**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 a His-tagged version of the *M. maripaludis* MCR at levels that exceed our milestone goal of 0.1 mg protein/g cell dw. We also reported successful cloning and expression of tagged versions of the ANME 2c and *M. marburgensis* MCRs in *M. maripaludis*, with the three subunits of the targeted enzymes and F430 cofactor being present. However, the expression 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.

In the last term, we have continued to resolve problems with obtaining sufficient expression levels of the his-tagged copy of the *M. maripaludis* Mcr. As soon as these cloning problems are resolved, we will delete the native MCR gene in the genome to demonstrate activity in vivo and provide proof of principle of our expression strategy. This will also provide a system for the mutagenesis of Mcr. We also demonstrated using RT-PCR that the mRNA levels of the heterologously expressed methanol methyltransferase from *Methanosarcina acetivorans* were low; therefore, we cloned and introduced a new construct with several important features that significantly enhance the expression levels. For example, we have demonstrated that the *M. acetivorans* methanol methyltransferase mRNA levels are similar to those of the native *mcr*. This is a major advance that will now be applied to the methanol methyltransferase from *Methanosphaera stadtmanae* and the heterologous Mcr’s from ANME and *M. marburgensis.*

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 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 2, our completed genome scale flux balance model of *M. maripaludis* is complete and predicts gene knockout lethality with over the 85% accuracy threshold. In the previous quarter, we completed additions of thermodynamic constraints to our model that predict free energy generation for predicted flux distributions. We demonstrated the efficacy of this method by predicting overall free energy for reverse methanogenesis without coupling to a reduction pathway, showing that the required equilibrium quotient for this scenario would be infeasible. This result suggested that achieving conversion of methane to methanol will indeed require an exergonic electron sink to offset the energetic cost of methane oxidation. After testing this first strain design, we intend to use the next quarter to automatically generate more strain designs and test the thermodynamic and stoichiometric feasibility of these designs using our model to create a ranked list of our top predicted strain designs.

Additionally, we have spent a significant portion of this quarter drafting a manuscript of our model. During the next quarter, we expect to submit this manuscript for publication.

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 considerably extended the molecular dynamics trajectories of the explicitly-solvated MCR protein. By employing covariance analysis and functional mode analysis, we have found that fluctuation of the Tyr337(OH)-CoM(S) hydrogen bond correlates with the rotational orientation of the CoM(CH3) toward the CoB proton. Therefore this concerted CoM+Tyr337 motion has a period of 0.20 microseconds and it might be key to C-H bond formation.

The potential of mean force calculations of the free energy profile for CoB(SH) insertion into MCRred1, initiated in the previous quarter, were not completed before the staffing transition from Dr. Smith to Dr. Raugei (July 1st, 2015), due to Dr. Smith’s change of employment. However the aforementioned MD and trajectory analyses address Milestone 4.2 (due September 2015) “apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics”.

Experimental data on the pKa value of catalytically relevant residues at the active site have become available. The identity of these residues is still unknown. In order to guide their identification, we have started p*K*a estimates based on Poisson-Boltzmann calculations and more accurate free energy perturbation molecular dynamics.

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 | 5/1/15 | 30% 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.** Cloning is underway and we are currently dealing with some technical difficulties with the cloning. |
| M1.4 | 7/1/15 | 60% complete | **Test activity of expressed *M. maripaludis* Mcr by deleting native Mcr. Demonstrate activity by viability of knock-out strain.** This step depends on M1.3; hence we will carry it out once M1.3 is complete. |
| M1.5. | 6/1/15 | 50% complete | **Determine mRNA levels for heterologous proteins. Achieve mRNA levels similar to native Mcr.** This has now been accomplished for the methanol methyltransferase of *Methanosarcina acetivorans*. |
| M1.6 | 8/1/15 | 90% 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.** A smaller expression plasmid has been constructed and is now in use. A strain has been constructed with the proteasome activator knocked out. The chaperonin has been cloned and will soon be introduced into *M. maripaludis*. |
| M1.10 | 5/1/15 | 50% 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.** This is 100% complete for the Methanosarcina methanol methyltransferase and we will do the same with the Methanosphaera genes. |
| 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 | 90% | **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 86.7% and we are in the process of re-measuring growth yields and ATP maintenance values with chemostat experiments. This process was delayed waiting for new chemostat equipment and is now moving forward with the vessels fully-operational. We will finish collecting these data within the next few weeks and expect to be well within the 20% error range for yield predictions. |
| M2.4 | 6/1/15 | 70% | **Deliver top 5-10 strain design predictions based on methanol yield to team for implementation.** We have successfully added free energy predictions to our model, allowing us to test the thermodynamic feasibility of proposed strain designs. This includes our first strain design, which was deemed to be energetically infeasible. We are finalizing our automated strain design pipeline, through which we are planning to iteratively generate stoichiometrically-feasible designs and test their energetic feasibility. This process should yield our desired top 5-10 designs to reasonably achieve reverse methanogenesis to methanol. |
| 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. |
| M4.2 | 9/1/15 | 80% Complete | **Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.** The long-timescale MD calculations for the red1 state of MCR are completed, and all that remains are detailed analyses of concerted motions further away from the active site, specifically, unstructured regions near Tyr337. Estimates of the acidity of the acidity of active site residues are currently underway to guide the experimental assignment of key functional residues. |
| 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 the red1 and ox1 reaction intermediates proposed by Ragsdale’s lab have been completed. QM/MM simulations are currently 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. |
| 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.** 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.** The goal is to introduce a cloned and his-tagged copy of the *M. maripaludis* Mcr and demonstrate activity by deleting the copy in the genome. This will be important as proof of principle of our expression strategy. It will also set up a system for the mutagenesis of the *M. maripaludis* Mcr, should we opt to focus on the *M. maripaludis* Mcr for biochemical studies. Currently this effort is delayed by technical difficulties with the cloning. We expect to resolve these soon.

**M1.5.** Since levels of expressed heterologous proteins were low, it was important to determine whether there was a deficiency in expression at the mRNA level. We are currently focusing on the methanol methyltransferase because it is critical in our overall objective and must come from a heterologous source (*M. maripaludis* contains no gene for this enzyme). Initial experiments using RT-PCR indicated that mRNA levels were indeed a problem. We therefore cloned and introduced a new construct that included intergenic sequences from *Methanococcus voltae* (closely related to *M. maripaludis*) and the transcriptional terminator from the gene encoding the highly expressed S-layer protein of *M. maripaludis*. RT-PCR (Fig. 1) showed that with the methanol methyltransferase from *Methanosarcina acetivorans*, we have been successful, and mRNA levels are similar to those of the native *mcr*. We will take similar measures with the methanol methyltransferase from *Methanosphaera stadtmanae* and the heterologous Mcr’s.



**Fig. 1.** RT-PCR of methanol methyltransferase (mta) genes from Methanosarcina acetivorans expressed in M. maripaludis. mtaC1, first gene in the operon. MA4380, last gene in the operon. The native mcr genes are shown for comparison. Lanes 3, 6, 10, and 13 show RT-PCR signals.

**M1.6.** A smaller replicative expression plasmid and a suitable host strain have been made and tested and are now being used routinely. The plasmid is equipped with a promoter and a transcriptional terminator and its utility was demonstrated by our successful expression of mRNA as shown above. A similar vector designed for integration into the genome instead of replication as a plasmid has also been constructed since it may afford greater expression levels in the polyploid *M. maripaludis*. A strain has been constructed with the proteasome activator knocked out. The chaperonin has been cloned and will soon be introduced into *M. maripaludis*. Soon we will test the effect of these measures on the levels of expressed heterologous proteins.

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| **M1.10.** Fig. 2 shows a recent Western blot demonstrating expression of the *Methanosarcina acetivorans* methanol methyltransferase. This is the protein obtained from the strain that generated the RT-PCR results shown above. We will test the measures implemented in M1.6 to see if they increase protein levels.  **Fig. 2.** Western blot showing a his-tagged subunit (MtaB1) the *Methanosarcina acetivorans* methanol methyltransferase expressed in *M. maripaludis*. HdrA is a his-tagged protein known to be expressed at high levels, shown for comparison. |  |

**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 and improved our metabolic model of *M. maripaludis S2* (Fig. 3)*.* Improving the model remains an ongoing process, though we have made minimal changes to the model in the last quarter because it already depicts of central metabolism accurately and thus fulfills its role as a platform for generating strain designs. Our efforts in model improvement have primarily been aimed at better integrating the model with publicly available databases by including cross references to these databases. We have also begun chemostat experiments to gather final pieces of growth yield validation data, which will complete the process of verifying our model’s predictive accuracy. These efforts in model integration and quantitative validation have been particularly salient as we prepare to submit our completed model for publication next quarter.

Our modeling focus has shifted to generating strain designs to guide our metabolic engineering approach, particularly by pairing stoichiometric analysis with thermodynamic constraints. Using our model, we predicted that the main stoichiometric challenges of reversing methanogenesis are removing hydrogen produced by reversal of heterosulfide reductase and generating reduced ferredoxin that can be used to pump out sodium ions through either the Eha or Ehb hydrogenase. The most trivial solution to this challenge would be to add a ferredoxin: hydrogen oxidoreductase that transfers electrons from hydrogen to ferredoxin. Indeed, adding this reaction to our model allowed us to predict methanol formation from methane; however, when adding in our method of free energy prediction, it became clear that this scenario was energetically infeasible. Though this trivial case has thus been ruled out as a feasible strain design, it demonstrated the efficacy of pairing traditional stoichiometric modeling with thermodynamic information. As we move into the final quarter of 2015, our main focus is currently on working with the SimOptStrain algorithm for rapid generation of strain designs. Our progress on getting this tool operational has been slower than expected, but we believe we are now close to achieving this goal point and will soon be able to use our model to automatically generate strain designs. Once armed with the ability for automatic strain design, we will begin an iterative process in the next quarter, whereby we rapidly predict strain designs, test those designs for thermodynamic feasibility in our model, then discard infeasible designs and repeat the cycle until we have a list of our top 5-10 designs.

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Figure 3*: Likelihood-based gap fill workflow*

**Milestones 3.x**

In previous quarterly reports, we demonstrated that, under single-turnover conditions, anaerobic methane oxidation (the reverse methanogenesis reaction) by the *M. marburgensis* MCR occurs at rates (0.8 s-1, 20 oC) that reach our target 1.0 s-1 (0.5 units/mg, at optimal conditions). This rate constant is 400-fold faster that that obtained by steady-state kinetics (kcat = 0.0025 s-1) (1). Thus, we performed experiments to validate both the presteady-state and steady-state rate constants.

Pre-steady state results indicate that CoBS-SCoM reacts with MCR in a three-step reaction (Fig. 4) in

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| which the first step is formation of the binary Michaelis complex with the heterodisulfide substrate and the second step is electron transfer, generating the Ni(II)-radical anion form of the substrate. Then, methane reacts to form methyl-SCoM and CoBSH. Our results indicate that the chemical (bond-breaking and –forming) steps in the MCR reaction occur over 100-fold faster than rate-limiting product release.  As mentioned above, methane oxidation by MCR | Fig. 4. Proposed two-step mechanism for reaction of MCR with CoBSSCoM in reverse methanogenesis. |

is thermodynamically unfavorable (Eq. 1). The only steady-state measurements that have been performed of the reverse reaction were based on NMR studies, which were subject to a number of assumptions, and the value of kcat was 0.0025 s-1 (1). Furthermore, these experiments were performed under conditions that favor the forward reaction. This value is 400-fold slower than the value measured by rapid kinetics and 40,000-fold slower than the kcat for the forward reaction. Thus, it is extremely important to measure the steady-state rate (the values of kcat and kcat/Km).

We have developed an independent method to directly monitor the reverse reaction. With this protocol, the reverse reaction is coupled to a reaction that is so exergonic that the reverse reaction becomes thermodynamically favorable (Fig. 5). This coupled reaction is the thiol:fumarate reductase (TFR) from *M. marburgensis* (Equation 2).

(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

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| 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 levels of this enzyme, with the | Figure 5. Rapid kinetics measurement of reverse methanogenesis by MCR |

activity of the purified *M. marburgensis* enzyme reaching 150 U/mg (2). The enzyme has also been found in *Methanococcus*, *Methanopyrus*, *Methanosarcina* and *Methanogenium* (2). We have recently purified this TFR to near homogeneity and coupled it to MCR. At the present time, we are confident that this assay will work, however, we need to optimize the reaction before we can state reliably what are the steady state kinetic parameters.

We now surmise 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.

Recent pH studies of the reverse reaction indicate that stabilization of the MCR:CoBS-•SCoM complex is controlled by protonation of a group within p*K*a value ~ 7.3. pH jump studies indicate that protonation of this group cannot be rapidly equilibrated with outside solvent. These studies are being integrated with the QM/MD work described below.

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| **Milestone 4.2: Apply potential energy function to identify amino acids that contribute to substrate binding thermodynamics.** In the last quarter we analyzed triplicate 500 ns molecular dynamics trajectories of the explicitly-solvated MCR protein. By employing covariance analysis and functional mode analysis, we have found a correlation between the Tyr337(OH)-CoM(S) hydrogen bond the relative orientation of CoM(CH3) toward the CoB proton. Figure 3 shows the mode of motion associated with CoM(CH3) approach to CoB(SH). When the Tyr337(O) is within H-bonding distance of CoM(S) (3.5 Å), the CoM methyl C atom points toward CoB(S) (3.7 Å). Likewise, when Tyr337 has broken contact with CoM(S) (6.2 Å), the CoM methyl carbon rotates way from CoB (5 Å). | Figure 6: Depiction of 0.2 microsecond motion driving the orientation of CoM(CH3) toward CoB(SH). The structure drawn with thick lines has a hydrogen bond between Tyr337(O) and CoM(S). In the structure drawn with grey lines, the hydrogen bond is broken and CoM(CH3) is oriented further away from CoB via 20 degree rotation. |

We hypothesize that this concerted CoM+Tyr337 motion (with period of 0.20 microseconds) might be a previously unrecognized and extremely important key for the formation/cleavage of the C-H bond of methane. We are currently exploring this hypothesis. Further assessment of the covariance and correlation analyses will also illuminate how other flexible protein residues relate to substrate binding, such as unstructured regions with transient hydrogen bonds to Tyr337.

**Milestone 4.3: Validate activation energies in MCR pathways using experimental data from Ragsdale’s lab.** We have completed the QM/MM optimization of the structures of the MCRox1,MCRred1 and CoBS-SCoM disulfide state reaction intermediates using hybrid quantum/classical mechanics. We have now all the starting point to explore possible reaction pathways.

The simulation of the CoB(SH) insertion into MCRred1 started in the previous quarter was not completed before the staffing transition from Dr. Smith to Dr. Raugei. We are currently addressing issues related to the identification of order parameters for the potential of mean force calculations that describe at best the insertion pathway.

We have started the calculation of the p*K*a values of residues at the active site in order to guide the assignment of key ionizable residues identified experimental in Ragsdale’s lab. The computational estimate of p*K*a values will exploit both traditional Poisson-Boltzmann methodologies and more accurate (but time-consuming) free energy perturbation molecular dynamics.

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

**Milestone 1.3-1.4. Test for genetic complementation of MCR deletion mutation in *M. maripaludis*.** We are making progress with enhanced expression of heterologously expressed proteins, as described above. Activation of MCR may still be a problem once the expression levels are solved. If so, one solution is to clone the species-specific activators from the hetelologous species. 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 1.10. Clone and express methanol methyltransferases:** We are much more optimistic about the MeTr, as described above. Efforts to further enhance expression are underway, again as described.

**Milestones 2.4.**

With our model essentially completed, our chief remaining challenges lies in generating reasonable strain designs. We have had some solver difficulties implementing the SimOptStrain strain design method but are currently working through these and expect to have a working algorithm soon. Once this is complete, we expect to be able to quickly generate new strain designs and test their energetic feasibility.

**Milestone 3.3.**

As described in Milestone 1.4, the key current issue is obtaining sufficient levels of the methanol:CoM MeTr. The Leigh lab is making progress on solving this problem and we will likely have sufficient MeTr to test activity and cobalamin incorporation. If the latter is a problem, we will assay activity in a construct that contains the appropriate ATP-dependent methyltransferase activation protein (MAP) (3).

**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.

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