**Technical Milestones and Deliverables**

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| **Prime Recipient** | The University of Michigan |
| **Award No.** | DE-AR0000426 |
| **Type of Funding Agreement** | Cooperative Agreement |
| **Competitive or Noncompetitive Award** | Competitive |
| **Funding Opportunity Announcement (if applicable)** | DE-FOA-0000881, REDUCING EMISSIONS USING METHANOTROPHIC ORGANISMS FOR TRANSPORTATION ENERGY (REMOTE) |

Please refer to Subpart A of Attachment 1 to this Award, especially Clause 6 (Federal Stewardship and Substantial Involvement).

# STATEMENT OF PROJECT OBJECTIVES

This is a multi-investigator and multidisciplinary Technology Development Project that aims to develop novel and transformational technology for the biological synthesis of methanol from methane, the major component of natural gas. This project combines the complementary expertise and skills of a team of four investigators: Stephen Ragsdale from the University of Michigan (UM, the Lead Institution) is the Contact PI and Coordinator for the Project. The co-investigators are John Leigh from the University of Washington (UW), Nathan Price from the Institute for Systems Biology (ISB) and Dayle Smith from Pacific Northwest National Laboratory (PNNL). Methanogenic archaea generate nearly all of the methane on earth and produce it at a level of 1 billion tons per year. It was recently found that methanogens also are involved in the anaerobic oxidation of methane. In fact, large amounts (0.3 billion tons per year) of methane are oxidized to CO2 in marine sediments by microbial communities, which consist of methanogens (ANME-1, ANME-2 or ANME-3) and sulfate- or nitrate-reducing bacteria.

Surprisingly, Methyl Coenzyme M Reductase (MCR) is the key enzymatic catalyst in both the anaerobic synthesis of methane and the oxidation of methane (AOM).

The primary project goal is to engineer a metabolic pathway for the conversion of methane to methanol at rates, efficiency and yield that meet the Technology Development goals for this ARPA-E program. To make the process thermodynamically favorable, we will also introduce sulfate or nitrate reduction pathway. The ultimate goal is to achieve methane conversion to methanol at a rate of at least 1g CH4/g cell dw/hr (0.5g CH4/L/hr).

John Leigh (UW) will introduce the appropriate genes for conversion of methane to methanol and sulfate to sulfide into the model methanogen, *M. maripaludis*. Nathan Price (ISB) will use genome-scale metabolic flux modeling of engineered *M. maripaludis* strains to ensure that enzymes in the engineered pathway can operate at high efficiency in the new organism. He will also reengineer other aspects of *M. maripaludis* metabolism to optimize the methane-to-methanol pathway. Stephen Ragsdale (UW) will perform biochemical and biophysical studies to determine and ensure the functionality of all introduced enzymes to efficiently and rapidly convert methane to methanol. Dayle Smith (PNNL) will use multi-scale computational chemistry techniques to calculate reaction pathway

thermodynamics and identify key enzymatic elements that control substrate binding, overall reaction thermodynamics and reversibility. This information will be used to design and optimize the enzymatic conversion of methane to methanol. The Project will be coordinated with Staff and Fellows the Office of Technology Transfer at the University of Michigan and with independent Consultants to develop a Technology to Market Plan. This OTT collaboration will address all intellectual property and technology transfer matters related to the project team.

Related to convergence of our research goals with the mission of ARPA-E, successful completion of the aims of this proposal will reduce US dependence on foreign energy sources, which will enhance US energy security. Because methane is such a clean burning fuel, its increased use in the transportation industry will lead to reduced emissions and increase energy efficiency. The project also aims to transform the way transportation fuels are distributed, which will stimulate domestic manufacturing.

# B. SCHEDULE OF TECHNICAL TASKS, MILESTONES, AND DELIVERABLES

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| WBS | Name (task, subtask, milestone) | Duration | Start | Finish |
|  |  | months |  |  |
| 1 | Engineer pathways for anaerobic bioconversion methane to liquid fuels - John Leigh (U. Washington) |  |  |  |
| 1.1 | Clone ANME MCR genes into Methanococcus maripaludis and verify expression | 5 | 1/23/14 | 6/1/14 |
| M1.1 | Confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw. Expressed protein will be oligo-His tagged for purification and blotting. |  | 6/1/14 | 6/1/14 |
| 1.2 | Clone Methanothermobacter marburgensis MCR genes into M. maripaludis and verify expression | 5 | 6/1/14 | 11/1/14 |
| M1.2 | Confirm presence of introduced genes and expressed protein by RT-PCR and Western blot, and obtain at least 0.1 mg protein/g cell dw. |  | 11/1/14 | 11/1/14 |
| 1.3. | Test expression strategy by introducing his-tagged *M. maripaludis* Mcr on a vector | 2 | 3/1/15 | 5/1/15 |
| M1.3 | Achieve expressed *M. maripaludis* Mcr protein level similar to native Mcr |  |  | 5/1/15 |
| 1.4 | Test activity of expressed *M. maripaludis* Mcr by deleting native Mcr | 2 | 5/1/15 | 7/1/15 |
| M.1.4. | Demonstrate activity by viability of knock-out strain |  |  | 7/1/15 |
| 1.5. | Determine mRNA levels for heterologous proteins | 3 | 3/1/15 | 6/1/15 |
| M.1.5. | Achieve mRNA levels similar to native Mcr |  |  | 6/1/15 |
| 1.6. | Implement measures to increase levels of heterologous proteins: Construct an *M. maripaludis* strain to support replication of a smaller expression plasmid, overexpress chaperonin, knock out proteasome activator | 5 | 3/1/15 | 8/1/15 |
| M.1.6. | Achieve introduction of gene for plasmid maintenance into *M. maripaludis* chromosome, achieve introduction of expressed chaperonin, achieve knock out of proteasome activator |  |  | 8/1/15 |
| 1.7. | Determine effect of measures to increase levels of heterologous proteins: re-introduce heterologous Mcr’s and methanol methyltransferases | 2 | 8/1/15 | 10/1/15 |
| M1.7 | Achieve increased levels of heterologous proteins as determined by Western blot |  |  | 10/1/15 |
| 1.8 | 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 |
| 1.10. | Clone Methanosarcina and Methanosphaera methanol methyltransferase genes into *M. maripaludis* and verify expression | 4 | 1/1/15 | 5/1/15 |
| 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 |
| 1.11 | Test for methanol methyltransferase activity in cell extract | 2 | 12/1/15 | 2/1/16 |
| 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 |
| 1.13 | 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 |
| M1.13 | 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 |
| 1.16 | Manipulate expression of electron flow pathways as necessary. Introduce modified MCRs | 2 | 9/1/16 | 11/1/16 |
| 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 |
| 1.17 | Test for conversion of methane to methanol and optimize metabolic fluxes as needed | 2 | 11/1/16 | 1/1/17 |
| 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. maripaludis* 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 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. | 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. | Sulfite 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. | Sulfite 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 |

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| **WBS** | **Task Title and Description** | |
| **1.0** | **Engineer pathways for anaerobic bioconversion methane to liquid fuels**. Our primary goal is to demonstrate conversion of methane to methanol in a model methanogen, *M. maripaludis*. To make the process thermodynamically favorable, we will also introduce sulfate reduction pathway. *M. maripaludis* contains MCR and HDR, which may be sufficient for conversion of methane to methyl- SCoM. Introduction of the methyltransferase will allow conversion to methanol. We will introduce the ANME MCR and HDR to determine if they are better suited for methane oxidation. In the second part, we will **engineer pathways to convert sulfate to sulfide.** This exergonic electron-accepting pathway will be introduced to render the overall methane to liquid fuel process exergonic. In natural consortia, this is achieved by a syntrophic process; we will introduce the pathway into *M. maripaludis*, whose growth is resistant to (and enhanced by) sulfide. (Note: recent reports suggest the possibility of using a non-canonical sulfate reduction pathway or a nitrate reduction pathway. These will be considered as alternatives to ATP sulfurylase and APS reductase.) | |
| **WBS** | **Sub-Task Title and Description** | **Milestone/Deliverable Title and Description** |
| 1.1 | Clone ANME MCR genes into *M. maripaludis* and verify expression |  |
| 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. |
| 1.2 | Clone *Methanothermobacter marburgensis* MCR genes into *M. maripaludis* and verify expression |  |
| 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. |
| 1.3 | Test for genetic complementation of MCR deletion mutation in *M. maripaludis* |  |
| M1.3 |  | Confirm activity of introduced enzymes by demonstrating viability of *M. maripaludis* MCR deletion mutants in expression strains |
| 1.4 | Clone *Methanosarcina* and *Methanosphaera* methanol methyltransferase genes into *M. maripaludis* and verify expression |  |
| M1.4 |  | 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. |
| 1.5 | Test for methanol methyltransferase activity in cell extract |  |
| M1.5 |  | Demonstrate activity of introduced enzymes by in vitro assay. Obtain at least 2x10-4 umoles CH3OH/g total cell protein/second. |
| 1.6 | Test for metabolic conversion of methanol to methane by methanogenesis from methanol, OR demonstrate conversion of methane to methanol using labeled substrates | |
| M1.6 | 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). (The activity level of 2x10-4 umoles/g total cell protein/second is based on an initial expression level of 0.1 mg protein/g cell dw, and an assumed initial specific activity of ANME MCR of 0.07 umoles/min/mg. The latter is calculated based on the CH4 oxidation rate and protein content of natural communities.) | |
| 1.7 | Increase expression level of MCR | |
| M1.7 | Obtain 50 mg MCR protein/g cell dw (10% of total cell protein). | |
| 1.8 | Clone genes for ATP sulfurylase and APS reductase into *M. maripaludis* and verify expression | |
| 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. | |
| 1.9 | Assay for ATP sulfurylase and APS reductase activities | |
| M1.9 | Demonstrate activity of introduced enzymes by in vitro assay. Obtain 5x10-5 umoles/g total cell protein/second. | |
| 1.10 | Clone sulfite reductase genes from related methanogens into *M. maripaludis* and verify expression | |
| 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. | |
| 1.11 | Clone sulfite reductase genes from sulfate reducers into *M. maripaludis* and verify expression | |
| 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. | |
| 1.12 | Assay for sulfite reduction to sulfide | |
| M1.12 | Demonstrate activity of introduced enzymes by in vitro assay. Obtain at least 5x10-5 umoles/g total cell protein/second. | |
| 1.13 | Manipulate expression of electron flow pathways as necessary. Introduce modified MCRs. | |
| M1.13 | Reach activities of sulfate to sulfide pathways of 1.25 umoles/g total cell protein/second.  Demonstrate improved fluxes by introduction of modified MCRs | |
| 1.14 | Test for conversion of methane to methanol and optimize metabolic fluxes as needed | |
| M1.14 | Achieve methane conversion to methanol at a rate of at least 1g CH4/g cell dw/hr (0.5g CH4/L/hr). | |
|  |  | |
| **2.0** | **Metabolic modeling and flux measurements.** Use genome-scale metabolic flux modeling of engineered *M. maripaludis* strains used for expression of methane to methanol/butanol and sulfate reducing pathways. This process will ensure that enzymes in the engineered pathway can operate at high efficiency in the new organism. Based on modeling and experimental outcomes, reengineer other aspects of *M. maripaludis* metabolism by performing genetic alterations to optimize methanol/butanol pathways in new metabolic context. We will perform associated metabolomic and metabolic flux experiments to iteratively test, refine, and validate the metabolic model. | |
|  | **Build genome scale flux balance and core metabolic flux models for *M. marapaludis* metabolism.** | |
| 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 | |
| 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 | |
| M2.1. | First genome-scale metabolic model for *M. maripaludis* capable of simulating growth and byproduct section with >75% accuracy | |
| 2.3 | **Genome scale flux balance and core metabolic flux models:** Build detailed core metabolic network model to enable metabolic flux measurements | |
| 2.4 | **Genome scale flux balance and core metabolic flux models:** Integrate detailed core metabolic flux model with the genome-scale flux balance model | |
| M2.2. | First model of core metabolism in M. marapaludis with atom-level detail for molecular transformations as needed for flux measurements. | |
| 2.5. | **Validate metabolic models against experimental data:** Perform measurements of growth rates, substrate uptake rates, and byproduct secretion rates | |
| 2.6. | **Validate metabolic models against experimental data:** Perform time series metabolomics measurements in batch cultures | |
| 2.7. | **Validate metabolic models against experimental data:** Compare all measurements under different conditions to model simulation | |
| 2.8. | **Validate metabolic models against experimental data:** Iteratively improve the model as needed | |
| M2.3. | Accuracy estimates for the metabolic model and iterative improvement with manual curation until model predictions are in  >85% agreement with data. | |
| 2.9. | **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 | |
| 2.10. | **Use the models to guide strain engineering for methanol production:** Simulate expected product conversion yields and compare with experiment | |
| M2.4. | Provide engineered strains with highest methanol production. | |
| 2.11 | **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 | |
| 2.12 | **Use the models to guide strain engineering for methanol production:** Measure metabolic fluxes in engineered strains and compare distributions with the computed optimum | |
| 2.13. | **Use the models to guide strain engineering for methanol production:** Iteratively design and refine network and test engineered strain to optimize for methanol production | |
| M2.5. | Provide 3 best engineered strains with highest methanol production based on metabolic modeling | |
| **3.0.** | **Biochemical optimization of enzymes involved in methane conversion to methanol.** Perform biochemical and biophysical studies to determine the functionality of enzymes to efficiently and rapidly convert methane to methanol. | |
| 3.1 | Determine endogenous *M. maripaludis* activities of MCR, HDR and Methyltransferase | |
| 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 | |
| **M.3.1.** | Deliver MCR with specific activity for the purified protein of >5 units/mg (after activation) in methane synthesis and 0.07 units/mg in methane oxidation, where one unit equals 1 micromol substrate converted per min. | |
| 3.3. | Perform site-directed mutagenesis and selection to increase the methane oxidation (methyl-SCoM production) specific activity of the chosen (most likely ANME) MCR by 20-fold. | |
| **M.3.2.** | Deliver an MCR with a specific activity of at least 1.4 units/mg in conversion of methane to methyl-SCoM. | |
| 3.4. | Perform site-directed mutagenesis and selection to improve the methane oxidation (methyl-SCoM production) specific activity of the chosen (most likely ANME) MCR 200-fold above the initial one (M3.1). | |
| **M.3.3.** | Deliver an MCR with a specific activity of at least 14 units/mg in conversion of methane to methyl-SCoM. | |
| 3.5 | Heterodisulfide Reductase (HDR): Determine kinetic parameters of the native and ANME HDRs in the forward & reverse direction | |
| **M.3.4.** | Deliver HDR with a specific activity for the purified protein of at least 20 units/mg. | |
| 3.6. | Methyl-SCoM:Methanol Methyltransferase (MeTr): Determine kinetic and physical (spectroscopic and metal binding) properties of the heterologously expressed MeTr in the forward & reverse direction | |

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| **M.3.5.** | Deliver MeTr with an activity for the purified protein of at least 20 units/mg for methanol production from methyl-SCoM |
| 3.7. | **Sulfate to sulfide module:** Measure background endogenous SO4-to-sulfide activity **(ATP sulfurylase, APS reductase and sulfite reductase)** |
| 3.8. | Sulfate to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed ATP sulfurylases |
| **M.3.6.** | Deliver ATP Sulfurylase with an activity for the purified protein of at least 20 units/mg |
| 3.9. | Sulfate to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed APS Reductase |
| **M.3.7.** | Deliver APS Reductase with an activity for the purified protein of at least 20 units/mg |
| 3.10. | Sulfate to sulfide module: Purify and assess kinetic and physical properties of heterologously expressed Sulfite Reductase |
| **M.3.8.** | Deliver Sulfite Reductase with an activity for the purified protein of at least 20 units/mg |
| 3.11. | Ensure that the chosen sulfate to sulfide enzymes couple to give predicted flux - in vitro and in vivo |
| 3.12. | Substitute enzymes from a single host if necessary - make adjustments based on in vivo flux measurements |
| **M.3.9.** | Deliver enzymes for the sulfate-to-sulfide module based on highest activity, at least 20 units/mg. |
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| 4.0. | **Molecular modeling of the ANME MCR and other enzymes involved in GTL.** Use multi-scale computational chemistry techniques to calculate reaction pathway thermodynamics with respect to differences in amino acid sequence for MCRs. Based on Aim 3 and molecular modeling outcomes identify key enzymatic elements that control MCR substrate binding, overall reaction thermodynamics and reversibility. |
| 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 |
| M4.1 | Reproduce experimental MCR structure 1MRO.pdb with a root-mean-squared deviation of less than 10 Ångstroms. |
| 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. |
| M4.2 | Identification of at least 10 amino acids modulating substrate binding |
| 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 |
| M4.3 | Validate activation energies in the two pathways using experimental data from Ragsdale’s lab to reach agreement within 5 kcal/mol |
| 4.4. | Predict effects of site-specific substitutions within methanogenic and methanotrophic (ANME) MCR on activation energies |
| M4.4 | Validate changes in activation energies for amino acid substitutions to reach agreement to within 5 kcal/mol with data from Ragsdale’s lab. |
| 4.5 | Predict effects of site-specific substitutions within methanogenic and methanotrophic (ANME) MCR on reaction reversibility |
| M4.5 | 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. |
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| 5.0 | **Technology Transfer and Intellectual Property:**  Work with Dr. Nadine Wong1 and others in OTT2 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 |
| M5.1. | Reach agreement and sign an IP sharing agreement among U. Mich, U. Washington, PNNL, and ISB.3 |
| 5.2. | Hire OTT fellow(s)4 to look at the ARPA-E REMOTE proposal and assess the relevant existing current patent landscape. |
| M5.2. | Develop a profile of the existing patents related to methane to methanol (and GTL) biotechnology. |
| M5.3. | File provisional patent for our plan to convert methane to methanol. |
| 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 |
| M5.4. | Develop a working Tech to Market plan |
| 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.2 |
| M5.5. | Develop a document that assesses the market for conversion of methane to methanol and to butanol (GTL) |
| 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.5 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. |
| M.5.6. | Hire an independent T2M Consultant to oversee the tech-to-market plan and forge relationships with technology partners. |
| 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. |
| M.5.7. | Develop a pitch deck. |
| 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. |
| M.5.8. | Meet with potential industrial partners |
| 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. |
| M.5.9. | Write proposal for follow-up funding of the project. |
| 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. |
| 5.10. | **Technoeconomic analysis:** Develop technoeconomic model based on the model that Ramon used when he developed the REMOTE program |
| M.5.10. | Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs |
| M.5.11. | Add data to the technoeconomic model and perform sensitivity analysis |
| M.5.12. | Complete the technoeconomic model |

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