Isolation of "Vadin CA 11", an Uncharacterized Methanogen of the Class Thermoplasmata From an Anaerobic Wastewater Treatment Reactor

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Introduction

Wastewater treatment plants account for approximately 3 percent of the United States' energy demand[1]. Conventional wastewater treatment plants use an activated sludge process in which oxygen is distributed through the wastewater via pumps. A potential energy-positive alternative is anaerobic wastewater treatment, which generates methane-rich biogas while producing less biosolids.

In order to understand how to design an AnWT that is energy positive, the biological reactors in the system need to be understood. At two AnWT in Colorado, namely Plum Creek and Mines Park, there are established microbial communities that consist of numerous anaerobic microbes, including methanogens. Methanogens are archaea that thrive in anaerobic conditions. They produce methane by reducing carbon dioxide in the presence of hydrogen or by fermenting acetate.

Vadin CA 11 is an uncharacterized methanogen of the Thermoplasmata family that could represent up to 7% of the microbial community. Based on its relatives, it is expected to metabolize methanol in the presence of hydrogen. Vadin CA 11's abundance is different in the four reactor compartments in the Mines Park AnWT. Further, Vadin CA 11's presence has been observed to change with temperature. The goal of this study was to isolate Vadin CA 11 from the Mines Park AnWT. From there, physiologies studies can be performed.

Isolation Process

Anaerobic samples were collected from compartments 2 and 3 of the 4-compartment Mines Park AnWT. These compartments were chosen based on their high relative abundance of Vadin CA 11 in past genome sequences. These samples were maintained anaerobic by using a Sheldon Manufacturing, Inc. Bactron X Anaerobic/ Environmental Chamber (**Figure 1)**, gas-tight serum bottles, anaerobic water, and nitrogen gas.

Enrichment cultures were prepared from both compartment 2 and 3 samples in serum vials. A growth medium was made that contained 10 mL filtered wastewater from their respective compartments, 125 uL bicarbonate, 150 uL selenite-tungstate solution[3] (0.5 g NaOH, 3.0 mg Na_2SeO_3 x 5 H_2O, 4.0 mg Na_2WO_4 x 2 H_2O in 100 mL of anaerobic water), 50 uL methanol-antibiotic solution, and an atmosphere of 1-2 psi carbon dioxide and 3-5 psi of hydrogen. 50 uL of centrifuged biomass from the compartments was then added to serum vials in a 5-log serial dilution. The goal of serial dilution was to dilute out other hydrogen- and methanol-consuming microbes except for Vadin CA 11. The vials were placed in an incubator at 30 degrees Celsius. The enrichment culture was sub-cultured as needed to ensure microbes had sufficient nutrients and to continue eliminating other methanogens. Sub-culture nutrient media was identical to that used in the enrichment culture except a nutrient, vitamin, and coenzyme M solution was substituted for AnWT filtrate.

The generation of methane in the headspace of the serum bottles indicates methanogenic activity. To determine if the cultures contained active methanogens, analysis of the headspace in the enrichment cultures was performed using gas chromatography mass spectrometry (GS-MS) (Hewlett Packard 6890 with Agilent 5973 Mass Selective Detector). Results are shown in **Figure 2.**

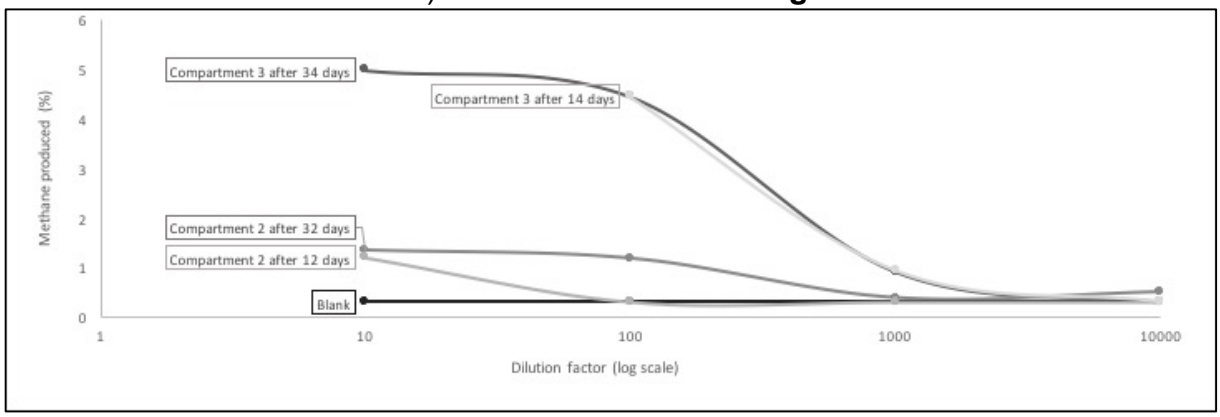

Figure 2: Development of methane in headspace of compartment 2 and 3 enrichment cultures at different dilutions.

There is more methanogenic activity in compartment 3 than compartment 2. This is consistent with weekly methane trends in the headspace of the reactor compartments. In this scenario, the microbial community in compartment 2 developed more slowly. Samples that produced methane were used to seed anaerobic, sterile roll tubes containing 0.135 g agar, 0.1 mL bicarbonate, 1-3 psi of carbon dioxide, 3-5 psi hydrogen gas, 0.03 mL macro nutrients, 0.1 mL trace element solution SL-10[4] (10.0 mL HCl (25%; 7.7 M), 1.50 g $FeCl_2$ x 4 H_2O, 70.0 mg $ZnCl_2$, 100.0 mg $MnCl_2$ x 4 H_2O, H_3BO_3, $CoCl_2$ x 6 H_2O, $CuCl_2$ x 2 H_2O, $NiCl_2$ x 6 H_2O, Na_2MoO_4 x 2 H_2O, and 100 mL anaerobic water), 0.1 mL vitamin ATCC 141[5] (2.00 mg biotin, 2.00 mg folic acid, 10.00 mg pyridoxine-HCl, 5.00 mg thiamine-HCl x 2 H_2O, 5.00 mg nicotinic acid, 5.00 mg D-Ca-pantothenate, 0.10 mg vitamin B_{12}, 5.00 mg p-Aminobenzoic acid, and 5.00 mg lipoic acid in 1000 mL anaerobic water), 0.1 mL methanol-antibiotic solution, 0.05 mL sodium sulphide, 0.1 mL co-enzyme M, 0.1 mL selenium-tungstate solution, 0.1 mL L-cysteine, 1-drop resazurin, and 0.1 mL sodium chloride. Microbial growth was observed in roll tubes and a variety of colonies were selected for further cultivation (**Figure 3**).

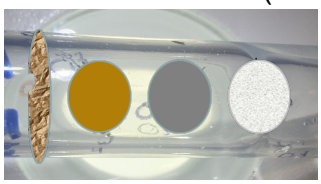

Figure 3: Colony morphology seen in roll tubes.

To verify that the samples taken contain the microorganism of interest, DNA was extracted from colonies growing in the roll tubes according to the DNeasy PowerLyzer PowerSoil Kit protocol[6]. Quantitative polymerase chain reaction of the sample DNA was performed in the Light cycler 480 II (Roche) using the Zymofemto bcc template with modifications (modifications include changing annealing temperature to 61 degrees Celsius, extension time to 30 seconds, analysis mode to include melt curve, and acquisition mode to continuous). The primers used were a de novo primers ‘Vadin CA 11R’ (5’-ACC GTC GGA TCC GTT CTA GC-3’) and a newly and 'Vadin CA 11F’ (5’-AGT CTA AAA CGT TCG TAG CCG G-3’). Analysis was done to determine the concentration of DNA in the samples (using Qubit 2.0 Fluorometer from Invitrogen).

Results

Vadin CA 11 is likely present due to the fact that the samples amplified between the positive and negative control (**Figure 4)**. The farthest left blue curve shows a culture of compartment 3 diluted 1000 fold. It has the lowest biomass to crossing point ratio and thus has a higher abundance of the amplicon than other samples. However, there is a potential for false positives.

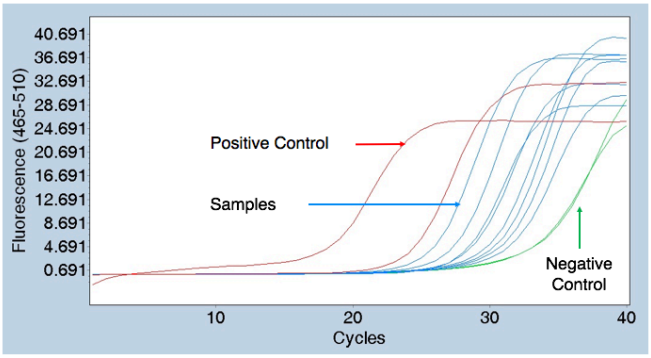

Figure 4- qPCR results for cultures of different compartments and dilutions.

Future Work

Standards will be made for qPCR to quantify the number of copies of amplicon per DNA sample. Roll tubes and subcultures will be repeated until a pure culture is obtained. High possibility Vadin CA 11 DNA samples will be sent off for Sanger sequencing to determine if they are a pure culture. Once a pure culture is obtained, physiology studies will be performed. This knowledge can inform decisions that impact the AnWt microbial community and the overall success of AnWt.

References

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[6]Bio-Rad. (n.d.). 2.3 DNA Preparation From Bacteria. In *Instruction Manual, InstaGene Matrix* (p. 4). Bio-Rad. Retrieved from http://www.bio-rad.com/en-us/product/instagene-matrix