Diversity of cultured bacteria from the perennial ice block of Scărişoara Ice Cave, Romania

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Abstract: Cave ice ecosystems represent a poorly investigated glacial environment. Diversity of cave ice bacteria and their distribution in perennial ice deposits of this underground glacial habitat could constitute a proxy for microbial response to climatic and environmental changes. Scărişoara Ice Cave (Romania) hosts one of the oldest and largest cave ice blocks worldwide. Here we report on cultured microbial diversity of recent, 400, and 900 years-old perennial ice from this cave, representing the first characterization of a chronological distribution of cave-ice bacteria. Total cell density measured by SYBR Green I epifluorescence microscopy varied in the 2.4 x 10^4 – 2.9 x 10^5 cells mL^-1 range. The abundance of cultured bacteria (5 x 10^2 – 8 x 10^4 CFU mL^-1) representing 0.3-52% of the total cell number decreased exponentially with the ice age, and was higher in organic rich ice sediments. Cultivation at 4˚C and 15˚C using BIOLOG EcoPlates revealed a higher functional diversity of cold-active bacteria, dependent on the age, sediment content and physicochemical properties of the ice. The composition dissimilarity of ice microbiota across the ice block was confirmed by growth parameter variations when cultivated in different liquid media at low and high temperatures. PCR-DGGE and sequencing of bacterial 16S rRNA gene fragments from the cultured ice samples led to the identification of 77 bacterial amplicons belonging to Gammaproteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, showing variation in distribution across the ice layers. Several identified OTUs were homologous to those identified in other glacial and karst environments and showed partial conservation across the ice block. Moreover, our survey provided a glimpse on the cave-ice hosted bacteria as putative biomarkers for past climate and environmental changes.

Keywords: ice cave; bacteria; biodiversity; 16S rRNA gene; Scărişoara

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INTRODUCTION

The increased interest in microbial communities from various frozen environments has focused on understanding their mechanism of adaptation and role in these habitats. In spite of the in depth investigation of the structural and functional microbial diversity in frozen environments (Price, 2007; Priscu et al., 2007; Margesin & Miteva, 2011; Gunde-Cimerman et al., 2012) such as polar ice sheets and glaciers (Miteva et al., 2004, Lanoil et al., 2009; Rehakova et al., 2010; Anesio & Laybourn-Parry, 2012), permafrost (Rivkina et al., 2004), mountain glacier forefields (Lapanje et al., 2012; Zumsteg et al., 2012), frozen lakes (Felip et al., 1995), sea ice (Deming, 2002), Arctic (Varin et al., 2010; Adams et al., 2014) and Antarctic permanent lake ice (Priscu et al., 1998; Dieser et al., 2010, Murray et al., 2012), very little is known about microbial communities present in cave-hosted perennial ice accumulations. Such habitats are found in caves from mid-latitude, mid-altitude mountains, where the combination of cave morphology and local climatic conditions allow for the year-round preservation of ice and associated azonal glacial climatic conditions (Perşoiu & Onac, 2012). These limited reports include isolation of bacterial species (Margesin et al., 2004) and diatom flora (Lauriol et al., 2006) from this type of habitat. Recent investigations of the microbial communities from volcanic ice caves formed on Mount Erebus, Antarctica, revealed a low diversity of bacteria and fungi in this extreme subsurface habitat (Tebo et al., 2015). However, no data on the chronological
distribution of bacteria in ice caves have been reported so far.

Scărișoara Ice Cave, located in the Bihor Mountains, NW Romania, (46°29′23″N 22°48′35″E, 1165 m asl) is a limestone cave hosting one of the oldest (>3,500 years) and largest (>100,000 m²) perennial underground ice blocks in the world (Holmlund et al., 2005; Perşoiu & Pazdur, 2011). Over the last century its underground ice block was extensively studied in order to understand climatic and glaciological processes (Racoviţă, 1927; Şerban et al., 1947; Racoviţă, 1994, Racoviţă & Onac, 2000). More recently, it became the subject of studies aiming to reconstruct climatic and environmental changes in the region (Onac et al., 2007; Feurdean et al., 2011; Perşoiu et al., 2011a; Perşoiu & Pazdur, 2011). Unlike surface glaciers and glacier caves, the perennial ice from this cave was formed by water freezing (Perşoiu et al., 2011a). Between spring and late autumn, water infiltrates in the Great Hall area (Fig. 1A,B), forming a shallow lake (up to 20 cm deep) on top of the existing ice block. During winter, the entire lake freezes resulting in a ~20-cm thick ice layer that traps at the bottom various sediments deposited during summer. Therefore, the resulted ice block consists of ice layers of variable thickness separated by organic- and inorganic-rich sediment layers (Perşoiu et al., 2011a; Perşoiu & Pazdur, 2011). Nitrifying bacteria were identified in the limestone area of Scărișoara Ice Cave more than six decades ago (Pop, 1949). Our recent investigation of the ice deposits from this cave reported the presence of cultured bacteria in one-year old ice stalagmites formed in the Little Reservation area (Fig. 1A, C), belonging to Pseudomonas, Bacillus and Paenibacillus genera (Hillebrand-Voiculescu et al., 2013). In addition, preliminary data indicated the presence of bacterial and eukaryotic SSU rRNA genes in samples collected from 1, 400 and 900 years old ice-block layers, and the occurrence of phototrophic microorganisms in sun- and light-exposed ice using epifluorescence microscopy (Hillebrand-Voiculescu et al., 2014).

Here, we investigated the structural and functional diversity of cultured bacteria throughout the perennial ice block of Scărișoara Cave in correlation with its physicochemical and chemical parameters, using BIOLOG EcoPlates, PCR-DGGE and phylogenetic analysis of bacterial 16S rRNA gene sequences from the culturable microbial fraction isolated from different locations of the subterranean ice. Our findings highlighted the heterogeneous distribution of cave ice-bacteria in sequential ice layers up to 900 yr-old characterized by various light exposure regimes (dark, indirect and direct sunlight) and organic matter content (clear ice and sediment-rich ice). This data, contributing to the microbial characterization of Scărișoara Ice Cave ecosystem, represents the first report on bacterial chronosequence in ice deposits from a limestone cave.

**MATERIALS AND METHODS**

**Ice sampling**

Ice samples of different ages were collected from C-dated ice layers of the perennial ice block from Scărișoara Ice Cave (Perşoiu & Pazdur, 2011). Five different samples of 1 (AD 2012), 400 (385 cal BP) and 900 (943 cal BP) years old ice were extracted from the ice block. Recent (1 year old) ice samples originated from the Great Hall from a sun-exposed site (sample 1-L) in the immediate vicinity of the entrance, and from an indirect light exposed area (sample 1-S), in the center of the cave (Fig. 1B). These samples were collected from the top of the ice block by vertical drilling, after removing ~5 cm of the superficial layer. The 400 (400-O), and 900 (900-O and 900-I) yr-old ice samples were collected from the Little Reservation ice wall (Fig. 1C) by horizontal drilling, after removing ~20 cm of the ice wall surface. Both 400-O and 900-O samples correspond to organic-rich ice layers, while 900-I represent clear ice (Fig. 1D). Sampling was carried out in triplicate from each location, under aseptic conditions (Hillebrand-Voiculescu et al., 2014). The ice surface was flamed for 5-10 s prior to
the drilling procedure, and both the outer and inner surfaces of the coring auger (5-cm diameter, 50-cm length) were decontaminated with 96% ethanol and flaming after each drilling step. Ice samples were collected in sterile 1-L flasks, in the presence of an open-flame laboratory torch.

**Physicochemical and chemical analyses**

Carbon and nitrogen contents of the ice samples (0.5 mL) melted at 4°C were determined using a Multi N/C 3100 elemental combustion analyzer (Analytik Jena, Jena, Germany). Total nitrogen content (TN) was measured using a furnace temperature of 850°C, and the carbon content, comprising total carbon (TC) and inorganic carbon (IC), was determined at 800°C. Total organic carbon content (TOC) was calculated by subtracting inorganic carbon IC from the total carbon TC values. The average values of the carbon and nitrogen contents, expressed as mg mL⁻¹ melted ice, and the standard deviations were calculated from three replicates. The pH, electrical conductivity (EC), and total dissolved solids (TDS) values of the melted ice samples were measured at 22°C using a Multiparameter HI9828 water quality meter (Hanna Instruments, Woonsocket, RI, USA).

**Cultivation and cell density of ice-contained bacteria**

Ice-contained heterotrophic bacteria were cultivated on various growth media: T1 (5 g L⁻¹ peptone, 0.15 g L⁻¹ ferric ammonium citrate, 0.2 g L⁻¹ MgSO₄·7H₂O, 0.05 g L⁻¹ CaCl₂, 0.05 g L⁻¹ MnSO₄·4H₂O, 0.01 g L⁻¹ FeCl₃·6H₂O (Bidle et al., 2007)), T2 (1 g L⁻¹ glucose, 1 g L⁻¹ peptone, 0.5 g L⁻¹ yeast extract, 0.2 g L⁻¹ MgSO₄·7H₂O, 0.05 g L⁻¹ MnSO₄·4H₂O (Bidle et al., 2007)), Luria broth (LB), and LB containing 10 g L⁻¹ glucose (LBG). Ice samples (0.5 mL) were thawed at 4°C and used for liquid media inoculation (1:10 v:v). Bacterial cultures were incubated at 4°C and 15°C with shaking for 20 and 17 days, respectively. Cell growth was monitored by measuring the OD₆₀₀ using a FluoroStar Omega plate reader (BGM Labtech, Ortenberg, Germany). The growth parameters, doubling time (DT), and lag time were calculated using the DoublingTime exponential least square fitting software (Roth, 2006), and the linear fit of the exponential phase slope, respectively. The average values and standard deviations were calculated from two experimental data sets.

The total cell density of ice bacteria was determined by enumeration of SYBR Green I labeled cells using epifluorescence microscopy (Noble & Fuhrman, 1998). Melted ice samples (2 ml) were sonicated for 30 minutes in the presence of 10% Tween 20 (Fluka Chemie GmbH, Buchs, Switzerland), and further incubated for 15 minutes with SYBR Green I Dye (Thermo Fisher Scientific, Waltham, MA, USA). After passing on 0.22 mm pore size filters (Merck Millipore, Billerica, MA, USA), the strain cells were counted using an AXIO Scope A1 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

The cell content of cultured heterotrophs from ice samples was determined by cultivation on R2A medium, commonly used for cultivation of heterotrophs from cold habitats (Reasoner & Geldreich, 1985; Miteva et al., 2004; Yu & Margesin, 2014), at both 4°C and 15°C. The plates were inoculated with 100 ml of melted ice diluted 1:1, 1:10, 1:100 and 1:1000 in sterile water, and incubated at 4°C and 15°C for 51 and 13 days, respectively. The average cell density and standard deviation values were calculated from triplicate data sets, and expressed as number of colony forming units (CFU) mL⁻¹ of melted ice.

**BIOLOG EcoPlates**

The functional diversity of culturable bacteria from recent and old ice layers was investigated using Biolog EcoPlates (Garland & Mills, 1991; Lehman et al., 1995). The 96-well microplates containing 31 different carbon sources in triplicate were inoculated with 300 ml of melted ice, and incubated at 4°C for 68 days and at 15°C for 46 days. The color development was monitored daily using a FluoroStar Omega plate reader (BGM Labtech, Ortenberg, Germany).

The carbon-source utilization pattern was analyzed based on the calculated parameters average well-color development (AWCD), substrate richness (R), Shannon-Weaver diversity index (H), and Shannon substrate evenness (E), computed for each well (i) as:

\[ R = \frac{\sum_{i=1}^{n} O_i}{S} \]

\[ H = -\sum_{i=1}^{n} p_i \ln p_i \]

where \( p_i = \frac{O_i}{\sum_{j=1}^{n} O_j} \), and \( E = H/R \) (Garland, 1997; Insam & Gobenna, 2004). These parameters were calculated using the average absorbance values of the triplicate readings, after subtracting the blank (absorbance on the well containing no C-substrate), and using a 0.25 (OD₆₀₀) threshold value for positive growth response. The computed parameters corresponded to 42-days growth at 4°C, and 30-days growth at 15°C, representing the shortest incubation time for reaching maximum AWCD (plateau phase) for all the samples (Pessi et al., 2012).

**DNA extraction and PCR amplification**

Genomic DNA was extracted from the melted ice samples cultivated on T1, T2, LB and LBG media using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) by a modified protocol including two initial cell lysis steps. The cell pellet from 4-mL culture was incubated for 1 hour at 37°C in the presence of 200 ml TE containing 20 units mutanolysin (Fermentas, Waltham, MA, USA) per gram of cell pellet, to disrupt the Gram-positive bacterial cell wall. The resulted extract was incubated for 30 min at 56°C with 12 units proteinase K per gram of cell pellet, in the presence of ZR bashing beads (Zymo Research Corporation, Irvine, CA, USA), using a Homogenization system SpeedMill PLUS (Analytic Jena), and further processed according to the manufacturer protocol.

PCR amplification of bacterial 16S rRNA gene fragments for DGGE analysis was performed using a Thermal Cycler C1000™ (Bio-Rad Laboratories, Hercules, CA, USA). The reaction consisted of an initial 2-min denaturation step at 95°C, 35 cycles of 30 s at 95°C, 1 min at 54°C, and 1 min at 72°C, and a final 5-min elongation step at 72°C (Muyzer et al., 1993). The amplification mixture contained 40–100 ng DNA template, 0.2 μM of forward (F357-
GC: 5'- CGC CCG CCG CCG GCC GGG GGG GGG GGC GGC CCT ACG GGA GCC AGC AG-3' and reverse (RS18: 5'-ATT ACC GCG GCT GCT GG-3') primers, 1xDreamTaq buffer containing 2 mM MgCl₂, 0.2 mM dNTP mix (Thermo Fisher Scientific), and 5 U DreamTaq DNA polymerase (Thermo Fisher Scientific), in a final volume of 50 ml. The presence and size of the PCR products were visualized by 1% agarose (w/v) gel electrophoresis.

**DGGE**

To assess the diversity of cultured ice-contained bacteria, DGGE analysis of the 16S rRNA gene fragments was performed using a DGGE-4801-220 system (CBS Scientific). Amplicons (0.5-1 mg) were loaded onto 8% denaturing polyacrylamide gels containing 37.5:1 acrylamide/ bisacrylamide (Carl Roth, Karlsruhe, Germany) and a 30-55% urea-formamid linear gradient, where 100% corresponded to 7 M urea (Serva, Heidelberg, Germany) and 40% formamide (Carl Roth, Karlsruhe, Germany). Samples were migrated for 4 h in TAE buffer (Lonza Group, Basel, Switzerland) at 60°C and 220 V. After staining for 90 min at 20°C with 0.1 μg ml⁻¹ ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA), the DNA fragments were excised from the gel, incubated in 20 ml sterile MilliQ water at 4°C for 48 h, and reamplified by PCR as described above. Amplicon sequencing was carried out using the R518 bacterial primer (Macrogen, Amsterdam, The Netherlands).

The sequences reported in this study (bacterial 16S rRNA DGGE fragments) were assigned the GenBank accession numbers KF85203-KF853221, KJ454416-KJ454425, and KP219085-KP219133.

**Sequence analysis**

The DNA sequences were edited using CodonCode Aligner (www.codoncode.com) and BioEdit (Hall, 2007) for eliminating the false gaps and sequencing errors.

**Results**

**Chemical analysis of melted ice samples** showed variations of the total carbon (TC), organic carbon (TOC) and total nitrogen (TN) contents (Table 1). The highest TC, TOC and TN content (~40 mg L⁻¹) were measured for 1-S and 400-O ice samples, while 1-L and 900-O/I samples had 4-fold lower values. The two organic-rich samples 1-S and 400-O also had the highest TOC content (~30 mg L⁻¹), representing 80% and 68% of the TC content, respectively. Moreover, a 3-fold lower TOC value was measured for the recent ice sample 1-L, while the oldest ice samples 900-O/I showed 5 to 8-fold lower values as compared to that of 1-S. The ice inorganic carbon IC content was generally low (5-14 mg L⁻¹), representing 20-31% of the TC content in 1-S and 400-O, and 45-64% in 1-L and 900-O/I samples. The highest TN content was found in 1-S and 400-O, which decreased by about 4-fold in 1-L and 900-O/I. The ratio of TC to TN varied widely, ranging from 1.2 to 10.6 mg L⁻¹.

**Statistical analysis**

The response of ice-embedded microbial communities to the used carbon source based on microbial growth (OD₅₉₀) on 31 different substrates at 4 and 15°C using BIOLOG EcoPlates, and their functional diversity (H) dependence on ice (physico) chemical parameters were analyzed by Principal Component Analysis (PCA) using the Excel add-in Multibase 2015 Solver (Numerical Dynamics, Japan).

The ordination plots were constructed using Past 3.0 software (Kemphle et al., 1989).

**Ice physicochemical and chemical properties**

In order to correlate the cell density and diversity of cultured bacteria with the physicochemical characteristics of the ice substrate, the carbon and nitrogen content, along with the pH, EC, and TDS parameters of recent and old ice samples were determined. The results (Table 1) indicated mildly alkaline pH values (7.5-7.6) for the recent (1-S, 1-L) and 400-O ice samples, with a slight increase for the older 900-O (pH 7.9) and 900-I (pH 8.0) samples. The electrical conductivity of melted ice samples varied with both the age and organic content of the sediment. Thus, the highest EC value (124 mS cm⁻¹) was measured for 1-S, showing a 2-fold decrease for both 1-L and 400-O samples. The lowest conductivity (15 - 17 mS cm⁻¹) was recorded for the oldest ice samples 900-O and 900-I. In accordance, TDS concentrations decreased with the ice age, ranging from 7 mg L⁻¹ to 62 mg L⁻¹, with the exception of 1-L and 400-O that showed similar TDS values (Table 1).

**Table 1. Physicochemical and chemical properties of ice samples.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>EC (μS cm⁻¹)</th>
<th>TDS (mg L⁻¹)</th>
<th>TC (mg L⁻¹)</th>
<th>IC (mg L⁻¹)</th>
<th>TOC (mg L⁻¹)</th>
<th>TN (mg L⁻¹)</th>
<th>TC/TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-S</td>
<td>7.48</td>
<td>124.2</td>
<td>61.9</td>
<td>41.97 ± 0.47</td>
<td>8.60 ± 0.07</td>
<td>33.38 ± 0.48</td>
<td>2.15 ± 0.03</td>
<td>19.52 ± 0.47</td>
</tr>
<tr>
<td>1-L</td>
<td>7.57</td>
<td>65.1</td>
<td>32.0</td>
<td>20.52 ± 0.44</td>
<td>9.25 ± 0.04</td>
<td>11.28 ± 0.45</td>
<td>0.53 ± 0.01</td>
<td>38.72 ± 0.44</td>
</tr>
<tr>
<td>400-O</td>
<td>7.40</td>
<td>61.6</td>
<td>30.3</td>
<td>43.72 ± 0.95</td>
<td>13.76 ± 0.07</td>
<td>29.96 ± 0.95</td>
<td>2.23 ± 0.03</td>
<td>19.60 ± 0.95</td>
</tr>
<tr>
<td>900-O</td>
<td>7.87</td>
<td>17.3</td>
<td>9.0</td>
<td>12.32 ± 0.30</td>
<td>5.84 ± 0.04</td>
<td>6.48 ± 0.30</td>
<td>0.62 ± 0.01</td>
<td>19.87 ± 0.30</td>
</tr>
<tr>
<td>900-I</td>
<td>8.03</td>
<td>15.0</td>
<td>7.0</td>
<td>8.46 ± 0.12</td>
<td>5.42 ± 0.01</td>
<td>3.03 ± 0.12</td>
<td>0.64 ± 0.09</td>
<td>13.22 ± 0.15</td>
</tr>
</tbody>
</table>

Chemical analysis of melted ice samples showed variations of the total carbon (TC), organic carbon (TOC) and total nitrogen (TN) contents (Table 1). The highest TC, TOC and TN content (~40 mg L⁻¹) were measured for 1-S and 400-O ice samples, while 1-L and 900-O/I samples had 4-fold lower values. The two organic-rich samples 1-S and 400-O also had the highest TOC content (~30 mg L⁻¹), representing 80% and 68% of the TC content, respectively. Moreover, a 3-fold lower TOC value was measured for the recent ice sample 1-L, while the oldest ice samples 900-O/I showed 5 to 8-fold lower values as compared to that of 1-S. The ice inorganic carbon IC content was generally low (5-14 mg L⁻¹), representing 20-31% of the TC content in 1-S and 400-O, and 45-64% in 1-L and 900-O/I samples. The highest TN content was found in 1-S and 400-O, which decreased by about 4-fold in 1-L and 900-O/I. The organic rich sediments 1-S, 400-O and 900-O presented similar TC/TN values of approximately 20 mg L⁻¹, while the clear recent ice sample 1-L had a 2-fold higher TC/TN ratio. The lowest TC/TN score (13 mg L⁻¹) was found for the 900-I ice deposit.
Growth and enumeration of cultured heterotrophic bacteria

Heterotrophic bacteria from Scărişoara ice samples were cultivated on both solid (R2A) and liquid (T1, T2, LB, and LBG) media at 4°C and 15°C, in order to calculate the cell density and growing parameters of the culturable bacterial communities present in the ice block.

Bacterial abundance

The total cell density of ice bacteria stained with SYBR green 1 and measured by epifluorescence microscopy varied in the 2.4 - 22.3 x 10^4 cells mL^-1 range (Table 2). The highest bacterial content was found in 1-S and 400-O ice samples, while both 900 yr-old ice samples (900-O/I) showed a ~10-fold decrease in microbial cell density. 1-L recent ice exhibited 2-fold lower cell content relative to that of the same aged ice sample 1-S.

When grown on R2A medium at 4°C and 15°C, the culturable cell number ranged from 0.7 x 10^2 CFU mL^-1 to 7.8 x 10^4 CFU mL^-1 (Table 2). At 4°C, 1-S, 1-L and 400-O samples contained 5 - 8 x 10^4 CFU mL^-1, while in the oldest ice 900-O and 900-I the cell density was 50-100-fold lower. The bacterial communities from the recent ice samples 1-L and 1-S also exhibited the highest cell density (1.4 x 10^4 CFU mL^-1) when cultivated at 15°C (Table 2), whereas the old ice layers showed a 10-fold (400-O), 100-fold (900-O), and 600-fold (900-I) lower culturable population at this temperature. The density of bacteria cultivated at 4°C was higher than that growing at 15°C for all analyzed ice sediments (Table 2), suggesting a higher viability of cold-active microorganisms, particularly in the old ice layers. Moreover, on R2A, the cell density of bacteria from organic-rich ice layers 1-S, 400-O and 900-O cultivated at both 4°C and 15°C showed an exponential decrease with the age of the ice (Fig. 2). The viability of the analyzed bacterial communities showed a significant decrease with the ice age (Table 2); when cultivated at 4°C, the highest viability was found in recent ice layers (35% for 1-S and 52% for 1-L), with a significant drop to 13% in 400 yr-old ice, and as low as 2-5% in 900 yr-old ice. We found less viability of culturable bacteria when grown at 15°C; varying from 10-19% in recent ice samples to 0.3-1.5% in both 400 and 900 yr-old ice, with the lowest cultured fraction found in 900-I sample (Table 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ice-contained cell density (cells mL^-1)</th>
<th>Cultured cell density 4°C (CFU mL^-1)</th>
<th>Cell viability 4°C (%)</th>
<th>Cultured cell density 15°C (CFU mL^-1)</th>
<th>Cell viability 15°C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-S</td>
<td>(2.2 ± 0.3) x 10^5</td>
<td>(7.8 ± 1.4) x 10^4</td>
<td>35.0</td>
<td>(4.3 ± 0.5) x 10^4</td>
<td>19.3</td>
</tr>
<tr>
<td>1-L</td>
<td>(1.0 ± 0.3) x 10^3</td>
<td>(5.2 ± 0.9) x 10^4</td>
<td>52.0</td>
<td>(1.0 ± 0.2) x 10^4</td>
<td>10.0</td>
</tr>
<tr>
<td>400-O</td>
<td>(2.9 ± 0.9) x 10^3</td>
<td>(3.7 ± 0.4) x 10^4</td>
<td>12.8</td>
<td>(4.4 ± 0.8) x 10^4</td>
<td>1.5</td>
</tr>
<tr>
<td>900-O</td>
<td>(2.9 ± 0.3) x 10^4</td>
<td>(1.0 ± 0.5) x 10^4</td>
<td>5.3</td>
<td>(4.0 ± 0.9) x 10^4</td>
<td>1.4</td>
</tr>
<tr>
<td>900-I</td>
<td>(2.4 ± 1.1) x 10^4</td>
<td>(0.5 ± 0.3) x 10^4</td>
<td>2.1</td>
<td>(0.7 ± 0.6) x 10^4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Fig. 2. Ice age dependence of cultured cell density. Cell density from each melted-ice sample cultivated on R2B-agar at 4°C (grey) and 15°C (black) was determined by serial dilution inoculations, as described in Methods. The calculated cell density (CFU mL^-1) of 1-year and 900-years old ice-contained microbiota represent the average values (Table 2) for ice samples of same age, 1-S, 1-L, and 900-O/I, respectively. Curve plot at 4°C (R = 0.956); 15°C (R = 0.999). Two sample test provided a variance value of 0.55.

Liquid media growth of ice microbiota

When the cave ice was cultivated for 20 days at 4°C and 15°C on various liquid media (T1, T2, LB and LBG), differences in microbial diversity throughout the cave ice block became apparent (Bidle et al., 2007); at 4°C only media containing yeast extract (T2, LB and LBG) were suitable for the growth of cave-ice microorganisms, with the exception of the 1-S sample, while at 15°C all samples contained culturable bacteria on all media. In accordance with this, the calculated lag time and doubling time (Table 3) showed variations with cultivation temperature and media composition. At 4°C, the lag time values varied from 4.4 to 10.9 days, with the exception of the 1-S sample cultivated on T1, which showed a delayed growth (13.4 days lag time). Under these conditions, the doubling time values ranged from 0.9 to 2.8 days. At 15°C, growth started after only 0.1 - 1.7 days (lag time), and the doubling time varied from 1 to 3.4 days.

Community-level physiological profile (CLPP) of cave-ice microorganisms

The calculated parameters AWCD, R, and H for the melted ice incubated at 15°C and 4°C using BIOLOG EcoPlates clearly indicated a higher functional diversity in 1-S and 400-O ice layers, corresponding to AWCD of 0.74 ± 0.02 and 0.94 ± 0.09, substrate richness R of 18.38 ± 3.23 and 24.08 ± 1.44, and...
Table 3. Growth parameters of cave ice microbiota cultivated at 4°C and 15°C. (The doubling time (DT) and Lag time values were calculated from the corresponding growth curves, as indicated in Materials and Methods).

<table>
<thead>
<tr>
<th>Medium</th>
<th>T (°C)</th>
<th>Sample</th>
<th>DT (day)</th>
<th>DT (day)</th>
<th>LB</th>
<th>LB G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-S</td>
<td>3.06 ± 0.01</td>
<td>13.40 ± 0.25</td>
<td>0.91 ± 0.10</td>
<td>4.45 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>1-L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.19 ± 0.10</td>
<td>8.97 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>400-O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.00 ± 0.05</td>
<td>9.04 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>900-O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.81 ± 0.06</td>
<td>8.94 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>900-I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.24 ± 0.09</td>
<td>7.95 ± 0.15</td>
</tr>
<tr>
<td>15</td>
<td>1-S</td>
<td>3.38 ± 0.30</td>
<td>0.59 ± 0.11</td>
<td>1.00 ± 0.05</td>
<td>0.96 ± 0.01</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1-L</td>
<td>1.47 ± 0.10</td>
<td>1.70 ± 0.02</td>
<td>1.38 ± 0.04</td>
<td>1.56 ± 0.12</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>400-O</td>
<td>2.13 ± 0.10</td>
<td>0.45 ± 0.08</td>
<td>1.68 ± 0.12</td>
<td>0.36 ± 0.07</td>
<td>1.24 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>900-O</td>
<td>1.50 ± 0.04</td>
<td>0.36 ± 0.02</td>
<td>1.20 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>1.26 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>900-I</td>
<td>1.24 ± 0.04</td>
<td>0.61 ± 0.09</td>
<td>1.05 ± 0.23</td>
<td>1.75 ± 0.14</td>
<td>1.14 ± 0.04</td>
</tr>
</tbody>
</table>

Shannon-Weaver diversity index H of 2.55 ± 0.02 and 2.7 ± 0.09, respectively (Fig. 3). In the case of the 1-L and 900-O/I samples, the AWCD, R and H values showed a significant decrease (up to 20-fold). The lowest values were obtained for 900-I (AWCD of 0.04 ± 0.01 and 0.08 ± 0.01, R of 1.04 ± 0.62 and 1.22 ± 0.51, and H of 0.18 ± 0.01 and 0.35 ± 0.11) at 15°C and 4°C temperature, respectively. All calculated parameters showed higher values for the microbial communities cultivated at 4°C as compared to 15°C. Evenness (E) values varied in the 0.88 ± 0.05 - 0.97 ± 0.12 interval, with lower average and higher standard deviation values for 900-O (0.74 ± 0.27) and 900-I (0.84 ± 0.12) samples cultivated at 4°C.

The carbon substrate utilization profile of the Scărişoara cave ice bacterial communities (Supplemental Table 4) indicated the use of Tween 40 by all samples, regardless their growth temperature. Interestingly, the extensive use of this non-ionic detergent was also found in the cases of all analyzed strains belonging to Sphingomonas sp. isolated from lake sediments of southern Finland (Rapala et al., 2005). 1-S and 400-O microbiota could utilize most of the substrates (24 and 26, respectively), with some variations with the growth temperatures, while a more limited number of substrates was used by 1-L (10) and 900-O/I (3/1). Principal component analysis (PCA) of the BIOLOG EcoPlates variation in bacterial growth (OD₅₉₅) at 4°C and 15°C on 31 different C-sources (Fig. 4) explained 64.84 % of the total variance (PC1). The ordination plot showed that the substrate utilization profiles of ice bacterial communities were location-specific; cultured bacteria from the 1-S and 400-O samples grown at 4°C and 15°C were distributed close to each other along PC1 axis, and distant from the other ice samples. The substrate utilization profiles of 900-O and 900-I samples formed a distinct group, in close proximity to the 1-L group, and independent of their growth temperature. Recent ice samples 1-S
and 1-L were separated along PC2 axis from the older (400-O and 900-O/I) ice layers. PC1 correlated with the carbohydrates D-xylose (r = 0.32) and D-mannitol (r = 0.35), the carboxylic acid 4-hydroxy benzoic acid (r = 0.31), and aminoacids L-asparagine (r = 0.33) and L-serine (r = 0.39). In addition, PC2 correlated positively (p < 0.05) with the carbohydrate α-D-lactose (r = 0.32) and carboxylic acid 4-hydroxy benzoic acid (r = 0.34), and negatively with both carbohydrates i-erythritol (r = -0.44) and D-mannitol (r = -0.44).

The PCA plot of the Shannon diversity index (H) calculated for cultured bacteria at 4°C and 15°C, in relation with the physicochemical and chemical parameters of ice samples (Fig. 5), explained 93.4% of the data variation for the separation on the first two axes. For the PC1 axis, all chemical parameters (Table 1) had approximately equal contribution to sample partition except for TC/TN, which had a large contribution to the sample separation along the PC2 axis. Therefore, sample 1-L showed a high score on PC2, being well separated from all other samples based on the TC/TN contribution. Samples 1-S and 400-O formed a separate group based on similar physicochemical characteristics. The oldest ice samples, 900-O and 900-I, were also grouped due to the pH of the ice substrate, which was more alkaline (Table 1). The Shannon-Weaver diversity H index of microbial communities grown at high (H^4) and low (H^3) temperatures appeared to be explained by EC (p < 0.04), TOC (p < 0.01) and IC (p < 0.03) carbon contents, and showed significant (p < 0.01) negative correlation with the pH.

**PCR-DGGE and phylogenetic composition of ice bacterial community**

The DGGE profile of PCR-amplified bacterial 16S rRNA gene fragments of ice microbial communities cultivated in different media at low and high temperatures (Fig. 6) indicated the presence of different species in all five samples. The distinct DGGE patterns of bacterial amplicons from T1, T2, LB and LBG cultures at 4°C (Fig. 6A) and 15°C (Fig. 6B) confirmed the diversity of culturable bacterial communities from each ice sample and their distinctiveness across the cave ice block. A total of 77 DGGE amplicons (Fig. 6) of cultured bacteria at 4°C (37) and 15°C (40) were excised from the gels, reamplified and sequenced, corresponding to 68 distinct bacterial OTUs showing 86-100% identity with environmental sequences (Supplemental Table 5). The closest identity to 18 OTUs originated from cold environments (glaciers, snow pits, ice nuclei, permafrost, Arctic and Antarctic soil, lake sediments, mats, etc.), with 3 from cave-related habitats (lava cave, cave drip water, and karst water rivulet), while 40 others OTUs corresponded to sequences from soil, dust and sand, ground water, coastal and deep sea sediments, rivers and streams, sea and lakes water, biofilms and sediments, thermal springs, etc. (Supplemental Table 5).

Eight bacterial OTUs were conserved among ice layers of different age and/or sediment content and demonstrated homology to *Pseudomonas*, *Serratia*, and *Rahnella* species, and uncultured clones (Supplemental Table 5). A soil bacterium 5V-07 [EU839205] homolog was found in 1-S and 900-O/I ice samples (SM4.1-S.46, SM15.900-O.84 and SM15.900-I.199) was cultivated at 4°C and 15°C, respectively. Also, two ice cave OTUs homologous to *Pseudomonas* sp. [FM161544] (SM15.1-S.93 and SM15.900-I.102) and *Rahnella* sp. [FM161540] (SM15.400-O.96 and SM15.900-O.98), were common to different aged ice layers (Supplemental Table 5). A glacier isolated *Serratia* sp. [LN680099] homolog was found in both 1-S and 900-O samples (SM15.1-S.80 and SM15.900-O.74) cultivated at 15°C. Recent (SM4.1-L.43) and 900 yr-old (SM15.900-I.101) clear ice samples contained a homologue of an Arctic thermal spring uncultured clone [JX257866]. The deep sea sediment homologue *Pseudomonas* sp. [AM111029] was encountered in both SM4.1-S.1 and SM15.900-I.64. In addition to various aged strata, common OTUs were also found in organic rich and clear ice samples of same age (SM4.900-O.10 and SM4.900-I.13), and in the recent ice samples SM4.1-S.37/44 (Supplemental Table 5, Fig. 6).
The similar migration pattern of several DGGE amplicons (Fig. 6) suggested the occurrence of additional bacterial strains common to different cave ice layers, such as the soil bacterium 5V-07 [EU839205], which was present in four of the five analyzed samples (1-S, 400-O and 900-O/I), while Pedobacter steynii [KF583713] (1-L/400-O), Bacillus sp. [KC160801] (1-L/400-O), bacterial clone VS16-38 [JX257866] homologue (1-S/900-I), Pseudomonas clone [AY881672] (400-O/900-I), and uncultured bacterium [FJ527575] (1-S/900-O) could be identified in two different aged ice samples.

The relative abundance of the identified OTUs in Scărişoara ice samples (Fig. 7) highlighted the composition heterogeneity of culturable bacterial communities throughout the cave’s ice block, indicating the ubiquitous presence of Gammaproteobacteria that dominated 1-S and 900-O/I ice layers, and of Firmicutes that was less represented, but with a major presence in 400-O. Bacteroidetes phylum was encountered only in 1-L and 900-O/I samples. Moreover, 1-L sample appeared to contain a higher number of taxa, comprising Actinobacteria and Betaproteobacteria in addition to Gammaproteobacteria, Firmicutes, and Bacteroidetes.

**DISCUSSION**

Our investigation revealed the presence of culturable bacteria in all analyzed samples of Scărişoara Cave ice block up to 900 yr-old, varying in the range of $5 \times 10^2 - 7.8 \times 10^4$ CFU mL$^{-1}$, similar to other glacial habitats (Skidmore et al., 2000; Lee et al., 2011; Bell, 2012). The viability of cultured bacteria, as compared to the total cell content ($2.2 \times 10^4 - 2.9 \times 10^5$ cell mL$^{-1}$), showed a remarkable drop in older ice strata, shifting from 35-52% culturability in recent ice to 0.3-5.3% in the 900 yr-old ice samples.

Cultivation in the presence of different substrates and at different temperatures revealed a various response in terms of growth lag time and doubling time of bacterial communities from all analyzed ice samples, demonstrating their compositional heterogeneity. The abundance of the cultured cave ice-contained bacteria appeared to be influenced by the age and physicochemical properties of the ice substrate, with a lesser impact of ice chemistry (carbon and nitrogen contents).

The cell density of culturable microbiota decreased exponentially with the age of the ice layer. The recent ice samples 1-S and 1-L exhibited the highest cell content when cultivated on R2A at low and high temperatures, while the lowest viable cell content was found in 900 yr-old ice. The viability of cells cultivated at 4°C was generally higher than that at 15°C in all the analyzed ice samples, suggesting a higher resilience of cold-adapted microorganisms in this habitat. A significant difference between bacterial communities cultured at 4°C and 15°C was observed in 400 yr-old ice, favoring low-temperature culturable microbiota.

Physicochemical parameters of the ice samples appear to have a strong effect on the culturable bacterial cell content of this habitat. The high cell density of heterotrophs from the sediment-rich ice samples 1-S and 400-O suggested that the activity and survival of cells embedded in cave-ice was influenced by the high sediment content and TDS content of the ice layers. No clear dependence of the cell content and functional variability of the
samples on the ice chemical characteristics was observed; the recent ice samples 1-S and 1-L showed a comparable culturability, despite of different TOC and TN concentrations and utilized carbon-sources (BIOLOG EcoPlates). Considering the similarities of 1-S and 400-O ice formation (Persoiu & Pazdur, 2011), this quantitative characteristic of cave-ice bacterial communities could be strongly related to the physicochemical characteristics of the cave ice layers. In the case of the common-origin ice layers 900-O and 900-I, both the chemical properties and cultured microbial contents do not showed significant variations, confirming a strong correlation of the bacterial content mainly with the ice age.

The C/N composition of the ice sediments varied considerably with the distance from the cave entrance. The distinct ice formation pattern of 1-S and 1-L samples, corresponding to surface-enriched organic sediments, and cryogenic cave carbonate enriched ice, respectively, was reflected in the total and organic/inorganic carbon and nitrogen contents of the two recent ice samples. Thus, the higher TOC and TN contents of 1-S could originate from both the direct influx of surface-derived organic matter, due to the proximity of the site to cave entrance, and from the activity and decomposition of photrophs flourishing in the sunlit supraglacial lake formed during the warm period (data not shown). Surprisingly, the inorganic carbon (IC) content of the two surface samples 1-S and 1-L was similar, indicating that the variations of dissolved carbonate and cryogenic cave carbonates formed in the two locations (Zak et al., 2008; Persoiu et al., 2011b) were removed in the supraglacial lake, with little or no contribution from bacteria to the calcification process.

As shown by Persoiu & Pazdur (2011), the ice formed near the cave entrance (sample 1-S, Fig. 1A) was rich in surface-originating materials (Feurdean et al., 2011), while that in the central part of the Great Hall (sample 1-L, Fig. 1A) contains mainly autogenic material composed of cryogenic cave carbonates and carbonates derived from the weathering of the cave walls (Zak et al., 2008).

In addition, climate at the time of ice layers formation appeared to play a role in geochemical composition of ice strata, and influence the culturable fraction of embedded bacterial communities. Thus, the climate associated to sample 900-O formation (~1050 AD, the peak of the Medieval Warm Period - MWP) was slightly warmer and drier than during the genesis of 400-O sample (~1550 AD, during the colder and wetter Little Ice Age - LIA). These climatic differences implied changes in the forest composition above the cave, with dominance of beech (Fagus sylvatica) during MWP, and of spruce (Picea abies) during the LIA, respectively (Feurdean et al., 2011). The high TOC and TN contents of the 400-O ice sample relative to 900-O could be related to the enrichment in carbon and nitrogen of the cave-surrounding soil formed during spruce-dominated forests period (LIA), as compared to the one formed during beech-dominated forests period (MWP) (Vesterdal et al., 2008).

The functional heterogeneity of cultured ice bacteria throughout the cave ice block was revealed by the various responses in terms of growth lag time and doubling time when cultivated at 4°C and 15°C on different substrates. The CLPP analysis using BIOLOG EcoPlates revealed an overall higher functional diversity of cold-active bacteria throughout the cave ice block, based on higher H values when cultivated at 4°C. Except for the general use of Tween 40 by all analyzed samples of the cave ice block (Rapala et al., 2005), the carbon-source utilization varied with the age and sediment content of the ice substrate. The functional heterogeneity estimated by BIOLOG EcoPlates cultivation showed a relatively grouped PCA distribution of ice bacterial communities based on the age of the ice, which was independent of their sediment content (900-O and 900-I samples) and light regime (1-S and 1-L samples). In the case of 1-S and 400-O samples, a correlation of cultured bacterial composition and functional heterogeneity with the physicochemical properties of the ice layers could be observed, in direct correlation with their relatively grouped PCA distribution.

In addition, the bacterial functional diversity of the cultured segment of ice microbiota appeared to be dependent also on the origins of water source that formed the different areas of Scărişoara ice block, as indicated by the large shift (2-5-fold reduction) of the H values of the 1-S vs.1-L and 400-O vs. 900-O samples, respectively, and their PCA distribution associated to the physicochemical parameters. Meanwhile, the ice samples of a high sediment-content (1-S, 400-O and 900-O) were characterized by the same TC/TN ratio, but had different cell numbers and culturable bacteria substantiating a moderate impact of the cave-ice chemistry on the microbial community structure from Scărişoara ice block. This cultivable ice-embedded microbiota appears to be dependent in particular on the age and physicochemical properties of the ice substrate.

The 77 identified bacterial sequences in cultured ice samples up to 900 years old were assigned to various species of Pseudomonas, Carnobacterium Rhahnella, Bacillus, Paenibacillus, Lysinibacillus, Sporosarcina, Flavobacterium, Pedobacter, Arthrobacter, Serratia, Yersinia, and unspecified uncultured bacteria. These strains were affiliated to Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria phyla, dominated by Gammaproteobacteria. Common bacterial strains with those described in various cold habitats (Segawa et al., 2010; Margesin & Miteva, 2011; Wong et al., 2011; Anesio & Laybourn-Parry, 2012) were found in Scărişoara cave ice block, confirming a common distribution of specific strains in glacial environments. Among these, the Arthrobacter strain SM4.1-L.33 [KF853212] identified in recent ice deposits (1-L sample) of Scărişoara cave and grown at 4°C corresponded (97% identity) to a strain isolated from a 25,000 years old permafrost ice wedge (Katayama et al., 2007). Facultative psychrophilic species belonging to this genus (Arthrobacter psychrophilenolicus) have also been identified in an Austrian alpine ice cave (Margesin et al., 2004).
Interestingly, the presence in old ice strata (400-O and 900-O) of a karst water-specific Rahnella species, an endophytic bacteria from spruce (Picea abies) seeds (Cankar et al., 2006), provides a putative microbial biomarker candidate for distinguishing between the two periods based on the quantitative representation of this bacterium in ice layers from dominating (LIA) or scarcer (WMP) spruce forests occurring in the surroundings of the cave at the time of the ice deposition (Feurdean et al., 2011). However, quantitative analysis of both Rahnella representation as well as climate and vegetation dynamics outside the cave is required in order to confirm this hypothesis. Another cave-associated bacterium was the cultured strain SM15.400-O.62 [KJ454424] from 400-O ice sample, homologous (99% identity) to a lava-cave bacterium clone, indicating the presence of specific bacteria for cave environments.

Conservation of bacterial species in increasing aged ice sediments, most of them as culturable organisms, was confirmed in the cases of different OTUs belonging to particular Pseudomonas and Paenibacillus genera, and uncultured bacteria from soil and Arctic thermal springs.

In addition to the identified cultured phyotypes, the high ratio (28.2% average value) of unclassified bacterial OTUs found in Scărișoara Cave ice block, reaching 42.8% in the case of 400-O sample, suggested a higher bacterial diversity of the cave ice microbiota. Also, the low identity score (86-96%) of several cultured ice bacterial sequences suggest the ability to identify novel species in this glacial habitat. Further investigations of environmental samples should provide a more accurate overview of the cave ice-microbiota, overcoming the limitations induced by cultivation and PCR amplification, and revealing also the autotrophic bacterial communities that are expected to be prominent in this type of habitat.

This first report on the time and space-dependence of cultured bacterial communities from a perennial cave ice block and continues to contribute to the characterization of Scărișoara Ice Cave, which is already well-documented glacial habitat from geological and palaeoclimatic perspectives (Racoviță & Onac, 2000; Persoiu & Pazdur, 2011). By identifying bacterial species, and highlighting differences on the abundance, distribution, and diversity of the cave ice-block-embedded bacterial communities for the last millennium, our data support the hypothesis of a close relationship between climatic-related source microbiota and the cave-ice bacterial community composition, allowing for identifying possible climate biomarkers in this underground glacial habitat.

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