**Reducing Emissions Using Methanotrophic Organisms For Transportation Energy**

**Anaerobic Bioconversion Of Methane To Methanol**

**A.    Executive summary**:

Our REMOTE 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). Nadine Wong in our technology transfer office is our T2M liason.Briefly, our scientific specific aims are:

1. To actively express the gene clusters encoding the MCRs from *M. marburgensis* and from the anaerobic methane oxidizers, ANME-1 and ANME-2, in a genetically tractable methanogen; to genetically engineer the pathways for converting methane to methanol and to couple this pathway to the sulfate reduction path, which makes this process thermodynamically favorable.
2. To use genome-scale metabolic flux modeling of the genetically engineered *Methanococcus maripaludis* strains, thus, ensuring that enzymes in the engineered pathway can operate at high efficiency in the new organism. We will also reengineer other aspects of *M. maripaludis* metabolism by performing genetic alterations to optimize the methanol pathway in its new metabolic context and perform metabolomic and metabolic flux experiments to iteratively test, refine, and validate the metabolic model.
3. To purify the newly introduced enzymes from *M. maripaludis* and conduct in vitro biochemical studies to test their functionality. We will also conduct pathway flux measurements and efficiency determinations aimed at optimizing methane oxidation to liquid fuels, and
4. To use computational studies to understand the mechanism of the anaerobic methane oxidizing enzyme, MCR, and to predict the effects of site-directed mutagenesis on the reaction mechanism.

We have made progress on each of these aims and all scientific aims are on track to timely completion. I have outlined challenges and risks associated with the work and identified alternative strategies should any of the current plans fail. We also are on budget.

With respect to Aim 1, we generated a codon-optimized version of the ANME-2C mcr genes and cloned them under control of the nif promoter. We detected a positive Western blot signal under N-fixing conditions, confirming regulated expression. We cloned codon-optimized versions of the *M. marburgensis* mcr genes and confirming expression by Western blotting. The Western signal appears stronger with the codon-optimized version than it did with the non-codon optimized version. Quantitation of yield is under way.

With respect to Aim 2, we built draft genome scale flux balance and core metabolic flux models for M. marapaludis metabolism and completed the semi-automated reconstruction of version 1.0 of the *M. maripaludis* model using a maximum likelihood orthology approach. Since completing the initial version, we have expanded and improved the model through manual curation based on biochemical, genetic, and physiological data from literature, improving the depiction of the electron transport chain, corrected the formate and acetate catabolism pathways, and added over 20% more reaction-associated genes.

With respect to Aim 3, we are culturing the two ANME strains for enzymatic measurements. In rapid kinetics experiments with the *M. marburgensis* enzyme, we observe a rate constant for MCR in methane ***oxidation*** of 0.8 s-1 (20 oC) and even more rapid reaction with methane. Thus, the key steps in the catalytic cycle appear to occur at >0.1 units/mg, near our goal of 0.5 units/mg.

For Aim 4, we calculated all force field parameters for cofactors of MCR, ANME-1, modified amino acids, CoM and CoB. We are using molecular dynamics and statistical thermodynamics to identify at least ten animo acids that modulate substrate binding to MCR and ANME-1. We built a potential energy model for MCR inter-atomic interaction potentials including calculating force field parameters for substrates and cofactors to calculate an MCR structure with < 1 Å difference from the initial crystal structure.

Regarding Aim 5 (technology to market), with Nadine Wong (in our OTT), we have engaged Michael Psarouthakis, who recently joined OTT as Senior Business Formation specialist. We reached agreement and signed an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB. We 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. We have completed a preliminary market assessment of the methane to methanol conversion market and identified several potential competing technologies in the market. We hired Rich Zvosec, President of High Hurdles, LLC, as a technology consultant. He has extensive experience in the biofuel space and with ARPA-E. The team is working through OTT with an attorney, Dr. David Casimir, Ph.D., J.D, (Casimir Jones based in Madison WI) to draft a patent describing the recent IP on the genetically engineered organisms.

Before addressing the Status of Milestones, I will directly reply to the three questions sent by Chad on July 7.

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| **M5.1 Agree and sign an IP sharing agreement among U. Michigan, U. Washington, PNNL, and ISB.** | **GREEN** |
| *The team reported that an IP sharing agreement was draft and shared with all parties*. **Question: Has the agreement been finalized and signed by everyone? Yes. See Section B.** | |
| **M5.2 Develop a profile of the existing patents related to methane to methanol (and GTL) biotechnology.** | **GREEN** |
| Completed with assistance from OTT Fellow, a preliminary search of patents related to the bioconversion of methane. | |
| **M5.3 File a provisional patent.** | **RED** |
| *The team reported that no IP has been filed. At the upcoming site visit, ARPA-E would like an update on the team’s IP strategy. If no IP has been filed at that time, the strategy should include a clear description of why that is, and the criteria the team will use to determine the right time for filing.* Nadine Wong and Steve Ragsdale met recently with attorney, Dr. David Casimir, who determined that there is sufficient IP at this stage to file a provisional patent. We are currently drafting this document. Also please see section B. | |

Related to the cloning of *M. marburgensis* MCR in *M. maripaludis*” it was stated*: “*although I think we only have confirmation of subunit A”. The A subunit is the last gene in the operon so the entire protein should be expressed in amounts at least as high as subunit A.

The next on-site visit will be Sept 5, 2014 at the University of Michigan.

**B.    Status of milestones due in the current quarter (Q2FY14) 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 | 90% 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 codon-optimized version of the ANME-2C *mcr* genes was cloned after the *nif* promoter. A positive Western blot signal was detected under nitrogen fixing conditions, confirming expression of the protein. Quantitation of yield is under way. |
| M1.2 | 11/1/14 | 90% 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 codon-optimized version of the *M. marburgensis* *mcr* genes was cloned after the *hmv* promoter. A positive Western blot signal was detected, confirming expression of the protein. The Western signal appears stronger with the codon-optimized version than it did with the non-codon optimized version. Quantitation of yield is under way. |
| 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 2). Our manual curation of the *M. maripaludis* draft model has improved the accuracy of the electron transport chain, corrected uptake pathways for formate and acetate, and added over 20% more genes to the model using biochemical, genetic, and physiological data from literature during this past quarter. |
| M3.1 | 10/1/14 | | 50% 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 are culturing the two ANME strains for enzymatic measurements. In rapid kinetics experiments with the *M. marburgensis* enzyme, we observe a rate constant for MCR in methane ***oxidation*** of 0.8 s-1 (20 oC) and even more rapid reaction with methane. This rate equates to >0.1 units/mg, near our goal of 0.5 units/mg. |
| M3.2 | 7/1/14 | | 20% 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 | | 100% Complete | **Agree and sign an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB.** We reached agreement and signed an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB. |
| 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 | | 20% complete | **File provisional patent for our plan to convert methane to methanol.** We are drafting a provisional patent and hope to have it filed by the time of the Sept 5, 2014 meeting. |
| M5.4 | 7/1/14 | | 70% complete | **Develop tech to market plan.** We have engaged Michael Psarouthakis, who recently joined OTT as Senior Business Formation specialist. 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 | | 100% Complete | **Hire an independent T2M Consultant to oversee the tech-to-market plan and forge relationships with technology partners.** We hired Rich Zvosec, President of High Hurdles, LLC, as technology consultant. He has experience in the biofuel space and with ARPA-E. |

**C.    Supporting data & additional information**

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| **Milestones 1.1- 1.2: Express Mcr proteins in *M. maripaludis***  Use of codon-optimized genes has resulted in successful expression of the ANME-2C and *Methanothemobacter marburgensis* Mcr proteins in *M. maripaludis*. The figure shows Western blots using anti His6 antibody to detect the tagged McrA subunits. The ANME-2C genes, cloned after the *nif* promoter for nitrogen fixation, show expression under nitrogen fixing conditions as expected (Fig. 1). The *M. marburgensis* genes are cloned after the constitutive *hmv* promoter. The use of the inducible *nif* promoter may be an effective strategy, and indeed we have not had success with the *hmv* promoter with the ANME 2-C genes. We are therefore investigating the use of the *nif* promoter for the *M. marburgensis* genes. We are currently scaling up growth of the Mcr expressing strains for the fermenter. We will measure the yield of protein when we begin purification. (In the figure, CbiX is a protein in *M. maripaludis* that contains a stretch of His residues and therefore also binds the antibody). | Figure 1. Expression of MCRs |

**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 2**).

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Figure 2*: 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 with Dr. Leigh, 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.

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 altered the model to correct formate catabolism for methane production and added 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 & Hendrickson et al., 2007, PNAS 104(21):8930-4). Once the network quality is sufficient, we will perform simulations to aid strain design efforts**.** The curation confirms that themethanogenesis pathway 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:**

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| We have optimized conditions for small-scale growth of the host strain *M. maripaludis*, containing the heterologously expressed proteins from *M. marburgensis* and the two ANME strains. We are now optimizing for large-scale growth at the 10L scale in order to determine endogenous activities of MCR, HDR and methyltransferase in *M. maripaludis* and to devise/revise conditions for activation of the proteins. | Macintosh HD:Users:sragsdal:Desktop:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:Quarterly Reports:second quarter:FIrst half rxn_MCR_CH4Ox.tif  Fig 3. Single turnover kinetics – methane oxidation |

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 have optimized the conditions for activating the *M. marburgensis* enzyme and for measuring its activity in the methane synthesis direction. We currently are 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 ± methane so that we can compare the activities of the two classes of MCRs (ANME and the methane producing enzyme).

With the *M. marburgensis* enzyme, we have observed a surprisingly fast rate constant for methane ***oxidation*** (0.8 s-1, 20 oC) (Fig. 3). Thus, the key steps in the catalytic cycle appear to occur at >0.1 units/mg, which is near our goal (0.5 units/mg). Our results have also opened the door to a new mechanism for methane synthesis/oxidation that has not previously been considered.

**Milestones 4.1-4.3**

**Milestone 4.1 :** Final classical force field parameters (potential energy terms) were calculated using Density Functional Theory for the MCR and ANME-1 nickel cofactors, substrates (CoM, CoB, SCoM-SCoB, and CoB 6, 7, and 8-carbon analogues), and modified amino acids.

To expand the model we have calculated MCR cofactor geometries and atom-centered charges for the reactive MCR-Red1 cofactor and substrates using a hybrid quantum/classical mechanical approach. Now we are progressing towards milestone 4.2.

**Milestone 4.2:** Currently we are making progress toward 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. Specifically, we are calculating biochemical properties for the MCR-Red1 state using the CoB6, CoB7 and CoB8 to understand how the substrate length relates to binding affinity and dynamics.

**Milestone 4.3:** Simultaneously, we are using hybrid classical/quantum mechanics to test a new reaction pathway discovered from the experiments in Aim 3.

**D.    Major risks to future milestones**:

**Milestones 1.1-1.2**

The ongoing challenge will be to produce useful levels of heterologously expressed proteins. In addition to codon optimization and the use of suitable promoters, strategies will include measures to improve mRNA stability and protein stability and solubility.

**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 are optimistic about use of the codon-optimized MCRs, which provide increased expression. Regarding the second concern, our recent results using single turnover kinetics indicate that the rates of methane oxidation are not as slow as expected; in fact, the chemical steps seem to be near our goal. We are developing methods for measuring steady-state kinetics to see where the bottleneck in the mechanism lies. One important effort is to identify the rate-limiting step so that we can work with D. Smith at PNNL to guide our mutagenesis efforts if we do indeed need to lower the activation barrier for that step. We also are considering strategies for random mutagenesis and selection for a higher activity of methane oxidation.

**Milestone 4.1-4.3**

**Milestone 4.1:** Our chances of success of achieving milestones 4.2-4.3 is maximized by the high-level derivation of force field parameters using Density Functional Theory achieved in this aim. The resulting potential energy terms validate the experimentally-measured structures of MCR and bound cofactors, therefore this milestone is complete (as described in the 1st quarter report).

**Milestone 4.2:** Our chances of success in predicting structural, dynamic and thermodynamic properties of MCR reactivity via classical mechanics are high based on the observations so far. MD calculations of MCR-Red1 bound to CoB6/7/8 cofactors have “equilibrated” (relaxed in the solvent environment under ambient conditions). Currently the > 100 ns MD simulations are running, and preliminary analysis indicates that the observed relative flexibility of the cofactors is due to differing numbers of hydrogen bonds to the protein environment.

**Milestone 4.3:** The work related to milestone 4.3 is very far ahead of schedule, due to the fact that a novel and promising mechanism was very recently proposed by Ragsdale’s lab. It is preferable to model this new reaction parallel with the experiments to optimizing links between Aims 3 and 4. For this reason we are embarking on this task ahead of schedule (FY16 as initially scheduled). This work is in the very preliminary stages, setting up and testing the appropriate computational parameters. The risk associated with this milestone is the possibility of being “scooped” by competing scientists.

**E.    Budget Summary:**

The project is on budget for the current period.