CrossMark

ORIGINAL PAPER

Growth of high-elevation *Cryptococcus* sp. during extreme freeze–thaw cycles

L. Vimercati[1] · S. Hamsher[1] · Z. Schubert[1] · S. K. Schmidt[1]

Abstract Soils above 6000 m.a.s.l. are among the most extreme environments on Earth, especially on high, dry volcanoes where soil temperatures cycle between -10 and 30 °C on a typical summer day. Previous studies have shown that such sites are dominated by yeast in the cryophilic *Cryptococcus* group, but it is unclear if they can actually grow (or are just surviving) under extreme freeze–thaw conditions. We carried out a series of experiments to determine if *Cryptococcus* could grow during freeze–thaw cycles similar to those measured under field conditions. We found that *Cryptococcus* phylotypes increased in relative abundance in soils subjected to 48 days of freeze–thaw cycles, becoming the dominant organisms in the soil. In addition, pure cultures of *Cryptococcus* isolated from these same soils were able to grow in liquid cultures subjected to daily freeze–thaw cycles, despite the fact that the culture medium froze solid every night. Furthermore, we showed that this organism is metabolically versatile and phylogenetically almost identical to strains from Antarctic Dry Valley soils. Taken together these results indicate that this organism has unique metabolic and temperature adaptations that make it able to thrive in one of the harshest and climatically volatile places on Earth.

Introduction

Recent research indicates that some of the most extreme soil ecosystems on Earth are found at high elevations in dry valleys and slopes of the high Andes (Schmidt et al. 2009, 2012). In fact, the driest high mountains on Earth occur just to the east of the Atacama Desert, where numerous, massive strato-volcanoes rise majestically from the Puna de Atacama (Costello et al. 2009). The highest of these volcanoes is the brooding mass of Volcán Llullaillaco (el. 6739 m.), the location of the highest elevation archeological sites on Earth (Reinhard 1999; Reinhard and Ceruti 2010) and "arguably the best naturally preserved assemblage of mummies found anywhere in the world" (Wilson et al. 2013). From a microbiological perspective these mummies are also of great interest because they showed almost no signs of decay after being buried without preserving chemicals for 500 years. The lack of natural decay of these mummies may indicate an environment that is too cold and dry for the proliferation of decaying microbes and inspired several microbiological expeditions to these mountains over the last 10 years (Costello et al. 2009; Lynch et al. 2012).

The present study builds on our initial studies of the biogeochemistry and microbiology of the high slopes of Volcán Llullaillaco and nearby Volcán Socompa (Costello et al. 2009; Lynch et al. 2012). These studies showed that the soils on these volcanoes contained microbial communities of extremely low diversity, except in areas near

Communicated by F. Robb.

✉ S. K. Schmidt
steve.schmidt@colorado.edu

L. Vimercati
lara.vimercati@colorado.edu

S. Hamsher
sarah.hamsher@colorado.edu

Z. Schubert
zachary.schubert@colorado.edu

1 Department of Ecology and Evolutionary Biology, University of Colorado, Campus Box 334, Boulder, CO 80309, USA

fumaroles (Costello et al. 2009). Perhaps most surprising was the extremely low diversity of eukaryotic microbes found in non-fumarolic soils on these volcanoes. The dominant eukaryotes in culture-independent studies of both volcanoes are related to endolithic and xerotolerant yeasts in the genus *Cryptococcus* (Basidiomycota) that are closely related to organisms isolated from soils of the Dry Valleys of Antarctica and the Himalayas (Schmidt et al. 2012). These yeasts from the Dry Valleys of Antarctica and the Himalayas have been known for many years (Goto and Sugiyama 1970; Vishniac and Hempfling 1979a), but their ecological role in extreme soils systems is still largely unknown. The genus *Cryptococcus* is very common in soils of extreme polar regions, dominating Antarctic eukaryotic libraries (Buzzini et al. 2012) and representing one third of species in the Arctic libraries (Buzzini et al. 2012). Recent studies in European and South American glaciers have also found an extremely high abundance of *Cryptococcus*, up to 39 % and 33 % of total yeast species, respectively (Buzzini et al. 2012). Given their ubiquity in extreme high-elevation and high-latitude soils, it could be that they are just dormant cells that are globally distributed from less extreme systems. Conversely, they may be important functional components of extreme soils, but little evidence of their functional role exists (Vishniac and Hempfling 1979b).

Daily temperature cycling across the freezing point is considered to be a key challenge for microbial growth and survival at high elevations and high latitudes. Previous studies have shown that freeze–thaw cycles frequency influence microbial communities in the cryosphere (Yergeau and Kowalchuk 2008) and limit net primary productivity in the extreme soils of the Dry Valleys of Antarctica, where daily fluctuations vary more than 20 °C per day during the austral summer (Cary et al. 2010). Mineral soils at high elevation on Llullaillaco and other nearby volcanoes can experience even more extreme diurnal temperature fluctuation than soils of the Antarctica Dry Valleys (Lynch et al. 2012; Schmidt 1999; Schubert 2014). The purpose of this study is to investigate: (1) whether *Cryptococcus* can grow during extreme freeze–thaw cycles typical of Volcán Llullaillaco; (2) the metabolic capabilities and growth rate of *Cryptococcus* across a range of temperatures and during repeated freeze–thaw cycles, and (3) the phylogenetic placement of this species in relation to *Cryptococcus* from other extreme environments.

Materials and methods

Sample collection

Soils used in this study were collected at an elevation of 6030 meters above sea level (m.a.s.l.) as described

elsewhere (Lynch et al. 2012). At this elevation soils are subject to year-round, freeze–thaw cycles and we endeavored to replicate these conditions in the laboratory. It is especially important to mimic the sub-zero cooling rates experienced in the field. Soil buffers changes in temperature compared to air temperatures and therefore the rate at which field soils cool at night is much slower than that of air. This is an important consideration when designing freeze–thaw experiments because the small volume of soil used in laboratory experiments can cool at a much faster rate than could ever occur in the field, and it has been shown that the sub-zero cooling rate of soils is one of the most important parameters controlling microbial survival during freeze–thaw stress (Henry 2007; Lipson et al. 2000; Schmidt et al. 2009). We, therefore, used a specially designed chamber (http://DarwinChamber.com) to mimic the sub-zero freezing rate experienced by field soils at a depth of 4 cm at high elevations. Figure 1 shows the rate of sub-zero soil cooling in the chamber compared to field data (4 cm depth) obtained from an elevation of 5800 m.a.s.l. on Volcán Llullaillaco during February (Lynch et al. 2012).

Five g of soils were used in each microcosm (four replicates per treatment). Treatments were: freeze–thaw with added water (70 % water holding capacity = freeze–thaw + water) and freeze–thaw at field water content (0.24 % water = freeze–thaw dry). An additional five replicates of untreated soils were used as controls. The chamber was run for 48 days, cycling up to a high of 27 °C during the day and a low of −9 °C at night with an amplitude of 36 °C in 24 h (Fig. 1).

DNA extraction, amplification and amplicon sequencing

Total DNA was extracted with Power Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA). Amplification of the eukaryotic 18S rRNA was performed using the oligonucleotide primers Euk_1391f/EukBr (Earth Microbiome Project, accessible at http://www.earthmicrobiome.org/emp-standard-protocols/18s/). All forward and reverse primers were modified to include a unique 12-nucleotide barcode. PCR reaction mixtures contained 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 1 µL of template and 12.5 µL of MM Gotaq Hot start Colorless Master Mix (Promega Corporation, Madison, WI, USA). The reaction volume was adjusted to a total of 25 µL with ultrapure DNase-/RNase-free water. Thermal cycles consisted of an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s; 57 °C for 60 s; and 72 °C for 90 s; with a final elongation step of 72 °C for 10 min. To prepare amplicons for sequencing, amplicon purification and normalization was done with Invitrogen SequalPrep Normalization Kit
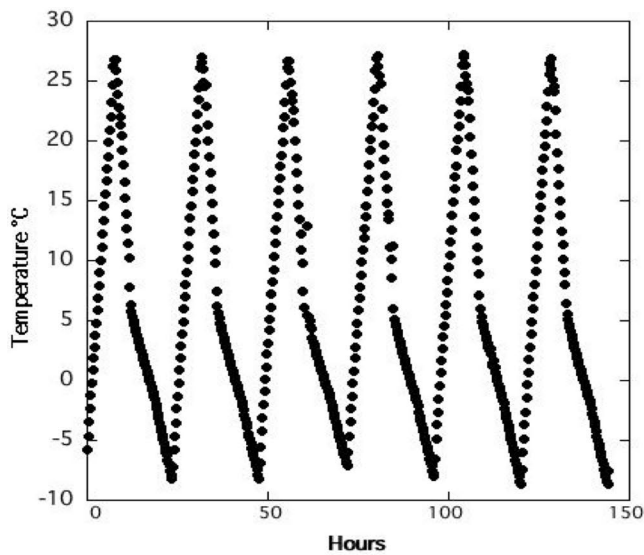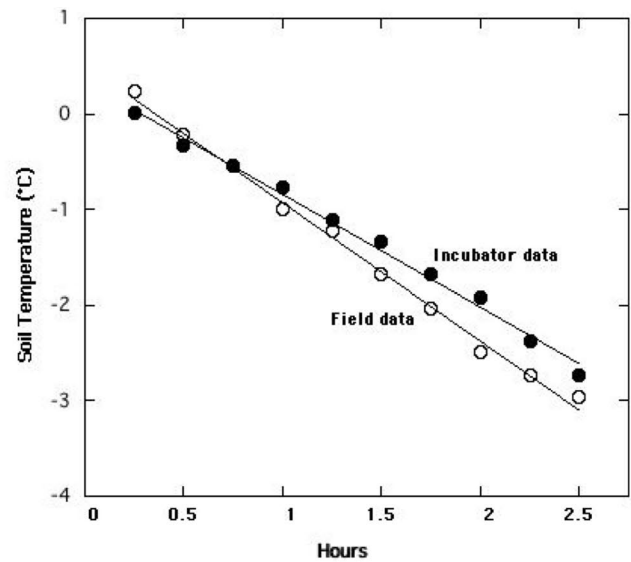
Fig. 1 Example of freeze–thaw cycles as recorded in the growth chamber used in this study. The chamber was cycling up to a high of 27 °C during the day and a low of −9 °C at night with an amplitude of 36 °C in 24 h (*left panel*). The *right panel* shows the period of most rapid sub-zero cooling in the chamber ("Incubator data") and in field soils (4 cm depth) on Volcán Llullaillaco ("Field data"). We endeavored to mimic field-cooling rates because this is a critical factor overlooked in most freeze–thaw experiments and is an important factor determining survival of microbes during natural freeze–thaw cycles (Lipson et al. 2000; Schmidt et al. 2009)

(Invitrogen Inc., CA, USA). Amplicons were combined into a single pool and sequenced using the Illumina MiSeq platform (BioFrontiers Institute, Boulder, Colorado) using pair-end 2 × 150 bp chemistry.

Sequencing and statistical analyses

Forward-oriented sequences were demultiplexed, quality filtered and processed using the QIIME pipeline (Caporaso et al. 2010b). Paired-end sequences did not work for these reads and only the read corresponding to the 1391F primer was used. This read was selected because it overlaps more with most sequences within the NCBI and SILVA databases (Darcy and Schmidt 2016). Singletons were excluded from further analysis and sequences with >97 % SSU rRNA gene sequence similarity were clustered into an OTU via UCLUST. Representative sequences for each OTU were chosen for classification and the Silva 104 reference database was employed to assign taxonomy identification to each single OTU. Sequences were aligned with PyNAST (Caporaso et al. 2010a) and a phylogeny was built with the FastTree algorithm (Price et al. 2009). OTU tables were rarified to 1488, the lowest number of sequences in a sample and were used to assess *Alpha* diversity and relative abundance of all taxa. To evaluate the significance of phylogenetic differentiation across different conditions, pairwise distance matrices based on weighted Unifrac (Lozupone and Knight 2005) were generated for the entire communities. A one-way Analysis of Similarity test (ANOSIM) from the R vegan package (Oksanen et al. 2013) was used to test significance of difference in *Beta* diversity between untreated samples and treatments (freeze–thaw and freeze–thaw + water addition). Additionally, principal coordinate analysis (PCoA) ordination was constructed based on both OTU tables and weighted Unifrac distance matrixes to visualize differences among community compositions of treatments among samples.

Culture isolation

To obtain isolates approximately 0.05 g of soil that had been exposed to 48 days of freeze–thaw cycling (as described above) were streaked onto agar media containing 4 g of Llullaillaco soil; 1 g of KH_2PO_4; 1 g of $MgSO_4$ $7H_2O$; 0.04 g of NH_4NO_3; 0.1 g of carboxymethyl-cellulose; 0.05 g of chitin; 0.1 g of tryptone; 0.1 g of yeast extract and 15 g of agar per liter of water. The pH of the medium was 5.5 and all cultures were incubated at 6 °C. Isolates were subcultured several times to obtain pure cultures.

Growth rates

To establish the optimal growth temperature of the *Cryptococcus* isolate, subsamples of the isolated cultures from liquid medium were grown at different temperatures. Three replicates were used at each temperature tested (−6, −2, 4, 10, 16, 22, 27 °C). All inoculums (grown at 6 °C) were taken from the same tube to insure that the yeast used for

all the temperature treatments were at the same metabolic state at the beginning of the experiment. Growth was measured through absorbance at 630 nm to assess cell density changes in a fixed volume. Absorbance was measured in a 96-well plate using Synergy HT Multi-Detection Microplate reader (Biotek, Winooski, Vermont). Correction was made for negative controls, which consisted of sterile media. Data were fitted using an exponential growth function (Darcy and Schmidt 2016) using Kaleidagraph (Synergy Software, Reading, PA) and the maximum specific growth rate (μ) was determined for each growth temperature. Growth of the *Cryptococcus* isolate in liquid medium was also tested under freeze–thaw cycles following the same cycle program described above. Growth data at different temperatures were fitted with a simplified form of the integrated Schoolfield equation (Schmidt et al. 2008; Schoolfield et al. 1981) to test if growth cultures follow Arrhenius temperature responses (growth rate is exponentially proportional to temperature) at low temperature and growth inhibition at high temperatures.

Cryptococcus sequencing and phylogenetics

Biomass for sequencing isolates was grown on agar plates in permissive medium (as described above). The small subunit ribosomal DNA (SSU) was amplified directly from individual yeast colonies without prior DNA extraction using 4Fa-Short (5′-ATCCGGTTGATCCTGC-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR was performed with the AmpliTaq Gold® 360 kit (Thermo Fisher Scientific, Waltham, USA) following manufacturer's protocols. The PCR profile was: 95 °C initial denaturation for 10 min; 35 cycles of 95 °C, 1 min; 53 °C, 30 s; 72 °C, 2.5 min; with a final elongation step of 72 °C for 10 min. PCR products were cleaned using the QIAquick Gel Extraction kit (Qiagen Inc., Valencia, USA) according to manufacturer's protocols and sent to Functional Biosciences (Madison, WI, USA) for subsequent sequencing using the T7 and M13 primers. The sequences were edited into contigs using Geneious 5.4.6 (Drummond et al. 2011). Edited sequence was identified with a National Center for Biotechnology Information (NCBI) BLASTN search and the sequence was a match (100 %) to an uncultured eukaryote clone (JX099190) from Lynch et al. (2012). To place the yeast in phylogenetic context, published sequences from Lynch et al. (2012) and sequences from two outgroup taxa were downloaded from NCBI and aligned using the MUSCLE algorithm (Edgar 2004) in Geneious 5.4.6 (Drummond et al. 2011). Sequences were clustered with select database guide sequences into 97 % identity operational taxonomic units (OTUs) using the average neighbor algorithm implementation in mothur (Schloss et al. 2009). The resulting alignment of 1879 sites and 21 sequences was

used for Bayesian inference (BI) and maximum likelihood (ML) analyses. The BI analysis was performed in MrBayes version 3.2 (Huelsenbeck and Ronquist 2001) using the GTR+G+I model and default priors. The BI analysis was run to convergence for 5 M generations with 25 % burn-in and chains sampled every 1000 generations. Changes in temperature did not change tree topology or resulting posterior probabilities so the default of 0.1 was utilized. The ML analysis was conducted using a general time reversible (GTR) model of evolution with a gamma distribution (*g*) and a proportion of invariable sites (*I*) with PhyML version 3.0 (Guindon et al. 2010) in SeaView version 4.3.4 (Gouy et al. 2010). Node support was estimated using 500 bootstrap replicates.

Substrate utilization

For characterization of potential substrate utilization by the isolated *Cryptococcus* sp., Biolog YT microplates (BIOLOG®, Hayward CA) were used. *Cryptococcus* grown in permissive medium was pelleted and resuspended in sterile water. Plates were inoculated with 100 μL of *Cryptococcus* cell suspension and were incubated at 10 °C for 4 days in the dark. Sterile water was used for control wells. After this period metabolism of each compound was measured by both visual inspection of a colorimetric indicator and through absorbance (630 nm) measured using a 96-well plate using Synergy HT Multi-Detection Microplate reader (Biotek, Winooski, Vermont). Compounds with absorbance values 1.5–2 times greater than that of sterile water were considered as potential carbon sources.

Results

Community shifts during freeze–thaw cycles

An experiment was conducted to determine the effects of repeated freeze–thaw cycles on the structure of the eukaryotic community and the relative abundance of *Cryptococcus* phylotypes in the community. Initially, five replicate soil samples from Llullaillaco showed high variability in community structure (Fig. 2). In contrast, after 2 months of freeze–thaw cycling, microcosms held at 70 % of water holding capacity (freeze–thaw + water) and at field moisture levels (freeze–thaw dry) showed significantly altered microbial communities that were dominated by a *Cryptococcus* phylotype (Fig. 2). ANOSIM analysis (Oksanen et al. 2013) showed that *Beta* diversity of the communities was significantly different between untreated microcosms and microcosms subjected to freeze–thaw cycles both for freeze–thaw + water and freeze thaw dry samples [with a Global *R* of 0.2 ($p < 0.05$) for both comparisons]. The
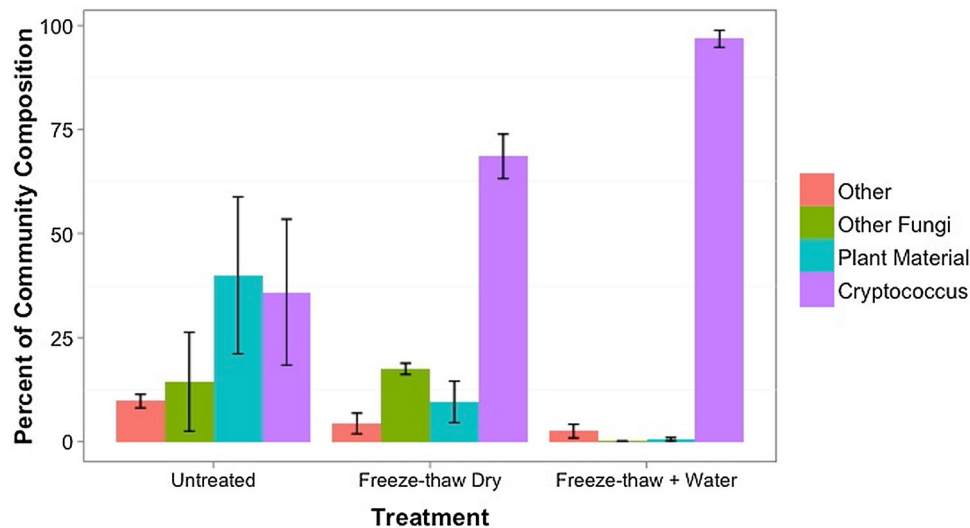
Fig. 2 Relative abundance of eukaryotic phylotypes after 48 days of freeze–thaw cycles, based on PCR amplifications of the 18S rRNA gene using Euk1391f/EukBr primer sets. The microbial community differed significantly between untreated and freeze–thaw dry samples and between untreated and freeze–thaw + water samples [ANOSIM, Global R of 0.2 ($p < 0.05$) for both comparisons]. Plant material represents Aeolian deposited material because no plants grow at elevations above 5000 m.a.s.l. on Llullaillaco and these soils were collected at elevation of 6030 m.a.s.l. Each *bar* is the mean of four replicate microcosms (*error bars* are standard error of the mean)

observed community shift was obviously driven by the complete dominance of *Cryptococcus* in both treatments. While these data indicate that *Cryptococcus* likely grew during the experiment, it is important to note that these are relative abundance data and therefore are not definitive proof of growth.

Phylogenetics and growth of *Cryptococcus* cultures

The yeast isolated from Llullaillaco after 48 days of freeze–thaw had the identical long-read 18S (1879 sites) as the dominant phylotype from Volcán Llullaillaco (Genbank# JX099190). These sequences indicate that the isolated yeast clusters within a *Cryptococcus* clade and its most closely related organisms are *C. friedmanni*, *C. albidus*, *C. antarcticus*, *C. bhutanensis* and *C. vishniacii*. This clade contains members that have been found only at high altitude or high latitude with the exception of *C. albidus* that has been found also in more temperate sites (Fig. 3).

To determine if the *Cryptococcus* sp. isolate could grow during extreme freeze–thaw cycles, replicate cultures were subjected to the same freeze–thaw cycles described above. Under these conditions, the culture grew at a rate of 0.013 h^{-1} (Fig. 4). Experiments were also done to determine the optimal growth temperature of the isolate and to compare its growth rate at various temperatures to the rate under freeze–thaw cycling (Fig. 5). The isolate was able to grow at the lowest temperatures tested (−2 and −6 °C) but not at 27 °C and the growth rate at 0 °C was approximately
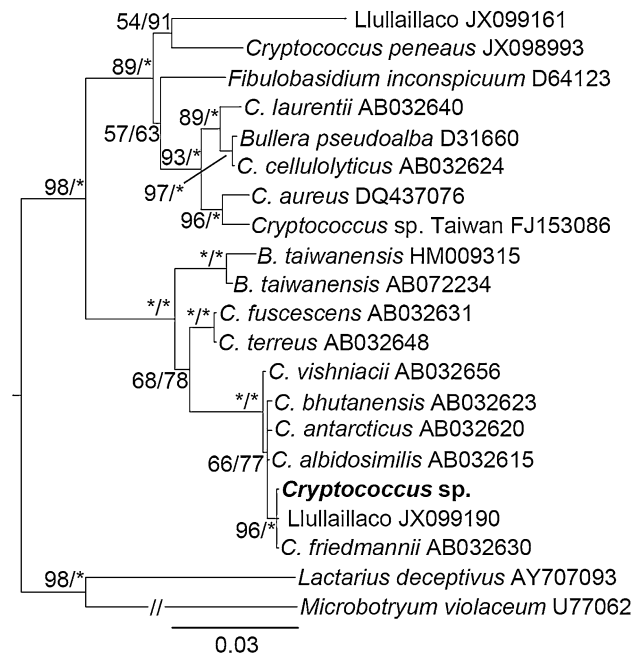

Fig. 3 Bayesian consensus tree of SSU data from environmental sequences from Llullaillaco volcano (JX099161 and JX099190) and representatives of *Cryptococcus* isolates including the *Cryptococcus* used in this study (in *bold type*). Node support is given as maximum likelihood bootstrap values (500 bootstrap replicates)/Bayesian posterior probability (as a percent). Note that the closest phylotypes to the *Cryptococcus* isolate used in this study are an environmental sequence (JX099190) from Volcán Llullaillaco and *C. friedmannii* (AB032630) from Antarctica. Data are from this study, Schmidt et al. (2012), and Lynch et al. (2012)
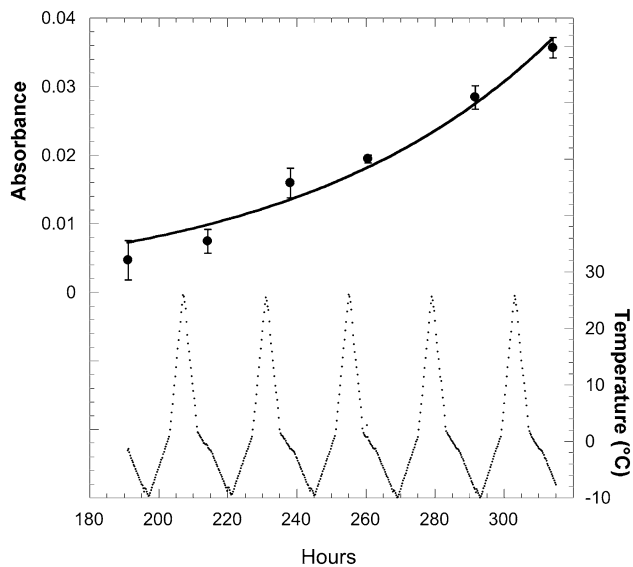
Fig. 4 Exponential growth of the *Cryptococcus* sp. isolate (*top panel*) during freeze–thaw cycles of 27 to −10 °C as measured with data loggers in the growth chamber (*bottom panel*). Temperatures in the actual growth medium (measured by thermocouple) were comparable to these in the chamber, but showed a lag of about 1 h (data not shown). All cultures froze solid every night and completely melted every day. It is not clear from these data if growth was continuous or occurred only during periods of liquid water since all sub-samples were taken when cultures were melted. *Each point represents the mean of four replicate cultures (error bars are standard error of the mean)*
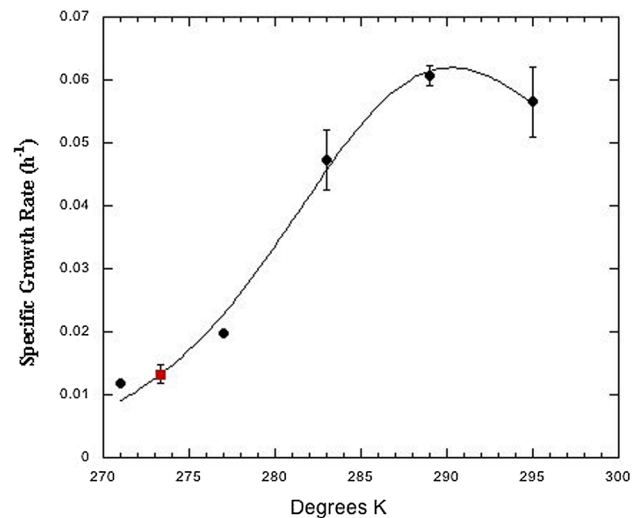

Fig. 5 Temperature response curve for *Cryptococcus* growth rates over a range of temperatures. The rates plotted are the maximum specific growth rate (μ with units of h^{-1}) observed at each temperature. The curve fit is a non-linear regression fit of the Schoolfield equation (Schoolfield et al. 1981) to the data. R^2 values for curve fit is >0.98. *Each point represents the mean of three replicate cultures (error bars are standard error of the mean). The red point represents the growth rate of Cryptococcus during freeze–thaw cycles (from the curve in Fig. 4). The x axis is in degrees Kelvin and can be converted to degrees Centigrade by subtracting 273.15 from each value*

the same as the rate under freeze–thaw conditions (Fig. 5). The Schoolfield equation (Schmidt et al. 2008; Schoolfield et al. 1981) showed that rates exhibited Arrhenius behavior (growth rate being exponentially proportional to temperature) at low temperatures and enzyme inhibition at temperatures above the optimal temperature of about 17 °C (Fig. 5).

Substrate utilization

Table 1 shows the results obtained from three independent replicate experiments of substrate utilization by the *Cryptococcus* isolate using Biolog YT microplates (BIOLOG®, Hayward CA).

The *Cryptococcus* isolate was able to utilize many sugars and other plant-derived compounds including aromatic compounds (arbutin and salicin), a hydrolysable tannin (quinic acid) and plant-derived polymers (cellulose and dextrin) (Table 1).

Discussion

Field data from Volcán Llullaillaco and other nearby volcanoes show extraordinary temperature fluctuation faced

by microbial life in these high elevation soils (Lynch et al. 2012; Schmidt 1999; Schubert 2014), but it is currently not known whether organisms found at these sites are able to grow there or if they are just dormant cells deposited by Aeolian processes. Therefore, the first goal of this study was to determine if the dominant eukaryotic organism found in these soils by previous workers (Costello et al. 2009; Lynch et al. 2012) could increase in relative abundance during freeze–thaw cycles similar to those that would occur just under the surface of the soil (4 cm depth). Exposure of soil microcosms to repeated freeze–thaw cycles at field moisture levels (freeze–thaw dry) and in the presence of added water (freeze–thaw + water) resulted in a significantly different community structure (ANOSIM) compared to untreated microcosms. The community shift was driven by *Cryptococcus*, especially in samples subjected to freeze–thaw cycles with added water (freeze–thaw + water). These results provide evidence that *Cryptococcus* has the capacity to grow during freeze–thaw cycles, and that it may be able to mainly do that during periods of higher soil moisture. Such periods of higher soil moisture do occur on Llullaillaco and other nearby volcanoes during snow-melt after infrequent snow storms despite the tendency for snow to sublimate directly back to the atmosphere at extreme elevations (Schubert 2014). At the same time, its prevalence also

Table 1 Potential carbon sources used by *Cryptococcus* sp. isolate measured with Biolog YT microplates

Potential carbon source	Utilization
2-Keto-D-gluconic acid	++
Acetic acid	0
Acetoin plus D-xylose	+
Adonitol	++
Alpha-D-lactose plus D-xylose	++
Alpha-D-glucose	++
Alpha-keto-glutaric acid	+
Alpha-methyl-D-glucoside	++
Amygdalin	0
Arbutin	++
Beta-methyl-D-glucoside	++
Bromo succinic acid	++
D-Arabinol	+
D-Arabinose	++
D-Arabitol	0
D-Cellobiose	++
D-Galactose	+
D-Galactose plus D-xylose	+
D-Gluconic acid	++
D-Glucosamine	0
D-Glucuronic acid plus D-xylose	++
D-Mannitol	++
D-Melezitose	++
D-Melibiose	0
D-Melibiose plus D	+
D-Psicose	+
D-Raffinose	0
D-Ribose	+
D-Sorbitol	++
D-Trehalose	++
D-Xylose	++
Dextrin	++
Dextrin plus D-xylose	++
Formic acid	
Fumaric acid	++
Gamma-amino-butyric acid	+
Gentiobiose	++
Glycerol	++
i-Erythritol	0
Inulin	0
L-Arabinose	++
L-Aspartic acid	0
L-Glutamic acid	++
L-Malic acid	++
L-Proline	0
L-Rhamnose	++
L-Sorbose	0
m-Inositol plus D-xylose	+

Table 1 continued

Potential carbon source	Utilization
Maltitol	++
Maltose	++
Maltotriose	++
N-Acetyl-D-glucosamine	0
N-Acetyl-L-glutamic acid plus D-xylose	+
Palatinose	++
Propanediol plus D-xylose	+
Propionic acid	0
Quinic acid plus D-xylose	++
Salicin	+
Stachyose	0
Succinic acid	0
Succinic acid mono-methyl ester	+
Succinic acid mono-methyl ester plus D-xylose	+
Sucrose	++
Turanose	++
Tween 80	+

Cryptococcus sp. isolate growth following metabolization of potential carbon sources was measured through absorbance (Synergy HT Multi-Detection Microplate reader)

Utilization categories are: less than control absorbance (0); 1.5X control absorbance (+); and 2X control absorbance (++). Metabolization of each potential carbon source was measured in three independent replicate experiments

in freeze–thaw dry samples leads to the hypothesis that *Cryptococcus* may be partially active even at extremely low water content.

To confirm that *Cryptococcus* can grow during freeze–thaw cycles we isolated a strain of *Cryptococcus* from Llullaillaco soils and tested its ability to grow during freeze–thaw cycles in our freeze–thaw incubator. Before doing these experiments we confirmed that the isolate we used was a strong genetic match to the *Cryptococcus* phylotypes in soils from Llullaillaco. Figure 3 shows that the isolate used in our experiments is almost genetically identical to environmental sequences obtained directly from Llullaillaco soils by Lynch et al. (2012) and to *Cryptococcus* "species" isolated from Antarctic soils (*C. friedmannii, C. antarcticus, C. vishniacii*) and the Himalayas (*C. bhutanensis*). The close identity of all of these cryospheric strains of *Cryptococcus* is interesting in its own right and indicates that further phylogeographic work with these organisms may yield new insights into the functional significance of *Cryptococcus* in vast regions of the cryosphere. However, for the present study, Fig. 3 simply proves that we are working with a relevant strain of this organism because it is closely affiliated with environmental DNA sequences and isolates from extreme environments (1879 bp, 99 % similarity).

The primary purpose of our study was to establish if *Cryptococcus* could actively grow under low temperatures and during extreme freeze–thaw cycles commonly observed at high elevation sites. Experiments with our isolated strain of *Cryptococcus* sp. showed that it can grow at the lowest constant temperature tested (−6 °C), and even more surprisingly, that it could grow during freeze–thaw cycles with temperatures ranging from a high of 27 °C to a low of −10 °C (Fig. 4). To our knowledge this is the first definitive demonstration of growth of an organism during repeated extreme freeze–thaw cycles and lends even stronger support to our hypothesis that *Cryptococcus* can function in high altitude environments where daily freeze–thaw cycles occur year-round. The fact that our strain can grow at temperatures below zero is also an indication that it may be adapted to life at high elevations; however, many other studies have demonstrated the growth of a wide variety of yeasts at sub-zero temperatures (reviewed in Buzzini et al. 2012), whereas this study is the first to show growth during extreme temperature cycles.

We conducted further experiments with this organism and determined that its temperature optimum for growth is about 17 °C (Fig. 5) and that it could not grow at a constant temperature of 27 °C. It can, therefore, be classified as a psychrotroph rather than a true psychrophile (Morita 1975). Being a psychrotroph makes sense for an organism that experiences extreme temperature fluctuations in the field, while true psychrophiles would be better suited to the more stable temperature regimes such as in permafrost (Rhodes et al. 2013). There is also evidence that adaptation to oligotrophic conditions (e.g., nitrogen and carbon starvation) and other types of stress, including osmotic stress, may pre-adapt yeast to freeze–thaw tolerance (Park et al. 1997). In other words it is very likely that cross-resistance to many types of stress may pre-adapt cells to freeze–thaw stress in both Eukarya and Bacteria (Park et al. 1997; Leenanon and Drake 2001; Bang and Drake 2002; Dubernet et al. 2002). This sort of cross-resistance may be especially relevant to the extreme soils of Llullaillaco where high UV radiation, extreme desiccation, low nutrient availability and freeze–thaw cycles co-occur every day of the year at elevations above 6000 m.a.s.l.

Our experiments with varying temperatures and water levels (Figs. 2, 4) give a preliminary indication of how *Cryptococcus* may be able to function in extreme high elevation soils, but it is still unclear what substrates they have access to for carbon and energy in these extremely oligotrophic soils. The soils we have studied so far on Llullaillaco contain among the lowest carbon contents of any soils yet studied on Earth even lower than soils from the Dry Valleys of Antarctica (Lynch et al. 2012). However, Aeolian plant and animal matter is routinely transported from lower elevations to high elevations and can support simple ecosystems in plant-free, high-elevation ecosystems (Swan 1992). To survive in an Aeolian-supported environment, one might expect organisms that are generalist in their metabolic requirements so that they can take advantage of whatever nutrients come their way. Our preliminary work (Table 1) with *Cryptococcus* hints that it is indeed a generalist, as suggested before for this genus (Benham 1956), at least in terms of the breadth of carbon substrates that it can utilize for growth (Table 1). *Cryptococcus* is able to grow oxidizing sugars such as glucose, maltose, maltotriose, gentiobiose, cellobiose and trehalose and may therefore be able to grow in response to sugars released following breakdown of Aeolian deposited plant material. The freezing and thawing of soil may be key in increasing nutrients and carbon availability since it has long been known that freeze–thaw cycles damage and destroy microbial cells (Skogland et al. 1988) providing nutrients to surviving microbes. It is therefore possible that *Cryptococcus* also thrives thanks to nutrients and carbon released following freeze–thaw cycles. Uptake of trehalose may also help *Cryptococcus* adapt to anhydrobiosis. Trehalose accumulation is known to be a strategy employed by both bacteria (Welsh and Herbert 1999) and eukaryotes (Gadd et al. 1987) to cope with desiccation stress. *Cryptococcus* can also use plant aromatic compounds such as arbutin and salicin (Table 1) further indicating that it may depend on Aeolian deposited plant material. It is also relevant that our deep sequencing of the 18S gene at this site revealed the presence of exogenous plant material in the soils used in our soil experiment, but that this plant material was not detected after incubation in the presence of water, perhaps indicating that *Cryptococcus* was involved in the degradation of this material (Fig. 2). In contrast, the inability of *Cryptococcus* to oxidize *N*-acetyl-glucosamine (the monomer from chitin) suggests that this organism may not be involved in the breakdown of Aeolian deposited arthropods—another large source of carbon to many Aeolian habitats (Swan 1992). Overall, the assimilation profile of our isolate is very similar to that of the Antarctic yeast *Cryptococcus friedmannii*, whose 18S rRNA is also the closest match to our isolate (Fig. 3). For example, *C. friedmannii*, can also utilize cellobiose and Vishniac (1985) claimed that this trait distinguished it from all the other basidiomycetous yeasts. Vishniac (1985) proposed that *C. friedmannii* is a secondary consumer within cryptoendolithic communities, but to our knowledge there are no cryptoendolithic communities (or in fact any known phototrophs) in the volcanic glass soils of Llullaillaco and therefore our working hypothesis is that *Cryptococcus* on Llullaillaco are primarily supported by Aeolian plant material and not autochthonously produced organic carbon.

Conclusion

Taken together, our data indicate that *Cryptococcus* species on Volcán Llullaillaco are well adapted to a life in soils that undergo extreme diurnal temperature fluctuations and exogenously supplied carbon compounds. During freeze–thaw cycles *Cryptococcus* was able to grow at a specific growth rate of 0.013 h^{-1} that corresponds to a doubling time of 50 h or about 2 days. This means that in the presence of some exogenous plant matter, *Cryptococcus* populations could potentially double in size every 2 days as long as snow melt water was available. At present we don't know how many such periods of water availability would occur per year at 6000 m.a.s.l., but climbers and scientists have observed such periods on Llullaillaco and other nearby volcanoes (Halloy 1991; Costello et al. 2009; Schmidt 1999, personal observation 2009). Therefore, our working hypothesis is that *Cryptococcus* (and perhaps other soils microbes) are able to persist in the extreme soils above 6000 m.a.s.l. by increasing in numbers during rare times of water and substrate availability and then entering long periods of dormancy in between such events. More work is needed on the survival strategies and in situ dynamics of these hardy yeasts to confirm this hypothesis.

References

Bang W, Drake MA (2002) Resistance of cold-and starvation-stressed *Vibrio vulnificus* to heat and freeze–thaw exposure. J Food Protect 65:975–980

Benham RW (1956) The genus *Cryptococcus*. Bacteriol Rev 20:189–199

Buzzini P, Branda E, Goretti M, Turchetti B (2012) Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol 82:217–241

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266–277

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010b) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Cary SC, McDonald IR, Barrett JE, Cowan DA (2010) On the rocks: the microbiology of Antarctic Dry Valley soils. Nat Rev Microbiol 8:129–138. doi:10.1038/nrmicro2281

Costello EK, Halloy SRP, Reed SC, Sowell P, Schmidt SK (2009) Fumarole-supported islands of biodiversity within a hyperarid, high-elevation landscape on Socompa Volcano, Puna de Atacama, Andes. Appl Environ Microbiol 75:735–747

Darcy JL, Schmidt SK (2016) Nutrient limitation of microbial phototrophs on a debris-covered glacier. Soil Biol Biochem 95:156–163

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R (2011) Geneious, version 5.4. Geneious, Auckland, New Zealand. http://www.geneious.com. Accessed 15 Sep 2015

Dubernet S, Panoff JM, Thammavongs B, Guéguen M (2002) Nystatin and osmotica as chemical enhancers of the phenotypic adaptation to freeze–thaw stress in *Geotrichum candidum* ATCC 204307. Int J Food Microbiol 76:215–221

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Gadd GM, Chalmers K, Reed RH (1987) The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 48:249–254

Goto S, Sugiyama J (1970) Studies on Himalayan yeasts and molds (IV). Several asporogenous yeasts, including two new taxa of *Cryptococcus*. Can J Bot 48:2097–2101

Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221–224. doi:10.1093/molbev/msp259

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321. doi:10.1093/sysbio/syq010

Halloy S (1991) Islands of life at 6000 m altitude: the environment of the highest autotrophic communities on Earth (Socompa Volcano, Andes). Arctic Alpine Res 23:247–262

Henry HA (2007) Soil freeze–thaw cycle experiments: trends, methodological weaknesses and suggested improvements. Soil Biol Biochem 39:977–986

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Leenanon B, Drake MA (2001) Acid stress, starvation, and cold stress affect poststress behavior of *Escherichia coli* O157: H7 and non-pathogenic *Escherichia coli*. J Food Protect 64:970–974

Lipson DA, Schmidt SK, Monson RK (2000) Carbon availability and temperature control the post-snowmelt decline in alpine soil microbial biomass. Soil Biol Biochem 32:441–448

Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–8235

Lynch RC, King AJ, Farías ME, Sowell P, Vitry C, Schmidt SK (2012) The potential for microbial life in the highest elevation (>6000 m.a.s.l.) mineral soils of the Atacama region. J Geophys Res 117:G02028

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MH, Wagner H (2013) Vegan: Community Ecology Package. R Package version 2.0-10. http://CRAN.R-project.org/package=vegan. Accessed 16 Sep 2015

Park JI, Grant CM, Attfield PV, Dawes IW (1997) The freeze–thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal transduction pathway. Appl Environ Microbiol 63:3818–3824

Price MN, Dehal PS, Arkin AP (2009) FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26:1641–1650. doi:10.1093/molbev/msp077

Reinhard J (1999) Frozen in time. Natl Geogr 196:36–55

Reinhard J, Ceruti MC (2010) Inca rituals and sacred mountains: a study of the world's highest archaeological sites. UCLA Cotsen Institute of Archaeology Press, Los Angeles

Rhodes M, Knelman J, Lynch R, Darcy JL, Nemergut DR, Schmidt SK (2013) Alpine and arctic soil microbial communities. The prokaryotes. Springer, Berlin, pp 43–55. doi:10.1007/978-3-642-30123-0_37

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541

Schmidt D (1999) Das Extremklima Der Nordchilenischen Hochatacama Unter Besonderer Berücksichtigung Der Höhengradienten. Dresdener Geographische Beiträge 4:1–122

Schmidt SK, Wilson KL, Meyer AF, Gebauer MM, King AJ (2008) Phylogeny and ecophysiology of opportunistic "snow molds" from a subalpine forest ecosystem. Microb Ecol 56:681–687

Schmidt SK, Nemergut DR, Miller AE, Freeman KR, King AJ, Seimon A (2009) Microbial activity and diversity during extreme freeze–thaw cycles in periglacial soils, 5400 m elevation, Cordillera Vilcanota, Perú. Extremophiles 13:807–816. doi:10.1007/s00792-009-0268-9

Schmidt SK, Naff C, Lynch R (2012) Fungal communities at the edge: ecological lessons from high alpine fungi. Fungal Ecol 5:443–452. doi:10.1016/j.funeco.2011.10.005

Schoolfield RM, Sharpe PJ, Magnuson CE (1981) Non-linear regression of biological temperature-dependent rate models based on absolute reaction-rate theory. J Theor Biol 88:719–731. doi:10.1016/0022-5193(81)90246-0

Schubert ZR (2014) Dew formation and water availability at high elevation in the Atacama Desert, Chile. Undergraduate Honors Thesis. Paper 192. University of Colorado, Boulder

Skogland T, Lomeland S, Goksøyr J (1988) Respiratory burst after freezing and thawing of soil: experiments with soil bacteria. Soil Biol Biochem 20:851–856. doi:10.1016/0038-0717(88)90092-2

Swan LW (1992) The Aeolian biome. Bioscience 42:262–270. doi:10.2307/1311674

Vishniac HS (1985) *Cryptococcus friedmannii*, a new species of yeast from the Antarctic. Mycologia 77:149–153. doi:10.2307/3793260

Vishniac HS, Hempfling WP (1979a) *Cryptococcus vishniacii* sp. nov., an Antarctic yeast. Int J Syst Bacteriol 29:153–158. doi:10.1099/00207713-29-2-153

Vishniac HS, Hempfling WP (1979b) Evidence of an indigenous microbiota (yeast) in the Dry Valleys of Antarctica. J Gen Microbiol 112:301–314

Welsh DT, Herbert RA (1999) Osmotically induced intracellular trehalose, but not glycine or betaine accumulation promotes desiccation tolerance in *Escherichia coli*. FEMS Microbiol Lett 174:57–63

Wilson AS, Brown EL, Villa C, Lynnerup N, Healey A, Ceruti MC, Reinhard J, Previgliano CH, Araoz FA, Gonzalez Diez J, Taylor T (2013) Archaeological, radiological, and biological evidence offer insight into Inca child sacrifice. PNAS 110:13322–13327. doi:10.1073/pnas.1305117110

Yergeau E, Kowalchuk GA (2008) Responses of Antarctic soil microbial communities and associated functions to temperature and freeze–thaw cycle frequency. Environ Microbiol 10:2223–2235. doi:10.1111/j.1462-2920.2008.01644.x