Excursion into Microbial Diversity using the Nitrogen Cycle as a Model

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INTRODUCTION

Historically, microbiology has been studied using pure cultures either of disease-associated organisms or the laboratory workhorse, Escherichia coli. More recently, microbiologists have been returning to the environment to study consortiums of microorganisms in terms of their relationships with each other and their impact on the Earth. An example of a known consortium is the microorganisms involved in the nitrogen cycle (Figure 1; Schlegel, 1993).

Fig. 1.4. The nitrogen cycle.

Nitrogen exists in the environment in either an inorganic or organic form. Inorganic nitrogen includes N₂ gas which comprises 80% of the Earth’s atmosphere. Nitrogen gas can be converted into a biological form by free-living and symbiotic microorganisms in a process called nitrogen fixation. Nitrogen fixation is a reductive process transferring 6[H] to N₂ to form ammonia and releasing 2 [H] as H₂. The ammonia is used in amino acid biosynthesis and incorporated into proteins. When death occurs, proteolysis regenerates the ammonia which can be used by the nitrifying bacteria for energy.

Nitrification, the biological conversion of reduced forms of nitrogen to nitrate, is a two step process. The chemolithoautotrophic (use ammonia for energy, CO₂ as a carbon source) genera (Nitrosomonas, Nitrosolobus, Nitrosospira, Nitrosovibrio, and Nitrosococcus) that oxidize ammonia to NO₂⁻ under aerobic conditions. NO₂⁻ is subsequently reduced to N₂ by denitrifying bacteria (Nitrobacter, Nitrococcus, Nitrospina, Nitrospira) that use the NO₃⁻ as alternative electron acceptors to oxygen, and are most effective in the absence of oxygen (Mulder et. al, 1995).

In theory, ammonium can also be used as an inorganic electron donor for denitrification (3 NO₃⁻ + 5NH₄⁺ → 4 N₂ + 9 H₂O + 2 H⁺, ΔG°' = -297 kJ/mol NH₄⁺), which is nearly as favourable as in the aerobic nitrification process (ΔG°' = -349 kJ/mol NH₄⁺).
NH₄⁺(Mulder et al., 1995). Reports from Kuenen’s laboratory in The Netherlands (Mulder et al., 1995; van de Graaf et al., 1995; van de Graaf et al., 1996) were the first to describe this process to explain an increased removal of ammonia from a laboratory-scale denitrification reactor, but no organism has been identified yet. Enrichment cultures yielded a dominant organism that was Gram-negative.

Enrichment cultures are one way to characterize microbial diversity. The enrichment culture aims to select for a microbe or microbes that are able to thrive under certain conditions. This approach can be used to obtain cultures that are able to degrade a certain chemical (either for metabolism or co-metabolism), produce a desired product, live under extreme conditions, or utilize a predominant oxidant (Kurt Hanselmann, Microbial Diversity, class notes). To obtain these goals, several strategies can be used such as the source of the inoculum, composition of the medium, physical pretreatment of the inoculum, selection of incubation conditions, culture transfer frequency and state, and/or growth promotion/inhibition by additives (Kurt Hanselmann, Microbial Diversity, class notes).

The second way to characterize microbial diversity as emerged with the development of molecular techniques. Use of the polymerase chain reaction (PCR) has proven to be a powerful technique in the isolation of microbial communities that may not be amenable to culture techniques. Sequence analysis of PCR amplified 16s rRNA can suggest possible enrichment conditions that might not have been thought of from initial characterization of the study site. One disadvantage of PCR-based techniques is that there is no pure culture to continue characterization of the microbial population.

The purpose of this research project was to make use of both these approaches to study the presence of ammonia oxidizers in the Great Sippewissett Salt Marsh. Much of the experimental design was based on the work of others. Enrichment cultures were prepared using published media recipes and PCR techniques were dependent on the probes designed by others. Two isolates were obtained, but have not been identified since their appearance on plates takes 4-8 days. PCR techniques were used to identify 5 members of the enrichment culture and have been sent for sequencing. To date, the sequencing results have not returned.

MATERIALS AND METHODS

Media: Synthetic Medium (van de Graaf et al., 1996) consisted of 1.25 ml 0.4 M (NH₄)₂SO₄, 34.5 mg NaNO₂, 50 mg KHCO₃, 2.72 mg KH₂PO₄, 0.5 g EDTA (sodium salt) per 100 ml of distilled, deionized water. The salt solution was dispensed into 30 ml serum bottles (20 ml) and 100 ml into medicine bottles and autoclaved for 15 minutes at 121°C, cooled to 60°C and 1 ml of 100 mM MgSO₄·7H₂O, 1 ml of 100 mM CaCl₂, and 1 ml of lab trace elements were added per 100 ml. Serum bottles were sealed with mushroom stoppers and crimped. The head space was evacuated and flushed with H₂/CO₂ gas 3 times to generate an anaerobic environment. In addition, 10 ml was dispensed into sterile 150 mm x 16 mm test tubes, capped with Kaput closures. Agar
plates were made by adding 1.5 g Plant Cell Culture Reagent Grade Agar (Sigma) per 100 ml media.

Simple Media (Abeliovich, 1985) consisted of 1.35 g Na₂HPO₄, 0.07 g KH₂PO₄, 0.05 g NaHCO₃, 0.0014 g FeCl₃.6H₂O per 100 ml of deionized, distilled water. The medium was dispensed 100 ml into medicine bottles and autoclaved for 15 minutes at 121°C, cooled to 60°C and 1 ml of 100 mM MgSO₄.7H₂O, 1 ml of 100 mM CaCl₂, and 1 ml of 0.4 M (NH₄)₂SO₄ were added. A white precipitate formed. The completed medium was dispersed (10 ml) into sterile 150 mm x 16 mm test tubes, capped with Kaput closures.

NH₄-oxidizing bacteria media (Gerhardt et al., 1994) consisted of 0.0087 g K₂HPO₄, 0.001 g EDTA (sodium salt), 1.5 ml of 100 mM MgSO₄.7H₂O, 0.1 ml of 100 mM CaCl₂, 2.5 ml of 0.4 M (NH₄)₂SO₄, and 1 ml of the lab trace elements in 25 ml deionized, distilled water and 70 ml seawater. The pH was adjusted to 7.5 with 0.5 M K₂CO₃, and 0.25 ml of 0.5% phenol red was added. The medium was dispensed 100 ml into medicine bottles and autoclaved for 15 minutes at 121°C. The sterile medium was dispersed (10 ml) into sterile 150 mm x 16 mm test tubes, capped with Kaput closures. Solid medium was made by adding 1.5 g Plant Cell Culture Reagent Grade Agar (Sigma) per 100 ml media and omitting the phenol red.

Modified Winogradsky’s Medium (Atlas & Parks, 1997) consisted of 0.5 g CaCO₃, 0.1 g (NH₄)₂SO₄, 0.1 g K₂HPO₄, 0.1 g NaCl, 0.05 g MgSO₄.7H₂O, and 0.04 g FeSO₄ added to 100 ml of deionized, distilled water and mixed thoroughly. The flask was gently heated until dissolved, but not autoclaved and distributed (10 ml) into sterile 150 mm x 16 mm test tubes, capped with Kaput closures, swirling the flask to suspend precipitate.

Lab Trace Elements consisted of 7.8 mM nitrilotriacetic acid (NTA), 0.51 mM Fe(NH₄)₂(SO₄)₂.6H₂O, 1.2 mM Na₂SeO₃, 0.42 mM CoCl₂.6H₂O, 0.53 mM MnSO₄.2H₂O, 0.41 mM Na₂MoO₄.2H₂O, 0.3 mM Na₂WO₄.2H₂O, 0.35 mM ZnSO₄.7H₂O, 0.17 mM AlCl₃.6H₂O, 0.11 mM NiCl₂.6H₂O, 0.16 mM H₃BO₃, 40 μM CuSO₄.5H₂O. To make: dissolve NTA in 800 ml deionized, distilled water, then adjust pH to 6.5 with KOH. Alternatively, use 2.16 g Na₃NTA, the trisodium salt, and omit pH adjustments. Dissolve minerals in order, adding the next mineral only after the previous one dissolves. Adjust the pH to 7.0 and dilute to 1 L. Be sure to make enough trace element solution to last 1-2 years. Recommended dilution is 1:100.

**Inoculum:** Several mat samples were taken from various places in Sippewissett Marsh using small petri dishes and a spatula. The mat was either scraped with the spatula and placed in a sterile dish or the dish was pressed directly into the map. Inoculums were prepared by placing some of the sample in a 1.5 ml eppendorf tube (up to 0.5 ml mark approximately) and adding 1 ml of PBS pH 7.4. The samples were quickly vortexed and sonicated using the Blintzonic sonicator for 10 minutes. Samples #2, 3, and 5A were mechanically dispersed using a pasteur pipette after sonication. Four drops of the
suspension from a pasteur pipette was used to inoculate media. Media was incubated at room temperature in the dark. Samples are described in Table 1.

Table 1: Description of samples and where they were taken for use in inoculating medium.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>A cyanobacteria mat taken from near the beach part to the salt marsh. Consists mostly of sand, with thin layer of bacteria on top. The sand is green.</td>
</tr>
<tr>
<td>2</td>
<td>A thin, compact mat composed of soil taken near high nitrogen plots within marsh. Very tough and a piece was cut out with a spatula. Green layer on top, brown soil underneath, about 8 mm thick in total.</td>
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<tr>
<td>3</td>
<td>A thin mat taken near the high nitrogen plots used by other scientists. This mat consists of an upper green layer and a lower pink layer, followed by a brown soil underneath, about 2 mm thick in total. Also needed to be cut with spatula to place in dish.</td>
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<tr>
<td>4</td>
<td>A sample of multi-coloured sand taken near the beach by pressing petri dish into sand. Top layer white (3 mm), green layer (3 mm), pink layer (2 mm), brownish gray layer (≥ 5 mm)</td>
</tr>
<tr>
<td>5</td>
<td>Another mat sample taken near the beach. The upper green layer is very compact (2 mm), lower pink layer interspersed amongst sand (3 mm), lower brownish layer (1 mm) followed by sand</td>
</tr>
<tr>
<td>5A</td>
<td>The green layer from above obtained by carefully scraping the mat</td>
</tr>
<tr>
<td>5B</td>
<td>The pink layer from above obtained by carefully scraping the mat</td>
</tr>
<tr>
<td>5C</td>
<td>The brown/gray layer from above obtained by carefully scraping the mat</td>
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PCR of 16S rRNA genes: A bead beater procedure was used to isolated DNA from enrichment cultures. A 1 ml aliquot from a enrichment culture showing turbid growth was pelleted by centrifugation in an eppendorf tube (5 minutes at 13,000 x g). The supernatant was removed and the cell pellet was resuspended in 100 μl of 10 mM Tris buffer, pH 7.6 and transferred to a bead beater tube. A capful of glass beads was added to the bead beater tube with 100 μl of phenol. The sample was placed in the bead beater for 1 minute on the homogenize setting. The upper aqueous top layer was transferred to a 0.5 ml tube and centrifuged for 5 minutes at 13,000 x g. The upper layer was transferred to a new tube and a 100 μl of chloroform was added to the tube, vortexed briefly and centrifuged for 5 minutes at 13,000 x g. The upper layer was transferred to a new tube and 10 μl of 3 M sodium acetate and 100 μl of isopropanol were added to the tube. The contents of the tube were well mixed and the DNA allowed to precipitate on ice for 10 minutes. The DNA was pelleted by centrifugation for 5 minutes at 13,000 x g. The pellet
was washed with 200 µl 70% ethanol and dissolved in 10 µl of sterile deionized, distilled water.

The PCR reaction was run with 1 µl DNA, 5 µl 10X enzyme buffer, 6 µl 25 mM MgCl₂, 4 µl 2.5 mM dNTP mix, 2 µl 20 mM universal 16S rRNA forward primer, 1 µl universal 16S rRNA reverse primer, 31 µl sterile deionized, distilled water (total reaction volume 50 µl), and 1 bead of Promega TaqBead polymerase in 0.2 ml PCR tubes. The thermocycler was programmed for 94°C for 5 minutes, 30 x (94°C for 45 seconds, 53°C for 45 seconds, 72°C for 90 seconds), and held at 4°C. After PCR, 5 µl was loaded with 1 µl loading dye onto 1% TBE agarose gel, and run for 60 minutes at 100 V.

TOPO cloning of enrichment amplified 16S rRNA genes: The TOPO cloning kit was provided by Invitrogen. The TOPO cloning reaction was performed according to manufacturer’s suggestions. Briefly, the 16S rRNA gene PCR product was cloned into the pCR-TOPO vector and transformed into One Shot competent cells provided with the kit. The transformants were plated onto LB with 50 µg/ml kanamycin plates with 40 µl of 40 mg/ml Xgal and 100 mM IPTG. The plates were incubated at 37°C overnight and 40 white colonies were transferred to a new LB/50 kanamycin plate. Inserts were checked and amplified in 10 clones using the TOPO primers for PCR. DNA was released from the cells by resuspending them in water and boiling them in the microwave for 5 minutes on high. PCR reactions consisted of 5 µl 10X enzyme buffer, 4 µl dNTP, 2 µl primers, 5 µl DNA, 1 µl Vent polymerase and 33 µl deionized distilled water. The thermocycler was programmed for: 94.5°C for 5 minutes, 30 x (94.5°C for 45 seconds, 53.5°C for 45 seconds, 72.5°C for 100 seconds), and held at 4°C overnight.

Restriction Fragment Linear Polymorphism (RFLP) Analysis of TOPO clones: The TOPO clones that amplified in the above reaction were further analyzed by RFLP. The amplified DNA was digested with the enzymes: MspI, HaeIII, and HinP1 according to manufacturer’s instructions. Digests were run on a 1% TBE agarose gel at 100 V for 1 hour. Unique clones were sent for sequencing.

PCR of DNA from enrichment cultures using NitA and NitB primers: DNA was isolated from the enrichment cultures as described above. The NitA and NitB primers are as published by Voytek and Ward (1995). The reaction was carried out as previously published (Ward et al., 1997).

RESULTS

Enrichment cultures: Only inoculum #3 yielded growth in the medium used after 3 days. A variety of cell morphotypes was observed under the microscope and are summarized in Table 2 below. A 100 µl aliquot from each enrichment was plated on solid media of the synthetic medium or of the NH₄-oxidizing bacteria medium since the other
two media formed a precipitate or could not be autoclaved. After 3 days, two colony types were seen on the NH₄-oxidizing bacteria medium from an original inoculum from the same medium. The first colony was small, round and transparent. The second colony was irregular in shape and a whitish beige in colour. Both colony types were restreaked to NH₄-oxidizing bacteria medium and growth was observed for the big colony type after 4 days. A wet mount was made and shown in Figure 2B. These cells did not show motility. The colony was restreaked to NH₄-oxidizing bacteria medium with 0.02% yeast extract and incubated for 4 days. A wet mount showed similar cell morphology. The small colony type did not grow within the time allowed.

After 5 days, small, opaque colonies were seen on the NH₄-oxidizing bacteria medium from an original inoculum from the modified Winogradsky medium. These colonies were streaked to solid NH₄-oxidizing bacteria medium and solid synthetic medium. Small colonies appeared on the NH₄-oxidizing bacteria medium after 8 days. A wet mount was made and shown in Figure 2A. These cells were motile.

Table 2: Summary of cell morphotypes seen in the various enrichment media for inoculum #3, a thin microbial mat near the high nitrogen plots

<table>
<thead>
<tr>
<th>Medium</th>
<th>Description of morphotypes</th>
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<tbody>
<tr>
<td>Synthetic (anaerobic)</td>
<td>Large globular, small fat rods, diplococci, single cocci</td>
</tr>
<tr>
<td>Synthetic (aerobic)</td>
<td>Long narrow rods, short narrow rods, large globular, curved rods both slightly curved and U-shaped, single cocci</td>
</tr>
<tr>
<td>Simple</td>
<td>Large globular, short rods in pairs, slightly curved spirochetes, short rods that were very motile</td>
</tr>
<tr>
<td>NH₄-oxidizing bacteria medium</td>
<td>rods in chains of 4, large globular, curved rods, short narrow rods, single cocci</td>
</tr>
<tr>
<td>Modified</td>
<td>Curved rods, cocci in pairs with possible capsule surrounding, short thin rods, long thin rods, short fat rods</td>
</tr>
</tbody>
</table>
Figure 2A: Wet mount of small, opaque colonies from enrichment using Modified Winogradsky's medium and streaked to NH$_4$-oxidizing bacteria medium. Growth occurred after 8 days. The rods were motile.

Figure 2B: Wet mount of large colony's from an enrichment using NH$_4$-oxidizing bacteria medium. The cells were not motile.
Figure 3: RFLP analysis of samples 2 (Lanes 2-4), 4 (Lanes 5-7), 21 (Lanes 8-10), 24 (Lanes 11-13), and 12 (Lanes 14-16). TOPO clone inserts were amplified using PCR. Resulting DNA was digested with restriction enzymes and run on a 1% TBE agarose gel. Enzymes were MspI (Lanes 2, 5, 8, 11, and 14), HaeIII (Lanes 3, 6, 9, 12, and 15), and HinfI (Lanes 4, 7, 10, 13, and 16). Lane 1 is a 100 bp ladder.
Molecular Techniques: The TOPO cloning was very effective, producing a large number of clones with inserts. However, the PCR amplification of the individual clones was not very good, with only 5 out of the 10 clones chosen showing a PCR product. However, when digested with restriction enzymes, all the clones showed different pattern (Figure 3). Each of the clones were sent for sequencing. There was no amplification with the NitA/NitB primers, suggesting that no ammonia oxidizers were present in the enrichment culture. However, a positive control was not run so that this result cannot be confirmed.

CONCLUSIONS

This course was an excellent opportunity to explore the world of microbial diversity on a hands on level. Some of the key lessons I learned in the course are: 1. You always need a place to start. 2. Be aware of your limitations in the techniques you are using, as none are perfect. 3. As a discipline, microbial ecology is a constantly evolving hypothesis, in that the more experiments that are done, the more questions remain unanswered. 4. Nothing, no matter how small, lives in a world of isolation. Everything depends on everything else.

REFERENCES


Late-breaking research
clones in RFLP identified as

*Acinetobacter anitratus*
*Beta proteobacterium*
*Chromatium sp.*
*Pseudomonas agofermas*
*Sphingomonas sp.*

Using sequence analysis of rRNA genes.