**Team Michigan Milestone Revisions:**

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 (1).

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 (2). 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 (3), 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 (5).

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

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

Previous experiments indicate that steady-state catalysis of reverse methanogenesis by MCR occurs at a slow rate of 0.0025 s-1 at 60 oC (6). 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.

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 (3).

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 (5).

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

In the first year of the project, we successfully built a genome-scale metabolic model of *M. maripaludis S2* with an accurate representation of the Wolfe Cycle, the catabolic pathway in our organism (7). The uniqueness of the intermediates involved in the methanogenic pathway (8) 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. We propose to instead focus our metabolomics runs on measuring 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 try to find a way to measure methanogenic intermediates. The White group has expertise in identifying unique compounds in methanogenic archaea, including key synthesis steps for methanogenic coenzymes (9), 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.

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 (10). We are not equipped to measure hydrogen uptake rates, but we still plan to measure growth rate, growth yield, and byproduct secretion using gas chromatography. 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. We are uncertain that the existing curve is accurate and propose to create a new OD v. dry cell weight curve. This short experiment would greatly aid our efforts because it would enable us to calculate accurate growth yield measurements and give us high-quality standards to measure against predictions from our model. We estimate t and have also have also data from these experiments by the e John Leigh has contacted the White group at Virginia tech

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