Extremophilic fungi in arctic ice: a relationship between adaptation to low temperature and water activity

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Abstract

Little is known about fungal diversity in extremely cold regions. Low temperatures induce the formation of ice crystals and therefore also the creation of low water activity (aw). These are the dominant factors in external chemistry that influence microbial biota in cold regions. Therefore, we have used selective low water activity media plus low incubation temperatures for the isolation of fungi from an Arctic environment. In comparison with the highest values of colony forming units (CFU) obtained on mesophilic media, considerably higher fungal CFU per litre of water were detected on low aw media, ranging from 1000 to 3000 l−1 in seawater, 6000 to 7000 l−1 in melted sea ice and up to 13,000 l−1 in melted glacier ice. The dominant taxa were ascomycetous and basidiomycetous yeasts, melanized fungi, mainly represented by the genera Cladosporium and Aureobasidium plus different species of the genus Penicillium. Preliminary taxonomic analyses revealed several new species and varieties. Further characterisations are needed to determine whether this diversity is due to geographic isolation, ecological conditions or independent evolutionary origin.

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1. Introduction

Life in extreme environments is dominated by microorganisms. The Arctic and Antarctic regions have over the last decade been investigated mainly for the presence of psychrophilic bacteria and Archaea, and occasionally for algae, but rarely for fungi (Abyzov, 1993; Vishniac, 1993; Nienow and Friedman, 1993; Broady, 1993; Ma et al., 1999a). Recently it has been shown that fungi are not only able to sustain, but also to propagate at different environmental extremes such as hypersaline waters (Gunde-Cimerman et al., 2000), dry rock surfaces (Steflinger, 1998) and ocean depths (Lopez-Garcia et al., 2001). Furthermore, fungi have occasionally been isolated from extremely cold environments, such as vegetation (Babjeva and Reshetova, 1998; Tosil et al., 2002), permafrost (Golubev, 1998; Vishniac, 1993; Nienow and Friedman, 1993; Broady, 1993; Soinam et al., 2000), water (Broady and Weinstein, 1998; Botha and Wolfaardt, 2000), snow (Abyzov, 1993) and glacier ice (Abyzov, 1993; Reeve et al., 2002; Ma et al., 1999a). Media used for these isolations were of different compositions, but in all cases they supported mesophilic growth, with a water activity (aw) value of ≈1.0.

Active microbial growth in extremely cold environments is under the influence of ice formation and consequently of little biologically available liquid water. Thus, water activity (rather than extremely low temperatures) in habitats such as snow, sea ice and glacier ice, is the dominant factor in external chemistry that influences microbial activity. During freezing and binding of water in ice crystals, ions are expelled and ion concentration in the remaining liquid water increases.

Therefore, in the present experiments, isolation conditions were designed to accommodate xerotolerant/halotolerant fungi from ice by using media with high
concentrations of salt or sugar, and thus low water activity. These media should give a selective advantage to culturable microorganisms that are adapted to ice, thereby possibly enabling the isolation of higher fungal CFU numbers than previously have been reported.

2. Materials and methods

2.1. Isolation site

Kongsfjorden is one of the large fjords found on the western coast of Spitsbergen, in the Svalbard Archipelago, located at 79°N, 12°E. It is 26 km long and 8 km wide and stretches from ESE to WNW at the Greenland Sea. The majority of the drainage basin is covered by glaciers, which calve pieces of glacier ice into the fjord throughout the year. The annual mean temperature is around −5 °C, but the water is warmer and less salty than the open sea during the summer. The fjord water temperature is ≥0 °C at the end of May and 3.8 °C by the end of August. The mean salinity is from 34.00 to 35.00 PSU. Lowering of salinity can occur in summer and near the surface (Ito and Koduh, 1997).

2.2. Isolation methods

Samples of seawater were taken at six different locations within the fjord, while sea ice was collected close to the head of the fjord. Twenty pieces of floating glacier ice were collected in the fjord and glacier ice was sampled directly from the Kronebreen glacier. All samples, with the exception of sea ice, were collected in June and August 2001. Sea-ice samples were incubated on the media. After sampling, the ice was melted aseptically at room temperature. Filtration of 10–100 ml aliquots of water was performed immediately on Millipore membrane filters (0.45 μm pore size) and placed on two enumeration and 10 different selective agar media having either high salt or high sugar concentrations. A drop of the original sample was applied on the membrane and dispersed with a Drigalski spatula. For every ice sample and medium at least four and up to 10 aliquots were filtered in parallel, and the average numbers of CFU were calculated (Gunde-Cimerman et al., 2000). Differences in CFU/100 ml among replicate aliquots were up to 5%. Plates were incubated for up to 14 weeks at 10, 15 and 22 °C. The CFU on enumeration media were counted after 3, 5, 7, 14, 30, 60 and 100 days of incubation. The following solid agar media were used.

For enumeration of CFU per 1000 ml of water, a general purpose medium DRBC (a_w = 1.000) (King et al., 1979), a general-purpose enumeration medium and a medium for detection of moderate xerophiles DG-18 (a_w = 0.946) (Hocking and Pitt, 1980) were used. The selective media containing sugar used for isolation of fungi were: 10% glucose−12% NaCl agar (MY10-12, a_w = 0.880) and malt yeast extract medium with 20/35/50% glucose (MYG, a_w = 0.941–0.890). For salt-based media, different NaCl concentrations (5/10/15/17/24/30%) were added to malt extract agar (MEA, a_w = 0.951–0.782). For the prevention of bacterial growth, chloramphenicol (50 mg/l) was added to all media. Water activities (a_w) of the media, as a measure of the unbound water which is available for the growth of microorganisms (Northolt et al., 1995), were determined using the EX-F Culture Collection of the Department of Biology, Biotechnical faculty, University of Ljubljana, Slovenia.

2.3. Taxonomy

Isolated melanized fungi were identified by their cellular morphology and physiology, and by the sequencing of their ITS rDNA (Hoog et al., 1999; Zalar et al., 1999) to the genus and in some cases species level. Isolates of the genera Penicillium have been identified to the species level by morphology, physiology and secondary metabolite profiles using HPLC (Frisvad and Thrane, 1995). Isolates of non-melanized yeasts have not yet been identified. All isolated strains have been deposited in the EX-F Culture Collection of the Department of Biology, Biotechnical faculty, University of Ljubljana, Slovenia.

2.4. Determination of environmental parameters

For all water samples, the pH (ISO 10253: 1994E electrochemical method), temperature, and Na^+, Mg^{2+} and K^+ cation concentrations (ICP, AES Thermo Jarell Ash), total phosphorus content (determined spectrophotometrically after mineralization with persulfate, ISO 6878/1: 1986E) and water activity (a_w) (CX-1 system, Campbell Scientific Ltd) were determined (Gunde-Cimerman et al., 2000).

3. Results

3.1. Environmental parameters

The pH of the water, from which isolates were obtained, varied between 7.1 and 7.4 in all samples. The highest cation concentrations were determined for seawater and the lowest for glacier ice, where they ranged for sodium from 5 to 340 mg/kg, for potassium from 20 to 310 mg/kg and for magnesium from 70 to 550 mg/kg, respectively. The highest phosphorus content was determined in glacier-ice samples and the lowest in the remainder (<1.00).

3.2. Isolation of fungi on enumeration media

The results obtained on enumeration media are presented in Fig. 1. The total number of fungal CFU on
DRBC, a general-purpose medium with \( a_w = 1.000 \), was \( \approx 3000 \) CFU/l in seawater and 5000 CFU/l in glacier ice, but only up to 200 CFU/l in sea ice. When estimates of CFU were performed using an enumeration medium for xerophiles, with \( a_w \) lowered to 0.946, the numbers of CFU increased most for sea and glacier ice. The highest individual values reached 13,000 CFU/l, whereas the mean values for glacier ice were in the range of 8000–9000 CFU/l and for sea ice from 6000 to 7000 l\(^{-1}\). The lowest values, \( \approx 1500 \) CFU/l, were detected in seawater.

With the exception of seawater in all cases, the CFU numbers obtained on \( a_w = 1.0 \) medium were always considerably higher at 22 °C than at 15 °C. The incubation time was generally shorter on the low-\( a_w \) medium (2–3 days) than on the mesophilic medium (3–5 days) and always shorter at 22 °C. The majority of fungi detected on both enumeration media were represented by non-melanized yeasts, although the proportion of melanized yeasts increased considerably in glacier ice samples in comparison with sea ice and seawater. The genus *Penicillium* appeared in seawater as well as in glacier ice.

### 3.3. Isolation of fungi on selective sugar media

The population dynamics of fungi on sugar-based media are presented in Fig. 2. On selective media, with lowered \( a_w \) due to increased sugar concentrations, the highest CFU values of fungi were detected in glacier ice. They ranged from 6000 to 8000 CFU/l, which was considerably higher than in seawater, where CFU values never exceeded 500 l\(^{-1}\) and were generally lower. In all cases, the highest CFU values were obtained on medium
with 20% glucose \( (a_w = 0.941) \) and the lowest on 50% glucose \( (a_w = 0.890) \), while no fungi were detected on the medium with even lower \( a_w \) (data not shown). Non-melanized yeasts dominated in all samples, although the ratio of melanized to non-melanized fungi increased considerably in the glacier and sea-ice samples, where they occasionally represented up to 90% of all mycobiota, whilst they were extremely rare in seawater. The majority of non-melanized yeasts was detected on 20% glucose \( (a_w = 0.941) \), considerably less on 35% glucose \( (a_w = 0.915) \), whereas they almost completely disappeared from 50% glucose \( (a_w = 0.890) \), where the melanized yeast-like genus *Aureobasidium* sp. and *Cladosporium* spp. dominated. The third group of isolated mycobiota was represented by filamentous fungi, mainly different species from the ubiquitous genus *Penicillium*. They appeared more frequently in those ice samples with higher quantities of sediment, where they occasionally represented up to 25% of all isolated fungi.

### 3.4. Isolation of fungi on selective saline media

The population dynamics of fungi on salt-based media are presented in Fig. 3. On selective saline media, the highest individual fungal CFU, up to 13,000 l\(^{-1}\) in seawater, were detected again in the glacier-ice samples and the lowest (up to 2000 l\(^{-1}\)) in seawater. The highest values were obtained in both cases on the medium with 5% NaCl \( (a_w = 0.951) \). With increasing salinity, the number of fungal CFU decreased. Therefore, the upper salinity range for the detection of fungi was 24% NaCl \( (a_w = 0.828) \), with CFU values only up to 5 CFU/l. At lower salinities non-melanized yeast represented the majority, but with increasing salinity the proportions changed in favour of melanized fungi and *Penicillium* spp., with *P. crustosum* again being the most frequently isolated species.

### 3.5. Biodiversity of isolated *Penicillium* spp

The biodiversity and ratios of individual *Penicillium* species are presented in Fig. 4. The most frequently isolated *Penicillium* species was *P. crustosum*. In comparison with other *Penicillium* species, its CFU numbers increased from \( \approx 80 \) l\(^{-1}\) in seawater, to \( \approx 200 \) l\(^{-1}\) in sea ice, to \( \approx 1000 \) l\(^{-1}\) in glacier ice, respectively. The diversity of species was the highest in seawater, but decreased in sea ice and glacier ice, where fewer species were detected, representing higher proportions. The detected species were *P. groenlandense*, *P. chrysogenum*, *P. nalgiovense*, *P. commune*, *P. lanosum*, *P. echinulatum*, *P. palitans*, *P. brevicompactum*, *P.olsonii* and *P. decumbens*, plus three species that are probably new to science and have tentatively been named *P. nordicum*, *P. arcticum* and *P. svalbardense*.

### 4. Discussion

Atmospheric circulation over polar regions provides air-mass exchange with lower latitudes. Microorganisms transported on terrestrial dust and in precipitation become embedded in the ice formed from falling snow. Ice is therefore a natural air-sampling medium, which can entrap microorganisms that may have originated in the vicinity of the deposited ice, or they may have traveled from far away. Glacial ice may also contain organic matter of Aeolian origin as well as layers of sand and other inclusions (Abyzov, 1993; Ma et al., 1999a). It can be considered an excellent matrix for long-term preser-
vation of microorganisms and allows, as such, the study of both contemporary and ancient microbial diversity. Thus, sampling of glacier ice has repeatedly demonstrated the presence of microorganisms, characteristic to the polar regions, as well as species from temperate and tropical regions in small numbers (Ma et al., 1999a,b).

Ice-structure models suggest the presence of liquid channels around ice crystals, formed due to expelled salts as the ice crystals freeze together. Shifts in salinities in the ice occur on a micro level, while on a macro level areas of dry and wet alternate (Priscu et al., 1998). Thin films of liquid water around embedded grains of sediments and cryoconite holes have been reported as being potential microniches for active microbial growth in ice (Reeve et al., 2002; Felip et al., 1995; Sawstrom et al., 2002; Wharton et al., 1985).

We have surmised that certain fungi adapted to low water activity and high salt concentrations as well as sustaining low temperatures, could grow at these conditions, thus maintaining a continuous colonization of the ice. Fungi have so far not been much studied in natural, extremely cold environments. Although viable fungi have been discovered in some samples from ice layers up to 140,000 years old (Ma et al., 1999a), the numbers found were low and did not exceed a few hundred CFU/l.

Halophilic and xerophilic fungi have recently been isolated from rocks and natural hypersaline environments. It is interesting to speculate whether these species share some features with those isolated from ice. Whereas salinity creates both ionic and osmotic stress drought, low temperature per se cause osmotic stress. Freezing leads to cellular dehydration due to reduced water absorption and conduction, whereas high salinity causes the same effects due to osmotic imbalances. Compatible solutes are known to accumulate in response to certain physical stresses, such as desiccation and high salinity, but have so far been ignored with regard to protection against freezing temperatures. Since protection against dehydration damage is correlated with intracellular accumulation of compatible solutes, an increase in the amount of non-freezable water may arise through the production of such solutes as low molecular weight sugar alcohols (polyols) or sugars (trehalose). Thus, there may be a relation between resistance to drought, high salinity and cold in fungal species.

Therefore, using low water activity media for the isolations of halophilic/xerophilic fungi from hypersaline waters, we expected to obtain higher CFU numbers than previously reported from ice samples. This has now been confirmed by the present study and is supported by the fact that the main groups of isolated fungi that were isolated in Kongsfjorden appear as well in solar salterns of the Mediterranean coast (Gunde-Cimerman et al., 2000). These isolates included melanized fungi that were mainly represented by the oligotrophic genus Cladosporium, and were taxonomically and phylogenetically closely related to black yeast-like hyphomycetes, mainly from the genus Aureobasidium. Both genera have previously been sporadically isolated from the polar regions, specifically C. sphaerospermum, C. herbarum and A. pullulans (Abyzov, 1993; Ma et al., 1999a).

Among the non-melanized fungi that were isolated, it was different species of the known food-borne xerophilic genera that predominated, particularly the cosmopolitan genus Penicillium, in accordance with the fact that
many species of this genus prefer growth at lower temperatures and can be isolated in alpine and tundra soils as well as in permafrost layers. The diversity and occurrence of *Penicillium* species in the ice samples from Kongsfjorden revealed a hitherto unparalleled richness and abundance.

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**References**


