**Third Quarter (April 1-June 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. Below, I have outlined challenges and risks associated with the work and identified alternative strategies should any of the current plans fail. Although the major aims (above) remain intact, I proposed some changes in approach that should facilitate accomplishing the GTL goals. We also are on budget.

With respect to Aim 1, we 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 the initial 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 added incremental improvements to our model and created a gene knockout panel based on literature data to test the model’s ability to accurately predict gene knockout lethality. Our model is above the 85% accuracy threshold we set for this metric and we are awaiting parts for the chemostats in the Leigh lab to further test our model’s predictions for growth yield and byproduct secretion rates as well as results from our metabolomics harvests. We have made progress on generating engineering designs by successfully predicting methanogenesis from methanol and reverse methanogenesis to create methanol. The next step is to add thermodynamic constraints to our model, which is clearly a a vital step for providing feasible strain designs as energetics are at the heart of this engineering effort. Therefore, we have implemented a new method of applying free energy values to the model’s extracellular metabolites to ensure that our engineering solutions fulfill energetic requirements. In the next quarter, we expect to use our thermodynamically-constrained model to provide multiple different strain designs for converting methane to methanol.

With respect to Aim 3, we validated the previously reported and surprisingly rapid rates (>0.8 s-1) of anaerobic methane oxidation by the thermophilic *M. marburgensis* MCR under single-turnover conditions. We have developed a novel and straightforward steady-state assay for reverse methanogenesis that couples Thiol:Fumarate Reductase (TFR) to MCR and have partially purified this enzyme. We have been successful in obtaining some activation of the *M. maripaludis* MCR using formate and CO, but the activity is well below that of *M. marburgensis*.

With respect to Aim 4, we have calculated the structures of the MCRox1 and MCRred1 reaction intermediates using hybrid quantum/classical mechanics. The MCRred1 structure has not been measured experimentally; therefore our QMMM structure is an important and novel discovery. MD simulations show fluctuations of the CoBSH substrate that may promote C-H bond formation (or cleavage in the reverse direction).

 We also deployed Potential of Mean Force (PMF) molecular dynamics to calculate the free energy surface for CoB(SH) insertion into MCRred1. These results are expected to quantitatively predict how structure and dynamics of MCR dictate CoB uptake and binding and provide insights into the relative binding affinities of CoB in the presence and absence of CoM.

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.

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| --- | --- | --- | --- |
| **WBS** | **Due Date** | **Status** | **Summary** |
| M1.3 | 12/31/14 | 30% 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.**  As explained in the last report, this has been unsuccessful with the heterologous *M. marburgensis* MCR. Our focus now is to demonstrate that complementation will work with the *M. maripaludis* Mcr, allowing the native gene to be deleted. This construct is being made. |
| M1.4 | 4/30/15 | 60% 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.**  As stated in the previous report, we have successfully cloned the *Methanosphaera* methanol methyltransferase genes into *M. maripaludis* and verified expression by Western blot. We have now conducted RT-PCR and have found that mRNA levels are low. Two new constructs are under way, one containing *Methanosphaera* methanol methyltransferase genes where we expect improved expression, and one containing *Methanosarcina* methanol methyltransferase genes. |
| M1.5. | 6/1/15 | 0% | 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 | 10% | 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). We have initiated cultures of the strain expressing the Methanosphaera methanol methyltransferase for growth and methanogenesis on H2 and methanol.  To date, no growth has been observed.  These efforts will be continued after we have obtained higher expression of the methyltransferase protein. |
| M1.15 | 3/1/16 | 50% complete | **Clone the *M. maripaludis* MCR genes into *M. maripaludis* and verify expression.** A plasmid containing the *M. maripaludis* MCR genes was obtained from Arzeda and introduced into *M. maripaludis*. An attempt to then delete the native MCR was unsuccessful. A new construct is being made. |
| 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. |
| M2.3 | 6/1/15 | 80% | **Deliver improved model that enables predictions with >85% predictive accuracy for knockout lethality, and <20% error for wildtype growth and byproduct yield predictions.** We have improved our model’s predictive accuracy for knockout lethality and have achieved 90% agreement of our predictions with experimental results. Our comparison with growth and yield measurements has been delayed because of chemostat equipment failure. Once the chemostats have been returned to their fully-operational state, we will resume our growth experiments and gather data that will allow us to test the accuracy of our model’s predictions. |
| M2.4 | 6/1/15 | 50% | **Deliver top 5-10 strain design predictions based on methanol yield to team for implementation.** We have successfully proposed a design to achieve reverse methanogenesis to methanol, but we are wary that this proposed design may not be energetically feasible. Rather than propose our designs based purely on stoichiometry, we have created a novel method of representing free energy output of our model and are in the process of applying this method to our strain designs. Though this represents a slight delay in achieving the milestone, we are confident that is a vital detail to add and that it will help us create more meaningful strain designs. We aim to get the designs out to the full team shortly (before next update). |
| M3.2 | 6/30/15 | 100% 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 have a specific activity higher than our target. 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. |
| 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. As described in the M1.x section, we have been having trouble with heterologous expression of the methyl-SCoM:methanol MeTr. As soon as this is accomplished, we will be able to proceed with these characterizations. |
| 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 | 65% Complete | **Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.**  We are currently performing Potential of Mean Force molecular dynamics (PMF) calculations on the red1 state of MCR to calculate the free energy surface for CoB insertion into MCR. These results will reveal the role and relative importance of MCR residues in binding and transporting CoB to the active site. (See Supporting Information) |
| M4.3 | 2/1/16 | 20% 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. In addition to the red1 state, the ox1 state has been optimized. Currently QM/MM is being applied to the CoBS-SCoM intermediate. |
| M5.7 | 2/1/15 | 80% 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 | 40% Complete | **Meet with potential industrial partners**. Though we did have conversations with several potential partners, mostly venture funds people, we agree with the advice of Rich Zwosec, our consultant, that we should not initiate meetings with potential partners until we have demonstrated robust conversion of methanol to methane. This can be accomplished as soon as we can heterologously express adequate levels of the methanol:CoM MeTr. This is currently a major focus and progress toward this end is described under the Milestone 1.X sections. **The Date should have been adjusted to 12/1/16 and not come up as a current deadline.** |
| M5.10 | 9/1/15 | 0% complete | **Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs.** Based on the approval reached last quarter, **this deadline should have been adjusted to 9/1/15 and not come up as a current deadline**. |

**C.    Supporting data & additional information**

**Milestones 1.x**

Although we have successfully expressed heterologous Mcr and methanol methyltransferase in *M. maripaludis*, protein levels have been low. In addition, we have recently obtained preliminary data indicating that the mRNA levels for heterologous genes are also low. We have therefore determined that it is important to construct improved expression systems.

* For cloning and expression on a replicative vector, our current vector is larger than 10 kb. We are constructing several versions of a smaller replicative vector that is only 5 kb. To do this, a gene required for plasmid replication has been moved into the genome, creating a new host strain. Transformation of the new host strain with the new vector suggests that this new system is working well, and with a markedly improved frequency of transformation.
* To improve the stability of expressed proteins, we are cloning an in-frame deletion of a proteasome-activating nucleotidase, and an extra copy of a chaperonin, both of which will also be introduced into the host strain.
* Since we have now found that mRNA levels of heterologous genes are low, we are making a new construct of the *Methanosphaera* methanol methyltransferase genes that contains intergenic sequences and a transcriptional terminator from *M. maripaludis*. The previous construct contained *Methanosphaera* intergenic sequences and no terminator. The *Methanosarcina* methanol methyltransferase operon currently under construction will also contain intergenic sequences and a transcriptional terminator from *M. maripaludis*.

**Milestones 2.3-2.4: Build and refine a draft metabolic model for *M. maripaludis***

In previous reports, we have described in detail the process by which we built our metabolic model of *M. maripaludis S2.* Though improving the model is an ongoing process, we have made minimal changes to the model in 2015 because it is already an accurate depiction of central metabolism and thus fulfills its role as a platform for generating strain designs. At this stage in the project, we have been chiefly concerned with making incremental model improvements and using the model as a metabolic engineering tool. These two aims have been our foci during the last quarter

We have made minor revisions to some biosynthesis reactions based on newly available literature and are pleased to report that our model predicts lethal gene knockouts with 90% accuracy compared to experimental results reported in literature. Evaluating our model’s prediction accuracy for growth yields and byproduct secretion has been slightly delayed by chemostat equipment failure, but we should be able to measure these quantities soon after the chemostats are back online. We have successfully predicted methanogenesis from methanol and hydrogen by inserting methanol methyltransferase genes, reflecting the current experimental efforts to achieve this conversion. Subsequently, we have predicted reverse methanogenesis from methane to methanol by adding an enzymatic reaction to reduce ferredoxin with electrons from hydrogen to our model. This prediction suggested the importance of reducing ferredoxin in our strain design, but it also did not represent a thermodynamically feasible solution to our system. Rather than generate all of our strain designs based purely on stoichiometry, as we have done in this particular solution, we have elected to add a novel form of predicting free energy to our model as a novel constraint. By calculating overall free energy based on standard free energies of formation and concentrations of external metabolites, we are confident that we can predict strain designs that fulfill both stoichiometry and thermodynamics. We are in the final stages of applying this new method to our model and expect to be able to use SimOptStrain algorithm to generate our top 5-10 strain designs during the following quarter.

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Figure 2*: Likelihood-based gap fill workflow*

**Milestones 3.x**

In the previous quarterly report, we described kinetic data showing that, under single-turnover conditions, anaerobic methane oxidation (the reverse methanogenesis reaction) by the *M. marburgensis* MCR occurs at surprisingly rapid rates (0.8 s-1, 20 oC), essentially equaling our goal of 1.0 s-1 (0.5 units/mg). In these experiments, we reacted MCR with CoBSSCoM and used stopped flow spectrophotometry to follow the conversion of the active Ni(I)-enzyme (with a 390 nm absorbance peak) to the Ni(II)/(III) state (at 420 nm). This rate constant is 400-fold faster that that obtained by steady-state kinetics (kcat = 0.0025 s-1) (3). Because of these unusual results, we have been performing experiments to validate both the presteady-state and steady-state rate constants.

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| During this last term, we developed an independent method to validate the rate constant obtained from the stopped-flow experiments. We performed chemical quench experiments to measure the rate of formation of CoBSH, the product of the single-turnover reaction of MCR with CoBSSCoM (Figure 1). The reaction mixture was incubated for various time periods and quenched with 6 M Guanidine-HCl to release any product formed. Then, the amount of CoBSH formed was measured by incubating with Ellman’s reagent, which reacts with thiolates to form a yellow adduct. Finally the amount of CoBSH formed was plotted versus time and fit to a single exponential curve, revealing a limiting rate constant of 5 s-1.  There are important implications of the single-turnover and steady- | Figure 1. Rapid kinetics measurement of reverse methanogenesis by MCR. |

state results. If the steady-state (kcat) and pre-state (kmax) rate constants are both correct, the chemical (bond-breaking and –forming) steps in the MCR reaction must occur over two orders-of-magnitude faster than the rate-limiting product release.

To validate the steady-state measurement, we have developed a straightforward assay (reaction 3 and Figure 2) in which the thermodynamically unfavorable oxidation of methane (reaction 1) is coupled to the exergonic reduction of fumarate with HS-CoM and HS-CoB (reaction 2), catalyzed by cytoplasmic

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| thiol:fumarate reductase (TFR). This enzymatic system is also proposed to serve as electron acceptor to drive anaerobic methane oxidation (below). The TFR coding sequence is present in *M. maripaludis*, but this enzyme has not been yet isolated or studied. However, cell extracts of *M. marburgensis* (0.7 U/mg, and *M. thermophila* strain ∆H (0.6 U/mg) have high | Figure 2. Rapid kinetics measurement of reverse methanogenesis by MCR |

levels of this enzyme, with the activity of the purified *M. marburgensis* enzyme reaching 150 U/mg (4). The enzyme has also been found in *Methanococcus*, *Methanopyrus*, *Methanosarcina* and *Methanogenium* (4). Purifying and characterizing this TFR and coupling it to MCR is one of the revised project goals (3.8). So far, we have enriched the *M. marburgensis* enzyme 20-fold.

(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

As described under the milestone revisions, we feel that the three most fruitful approaches to drive reverse methanogenesis is to couple methane oxidation to (a) fumarate reduction by TFR (b) sulfite reduction or (c) using an electrochemical system. Thus, the enzymatic studies on TFR that are underway are important in our plans to use TFR as an electron acceptor to drive the GTL process.

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| **Milestone 4.2: Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.**  We are currently performing Potential of Mean Force molecular dynamics (PMF) calculations on the red1 state of MCR to calculate the free energy surface for CoB insertion into MCR. These results will reveal the role and relative importance of MCR residues in binding and transporting CoB to the active site.  **Milestone 4.3:** In the previous term, we calculated | |  |
| the structures of the MCRox1 and MCRred1 reaction intermediates using hybrid quantum/classical mechanics. The key interatomic distances involving nickel and substrate sulfur atoms for the MCRox1 are within 0.05Å of those from the MCRox1 crystal | Figure 3: Depiction of a substrate channel in the average structure of MCRred1 from molecular dynamics. Protein chains are depicted as ribbons, the active site as black spheres, and the channel cavity is shown as cyan spheres. | |

structure, demonstrating that the computational approach was successful. The MCRred1 structure has not been measured experimentally; therefore our QMMM structure is an important and novel discovery. The nickel-S(CoM) distance is longer in the reduced state, which is expected due to the lower-magnitude nickel charge. The distance between the hydrogen of CoB(SH) and the methyl carbon of H3C-SCoM is 2.8 Å, which suggests that the sulfur of CoB must move an additional 1.7 A for the H-C bond of methane to be formed. MD simulations show fluctuations of this magnitude accompany rotation of the thiolate moiety of the CoB. Furthermore, experimental measurements in the Thauer lab demonstrate that the sulfur of CoBSH moves ~2.0 Å towards the Ni in the Ni(I)-MCRred2 state (5), which would be consistent with our computed rotational changes. Presently we are running QMMM geometry optimizations of additional reaction intermediates, including the CoBS-SCoM disulfide state.

 In the last quarter, we deployed Potential of Mean Force (PMF) molecular dynamics to calculate the free energy surface for CoB(SH) insertion into MCRred1. These results will reveal the role and relative importance of MCR residues in binding and transporting CoB to the active site. Specifically, these results we will determine, in a high-precision quantitative manner, how structure and dynamics of the residues lining the substrate channel dictate CoB uptake and binding, and will also provide insights into the relative binding affinities of CoB in the presence and absence of CoM.

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

**Milestone 1.3. Test for genetic complementation of MCR deletion mutation in *M. maripaludis*.** The difficulties with obtaining active Mcr from a heterologous species have to do with expression levels and activation of MCR. Recent evidence suggests that activation requires species-specific mechanisms. One solution is to clone the species-specific activators from the hetelologous species. However, activation is only partially understood. Another solution is to work with the *M. maripaludis* Mcr, as in the proposed milestone changes. Efforts are under way to improve expression levels of heterologous genes as described above.

**Milestone 1.4. Clone and express methanol methyltransferases:**

No change from previous report: Similar 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.

**Milestone 1.15. Clone and express the *M. maripaludis* Mcr:**

Our initial attempt used a construct obtained from Arzeda that had been made for their specific goals. Since this was unsuccessful, we are making another expression construct. Possible complications may arise from recombination between the introduced and native genes.

**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 3.3.**

As described in Milestone 1.4, the key current issue is obtaining sufficient levels of the methanol:CoM MeTr. This is being addressed as described under that section. Another 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) and the HgcA, which is a mercury methylase, from Desulfovibrio. In recent studies, by alteration of growth conditions, we have obtained CFeSP with at least 60% cobalamin and FeS incorporation in vivo. For HgcA, it appears that the form of cobalamin is key to the incorporation. 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.

**Section III. Changes in Approach –**

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 we present the revised milestone schedule and describe the proposed revisions to projects 1, 2, and 3.

**Proposed Changes to the Task Table**:

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| 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 |

**Description and Explanation of the Proposed Changes in Approach**

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* *(4)* and the successful expression in *M. maripaludis* of a F420-dependent sulfite reductase from a closely related species (11).

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 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 (12). 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 (13) 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 (14), 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 (15). 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.

3. 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 (3). 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 (4). The enzyme has also been found in many strains of methanogens including *Methanococcus*, *Methanopyrus*, *Methanosarcina* and *Methanogenium* (4). 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* *(4)* 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 (11).

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