CHAPTER 7: ANCIENT CYANOBACTERIAL DNA FROM SEDIMENT CORES AT BEAK ISLAND (ANTARCTIC PENINSULA): CHALLENGES FOR A MOLECULAR APPROACH.

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Abstract

The BelSPO project HOLANT aims to determine how the climate of coastal (Sub-)Antarctic regions has varied during the Holocene and assess the impact of climate anomalies on ecosystem functioning and biodiversity. Using lake sediment records, we tracked the biodiversity changes during the last ca 3500 years, in 2 coastal Antarctic lakes, BK1 and BK2 in Beak Island, Antarctic Peninsula.

Cyanobacteria are the dominant benthic phototrophs in Antarctic lakes ecosystems (Vincent WF, 2000) but they do not leave recognizable fossils, making it difficult to assess their response to past climate variability. Nevertheless, DNA can be preserved for long periods under constantly low temperatures (Willerslev et al., 2005). Hence, changes in cyanobacterial diversity were analysed on the basis of 16S rRNA gene sequences in DNA extracted from the lake sediment cores.

Two sediment cores taken from lakes BK1 and BK2 in Beak Island (Trinity Peninsula, Antarctic Peninsula) were analysed. The cores were divided in two sections (lacustrine and marine). Changes in cyanobacterial diversity occurring within the lacustrine section, when the lake was isolated from the sea, were studied. The estimated age of the BK1 sediment core (0-48 cm below surface) ranged from modern to 3500 years old, and that from BK2 (1–156 cm below surface) ranged from 300 to 3500 years old. All steps for the analysis of fossil DNA were completed in a laminar flow hood and the extraction involved several steps and procedures to prevent contamination. DNA was extracted using the FastDNA Spin Kit for Soil (Qbiogene, USA) and amplified using primers 16S369F (GGCAGCAGTGGGGAATTTTC), 16S784R and 23S30R (Boutte et al., 2006). DGGE was performed using specific primers for cyanobacteria 16S369F, 16S781RGC(a) and 16S781RGC(b) (Boutte et al., 2006) to obtain the main genotypes present in each layer. The sequenced bands were compared to public databases (Genbank, RDPII [http://rdp.cme.msu.edu/]) to find their closest relatives. Cyanobacterial sequences were grouped in OTUs (Taton et al., 2003) and plastid sequences in clusters.

Sediment core samples may contain low concentrations of ancient DNA (aDNA), impeding the PCR detection. In addition, living bacteria can develop in the core and their "modern" DNA can effectively compete with aDNA during PCR amplification.

To address this problem, we tested two approaches: i) the optimization of the direct PCR method, and ii) the use of a Multi Displacement Amplification (MDA) as a preliminary step before PCR.

i) To improve the direct PCR method, we designed a new primer, 16S369F instead of the 16S378F primer used for « modern » cyanobacterial communities (Taton et al., 2003; Boutte et al., 2006). In silico analysis with the Probe Match option (RDPII) showed a similar cyanobacterial specificity for both primers. However, the discriminating position giving a mismatch with the bacterial sequences was in the middle of the primer for 16S378F, but at the 3´end for 16S369F and therefore was more effective. Our experimental results confirmed that the use of the 16S369F primer facilitates the amplification of cyanobacterial aDNA. We also increased the concentration of polymerase from 1 to 1.2 Units per reaction and increased the $MgCl_2$ concentration from 1.5 to 2.0 mM.

The quality of DNA was tested after extraction. The amplification of long fragments of the ribosomal operon with primers 16S369F-23S30R (ca. 1800 bp) was difficult or impossible, probably because of the fragmentation of fossil DNA, as shown by Willerslev et al. (2005). However, the amplification of smaller fragments with primers 16S369F-16S784R (ca. 450 bp) was generally possible with direct PCR.

The study by direct PCR of the sediment core layers which contained enough aDNA to be amplified, showed a low turnover of cyanobacterial species for both lakes. Amplification was successful for almost all the layers, obtaining a picture of the diversity from modern times to ca. 3500 years of age. Lake BK1 showed a compartmented diversity, with filamentous cyanobacteria related to *Anabena, Nostoc* and filamentous Oscillatoriales in the upper 2.5 cm, which disappeared in the rest of the core. In Lake BK2, filamentous cyanobacteria were found in all the layers, until ca 3500 years BP. Unicellular *Synechococcus* related (99% 16S rRNA similarity) to a *Synechoccocus* sp. (EF627920) from the Baltic Sea, was extensively detected in both cores, maybe due to its higher resistance to DNA degradation. In addition, a new plastid cluster related to green algae was also found all over the cores.

ii) MDA is an isothermal amplification reaction which can increase the copy number of DNA and simultaneously dilute inhibitory substances co-purified with this DNA. The reaction starts by annealing short random primers to the template and the DNA synthesis is carried out by the high fidelity phi29 polymerase. Short primers are required to increase the number of hybridization sites, and avoid preferential targets or biases in the representation of some genomic regions. MDA can amplify a few femtograms of DNA in a bacterium (Lasken, 2007) and allows the amplification of total genomes and Whole Genome Amplification from environmental samples (Gonzalez et al., 2005).

Thus, samples BK1-12 (24 cm depth) and BK1-14 (36 cm depth), for which direct PCR was unsuccessful, were tested by MDA as an initial step before PCR amplifications. The MDA was performed with three protocols. The first one used the REPLI-g kit, following the manufacturer' instructions (Qiagen, Netherlands). A 'home made' reaction mix was used for the other two protocols, composed of phi29 polymerase (Fermentas, Germany), pyrophosphatase (New England Biolabs, UK), dNTPs (Sphaero Q, Netherlands), reaction buffer (Fermentas, Germany) and primers. The reaction mix was incubated with the aDNA for 18h at 30ºC for amplification and then, inactivated 3´at 65ºC. The two protocols used different primer sets. The first primer set consisted of exo-resistant random primers (Fermentas, Germany). They are

a mixture of single-stranded random oligonucleotides (heptamers) with two 3´-terminal phosphorothioate (PTO) modifications that are resistant to the 3´-5´exonuclease activity of the phi29 polymerase. The second primer set used HIP specific primers (also PTO modified) to try to improve the cyanobacterial specificity of the reaction to the detriment of chloroplast or other bacterial DNA during the MDA.

Highly Iterated Palindromes (HIPs) are repetitive DNA elements. According to Robinson (1995), octameric palindromes called HIP1 (GCGATCGC) are over-represented in cyanobacteria but rare in other bacteria. We also designed two new primers, GCGATCGCNA (HIPNA) and GCGATCGCNT (HIPNT). The addition of two nucleotides at the 3'end had been advised by Smith et al. (1998) to generate reproducible banding patterns to distinguish related cyanobacterial strains. Results showed that HIPNT primers did not seem to have a sufficient number of annealing sites to allow a correct amplification of complete genomes. HIPNA did not give better amplification patterns than the octameric primer from Robinson (1995), and therefore the HIP1 primer was finally selected for the test.

Preliminary MDA results showed amplification of the 2 samples, with different results depending of the protocol. The use of random primers gave the highest yield of bacterial DNA, and most sequences belonged to the Firmicutes phylum whereas the 'home made' MDA based on HIP1 primer and the MDA realised with the REPLI-g kit did not amplify bacterial sequences. REPLI-g use random hexamers but their exact sequences are not provided by the manufacturer. Interestingly, one OTU related to *Leptolynbgya* was detected in the sample BK1-14 (36 cm depth) with the REPLI-g method whereas, with direct PCR, filamentous OTUs were only detected in the upper layers (the first 2.5 cm) of the core. Further experiments must be done to elucidate if this OTU is really only present in this layer or if it is also present in the other layers in such low concentrations that it cannot be detected by direct PCR. Indeed, taxa representing less that 1% of the total community are lost with DGGE (Forney et al., 2004).

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