Anaerobic and aerobic cultivation of marine pelagic bacteria.

Miniproject

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Marine Biological Laboratory

Woods Hole, MA

Lasse Riemann
lriemann@vip.cybercity.dk
Freshwater Biological Laboratory
University of Copenhagen
51 Helsingørsgade
3400 Hillerød
Denmark

Background

Preliminary data from my Ph.D. work indicate that 40-50 % of pelagic marine bacteria has a PTS system (Phospotransferase-system). This system is characteristic of anaerobic and facultative anaerobic bacteria, but seems to be lacking in many aerobes. Use of PTS is energetically unfavorable under aerobic conditions; however, the widespread presence of this carbohydrate uptake system in the aerobic water column may be explained by the idea of "particle-specialist" bacteria. According to this idea a number of bacteria are waiting inactively in the water column as free-living bacteria. As they encounter, colonize and proliferate on particulate matter they express high growth rates as well as high ectoenzymatic activities and high densities are obtained. As suggested in the literature, anaerobic/microaerobic conditions may prevail on these particles; i.e., here possession of a PTS would be favorable.

The present mini project provides a comparison between amplifiable constituents of a natural seawater community with the corresponding culturable fraction from the same seawater sample. One aim was to elucidate whether facultative anaerobes are an important fraction of culturable pelagic marine microbial communities. This was expected as an overlap in composition between bacteria isolated from aerobic and anaerobic conditions.

In addition, I was interested in comparing the culturable fraction with a clone library from the same seawater sample. Whether culturable bacteria are an important part of pelagic bacterial communities is a matter of dispute. Some papers report a limited overlap between clone libraries and cellular isolates from the same sample (Suzuki et al. 1997), while other reports have shown dominance of culturable bacteria in natural marine communities (Rehnstam et al. 1993). This project will provide new information on this issue. In the present study, anaerobic conditions as well as aerobic conditions were applied. The background is that using traditional aerobic plating many bacteria may be overgrown by opportunistic "plate specialists", which are quantitatively unimportant in the natural community. I hoped that the use of anaerobic conditions would select for facultative anaerobes, which would have been overgrown under aerobic conditions.

It was hypothesized that there would be only a limited overlap between the clone library and the culturable bacteria despite the additional use of anaerobic conditions. A larger overlap between isolates cultured under aerobic/anaerobic conditions was anticipated.

The comparison between the clone library and cellular isolates was done by restriction fragment length polymorphism (RFLP) analysis. Using appropriate restriction enzyme digests of PCR amplified 16S rRNA genes different operational taxonomic units can be distinguished. This method has been used at the community level (Acinas et al. 1997); however, it is mostly used as a method allowing for rapid differentiation among species and sub-species prior to or instead of more laborious methods; e.g., sequencing or phenotypic analyses. Lee et al. (1997) used a broad spectrum of restriction enzymes in their analyses of bacteria in the genera *Clavibacter* and *Rathayibacter* (coryneform bacteria). They found that the combined use of two key restriction enzymes (*Hae*III, *Hha*I) was sufficient to differentiate between the 14 closely related species and subspecies analyzed. In a comprehensive study of planktonic Archaea in different oceanic provinces, Massana et al. (2000) screened 40 clones per library in 8 libraries by using the tetrameric restriction enzymes *Hae*III and *Rsa*I. Clones with identical RFLP patterns were grouped into OTU's (Operational Taxonomic Unit) and by sequencing they concluded that clones belonging to the same OTU had an average level of sequence similarity of 97.7 %. Further, it was found that dendrograms constructed from RFLP patters had the same topology as the phylogenetic tree constructed by using sequences.

Materials and methods

The experimental part is outlined below:

Clone library
Natural sample → filtration (0.2 μm) → DNA extraction → PCR (Bacterial primers) → cloning → PCR/RFLP of clone library (M13 primers) → PCR (Bacterial primers) → RFLP pattern library

Cultivation
Natural sample → dilution to extinction $10^{0} - 10^{-4}$ /plating/ ±O2 → single colony isolations → PCR (Bacterial primers) - RFLP → RFLP pattern library

A 1-liter seawater sample was obtained from surface waters off Woods Hole Harbor. In the laboratory, 200 ml was filtered onto a 47 mm, 0.22 µm pore-sized nylon filter (MSI) and immediately frozen at –80°C. Thereafter, serial dilutions (10^0 –10^{-4}) were made in sterile seawater and 100 µl was spread on dilutions (1/1, 1/5, 1/10) of Zobell seawater medium (per liter: 5 g peptone, 1 g yeast extract, 15 g agar in 80:20% GF/F filtered seawater: dd H_2O). A full set of plates was incubated at room temp. under aerobic and anaerobic conditions, respectively.

Nucleic acids were extracted from half a filter, cut in pieces, using the Ultraclean Soil DNA Kit (Mo Bio). Cells on the filters were disrupted by 2 min beat beating. Bacterial 16S rDNA was PCR amplified using primers 8F and 1492R and 57 ng DNA template using the following conditions: denaturation (95°C, 30 sec), annealing (55°C, 30 sec), extension (72°C, 60 sec) for 30 cycles, followed by a final 5 min extension. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) following manufacturers instructions. 60 insert-containing clones were PCR amplified using M13 primers, diluted 20-fold and re-amplified using Bacterial primers. Products were digested for 2 hrs at 37°C with the tetrameric restriction enzymes *Rsa*I (GTAC) and *Hpa*II (CCGG) (both from Promega) and the following ingredients: 5 µl PCR products, 1X Multi Core buffer, 0.1 M acetylated BSA, 5 units of enzyme, final vol. 10 µl). Products and 100 bp DNA ladders were run on 1.7% Metaphor agarose gels in 0.5X TBE buffer at 70 V. Gels were stained with EtBr and RFLP patterns were aligned and compared by eye.

Approximately 20 isolates from the 10^0 dilution of 1/1, 1/5, and 1/10 Zobell media with and without oxygen (≈120 isolates total) were clean-streaked 3 times before being PCR amplified. Isolates with different colony morphologies were picked first, then random colonies. Prior to PCR amplification a bit of each colony was transferred to 10 µl sterile water, heated to 95°C for 5 min, spun down at 13,000 rpm for 1 min, and 0.5 µl supernatant was added to each PCR tube (25 µl vol.). Successful amplification was confirmed by agarose electrophoresis (1%) before any restriction digest.

For each sample set (clones, +O_2, -O_2) the number of unique RFLP patterns and the number of identical RFLP patterns were determined for each restriction enzyme. Then patterns from the two enzymes were compared and unique clones/ isolates were determined.

Results

The aim was to compare 60 clones with 60 isolates grown aerobically and 60 isolates grown anaerobically. Due to unsuccessful isolation, PCR amplifications or digests the numbers were significantly reduced. RFLP patterns for both *Rsa*I and *Hpa*II were obtained from 59 clones, 39 isolates grown anaerobically, and 41 isolates grown aerobically.

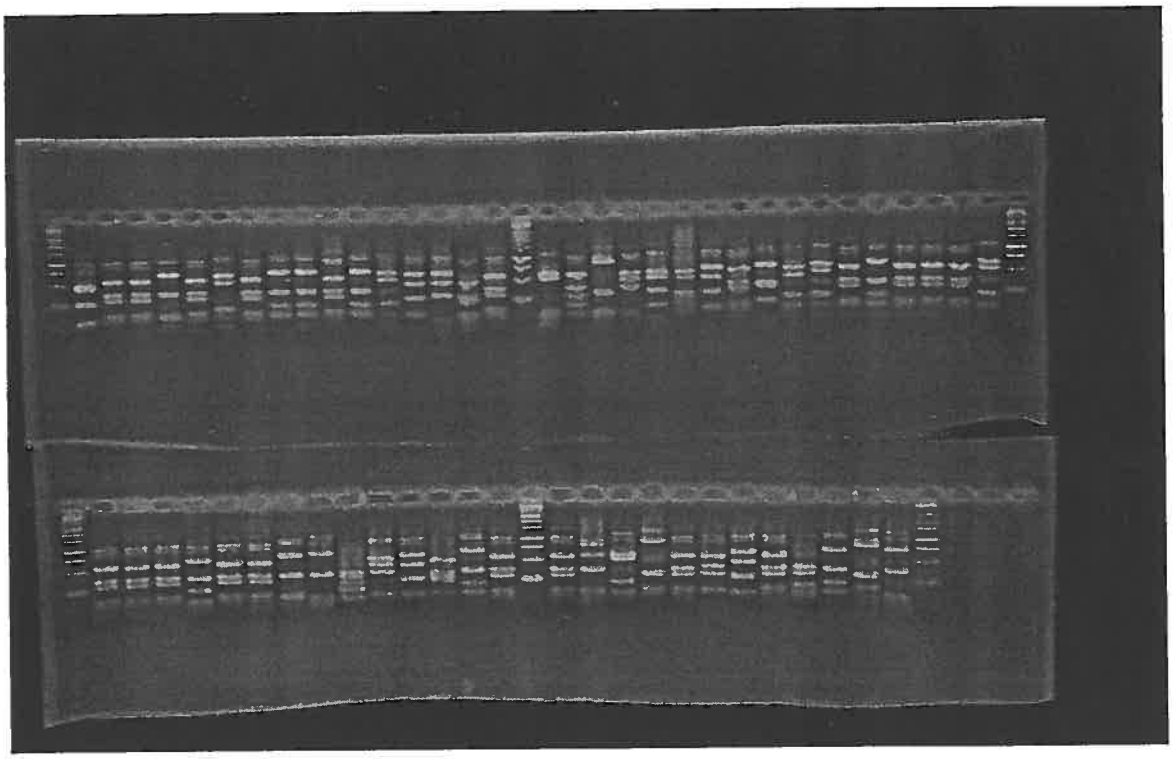

Fig. 1. The *Hpa*II digest of the PCR amplified clones using Bacterial primers. The gel was 1.7% Metaphor agarose.

As expected colonies formed a lot faster on the non-diluted Zobell media as compared to the diluted. Further, colonies grew slower at anaerobic conditions. Obvious differences in colony morphologies were observed between the 10-fold diluted media and the non-diluted media.

From the clone library, digestion with the two enzymes yielded 34 distinct RFLP banding patterns. In Fig. 1, the RFLP patterns of the *Hpa*II digest of the clones is given as an example. Sixteen clones had identical banding patterns. Six identical isolates were found among the 39 isolates, which were grown anaerobically. Five of these were grown on 10-fold diluted Zobell. Three replicates of three isolates were found among the 41 isolates grown aerobically. Six of these were on 1/10 Zobell.

No overlap for both *Rsa*I and *Hpa*II banding patterns were found between clones and the 39 isolates grown at anaerobic conditions. Further, no obvious overlaps were observed between isolates grown at anaerobic and aerobic conditions.

Discussion

A major concern in the present study was whether the combination of the two restriction enzymes applied could distinguish between different bacterial phylotypes. Due to the time constraints of this project no controls on this issue were performed. Comparative sequencing of clones/isolates with unique and/or identical RFLP patterns would have been and obvious control.

Previous use of different restriction enzymes than used here indicates that the combination of two restriction enzymes may in most cases distinguish between different microbial species/strains/clones (Lee et al. 1997, Massana et al. 2000). Moyer et al. (1996) conducted an assessment of 10 tetrameric restriction enzymes by using a computer simulated RFLP analysis for over 100 proximally and distally related bacterial small-subunit (SSU) rRNA gene sequences. Of the examined enzymes *Rsa*I was among the three most efficacious at detecting and differentiating bacterial SSU rRNA genes. Further, Moyer et al. found that the combination of two restriction enzymes on average could distinguish between 96% of the SSU's surveyed. Thus, my use of two enzymes should be able to distinguish between almost all clones/isolates.

The main purpose of the present project was to compare RFLP patterns from a clone library with those of isolates grown aerobically or anaerobically. Doing this comparison almost no overlaps were observed. While this could theoretically be true the many uncertainties in the present approach should be emphasized. For instance, it was very difficult to compare RFLP patterns by eye and even assigning molecular size to certain bands was sometimes hard. In addition, some digests seemed to be incomplete and on some gels DNA ladders had different vertical migration across the gel.

RFLP analysis provides an easy and inexpensive way to compare a large number of clones and isolates. Many of the uncertainties in my use of this method can be directly assigned to my inexperience with the method. For instance, more ladders should have been used, gel pictures should have been close-ups and the gels should in general have run longer (up to 4-5 hrs instead of 2-3 hrs). I believe that if the present approach had been coupled to gel analysis software, use of more DNA ladders per gel, longer runs, and complete digests then it would have provided useful information. However, the way I did this combined with the present "by eye analysis" it is not a feasible or reliable technique.

A few interesting observations were done. Sixteen of the 59 clones analyzed had identical RFLP patterns. If it is assumed that 1) the combination of the *Rsa*I and *Hpa*II restriction enzymes can distinguish all clones; 2) that all bacteria were PCR amplifiable with the used primers, and 3) that PCR amplification gives a quantitative view of the original sample, then 27% of the bacteria in the sample had identical 16S rDNA. Dominance of a limited number of bacterial phylotypes in pelagic marine waters has previously been observed in a few cases (Rehnstam et al. 1993).

In the literature, the importance of using nutrient poor media when culturing marine heterotrophic bacteria has often been shown. In my culturing efforts, this could be directly confirmed by the observation of obvious differences in colony morphologies for isolates grown on un-diluted versus 10-fold diluted Zobell media.

For future work involving PCR amplification of bacterial isolates it should be emphasized that the 95°C heating step prior to addition of cells to the PCR mixture was essential. Without the heating step 9 of 60 PCR reactions worked, while with the heating step 120 of 120 reactions amplified successfully.

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