Ecophysiology of prokaryotes
WS 2010/11

AG Cypionka / Paleomicrobiology
28. 02. 2011 – 25.03. 2011
Seminar plan

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<th>We 02.03.</th>
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Seminar list

Project A


Project B


Project C


Project D


**Project E**


**Project F**


Project A (Judith Lucas)

Diversity, abundance and isolation of *Roseobacter*-affiliated bacteria from marine sediments

Background
The *Roseobacter* clade is a major lineage of the family *Rhodobacteraceae* of the *Alphaproteobacteria*. Its members form one of the most abundant and successful groups in the marine environment. No other phylogenetically coherent clade occurs in such a variety of marine habitats and ecosystems of different geographic regions and has such a diverse physiology. Members of the *Roseobacter* clade have been detected in the water column, in biofilms, oxic and anoxic sediments and in association with other organisms (Buchan et al. 2005, Wagner-Döbler & Biebl 2006). Although this phylogenetic lineage exhibits a broad metabolic versatility, members of the *Roseobacter* clade are generally considered to be obligate aerobes. Nevertheless, there are some exceptions with a facultatively anaerobe metabolism (e.g. *Roseobacter denitrificans*, *Roseovarius crassostreae*, *Dinoroseobacter shibae*) and others are even capable of performing aerobic anoxygenic photosynthesis (AAP).

Sediments have been identified as the third-most important habitat for the *Roseobacter*-clade (Buchan et al., 2005). Molecular surveys showed that this group contributes with an average of 3% to the microbial communities thriving in marine surface sediments (e.g. Li et al. 1999, Madrid et al. 2001, Mills et al. 2003), but they were also detected in deep anoxic layers. However, the abundance, distribution and metabolic potential of sediment-dwelling *Roseobacter* have not been studied systematically so far.

To elucidate their role in marine sediments in comparison to other surfaces, algal and sediment samples were taken from Helgoland island and analyzed on different phylogenetic levels. DGGE was performed using primers specific for *Bacteria*, *Rhodobacteraceae* and *Phaeobacter* sp.. All phylogenetic groups were analyzed by quantitative PCR. To confirm the results, numbers of *Bacteria* and *Rhodobacteraceae* were determined by CARD-FISH.

During another extended cultivation study (Köpke et al., 2005), five facultatively anaerob *Roseobacter*-affiliated strains were isolated from tidal-flat sediments. One strain NB43 seems to form a new genus. Some physiological analyses like determination of the optimal growth temperature and ph still have to be done.

Aim of the project:
While investigated numbers of *Roseobacter*-affiliated bacteria within the Helgoland samples are unlikely high (CARD-FISH: ~ 20% qPCR: 40-60%), *Phaeobacter* sp. could hardly be found by applying specific PCR. Questions and tasks which will be addressed are:

- Does the DNA-concentration influence the quantification via qPCR?
- And if so, is there a correlation between DNA-concentration and cell-quantification?
- Verification of qPCR results via CARD-FISH
- Detection of *Phaeobacter* sp. with new specific primers.
- Determination of temperature- and pH-Optimum of the tidal-flat isolate NB43.
Methods/ working program

DNA extraction and quantification
DNA will be extracted from Helgoland sediments by using the MolBio-Kit for soil and quantified afterwards via PicoGreen on a microtiter plate reader. General primers for the bacterial 16S rRNA gene will be used to make sure that the extracted DNA is amplifiable.

PCR
A PCR using primers PHA-16S-129f and PHA-ITS-111r specific for *Phaeobacter* will be applied to detect *Phaeobacter* sp. in the Helgoland samples.

Real-time PCR
The extracted and quantified DNA serves as a template to determine the abundance of *Rhodobacteraceae* and *Phaeobacter* sp. by qPCR. The template DNA will be diluted (1:100, 1:200, 1:300, 1:400) to analyze the influence of different template concentrations on the cell quantification.

CARD-FISH
To verify and prove the qPCRs results, CARD-FISH will be performed using a *Rhodonacteraceae* specific probe (HRP-Ros536).

Determination of growth parameters
In order to determine optimal growth temperature and pH for NB43 growth curves should be recorded. Growth of NB43 will be monitored through cell counting after staining with SybrGreen I as direct detection and quantification method. In addition the turbidity will be measured photometrically (OD436).

Time schedule:
First week: Monday: General introduction
Tuesday: DNA-extraction/Quantification of DNA-amount
Wednesday: PCR/Preparation of qPCR-standard
Thursday: Preparation dilution series and qPCR Roseobacter
Friday: evaluation qPCR/2nd qPCR Roseobacter
Second week: CARD-FISH, SybrGreenI-cell counts, Phaeobacter PCR
Third week: Preparation of medium/ Growth curves temperature-optimum
Fourth week: Preparation of buffers and media/Growth curves pH-optimum

Literature:
Project B (Monika Sahlberg)

Abundance of *Rhizobium radiobacter* and *Roseobacter* sp. within sediments of the Benguela upwelling system

**Background**

The marine deep biosphere is known to be the largest biotope on Earth. Estimates of prokaryotic cells indicate that it harbours 55-85% of the Earth’s prokaryotic biomass and 30% of the total living biomass (Jørgensen and D’Hondt, 2006). One goal of the Integrated Ocean Drilling Program (IODP) is to investigate these microorganisms and to understand the nature of the complex microbial ecosystem that inhabits the Earth’s subseafloor. Within the frame of IODP several studies have shown that there are certain phyla which seem to dominate the subseafloor communities. Therefore, we have chosen four model organisms to trace their abundance along different sediment cores. Two of them are members of the alphaproteobacterial phyla *Rhizobium* and *Roseobacter*.

1) *Rhizobium radiobacter*: This species represents the most often cultured deep-biosphere bacterium, worldwide (Süß *et al.* 2006). Members of this species are facultative anaerobes which have been detected in shallow and deep sediments. Further, this group shows a relative increase with depth. Therefore, it is hypothesized that this group is able to adapt and to grow in the deep subsurface.

2) *Roseobacter*: The *Roseobacter* clade is distributed worldwide, contributing significantly to the communities in the water column (Selje *et al.* 2004). Some of them have also been isolated from the upper layers of sediment. Furthermore, this species was found to have certain capabilities of anaerobic growth. It is suggested that this group enters the sediments only by the sedimentation process. They are buried, may survive for some time, but will not thrive in deeper and older layers.

**The Benguela upwelling area – origin of the samples**

Generally, upwelling areas are characterized by a high productivity and the Benguela upwelling area constitutes one of the four major coastal upwelling systems in the world. Primary production is highly stimulated due to the circulation-driven upwelling of nutrient-rich waters from the deep sea. The extremely high contents of organic carbon of up to 20% dry weight (Mollenhauer *et al.* 2002) provoke elevated microbial activities and metabolic rates (Glud *et al.* 1994; Niewöhnner *et al.* 1998; Emeis *et al.* 2004).

A steep slope of the continental margin with decreasing sedimentation rates at increasing water depths allows studying a variety of sedimentary systems within a relatively narrow area. By the use of molecular techniques we want to get a hint to the question, to which extend the model organisms respond to increasing water depths and varying content of organic matter. Highest organic matter contents have been observed in shelf sediments, indicating that the maximum of primary production takes place in waters overlying the continental shelf. A second maximum in organic carbon content is located at the upper slope, where a high input of organic matter occurs due to an elevated lateral transport (Mollenhauer *et al.* 2002).
Figure 1: Sampling station located along a transect perpendicular to the coast of Namibia within the Benguela upwelling system. S: sampling station.

Figure 2: Total cell numbers counted onboard the RV Meteor. The site-specific decrease with sediment depth is a response to geochemical settings within the sediments of the transect.
**Aim of this project**
In this project we will determine the abundance of *R. radiobacter* and *Roseobacter* sp. within sediments from different sampling sites of the Benguela upwelling area in order to investigate their distribution from shallow to deep sediments. Samples were taken along a transect off the coast of Namibia during Meteor-expedition M76/1 in 2008 (Figure 1). Since total cell numbers were determined onboard the RV Meteor (Figure 2), we will analyse the sediment samples for comparison by the application of quantitative (real-time) PCR as a molecular biological tool.

**Methods**
- DNA extraction from sediments
- DNA quantification with PicoGreen
- PCR in order to create a standard for the quantitative PCR
- Quantitative (real-time) PCR
- DGGE

**Time schedule:**
**First week:**
- Monday: General introduction
- Tuesday: DNA-extraction
- Wednesday: DNA-extraction/Quantification of DNA-amount
- Thursday: PCR (*Bacteria*)
- Friday: PCR/Preparation of qPCR-standard

**Second week:**
- qPCR *Bacteria*, Preparation of qPCR-standards for *Roseobacter and R. radiobacter*

**Third week:**
- qPCR *Roseobacter, R. radiobacter and Alphaproteobacteria*

**Fourth week:**
- DGGE *Alphaproteobacteria*

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**Table 1:** Origin of sediment samples from the Namibian coast sampled during RV Meteor cruise M76/1.

<table>
<thead>
<tr>
<th>Site/device</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Water depth [m]</th>
<th>Sediment depth [cmbsf]</th>
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<tr>
<td>128 06-MUC-2</td>
<td>25°00.00’S</td>
<td>14°23.36’E</td>
<td>133</td>
<td>S, 40</td>
</tr>
<tr>
<td>128 06-GC-8</td>
<td>25°00.00’S</td>
<td>14°23.36’E</td>
<td>133</td>
<td>150, 245</td>
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<tr>
<td>128 07-MUC-1</td>
<td>25°20.65’S</td>
<td>13°46.47’E</td>
<td>299</td>
<td>S, 30</td>
</tr>
<tr>
<td>128 07-GC-2</td>
<td>25°20.65’S</td>
<td>13°46.47’E</td>
<td>299</td>
<td>150, 250, 350</td>
</tr>
<tr>
<td>128 02-MUC-1</td>
<td>25°30.00’S</td>
<td>13°27.01’E</td>
<td>792</td>
<td>S, 30</td>
</tr>
<tr>
<td>128 02-GC-3</td>
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<td>150, 340, 430</td>
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<tr>
<td>128 03-MUC-3</td>
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<td>13°04.20’E</td>
<td>1938</td>
<td>S, 20</td>
</tr>
<tr>
<td>128 03-GC-1</td>
<td>25°45.06’S</td>
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<td>1942</td>
<td>150, 390, 450</td>
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<tr>
<td>128 08-MUC-1</td>
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<td>3794</td>
<td>S, 20</td>
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<tr>
<td>128 08-GC-2</td>
<td>26°22.13’S</td>
<td>11°53.46’E</td>
<td>3794</td>
<td>150, 390, 450</td>
</tr>
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</table>

Water was sampled at all sites at 5m and directly above the seafloor.
S = sediment surface (0-5 cm)
Literature
**Project C (Maya Soora)**

**Influence of light on the survival of Dinoroseobacter shibae during starvation**

**Background**

*Dinoroseobacter shibae* was isolated from a culture of the marine dinoflagellate *Prorocentrum lima* (Biebl *et al.*, 2005). The strains belong to the aerobic anoxic phototrophic bacteria (AAPB), which were discovered in 1979 by Shiba *et al.* and have been studied with high efforts since then. AAPB are related to the facultative anaerobic purple-non-sulfur bacteria, but developed some major differences during their evolution: AAPB are able to perform light-driven energy conservation only under oxic conditions and without the production of elemental oxygen (anoxygenic). Like the purple-non-sulfur bacteria, AAPB contain bacteriochlorophyll a (BChl a) as light-absorbing pigment, but in significantly lower cellular content, while an unusually high amount of carotenoids can be found, which mainly determine the color of the cells and play an important role in preventing photo-oxidative damage to the cells. Pigment production in AAPB is only active under oxic conditions and, even more important, influenced strongly by light: Even low light intensities inhibit the production of pigments completely (Yurkov and Beatty, 1998). AAPs are capable of using light as a source of energy under oxic conditions without the generation of oxygen. Light was shown to induce ATP formation and proton translocation by the cells (Holert *et al.*, 2010). However, the cells do not grow by light energy alone. Instead there is only a certain level of light-dependent increase in the amount of biomass, protein and pigment concentrations (Biebl *et al.*, 2006).

In this project, the major focus would be under which conditions does light have the maximum competitive advantages for the bacteria. Accordingly, we test that the role of light energy for the cellular metabolism is proportional to the degree of starvation. Batch cultures of *D. shibae* will be maintained for several days under starvation in a) the dark, b) under continuous illumination and c) under dark-light cycles. The physiological fitness will be recorded by analysing respiration, chemiosmotic proton translocation and the adenylate energy charge.

**Aim of the project**

By analyzing the physiological response to light we could answer:

- Whether the cells stay viable and how fast various components of the biomass are degraded?
- How the cells survive and keep the capability of rapid recovery during prolonged periods of starvation in the dark or under light-dark cycles?
- What activities does the cells show when substrate or light are supplied again?
- Is light used for the build-up of reserve material or is it used for biosynthesis of protein and cell division?

**Methods**

- **Preparation of sea water medium**
  
  The essential part of the project is the cultivation of *D. shibae* in chemically defined medium which is similar to sea water and finally added with succinate as substrate.
• **Analysis of biomass parameters**
  The physiological experiments are based on the analyses of several biomass-related parameters that will be used by standard methods. These are substrate consumption, cell number, dry mass, contents of protein, bacteriochlorophyll \(a\).

• **Determination of metabolic activities**
  The metabolic activities to be measured include substrate-stimulated and endogenous respiration in the light and in the dark, and electron-transport driven proton translocation under the same conditions.

• **Assessment of the energetic state**
  The energy charge will be measured by the luciferin-luciferase test [ATP bioluminescence Assay Kit CLS II (Boehringer Mannheim)] and a luminometer. As the adenylates showed intracellular concentration changes of more than 10 mM per min, it is important to stop the metabolism instantaneously. This is achieved by adding ice-cold perchloric acid to the assays. The principle behind is that the luciferin reacts with oxygen to emit light. The luciferase acts as a catalyst to speed up the reaction, which is mediated by ATP. The so generated light is determined using luminometer. A recorder at the outlet of the luminometer collects measured values in mV.

  \[
  \text{Luciferase} \quad \text{Luciferin} + \text{ATP} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{Light}
  \]

**Time schedule**

**First week:**
- First and second day: General introduction and handling of instruments; preparation of media and buffers.
- 3\textsuperscript{rd} day: Inoculation and incubate under different conditions

**Second week:**
- Analysis of biomass and energetic parameters

**Third week:**
- Analysis of biomass and energetic parameters

**Fourth week:**
- Analysis of biomass and energetic parameters and present the results

**Literature**


Project D (Tim Engelhardt)

Characterization of bacteriophages in sulfate-reducing bacteria (SRB) from deep-subsurface environments

Background
Viruses are the most abundant biological entities on earth and are known to greatly affect microbial ecology in all environments. Bacterioplankton is approximately on the order of $10^6$ to $10^7$ cells per milliliter of seawater and their number increases up to $10^9$ cells per gram in nutrient-rich sediments layers. The viral population in respective habitats is typically one order of magnitude higher\(^1\). Viruses have an influence on bacterial and archaeal mortality and subsequently shaping the microbial communities. According to the “killing the winner”-theory, viruses diversify indigenous microorganisms by providing niches for slow growing or less adapted organisms. The lysis of host organisms due to viral infections and the release of cell compounds affect biogeochemical cycles and may provide nutrients for other inhabitants\(^2,3,4\) (viral shunt). In addition, viruses contribute to genetic adaptation of microorganisms to the given environmental conditions via horizontal gene transfer and may increase the survivability of lysogenized host cells\(^5\).

Deep subsurface environment are typically characterized by anoxic conditions and nutrient limitation. Indigenous microorganisms are often prone to starvation\(^6\). The viral impact on these communities is less understood, as viral activity is basically linked to microbial activity. Considering the viral reproduction cycles, the lysogeny appears to be a better strategy and is favored under oligotrophic and anoxic conditions. Furthermore, viruses become an more important mortality factor in this environments due to the absence of grazers\(^7,8\).

In the frame of the practical course we will examine the characteristics of lysogenic and lytic phage-host interactions with a selection of subsurface SRB isolates. Desulfovibrio aespoeensis strain P20, D. indonesiensis strain P23 and Desulfotignum balticum strain P18 were isolated from the sediment column of IODP Site U1301\(^9\). The sampling site is located at the eastern flank of the Juan de Fuca Ridge, Northeast Pacific, which exhibits a deep sulfate-containing zone due to crustal-fluid diffusion from below. The type strain of Desulfovibrio aespoeensis was isolated from deep granitic groundwater sampled at depth of 600m\(^10\). This organism was found to be sensitive to lytic infection of the indigenous podovirus HEy2\(^11\). In addition, prophage-like sequences was found in its genome. This organism will provide a reference for the analysis of lysogenic phage-host interactions and will be subject for a comparison of Desulfovibrio-affiliated SRBs from different subsurface environments.

Aim of the study
During the practical course we will perform induction experiments under anoxic conditions with Desulfovibrio aespoeensis\(^T\) and strain P20 to test, if the isolates harbor inducible prophages. This approach will give informations about the latent period and the burst size of the respective phage. Examination of the morphology and the genome sizes of the obtained phages will further contribute to the characteristics of the phage-host system.

In a second experiment we will test if the different strains of Desulfovibrio spp. and Desulfotignum balticum are sensitive to lytic infection. Phage extract from corresponding sediment depths of the isolates will be prepared. Plaque assays will be done under anoxic conditions to detect and isolate possible lytic phages. In addition, morphology and genome sizes will be analysed.
Work schedule

Induction experiment
(\textit{D. aespoesensis}$^1$ + P20)

Plaque assays
(P20 + P23 + P18)

Direct counting via SybrGreenI (Cells +VLPs)

Phage purification

Phage production in liquid culture

Phage purification

Transmission Electron Microscopy
Puls-field gel electrophoresis

Time schedule

First week: Preparation of materials, Phage extraction, Plaque assays
Second week: Induction experiment + VLP-/cell counts, Phage purification + preparation of phage concentrate
Third week: Puls-field gel electrophoresis (PFGE)
Fourth week: Transmission electron microscopy (TEM)

Literature

**Project E (Bert Engelen)**

**Microbial abundance and diversity within a subterranean gas-storage facility**

**Background**
Subterranean gas and oilfields are hotspots of the terrestrial deep-biosphere. Especially the disturbance of the system by human activities can lead to a stimulation of indigenous microorganisms. Contamination by the exploration process can additionally introduce surface bacteria that form growing populations within the subsurface. 

As these reservoirs represent anoxic environments, fermenting bacteria are growing on the degradation of hydrocarbons. Their fermentation products are further metabolized by other anaerobic microorganisms. Therefore, the reservoirs are characterized by complex microbial communities building up an anaerobic foodweb. One important group of bacteria that causes immense technical and financial problems for the oil and gas industry are sulfate reducers. On the one hand, the microbially mediated formation of H$_2$S can result in a bad quality of the gas itself. On the other hand, the chemical reaction with iron or other heavy metals might clog the pore space within the reservoir or industrial gas filters.

The described issues are not only problematic for natural gas-fields but also for subterranean gas-storage facilities. In this case, gas from other areas (e.g. Siberia) is pumped via pipelines to exploited gas-fields where it is stored until further usage. One week before the start of the course, we had the opportunity to sample one of these storage facilities, located in southern Germany. The company has asked for a detailed microbial community analysis to understand which processes are going on in their gas-field. The trigger to commence this investigation was a gas filter that showed iron-sulfide precipitates. We have a strong indication that microbial sulfate reduction is the reason for this. The samples will be analysed during the practical course in projects E and F. While project F focuses on the cultivation of sulfate-reducing bacteria, in this project, we will quantify *Archaea*, *Bacteria* and sulfate reducers by qPCR and will determine their diversity by denaturing gradient gel electrophoresis (DGGE).

**Aim of this study**
Analyzing the above-described material, we want to answer the questions:

- How many *Archaea* and *Bacteria* are present within the subterranean gas-storage facility?
- How abundant are sulfate-reducing bacteria?
- What is the diversity of the different phylogenetic and metabolic groups?

**Methods / Working program**

**DNA extraction and quantification and control of purity**
DNA will be extracted from different material (water and pump-pit samples) by using the FastDNA®Spin® Kit for soil and quantified afterwards via PicoGreen on a microtiter plate reader. General primers for the bacterial 16S rRNA gene will be used to make sure that the extracted DNA is amplifiable.

**Quantitative PCR**
The extracted and quantified DNA serves as a template to determine the abundance of *Bacteria, Archaea* and sulfate reducers (dsrA-gene) by qPCR. The results will be compared to direct counts performed in project F.
**Molecular screening by DGGE**

The DNA will be amplified by specific PCR for the above described phylogenetic and metabolic groups by using primers that are feasible for DGGE analysis. DGGE will be performed to determine their diversity. Several bands will be extracted, reamplified and send for commercial sequence analysis.

**Time schedule:**

**First week:**
- Monday: general introduction
- Tuesday: DNA extraction
- Wednesday: Further DNA extraction and quantification
- Thursday + Friday: 16S rRNA gene amplification

**Second week:**
- qPCR

**Third week:**
- specific DGGE-PCR and DGGE

**Fourth week:**
- DGGE and reamplification of bands, sequence analysis (if possible)

**Literature:**


Project F (Heribert Cypionka)

Cultivation of sulfate-reducing bacteria from a subterranean gas-storage facility

Background
As described for project E, subterranean gas-fields are hotspots of the terrestrial deep-biosphere, characterized by complex anaerobic foodwebs. While project E focuses on the molecular microbial community analysis of a subterranean gas-storage facility, project F will use the same material to cultivate sulfate-reducing bacteria (SRB). This group of bacteria causes immense technical and financial problems for the gas industry. On the one hand, the microbially mediated formation of H₂S can result in a bad quality of the gas itself. On the other hand, the chemical reaction with iron or other heavy metals might clogg the pore space within the reservoir or industrial gas filters. The latter was the trigger to commence this investigation. The samples that were taken one week before the start of the course will be used to stimmulate growth of sulfate-reducing bacteria.

Aim of this study
Analyzing the above-described material, we want to answer the questions:

- What is the total cell number within samples of the subterranean gas-storage facility?
- Which sulfate-reducing bacteria can be stimmulated to grow and what is their proportion?
- Can we simulate the precipitation of iron-sulfur complexes by specific cultivation techniques?

Methods / Working program

Total cell counting
Total cell numbers will be determined by flurescence microscopy using SybrGreen as nucleic acid dye (Lunau et al. 2005). Cell counting will be performed by the application of the program CountThem.

Most probable number technique
The fraction of SRB that can be stimmulated to grow will be determined by anoxic incubations in serial dillutions.

Incubation experiments
The in-situ sulfur precipitation will be simulated in H₂S-oxidant gradient cultures. Fe(III)-salts and O₂ will be used as oxidants in agar and the gaseous phase, respectively. H₂S will be produced by pure cultures of SRB or enrichments from the gas-storage facility.

Time schedule:

First week: Monday: General introduction and desing of the experiments
           Tuesday: Preparation of culture media
           Wednesday: Inoculation of MPN-cultures
           Thursday + Friday: Set up of precipitation expetiment
Second week: Total cell counting, monitoring of growth
Third week: Monitoring of growth, (additional precipitation set up)
Fourth week: Analysis of MPN-cultures
Literature:


