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Biodiversity and chronological distribution of microorganisms in perennial ice deposits from Scarisoara Ice Cave (Romania)

Synthetic scientific report
(2012-2013)

Our investigation of the perennial ice block from Scarisoara Ice Cave represents the first study of the microbial diversity from cave glaciers in correlation with the age and geoclimatic characteristics of the ice substrate.

The results obtained correspond to all objectives and activities established for 2012 and 2013 steps, in accordance with the additional 2013 contract document: (1) **Ice sampling of different age from Scarisoara ice block**; (2) **Physiological study of the microbial communities from cave ice deposits.** *2.1 Cultivation of microorganisms from melted ice in (A) liquid media and (B) solid media; 2.2 Microbial characterization by epifluorescence microscopy; 2.3 Evaluation of autotrophic carbon and nitrogen fixation*; (3) **Filogenetic study of the microbial communities from ice deposits;** *3.1 Construction of SSU-rRNA gene libraries; 3.2 Evaluation of the gene library diversity using DGGE and ARDRA analyses*; *3.3 DNA sequencing of SSU rRNA gene libraries*; *3.4. Phylogenetic analysis*; **(4) Geochemical analyses of cave ice sediments**

Objective 1. Ice sampling of different age from Scarisoara ice block

Ice samples were collected from different locations of the Great Hall and Little Reserve sectors of Scarisoara Ice Cave during 5 field trips (March, June, September 2012 and February, March 2013). Sampling was performed under aseptic conditions. Ice samples were collected from locations (ice layers) previously carbon dated (Perşoiu et al., 2010), corresponding to different aged ice: *1-S* (1 year, sun exposure), *1-L* (1 year, indirect light exposure) from the Great Hall, and from dark locations in the Little Reserve: *1-D* (1 year), *400-O* (400 years, organic-rich sediment), *900-O* (900 years, organic-rich sediment), and *900-I* (900 years, clear ice). Water samples (*A-S*) were collected from the supraglacial pond

formed at the surface of the ice block from an area exposed to direct sunlight from the Great Hall.

To avoid sample contamination, a 20-cm surface layer was removed from the ice block and flamed. Ice cores (0.5 cm long x 3 cm diameter) were extracted using a manual drill previously sterilized, transferred to 1-L sterile flasks, and stored at -20°C. Water samples were collected using sterile 60 ml syringes. An electric drill (10 cm diameter ice cores) particularly manufactured for this project was used for vertical drilling in the Great Hall up to 10-m depth in the ice block during the 2013 field trips.

Objective 2. Physiological study of the microbial communities from cave ice deposits

2.1 Cultivation of microorganisms from melted ice in (A) liquid media and (B) solid media

(A) Microorganisms from ice sampels of different age were cultivated at 4°C and 15°C on liquid media used for heterotrophic bacteria growth: Luria Bertani (LB), LB suplemented with 10% glucose (LBG), and mineral media supplemented with glucose, peptone, yeast extract and/or casaminoacids T1, T2 and T3 (Bidle *et al.*, 2007) under static and stirring conditions. Bacterial growth was monitored spectrophotometrically using FluoStar Omega plate reader (BGM Labtech) at OD_{600} . Growth curve profiles of the 1-S, 1-L, 400-O, 900-O and 900-I ice samples cultivated at 4°C and 15° indicate a variation of the growth parameters Doubling Time (1 – 3.4 days) and Lag Time (0.1 – 11 days) dependent on the ice age and growth temperature, confirming the structural diversity variation of the contained microbial communities from different ice samples, and between the ones adapted to different growth temperatures from each ice sample.

(B) Functional diversity of ice-contained microbiota was evaluated by cultivation at 4°C and 15°C on solid LB medium, and by using the BIOLOG EcoPlates system based on growth preference on 31 different carbon-sources. In the first case, the cell density of cultured microbiota was determined using the serial dilution inoculation procedure, after incubation for variable times up to 6 weeks. The results showed a culturable cell content ranging from 200 to 20,000 CFU/mL at 4°C, and from 50 to 320,000 CFU/mL at 15°C. For the second experiment, the BIOLOG EcoPlates plates inoculated with 0.3 ml melted ice from each ice sample were incubated at 4°C and 15°C, and the cell growth was monitored at OD_{590} using a FluStar Omega (BGM Labtech) plate reader. The statistical growth parameters AWCD, richness (R), Shannon diversity index (H) and Shannon evenness index (E) were calculated for each sample and growth temperature (Pessi *et al.*, 2012), revealing a high functional

diversity and a broader carbon-source type utilization by the microorganisms from 1-S and 400-O samples, at both 4°C and 15°C, while the lowest diversity was recorded for the oldest clear ice 900-I sample.

2.2 Microbial characterization by epifluorescence microscopy

Ice samples 1-S and 1-L were cultivated for 2 months at 7°C in BG_{11} medium specific for cyanobacteria growth, in the presence of light using microplates. Phototrophic (cyanobacteria and algae) and heterotrophic microorganisms, were analyzed by fluorescence microscopy using A1 microscop (Zeiss), either in natural fluorescence (chlorophyll or pigments), labeled with SybrGreen (total prokaryotic and eukaryotic cells) or with ethidium homodimer (indicating altered plasma membrane, dead cells). The results indicate the presence of unicellular and filamentous fluorescent cells, and overlapped viable cells dominating the total (Syber Green labeled) microbial community in the two recent ice samples exposed to light and sun, 1-S and 1-L. No viable phototrophs were visualized in 400-O, 900-O and 900-I ice samples collected from dark sediments of 400 and 900 years old, respectively.

2.3 Evaluation of autotrophic carbon and nitrogen fixation

The response of sun-exposed ice microbiota to the presence and absence of nitrogen was analyzed in correlation with the presence of viable phototrophic microorganisms determined in this ice sample. When cultivated in BG_{11} medium at 7°C, the growth doubling time of microbial populations containing both fototrophs and heterotrophs from 1-S melted ice sample is of 10 days. In the absence of nitrogen source, when cultivated in BG_0 medium, the growth rate is extremely reduced. In this case, after 2 months incubation the culture did not reach stationary phase. This result is due to either the absence of atmospheric nitrogen fixation by phototrophic microorganisms, or to an extremely low growth rate of ice microbiota that precluded a sensible growth and cell division under these conditions.

3. Filogenetic study of the microbial communities from ice deposits

3.1 Construction of SSU-rRNA gene libraries

A 16S rRNA gene library was obtained from the culturable bacteria from 1-D and 900-O ice samples. After cultivation of melted ice in 10 mL LB liquid media at 15°C for 7 days, the total genomic DNA was extracted using DNAeasy Blood and Tissue kit (Qiagen) in the presence of mutanolysin for enhancing the efficiency of cell wall lysis. The concentration

and purity of resulted DNA were determined spectrophotometrically (Nanodrop1000), resulting pure samples of 80-350 ng/µl.

Bacterial 16S rRNA genes were amplified by PCR using universal B8F and B152R primers, following a touch-down reaction: 2 min /95°C, 5 cycles of 1 min/95°C, 1.5 min/62-57°C, and 2 min/72°C, the annealing temperature being reduced by for each cycle, and 25 cycles of 1 min/95°C, 1.5 min/60°C, 2 min/72°C, with a final extension step of 10 min/72°C.

The resulted amplicons were cloned in *pGEM-T Easy* (Promega) cloning vector and amplified in *E. coli* XL1Blue. The resulted colonies were analyzed by ARDRA and their sequence was partially determined. The 16S rRNA gene library obtained from cultured 900-O ice sample contained 182 bacterial clones of phychrotolerant bacteria cultivated in LB at 15°C. In the case of recent ice sample 1-D cultivated under the same conditions, the resulted 16S rRNA gene library contained 154 clones.

3.2 Evaluation of the gene library diversity using DGGE and ARDRA analyses;

DGGE (Denaturing Gradient Gel Electrophoresis)

The structural diversity of prokaryotic (*bacterial and archaeal) and eukaryotic (total and fungi) microbial communities* from 1-S, 1-L, 400-O, 900-O and 900-I ice samples was analyzed by DGGE analysis of 16S rRNA genes amplified from uncultured and cultured ice microbiota. Total DNA was extracted from melted ice and from microbial cultures on T1, T2, LB, LBG media. Fungi diversity was analyzed from ice samples and LB/LBG cultures. About 500 ml melted ice was filtered on 22 µm Millipore sterile filters and genomic DNA was extracted using DNAeasy Blood and Tissue kit (Qiagen) in the presence of mutanolysin to ensure Gram positive and archaeal strains cell lysis. In the cases of liquid cultures on different media, total DNA was extracted from 5 ml culture after 7-20 days incubation at 15°C. SSU rRNA genes were amplified by PCR using specific primers including one with GC-clamp extensions: F357-GC and R518 for bacteria; NS1-GC and NS2 for fungi, and Euk1A-GC and Euk516for eukaryotic microorganisms. The DNA amplicon pools were separated by electrophoresis using urea/formamide denaturant gradient of 30-55% for bacteria (16S rRNA) and 20-35% for eukaryotes (18S rRNA). For archaea, 16S rRNA genes were amplified in two steps (nested PCR), using A8F/1492r primers in the first one, and PARCH340f_GC/PARCH519 primer pairs containing GC clamp in the second one, and the amplified DNA from the first reaction as matrix. The DNA fragments were analyzed on20-35%, 30-55% and 20-80% gradient DGGE electrophoresis.

The DGGE profile (distribution and number of DNA fragments) of amplified SSU rRNA genes from each sample corresponding to uncultured (ice extracted) and cultured (T1, T2, LB, LBG media) microorganisms indicated a high structural diversity (number of bands) and composition (migration profile) of each of the bacteria and fungi/total eukayotic communities, and a lower diversity of the archaeal one. The DNA fragments were extracted from the gel, re-amplified using the same primers, and sequenced to identify the nucleotide affiliation, in order to determine the species composition of Scarisoara cave ice microbiota.

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The molecular diversity of bacterial 16S rRNA gene library of 1-D and 900-O samples cultivated in LB at 15°C was screened using ARDRA analysis based on DNA profile variations of digested clones using different restriction enzymes. Isolated plasmids corresponding to 900-O gene library were treated with cu *HinfI/HaeIII*, and *RsaI* restriction enzymes (Fermentas) and analyzed by electrophoresis on 2% agarose gels. The 100-119 and 95-99 library clones indicate various restriction profiles, and were further used as a selective clone pool for gene sequencing. In the case of 1-D sample the restriction digestion using the same procedure led to identification of 32 different ARDRA profile clones that were selected for further sequencing, in order to identify the species diversity of ice-contained microbiota.

3.3 DNA sequencing of SSU rRNA gene libraries

The nucleotide sequences of the bacterial 16S rRNA gene library from 900-O ice sample cultivated at 15°C in LB medium were partially determined using a Genetic Analyzer ABIPrism 3500 (Applied Biosystem). The resulted sequences were analyzed with BLAST-NCBI platform indicating the presence of homologous species (80-98% identity) with *Pseudomonas* strains (*P. mandelii (98%), P. syringae (80%), P. Sp 11,* a psychrotolerant species from alpine soils (98%)*, P. brassicacearum* (86%)), *Antarctic bacterium RO2* (96%), and uncultured bacteria pLW-23 identified in Washington Lake fresh water (98%).

The archaeal species composition of ice samples was investigated by PCR-DGGE analysis and sequencing of 16S rRNA specific amplicons using PARCH340f-CG and PARCH519r primers. The identification of five methanogenic phylotypes homologous to *Methanotorris igneus* Kol 5, *Methanocaldococcus vulcanius* M7, *Methanocaldococcus jannaschii* DSM 2661, *Methanocaldococcus* sp. FS406-22, *Methanocaldococcus fervens* AG86 in the 1-S, 1-L and 900-I samples of Scarisoara ice block indicated the presence of

microorganisms belonging to the Archaea domain of life in this ecosystem, and their distribution in both recent and old ice layers of Scarisoara cave glacier.

3.4. Phylogenetic analysis

Phylogenetic analysis of bacterial 16S rRNA amplicons of microbial communities from ice and cultured 1-S, 1-L, 400-O, 900-O, 900-I Scarisoara samples was performed using BLAST-NCBI and LIRMM (www. phylogeny.fr) bioinformatic tools. The results indicate the presence of 63 different phylotypes belonging to Proteobacteria, Firmicutes, Actinobacteria and Chlorobi, and 53 phylotypes showing no homology to already identified species. Some of these strains show the highest sequence identity score with bacteria reported in similar environments (soil, water, sediments, etc) common to sold habitats (Arctic, Antarctic, alpine glaciers, etc).

4. Geochemical analyses of cave ice sediments

Chemical analysis of A-S, 400-O, 900-O and 900-I water and ice samples were carried out to determine their *chemical oxygen demand (COD), nitrate, calcium, sulfate and chloride ion contents*. The calculated values (mg/L ice or water) of these parameters decreased with the age of ice between 400 and 900 years old samples with similar sediment content (400-O and 900-O). In addition, the presence of organic substrate in ice deposits of same age was associated with higher COD, nitrates and calcium ion contents, in the case of 900-O and 900-I samples, respectively.

Measurements of total carbon (TC), inorganic carbon (IC, organic carbon (TOC) and total nitrogen (TN) contents of melted ice samples 1-S, 1-L, 400-O, 900-O, 900-I indicated the highest carbon and nitrogen values for 1-S and 400-O, that increase with the age of ice. The TC/TN ratio is identical for ice samples with similar organic content (1-S, 400-O and 900-O), and showed higher values for clear ice samples 1-L and 900-I.

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