**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.

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| **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 F430 that differs from methanogens. (In contrast, the ANME-1 Mcr has a modified F430, 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 tools and is integrated into the DOE KnowledgeBase framework (**Figure 1**).

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**Figure 1*: Likelihood-based gap fill workflow***

Our draft model was able to predict growth and, after a small amount of curation, successfully predict methane production using reasonable methanogenesis pathways. However, the biochemical representation of metabolism was 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 is the current focus of our work.

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 *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). Through these interactions, we have updated ATP metabolism and added specific ferredoxin types to more accurately represent the *M. maripaludis* electron transport chain. Furthermore, we have identified and corrected numerous incorrect gene annotations in the draft model, improving our network’s accuracy while adding over 20% more genes to our model.

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. We have also altered the model to correct formate catabolism for methane production and add the acetate uptake pathway. 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 themethanogenesis 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 CH3-SCoM conversion to methane (following decrease in radioactivity of 14CH3-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).

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| **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.