparent K_m for each coenzyme **(Fig. 5)**. Neither MaTrx2 nor MaTrx6Δsp at twice the concentration of MaTrx7 resulted in reduction of insulin above background. It is not surprising that MaTrx6 is not directly reduced by MaTrxR, since MaTrx6 is probably extracellular. On the other hand, MaTrx2 is likely cytoplasmic, but these data revealed MaTrx2 is not a redox partner to MaTrxR. Interestingly, EcTrx1, at twice the concentration of MaTrx7, exhibited MaTrxR-dependent insulin reduction activity (**Fig. 5**), albeit 100-fold lower than MaTrx7. The MaTrxR-EcTrx1 activity with NADPH was 2.0 ± 0.26 (ΔA650/min^2 × 10^{-3})/mg compared to MaTrxR-MaTrx7 with NADPH of 223 ± 30 (ΔA650/min^2 × 10^{-3})/mg. The DTNB reductase assay is commonly used to determine TrxR-Trx reaction kinetic parameters; but, since MaTrxR has

DTNB reductase activity, this assay could not be used to determine the MaTrxR-MaTrx7 kinetic parameters. However, since MaTrxR could not reduce oxidized glutathione (data not shown), the GHOST assay, which uses oxidized glutathione as a substrate for Trx [23], was utilized to determine the MaTrxR-MaTrx7 kinetic parameters **(Table 3)**. The apparent K_m value for MaTrx7 is higher than that observed for *E. coli* and yeast Trxs [33, 34], but is comparable to K_m values obtained for Trxs from other archaea [8, 9]. These results revealed that *M. acetivorans* contains a complete NADPH-dependent thioredoxin system comprised of MaTrxR and at least MaTrx7. For MaTrx2 and MaTrx6 to function *in vivo* these Trxs must be linked to a redox partner other than MaTrxR.

Discussion

Methanogens are strictly anaerobic prokaryotes that were likely present prior to the appearance of oxygen on earth. Methanogens are specialists, only capable of growth by methanogenesis, which requires unique cofactors, coenzymes, and enzymes. Methanogens lack glutathione [35-37], but contain small thiol-containing coenzymes, such as CoA, coenzyme M, and coenzyme B [11]. Moreover, the primary electron carriers in methanogens are F_{420} and ferredoxin, instead of NAD/NADP, which are used by the majority of other organisms. Therefore, it is plausible that methanogens may contain variant thioredoxin systems. An understanding of the thioredoxin system(s) in methanogens may provide insight into the evolution and diversification of the thioredoxin system. Recent evidence revealed MjTrx1 from *M. jannaschii* is capable of reducing disulfides in numerous oxidized *M. jannaschii* proteins, including enzymes directly involved in methanogenesis and biosynthesis [12]. This result indicates Trx likely played a fundamental role in cells before the rise of atmospheric oxygen levels. MjTrx1 is a member of methanogen Trx clade C and is distinct from Trx homologues

found in other methanogens (**Fig. 1**). The other Trx in *M. jannaschii* (MjTrx2) is a member of clade A and was shown to have limited insulin disulfide reduction activity [12], indicating it may not function as a true Trx. Thus, *M. jannaschii* and the majority of the *Methanococci* may have one primary Trx. In contrast, the *Methanomicrobia* contain 2-4 times as many Trxs as the *Methanococci*, all of which appear distinct from MjTrx1 (**Fig. 1**). For example, *M. acetivorans*, a member of the *Methanomicrobia* and the focus of this study contains at least five distinct Trx homologues. Why do some methanogens apparently have a need for additional Trxs?

Members of the *Methanomicrobia*, specifically the *Methanosarcinales*, are the most metabolically diverse methanogens, capable of hydrogenotrophic (CO_2-reducing), methylotrophic, and aceticlastic methanogenesis [11]. *Methanomicrobia* typically have larger genomes than the *Methanococci* and *Methanobacteria*, which are restricted to hydrogenotrophic methanogenesis. *M. acetivorans* possesses the largest genome of any methanogen, and is capable of growing by methylotrophic and aceticlastic methanogenesis [38]. Although, *M. acetivorans* is incapable of hydrogenotrophic methanogenesis, it can conserve energy by CO-dependent reduction of CO_2 to CH_4 [27, 39]. The growth of *M. acetivorans* with different substrates (CO, methanol, and acetate) requires large-scale changes in protein and gene expression, including electron carriers, electron transport system components, and methanogenesis enzymes [27, 28, 40]. Thus, *M. acetivorans*, and other members of the *Methanomicrobia* may have acquired additional Trxs that rely on different redox partner(s) and are specific for different targets to correlate with changes in electron carriers and enzymes used during growth with CO, methylated substrates, and acetate. For example, F_{420} is the primary electron carrier used during growth with methanol, whereas ferredoxin is the primary electron carrier during growth with acetate [41].

We show here that *M. acetivorans* contains a complete thioredoxin system comprised of NADPH, MaTrxR, and at least MaTrx7. MaTrx7 is a member of methanogen Trx Clade D, which contains Trxs only found in the *Methanosarcinales*. Given the gene location and lack of activity with MaTrx2 and MaTrx6, MaTrxR is likely specific for MaTrx7. MaTrxR-MaTrx7 may have been acquired from bacteria to carry out a function specific to members of the *Methanosarcinales*. Outside of the *Methanosarcinales*, the amino acid sequence of MaTrxR has highest identity to TrxR (TOL2_C00640) from *Desulfobacula toluolica* Tol2 (51%), an anaerobic sulfate-reducing bacterium [42]. Interestingly, *D. toluolica* Tol2 and several other sulfate-reducing bacteria have the same gene arrangement (*trx-trxR*) as in the *Methanosarcinales*. Thus, it is possible MaTrxR and MaTrx7 were acquired from sulfate-reducing bacteria, which is consistent with the previous proposal that gene acquisition from anaerobic bacteria led to the evolution of the *Methanomicrobia* [43, 44]. Enzyme assays revealed MaTrxR is specific for NADPH and cannot be reduced by $F_{420}H_2$. NADP is not directly reduced by methanogenesis enzymes, signifying reducing equivalents are not directly transferred to MaTrxR from a methanogenesis enzyme. However, methanogens contain enzymes that could mediate electron transfer from $F_{420}H_2$ or reduced ferredoxin to NADP. $F_{420}H_2$:NADP oxidoreductase (Fno) catalyzes the reversible hydride transfer from $F_{420}H_2$ to NADP. Fno functions to produce NADPH for biosynthesis in the majority of methanogens [45, 46], consistent with the primary function of Trx in most cells. The genome of *M. acetivorans* encodes one Fno (MA4235) that may be responsible for the generation of NADPH from $F_{420}H_2$ needed by MaTrxR in *M. acetivorans*. Ferredoxin is reduced by carbon monoxide dehydrogenase/acetyl-CoA synthase with electrons supplied by the oxidation of the carbonyl group of acetate [47]. For MaTrxR to function in *M. acetivorans* during growth with acetate,

reduced ferredoxin would likely need to directly or indirectly supply electrons to NADP. Ferredoxin:NADP oxidoreductases (Fnr) are flavoenzymes that catalyze the reversible transfer of reducing equivalents from ferredoxin to NADP, and are common in plants and bacteria [48]. *M. acetivorans* contains a homolog of NADH-dependent reduced ferredoxin:NADP oxidoreductase (NfnAB), encoded by *ma3786-87*. In *Clostridia* and related anaerobic bacteria, NfnAB catalyzes an electron bifurcation reaction, whereby the endergonic reduction of NADP with NADH is coupled to the exergonic reduction of NADP with reduced ferredoxin [49]. Interestingly, among methanogens, NfnAB appears restricted to the *Methanomicrobia* [50]. However, NfnAB has not been characterized from a methanogen, so its precise function is not clear. Nonetheless, *M. acetivorans* would likely not use NfnAB to generate NADP with NADH, since NADH is not directly produced during methanogenesis. In *M. acetivorans*, and other *Methanomicrobia*, NfnAB may catalyze the exergonic reduction of NADP with reduced ferredoxin to supply MaTrxR and other NADPH-dependent biosynthetic enzymes with NADPH.

Although only MaTrx2, MaTrx6, and MaTrx7 were tested as substrates for MaTrxR, the lack of activity with MaTrx2 and MaTrx6, along with the conserved *trx-trxR* gene arrangement in the *Methanosarcinales*, indicates MaTrx7 is likely the only Trx substrate of MaTrxR. MaTrx7 has a unique active site (CTAC) which likely contributes to specific interactions with MaTrxR. Both MaTrx2 and MaTrx6 have the conventional Trx active site (CGPC), as found in EcTrx1. Thus, it was surprising that MaTrxR was unable to reduce MaTrx2 or MaTrx6, but could reduce EcTrx1, albeit not as efficiently as MaTrx7 (**Fig. 5**). This result suggests that interaction of TrxR with Trx is controlled by more than just the active site region of Trx. Indeed, examination of the specificity of yeast TrxR for the three yeast Trxs revealed three interaction loops within Trx [34], two of which are found in all MaTrxs. In particular, interaction loop 3 is more similar between

MaTrx7 and EcTrx1, than between MTrx7 and MaTrx2 or MaTrx6 (**Fig. S2**), which may explain why MaTrxR is able to reduce EcTrx1, but not MaTrx2 or MaTrx6. Given the differences in activity of MaTrxR with MaTrxs and EcTrx1, the *M. acetivorans* thioredoxin system could provide an attractive model to understand the specificity of TrxRs and Trx for redox partners.

MjTrx1 was shown to target a large number of oxidized proteins in *M. jannaschii*, consistent with MjTrx1 as the primary, if not only Trx, in *M. jannaschii* [12]. However, the redox partner to MjTrx1 has not been identified, so it is unclear what protein(s) provides reducing equivalents *in vivo* to MjTrx1. *M. jannaschii* contains a TrxR homolog (Mj1356), but experiments by Lee *et al* showed that recombinant Mj1356 was incapable of reducing MjTrx1 with NADPH [15]. However, it was not clear if the lack of reduction was due to the inability of MJ1356 to be reduced by NADPH or the lack of interaction with MjTrx1. Nonetheless, these results indicate that *M. jannaschii*, and possibly all *Methanococci*, have a thioredoxin system not dependent on NADPH, unlike members of the *Methanomicrobia*. In *M. jannaschii*, the reduction of MjTrx1 may be directly linked to reduction by methanogenesis electron carriers ($F_{420}H_2$ or reduced ferredoxin). Similarly, the reduction of the other MaTrxs could be directly linked to $F_{420}H_2$ or reduced ferredoxin. Overall, linking the reduction of cytoplasmic Trxs in *M. acetivorans* to different electron carriers may allow *M. acetivorans* to control the specificity and activity of redox proteins within the cell under different growth conditions. Changes in MaTrx abundance may also provide a mechanism to modulate electron transfer during changing growth conditions. For example, expression of MaTrx2 was shown to be up-regulated in acetate-grown cells compared to methanol-grown cells of *M. acetivorans* [28].

Interestingly, not all of the Trx homologues in *M. acetivorans* are cytoplasmic. MaTrx3 and MaTrx6 (clade E Trxs, **Fig. 1**) contain an N-terminal signal peptide (**Fig. S2**), indicating each is

likely targeted across the membrane. Clade E Trxs are only found in the *Methanomicrobia*, which contains the only methanogens that harbor cytochromes, including cytochrome *c*. *M. acetivorans* in particular has been shown to use a cytochrome *c* as part of an Rnf complex to facilitate transfer of electrons from ferredoxin to heterodisulfide reductase [51, 52]. However, the machinery responsible for cytochrome *c* maturation in methanogens has not been identified. The gene encoding MaTrx6 is adjacent to *ccdA* in the genome of *M. acetivorans*, suggesting MaTrx6 may receive reducing equivalents from CcdA to reduce disulfides in apo-cytochrome *c*, similar to the process found in bacteria [24].

Conclusions

Results from this study revealed that methanogens contain Trx homologues distributed within at least eight clades, with the *Methanococci* and *Methanobacteria* restricted to Trxs within 1-2 clades, while the *Methanomicrobia* contain Trxs from >2 clades. The characterization of thioredoxin system components from *M. acetivorans*, provides the first insight into the role of the thioredoxin system in the metabolically diverse cytochrome-containing methanogens. Importantly, we demonstrate that *M. acetivorans* contains a complete NADPH-dependent thioredoxin system (MaTrxR-MaTrx7), providing the first experimental evidence for the presence of this system in methanogens. The use of a NADPH-dependent thioredoxin system may be specific to *Methanomicrobia*, but additional experimentation is needed to understand how widespread the NADPH-dependent system is. *M. acetivorans* contains at least two Trx homologues not directly reduced by the only TrxR, revealing *M. acetivorans* has a diverse thioredoxin system, whereby the multiple and differentially-located Trx homologue are likely linked to different redox partners. The detailed understanding of the metabolism of *M. acetivorans*, combined with its genetic system, makes *M. acetivorans* a particularly attractive

model to investigate what appears to be a complex thioredoxin system network in cytochrome-containing *Methanomicrobia*, when compared to cytochrome-lacking *Methanococci* and *Methanobacteria*.

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Figures and Tables

Figure 1

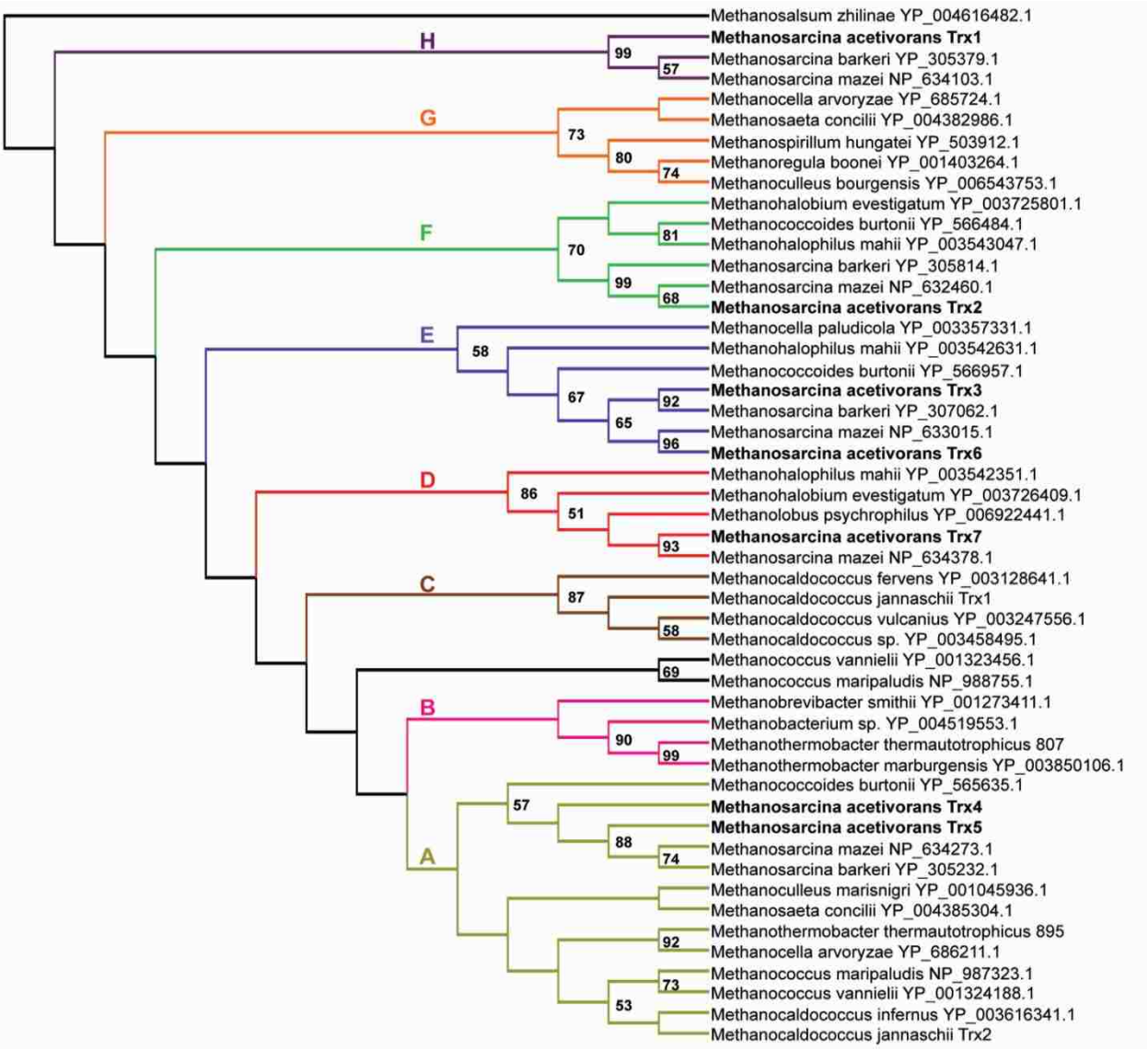


Fig. 1. Phylogenetic analyses of methanogen Trx homologues. A simplified phylogenetic tree based on the complete tree (Fig. S1). Clades (A-H) are labelled and differently colored. Numbers above nodes represent maximum-likelihood bootstrap values; only values >50% are shown.

Table 1. Thioredoxin system homologues encoded in the genome of *M. acetivorans* C2A.

Gene ID	homologue designation	Predicted location	Trx homologue clade	pI/MW
MA1368	MaTrxR	Cytoplasm	-	5.8/34.0
MA0965	MaTrx1	Cytoplasm	H	4.7/15.0
MA3212	MaTrx2	Cytoplasm	F	5.1/10.4
MA3702	MaTrx3	Extracellular/membrane	E	4.7/19.6
MA3938	MaTrx4	Cytoplasm	A	5.4/8.4
MA3942	MaTrx5	Cytoplasm	A	8.5/8.7
MA4254	MaTrx6	Extracellular/membrane	E	4.2/17.7
MA4683	MaTrx7	Cytoplasm	D	5.6/9.2

Table 2. MaTrxR activity with different electron donors and acceptors.

e^- donor	e^- acceptor	Specific activity
NADPH	DTNB	0.3 ± 0.005[a]
NADH	DTNB	0.5 ± 0.005[a]
NADPH	O_2	2.9 ± 0.07[b]
NADH	O_2	0.13 ± 0.01[b]

[a] µmol TNB min^{-1} mg^{-1} TrxR
[b] µmol NAD min^{-1} mg^{-1} TrxR

Table 3. Kinetic parameters of MaTrxR.

substrate	K_m (µM)	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})
NADH[a]	736 ± 57	49	0.067
NADPH[a]	6.3 ± 0.5	39	6.2
MaTrx7[b]	86 ± 5	70.5	0.82

[a]Measured using the DTNB assay as described in materials and methods
[b]Measured using the GHOST assay with NADPH as described in materials and methods

Figure 2

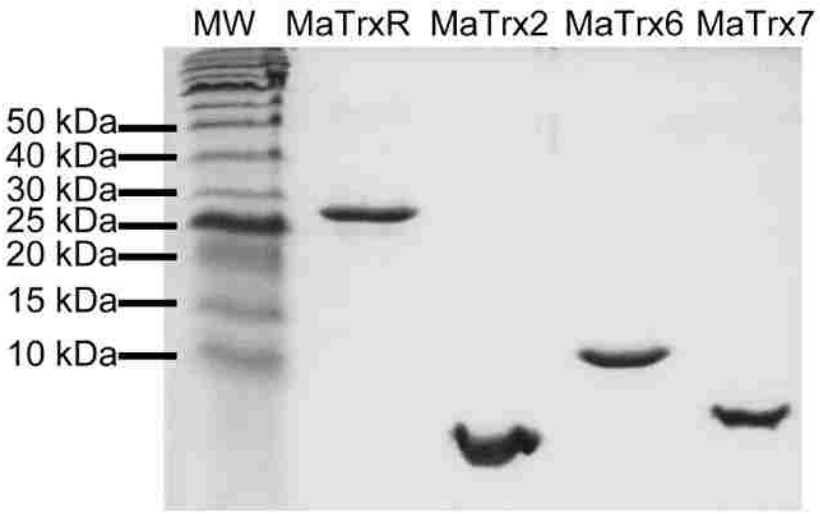


Fig. 2: SDS-PAGE analysis of recombinant proteins purified from *E. coli*. The purified recombinant proteins (3 µg each) were separated on 15% SDS-PAGE. MW, Marker lane.

Figure 3

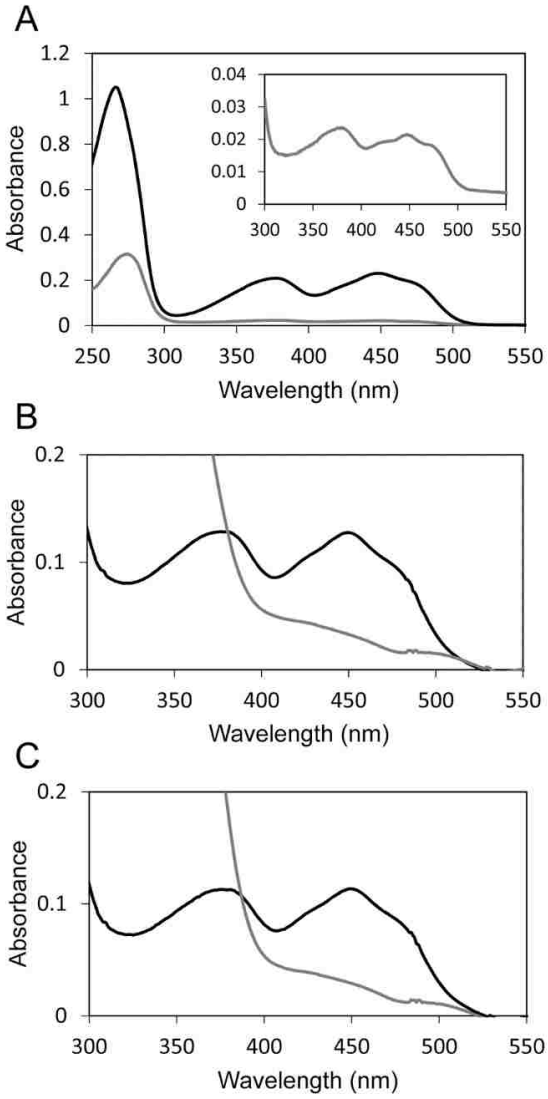


Fig. 3. Spectroscopic analysis of MaTrxR. (**A**) UV-visible spectrum of 10 µM as-purified MaTrxR (gray line) and FAD-reconstituted MaTrxR (black line). Inset: magnified spectrum of as-purified MaTrxR. (**B**) Spectrum of 6.3 µM MaTrxR before (black line) and after (gray line) the addition of 70 µM NADPH under anaerobic conditions. (**C**) Spectrum of 6.3 µM MaTrxR before (black line) and after (gray line) the addition of 110 µM NADH under anaerobic conditions. All spectra were of MaTrxR in 50 mM Tris-HCl pH 7.5, 150 mM NaCl.

Figure 4

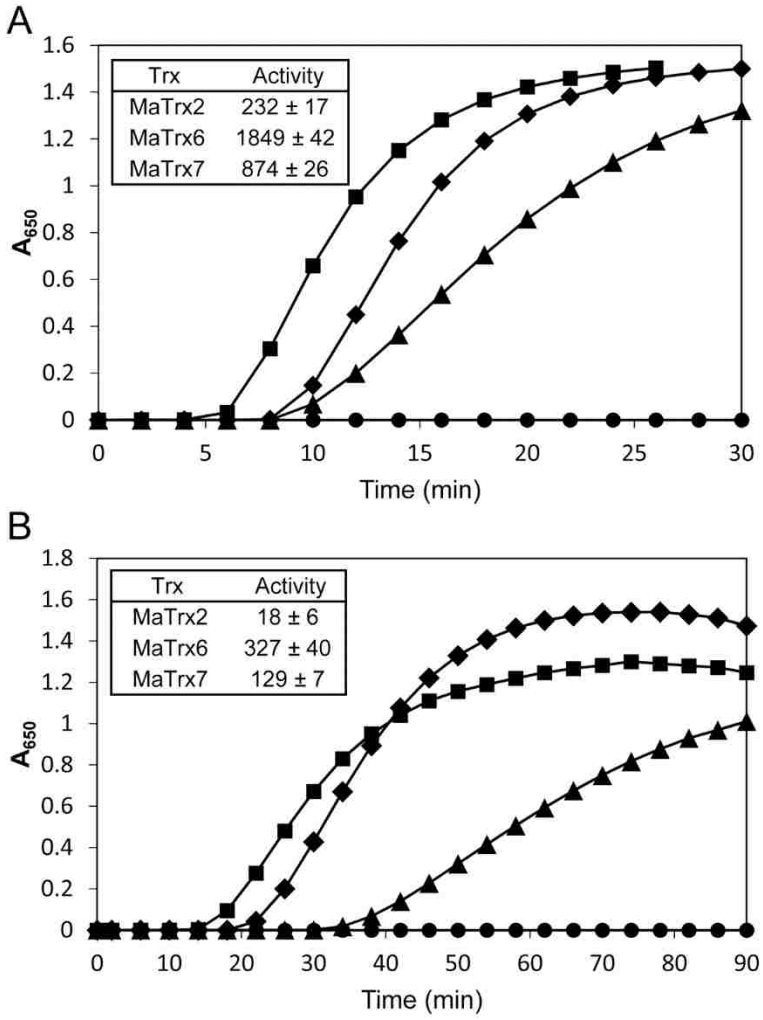


Fig. 4. Comparison of the reduction of insulin catalyzed by MaTrxs. (**A**) DTT-dependent activity: 9 µM MaTrx2 (triangles), 3 µM MaTrx6ΔSp (squares), and 6 µM MaTrx7 (diamonds) were added to 0.33 mM DTT and 0.13 mM insulin in 100 mM KPO_4 pH 6.8 under anaerobic conditions. The complete reaction without the addition of thioredoxin was included as a negative control (circles). Absorbance at 650 nm at 2 min intervals is shown. The data are the mean ± SD of triplicate reactions and the specific activity ($\Delta A650/min^2 \times 10^{-3}/mg$) of each thioredoxin is shown in the inset. (**B**) Lipoamide-dependent activity: 12 µM MaTrx2 (triangles), 3 µM MaTrx6ΔSp (squares), and 6 µM MaTrx7 (diamonds) were added to 0.33 mM NADH, 4 units lipoamide dehydrogenase, 0.05 mM lipoamide and 0.13 mM insulin in 100 mM KPO_4 pH 6.8 under anaerobic conditions. The complete reaction without the addition of thioredoxin was included as a negative control (circles). Absorbance at 650 nm at 4 min intervals is shown. The data are the mean ± SD of triplicate reactions and the specific activity ($\Delta A650/min^2 \times 10^{-3}/mg$) of thioredoxin is shown in the inset.

Figure 5

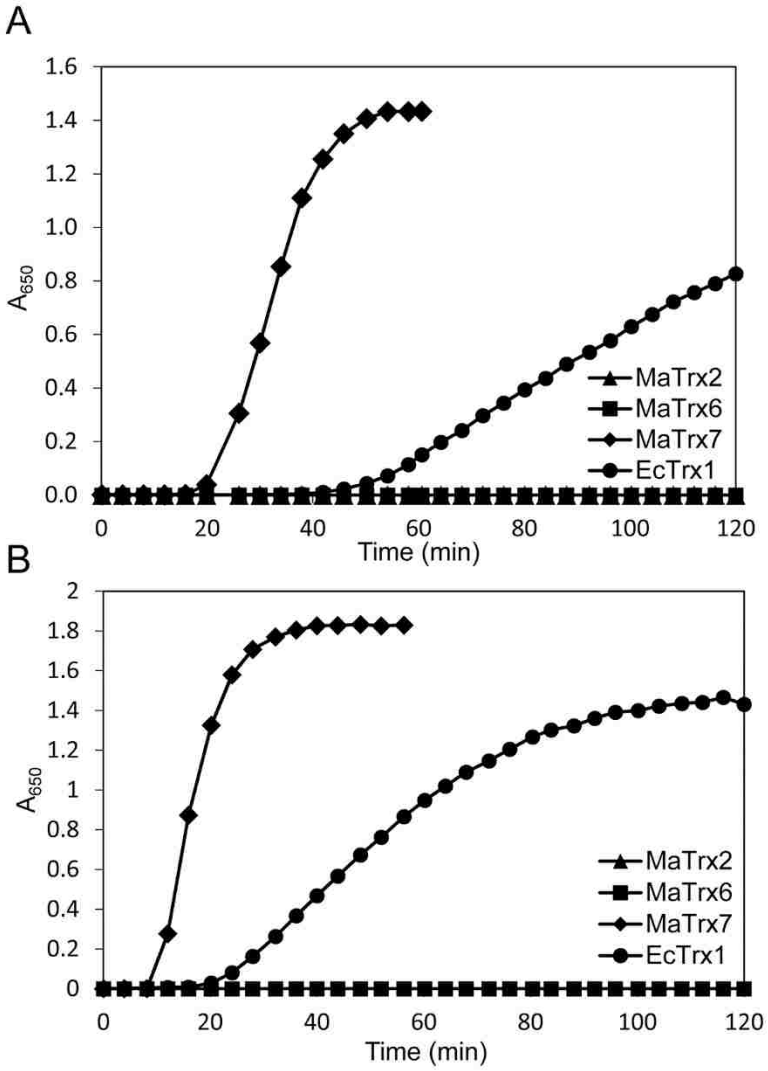


Fig. 5. Comparison of the reduction of insulin catalyzed by the M. acetivorans thioredoxin system components. (A) NADPH-dependent activity: 10 µM MaTrx2, 10 µM MaTrx6ΔSp, 5 µM MaTrx7, or 10 µM EcTrx1 were added to 0.35 mM NADPH, 1 µM MaTrxR, and 0.13 mM insulin in 100 mM KPO_4 pH 6.8 under anaerobic conditions. The complete reaction without the addition of thioredoxin was included as a negative control (not shown). Absorbance at 650 nm at 4 min intervals is shown. The data are the mean of triplicate reactions. **(B)** NADH-dependent activity: 10 µM MaTrx2, 10 µM MaTrx6ΔSp, 5 µM MaTrx7, or 10 µM EcTrx1 were added to 1 mM NADH, 1 µM MaTrxR, and 0.13 mM insulin in 100 mM KPO4 pH 6.8 under anaerobic conditions. The complete reaction without the addition of thioredoxin was included as a negative control (not shown). Absorbance at 650 nm at 4 min intervals is shown. The data are the mean of triplicate reactions.

Table S1. Identification of additional thioredoxin homologues in the genomes of sequenced methanogens.

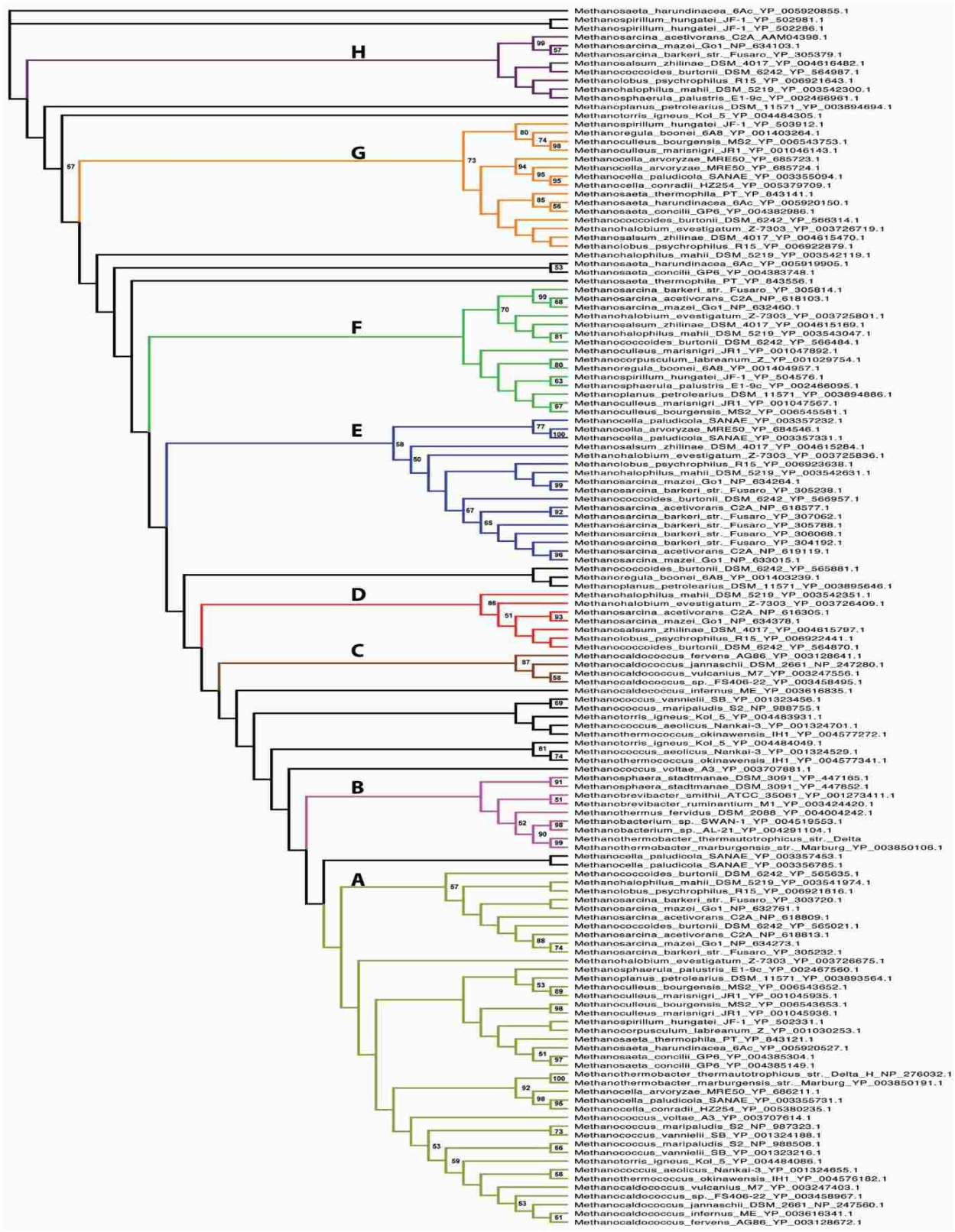
Species	Trx homolog Accession no.[a]	ORF
Methanosarcina acetivorans C2A	NP_616305.1 NP_618813.1 NP_619119.1 NP_618103.1 NP_618809.1 NP_618577.1 NP_615918.1	MA4683 (MaTrx7) MA3942 (MaTrx5) MA4254 (MaTrx6) MA3212 (MaTrx2 MA3938 (MaTrx4) MA3702 (MaTrx3 MA0965 (MaTrx1)
Methanosarcina barkeri str. fusaro	YP_303720.1 YP_305814.1 YP_305788.1 YP_305232.1 YP_305379.1 YP_306068.1 YP_304192.1 YP_307062.1 YP_305238.1	Mbar_A0155 Mbar_A2310 Mbar_A2283 Mbar_A1710 Mbar_A1859 Mbar_A2577 Mbar_A0633 Mbar_A3617 Mbar_A1716
Methanosarcina mazei Go1	NP_632761.1 NP_632460.1 NP_634273.1 NP_634378.1 NP_633015.1 NP_634264.1 NP_634103.1	MM_0737 MM_0436 MM_2249 MM_2354 MM_0991 MM_2240 MM_2079
Methanococcoides burtonii DSM 6242	YP_566484.1; YP_564870.1; YP_564987.1; YP_565635.1; YP_565021.1; YP_566957.1; YP_565881.1; YP_566314.1;	Mbur_1851 Mbur_0102 Mbur_0227 Mbur_0943 Mbur_0264 Mbur_2353 Mbur_1209 Mbur_1670
Methanolobus psychrophilus R15	YP_006921816.1; YP_006923638.1; YP_006922441.1; YP_006922879.1; YP_006921643.1	Mpsy_0235 Mpsy_2064 Mpsy_0864 Mpsy_1303 Mpsy_0062
Methanohalophilus Mahii DSM 5219	YP_003542631.1; YP_003542351.1; YP_003541974.1; YP_003542119.1; YP_003543047.1 YP_003542300.1	Mmah_1490 Mmah_1202 Mmah_0806 Mmah_0954 Mmah_1907 Mmah_1149

Table S1 continued

Species	Trx homolog Accession no.[a]	ORF
Methanosalsum zhilinae	YP_004615470.1; YP_004615797.1; YP_004616482.1; YP_004615169.1 YP_004615284.1	Mzhil_0376 Mzhil_0711 Mzhil_1419 Mzhil_0069 Mzhil_0186
Methanohalobium evestigatum Z-7303	YP_003725801.1; YP_003726675.1; YP_003726409.1; YP_003726719.1 YP_003725836.1	Metev_0073 Metev_0989 Metev_0712 Metev_1035 Metev_0109
Methanocella conradii HZ254	YP_005379709.1; YP_005380235.1 YP_005381598.1	Mtc_0423 Mtc_0960 Mtc_2347
Methanocella paludicola SANAE	YP_003355731.1; YP_003357232.1; YP_003355094.1; YP_003357453.1; YP_003356785.1 YP_003357331.1	MCP_0676 MCP_2177 MCP_0039 MCP_2398 MCP_1730 MCP_2276
Methanosphaerula palustris E1-9c	YP_002466095.1; YP_002467560.1 YP_002466961.1	Mpal_1024 Mpal_2567 Mpal_1934
Methanoplanus petrolearius DSM 11571	YP_003894886.1; YP_003893564.1; YP_003895646.1 YP_003894694.1	Mpet_1695 Mpet_0352 Mpet_2464 Mpet_1499
Methanosaeta harundinacea 6Ac	YP_005920527.1; YP_005920150.1; YP_005919905.1 YP_005920855.1	Mhar_1543 Mhar_1159 Mhar_0911 Mhar_1875
Methanospirillum hungatei JF-1	YP_502331.1; YP_503912.1; YP_502981.1; YP_504576.1 YP_502286.1	Mhun_0860 Mhun_2493 Mhun_1531 Mhun_3175 Mhun_0815

[a]Highlighted sequences were not identified in a previous study (Susanti et al. 2014)

Figure S1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D
C
B
A

Figure S1 continued

Fig. S1. A phylogenetic tree based on maximum likelihood analysis of 140 Trx homologue sequences from sequenced methanogen genomes. Trx clades (A-H) are labelled and differently colored. Numbers above nodes represent maximum-likelihood bootstrap values; only values >50% are shown.

Figure S2

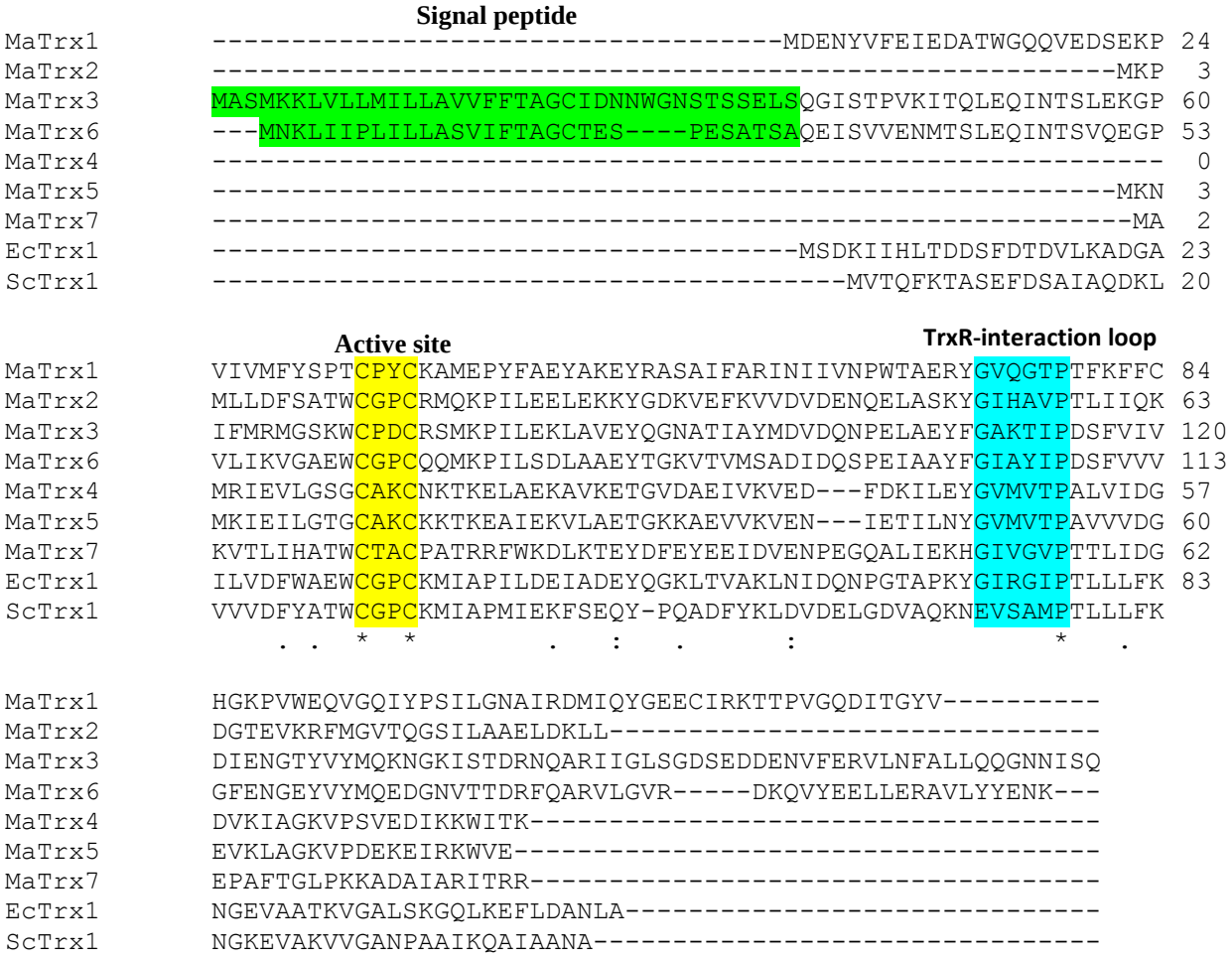

Fig. S2. Amino acid sequence alignment of MaTrxs with Trx from *E. coli* (EcTrx1) and *S. cerevisiae* (ScTrx1). Invariant residues are indicated by an asterisk. The active site is highlighted in yellow and the putative signal peptide of MaTrx3 and MaTrx6 is highlighted in green. The box denotes the flexible loop region 3 identified as critical for interaction with TrxR (Oliveira et al. 2010).

Figure S3

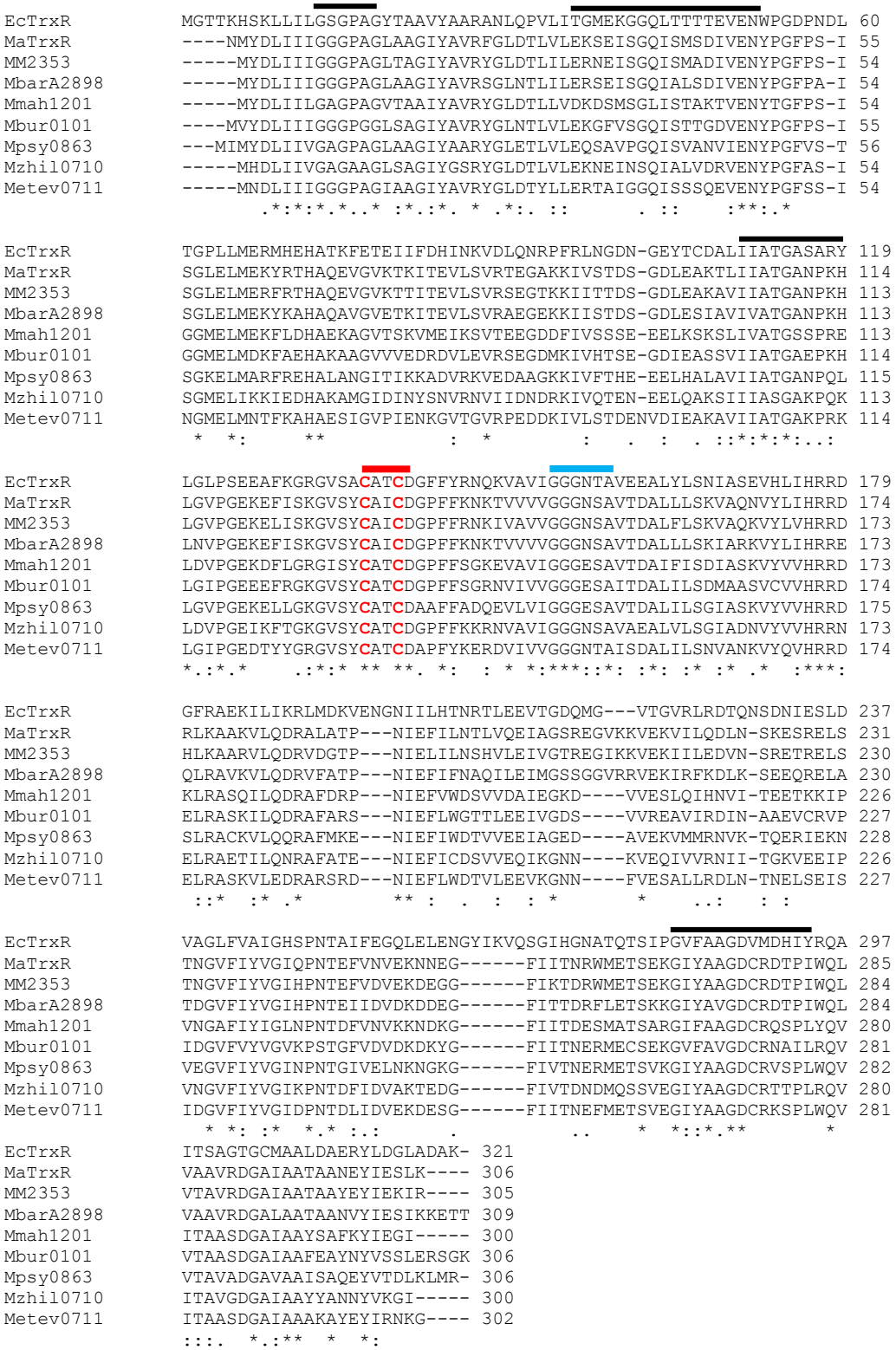
EcTrxR MGTTKHSKLLILGSGPAGYTAAVYAARANLQPVLITGMEKGGQLTTTTEVENWPGDPNDL 60
MaTrxR ----NMYDLIIIGGGPAGLAAGIYAVRFGLDTLVLEKSEISGQISMSDIVENYPGFPS-I 55
MM2353 -----MYDLIIIGGGPAGLTAGIYAVRYGLDTLILERNEISGQISMADIVENYPGFPS-I 54
MbarA2898 -----MYDLIIIGGGPAGLAAGIYAVRSGLNTLILERSEISGQIALSDIVENYPGFPA-I 54
Mmah1201 -----MYDLIILGAGPAGVTAAIYAVRYGLDTLLVDKDSMSGLISTAKTVENYTGFPS-I 54
Mbur0101 ----MVYDLIIIGGGPGGLSAGIYAVRYGLNTLVLEKGFVSGQISTTGDVENYPGFPS-I 55
Mpsy0863 ---MIMYDLIIVGAGPAGLAAGIYAARYGLETLVLEQSAVPGQISVANVIENYPGFVS-T 56
Mzhil0710 -----MHDLIIVGAGAAGLSAGIYGSRYGLDTLVLEKNEINSQIALVDRVENYPGFAS-I 54
Metev0711 -----MNDLIIIGGGPAGIAAGIYAVRYGLDTYLLERTAIGGQISSSQEVENYPGFSS-I 54
EcTrxR TGPLLMERMHEHATKFETEIIFDHINKVDLQNRPFRLNGDN-GEYTCDALIIATGASARY 119
MaTrxR SGLELMEKYRTHAQEVGVKTKITEVLSVRTEGAKKIVSTDS-GDLEAKTLIIATGANPKH 114
MM2353 SGLELMERFRTHAQEVGVKTTITEVLSVRSEGTKKIITTDS-GDLEAKAVIIATGANPKH 113
MbarA2898 SGLELMEKYKAHAQAVGVETKITEVLSVRAEGEKKIISTDS-GDLESIAVIVATGANPKH 113
Mmah1201 GGMELMEKFLDHAEKAGVTSKVMEIKSVTEEGDDFIVSSSE-EELKSKSLIVATGSSPRE 113
Mbur0101 GGMELMDKFAEHAKAAGVVVEDRDVLEVRSEGDMKIVHTSE-GDIEASSVIIATGAEPKH 114
Mpsy0863 SGKELMARFREHALANGITIKKADVRKVEDAAGKKIVFTHE-EELHALAVIIATGANPQL 115
Mzhil0710 SGMELIKKIEDHAKAMGIDINYSNVRNVIIDNDRKIVQTEN-EELQAKSIIIASGAKPQK 113
Metev0711 NGMELMNTFKAHAESIGVPIENKGVTGVRPEDDKIVLSTDENVDIEAKAVIIATGAKPRK 114
EcTrxR LGLPSEEAFKGRGVSACATCDGFFYRNQKVAVIGGGNTAVEEALYLSNIASEVHLIHRRD 179
MaTrxR LGVPGEKEFISKGVSYCAICDGPFFKNKTVVVVGGGNSAVTDALLLSKVAQNVYLIHRRD 174
MM2353 LGVPGEKELISKGVSYCAICDGPFFRNKIVAVVGGGNSAVTDALFLSKVAQKVYLVHRRD 173
MbarA2898 LNVPGEKEFISKGVSYCAICDGPFFKNKTVVVVGGGNSAVTDALLLSKIARKVYLIHRRE 173
Mmah1201 LDVPGEKDFLGRGISYCATCDGPFFSGKEVAVIGGGESAVTDAIFISDIASKVYVVHRRD 173
Mbur0101 LGIPGEEEFRGKGVSYCATCDGPFFSGRNVIVVGGGESAITDALILSDMAASVCVVHRRD 174
Mpsy0863 LGVPGEKELLGKGVSYCATCDAAFFADQEVLVIGGGESAVTDALILSGIASKVYVVHRRD 175
Mzhil0710 LDVPGEIKFTGKGVSYCATCDGPFFKKRNVAVIGGGNSAVAEALVLSGIADNVYVVHRRN 173
Metev0711 LGIPGEDTYYGRGVSYCATCDAPFYKERDVIVVGGGNTAISDALILSNVANKVYQVHRRD 174
EcTrxR GFRAEKILIKRLMDKVENGNIILHTNRTLEEVTGDQMG---VTGVRLRDTQNSDNIESLD 237
MaTrxR RLKAAKVLQDRALATP---NIEFILNTLVQEIAGSREGVKKVEKVILQDLN-SKESRELS 231
MM2353 HLKAARVLQDRVDGTP---NIELILNSHVLEIVGTREGIKKVEKIILEDVN-SRETRELS 230
MbarA2898 QLRAVKVLQDRVFATP---NIEFIFNAQILEIMGSSGGVRRVEKIRFKDLK-SEEQRELA 230
Mmah1201 KLRASQILQDRAFDRP---NIEFVWDSVVDAIEGKD----VVESLQIHNVI-TEETKKIP 226
Mbur0101 ELRASKILQDRAFARS---NIEFLWGTTLEEIVGDS----VVREAVIRDIN-AAEVCRVP 227
Mpsy0863 SLRACKVLQQRAFMKE---NIEFIWDTVVEEIAGED----AVEKVMMRNVK-TQERIEKN 228
Mzhil0710 ELRAETILQNRAFATE---NIEFICDSVVEQIKGNN----KVEQIVVRNII-TGKVEEIP 226
Metev0711 ELRASKVLEDRARSRD---NIEFLWDTVLEEVKGNN----FVESALLRDLN-TNELSEIS 227
EcTrxR VAGLFVAIGHSPNTAIFEGQLELENGYIKVQSGIHGNATQTSIPGVFAAGDVMDHIYRQA 297
MaTrxR TNGVFIYVGIQPNTEFVNVEKNNEG------FIITNRWMETSEKGIYAAGDCRDTPIWQL 285
MM2353 TNGVFIYVGIHPNTEFVDVEKDEGG------FIKTDRWMETSEKGIYAAGDCRDTPIWQL 284
MbarA2898 TDGVFIYVGIHPNTEIIDVDKDDEG------FITTDRFLETSKKGIYAVGDCRDTPIWQL 284
Mmah1201 VNGAFIYIGLNPNTDFVNVKKNDKG------FIITDESMATSARGIFAAGDCRQSPLYQV 280
Mbur0101 IDGVFVYVGVKPSTGFVDVDKDKYG------FIITNERMECSEKGVFAVGDCRNAILRQV 281
Mpsy0863 VEGVFIYVGINPNTGIVELNKNGKG------FIVTNERMETSVKGIYAAGDCRVSPLWQV 282
Mzhil0710 VNGVFIYVGIKPNTDFIDVAKTEDG------FIVTDNDMQSSVEGIYAAGDCRTTPLRQV 280
Metev0711 IDGVFIYVGIDPNTDLIDVEKDESG------FIITNEFMETSVEGIYAAGDCRKSPLWQV 281
EcTrxR ITSAGTGCMAALDAERYLDGLADAK- 321
MaTrxR VAAVRDGAIAATAANEYIESLK---- 306
MM2353 VTAVRDGAIAATAAYEYIEKIR---- 305
MbarA2898 VAAVRDGALAATAANVYIESIKKETT 309
Mmah1201 ITAASDGAIAAYSAFKYIEGI----- 300
Mbur0101 VTAASDGAIAAFEAYNYVSSLERSGK 306
Mpsy0863 VTAVADGAVAAISAQEYVTDLKLMR- 306
Mzhil0710 ITAVGDGAIAAYYANNYVKGI----- 300
Metev0711 ITAASDGAIAAAKAYEYIRNKG---- 302

Figure S3 continued

Fig. S3. Amino acid sequence alignment of TrxRs from *Methanosarcinaceae* with TrxR from *E. coli* (EcTrx1). Invariant residues are indicated by an asterisk. The active site is indicated by the red line, with the active site cysteines in red. The FAD binding regions are indicated by the black lines and the NADPH binding region is indicated by the blue line.

Appendix 1.1: Lead Author Confirmation Letter for Chapter I

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter I, titled "Molecular Characterization of the Thioredoxin System from *Methanosarcina acetivorans*" of A. C. McCarver's dissertation was submitted for publication in FEBS Journal in 2014 with coauthor D. J. Lessner.

I, Dr. Daniel J. Lessner, advisor of Addison C. McCarver, confirm Addison C. McCarver was first author and completed at least 51% of the work for this manuscript.

Dr. Daniel J. Lessner
Associate Professor
Department of Biological Sciences
University of Arkansas

Date

Chapter II

Methanosarcina acetivorans Utilizes a Single NADPH-dependent Thioredoxin System and Contains Additional Thioredoxin Homologs with Distinct Functions.

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Abstract

The thioredoxin system plays a central role in the intracellular redox maintenance in the majority of cells. The canonical system is comprised of an NADPH-dependent thioredoxin reductase (TrxR) that reduces the disulfide reductase thioredoxin (Trx). Although Trx is encoded in almost all sequenced genomes of methanogens, its incorporation into their unique physiology is not well understood. *Methanosarcina acetivorans* contains a single TrxR (MaTrxR) and seven Trx (MaTrx1-7) homologs. We previously showed that MaTrxR and at least MaTrx7 comprise a functional NADPH-dependent thioredoxin system. Here, we report the characterization of all seven recombinant MaTrxs. MaTrx1, MaTrx3, MaTrx4, and MaTrx5 lack appreciable disulfide reductase activity, unlike previously characterized MaTrx2, MaTrx6, and MaTrx7. Enzyme assays demonstrated that of the MaTrxs, only the reduction of disulfide-containing MaTrx7 is linked to the oxidation of reduced coenzymes. NADPH is shown to be supplied to the MaTrxR-MaTrx7 system through the oxidation of the primary methanogen electron carriers $F_{420}H_2$ and ferredoxin, indicating it serves as a primary intracellular reducing system in *M. acetivorans.* Bioinformatic analyses also indicate that the majority of methanogens likely utilize a NADPH-dependent thioredoxin system. The remaining MaTrxs may have specialized functions. MaTrx1 and MaTrx3 exhibited thiol oxidase activity. MaTrx3 and MaTrx6 are targeted to the membrane of *M. acetivorans* and likely function in the formation and the reduction of disulfides in membrane and/or extracellular proteins, respectively. This work provides insight into the incorporation of Trx into the metabolism of methanogens, and reveals that methanogens contain Trx homologs with alternative properties and activities.

Introduction

The strictly anaerobic methane-producing archaea (methanogens) are the only cellular organisms capable of biological methane production (methanogenesis), an important step in the global carbon cycle [1, 2]. Methanogens are ubiquitous microbes, found in diverse environments, ranging from the human gastrointestinal tract to the Antarctic [2, 3]. No matter the environment, significant methane production by methanogens only occurs under strictly anaerobic conditions due to the requirement of a large number of redox-sensitive enzymes, coenzymes, and cofactors for methanogenesis [4]. Thus, methanogens require an intracellular electron transfer system(s) to maintain a reduced intracellular environment. Although the enzymes and factors involved in energy-conserving electron transfer reactions during methanogenesis have been fairly well-characterized [2, 4, 5], the enzymes and factors involved in intracellular electron transfer for redox maintenance, biosynthesis, and protection from oxidative stress are less understood.

Thiol-disulfide oxidoreductases play a central role in the intracellular redox maintenance of cells. In particular, the vast majority of cells rely on thioredoxin (Trx), a small (~12 kDa) thiol-disulfide oxidoreductase, to maintain a reduced intracellular environment [6]. The canonical thioredoxin system is comprised of a thioredoxin reductase (TrxR), which uses reducing equivalents from NADPH, generated from metabolism, to reduce the active site cysteines within a CXXC motif of Trx. Trx primarily catalyzes the reduction of disulfides, but can also provide reductant for other enzymes. As such, Trxs are typically capable of reducing disulfides in a diverse number of proteins and are involved in physiological processes, in addition to general redox maintenance and protection during oxidative stress [7]. For example, Trx provides reducing equivalents to biosynthetic enzymes, such as ribonucleotide reductase [7]. There are a number of more complex and specialized Trx-related systems. In particular, many cells also contain

glutaredoxin (Grx), in addition to Trx. Grx is structurally and functionally similar to Trx, but receives reducing equivalents from glutathione that is reduced by a NADPH-dependent glutathione reductase [8]. However, the glutaredoxin system is primarily found in aerobes and is typically not present in strict anaerobes [8]. Methanogens do not contain glutathione, indicating the lack of a functional glutaredoxin system [9-11]. Recent evidence showed that Trx homologs are present in almost every sequenced methanogen genome, indicating Trx is likely the primary thiol-disulfide oxidoreductase involved in redox maintenance in methanogens [12, 13]. Indeed, Trx was shown to target a large number of proteins, including those involved in methanogenesis, in the methanogen *Methanocaldococcus jannaschii* [13]. Yet, how Trx is assimilated into the metabolism of methanogens, in particular the enzyme(s) and coenzyme(s) involved in providing reducing equivalents to Trx, is less understood. Importantly, NADPH is not directly generated by methanogenesis. Instead, reduced coenzyme $F_{420}H_2$, a 5'- deazaflavin derivative, and reduced ferredoxin are produced during methanogenesis [2, 4]. Methanogens may therefore directly use $F_{420}H_2$ and/or reduced ferredoxin to provide reducing equivalents to Trx or alternatively generate NADPH from the oxidation of $F_{420}H_2$ and ferredoxin.

Previous work by our group has demonstrated that the methanogen, *Methanosarcina acetivorans*, contains seven Trx homologs (MaTrx1-7) and a single TrxR (MaTrxR). Recombinant MaTrx2, MaTrx6, and MaTrx7 have catalytic disulfide reductase activity, and recombinant MaTrxR is specific for NADPH as an electron donor [12]. Of the three characterized MaTrxs, only MaTrx7 was reduced by MaTrxR, indicating *M. acetivorans* possesses a canonical Trx system comprised of NADPH, MaTrxR, and at least MaTrx7. Here, we report the characterization of all seven MaTrxs, including reduction by MaTrxR, analyses of electron donors, and alternative activities. Results support that the NADPH-dependent thioredoxin system, comprised of MaTrxR-

MaTrx7, is likely the general intracellular reducing system in *M. acetivorans*. Bioinformatic analyses indicate that the majority of methanogens contain a NADPH-dependent TrxR, suggesting that the canonical Trx system is used by the majority of extant methanogens, with the exception of some Methanococci, including *M. jannaschii*. The remaining MaTrxs likely have specialized functions, including two (MaTrx3 and MaTrx6) that are associated with the membrane of *M. acetivorans*.

Materials and Methods

Cloning of *M. acetivorans* Trx homolog genes: The genes encoding MA_RS05020 (MaTrx1), MA_RS19290 (MaTrx3), MaTrx3ΔSp (deleted of signal peptide amino acids 1-34), MA_RS20550 (MaTrx4), and MA_RS20570 (MaTrx5) were cloned into the *E. coli* expression vector pET28a as previously described for MaTrx2, MaTrx6, MaTrx6Δsp, and MaTrx7 [12]. Plasmids containing *matrx1*, *matrx3*, *matrx3ΔSp, matrx4*, and *matrx5* were verified by DNA sequencing and named pDL342, pDL343, pDL344, pDL345, and pDL346, respectively.

Purification of recombinant proteins: Proteins were expressed in *E. coli* Rosetta DE3 (pLacI) transformed with pDL342, pDL343, pDL344, pDL345, or pDL346. Each *E. coli* expression strain was grown in Luria broth medium and protein expression induced with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} of 0.5-0.7. The induced cultures were incubated at 25 °C for 16 hrs. The cells were harvested, and recombinant protein purified as described previously [12]. Purified recombinant protein was stored in buffer A (50 mM TRIS, 150 mM NaCl pH 7.2) at -80 °C.

Generation of oxidized and reduced MaTrxs: MaTrxs were incubated anaerobically at 25 °C for 20 min in buffer A containing either a 10:1 molar excess of H_2O_2 or DTT, to generate oxidized MaTrx ($MaTrx_{ox}$) and reduced MaTrx ($MaTrx_{red}$), respectively. After incubation, residual H_2O_2 or DTT was removed by buffer exchange with a NAP-5 column (GE Healthcare). The number of thiols were quantified in each MaTrx sample using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The standard assay contained 175 µM DTNB in buffer A and MaTrx (5-60 µM). After 15 min of anaerobic incubation, the absorbance at 412 nm was used to calculate the thiol concentration using ε_{412}= 14,150 m^{-1} cm^{-1} [14]. $MaTrx_{ox}$ samples were analyzed by non-reducing 15 % SDS-PAGE for the presence of oligomers due to the formation of intermolecular disulfides.

Enzyme activity assays with *M. acetivorans* cell-free lysate: *M. acetivorans* was grown in high-salt (HS) medium supplemented with 125 mM methanol and 0.025 % Na_2S (w/v) to an OD_{600} of 0.75 [15]. Unless stated otherwise, all subsequent manipulations were done inside an anaerobic chamber (COY laboratories). Cells were harvested by centrifugation for 10 min at 16,000 x *g* and 10 °C. The cell pellet was resuspended in buffer A supplemented with protease inhibitors (1 mM benzamidine and 1 mM PMSF). Cell suspensions were stored at -80 °C in anaerobic vials. Cell suspensions were thawed on ice and sonicated to lyse cells. Cell lysate was clarified by centrifugation at 16,000 x *g* for 10 min at 10 °C and the soluble fraction stored at -80 °C in anaerobic vials.

$F_{420}H_2$:NADP oxidoreductase (Fno) activity in *M. acetivorans* cell lysate was determined by measuring the NADP-dependent oxidation of $F_{420}H_2$. F_{420}, provided by Dr. Lacy Daniels (Texas A&M University, Kingsville, TX, USA), was chemically reduced to $F_{420}H_2$ using sodium borohydride as previously described [16]. Fno activity assays were performed in buffer A

containing *M. acetivorans* lysate (100 µg total protein) and 70 µM $F_{420}H_2$. After equilibration, reactions were initiated by the addition of 1 mM NADP or an equivalent volume of buffer A as a control. The oxidation of $F_{420}H_2$ was monitored at 420 nm for 10 min. The rate of $F_{420}H_2$ oxidation was determined using ε_{420} = 40,000 m^{-1} cm^{-1} [17].

To test for the presence of a functional ferredoxin:NADP reductase (Fnr), CO-dependent reduction of NADP by *M. acetivorans* was assayed. Lysate was prepared from acetate-grown cells as described above. To exclude the possibility of F_{420} mediating electron transfer between ferredoxin and NADP [18], low molecular weight compounds were removed from the lysate by two consecutive 6-fold concentration and dilution steps using buffer A with a 10 kDa MW Nanosep spin column (Pall Corporation). Cell lysate was pre-incubated with either CO or N_2 by transferring lysate to a 2 mL serum vial and flushing the headspace with CO or N_2 for 2 mins, followed by incubation on ice for 30 min. Assays were performed in a sealed quartz cuvette containing 400 µL of buffer A with a headspace of either N_2 or CO. Lysate (100 µg) was added to the cuvettes and reactions were initiated by the addition of 500 µM NADP to each sealed cuvette. The amount of NADPH produced over time was determined using ε_{340} = 6,220 m^{-1} cm^{-1}.

$MaTrx_{ox}$-dependent oxidation of NADH, NADPH, and $F_{420}H_2$ by *M. acetivorans* cell lysate was measured spectrophotometrically by monitoring the change in absorbance at 340 nm for NAD(P)H or 420 nm for $F_{420}H_2$. Assays were performed under anaerobic conditions in cuvettes containing 300 µg cell lysate, 70-100 µM $MaTrx1\text{-}7_{ox}$, and either 100 µM NAD(P)H or 70 µM $F_{420}H_2$ in buffer A (total volume of 100 µL). Assay mixtures were incubated in the absence of $MaTrx_{ox}$ until a stable baseline was obtained, then reactions were initiated by the addition of $MaTrx_{ox}$. The amount of NAD(P)H or $F_{420}H_2$ consumed over time was determined using ε_{340} = 6,220 m^{-1} cm^{-1} and ε_{420} = 40,000 m^{-1} cm^{-1}, respectively.

MaTrx activity assays. Disulfide reductase activity of each MaTrx was measured with DTT as an electron donor and insulin as the substrate as previously described [12]. Specific activity is reported as $\Delta A_{650} \cdot \min^{-2} \times 10^{-3}$ after subtraction of the background rate of insulin reduction by DTT alone.

Disulfide isomerase and thiol oxidase activity of MaTrxs was determined using bovine pancreatic RNaseA (Amresco) as a substrate. Reduced RNaseA (rRNasA) was specifically used as the substrate to measure thiol oxidase activity, and was generated similar to as previously described [19], except that 20 mg of RNaseA was brought into an anaerobic chamber and dissolved in 2 mL of 6 M guanidine HCl, 2 mM EDTA, 50 mM TRIS-HCl (pH 9.0), and 0.2 M DTT. After 2 hrs of incubation, the rRNaseA was buffer-exchanged into buffer A using a PD-10 column (GE Healthcare) and stored in sealed vials at -80 °C. Scrambled RNaseA (scRNaseA) was used as the substrate to measure disulfide isomerase activity and was generated as described previously [20]. The *in vitro* refolding of rRNaseA and scRNaseA to generate active RNaseA was performed as described [19]. Briefly, refolding buffer contained 5 µM rRNaseA or scRNaseA, 1 mM glutathione (GSH), and 1.11 mM oxidized glutathione (GSSG) yielding a redox potential of -150 mV based on the Nernst equation. *Escherichia coli* DsbA was used as a positive control and was provided as a gift from Dr. James Bardwell (University of Michigan, Ann Arbor, Michigan). RNase refolding assays contained 20 µM DsbA or MaTrx1-7. All refolding assays were performed in triplicate for 3 min at 15 °C and RNaseA activity after incubation with DsbA or MaTrx1-7 was compared to activity recovered in refolding buffer alone to account for non-enzymatic disulfide formation or rearrangement due to GSH/GSSG. RNaseA activity was determined by monitoring the amount of RNA degraded over time using the methylene blue assay as previously described [21]. The RNA substrate was prepared by dissolving 100 mg of Torula yeast RNA (Sigma) in 10 mL of anaerobic

0.1 M MOPS-HCL (pH 7.5), 2 mM EDTA (buffer B). Preparation of methylene blue binding buffer was done by adding 1 mg of methylene blue to 100 mL of anaerobic buffer B and the absorbance at 688 nm adjusted to 0.5 ± 0.02 with buffer B. A standard curve of methylene blue bound to RNA was generated using a range of 0-1000 µg of RNA, and the absorbance at 688 nm was determined. The excel kinetics modeling add-in was used to obtain the maximum absorbance change (V_{max}) and the concentration of RNA needed to obtain ½ V_{max} (K_m). The Michealis-Menten equation was used to calculate the concentration of RNA (S) at a given absorbance (V).

Generation of *M. acetivorans* strains expressing FLAG-tagged MaTrx3 or MaTrx6 for localization analysis. PCR was used to amplify *matrx3*, *maTrx3ΔSp*, *matrx6*, and *maTrx6ΔSp*. Each forward primer contained a 5' *Nde*I site and each reverse primer encoded a C-terminal FLAG tag followed by a *Hind*III site. The PCR product was digested with *Nde*I and *Hind*III and ligated with similarly digested pJK027A [22]. The resulting plasmids containing *matrx3-FLAG*, *maTrx3ΔSp-FLAG*, *matrx6-FLAG*, and *maTrx6ΔSp-FLAG* were named pDL350, pDL353, pDL348, and pDL349 respectively. *M. acetivorans* strain WWM73 was transformed with pDL350, pDL353, pDL348, and pDL349 as described previously [23]. Successful integration of the plasmid into the chromosome of each strain was determined as described [22], and the resulting strains were named DJL80 (MaTrx6-FLAG), DJL81 (MaTrx6ΔSp-FLAG), DJL82 (MaTrx3-FLAG), and DJL83 (MaTrx6ΔSp-FLAG). These strains allow for the tetracycline-inducible chromosomal expression of each MaTrx-FLAG.

Immunodetection of FLAG-tagged MaTrx3 and MaTrx6 in membrane and soluble fractions of *M. acetivorans* strains. Cultures of strains DJL80-83 were grown in HS medium (100 ml)

supplemented with 125 mM methanol and 0.025 % Na_2S (w/v). Tetracycline (100 µg/ml) was added where indicated to induce expression of the MaTrx-FLAG in each strain. The cells were harvested by centrifugation when the OD_{600} reached 0.5-0.75. Pelleted cells were resuspended in a volume of buffer A containing 1 mM benzamidine and 1 mM PMSF, and normalized based on OD_{600}. Cell suspensions were stored in microfuge tubes at -80 °C.

Methanogen cell membrane and soluble (cytoplasmic) fractions were separated in a manner similar to that described previously [24]. Frozen cells of strains DJL80-83 were lysed by five freeze/thaw cycles. After lysis, 10 units of RQ1 DNase 1 (Promega) was added to each tube and incubated at 37 °C for 25 min, followed by centrifugation at 10,500 x *g* for 5 min at 10 °C. The supernatant was removed and centrifuged a second time. The supernatant (soluble fraction) was removed and membranes were pelleted by centrifugation at 70,000 x *g* for 1 hr at 10 °C in 1.5 mL safe-lock Eppendorf tubes. The pellet (membrane fraction) was washed in 500 µL of 25 mM TRIS (pH 7.5). Both the membrane fraction and the soluble fraction were separately centrifuged a second time at 70,000 x *g* for 1 hr at 10 °C to further remove contaminating proteins from each fraction. The supernatant was removed from the centrifuged soluble fractions and was used as the final soluble fraction for each strain. The pellet from each centrifuged membrane fraction was resuspended in a small volume of 25 mM TRIS, 8 M urea (pH 7.5), then diluted to 150 µL with 25 mM TRIS (pH 7.5) and was used as the final membrane fraction for each strain. Total protein was quantified for all fractions using the Bradford assay with BSA as a standard. For detection of each MaTrx-FLAG by Western blot, identical amounts of protein for the membrane fractions and soluble fractions of strains DJL80/DJL81 and DJL82/DJL83 were analyzed by 15% SDS-PAGE. Protein was transferred to a PVDF membrane and Western blotting was performed using standard protocols using an α-FLAG tag primary antibody (Rockland Immunochemicals), and a HRP-

conjugated secondary antibody (Promega). An enhanced chemiluminescent substrate (Thermo Scientific) was used for detection.

Determination of the cytochrome *c* content of membrane fractions. *M. acetivorans* strains DJL80-83 were grown with methanol to late log phase, cells harvested, and membrane fractions generated as described above. The production of the previously detected 25 kDa cyt *c* in *M. acetivorans* [25] was quantified by SDS-PAGE analysis followed by densitometry of bands in heme-stained gels similar to described [26]. Loaded samples (26 µg) were normalized based on total protein. SDS-PAGE analysis was carried out on 15% polyacrylamide gels run at 90 V at 10 °C. Gels were stained for covalently bound heme using *o*-diansidine as previously described [27]. Due to observed differences is staining efficiencies between gels, the cytochrome *c* content of membrane fractions from each strain grown under non-inducing and inducing conditions was compared in a single gel. Each gel was loaded with duplicate samples of membrane fractions generated from two independent cultures for each condition (total samples = 4). Gels were imaged using a UMAX Powerlook 2100XL tri-linear CCD scanner at 400 dpi. Intensity of the band corresponding to the 25 kDa cyt *c* was determined using ImageJ. Using the method described by Gassmann *et al* [28], only the central 30% of the band was used to calculate intensity. For comparison, the intensity of cyt *c* determined in membrane fractions of each non-induced culture was set to 100 arbitrary units.

Results

Analysis of MaTrx disulfide reductase activity and reduction by MaTrxR. Recombinant MaTrx1, MaTrx3Δsp, MaTrx4, and MaTrx5 were each purified to homogeneity as described for recombinant MaTrx2, MaTrx6Δsp, and MaTrx7 [12]. Each MaTrx was assayed for disulfide

reductase activity with insulin as a substrate. Unlike MaTrx2, MaTrx6Δsp, and MaTrx7, none of the additional MaTrxs exhibited significant insulin disulfide reductase activity (**Fig. 1**). Although MaTrx3Δsp exhibited low activity, it is likely not physiologically relevant when compared to the activities of MaTrx2, MaTrx6Δsp, and MaTrx7.

Using insulin disulfide reduction assays, MaTrxR was previously shown to reduce MaTrx7, but not MaTrx2 or MaTrx6Δsp [12]. The lack of insulin disulfide reduction by MaTrx1, MaTrx3Δsp, MaTrx4, and MaTrx5 occludes using the insulin reduction assay. Thus, MaTrxs with active site disulfides were generated to test as substrates for MaTrxR. H_2O_2 was used to generate MaTrxs with oxidized active site cysteines ($MaTrx_{ox}$). With the exception of MaTrx1, the number of thiols per monomer for each $MaTrx_{ox}$ was close to zero (**Table 1**), consistent with complete thiol oxidation. MaTrx1 contains four cysteines, unlike the other MaTrxs, and the data indicate that at least two of these cysteines cannot be oxidized by H_2O_2. This is possibly due to these cysteines being inaccessible (i.e. buried in the protein) to H_2O_2. Importantly, non-reducing SDS-PAGE of each $MaTrx_{ox}$ showed each protein was monomeric (**Fig. 2**), consistent with the presence of intramolecular active site disulfides after oxidation with H_2O_2. If intermolecular disulfides were formed during oxidation with H_2O_2 then higher molecular weight species (e.g. dimers) would have been observed by non-reducing SDS-PAGE. Moreover, similar levels of disulfide reductase activity were observed with $MaTrx2_{ox}$, $MaTrx6\Delta sp_{ox}$, and $MaTrx7_{ox}$ compared to non-oxidized samples (data not shown), indicating that the proteins were not damaged beyond active site oxidation by incubation with H_2O_2. Incubation of each MaTrx with DTT resulted in an increase in the number of thiols detected (**Table 1**), revealing each protein contains cysteines capable of thiol-disulfide exchange. Each $MaTrx_{ox}$ was then tested for reduction by MaTrxR. Only the addition of $MaTrx7_{ox}$ to MaTrxR resulted in significant NADPH oxidation (**Table 1**). These results suggest

that MaTrx3Δsp, MaTrx4, and MaTrx5 are capable of thiol-disulfide exchange, but are likely not disulfide reductases. Moreover, MaTrxR is specific for MaTrx7 and is incapable of reducing any of the additional MaTrxs.

Examination of the ability of NADH, NADPH, and $F_{420}H_2$ to supply electrons for the reduction of MaTrxs. Of the seven MaTrxs, only $MaTrx7_{ox}$ was reduced by MaTrxR, indicating that if the other MaTrxs function as specific disulfide reductases, they must have a different redox partner(s) and/or electron donor(s). Thus, to test for the presence of enzymes that mediate the oxidation of the electron carriers NADH, NADPH, or $F_{420}H_2$ and reduction of the disulfide in $MaTrxs_{ox}$, oxidation assays with cell lysates were performed. Only the addition of $MaTrx7_{ox}$ to *M. acetivorans* cell lysate resulted in the statistically significant oxidation of both NADPH (331 ± 18 pmol min^{-1} mg^{-1}) and $F_{420}H_2$ (138 ± 12 pmol min^{-1} mg^{-1}). All assays with other oxidized MaTrxs did not result in activity that was statistically significant above the background. The addition of $MaTrx7_{red}$ to cell lysate also did not result in the significant oxidation of either NADPH or $F_{420}H_2$, confirming oxidation of both electron donors was due to the reduction of the disulfide in $MaTrx7_{ox}$. These data indicate that reduction of the other MaTrxs is not linked to the oxidation of NADH, NADPH, or $F_{420}H_2$. Since both NADPH and $F_{420}H_2$ were oxidized by the addition of $MaTrx7_{ox}$ to cell lysate, it is possible that $MaTrx7_{ox}$ is reduced by an unknown enzyme that directly oxidizes $F_{420}H_2$. More likely, the oxidation of $F_{420}H_2$ to generate NADPH needed by MaTrxR, is mediated by the Fno homolog (MA_RS22115) encoded in the genome of *M. acetivorans*. The addition of recombinant MaTrxR to cell lysate resulted in a five-fold increase in the rate of $MaTrx7_{ox}$-dependent $F_{420}H_2$ oxidation (787 ± 60 pmol min^{-1} mg^{-1}), consistent with the transfer of reducing equivalents from $F_{420}H_2$ to $MaTrx7_{ox}$ involving MaTrxR and therefore NADPH. Overall, these

results confirm the *in vivo* reduction of $MaTrx7_{ox}$ is dependent on MaTrxR, and link the production of NADPH to the oxidation of $F_{420}H_2$, through the activity of Fno, presumably the product of the MA_RS22115 gene.

***M. acetivorans* can generate NADPH for MaTrxR from the activities of Fno and Fnr.** The results from the incubation of $MaTrx7_{ox}$ with cell lysates indicate that NADPH is produced by the oxidation of $F_{420}H_2$, consistent with the activity of Fno. To confirm *M. acetivorans* contains a functional Fno, cell lysates were examined for NADP-dependent $F_{420}H_2$ oxidation. Lysate from *M. acetivorans* cells exhibited NADP-dependent $F_{420}H_2$ oxidation at a rate of 6.8 ± 1 nmol min^{-1} mg^{-1}, revealing the presence of a functional Fno. During growth of *M. acetivorans* with acetate and CO, ferredoxin is the primary electron carrier [1, 29]. Thus, *M. acetivorans* would likely need to generate NADPH for MaTrxR from the oxidation of ferredoxin. MA_RS19715-19720 in the genome of *M. acetivorans* encodes a homolog of a two subunit Fnr, similar to NfnAB from Clostridia [30]. CO-dependent reduction of NADP by cell lysates, as described in the methods, was used to examine for the presence of Fnr activity in *M. acetivorans*. Carbon monoxide dehydrogenase (CODH) from *Methanosarcina* oxidizes CO to CO_2 with the concomitant reduction of ferredoxin [31, 32]. An approximately three-fold higher rate of NADP reduction was observed when *M. acetivorans* cell lysates were provided CO compared to N_2 as a control (**Fig. 3**). These results reveal that *M. acetivorans* can generate NADPH by the oxidation of $F_{420}H_2$ or reduced ferredoxin.

The majority of sequenced methanogens encode NADPH-dependent TrxR, Fno, and Fnr. To assess the prevalence of a NADPH-dependent thioredoxin system in methanogens, we analyzed

sequenced methanogen genomes currently available in the NCBI database for the presence of TrxR, Fno, and Fnr. Using MaTrxR as a BLAST search query, a TrxR homolog with >30 % identity and >70% coverage was found in 64 of the 75 analyzed methanogen genomes (83%). **Table S1** shows the prevalence of TrxR among methanogens at the genus level. We next assessed the electron donor preference of the methanogen TrxR homologs by aligning the active site region of the 64 sequences (**Fig. S1**) and examining them for the presence of the NADPH-binding motifs GXGXXA [33] and VXXXHRRRDXXRA, an arginine-rich sequence found in *E. coli* TrxR [34]. Characterized archaeal, bacterial, and eukaryotic TrxRs that have the consensus GXGXXA motif can accept reducing equivalents from NADPH [35-39]. However, the TrxR from the archaeon *Thermoplasma acidophilum* lacks the consensus GXGXXA motif and cannot accept reducing equivalents from NADPH even though it can reduce *T. acidophilum* Trx [34]. The physiological electron donor to *T. acidophilum* TrxR is unknown. Thus, conservation of the GXGXXA motif appears critical to the ability of TrxR to use NADPH as an electron donor. The GXGXXA motif is present in 55 of the 64 methanogen TrxR homologs (86%) (**Fig. S1**), indicating the majority of methanogens contain a NADPH-dependent TrxR. However, the GXGXXA motif is not conserved in TrxR homologs from certain Methanococci. Thus, NADPH is likely not the electron donor to TrxR in a small subset of the methanogens.

Using *M. acetivorans* MA_RS22115 (Fno), a BLAST search of methanogen genomes revealed that all TrxR-containing methanogens encode a homolog of Fno (**Table S1**). Likewise, using *M. acetivorans* MA_RS19715 (Fnr), a BLAST search of methanogen genomes revealed that all TrxR-containing methanogens, with the exception of species of the genera *Methanothermus* and *Methermicoccous*, encode a homolog of Fnr (**Table S1**). These results

suggest all methanogens have the ability to direct reducing equivalents generated from methanogenesis ($F_{420}H_2$ and reduced ferredoxin) to a NADPH-dependent thioredoxin system.

MaTrx1 and MaTrx3 have thiol oxidase activity similar to *E. coli* DsbA. A number of Trx-like proteins are involved in activities other than disulfide reduction, including protein disulfide isomerase and disulfide-forming (thiol oxidase) activities [40]. For example, DsbA is a Trx-like protein found in the periplasm of *E. coli* that is capable of both rearranging (disulfide isomerization) and forming disulfides by the oxidation of thiols in proteins [41]. Thus, since four of the seven MaTrxs lack insulin disulfide reductase activity, but appear capable of thiol-disulfide exchange, the MaTrxs were tested for disulfide isomerase and thiol oxidase activities using RNase as a substrate. RNase requires disulfides in the correct configuration for activity; thus, RNase with incorrect disulfides (scrambled) and RNase with thiols (reduced) can be used as substrates to measure the disulfide isomerase and thiol oxidase activities, respectively [19]. In comparison to *E. coli* DsbA, which was included as a positive control, none of the MaTrxs exhibited statistically significant disulfide isomerase activity with scrambled RNase above the control level determined with buffered GSH/GSSG alone (**Fig. 4A**). However, both MaTrx1 and MaTrx3Δsp showed statistically significant thiol oxidase activity with reduced RNase, with the activity of MaTrx3Δsp comparable to that of DsbA (**Fig. 4b**). These results reveal that MaTrx1 and MaTrx3 are thiol-disulfide oxidoreductases, but likely serve as thiol oxidases to form disulfides rather than reduce disulfides, similar to DsbA. The enzymatic activities of MaTrx4 and MaTrx5 are unknown.

MaTrx3 and MaTrx6 contain a signal peptide that localizes each protein to the membrane of *M. acetivorans*. Both MaTrx3 and MaTrx6 contain an N-terminal signal peptide predicted to

target each protein across the membrane of *M. acetivorans* [12]. Consistent with this prediction, expression of recombinant MaTrx3 and MaTrx6 in *E. coli* results in accumulation of each protein primarily in the insoluble fraction, whereas expression of recombinant MaTrx3Δsp and MaTrx6Δsp are found in the soluble fraction of *E. coli* lysates (data not shown). To examine the importance of the signal peptide to the localization of MaTrx3 and MaTrx6 directly in *M. acetivorans*, strains were generated capable of expressing MaTrx3, MATrx3Δsp, MaTrx6, or MaTrx6Δsp with a C-terminal FLAG tag (MaTrx-FLAG) to allow immunodetection by Western Blot. Strain DJL80 contains MaTrx6-FLAG, strain DJL81 contains MaTrx6Δsp-FLAG, strain DJL82 contains MaTrx3-FLAG, and strain DJL83 contains MaTrx3Δsp-FLAG. Growth of each strain under inducing conditions (+ tetracycline) did not alter growth rate or yield, but led to the immunodetection of a protein consistent with the predicted molecular weight of each MaTrx-FLAG that was absent in cells grown under non-inducing conditions (data not shown). These data indicate that each MaTrx-FLAG is expressed and does not alter the general growth of each strain. Therefore, lysate from induced cells of each strain was separated by centrifugation into soluble and membrane fractions, followed by Western blot analysis using anti-FLAG antibodies. MaTrx6Δsp-FLAG was only detected in the soluble fraction of DJL81 cells, whereas MaTrx6-FLAG was detected in both the membrane and soluble fractions of DJL80 cells (**Fig. 5A**). Similarly, MaTrx3Δsp-FLAG was only detected in the soluble fraction of DJL83 cells, whereas MaTrx3-FLAG was detected in both the membrane and soluble fractions of DJL82 cells (**Fig. 5B**). These results demonstrate that the N-terminal signal peptide of MaTrx6 and MaTrx3 directs each protein to the membrane of *M. acetivorans*. This is the first evidence of membrane-localized Trx homologs in a methanogen.

Expression of FLAG-tagged MaTrx3 and MaTrx6 alters the level of cytochrome *c* in *M. acetivorans*. Among methanogens, homologs of MaTrx3 and MaTrx6 are restricted to the Methanomicrobia [12], the only methanogens that contain cytochromes, including cytochrome *c* [2]. In other organisms, extracellular and membrane-associated Trx-like proteins serve key roles in the maturation of cytochrome *c*, which has heme covalently attached to thiols of cysteines typically within a CXXCH motif [26, 42]. Thus, to provide initial insight into the potential for MaTrx3 and MaTrx6 to play a similar role in *M. acetivorans*, the effect of the increased expression of FLAG-tagged MaTrx3 and MaTrx6 on the level of cytochrome *c* was examined in *M. acetivorans* strains DJL80-83. The genome of *M. acetivorans* encodes three predicted cytochrome *c* proteins, two of which have been experimentally detected [25]. A 25 kDa cytochrome *c* is produced during growth with methanol or acetate and a 55 kDa cytochrome *c* is produced only during growth with acetate [25]. The effect of the expression of FLAG-tagged MaTrx3 and MaTrx6 on the level of the 25 kDa cytochrome *c* in methanol-grown cells was determined by densitometry of bands in heme-stained SDS-PAGE gels. Gels were loaded with the membrane fraction of cells grown under conditions that do not induce (- tetracycline) or induce (+ tetracycline) expression of each FLAG-tagged MaTrx. A similar level of cytochrome *c* was detected in the membrane fractions from induced and non-induced cells of strains DJL81 and DJL83 (**Table 2**), which express MaTrx6Δsp-FLAG or MaTrx3Δsp-FLAG in the cytoplasm, respectively. In contrast, the membrane fraction of induced DJL80 cells that express MaTrx6-FLAG, shown to localize to the membrane (**Fig. 5**), contain approximately 50% less cytochrome *c* than non-induced cells of DJL80. Furthermore, the membrane fraction of induced cells of DJL82 that express MaTrx3-FLAG, also shown to localize to the membrane (**Fig. 5**), contain approximately 50% more cytochrome *c* than non-induced DJL82 cells. Thus, the expression of

membrane-localized MaTrx3-FLAG and MaTrx6-FLAG alters the level of the heme-containing 25 kDa cytochrome *c* in *M. acetivorans*. However, altered cytochrome *c* content as a result of indirect effects, such as changes in membrane protein content due to the presence of MaTrx3-FLAG and MaTrx6-FLAG, cannot be ruled out. Though, it is important to note that expression of MaTrx6-FLAG had an opposite effect on the level of cytochrome *c* (decreased), compared to expression of MaTrx3-FLAG, which resulted in an increase in cytochrome *c*. This difference indicates that the catalytic activities of MaTrx6 (disulfide reductase) and MaTrx3 (thiol oxidase) may play a role in the altered cytochrome *c* levels.

Discussion

The majority of sequenced methanogens encode a TrxR homolog, and all methanogens, with the exception of *Methanopyrus kandleri*, contain at least one Trx homolog, underscoring the importance of the thioredoxin system to methanogen physiology [12, 13]. Among methanogens, *Methanosarcina* species encode the highest number of Trx homologs (six to eight), yet typically encode a single TrxR homolog [12]. Results from the biochemical characterization of MaTrxR and MaTrx1-7 in this study confirm that NADPH-dependent MaTrxR is specific for MaTrx7 [12] and reveal that the remaining MaTrxs have distinct properties, and thus likely different functions. The model shown in **Fig. 6** illustrates the proposed role(s) of the MaTrxs in *M. acetivorans*. MaTrx7 appears to be the primary intracellular reducing Trx in *M. acetivorans*. MaTrx7 is the only MaTrx reduced by MaTrxR, and experiments with cell lysates support the *in vivo* reduction of MaTrx7 by MaTrxR. Reducing equivalents can be provided during methanogenesis with all growth substrates used by *M. acetivorans* through Fno and/or Fnr activities (**Fig. 6**), supporting the assimilation of a NADPH-dependent thioredoxin system into the physiology of *M. acetivorans*. The proteins targeted for reduction by MaTrx7 in *M. acetivorans* are largely unknown. However,

MaTrx7 was shown to reduce the disulfides in the redox-sensitive transcription repressor MsvR, which activates DNA binding [43]. Moreover, recent results from a MaTrx7 pull-down experiment reveal that MaTrx7 is capable of reducing disulfides in several hundred *M. acetivorans* proteins (unpublished results), consistent with MaTrx7 serving as general disulfide reductase. Thus, the NADPH-dependent MaTrxR-MaTrx7 system likely serves as a general reducing system in *M. acetivorans*.

Results from comparative bioinformatic analyses also indicate that the TrxR homolog in the majority of methanogens, with the exception of a subset of Methanococci, is likely specific for NADPH. Thus, a NADPH-dependent thioredoxin system is likely used by the majority of methanogens. One potential benefit to using NADPH to directly reduce TrxR, instead of the primary methanogenesis electron carriers F_{420} or ferredoxin, is to provide specificity and minimize competition for reducing equivalents needed for energy conservation. This separation of electron donors is similar to that used by the vast majority of cells, which use NADH for catabolism and NADPH for anabolism. However, some species from deeper methanogen lineages (e.g. *M. jannaschii*) may use $F_{420}H_2$ or ferredoxin directly to reduce Trx, as these methanogens lack the conserved NADPH binding site in the encoded TrxR homolog. Indeed, during review of this manuscript it was demonstrated that $F_{420}H_2$ serves as the electron donor to *M. jannaschii* TrxR, which in turn reduces functional Trx1 [44].

Recent evidence has also revealed that methanogens contain additional Trx-related proteins that function in intracellular redox physiology. In *M. acetivorans*, ferredoxin:disulfide reductase (Fdr), a protein homologous to ferredoxin:thioredoxin reductase (Ftr) from plants, can reduce protein disulfides with reducing equivalents provided directly from ferredoxin [45]. In the same study, Fdr was shown to be incapable of reducing MaTrx2, the only intracellular MaTrx other than

MaTrx7 with disulfide reductase activity. More recently, Fdr from *Methanosarcina barkeri* was shown to be specific for NrdH, a Trx-like protein, that reduces the active-site disulfide in the unusual anaerobic ribonucleotide reductase NrdD found in some methanogens [46]. The Fdr-NrdH system is not ubiquitous in methanogens, and appears to be an intracellular reducing system that is specific for a subtype of anaerobic ribonucleotide reductase restricted to methanogens from the orders Methanomicrobiales and Methanosarcinales [46]. In addition, the genome of some methanogens encode Grx-like proteins, even though methanogens lack glutathione. A Grx-like protein from *M. acetivorans* was named methanoredoxin (Mrx) based on the ability to use coenzyme M, as well as glutathione, as a direct source of reductant [47]. Coenzyme M is a low molecular weight thiol found in all methanogens where it is directly involved in methanogenesis [2]. However, Mrx homologs are only found in roughly 50% of methanogen species with sequenced genomes [47], indicating disulfide reduction by Mrx may also serve a more specialized, rather than general function. The disulfide-containing targets of Mrx have not been identified. It is common for organisms to have more than one intracellular reducing system. For example, *E. coli* contains both Trx and Grx [8]. This appears true of at least some methanogens as well, in particular members of Methanosarcinales. Nonetheless, despite the unique physiology of methanogens, one that relies heavily on ferredoxin and coenzyme F_{420} as electron carriers, rather than NAD(P), the accumulated results indicate that the majority of methanogens likely use a canonical NADPH-dependent thioredoxin system.

In addition to a canonical thioredoxin system comprised of MaTrxR-MaTrx7, *M. acetivorans* contains four additional intracellular Trx homologs. MaTrx1 is unique among the MaTrxs as it contains two additional cysteines [12] and an active site motif (CPYC) similar to Grx [8]. Also, unlike the other MaTrxs, not all of the cysteines in MaTrx1 were capable of thiol-

disulfide exchange. MaTrx1 also lacked disulfide reductase activity, instead having low, but detectable, thiol oxidase activity. These results indicate that MaTrx1 is possibly an intracellular disulfide-forming enzyme (**Fig. 6**), but the importance of such an activity to methanogens is unclear. MaTrx2 has disulfide reductase activity but is not reduced by MaTrxR, and experiments with cell lysates did not link the reduction of disulfide-containing MaTrx2 to the oxidation of NADH, NADPH, or $F_{420}H_2$. Thus, the redox partner(s) to MaTrx2 is unknown (**Fig. 6**). MaTrx2 homologs appear restricted to *Methanosarcina* [12] and may have a specialized function in these methanogens. MaTrx4 and MaTrx5 are similar to one another and have the same CAKC active site motif [12]. Although the cysteines of both proteins could be oxidized and reduced, consistent with thiol-disulfide exchange activity, neither protein exhibited disulfide reductase, disulfide isomerase, or thiol oxidase activities. Thus, the function and role(s) of MaTrx4 and MaTrx5 are also unclear and may be unrelated to known Trx functions.

Results from the expression of FLAG-tagged MaTrx3 and MaTrx6 demonstrate that the N-terminal signal sequence targets both proteins to the membrane of *M. acetivorans*, providing the first experimental evidence that methanogens possess membrane-associated Trx proteins. The signal peptide is also likely retained, as evidenced by the presence of full-length product detected by Western analysis in membrane fractions (**Fig. 5**). Thus, the N-terminal sequence of both MaTrx3 and MaTrx6 likely serves to anchor each protein to the membrane, most likely on the extracellular surface. Although, MaTrx3 and MaTrx6 are homologous proteins and are within the same methanogen Trx clade [12], they are clearly not functionally equivalent. Experimental results support each protein is capable of thiol-disulfide exchange; however, MaTrx6 is a disulfide reductase, whereas MaTrx3 is a thiol oxidase. The cellular location and activities indicate MaTrx3 and MaTrx6 function in the formation and reduction of disulfides in membrane and/or extracellular

proteins, respectively. *M. acetivorans* contains several membrane-associated and extracellular proteins with cysteines that are required for growth. For example, there are multiple CXXCH motifs in the 55 kDa cytochrome *c* encoded by MA_RS03460 that is involved in electron transfer by the Rnf complex that is required for growth of *M. acetivorans* with acetate [48]. In bacteria and eukaryotes extracellular thiol oxidases and disulfide reductases are documented to oxidize and reduce the CXXCH cysteines in apo-cytochrome *c* to stabilize and prepare the protein for heme insertion [26, 42, 49]. The involvement of these enzymes in the maturation of cytochrome *c* in methanogens has not been demonstrated. However, the altered levels of cytochrome *c* in the membranes of *M. acetivorans* as a result of the expression of FLAG-tagged MaTrx3 and MaTrx6 provides indirect evidence that MaTrx3 and MaTrx6 may play a role in the steps leading to heme insertion in apo-cytochrome *c* in this methanogen.

For MaTrx3 and MaTrx6 to function at the membrane of *M. acetivorans*, a membrane-associated redox partner(s) would be required to serve as an electron donor to MaTrx6 and an electron acceptor to MaTrx3. One probable redox partner to MaTrx6 is MA_RS22215, which encodes a homolog of CcdA. MaTrx6 is encoded upstream, and possibly co-transcribed, with MA_RS22215. This gene arrangement is conserved in all Methanosarcinales [12]. In bacteria, CcdA functions to transfer reducing equivalents from intracellular Trx across the membrane to support the catalytic disulfide reductase activity of an extracellular Trx homolog. For example, in *Bacillus subtilis* CcdA provides reductant to membrane-anchored ResA, a Trx homolog that is responsible for reducing the CXXCH disulfide in apo-cytochrome *c* [50]. Thus, it seems reasonable to propose that the CcdA homolog in *M. acetivorans* serves a similar role, supplying reductant to MaTrx6 from MaTrx7, the primary intracellular Trx (**Fig. 6**). For redox partners to MaTrx3, there are no obvious candidates encoded in the genome. A few systems have been

characterized that use a thiol oxidase to catalyze the specific formation of disulfides in extracellular and/or periplasmic proteins. For example, DsbA is a Trx homolog that oxidizes thiols to disulfides in proteins, including the CXXCH motif of apo-cytochrome *c*, to increase protein stability in the periplasm of *E. coli* [26]. DsbA is re-oxidized by the cytoplasmic membrane protein DsbB, which then transfers electrons to the membrane-bound quinone pool [51]. The genome of *M. acetivorans* lacks genes for homologs of proteins known to serve a redox partners to extracellular thiol oxidases, such as DsbB. Determining the importance of MaTrx3 and MaTrx6 to the physiology of *M. acetivorans*, including the identification of redox partners and target proteins, will require additional experimentation and is currently underway.

Conclusions

The results presented here reveal that *M. acetivorans* contains seven Trx homologs with different functional properties and cellular locations. NADPH-dependent MaTrxR is specific for MaTrx7, and MaTrxR-MaTrx7 likely comprise the general intracellular reducing system in *M. acetivorans*. Reducing equivalents are provided to the MaTrxR-MaTrx7 system through the oxidation of the primary methanogen electron carriers, $F_{420}H_2$ and ferredoxin. Bioinformatic analyses indicate that the majority of methanogens also likely use a NADPH-dependent thioredoxin system. MaTrx3 and MaTrx6 are localized to the membrane of *M. acetivorans*, and function to generate or reduce membrane and/or extracellular proteins, respectively. The physiological function(s) of MaTrx1, MaTrx2, MaTrx4, and MaTrx5 are unclear, and will require additional experimentation to elucidate what roles these Trx homologs serve in the physiology of *M. acetivorans*.

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Figures and Tables

Figure 1.

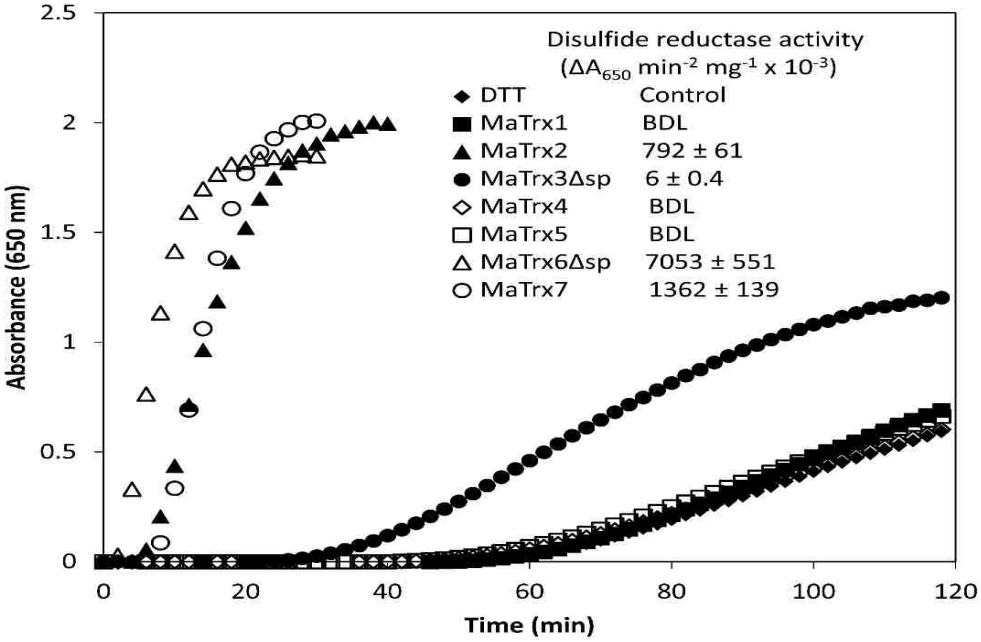


Fig. 1. Insulin disulfide reductase activity of MaTrxs. Assays were performed in anaerobic buffer containing DTT (1 mM) alone or with: MaTrx1 (20 µM), MaTrx2 (10 µM), MaTrx3 (20 µM), MaTrx4 (20 µM), MaTrx5 (20 µM), MaTrx6 (5 µM), MaTrx7 (5 µM).

Table 1. MaTrx thiol-disulfide exchange ability and specificity of MaTrxR.

MaTrx	Total cysteines	$MaTrx_{ox}$ Thiols[a]	$MaTrx_{red}$ Thiols[b]	NADPH oxidation[c] (MaTrxR + $MaTrx_{ox}$)
MaTrx1	4	3.20 ± 0.35	4.58 ± 0.3	BDL
MaTrx2	2	0.05 ± 0.02	1.66 ± 0.77	ND
MaTrx3Δsp	2	0.05 ± 0.03	2.04 ± 0.31	BDL
MaTrx4	2	0.06 ± 0.14	1.04 ± 0.07	20 ± 2
MaTrx5	2	0.11 ± 0.03	1.83 ± 0.17	BDL
MaTrx6Δsp	2	0.21 ± 0.05	1.14 ± 0.31	ND
MaTrx7	2	0.13 ± 0.04	1.4 ± 0.08	829 ± 134

[a]Thiols per MaTrx monomer after incubation with H_2O_2 as determined by the DTNB assay.
[b]Thiols per MaTrx monomer after incubation with DTT as determined by the DTNB assay.
[c]nmol NADPH oxidized min^{-1} mg^{-1} oxidized MaTrx.
BDL: below detection limit.
ND: not determined

Figure 2

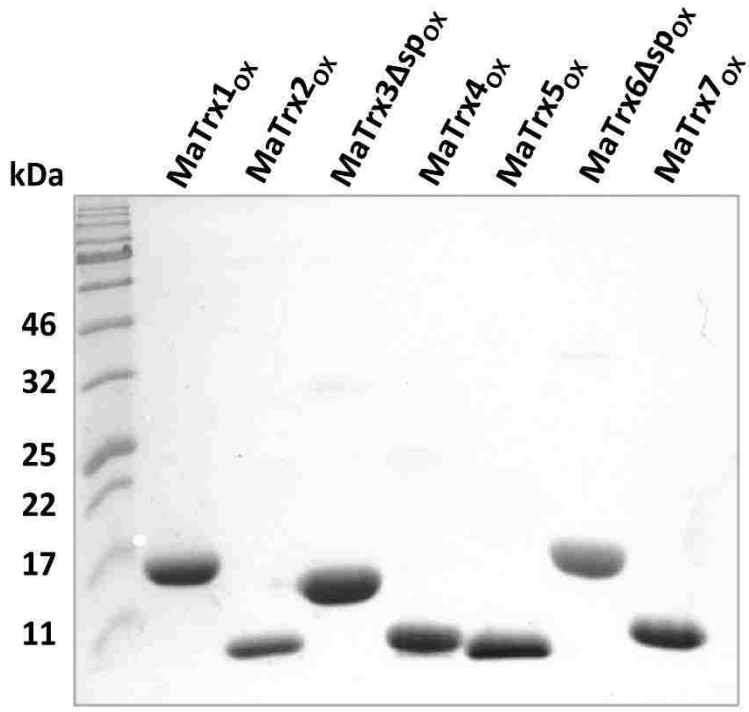


Fig. 2. Non-reducing SDS-PAGE of H_2O_2-oxidized MaTrxs. Each $MaTrx_{ox}$ (10 μg) was separated by 15% SDS-PAGE in the absence of a reducing agent.

Figure 3

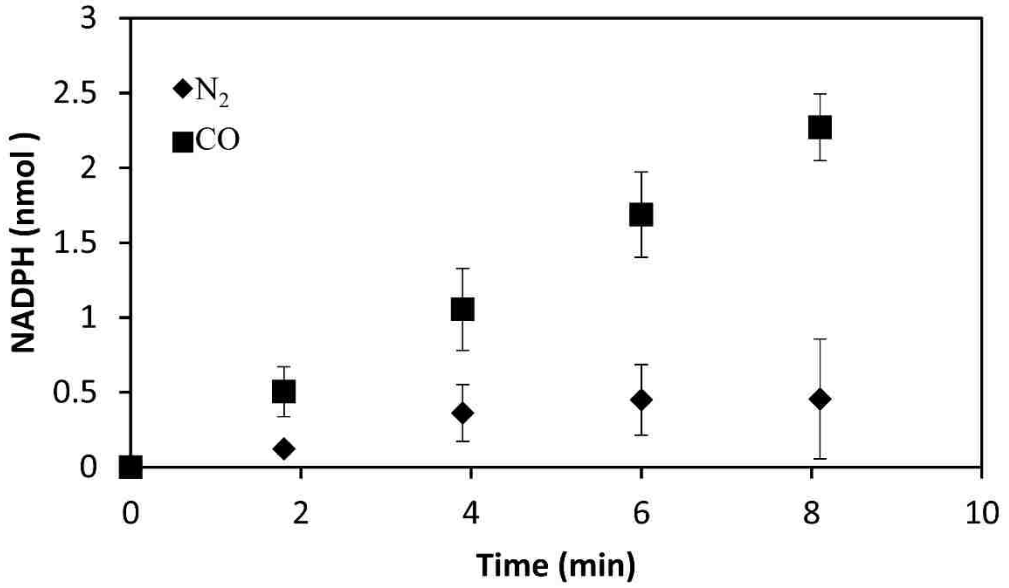


Fig. 3. CO-dependent reduction of NADP by *M. acetivorans* cell lysate. Assays were performed in sealed anaerobic cuvettes containing lysate (100 µg), NADP (0.5 mM), and a headspace of either N_2 or CO. Data points are the mean (± SD) of triplicate assays.

Figure 4

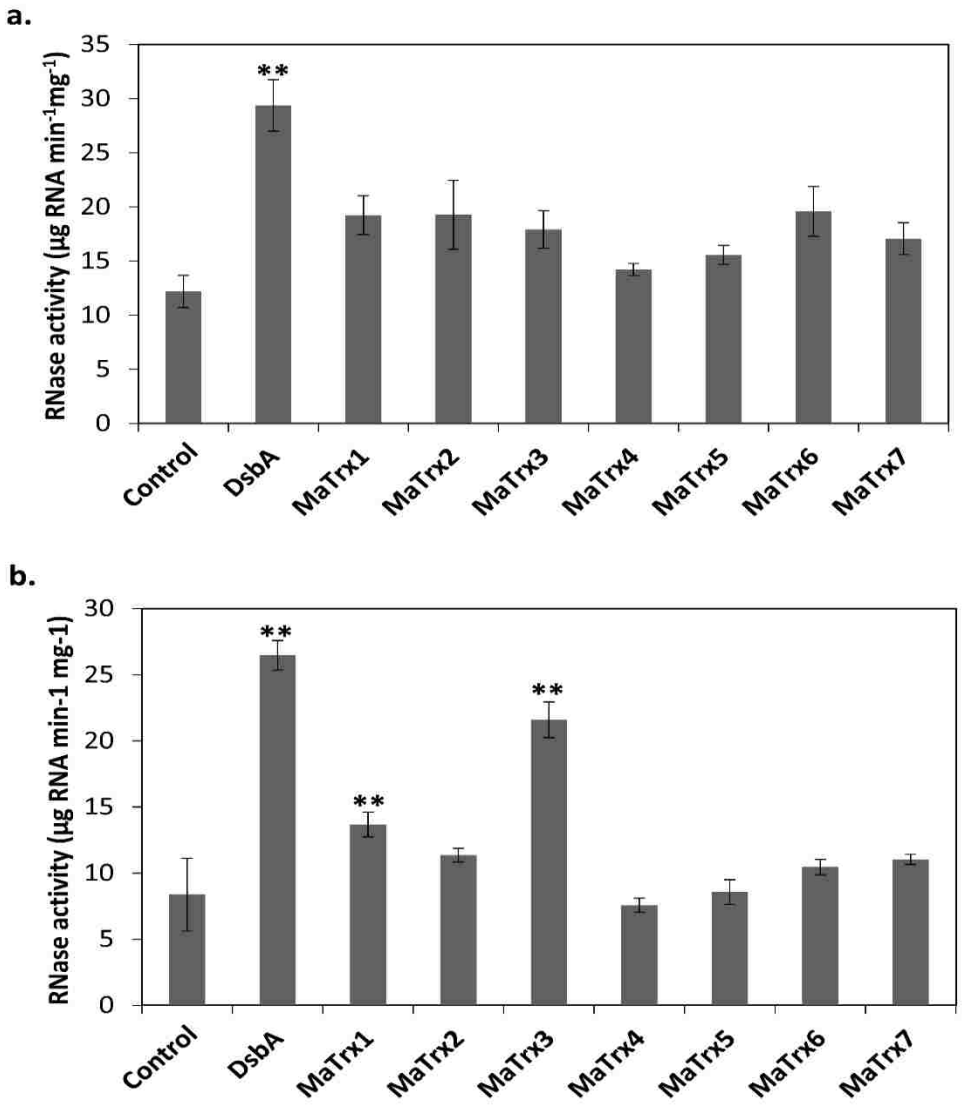


Fig. 4. Analysis of disulfide isomerase and disulfide-forming activities of MaTrxs using scrambled and reduced RNase. (A) RNase activity of disulfide-scrambled RNase after incubation in GSH/GSSG redox buffer alone (control), with DsbA, or with the indicated MaTrx. (B) RNase activity of reduced RNase after incubation in GSH/GSSG redox buffer alone (control), with DsbA, or with the indicated MaTrx. Data points are the mean (± SD) of triplicate assays. **P ≤ 0.002 (t-test).

Figure 5

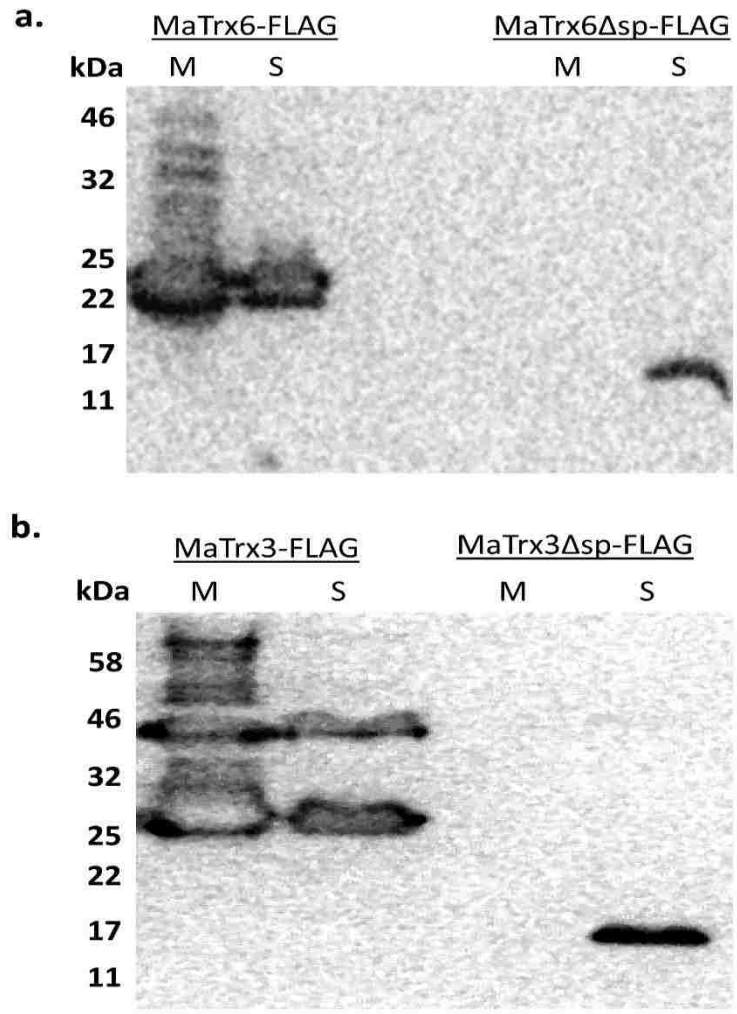


Fig. 5. Western blot analysis of fractionated cell lysates from *M. acetivorans* strains expressing FLAG-tagged MaTrx3 and MaTrx6. (A) Western blot analysis using anti-FLAG antibodies of membrane (M) and soluble (S) fractions of DJL80 cells containing MaTrx6-FLAG (18.8 kDa) and DJL81 cells containing MaTrx6Δsp-FLAG (15.8 kDa). Total protein in samples: M-7.5 µg, S-50 µg. (B) Western blot analysis using anti-FLAG antibodies of membrane (M) and soluble (S) fractions of DJL82 cells containing MaTrx3-FLAG (20.3 kDa) and DJL83 cells containing MaTrx3Δsp-FLAG (16.7 kDa). Total protein in samples: M-5 µg, S-35µg.

Table 2. The effect of the expression of FLAG-tagged MaTrx3 and MaTrx6 on the levels of the 25 kDa cytochrome *c* in membrane fractions of methanol grown *M. acetivorans*.

Strain	Tetracycline	Level of cyt *c*[a]
DJL80 (MaTrx6-FLAG)	-	100 ± 10
	+	57 ± 16*
DJL81 (MaTrx6Δsp-FLAG)	-	100 ± 13
	+	123 ± 37
DJL82 (MaTrx3-FLAG)	-	100 ± 25
	+	170 ± 18*
DJL83 (MaTrx3Δsp-FLAG)	-	100 ± 30
	+	138 ± 13

[a]The levels of cyt *c* were determined by densitometry of heme-stained SDS-PAGE gels. Gels were loaded with replicate samples of normalized membranes purified from two independent cultures. The amount of cyt *c* produced in non-induced cells (- tetracycline) is set to 100 arbitrary units.

*Significantly different from the level of cyt *c* in non-induced cells (-tetracycline), $P \leq 0.05$ (t-test).

Figure 6

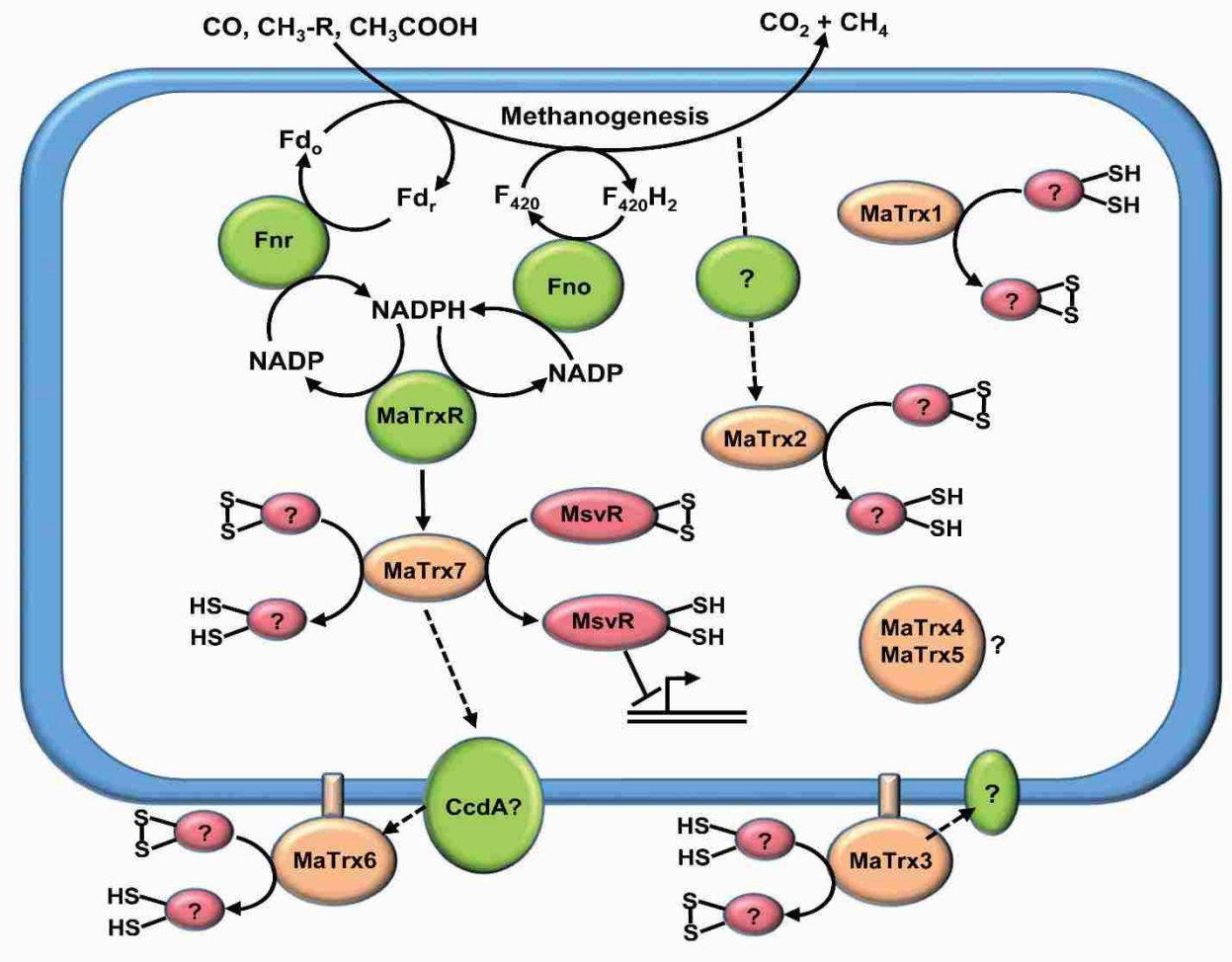


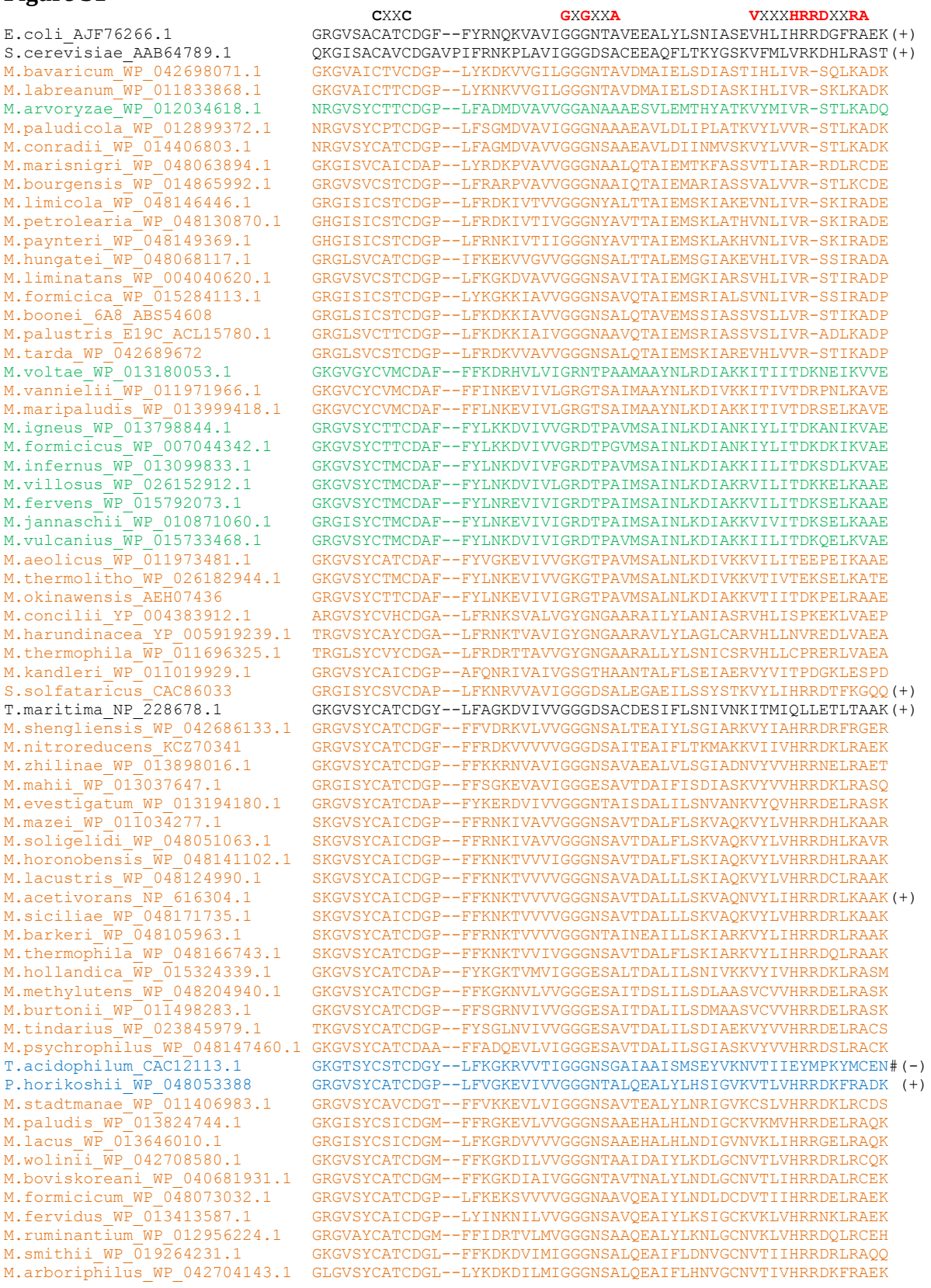
Fig. 6. Model showing the location and proposed function(s) of MaTrxs in *M. acetivorans*. Solid lines indicate detected activities and dashed lines indicate putative activities. Question marks denote unknown or proposed protein(s) or factor(s). MaTrx7 was previously shown to reduce disulfides in oxidized MsvR, activating binding of DNA. Fd_o: oxidized ferredoxin, Fd_r: reduced ferredoxin, Fnr: ferredoxin:NADP oxidoreductase, Fno: $F_{420}H_2$:NADP oxidoreductase.

Table S1. Prevalence of selected intracellular reducing components encoded in methanogen genomes.

Genus	TrxR	Fno	Fnr
Methanopyrus	+	+	+
Methanococcus	+	+	+
Methanothermococcus	+	+	+
Methanocaldococcus	+	+	+
Methanotorris	+	+	+
Methanobacterium	+	+	+
Methanobrevibacter	+	+	+
Methanosphaera	+	+	+
Methanothermobacter	+	+	+
Methanothermus	+	+	-
Methanolinea	+	+	+
Methanomicrobium	-	+	+
Methanoculleus	+	+	+
Methanofollis	+	+	+
Methanolacinia	+	+	+
Methanoplanus	+	+	+
Methanocorpusculum	+	+	+
Methanospirillum	+	+	+
Methanosarcina	+	+	+
Methanococcoides	+	+	+
Methanohalobium	+	+	+
Methanohalophilus	+	+	+
Methanolobus	+	+	+
Methanosalsum	+	+	+
Methanomethylovorans	+	+	+
Methanosaeta	+	+	+
Methermicoccus	+	+	-
Methanocella	+	+	+
Methanomassiliicoccales	+	+	+

A plus sign (+) indicates one or more species within a genus encode a homolog (>30 % identity and >70% coverage to *M. acetivorans* TrxR, Fno, and Fnr).
A dash (-) indicates the absence of an encoded homolog from all species within a genus.

Figure S1


CXXC
GXGXXA
VXXXHRRDXXRA
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S.cerevisiae_AAB64789.1 QKGISACAVCDGAVPIFRNKPLAVIGGGDSACEEAQFLTKYGSKVFMLVRKDHLRAST(+)
M.bavaricum_WP_042698071.1 GKGVAICTVCDGP--LYKDKVVGILGGGNTAVDMAIELSDIASTIHLIVR-SQLKADK
M.labreanum_WP_011833868.1 GKGVAICTTCDGP--LYKNKVVGILGGGNTAVDMAIELSDIASKIHLIVR-SKLKADK
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M.paludicola_WP_012899372.1 NRGVSYCPTCDGP--LFSGMDVAVIGGGNAAAEAVLDLIPLATKVYLVVR-STLKADK
M.conradii_WP_014406803.1 NRGVSYCATCDGP--LFAGMDVAVVGGGNSAAEAVLDIINMVSKVYLVVR-STLKADK
M.marisnigri_WP_048063894.1 GKGISVCAICDAP--LYRDKPVAVVGGGNAALQTAIEMTKFASSVTLIAR-RDLRCDE
M.bourgensis_WP_014865992.1 GRGVSVCSTCDGP--LFRARPVAVVGGGNAAIQTAIEMARIASSVALVVR-STLKCDE
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M.lacus_WP_013646010.1 GRGISYCSICDGM--LFKGRDVVVVGGGNSAAEHALHLNDIGVNVKLIHRRGELRAQK
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M.boviskoreani_WP_040681931.1 GRGVSYCATCDGM--FFKGKDIAIVGGGNTAVTNALYLNDLGCNVTLIHRRDALRCEK
M.formicicum_WP_048073032.1 GRGVSYCATCDGP--LFKEKSVVVVGGGNAAVQEAIYLNDLDCDVTIIHRRDELRAEK
M.fervidus_WP_013413587.1 GRGVSYCAICDGP--LYINKNILVVGGGNSAVQEAIYLKSIGCKVKLVHRRNKLRAEK
M.ruminantium_WP_012956224.1 GRGVAYCATCDGM--FFIDRTVLMVGGGNSAAQEALYLKNLGCNVKLVHRRDQLRCEH
M.smithii_WP_019264231.1 GKGVSYCATCDGL--FFKDKDVIMIGGGNSALQEAIFLDNVGCNVTIIHRRDRLRAQQ
M.arboriphilus_WP_042704143.1 GLGVSYCATCDGL--LYKDKDILMIGGGNSALQEAIFLHNVGCNVTIVHRRDKFRAEK

Figure S1 cont.

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                                    CXXC                GXGXXA                VXXXHRRDXXRA
M.marburgensis_WP_013295913.1  GRGVCYCATCDGP--LYRGRKVLMVGGGNSAAQEAVFLKNIGCDVSIVHRRDELRADR
M.thermautotro_WP_048060886.1  GRGVCYCATCDGP--LYKGRKVLMVGGGNSAAQEAVFLKNIGCDVSIVHRRDELRADK
M.intestinalis_WP_020449568.1  GKGVSYCASCDAG--FYKNKIVGVVGDGSEAGESAVLLSKYASNVYWISSGR---SVS
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Fig. S1. Amino acid alignment of the active site region of methanogen TrxR homologs with characterized TrxR from bacteria, eukaryotes, and other archaea. BLASTP searches for putative TrxR in methanogens were performed using MaTrxR as a query against available sequenced methanogen genomes (NCBI). The highest scoring protein (>30 % identity and >70 % coverage) for each species was included and aligned with selected non-methanogen TrxR sequences using Clustal Omega. Only the region encompassing the active site cysteine motif (CXXC) and NADPH binding site motifs are shown. Non-archaeal sequences are colored black. Non-methanogen archaeal sequences are colored blue. Methanogen sequences that contain the GXGXXA motif are colored orange. Methanogen sequences lacking the GXGXXA motif are colored green. Non-methanogen sequences lacking the GXGXXA motif are indicated by a # sign. Based on previous studies (references 34-39), sequences that encode TrxR capable or incapable of using NADPH are indicated by (+) and (-) signs, respectively.

Appendix 2.1: Lead Author Confirmation Letter for Chapter II

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter II, titled "*Methanosarcina Acetivorans* Utilizes a Single NADPH-dependent Thioredoxin System and Contains Additional Thioredoxin Homologues with Distinct Functions." of A. C. McCarver's dissertation was submitted for publication in Microbiology-Sgm in 2016 with coauthors F. H. Lessner, J. M. Soroeta, and D. J. Lessner.

I, Dr. Daniel J. Lessner, advisor of Addison C. McCarver, confirm Addison C. McCarver was first author and completed at least 51% of the work for this manuscript.

Dr. Daniel J. Lessner
Associate Professor
Department of Biological Sciences
University of Arkansas

Date

Chapter III

The NADPH-dependent Thioredoxin System in *Methanosarcina acetivorans* is a Global Thiol Repair System for Oxidatively Damaged Proteins

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Abstract

The cytoplasm of cells from all organisms, including aerobes, is highly reduced. Thus, all cells require an intracellular system to maintain redox homeostasis. The vast majority of extant cells from all three domains of life use the thioredoxin system. Thioredoxin (Trx) is a small protein with a Cys-XX-Cys active site that reduces cysteine disulfides of target proteins. Trx can influence the structure/activity of proteins, serve as an electron source for enzymes, and repair oxidatively damaged proteins. The canonical Trx is reduced by thioredoxin reductase that is NADPH-dependent. However, strictly anaerobic methane producing archaea (methanogens) do not directly generate NAD(P)H during methanogenesis, instead the deazaflavin F_{420} and ferredoxin are directly reduced. The thioredoxin system of model methanogen *Methanosarcina acetivorans* has been determined to be a NADPH-dependent system comprised of MaTrxR and MaTrx7 that obtain NADPH from the oxidation of the primary reducing equivalents F_{420} and ferredoxin to reduce $NADP^+$ [1, 2]. Moreover, our work has shown that many methanogens likely employ the use of a NADPH-dependent thioredoxin system rather than the F_{420} dependent thioredoxin system found in methanogens belonging to the class Methanococci [2, 3]. However, it still remains unclear the role(s) of a NADPH-dependent thioredoxin system in the physiology of methanogens. The work shown here addresses elucidating candidate target proteins to MaTrx7 through the use of Trx affinity chromatography, where aerobically exposed *M. acetivorans* cell lysate was supplied to a single cysteine residue mutant form of MaTrx7 bound to resin. The proteins that bound to the MaTrx7 resin were sequenced using mass spectrometry, and in total over 700 proteins were identified, including many proteins involved in methanogenesis. *M. acetivorans* cell lysate was capable of reducing $NADP^+$ after exposure to oxygen without supplication of additional exogenous electron donor, supporting that *M. acetivorans* possesses

electron donor pools that are oxygen insensitive. Cumulatively, the work supports that the MaTrxR-MaTrx7 system can serve as a general redox maintenance system after oxygen exposure, and thus would allow for the repair of methanogenesis enzymes needed to resume growth.

Introduction

Intracellular thiol redox maintenance systems are critical to all forms of life, as they are responsible for catalytic reduction of many enzymatic pathways such as biosynthesis and for reductive repair of unwanted disulfide bonds. One type of thiol redox maintenance system, the thioredoxin system, is ubiquitous to aerobes and anaerobes alike. The thioredoxin system is comprised of a thioredoxin reductase (TrxR) that oxidizes NADPH to reduce one or more Trxs. Reduced Trx functions to reduce a multitude of proteins that can be dependent upon Trx or proteins that have had damaging disulfide bonds formed as a result of oxidation. While NADH is also generated in many cells utilizing the NADPH-dependent thioredoxin system, these cells reserve NADH for catabolic processes and energy conservation, thus allowing the cell to determine the state of catabolism/energy conservation while regulating anabolism/redox state. The redox state of the cell is kept low during normal growth by keeping excess reductants available and proteins reduced, thus the levels of intracellular NADPH exceed that of the oxidized form $NADP^+$, allowing the thioredoxin system to readily access NADPH needed for the reduction of target proteins [4]. During events of oxidative stress, the levels of intracellular $NADP^+$ increase and signal to the cell to the cell to increase the levels of key enzymes for NADPH production [5]. Many of these NADPH generating enzymes are targets for the thioredoxin system, indicating that Trx plays a direct regulatory role in the levels of NADPH in the cell and ultimately the intracellular redox state [6].

Although a multitude of eukaryotic and prokaryotic thioredoxin systems have been studied extensively, there remains a gap in how the thioredoxin system functions in the strictly anaerobic, methane producing archaea (methanogens). Indeed, strict anaerobes live in a more reduced environment than aerobes, but the need for redox maintenance systems are equally

important as many of these organisms contain a greater proportion of redox sensitive enzymes, coenzymes, and cofactors that are in lower abundance in aerobes due to the increase of encounters with oxygen and reactive oxygen species. Thiol redox systems like the thioredoxin system may be of increased demand in methanogens because they are estimated to utilize more FeS clusters, which are coordinated by reduced cysteine residues, than other prokaryotes [7]. Recent evidence indicates that the thioredoxin system components are ubiquitous to methanogens with homologs of TrxR and multiple Trxs encoded in the genomes of nearly all methanogens, but the source of electrons for methanogen thioredoxin systems remained unclear [1, 8]. Methanogens do not utilize NADPH as the primary electron donor during central carbon metabolism (methanogenesis), but rather coenzyme F_{420} and the small FeS containing protein ferredoxin. Recent work has shown the thioredoxin system from the deeply rooted hyperthermophillic methanogen *Methanocaldococcus jannaschii* and other Methanococci species contain F_{420}-dependent TrxRs [3]. MjTrxR reduces MjTrx1 that is then capable of reducing a multitude of proteins involved in methanogenesis, replication, and the oxidative stress response, suggesting that the *M. jannaschii* thioredoxin system is necessary for redox homeostasis [8]. The F_{420}-dependent thioredoxin system in *M. jannaschii* is directly integrated into the physiology by utilizing one of the primary electron carriers generated during methanogenesis, thus allowing the *M. jannaschii* thioredoxin system to function and respond based on the state of methanogenesis. The reduction of F_{420} is primarily produced during methanogenesis by F_{420}-reducing hydrogenases, but also to a lesser extent by H_2-dependent methylenetetrahydromethanopterin dehydrogenase (Hmd) and $F_{420}H_2$-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) by means of an alternative pathway using methanogenesis intermediates [9]. The utilization of F_{420} dependent thioredoxin systems in

Methanococci allows for redox regulation that functions similarly to NADPH/$NADP^+$ where the cell can determine the redox state based on the amount of reduced F_{420}. Moreover, utilizing F_{420} instead of reduced Fd is likely due to Fd being used for energy conservation similar to NADH in *E. coli*. However, in the event of oxidative stress the rate of methanogenesis is dramatically reduced or even halted [10], thus the *M. jannaschii* thioredoxin system is limited to thiol redox maintenance when oxidative stress is minimal to none due to the lack of sufficient F_{420} production.

Methanosarcina species, however, utilize a F_{420} dependent dehydrogenase that helps to generate an ion gradient for energy conservation, and thus a F_{420} dependent thioredoxin system would funnel electrons away from energy conservation to be used for reductive biosynthesis and repair ultimately causing unregulated overlap between the two pathways. Some *Methanosarcina* species are also capable of utilizing a wider range of substrates from methylated compounds to acetate fermentation, which accounts for the majority of biologically produced methane. An increase in growth substrate utilization necessitates increased regulation over electron donor pools. For instance, reduced Fd is hypothesized to be the primary electron carrier during acetate growth while F_{420} serves as the primary carrier during methylotrophic growth, and thus a thiol redox maintenance system relying solely upon reduced F_{420} would be hindered during growth on acetate. Evidence from the recently evolved mesophilic methanogen *Methanosarcina acetivorans* supports that a NADPH dependent thioredoxin system exists in methanogens, and is likely the predominant system among all methanogens except Methanococci species [1, 2]. Methanogens may have evolved multiple redox systems like the NADPH dependent thioredoxin system, the Ferredoxin thioredoxin system, and the methanoredoxin system when the earth became oxygenated as a response to an increase in the number of oxidative stress events [11, 12].

Generating NADPH as a secondary electron carrier would effectively provide additional means of regulation in the cell between redox maintenance and energy conservation pathways, and indeed reduction of $NADP^+$ can be accomplished in nearly all methanogens by oxidizing the primary reducing equivalents generated during methanogenesis by utilizing of F_{420}:$NADP^+$ oxidoreductase (Fno) and Ferredoxin:$NADP^+$ reductase (Fnr) [2]. Oxidizing both Fd and F_{420} to produce NADPH effectively couples the NADPH dependent thioredoxin system to central carbon metabolism of methanogens. Additionally, most of the enzymes responsible for reducing Fd and F_{420} are oxygen sensitive, and utilizing a secondary electron donor would allow to oxidize a different source of electrons using enzymes that may be oxygen insensitive. Despite the apparent advantages of utilizing a secondary electron carrier for redox maintenance, the role(s) of the NADPH-dependent thioredoxin system in methanogens remains to be elucidated. The focus of the work presented here is to assess whether the *M. acetivorans* thioredoxin system could serve as a general thiol redox maintenance system in *M. acetivorans* and if the production of NADPH is limited to anaerobic conditions.

Materials and Methods

Cloning, overexpression, and purification of MaTrx7C15S: MA_RS07110 (MA4683) was cloned into pET28a as reported previously [1] and the resulting plasmid named pDL336. Site directed mutagenesis was performed on pDL336 using the Quickchange II kit (Agilent) to change the codon for the amino acid 15, the resolving cysteine residue [13], to encode for serine (MaTrx7C15S) using the primer sequences 5'-tcacgctacctggagcacggcatgtcc-3' and 5'-ggacatgccgtgctccaggtagcgtga-3' as the forward and reverse primers respectively. The resulting plasmid encoding MaTrx7C15S was sequence verified and named pDL341. pDL341 was transformed into Rossetta DE3 *Escherichia coli* cells for overexpression and purification as

previously reported [1]. MaTrx7C15S was purified to >95% homogeneity by SDS PAGE, and stored in 50 mM TRIS 150 mM NaCl pH 7.2 (buffer A) at -80 °C. Protein concentrations were determined using the Bradford assay with BSA as a standard.

Binding MaTrx7C15S to cyanogen bromide agarose: A total of 0.14 g dry weight of cyanogen bromide activated agarose (GE Healthcare) was prepared for binding per the manufacturer's protocol yielding 0.5 mL of rehydrated agarose. 1.25 mg of MaTrx7C15S diluted in 750 µL of 0.1 M $NaHCO_3$ 0.5 M NaCl at pH 8.3 (coupling buffer) was used to resuspend the previously prepared cyanogen bromide agarose and nutated for 2hrs in a 2 mL Eppendorf tube at RT. Once bound, the MaTrx7C15S coupled agarose was transferred to a chromatography column and residual MaTrx7C15S washed from the agarose using 5 column volumes of coupling buffer. To block any remaining uncoupled agarose the columns were washed with 2 column volumes of 0.1 M TRIS at pH 8. The columns were then prepared for storage per the manufacturer's protocol and stored overnight at 4° C in buffer A for use the following morning.

Growth and generation of *M. acetivorans* cell free lysate: *M. acetivorans* was grown in 300 mL of high-salt (HS) medium supplemented with 125 mM methanol and 0.025 % Na_2S (w/v) to an OD_{600} of 0.5 [14]. Cells were harvested by centrifugation for 10 min at 8,600 x *g* and 4 °C. The cell pellet was resuspended in 2 mL of anaerobic buffer A supplemented with protease inhibitors (1 mM benzamidine and 1 mM PMSF). Cell suspensions were stored at -80 °C in anaerobic vials. The cells were lysed using 5 cycles of freezing at -80° C and thawing to 30° C in a warm water bath, and then 10 units of DNase I were added to the vial using a Hamilton syringe and then incubated at 37 °C for 1 hr. *M. acetivorans* lysate was then clarified by centrifugation at 16,000 x g for 10 min at RT and the soluble fraction stored at -80 °C in anaerobic vials at a concentration of 8.4 mg $\cdot$ mL^{-1}.

Purification of *M. acetivorans* target proteins to MaTrx7C15S using reduced and alkylated MaTrx7C15S resin: 1mL of MaTrx7C15S resin was allocated into two 0.5 mL chromatography columns. Unless stated otherwise, all subsequent manipulations were done inside an anaerobic chamber (COY laboratories). Both columns were washed with 5 column volumes (CV) of anaerobic buffer A, and then reduced 4 CV of 100 mM dithiothreitol (DTT) in buffer A, allowing 2 CV to fully enter the agarose bed before incubation for 20 min at RT in the dark. After reduction, one MaTrx7C15S bound column was acetylated using iodoacetamide (IAA) to serve as the negative control. Alkylation was accomplished by washing the column with 5 column volumes of 20 mM TRIS 500 mM NaCl at pH 8 (buffer B) to increase the pH. Then the column was treated with 5 CV of 10 mM IAA in buffer B and incubated for 30 min at RT in the dark followed by washing with 5 column volumes of buffer A. Both columns remained in buffer A until incubation with *M. acetivorans* lysate.

M. acetivorans lysate was oxidized by exposure to atmospheric oxygen for 2hr at RT and mixed gently using a pipettor every 30 min to distribute air. 900 µL of oxidized *M. acetivorans* lysate was added to the reduced MaTrx7C15S column and the IAA MaTrx7C15S control column after allowing buffer A to completely flow through the columns leaving 400 µL of lysate atop of the resin. The lysate and resin was mixed and the slurry removed to a 2 mL Eppendorf tube, which was then nutated for 30 min at RT. After incubation of the MaTrx7C15S resin with lysate the slurry was added back to their respective columns and repacked onto the column by allowing the lysate to flow through. Columns were washed with 6 CV of 50 mM TRIS 1M NaCl pH 7.2, 3 mL of 50 mM TRIS 2M NaCl pH 7.2, and finally with buffer A. To elute thiol bound proteins, 2 CV of buffer A supplemented with 100 mM DTT was added to each column and 1 CV entered the resin bed before the flow was stopped for a 20 min incubation at RT in the dark. DTT eluted

proteins were collected from each column for Qubit protein determination and SDS PAGE analysis. A total of 350 µL of the remaining DTT eluate from the columns were lyophilized and resuspended in 40 µL of buffer A. 13 µL of each concentrated eluate was loaded onto a freshly prepared 4-20% gradient SDS PAGE gel, the gel fixed with 40 % methanol and 10 % acetic acid in water after running, and ultimately stained with the blue silver Coomassie staining protocol [15], and the bands excised as depicted in figure 1 for LC-MS/MS analysis. The sample numbers from column A in S1 that correspond to the numbers depicted in Fig. 1 were used to validate the size of the identified proteins based on the location in the gel using the molecular weight marker and the predicted molecular weight in S1. Excisions of bands from the IAA MaTrx7C15S gel lane were not shown in Fig. 1, but were documented and utilized to validate identified proteins in the same way. All identified proteins exhibiting a molecular weight outside of the range of their respective gel slice were removed from S1.

LC-MS/MS analysis, database search, and data parsing: In-gel digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis with an Orbitrap Velos mass spectrometer (Thermo, San Jose, CA) in the Proteomic Facility at the University of Arkansas for Medical Sciences (Little Rock, AR) was performed as described previously [16]. Mascot (Version 2.5.1; Matrix Science, Boston, MA) was used to search against the UniProt/SwissProt database for *M. acetivorans* (April, 2015; 4468 entries) using LC-MS/MS data. The parameters for database searching were: (i) 2.0 ppm mass error tolerance for MS and 0.5 Da for MS/MS, (ii) a maximum of 2 missed cleavages, (iii) fixed modification of carbamidomethylation of cysteine residues, and (iv) variable modifications of acetylation at peptide N terminus and oxidation of methionine residues. Search results were further processed by Scaffold software (version 4.4.5; Proteome Software, Portland, OR) for viewing protein and peptide identification information. In

the Scaffold analysis, protein identification probability with at least two peptides was set to 99%, and the peptide identification probability was set to 90%. Multiple identifications of a single protein were removed from S1 and the representative proteins with the highest unique peptides, unique spectrum, and total spectrum counts remained for further comparison against the identified proteins the IAA MaTrx7C15S gel lane in Fig. 1. Proteins identified in the IAA MaTrx7C15S gel lane were omitted from S1 if the total unique peptides, unique spectrum, and spectrum counts were higher than those identified in the MaTrx7C15S gel lane.

Measuring intrinsic $NADP^+$ reduction and CODH activity of *M. acetivorans* cell lysate:

Cells were grown in MeOH to an OD at 600 nm of 0.574 and harvested as previously mentioned. The cell lysate was generated anaerobically via sonication using 5 pulses at 5 seconds each with a 3 min rest on ice in between pulses. Soluble lysate was fractionated by centrifugation 16,000 x g for 10 min at RT. The resulting lysate was stored in anaerobic vials at -80 °C. At the time of the assay, the lysate was removed anaerobically from the vial and split into two 1.5 mL Eppendorf tubes and a protein determination was performed using the Qubit assay. One tube of lysate remained anaerobic at RT, while the other exposed to atmospheric oxygen for 1 hr at RT. Both aliquots of lysate were mixed by gentle pipetting after 30 min. $NADP^+$ reduction assays were performed using 300 µg of lysate with 1 mM $NADP^+$ in buffer A at a total of 100 µL. Reactions were initiated with the addition of $NADP^+$. The amount of NADPH produced over time was determined using ε_{340} = 6,220 m^{-1} cm^{-1} for each treatment in triplicate. CODH reduction assays were performed using methyl viologen (MV) as a surrogate electron acceptor for ferredoxin. Cell lysate was pre-incubated with CO by transferring lysate to a 2 mL serum vial and flushing the headspace with CO for 2 mins, followed by incubation on ice for 30 min. Assays were performed in a sealed quartz cuvette containing a total of 400 µL using buffer A as

a diluent with a headspace of CO. Lysate (100 µg) was added to the cuvettes and reactions were initiated by the addition of 8 mM MV and the reaction monitored at 603 nm over time. The amount of MV reduced over time was determined using ε_{603} = 11,300 m^{-1} cm^{-1} [17].

Results and Discussion

Intrinsic NADPH production of aerobically exposed *M. acetivorans* cell lysate: To date, the ability of *M. acetivorans* to produce NADPH after oxidation had not been assessed. While determining whether the molecular mechanisms in *M. acetivorans* possessed the ability to produce NADPH after exposure to oxygen, it was observed that $NADP^+$ could be reduced at a relatively large quantity albeit a slow rate (fig. 2a) without a supply of reduced F_{420} and Fd. Moreover, the activity was not drastically altered even after 1 hr exposure to atmospheric oxygen (fig. 2a), supporting the ability to reduce $NADP^+$ post oxidative stress. However, the reduction of methyl viologen, a surrogate for Fd, through the oxidation of carbon monoxide (carbon monoxide dehydrogenase activity) had completely ceased after exposure to oxygen (fig. 2b), indicating that reduced Fd generated during central metabolism is not the source of electrons for reducing $NADP^+$ post oxidation. During methanogenesis the enzymes methylene-H4MPT reductase (Mtr) and F_{420} -dependent methylene-H4MPT dehydrogenase are responsible for reducing F_{420} (fig. 3). Mtr in *M. acetivorans* may be capable of producing the reduced F_{420} needed by Fno to reduce $NADP^+$ since Mtr from *Methanobacterium thermoautotrophicum* is functional after exposure to oxygen [18]. However, the substrate methyl-CoM used by Mtr to reduce F_{420} would be in limited supply as the enzyme methanol-5-hydroxybenzimidazolylcobamide methyltransferase (Mta), which is responsible for the production of the substrate methyl CoM for Mtr, has been shown to be sensitive to oxygen in *Methanosarcina barkeri* [19]. Ultimately the amount of NADPH produced both anaerobically

and aerobically exposed lysates suggests that it is unlikely that methanogenesis intermediates alone provide the electrons needed since many of these enzymes are oxygen sensitive. Rather, it is likely that *M. acetivorans* has an alternative carbon source that can be oxidized in the event of oxidative stress to produce NADPH for the *M. acetivorans* thioredoxin system. Recent work investigated the concentrations of metabolites within the glycogen synthesis/degredation pathway of *M. acetivorans,* and indicated that many of these enzymes are not sensitive to oxygen [20]. In particular, the $NADP^+$ dependent enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was shown to be oxygen insensitive and the levels of glyceraldehyde 3-phosphate (G3P) during mid log phase growth (similar to the NADPH production study shown in fig. 2a) were high enough to supply the majority of the NADPH produced in this study (1.6 nmol mg^{-1} of protein of G3P) [20]. The remaining NADPH produced in this study could be attributed to dihydroxyacetone phosphate and fructose 1,6 bisphosphate that can be readily converted to G3P through the oxygen insensitive activity of triosephosphate isomerase and aldolase respectively (Fig 2). Cumulatively, the data suggests that *M. acetivorans* uses central carbon metabolism to provide NADPH to the *M. acetivorans* system during anaerobic growth and can utilize the carbon intermediates from glycogen synthesis/degradation to repair oxidative damage (fig. 4). Such a distinction between the sources of carbon for the production of reducing equivalents is not likely to be observed in Methanococci species as they appear to utilize F_{420} dependent thioredoxin systems that overlap with central carbon metabolism, and could explain why species like those belonging to the order Methanosarcinales are more aerotolerant than the more ancestral members of the class Methanococci.

Purification of MaTrx7C15S and binding to CNBr resin: MaTrx7C15S was purified to homogeneity at comparable levels to that of wild type MaTrx7 when each are expressed in *E.*

coli. Mutating the resolving cysteine residue (C15) to serine was verified by DNA sequencing of the plasmid, which was further supported by the result that MaTrx7C15S had approximately 45-fold less disulfide reductase activity base on a DTT dependent insulin assay (data not shown). MaTrx7C15S successfully bound to CNBr as MaTrx7C15S was undetectable in the flow through of the buffer solution post incubation with resin.

Determination of potential MaTrx7 repair/target proteins: In an effort to best simulate oxidative stress in the environment, *M. acetivorans* cell lysate was oxidized using atmospheric oxygen, rather than chemical oxidants like H_2O_2. Many of the enzymes and proteins in methanogens contain FeS clusters or other oxygen sensitive metals that when oxidized propagate the formation of peroxides and radicals per the Fenton reaction, and thus rapid disulfide bonds can be formed by exposing lysate to oxygen. Following the incubation of oxidized lysate with MaTrx7C15S resin, each column was washed with up to 2 M NaCl until no further proteins were eluted (verified visually on SDS-PAGE gel not shown), thereby removing the majority of ionically bound proteins. The effective removal of ionically bound proteins was corroborated when the sequence data indicated that only 46 of the 711 potential MaTrx7 targets did not contain any cysteine residues (table S1).

Alkylation of the attacking cysteine residue was performed in lieu of binding and generating the MaTrx7C12S/C15S double mutant, since heterologous over-expression of two different proteins could lead to variations between samples. Alkylation of reduced MaTrx7C15S resin effectively blocked the remaining cysteine residue from targeting *M. acetivorans* proteins as the DTT eluate from the negative column only yielded approximately 16.4 µg as opposed to the 129.6 µg of total protein observed from the reduced MaTrx7C15S resin. The resulting DTT eluate containing thiol mediated target proteins was analyzed and sequenced for both the

negative and experimental columns, which contained a total protein count of 238 and 736 respectively (table S1) after removing duplicates. Despite the total protein of the being approximately 8-fold less protein in the negative control eluate than the experimental eluate, the LC-MS/MS analysis was still able to detect one third of the proteins determined in the LC-MS/MS analysis for the experimental sample due to the instrument's high sensitivity. The most likely explanation for the detection of nearly a third of the total potential targets within the negative sample is that the alkylation of the reduced MaTrx7C15S resin did not alkylate 100 % of the resin, but rather a majority of the resin given the effective 8-fold reduction of total protein. The residual reduced MaTrx7C15S resin would be capable of reducing the same proteins as the non-alkylated MaTrx7C15S resin, so a threshold for the identification of MaTrx7 targets was set for the experimental sample (outlined in the methods section) where any protein with a signal weaker than the negative control was omitted leaving 711 potential target proteins for MaTrx7 (table S1).

While Trx affinity chromatography remains still to be one of the most effective methodologies for purifying Trx targets, the methodology does pose several limitations as discussed in length [21]. Particularly, non-specific disulfide bond reduction by the extremely reactive cysteinyl residue in Trx makes it difficult to elucidate *in vivo* targets dependent upon Trx as opposed to oxidatively damaged proteins undergoing reduction. However, the limitations of Trx affinity chromatography are advantageous in this study as the approach allows for high throughput analysis of all the possible proteins that may be repaired by the *M. acetivorans* thioredoxin system after an oxidative stress event. Secondly, any previously identified *in vivo* targets to Trx from other organisms that are observed in this study will likely be *in vivo* targets proteins in *M. acetivorans*, thus outlining possible specific *in vivo* thiol targets to MaTrx7 for

future studies. Together, the identification of potential repair and target proteins in *M. acetivorans* support the *M. acetivorans* thioredoxin system's role as a general oxidative stress repair and redox maintenance system.

Similar to identified targets of Trx in other species, many proteins are involved in methanogenesis and glycogen synthesis/degradation were identified [22], supporting that the MaTrx system can repair and possibly thiol redox regulate central carbon metabolism in the event of oxidative stress (Fig. 2). Furthermore, some of the proteins involved in methanogenesis and glycogen synthesis/degradation have been shown in other organisms to have an activity bolstered or regulated by Trx (Fig. 2). In addition to the role of Trx in central metabolism there were oxidative stress response and repair proteins, FeS cluster biogenesis, and replication proteins identified, further supporting the role of the *M. acetivorans* thioredoxin system in general thiol redox maintenance (table 1).

Methanogenesis and energy conservation: The majority of enzymes involved in methanogenesis are extremely oxidant sensitive due to the presence of FeS clusters thereby stopping the production of primary reducing equivalents (F_{420} and Fd) during oxidative stress events. The *M. acetivorans* thioredoxin system could function post oxidative stress to repair central carbon metabolism since it depends upon NADPH, which was shown to be effectively produced even after oxidative stress (fig. 4a). The role of *M. acetivorans* thioredoxin system in methanogenesis repair is supported by MaTrx7 reduction of every methanogenesis enzyme involved in methyl substrate and acetate substrate growth.

The *M. acetivorans* thioredoxin system also functions in the repair and potentially even regulates energy conservation enzymes such as Hdr, Fpo, and ATP synthase. In plants, the Trx

system can alter the activity of ATP synthase by reducing a pair of exposed cysteine residues [23]. Therefore, it is plausible that the *M. acetivorans* thioredoxin system plays a repair role of oxygen sensitive enzymes like Hdr and Fpo to regenerate the ion gradient needed for energy conservation while also a regulatory role of one or more of these enzymes during growth to modulate ATP levels inside the cell similar to the thioredoxin system in plants [24].

FeS cluster biogenesis: The FeS biogenesis system in *M. acetivorans* is comprised of a putative cysteine desulfurase (IscS2) and iron sulfur cluster scaffolding proteins (IscU1, 2, SufB1, 2, SufC1, 2). The sulfur group of cysteine is removed and retained by IscS2 thereby making alanine. The scaffolding proteins then obtain Fe and coordinate the assembly of the cluster. Assembled clusters then are relayed from the scaffolding proteins to carriers and target proteins. All of the required FeS cluster proteins are utilizing cysteine residues to facilitate reaction and relay, and the cysteines must be reduced to coordinate the necessary components. Therefore, it is conceivable that the *M. acetivorans* thioredoxin system could play a role in the reduction of cysteine residues for FeS cluster transfer and assembly, especially since several FeS cluster biogenesis proteins have been identified as targets of Trx in *Arabidopsis thaliana* [25]. FeS cluster biogenesis systems would be necessary post-oxidative stress, and the ability of the *M. acetivorans* thioredoxin system to obtain reducing equivalents for the reductive repair of FeS cluster containing proteins could be the critical step towards the assembly of FeS clusters and ultimately the activity of the effected enzymes.

Oxidative stress response: Several well-known oxidative stress response and repair proteins were identified as potential targets, of which three have an activity determined to be dependent upon Trx. Methanogen-specific transcriptional regulator MsvR from *M. acetivorans* for instance is theorized to be a part of the oxidative stress response by binding DNA when reduced by either

DTT or *M. acetivorans* thioredoxin system *in vitro* thereby preventing transcription of certain genes that remain to be elucidated [26]. The MsvR homolog from *Methanothermobacter thermautotrophicus* is responsible for the regulation of an oxidative stress operon, and it is likely the MsvR from *M. acetivorans* performs a similar function [27]. After an oxidative stress event occurs a thiol redox system alone would not be sufficient as more specific repair processes would be needed such as the repair of methionine sulfoxides and peroxides generated from the oxidation of metals. Methionine sulfoxide reductase (Msr) is an *in vivo* target of Trx in many organisms [6, 28, 29]. Msr uses Trx to catalytically reduce methionine sulfoxides to methionine thereby mitigating the potential oxidative damage caused by sulfoxides in the cell. Peroxides generated during oxidative stress can be reduced by peroxiredoxins (Prx) to H_2O. Prx is generally reduced by a Trx or dithiol compound such as glutathione, but due to the absence of glutathione in methanogens Trx remains the likely electron donor to Prx. Collectively, MaTrx7 could play a central role in the oxidative stress response and repair similar to most organisms studied to date.

Replication and translation: The ability of a redox maintenance system to tightly regulate metabolism is indeed critical to the growth of the organism, but regulation of replication and translation activities post oxidation in the cell would prevent using energy and resources during oxidative stress events that prohibit growth. Potential target proteins of replication and translation functions were identified. Interestingly only the sliding clamp subunit of DNA polymerase was identified which contains two cysteine residues. DNA polymerase is only functional as a complex, so the identification of only one subunit in the dataset suggests that the other subunits were disassociated due to either oxidation during the treatment of the lysate or washing with the column with high salt pre-elution. It is likely that MaTrx7 is performing a

specific regulatory or repair role the sliding clamp subunit of DNA polymerase. Similar mechanisms have been observed in the Bacteriophage T7 polymerase, which has been shown to have an activity regulated by Trx by the interaction with a Trx binding domain (TBD) near the thumb of the sliding clamp [30].

The previously studied RNA polymerase (RNAP) in *M. acetivorans* was identified as a target of MaTrx7. RNAP has been determined to be an oxygen labile enzyme losing the FeS clusters in domain 3 of subunit D post oxidation thereby altering the interaction of DL with the remaining subunits or with transcription factors [31]. The resulting changes in RNAP may change the transcription profile or limit the activity of RNAP in an oxidized environment. After the loss of FeS clusters in RNAP it is likely disulfide bonds were formed preventing the repair of the enzyme, and thus MaTrx7 may serve a role in RNAP repair. One example of redox sensing could be to have RNAP change the transcriptome when RNAP is oxidized; an activity that cannot be reversed until the redox state of the cell is brought back towards homeostasis by an increase in reduced MaTrx7.

Conclusions

The data obtained to date suggests the *M. acetivorans* thioredoxin system is the primary thiol redox maintenance system that functions to repair oxidatively sensitive enzymes while also reducing a set of specific target proteins similar to the thioredoxin systems of many anaerobes and aerobes observed today. The *M. acetivorans* thioredoxin system utilizes the secondary electron carrier NADPH that can be produced through the oxidation of the primary electron carriers that are generated during methanogenesis (anaerobic growth). As a result of oxidative stress the ability to reduce/repair the cell from primary electron carriers alone is insufficient, during which *M. acetivorans* is capable of generating NADPH from an oxygen insensitive

alternative carbon source. Glycogen synthesis/degradation intermediates generated during methanogenesis are the most probable sources of carbon to be oxidized as they are readily available, abundant, and the enzymes responsible for their oxidation are insensitive to oxygen. Since the majority of methanogens contain TrxR that appear to bind NADPH, it is likely that methanogens evolved the NADPH dependent thioredoxin system and the ability to generate the secondary electron carrier NADPH aerobically as a response to the increase in the number of oxidative stress events.

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Figures and Tables

Figure 1

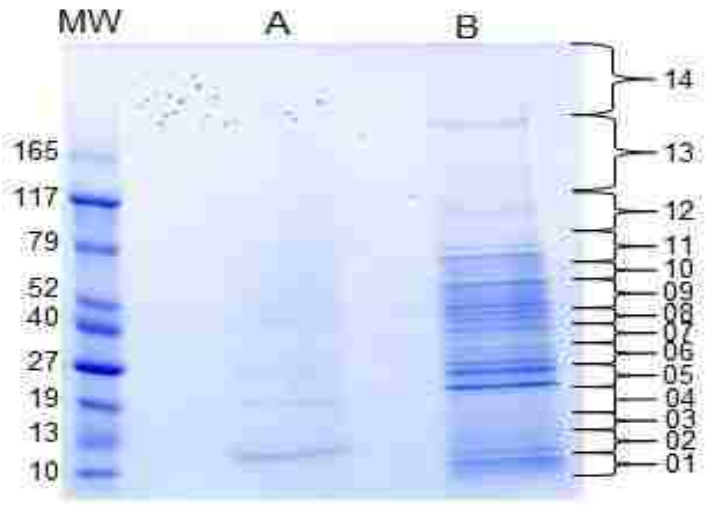

Figure 1. SDS-PAGE gradient gel of *M. acetivorans* proteins eluted with DTT from resin containing A) iodoacetamide-treated MaTrx7C15S and B) MaTrx7C15S. MW: molecular weight marker (kDa). Numbered brackets denote individual gel slices that were analyzed.

Figure 2

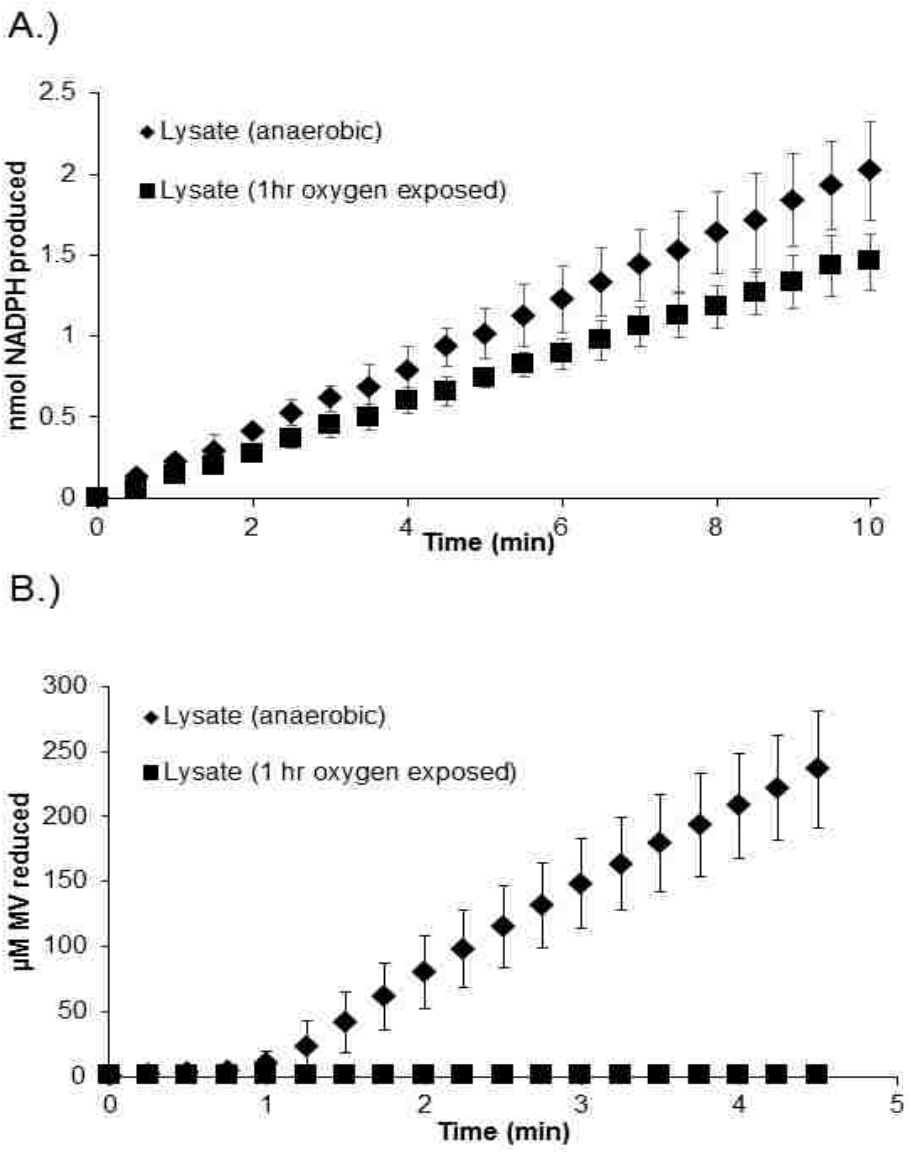

Figure 2. Comparison of the effect of oxygen exposure on the intrinsic NADP-reductase activity (A) and CODH activity (B) of cell lysate from methanol grown *M. acetivorans* Cell lysate was maintained anaerobic or was exposed to air for one hour then made anaerobic again. NADP-reductase and CODH activities were measured using NADP and methyl viologen (MV) respectively, as described in Methods. Each data point is the mean of triplicate assays and error bars show standard deviation.

Figure 3

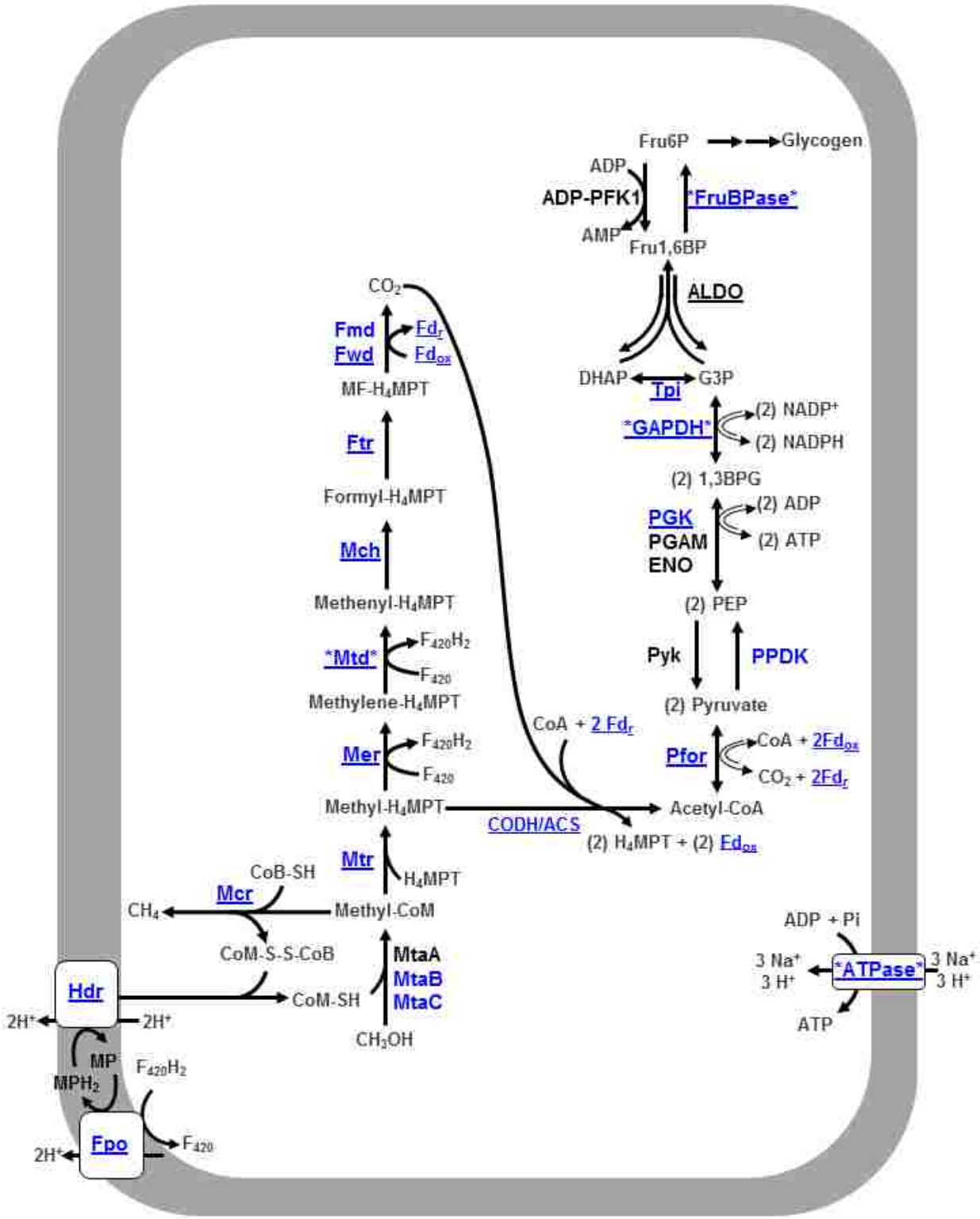
Fru6P
Glycogen
ADP
ADP-PFK1
AMP
FruBPase
Fru1,6BP
ALDO
DHAP
G3P
Tpi
GAPDH
(2) NADP+
(2) NADPH
(2) 1,3BPG
PGK
PGAM
ENO
(2) ADP
(2) ATP
(2) PEP
Pyk
PPDK
(2) Pyruvate
Pfor
CoA + 2Fdox
CO2 + 2Fdr
CoA + 2 Fdr
CODH/ACS
(2) H4MPT + (2) Fdox
Acetyl-CoA
CO2
Fmd
Fwd
Fdr
Fdox
MF-H4MPT
Ftr
Formyl-H4MPT
Mch
Methenyl-H4MPT
Mtd
F420H2
F420
Methylene-H4MPT
Mer
Methyl-H4MPT
Mtr
H4MPT
CoB-SH
Mcr
CH4
Methyl-CoM
CoM-S-S-CoB
MtaA
MtaB
MtaC
CoM-SH
CH3OH
Hdr
2H+
2H+
MP
MPH2
F420H2
Fpo
2H+
F420
ADP + Pi
3 Na+
3 H+
ATPase
3 Na+
3 H+
ATP

Figure 3 continued

Figure 3. Model of central metabolism of *M. acetivorans* and the corresponding proteins identified in the MaTrx7C15S eluate. Enzymes in blue denote enzymes identified in the MaTrx7C15S eluate (Fig. 1). An underline denotes this enzyme was identified in other studies as a target of Trx in another organism(s). An asterisk denotes the activity of the enzyme from other organisms has been shown to be regulated or dependent upon Trx or thiol-containing compounds. Corresponding references for enzymes are indicated in table 1. Abbreviations: ADP-PFK1, ADP-dependent phosphofructokinase 1; ALDO, fructose 1,6-bisphosphate aldolase; ATPase, ATP synthase; CODH/ACS, acetyl-CoA decarbonylase/synthase; ENO, enolase; Fd, ferredoxin; Fmd and Fwd, molybdenum- and tungsten- dependent formylmethanofuran dehydrogenase; Fpo, F_{420} dehydrogenase; FruBPase, Fructose 1,6-bisphosphotase; Ftr, formylmethanofuran-H_4MPT; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hdr, heterodisulfide reductase; Mch, methenyl-H4MPT cyclohydrolase; Mcr, methyl-coenzyme M reductase; Mer, methylene-H4MPT reductase; Mta, methanol-5-hydroxybenzimidazolylcobamide methyltransferase; Mtd, F_{420} -dependent methylene-H4MPT dehydrogenase; Mtr, methyl-H_4MPT coenzyme M methyltransferase; Pfor, pyruvate-ferredoxin oxidoreductase; PGAM, 3-phosphoglycerate mutase; PGK, 3-phosphoglycerate kinase; PPDK, pyruvate phosphate dikinase; Pyk, pyruvate kinase; Tpi, triosephosphate isomerase.

Figure 4

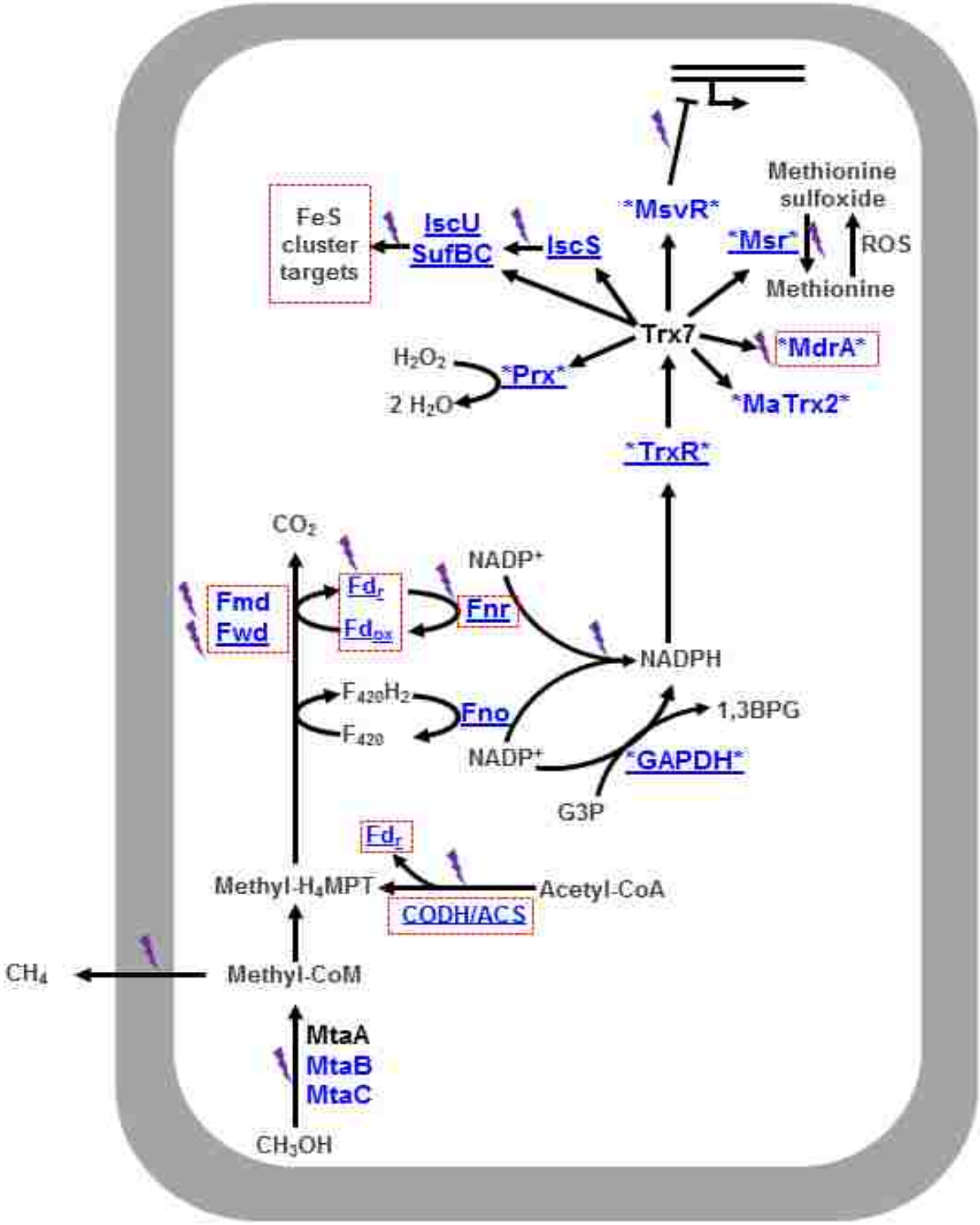
Methionine sulfoxide
ROS
Methionine
MsvR
Msr
FeS cluster targets
IscU
SufBC
IscS
Trx7
MdrA
H_2O_2
Prx
2 H_2O
MaTrx2
TrxR
CO_2
NADP+
Fmd
Fwd
Fd_r
Fd_{ox}
Fnr
NADPH
$F_{420}H_2$
F_{420}
Fno
1,3BPG
NADP+
GAPDH
G3P
Fd_r
Methyl-H_4MPT
Acetyl-CoA
CODH/ACS
CH_4
Methyl-CoM
MtaA
MtaB
MtaC
CH_3OH

Figure 4 continued

Figure 4. Proposed models depicting the role of MaTrx7 during anaerobic growth and the pathways inhibited by oxygen/oxidative stress (purple lightning symbols) with possible pathways used to generate NADPH for reduction of MaTrx7. Proteins in blue are potential MaTrx7 targets. Underlined proteins are Trx targets that have been identified in other organisms, and proteins with asterisks have been shown to have an activity regulated or dependent upon Trx or thiol containing compounds. Dashed red boxes indicate FeS cluster containing proteins. Corresponding references have been indicated in table 1. Abbreviations: CODH/ACS, acetyl-CoA decarbonylase/synthase; Fd_{ox} and Fd_r, oxidized and reduced ferredoxin; Fno, $F_{420}H_2$: $NADP^+$ oxidoreductase; Fnr, ferredoxin $NADP^+$ oxidoreductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IscS, cysteine desulfurase; MdrA, Methanosarcina disulfide reductase; Msr, methionine sulfoxide reductase; MsvR, redox-sensing transcriptional regulator; Prx, peroxiredoxin.

Table 1

Biological function	Locus tag (Cysteine count)	Protein name	References
ATP synthesis			
	MA_4158 (5) MA_4159 (1) MA_4156 (0) MA_4155 (1) MA_4157 (0)	*V-type ATP synthase α chain, β chain, subunit C, subunit E, subunit F	6,8,22,29
Fe-S cluster biogenesis			
	MA_0236 (4)	Cysteine lyase	
	MA_2718 (4)	*Cysteine desulfurase IscS2	29
	MA_0807 (3)	IscU1	29
	MA_2717 (4)	IscU2	29
	MA_0936 (5)	SufB1	
	MA_4407 (4)	SufB2	
	MA_0937 (3)	SufC1	
	MA_4406 (6)	SufC2	
Metabolic processes			
	MA_0072 (11)	*Anaerobic ribonucleotide-triphosphate reductase	6
	MA_4218 (13)	Archaeal glutamate synthase [NADPH]	22
	MA_2720 (2)	Cysteine synthase	22
	MA_4235 (4)	$F_{420}H_2$-dependent $NADP^+$ reductase	
	MA_3786 (7)	Ferredoxin $NADP^+$ reductase	22
	MA_3344 (6)	*Fructose 1,6-bisphosphotase	24,22
	MA_3345 (5)	*Glyceraldehyde-3-phosphate dehydrogenase 2	6,24,25
	MA_2690 (8)	Phosphoenolpyruvate carboxylase	6
	MA_3592 (1)	Phosphoglycerate kinase 2	6,8
	MA_0608 (12)	Pyruvate phosphate dikinase	22
	MA_0032 (4) MA_0031 (7) MA_0033 (9) MA_0034 (0)	*Pyruvate:ferredoxin oxidoreductase subunit α, β, δ, γ	8
	MA_4607 (4)	Triosephosphate isomerase	6,8

Table 1 continued

Biological function	Locus tag (Cysteine count)	Protein name	References
Methanogenesis			
	MA_3733 (3)	5,10-methylenetetrahydromethanopterin reductase	8
	MA_1016 (29) MA_3860 (28) MA_4399 (28) MA_1014 (7) MA_3862 (7) MA_1012 (2) MA_3864 (2) MA_1015 (0) MA_3861 (0) MA_1011 (6) MA_3865 (6)	Carbon monoxide dehydrogenase/acetyl-CoA synthase α1, α2, α3, β1, β2, δ1, ε1, ε2, γ	8
	MA_2868 (37) MA_4237 (15) MA_4236 (10)	CoB--CoM heterodisulfide reductase 1 subunit A, B, C	
	MA_0688 (18)	CoB--CoM heterodisulfide reductase 2 subunit D	
	MA_4430 (5)	*F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase	8
	MA_0683 (9)	Ferredoxin[c]	
	MA_0010 (4)	Formylmethanofuran-tetrahydromethanopterin formyltransferase	8
	MA_0455 (15) MA_0456 (3) MA_4392 (12)	Methanol-5-hydroxybenzimidazolylcobamide methyltransferase isozyme 1, 2 MtaB,C	
	MA_1710 (8)	Methenyltetrahydromethanopterin cyclohydrolase	8
	MA_4546 (7) MA_4550 (5) MA_4549 (1) MA_4548 (4) MA_4547 (2)	Methyl coenzyme M reductase, subunit α, β, C, D, γ	8
	MA_4379 (6)	Methylcobamide:CoM methyltransferase isozyme M	
	MA_0306 (7) MA_0309 (14) MA_0307 (3) MA_0308 (4) MA_0304 (6) MA_0305 (33)	Molybdenum-dependent Formylmethanofuran dehydrogenase, subunits A,B, C, D, E, F	
	MA_0272 (3) MA_0270 (0) MA_0269 (3)	Tetrahydromethanopterin S-methyltransferase subunit A, G, H	8
	MA_0834 (9) MA_0832 (5) MA_0835 (3)	Tungsten-dependent Formylmethanofuran dehydrogenase, subunits B, C, D	8

Table 1 continued

Biological function	Locus tag (Cysteine count)	Protein name	References
Oxidative stress response and repair			
	MA_3736 (4)	*Methanosarcina disulfide reductase MdrA	
	MA_1458 (10)	*MsvR	26
	MA_1431 (2) MA_0449 (5)	*Peptide methionine sulfoxide reductase MsrA, B	6,26,27
	MA_4103 (4)	*Peroxiredoxin	8,25
	MA_1368 (3)	*Thioredoxin reductase (NADPH)	22
	MA_3212 (2)	*Thioredoxin MaTrx2	22
Replication, translation, transcription, and post transcriptional modification			
	MA_1478 (1)	*DnaK	
	MA_1263 (13) MA_1264 (7) MA_1265 (4) MA_1111 (13) MA_1266 (0) MA_0721 (1) MA_0598 (4) MA_4672 (4)	DNA-directed RNA polymerase subunit A, B, B', D, H, L, N, P	8,22
	MA_0110 (2)	DNA polymerase sliding clamp	
Sulfur metabolism			
	MA_0685 (6)	*Sulfite reductase	22

Table 1. A condensed list of MaTrx7 potential target proteins based on the S1 file. The proteins have been grouped by biological functions, and the protein names with an asterisks preceding the name have been found to have an activity that is regulated or modulated by Trx or sulfide containing compounds. Corresponding references are listed in the right most column.

Chapter IV

The *Methanosarcina acetivorans* Thioredoxin System Activates DNA Binding of the Redox-Sensitive Transcriptional Regulator MsvR

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Abstract

The production of biogas (methane) by anaerobic digestion is an important facet to renewable energy, but is subject to instability due to the sensitivity of strictly anaerobic methanogenic archaea (methanogens) to environmental perturbations, such as oxygen. An understanding of the oxidant-sensing mechanisms used by methanogens may lead to the development of more oxidant tolerant (i.e. stable) methanogen strains. MsvR is a redox-sensitive transcriptional regulator that is found exclusively in methanogens. We show here that oxidation of MsvR from *Methanosarcina acetivorans* (MaMsvR) with hydrogen peroxide oxidizes cysteine thiols, which inactivates MaMsvR binding to its own promoter (P_{msvR}). Incubation of oxidized MaMsvR with the *M. acetivorans* thioredoxin system (NADPH, MaTrxR, and MaTrx7) results in reduction of the cysteines back to thiols and activation of P_{msvR} binding. These data confirm that cysteines are critical for the thiol-disulfide regulation of P_{msvR} binding by MaMsvR and support a role for the *M. acetivorans* thioredoxin system in the *in vivo* activation of MaMsvR. The results support the feasibility of using MaMsvR and P_{msvR}, along with the *Methanosarcina* genetic system, to design methanogen strains with oxidant-regulated gene expression systems, which may aid in stabilizing anaerobic digestion.

Introduction

Methane-producing archaea (methanogens) are strict anaerobes that are the rate-limiting step in biogas production [1]. Members of the genus *Methanosarcina* are particularly important

in biogas production, due to their ability to produce methane with multiple substrates, including CO_2, methylated compounds (ex. methanol), and acetate, where all other methanogens are restricted to hydrogenotrophic ($H_2 + CO_2$) methanogenesis [2]. Environmental perturbations can significantly impact the ability of methanogens to produce methane. For example, many methanogenesis enzymes are oxygen sensitive resulting in a loss of energy conservation and methane production upon exposure to oxygen [3]. The partial reduction of oxygen by flavoenzymes and metalloenzymes results in the production of reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which target many macromolecules within cells [4]. For example, O_2^- and H_2O_2 oxidize Fe-S cluster containing enzymes and thiols of cysteine residues in proteins, causing Fe-S cluster degradation and formation of deleterious disulfides, which leads to loss of metabolism [5]. For anaerobes, such as methanogens, to overcome oxygen exposure requires mechanisms to decrease the production of ROS and/or actively remove ROS, as well as repair oxidatively-damaged proteins. Indeed, many methanogens can survive oxygen exposure; however, methane production is severely inhibited [6-9]. In particular, members of the *Methanosarcinales* appear to be some of the most aerotolerant methanogens [8], which is likely due to a large number of putative antioxidant and repair proteins encoded in the genomes of sequenced *Methanosarcinales*.[10-13] However, information on the mechanisms used by methanogens, including the *Methanosarcinales*, to sense and respond to oxygen (oxidative stress) is limited. An understanding of the oxidant-sensing mechanism used by *Methanosarcina* sp., along with their robust genetic system [14], may lead to the development of strains with increased oxidant tolerance.

Many cells use the formation of disulfides in proteins to monitor the cellular redox state and the presence of deleterious ROS. For example, OxyR is a transcriptional regulator found in

Escherichia coli and other bacteria that is used to monitor the presence of H_2O_2 [15]. Redox-sensing thiols of cysteine residues in OxyR are oxidized in the presence of H_2O_2, resulting in OxyR activation which increases the expression of H_2O_2 scavengers, Fe-S cluster repair enzymes, and thiol redox buffer systems. Once H_2O_2 levels have been reduced, OxyR is inactivated by the reduction of the disulfides by glutaredoxin 1, with reductant supplied by glutathione/glutathione reductase and NADPH [16]. H_2O_2-sensing transcriptional regulators have also been identified in eukaryotes [17]. Recent evidence has revealed the presence of a redox-sensing transcriptional regulator (MsvR) in methanogens. In *Methanothermobacter thermautotrophicus*, MsvR regulates expression of $F_{420}H_2$ oxidase (FpaA) by redox-sensitive binding to the promoter region of *fpaA* [18]. MsvR also regulates its own expression in *M. thermautotrophicus*. MsvR functions as a negative regulator to repress expression of *fpaA* and *msvR* under reducing conditions. Oxidation of MsvR results in the induction of *fpaA* and *msvR*. More recently, MsvR from *Methanosarcina acetivorans* (MaMsvR), a member of the *Methanosarcinales*, was shown to bind to its own promoter (P_{msvR}) only under reducing conditions [19]. Formation of disulfide(s) within the C-terminal V4R effector domain of MaMsvR were shown to abrogate binding of MaMsvR to the P_{msvR} promoter region. For MaMsvR to function as a redox-sensing transcriptional regulator, P_{msvR} binding by MaMsvR would need to be restored by disulfide reduction to reset the system once oxygen/ROS is removed. Reduction of MaMsvR disulfides and activation of P_{msvR} binding can be accomplished *in vitro* by the addition of the disulfide-reducing agent dithiothreitol (DTT); however, the physiological reducing system is unknown. In the majority of organisms, disulfide reduction is mediated by thioredoxin and/or glutaredoxin systems [20].

Methanogens lack glutathione and therefore likely do not possess functional glutaredoxin systems [21, 22]. However, recent evidence indicates the majority of methanogens contain thioredoxins (Trx) [23, 24], which are small (~12 kDa) proteins that possess a CXXC active site motif necessary for disulfide oxidoreductase activity [20]. In the canonical system, Trx receives reducing equivalents from thioredoxin reductase (TrxR) with NADPH as the electron donor. Thus, the thioredoxin system could serve as the MsvR disulfide reducing system. *M. acetivorans* contains seven putative Trx homologs (MaTrx1-7) and a single TrxR homolog (MaTrxR). Recent evidence revealed that *M. acetivorans* contains at least three functional Trxs (MaTrx2, MaTrx6, and Matrx7) and a complete NADPH-dependent thioredoxin system comprised of MaTrxR and MaTrx7 [23]. We show herein that the *M. acetivorans* NADPH-dependent thioredoxin system can reduce disulfides in oxidized MaMsvR and restore P_{msvR} -binding activity, indicating that the thioredoxin system is the physiological MaMsvR disulfide reducing system. Thus, it may be feasible to use P_{msvR} along with the *Methanosarcina* genetic system to design *Methanosarcina* strains with oxidant-responsive genes, which may increase the stability of biomethanation.

Materials and Methods

Protein purification and manipulation. Recombinant MaTrxR and MaTrx7 were expressed in *E. coli* and purified to homogeneity as previously described [23]. Strep-tagged MaMsvR was expressed in *E. coli* and purified to homogeneity as previously described [19]. H_2O_2-oxidized MaMsvR ($MaMsvR_{ox}$) was prepared by incubation of MaMsvR with 100-fold molar excess of H_2O_2 in buffer A (20 mM Tris pH 8, 15 mM $MgCl_2$, 120 mM KCl, 12.5 μg/mL heparin, 10% glycerol) for 30 mins. Residual H_2O_2 was removed by buffer exchange into buffer A using a NAP5 column (GE Healthcare). DTT-reduced samples of MaMsvR were prepared by incubating

100 μM $MaMsvR_{ox}$ in buffer A containing 10 mM DTT for 20 min at room temperature. Residual DTT was removed using a NAP5 column. The ability of the thioredoxin system to reduce $MaMsvR_{ox}$ was assayed by incubation of 10 μM $MaMsvR_{ox}$ with 1 mM NADPH, 0.5 μM MaTrxR, and 2.5 μM MaTrx7 for 1 hour at 37 °C in buffer A. Protein concentrations were determined by both the Bradford assay and using fluorescence with a Qubit protein assay following the manufacturer's instructions (Invitrogen).

Electrophoretic mobility shift assay (EMSA). Complimentary 50-bp oligonucleotides containing P_{msvR} were synthesized (Integrated DNA technologies) and annealed to generate the P_{msvR} DNA probe used in all EMSAs [19] . DNA-binding reactions were prepared by incubating 100 nM P_{msvR} with 8 μM MaMsvR in buffer A for 20 min at 37°C. Binding reactions were loaded onto a pre-run 6% polyacrylamide gel in 0.5X TBE buffer and electrophoresed for 75 min at 75 V at 10 °C. Gels were stained using SYBR gold (Life Technologies) and visualized using a Gel-Doc XR+ system (Bio-Rad Technologies).

Quantitation of thiols in MaMsvR. Aliquots of MaMsvR-containing samples used in EMSAs were analyzed for total thiol content using DTNB [25]. MaMsvR was denatured and thiols quantified by the addition of 10 μL of MaMsvR-containing sample to 90 μL of 6M guanidine-HCl in 100 mM KPO_4, pH 7.8 containing 175 μM DTNB. Samples were incubated anaerobically for 15 min at room temperature and the absorbance at 412 nm was recorded. The number of thiols per MaMsvR monomer was calculated based on the concentration of TNB using ε_{412} =13,700 M^{-1} cm^{-1} [25]. All samples were analyzed in triplicate. The background amount of thiols contributed by the denatured thioredoxin system was determined in samples containing NADPH, MaTrxR, and MaTrx7, but without MaMsvR.

Results and Discussion

Reduction of MaMsvR disulfides and activation of MaMsvR DNA-binding by thioredoxin. MaMsvR contains ten cysteine residues, with two located in the DNA-binding domain, four in the V4R domain, and the remaining four located in the linker domain [19] . The cysteines within the V4R domain (C206, C225, C232, and C240) are postulated to function in redox-sensing, whereby thiol-disulfide exchange causes conformation changes which alter the ability of MaMsvR to bind an inverted repeat sequence motif (TTCGN$_{7\text{-}9}$CGAA) upstream of P_{msvR}. Three of the residues (C206, C232, and C240) are conserved in all MsvR homologs [18]. Specifically, C206 was shown to be critical for redox-sensitive binding of MaMsvR to P_{msvR}, because a MaMsvR C206A variant was able to bind to P_{msvR} under non-reducing conditions, whereas the wild-type MaMsvR is unable [19]. Previous results also revealed C225 was not involved in redox-sensing, while C232 and C240 impact MsvR binding to P_{msvR}, but the precise role of these cysteines is unclear. Thus, C206 is likely, and C232/C240 are possibly, involved in thiol-disulfide formation which serves to control DNA-binding by MaMsvR.

EMSA and thiol quantitation experiments were used to examine the role of thiol-disulfide exchange in controlling DNA binding by MaMsvR. First, MaMsvR was incubated with 100-fold molar excess of H_2O_2 to generate H_2O_2-oxidized MaMsvR ($MaMsvR_{ox}$). Quantitation of the thiol content of $MaMsvR_{ox}$ under denaturing conditions revealed that four of the cysteines were not oxidized by H_2O_2 (**Table 1**), indicating some cysteines are inaccessible to H_2O_2, and likely do not participate in redox-sensing. Importantly, $MaMsvR_{ox}$ was incapable of binding to the P_{msvR} region as revealed by the lack of shift when examined by EMSA (**Fig. 1, lane 2**). This result indicates that oxidation of the thiols of six cysteine residues is sufficient to inactive MaMsvR DNA binding. The subsequent treatment of $MaMsvR_{ox}$ with DTT resulted in detection of

approximately nine thiols (**Table 1**), consistent with the total number of cysteines present in MaMsvR. Moreover, incubation of $MaMsvR_{ox}$ with DTT restored binding to P_{msvR} (**Fig. 1, lane 3**). This result is consistent with H_2O_2 causing the oxidation of six thiols to disulfides, which causes reversible inactivation MaMsvR binding to P_{msvR}. The remaining four thiols are likely buried within the folded protein and are inaccessible to H_2O_2 or DTT, and therefore do not participate in thiol-disulfide exchange.

Similar experiments were performed to determine if the *M. acetivorans* thioredoxin system could also activate DNA-binding of $MaMsvR_{ox}$. Incubation of $MaMsvR_{ox}$ with NADPH, MaTrxR, and MaTrx7 (complete thioredoxin system) activated binding of $MaMsvR_{ox}$ to P_{msvR} (**Fig. 1, lane 6**). The complete thioredoxin system alone did not cause a shift of P_{msvR} in the EMSA (**Fig. 1, lane 4**) and NADPH/MaTrxR in the absence of MaTrx7 also failed to activate binding of $MaMsvR_{ox}$ to P_{msvR} (**Fig. 1, lane 5**). Moreover, incubation of $MaMsvR_{ox}$ with the complete thioredoxin system resulted in the detection of ten thiols (**Table 1**), consistent with all the H_2O_2-generated disulfides in MaMsvR being surface exposed and accessible to reduction by MaTrx7. Taken together these results demonstrate that the *M. acetivorans* NADPH-dependent thioredoxin system can activate P_{msvR} binding in oxidized MaMsvR and that MaTrx7 is required for the reduction of disulfides in oxidized MaMsvR. The reduction of MaMsvR by MaTrx7 is the first evidence of thioredoxin playing a role in the regulation of the activity of a transcription regulator in a methanogen. The activation of MaMsvR DNA binding by MaTrx7 also integrates P_{msvR} regulation by MsvR into the physiology of *M. acetivorans*, which supports the future use of P_{msvR} in engineering oxidant-responsive gene expression strains. For example, we have previously demonstrated that increased expression of catalase protects *M. acetivorans* from H_2O_2 [26].

Proposed model of MaMsvR regulation in *M. acetivorans*. Based on results from previous studies [18, 19] and herein, we propose the following model (**Fig. 2**) for the regulation of the P_{msvR} binding activity of MaMsvR by thiol-disulfide exchange involving the thioredoxin system. Exposure of *M. acetivorans* to oxidants (ex. H_2O_2) results in oxidation of critical cysteines in MaMsvR to disulfides. Based on previous studies, C206 plays a crucial role, likely forming an intermolecular disulfide between MaMsvR monomers [19]. However, under the conditions tested here, at least six cysteines are involved in H_2O_2-induced disulfide formation, which may generate three intra-molecular, six inter-molecular, or some combination of intra/inter-molecular disulfides. Nonetheless, the formation of disulfides likely causes a conformational change in MaMsvR, such that MaMsvR is no longer able to bind P_{msvR}, which allows for RNAP to bind and transcription to proceed. Removal of oxidant and/or an influx of electron donor would allow for the reduction of MaMsvR disulfides by MaTrx7, with reducing equivalents supplied by MaTrxR and NADPH. The *in vitro* results presented here demonstrate that MaTrx7 can specifically reduce disulfides in MaMsvR, but we cannot rule out that the additional MaTrxs or other proteins also participate in the *in vivo* reduction of disulfides in MaMsvR and may do so under different conditions. However, the target specificity and the redox partner(s) of the other MaTrxs is currently unknown [23]. The data presented here link the regulation of MaMsvR to the redox status of *M. acetivorans* and the availability of reducing equivalents (e.g. NADPH). The results also reveal that methanogens have oxidant sensing systems which are integrated into metabolism in a manner similar to systems identified in bacteria and eukaryotes. Ultimately, due to the thioredoxin-dependent reversible P_{msvR} binding by MaMsvR it may be feasible to engineer strains with oxidant-inducible genes (e.g. catalase) using P_{msvR} in an effort to generate oxidant tolerant strains without an increased energy demand that would come from constitutive gene expression.

Acknowledgements.

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Figures and Tables

Figure 1

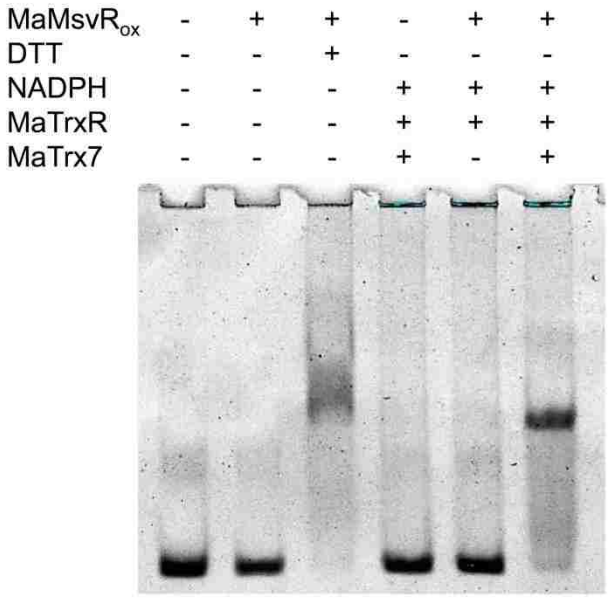

Fig. 1. Activation of MaMsvR P$_{msvR}$ **binding by the *M. acetivorans* thioredoxin system**. EMSA performed with P$_{msvR}$ and the addition of the indicated components as described in materials and methods.

Figure 2

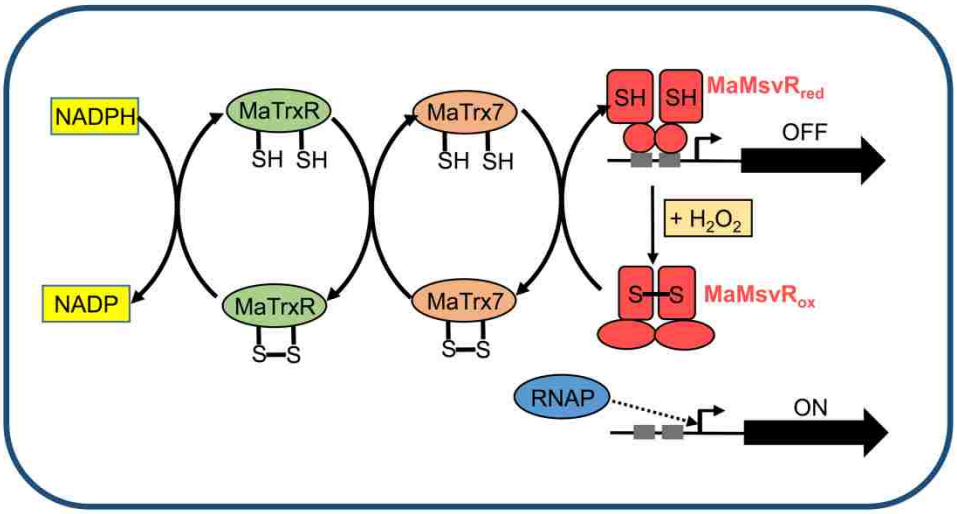

Fig. 2. Proposed model of MaMsvR activation by the NADPH-dependent MaTrxR-MaTrx7 thioredoxin system in *M. acetivorans*. H_2O_2 causes the oxidation of thiols (SH) to disulfides which inactivates MaMsvR DNA binding, allowing transcription by RNAP. MaTrx7 receives reducing equivalents from NADPH/MaTrxR to reduce the disulfides to thiols and restore MaMsvR DNA binding.

Table 1. Quantitation of MaMsvR thiols.

Sample[a]	Thiols
$MaMsvR_{ox}$	4.0 ± 0.6
$MaMsvR_{ox}$ + DTT	9.0 ± 1.5
$MaMsvR_{ox}$ + NADPH/MaTrxR	4.8 ± 0.1
$MaMsvR_{ox}$ + NADPH/MaTrxR/MaTrx7	9.9 ± 1.0

[a]samples were processed and thiols quantified using DTNB as described in the Materials and Methods

Appendix 4.1: Co-Author Confirmation Letter for Chapter IV

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter IV, titled "The *Methanosarcina acetivorans* Thioredoxin System Activates DNA Binding of the Redox-sensitive Transcriptional Regulator MsvR." of A. C. McCarver's dissertation was submitted for publication in the Journal of Industrial Microbiology & Biotechnology in 2015 with R. C. Sheehan as primary author and co-authors A.C. McCarver, C. E. Isom, E. A. Karr, and D. J. Lessner.

I, Dr. Daniel J. Lessner, advisor of Addison C. McCarver, confirm Addison C. McCarver was co-author to the primary author Ryan C. Sheehan, a former graduate student at the University of Arkansas.

Dr. Daniel J. Lessner
Associate Professor
Department of Biological Sciences
University of Arkansas

Date

Conclusions

The study of methanogen physiology has become increasingly important as greenhouse gasses rise and encourage global climate change. Furthermore, as the human population on earth grows the need for additional sources of fuel to power human infrastructure increases, in particular sources of fuel that burn more cleanly than fossil fuels. The study of methanogen physiology offers insight into possible mechanisms for increasing the production of biologically sourced fuels (biofuels), while simultaneously providing insight into possible methods to regulate methanogenesis and ultimately part of global climate change. Although the primary molecular pathway responsible for contributing to global climate change, methanogenesis, has been the target of study for nearly three decades, secondary contributors such as increases in oxygen tolerance among methanogens have not been studied so extensively. Since nearly two thirds of biologically produced methane originates from acetate, a substrate that can only be used by some methanogens belonging to the class Methanomicrobia that are among the most aerotolerant species of methanogens, it is conceivable that the mechanisms behind oxygen detoxification and repair play a critical role in the ability of these methanogens to effectively grow. Members of Methanomicrobia inhabit environments ranging from the hind gut of ruminants to deep anoxic marine environments, so the frequency in which these organisms experience oxidative stress varies depending upon the proximity to aerobic environments. Effective growth on acetate could be limited by the ability to remove oxygen and repair oxidative damage, and thus the study of these pathways could elucidate methods for limiting growth of these methanogens in areas where the habitat is anthropogenic (i.e. cattle farms and rice paddy fields) in order to ultimately mitigate anthropogenic greenhouse gas production.

The bulk of methanogen physiology relies on FeS clusters in proteins, which are cooridinated by reduced cysteine residues. FeS clusters are sensitive to oxygen, and since the metabolism of all methanogens are based on functional FeS clusters then there would need to be a system that could obtain electrons post-oxidative stress for the purporse of reducing disulfides left begind after the oxidation of FeS clusters. The NADPH-dependent thioredoxin system is ubiquitous and performs a critical role of maintaining reduced disulfides. Since methanogens use reduced ferredoxin and F_{420} as electron carriers genereated by methanogenesis, they would need a mechanism for generating NADPH during methanogenesis and in the absence of methanogenesis (oxidative stress) if the NADPH-dependent thioredoxin system is to play a role in oxidative stress repair.

The work presented in this dissertation examined the role of a NADPH-dependent thioredoxin system in the model methanogen *M. acetivorans*. *M. acetivorans* is capable of growth on all methanogenesis substrates, and thus serves as an excellent model for the examination of the role of a thiol redox pathway in oxidative stress repair. The study of the NADPH-dependent thioredoxin system in *M. acetivorans* was examined in four parts: 1) the presence of thioredoxin system components in methanogens and initial characterization of the NADPH-dependent thioredoxin system in *M. acetivorans* 2) additional characterization of the remaining thioredoxins and examination of NADPH-dependent thioredoxin reductases in methanogens 3) assessing the ability of the NADPH-dependent thioredoxin system to serve as a broad thiol redox repair system in *M. acetivorans* 4) the ability of the NADPH-dependent thioredoxin system to perform a redox regulatory role by modulating the activity of the transcriptional regulator MsvR. The work performed in *M. acetivorans* can be applied to methanogens as a whole, and has been in some aspects of this dissertation supporting that

methanogens appear to predominantly utilize NADPH-dependent thioredoxin systems. NADPH-dependent thioredoxin systems appear absent in more ancestral methanogens suggesting that this system may have evolved within methanogens. NADPH production and broad reduction of *M. acetivorans* proteins by MaTrx7 post-oxidation supports that the system can function post-oxidative stress. Cumulatively, the work presented in this dissertation supports that the primary thiol redox maintenance system in methanogens is the canonical NADPH-dependent thioredoxin system observed in many previously studied organisms. The knowledge gained in this study can provide a foundation for a means to better manage methanogen growth in unwanted environments such as cattle farms, or in environments where effective growth has historically been the limiting factor behind successful fermentation vessels for biofuel production. Furthermore, insight into the evolution of thiol redox maintenance systems from early, anaerobic earth to the more oxidizing environment inhabited today would further the understanding of the evolution of life.

Appendix 1 Institutional Biosafety Committee Approval

September 15, 2017

MEMORANDUM

TO: Dr. Daniel Lessner

FROM: Ines Pinto, Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 18016

PROTOCOL TITLE: Unraveling the role of iron-sulfur clusters in RNA polymerase

APPROVED PROJECT PERIOD: Start Date September 14, 2017 Expiration Date September 13, 2020

The Institutional Biosafety Committee (IBC) has approved Protocol 18016, "Unraveling the role of ironsulfur clusters in RNA polymerase". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Appendix 1.1 Institutional Biosafety Committee Approval

UNIVERSITY OF ARKANSAS

628 SCEN • Department of Biological Sciences • Fayetteville, Arkansas 72701 • (479) 575-2239 e-mail:dlessner@uark.edu • web site: http://biology.uark.edu/

November 20, 2017

This letter is to confirm the recombinant DNA used in the research conducted by Addison McCarver in the dissertation entitled "*Understanding intracellular redox regulation in methanogens: characterization of the components and targets of the NADPH-dependent thioredoxin system from Methanosarcina acetivorans*" was approved by the Institutional Biosafety Committee in protocols #09103 (previous) and #18016 (current).

Sincerely,

Daniel J. Lessner, Ph.D.
Associate Professor