**Second Quarter (January 1-March 31, 2015) Report – Team Michigan**

**Reducing Emissions Using Methanotrophic Organisms For Transportation Energy (REMOTE)**

**Anaerobic Bioconversion Of Methane To Methanol**

**A.    Executive summary**:

Our REMOTE project aims to develop transformational technologies for bioconversion of methane to liquid fuels. We plan to engineer the genetically tractable methanogen, *Methanococcus maripaludis*, with genes that encode the conversion of methane to methanol. Because this process is thermodynamically unfavorable, we also will include the genes encoding sulfate or nitrate reduction.

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 *M. 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 have previously reported successful cloning and expression of tagged versions of the ANME 2c and *M. marburgensis* MCRs in *M. maripaludis* as well as a His-tagged version of the *M. maripaludis* MCR at levels that exceed our milestone goal of 0.1 mg protein/g cell dw and demonstrated the three subunits of the targeted enzymes are present and that the F430 cofactor is incorporated.

In the last quarter, we determined that the levels of the heterologously expressed ANME and *M. marburgensis* enzymes are lower than our target levels and do not complement the native enzyme; thus, we are working to increase these levels, in the event that the *M. maripaludis* MCR does not work sufficiently well in catalyzing the reverse reaction. We successfully cloned the heterologousmethanol methyltransferase genes into *M. maripaludis* and verified expression by Western blot.

With respect to Aim 2, we have previously reported construction and completion of a genome scale flux balance model for *M. marapaludis* metabolism using a maximum likelihood orthology approach and refinement through manual curation based on biochemical, genetic, and physiological data from literature.

In the last quarter, we took steps to further refine our model outside of the well-characterized methanogenesis pathway Though the core metabolism of our model remains largely unchanged, we have improved the model’s portrayal of various uptake and biosynthesis pathways, as well as revising our biomass composition. We have also concluded harvests for targeted metabolomics experiments and submitted cells for analysis; we expect to receive these data in the next quarter. We have begun experiments to measure dry cell weights and growth yields which, coupled with the results of our metabolomics experiments, will give us a more robust set of data to use for model training and validation. We have started using our model to probe possible engineering strategies to alter *M. maripaludis* for conversion of methane to methanol and expect to have preliminary predictions in the next quarter.

With respect to Aim 3, we previously demonstrated expression and F430 incorporation in the ANME, *M. marburgensis* and *M. maripaludis* MCRs and reported surprisingly rapid rates (>0.8 s-1 under non-optimal temperatures of 20 oC) of anaerobic methane oxidation by the thermophilic *M. marburgensis* MCR under single turnover conditions. We also had shown that this rate constant is near our goal (0.5 units/mg).

In the last quarter, we found that in vitro activation of the heterologously expressed MCR has not been successful. On the other hand the *M. maripaludis* MCR was shown to be highly active in both forward and reverse directions. Thus, we expect that the native enzyme will support methane oxidation to methanol and do not expect to need to genetically engineer an enzyme from ANME.

With respect to Aim 4, we previously generated a model for the active MCRred1, modeled binding of the substrate CoB to the enzyme, and demonstrated that CoB explores multiple rotamers that allow the CoB(SH) to approach the CoM methyl group. We also observed significant fluctuations in the Tyr333(OH)-CoM(S) distances indicating that MCR fluctuations modulate the proton affinity of CoM.

In the last quarter, we successfully reproduced the MCR structure, with a difference of less than 1 Å beween the calculated and the experimental crystal structure. We also have applied a potential energy function to identify amino acids that contribute to substrate binding. We also calculated the active MCRred1 structure and have revised the computational program to accommodate the number of atoms required to identify catalytically-important groups in the MCR substrate binding channel and active site and to analyze concerted motions of these groups that are key to catalysis of methane formation and oxidation. These are key upgrades that will allow us to theoretically validate activation energies in proposed MCR pathways using experimental data from Ragsdale’s lab and to perform QM/MM geometry optimizations of reaction intermediates proposed by Ragsdale’s lab.

With respect to Aim 5, we previously finalized an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB (participating institutions), developed a profile of the existing patents related to methane to methanol (and GTL) biotechnology, completed a preliminary market assessment of the methane to methanol conversion market and identified several potential competing technologies in the market. We also hired a consultant (Rich Zvosec of High Hurdles), who has extensive experience in the biofuel space. We developed an IP strategy document that sets forth a protocol and strategy for best practices capture of IP generated during the project.

In the past quarter, we filed a provisional patent application on generation of a new organism that contains the MCR gene, developed our initial T2M plan, and are beginning to develop a pitch deck to use in meetings with potential partners, funders, etc.

**B.    Status of milestones due in the current quarter (Q2FY15) and status of any overdue milestones**. I included all milestones that being currently worked on. No milestones are overdue. Changes requested and discussed with staff at the last project report are highlighted below (boldface & Red).

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| **WBS** | **Due Date** | **Status** | **Summary** |
| M1.3 | 12/31/14 | 20% complete | **Test for genetic complementation of MCR deletion mutation in *M. maripaludis*. Confirm activity of introduced enzymes by demonstrating viability of *M. maripaludis* MCR deletion mutants in expression strains.**  This has been attempted for the *M. marburgensis* MCR-expressing strain. To date, the deletion mutation could not be obtained. This will likely require cloning and expression of species-specific activation genes, and increased expression levels of the heterologous MCR’s. These efforts are under way. |
| M1.4 | 4/30/15 | 40% complete | **Clone *Methanosarcina* and *Methanosphaera* methanol methyltransferase 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.**  We have successfully cloned the *Methanosphaera* methanol methyltransferase genes into *M. maripaludis* and verified expression by Western blot. |
| M1.5. | 6/1/15 |  | Demonstrate activity of introduced methanol methyltransferase by in vitro assay. Obtain at least 2x10-4 umoles CH3OH/g total cell protein/second. |
| M1.6 | 7/1/15 |  | Demonstrate 2x10-4 umoles CH3OH to CH4/g total cell protein/second, OR show flux of CH4 to CH3OH (the latter will be limited at this stage due to thermodynamic limitations). |
| M1.15 | 3/1/16 | 50% complete | **Clone the *M. maripaludis* MCR genes into *M. maripaludis* and verify expression.** No new progress this quarter. |
| M2.1 | 12/1/14 | 100% 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 M. maripaludis model using our maximum likelihood orthology approach (See Section C, Figure 2) and literature-based manual curation. Our model accurately depicts methanogenesis and can reasonably predict growth and byproduct secretion as compared to experimental data. We are setting up metabolomics experiments to gather additional model validation data and will be using SimOptStrain with our model to predict viable engineering designs for conversion of methane to methanol. |
| M2.2 | 2/1/15 | 10% | **Deliver first model of core metabolism in *M. marapaludis*** **with atom-level detail for molecular transformations as needed for flux measurements**. Our genome-scale metabolic model depicts a clear picture of core metabolism with regard to primary metabolites,methanogenesis intermediates, and methanogen-specific coenzymes. We are working to confirm metabolite presence through metabolomics experiments. |
| M2.3 | 6/1/15 |  | Deliver improved model that enables predictions with >85% predictive accuracy for knockout lethality, and <20% error for wildtype growth and byproduct yield predictions |
| M3.2 | 6/30/15 | 50% Complete | **Deliver an HDR with a specific activity (purified protein) of at least 10 units/mg.** The HDR that is native to our genetic host, *M. maripaludis*, was assayed and found to be similarly active to the enzyme from *M. marburgensis* (Table I).  Because the enzyme is highly active in both forward and reverse directions, we expect that the native enzyme will support methane oxidation to methanol. Therefore, we do not expect to need to genetically engineer an enzyme from ANME. We have not yet determined coupling to MCR and the MeTr. |
| M3.3 | 12/1/15 | 0% complete | **Deliver an MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM**: Determine kinetic and physical properties of the heterologously expressed methyl-SCoM:methanol MeTr in the forward & reverse direction. choose the MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM. |
| 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. |
| M4.2 | 9/1/15 | 40% Complete | **Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.**  We are continuing the explicit-solvent classical molecular dynamics simulations for longer simulation times while developing and testing trajectory analysis tools. In addition to the red1 states, we are setting up simulations for additional catalytic states. The parallel GROMACS software used for the MD calculations is very fast, yet the previous version of the program was not sufficient for the computationally-demanding exploration of concerted motions via covariance analysis. PNNL computer scientists were recruited to fix the program by re-writing sections of code, adding parallelization and customizing program settings for the lab supercomputer. The program is now able to accommodate the number of atoms required to identify catalytically-important groups in the MCR substrate binding channel and active site and to analyze concerted motions of these groups that are key to catalysis of methane formation and oxidation. |
| M4.3 | 2/1/16 | 5% complete | **Validate activation energies in MCR pathways using experimental data from Ragsdale’s lab.**  QM/MM geometry optimizations of reaction intermediates proposed by Ragsdale’s lab are currently underway. The CoB7 red1 state has been geometry optimized (Supporting Information) and optimization of the other reaction intermediates are underway. |
| M5.7 | 2/1/15 | 40% Complete | **Develop a pitch deck.** Ppt presentation tailored to use in meetings with potential partners, funders, etc. Slides will focus more on the market/commercial aspect rather than the technical. This would include market size, value proposition etc. |
| M5.8 | 12/1/16 | 0% complete | **Meet with potential industrial partners**. Partner/funding outreach and engagement: Begin developing proposals for industrial and federal funding, based on the analysis of target companies and federal funding options. |
| M5.9 | 4/1/16 |  | Write proposal for follow-up funding of the project. |
| M5.10 | 9/1/15 | 0% | **Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs.** We have hired a consultant (Rich Zwotek) and will engage him over the next quarter to develop the technology to market plan, pitch deck as well as to start drafting the technoeconomic analysis. |

**C.    Supporting data & additional information**

**Milestones 1.3-1.4: Express MCR proteins in *M. maripaludis.***

**Milestone 1.3:** **Test for genetic complementation of MCR deletion mutation in *M. maripaludis*.** This has not been successful after two attempts with the *M. marburgensis*-expression strain. As stated in the last update, possible problems include that the expression level is not yet similar to the level for the native MCR, that the proteins that activate the MCR are species specific, that the heterologous MCR is not properly covalently modified, or that the F430 cofactor of the host strain does not function in the heterologous enzyme. Measures to increase expression include cloning the genes into the chromosome (*M. maripaludis* is polyploid) instead of using the low-copy replicative plasmid. The construction of the vector containing the *M. marburgensis mcr* genes for markerless integration is ongoing. A previous attempt to integrate ANME2c *mcr* genes into the chromosome proved successful. This was done through We are currently pursuing the markerless approach, which has been shown to be successful for the *Methanosphaera* methyltransferase artificial operon (see below). We have also continued to work on the cloning of heterologous activation genes. Efforts are underway to construct both codon-optimized and non-optimized versions of the *M. marburgensis* and ANME2cMCR activation genes. After incorporating both *mcr* operons and the associated activation genes into the chromosome for each MCR system, another attempt to knock out the *M. maripaludis mcr* will be initiated. A successful knockout will imply that the heterologous MCR is functional.

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| **Milestone 1.4:** **Clone *Methanosarcina* and *Methanosphaera* methanol methyltransferase genes into *M. maripaludis* and verify expression.** We have successfully cloned the *Methanosphaera* methanol methyltransferase genes, along with an activation gene, in an artificial operon and introduced the construct into *M. maripaludis*, where it has integrated into the genome. We have verified expression by Western blot (Fig. 1). Since the His tag is on the protein expressed from the last gene in the operon, the positive Western signal suggests that all four proteins are expressed. This initial construct uses the *nif* promoter, and we do not yet have a quantitative measure of expression. A construct that uses the constitutive *hmv* promoter is in the process of being introduced into *M. maripaludis*. Once this process is completed, we will make quantitative expression measurements.  Fig. 1. Western blot showing expression of the methanol methyltransferase protein MapB. CbiX is an unrelated oligohistidine-containing protein expressed in *M. maripaludis*. Oligo-His antibody was used. |  |

**Milestones 2.1-2.2: Build and refine a draft metabolic model for *M. maripaludis***

We built a draft metabolic model of *M. maripaludis S2* using the ModelSEED automated model-building pipeline with our novel likelihood-based gap filling approach that maximizes consistency of gap-filling.

results with available genomic data (1). =We refined the draft model by correcting the electron transport chain, augmenting the number of viable carbon and nitrogen sources, adding paths for unique coenzyme syntheses, altering the biomass composition and major biosynthetic pathways, and modifying our gene-protein-reaction relationships to accurately reflect recent literature. Our completed model accurately depicts core metabolism of *M. maripaludis* and predicts reasonable growth yields on all common defined media conditions. Furthermore, our literature-based refinements have increased the model’s genome coverage by adding over 27% more genes. We have worked with the Leigh group to harvest cells for targeted metabolomics experiments on high- and low-hydrogen conditions, which will allow us to better understand the metabolites present during steady-state growth under these conditions. We are currently running experiments to measure growth yield and cell dry weight data; these results, coupled with those from the metabolomics experiments, will form the validation set for our model. Concurrently, we have begun to manually simulate strategies for engineering *M. maripaludis* to produce methanol from methane and we are adapting the SimOptStrain bi-level optimization method (2) to assist in this process.

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Figure 2*: Likelihood-based gap fill workflow*

**Milestone 3.2. Deliver an HDR with a specific activity (purified protein) of at least 10 units/mg that couples effectively to the MCR and methyltransferase**.

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| The HDR that is native to our genetic host, *M. maripaludis*, was assayed and found to be at least as active as the well-studied *M. marburgensis* enzyme (Table I). Because this enzyme has an activity that exceeds our requirements, in both forward and reverse directions, we expect that the native *M. maripaludis* enzyme will support methane oxidation to methanol. Therefore, we do not expect to need to genetically engineer an enzyme from ANME. We have not yet determined coupling to MCR and the MeTr. This will be done in an vivo assay of methanol conversion to methane (Milestone 1.4). | **Macintosh HD:Users:sragsdal:Desktop:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:Quarterly Reports:Quarter 1_2015:HCR assay.tif** |

**Milestone 3.3. Methyl-SCoM:Methanol Methyltransferase (MeTr): Determine kinetic and physical properties of heterologously-expressed methyl-SCoM:methanol MeTr in forward & reverse direction. Choose MeTr with specific activity >10 U/mg for methyl-SCoM conversion to methanol**. The Leigh laboratory has cloned the *Methanosphaera* methanol methyltransferase (MtaABC) into *M. maripaludis*. As shown by studies of the *M. barkeri* system, MtaB is a zinc metalloenzyme, while MtaC contains the cobalamin (3). MtaA binding MtaBC and CoM. We will purify and begin kinetic studies of

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| this methyl-SCoM:methanol methyltransferase (MtaABC) (Figure 3) as soon as the Leigh lab has optimized the expression levels. One of the subunits contains a His tag, so we expect to be able to purify this complex using affinity purification on a Ni-NTA column. Activity of the MtaBC component will be measured in both forward and reverse directions. For this, we will assay the conversion of methanol to methylcobalamin as described (4) and will | Macintosh HD:Users:sragsdal:Desktop:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:Quarterly Reports:Quarter 1_2015:MtaABC.tif  Figure 3. Reaction catalyzed by MtaABC |

expect a specific activity of ~750 U/mg and Km values for CoM and methanol of 10 mM and 50 mM, respectively, similar to the published values for the *M. barkeri* enzyme. This is assayed by following the methylation of free cob(I)alamin by methanol in an assay mixture containing 50 mM Mops/KOH, pH 7.2, 100 pM hydroxocobalamin (Sigma), *5* mM Ti(III)citrate, 100 mM methanol with the reaction initiated by adding MeTr and followed by measuring the increase in absorbance at 528 nm *(ε*= 6.1 mM-1cm-1) and the decrease in absorbance at 388 nm *(ε*= 23.2 mM-1cm-1). The MtaA will be assayed as described (5) by following the transfer of the methyl group of methylcob(III)alamin to coenzyme M to generate methyl-CoM in an assay mixture containing 50 mM Mops/KOH, pH 7.2, 300 pMmethylcobalamin and 1 mM coenzyme M, and initiating the reaction by addition of MeTr. Activity will monitored by following thc decrease in absorbance at 528 nm ***(Δε*** = 6.1 mM-1cm-1) due to demethylation of methyl-Co(III) and the increase in absorbance at 388 nm due to increase in Co(I) ***(Δε*** = 23.2 mM-1cm-1). The overall reaction will be measured in the direction of methane formation by following the transfer of 14CH3 from 14CH3-OH to CoMSH to generate 14CH3-SCoM. The opposite reaction will be measured by following the CoMSH-dependent conversion of 14CH3-SCoM to 14CH3-OH.

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| **Milestone 4.3-4.5: Hybrid quantum/classical mechanics calculations of MCR reaction intermediates.**  We are currently running QM/MM geometry optimizations of the reaction intermediates recently proposed by Ragsdale’s lab. To date the red1 structure has converged (Figure 4). The nickel and CoM sulfur electron densities are similar to those previously calculated by Siegbahn (Chem. Eur. J. 2012, 18, 6309 – 6315), however the key inter-atomic distances are ~0.1 Å closer due to the more realistic model using un-truncated groups and inclusion of the solvated protein matrix.  Figure 4: QM/MM optimized structure of catalytic core atoms of MCRred1. For clarity only QM atoms are shown. |  |

**Milestones 5.1-5.8 (technology to market)**

Nadine Wong in our (U. Mich) Office of Technology Transfer is our primary liaison and Rich Zvosec (High Hurdles) is our consultant. Based on the recent technical milestones from John Leigh's lab, on December 10, 2014, David A. Casimir, J.D., Ph.D. of Casimir Jones, S.C. filed a provisional patent application to cover the expression of MCR proteins in *M. maripaludis*.  We developed an IP strategy document that sets forth a protocol and strategy for best practices capture of IP generated during the project. The plan has identified the key technical milestones that are likely to merit invention reporting/patent filing and will be tracked by the team.  We have hired Rich Zvosec (High Hurdles) as our consultant. Dr. Zvosec has developed a technology to market plan, which is being used to develop a pitch deck for interactions with technology partners. The T2M plan includes potential industrial partners that we will approach at the appropriate stage. We feel that we are on track to initiate these interactions when we have demonstrated conversion of methanol to methane in the recombinant *M. maripaludis* system. We plan to work with TJ Augustine at ARPA-E as we fully develop the technoeconomic analysis and have sent our draft analysis to Dr. Augustine and other project coordinators at ARPA-E for input.

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

**Milestone 1.3.** See supporting data and additional information above.

**Milestone 1.4**

Simlilar to MCR, challenges for the methanol methyltransferase include achieving sufficient expression levels and a probable requirement for activation of the cobalamin. Efforts to optimize expression are similar to those for MCR. Activation genes from the source organism are being included in our expression clones.

**Milestones 2.1 and 2.2.**

With our model completed our remaining challenges lie in obtaining model validation data; namely, there is currently no established metabolomics method for methanogen-specific intermediates. The Price and Leigh laboratories are working together with metabolomics experts aiming to devise a new method that will overcome this obstacle.

**Milestone 3.2.**

The riskiest part of this goal is the effective coupling of the *M. marapaludis* HDR to the *M. marapaludis* MCR and *Methanosphaera* MeTr. One advantage to using the homologous *M. marapaludis* HDR and MCR is that they naturally couple together in methane formation. We might have issues if we need to express the ANME MCR enzymes and in that case will need to screen for and engineer into *M. marapaludis* the appropriate heterologous HDR. Here it should be pointed out that, even though we are observing expression and F430 loading of the heterologous MCRs in *M. marapaludis*, the activation system used for in vitro assays of the *M. marburgensis* and the ANME MCR has not so far been successful. As described in the Milestone 1 section, efforts are underway in the Leigh laboratory to increase expression of these heterologous MCRs and to construct both codon-optimized and non-optimized versions of the *M. marburgensis* and ANME2cMCR activation genes.

**Milestone 3.3.**

One potential issue with heterologous expression of the methanol:CoM MeTr is incorporation of cobalamin. This has been problematic with expression of various corrinoid proteins in *E. coli*, for example the CFeSP from *M. thermoaceticum* (6). In recent studies in Ragsdale’s lab, by alteration of growth conditions, we have obtained CFeSP with at least 60% cobalamin and FeS incorporation in vivo. There also may be issues with activation of the MeTr due to lack of the appropriate ATP-dependent methyltransferase activation protein (MAP) (7), studied most fully in *M. barkeri*, which may need to be cloned into the *M. marapaludis* host.

**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. Analysis of sub-microsecond MD data collected so far for MCR-Red1 bound to CoB6/7/8 cofactors are stable.

**Milestone 4.3:** The riskiest aspect of M4.3 is the calculation of barriers between reaction intermediates, which is highly sensitive to the initial geometry of the proposed reaction intermediate structure. As stated in the last report, there is also a risk of being “scooped” by competing scientists, however the computational resources and the approach we are using is more sophisticated than others previously applied to this system.

**Milestones 5.1-5.8:** The risks related to the T2M plans would be tied to our technical progress. For instance, our timing to approach potential industrial partners or to complete a technoeconomic analysis will be depend on demonstration of the conversion of methanol to methane in the recombinant *M. maripaludis* system.

SUGGESTED REVISIONS TO THE PROJECT OBJECTIVES AND MILESTONES:

Now that we are over one year into the project, we have recognized that there are alternatives to our original plan that are more likely to lead us (more rapidly) to the GTL objective.

For one example, we have recognized that there are potential problems with the sulfate to sulfide pathway that we should be able to avoid. In nature, several pathways have been shown to couple to reverse methanogenesis, so we propose to replace the use of sulfate as electron acceptor with the use of sulfite or the fumarate. This revision would avoid one of the issues that was brought up in the REMOTE meeting - that the sulfate-to-sulfide pathway requires an additional ATP, which is not a requirement for the two suggested alternative electron acceptor pathways.

Another example is the rate of the reverse reaction. At the outset, we all assumed that the reverse methanogenesis pathway was rate limited by the MCR reaction and that the bond making and bond breaking steps in this enzymatic reaction were both kinetically and thermodynamically unfavorable. However, as we have presented, our kinetic studies that indicate that the chemistry of the reverse MCR reaction is not as kinetically limiting as we had initially thought. There are many ramifications of these experiments.  So, we have recognized the importance of being very clear about rates of the reverse reaction and are developing methods and doing experiments to clarify this issue. Our recent experiments indicate that steady-state (what will be happening in the methanogenic cell) is limited by product release, so it is very important to understand the interactions between MCR, the rate limiting enzyme in the pathway, and CoB, the product that forms an inhibitory nonproductive complex. So, we are directing attention through our enzymology experiments and computational experiments (in Dayle Smith's lab) to characterize the interactions between MCR. Once we know about the interactions, we can make a plan to make variants that would lead to faster product release, which we think would have a direct effect on increasing the rate of the GTL process.

Thus, below (Section A) we describe the proposed revisions to projects 1, 2, and 3 and then (Section B), present the revised milestone schedule.

**Section A: Description and explanation of Proposed Revisions to “Anaerobic Bioconversion Of Methane To Methanol”**

1. Description and explanation of Proposed Revisions in Project 1 (J. Leigh):

In the first year of the grant we have found that heterologous genes can be expressed, but that the protein levels are quite low. At this juncture it seems wise to add experiments to determine why this is the case and to start taking measures that ought to increase expressed protein levels. Therefore we have added milestones to test the overall approach by expressing the *M. maripaludis* enzyme itself using the same system, to determine whether the problem lies at the mRNA or post-mRNA level, and to try to improve protein stability by overexpressing the chaperonin and knocking out a proteasome activator. As an insurance policy, we also keep open the possibility of using the *M. maripaludis* Mcr itself. Also, our REMOTE-funded partners at Arzeda have had success with an improved replicative plasmid that we propose to implement by moving the plasmid stability gene into the chromosome (8).

Developments in the literature and in our thinking have led to a reevaluation of what will be required not only to express heterologous proteins, but to activate the enzymes. Mcr requires several activation proteins, some of which may be species-specific (9). We propose alternative milestones, one involving cloning in species-specific activators, and the other using the *M. maripaludis* Mcr itself. The latter alternative circumvents any persistent problems with protein levels as well. In addition, the methanol methyltransferase may require an activator.

Our thinking has also matured regarding the electron-sink pathway to make the methane to methanol process thermodynamically favorable. Our original plan was based on the known association of sulfate reducers with ANME organisms. However, the complexity of the sulfate reduction pathway, the requirement for ATP to activate sulfate, and potential challenges in expressing heterologous enzymes suggest a rethinking of this goal may be in order. We propose to replace the milestones involving sulfate reduction with three alternative approaches: use of electrical charge, use of fumarate reduction, and use sulfite reduction. The electrochemical approach is suggested by the finding that *M. maripaludis* can use an electrical charge for methanogenesis (10), while the use of fumarate or sulfite reduction is suggested by the known HSCoM and HSCoB-utilizing fumarate reduction reaction that already exists in *M. maripaludis* *(11)* and the successful expression in *M. maripaludis* of a F420-dependent sulfite reductase from a closely related species (12).

Because we wish to insert milestones to improve protein levels and activation of heterologous enzymes (including the methanol methyltransferase), we propose to delay certain milestones by 8 months (activity of methanol methyltransferase and flux between methanol and methane).

2. Description and explanation of Proposed Revisions in Project 3 (S. Ragsdale):

Previous experiments indicate that steady-state catalysis of reverse methanogenesis by MCR occurs at a slow rate of 0.0025 s-1 at 60 oC (13). This was an important experiment that established the role of MCR in this process. However, it was a complicated isotope-labelling experiment in which formation of 13CH3-SCoM from 13CH4 and CoMS-SCoB in the presence of 12CH3-S-CoM. We have measured a much faster rate constant of 0.8 s-1 under presteady-state conditions. Because presteady-state rate constant for reverse methanogenesis is markedly faster than the steady-state rate, one could conclude that the reaction is limited by product (CoBSH or Methyl-SCoM) release. It is crucial to ensure that the steady-state rates are confirmed by another method, because future plans for optimizing the MCR reverse reaction rely on having unambiguous and validated values for this rate constant.

Given that the experiments just described indicate that steady-state kinetics (what will be happening in the methanogenic cell) is limited by product release, it is very important to understand the interactions between MCR, the rate limiting enzyme in the pathway, and CoB, the product that forms an inhibitory nonproductive complex. So, the Ragsdale lab is directing attention through our enzymology experiments and computational experiments (in Dayle Smith's lab) to characterize the substrate binding thermodynamics (enthalpic, entropic, solvent and steric contributions) in Aim 4.2 and in Milestone 4.2 (Identification of at least 10 amino acids modulating substrate binding). Once we have identified these key interactions, we will generate variants that will lead to weaker binding, thus to faster product release. We will then experimentally determine the steady-state and presteady state kinetics for the MCR reaction and test our hypothesis by comparing these rates with those of the wild-type protein.

As described at the site visit, we plan to measure the reverse reaction by coupling the endergonic anaerobic oxidation of methane (reaction 1) with the exergonic reduction of fumarate with HS-CoM and HS-CoB catalyzed by cytoplasmic thiol:fumarate reductase (reaction 2) from hydrogenotrophic methanogens (Figure 1). Cell extracts of *M. marburgensis* (0.7 U/mg, and *M. thermophila* strain ∆ H (0.6 U/mg) have quite high levels of this enzyme, with the activity of the purified *M. marburgensis* enzyme reaching 150 U/mg (11). The enzyme has also been found in many strains of methanogens including *Methanococcus*, *Methanopyrus*, *Methanosarcina* and *Methanogenium* (11). The coding sequence is also present in *M. maripaludis* , but the enzyme has not been yet isolated or studied from our host strain. As described below, we also plan to study the properties of this purified enzyme and its coupling as the electron donor to couple to and drive methane oxidation.

(1) CH4 + CoM-S-S-CoB ⇌CH3-S-CoM + HS-CoB ∆Go = + 30 kJ/mol

(2) HS-CoM + HS-CoB + fumarate → CoM-S-S-CoB + succinate ∆Go´ = - 35 kJ/mol

(3) CH4 + H-S-CoM + fumarate →CH3-S-CoM + succinate ∆Go´ = - 5 kJ/mol



Thus our new project goal (3.8.), as described in the attached file (Michigan milestone revisions 3-5-2015 w revised dates.docx) is to Evaluate steady-state kinetics for the reverse MCR reaction, determining its kinetic parameters and establishing the rate-limiting step. The associated milestones are:

* M3.8 Provide the steady-state rate of the reverse reaction
* M3.9 Establish if product release or chemistry is rate-limiting in AOM

Revision of the electron transfer pathway goals, i.e., to replace the milestones involving sulfate reduction with three alternative approaches, also requires some changes in the objectives and milestones for the enzymology section in Project 3. We feel that the three most fruitful approaches are to couple methane oxidation to an electrochemical system, to fumarate reduction or to sulfite reduction. As mentioned above, *M. maripaludis* has been shown to couple methaneogenesis to an electrochemical cell (10).

We propose to perform in vitro studies to characterize fumarate reduction by the thiol:fumarate reductase and its potential coupling to methane oxidation by MCR. These experiments are closely linked to the in-vitro experiments described above to assay methane oxidation by MCR. We will perform enzymatic studies to characterize the *M. marburgensis* *(11)* and *M. maripaludis* thiol:fumarate reductases and optimize their interaction with the MCRs. We think it is best to begin studies with the *M. marburgensis* thiol:fumarate reductase because it has very high activity and may couple best to *M. marburgensis* MCR, which at present is the best system for in vitro studies of methane oxidation. It also is important to study the coupling between the analogous enzymes from our host organism.

We already had described plans to study the properties of sulfite reductase and note that successful expression in *M. maripaludis* of a F420-dependent sulfite reductase from a closely related species has been demonstrated (12).

3. Description and explanation of Proposed Revisions in Project 2 (N. Price):

In the first year of the project, we successfully built a genome-scale metabolic model of *M. maripaludis S2* including an updated accurate representation of the Wolfe Cycle, a primary catabolic pathway in our organism (14). When we had initially written the proposal a couple of years ago, the idea had been to also do a detailed model to track at the atom level transitions of a subset of metabolism and then use this approach coupled with isotopomer labeling to make flux calculations. This has proven to be much more challenging than anticipated. The uniqueness of the intermediates involved in the methanogenic pathway (15) presents a challenge for performing targeted metabolomics and fluxomics experiments because measuring these compounds would require developing novel assays and cannot be performed by our metabolomics collaborators – where the techniques have been focused on more conventional central carbon metabolism in other species (e.g. E. coli, yeast, humans). What this means is that there is a (likely multi-year) development program needed before this will really be feasible for an organism like M. maripaludis. We propose to instead to more-profitably focus our efforts on metabolomics, which we believe will better advance the present project. We will measure these metabolites on a common targeted LC-MS platform; this will help us shed light on crucial biosynthetic pathways and allow us to improve the model around central catabolism. We are currently collecting cells from a chemostat under high- and low-H2 conditions and expect to submit these cells for processing by the end of March.

John Leigh has also contacted the White group at Virginia Tech, another group that works with *M. maripaludis,* to work with us as we find ways to better measure methanogenic intermediates. The White group has expertise in identifying unique compounds in methanogenic archaea, including key synthesis steps for methanogenic coenzymes (16), and they are interested in running metabolite measurements on samples of our chemostat cultures. We propose to collaborate with the White group by sending them samples of our cells under low- and high-H2 conditions so that they can help us analyze the levels of metabolites that we cannot measure in an ordinary targeted LC-MS or GC-MS.

In the process of validating our model on experimental data, we have encountered a relative dearth of information and have relied on very few measured growth yields to guide model development (17). We are not equipped to measure hydrogen uptake rates, but we will be measuring additional growth rate, growth yield, and byproduct secretion using gas chromatography. These experiments will be key for continuing to improve the model and for testing strain designs from model predictions. For growth yield calculations, we calculate dry cell weight by measuring optical density (OD) and relating this number to dry cell weight using a calibration curve. Expanding these experiments, coupled with the metabolomics, will greatly aid our efforts because it will give us higher-quality standards against which to measure predictions from our model.

**Section B: Proposed Revisions to SCHEDULE OF TECHNICAL TASKS, MILESTONES, AND DELIVERABLES**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| WBS | Name (task, subtask, milestone) | Duration | Start | Finish |
|  |  | months |  |  |
| 1 | Engineer pathways for anaerobic bioconversion methane to liquid fuels - John Leigh (U. Washington) |  |  |  |
| 1.1 | Clone ANME MCR genes into Methanococcus maripaludis and verify expression | 5 | 1/23/14 | 6/1/14 |
| M1.1 | 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. |  | 6/1/14 | 6/1/14 |
| 1.2 | Clone Methanothermobacter marburgensis MCR genes into M. maripaludis and verify expression | 5 | 6/1/14 | 11/1/14 |
| M1.2 | 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. |  | 11/1/14 | 11/1/14 |
| 1.3. | Test expression strategy by introducing his-tagged *M. maripaludis* Mcr on a vector | 2 | 3/1/15 | 5/1/15 |
| M1.3 | Achieve expressed M. maripaludis Mcr protein level similar to native Mcr |  |  | 5/1/15 |
| 1.4 | Test activity of expressed M. maripaludis Mcr by deleting native Mcr | 2 | 5/1/15 | 7/1/15 |
| M.1.4. | Demonstrate activity by viability of knock-out strain |  |  | 7/1/15 |
| 1.5. | Determine mRNA levels for heterologous proteins | 3 | 3/1/15 | 6/1/15 |
| M.1.5. | Achieve mRNA levels similar to native Mcr |  |  | 6/1/15 |
| 1.6. | Implement measures to increase levels of heterologous proteins: Construct an M. maripaludis strain to support replication of a smaller expression plasmid, overexpress chaperonin, knock out proteasome activator | 5 | 3/1/15 | 8/1/15 |
| M.1.6. | Achieve introduction of gene for plasmid maintenance into M. maripaludis chromosome, achieve introduction of expressed chaperonin, achieve knock out of proteasome activator |  |  | 8/1/15 |
| 1.7. | Determine effect of measures to increase levels of heterologous proteins: re-introduce heterologous Mcr’s and methanol methyltransferases | 2 | 8/1/15 | 10/1/15 |
| M1.7 | Achieve increased levels of heterologous proteins as determined by Western blot |  |  | 10/1/15 |
| ~~1.3~~ | ~~Test for genetic complementation of MCR deletion mutation in M. maripaludis~~ | ~~2~~ | ~~11/1/14~~ | ~~1/1/15~~ |
| ~~M1.3~~ | ~~Confirm activity of introduced enzymes by demonstrating viability of M. maripaludis MCR deletion mutants in expression strains~~ |  | ~~1/1/15~~ | ~~1/1/15~~ |
| 1.8 | Introduce genes for activation of heterologous Mcr’s as necessary | 6 | 1/1/16 | 7/1/16 |
| M1.8 | Demonstrate activation of heterologous Mcr’s by viability of M. maripaludis Mcr knock-out strain, or use M. maripaludis Mcr |  |  | 7/1/16 |
| 1.9 | Increase expression level of MCR or use M. maripaludis Mcr (already expressed at high level) | 18 | 7/1/15 | 1/1/17 |
| M1.9 | Obtain 50 mg MCR protein/g cell dw (10% of total cell protein) or show that M. maripaludis Mcr will catalyze reverse reaction. |  | 1/1/17 | 1/1/17 |
| ~~1.4~~  1.10. | Clone Methanosarcina and Methanosphaera methanol methyltransferase genes into M. maripaludis and verify expression | 4 | 1/1/15 | 5/1/15 |
| ~~M1.4~~  M1.10. | 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. |  | 5/1/15 | 5/1/15 |
| ~~1.5~~  1.11 | Test for methanol methyltransferase activity in cell extract | 2 | 12/1/15 | 2/1/16 |
| M1.5  M1.11 | Demonstrate activity of introduced enzymes by in vitro assay. Obtain at least 2x10-4 umoles CH3OH/g total cell protein/second. |  |  | 2/1/16 |
| 1.12 | Clone activators of methanol methyltransferases into M. maripaludis | 2 | 10/1/15 | 12/1/15 |
| M1.12 | Verify expression of activators of methanol methyltransferases |  |  | 12/1/15 |
| ~~1.6~~  1.13 | Test for metabolic conversion of methanol to methane by methanogenesis from methanol, OR demonstrate conversion of methane to methanol using labeled substrates | 1 | 2/1/16 | 3/1/16 |
| ~~M1.6~~  M1.13 | Demonstrate 2x10-4 umoles CH3OH to CH4/g total cell protein/second, OR show flux of CH4 to CH3OH (the latter will be limited at this stage due to thermodynamic limitations). |  |  | 3/1/16 |
| 1.14 | Clone gene for F420-dependent sulfite reductase from a related methanogen into M. maripaludis | 3 | 1/1/16 | 4/1/16 |
| M1.14 | Verify expression of F420-dependent sulfite reductase |  |  | 4/1/16 |
| 1.15 | Test alternative strategies for thermodynamic electron sink: methane oxidation on an electrode, methane oxidation with fumarate, or methane oxidation with sulfite | 8 | 3/1/16 | 11/1/16 |
| M1.15 | Demonstrate methanol from methane on an electrode, with fumarate, or with sulfite |  |  | 11/1/16 |
| ~~1.8~~ | ~~Clone genes for ATP sulfurylase and APS reductase into M. maripaludis and verify expression~~ | ~~5~~ | ~~7/1/15~~ | ~~12/1/15~~ |
| ~~M1.8~~ | ~~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.~~ |  | ~~12/1/15~~ | ~~12/1/15~~ |
| ~~1.9~~ | ~~Assay for ATP sulfurylase and APS reductase activities~~ | ~~2~~ | ~~12/1/15~~ | ~~2/1/16~~ |
| ~~M1.9~~ | ~~Demonstrate activity of introduced enzymes by in vitro assay. Obtain 5x10-5 umoles/g total cell protein/second.~~ |  | ~~2/1/16~~ | ~~2/1/16~~ |
| ~~1.10~~ | ~~Clone sulfite reductase genes from related methanogens into M. maripaludis and verify expression~~ | ~~2~~ | ~~2/1/16~~ | ~~4/1/16~~ |
| ~~M1.10~~ | ~~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.~~ |  | ~~4/1/16~~ | ~~4/1/16~~ |
| ~~1.11~~ | ~~Clone sulfite reductase genes from sulfate reducers into M. maripaludis and verify expression~~ | ~~4~~ | ~~4/1/16~~ | ~~8/1/16~~ |
| ~~M1.11~~ | ~~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.~~ |  | ~~8/1/16~~ | ~~8/1/16~~ |
| ~~1.12~~ | ~~Assay for sulfite reduction to sulfide~~ | ~~1~~ | ~~8/1/16~~ | ~~9/1/16~~ |
| ~~M1.12~~ | ~~Demonstrate activity of introduced enzymes by in vitro assay. Obtain at least 5x10-5 umoles/g total cell protein/second..~~ |  | ~~9/1/16~~ | ~~9/1/16~~ |
| ~~1.13~~  1.16 | Manipulate expression of electron flow pathways as necessary. Introduce modified MCRs | 2 | 9/1/16 | 11/1/16 |
| ~~M1.13~~  M.1.16 | Reach activities of electron sink pathways of 1.25 umoles/g total cell protein/second. Demonstrate improved fluxes by introduction of modified MCRs |  | 11/1/16 | 11/1/16 |
| ~~1.14~~  1.17 | Test for conversion of methane to methanol and optimize metabolic fluxes as needed | 2 | 11/1/16 | 1/1/17 |
| ~~M1.14~~  M1.17 | Achieve methane conversion to methanol at a rate of at least 1g CH4/g cell dw/hr (0.5g CH4/L/hr). |  | 1/1/17 | 1/1/17 |
| 2 | Metabolic modeling and flux measurements - Nathan Price (ISB) |  |  |  |
| 2.1 | 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 | 3 | 1/23/14 | 2/1/14 |
| 2.2 | Genome scale flux balance and core metabolic flux models: Manual curation of M. maripaludis model based on biochemical, genetic, and physiological data from literature | 8 | 2/1/14 | 10/1/14 |
| M2.1 | Milestone: Deliver first genome-scale metabolic model capable of simulating growth and byproduct section with >75% accuracy |  | 10/1/14 | 10/1/14 |
| 2.3 | Validate genome-scale metabolic model against experimental data: Perform measurements of growth rates and yields, byproduct secretion rates, and dry cell weight | 9 | 3/1/14 | 12/1/14 |
| .2.5 | Validate metabolic models against experimental data: Perform targeted metabolomics measurements on methanogenic intermediates in steady-state chemostat cultures | 5 | 3/1/15 | 11/1/15 |
| 2.6. | Validate metabolic models against experimental data: Compare all measurements under different conditions to model simulation | 3 | 11/1/14 | 12/1/14 |
| 2.7. | Validate metabolic models against experimental data: Iteratively improve the model as needed; Perform targeted metabolomics measurements on common metabolites in steady-state chemostat cultures | 4 | 12/1/14 | 6/1/15 |
| M2.3 | Milestone: Deliver improved model that enables predictions with >85% predictive accuracy for knockout lethality, and <20% error for wildtype growth and byproduct yield predictions |  | 6/1/15 | 6/1/15 |
| 2.8. | Use the models to guide strain engineering for methanol production: Introduce engineered pathways for methane to methanol and sulfate to sulfide into the integrated metabolic model | 1 | 6/1/15 | 7/1/15 |
| 2.9. | Use the models to guide strain engineering for methanol production: Simulate expected product conversion yields and compare with experiment | 1 | 7/1/15 | 6/1/15 |
| M2.4 | Milestone: Deliver top 5-10 strain design predictions based on methanol yield to team for implementaton |  | 6/1/15 | 6/1/15 |
| 2.10 | Use the models to guide strain engineering for methanol production: Evaluate alternative designs and identify additional alterations to help optimize metabolic fluxes for production of methanol | 2 | 6/1/15 | 8/1/15 |
| 2.11 | Use the models to guide strain engineering for methanol production: Measure metabolic fluxes in engineered strains and compare distributions with the computed optimum | 3 | 8/1/15 | 11/1/15 |
| 2.12. | Use the models to guide strain engineering for methanol production: Iteratively design and refine network and test engineered strain to optimize for methanol production | 15 | 11/1/15 | 10/31/16 |
| M2.5 | Milestone: Provide 3 best engineered strains with highest methanol production based on metabolic modeling |  | 10/31/16 | 10/31/16 |
|  |  |  |  |  |
| 3 | Biochemical optimization of enzymes involved in methane conversion to methanol - Steve Ragsdale (U. Mich) |  |  |  |
| 3.1 | Determine endogenous *M. maripaludis* activities of MCR, HDR and Methyltransferase | 3 | 1/23/14 | 2/1/14 |
| 3.2 | 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 | 10 | 2/1/15 | 6/30/15 |
| M3.1 | Milestone: 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. |  | 11/1/14 | 11/1/14 |
| 3.3. | Heterodisulfide Reductase (HDR): Determine kinetic parameters of the native ~~and ANME~~ HDRs in the forward & reverse direction | 6 | 1/23/14 | 7/1/14 |
| M3.2 | Milestone: choose the HDR with a specific activity of at least 10 units/mg. |  | 6/30/15 | 6/30/15 |
| 3.4. | Methyl-SCoM:Methanol Methyltransferase (MeTr): Determine kinetic and physical properties of the heterologously expressed MeTr in the forward & reverse direction | 12 | 10/1/14 | 10/1/15 |
| M3.3 | Milestone: choose the MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM |  | 10/1/15 | 10/1/15 |
| 3.5. | ~~Sulfate~~ Sulfite to sulfide module (ATP sulfurylase, APS reductase and sulfite reductase): Measure background endogenous sulfite-to-sulfide activity | 3 | 7/1/15 | 10/1/15 |
| ~~3.6.~~ | ~~Sulfate to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed ATP sulfurylases~~ | ~~9~~ | ~~11/1/15~~ | ~~7/1/16~~ |
| ~~M3.4~~ | ~~Milestone: choose the ATP Sulfurylase with a specific activity of at least 10 units/mg.~~ |  | ~~7/1/16~~ | ~~7/1/16~~ |
| ~~3.7.~~ | ~~Sulfate to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed APS Reductase~~ | ~~7~~ | ~~4/1/16~~ | ~~10/1/16~~ |
| ~~M3.5~~ | ~~Milestone: choose the APS Reductase with a specific activity of at least 10 units/mg.~~ |  | ~~10/1/16~~ | ~~10/1/16~~ |
| 3.8. | Sulfite to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed Sulfite Reductase | 6 | 5/1/16 | 10/1/16 |
| M3.6 | Milestone: choose the Sulfite Reductase with a specific activity of at least 10 units/mg. |  | 10/1/16 | 10/1/16 |
| 3.9. | Ensure that the chosen sulfate to sulfide enzymes couple to give predicted flux - in vitro and in vivo | 4 | 9/1/16 | 1/1/17 |
| 3.10. | Substitute enzymes from a single host if necessary - make adjustments based on in vivo flux measurements | 4 | 9/1/16 | 1/1/17 |
| M3.7 | Milestone: choose enzymes for the sulfite-to-sulfide module based on highest activity (>10 units/mg) |  | 1/1/17 | 1/1/17 |
| 3.8. | Evaluate steady-state kinetics for the reverse MCR reaction, determining its kinetic parameters and establishing the rate-limiting step. | 6 | 2/1/15 | 8/1/15 |
| M3.8 | Provide the steady-state rate of the reverse reaction |  | 8/1/15 | 8/1/15 |
| M3.9 | Establish if product release or chemistry is rate-limiting in AOM |  | 8/1/15 | 8/1/15 |
| 3.9. | Characterize the coupling between the *M. marburgensis* MCR and the thiol:fumarate reductase *M. maripaludis* thiol:fumarate reductase and its interaction with the *M. maripaludis* MCR | 12 | 3/1/15 | 3/1/16 |
| 3.10 | Characterize the *M. maripaludis* thiol:fumarate reductase and its interaction with the *M. maripaludis* MCR | 8 | 7/1/15 | 3/1/16 |
| M3.10 | Establish the optimum conditions for coupling the MCR and the thiol:fumarate reductase |  |  | 3/1/16 |
| 3.11. | Experimentally determine the steady-state and presteady state kinetics and CoBSH binding constants in the MCR reaction for the MCR variants (generated on the basis of D. Smith’s computational work) versus the wild-type protein. |  |  |  |
| M.3.11 | Generate MCR variants that exhibit weaker binding of CoBSH. |  |  | 5/26/16 |
| M.3.12 | Deliver an MCR with faster steady-state rates for the reverse MCR reaction. |  |  | 5/26/16 |
|  |  |  |  |  |
| 4 | Molecular modeling of the ANME MCR and other enzymes involved in GTL - Dayle Smith (PNNL) |  |  |  |
| 4.1 | 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 | 6 | 1/23/14 | 7/1/14 |
| M4.1 | Milestone: Reproduce experimental MCR structure 1MRO.pdb with a root-mean-squared deviation of less than 10 Ångstroms. |  | 7/1/14 | 7/1/14 |
| 4.2 | Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics (enthalpic, entropic, solvent and steric contributions): Perform > 100 ns explicit-solvent molecular dynamics simulations (MD) for solvated proteins in reactant and product states, Run trajectory analyses to calculate hydrogen bond networks, per-residue fluctuations, interaction potential energies, and Perform free energy perturbation calculations for point-mutations associated with Aim 3. | 15 | 7/1/14 | 10/1/15 |
| M4.2 | Milestone: Identification of at least 10 amino acids modulating substrate binding |  | 10/1/15 | 10/1/15 |
| 4.3 | Determine MCR reaction pathways and reversibility as dependent on identified key amino acids from Aim 3 and task 4.2: Calculate and compare reaction pathway activation energies in methanogenic MCR for organometallic and radical pathways using a more extensive and accurate model than studies previously published | 5 | 10/1/15 | 3/1/16 |
| M4.3 | Milestone: Validate activation energies in the two pathways using experimental data from Ragsdale’s lab to reach agreement within 5 kcal/mol |  | 3/1/16 | 3/1/16 |
| 4.4. | Predict effects of site-specific substitutions within methanogenic and methanotrophic (ANME) MCR on activation energies | 7 | 1/1/16 | 8/1/16 |
| M4.4 | Milestone: Validate changes in activation energies for amino acid substitutions to reach agreement to within 5 kcal/mol with data from Ragsdale’s lab. |  | 8/1/16 | 8/1/16 |
| 4.5 | Predict effects of site-specific substitutions within methanogenic and methanotrophic (ANME) MCR on reaction reversibility | 5 | 8/1/16 | 1/1/17 |
| M4.5 | Milestone: Validate activation energies against those from Ragsdale’s lab to reach agreement within 5 kcal/mol. This will quantitatively establish the catalytic role of specific point-mutations on reaction reversibility. |  | 1/1/17 | 1/1/17 |
|  |  |  |  |  |
| 5 | Technology Transfer and Intellectual Property: Work with Dr. Nadine Wong and others in Univ Mich. OTT to address IP and technology transfer matters related to the project team. |  |  |  |
| 5.1. | Develop an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB | 3 | 1/23/14 | 4/1/14 |
| M5.1. | Milestone: Reach agreement and sign an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB |  | 4/1/14 | 4/1/14 |
| 5.2. | Hire OTT fellow(s) to look at the ARPA-E REMOTE proposal and assess the relevant existing current patent landscape. | 3 | 1/1/14 | 4/1/14 |
| M5.2. | Milestone: Develop a profile of the existing patents related to methane to methanol (and GTL) biotechnology. |  | 4/1/14 | 4/1/14 |
| M5.3. | Milestone: File provisional patent for our plan to convert methane to methanol. |  | 4/1/14 | 4/1/14 |
| 5.3. | Technology to Market Plan: Work with Dr. Nadine Wong and Fellows from OTT and Tech Transfer Consultants (above) to develop Tech to Market Plan | 6 | 1/23/14 | 7/1/14 |
| M5.4. | Milestone: Develop tech to market plan |  | 7/1/14 | 7/1/14 |
| 5.4. | Market analysis: Use fellows within the OTT Fellows program to conduct market assessment of the technology area relevant to the conversion of methane to methanol and other liquid fuels | 3 | 7/1/14 | 10/1/14 |
| M5.5. | Milestone: Develop a document that assesses the market for conversion of methane to methanol and to butanol (GTL). |  | 10/1/14 | 10/1/14 |
| 5.4. | 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. | 6 | 1/23/14 | 7/1/14 |
| M5.6. | Milestone: Hire an independent T2M Consultant to oversee the tech-to-market plan and forge relationships with technology partners. |  | 7/1/14 | 7/1/14 |
| 5.5. | Partner/funding outreach and engagement: Develop a pitch deck. Ppt presentation tailored to use in meetings with potential partners, funders, etc. Slides would focus more on the market/commercial aspect rather than the technical. This would include market size, value proposition etc. | 4 | 10/1/14 | 2/1/15 |
| M5.7. | Milestone: Develop a pitch deck |  | 2/1/15 | 2/1/15 |
| 5.6. | Partner/funding outreach and engagement: Travel to the annual ARPA-E summit. |  |  |  |
| 5.7. | Partner/funding outreach and engagement: Hire graduate student fellow(s) at OTT3 to develop a preliminary list of companies with technology in the methane to methanol and methanol to fuels areas. OTT and UM have ongoing relationships with several target companies such as BASF, Dow Chemical and can be used to initiate discussions with those partners. | 6 | 8/1/14 | 2/1/15 |
| M5.8. | Milestone: Meet with potential industrial partners. |  | 2/1/15 | 2/1/15 |
| 5.8. | Partner/funding outreach and engagement: Begin developing proposals for industrial and federal funding, based on the analysis of target companies and federal funding options. | 22 | 2/1/15 | 12/1/16 |
| M5.9. | Milestone: Write proposal for follow-up funding of the project. |  |  | 4/1/16 |
| 5.9. | Partner/funding outreach and engagement: Involve OTT to negotiate the confidentiality and material transfer agreements, work with ORSP to negotiate IP terms within a sponsored research agreement. | 17 | 7/1/15 | 12/1/16 |
| 5.10. | Technoeconomic analysis: Develop a technoeconomic model based on the model that Ramon used when he developed the REMOTE program | 28 | 6/1/14 | 10/1/16 |
| M5.10 | Milestone: Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs | 6 | 1/1/15 | 9/1/15 |
| M5.11. | Milestone: Add data to the technoeconomic model and perform sensitivity analysis | 12 | 9/1/15 | 9/1/16 |
| M5.12. | Milestone: Complete the technoeconomic model | 3 | 6/1/16 | 9/1/16 |

**REFERENCES**

1. Benedict, M. N., Mundy, M. B., Henry, C. S., Chia, N., and Price, N. D. (2014) Likelihood-based gene annotations for gap filling and quality assessment in genome-scale metabolic models, *PLoS computational biology* **10**, e1003882

2. Kim, J., Reed, J. L., and Maravelias, C. T. (2011) Large-scale bi-level strain design approaches and mixed-integer programming solution techniques, *PLoS One* **6**, e24162

3. Hagemeier, C. H., Krer, M., Thauer, R. K., Warkentin, E., and Ermler, U. (2006) Insight into the mechanism of biological methanol activation based on the crystal structure of the methanol-cobalamin methyltransferase complex, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18917-22

4. Sauer, K., Harms, U., and Thauer, R. K. (1997) Methanol:coenzyme M methyltransferase from Methanosarcina barkeri - Purification, properties and encoding genes of the corrinoid protein MT1, *Eur. J. Biochem.* **243**, 670-7

5. Harms, U., and Thauer, R. K. (1996) Methylcobalamin:coenzyme M methyltransferase isoenzymes MtaA and MtbA from Methanosarcina barkeri - Cloning, sequencing and differential transcription of the encoding genes, and functional overexpression of the mtaA gene in Escherichia coli, *Eur. J. Biochem.* **235**, 653-9

6. Lu, W. P., Schiau, I., Cunningham, J. R., and Ragsdale, S. W. (1993) Sequence and expression of the gene encoding the corrinoid/iron-sulfur protein from Clostridium thermoaceticum and reconstitution of the recombinant protein to full activity, *J. Biol. Chem.* **268**, 5605-14

7. Daas, P. J. H., Wassenaar, R. W., Willemsen, P., Theunissen, R. J., Keltjens, J. T., Vanderdrift, C., and Vogels, G. D. (1996) Purification and properties of an enzyme involved in the ATP-dependent activation of the methanol:2- mercaptoethanesulfonic acid methyltransferase reaction in Methanosarcina barkeri, *J. Biol. Chem.* **271**, 22339-45

8. Walters, A. D., Smith, S. E., and Chong, J. P. (2011) Shuttle vector system for Methanococcus maripaludis with improved transformation efficiency, *Appl Environ Microbiol* **77**, 2549-51

9. Prakash, D., Wu, Y., Suh, S. J., and Duin, E. C. (2014) Elucidating the process of activation of methyl-coenzyme M reductase, *J Bacteriol* **196**, 2491-8

10. Lohner, S. T., Deutzmann, J. S., Logan, B. E., Leigh, J., and Spormann, A. M. (2014) Hydrogenase-independent uptake and metabolism of electrons by the archaeon Methanococcus maripaludis, *The ISME journal* **8**, 1673-81

11. Heim, S., Kunkel, A., Thauer, R. K., and Hedderich, R. (1998) Thiol:Fumarate reductase (Tfr) from Methanobacterium thermoautotrophicum - Identification of the catalytic sites for fumarate reduction and thiol oxidation, *Eur. J. Biochem.* **253**, 292-9

12. Johnson, E. F., and Mukhopadhyay, B. (2008) Coenzyme F420-dependent sulfite reductase-enabled sulfite detoxification and use of sulfite as a sole sulfur source by Methanococcus maripaludis, *Appl Environ Microbiol* **74**, 3591-5

13. Scheller, S., Goenrich, M., Boecher, R., Thauer, R. K., and Jaun, B. (2010) The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane, *Nature* **465**, 606-8

14. Thauer, R. K. (2012) The Wolfe cycle comes full circle, *Proc. Natl. Acad. Sci. U.S.A* **109**, 15084-5

15. Deppenmeier, U. (2002) The unique biochemistry of methanogenesis, *Prog. Nucleic Acid Res. Mol. Biol.* **71**, 223-83

16. Graham, D. E., and White, R. H. (2002) Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics, *Natural product reports* **19**, 133-47

17. Costa, K. C., Yoon, S. H., Pan, M., Burn, J. A., Baliga, N. S., and Leigh, J. A. (2013) Effects of H2 and Formate on Growth Yield and Regulation of Methanogenesis in Methanococcus maripaludis, *J. Bacteriol.* **195**, 1456-62