**FY 2016: Q2 Report (January 1st – March 31, 2015) – 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 Simone Raugei (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 an appropriate 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.

**Aim 1:** The minimum requirement to obtain metabolic flux from methanol the methane will be the introduction of active methanol methyltransferase. For this reason we have been concentrating much of our effort on this goal, and have successfully expressed the *Methanosarcina acetivorans* protein in *M. maripaludis*. Western blots suggest we should have sufficient levels of expression that the Ragsdale lab can determine the activity and cobalamin incorporation. In addition, efforts are underway to increase protein levels, and to activate the enzyme in vivo if necessary.

With regard to the methylreductase, we will continue efforts to express the *M. marburgensis* and ANME enzymes in *M. maripaludis*, and to add activator genes as necessary. In addition, we are concentrating on the *M. maripaludis* enzyme itself since activation should not be an issue. Multiple strategies concentrating on the expression the McrA subunit should enable us to produce variants of this subunit for biochemical analyses.

**Aim 2:** Our completed genome scale flux balance model of *M. maripaludis* has already reached its accuracy targets for predicting gene knockout lethality and growth yields over a range of methane secretion rates. Moreover, we have previously reported that adding a ferredoxin:hydrogen oxidoreductase allows us to predict stoichiometrically feasible conversion of methane to methanol. The remaining challenge is to use our model to predict energetically feasible methane oxidation via the addition of reduction pathways. In the past quarter, we addressed this challenge by simulating methane oxidation in concert with three different reduction pathways commonly found in nature: sulfate reduction to sulfide, nitrate reduction to nitrite, and ferric ion reduction to ferrous ions. Based upon these simulations, we predicted only nitrate reduction to be both stoichiometrically and energetically feasible for coupling to methane oxidation. Our model suggests that at standard 1 mM metabolite effective concentrations, 0.66 mol NO3 reduced/mol CH4 oxidized would be sufficient to drive our desired conversion.

**Aim 3:** With respect to Aim 3, we are working with the Leigh laboratory to quantify expression levels to aid in optimizing our assay. We also are trouble-shooting the steady-state assay for reverse methanogenesis that couples Thiol:Fumarate Reductase (TFR) to MCR. Although we have purified this enzyme and demonstrated its activity and cofactor composition, coupling of the enzyme to MCR has been problematic.

**Aim 4:** With respect to Aim 4, we have continued our investigation on residues at the active site that potentially modulate the catalytic activity of MCR. Specifically, we carried out an analysis of the interaction which favors the bind of CoBSH. We found that a triad of charged residues (Arg225, Lys256 and His257) anchors the charged phosphate tail of CoBSH. In addition, Val482 keeps the head of CoB in place via hydrogen bonding between the backbone amide N-and the CoB thiol sulfur atom. Preliminary free energy calculations indicate that the charged triad is the largest contributor to the CoB binding. Breaking of the interaction with the charged CoBSH tail causes the fast dissociation of CoBSH.

We also completed a series of quantum chemical calculations aimed at assessing the relative energetics of the initial steps of methane release from methyl-CoM. We confirmed that the breaking of methyl-CoM bond follows a homolytic route with the formation of a transient methyl radical, which promptly abstract an H atom from the CoBSH thiol group, and the Ni(II)-SCoM cofactor state. We could not locate any pathway associated to the heterolytic dissociation of the bond with the formation of a methyl anion and the Ni(III)-SCoM cofactor state. Time-dependent density functional theory calculations suggested that the anionic pathway should be accessible only via on an excited state wavefunction. Additional calculations indicated that Ni(III)-SCoM state is inaccessible because its very positive reduction potential, *E*0’. We obtained a conservative estimate of *E*0’ = 1.4 V vs. NHE. Indeed, with a calculated redox potential for the CoBS•/CoBS- couple in the enzyme cavity of *E*0’ = 0.0 V vs. NHE, Ni(III)-SCoM would promptly oxidize CoBS- during turnover.

**Aim 5:** 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.

We have 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. We are set to begin talking to potential investors and sources of future funding as soon as we are able to meet the important guideline of converting methanol to methane using our genetic system. Meeting this goal should also allow us to file a new patent application.

**B.    Status of milestones due in the current quarter (Q1FY16) 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 | 5/1/15 | 50% complete | **Test expression strategy by introducing his-tagged *M. maripaludis* Mcr on a vector. Achieve expressed *M. maripaludis* Mcr protein level similar to native Mcr.**  We successfully cloned the complete *M. maripaludis* Mcr operon on a replicative plasmid and introduced the construct into *M. maripaludis*. We are continuing work on this milestone by introducing *M. maripaludis* *mcrA* (“codon-optimized”) using replicative and integrative vectors. |
| M1.4 | 7/1/15 | 50% complete | **Test activity of expressed *M. maripaludis* Mcr by deleting native Mcr. Demonstrate activity by viability of knock-out strain**  The initial attempt was unsuccessful. We will try again as we progress with Milestone M1.3. |
| M1.6 | 8/1/15 | 80% complete | **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. Achieve introduction of gene for plasmid maintenance into *M. maripaludis* chromosome, achieve introduction of expressed chaperonin, achieve knock out of proteasome activator.**  Construction of smaller replicative plasmids and a host strain with the plasmid maintenance gene are complete. A proteasome activator has been knocked out. Expression of the chaperonin, and a knock-out of another potential proteasome activator are underway. |
| M1.7 | 10/1/15 | 50% complete | **Determine effect of measures to increase levels of heterologous proteins: re-introduce heterologous Mcr’s and methanol methyltransferases. Achieve increased levels of heterologous proteins as determined by Western blot.**  A knockout of a proteasome activator appeared to have no effect on the level of the methanol methyltransferase. The effects of the other measures of milestone M1.6 will be tested. |
| M1.11 | 2/1/16 | 50% complete | **Test for methanol methyltransferase activity in cell extract. Demonstrate activity of introduced enzymes by in vitro assay. Obtain at least 2x10-4 umoles CH3OH/g total cell protein/second.**  An initial assay showed no activity. We are re-checking expression levels, obtaining a positive control, and trouble-shooting the assay. |
| M1.12 | 12/1/15 | 90% complete | **Clone activators of methanol methyltransferases into *M. maripaludis*. Verify expression of activators of methanol methyltransferases.**  The activator was cloned and introduced as part of an artificial operon with the *mta* genes for the methanol methyltransferase. Expression along with the *mta* genes is assumed and this will be confirmed. |
| 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 and literature-based manual curation. Our model accurately depicts methanogenesis and can reasonably predict growth and byproduct secretion as compared to experimental data. We have now fully drafted the manuscript about this model and it’s experimental validation, which we expect to finalize and submit for publication within the next month or so. |
| M2.3 | 6/1/15 | 100% complete | **Deliver improved model that enables predictions with >85% predictive accuracy for knockout lethality, and <20% error for wildtype growth and byproduct yield predictions.** Our model’s predictive accuracy for knockout lethality is 90% and our new growth yield data allowed us to train our model such that it predicts growth well within the 20% threshold over a range of different methane secretion rates. |
| M2.4 | 6/1/15 | 80% complete | **Deliver top 5-10 strain design predictions based on methanol yield to team for implementation.** After testing multiple possible electron sinks, simulations with our model have suggested that nitrate reduction to nitrite could feasibly couple to methane oxidation, provided that a ferredoxin:hydrogen oxidoreductase is also added to the organism. Other reduction pathways commonly found in methanotrophic consortia were predicted as infeasible, thus our next step will be to push beyond these previously observed pathways to predict additional feasible strain designs. |
| M3.3 | 12/1/15 | 50% 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 directions. As described in the M1.11 and M1.12, the Leigh group has achieved heterologous expression of this enzyme; however, we did not detect activity. We are expressing a reductive activase along with the methyl-SCoM:methanol MeTr and working on the assay. |
| M4.2 | 10/1/15 | 90% Complete | **Identify at least 10 amino acids modulating substrate binding**  We have identified the most enzyme residues that mostly contribute to the binding of CoBSH. Free energy calculations are underway to estimate the relative contribution of each residue to substrates binding. |
| 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. | 2/1/15 | 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. |
| M5.9 | 8/1/15 | 0% complete | **Write proposal for follow-up funding of the project**. We have identified one potential source of follow-up funding and will be discussing this in the next month. |
| M5.10 | 9/1/14 | 30% complete | **Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs.**  We have developed a preliminary model and will discuss this with ARPA-E project officers at the Remote meeting to ensure this meets their criteria. As described above, we are awaiting some experimental results on the conversion of methanol to methane and the targeted reverse reaction before proceeding with this. It will be helpful as the models from other groups become available to share these. |
| M5.11 | 9/1/15 | 0% complete | **Add data to the technoeconomic model and perform sensitivity analysis**  As described above, we are awaiting some experimental results on the conversion of methanol to methane and the targeted reverse reaction before proceeding with this. It will be helpful as the models from other groups become available to share these. |

**C.    Supporting data & additional information**

**Milestones 1.x**

**M1.3 and M1.4.** Based on the results of our experiments so far, the best strategy for expressing variants of the *M. maripaludis* Mcr is to concentrate of the McrA subunit, which is the most relevant for expressing mutant alleles for biochemical analysis. We are changing the DNA sequence using a codon-optimization process to decrease the likelihood of unwanted homologous recombination with the native gene. We are also pursuing several strategies for maintaining the gene on replicative vectors and integrated into sites on the genome.

**M1.6 and M1.7.** These milestones address efforts to optimize expression of heterologous proteins and to increase their stability in *M. maripaludis*. Several of these measures have been completed and we are continuing to make progress in constructing and testing a variety of vectors, integration strategies, and promoters to optimize expression. With regard to protein stability, we have become aware of a second protein that might function as a proteasome activator and are knocking it out, as well as trying to knock out the proteasome itself (it might be essential for viability).

**M1.11.** The methanol methyltransferase assay may require some optimization and we have only tried it once with cell extract of *M. maripaludis* expressing the methanol methyltransferase genes. We are obtaining cultures of *Methanosarcina acetivorans* and will make cell extract to use as a positive control. We will also work with the Ragsdale lab on the assay.

**M1.12.** A gene encoding an activator of the methanol methyltransferase gene was identified in *Methanosarcina acetivorans* and included in the artificial operon with the genes for the enzyme. Since Western blots for the B-subunit of the enzyme were positive it is likely that the activator is also expressed.

**Milestones 2.x**

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

We have previously described our metabolic model of *M. maripaludis S2* and the iterative process of

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| manual curation. In the previous quarter, we also described our model’s ability to robustly predict measured growth yields within or close to the 95% confidence interval across a | Figure 2*: Growth yield predictions compared to 95% confidence interval of measured values* |

range of methane evolution rates (Fig. 2). This growth yield validation falls well within our goal of <20% prediction error and, together with our previously described knockout validation data, demonstrates the high quality of our model.

Having already demonstrated that adding a ferredoxin:H2 oxidoreductase could stoichiometrically but not energetically enable reverse methanogenesis, we tested hypothetical additions to our solution. Namely, we tested 3 reduction pathways previously found in methanotrophic consortia: sulfate reduction to sulfide; nitrate reduction to nitrite; and ferric ion reduction to ferrous ions. Of these possible pathways, shown below in Figure 3, we predicted that only nitrate reduction would be a feasible strain design. Although iron reduction would be the most thermodynamically favorable of these pathways (ΔG ≈ -207 kJ/mol), the absence of water in this reaction creates a mass imbalance in our model. Thus even though we achieved energetic feasibility, we could not simultaneously achieve stoichiometric feasibility. Sulfate reduction did not suffer from this same problem as each mole of reduced sulfate produced 4 moles of water. However, the initial reaction in this pathway consumes 1 mole of ATP, the metabolite most prominently required for biomass. Because sulfate reduction produces the least negative free energy (ΔG ≈ -92 kJ/mol), the amount of sulfate that must be reduced in order to offset the energy of methane oxidation actually consumes all the ATP produced through reverse methanogenesis, rendering this pathway infeasible in the model.

Unlike the aforementioned pathways, nitrate reduction was predicted to be feasible because it produces water to achieve mass balance in the model and has a sufficiently negative free energy (ΔG ≈ -146 kJ/mol) to offset the free energy of reverse methanogenesis without using up too many electron carriers. At standard effective metabolite concentrations of 1 mM, we predict that 0.66 moles NO3 reduced per mole CH4 oxidized would be sufficient to cross the threshold of ΔG ≤ 0 and would produce about 0.88 moles of CH3OH. Furthermore, a higher flux bound on nitrate or more favorable concentration gradient could easily drive this value much lower. This provides the first strain design predicted by our model to be completely feasible for methane oxidation to methanol. Notably, our predictions thus far have generally been restricted to the realm of transformations known to occur in methanotrophic consortia in nature. These predictions have informed our possible future candidate pathways by demonstrating the importance of generating water and of managing electron carrier usage in our solutions. Moving forward, we plan to test many more possible pathways while adhering to these criteria.

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|  | Figure 3: Candidate reduction pathways for making conversion of methane to methanol energetically feasible |

**Milestones 3.x**

**Milestones 3.3. Deliver an MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM**

We have been working closely with the Leigh group to measure kinetics for the methyltransferase and the MCR reactions. As described above, we seem to now have sufficiently high expression of the MeTr that will allow us to measure activity and cofactor incorporation. We have so far not detected activity of the heterologously expressed methyltransferse and are working with the Leigh laboratory to quantify expression levels to aid in optimizing our assay. The activity of the homologously expressed protein is known, so we will compare those rates with ours.

With respect to Aim 3, we are trouble-shooting the steady-state assay for reverse methanogenesis that couples Thiol:Fumarate Reductase (TFR) to MCR. Although we have purified this enzyme and demonstrated its activity and cofactor composition, coupling of the enzyme to MCR has been problematic.

We have nearly completed our mechanistic studies of the reverse MCR reaction and will be preparing a manuscript describing this work, which will involve experimental work from our lab and computational work in collaboration with the Raugei group at PNNL. Also in collaboration with Raugei, we are gaining an excellent experimental-computational picture of the interaction between CoBSH and MCR.

**Milestones 4.x**

**Milestone 4.2: Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.**

In the last quarter we continued our analysis of molecular dynamics trajectories of the explicitly-solvated MCR protein to complete the identification of residues contributing to the binding of substrates (Figure 8). Simulations revealed that a network of positively charged residues (Arg225, Lys256, His257 and Arg270) that strongly interact the ionized phosphate and carboxylic groups of the CoBSH tail. The backbone of the active site Tyr367 also participate to this network of interactions being hydrogen bonded to both Lys256 and Arg270. In addition, the thiol head of CoBSH is held in place by a hydrogen bond with the amide group of Val482. We also found that Tyr367 is kept rigidly in place by a hydrogen bond with the NH group of the beta-lactam ring of the F430 cofactor. In contrast the side chain other active site tyrosine (Tyr333) does not interact with protein residues and it is consequently more flexible. We currently carrying out free energy calculations aimed at quantify the contribution of each single residue to

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| binding of the substrate.  **Milestone 4.3: Validate activation energies in MCR pathways using experimental data from Ragsdale’s lab.** The QM calculations started in the previous quarter were reanalyzed to understand at an electronic level why the heterolytic CoM(S-CH3) bond scission (anionic catalytic mechanism) is not feasible and only the homolytic scission is viable (radical catalytic mechanism). This additional analysis suggested that the heterolytic mechanism should be accessible only via an excited electronic system of the system. From a purely thermodynamic standpoint, we showed that the Ni(III)-SCoM intermediate, which would be generated from an anionic mechanism, is a relatively strong oxidant (calculated *E*0’ = 1.4 V vs. NHE) and would promptly oxidize the CoBS- species formed from proton abstract by the methyl radical (calculated *E*0’ = 0.0 V vs. NHE). | Macintosh HD:Users:raug510:Desktop:Untitled.png  Figure 8. Hydrogen bonded interactions between active site residues at room temperature. Red sticks indicate hydrogen bonds |

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

**Milestones 1.x.** Cloning and expressing an operon containing the entire set of genes for the Mcr protein, and knocking out the native copy, proved challenging. With our new focus on just the McrA subunit, and with our implementation of a variety of strategies for integration and expression, we are optimistic that this goal can be accomplished. Expressing the McrA subunit should enable us to move forward with biochemical analysis of mutant versions of the methylreductase. With regard to the methanol methyltransferase, further efforts to increase expression and stability, and optimization of the assay, should enable us to demonstrate activity.

**Milestones 2.4.**

With our model essentially completed, our chief remaining challenges lies in generating reasonable strain designs. We have had difficulties implementing the SimOptStrain strain design method and using Kbase gap-filling as a means for generating new designs. also with using the Kbase for gapfilling. We will continue to work with these platforms to try to quickly generate new strain designs and test their energetic feasibility. However, rather than relying solely on these tools, we will also be extending our manual efforts to identify and test promising candidate pathways to pair with methane oxidation.

**Milestone 3.3.**

As described in Milestone 1.4, a key current issue is obtaining sufficient levels of active heterologously expressed methanol:CoM MeTr and MCR. The Leigh lab has recently made significant progress on solving this problem and we are likely to be in position to now to test activity and cobalamin incorporation for the MCR. We are also assaying activity in a construct that contains the ATP-dependent methyltransferase activation protein (MAP) (1).

**Milestone 4.2:** Our chances of success in predicting structural, dynamic and thermodynamic properties of MCR reactivity via classical mechanics are highly based on the observations so far. Analysis of sub-microsecond MD trajectories collected so far indicates that in MCR-Red1 cofactors are stably bound and the motion of the cofactors is highly correlated with the motion of residues at the active site, in particular to Tyr337.

**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 previous reports, 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 –** None.

**REFERENCES**

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