Applicant Name: Ragsdale, S.W.
Control Number: 0881-1501

TECHNICAL VOLUME - TECHNOLOGY DEVELOPMENT PROJECT
Reducing Emissions Using Methanotrophic Organisms For Transportation Energy
ANAEROBIC BIOCONVERSION OF METHANE TO METHANOL

I. TECHNICAL APPROACH:

This Technology Development Project aims at developing novel and transformational technology for the biological synthesis of liquid fuels from methane, the major component of natural gas. Accounting for 22% of U.S. energy consumption, methane is the simplest organic compound and has the highest energy content of any carbon-based fuel. Widely mined and used as a fuel for heating and cooking, methane also is used by the chemical industry to produce synthesis gas, to generate electricity, and to serve as a vehicle fuel in the form of compressed or liquid natural gas. Methane utilization also has environmental ramifications because it is a potent greenhouse gas whose levels have doubled over the past two centuries (1).

A class of microbes called methanogens generate nearly all of the methane on earth and produce it at a level of 1 billion tons per year. Much of the methane is recaptured and used as an energy source by methano*trophic* microbes. The process of methane oxidation by aerobic bacteria has been known for over a century. Over the last decade, the surprising discovery emerged that methane can also be oxidized anaerobically. In fact, large amounts (0.3 billion tons per year) of methane are oxidized to CO_2 in marine sediments by microbial communities, which consist of methanotrophic archaea (ANME-1, ANME-2 or ANME-3) and sulfate- or nitrate-reducing bacteria. A recent surprising finding is that Methyl Coenzyme M Reductase (MCR), the key enzymatic catalyst in the anaerobic synthesis of methane, also catalyzes the oxidation of methane (AOM). This protein is unique to methanogens and anaerobic methane oxidizers.

The project, aimed at synthesizing liquid fuels from methane, 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). 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. The secondary aim is to further convert the methanol to butanol.

The current state-of-the-art process for the conversion of natural gas to liquid fuels utilizes a chemical process (Fischer-Tropsch chemistry), which is limited by high capital costs and low conversion efficiencies. Another feasible route is the use of aerobic methane oxidizing bacteria in a bioengineering process; however, this is calculated to yield energy efficiency less than 51%. Being a major catabolic pathway with demanding thermodynamics, anaerobic methane oxidation must be highly energy efficient - a designed anaerobic methane-to-butanol pathway would reach a theoretical energy efficiency of 77%. Based on studies of methane synthesis, the anaerobic pathway should quantitatively (1 mol/mol) convert methane to methanol.

We will genetically engineer a metabolic pathway to convert methane to methanol and/or butanol. We will optimize this process by a rational approach involving metabolic flux modeling and adjustment of the rates of the individual enzymatic catalysts involved in converting methane to the desired liquid fuels.

II. R&D TASKS:

This proposal describes a multidisciplinary Technology Development Project aimed at developing novel and transformational technologies for bioconversion of methane to liquid fuels." In the announcement for the ARPA-E Funding Opportunity (DE-FOA-0000881) on "Reducing emissions using methanotrophic organisms for transportation energy (REMOTE) program it was stated that "efficient, cost-effective conversion of methane to a liquid fuel at any scale of production would be transformative in enabling natural gas as a transportation fuel." The foundation of our strategy involves using a microbial system that catalyzes the anaerobic oxidation of methane. 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). The project goal is to engineer a metabolic pathway that meets the Technology Development performance metrics, i.e., a rate exceeding 1 g of fuel/g cell dry weight/hr, energy efficiency greater than 64% and carbon yield of at least 67%.

In nature, large amounts (0.3 billion tons per year) of methane are oxidized to CO_2 in marine sediments by consortia consisting of methanotrophic archaea (ANME-1, ANME-2 or ANME-3) and sulfate-reducing bacteria (2-5). The key enzymatic catalyst in the anaerobic oxidation of methane (AOM) is Methyl Coenzyme M Reductase (MCR), a protein that is unique to methanogens and anaerobic methane oxidizers. Although the role of MCR as the key enzyme in methane synthesis (1 billion tons/year) has been known for decades, its role in AOM was discovered only recently (3,6).

The specific aims of this project are:

(1) To actively express the gene clusters encoding the MCRs from ANME-1 and ANME-2 in a genetically tractable methanogen and to genetically engineer the pathways for converting methane to methanol and to butanol. These pathways will be coupled to the dissimilatory sulfate reduction path to make the gas to liquid fuel process thermodynamically favorable.

(2) To use genome-scale metabolic flux modeling of the genetically engineered *Methanococcus maripaludis* strains that will be used for expression of the methane to methanol/butanol and sulfate reducing pathways. This process is to ensure that enzymes in the engineered pathway can operate at high efficiency in the new organism. Based on the modeling and experimental outcomes, we will also reengineer other aspects of *M. maripaludis* metabolism by performing genetic alterations, i.e., knock-outs, to optimize the methanol/butanol pathways in its new metabolic context. We will also perform associated 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.

(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.

III. R&D STRATEGY:

III.A. Innovation and Impact

This Technology Development Project aims at meeting major objectives and addressing major challenges described in the ARPA-E Funding Opportunity (DE-FOA-0000881) on "Reducing emissions using methanotrophic organisms for transportation energy (REMOTE)" by developing a novel and transformational technology for the biological synthesis of liquid fuels from methane. Accounting for 22 percent of the energy consumption of the U.S., methane, the major component (~87 percent) of natural gas, is the simplest organic compound and produces more heat per mass unit (55.7 kJ/g) than any other hydrocarbon. Responsible for more than 90% of the earth's atmospheric methane (the balance being generated by geochemical reactions (7)), methano*genic* microbes produce 1 billion tons of methane per year (8). Methane is widely mined and used as a fuel for heating, cooking and generation of electricity and is a clean fuel, emitting less sulfur, carbon, and nitrogen than coal or oil, and leaves little ash. In the chemical industry, methane is used to produce synthesis gas (syngas, a mixture of CO and H_2) and as a fuel for electricity generation. Methane is also used as a vehicle fuel in the form of compressed (CNG) or liquid (LNG) natural gas, especially in Asia and South America, and ARPA-E has announced its "Methane Opportunities for Vehicular Energy" (MOVE) program to address the refueling infrastructure and the high cost of compression and storage for the widespread use of CNG and LNG in the transportation industry. The rationale for the REMOTE program is to avoid the high costs of gas storage and distribution and to match our nation's distribution infrastructure by developing efficient, cost-effective processes for the conversion of methane to a liquid fuel that combine high energy density with broad compatibility across all modes of transportations.

Methane is not only used as a source of energy for human consumption. In nature, methano*trophic* microbes capture emitted methane and oxidize it as a source of cell carbon and energy as a key part of the global carbon cycle (9). Methane oxidation by aerobic bacteria (Eq 1) has been known for over a century (10); while, it has been recognized only recently that methane can also be oxidized anaerobically (the process that is the focus of this proposal, Equations below). The anaerobic oxidation of methane (AOM) occurs in various sedimentary deep sea and shallow lagoon settings at a depth interval known as the sulfate–methane transition zone (11). Fluorescence in situ hybridization studies and phylogenetic analyses reveal that methane-oxidizing archaea and sulfate-reducing bacteria are closely associated in this zone (12,13) indicating that methane oxidation involves a consortium of microbes, one population catalyzing AOM and another oxidizing sulfate. In nature, large amounts (0.3 billion tons per year) of methane are oxidized to CO_2 in marine sediments methanotrophic archaea (ANME-1, ANME-2 or ANME-3) and sulfate-reducing (2-5) or nitrate-reducing (14) (though the nitrate respiration appears to actually result from *aerobic* methane oxidation (15)) bacteria. While methanotrophs are efficient at capturing emitted methane, over the past two centuries since the industrial revolution, the methane balance point has tipped in favor of methane synthesis leading to a doubling of the atmospheric methane concentration during this time period. Rising methane concentrations has environmental ramifications because it is a potent greenhouse gas that is 21 times more effective at trapping heat in the atmosphere than carbon dioxide (16).

We propose to develop a novel and transformational bioengineering technology for the biological synthesis of liquid fuels (e.g., methanol) from methane. We will pursue this in two modular stages (Fig. 1). In Stage 1, we will engineer a model methanogenic bacterium, *Methanococcus maripaludis,* to convert methane to methanol (eq. 1). In Stage 2, to make this

process thermodynamically favorable, we will introduce the gene module for conversion of sulfate to sulfide. This mimics natural AOM, except that, instead of oxidizing methane completely to CO_2, the process will be stopped at methanol. Once this is accomplished, we will have achieved our major goal: establishment of a transformational platform for converting methane to a liquid fuel. A third stage of this technology, which may be beyond the scope of this proposal and shown below in Fig. 3, is conversion of the methanol to butanol.

$CH_4 + H_2O \rightarrow CH_3OH + H_2$ $\Delta G^{0'} = 112.5$ kJ/mol (Eq. 1)

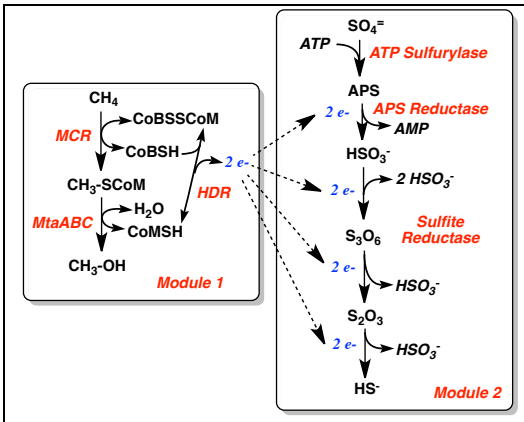

Fig. 1. Engineered pathway to convert methane to methanol.

The current state-of-the-art for the conversion of natural gas to liquid fuels is a chemical process (GTL) utilizing Fischer-Tropsch chemistry. While this proven technology increases volumetric energy density and avoids the costs of gas storage and distribution, it is limited by high capital costs and low conversion efficiencies. FT-GTL suffers from high costs and low efficiencies because it is a complex, multi-step process that involves converting methane to synthesis gas (syngas), converting the syngas to a hydrocarbon and (3) separating and upgrading the diverse products. Another drawback is that the process requires numerous temperature and pressure changes.

Our proposed solution departs significantly from currently available technology and differs from others under investigation in the field. We propose to use a novel bioengineering approach to convert methane to liquid fuels. The foundation of our strategy involves engineering a model methanogen to catalyze the anaerobic oxidation of methane to methanol. To make this process thermodynamically favorable, we will also engineer the sulfate reduction pathway. Once this is accomplished, we will engineer the organism with the genes required to convert methanol to butanol. Anaerobic methane oxidation is highly energy efficient. The REMOTE FOA states that the theoretical energy efficiency of a designed anaerobic methane-to-butanol pathway is 77%, while aerobic methane oxidation (e.g., using methane monooxygenase) leads to energy efficiency less than 51%, because of inefficiency both in the activation of methane (66%) and in the conversion of formaldehyde (product of methane activation) into fuel (78%). This higher efficiency of the anaerobic process is due at least in part to the lower ATP yield of anaerobic respiration causing growth of anaerobic microbes to be limited by catabolism. For example, O_2-dependent (aerobic) methane oxidation to CO_2 (Eq. 2) proceeds with a $\Delta G^{0'}$ of -817 kJ mol^{-1}, while AOM coupled to sulfate reduction (Eq 3) has a $\Delta G^{0'}$ of only -21 kJ mol^{-1}. Thus, to survive, anaerobes must convert much more substrate to product than aerobes.

$$CH_4 + 4O_2 \rightarrow 2H_2O + CO_2 \quad \Delta G^{0'} = -817 \text{ kJ/mol (Eq. 2)}$$

$$CH_4 + SO_4^{=} + H^{+} \rightarrow HS^{-} + 2H_2O + CO_2 \quad \Delta G^{0'} = -21.2 \text{ kJ/mol (Eq. 3)}$$

The key enzymatic catalyst in AOM is Methyl Coenzyme M Reductase (MCR), a protein that is unique to methanogens and anaerobic methane oxidizers (17-19) and catalyzes the first step in methane oxidation (Eq 4). The MCR reaction is tightly coupled, with methyl-SCoM being converted into methane (in the methane synthesis direction) with 100% carbon conversion efficiency. Therefore, based on the principle of microreversibility, there should be 100% conversion of methane into methyl-Coenzyme M. Furthermore, the methanol methyltransferase reaction that catalyzes the conversion of methyl-SCoM to methanol is tightly coupled and, thus, cumulatively, this combined process should quantitatively convert methane to methanol. *Thus,*

performance of the proposed solution represents a significant advance relative to the state of the art. While the role of MCR as the key enzyme in methane synthesis (1 billion tons/year) has been known for decades, its role in AOM was discovered only recently (3,6,20). MCR is not present in aerobic methanotrophs (10), in which the key methane-oxidizing enzyme is methane monooxygenase, which occurs as a soluble iron or a membrane-bound copper enzyme (21).

$$CH_4 + CoBS\text{-}SCoM \rightarrow CH_3\text{-}SCoM + CoBSH \quad \Delta G^{0'} = +30 \text{ kJ/mol (Eq. 4)}$$

As mentioned above, in nature, AOM appears to involve a syntrophic process (Eq. 3) in which endergonic AOM by a methanogen (Eq. 5) is coupled to and driven by the exergonic reduction of sulfate by a sulfate reducer (Eq. 6) (12,13). Organisms related to ANME-2 also have been shown to couple AOM (Eq 3) to the microbial reduction of nitrate to dinitrogen gas (Eq 7), yielding a significantly more favorable energy balance (Eq 8) (14). We plan to develop an integrated process of methane oxidation by consolidated bioengineering in which the oxidation of methane and the coupled exergonic process occurs in a single organism.

$$CH_4 + 2H_20 \rightarrow 4H_2 + CO_2 \quad \Delta G^{0'} = +131 \text{ kJ mol}^{-1} \text{ (Eq. 5)}$$

$$S0_4^{=} + 4H_2 + H^+ \rightarrow HS^- + 4H_20 \quad \Delta G^{0'} = -152.2 \text{ kJ/mol (Eq. 6)}$$

$$N0_3^- + 2H^+ + 4H_2 \rightarrow N_2 + 2H_2O \quad \Delta G^{0'} = -436.4 \text{ kJ/mol (Eq. 7)}$$

$$5\ CH_4 + 8\ N0_3^- + 8H^+ \rightarrow 4\ N_2 + 5CO_2 + 14H_2O \quad \Delta G^{0'} = -765 \text{ kJ/mol } (-133 \text{ kJ/mol } CH_4) \text{ (Eq. 8)}$$

The proposed metabolic engineering project is innovative in using a recently discovered pathway of anaerobic methane oxidation. We will integrate the coupled processes of AOM and sulfate reduction in a single organism. In addition, we will build a genome-scale flux balance model to address methane oxidation in an anaerobic methanogen and better understand the mass and energy balances for these systems. In particular, this model will help tune the metabolic network so that the newly introduced engineered pathways can be fully utilized within the constraints of the organism's metabolic needs. Such an approach will be valuable in utilizing methanogenic organisms for the transformation of methane, as these organisms have mastered one-carbon metabolism for both fuel production and fuel utilization. The project also brings together researchers with complementary expertise in biochemistry, genetics, synthetic and computational chemistry, and metabolic modeling. Thus, the project synthesizes scientific principles from disparate fields that do not typically intersect. The program also meets the intent of ARPA-E to bring together researchers in government labs, private industry, and academia.

Besides our major goal of meeting the specific goals and performance metrics of the REMOTE program, our technology development will significantly impact several of the key ARPA-E mission areas. One of the reviewers stated that our "*proposed work represents a highly innovative and high-risk approach to engineering novel organisms capable of converting methane to liquid fuels under anaerobic conditions"*. Funding "Innovative" and "high-risk" research is a key mission of ARPA-E and we appreciate that the reviewers recognize the transformational possibilities of our proposal, which truly challenges the current state-of-the-art methods in converting gas to liquid fuel and develops technology that ill bridge the gap between basic energy research and development/industrial innovation.

Furthermore, our project involves collaborators from a government laboratory, a research institute, and two academic institutions, another stated intention of ARPA-E funding. We feel that the members of this research team have cross-disciplinary skill sets and are the most qualified people in the world to achieve success in this project, for reasons described in Section III.C. On the other hand, if we find that it is important to enlist others with specialized expertise that we did not anticipate during preparation of this proposal, we will shift budgets accordingly

to ensure that we can add those collaborators. Similarly, if we find that one of the subcomponent Tasks is no longer required or does not prove to be as promising as anticipated, we will phase out those programs so that the project retains only those projects whose promise remains real.

The proposal also tackles the most challenging aspects of the gas to liquid fuel technology – the bioconversion of methane to methanol coupled to sulfate reduction – by using recently discovered enzymes that naturally catalyze this process, but which have never yet been found together in a single organism. If the proposed transformational technology is successfully developed, it will be of tremendous commercial interest and application.

Aim 1: *Engineer the pathways for anaerobic bioconversion methane to liquid fuels*
Under Aim 1, John Leigh (Univ. of Washington) will engineer the five-gene cluster encoding the MCR from two anaerobic methane oxidizers (ANME-1 and ANME-2) into *Methanococcus maripaludis*, which is a premier model for hydrogenotrophic methanogens (22) that Leigh's lab has worked with for 20 years. He also will engineer the metabolic pathway required for the conversion of methane into methanol (eventually to butanol) into this same species.

John Leigh's lab has a long history in the development and use of *M. maripaludis* as a model species of methanogen (22). All methanogens require strictly anaerobic, reduced conditions for their growth and manipulation. Most species are grown on hydrogen and carbon dioxide as their primary substrates for energy-generating metabolism. A laboratory must be equipped for these requirements. With the necessary equipment, working with these organisms becomes second nature. The advantages of *M. maripaludis* lie in its rapid and facile growth in the laboratory and in its amenability to genetic manipulation. With a doubling time near two hours at its optimum growth temperature of 37°C, *M. maripaludis* yields liquid cultures over night, and colonies on agar medium in two or three days. As a result, experiments progress rapidly, and, key to the goal of this project, metabolic flux is rapid. The growth advantage of *M. maripaludis* extends to use of fermenters for large-scale cultivation and the use of chemostats for continuous culture (23). Leigh's lab made it a priority to develop continuous culture techniques for *M. maripaludis* because they allow one to rigorously and reproducibly control nutritional conditions and to eliminate variations of growth rate and cell density when comparing cultures. Perhaps unique among laboratories studying methanogens, the continuous culture approach is now routine in Leigh's lab. The only other group of methanogens that comprise a laboratory model due to their genetic manipulability are species of *Methanosarcina*. However, these organisms grow much more slowly and require prolonged incubations for growth of cultures.

Genetic manipulations in Archaea are traditionally difficult due to their non-susceptibility to many antibiotics used against Bacteria and their specialized growth conditions. However, genetic manipulations in *M. maripaludis* are facilitated by a high-efficiency transformation procedure, the use of two effective antibiotic selections (for puromycin and neomycin), and the use of 6-azauracil for counterselection. These tools result in the ability to use a "pop-in pop-out" approach to make markerless mutations, which consist of in-frame deletions or other kinds of mutations that are not encumbered by residuals such as antibiotic resistance markers. Perhaps the best illustration of our use of this approach is in our recent construction of a strain containing seven different in-frame deletion mutations and an additional mutation that induced the expression of an otherwise cryptic pathway (24).

Several different recombinant vectors have been developed that allow *M. maripaludis* to be used for the heterologous expression of anaerobic proteins and for the generation of site-

specific variants (Table 1). The vectors can integrate into the genome or can remain as separate replicons and have been equipped with antibiotic selection markers, promoters for the expression of cloned genes, and oligo-his tag moieties for the tagging of proteins. Commonly used vectors in the lab include multicopy replicative vectors and integrative vectors that insert into the genome in single copy. Strong promoters are present to drive expression of recombinant genes and are constitutive (histone and S-layer promoters) or inducible by starvation for nitrogen (nif promoter). Two antibiotic resistance markers are used, for puromycin (pur) or neomycin (neo), and oligo-his moieties are present in some vectors for tagging proteins on their N- or C-termini.

Table 1. Vectors in common use in the Leigh lab.

Replicative	Promoter	Antibiotic	Other Features
pWLG series	Histone promoter	pur or neo	N- or C-terminal his tag
pMEV1.1.2	S-layer promoter	pur	
pHW40	Nif promoter	pur or neo	
Integrative			
pJK3	*Methanosarcina* mcr promoter	pur	Contains site for insertion of homologous sequence
pIJA03NH	Various promoters can be inserted	neo	Integrates into the argH site
pBLprt	Various promoters can be inserted	neo or markerless	Integrates into the upt site

Using these vectors, the Leigh laboratory has been successful in expressing genes either for genetic complementation purposes or for production of active proteins for biochemical or structural studies. We routinely grow expression strains in fermenters and purify expressed oligo-his tagged proteins using a purification pipeline featuring anaerobic Ni-affinity chromatography and an FPLC housed inside an anaerobic chamber. For example, we conducted extensive biochemical studies of the nitrogenase and the nitrogenase inhibitor NifI from *M. maripaludis*, using protein expressed in *M. maripaludis* (25) and, in recent unpublished work, determined the crystal structure of the heterologously expressed *Methanocaldococcus* sp. NifI. As an example of yields, we obtained 1.2 mg *Methanocaldococcus* NifI protein per g cell dry weight. We also expressed and purified the transcriptional regulator NrpR and, with special significance to this proposal, we have expressed and purified the heterodisulfide reductases from *M. maripaludis* and the methanogen *Methanothermobacter marburgensis* in the *M. maripaludis* system (Duin and Leigh, unpublished). These examples illustrate our approach to the introduction and expression of proteins that we will use here.

Mcr is the central enzyme of methane oxidation and will be our first priority for expression. The Mcr will be studied in Ragsdale's lab and will catalyze the first step in the methane oxidation pathway to be engineered. Anke Meyerdierks (Max Plank Institute) has agreed to send us cosmids containing the genes for the 5-subunit Mcr proteins from ANME-1 and ANME-2 organisms, and cloning of these genes in the *M. maripaludis* system should be straightforward. The two ANME Mcr proteins are known to harbor some minor differences in post-translational amino acid modifications compared to each other and to methanogenic Mcr

proteins, and ANME-1 has a methyl-thio modification on the coenzyme F_{430}. We will include both ANME Mcr proteins in our experiments to increase the likelihood of obtaining active protein. In addition, Mcr from the methanogen *Methanothermobacter marburgensis* has been shown to catalyze both methane synthesis and methane oxidation to methyl-SCoM. Because this protein, expressed and purified from the native organism, has been extensively studied in the Ragsdale laboratory and we will clone and express it as well. We will confirm expression of the genes for Mcr by RT-PCR and confirm the presence of the protein by Western blot. In addition, the *M. maripaludis* system allows one to confirm the presence of active protein by genetic complementation. Thus, if the expressed Mcr is active, we should be able to delete the native Mcr using our standard genetic techniques.

The ANME Mcr involved in AOM has been shown to involve the same sulfhydryl coenzymes, HSCoM, HSCoB, and the heterodisulfide CoMSSCoB, as do methanogens (6). Therefore, the product of methane oxidation is expected to be methyl-SCoM and HSCoB. To effect conversion of methyl-SCoM to methanol, we will clone and express the extensively characterized *Methanosarcina barkeri* methyltransferase *mtaABC* genes. After transfer of the methyl group, the products will be HSCoM, HSCoB, and methanol. Expression will be confirmed by RT-PCR and Western blot, and activity confirmed by assaying for methylation of HSCoM with methanol.

The introduction of the methane-oxidizing Mcr and the methanol-producing Mta described above will complete a pathway for conversion of methane to methanol. This pathway must be coupled to an exergonic electron-accepting pathway that will render the overall process exergonic. In natural consortia, methane oxidation is coupled to dissimilatory sulfate reduction in the bacterial partner, or possibly to zero-valent sulfur production in the ANME organism (26). Enzymes of the canonical dissimilatory sulfate reduction pathways have been purified from a methane-oxidizing consortium (27), whereas the proposed zero-valent sulfur production was suggested to involve an uncharacterized non-canonical pathway of sulfate reduction. Our plan is to couple the methane oxidation pathway to the canonical sulfate reduction pathway as outlined in Fig. 1 above. How this coupling will work is shown in Fig. 2. First, heterodisulfide reductase (Hdr) operating in the reverse direction oxidizes HSCoM and HSCoB to produce reduced ferredoxin (Fd), and H_2 via the hydrogenase Vhu. Reduced ferredoxin is then used by the energy-converting hydrogenase Eha to produce H_2 in an energy-conserving reaction that produces a chemiosmotic membrane potential. The H_2 produced will be used to reduce sulfate. The pathway is expected to be thermodynamically feasible at the ambient low partial pressure of H_2. Furthermore, ATP is produced chemiosmotically to activate sulfate and to support growth. This mechanism for the generation of a chemiosmotic potential and ATP production is proposed to occur naturally in *Methanosphaera stadtmanae* (28). Alternatively, in *M. maripaludis* we recently discovered a pathway that would achieve the same result (24). By inducing expression of the gene for glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR), a cycle occurred in which GAPOR coupled with steps in gluconeogenesis resulting in electron flow from NADPH to ferredoxin, hydrolyzing ATP. In reverse, reduced Fd would reduce NADP in a cycle producing ATP. NADPH could then be used to reduce F_{420}, from which H_2 would be produced.

The enzymes for the scheme shown in Fig. 2 for coupling of methane oxidation to sulfate reduction are already present in *M. maripaludis*, except for those of sulfate activation and reduction. To add the sulfate-reducing pathway, we will clone and express the ATP sulfurylase (Sat), APS reductases (Apr), and sulfite reductases (Dsr) of sulfate reducers, and verify

expression by RT-PCR and Western blot. (The intermediate, sulfite, is toxic to methanogens; however, sulfite reductase activity can render the organisms sulfite-resistant (29)). The electron donors for Apr and Dsr in vivo are not well known. It has been proposed that electrons for Apr flow by reverse electron bifurcation (electron confurcation) through an enzyme present in sulfate reducers that is homologous to Hdr, QmoABC QmoABC (30). Electron flow to sulfite may occur through a complex of Dsr proteins and also involve Hdr-like proteins. We will clone and express these proteins as necessary. To provide electrons to Qmo and to the Dsr complex, we will manipulate electron flow pathways in *M. maripaludis* as necessary. Pathways are known in *M. maripaludis* for electron flow between H_2, Fd, NAD(P), and F_{420}, making it possible to provide a variety of electron donors (24). In addition, sulfate reducers in the ANME consortium may use HSCoM and HSCoB to reduce APS or sulfite, or may reduce sulfate without activation to APS using electrons from the low-potential Fd (28). To better address these possibilities, it is a long-term goal to express and characterize the enzymes of sulfate reduction from the sulfate-reducing partner of the ANME consortium. As a further alternative, for the reduction of sulfite, we will exploit an assimilatory sulfite reductase already present in methanogens. This enzyme, Fsr, is an F_{420}-dependent sulfite reductase that is present in *Methanocaldococcus jannaschii*, and when expressed in *M. maripaludis*, conferred resistance to sulfite, which it converted to sulfide (29). Fsr is also present in *M. maripaludis* relatives *Methanothermococcus thermolithotrophicus* and the mesophilic *Methanococcus aeolicus* (31), suggesting the gene is available from several sources.

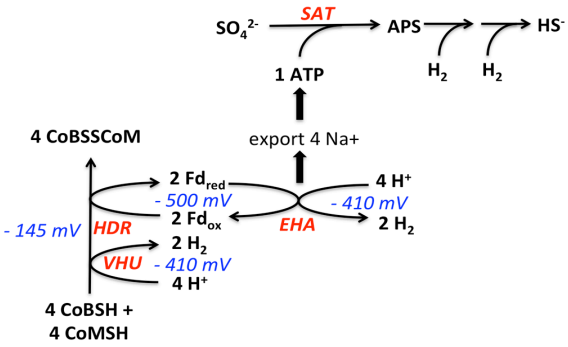

Fig. 2. Scheme for coupling CH_4 oxidation to $SO_4^=$ reduction. Redox potentials shown for standard conditions, i.e. 1 atm (100 kPa) H_2. The pathway should be thermodynamically feasible at an expected H_2 partial pressure of < 10 Pa.

Our primary goal is to demonstrate conversion of methane to methanol. A secondary goal and the third phase of gene introduction would involve cloning the genes encoding the conversion of methanol to butanol. A suitable pathway (Fig. 3) involves conversion of methanol and CO_2 to acetyl-CoA through the Wood-Ljungdahl pathway, which is present in *M. maripaludis*. The acetyl-CoA would then be converted to butanol through acetoacetyl-CoA, hydroxybutyryl-CoA, butenyl (crotonyl)-CoA, butyryl-CoA and butanol. This pathway is present in solvent-producing clostridia, such as *Clostridium carboxidivorans*, which possesses the Wood-Ljungdahl pathway along with the metabolic pathway for butanol production (32). This pathway also exists in *C. acetobutylicum* and has been successfully engineered into *E. coli* (33) and *S. cerevisiae* (34). In *C. carboxidivorans* and other solventogenic clostridia, the genes involved in the conversion of

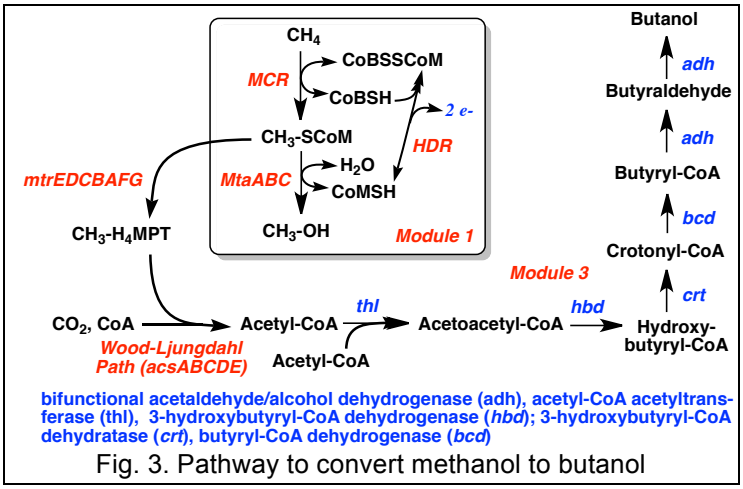

Fig. 3. Pathway to convert methanol to butanol

acetyl-CoA to butyryl-CoA are in a gene cluster on the chromosome. To assess activity of this system, the Price and Leigh labs (Task 2) will measure the methane, methanol, and butanol flux and the Ragsdale's lab (Task 3) will perform steady-state kinetic measurements of the individual enzymes. The Price lab will also incorporate this added butanol production pipeline into the genome-scale model for *M. maripaludis* to aid with strain design. We will also generate strains lacking the sulfate reduction pathway – in the absence of this electron transfer sequence, *M. maripaludis* should convert butanol to methane and, in its presence, would convert methane to butanol. We will also determine the effect of a knock out (KO) of the methylene-H_4MPT reductase gene (mer) (35), because this gene is involved in oxidation of methyl-SCoM to CO_2. We will determine if this KO increases flux to methanol in the absence of the methanol-to-butanol pathway, and towards butanol in its presence. In the presence of the sulfate reduction path, we expect to achieve optimal flux of methane to methanol (or butanol) in the *mer*$^-$ strains.

Aim 2: *Metabolic modeling and flux measurements*

In Aim 2, Nathan Price's laboratory will perform genome-scale metabolic modeling of the genetically engineered *M. maripaludis* strains, adding to the model the ANME genes for anaerobic methane oxidation and the other genes needed to convert methane to methanol and to butanol. The model will be used to predict key system-wide performance metrics, including the potential flux of methane to liquid fuels. The project goal is to engineer a metabolic pathway that meets the Technology Development performance metrics, i.e., a rate exceeding 1 g of fuel/g cell dry weight/hr, energy efficiency greater than 64% and carbon yield of at least 67%.

The genome-scale metabolic reconstruction approach that the Price lab will use to study *M. maripalidus* has been successfully used to study over 100 different organisms with diverse applications in biotechnology (reviewed in (36)). **Critically for this proposal, flux-based models have proven especially useful to guide strain improvements.** For example, the use of genome-scale models to guide improvement of valine production in *E. coli* resulted in substantially higher titers (37). A wide variety of algorithms has been developed to guide strain design from well-curated metabolic network models (see (38) for review). In addition, the Price lab has extensive expertise in metabolic network reconstruction, curation, and analysis using best practices in the field (39). We have already produced curated genome-scale metabolic models for a number of organisms, including two methanogens. Under previous funding by the DOE, we reconstructed the first highly curated genome-scale metabolic model for *Methanosarcina acetivorans* C2A (40) and substantially updated the previously existing metabolic model for *Methanosarcina barkeri* Fusaro (41), representing the non-H_2- and H_2-utilizing sub-types of *Methano-sarcina*, respectively. Both models are 96% accurate in predicting the results of gene knockout lethality experiments and are able to accurately and

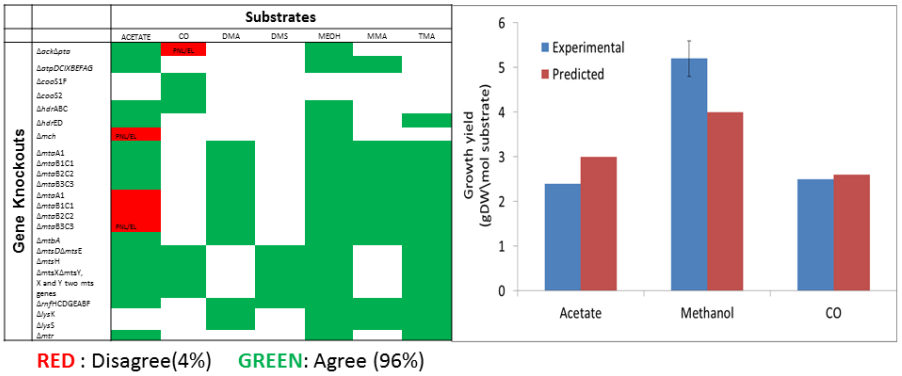

Figure 4. Price Lab reconstruction of *M. acetivorans* accurately predicted gene knockout lethality (left) and growth yield (right).

quantitatively predict growth phenotypes on different substrates (Fig. 4).

The first task will be to build a genome-scale metabolic reconstruction of *M. maripaludis* on which to base the metabolic simulations. A curated model of *M. maripalidus* core metabolism already exists, consisting of 82 reactions and 72 metabolites (42). In order to expand this model to the genome scale, we will leverage our knowledge of methanogens in *Methanosarcina* as well as automated reconstruction tools, and apply curation efforts to improve the model quality. Automated reconstructions of *M. maripalidus* have been created in both the paths2models project (43) and the ModelSEED project (44). Our lab has extensive experience working with the ModelSEED tools and the corresponding DOE KnowledgeBase (KBase) platform and are working closely with this team to release an improved gap-filling and annotation module within the KBase platform that maximizes genomic evidence for the filled gaps (we will submit our joint paper on this effort and publically release the tool by the end of the summer). We will also build our curated *M. maripaludis* model and distribute it within the DOE Kbase framework.

Automated reconstruction tools provide an advanced starting point for building metabolic reconstructions, but curation will be necessary to turn the reconstruction into models that can be used to make useful predictions and accurately compute selected phenotypes (e.g., growth rate, metabolic fluxes, gene essentiality). For example, to our knowledge, the path2models reconstruction is not yet capable of simulating growth, and neither path2models nor the ModelSEED model correctly predicts byproduct secretion correctly without additional curation. **Subsequently, the first focal point of our modeling efforts will be the curation of a functional and predictive genome-scale metabolic network model for *M. maripaludis*.** The additional detail to be added during the manual curation includes: 1) assurance that all reactions are mass and charge balanced; 2) identification and addition of missing reactions needed to accomplish known functions (e.g., production of amino acids); 3) inclusion of transporters and spontaneous reactions; and 4) generation of plausible biomass equations (i.e., stoichiometric composition of the biomass of *M. maripaludis* used as an objective function for modeling growth). Constraints on fluxes will also be added by determining thermodynamic directionality and incorporating experimental flux measurements, particularly update and secretion rates.

We will validate the accuracy of the curated model prior to using it as a tool for strain design. We will validate initially by comparing model predictions with existing experimental data and by generating our own data tailored to the specific conditions we are studying. Existing experimental datasets for *M. maripalidus* include a comprehensive set of gene knockout phenotype data determined by transposon mutagenesis (45), and continuous growth data under a variety of nutrient-limiting conditions (46). To add to this existing corpus of data, with John Leigh our team will perform routine measurements of growth rates and external metabolite changes over time under a number of different conditions. These sources combined will provide data of precisely the same type as we used to perform initial validations of the metabolic models we have already built for *M. acetivorans* and *M. barkeri*. With John Leigh and our collaborator Dan Raftery (see letter of support), a set of metabolomics experiments will also be performed for growth and product generation on different concentrations of CO_2 and H_2 to identify metabolites present in the cell. Metabolite identification will be used to help complete pathways within the organism that are missing from the genetic analyses. In this way, we will improve the model until we achieve a high degree of accuracy in matching available phenotype data.

The curated metabolic model will be used to guide strain design by predicting the outcome of introducing the newly engineered pathways, as well as additional genetic

perturbations (e.g., gene deletions/ additions) to the system that will help maximize the production of methanol and butanol from methane. Existing algorithms such as OptStrain (47) will be used to suggest perturbations that improve expected maximum yields in a network context and couple production of methanol and butanol to the organism's growth and survival. Correct algorithmic predictions will lead to improved strain design, and those that are incorrect will lead to additional curation and improvement of the model's accuracy for use in making subsequent predictions. In this manner, the model provides a powerful means of iterative strain improvement. Using genome-scale models is particularly advantageous for strain design because coupling production to growth will almost certainly involve alterations to aspects of metabolism other than the introduced engineered pathways to make their use align with the regulation and selection pressures active on the organism.

As our designed strain is progressing, we, in collaboration with John Leigh, will also employ a powerful flux measuring approach that will evaluate experimentally and within the context of the model the fluxes that are being utilized by *M. maripaludis* and, by iteratively probing these fluxes, we will guide the additional modifications that are needed to optimize production. Metabolic flux analysis (MFA) (48-50) is an important technique that uses stable isotope labeling for measuring the flux of the metabolic pathways and reactions that occur within a cell. The microorganism is grown on a carbon source that includes at least one isotopic label (usually ^{13}C). The cell is allowed to reach a steady state. Due to the existence of both a labeled and unlabeled carbon source, compounds form isotopomers with both labeled and unlabeled carbon atoms. The isotope pattern of output metabolites is then measured with an appropriate isotope labeling measuring technique, usually GC-MS (gas chromatographer coupled with a mass spectrometer). By measuring the isotopomer distribution of metabolites, the flux through each reaction in the network can be determined. Comprehensive flux modeling requires a more detailed network model that maps each of the atom transitions in a set of reactions to determine which atom in each of the reactants maps to each of the atoms in the products (48-50). With the initially validated model, we will thus perform flux measurements with a hybrid of the MFA and flux balance approaches. We will use the detailed MFA model for a central (core metabolism) portion of the network (as is typical in this field) to calculate the flux distribution that best describes the observed isotope-labeling pattern. However, since this more detailed model cannot account for all possible metabolic fluxes (information and computation limited) we will use the genome-scale flux balance model to evaluate the potential effect of reactions that are not included in the detailed model. This step will enable us to add error bars on the flux measurements because of the missing reactions (something that is too often overlooked in these types of measurements). Overall, this approach will enable us to determine the reaction fluxes through the network during our designs, which will allow us to make additional modifications to *M. maripaludis* through an iterative approach to direct metabolic flux towards efficient use of the desired pathways. In this way, we will be able to provide a wealth of information to achieve and enhance the production of methanol/butanol by *M. maripaludis*.

Aim 3: *Biochemical characterization of enzymes involved in methane conversion to liquid fuels*
In Aim 3, Ragsdale's laboratory (Univ. of Michigan) will conduct in vitro biochemical studies to determine quantitative performance metrics and functionality of the *M. maripaludis*-expressed enzymes. First, they will determine specific activities in the cell lysate. In a few cases, e.g., MCR and HDR, there will be a significant background activity from the native protein and the activity

will not necessarily reflect the activity of the heterologously expressed protein. In these cases, they will have to rely on measurements made on the purified enzymes.

With respect to the enzymes involved in converting methane to methanol, the Ragsdale laboratory has extensive experience in characterizing the kinetic and spectroscopic properties of MCR (51-54), HDR (55-57) and corrinoid-dependent methyltransferases (58-62). We will purify and characterize the heterologously expressed MCR, HDR and MtaABC and determine essential Michaelis parameters (K_m, k_{cat}, k_{cat}/K_m) for each of these enzymes. The Michaelis parameters will be used as input data for the metabolic flux modeling studies conducted by the Price laboratory. These values will also indicate how actively the enzymes have been expressed. For example, the purified enzymes would be expected to exhibit values similar to those for the *M. marburgensis* proteins. MCR activity will be determined in the forward direction by following methane formation (gas chromatography) (63) or measuring, by liquid scintillation counting, the depletion of radioactivity from solution as $^{14}CH_3$-SCoM is converted to insoluble $^{14}CH_4$ (64). Activity in the reverse direction will be determined by proton-NMR, measuring the formation of $^{13}CH_3$-SCoM from $^{13}CH_4$ and CoMS-SCoB in the presence of $^{12}CH_3$-SCoM as described (20)

The published K_m value for methyl-SCoM ranges from 0.6 to 5.4 mM (64-67). This relatively wide range of values could result from differences in MCR preparation (presence of HSCoM (64,67) or methyl-SCoM (66) during purification) or differences in conditions in which the activity assays were performed, i.e., reductant (DTT vs. Ti(III) citrate), buffer (potassium phosphate vs. MOPS) and pH (6.7, 7.0 or 7.6). The published K_m value for CoBSH varies from 0.1 to 0.5 mM (64,66) and we discovered that this value is strongly pH dependent, with a value of 110 ± 0.6 μM at pH 7.6. The k_{cat} value, corrected to 1.0 spin/mol enzyme, is also strongly pH dependent with a value of 250 s^{-1} (specific activity of 100 U mg^{-1} at pH 7.6) (68). The value of k_{cat}/K_m for methyl-SCoM has been independently determined to be 40 mM^{-1} s^{-1} at pH 7.6 (64), which agrees with the K_m and V_{max} values. In addition, the Ragsdale laboratory has developed ways to activate MCR to its active Ni(I) state (63,69). Assessment of this parameter is made by following the intensity of the characteristic UV-visible (strong peak at 425 nm for inactive Ni(II) and 390 for Ni(I)) and EPR (characteristic g-values of 2.24 and 2.065 for Ni(I), while Ni(II)) is EPR-silent, as described (63).

HDR will be purified and activity will be measured at 56 °C following the CoBSSCoM-dependent oxidation of reduced methyl viologen (56). At saturating concentrations of heterodisulfide, the specific activity of the purified *M. marburgensis* enzyme was 270 units mg^{-1} and the apparent K_m value for the heterodisulfide was 0.5 mM (56). For the MtaABC system, we will assay the conversion of methanol to methylcobalamin as described (70) and will expect a specific activity of ~750 U/mg and K_m values for CoM and methanol of 10 mM and 50 mM, respectively, similar to the published values for the *M. barkeri* enzyme.

Similarly, for characterizing the sulfate reduction pathway module (Fig. 1), the Ragsdale laboratory will purify and determine the kinetic parameters for the tagged versions of ATP sulfurylase, APS reductase and sulfite reductase. These enzymes have been characterized in a number of sulfate-reducing bacteria, i.e., Desulfovibrio, Desulfotomaculum, and Archaeoglobus (71,72). In dissimilatory sulfate reduction (Fig. 1), ATP sulfurylase catalyzes the activation of inorganic sulfate by ATP to give pyrophosphate and adenosine 5'-phosphosulfate (APS); then APS reductase catalyzes the reduction of APS to AMP and sulfite, which is reduced to sulfide by sulfite reductase. Cloning and expression of the ATP sulfurylase (MgATP-sulfate adenylyltransferase) in *M. maripaludis* is not expected to be problematic, given that the archaeal

(*Archaeoglobus fulgidus*) protein has been actively expressed in E. coli (72). The enzyme will be assayed in the thermodynamically favorable direction by following ATP generation from the reaction of APS with pyrophosphate (72). ATP is measured by a hexokinase and glucose-6-phosphate dehydrogenase-coupled spectrophotometric assay, following NADPH formation (ε_{340} = 6.22 mM^{-1} cm^{-1}), where 1 mol of NADP is reduced per mole of ATP present. APS reductase will be assayed as described (73) in the direction of APS formation from sulfite and AMP with ferricyanide or cytochrome c as the artificial electron acceptor. This enzyme contains noncovalently bound FAD and iron-sulfur clusters, which will be assessed by UV-visible and EPR spectroscopy to ensure that the enzyme is properly assembled. Sulfite reductase activity will be measured as described (73) in the direction of sulfite reduction by following the decrease in absorbance of reduced (Ti(III)citrate or electrochemically) methyl viologen (ε_{600} = 13.0 mM^{-1} cm^{-1}) as the electron donor. The enzyme contains siroheme and six [4Fe-4S] clusters, which will be assessed by UV-visible and EPR spectroscopy. Work described above under Aim 1 demonstrates that *M. maripaludis* contains siroheme (29).

We will measure activities in the *M. maripaludis* strain containing the methanol to butanol pathway of the tagged butanol dehydrogenase and butyraldehyde dehydrogenase (74,75), acetyl-CoA acetyltransferase (76), 3-hydroxybutyryl-CoA dehydrogenase (77); 3-hydroxybutyryl-CoA dehydratase (crotonase) (77) and butyryl-CoA dehydrogenase (77).

Aim 4: *Molecular modeling of the ANME MCR and other enzymes involved in GTL*

In Aim 4, Dayle Smith (PNNL) will use molecular modeling calculations to understand the mechanism of the anaerobic methane oxidizing MCR and to predict the effects of differences among the various MCRs on their respective reaction mechanisms and catalytic bias (toward either utilizing or producing methane), in consultation with Dr. Appel. She will also test the effects on the reaction mechanism of site-directed mutagenesis of the specific residues that differ among the MCRs. These studies will be closely integrated with those described under Aim 3.

Molecular modeling techniques are an invaluable set of tools to delve into the mechanistic details underlying the reversible MCR reaction mechanism, owing to the capacity of these methods to validate or predict structural and thermodynamic properties, such as substrate binding orientation, geometries and electronic structure of reaction intermediates, thermodynamics of steps in the reaction pathway, and the roles of specific protein residues and atomic substitutions, while controlling for all relevant chemical and biochemical factors including experimental conditions (i.e., pH and solvent composition), structural aspects (i.e. amino acid sequence and substrate composition), and subatomic properties (i.e. electron configuration). Dr. Smith has extensive experience using theoretical quantum, classical and statistical physics to provide a multi-scale understanding of structural, dynamic and thermodynamic properties of metal-containing proteins. The insights gleaned from her work on cytochrome, nitrogenase and hydrogenase enzymes (with multiple, coupled protein-embedded iron, nickel and molybdenum chemical species) are directly applicable to MCRs (78-83). The key performance metrics of this aim are: determination of the roles of MCR active site residues on reaction thermodynamics and reversibility; suggest novel reaction pathways and branches; potential energy profiles for existing and novel pathways with various MCR structures.

Molecular modeling methods can predict biochemical properties using classical mechanics (CM), quantum mechanics (QM), or both. CM does not account for interactions between sub-atomic electrons and nuclei and instead treats them in an average way for each

atom, an approximation that makes it possible to model the structure, motion, and binding thermodynamics of large, solvated protein systems over long (up to microsecond) timescales. Quantum mechanics (QM) is the appropriate approach to describe actual chemical changes such as bond breaking/formation and electron transfer between reactants and products. Although QM is more accurate than CM, it is much more computationally-intensive and therefore limited to a smaller number of atoms. Used together, these methods will provide the necessary understanding of the interplay between active site structure, protein structure and motion, and methane oxidation by MCR. Molecular modeling of methane oxidation by MCR will be carried out using a multi-scale approach to describe three coupled spacial domains: 1) the MCR protein, cofactor and substrates and the solution environment, treated with classical mechanics (CM); 2) the reaction sub-species directly involved the breaking/formation of bonds forming reaction intermediates involving F430, SCoM and SCoB, treated with quantum mechanics (QM), and 3), the interface between the QM region and the surrounding protein and solvent atoms, treated with a combination of CM and QM.

Initial MCR-substrate structures: Dr. Ragsdale has proposed mechanistic roles for the amino acids near F430 in the MCR binding cleft. Tyr333, Tyr367, Arg120, Gln147, and the thioglycine TGly445 (Figure 5) may drive the MCR methane oxidation reaction and influence reversibility by controlling the binding orientation of the SCoB and SCoM substrates and stabilization of intermediates, and therefore the reaction thermodynamics and yields. All molecular modeling methods require specification of atomic coordinates, and the structures of the engineered MCRs are unknown. Initial atomic coordinates for engineered MCRs will be built from the MCR- substrate structure from x-ray crystallography (PDB ID 1MRO.pdb). The structures (initial coordinates) for MCRs with different amino acid sequences (native and engineered MCRs) will be built using sequence alignment with the structure building program SwissMol.

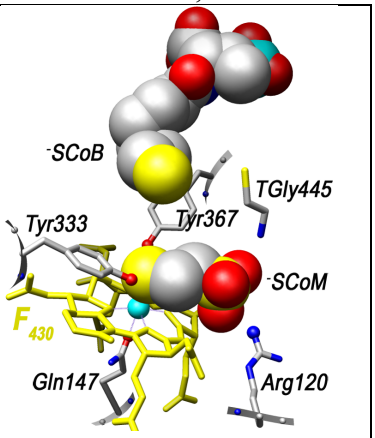

Fig. 5. MCR active site

Comparison of native versus engineered MCR structures, dynamics and substrate binding: Using the aforementioned initial atomic coordinates for the native and engineered MCR-substrate complexes being studied by Dr. Ragsdale's lab, Dr. Smith will build a realistic solvent environment by surrounding the protein, cofactor and substrates in a cubic box with a minimum distance of 15 Å between solute atoms and each side of the box in order to model free and complexed MCR in a realistic manner. Based on an initial set-up of 1MRO.pdb, this solvated system consists of approximately 38,000 MCR-substrate atoms and 100,000 water molecules. Using the CM inter-atomic description, the interactions between atoms will be described using the AMBER potential energy function (Eq. 9), which includes both bonded (bonds, angles and dihedral angles) and non-bonded contributions (Coulomb and Lennard-Jones potentials). From this potential energy function, the equation-of-motion can be iteratively solved to predict the motion of the solvated MCR-substrate complex in any stage of the reaction to calculate the interactions between particles as a function of time, temperature, pressure and volume.

$$U = \sum_{bonds} k_r (r - r_0)^2 + \sum_{angles} k_\theta (\theta - \theta_0)^2 + \sum_{dihedrals} k_\varphi \left[1 + \cos(n\varphi + \varphi_0)\right] + \sum_i \sum_{i \neq j} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] + \sum_i \sum_{i \neq j} \frac{q_i q_j}{\varepsilon_0 r_{ij}} \quad \text{Eq 9}$$

The potential energy terms for the protein residues and solvent species have been

developed and tested, however it will be necessary to derive the parameters for the thioglycine, F430, SCoM and SCoB species reactants, products and intermediates. Using the same approach as a previous study of [NiFe] hydrogenase, potential energy terms for these species will be derived using density functional theory (DFT) calculations on small molecular models (78).

In order to examine role of overall MCR amino acid composition on the binding orientation and binding thermodynamics, Dr. Smith will use the derived potential energy function (Eq. 1) to run > 100 nanosecond molecular dynamics simulations on complexes of substrate with the native and engineered MCRs and use trajectory analysis methods to assess the steric and electrostatic effects of MCR on substrate binding. All CM calculations will be performed using the GROMACS software implementation for parallel computers. For instance, from MD trajectory analysis we can calculate substrate binding poses and the relative enthalpic and entropic contributions to binding due to residues at the locations of Tyr333, Tyr367, Arg120, Gln147, and TGly445 and the effects of substitutions at positions away from the binding cleft on MCR stability and secondary structure. The MD analyses will also enable us to propose additional mutation sites. Using classical free energy perturbation (FEP) with MD Dr. Smith will calculate relative substrate binding free energies of native and engineered MCR. By individually perturbing residues from native to mutated amino acids in the substrate-bound and free MCR, she will determine the thermodynamic importance of each mutation and thereby rank them in importance for ligand binding and stabilization of reaction intermediates. In this manner, we can determine short- and long-range, enthalpic and entropic contributions of the proposed amino acid substitutions on the reaction thermodynamics.

*Reaction pathway thermodynamics of methane oxidation for native and engineered MCRs:*A theoretical description of the forward and reverse reaction energy profiles requires the use of quantum mechanical methods to account for electron redistribution and bond breaking and formation. In particular, we are interested in understanding the role of active site residues on the thermodynamics of the reverse reaction pathway: the conversion of methane to methanol.

A previous QM study of both forward and reverse pathways concluded that a reverse pathway involving an intermediate methyl radical species is more feasible than the mechanism involving a nickel-methyl intermediate (84). These calculations, though insightful, included a limited set of truncated active site sidechains (Tyr333, Tyr367, and Gln147) and neglected the rest of the protein matrix (including Arg120 and TGly445) and used a continuum solvent model rather than explicit water molecules.

We propose to gain a more realistic and robust understanding of the proposed mechanisms by calculating forward and reverse reaction potential energy profiles including all protein and explicit solvent atoms using a combined quantum mechanical/classical mechanical procedure with a QM description of the nickel porphyrin cofactor and the directly-interacting protein groups and a CM description of the protein and solvent matrix. The expected result from this more thorough treatment of the active site is that reaction pathways which require charged species are more likely to be lower in energy when all of the residues are included. In the MCR-substrate structure in 1MRO.pdb, there are 12 water molecules embedded between the species shown in Figure 2. Since water molecules interact directly with the groups involved in the reaction, several of which proceed through charged and radical intermediates, an explicit solvent model is far superior to a continuum model. Therefore the energy of the ionic steps may become more favorable, and the relative reaction pathway energies and barriers can be more accurately compared using this proposed methodology.

In consultation with Dr. Appel, Dr. Smith will use the structural and thermodynamic data to test and propose likely mechanism pathways optimized for formation of methanol that include substrate-triggered conformational changes. Initially the two predominant pathways that have been reported will be considered, however, as the overall project proceeds, additional pathways and branches will be explored as appropriate. Identifying and developing an understanding of the potential opportunities to facilitate the activation and conversion of methane will be the overall focus of this aim. Dr. Appel has extensive experience in the consideration and construction of catalytic pathways based upon the relative energies of the potential intermediates. Using a similar approach, Dr. Appel will facilitate understanding possible pathways and the relative influence of each catalytic step in order to develop an understanding of what the key features are in the enzymes, and therefore how to improve the overall catalytic process.

III.B. Feasibility

This Technology Development Project aims at meeting major objectives and addressing major challenges described in the ARPA-E FOA-0000881 on "Reducing emissions using methanotrophic organisms for transportation energy (REMOTE)" by developing a novel and transformational technology for the biological synthesis of liquid fuels from methane. The project goal is to engineer a metabolic pathway that converts methane to methanol that meets the Technology Development performance metrics, i.e., a rate exceeding 1 g of fuel/g cell dry weight/hr, energy efficiency greater than 64% and carbon yield of at least 67%.

The feasibility of our strategy to engineer a pathway for conversion of methane to methanol into the genetically tractable model methanogen, *Methanococcus maripaludis* is supported by several important criteria. We have developed a three-stage pathway incorporation strategy that will be tested and optimized with respect to thoughtfully designed performance metrics at each stage. Expression of each gene will be assessed by Western analysis and by RT-PCR in the Leigh laboratory. Active expression will be tested by enzymatic assays of the expressed protein and by functional in vivo assays in the Ragsdale and Leigh laboratories. All gene incorporation experiments will be preceded by and correlated with metabolic flux modeling experiments that are integrated with measurements in the Ragsdale laboratory of the kinetic parameters for each enzyme and assessments in the Price laboratory of substrate and product flux.

Feasibilty of our strategy can also be measured by our choice of developing the process in a well-studied model methanogen instead of *E. coli* or a genetically tractable non-methanogenic anaerobe. The host organism, *M. maripaludis*, is a premier model for hydrogenotrophic methanogens (22) that Leigh's lab has worked with for 20 years. This choice was guided by the successes of the Leigh group in actively expressing methanogenic genes. It was also based on the recognition of the many potential difficulties associated with heterologous synthesis of the essential nickel-cofactor of MCR, Factor F_{430}. Most of the genes involved in F_{430} synthesis have not been identified. As stated in a recent review, "Based on the observation that coenzyme F430 is derived from either precorrin-2 *or* sirohydrochlorin and proceeds via the *seeD* intermediate, *a biosynthetic pathway has been postulated* … . There are likely to be between 6 and 8 enzymes required in the transformation of precorrin-2 into F430", none of which have been identified (85). Furthermore, MCR contains several posttranslational modifications (86,87), which are generated by unidentified enzymes. While engineering an active form of MCR into a nonmethanogen would at this stage be impossible, expression of the active enzyme in a methanogen is rather straightforward. Ragsdale's laboratory has succeeded in active expression

of the *M. marburgensis* MCR into *Methanosarcina acetivorans* using the genetic system that was developed in Metcalf's laboratory, albeit expression levels were lower than in the native organism (88,89). Thus, expressing these genes in *M. maripaludis* is feasible and we will optimize expression levels of the various MCRs as described in Aim 1.

Our step-wise gene incorporation strategy is designed like a three-legged stool, which will support the eventual success of the project: (1) methane to methanol (2) sulfate to sulfide (3) methanol to butanol. For the methane to methanol subcomponent, we will first introduce the MtaABC genes encoding the three subunits (MtaA, MtaB, and MtaC) of the methanol:coenzyme M methyltransferase, which catalyzes conversion of methyl-SCoM to methanol (90). This modification is expected to transform *M. maripaludis* into a methylotroph, capable of converting methanol to methane, a trait usually associated with methanogens like Methanosarcina. Though this is the reverse of the desired reaction, successful conversion of methanol to methane would be proof of principle that the enzyme is active. With reference to Fig. 1, *M. maripaludis* already contains MCR and HDR, which are key enzymes in catalyzing the final steps in methanogenesis. We will then begin introducing the various five-gene clusters encoding the MCRs from two anaerobic methane oxidizers (ANME-1 and ANME-2) and from the methanogen *M. marburgensis*. To explore physiological function of the heterologously expressed protein, we will knock out the native MCR and measure if the introduced enzyme can complement the native protein. We will compare/contrast the kinetic parameters of these actively expressed MCRs and integrate these measurements with the metabolic flux model. So far, none of the catalytic properties of the ANME MCRs have been measured. An important questions is whether the methanotrophic MCR exhibits a different catalytic bias (defined as $(k_{cat}/K_m)[substrate]_{forward}\}/\{(k_{cat}/K_m)[substrate]_{reverse}$) than the methanogenic enzyme. It will be essential to incorporate the genes encoding the most active methane oxidizing MCR for this project. For example, without such a bias, any of the MCRs (even the one from the native *M. maripaludis*) should promote methane oxidation. It is well recognized that hydrogenases (91) and fumarite reductases (92), as well as other systems, exhibit marked catalytic biases.

$$CH_3\text{-SCoM} + H_2O \rightarrow CH_3OH + HSCoM \quad \Delta G^{0'} = +27 \text{ kJ/mol} \quad \text{Eq. 10}$$

In the second phase of gene introduction, to allow conversion of methane to methanol to be thermodynamically favorable, we will then introduce the dissimilatory sulfate reduction pathway. One might be concerned about the generation of toxic levels of sulfide; however, growth of *M. maripaludis* is enhanced by sulfide, which is a sulfur source for iron-sulfur cluster biosynthesis and also is routinely used as a reductant to keep the redox potential at the optimal levels for growth of the organism.

The third phase of gene introduction involves cloning the genes encoding the conversion of methanol to butanol. To assess activity of this system, the Price lab (Task 2) will measure the methane, methanol, and butanol flux and Ragsdale's lab (Task 3) will perform steady-state kinetic measurements of the individual enzymes. We will also generate strains lacking the sulfate reduction pathway - in the absence of this electron transfer sequence, *M. maripaludis* should be "butanol-otrophic" (convert butanol to methane) and it its presence, it would convert methane to butanol. We will also determine the effect of a knock out (KO) of the methylene-H_4MPT reductase gene (mer) (35), because this gene is involved in oxidation of methyl-SCoM to CO_2. We will determine if this KO increases flux to methanol in the absence of the methanol-to-butanol pathway, and towards butanol in its presence. In the presence of the sulfate reduction path, we expect to achieve optimal flux of methane to methanol (or butanol) in the mer^- strains.

III.C. Performance Team

The project involves a team of four investigators with complementary expertise, experience and skills that make us uniquely qualified to successfully execute the project plan: John Leigh (Univ. Washington), Nathan Price (Institute for Systems Biology), Stephen Ragsdale (Univ. Michigan) and Dayle Smith (PNNL). This includes the experience of Dr. Leigh in the genetics and physiology of methanogens to integrate the necessary genes into the methanogenic host, the expertise of Dr. Price to provide the required metabolic flux modeling to place the engineered pathways into the context of the full microbial metabolic network, the skills of Dr. Ragsdale in characterizing metalloenzymes including the key enzymes involved in this proposed pathway, and the experience of Dr. Smith in determining how protein structure and dynamics modulate metalloenzymatic catalysis and multi-center electron transport.

The Applicant, Ragsdale, has been studying the biochemistry and metabolism of anaerobic microbes, including methanogens, for over 25 years. His laboratory routinely cultures cells and purifies enzymes from strict anaerobes. The proposed work synergizes well with Dr. Ragsdale's current work funded by the DOE (ER15931). Much of his work has focused on determining the enzymatic mechanisms of metalloenzymes as individual enzymes and within large macromolecular complexes, including MCR, HDR and B_{12}-dependent methyltransferases that play a key role in the proposed methane-to-methanol pathway. His expertise in steady-state and transient kinetics and in spectroscopic methods complements well that of his team members. Ragsdale has successfully administered various NIH-, DOE- and NSF-funded projects (e.g. staffing, research protections, budget), collaborated with many other researchers and labs, and produced over 180 peer-reviewed publications. He has been consulting for many years with biotech companies (e.g., Genomatica, Inc.), who are attempting to engineer special pathways in anaerobic microbes. His laboratory includes specialized equipment for spectroscopy (e.g., EPR, UV-vis), electrochemistry, transient and steady-state kinetics and for performing anaerobic manipulations (e.g., five anaerobic chambers), growing bacteria and preparing cell extracts.

Dr. Leigh has worked on the biochemistry and genetics of methanogenesis for over 20 years. He has been instrumental in the developing genetic approaches in methanogens and has applied them extensively to the analysis of electron flow and energy conservation, uncovering the roles of several hydrogenases and dehydrogenases. He also has heterologously expressed and purified several proteins in *Methanococcus maripaludis*. The proposed work synergizes well with Dr. Leigh's current work funded by the DOE (ER15709). Other work in Leigh's lab has focused on regulation of methanogenesis and nitrogen fixation and has been funded by NIH and NSF. Collaborations have involved studies of the systems biology of methanogenesis, the structure of nitrogenase regulatory complexes, and the proteomics of *M. maripaludis*. His laboratory is equipped for anaerobic microbiology, genetics, biochemistry and standard molecular biology. Specialized equipment includes two anaerobic chambers, one containing an FPLC, stations for use of anaerobic gasses, "pressure cooker" vessels for anaerobic incubation of agar plates, and three fermenters/chemostats modified in-house for growth of methanogens.

Dr. Smith has wide expertise in combining quantum, classical and statistical mechanical methods to model the chemical and thermodynamic properties of metalloproteins (78,80), including elucidating the binding and chemical steps and catalytic roles of specific amino acids. Dr. Smith is currently contributing two DOE funded projects (Office of Basic Energy Science and Biological and Environmental Research) applying quantum, classical and statistical

mechanical methods to discover how protein structure and dynamics modulate metalloenzymatic catalysis and multi-center electron transport. To accomplish the project tasks, Dr. Smith will utilize the extensive supercomputing resources at PNNL, such as the Chinook supercluster with 2310 HP dual-socket, quad-core AMD nodes, the Olympus cluster with 604 32-processor nodes and a peak processing speed 162 Teraflops, and a desktop 2-processor Linux computer for data analysis and visualization. PNNL also provides expert hardware and software support for troubleshooting, software installation and parallel optimization.

Dr. Price is a leader in the field of metabolic modeling and has reconstructed genome-scale metabolic models for two methanogenic species, each showing 96% accuracy when compared to growth phenotype data. Additionally, his method for integrating gene regulatory and metabolic networks (Probabilistic Regulation of Metabolism) is the method being implemented into DOE's Knowledgebase. Dr. Price's current computational resources include: 120 centralized servers; 25 workstations for individual researchers; a centralized computer cluster running Sun Grid Engine; 500 terabytes of online data storage and internet access through a gigabit connection to the Pacific Northwest Gigapop. The Price Lab has its own 40TB server and 30 personal computers with an additional 100-node computational cluster owned by the lab and maintained in the School of Chemical Sciences at Univ. of Illinois, where Dr. Price is an adjunct professor.

References:

1. Lashoff, DA & Ahuja, D. (1990) *Nature* **344**, 213-42
2. Hallam, SJ et al. (2004) *Science* **305**, 1457-62
3. Kruger, M et al. (2003) *Nature* **426**, 878-81
4. Nauhaus, K, Boetius, A, Kruger, M & Widdel, F. (2002) *Environ Microbiol* **4**, 296-305
5. Michaelis, W et al. (2002) *Science* **297**, 1013-5
6. Shima, S et al. (2012) *Nature* **481**, 98-101
7. Atreya, SK, Mahaffy, PR & Wong, A. (2007) *Planet Space Sci* **55**, 358–69
8. Thauer, RK. (1998) *Microbiol* **144**, 2377-406
9. Ragsdale, SW. (2007) *J Inorg Biochem* **101**, 1657-66
10. Hanson, RS & Hanson, TE. (1996) *Microbiol Rev* **60**, 439-71
11. Valentine, DL & Reeburgh, WS. (2000) *Environ Microbiol* **2**, 477-84
12. Orphan, VJ, House, CH, Hinrichs, KU, McKeegan, KD & DeLong, EF. (2001) *Science* **293**, 484-7
13. Orphan, VJ, House, CH, Hinrichs, KU, McKeegan, KD & DeLong, EF. (2002) *Proc Natl Acad Sci USA* **99**, 7663-8
14. Raghoebarsing, AA et al. (2006) *Nature* **440**, 918-21
15. Ettwig, KF et al. (2010) *Nature* **464**, 543-8
16. Schimel, J. (2000) *Nature* **403**, 375, 7
17. Zhou, Y, Sliwa, DA & Ragsdale, SW. (2012) in *Handbook of Porphyrin Science* (Kadish, KM, Smith, KM & Guilard, R eds.), World Scientific Publishing. pp
18. Jaun, B & Thauer, RK. (2007) in *Nickel and its Surprising Impact in Nature* (Sigel, A, Sigel, H & Sigel, RKO eds.), John Wiley and Sons, West Sussex, England. pp 323-56
19. Shima, S & Thauer, RK. (2005) *Curr Opin Microbiol* **8**, 643-8
20. Scheller, S, Goenrich, M, Boecher, R, Thauer, RK & Jaun, B. (2010) *Nature* **465**, 606-8
21. Hakemian, AS & Rosenzweig, AC. (2007) *Annual review of biochemistry* **76**, 223-41
22. Leigh, JA, Albers, SV, Atomi, H & Allers, T. (2011) *FEMS Microbiol Rev* **35**, 577-608

23. Haydock, AK, Porat, I, Whitman, WB & Leigh, JA. (2004) *FEMS Microbiol Lett* **238**, 85-91
24. Costa, KC, Lie, TJ, Jacobs, MA & Leigh, JA. (2013) *MBio* **4**
25. Dodsworth, JA & Leigh, JA. (2006) *Proc Natl Acad Sci USA* **103**, 9779-84
26. Milucka, J et al. (2012) *Nature* **491**, 541-6
27. Basen, M et al. (2011) *Environ Microbiol* **13**, 1370-9
28. Thauer, RK, Kaster, AK, Seedorf, H, Buckel, W & Hedderich, R. (2008) *Nat Rev Microbiol* **6**, 579-91
29. Johnson, EF & Mukhopadhyay, B. (2008) *Appl Environ Microbiol* **74**, 3591-5
30. Grein, F, Ramos, AR, Venceslau, SS & Pereira, IA. (2013) *Biochim Biophys Acta* **1827**, 145-60
31. Susanti, D & Mukhopadhyay, B. (2012) *PLoS One* **7**, e45313
32. Bruant, G, Levesque, MJ, Peter, C, Guiot, SR & Masson, L. (2010) *PLoS One* **5**, e13033
33. Atsumi, S et al. (2008) *Metab Eng* **10**, 305-11
34. Steen, EJ et al. (2008) *Microb Cell Fact* **7**, 36
35. Vaupel, M & Thauer, RK. (1995) *Eur J Biochem* **231**, 773-8
36. Milne, CB, Kim, PJ, Eddy, JA & Price, ND. (2009) *Biotechnol J* **4**, 1653-70
37. Park, JH, Lee, KH, Kim, TY & Lee, SY. (2007) *Proc Natl Acad Sci USA* **104**, 7797-802
38. Zomorrodi, AR, Suthers, PF, Ranganathan, S & Maranas, CD. (2012) *Metab Eng* **14**, 672-86
39. Thiele, I & Palsson, BO. (2010) *Nat Protoc* **5**, 93-121
40. Benedict, MN, Gonnerman, MC, Metcalf, WW & Price, ND. (2012) *J Bacteriol* **194**, 855-65
41. Gonnerman, MC, Benedict, MN, Feist, AM, Metcalf, WW & Price, ND. (2013) *Biotechnol J*
42. Stolyar, S et al. (2007) *Mol Syst Biol* **3**, 92
43. http://code.google.com/p/path2models/.
44. Henry, CS et al. (2010) *Nat Biotechnol* **28**, 977-82
45. Sarmiento, F, Mrazek, J & Whitman, WB. (2013) *Proc Natl Acad Sci U S A* **110**, 4726-31
46. Hendrickson, EL et al. (2008) *J Bacteriol* **190**, 2198-205
47. Pharkya, P, Burgard, AP & Maranas, CD. (2004) *Genome Res* **14**, 2367-76
48. Zamboni, N, Fendt, SM, Ruhl, M & Sauer, U. (2009) *Nat Protoc* **4**, 878-92
49. Wiechert, W. (2001) *Metab Eng* **3**, 195-206
50. Walther, JL, Metallo, CM, Zhang, J & Stephanopoulos, G. (2012) *Metab Eng* **14**, 162-71
51. Sarangi, R, Dey, M & Ragsdale, SW. (2009) *Biochem* **48**, 3146-56
52. Ragsdale, SW. (2003) in *The Porphyrin Handbook* (Kadish, KM, Smith, KM & Guilard, R eds.), Academic Press, New York. pp 205-28
53. Li, X, Telser, J, Hoffman, BM, Gerfen, G & Ragsdale, SW. (2010) *Biochem* **49**, 6866-76
54. Cedervall, PE, Dey, M, Pearson, AR, Ragsdale, SW & Wilmot, CM. (2010) *Biochem* **49**, 7683-93
55. Bäumer, S et al. (1998) *FEBS Lett* **428**, 295-8
56. Simianu, M, Murakami, E, Brewer, JM & Ragsdale, SW. (1998) *Biochem* **37**, 10027-39
57. Murakami, E, Deppenmeier, U & Ragsdale, SW. (2001) *J Biol Chem* **276**, 2432-9
58. Kung, Y et al. (2012) *Nature* **484**, 265-9
59. Doukov, TI, Hemmi, H, Drennan, CL & Ragsdale, SW. (2007) *J Biol Chem* **282**, 6609-18
60. Lu, WP, Becher, B, Gottschalk, G & Ragsdale, SW. (1995) *J Bacteriol* **177**, 2245-50
61. Seravalli, J, Zhao, S & Ragsdale, SW. (1999) *Biochem* **38**, 5728-35
62. Zhao, SY & Ragsdale, SW. (1996) *Biochem* **35**, 2476-81
63. Becker, DF & Ragsdale, SW. (1998) *Biochem* **37**, 2639-47

64. Horng, Y-C, Becker, DF & Ragsdale, SW. (2001) *Biochem* **40**, 12875-85
65. Ellermann, J, Hedderich, R, Böcher, R & Thauer, RK. (1988) *Eur J Biochem* **172**, 669-77
66. Bonacker, LG, Baudner, S, Mörschel, E, Böcher, R & Thauer, RK. (1993) *Eur J Biochem* **217**, 587-95
67. Goenrich, M et al. (2004) *J Biol Inorg Chem* **9**, 691-705
68. Goubeaud, M, Schreiner, G & Thauer, RK. (1997) *Eur J Biochem* **243**, 110-4
69. Zhou, Y, Dorchak, AE & Ragsdale, SW. (2013) *Front Microbiol* **69**, in press
70. Sauer, K, Harms, U & Thauer, RK. (1997) *Eur J Biochem* **243**, 670-7
71. Gavel, OY et al. (1998) *Biochemistry* **37**, 16225-32
72. Sperling, D, Kappler, U, Truper, HG & Dahl, C. (2001) *Methods Enzymol* **331**, 419-27
73. Dahl, C & Truper, HG. (2001) *Methods Enzymol* **331**, 427-41
74. Dürre, P, Kuhn, A, Gottwald, M & Gottschalk, G. (1987) *Appl Microbiol Biotechnol* **26**, 268–72
75. Fontaine, L et al. (2002) *J Bacteriol* **184**, 821-30
76. Wiesenborn, DP, Rudolph, FB & Papoutsakis, ET. (1988) *Appl Environ Microbiol* **54**, 2717-22
77. Boynton, ZL, Bennet, GN & Rudolph, FB. (1996) *J Bacteriol* **178**, 3015-24
78. Smith, DMA, Xiong, Y, Straatsma, TP, Rosso, KM & Squier, TC. (2012) *Journal of Chemical Theory and Computation* **8**, 2103-14
79. Smith, DMA, Straatsma, TP & Squier, TC. (2012) *Biophys J* **103**, 1576-84
80. Smith, DMA, Rosso, KM, Dupuis, M, Valiev, M & Straatsma, TP. (2006) *J Phys Chem B* **110**, 15582-8
81. Smith, DMA, Dupuis, M, Vorpagel, ER & Straatsma, TP. (2003) *J Am Chem Soc* **125**, 2711-7
82. Smith, DMA, Dupuis, M & Straatsma, TP. (2005) *Mol Phys* **103**, 273-8
83. Smith, DMA, Danyal , K & Seefeldt, LC. (2013) *Biochem* **(in progress)**
84. Chen, S-L, Blomberg, MRA & Siegbahn, PEM. (2012) *Chemistry-a European Journal* **18**, 6309-15
85. Warren, MJ, Deery, E & Rose, R-S (eds). (2009) *Biosynthesis of Siroheme and Coenzyme F430*, Vol. 1, Landes Bioscience and Springer Science+Business Media, Austin, TX
86. Ermler, U, Grabarse, W, Shima, S, Goubeaud, M & Thauer, RK. (1997) *Science* **278**, 1457-62
87. Selmer, T et al. (2000) *J Biol Chem* **275**, 3755-60
88. Metcalf, WW, Zhang, JK, Apolinario, E, Sowers, KR & Wolfe, RS. (1997) *Proc Natl Acad Sci U S A* **94**, 2626-31
89. Guss, AM, Rother, M, Zhang, JK, Kulkarni, G & Metcalf, WW. (2008) *Archaea* **2**, 193-203
90. Hagemeier, CH, Krer, M, Thauer, RK, Warkentin, E & Ermler, U. (2006) *Proc Natl Acad Sci U S A* **103**, 18917-22
91. Parkin, A, Cavazza, C, Fontecilla-Camps, JC & Armstrong, FA. (2006) *J Am Chem Soc* **128**, 16808-15
92. Ackrell, BA, Armstrong, FA, Cochran, B, Sucheta, A & Yu, T. (1993) *FEBS Lett* **326**, 92-4
93. Lynd, LR et al. (2008) *Nat Biotechnol* **26**, 169-72
94. Lynd, LR, van Zyl, WH, McBride, JE & Laser, M. (2005) *Curr Opin Biotechnol* **16**, 577-83
95. Appel, A et al. (2013) *Chemical Reviews*, in press.

IV. TECHNOLOGY-TO-MARKET STRATEGY

The proposed technology is not being pursued by industry today for several reasons. Anaerobic oxidation of methane (AOM) was only recently discovered/validated and the organisms involved in AOM were identified only in the last decade and have not yet been cultured. Furthermore, this is a coupled syntrophic process involving the ANME organisms for endergonic methane oxidation and sulfate reducing microbes for the exergonic reduction of sulfate. Therefore, for this process to be feasible, one must either culture both organisms under syntrophic conditions or genetically engineer a single organism to catalyze both processes. We have chosen the latter consolidated bioprocessing (CBP) approach because, as outlined by Lynd and coworkers for ethanol production from cellulose (93,94), this strategy allows a single organism to catalyze the conversion of substrate to product in a single process step. By CBP, naturally occurring cellulolytic microorganisms have been engineered to improve yield and titer of product (94). Another reason this strategy has not been pursued yet at an industrial scale is that genetic systems to transform DNA into methanogenic organisms has only recently been developed.

The FOA states that "efficient, cost-effective conversion of methane to a liquid fuel at any scale of production would be transformative in enabling natural gas as a transportation fuel." We plan to achieve lab-scale bioconversion of methane to methanol. Indeed there are significant challenges in each of our goals. Task 1 appears to include the most formidable challenges - heterologous expression of the three enzymes required for anaerobic conversion of methane to methanol and the three enzymes involved in sulfate reduction to sulfide. However, we are using a host organism that already actively generates two of the key enzymes in the process and there is evidence that the sulfate-to-sulfide process can be successfully incorporated into this organism. Most importantly, we have enlisted a highly experienced team of investigators who will be supporting this key aim with state-of-the art metabolic flux analysis and molecular and metabolic modeling as well as biochemical experiments that will help to trouble-shoot problems and optimize the system at each level of this modular approach to achieve a consolidated bioprocessing system with the desired efficiency and rate.

We have not yet approached industrial sponsors of this research because we feel that it is too early to involve such entities. We believe that the development of this technology would be of interest to numerous industrial companies, such as DuPont, Genomatica, BASF, Dow. Additionally, several funding agencies (such as DOE or SBIR) would have interest in advancing this technology following our initial development. The individual researchers have experience working with industry to design metabolic pathways. For example, the Prime Recipient, Ragsdale, has been a consultant with Genomatica for three years in an unrelated project. Genomatica achieved enviable success in bioengineering 1,4-butanediol (BDO) by a process that made 5 million pounds of this chemical in late 2012 and has been licensed by BASF, the world's #1 producer of BDO. In addition, the thesis advisor of co-PI Nathan Price founded Genomatica. With the successful completion of our goals, our team will be ready to approach an industrial collaborator to increase the scale of the process and perhaps to license the technology.

At the industrial stage, resources will be needed to optimize the process. For example, various sources of the enzymes can be expressed in the *M. maripaludis* platform to find the combination of genes that produces the highest yield of product at optimal efficiency. In some

cases, for highest efficiency of product formation, protein-protein interactions need to be enhanced and often the expression levels and stoichiometry of proteins can be optimized.

Methane is widely mined and there is a distribution system set up for moving methane around the country (world). Using this technology described here, the methane, a flammable gas, can be oxidized in the absence of O_2, which would be advantageous from a safety perspective.

V. BUDGET SUMMARY:

OVERALL SUMMARY: As described in the proposal, the four tasks are interrelated to reach each of the milestones in the proposal. The budget reflects this relationship. To review the milestones as they are expressed in the Feasibility Section (III.B.), we are pursuing a step-wise gene incorporation strategy by sequentially introducing the following pathway modules: (1) methane to methanol (2) sulfate to sulfide (3) methanol to butanol. Leigh is introducing the genes into *M. maripaludis* while Price is assessing the effect of the genes on flux of methane, methanol, and other metabolites as well as working with Leigh to reengineer *M. maripaludis* for optimal activity and efficiency of the desired pathway. Contemporaneously, Ragsdale is determining the activity of each of the pathway enzymes in the various engineered strains, while Smith is informing us about the characteristics of the ANME MCR and about issues of catalytic bias in the ANME versus methanogenic MCR, the enzyme at the crux of the methanogenesis and methanotrophic pathway. We expect that each of these laboratories will require a similar budget over each of the three years because of the interdependence of the various laboratories for ultimate success of the project. Below, all costs are presented per annum.

PERSONNEL: Each of the PIs is requesting to direct their laboratory's tasks: Ragsdale, Leigh, Price, and Smith with 2.0, 3.6, 3.5, and 0.6 months, respectively. Note that U-M, UW, and ISB are providing funds to cost-share this (and other) component of the budget.

Each of the laboratories requests full-time support for postdoctoral associates: Ragsdale, Leigh, Price, and Smith with 1, 1, 2 and 1 postdoctors each. The Leigh laboratory is also requesting full-time support for two Research Scientists. The request for three senior-level researchers (postdoctor and research scientists) in Leigh's lab is justified because of the large number of genes that need to be introduced. Thus, there will be many *M. maripaludis* strains that need to be generated and maintained. The two postdoctors in the Price lab will work together, one on the computational and the other on the experimental aspects of the metabolic flux modeling experiments.

U-M (Ragsdale) is also requesting support for a graduate students and an undergraduate student to join the postdoctor in assessing the properties of the cloned genes. Many of these will be steady-state kinetic measurements and this data will be interfaced with the metabolic modeling and cloning experiments being performed by the Price and Smith laboratories. These researchers will also be interfacing closely with the Smith laboratory to assess differences in properties of the ANME versus methanogenic MCRs. There will also be tight coordination in performing flux measurements and assessing how closely the predicted properties of the various enzymes in the three modules match with those experimentally determined.

FRINGE BENEFITS: The costs of fringe benefits vary among the institutions: U-M (Ragsdale) at 30% (faculty & postdoc) and 8% (undergrad); UW (Leigh) at 26.90% (faculty), 23.40% (postdoc) and 34.00% (senior scientist); Price (ISB) at 41.7%, and Smith (PNNL) with 32.5% (Res. Scientist) and 16.4% (Postdoc).

TRAVEL: For the U. Mich travel budget, this request is broken into two components. U-M (Ragsdale) is requesting $3,000 (under "Travel") for him, the postdoctor, and one graduate student attend one meeting per year to disseminate research results and to travel to the state of

Washington to meet with the co-investigators and their laboratories and discuss results in the various laboratories. He also is requesting $2000 per year (under "Other Expenses/TT&O") to attend the annual ARPA-E meeting. Leigh is requesting $5100 to attend project-specific conferences to disseminate research and Price is requesting $6180 per year for him and postdocs to attend project-specific conferences to disseminate research results. Smith is requesting $3,921 to travel twice/year to Ann Arbor to meet and share results with Ragsdale and researchers in his lab.

EQUIPMENT: U-M (Ragsdale) is requesting funds in Year 1 to purchase a fermenter ($45,000) and a flow-through centrifuge ($38,000) To culture and process the cells to purify and characterize all of the enzymes (several forms of MCR, HDR, MeTr, sulfate reduction enzymes, enzymes involved in conversion of methanol to butanol) described in this project. ISB (Price) requests funds ($21,234 in Year 1) to purchase a COY Type B Anaerobic Chamber to perform characterization and validation experiments related to the metabolic flux measurements.

SUPPLIES: U-M (Ragsdale) is requesting funds ($23,000/yr) to cover the cost of materials and supplies for the postdoctor, graduate student, and undergraduate to perform the enzyme characterization experiments. UW (Leigh) is requesting $40,000 to cover to cover project-specific costs of DNA sequencing, service contracts, equipment repairs, and dishwashing services for a postdoctor and two senior investigators. ISB (Price) requests ~31,000/yr plus ~$8,000 in year 1 to cover experimental flux measurements. PNNL (Smith) has no supply request for their computational work.

OTHER DIRECT COSTS: UM (Ragsdale) requests funds (~$23,000/yr = $25K-$2K travel, above) to cover tuition, publication and instrumentation costs. ISB (Price) requests funds (~$25,000/yr) to cover costs of metabolomics and mass spectrometric studies and large-scale computational work. PNNL (Smith) requests ~$2500/yr to cover costs to support the computational work for the postdoc and PI.

COST SHARE: Combined, three of the institutions have provided a 5% cost share ($201,028 total) for this project ($4,016,701 total). UM will provide cost sharing ($115,500) for PI salary, travel, and research supplies. UW is contributing $75,528 and ISB will provide $10,000 in the form of PI effort and waived F&A.

INDIRECT COSTS: These costs vary among the institutions: UM – 55.5% (MTDC), UW – 54.5%, ISB – 80%, and PNNL – 43.9%.

VI. QUALIFICATIONS, EXPERIENCE, & CAPABILITIES

Stephen W. Ragsdale

Education/Training

B.S. Chemistry & Biochemistry, 1979, The University of Georgia, Athens, GA.
Ph.D. Biochemistry, 1983, The University of Georgia, Athens, GA, Lars G. Ljungdahl, Georgia Power Distinguished Professor of Biochemistry, advisor.
Postdoctor, 1984-1987, Case Western Reserve Univ., Cleveland, OH. Harland G. Wood, advisor, now deceased.

Employment History

Assistant Professor, Department of Chemistry, University of Wisconsin-Milwaukee 1987-91.
Associate Professor, Department of Biochemistry, University of Nebraska, 1991-96.
Professor, Department of Biochemistry, University of Nebraska, 1996-2007.
Charles E. Bessey Professor, 2003-07, University of Nebraska.
Director, Molecular Bioscience & Biotechnology Integrated Grad. Training Program, 2006-07.
Chairperson, UNL Research Council (2003-2004, 2004-2005)
Professor, Department of Biological Chemistry, University of Michigan, 2007-

Awards and honors

Merit Award from NIH on GM39451
Provost's Teaching Innovation Prize, Finalist ('11, '12)
Inducted into the Univ of Michigan League of Research Excellence ('11)
Inducted into the Univ of Michigan League of Educational Excellence ('12)
Elected Fellow of the American Academy for the Advancement of Science ('09)
Ljungdahl Lectureship, University of Georgia, Athens, GA. Oct. 2009.
Frederick J. Bollum Endowed Biochemistry Lectureship, University of Minnesota, May 2009
Elected Fellow of the American Academy of Microbiology ('06)
Outstanding Research and Creativity Award from UN System (2003)
Charles E. Bessey Professorship (2003)
Shaw Scholar Award, Milwaukee Foundation (1987-92)
Public Health Service National Research Service Award from NIH (1985-86)

10 peer-reviewed publications specifically related to the proposed R&D project

Zhou, Y., Dorchak, A.E., and Ragsdale, S.W. (2013) In vivo activation of methyl-coenzyme M reductase by carbon monoxide, *Frontiers in Microbiology 4*, 69. PMC3612591.

Kung, Y., Ando, N., Doukov, T.I., Blasiak, L. C., Bender, G., Seravalli, J., Ragsdale, S.W., and Drennan, C.L. (2012) Visualising molecular juggling within a B_{12}-dependent methyltransferase complex, Nature **484**: 265-269.

Cedervall, P.E., Dey, M., Li, X., Sarangi, R, Hedman, Ragsdale, S.W., and Wilmot, C.M. (2011) Structural analysis of a Ni-methyl species in methyl-coenzyme M reductase from *Methanothemobacter marburgensis*, *Journal of the American Chemical Society*, **133**: 5626-8. PMC3086036

Dey, M., Xi, X., Kunz, R. C., and Ragsdale, S. W. (2010) Detection of Organometallic and Radical Intermediates in the Catalytic Mechanism of Methyl-Coenzyme M Reductase Using the

Natural Substrate Methyl-Coenzyme M and a Coenzyme B Substrate Analog, *Biochemistry* ***49***: 10902-10911. PMID: 21090696

Cedervall, P.E., Dey, M., Pearson, A.R., Ragsdale, S.W., and Wilmot, C.M. (2010) Structural Insight into Methyl-Coenzyme M Reductase Chemistry using Coenzyme B Analogues, *Biochemistry* **49:** 7683–7693. PMID: 20707311

Li, X., Telser, J., Kunz, R.C., Hoffman, B.M., Gerfen, G., and Ragsdale, S.W. (2010) Observation of organometallic and radical intermediates formed during the reaction of methyl-coenzyme M reductase with bromoethanesulfonate, *Biochemistry* **49:** 6866-6876. PMC2919591.

Sarangi, R., Dey, M., and Ragsdale, S.W. (2009) Geometric and Electronic Structures of the Ni(I) and Methyl-Ni(III) Intermediates of Methyl-Coenzyme M Reductase, *Biochem.* ***48***: 3146-3156. PMC2667316.

Kunz, R. C., Dey, M., and Ragsdale, S.W. (2008) Characterization of the Thioether Product Formed From the Thiolytic Cleavage of the Alkyl-Nickel Bond in Methyl-Coenzyme M Reductase *Biochemistry* ***47*****:** 2661-2667. PMID: 18220418.

Dey, M., Kunz, R. C., Lyons, D. M., and Ragsdale, S.W. (2007) Characterization of Alkyl-Nickel Adducts Generated by Reaction of Methyl-Coenzyme M Reductase with Brominated Acids *Biochemistry*, **46**: 11969-11978. PMID: 17902704.

Dey M, Telser, J, Kunz R.C., Lees, N.S., Ragsdale, S.W., Hoffman, B. (2007) Biochemical and spectroscopic studies of the electronic structure and reactivity of a methyl-Ni species formed on Methyl-Coenzyme M Reductase, *Journal of the American Chemical Society,* **129**: 11030-2. PMID: 17711283

10 other peer-reviewed publications demonstrating capabilities in the broad field

Wang, V.C., Can, M., Pierce, E., Ragsdale, S.W., and Armstrong, F.A. (2013) A unified electrocatalytic description of the action of inhibitors of nickel carbon monoxide dehydrogenase, *Journal of the American Chemical Society* **135**: 2198-2206.

Appel, A., Bercaw, J., Bocarsly, A., Dobbek, H., DuBois, D., Dupuis, M., Ferry, J., Fujita, E., Hille, R., Kenis, P., Kerfeld, C., Morris, R., Peden, C., Portis, A., Ragsdale, S.W., Rauchfuss, T., Reek, J., Seefeldt, L., Thauer, R., and Waldrop, G. (2013) Frontiers, Opportunities, and Challenges in Biochemical and Chemical Catalysis of CO_2 Fixation, *Chemical Reviews*, in press.

Bender, G., and Ragsdale, S. W. (2010) Evidence That Ferredoxin Interfaces with an Internal Redox Shuttle in Acetyl-CoA Synthase during Reductive Activation and Catalysis, *Biochemistry* **50:** 276–286. PMC3077469.

Pierce, E., Becker, D.F., and Ragsdale, S.W. (2010) Identification and Characterization of Oxalate Oxidoreductase, a Novel Thiamine Pyrophosphate-dependent 2-Oxoacid Oxidoreductase that Enables Anaerobic Growth on Oxalate, *Journal of Biological Chemistry* **285**: 40515-40524. PMC3003350.

Bender, G., Stich, T.A., Yan, L., Britt, R.D., Cramer, S.P., and Ragsdale, S.W. (2010) Probing the catalytic mechanism of acetyl-CoA synthase by infrared and EPR characterization of the photolyzed Ni(I)-CO intermediate, *Biochemistry* **49:** 7516–7523. PMC2932805.

Kung, Y., Doukov,T.I., Seravalli, J., Ragsdale, S.W., Drennan, C.L. (2009) Crystallographic snapshots of cyanide- and water-bound C-clusters from bifunctional carbon monoxide

dehydrogenase/ acetyl-CoA synthase, *Biochemistry **48***: 7432-40. PMC2721637.

Ragsdale, S.W. (2009) Nickel-based enzymatic systems, *Journal of Biological Chemistry*. **284**: 18571-18575. PMC2707248.

Seravalli, J and Ragsdale, S.W. (2008) ^{13}C-NMR Characterization of an Exchange Reaction Between CO and CO_2 Catalyzed by Carbon Monoxide Dehydrogenase, *Biochemistry* **47**, 6770-6781. PMC2664834.

Pierce, E., Xie, G., Barabote, R. D., Saunders, E., Han, C. S., Detter, J. C., Richardson, P., Brettin, T. S., Das, A., Ljungdahl, L. G., and Ragsdale, S.W. (2008) The Complete Genome Sequence of *Moorella thermoacetica*, *Environmental Microbiology* **10**: 2550-2573. PMC2575129.

Banerjee, R**.** and Ragsdale, S.W. (2003) B_{12} Biochemistry. *Annual Review of Biochem*istry, 72: 209-47.

10 non-peer reviewed publications & patents demonstrating capabilities in the broad field

Zhou, Y., Sliwa, D.A., and Ragsdale, S.W. (2013) Biochemistry of Methyl-CoM Reductase and Coenzyme F_{430}, In *Handbook of Porphyrin Science* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.), World Scientific Publishing, in press.

Bender, G., Pierce, E., Hill, J.A., Darty, J.E., and **Ragsdale, S.W.** (2011) Metal Centers in the Anaerobic Microbial Metabolism of CO and CO_2, *Metallomics* **3**: 797-815.

Ragsdale, S. W., Yi, L., Bender, G., Gupta, N., Kung, Y., Yan, L., Stich, T.A., Doukov, T., Leichert, L., Jenkins, P.M., Bianchetti, C.M., George, S.J., Cramer, S.P., Britt, R.D., Jakob, U., Martens, J.R., Phillips, G.N., and Drennan, C.L. (2012) Redox, haem and CO in enzymatic catalysis and regulation, *Biochem Soc Trans* **40**: 501-507.

Ragsdale, S.W. and Yi, L. (2011) Thiol/Disulfide Redox Switches as Regulators of Heme-Dependent Processes, *Antioxidants & Redox Signaling.* **14**: 1039-47. PMC3042308

Dey, M., Li, X., Zhou, Y., and Ragsdale, S.W. (2009) Evidence for Organometallic Intermediates in Bacterial Methane Formation Involving the Nickel Coenzyme F430, *In* Metal Ions in Life Sciences, Vol. 7. Sigel, A. & H. Sigel, Eds. Royal Society of Chemistry. Cambridge, UK., pages 71-110.

Ragsdale, S.W. (2008) Enzymology of the Wood-Ljungdahl Pathway of Acetogenesis, *Annals of the N. Y. Academy of Sciences,* **1125**: 129-136. PMID: 18378591.

Seravalli, J., and **Ragsdale, S. W.** (2007) Electron Paramagnetic Resonance in R. Banerjee (ed.), *Redox Biochemistry*, Wiley and Sons, Hoboken, NJ, Chpt 6.2, pp. 237-247.

Ragsdale, S.W., and Pierce, E. (2008) Acetogenesis and the Wood-Ljungdahl Pathway of CO_2 Fixation, *Biochim Biophys Acta - Proteins and Proteomics **10***: 1873-1898. PMCID: PMC2646786.

Ragsdale, S.W. (2006) Metals and their scaffolds in catalyzing difficult reactions. Chemical Reviews, **106**: 3317-37.

Ragsdale, S.W. (2006) One-carbon chemistry: CO_2, CO, CH_4, formate: 1. Reductive chemistry. In, I. Bertini, H. Gray, E.I. Stiefel, J. S. Valentine (ed.), *Biological Inorganic Chemistry: Structure and Reactivity*. University Science Books. Chpt 12, pp. 452-467.

John A. Leigh, Ph.D.

Education/training:

Swarthmore College	Biology	B.S., 1976
University of Illinois	Microbiology	M.S., 1979
University of Illinois	Microbiology	Ph.D., 1983
Massachusetts Institute of Technology	Molecular Genetics	Postdoctoral Fellow, 1983-85

Employment history:

2005-present	Professor, Department of Microbiology, University of Washington
1992-2005	Associate Professor, Department of Microbiology, University of Washington
1985-1992	Assistant Professor, Department of Microbiology, University of Washington

Awards and Honors

1986	Presidential Young Investigator Award
1986	Searle Scholarship
2006	Elected to Fellowship in the American Academy of Microbiology

Peer-reviewed publications related to project:

Lie T. J., K. C. Costa, D. Pak, V. Sakesan, and J. A. Leigh. 2013. Phenotypic evidence that the function of the [Fe]-hydrogenase Hmd in *Methanococcus maripaludis* requires seven *hcg* (*hmd*-cooccuring genes) but not *hmdII*. FEMS Microbiol. Lett. (in press).

Costa, K. C., T. J. Lie, M. A. Jacobs, and J. A. Leigh. 2103. H_2-independent growth of the hydrogenotrophic methanogen *Methanococcus maripaludis*. MBio 4: doi: 10.1128/ mBio.00062-13.

Costa, K. C, S. H. Yoon, M. Pan, J. A. Burn, N. S. Baliga, and J. A. Leigh. 2013. Effects of H_2 and formate on growth yield and regulation of methanogenesis in *Methanococcus maripaludis*. J. Bacteriol. 195: 1456-1462.

Lie, T. J., K. C. Costa, B. Lupa, S. Korpole, W. B. Whitman, and J. A. Leigh. 2012. Essential anaplerotic role for the energy-converting hydrogenase Eha in hydrogenotrophic methanogenesis. Proc. Natl. Acad. Sci. USA 109:15473-15478.

Costa K. C., P. M. Wong, T. Wang, T. J. Lie, J. A. Dodsworth, I. Swanson, J. A. Burn, M. Hackett, and J. A. Leigh. 2010. Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase. Proc. Natl. Acad. Sci. USA 107:11050-11055.

Lupa, B., E. L. Hendrickson, J. A. Leigh, and W. B. Whitman. 2008. Formate-dependent H_2 production by the mesophilic methanogen *Methanococcus maripaludis*. Appl. Environ. Microbiol. 74:6584-6590.

Hendrickson, E. L., and J. A. Leigh. 2008. Roles of coenzyme F_{420}-reducing hydrogenases and hydrogen- and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F_{420} and production of hydrogen during methanogenesis. J. Bacteriol. 190:4818-4821.

Hendrickson, E. L., Y. Liu, G. Rosas-Sandoval, I. Porat, D. Söll, W. B. Whitman, and J. A. Leigh. 2008. Global responses of *Methanococcus maripaludis* to specific nutrient limitations and growth rate. J. Bacteriol. 190:2198-2205.

Hendrickson, E. L., A. K. Haydock, B. C. Moore, W. B. Whitman, and J. A. Leigh. 2007. Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. Proc. Natl. Acad. Sci. USA 104:8930-8934.

Stolyar, S., S. Van Dien, K. L. Hillesland, N. Pinel, T. J. Lie, J. A. Leigh, and D. A. Stahl. 2007. Metabolic modeling of a mutualistic microbial community. Mol. Syst. Biol. 3:92.

Xia, Q., E. L. Hendrickson, T. Wang, R. J. Lamont, J. A. Leigh, and M. Hackett. 2007. Protein abundance ratios for global studies of prokaryotes. Proteomics 7: 2904-2919.

Peer-reviewed publications in broad field

Yoon, S. H., D. J. Reiss, J. C. Bare, D. Tenenbaum, M. Pan, J. Slagel, R. L. Moritz, S. Lim, M. Hackett, A. L. Menon, M. W.W. Adams, A. Barnebey, S. M. Yannone, J. A. Leigh, and N. S. Baliga. 2011. Parallel evolution of transcriptome architecture during genome reorganization. Genome Research 21:1892-1904.

Leigh, J. A., S. V. Albers, H. Atomi, and T. Allers. 2011. Model organisms for genetics in the domain archaea: methanogens, halophiles, thermococcales and sulfolobales. FEMS Microbiol. Rev. 35: 577-608.

Wisedchaisri, G., D. M. Dranow, T. J. Lie, J. B. Bonanno, Y. Patskovsky, S. A. Ozyurt, J. M. Sauder, S. C. Almo, S. R. Wasserman, S. K. Burley, J. A. Leigh, and T. Gonen. 2010. Structural underpinnings of nitrogen regulation by the prototypical nitrogen-responsive transcriptional factor NrpR. Structure 18: 1512-1521.

Dodsworth, J. A., L. Li, S. Wei, B. P. Hedlund, J. A. Leigh, and P. de Figueiredo. 2010. Interdomain conjugal transfer of DNA from Bacteria to Archaea. Appl. Environ. Microbiol. 76:5644-5647.

Lie, T. J., E. L. Hendrickson, U. M. Niess, B. C. Moore, A. K. Haydock, and J. A. Leigh. 2010. Overlapping repressor binding sites regulate expression of the *Methanococcus maripaludis* $glnK_1$ operon. Molecular Microbiol. 75:755-762.

Goldman, A. D., J. A. Leigh, and R. Samudrala. 2009. Comprehensive computational analysis of Hmd enzymes and paralogs in methanogenic Archaea. BMC Evolutionary Biol. 9:199.

Xia, Q., T. Wang, E. L. Hendrickson, T. J. Lie, M. Hackett, and J. A. Leigh. 2009. Quantitative proteomics of nutrient limitation in the hydrogenotrophic methanogen *Methanococcus maripaludis*. BMC Microbiol. 9:149

Dodsworth, J. A., and J. A. Leigh. 2007. NifI inhibits nitrogenase by competing with Fe protein for binding to the MoFe protein. Biochem. Biophys. Res. Commun. 364:378-382.

Lie, T. J. and J. A. Leigh. 2007. Genetic screen for regulatory mutations in *Methanococcus maripaludis* and its use in identification of induction-deficient mutants of the euryarchaeal repressor NrpR. Appl. Environ. Microbiol. 73:6595-6600.

Lie, T. J., J. A. Dodsworth, D. Nickle and J. A. Leigh. 2007. Diverse homologs of the archaeal

repressor NrpR function similarly in nitrogen regulation. FEMS Microbiol. Lett. 271:281-288.

Non-peer reviewed publications

Leigh, J. A. and W. B. Whitman. 2011. Archaeal genetics. *In* Brenner's Online Encyclopedia of Genetics. S. Maloy and K. Hughes, eds. Academic Press, Oxford.

Leigh, J.A. 2011. Growth of methanogens under defined hydrogen conditions. Methods Enzymol. 494:111-8.

Sarmiento, F. B., J. A. Leigh, and W. B. Whitman. 2011. Genetic systems for hydrogenotrophic methanogens. Methods Enzymol. 494:43-73.

Leigh, J. A., D. A. Stahl, and J. T. Staley. Evolution of metabolism and early microbial communities. In: W. T. Sullivan III and J. A. Baross (eds.), Planets and Life, The Emerging Science of Astrobiology. Cambridge University Press, 2007.

Leigh, J. A. Genomics of diazotrophic Archaea. In: R. Palacios and W. E. Newton, (eds.), Genomes and Genomics of Nitrogen Fixing Organisms. Springer, 2005.

Leigh, J. A. Regulation of nitrogen fixation in methanogenic Archaea. In: W. Klipp, B. Masepohl, J. R. Gallon and W. E. Newton (eds.), Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria. Kluwer Academic Publishers, 2004.

Leigh, J. A. Evolution of energy metabolism. In: Biodiversity of Microbial Life: Foundation of Earth's Biosphere, J. T. Staley and A.-L. Reysenbach, eds. (Wiley-Liss, New York), 2002.

Leigh, J. A., P. S. Kessler, and C. Blank. Regulation of *nif* gene transcription in *Methanococcus maripaludis*. In: Biological Nitrogen Fixation for the 21st Century, C. Elmerich, A. Kondorosi, and W. E. Newton (eds.), Kluwer Academic Publishers, Boston, 1998.

Nathan D. Price

Education/Training

B.S. Chemical Engineering, 2000, Brigham Young University, Provo, UT

Ph.D. Bioengineering, 2005, University of California, San Diego, CA, Bernhard Palsson, advisor.

Postdoctoral Fellow, 2005-2007, Institute for Systems Biology, Seattle, WA, Leroy Hood, advisor.

Employment History

Assistant Professor, University of Illinois, Urbana-Champaign; Department of Chemical and Biomolecular Engineering (primary), Institute for Genomic Biology, Center for Biophysics and Computational Biology, Center for Advanced BioEnergy Research, Department of Bioengineering, Neuroscience program, Department of Computer Science, 2007-2011.

Adjunct Faculty, University of Illinois, Urbana-Champaign, 2011–present.

Associate Professor, Institute for Systems Biology, Seattle, WA, 2011–present.

Affiliate Associate Professor & Member of Graduate College, University of Washington, Departments of Bioengineering, Computer Science & Engineering, 2011–present.

Associate Director, Institute for Systems Biology, Seattle, WA, 2013-present.

Awards and Honors

Camille Dreyfus Teacher-Scholar, 2011

Young Investigator Award, Roy J Carver Charitable Trust, 2010

National Science Foundation CAREER Award, 2009

National Cancer Institute Howard Temin Pathway to Independence Award, 2008

Tomorrow's PIs, Genome Technology, 2007

Sam E. and Kathleen Henry Postdoctoral Fellowship, American Cancer Society, 2006-2007

10 peer-reviewed publications specifically related to the proposed R&D project (total >65, h-index = 29, Google Scholar)

Benedict, M.N., Gonnerman, M.C., Metcalf, W.W., and Price, ND, Genome-scale metabolic reconstruction and hypothesis testing in the methanogenic archaeon Methanosarcina acetivorans C2A, (2012) *Journal of Bacteriology,* 194:855-65

Gonnerman MC, Benedict MN, Feist AM, Metcalf WW, **Price, ND**. Genomically and biochemically accurate metabolic reconstruction of Methanosarcina barkeri Fusaro, iMG746. (2013) *Biotechnol J*. Epub ahead of press.

Ezeji, T., Milne, C.B., **Price, N.D.**, and Blaschek, H.P., Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol producing microorganisms, A*pplied Microbiology and Biotechnology,* 85:1697-1712 (2010)

Chandrasekaran, S, and **Price, ND**. Probabilistic integrative modeling of genome-scale metabolic and regulatory networks in Escherichia coli and Mycobacterium tuberculosis, *Proc Nat Acad Sci USA*, 2010; 107:17845-50

Simeonidis, E, Chandrasekaran, S, and **Price, ND**, A guide to integrating transcriptional regulatory and metabolic networks using Probabilistic Regulation of Metabolism (PROM), (2013) *Methods in Molecular Biology*, 985:103-12

Milne, C.B., Eddy, J.A., Raju, R., Ardekani, S., Kim, P.-J., Senger, R.S., Jin, Y.-S., Blaschek, H.P., and **Price, ND**, Metabolic network reconstruction and genome-scale model of butanol-producing strain Clostridium beijerinckii NCIMB 8052, (2011) *BMC Systems Biology*, 5:130. *Highly Accessed* (special distinction given to the most downloaded papers in the journal)

Price, ND, Reed, J.L., and Palsson, B.O., Genome-scale models of microbial cells: evaluating the consequences of constraints, (2004) *Nature Reviews Microbiology*, 2: 886-897.

Milne, C.B., Kim, P.J., Eddy, J.A., and **Price, N.D.**, Accomplishments of genome-scale *in silico* modeling in industrial and medical biotechnology, (2009) *Biotechnology Journal*, 4:1653-1670

Wang, Y., Li, X. Milne, C.B., Jansson, H., Lin, W., Phan, G., Jin, Y.S., **Price, N.D.**, and Blaschek, H.P., Development of a gene knockout system using mobile group II introns (Targetron) and genetic disruption of acid production pathways in *Clostridium beijerinckii*, (2013) *Applied and Environmental Microbiology*, Accepted.

Ghosh, A., Zhao, H., and **Price, N.D.**, Genome-scale consequences of cofactor balancing in engineered pentose utilization pathways in *Saccharomyces cerevisiae*, *PLoS ONE 6(11):* e27316 (2011)

10 other peer-reviewed publications demonstrating capabilities in the broad field

Kim, P.-J., and Price, ND, Macroscopic kinetic effect of cell-to-cell variation in biochemical networks, (2010) *Physical Review Letters*, 104:148103.

Thiele, I, Swainston, N, Fleming, RMT, Hoppe, A, Sahoo, S, …**Price, ND**…, Goryanin, I, Nielsen, J, Westerhoff, HV, Kell, DB, Mendes, P, and Palsson, BO, A community-driven global reconstruction of human metabolism, (2013) *Nature Biotechnology,* 31:419-25

Wang, Y, Eddy, JA, and **Price, ND** Reconstruction of genome-scale metabolic models for 126 human tissues using mCADRE, (2012) *BMC Systems Biology*, 6:153. Highly Accessed (special distinction given to the most downloaded papers in the journal)

Sangar, V., Eddy, J.A., Simeonidis, E., and **Price, N.D.**, Understanding aberrant energy metabolism in disease through mechanistic modeling, (2012) *Frontiers in Computational Physiology and Medicine*, 3:404

Ko, Y., Ament, S.A., Eddy, J.A., Caballero, J., Earls, J.C., Hood, L., and **Price, N.D.**, Cell type-specific genes show striking and distinct patterns of spatial expression in the mouse brain, (2013) *Proceedings of the National Academy of Sciences USA*, 110:3095-3100

Kim PJ, Price ND. (2011) Genetic co-occurrence network across sequenced microbes. *PLoS Comput Biol*. 7:e1002340.

Chandrasekaran, S., Ament, S.A., Eddy, J.A., Rodriguez-Zas, S., Schatz, B.R., **Price, N.D.**[*] and Robinson, G.E.[*], Behavior-Specific Changes In Transcriptional Modules Lead To Distinct And Predictable Neurogenomic States, (2011) *Proceedings of the National Academy of Sciences USA*,

108:18020-5. *Co-corresponding authors

Nykter, M., **Price, N.D.**, Larjo, A., Aho, T., Kauffman, S.A., Yli-Harja, O., and Shmulevich, I., Critical Boolean networks exhibit maximal information diversity in structure-dynamics relationships, (2008) *Physical Review Letters*, 100:058702

Nykter, M., **Price, N.D.**, Aldana, M., Ramsey, S., Kauffman, S.A., Hood, L., Yli-Harja, O., and Shmulevich, I., Gene expression dynamics in the macrophage exhibit criticality, (2008) *Proceedings of the National Academy of Sciences USA*, 105:1897-1900

Labhsetwar, P., Cole, J., Roberts, E., **Price, N.D.**, and Luthey-Schulten, Z., Heterogeneity in protein expression induces metabolic variability in a modeled *Escherichia coli* population, (2013) *Proceedings of the National Academy of Sciences USA*, Accepted

1 non-peer-reviewed publication demonstrating capabilities in the broad field

Blaschek, H.P., Ezeji, T., and **Price, N.D.**, Present and future possibilities for the deconstruction and utilization of lignocellulosic biomass, Handbook of Bioenergy

Dayle M. A. Smith

Education/Training
B.S., Chemistry (1995), The Evergreen State College, Olympia, WA
Ph.D., Physical Chemistry (2001), University of Arizona, Tucson, AZ. Ludwik Adamowicz, Professor of Chemistry and Physics, advisor.
Postdoctoral Research Associate, 2001-2003, Pacific Northwest National Laboratory, Richland, WA. Tjerk P. Straatsma, Laboratory Fellow, advisor.

Employment History
Assistant Professor, Whitman College, Walla Walla, WA 2003-2010
Senior Research Scientist, Pacific Northwest National Laboratory, Richland, WA 2010-present

Awards and Honors
Merck/UNCF Ph.D. fellowship (1998)

10 peer-reviewed publications specifically related to the proposed R&D project
Smith, D.M.A., Straatsma, T.P., Squier, T.C., Retention of Conformational Entropy upon Calmodulin Binding to Target Peptides Is Driven by Transient Salt Bridges. Biophysical Journal 2012, 103 (1), 1576-1584.

Smith, D.M.A., Xiong, Y, Straatsma, T.P., Rosso, K.M., and Squier, T.C., Classical Force Field Development and Molecular Dynamics of [NiFe] Hydrogenase. Journal of Chemical Theory and Computation 2012, 8 (6), 2103–2114

Smith, D.M.A., Xiong, Y, Straatsma, T.P., Rosso, K.M., and Squier, T.C., Classical Force Field Development and Molecular Dynamics of [NiFe] Hydrogenase. Prep. Pap.-Am. Chem. Soc., Div. Fuel Chem. 2012, 57 (1), 575-576

Smith, D. M. A.; Rosso, K. M.; Dupuis, M.; Valiev, M.; Straatsma, T. P., Electronic coupling between heme electron-transfer centers and its decay with distance depends strongly on relative orientation. Journal of Physical Chemistry B 2006, 110, (31), 15582-15588.

Smith, D. M. A.; Dupuis, M.; Straatsma, T. P., Multiplet splittings and other properties from density functional theory: an assessment in iron-porphyrin systems. Molecular Physics 2005, 103, (2-3), 273-278.

Smith, D. M. A.; Dupuis, M.; Vorpagel, E. R.; Straatsma, T. P., Characterization of electronic structure and properties of a bis(histidine) heme model complex. Journal of the American Chemical Society 2003, 125, (9), 2711-2717.

10 other peer-reviewed publications demonstrating capabilities in the field
Stack, A. G.; Rosso, K. M.; Smith, D. M. A.; Eggleston, C. M., Reaction of hydroquinone with hematite II. Calculated electron-transfer rates and comparison to the reductive dissolution rate. Journal of Colloid and Interface Science 2004, 274, (2), 442-450.

Rosso, K. M.; Smith, D. M. A.; Wang, Z. M.; Ainsworth, C. C.; Fredrickson, J. K., Self-exchange electron transfer kinetics and reduction potentials for anthraquinone disulfonate. Journal of Physical Chemistry A 2004, 108, (16), 3292-3303.

Rosso, K. M.; Smith, D. M. A.; Dupuis, M., Aspects of aqueous iron and manganese (II/III) self-exchange electron transfer reactions. Journal of Physical Chemistry A 2004, 108, (24), 5242-5248.

Rosso, K. M.; Smith, D. M. A.; Dupuis, M., An ab initio model of electron transport in hematite (alpha-Fe2O3) basal planes. Journal of Chemical Physics 2003, 118, (14), 6455-6466.

Smith, D. M. A., Excess Electrons in DNA: A Computational Study. In Theoretical Prospects of Negative Ions, Kalcher, J., Ed. Research Signpost: 2002.

Smith, D. M. A.; Adamowicz, L., A dynamic model for electron transport in DNA. Journal of Physical Chemistry B 2001, 105, (38), 9345-9354.

Smith, D. M. A.; Jalbout, A. F.; Smets, J.; Adamowicz, L., Cytosine anions: ab initio study. Chemical Physics 2000, 260, (1-2), 45-51.

Desfrancois, C.; Abdoul-Carime, H.; Carles, S.; Periquet, V.; Schermann, J. P.; Smith, D. M. A.; Adamowicz, L., Experimental and theoretical ab initio study of the influence of N-methylation on the dipole-bound electron affinities of thymine and uracil. Journal of Chemical Physics 1999, 110, (24), 11876-11883.

McCarthy, W. J.; Smith, D. M. A.; Adamowicz, L.; Saint-Martin, H.; Ortega-Blake, I., An ab initio study of the isomerization of Mg- and Ca-pyrophosphates. Journal of the American Chemical Society 1998, 120, (24), 6113-6120.

Alexandrov, V.; Smith, D. M. A.; Rostkowska, H.; Nowak, M. J.; Adamowicz, L.; McCarthy, W., Theoretical study of the O-H stretching band in 3-hydroxy-2-methyl-4-pyrone. Journal of Chemical Physics 1998, 108, (23), 9685-9693.

Aaron M. Appel
Education/Training
B.S. Chemistry, 2000, Washington State University, Pullman, WA.
Ph.D. Inorganic Chemistry, 2005, University of Colorado, Boulder, CO.
Postdoctoral Fellow, 2005-2008, Pacific Northwest National Laboratory. James A. Franz, advisor.

Employment History
Senior Research Scientist, Pacific Northwest National Laboratory, 2008-2013.
Senior Scientist, Pacific Northwest National Laboratory, 2013-

Awards and honors
Member of the International Scientific Committee for the International Conference on Carbon Dioxide Utilization (2013-present)
Outstanding Performance Award, Pacific Northwest National Lab, December 2008
Outstanding Performance Award, Pacific Northwest National Lab, February 2006
Outstanding Second Year Graduate Student Award, University of Colorado, May 2003
Graduate Teaching Excellence Award, University of Colorado, Spring 2002
Outstanding Undergraduate Award, Phi Lambda Upsilon Honor Society at WSU, 2000
Honors College and College of Science Summer Internship, WSU, 2000
President's Honor Roll, WSU, 1996-2000

Peer-reviewed publications specifically related to the proposed R&D project
Appel, A., Bercaw, J., Bocarsly, A., Dobbek, H., DuBois, D., Dupuis, M., Ferry, J., Fujita, E., Hille, R., Kenis, P., Kerfeld, C., Morris, R., Peden, C., Portis, A., Ragsdale, S.W., Rauchfuss, T., Reek, J., Seefeldt, L., Thauer, R., and Waldrop, G. (2013) Frontiers, Opportunities, and Challenges in Biochemical and Chemical Catalysis of CO_2 Fixation, *Chemical Reviews*, in press.

Horvath, S., Fernandez, L. E., **Appel**, **A. M.**, Hammes-Schiffer, S. (2013) pH-Dependent Reduction Potentials and Proton-Coupled Electron Transfer Mechanisms in Hydrogen-Producing Nickel Molecular Electrocatalysts, *Inorganic Chemistry* **52:** 3643-3652.

Jain, A., Reback, M. L., Lindstrom, M. L., Thogerson, C. E., Helm, M. L., **Appel, A. M.**, Shaw, W. J. (2012) Investigating the Role of the Outer-Coordination Sphere in $[Ni(P^{Ph2}N^{Ph-R2})_2]^{2+}$ Hydrogenase Mimics, *Inorganic Chemistry* **51:** 6592-6602.

Lense, S., Ho, M. H., Chen, S. T., Jain, A., Raugei, S., Linehan, J. C., Roberts, J. A. S., **Appel, A.** M., Shaw, W. (2012) Incorporating Amino Acid Esters into Catalysts for Hydrogen Oxidation: Steric and Electronic Effects and the Role of Water as a Base, *Organometallics* **31:** 6719-6731.

Roberts, J. A**.**, **Appel, A. M.**, DuBois, D. L., Bullock, R. M. (2011) Comprehensive Thermochemistry of W–H Bonding in the Metal Hydrides $CpW(CO)_2(IMes)H$, $[CpW(CO)_2(IMes)H]^{\bullet+}$, and $[CpW(CO)_2(IMes)(H)_2]^{+}$. Influence of an *N*-Heterocyclic Carbene Ligand on Metal Hydride Bond Energies, *Journal of the American Chemical Society* **133:** 14604-14613.

Appel, A. M., Lee, S.-J., Franz, J. A., DuBois, D. L., Rakowski DuBois, M. (2009) Free Energy Landscapes for S-H Bonds in $Cp^*_2Mo_2S_4$ Complexes, *Journal of the American Chemical Society*

131: 5224-5232.

Appel, A. M., Lee, S.-J., Franz, J. A., DuBois, D. L., Rakowski DuBois, M., Twamley, B. (2009) Determination of S-H Bond Strengths in Dimolybdenum Tetrasulfide Complexes, *Organometallics* **28:** 749-754.

Fraze, K., Wilson, A. D., **Appel, A. M.**, Rakowski DuBois, M., DuBois, D. L. (2007) Thermodynamic Properties of the Ni-H Bond in Complexes of the Type $[HNi(P_2^RN_2^{R'})_2](BF_4)$ and Evaluation of Factors That Control Catalytic Activity for Hydrogen Oxidation/Production, *Organometallics* **26:** 3918-3924.

Other peer-reviewed publications demonstrating capabilities in the broad field

Hoffert, W.A., Mock, M. T., A**ppel**, **A. M.**, Yang, J. Y. (2013) Incorporation of Hydrogen-Bonding Functionalities into the Second Coordination Sphere of Iron-Based Water-Oxidation Catalysts, *European Journal of Inorganic Chemistry* DOI: 10.1002/ejic.201201499.

Seu, C. S., **Appel, A. M.**, Doud, M. D., DuBois, D. L., Kubiak, C. P. (2012) Formate oxidation via ß-deprotonation in $[Ni(P^R_2N^{R'}_2)_2(CH_3CN)]^{2+}$ complexes, *Energy & Environmental Science* **5:** 6480-6490.

O'Hagan, M., Ho, M. H., Yang, J. Y., **Appel, A. M.**, DuBois, M. R., Raugei, S., Shaw, W. J., DuBois, D. L., Bullock, R. M. (2012) Proton Delivery and Removal in $[Ni(P^R_2N^{R'}_2)_2]^{2+}$ Hydrogen Production and Oxidation Catalysts, *Journal of the American Chemical Society* **134:** 19409-19424.

Galan, B. R., Schoffel, J., Linehan, J. C., Seu, C. S., **Appel, A. M.**, Roberts, J. A. S., Helm, M. L., Kilgore, U. J., Yang, J. Y., DuBois, D. L., Kubiak, C. P. (2011) Electrocatalytic Oxidation of Formate by $[Ni(P^R_2N^{R'}_2)_2(CH_3CN)]^{2+}$ Complexes, *Journal of the American Chemical Society* **133:** 12767-12779.

Appel, A. M., Pool, D. H., O'Hagan, M. J., Shaw, W. J., Yang, J. Y., Rakowski DuBois, M., DuBois, D. L., Bullock, R. M. (2011) $[Ni(P^{Ph}_2N^{Bn}_2)_2(CH_3CN)]^{2+}$ as an Electrocatalyst for H_2 Production: Dependence on Acid Strength and Isomer Distribution, *ACS Catalysis* **1:** 777-785.

Kilgore, U. J., Roberts, J. A., Pool, D. H., **Appel, A. M.**, Stewart, M. P., Rakowski DuBois, M., Dougherty, W. G., Kassel, W. S., Bullock, R. M., DuBois, D. L. (2011) $[Ni(P^{Ph}_2N^{C6H4X}_2)_2]^{2+}$ Complexes as Electrocatalysts for H_2 Production: Effect of Substituents, Acids, and Water on Catalytic Rates," *Journal of the American Chemical Society* **133:** 5861–5872.

Franz J. A., Lee, S.-J., Bowden, T.A., Alnajjar, M. S., **Appel, A. M.**, Birnbaum, J. C., Bitterwolf, T. E., Dupuis, M. (2009) Activation of the S−H Group in Fe(μ_2-SH)Fe Clusters: S−H Bond Strengths and Free Radical Reactivity of the Fe(μ_2-SH)Fe Cluster, *Journal of the American Chemical Society* **131:** 15212-15224.

Appel, A. M., Lee, S.-J., Franz, J. A., DuBois, D. L., Rakowski DuBois, M., Birnbaum, J. C, Twamley, B. (2008) Formation and Reactivity of a Persistent Radical in a Dinuclear Molybdenum Complex, *Journal of the American Chemical Society* **130:** 8940-8951.

Appel, A. M., DuBois, D. L., Rakowski DuBois, M. (2005) Molybdenum-Sulfur Dimers as Electrocatalysts for the Production of Hydrogen at Low Overpotentials, *Journal of the American*

Chemical Society **127:** 12717-12726.

Appel, A. M., Newell, R., DuBois, D. L., Rakowski DuBois, M. (2005) Concentration of Carbon Dioxide by Electrochemically Modulated Complexation with a Binuclear Copper Complex, *Inorganic Chemistry* **44:** 3046-3056.

Non-peer reviewed publications & patents demonstrating capabilities in the broad field

Reike, P., Coffey, G., **Appel, A. M.** (January, 2013) Separation and/or Sequestration Apparatus and Methods, US Patent Application number 13/748,463.

Appel, A. M., Strohm, J. J. (April, 2012) Systems and Methods for Removing Components of a Gas Mixture. US Patent Application number 13/440,683.

VII. PARTICIPATING ORGANIZATIONS:

The proposed project integrates well with the goals of our respective institutions and taps the unique qualifications, expertise and facilities of the Project Team members. At the University of Michigan (U-M), the project meshes with the research thrusts of the University of Michigan Energy Institute (UMEI), of which Ragsdale is an adjunct faculty member. UMEI builds on U-M's strong energy research heritage at the heart of the nation's automotive industries. UMEI is contributing to the cost-share by providing a summer undergraduate fellowship. Ragsdale's long-term research goals align well with those of this proposed project, as he has studied aspects of bioenergy (CO and CO_2 fixation, methanogenesis, acetogenesis) for many years and has been continuously funded by DOE since 1987, including current work (ER15931).

The University of Washington is ranked number one among public universities nationally in receipt of federal research and training funds, and since 1975 has been in the top five for public and private universities. Current research awards include $30 million from the DOE (http://www.washington.edu/research/energy/). Leigh has worked with methanogenic Archaea since 1991 and has been continuously funded by the DOE since 2001, including current work (ER15709). Leigh's group contributed substantially to the development of *Methanococcus maripaludis* as a model organism, in which he has studied genetics, genomics, regulation, methanogenesis, and energy conservation. The equipment and expertise in Leigh's lab makes it ideally suited to the expression of proteins and the engineering of pathways in *M. maripaludis*.

The proposed research is synergistic with three of the areas of expertise at Pacific Northwest National Laboratory: computational chemistry, catalysis and biological energy transformation. PNNL has state-of-the art computational facilities and support staff and is home to the popular NWChem massively-parallel computational chemistry software, which will be used for this project (Smith is one of the developers). The fundamental research of the Institute for Integrated Catalysis (Appel) also overlaps with the project in identification of catalytic sites involved in elementary catalytic processes. The proposed research is also consistent with the activities of the Computational Biology & Bioinformatics group (Smith) in the Computational Sciences & Mathematics Division, particularly in the area of biological molecular modeling. Smith's research focuses on using physics methods to predict the chemical properties of metalloproteins for processes relevant to energy such as H_2 production and electron transport.

The proposed research matches the goals and mission of the Institute for Systems Biology (ISB), which include leveraging systems biology for environmental sustainability, a major area of interest for Profs. Price and Baliga. Within the ISB are major national Centers (e.g., the NIH-funded center for Systems Biology) that cultivate a multidisciplinary, interactive and focused Institutional culture where the needs of systems biology drive the integrated development of data-gathering technologies and computational tools. ISB embodies a philosophy, environment and administrative structure that in which researchers collaborate across disciplinary boundaries to share and leverage knowledge and expertise with partners in academia and industry. ISB is a scientific catalyst and a hub of strategic partnerships driving the impacts of systems biology on the environment. The Institute supports 10 faculty groups, 3 part-time faculty, 30 senior research scientists, 7 administrative departments and a K-12 science education group, collectively consisting of over 300 staff members. Recently ranked by SCIMAGO as 4th in the world for average impact of papers, ISB has been ranked repeatedly over the past decade as one of the 10 best places for postdoctoral fellows to work. Dr. Price has worked in modeling of metabolic networks since 2001, and in particular in the metabolic modeling of methanogens since 2009.

VIII. PRIOR COLLABORATION:

Though none of the team members have formerly collaborated on a research project, there have been significant interrelationships. Dr. Leigh and Dr. Price collaborate together closely through both being on the scientific advisory board for Trelys, Inc, a startup company focusing on re-engineering methanogen metabolism to make substrates for commercial products. Their SAB involvement involves monthly meetings where they help to advise the scientists working at Trelys. A member of Dayle Smith's team, Aaron Appel, and Ragsdale worked together on a DOE panel and were coauthors on a review related to CO_2 reduction (95). John Leigh and Steve Ragsdale recently initiated a collaboration to express the methyl-CoM Reductase from *M. marburgensis* (though nothing has been published yet related to this work).

IX. MANAGEMENT PLAN:

The organizational structure of the project involves Ragsdale as the Contact PI and Project Coordinator. Leigh, Price, Ragsdale and Smith are each responsible for one Specific Aim.

John Leigh will lead Task 1 involving active expression of the gene clusters encoding the MCRs from a methanogen (M. marburgensis) and from anaerobic methanotrophs (ANME-1 and ANME-2 in a genetically tractable methanogen. He also is responsible for genetic engineering of the pathways for converting methane to methanol and to butanol. Within the same organisms, he will also incorporate the dissimilatory sulfate reduction pathway to make the conversion of methane to methanol/butanol thermodynamically favorable.

Nathan Price will lead Task 2 to perform metabolic modeling and associated characterization and validation experiments of the genetically engineered *Methanococcus maripaludis* strains that will be used for expression of the methane to methanol/butanol and sulfate reducing pathways. He will also lead the effort to perform metabolomic and metabolic flux tracer experiments to aid in model refinement for improved accuracy. Based on the modeling and experimental outcomes, he will work with the Leigh laboratory to reengineer aspects of *M. maripaludis* metabolism by performing genetic alterations, i.e., knock-outs, to optimize the methanol/butanol pathways in its new metabolic context.

Stephen Ragsdale will lead the Task 3 to purify the newly introduced enzymes from *M. maripaludis* and conduct in vitro biochemical and biophysical studies to test their functionality.

Dayle Smith will lead Task 4 to perform computational studies to understand the mechanism of methanogenic and the methane-oxidizing MCR, and to predict the effects of differences among the various MCRs on the reaction mechanism.

As experts in each of these areas, the co-PIs will make the major autonomous decisions on the scientific and technical direction related to each of these aims. However, this is a highly collaborative process and we will all be kept abreast of and comment on each other's research findings through on-line journal clubs that will take place in monthly meetings on Google + Hangout. This format allows for facile video conference calls including up to 14 other Google+ users (the four principal investigators and members of their laboratories) as well as messaging, emails, sharing of documents, screen sharing, and Google Docs collaboration. It allows connections among computers, Android and Apple devices. We will also schedule a yearly meeting on the day before or after the yearly ARPA-E meeting.

X. MULTI-INVESTIGATOR PROJECTS:

Roles of Participants: For multi-organizational or multi-investigator projects, describe succinctly: the roles and the work to be performed by each PI and Key Participant; business agreements between the Applicant and each PI and Key Participant; and how the various efforts will be integrated and managed.

Multiple PIs: Standalone Applicants and Project Teams are required to disclose if the project will include multiple PIs. If multiple PIs will be designated, identify the, and provide a "Coordination and Management Plan" that describes the;

This is a multi-investigator project that enlists four researchers from a government laboratory, a research institute, and two universities. Stephen W. Ragsdale will be the Contact PI and Project Coordinator. This multiple-organizational management concept is important because each of the investigators has specialized and complementary knowledge and training related to the four specific aims of the project. We feel that we have enlisted the four best people in the world to accomplish the technological development performance goals of this project. Our team includes John Leigh (Univ. Washington), Nathan Price (Institute for Systems Biology), Stephen Ragsdale (Univ. Michigan) and Dayle Smith (PNNL), each of whom is responsible for one of the four specific aims of the proposal.

John Leigh's laboratory will actively express the gene clusters encoding the MCRs from a methanogen (M. marburgensis) and from anaerobic methanotrophs (ANME-1 and ANME-2 in a genetically tractable methanogen and to genetically engineer the pathways for converting methane to methanol and to butanol. Within the same organisms, he will also incorporate the dissimilatory sulfate reduction pathway to make the conversion of methane to methanol/butanol thermodynamically favorable. ***Nathan Price's laboratory*** will perform metabolic modeling and and associated characterization and validation experiments of the genetically engineered *Methanococcus maripaludis* strains that will be used for expression of the methane to methanol/butanol and sulfate reducing pathways. This process is to ensure that enzymes in the engineered pathways can operate at high efficiency in *M. maripaludis* by modifying the metabolic network such that utilization of the newly engineered pathway by the organism is optimized. This work will also involve some metabolomic and metabolic flux tracer experiments to aid in model refinement for improved accuracy. These experiments in conjunction with the model will also help to identify where metabolic flux is active as we work to adjust the flux pattern through the newly engineered pathways. Based on the modeling and experimental outcomes, he will reengineer aspects of *M. maripaludis* metabolism by performing genetic alterations, i.e., knock-outs, to optimize the methanol/butanol pathways in its new metabolic context. ***Stephen Ragsdale's laboratory*** will purify the newly introduced enzymes from *M. maripaludis* and conduct in vitro biochemical studies to test their functionality. They will also

conduct pathway flux measurements and efficiency determinations aimed at optimizing methane oxidation to liquid fuels. ***Dayle Smith's laboratory*** will perform computational studies to understand the mechanism of the anaerobic methanogenic and the methane-oxidizing enzyme, MCR, and to predict the effects of differences among the various MCRs on the reaction mechanism.

The organizational structure of the project involves Ragsdale as the Contact PI and Project Coordinator. Leigh, Price, Ragsdale and Smith are each responsible for one of the Specific Aims of the proposal and, as experts in each of these areas, will make the major autonomous decisions on the scientific and technical direction related to each of these aims. However, this is a highly collaborative process and we will all be kept abreast of and comment on each other's research findings through on-line journal clubs that will take place in monthly meetings on Google + Hangout. This format allows for facile video conference calls including up to 14 other Google+ users (the four principal investigators and members of their laboratories) as well as messaging, emails, sharing of documents, screen sharing, and Google Docs collaboration. It allows connections among computers, Android and Apple devices. We will also schedule a yearly meeting on the day before or after the yearly ARPA-E meeting.

XI. INTELLECTUAL PROPERTY STRATEGY:

The University of Michigan, as lead organization, and the collaborating organizations (University of Washington, Institute for Systems Biology, and Pacific Northwest National Laboratory) are aware of and committed to focusing the research described in this proposal to develop a novel and transformational bioengineering technology for the biological synthesis of liquid fuels from methane. The collaborating organizations intend to make data and intellectual property resulting from performance of the proposed project available for the benefit of the scientific community and the public in accordance with the NIH Grants Policy Statement and the Principles and Guidelines for Recipients of NIH Research Grants and Contracts on Obtaining and Disseminating Biomedical Research Resources: Final Notice, December 1999 and 10 CFR 600.136 - Intangible property.

Furthermore, the collaborating organizations are aware of and committed to supporting the NIH Final Statement on Sharing Research Data that results from performance of the proposed project. The collaborating organizations believe that data sharing is essential for expedited translation of research results into knowledge, products, and procedures to improve human health and will assure the timely release and sharing of data no later than the acceptance for publication of the main findings from the final dataset. The Principal Investigator in conjunction with other collaborating investigators will review publications resulting from this project to attribute authorship according to standard academic principles that appropriately recognize the contributions of each essential co-author.

The collaborating institutions agree to use the Uniform Biological Materials Transfer Agreement to distribute appropriate research materials to the research community. If necessary a contract will be developed with a distributor to assist with the dissemination of data and research tools to the broader research community.

With respect to licensing patented inventions developed through project activities, the collaborating institutions expect to elect title as appropriate under the Bayh-Dole Act to inventions made with federal funds by their investigators.

The Principal Investigator and all collaborating investigators will seek advice from representatives of their associated tech transfer offices on how best to share data and research resources with each other and the general scientific community. These representatives will evaluate intellectual property developed in the performance of this project to assess the impact, market potential, and strategies to be used to maximize benefits of the intellectual property for the general public, the academic and scientific community and the institutions. It is our intent to consider filing patent applications only on discoveries where there is a clear demonstration that patent protection and commercialization of these discoveries will result in a benefit to the public and will not conflict with the goals of DOE or our institutions to make unique research resources widely accessible and available to the research community in a timely manner to encourage new discovery and innovation leading to long term health benefits. The collaborating institutions have active licensing programs and can draw on their experience, contacts and market knowledge to transfer new technologies to industry for commercialization.

The University of Michigan's Office of Technology Transfer (http://techtransfer.umich.edu/) has been in existence for over twenty years and currently handles 400 invention disclosures or more per year from faculty in multiple departments and colleges. It is committed to transferring faculty inventions to the marketplace for the benefit of the public and enters into more than 100 license and option agreements each year, including an average of 8-10 spin off companies each year with technologies and products in diverse as battery technologies to new treatments for cancer and medical devices.

Since 2005, The University of Washington Center for Commercialization (C4C: http://depts.washington.edu/uwc4c/about-c4c/) has supported the commercialization of more than 100 projects, provided comprehensive mentoring and over $4 million in grants, and helped spin out new companies. Example companies include, Fate Therapeutics, EnerG2, MicroGREEN Polymers, and Farecast.

The Pacific Northwest technology transfer office has a long-standing history of developing technologies that successfully translate into industrial products, services, and consumer goods (http://www.pnl.gov/business/tech_transfer.aspx).

The Institute for Systems Biology (ISB) is committed to ensuring that discoveries resulting from its research will be used to benefit society. ISB regularly evaluates its research discoveries to determine whether they are appropriate to patent and, if so, how they can best be commercialized. ISB technologies have formed the basis for start-up companies, including Cytopeia, Nanostring and Integrated Diagnostics, in which ISB has taken an equity interest. (http://www.systemsbiology.org/intellectual-property)

The respective technology transfer offices of the collaborating institutions will guide discussion and decisions about various exclusive versus nonexclusive licensing strategies. It is the practice of the collaborating institutions to reserve a research use license for any resulting inventions in the final negotiated commercialization license. Government rights in such inventions will be reserved as well. When possible such reservations will include the right to share such inventions with others for noncommercial purposes. The insight and collective conscience of the faculty involved in this project can be invaluable to the technology transfer offices in tackling difficult decisions that weigh potential for monetary benefit against potential wide–spread scientific or academic use and similar issues.