REDUCING EMISSIONS USING METHANOTROPHIC ORGANISMS FOR TRANSPORTATION ENERGY (REMOTE)
DE-FOA-0000881

Award: DE-AR0000426
Prime Recipient: University of Michigan
Project Title: Anaerobic Bioconversion of methane to methanol
Principle Investigator: Professor Stephen Ragsdale 734-615-4621 sragsdal@umich.edu
Date of Report: 4/18/2014
Reporting Period: 1/1/2014 to 4/1/2014

I. Accomplishments and Milestone Update

Subtask 1.1 (1.1) ***Sub-Tasks 1.1***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status:
Description: Clone ANME MCR genes into Methanococcus maripaludis and verify expression

Subtask 1.1.1 (M1.1) ***Milestone M1.1***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status: The mcr genes from an ANME-2C organism were PCR-amplified and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into M. maripaludis. Unfortunately, expression was not sufficient to detect the His tag by Western blot. To improve expression we are now cloning a codon-optimized version.
Description: Confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw.

Subtask 1.2 (1.2) ***Sub-Tasks 1.2***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status:
Description: Clone Methanothermobacter marburgensis MCR genes into M. maripaludis and verify expression

Subtask 1.2.1 (M1.2) ***Milestone M1.2***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status: The mcr genes were PCR-amplified from M. marburgensis DNA and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into M. maripaludis, where expression was sufficient to detect the His tag by Western blot. The expression level has not yet been measured, but from the intensity of the Western it is low. To improve expression we are now cloning a codon-optimized version.
Description: Confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw.

Subtask 2.1 (2.1) ***Sub-Task 2.1***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status:
Description: Build genome scale flux balance and core metabolic flux models for M. marapaludis metabolism: Semi-automated reconstruction of M. maripaludis model using maximum liklihood orthology approach

Subtask 2.2 (2.2) ***Sub-Task 2.2***

Start Date: End Date: Percentage Complete: 75
Feedback to Performer:
Summary Status:
Description: Genome scale flux balance and core metabolic flux models: Manual curation of M. maripaludis model based on biochemical, genetic, and physiological data from literature

Subtask 2.2.1 (M2.1) ***Milestone M2.1***

Start Date: End Date: Percentage Complete: 75

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Feedback to Performer:

Summary Status: We have completed the semi-automated reconstruction of version 1.0 of the M. maripaludis model using our maximum likelihood orthology approach. (See Section C, Figure 1). We have begun the manual curation of the M. maripaludis draft model based on biochemical, genetic, and physiological data from literature.

Description: Milestone: Deliver first genome-scale metabolic model capable of simulating growth and byproduct section with >75% accuracy

Subtask 3.1 (3.1) ***Sub-Task 3.1***

Start Date: End Date: Percentage Complete: 25

Feedback to Performer:

Summary Status:

Description: Determine endogenous M. maripaludis activities of MCR, HDR and Methyltransferase

Subtask 3.2 (3.2) ***Sub-Task 3.2***

Start Date: End Date: Percentage Complete: 25

Feedback to Performer:

Summary Status:

Description: Methyl-SCoM Reductase (MCR): Characterize kinetic parameters, assess kinetic bias, and measure biophysical properties of heterologously expressed M. marburgensis and ANME MCRs in the forward & reverse direction

Subtask 3.2.1 (M3.1) ***Milestone M3.1***

Start Date: End Date: Percentage Complete: 25

Feedback to Performer:

Summary Status: We have received two strains containing the heterologously expressed MCR from John Leigh's group and are growing the cells to test activity. One strain contains a his-tagged MCR and the other lacks a purification tag.

Description: Milestone: Deliver MCR witha specific activity for the purified protein of at least 5 units/mg (after activation) in the methane synthesis direction and 0.5 units/mg in methane oxidation.

Subtask 3.3 (3.3) ***Sub-Task 3.3***

Start Date: End Date: Percentage Complete: 10

Feedback to Performer:

Summary Status:

Description: Heterodisulfide Reductase (HDR): Determine kinetic parameters of the native and ANME HDRs in the forward & reverse direction

Subtask 3.3.1 (M3.2) ***Milestone M3.2***

Start Date: End Date: Percentage Complete: 10

Feedback to Performer:

Summary Status: We will assay HDR in the cells sent from John Leigh's group.

Description: Milestone: choose the HDR with a specific activity of at least 10 units/mg.

Subtask 4.1 (4.1) ***Sub-Task 4.1***

Start Date: End Date: Percentage Complete: 100

Feedback to Performer:

Summary Status:

Description: Build classical physics potential energy model for MCR inter-atomic interaction potentials: Derive atom-centered charges, equilibrium coordinates and force constants for the four non-protein molecules F430, CoBSH, SCoM, CoBS-SCoM

Subtask 4.1.1 (M4.1) ***Milestone M4.1***

Start Date: End Date: Percentage Complete: 100

Feedback to Performer:

Summary Status: We have calculated all force field parameters for substrates and preliminary force field parameters for the MCR and ANME-1 cofactors. The calculated 1MRO.pdb structure differs by less than 1 Å from the initial crystal structure.

Description: Milestone: Reproduce experimental MCR structure 1MRO.pdb with a root-mean-squared deviation of less than 10 Ångstroms.

Subtask 5.1 (5.1) ***Sub-Task 5.1***

Start Date: End Date: Percentage Complete: 90

Feedback to Performer:

Summary Status:

Description: Develop an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB

Subtask 5.2 (M5.1) ***Milestone M5.1***

Start Date: End Date: Percentage Complete: 90

Feedback to Performer:

Summary Status: An IP sharing agreement was drafted and shared with all institutions in December 2013. The final institution has provided feedback in mid-March. Final discussions are being planned for the first week of April and we expect that this agreement will be finalized and signed by 4/30/14.

Description: Milestone: Reach agreement and sign an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB

Subtask 5.3 (5.2) ***Sub-Task 5.2***

Start Date: End Date: Percentage Complete: 50

Feedback to Performer:

Summary Status:

Description: Hire OTT fellow(s) to look at the ARPA-E REMOTE proposal and assess the relevant existing current patent landscape.

Subtask 5.3.1 (M5.2) ***Milestone M5.2***

Start Date: End Date: Percentage Complete: 100

Feedback to Performer:

Summary Status: With the help of an OTT Fellow, a preliminary search of the patents related to the bioconversion of methane to methanol and to butanol has been completed. We expect to conduct further searches as new inventions are developed during the research.

Description: Milestone: Develop a profile of the existing patents related to methane to methanol (and GTL) biotechnology.

Subtask 5.4 (5.3) ***Sub-Task 5.3***

Start Date: End Date: Percentage Complete: 70

Feedback to Performer:

Summary Status:

Description: Technology to Market Plan: Work with Dr. Nadine Wong and Fellows from OTT and Tech Transfer Consultants (above) to develop Tech to Market Plan

Subtask 5.4.1 (M5.4) ***Milestone M5.4***

Start Date: End Date: Percentage Complete: 70

Feedback to Performer:

Summary Status: We have completed a preliminary market assessment of the methane to methanol conversion market. We have also identified several potential competing technologies in the market.

Description: Milestone: Develop tech to market plan

Subtask 5.6 (5.6) ***Sub-Task 5.6***

Start Date: End Date: Percentage Complete: 50

Feedback to Performer:

Summary Status:

Description: Partner/funding outreach and engagement: Work through the Univ of Michigan Office of Technology Transfer (OTT) to identify and then hire an independent consultant for scouting and forging relationships with technology partners. During the first quarter, we will identify consultant candidates with the thought that that person would be onboard by the 2nd quarter and ramping up from there on.

Subtask 5.6.1 (M5.6) Milestone M5.6

Start Date: End Date: Percentage Complete: 50

Feedback to Performer:

Summary Status: We have identified and engaged two potential consultants to work on this project. Both consultants have experience in the biofuel space and one of them was part of a previous ARPA-E funded team. We expect to select a consultant by 5/1/14.

Description: Milestone: Hire an independent T2M Consultant to oversee the tech-to-market plan and forge relationships with technology partners.

Milestones and Metrics:

	Name	Status	Start	End
M1.1	Milestone M1.1	The mcr genes from an ANME-2C organism were PCR-amplified and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into M. maripaludis. Unfortunately, expression was not sufficient to detect the His tag by Western blot. To improve expression we are now cloning a codon-optimized version.		
M1.2	Milestone M1.2	The mcr genes were PCR-amplified from M. marburgensis DNA and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into M. maripaludis, where expression was sufficient to detect the His tag by Western blot. The expression level has not yet been measured, but from the intensity of the Western it is low. To improve expression we are now cloning a codon-optimized version.		
M2.1	Milestone M2.1	We have completed the semi-automated reconstruction of version 1.0 of the M. maripaludis model using our maximum likelihood orthology approach. (See Section C, Figure 1). We have begun the manual curation of the M. maripaludis draft model based on biochemical, genetic, and physiological data from literature.		
M3.1	Milestone M3.1	We have received two strains containing the heterologously expressed MCR from John Leigh's group and are growing the cells to test activity. One strain contains a his-tagged MCR and the other lacks a purification tag.		
M3.2	Milestone M3.2	We will assay HDR in the cells sent from John Leigh's group.		

M4.1	Milestone M4.1	We have calculated all force field parameters for substrates and preliminary force field parameters for the MCR and ANME-1 cofactors. The calculated 1MRO.pdb structure differs by less than 1 Å from the initial crystal structure.
M5.1	Milestone M5.1	An IP sharing agreement was drafted and shared with all institutions in December 2013. The final institution has provided feedback in mid-March. Final discussions are being planned for the first week of April and we expect that this agreement will be finalized and signed by 4/30/14.
M5.2	Milestone M5.2	With the help of an OTT Fellow, a preliminary search of the patents related to the bioconversion of methane to methanol and to butanol has been completed. We expect to conduct further searches as new inventions are developed during the research.
M5.4	Milestone M5.4	We have completed a preliminary market assessment of the methane to methanol conversion market. We have also identified several potential competing technologies in the market.
M5.6	Milestone M5.6	We have identified and engaged two potential consultants to work on this project. Both consultants have experience in the biofuel space and one of them was part of a previous ARPA-E funded team. We expect to select a consultant by 5/1/14.

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Reducing Emissions Using Methanotrophic Organisms For Transportation Energy
ANAEROBIC BIOCONVERSION OF METHANE TO METHANOL

A. Executive summary:

Our project aims to develop transformational technologies for bioconversion of methane to liquid fuels. Enlisting researchers from a government laboratory, a research institute, and two universities, the project involves a team of four investigators with complementary expertise and the skills needed to successfully execute the project plan: John Leigh (Univ. Washington), Nathan Price (Institute for Systems Biology), Stephen Ragsdale (Univ. Michigan) and Dayle Smith (PNNL).

We have made progress on each of these aims as described in more detail below and I feel that all scientific aims are on track to timely completion. I have outlined some of the challenges and risks associated with the work. I feel that we have identified alternative strategies should any of the current plans fail. We also are on budget.

The mcr genes from M. marburgensis and an ANME-2C organism were PCR-amplified and cloned into a replicative expression vector and we are working towards optimizing expression of these protein to reach our goal of expression levels of at least 0.1 mg protein/g cell dw. We have built draft genome scale flux balance and core metabolic flux models for M. maripaludis metabolism and completed the semi-automated reconstruction of version 1.0 of the M. maripaludis model using our maximum likelihood orthology approach. We also have begun the manual curation of the M. maripaludis draft model based on biochemical, genetic, and physiological data from literature. We are culturing the two strains from the Leigh lab (above) and working to deliver MCR with a specific activity for the purified protein of at least 5 units/mg (after activation) in the methane synthesis direction and 0.5 units/mg in methane oxidation. We have built a potential energy model for MCR inter-atomic interaction potentials including calculating all force field parameters for substrates and preliminary force field parameters for the MCR and ANME-1 cofactors to yield a calculated MCR structure that is less than one Ångstrom different than the initial crystal structure.

Regarding the technology to market goals, we have reached agreement and signed an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB. We expect that this agreement will be finalized and signed by 4/30/14. We have developed a profile of the existing patents related to methane to methanol (and GTL) biotechnology and expect to conduct further searches as new inventions are developed during the research. In addition, we have completed a preliminary market assessment of the methane to methanol conversion market and identified several potential competing technologies in the market. We have identified and engaged potential consultants with experience in the biofuel space for this project and plan to be able to select a consultant by 5/1/14.

B. Status of milestones due in the current quarter (Q1FY14) and status of any overdue milestones. I have included all milestones that are being currently worked on. Note that no milestones are overdue.

WBS	Due Date	Status	Summary
M1.1	6/1/14	75% Complete	**Clone ANME MCR genes into *M. maripaludis* and confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw. Expressed protein will be oligo-His tagged for purification and blotting.** The *mcr* genes from an ANME-2C organism were PCR-amplified and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into *M. maripaludis*. Unfortunately, expression was not sufficient to detect the His tag by Western blot. To improve expression we are now cloning a codon-optimized version.
M1.2	11/1/14	75% Complete	**Clone *Methanothermobacter marburgensis* MCR genes into *M. maripaludis* and verify expression. Confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw.** The *mcr* genes were PCR-amplified from *M. marburgensis* DNA and cloned into a replicative expression vector with a His tag. This construct was successfully transformed into *M. maripaludis*, where expression was sufficient to detect the His tag by Western blot. The expression level has not yet been measured, but from the intensity of the Western it is low. To improve expression we are now cloning a codon-optimized version.
M2.1	12/1/14	75% Complete	**Build draft genome scale flux balance and core metabolic flux models for *M. maripaludis* metabolism: Deliver first genome-scale metabolic model capable of simulating growth and byproduct section with >75% accuracy.** We have completed the semi-automated reconstruction of version 1.0 of the *M. maripaludis* model using our maximum likelihood orthology approach. (See Section C, Figure 1). We have begun the manual curation of the *M. maripaludis* draft model based on biochemical, genetic, and physiological data from literature.
M3.1	10/1/14	25% Complete	**Deliver MCR with a specific activity for the purified protein of at least 5 units/mg (after activation) in the methane synthesis direction and 0.5 units/mg in**

			methane oxidation. We have received two strains containing the heterologously expressed MCR from John Leigh's group and are growing the cells to test activity. One strain contains a his-tagged MCR and the other lacks a purification tag.
M3.2	7/1/14	10% Complete	**Milestone: choose the HDR with a specific activity of at least 10 units/mg.** We will assay HDR in the cells sent from John Leigh's group.
M4.1	7/1/14	100% Complete	**Reproduce experimental MCR structure 1MRO.pdb with a root-mean-squared deviation of less than 10 Ångstroms.** We have calculated all force field parameters for substrates and preliminary force field parameters for the MCR and ANME-1 cofactors. The calculated 1MRO.pdb structure differs by less than 1 Å from the initial crystal structure.
M5.1	4/1/14	90% Complete	**Agree and sign an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB.** An IP sharing agreement was drafted and shared with all institutions in December 2013. The final institution has provided feedback in mid-March. Final discussions are being planned for the first week of April and we expect that this agreement will be finalized and signed by 4/30/14.
M5.2	4/1/14	100% Complete	**Develop a profile of the existing patents related to methane to methanol (and GTL) biotechnology.** With the help of an OTT Fellow, a preliminary search of the patents related to the bioconversion of methane to methanol and to butanol has been completed. We expect to conduct further searches as new inventions are developed during the research.
M5.3	4/1/14	Incomplete	**File provisional patent for our plan to convert methane to methanol.** Given the very early stage of the research, no patent filings have been completed. We will file patent applications as more data is obtained and further validation of the pathway is completed.
M5.4	7/1/14	70% complete	**Develop tech to market plan.** We have completed a preliminary market assessment of the methane to methanol conversion market. We have also identified several potential competing technologies in the market.
M5.6	7/1/14	50% Complete	**Hire an independent T2M Consultant to oversee the tech-to-market plan and forge relationships with technology partners.** We have identified and engaged two potential consultants to work on this project. Both consultants have experience in the biofuel space and one of them was part of a previous ARPA-E funded team. We expect to select a consultant by 5/1/14.

C. Supporting data & additional information

Milestones 1.1- 1.2: Build and refine a draft metabolic model for *M. maripaludis*

We identified *mcr* genes for three ANME organisms: ANME-1, ANME-2A, and ANME-2C. Anke Meyerdierks kindly sent us fosmid DNA for all three. For cloning, we have focused initially on ANME-2C, since five *mcr* genes are present in a single contiguous order in an operon, and the enzyme is not thought to harbor a form of the coenzyme F_{430} that differs from methanogens. (In contrast, the ANME-1 Mcr has a modified F_{430}, as well as unusual modifications of certain amino acids.) We successfully cloned the five-gene operon (*mcrBDCGA*) onto a replicative vector in two forms, one with a His tag on the N-terminus of McrB and one with a His tag on the C-terminus of McrA. In both cases the promoter is the high-expressing hmv promoter. We successfully introduced both clones into *M. maripaludis*. Unfortunately, we did not detect any expression of the His tag by Western blot.

We cloned the *M. marburgensis mcr* genes onto a replicative vector, successfully introduced them into *M. maripaludis*, and detected low expression by Western blot for the His tag on C-terminus of McrA. Since expression was low, we are in the process of cloning a codon-optimized version.

We also generated a C-terminal McrA His tag in the native gene of *M. maripaludis* itself. This may be useful since expression of the native gene is naturally high.

Milestones 2.1: Build and refine a draft metabolic model for *M. maripaludis*

We have built a draft metabolic model of *M. maripaludis* S2 using the ModelSEED with our likelihood-based gap filling approach. The ModelSEED is an automated pipeline for building functional draft genome-scale metabolic models for microbes. The pipeline includes steps to build an incomplete network from gene annotations and to fill gaps in the network in order to make it complete enough to perform simulations. The Price lab has developed a novel gap-filling approach to maximize consistency of gap-filling results with available genomic data, given metrics of the ambiguity in gene annotations (manuscript in preparation). The implementation of this approach closely interfaces with the ModelSEED

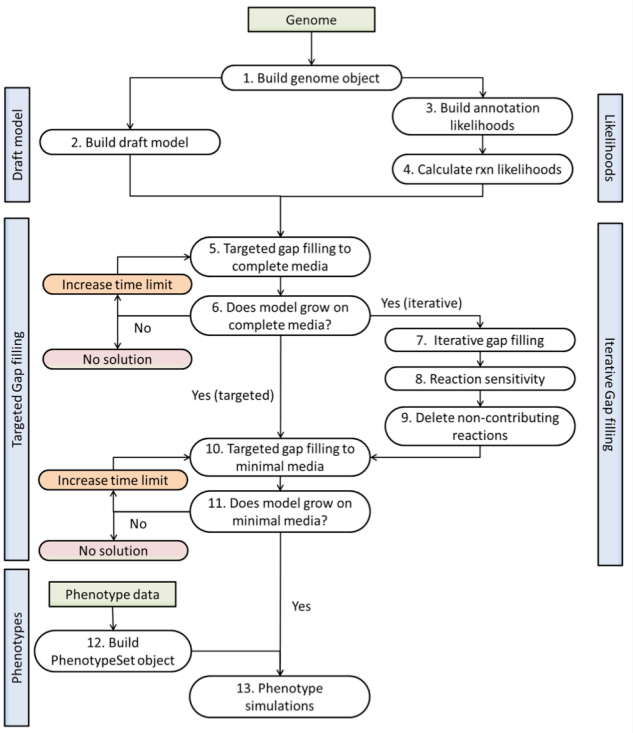

Figure 1*: Likelihood-based gap fill workflow*

tools and is integrated into the DOE KnowledgeBase framework (**Figure 1**).

Our draft model is able to predict growth and, after a small amount of curation, successfully predicts methane production using reasonable methanogenesis pathways. However, the biochemical representation of metabolism is incomplete in this model, due to possibly incorrect annotations and incomplete representation of archaeal metabolism in the ModelSEED biochemistry database and the SEED subsystems. This will be the focus of our work going forward.

We are aware of a recently published metabolic model of *M. maripaludis* (Goyal, et al., Mol Biosyst, Feb 20, 2014). This offers a possibility of accelerating our work. However, we have found numerous problems with that model, including incorrect methanogenesis pathways, which demonstrate that further curation is necessary. We are currently in the process of curating our draft model with using information from citations in the published model, biochemical databases such as MetaCYC and other literature sources that were not identified in the published model to improve the quality of the network. We are also combing through our in process *M. maripaludis* model iteratively with *M. maripaludis* expert John Leigh, a process we have followed earlier in high quality reconstructions of other methanogens (Benedict et al., 2012, J Bacteriol, 194(4):855-65 and Gonnerman et al, 2013, Biotech J, 8(9):1070-9).

At present, we have curated the metabolic pathways for generation of precursor metabolites and energy fermentation, including glycolysis, methanogenesis, pentose phosphate pathways, chemoautotrophic energy metabolism and hydrogen production. In this case, databases such as BioCyc, KEGG, NCBI and Brenda are used, and much more accurate information is being drawn from the literature about the strain (Hendrickson et al., 2004, J Bacteriol, 186(20):6956-696 and Hendrickson et al., 2007, PNAS 104(21):8930-4). Once the quality of the network is sufficient, we will perform simulations to aid strain design efforts**.** The curation confirms that the methanogenesis pathway, for which there are obvious flaw in the published model, is a key point, not only to methane production, but also to the growth and survival of the strain as part of the core carbon metabolism.

Milestones 3.1- 3.2:

We are optimizing conditions for culturing the host strain *M. maripaludis*, containing the heterologously expressed proteins. Once we have the conditions optimized for large-scale growth at the 10L scale, we will harvest cells and determine the endogenous activities of MCR, HDR and methyltransferase in *M. maripaludis*.

We have synthesized large quantities of the substrates, Coenzyme B and the heterodisulfide CoB-SS-CoM to assay both MCR and HDR. In the reverse direction, MCR is assayed using CoB-SS-CoM and methane as substrates. We been working with the native *M. marburgensis* MCR and have optimized the conditions for activating the enzyme (up to 90% of full activity) and for measuring its activity by determining methane directly (gas chromatography) and by measuring CH_3-SCoM conversion to methane (following decrease in radioactivity of $^{14}CH_3$-SCoM). We are assessing a novel way for measuring the reverse reaction by spectroscopically measuring conversion of the active Ni(I) enzyme to the Ni(II)/Ni(III) state in the presence of CoB-SS-CoM and methane so that we can compare the two classes of MCRs (ANME and the methane producing enzyme).

Milestone 4.1

Initial classical force field parameters (potential energy terms) were calculated for the MCR and ANME-1 nickel cofactors, substrates (CoM, CoB, SCoM-SCoB, and CoB analogues), and the MCR modified amino acids. The potential energy terms include "bonded" parameters (bond lengths, angles, dihedral periodicities and phases, and corresponding harmonic force constants), and "non-bonded" parameters (atom-centered point charges and Lennard-Jones radii and well depths).

The bonded parameters for all internal coordinates except those involving nickel were derived using the Generalized AMBER Force Field (GAFF). The nickel-porphyrin internal coordinates were taken from the initial structure 1MRO.pdb and force constants were copied from an iron-porphyrin force field. Atom-centered charges were calculated by fitting a quantum mechanical electrostatic potential to atom point-charges using Density Functional Theory (DFT).

A high-quality force field will reproduce and predict the structure, fluctuations and thermodynamic properties of a molecular system. Using Protein Data Bank structures for MCR with CoM+CoB (including C5, C6, C8 and C9 CoB analogues) and MCR with the SCoM-SCoB product with the novel force field parameters, we performed steepest-descent energy minimization to test the force field's ability to correctly model each molecular system. The root-mean-squared deviation between calculated and initial atomic coordinates is the measure of force field quality. Our M4.1 RMSD benchmark was an RMSD of 10 Ångstroms for 1MRO.pdb, and the model exceeded our expectations, producing RMSD values on the order of 0.1 Ångstroms (see table below).

PDB structure	**System**	**Cofactor**	**Root-mean-squared deviation (RMSD) of selected atoms relative to initial PDB structure (Å)**	
			Cα atoms	*Cofactor+substrate*
1MRO	MCR	F43	0.02	0.07
3M1V	MCR	F43	0.07	0.18
3M2R	MCR+COM+COB C5 analog	F43	0.06	0.12
3M2U	MCR+COM+COB C6 analog	F43	0.05	0.13
3M2V	MCR+COM+COB C8 analog	F43	0.06	0.15
3M30	MCR+COM+COB C9 analog	F43	0.07	0.18
3M32	MCR+disulfide product	F43	0.04	0.07

Currently we are deriving parameters for the modified ANME-1 amino acids 7-hydroxyl-L-tryptophan and S-oxymethionine. Once that is completed we can run the same initial calculations for ANME-1 as described above for the MCRs.

To improve the model we are now calculating MCR and ANME-1 cofactor geometries and atom-centered charges for Ni(I), Ni(II) and Ni(III) oxidation states using a combined QM/MM approach, which will complete milestone 4.1. Following that we will progress towards milestone 4.2, using molecular dynamics and statistical thermodynamics to identify at least ten amino acids that modulate substrate binding to MCR and ANME-1.

D. Major risks to future milestones:

Milestones 1.1-1.2
We have observed that the heterologously expressed MCRs from methanogens and anaerobic methanotrophs (ANMEs) exhibit low expression. It is likely that the problem is due to the presence of nonoptimal codons in the heterologously expressed protein. We are currently cloning a codon-optimized version and will compare expression and activity relative to the non-optimized genes.

Milestones 2.1-2.2
We have done the network reconstruction process many times before and so I don't anticipate any significant risk to not completing this milestone. The one issue that comes up with these reconstructions however is that the early stages generally move much faster than the last stages in terms of getting to a model that has high accuracy. Often one can build 90% of the model quite quickly, and it is the final 5-10% of the genes and the iterative comparison with data that takes all the time. We have developed a number of tools to help accelerate these processes (this is the fastest we have ever delivered a working draft genome-scale metabolic model), but I want to emphasize that while I am very happy with our progress so far, there is still a lot of curation needed to get it to the level of predictive power that we need here.

Milestones 3.1-3.2
The most serious concerns at present are (1) the low expression of the heterologously expressed MCRs from methanogens and anaerobic methanotrophs (ANMEs) and (2) the low inherent activity of methane oxidation relative to methane synthesis. Regarding the first concern, we will soon be testing activity and expression of the codon-optimized MCRs, which we hope will have increased expression and activity. Regarding the second concern, we are relying on the hypothesis that there has been no selective pressure for organisms to naturally develop a higher methane oxidation activity. It will be important to have an unambiguous map of the catalytic cycle, especially the rate-limiting step (RLS) so that we can work with D. Smith at PNNL to guide our mutagenesis efforts to lower the activation barrier for that RLS. We also are considering strategies for random mutagenesis and selection for a higher activity of methane oxidation.

Milestone 4.1
Our chances of success are maximized by a high-level derivation of force field parameters using Density Functional Theory. The MCR structure is similar to others we have modeled, especially the Mo-dependent nitrogenase, and based on this experience we don't expect any unusual behavior during molecular dynamics.

E. Budget Summary:

The project is on budget for the current period.

II. Issues, Risks, and Mitigation

N/A

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III. Changes in Approach

N/A

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

IV. Key Personnel

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Stephen W Ragsdale, Professor, Department of Biological Chemistry, University of Michigan Medical School, 1150 W. Medical Center Dr., 5301 MSRB III (5220D, my office), Ann Arbor, MI 48109-0606. Phone: 734-615-4621; Lab: 615-6150, main office: 734-763-6489, Fax: 734-763-4581,email: sragsdal@umich.edu, website: http://www.biochem.med.umich.edu/?q=ragsdale. Ragsdale is expert in biochemistry and enzymology of methanogenesis. Much of his work has focused on determining the enzymatic mechanisms of metalloenzymes involved in methanogenesis His laboratory includes specialized equipment for spectroscopy, electrochemistry, transient and steady-state kinetics and for performing anaerobic manipulations.

John Leigh, Department of Microbiology, University of Washington, Seattle, WA 98195-7735, J117a Health Sciences Bldg., 206-685-1390. Email: leighj@u.washington.edu. Dr. Leigh is expert in the biochemistry and genetics of methanogenesis. He has developed and applied genetic approaches in methanogens to the analysis of electron flow and energy conservation. He has developed methods to heterologously express and purifiy proteins in *Methanococcus maripaludis*. He also studies regulation of methanogenesis in projects funded by NIH and NSF.

Dr. Nathan D. Price, Institute for Systems Biology, 401 Terry Avenue North, Seattle, WA 98109, Phone: 206-732-1204. Email: nprice@systemsbiology.org. Dr. Price is a leader in the field of metabolic modeling and has reconstructed genome-scale metabolic models for two methanogenic species, each showing 96% accuracy when compared to growth phenotype data. Additionally, his method for integrating gene regulatory and metabolic networks (Probabilistic Regulation of Metabolism) is the method being implemented into DOE's Knowledgebase.

Dayle Smith, Senior Research Scientist, Pacific Northwest National Laboratory, PO Box 999, MSIN: J4-33, Richland, WA 99352, Tel: (509) 375-4358, Fax: (509) 375-7637, email: DayleMA.Smith@pnnl.gov. Dr. Smith has wide expertise in combining quantum, classical and statistical mechanical methods to model the chemical and thermodynamic properties of metalloproteins including elucidating the binding and chemical steps and catalytic roles of specific amino acids.

V. Project Output

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

A. Publications

Journal Article:

N/A

Paper:

N/A

Other:

N/A

B. Technologies / Techniques

N/A

C. Status Reports

N/A

D. Media Reports

N/A

E. Invention Disclosures

N/A

F. Patent Applications:

N/A

G. Licensed Technologies

N/A

H. Partnerships Formed

N/A

I. Websites Featuring Project Work or Results

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N/A

J. Other Products

N/A

K. Awards, Prizes, and Recognition

N/A

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VI. Follow-On Funding

N/A

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VII. Recipient and Principal Investigator Disclosure

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

N/A

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

VIII. Conflicts of Interest within Project Team

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

Project Team Conflict of Interest Exists: No

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

IX. Performance of Work in the United States

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

Work has been performed outside of the United States: No

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

X. Project Schedule Status

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Task Title	Task Number	Milestone	Schedule				
			Start Date	Finish Date	Actual Start	Actual Finish	Estimated % Complete
Task 1.0	**1.0**		**1/23/2014**	**1/1/2017**			**0%**
Sub-Tasks 1.1	1.1		1/23/2014	6/1/2014			75%
Milestone M1.1	M1.1	▼	6/1/2014	6/1/2014			75%
Sub-Tasks 1.2	1.2		6/1/2014	11/1/2014			75%
Milestone M1.2	M1.2	▼	11/1/2014	11/1/2014			75%
Sub-Task 1.3	1.3		11/1/2014	1/1/2015			0%
Milestone M1.3	M1.3	▼	1/1/2015	1/1/2015			0%
Sub-Task 1.4	1.4		1/1/2015	5/1/2015			0%
Milestone M1.4	M1.4	▼	5/1/2015	5/1/2015			0%
Sub-Task 1.5	1.5		5/1/2015	6/1/2015			0%
Milestone 1.5	M1.5	▼	6/1/2015	6/1/2015			0%
Sub-Task 1.6	1.6		6/1/2015	7/1/2015			0%
Milestone M1.6	M1.6	▼	7/1/2015	7/1/2015			0%
Sub-Task 1.7	1.7		7/1/2015	1/1/2017			0%
Milestone M1.7	M1.7	▼	1/1/2017	1/1/2017			0%
Sub-Task 1.8	1.8		7/1/2015	12/1/2015			0%
Milestone M1.8	M1.8	▼	12/1/2015	12/1/2015			0%
Sub-Task 1.9	1.9		12/1/2015	2/1/2016			0%
Milestone M1.9	M1.9	▼	2/1/2016	2/1/2016			0%
Sub-Task 1.10	1.10		2/1/2016	4/1/2016			0%
Milestone M1.10	M1.10	▼	4/1/2016	4/1/2016			0%
Sub-Task 1.11	1.11		4/1/2016	8/1/2016			0%
Milestone M1.11	M1.11	▼	8/1/2016	8/1/2016			0%
Sub-Task 1.12	1.12		8/1/2016	9/1/2016			0%
Milestone M1.12	M1.12	▼	9/1/2016	9/1/2016			0%
Sub-Task 1.13	1.13		9/1/2016	11/1/2016			0%
Milestone M1.13	M1.13	▼	11/1/2016	11/1/2016			0%
Sub-Task 1.14	1.14		11/1/2016	1/1/2017			0%
Milestone M1.14	M1.14		1/1/2017	1/1/2017			0%
Task 2.0	**2.0**		**1/23/2014**	**10/31/2016**			**0%**
Sub-Task 2.1	2.1		1/23/2014	4/1/2014			75%
Sub-Task 2.2	2.2		4/1/2014	12/1/2014			75%
Milestone M2.1	M2.1	▼	12/1/2014	12/1/2014			75%
Sub-Task 2.3	2.3		5/1/2014	2/1/2015			0%
Sub-Task 2.4	2.4		1/1/2014	2/1/2015			0%

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Milestone M2.2	M2.2	▼	2/1/2015	2/1/2015			0%
Sub-Task 2.5	2.5		2/1/2014	6/1/2014			0%
Sub-Task 2.6	2.6		6/1/2014	11/1/2014			0%
Sub-Task 2.7	2.7		11/1/2014	2/1/2015			0%
Sub-Task 2.8	2.8		2/1/2015	7/1/2015			0%
Milestone M2.3	M2.3	▼	7/1/2015	7/1/2015			0%
Sub-Task 2.9	2.9		6/1/2015	7/1/2015			0%
Sub-Task 2.10	2.10		7/1/2015	8/1/2015			0%
Milestone M2.4	M2.4	▼	8/1/2015	8/1/2015			0%
Sub-Task 2.11	2.11		8/1/2015	10/1/2015			0%
Sub-Task 2.12	2.12		10/1/2015	1/1/2016			0%
Sub-Task 2.13	2.13		1/1/2016	10/31/2016			0%
Milestone M2.5	M2.5	▼	10/31/2016	10/31/2016			0%
Task 3.0	**3.0**		**1/23/2014**				**0%**
Sub-Task 3.1	3.1		1/23/2014	4/1/2014			25%
Sub-Task 3.2	3.2		2/1/2014	12/1/2014			25%
Milestone M3.1	M3.1	▼	12/1/2014	12/1/2014			25%
Sub-Task 3.3	3.3		1/23/2014	7/1/2014			10%
Milestone M3.2	M3.2	▼	7/1/2014	7/1/2014			10%
Sub-Task 3.4	3.4		12/1/2014	12/1/2015			0%
Milestone M3.3	M3.3	▼	12/1/2015	12/1/2015			0%
Sub-Task 3.5	3.5		7/1/2015	11/1/2015			0%
Sub-Task 3.6	3.6		11/1/2015	8/1/2016			0%
Milestone M3.4	M3.4	▼	8/1/2016	8/1/2016			0%
Sub-Task 3.7	3.7		4/1/2016	11/1/2016			0%
Milestone M3.5	M3.5	▼	11/1/2016	11/1/2016			0%
Sub-Task 3.8	3.8		6/1/2016	12/1/2016			0%
Milestone M3.6	M3.6	▼	12/1/2016	12/1/2016			0%
Sub-Task 3.9	3.9		9/1/2016	1/1/2017			0%
Sub-Task 3.10	3.10		9/1/2016	1/1/2017			0%
Milestone M3.7	M3.7	▼	1/1/2017	1/1/2017			0%
Task 4.0	**4.0**		**1/23/2014**	**1/1/2017**			**0%**
Sub-Task 4.1	4.1		1/23/2014	7/1/2014			100%
Milestone M4.1	M4.1	▼	7/1/2014	7/1/2014			100%
Sub-Task 4.2	4.2		7/1/2014	10/1/2015			0%
Milestone M4.2	M4.2	▼	10/1/2015	10/1/2015			0%
Sub-Task 4.3	4.3		10/1/2015	3/1/2016			0%
Milestone M4.3	M4.3	▼	3/1/2016	3/1/2016			0%
Sub-Task 4.4	4.4		1/1/2016	8/1/2016			0%
Milestone M4.4	M4.4	▼	8/1/2016	8/1/2016			0%

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Sub-Task 4.5	4.5		8/1/2016	1/1/2017			0%
Milestone M4.5	M4.5		1/1/2017	1/1/2017			0%
Task 5.0	**5.0**		**1/23/2014**	**12/1/2016**			**0%**
Sub-Task 5.1	5.1		1/23/2014	4/1/2014			90%
Milestone M5.1	M5.1	▼	4/1/2014	4/1/2014			90%
Sub-Task 5.2	5.2		1/1/2014	4/1/2014			50%
Milestone M5.2	M5.2	▼	4/1/2014	4/1/2014			100%
Milestone M5.3	M5.3	▼	4/1/2014	4/1/2014			0%
Sub-Task 5.3	5.3		1/23/2014	7/1/2014			70%
Milestone M5.4	M5.4	▼	7/1/2014	7/1/2014			70%
Sub-Task 5.4	5.4		7/1/2014	10/1/2014			0%
Milestone M5.5	M5.5	▼	10/1/2014	10/1/2014			0%
Sub-Task 5.6	5.6		1/23/2014	7/1/2014			50%
Milestone M5.6	M5.6	▼	7/1/2014	7/1/2014			50%
Sub-Task 5.7	5.7		10/1/2014	2/1/2015			0%
Milestone M5.7	M5.7	▼	2/1/2015	2/1/2015			0%
Sub-Task 5.8	5.8		4/1/2016	4/1/2016			0%
Sub-Task 5.9	5.9		8/1/2014	2/1/2015			0%
Milestone M5.8	M5.8	▼	2/1/2015	2/1/2015			0%
Sub-Task 5.10	5.10		2/1/2015	12/1/2016			0%
Milestone M5.9	M5.9	▼	8/1/2015	8/1/2015			0%
Sub-Task 5.11	5.11		7/1/2015	12/1/2016			0%
Sub-Task 5.12	5.12		6/1/2014	10/1/2016			0%
Milestone M5.10	M5.10	▼	9/1/2014	9/1/2014			0%
Milestone M5.11	M5.11	▼	9/1/2015	9/1/2015			0%
Milestone M5.12	M5.12		12/1/2016	12/1/2016			0%

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XI A. Budget Status Lead Organization

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Cost Type Name	Total Project Cost	Quarterly Invoiced Amounts	Cumulative Invoiced To Date	Remaining Balance
PersonnelCharges	$236,606.97	$0.00	$0.00	$236,606.97
FringeBenefitsCharges	$63,379.80	$0.00	$0.00	$63,379.80
TravelCharges	$12,600.00	$0.00	$0.00	$12,600.00
EquipmentCharges	$87,372.89	$0.00	$0.00	$87,372.89
SuppliesCharges	$60,000.00	$0.00	$0.00	$60,000.00
ContractualCharges	$1,813,835.63	$0.00	$0.00	$1,813,835.63
ConstructionCharges	$0.00	$0.00	$0.00	$0.00
OtherCharges	$159,000.00	$0.00	$0.00	$159,000.00
IndirectCharges	$321,894.67	$0.00	$0.00	$321,894.67
Total	$2,754,689.96	$0.00	$0.00	$2,754,689.96
FederalShare	$2,552,533.96	$36,678.57	$36,678.57	$2,515,855.39
PerformerShare	$202,156.00	$0.00	$0.00	$202,156.00
Cost Share %	7%	0%	0%	7%

Cost Type Name	Quarterly Invoiced Amounts	Cumulative Invoiced To Date
TT&O PersonnelCharges	$0.00	$0.00
TT&O FringeBenefitsCharges	$0.00	$0.00
TT&O TravelCharges	$0.00	$0.00
TT&O EquipmentCharges	$0.00	$0.00
TT&O SuppliesCharges	$0.00	$0.00
TT&O ContractualCharges	$0.00	$0.00
TT&O ConstructionCharges	$0.00	$0.00
TT&O OtherCharges	$0.00	$0.00
TT&O IndirectCharges	$0.00	$0.00
Total	$0.00	$0.00

Agree that the information provided is accurate and correct: Yes

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XI B. Budget Status Sub Organization

May contain trade secrets or commercial or financial information that is privileged or confidential and exempt from public disclosure.

Award Number: WAS___PNNL
Organization Name:

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Cost Type Name	Total Project Cost	Quarterly Invoiced Amounts	Cumulative Invoiced To Date	Remaining Balance
PersonnelCharges	$171,490.93	$0.00	$0.00	$171,490.93
FringeBenefitsCharges	$53,833.00	$0.00	$0.00	$53,833.00
TravelCharges	$5,926.00	$0.00	$0.00	$5,926.00
EquipmentCharges	$0.00	$0.00	$0.00	$0.00
SuppliesCharges	$0.00	$0.00	$0.00	$0.00
ContractualCharges	$0.00	$0.00	$0.00	$0.00
ConstructionCharges	$0.00	$0.00	$0.00	$0.00
OtherCharges	$606.00	$0.00	$0.00	$606.00
IndirectCharges	$226,161.00	$0.00	$0.00	$226,161.00
Total	$458,016.93	$0.00	$0.00	$458,016.93
FederalShare	$458,016.93	$0.00	$0.00	$458,016.93
PerformerShare	$0.00	$0.00	$0.00	$0.00
Cost Share %	0%	0%	0%	0%

Agree that the information provided is accurate and correct: Yes

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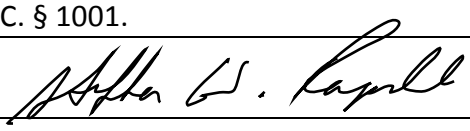
XII. Certification of Compliance

I have the authority to make the following certification on behalf of the Prime Recipient named above. On behalf of the Prime Recipient, I further certify that the information provided in this Research Performance Progress Report is accurate and complete as of the date shown below. I understand that false statements or misrepresentations may result in civil and/or criminal penalties under 18 U.S.C. § 1001.	
SIGNATURE: 	DATE: 4-16-14
TYPED NAME: Stephen W. Ragsdale	
TITLE: Professor, Dept. of Biol. Chemistry	
ORGANIZATION: University of Michigan	