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Evaluation of archeal growth on complex media and antibiotic regimes

Melanie Miller
University of Northern Iowa

EVALUATION OF ARCHEAL GROWTH ON COMPLEX MEDIA AND ANTIBIOTIC REGIMES

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Melanie Miller

University of Northern Iowa

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Date	Marek K. Sliwinski, Honors Thesis Advisor, Biology Department
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Date	Dr. Jessica Moon, Director, University Honors Program

I. Introduction

The first Archaea that were studied by scientists came from extreme environments such as fissures at the bottom of oceans where hydrothermal vents formed. It was perhaps surprising that later sampling of environmental DNA revealed Archaea inhabit normal environments as well, for example they are globally distributed in soils. These mesophilic soil species were only recently grown in laboratory pure cultures. In this study, we will expand on this work by feeding the community of soil microbes with various dilutions of tryptic soy broth and other complex media in combination with an antibiotic regime. This will allow us to compare how Archaea in the soil react to culture media with a variety of carbon and nitrogen sources. Determining whether premixed media can stimulate the growth of Archaeal or that premixed media is toxic to Archaea, will direct future researchers on how to grow and study the soil archaeal community in a controlled setting.

II. Literature Review

Archaea, one of the three domains of life, were first recognized as distinct from Bacteria in the late 1970's, making them relatively new to the scientific community (Woese et al., 1977). They were known for inhabiting some of the world's most extreme environments such as hot springs, acidic pools, thermal vents in the deep ocean, and even animal guts (Dridi et al., 2011). Based on their extremophile reputation, it was a surprise when scientists found archaea of the phylum Thaumarchaeota in soil by directly sampling DNA (Bintrim et al., 1996), but their functions and properties were still a mystery because they would not grow on standard cultivation media.

Further investigation found that these archaeans were in association with the tomato plant rhizoplane (Simon et al., 2000) and with the rhizosphere of diverse plant species (Sliwinski et al., 2004). With this information, researchers were able to grow enrichments of Thaumarchaeota in the laboratory using a media that included root extract from a tomato plant, making it a complex media, that is, the exact chemical composition of the media is undefined. Along with the tomato root extract, the media contained several antibiotics that inhibited the growth of bacteria but did not inhibit the growth of archaea (rifampin, carbenicillin, streptomycin, cephalothin, clindamycin). Ultimately, this approach was successful at enriching the archaeans from ~2% of the total microbes to ~20% of the total (Simon et al., 2005). More recently, a pure culture was obtained of the most common archaeal species that inhabits mesophilic soil, *Nitrosocosmicus oleophilus*, using defined media (Jung et al., 2016). This exciting discovery is allowing researchers to further study the nitrogen cycle (Gwak et al., 2020), the intricacies of archaeal cellular structure (Jung et al., 2016), and how archaea interact with other microbes in the soil ecosystem (Zhao et al., 2020).

Cultivation is a major key in furthering archaeal knowledge and research. Isolation of an archaeal strain involves three steps: collecting a sample, enrichment, and isolation. The most success has been found when original environmental conditions are mimicked for the enrichment and isolation (De la Torre et al., 2008). Additionally, there are two major techniques used for the enrichment and isolation; the first being "single colony picking by repetitive plate streaking on solid medium" (Button et al., 1993) or "serial dilution to extinction in liquid medium" (Schut et al., 1993). Serial dilution is exactly what Jung and his colleagues did in order to isolate the MY3 strain of ammonia oxidizing archaea. In this study, a sample of coal tar contaminated soil was taken and put into an enrichment culture. It was then transferred repeatedly to an artificial freshwater medium containing the antibiotics ampicillin, kanamycin, streptomycin, and clarithromycin until the MY3 strain was completely isolated. This strain is both mesophilic and neutrophilic (Jung et al., 2016). Nutrient levels were kept to a minimum since bacteria dominate nutrient rich environments (Hou et al., 2013). There are still many terrestrial archaea that have not been successfully grown in culture (Quaiser et al., 2002).

In addition to growth on defined media, complex media has been used with great success in the study of archaea. One study looked into the specific mediums needed to isolate several archaeans from the human digestive microbiota. Each of the five archaea looked into had previously required their own medium in order to be isolated. However, a group of researchers found and patented an "SAB medium" that was used to isolate all five archaean. This discovery improved the ease and speed of the detection of methanogenic archaea in specimens from the clinic as well as from the environment (Khelaifia et al., 2013).

III. Materials and Methods

A. Media Preparation

Each of the antibiotic stock solutions was prepared, filter sterilized, placed into microcentrifuge tubes and placed into the freezer. The antibiotics used and their concentrations are the following: Clindamycin Hydrochloride Monohydrate (50 mg/ml), Kanamycin Sulfate (50 mg/ml), Clarithromycin (2 mg/ml), Ampicillin (50 mg/ml), Carbenicillin (50 mg/ml), Streptomycin (50 mg/ml), Tetracycline (10 mg/ml), Gentamicin Sulfate (15 mg/mL), and Rifampicin (50 mg/ml). The following fungicide stock solutions were also prepared. They were not filter sterilized and stored in a refrigerator. The fungicides used were Nystatin (50 mg/mL) and Cycloheximide, 95% (100 mg/mL).

An artificial freshwater medium was used to grow the archaea. One liter of this solution was made using 1g NaCl, 0.4g $MgCl_2$ x $6H_2O$, 0.1g $CaCl_2$ x $2H_2O$, 0.2g

KH_2PO_4, 0.5g KCl, 10 mL HEPES buffer at pH 7.5, 1mL 7.5M FeNaEDTA, and 1mL vitamin solution. The vitamin solution contained monobasic sodium phosphate, dibasic sodium phosphate, p-aminobenzoic acid, D(+) - biotin, nicotinic acid, calcium D(+) pantothenate, and pyridoxine hydrochloride.

B. Collection Strategy of Microbial Communities

Soil was taken from the Daryl Smith Prairie on the University of Northern Iowa campus. The exact soil extraction site is shown below in Figure 1.

Figure 1. Daryl Smith Prairie Soil Sample Location

This soil was sifted to remove any large rocks, roots, etc. 0.05g of soil was weighed out into a conical tube. Deionized water was added into the tube until it reached a volume of 50 mL. The tube was then vortexed and centrifuged for 10 minutes at 10 rcf. The supernatant was transferred into a clean conical tube and centrifuged again for 10 minutes at 10 rcf. This was repeated unto there were no longer pellets appearing at the bottom of the tube. The supernatant was transferred into a clean conical tube and centrifuged for 10 minutes at 7000 rcf. The supernatant was discarded and the pellet was resuspended in deionized water. It was then vortexed.

C. Cultivation Techniques

Five different treatments were used. The first treatment contained 1% tryptic soy broth (TSB) and artificial freshwater media (AFM). The second treatment contained 10% tryptic soy broth and artificial freshwater media. The third contained 1% tryptic soy broth with water. The fourth contained 10% tryptic soy broth and water. The fifth, the negative control, contained artificial freshwater media with no tryptic soy broth. 5mL of each control was prepared. Then, 5uL of

the following antibiotics was added to each treatment to target any bacteria that may have been present: clindamycin hydrochloride monohydrate, kanamycin sulfate, clarithromycin, ampicillin, carbenicillin, streptomycin, tetracycline, gentamicin sulfate, and rifampicin. 5uL of nystatin and 5uL of cycloheximide were added to each treatment to target any fungi that may have been present. The working concentrations of the antibiotics and fungicides are shown in Table 1. 5uL of the archaea mixture obtained from the soil inoculation was added to each treatment. Finally, 5uL of sodium bicarbonate (1M), a carbon source, and 5uL of ammonia chloride (1M), a nitrogen source, was added to each treatment. Each treatment was then placed into three separate small centrifuge tubes and stored at 30°C.

Table 1. Working Concentrations of Antibiotics and Fungicides Used

Antibiotic/Fungicide	**Working Concentration**
Clindamycin Hydrochloride Monohydrate	50 ug/ml
Kanamycin Sulfate	50 ug/ml
Clarithromycin	2 ug/ml
Ampicillin	50 ug/ml
Carbenicillin	50 ug/ml
Streptomycin	50 ug/ml
Tetracycline	10 ug/ml
Gentamicin Sulfate	15 ug/ml
Rifampicin	50 ug/ml
Nystatin	50 ug/ml
Cycloheximide	100 ug/ml

Each week, the cultures were restarted using the same procedure as above. However, instead of using the archaeal mixture obtained from the soil inoculation as the source for archaea, 10% of the previous week's culture was added into the new culture. The remaining 90% was centrifuged at 19090 rcf for five minutes.

The supernatant was then removed and the pellet was frozen. This procedure was repeated for four weeks.

D. DNA Testing

Each week, every sample was viewed under a digital microscope to ensure that there were still organisms growing in each sample. Videos of each sample were recorded. At the end of the 4 week experiment, DNA testing was conducted on the frozen pellets. Extractions were performed with the Omega E.Z.N.A.® Soil DNA Kit following the manufacturer's instructions. A polymerase chain reaction (PCR) was run using Promega GoTaq following manufacturer's instructions for every time point using archaeal primers. Primer sequences were as follows.
Archaeal primers:
A109F ACKGCTCAGTAACACGT
M13-519R CACGACGTTGTAAAACGACTTACCGCGGCGGCTG
The presence of a positive PCR test was determined with agarose gel electrophoresis. Samples that were positive for Archeaa were measured for diversity using DNA profiling (Swanson et al., 2013).

IV. Results

Five samples amplified for archaea using the archaeal primers in the PCR: 2, 7, 14, 18, and 21. Sample 2 had a growth period of one week with the treatment of 1% TSB and AFM. Sample 7 had a growth period of two weeks with the treatment of 1% TSB and AFM. Both samples 2 and 7 had dark bands indicating more archaea than the other samples. Sample 14 had a growth period of three weeks with the treatment of 1% TSB and water. Sample 18 had a growth period of four weeks with the treatment of 10% TSB and AFM. Sample 21 was the negative control, DNA-free water. It amplified implying that there was contamination in it. These sample results are summarized in Table 2. The other samples did not have evidence of archaea. This is shown below in Figure 2.

Table 2. PCR Sample Results

Sample number	**Growth Period**	**Treatment**	**+/- PCR**
1	One week	AFM	-
2	One week	1% TSB with AFM	+
3	One week	10% TSB with AFM	-
4	One week	1% TSB with water	-
5	One week	10% TSB with water	-

6	Two weeks	AFM	-
7	Two weeks	1% TSB with AFM	+
8	Two weeks	10% TSB with AFM	-
9	Two weeks	1% TSB with water	-
10	Two weeks	10% TSB with water	-
11	Three weeks	AFM	-
12	Three weeks	1% TSB with AFM	-
13	Three weeks	10% TSB with AFM	-
14	Three weeks	1% TSB with water	+
15	Three weeks	10% TSB with water	-
16	Four weeks	AFM	-
17	Four weeks	1% TSB with AFM	-
18	Four weeks	10% TSB with AFM	+
19	Four weeks	1% TSB with water	-
20	Four weeks	10% TSB with water	-
21	Negative Control	DNA-free water	+

Figure 2. PCR Results Using Archeal Primers

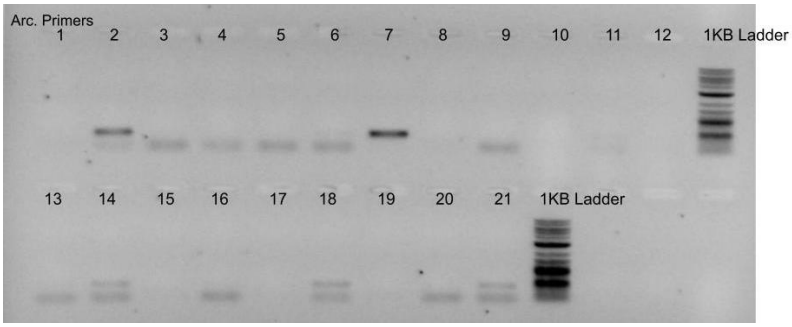
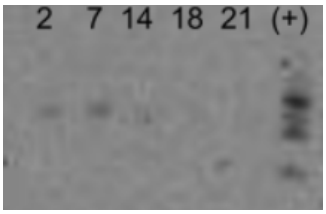


Figure 3. DNA Profiling Results



DNA profiling was done on the five samples that amplified for archaea in the PCR as well as soil from the Daryl Smith Prairie to measure species diversity in the cultures and to compare with the original diversity in the starting soil sample (Figure 3). The cultures each had just one identical band, while the soil from the Daryl Smith Prairie had many bands.

The experiment was repeated using two positive controls: salt water biofilm and a freshwater biofilm which have been known to have archaea (Schut et al., 1993). Prairie soil and centrifuged prairie soil were also included. The negative control did not have contamination (Figure 4). The fresh water band was darker than the saltwater band. The soil band was darker than the centrifuged soil band. The darker the band, the more archaea that is present. In this experiment, after one week, the freshwater with AFM amplified and the saltwater with 1% TSB and AFM amplified. None of the other samples amplified. This is shown in Figure 5 and Table 3.

Figure 4. PCR of Starting Inoculums

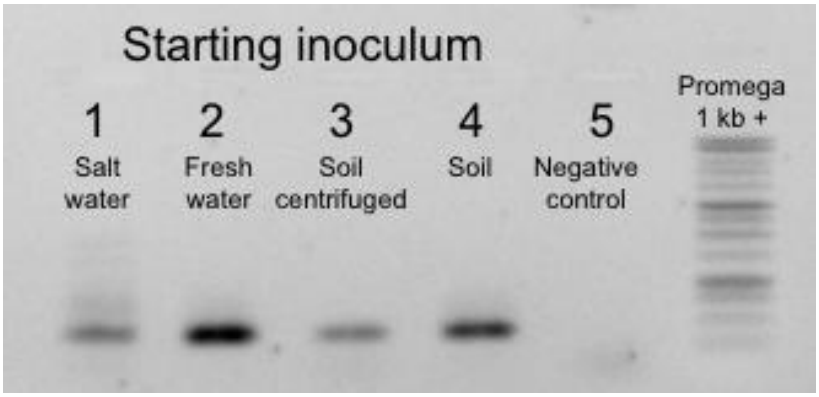


Figure 5. PCR Results of Repeated Cultivation Experiment

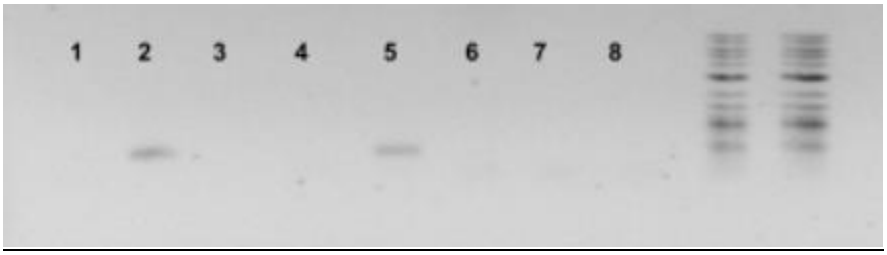


Table 3. PCR Results of Repeated Cultivation Experiment

Sample number	Growth Period	Treatment	+/- PCR
1	One week	Saltwater inoculum with AFM	-
2	One week	Freshwater inoculum with AFM	+
3	One week	Centrifuged soil inoculum with AFM	-
4	One week	Soil inoculum with AFM	-
5	One week	Saltwater inoculum with 1% TSB and AFM	+
6	One week	Freshwater inoculum with 1% TSB and AFM	-
7	One week	Centrifuged soil inoculum with 1% TSB and AFM	-
8	One week	Soil inoculum with 1% TSB and AFM	-

V. Discussion

For our experiments, we started with a soil sample from the Daryl Smith Prairie as the initial source of archaea. Previously, archaea were found at this sampling location (Swanson et al., 2013). A soil centrifugation step was performed because past students have found that this is an effective way to obtain a sample of archaea from soil with reduced fungal contamination (data not shown).

A few different species of Archaea have been cultivated from soil including the MY3 strain of ammonia oxidizing archaea (Jung et al., 2016). This isolate was grown using artificial freshwater media and defined sources of carbon and nitrogen. To limit bacterial contaminants, they also added a combination of ampicillin, kanamycin, streptomycin, and clarithromycin. In our study, we used artificial freshwater media with these antibiotics plus five additional antibiotics. It was found in our preliminary testing that using the combination of nine antibiotics may limit the amount of bacteria more effectively, when starting new cultures from prairie soil (data not shown). It was also found in the

preliminary testing that without fungicides, some cultures start to grow mold. Two fungicides, nystatin and cycloheximide, were used to combat this.

In our experiments we also added TSB, an undefined source of nutrients, at various concentrations because soil microbes are known to respond to variable nutrients (Koorem et al., 2014). Three different dilutions of TSB were used in an attempt to mimic different environments and select for different archaeal species. Water was used in place of AFM in some treatments to test if the TSB alone, without AFM, could enable archaea growth. When looking under the microscope each week at the samples, motile microbial cells were seen in all samples. However, archaeal DNA was not detected in all of the samples when doing the PCR testing. The sensitivity of the DNA testing may not have been enough to detect archaea. The DNA kits used did not have enough starting sample mass from the initial cultures, in the future, we will grow the cultures longer to collect larger cell pellets for testing.

The PCR for the first experiment showed that archaea was present in five different samples including the negative control. This contamination was not viewed in all of the other samples indicating the starting media was not contaminated and likely occurred at the time of the PCR set up. The experiment was repeated using two positive controls: salt water biofilm and a freshwater biofilm which have been known to have archaea (Schut et al., 1993). This second experiment showed no contamination in the negative control and the positive controls amplified.

The DNA profiling showed that only one type of archaea was present in each culture that gave a positive PCR result. However, the positive control, which was soil from the Daryl Smith Prairie that was used as the starting inoculum for those samples, showed many types of archaea were initially added. This indicates that after only one week, the diversity of archaea is greatly reduced in our culture media.

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