4-26-2020

Understanding Chemolithotrophic Reduction Mechanisms from the Dark Marine Biosphere

Anna Gwendolyn Blair
University of Dayton

Understanding Chemolithotrophic Reduction Mechanisms from the Dark Marine Biosphere

Honors Thesis

Anna Gwendolyn Blair

Department: Chemistry

Advisor: Justin Biffinger, Ph.D.

April 2020

Understanding Chemolithotrophic Reduction Mechanisms from the Dark Marine Biosphere

Honors Thesis

Anna Gwendolyn Blair

Department: Chemistry

Advisor: Justin Biffinger, Ph.D.

April 2020

Abstract

As greenhouse gas emissions contribute to global warming and an increase in CO_2 concentration in the earth's atmosphere, the scientific community is under pressure not just to examine new technologies to reduce emissions, but also to consider the effect that increased CO_2 concentration has on our terrestrial and marine ecosystems. In marine ecosystems, atmospheric CO_2 dissolves and reacts with water to form carbonic acid. This diprotic acid then dissociates, contributing to a lowered pH of ocean water and affecting all levels of marine life. Fortunately, nature already has carbonate reduction mechanisms in place that can reduce the harmful effects of ocean acidification. Being able to identify individual bacteria in biological carbonate-fixing consortia can lead to adaptive systems engineered around biofilms. In this study, environmental samples taken from defined sites the dark marine biosphere (ocean depth of 2100-2300 meters) in the Gulf of Mexico will be grown in a number of selective medias with defined carbonate contents. Bacterial samples will then be analyzed using ion chromatography to measure carbonate consumption as a function of time. The data collected thus far suggests that by selectively pressuring environmental consortia from the dark marine biosphere toward the purpose of fixing carbon, mechanisms and pathways can be generated to control the level of CO_2 in the marine environment.

Acknowledgements

I would like to thank Dr. Justin Biffinger for his mentorship throughout this project, the University Honors Program for funding and support, and the Department of Chemistry, College of Arts and Sciences – Dean's Summer Fellowship, and DARPA for funding for this project.

University *of* Dayton

Table of Contents

Table of Figures

Table of Tables

Introduction

The dark marine biosphere (DMB) is an environment that is permanently separated from light-driven energy production mechanisms. Survival in the DMB defies the normal rules for life: no light, extremes in temperature, oxygen limitation, and sparse sources of energy with ever increasing concentrations of carbon dioxide (CO_2). Despite these extremes, ~70% of the Earth's microorganisms live in and are adapted to generate cellular energy in the sub-seafloor sediments [1], yet only ~5% of the sea floor has been explored [2]. The DMB covers more than two-thirds of the Earth's area and is known as the most isolated region of the largest CO_2 sink [3]. This biosphere has the largest capacity to absorb and convert CO_2 based on not just surface area but also passive and active pumping mechanisms from microbial activity in the sediment and surrounding water. Thus, the microbiome (all microorganisms, their genetic elements and their abiotic interactions) of the DMB is actively adapting to ever increasing concentrations of CO_2 and must adjust to this environment for survival. Therefore, this biosphere is the greatest untapped resource for the discovery of new carbon fixation pathways and could provide evidence for how some of the most untouched areas on earth are responding to global climate change.

Synthetic biology is used as a biotechnological technique to create solutions for novel biological agents, intermediates and even fuels. However, many of these genetic pathways are transposed from the target organisms to an easily mutable model; but this is the inherent limitation to all synthetic biology approaches. Our ability to predict and model pathways better than the organisms themselves is still in its infancy. Thus, large advancements to the synthetic biology field will be contained in discovering new microorganisms and identifying the metabolic mechanisms and key enzymes they manipulate to survive. The DMB is currently the major geological sink for inorganic carbon and organisms from this extreme environment must adapt by utilizing novel metabolic pathways for survival. This untapped resource of unique microbes will lead to transformative biotechnological processes superior to current system biology approaches and models for carbon fixation.

Despite the potential discoveries in the DMB that could lead to carbon sequestration technologies, the majority of bioenergy studies remain focused on

harnessing the process and byproducts (*e.g.*, cellulosic biomass) of oxygenic photosynthetic microbial carbon fixation. Photosynthesis is highly evolved but restricted by numerous limiting factors (nutrients, oxidizing conditions, light limitation/inhibition, 2D geometries, and defined enzyme systems). Given the variability and austerity of the DMB, the microbiome and metabolic potential in the DMB has evolved in a 3D space. Unlike current photosynthetic biological systems (such as algae) which are limited by unfavorable energy balances or water limitation, the DMB can offer highly efficient dark carbon fixation pathways which can lead to revolutionary technologies.

This program used selective electron donor/acceptor pressure in anaerobic marine media to stimulate consortia from DMB marine sediments by coupling growth conditions that would stimulate anammox (anaerobic oxidation of ammonium) with dark carbonate fixation (reduction of carbonate) (Figure 1). In saltwater, the soluble form of CO_2 is ultimately carbonate and is governed by solubility constants, acidity, and the temperature primarily [4, 5] so carbonate conversion was the principal focus of this work. Anammox is a strategy for ammonia removal from wastewater under anoxic conditions and could be part of the natural cycling of ammonia in the anaerobic DMB. Anammox is carried out by several bacterial species that have yet to be classified taxonomically [6, 7].

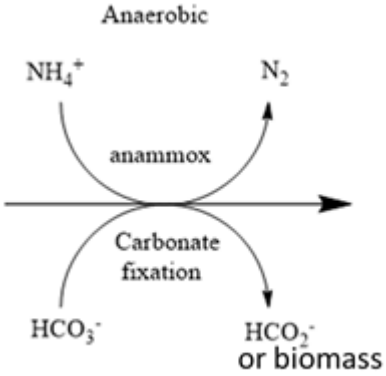

Figure 1 *Schematic for proposed conditions to stimulate consortia suitable for dark carbon fixation from environmental sediment samples.*

The goal of this project is to isolate, contour, identify, and categorize novel microbial consortia from the DMB capable of concentrating and fixing inorganic carbon in seawater from 4 to 25°C. The isolation of consortia and single strains that could contribute to unprecedented rates of CO_2 fixation would be a major advancement toward applicability in clean coal and biotechnology solutions to CO_2 reduction.

Methodology

This project was carried out in 3 phases. **Phase 1** entailed the acquisition and initial culturing of environmental sediment samples from the DMB. Samples were collected on board R/V *Point Sur* during a research expedition which took place June 11-16, 2017 (PS 17-27) Surface sediment samples were collected from three deep sites during this research expedition. Sediment samples were sliced on board R/V *Point Sur* after collection at 2 cm intervals and stored in a 15 mL falcon tube for future studies. Thirty total sediment slices from the three sites were collected.

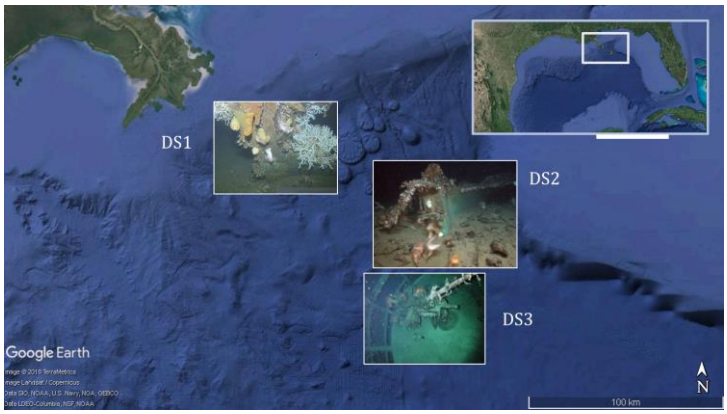

Figure 2 *Location of deep site samples used in Phase 1*

Sediment cores are stored in 2cm increments (0-2cm depth, 2-4cm depth, etc.) at 4℃ (the approximate temperature of the samples' natural environment) and are cultured anaerobically in a number of minimal media containing a vitamin solution and cocktail of electron acceptors at both 4℃ and 25℃. Sediment is cultured in duplicate, with one of each duplicate kept in the dark.

We suggested that coupling anammox and carbonate fixation would be a path to stimulate and isolate dark carbonate fixing anaerobic microorganisms from the sediment samples. The high throughput growth experiments were performed in selected media formulations (Table 1) using ammonium and carbonate as the core carbon and nitrogen source. All three solutions used to make the modified ONR7A medium were autoclaved (121°C, 30 min) separately and added after they had cooled to room temperature. Six samples (Labeled DS1, DS2, and DS3 of native sediment and DS1, DS2, and DS3 sediment exposed to marine broth (BD 2216)) were suspended in a modified formulation

of ONR7A. The modified medium had a salinity of 35% and is consistent with the salinity of saltwater. Since this medium had many of the common electron acceptors removed from it, the salinity of the base salt formulation was maintained with sodium and potassium chloride salts. Growth was confirmed in these experiments using the change in optical density at 600 nm; a common indicator of change in turbidity and cellular growth. These microbial isolates were cultured on agar plates formulated from the same saltwater medium that showed growth of the consortium. The original cultures were diluted by 10-fold until single colonies could be isolated from agar plates.

Solution 1	**Quantity added**	**Units**	**Formula Weight**	**Concentration (mM)**
NaCl	22.79	g	58.44	389.97
KCl	0.72	g	74.55	9.66
NaBr	0.083	g	102.89	0.81
H_3BO_3	0.027	g	61.83	0.44
NaF	0.0026	g	41.99	0.06
$Na_2HPO_4 \cdot 7\ H_2O$	0.0890	g	268.07	0.33
TAPSO	1.30	g	259.28	5.01
H_2O	500.00	ml		
Adjusted to pH 7.6 with NaOH				
Solution 2	Quantity added	Units	Formula Weight	Concentration (mM)
$MgCl_2 \cdot 6\ H_2O$	1.0165	g	203.3	5.00
$CaCl_2 \cdot 2\ H_2O$	0.1830	g	147.01	0.90
$SrCl_2 \cdot 6\ H_2O$	0.0183	g	266.62	0.09
NaCl	7.30	g	58.44	125.00
KCl	0.2386	g	74.55	3.20
H_2O	450.00	ml		
Solution 3	Quantity added	Units	Formula Weight	Concentration (mM)
$FeCl_2 \cdot 4\ H_2O$	0.0020	g	198.81	0.01
H_2O	50.00	ml		

Table 1 *Salt composition of the three solutions used to formulate Modified ONR7A growth media.*

Phase 2 of the project integrated quantitative analytical techniques using ion chromatography (IC). The CO_2 concentration and rate of fixation based on carbonate consumption and the resulting reduced products was analyzed in parallel with nitrate and sulfate reduction using a Dionex ICS5000 designed specifically for the analysis of carbonate and bicarbonate in saltwater.

We developed ion chromatographic methods (with conductivity detection) to analyze the changes in bicarbonate and acetate concentration from a total of 650 saltwater medium growth samples from our growth experiments. All ion chromatography samples from growth experiments and controls were centrifuged (5 min, 13k rpm) and passed through a 0.2 µm polycarbonate syringe filter. All samples were injected twice, and the average concentrations of the selected ions were calculated from calibration curves of samples with a known concentration of the ion of interest. The injection volumes were 25 µL for carbonate/acetate analysis and 10 µL for anion analysis. The use of ion exclusion chromatography was necessary because the bulk salts from the saltwater are eluted with the solvent front allowing for organic acids like carbonate and acetate to be analyzed directly.

In **Phase 3** of the project, metagenomic data was gathered from the growth experiments. Metagenomic DNA was collected and sequenced to help better characterize the consortia dynamics that influence carbonate fixation occurring in the native samples.

Results and Data Analysis

Successful growth experiments from Phase 1 were used for ion chromatography to detect carbon fixation. A representative chromatograph from the separation of carbonate from the same saltwater sample over 15 days is shown in Figure 3. This experiment confirmed that not only the sampling method was stable but also that residual atmospheric carbon dioxide was not entering the sampling vials and leading to inconsistent results. Acetate had a retention time of 12.3 minutes and this method resulted in the calculation of both acetate and carbonate concentration changes from a single injection (Figure 4).

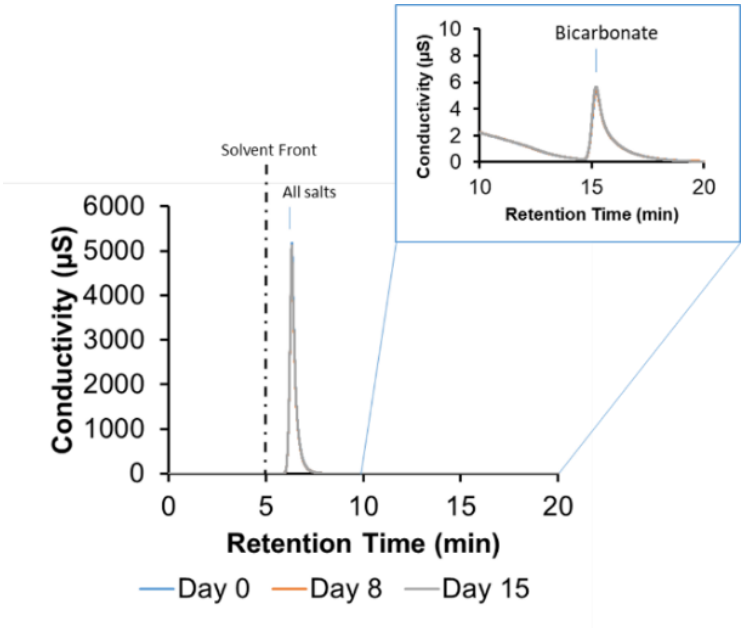

Figure 3 *Ion Chromatography results from the injection of 1 mM sodium bicarbonate in defined ONR7A growth medium.*

Figure 14 footnote: Chromatography Conditions; Temperature = 30°C, Flow Rate: 0.8 mL/min; Mobile phase: 1mM HNO_3; Ion Suppression: 5mM Tetrabutylammonium hydroxide with ACRS-ICE 500 suppressor; Column: Ionpac ICE-AS1 (9x250mm). Injection Volume: 25 µL

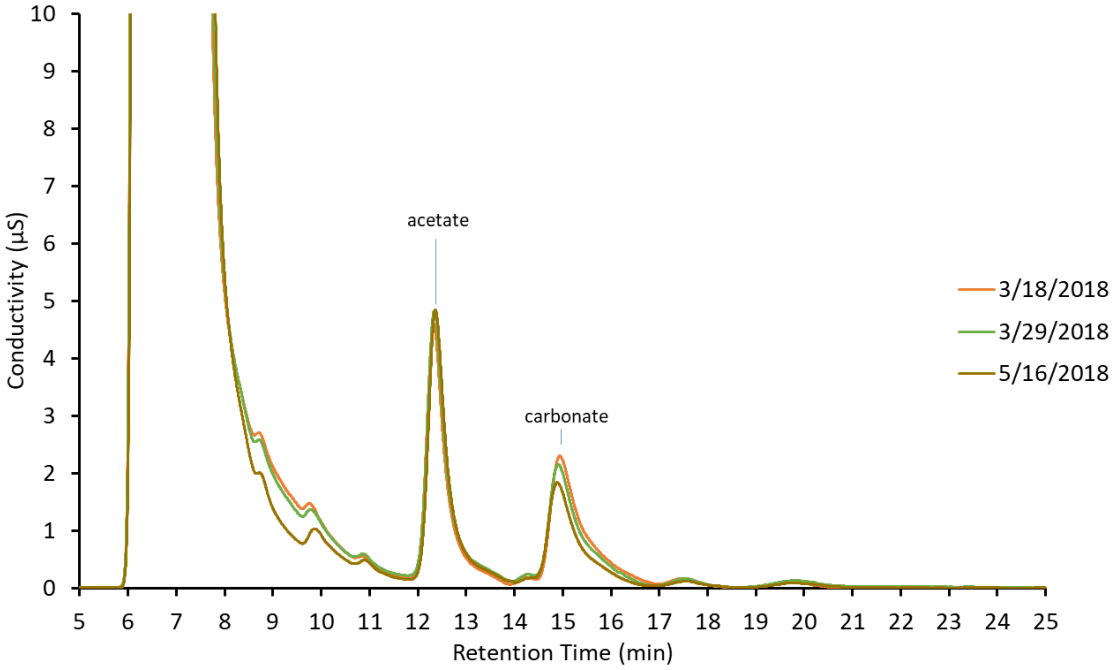

Figure 4 *Representative ion chromatography data of modified ONR7A growth medium from DS3 (Room Temperature) growth sample over 35 days.*

Figure 15 footnote: Chromatography Conditions; Temperature = 30°C, Flow Rate: 0.8 mL/min; Mobile phase: 1mM HNO_3; Ion Suppression: 5mM Tetrabutylammonium hydroxide with ACRS-ICE 500 suppressor; Column: Ionpac ICE-AS1 (9x250mm). Injection Volume: 25 µL

The total volume removed in the original samples for ion chromatography was 1 mL. This sample size only allowed for a maximum of 5-10 injections to collect the required data for the program. Thus, we calculated the relative uncertainty in our analysis of carbonate to determine if we were observing a statistical decrease in the concentration of carbonate over time using 2 injections. Also, carbonate was used in two different concentration ranges (10 mM and below 0.7 mM) from the growth experiments. The results from this analysis are shown in Table 2. Our data concluded we could predict if there was a decrease in carbonate concentration by 3% with cultures using a carbonate concentration of 9 mM and 4.7% for cultures with a concentration of 0.7 mM. The difference in the concentration change of carbonate needs to be >3.0% to be 95% confident that the sample concentration is lower than another sample using the Student's t-test assuming equal variances. This also confirmed our method was accurate enough to screen for small changes in carbonate concentrations over time.

	High Concentration (~9 mM)	Low Concentration (~0.7 mM)
Average	9.030 mM	0.6754 mM
n	5	5
Standard Deviation	0.275	0.0328
Confidence Interval (95%)	0.341	0.0407
% Relative Uncertainty	3.0%	4.7%

Table 2 *Calculation of relative uncertainty from 5 identical 25 µL injections of carbonate containing salt water using two different concentration ranges.*

With this quantitative analysis completed, we then processed 450 samples generated in previous growth experiments. These samples were injected twice and the averages and standard deviations in the concentration of carbonate and acetate were used to develop a predictive model for growth conditions resulting in carbonate fixation. We used any decrease in concentration, significant at a 95% level, to define if the sample was a potential consortium for carbonate fixation. The result was a table with the locations and growth conditions for the sample and designation for if the sample was a candidate

for organisms that could fix carbonate under our defined conditions. Samples indicated in green in the table were viable candidates that could fix carbonate under our defined conditions and were used in future experiments.

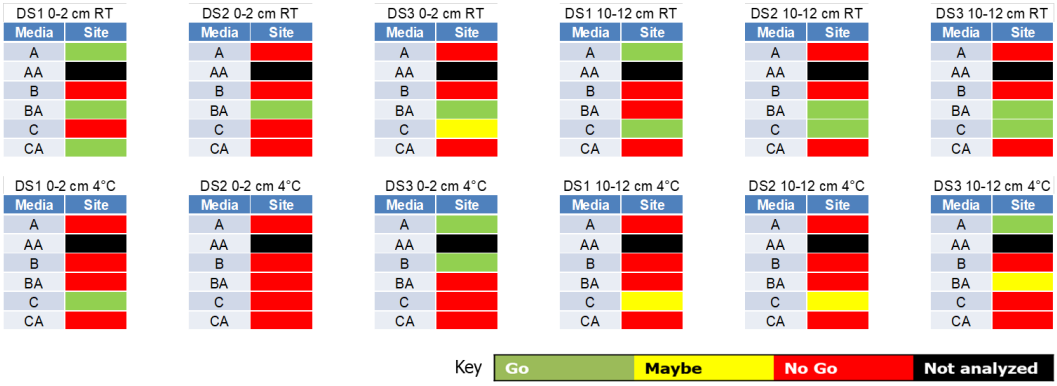

Figure 5 *Summary of Go/No-Go with different cultures and media conditions.*

DNA from three native samples (DS1, DS2 and DS3; 0-2 cm sediment slices) was isolated and sent to Genewiz (NJ, USA) for metagenomic analysis (Figure 6). DS2 was unique with 21% of the microbes unclassified at the genus level. The diversity of the samples is seen with the large 'other' category ranging from 44-53% of the total metagenome.

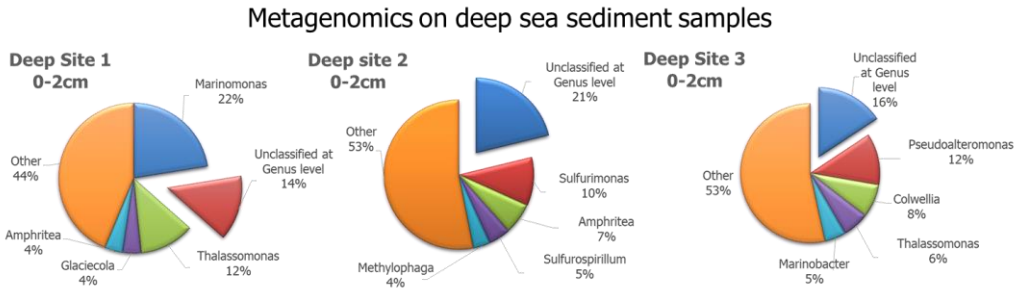

Figure 6 *Metagenomics on select deep sea sediment samples.*

Instead of looking at the microbial community, we narrowed our studies down to one microbe from DS3 which is a 99.87% match to *Marinobacter hydrocarbonoclasticus* based on 16S rRNA match. In order to sequence the whole genome, we cultured the isolate on MB agar, grew up a colony in MB broth and then extracted the genomic DNA (Figure 46). The purified DNA was sent to Seqmatic for whole genome sequencing.

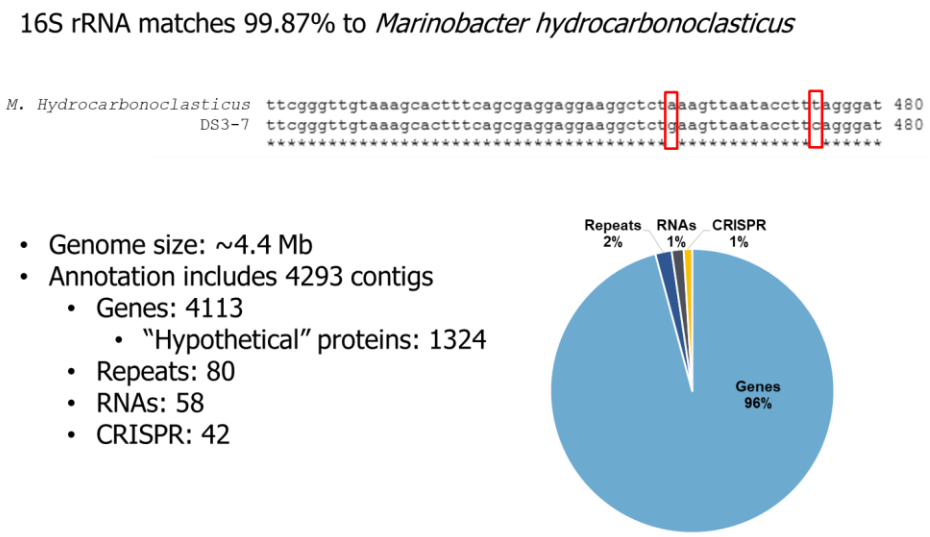

Figure 7 *Summary of Marinobacter hydrocarbonoclasticus* whole genome sequence.

It was important to determine the pathway this microbe is using to sequester carbon. In Tables 3 through 8, each of the six known carbon fixation pathways are shown. The genes written in black are genes that 'match' our *marinobacter* isolate and are present in the carbon fixation pathway. The genes written in red are absent from our *marinobacter* isolate, but present in the known carbon fixation pathway. After looking at all the genes identified in the six known carbon fixation pathways, it became clear this microbe is using an unknown mechanism to sequester carbon. There was not one pathway that had all the genes encoded within the whole *marinobacter* genome.

Reductive Calvin-Benson cycle (rCB)

Gene	Protein
facA	Acetyl-coenzyme A synthetase
fbaB	Fructose-bisphosphate aldolase class I
fbaA	Fructose-bisphosphate aldolase class II
gapA	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase
maeB	NADP-dependent malic enzyme
pckA	Phosphoenolpyruvate carboxykinase (ATP)
pgk	Phosphoglycerate kinase
prkB	Phosphoribulokinase
ppdK	Pyruvate phosphate dikinase
rpiA	Ribulose 5-phosphate isomerase A
rpe	Ribulose-phosphate 3-epimerase
sucC	Succinyl-CoA ligase (ADP-forming) beta chain
tktA	Transketolase
tpiA	Triosephosphate isomerase
xfp	Xylulose-5-phosphate phosphoketolase
rbcL	Ribulose-bisphosphate carboxylase large chain
rbcS	Ribulose-bisphosphate carboxylase small chain
rpiB	Ribose 5-phosphate isomerase B
yggF	Fructose-1,6-bisphosphatase I
glpX	Fructose-1,6-bisphosphatase II

Table 3 *Genes involved in the reductive Calvin-Benson cycle.*

Reductive acetyl-CoA cycle (rAC)

Gene	Protein
acnA	Aconitate hydratase
acnB	Aconitate hydratase 2
fumA	Fumarate hydratase class I, aerobic
fumC	Fumarate hydratase class II
folD	Methylenetetrahydrofolate dehydrogenase (NADP+)
metF	Methylenetetrahydrofolate reductase
ppc	Phosphoenolpyruvate carboxylase
ppsA	Phosphoenolpyruvate synthase
ppdK	Pyruvate phosphate dikinase
pfo	Pyruvate-flavodoxin oxidoreductase
sucD	Succinyl-CoA ligase (ADP-forming) alpha chain
mdh	Malate dehydrogenase
cdhD	Acetyl-coA decarbonylase/synthase complex, subunit delta
cdhE	Acetyl-coA decarbonylase/synthase complex, subunit gamma
cooS	Carbon-monoxide dehydrogenase catalytic subunit
fhs	Formate-tetrahydrofolate ligase
pycA	Pyruvate carboxylase, subunit A
pycB	Pyruvate carboxylase, subunit B
coxS	Carbon-monoxide dehydrogenase, small subunit
coxM	Carbon-monoxide dehydrogenase, medium subunit
coxL	Carbon-monoxide dehydrogenase, large subunit
acsB	Acetyl-CoA synthase, subunit alpha

Table 4 *Genes involved in the reductive acetyl-CoA cycle.*

Reverse tricarboxylic acid cycle (rTCA)

Gene	Protein
acnA	Aconitate hydratase
acnB	Aconitate hydratase 2
fumA	Fumarate hydratase class I, aerobic
fumC	Fumarate hydratase class II
icd	Isocitrate dehydrogenase
ppc	Phosphoenolpyruvate carboxylase
ppsA	Phosphoenolpyruvate synthase
ppdK	Pyruvate phosphate dikinase
pfo	Pyruvate-flavodoxin oxidoreductase
sdhC	Succinate dehydrogenase cytochrome b-556 subunit
sdhA	Succinate dehydrogenase flavoprotein subunit
sdhD	Succinate dehydrogenase hydrophobic membrane anchor protein
sdhB	Succinate dehydrogenase iron-sulfur protein
mdh	Malate dehydrogenase
porA	Pyruvate ferredoxin oxidoreductase, alpha subunit
porB	Pyruvate ferredoxin oxidoreductase, beta subunit
porD	Pyruvate ferredoxin oxidoreductase, delta subunit
porG	Pyruvate ferredoxin oxidoreductase, gamma subunit
korA	2-oxoglutarate ferredoxin oxidoreductase, alpha subunit
korB	2-oxoglutarate ferredoxin oxidoreductase, beta subunit
korD	2-oxoglutarate ferredoxin oxidoreductase, delta subunit
korC	2-oxoglutarate ferredoxin oxidoreductase, gamma subunit
frdA	Fumarate reductase flavoprotein subunit
frdB	Fumarate reductase iron-sulfur subunit
acly	ATP citrate (pro-S)-lyase
pycA	Pyruvate carboxylase, subunit A
pycB	Pyruvate carboxylase, subunit B

Table 5 *Genes involved in the reverse tricarboxylic acid cycle.*

3-hydroxypropionate bicycle (3HP)

Gene	Protein
accA	Acetyl-coenzyme A carboxyl transferase alpha chain
accD	Acetyl-coenzyme A carboxyl transferase beta chain
accB	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
accC	Biotin carboxylase of acetyl-CoA carboxylase
fumC	Fumarate hydratase class II
sdhC	Succinate dehydrogenase cytochrome b-556 subunit
sdhA	Succinate dehydrogenase flavoprotein subunit
sdhB	Succinate dehydrogenase iron-sulfur protein
mdh	Malate dehydrogenase
mcmA1	Methylmalonyl-CoA mutase, N-terminal domain
mcmA2	Methylmalonyl-CoA mutase, C-terminal domain
pccA	Propionyl-CoA carboxylase, alpha chain
pccB	Propionyl-CoA carboxylase, beta chain
mce	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase
meh	Mesaconyl-C4 CoA hydratase

Table 6 *Genes involved in the 3-hydroxypropionate bicycle.*

3-hydroxypropionate/4-hydroxybutyrate cycle (3HP-4HB)

Gene	Protein
atoB	Acetyl-CoA acetyltransferase
mcmA1	Methylmalonyl-CoA mutase, N-terminal domain
mcmA2	Methylmalonyl-CoA mutase, C-terminal domain
pccB	Propionyl-CoA carboxylase, beta chain
acrC	Acryloyl-CoA reductase
	3-hydroxypropionyl-CoA dehydratase
mce	Methylmalonyl CoA epimerase
	Malonic semialdehyde reductase
	4-hydroxybutyrate-CoA ligase
	3-hydroxypropionate-CoA ligase
	Acetoacetyl-CoA β-ketothiolase
	Succinyl/malonyl-CoA reductase
crt	Crotonyl-CoA hydratase
abfD	4-hydroxybutyryl-CoA dehydratase
	Succinic semialdehyde reductase

Table 7 *Genes involved in the 3-hydroxypropionate/4-hydroxybutyrate cycle*

Dicarboxylate/4-hydroxybutyrate cycle (D-HB)	
Gene	**Protein**
atoB	Acetyl-CoA acetyltransferase
fumA	Fumarate hydratase class I, aerobic
ppc	Phosphoenolpyruvate carboxylase
ppsA	Phosphoenolpyruvate synthase
sdhA	Succinate dehydrogenase flavoprotein subunit
sucD	Succinyl-CoA ligase (ADP-forming) alpha chain
sucC	Succinyl-CoA ligase (ADP-forming) beta chain
mdh	Malate dehydrogenase
porA	Pyruvate ferredoxin oxidoreductase, alpha subunit
porB	Pyruvate ferredoxin oxidoreductase, beta subunit
porD	Pyruvate ferredoxin oxidoreductase, delta subunit
porG	Pyruvate ferredoxin oxidoreductase, gamma subunit

Table 8 *Genes involved in the dicarboxylate/4-hydroxybutyrate cycle.*

Discussion and Conclusion

Sediment samples isolated from the DMB were successfully cultured in selective media and ion chromatography shows that some consortia were capable of fixing carbon. Using optimal growth conditions resulted in the growth of a microbe capable of sequestering carbonate from media. This microbe was identified as a *Marinobacter hydrocarbonoclasticus* with 16S sequence analysis. Whole genome sequencing was used to determine that the microbe does not contain a complete set of genes typically observed in published dark carbon fixation pathways. Future studies are required to determine the pathway this microbe is using to sequester carbonate. This microbe and the results from this program will lead to the discovery of a new, potentially energetically favorable, dark carbon fixation pathway which can be used downstream in synthetic biology techniques which will lead to transformative biotechnological processes superior to current system biology approaches and models for carbon fixation.

References

1. Edwards, K.J., K. Becker, and F. Colwell, *The Deep, Dark Energy Biosphere: Intraterrestrial Life on Earth*, in *Annual Review of Earth and Planetary Sciences, Vol 40*, R. Jeanloz, Editor. 2012. p. 551-568.
2. Orcutt, B.N., et al., *Microbial activity in the marine deep biosphere: progress and prospects.* Frontiers in Microbiology, 2013. **4**.
3. Parkes, R.J., et al., *A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere:geosphere interactions.* Marine Geology, 2014. **352**: p. 409-425.
4. Caumon, M.C., et al., *Experimental mutual solubilities of CO2 and H2O in pure water and NaCl solutions*, in *13th International Conference on Greenhouse Gas Control Technologies, Ghgt-13*, T. Dixon, L. Laloui, and S. Twinning, Editors. 2017. p. 4851-4856.
5. Lohrenz, S.E., et al., *Satellite estimation of coastal pCO(2) and air-sea flux of carbon dioxide in the northern Gulf of Mexico.* Remote Sensing of Environment, 2018. **207**: p. 71-83.
6. Kuenen, J.G., *Anammox bacteria: from discovery to application.* Nature Reviews Microbiology, 2008. **6**(4): p. 320-326.
7. Suneethi, S. and K. Joseph, *Batch culture enrichment of ANAMMOX populations from anaerobic and aerobic seed cultures.* Bioresource Technology, 2011. **102**(2): p. 585-591.