EXPRESSION OF SOLUBLE METHANE MONOOXYGENASE IN RECOMBINANT HOST ESCHERICHIA COLI FOR INDUSTRIAL BIOCONVERSION OF METHANE TO METHANOL

by

Miles Nelson

B.S. (Dickinson College) 2011

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

December 2019

Accepted:

_____	_____
Ricky Hirschorn, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
_____	
Jeffrey Rossio, Ph.D. Committee Member	
_____	_____
Craig Laufer, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

To my mother, who didn't get to read it. For the eye rolls, and the reminders, and the persistent love.

ACKNOWLEDGEMENTS

I would like to thank the staff at Hood College for their support and guidance in writing this proposal, especially Craig Laufer who helped as my advisor and gave me feedback throughout the process.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Expression of sMMO in recombinant host *E. coli* for Industrial Bioconversion of Methane to Methanol

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Nelson, Miles David	B.S.	N/A

3c. POSITION TITLE
Graduate Student

3d. MAILING ADDRESS *(Street, city, state, zip code)*
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3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: N/A FAX: N/A

E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☒ No ☐ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
01/30/20	01/30/22				350,000.00

9. APPLICANT ORGANIZATION
Name: Hood College
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE

PROJECT SUMMARY (See instructions):

Using the biological catalyst methane monooxygenases (MMOs), methane sequestration has the potential of both reducing greenhouse gas concentrations and producing a marketable fuel. Soluble methane monooxygenases (sMMOs), found in methanotrophic bacteria sequester methane under mild conditions and is one of the most viable biological catalysts for the methane to methanol reaction. The current understanding of *in vivo* sMMO shows it has several restraints inhibiting its industrial use. Currently one of the outstanding problems is successful expression of sMMO in a recombinant host such as *Eschericia coli*. In this proposal I hypothesize that 1) an undiscovered helper protein, or gene, aids in proper protein expression in its natural host and is expressed itself in low copper environments and 2) Co-expressing the novel protein(s) or gene(s) in *E. coli* will produce a recombinant expression system for sMMO.

RELEVANCE (See instructions):

Atmospheric methane sequestration and bioconversion into methanol for industrial use by using the soluble methane monooxygenase (sMMO) protein from *Methylococcus casulatus*. Current problem is successfully expressing sMMO in a recombinant host.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Miles Nelson	Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login) mdn6	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Dickinson College	B.S.	2011	Biochemistry and Molecular Biology
Hood College, Frederick, MD	M.S.	2020 (anticipated)	Biomedical Science

A. Positions and Honors

Associate Scientist 1, AstraZeneca

B. Selected peer-reviewed publications (in chronological order)

Walden, M., Crow, A., **Nelson, M. D.** and Banfield, M. J. (2013), Intramolecular isopeptide but not internal thioester bonds confer proteolytic and significant thermal stability to the *S. pyogenes* pilus adhesin Spy0125. Proteins. 82(3): 517-527

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College Biology department laboratories is the intended site for the experiments. It has the major equipment listed below and space requirements needed for the methods described. Steps involving DNA sequencing and MALDI-TOF would be performed by an outside contract lab on a per-order basis. The biological requirements are level 0 and do not require regulatory compliance.

Clinical:

N/A

Animal:

N/A

Computer:

The Hood College Biology department laboratories have a computer lab with sufficient data processing capabilities. Specific software programs required will be obtained using open software programs or grant funding.

Office:

N/A

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges
- PCR thermal cycler
- SDS-PAGE containers
- DNA Agarose containers
- Computers with internet connection
- Incubators
- -80degC Freezer
- 2-6 degC refrigerator
- Turbidimeter

The following consumables will be purchased with grant funding:

- Petri dishes
- Media components
- DNA ladders
- Protein ladders
- 2-dimensional gels, solutions for gels
- Various enzymes including DNAse, transposase, Cas9, etc.
- Various solutions such as methanol, naphthalene and o-dianisidine
- Antibiotics for screening assays
- Plasmids
- DNA extraction kits
- Sterile Erlenmeyer Flasks
- *M. capsulatus* Bath colony

SPECIFIC AIMS

1. **Identify a list of novel proteins and genes expressed by the native host when expressing soluble methane monooxygenase (sMMO) and not particulate methane monooxygenase (pMMO) using the copper switch.**

Identifying additional proteins or genes that interact with the expression system, like chaperones, would be the first step to transferring successful expression into a non-native host. Protein and gene expression levels that differ with and without copper would be identified to help differentiate interactions specific to the sMMO operon. In addition to the assembly of sMMO, the copper switch mechanism would be better identified. The differences could be seen at either the protein or genetic level when being compared.

2. **Screen these target proteins and genes for their successful contribution to sMMO activity in the native and non-native host.**

Other bacterial multicomponent monooxygenase (BMM) proteins have recently been expressed successfully in recombinant hosts where specific chaperons and chaperonins were co-expressed in the plasmid which aided in protein expression (Furuya *et al.* 2012; McCarl *et al.* 2018). A similar approach is suggested using a high throughput method of screening for successful recombinant expression where the set of target proteins, from objective one, is introduced into E*scherichia. coli* and the sMMO operon.

A complimentary gene knockout approach in *Methylococcus capsulatus* (Bath) would be taken to understand the impact of the targets on sMMO expression using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 for specific targets.

BACKGROUND AND SIGNIFICANCE

Methane has a greenhouse equivalency of roughly 25 times that of carbon dioxide, and its atmospheric concentration is currently nearing its historic high of 2,000 parts per billion. Overall, methane contributes about 1/3rd of the global warming potential, yet is often overlooked as a target for reducing emissions. Its atmospheric half-life is about 8 years, compared to carbon dioxide's half-life of 100 years. Although not a panacea to rising temperatures, if methane concentrations were to revert to pre-industrial levels, methane's effect on atmospheric GHG potential would be absent within 20 years (Boucher 2010), easily within a person's lifespan.

To achieve pre-industrial concentrations, either methane emissions can be reduced, or methane sinks can be increased. The primary emission sources of methane are natural decompositions such as in wetlands, the ocean, and the lower intestine of ruminants. The latter has increased substantially since cattle domestication. Methane can also be emitted in natural gas deposits, leaking natural gas infrastructure, and, in an alarming amount, thawing permafrost (Stolaroff *et al* 2012). With increasing meat-based diets requiring more ruminant agriculture, and positive climate feedback loops, methane emissions cannot be naturally offset by current methane sinks such as ground absorption, upper atmospheric decomposition, and natural methanotroph populations (Boucher 2010). Reducing

emissions is often a sought-after goal, but would require a shift in culture and economics that, while possible, may not give sufficient time before serious damage is done to the planet's current ecosystem.

Methane is economically valuable, but very costly to purify and compress for industrial use from the atmosphere (Haynes 2014). A natural sink for atmospheric methane is a type of bacteria called methanotrophs, which use methane as their primary source of carbon and energy. The first step of this metabolism uses a category of enzyme called methane monooxidases (MMO) which converts the methane gas into liquid methanol. Methanol is another economically valuable molecule easier to purify and transport than methane. A methanol-based economy has been described where methanol reduces and potentially replaces petroleum for energy (Olah 2005), and a detailed analysis of conversion to additional substrates from methane, such as n-butanol and formaldehyde, has been considered (Haynes, 2014). Additionally, the soluble form of MMO (sMMO) is active at ambient temperatures, and its structure-function relationship has been well characterized (Elango *et al.* 1997; Jae Lee 2016; Lock *et al.* 2017; Wang 2014).

Using the soluble form of methane monooxidase (sMMO) is a sought-after economic goal to the climate change problem. One of the major obstacles towards sMMO industrial scale up is that the protein cannot yet be actively expressed in a recombinant host such as *E. coli*. This expression in *E coli* would allow for a higher harvest yield and the use of glucose instead of methane as the primary carbon and energy feedstock.

PRELIMINARY REPORT / PROGRESS REPORT

There are two main types of MMO in methanotrophs, soluble (sMMO) and particulate (pMMO), both can be expressed in most methanotrophs, the most researched being *Methylococcus capsulatus* (Bath), and *Methylosinus trichosporium* OB3b. Both sMMO and pMMO offer pros and cons to their potential in being industrialized, but overall sMMO is better characterized, has a better chance of being expressed in *E. coli*, and is more amenable to be used in industrial situations due to its soluble nature, whereas pMMO is membrane bound and its active site has only recently been detailed (Ross *et al.* 2019).

To successfully grow a culture of Bath requires a source of methane sparged into a culture over a period of several days before harvesting the MMO (Strong *et al.* 2016; West *et al.* 1992). This can lead to expected blow-off methane not captured and metabolized by the cell, and the inclusion of methane to cell growth is not converted to the target methanol product. When scaled, this process becomes expensive, raises safety concerns, and is restricted by mass transfer of methane to cells (Lawton 2016a). If *E. coli* could be given a recombinant sMMO, glucose would be the primary carbon source, and the bacteria and sMMO would rapidly grow to optimal densities. The recombinant protein would then be collected and purified for storage and use in remote locations with high methane concentrations such as natural gas facilities, wetlands, or even the international space station (Galazka 2017).

Escherichia coli is a well characterized and a common host for expressing recombinant proteins. A large repertoire of *E. coli* strains is available for different recombinant DNA applications, including DNA replication, protein engineering, and

industrial scale up, making them an enviable host system for sMMO. Expressing recombinant proteins into *E. coli* often gives rise to a list of potential difficulties and is best overcome by understanding more of the native expression system for the protein of interest (Rosano 2014).

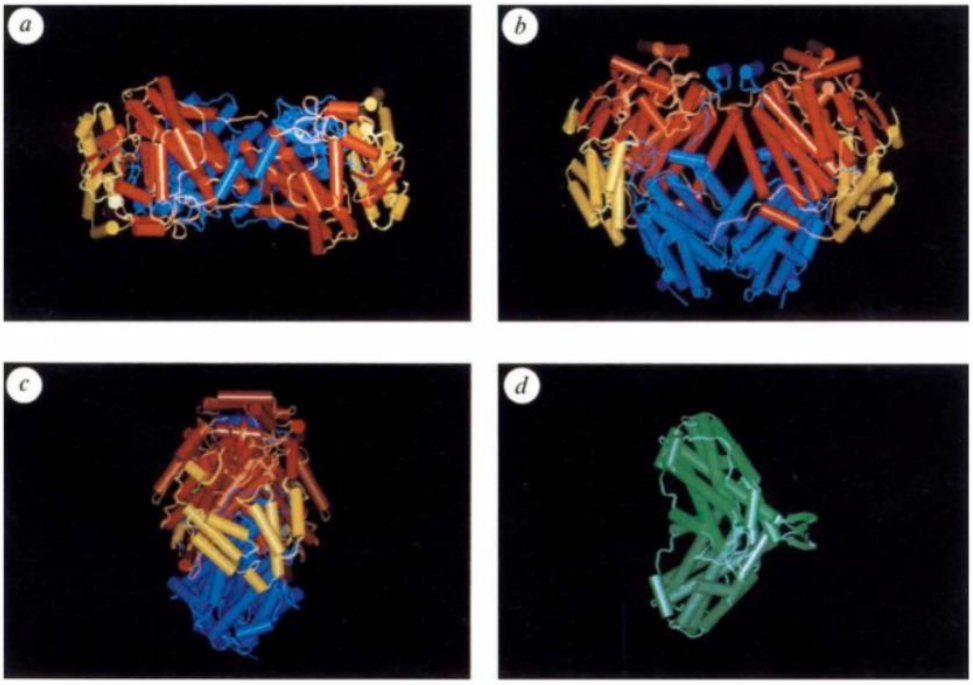

Figure 1. Structure of the hydroxylase (MMOH) protein of sMMO from various angles. Image D, shown in green, is a comparison to ribonucleotide reductase R2 which is also a dimer and has similarities for MMOH (Rosenzweig *et al.* 1993)

sMMO is a combination of three proteins: the hydroxylase (MMOH), a regulatory component (MMOB), and a reductase (MMOR) which contains two diiron active sites. MMOH is a dimer with three subunits $\alpha 2\beta 2\gamma 2$ forming a generally flat molecule with a large canyon intersecting the symmetrical axis (Figure 1). From the side it appears like a heart. The active sites are two non-heme diiron complexes in both of its alpha subunits

(Elango *et al.* 1997). The activity and mechanism of sMMO has been intensively studied and is a complex interaction between the hydroxylase (MMOH), two regulatory components (MMOBs), and a reductase (MMOR). The mechanism is generally understood to require the iron complex to become activated by MMOB, where it transitions through two intermediary phases, and oxidises the methane into methanol, then reduced to its native state by MMOR using NADH and FADH to repeat the process (Jae Lee 2016; Ross 2017).

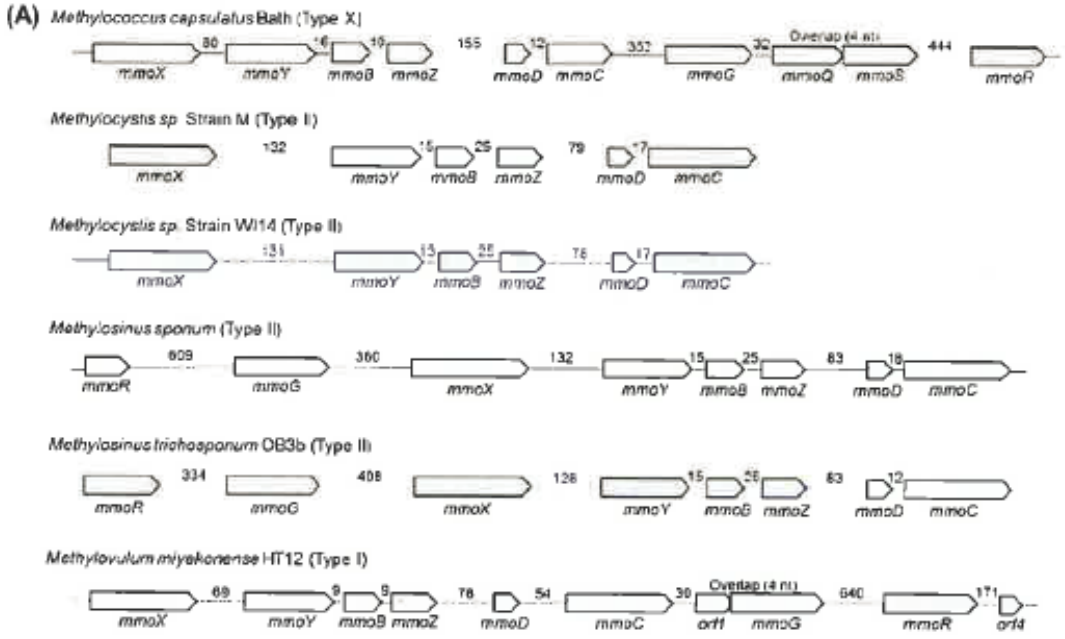

Figure 2. A map of the sMMO operon in various methanotrophs showing similarities to the organization of the subunits and genes (Jae Lee 2016).

The sMMO operon for native sMMO is conserved in sequential order between most sMMO expressing methanotrophs: mmoXYBZDC. The genes involved in expressing sMMO in Bath are in the mmoXYBZDCGQSR operon as shown in Figure 2. MMOH is

encoded by mmoX (alpha subunit), mmoY (beta subunit), and mmoZ (gamma subunit). MMOB is encoded by *mmoC,* and MMOR is encoded by *mmoZ.* So far MMOB and MMOR have been expressed in *E. coli* and shown to retain their activity (West *et al.* 1992). *mmoR* is a sigma54 dependent transcriptonal activator, and *mmoG* is most likely a groEL-like chaperone. Point mutations in either *mmoR* or *mmoG* prevents expression of sMMO (Scanlan *et al.* 2009). As of this report, no success has been found where MMOH is significantly expressed in a recombinant host and has retained its activity.

sMMO is categorized into the bacterial multicomponent monooxygenase (BMM) enzyme family and several features make it unique in its group. The BMM family can oxidize many substrates, with sMMO having the most diversity at roughly 50 substrates. This includes methanol, a smaller enzyme population, where the Q state in the catalytic reaction is unique (Osborne 2019, Fig 2). The N-terminal on MmoB is also not found on other BMMs (Wang 2014).

Other MOs in the BMM family in other methanotrophs have been successfully active when an additional helper protein is expressed (Furuya *et al.* 2013; McCarl *et al.* 2018). Assembly proteins have been found and conserved among many of the methane and butane MOs, such as GroEL-like proteins, like MMOD and PhK. These proteins have been shown to be duplicated into multiple copies in the operon and evolved for potentially specific parts of the MMOH subunits in the BMM family (Osborne 2019). This suggests that a yet unknown helper protein may have co-evolved with the sMMO operon to increase activity and may be unrelated to the theorized horizontal transfer of the proto-operon responsible for both sMMO and pMMO before methane metabolism was acquired (Osborne 2018). sMMO was successfully expressed in pMMO-only expressing

methanotrophs by Lloyd *et al.* (1999), albeit with low expression levels and in only 2 out of 248 strains of methanotroph. Both sMMO and pMMO were expressed concomitantly in these strains, showing that the presence of pMMO does not directly interact with sMMO expression levels.

The metabolism of methane in methanotrophs that have both sMMO and pMMO have what is referred to as the "Copper switch," and it is not yet fully described. A copper activated promoter activates the pMMO expression levels, and downregulates sMMO expression (Prior, 1985; Osborne 2018). Using this 'switch' may be a novel means to observe *in vivo* regulation between sMMO and pMMO without disrupting or altering the sMMO operon, including finding helper proteins that act in concert with the two enzymes.

I believe the recombinant expression problem has since been overlooked in academia due to its more industrial need. Additionally, any research into the problem, especially negative results, has been privatized and not freely open to the public for further study, along with the academic nature of not publishing negative results.

RESEACH DESIGN / METHODS

Aim 1. Discovery of Expression Helping Proteins Using 2D Gel Electrophoresis (Months 00-12)

The proposed 'copper-switch' in methanotrophs is in the control of expression between sMMO and pMMO. Both enzymes have differing structures and mode of activity suggesting that their expression, and potentially folding, requires a different set of helper proteins. The difference in this case would be the low vs. high copper environment where either sMMO (no copper) or pMMO (copper containing) is expressed. Using two cultures of native host, one would be copper restricted and the other would be given copper in its media. They would be confirmed on an SDS-PAGE to contain a significant difference in the amount of sMMO in the cytoplasm.

The two cultures would then be run on a 2-dimensional gel to create a "fingerprint" of each culture's soluble fraction. A separate gel can be run using the cell wall fraction after it is dissolved. The gel images would then be loaded into a computer program that analyzes the images for differences after normalizing the protein spacing from the control lines in the gel. It would also identify differences in abundance of proteins and create an estimate for expression level. When the two sets are compared to each other, target proteins can be identified, those that differ significantly between conditions, for further investigation. A similar approach was conducted on the surface proteins of Bath to find the involvement of multiple c-type cytochromes in the copper regulation pathway (Karlsen *et al.* 2008).

The identified proteins in this gel would be excised from the gel, dissolved, and submitted for mass spectroscopy to specify its mass and sequence. The protein would be compared to a protein BLAST search database, and the nucleotide sequence for the protein would be extrapolated. The resulting nucleotide sequence prediction can also be compared to the transposon interaction experiment results.

These procedures will also be conducted at different levels of copper concentrations in the media, and also by using strains of Bath without sMMO and new strains cultured in subsequent experiments with target genes knocked out. The results from these experiments will help identify targets and give perspective to subsequent targets in a rolling cross-examination.

Aim 2. Identifying genes by random transposon insertional gene inactivation (Months 4-20)

Transposons are sequences of DNA that can move within a genome by use of a transposase protein. They can be used to insert or disrupt genes at random and have previously been used in methanotrophs successfully (Ali 2009). Here the transposon would be allowed to integrate at random into the methanotroph genome in the hopes of disrupting a gene required for sMMO activity and locating a new regulatory pathway.

A transposon with a reporter element such as antibiotic resistance, would be transformed into a culture of methanotroph that is grown in media without copper. The media would also contain methanol to allow for the methanotroph's growth without

methane (Adegbola 2008). The culture would be plated on methanol and antibiotic plates to show successful transformation.

The colony forming units (CFU) would be tested for sMMO activity with both methanol and naphthalene and *o*-dianisidine colorimetric assay as described (Graham *et al.* 1992). The methanol would screen out non-sMMO interrupted genes needed for growth. A non-transformed control group of methanol grown methanotroph would also be tested as a positive control. The CFUs that do not turn red, meaning they are deficient of sMMO, would be analyzed for the site of the transposon insertion.

Additionally, the CFUs that turn red would be analyzed to identify any integration patterns of the transposable element. This would verify the probability of integration location, and that it is truly random or not.

To identify the integration site of the transposon, the genome of the colony would be digested with a DNAse and repaired into circular fragments. These fragments would be transformed into *E. coli* and plated and screened for the antibiotic resistance found on the transposon. The cDNA would be recovered and sent out for sequencing to identify the local genomic DNA. The sequence would be compared to the entire genome of Bath.

This procedure is expected to take time and may give a percentage of already known targets. If no new targets are identified in the first two rounds of selection, a *smmo* negative strain could be used, as described by Smith *et al.* (2002), and given the transposon. A sMMO containing plasmid with an alternative antibiotic marker would be introduced after the transposon transformation and selection. Selecting again for the new antibiotic resistance, the CFUs would be analyzed for sMMO activity. Those that do not show activity would be analyzed as before.

Aim 3. Knockout genes in native Bath using CRISPR/Cas9 (Months 8-22)

To confirm the involvement of the genes of interest identified in sMMO activity, a high throughput targeted knockout approach would be performed. Recently a procedure has been published using the CRISPR/Cas9 system to isolate a knockout *mmox* strain of Bath (Tapscott *et al.* 2019). This involves conjugation of Bath with pCas9, and an *E. coli* strain with pgRNA which contains the modified gRNA and a nonsense template to replace the gene of interest along with an endonuclease restriction site. The gRNA can be modified to the proteins of interest, and successful integration can be confirmed using the endonuclease enzyme and run on an agarose gel, along with screening for sMMO activity.

This system allows for screening a large number of targets found in previous assays, along with additional targets ascertained by further research. It would show direct effect of sMMO activity levels. These strains can also be subjected to 2D-PAGE and analysis, and growth conditions including copper rich environments.

Aim 4. Expressing the proteins of interest alongside sMMO in *E. coli* (Months 12-24)

A series of plasmids would be created for each of the proteins and genes of interest. These plasmids would be introduced alongside the sMMO plasmid containing the sMMO operon.

The identified target genes from Bath would be amplified from the host organism using PCR, run on an agarose gel, dissolved and inserted into a plasmid which would then be transduced into *E. coli,* along with a second plasmid containing the sMMO operon. The colonies of the *E. coli* would be screened using 2 antibiotic resistances, one from both of the plasmids.

The *E. coli* cultures would be introduced to a naphthalene and *o*-dianisidine colorimetric assay as described before (Graham *et al.* 1992). Successful *E. coli* colonies that have functioning sMMO and show a red pigment would be cultured to a larger scale and tested with methane metabolism as both a full cell, then and after lysing to see enzyme complex functionality in the supernatant. The supernatant would also be analyzed using SDS-PAGE.

Further study on the sMMO enzyme complex could proceed and the new protein(s) could be characterized. Comparing the sMMO complex and MMOH expression would be studied with the new genes of interest.

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