Isolation and Identification of Cold-Adapted Fungi in the Fox Permafrost Tunnel, Alaska

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Abstract

Permafrost microbiology is important for understanding biogeochemical processes, paleoecology, and life in extreme environments. Within the Fox, Alaska, permafrost tunnel, fungi grow on tunnel walls despite below freezing (-3°C) temperatures for the past 15,000 years. We collected fungal mycelia from ice, Pleistocene roots, and frozen loess. We identified the fungi by PCR, amplifying the ITS region of rRNA and searching for related sequences. The fungi within the tunnel were predominantly one genus, *Geomyces*, a cold-adapted fungi, and has likely “contaminated” the permafrost tunnel from outside. We were unable to obtain DNA or fungal isolates from the frozen loess, indicating fungal survival in permafrost soils can be strongly restricted. *Geomyces* can degrade complex carbon compounds, but we are unable to determine whether this is occurring. Results from this study suggest *Geomyces* may be an important colonizer species of other permafrost environments.

Keywords: Fox tunnel; fungi; *Geomyces*; ice wedge; loess; permafrost.

Introduction

The permafrost tunnel near Fox, Alaska, was constructed in the early 1960s to examine mining, tunneling, and construction techniques in permafrost. The tunnel was constructed, and continues to be maintained, by the U.S. Army Cold Regions Research and Engineering Laboratory. The tunnel consists of ice-cemented loess, massive ice, and ice wedges that have been dated from 12 kbp to 40 kbp. The permafrost present in the tunnel is syngenetic with multiple exposures of primary and secondary ice features (Bray et al. 2006, Shur et al. 2004). Fungal growth has been observed on the interior walls. The interior walls, ice wedges, and hanging roots within the tunnel are covered with white fungal mycelia all year. Although the temperature in the permafrost tunnel has remained below freezing (approximately -3°C) for at least the past 15,000 years (Katayama et al. 2007) microbial activity is still present. The tunnel is also very dry and there is no evidence of liquid water. As a consequence, the organisms that grow in the tunnel must be adapted to cold and dry environments.

Observational evidence, such as fungal growth appearing from drilled holes and nails, suggests that the fungal organisms were contaminants brought in from outside the tunnel either from air contamination or on sampling equipment. Fungal growth was observed not only on the loess-rich interior walls, but mycelia often completely carpeted ice wedges. The widespread nature of the fungi throughout the tunnel suggests that it can be transported by air currents. It may also be possible that the fungi occurs on ice surfaces and around drill holes because it is moisture-limited. When a hole is drilled, ice-cemented material is exposed and immediately starts to melt and then sublimate. Therefore, when a hole is drilled, moisture is liberated, and fungal growth at these sites should be possible.

Our research objective was to determine the identity of the fungal organism(s) covering the interior walls and ice wedges of the permafrost tunnel. Understanding their taxonomy could help us understand the microbiology of permafrost environments and determine the range of environmental parameters in which the some fungi can grow. A secondary objective was to determine whether the identified organisms have important characteristics related to biogeochemical processes or human health.

Methods

Soil sampling

To determine the identity of the fungal organisms throughout the permafrost tunnel and on different substrates, five samples were collected from interior walls, hanging roots, and ice wedges. Three samples were taken from the interior walls by scraping the fungus gently with the end of a sterile plastic centrifuge tube. When doing this it was common for loess particles to enter the tube (Figs. 1a, b, d). One sample was taken from an ice wedge in the ceiling in the side corridor (winze) of the tunnel (Fig. 1c). The mycelial “mat” was covering the ice wedge and was easily collected with tweezers and placed in a sterile centrifuge tube (Fig. 1c). The fourth sample was taken from a root hanging from the ceiling of the main adit where the adit and winze separate near the entrance (Fig. 1e). The root was covered in mycelia and the root was broken off with tweezers and placed in a sterile centrifuge tube. The tubes were frozen that same day before being shipped to Menlo Park. The fifth sample was ice that had accumulated in the winze. The ice was collected with a sterile hypodermic needle (Fig. 1f).
day and later transported to the USGS in Menlo Park for isolation and molecular characterization. We also sampled loess samples from the wall using a serrated metal drill corer (keyhole corer) that had been sterilized using ethanol. In the lab, we further attempted to minimize any potential contamination by scraping of the exterior of the core with sterile razor blades and sampling the interior of the frozen loess core. Carbon and nitrogen concentrations of loess were quantified on oven-dried material (105°C, 48 h) on a Carlo Erba C/N analyzer.

Soil preparation

Soil or fungal flocs were weighed out into 2 ml microcentrifuge tubes (Eppendorf Inc., Westbury, NY) in 0.5 g increments. Under sterile technique, 1.5 ml of DNase/RNase free water (Eppendorf Inc., Westbury, NY) was added to the soil samples or fungal mat. The soil slurry was vortexed for 10 minutes. The slurry was then centrifuged at 10,000 rpm for 1 minute. The supernatant was then serially diluted 10-fold, 100-fold and 1000-fold. These serial dilutions were then plated onto standard potato dextrose agar plates (Difco Inc., Lawrence, KS). As a control the wet soil from the previous step and the dry soil from the original sample were also plated onto potato dextrose agar plates (Difco Inc., Lawrence, KS).

Growth conditions

Plates of each dilution were grown at 0, 4, 20, and 37°C in the dark for 28–31 days. Fungal growth did not occur at 20 or 37°C but did occur at 0 and 4°C. Fungal isolates that appeared on the 4°C plates were isolated onto fresh potato dextrose plates. This step of removing isolates from plates took another 2–3 months of replica plating until there was no difference in isolate morphology. Pure cultures were tested for pH sensitivity at various pHs ranging from 2–8 on potato dextrose agar (Difco Inc., Lawrence, KS) over a period of one month. Growth was only observed at pH 5 and 6.

DNA isolation

Single isolates were removed from agar plates by scraping, placed in liquid nitrogen, and homogenized with a mortar and pestle. DNA was isolated from the homogenized samples following gram-positive bacteria DNA isolation protocol from a Purelink Genomic DNA mini kit (Invitrogen Inc., Carlsbad, CA). There was no fungal protocol with this kit. The isolated DNA was quantified using a standard Picogreen dsDNA assay (Invitrogen Inc., Carlsbad, CA). We also attempted to extract DNA from loess samples in bare (no mycelia) areas using a Powersoil DNA extraction kit (Mo Bio, Inc.), but agarose gel electrophoresis and the picogreen dsDNA assay showed that there was no measurable DNA in the extract. PCR was also tried but was not successful.

PCR conditions

Fungal DNA from isolates was amplified using ITS1-ITS4 primers in a PCR reaction using 0.5 to 1.0 μl of genomic fungal DNA. BSA at a final concentration of 1μg/ml was used to bind to common PCR inhibitors. Thermocycler parameters were an initial melting step 95°C (10 minutes one cycle), followed by 40 cycles of 95°C (1 minute), 53°C (30s), and 72°C (1 minute). PCR was concluded with a long extension step of 72°C (10 minutes), and then the PCR reactions were placed in the -20°C freezer.
Sequence analysis

Amplified DNA was sequenced at a commercial lab (MClab, San Mateo, CA), and results were imported into Geneious software (Biomatters Ltd., New Zealand). Sequences were examined for quality and Blast searched on the NCBI database. The three best matches were recorded.

Restriction analysis

Restriction analysis was performed on the PCR products in order to determine if the sequences were of the same or different organisms. PCR products were digested overnight with the enzyme CFO1. BSA was added at a concentration of 10 μg/μl to aid in enzymatic digestion. Digests were then run on a 3% gel for 90 min at 75 volts, with a 100 bp ladder (NEB Inc, Ipswich, MA), and negative controls. The gel was stained in ethydium bromide, rinsed for 20 min, and digitally photographed on a UV light table.

Results and Discussion

White mycelia are present on tunnel walls, ice wedges, and plant roots within the permafrost cave near Fox, Alaska, (Figs. 1a–f). The fungal organisms sampled and isolated from multiple locations within the Fox permafrost tunnel were all of the same genus, Geomyces. Two of the organisms were identified as Geomyces pannorum, while three other samples were closely associated with Geomyces strain FMCC-4 (Table 1). Restriction digests of the PCR products produced different restriction patterns for most of the organisms (Fig. 2). The restriction patterns for sample FG1 and FG2 were the only two samples to be identical to each other. Except for FG1 and FG2, all samples had unique restriction patterns, indicating that they had slightly different DNA sequences. Therefore, although they were all the same genus, they differed at the species or strain level.

The physiology of Geomyces is such that it is very well adapted to growth in the cold, dark, and dry conditions of the permafrost tunnel. Fungi are generally well suited for growing in dry habitats due to their hyphal growth form and unique forms of osmoprotection. Additionally, Geomyces is one of only a few fungal organisms known for growth below freezing, which would be a necessity for any organism living within this frozen environment (Ozerskaya et al. 2005, Panikov & Sizova 2007). Geomyces was not capable of growth at 20°C or above, but grew well, albeit slowly, at 4°C. Geomyces is considered to be a psychrotrophic fungi, which is an organism that can grow at 0°C, but its optimum growth rate is above 20°C. (Gilichinsky et al. 2005, Robinson 2001). Our results indicate that our isolates could not grow at 20°C or above, and therefore they should be considered psychrophilic fungi.

Geomyces is a commonly observed fungi in boreal and arctic ecosystems that can survive in permafrost environments due to its ability to grow at cold temperatures, its ability to withstand moisture stress and high salt tolerance (Lydolph et al. 2005, Robinson 2001). Geomyces has the ability to break down keratin, a compound contained within hair and nails, as well as cellulose, present in plant tissues (Freiedrich et al.). Because Geomyces is a keratinolytic fungi, it has been used as an indicator of the presence of ancient megafauna (due to the presence of hair and nails in some permafrost). The use of Geomyces as an “indicator species” is supported by this study because of the visible presence of ancient megafauna (generally bones) contained within the Pleistocene loess deposits (Willerslev et al. 2004). Cellulose may also be present in this permafrost environment due to the visible presence of plant roots, although Geomyces appeared no more dense on plant roots than on the loess tunnel walls.

Geomyces appeared to be capable of much more extensive growth on ice wedges compared to either roots or ice-cemented loess walls (compare Fig. 1c with others). The growth forms of Geomyces differed between the ice wedge and the permafrost tunnel walls. Geomyces often formed thin brittle mycelial sheets over the entire surface of the ice wedge that could be easily sampled with a small metal instrument. The ice wedges were dark in color and exhibited elevated DOC dissolved organic carbon concentrations (18.4 to 68.5 ppm; Douglas & Cai, unpublished data) which is probably a strong source of carbon substrate for the fungi.
On ice-cemented loess walls, *Geomyces* tended to have a spotty distribution. Growth was generally circular emanating from previously sampled areas, and the mycelia could not be easily sampled without breaking off loess particles from the walls. The growth of *Geomyces* on loess walls tended to be associated with disturbance or points of contamination, where people would, for example, place nonsterile sampling instruments on their surface. The loess walls had carbon and nitrogen concentrations of 2.75% and 0.25%, respectively, and had moisture contents ranging from 18 to 55%. Root samples were not examined for nutrient content, but organic tissues can generally range from 40 to 50% carbon and 1 to 5% N. Therefore, these substrates had enough C and N for microbial growth.

Surprisingly, no other genus of organism except *Geomyces* was isolated from the tunnel. It is possible that given our growth conditions for isolation, we selected for this organism, although we attempted isolation at multiple pH and temperature ranges. This does not preclude other organisms from being present in the tunnel, but it is a strong indication that all of the organisms visible within the tunnel are *Geomyces*. Restriction analysis showed that we isolated several species or strains of *Geomyces*. Although all of our isolates were of the same genus, restriction analysis showed that there were slight differences in their ITS or ribosomal sequences (Fig. 2). It is likely the fact that the isolated *Geomyces* fungi is common in boreal soils, can grow well at cold temperatures, and can be easily dispersed in air that allows it to be the dominant (or only) fungal organism visibly present on the walls of the permafrost tunnel.

We also attempted to isolate DNA from the ice-cemented loess walls in order to compare the fungal DNA present within the loess to the isolates. However, we were either unsuccessful at extracting DNA or the concentration of DNA within the ice-cemented loess walls was so low that we could not measure it, even using sensitive fluorometric techniques. We have been successful at extracting DNA from surface permafrost loess samples (1 m below the soil surface in boreal forests), which leads us to conclude that DNA concentrations within the tunnel loess walls were so low as to be unquantifiable. Our inability to extract DNA from loess in the Fox tunnel may have been due to the fact that few organisms survive frozen conditions for long periods of time (Panikov & Sizova 2007), and DNA quality in frozen soils is reduced over millennia (Willerslev et al. 2004).

We suspect that *Geomyces* in the Fox tunnel is a contaminant from outside the tunnel, rather than an organism that was present in the extant permafrost that has begun to grow on disturbed tunnel walls. *Geomyces* is a common soil organism associated with black spruce (Filion et al. 2004) and therefore is probably very prevalent in the surrounding environment. *Geomyces* growth is observed primarily around sampling holes, metal nails, and areas where the soil has been disturbed by human activity. This could have been caused by using nonsterile tools during sampling. Alternatively, small amounts of soil moisture that are released upon sampling the frozen walls (heat produced by friction between sampling equipment and wall) would permit water-limited fungi to grow rapidly in disturbed areas. The door to the Fox permafrost tunnel is often open and there is good air circulation through the tunnel. Therefore, it would be possible for organisms to contaminate the tunnel from outside.

There are some minor human health aspects to consider with regard to *Geomyces*. It is considered an indoor mold that reduces the air quality within some buildings. It is also found...
in household dust, damp walls, and archived paper such as in libraries. It is not considered dangerous. However, one variety of *Geomyces*, *Geomyces pannorum var pannorum* is suspected in creating slight skin and nail infections (Bloom et al. 2007).

**Cold-adapted fungi and biogeochemical cycling**

Carbon and nitrogen cycling within thawed permafrost soils is an important area of global change research (Zimov et al. 2006). This study highlights two factors that make permafrost environments unique microbiologically which, in turn, are important for biogeochemical cycling.

First, results from the permafrost tunnel and permafrost studies at the ground surface indicate that fungal abundance in permafrost soils is very low (Gilichinsky et al. 2005, Waldrop et al., unpubl.). Microbial diversity in permafrost soil is also likely to be restricted because fewer organisms are able to withstand frozen temperatures for long periods of time. Therefore, as permafrost soils thaw, what will be the fungal organisms that enter into this new biological niche? Likely it will be cold-adapted organisms not unlike *Geomyces* in this study. Therefore, the study of cold-adapted fungi in C and N cycling is an important area of future research.

Secondly, the temperature response of microbial activity in cold permafrost environments with low fungal diversity may have to be carefully evaluated. Normally, microbial activity increases with temperature, but cold-adapted fungi, such as *Geomyces*, may have faster growth rates and enzyme activities at low temperatures than at higher temperatures (Robinson 2001). An area of further study will be to determine the fungal organisms that act as primary colonizers of permafrost soils as they thaw. Could low microbial diversity in permafrost soils affect the efficiency or rate of biogeochemical processes as permafrost soils thaw? Given that soil microorganisms mediate biogeochemical cycles and the lack of data on permafrost microbiology, there is certainly a strong research need in this area.

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**References**


