Enriching for Acetogenic Spirochaetes

Abstract

Acetogenic spirochaetes have been isolated from many environments including the termite hindgut and microbial mats. The purpose of this project was to enrich for and isolate acetogenic spirochaetes from Trunk River, Woods Hole, MA as well as determine how the enrichment strategy, in which Rifamycin SV was added, influenced the growth of other acetogens such as those within the Firmicutes. Two spirochaete morphologies were seen using phase microscopy in enrichments containing Rifamycin SV but due to time constraints they were not isolated. Growth increased when a yeast extract/tryptone solution was added suggesting there was something limiting in the original medium. The addition of this solution also lead to the increase in rod shaped microorganisms suggesting that the Rifamycin SV had not killed all of the microorganisms in the enrichment but rather was just inhibiting growth. Quantitative PCR results did not show a general trend, but did suggest that in one enrichment containing Rifamycin SV there was an increase in Firmicutes after the yeast extract/tryptone solution was added again confirming that they were not killed, but rather inhibited in the enrichment medium.

Introduction

In general terms, acetogenic microorganisms are those microorganisms capable of reducing CO$_2$ to acetate via the acetyl Co-A pathway. Two of the most common acetogens isolated are those of the Firmicutes and Spirochaetes (Drake et al. 2002). While the Spirochaetes are well known for their dominance in the hindgut of termites (Pester and Brune 2006), they have been found in free-living forms in the environment (Margulis et al. 1993, Paster and Dewhirst 2000) including from a microbial mat from Sippewissett salt marsh, Woods Hole, Massachusetts (Teal et al. 1996).

There were two main goals associated with this project. The first goal was to determine if free-living spirochaetes could be isolated from Trunk River, Woods Hole, MA using an enrichment medium specifically designed to enrich for acetogenic spirochaetes. The second goal was to determine if the enrichment medium used was
actually killing other microorganisms, such as acetogenic Firmicutes, or just inhibiting their growth temporarily.

**Materials and Methods**

**Acetogenic Enrichments.** An anoxic sediment core and microbial mat sample were obtained from Trunk River, Woods Hole, MA. One gram of each was added to two different anaerobic enrichment treatments (four bottles in total). The first was a control enrichment, meant to enrich for any acetogenic microorganisms, as well as an enrichment containing 500ug/mL Rifamycin SV to enrich specifically for acetogenic spirochaetes. The basal medium consisted of a brackish seawater base (60%/40% freshwater/seawater base), contained minimal nutrients for growth, and bromoethanesulfonic acid (BES) to inhibit methanogenesis (see appendix A for medium details). Enrichments were purged so the headspace contained an 80% H₂/20% CO₂ mixture used as the electron donor and acceptor respectively.

Enrichments were incubated at room temperature in the dark and monitored over time microscopically. Hydrogen and carbon dioxide concentrations in the headspace were measured over time by gas chromatography and samples were periodically taken for acetate measurements by high performance liquid chromatography. Enrichments were transferred when substantial turbidity was seen and the headspace was refilled in enrichments when hydrogen or carbon dioxide was depleted. After 12 days a yeast extract/tryptone solution (final concentration of 250mg/L yeast extract/250mg/L tryptone) was added to the enrichments containing Rifamycin SV to stimulate growth.

**Quantitative PCR.** One milliliter samples were taken twice from all enrichment cultures in order to perform quantitative PCR. DNA extraction of these samples, as well as of 1g samples from the original inoculum, was carried out using the MoBio UltraClean Soil DNA Isolation Kit. As DNA yields contained high concentrations of humics a 1:10 dilution of the DNA was used for the PCR reactions.

Two primer sets were identified for use in quantitative PCR. The first set was specific for Firmicutes (Fierer et al. 2005): Lgc353f 5’-GCAGTAGGGAATCTTCCG-3’ and 519RB 5’-GTATTACCGCGGCTGCTGG-3’. The second set was specific for
formyltetrahydrofolate synthetase (FTHFS) which is conserved among acetogens and is a necessary enzyme in the acetyl Co-A pathway (Lovell and Hui 1991, Lovell and Leaphart 2005): FTHFS_F5 5’-TTYACWGGHAGYTTCCATGC-3’ and FTHFS_R5 5’-GTATTGDGTYTTRGCCATACA-3’. Standard curves were constructed by using these two primer sets on an acetogenic enrichment culture previously established and cloning them in E.coli. The PCR reaction was optimized for each primer set (the extension time of the FTHFS reactions was increased from 60 to 90 seconds) and the reactions were then run in conjunction with the enrichment DNA samples to determine copy number/mL in each enrichment.

Results and Discussion

Acetogenic Enrichments. Microscopic evaluation of enrichments over time showed that the control enrichments, meant to enrich for any acetogenic microorganism, was dominated by rods (Figure 1) of which some were short and fat, long and thin, motile, and non-motile. No spirochaetes were ever visualized in any of the control enrichments over the course of the study suggesting that they were outcompeted by these other microorganisms.

In the mat enrichment containing Rifamycin SV small, motile reflective microorganisms (a cross between a rod and coccus) that resembled Chromatium were observed. This was somewhat surprising as there were high concentrations of antibiotic and kept in the dark, but the enrichment was moved to the incubator with 660nm wavelength light on it in the hopes that maybe they could also be enriched as the light wavelength should not have impacted the growth of the spirochaetes greatly. Unfortunately, growth was only slightly stimulated. These

Figure 1. Phase contrast image of rods observed in control enrichments.
microorganisms were not seen in the sediment inoculated enrichment containing Rifamycin SV. Instead, this enrichment was observed to have some spirochaetes, but turbidity and growth never increased so a yeast extract/tryptone solution was added at which time spirochaetes in both the sediment and mat enrichment were detected. Two spirochaete cell morphologies were seen in both enrichments. One was a longer spirochaete that twitched slightly (Figure 2) and the other continually extended and retracted while anchoring itself (Figure 3). The later was viewed more frequently in the mat enrichment than the sediment enrichment. Unfortunately, the addition of yeast extract and tryptone also stimulated growth of other microorganisms as seen by the appearance of rods in these enrichments. This suggests that the Rifamycin SV is only inhibiting growth and not killing other microorganisms.
Hydrogen, carbon dioxide, and acetate data all showed similar trends (Figures 4-6, Appendix B). Hydrogen and carbon dioxide consumption as well as acetate production was highest in the control enrichments and lower for the enrichments containing Rifamycin SV. Interestingly, there was hydrogen and carbon dioxide consumption in all but one of the Rifamycin SV containing enrichments after the yeast extract/tryptone solution was added suggesting that the microorganisms may be fermenting the yeast extract or tryptone. Additionally, there was both consumption and production of carbon dioxide in the mat control original enrichment which may possibly be due to some secondary metabolism occurring.

Figure 4. Total hydrogen consumption and production over the entire enrichment period. Control original and with Rifamycin original enrichments are over a 17 day period, sediment enrichment with Rifamycin 1st transfer was over a 5 day period, and the rest of the enrichments were over a 9 day period.
Figure 5. Total hydrogen consumption and production over the entire enrichment period. Control original and with Rifamycin original enrichments are over a 17 day period, sediment enrichment with Rifamycin 1st transfer was over a 5 day period, and the rest of the enrichments were over a 9 day period.

Figure 6. Acetate Production in enrichments based on high performance liquid chromatography analysis. O – original; Tr. – Transfer.
**Quantitative PCR.** The quantitative PCR results did not show any significant trends. Results are presented at copy number/mL instead of cells/mL as the number of copies of the gene/cell was unknown for the microorganisms in these enrichments. Some interesting things to note include the increase in both FTHFS and Firmicutes copy number/mL in the mat control 1st transfer enrichment over time suggesting that the growth in the enrichment is both of acetogens and the Firmicutes (Figures 7-8). Additionally, the original mat enrichment with Rifamycin SV shows an increase in Firmicutes and a slight decrease in FTHFS copy number/mL which possibly suggests that when the yeast extract/tryptone solution was added growth of the Firmicutes was stimulated and growth of the acetogens was inhibited. Each sample was only run once with each set of primers due to reagent constraints but in the future it would be necessary to do multiple measurements to get a better understanding of what is really happening in the enrichments. Additionally, one problem that occurred was that the nuclease-free water used was contaminated and thus the negative controls had measureable copy numbers and so these studies should be repeated. However, the data is still useful as comparisons were made over time and among treatments of which all contained the same contaminated water and this should not have affected the results.

![Graph](image.png)

**Figure 7.** Copy number/mL of Firmicutes and FTHFS genes in control enrichments, without Rifamycin SV based on quantitative PCR analysis.
Acetogenic spirochaetes were successfully enriched for with the addition of Rifamycin SV and after the addition of a yeast extract/tryptone solution that stimulated growth. Two distinct morphologies were seen in enrichments inoculated from both the sediment and mat samples. Unfortunately, growth of other microorganisms was stimulated by the addition of the yeast extract and tryptone suggesting that Rifamycin SV does not kill the other microorganisms but rather inhibits their growth. This may have been supported by the quantitative PCR results for the mat original enrichment with Rifamycin SV in which there was an increase in Firmicutes copy number/mL after the addition of the yeast extract/tryptone solution. Isolation of the spirochaetes did not occur due to time constraints and in the future it may be necessary to add an additional nutrient to the medium to try to speed up growth of the spirochaetes as it appeared that something was lacking in this medium that stalled their growth.

**Literature Cited**


Appendix A

Basal Enrichment Medium (1L)
600 mL Freshwater Base
400 mL Seawater Base
1 mL 0.1% Resazurin Solution
5.98 g NaHCO3
20 mL 1M MOPS pH 7.2
0.27 g NH4Cl
0.17 g Potassium Phosphate
10 mL Trace Elements Solution
10 mL Vitamin Solution
0.078 g Cysteine-HCl
2 mL 0.2M Na2S
10 mL 2.5 M Bromoethanesulfonic Acid (BES)

Appendix B

Complete hydrogen data taken over time in sediment inoculated enrichments. ⋆- Headspace was refilled with H2/CO2 gas mix; ☯ - Yeast extract/tryptone solution
Complete hydrogen data taken over time in mat inoculated enrichments. ⋄- Headspace was refilled with H2/CO2 gas mix; ◇ - Yeast extract/tryptone solution (final concentration 250mg/L yeast extract and 250mg/L tryptone) was added to the enrichment.

Complete carbon dioxide data taken over time in sediment inoculated enrichments. ⋄- Headspace was refilled with H2/CO2 gas mix; ◇ - Yeast extract/tryptone solution (final concentration 250mg/L yeast extract and 250mg/L tryptone) was added to the enrichment.
Complete carbon dioxide data taken over time in mat inoculated enrichments. ★- Headspace was refilled with H2/CO2 gas mix; ◊ - Yeast extract/tryptone solution (final concentration 250mg/L yeast extract and 250mg/L tryptone) was added to the enrichment.