Microbial Diversity course
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Attempt to cultivate mesophile Archaea from marine sediments

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Introduction

The diversity and ecology of the Archaea domain remains a very intriguing question and a promising field of research. Indeed, it has first been observed that Archaea were thriving in very limited ecological niches, often associated with extreme temperature, salt concentration, or low pH. Methanogens, though very successful in mesophilic anaerobic environments, don’t display the same variety of energy metabolisms as most bacteria. Their growth is restricted to strict anaerobic environments; their substrate range is limited to CO2/H2, acetate and a few methylated compounds. Alternative energy metabolism such as anaerobic respiration (some halophiles and crenarchaeota) using a wide range of terminal electron acceptor such as nitrate (pyrobaculum, pyrococcus), iron III (pyrodictium), sulphate (Archaeoglobus), elemental sulfur (Ignicoccus) or aerobic respiration on sulfur or oxygen are all present in this domain. Some archaea are also able to carry fermentation and even thrive on light energy through the use of bacteriorhodopsin (Halobacterium). Thus, archaea possess in theory high diversity of energy metabolism. So why archael cells would display and use this metabolic diversity only in extreme environments?

However, several clue from molecular surveys, especially in marine environment, such as 16s clone library and fosmid cloning and sequencing show that Archaea are much more widespread than previously thought, even in mesophilic environments. The main problem that prevent further understanding of their metabolism and environmental contribution have been the inability to cultivate them.

An increasing interest for this environmental microbiology mystery, growing number of cultivation attempts, new cultivation methods, and valuable information from metagenomics should progressively reveal the environmental diversity of mesophilic Archaea and provide a better understanding of their contribution to various environments.

During this short personal project, my aim was the set-up of several enrichments, choosing defined energy metabolism, inoculum and carbon source, and monitor these for archaeal growth in non-extreme conditions.
Part 1: Is there mesophilic chemolithoautotrophs archaea in the marine sediments of Sippewisset salt marsh and Trunk river using anaerobic respiration as energy metabolism?

Both Sippewisset salt marsh and Trunk River are the place of intense organic matter burial from terrestrial plants and algae deposits. It is thus expected that a high fermentative activity is occurring in these environments, leading to high concentrations of CO2 and H2. Besides, these environments are regularly immerged by sea water during each tide. Hence, an important source of terminal electron acceptor such as SO42-, NO3-, Fe III able to sustain anaerobic respiration metabolism is provided to these sediments. Furthermore, sulphide-oxidizing bacteria such as *beggiaota* are thriving in both environments, giving rise to observable white deposits of elemental sulfur. S0 can in turn accept electrons during an anaerobic respiration process. It has been shown that such process is at the origin of prolific microbial growth in marine sediments. But is there any archaea able to grow by this metabolism?

During this experiment, I set up a series of enrichments, using anoxic layers of each of these sediments as inoculums, and each of the electron acceptor. To further define the expected metabolism, I decided to use CO2 as sole carbon source and H2 as the sole electron donor, thus selecting for chemolithoautotrophs. Each of these enrichment was carried at room temperature and 35°C, with and without antibiotic. The use of an antibiotic cocktail aimed at preventing bacterial growth, and trying to promote sole archaea growth. The enrichments were monitored for turbidity and with the microscope for 13 days.
Material and methods:

Sampling Site:
During 2 field trips, about 50 cm² of sediment have been collected in Trunk River and Great Sippewisset salt marsh. In the first location, a 50 ml falcon tube was filled with sediments from about 50 cm deep in a zone of intense methanogenesis. The sample were essentially black, organic rich mud and vegetal detritus. In the second location, 50 cm² of sediment were taken from the side of a river arm. The sediment were underlaying a field of halophile grass (probably *spartina*). The sample was composed of brown mud and numerous small decomposing roots.

Medium preparation:
For this experiment, I prepared an anoxic sea water base medium composed of:
- 750 mL of sea water from the Marine Biology Lab sea water tap. This water was then filtrated on whatmann filter and then on sterile 0.2 µm filter (nalgen).
- 250 ml of Deionized water.
- 5 ml of trace elements
- 1 ml of resazurin as anaerobic indicator.

At this stage, the medium was boiled for 10-20 min. in a pyrex flask, under N2/CO2 gas mix, and partially covered by rubber stopper to allow continuous gazing while boiling. The medium was then cooled on ice, still with continuous gas stream to allow redissolution of anoxic gas in seawater. The medium was brought in the anaerobic chamber and supplemented with:
- 4.2 g of NaHCO3 (for buffering) (fin. conc. 50mM)
- 1ml of 1M KH2PO4 (fin. conc. 1 mM)
- 0.8 g of NH4Cl (fin conc. 15 mM)
- 0.5 g cystein-HCl (fin conc. 2.8 mM)

When the medium color changed, 2 mL of O.2 M Na2S-9H2O (fin. conc.0.4 mM) were added. 30 ml of medium was dispensed in 120 ml bottle.

Beside, 4 concentrated solutions (50mM) solutions of electron acceptor were prepared:
- NO3- solution: 1.27 g NaN03 in 60 mL anaerobic water
- SO42- solution 2.13 g “
- Fe III solution 2.43 g
- S0 solution: env. 5 gr of grinded elemental sulfur was added to 60 ml of anaerobic water and heated 20 min. at 115 °C for fine suspension of sulfur particles.

A concentrated antibiotic cocktail solution was also made as follow:
- 0.3 g of Carbenicylin dissolved in 1 ml of anaerobic water.
- 0.3 g of Tetracyclin dissolved in 1 ml 50% ethanol
- 0.3 g of Ampicylin dissolved in anaerobic water
- 0.3 g of rifampycin dissolved in 1 ml ethanol
- 0.3 g of Streptomycin dissolved in anaerobic water

Each solution was vortex until dissolved and mixed with 60 ml anaerobic water. Further dissolution was achieve by shaking the bottle overnight at 30°C.

1 ml to each electron acceptor solution (final concentration 8 mM) and about 0.5 g of sulfur were added to 4 series of 8 media bottles. In each series, 1 ml of antibiotic solution was added 4 bottles (fin conc. 160 mg/L).

Within each 8 bottle series, about 1cm$^3$ of Sippewisset sample was added to 2 bottles of antibiotic and 2 bottles without), and idem for the Trunk river sample.

All bottles were pressurized at 5 Psi with H2/CO2 gaz mix.

For each combination of sample/electron donor, 1 bottle with antibiotic and 1 without was placed at 35 °C and another pair at room temperature.

The growth was followed by visible turbidity and microscopic observation.
Results

The result of each enrichment is given below, after 13 days of incubation.

<table>
<thead>
<tr>
<th></th>
<th>SO\textsubscript{4}\textsuperscript{2-}</th>
<th>S\textsuperscript{0}</th>
<th>NO\textsubscript{3}\textsuperscript{-}</th>
<th>Fe\textsuperscript{3+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ab</td>
<td>Ab</td>
<td>Ab</td>
<td>Ab</td>
</tr>
<tr>
<td>Trunk River</td>
<td>35°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Room T.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sippewisset</td>
<td>35°C</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Room T.</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ moderate growth, +/- visible cells but no significant growth, - no visible cells.

Discussion

The result of this experiment was not very conclusive, since most enrichment didn’t show significant growth after 13 days of incubation. The reasons for this may be an inappropriate inoculum: each sample was from a deep sediment layer. Considering the very high organic matter input in this two areas, it is probable that a rapid depletion of each electron acceptor occurs within the first centimeters of the sediments. The microbial community sampled was thus not naturally enriched for such metabolisms. This conclusion is supported by the very poor growth, even without antibiotic, whereas many bacteria from marine sediments are described to use these electron acceptors to oxidize H\textsubscript{2}.

Besides, and as observed elsewhere, antibiotic may partially inhibit the archaeal growth. Here, all the conditions for H\textsubscript{2}/CO\textsubscript{2} methanogenesis were present in the antibiotic containing bottles, and this method/media led to successful enrichments for methanogen during the course. However, none of the enrichments led to archaeal growth.

Finally, monitoring several enrichment by microscope is very time consuming, and don’t allow discrimination of archaea/bacteria in mixed communities. Hence, the quantitative PCR to monitor the archaea could be valuable in case of many parallel enrichments.
Alternatively, the FISH method with bacteria/archaea probes can also allow detection of even low archaeal growth in mixed communities.

The last and not the least possibility would be that there is no archaea able to use this electron acceptor in moderate temperature and salinity, while using H2 as an electron donor.

**Part II: DMSO/TMAO degrading communities in the Sippewisset and Trunk River marine sediments.**

Dimethylsulfide (DMS), and derivative such as DMSpropionate (DMSp), or DMSoxiide(DMSO) are involved in several processes in marine environments. DMSp is a key component of halophile plant colonizing salt marshes and of algae. This compatible solute that compensates the osmotic pressure created by the seawater on the cell cytoplasm. When released in the environment, DMSP enters a complex cycle where it is ultimately deposited in the sediment or released in the atmosphere, accounting for half of the gaseous sulfur released by the ocean: “the typical sea smell”. DMS has a very high greenhouse effect (30 times more than CO2) that makes this gas a good candidate for microbial mediated degradation studies. Is there any microbial community associated with DMSO rich sediments able to degrade this compound? Trimethyloxide (TMAO) is the counterpart of DMSP in the fishes: it compensates the osmotic pressure and is responsible for the typical fish smell. The question to be investigated here is: Is there a community degrading DMSO/TMAO in Sippewisset and Trunk River sediments where a high marine plant degradation take place? And is there methanogens involved in the demethylation of these compounds?

From Scwefel, et al.
We used also sponges extract as an inoculum by curiosity and because sponge extract was available from an other project.

When designing the media, we found interesting not to provide any N or S source. That way, any growth would require the complete degradation of DMSO and TMAO to CH4, H2S and NH3. These two compounds constituting in turn an S and N source for the community. No other C source than the methyl groups was provided and H2 was added as an electron donor. Here again, each enrichment was monitored for growth at 35°C or room temperature, with and without antibiotics.

**Material and methods:**

The anaerobic medium for this experiment was made using the “Widdel method”. This method allowed successful cultivation of many different anoxigenic phototrophs along the course. The media was design as follow:

1x *sea water base*

<table>
<thead>
<tr>
<th>NaCl</th>
<th>MgCl2.6H2O</th>
<th>CaCl2.2H2O</th>
<th>KCl</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>400g</td>
<td>60 g</td>
<td>3g</td>
<td>10g</td>
<td>20L</td>
</tr>
</tbody>
</table>

To 1L of this medium, 1 ml of 1000x trace elements solution was added, as well as 5 mL of 1M MOPS buffer (Good buffer!!) and 10 mL of 150 mM KH2PO4 as a phosphorus source. The media was autoclaved in a Widdel bottle for 1 h and then cooled in ice under N2 gas streaming.

2. 3 mL of DMSO and O.75 g of TMAO was added to the medium for a final concentration of 20 mM.

In order to provide a stoechiometric amount of reactant (see proposed equation in results/B) we distributed 22mL of media in 12 bottles and immediately flush them with N2 before capping them with rubber stopper. Later, half of them were supplemented with antibiotic mix as described in part one and the 3 inoculums were added in the anaerobic chamber before filling the headspace of each bottle with H2 at 1 atm. Again, half of the enrichments were placed at 35°C while the other half is stayed at room temperature.
 Results

The results for these enrichments are described in the table below:

<table>
<thead>
<tr>
<th></th>
<th>Trunk river</th>
<th>Sippewisset</th>
<th>Sponge extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td></td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Room temp.</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

 Discussion.

 A. Growth of sponge extract on antibiotics

At first, the growth—though slow—seemed particularly interesting. Although archaeal species in symbiosis with sponge have been described, none was cultivated so far. To test if the cell were indeed belonging to the archaea domain, I applied the Fish/Dapi method using the archaeal probe, the bacterial probe and a non hybridizing probe as a negative control. (See fig. 1). It appeared that the growing cells were bacteria and no hybridization signal was observed under epifluorescence microscope for archaeal fish assay. Beside, the Fish-stained cells cells represent close to 100% of the Dapi stained cells. Hence, instead of archaea, the enrichment selected for highly antibiotic resistant bacteria...

 B. Growth of the Sippewisset enrichment at 35°C

This enrichment was particularly interesting: a dense growth was observed after 12 days. About half of the cells were small irregular cocci, showing autofluorescence at 420 nm excitation showing that they were methanogens archaea (See fig 2). 7 ml of gas were consumed and GC measurments indicated that 1.5% of the headspace was methane. From
this data, we can deduce that either DMSO or TMAO or both were completely reduced to CH4. We propose for this process the following pathway:

1) anaerobic respiration
   \[ H_2 + (CH_3)_3NO \rightarrow (CH_3)_3N + H_2O \]
   \[ H_2 + (CH_3)_2SO \rightarrow (CH_3)_2S + H_2O \]

2) Methanogenesis
   \[ 3H_2 + (CH_3)_3N \rightarrow 3CH_4 + NH_3 \]
   \[ 2H_2 + (CH_3)_2S \rightarrow 2CH_4 + H_2S \]

According to this pathway, the ratio H2/CH4 is 7/5 in case of stoichiometric reaction. However, measured molar ratio is \(\frac{0.31}{0.066} = 0.44\). Hence, it is probable that the two metabolisms are not directly linked and are involving several organisms. It is worth mentioning that the media made with the “Widdel technique” is anaerobic enough to allow methanogens to grow. An archaeal 16s library has been done from this enrichment and will be sequenced (later...).

Further investigation for these methanogens would be
   1) the obtention of a pure culture of methanogens, maybe by providing DMS/TMA instead of their oxidized counterpart.
   2) The substrate range of this strain: H2/CO2, other methylated compound (methanol, betaine, etc...), and acetate.
   3) The oxygen sensitivity of this strain.
 Literature used for this work:


Emnitz, D; Kolb, S; Conrad, R
Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture
ENVIRONMENTAL MICROBIOLOGY, 7 (4): 553-565 APR 2005

DeLong, EE; Pace, NR
Environmental diversity of Bacteria and Archaea
SYSTEMATIC BIOLOGY, 50 (4): 470-478 AUG 2001

DeLong, EF
Everything in moderation: Archaea as 'non-extremophiles'
CURRENT OPINION IN GENETICS & DEVELOPMENT, 8 (6): 649-654 DEC 1998

Purificación Cabello, M. Dolores Roldán and Conrado Moreno-Vivián
Nitrate reduction and the nitrogen cycle in archaea
Microbiology 150 (2004), 3527-3546
Fig. 1: FISH/Dapi assay using Eubacterial probe. Archaeal and non hybridization probes didn’t show signal and are not shown here.

Cy3 labeled Eubacterial probe

Dapi staining (same field)

Fig 2: Phase contrast 1000x magnification / 420 nm autofluorescence of the Sippewisset enrichment at 35°C.