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ORIGINAL PAPER

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Molecular identification of Bacteria and Eukarya inhabiting an Antarctic cryoconite hole

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Abstract Inhabitants of a cryoconite hole formed in the Canada Glacier in the McMurdo Dry Valley region of Antarctica have been isolated and identified by small subunit (16S/18S) rDNA amplification, cloning, and sequencing. The sequences obtained revealed the presence of members of eight bacterial lineages (*Acidobacterium*, *Actinobacteria*, *Cyanobacteria*, *Cytophagales*, *Gemmimonas*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*) and metazoan (nematode, tardigrade, and rotifer), truffle (*Choiromyces*), ciliate (*Spathidium*), and green algal (*Pleurastrium*) Eukarya. Bacterial recovery was ~20-fold higher at 4 °C and 15 °C than at 22 °C, and obligately psychrophilic bacteria were identified and isolated. Several of the rDNA molecules amplified from isolates and directly from cryoconite DNA preparations had sequences similar to rDNA molecules of species present in adjacent lake ice and microbial mat environments. This cryoconite hole community was therefore most likely seeded by particulates from these local environments. Cryoconite holes may serve as biological refuges that, on glacial melting, can repopulate the local environments.

Keywords Antarctica · Cryoconite hole · Frozen ecosystem · Glacial ice · McMurdo Dry Valley · Refuge survival

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Introduction

Cryoconite holes are formed when particulates, lodged on the surface of a glacier, are warmed by solar irradiation and melt into the underlying ice (see Fig. 1B, C). They are unique environments that contain liquid water inoculated with materials released from the particulates and from the melted glacial ice. During the polar summer, photosynthesis by algae and cyanobacteria within the cryoconite hole can provide sufficient nutrients for complex community development, and light microscopy has documented the presence of bacteria, algae, diatoms, fungi, and rotifers in cryoconite hole ecosystems (Gerdel and Drouet 1960; Wharton et al. 1981, 1985; Kohshima 1989; DeSmet and Van Rompu 1994; Grøngaard et al. 1999; Takeuchi et al. 2000). However, there are no documented reports of molecular investigations of a cryoconite hole community. To address this deficiency, small subunit rDNA molecules have been amplified, cloned, and sequenced from bacterial isolates and from DNA preparations recovered directly from a cryoconite hole that formed in the Canada glacier in the McMurdo Dry Valley region of Antarctica (Fig. 1A). The results obtained confirm the presence of diverse species of both Bacteria and Eukarya in this permanently cold but seasonally active environment. They also confirm that cryoconite holes are most likely seeded by particulates from the local environment and could then serve as refuges for local species through the extreme cold of the Antarctic winter.

Materials and methods

Cryoconite sample recovery and handling

A 30-cm core of ice and underlying sediment was recovered from a frozen cryoconite hole that had formed in the Canada Glacier in the McMurdo Dry Valley region of Antarctica (Fig. 1C, D). The bottom ~5 cm of the core contained inorganic particulates and biomass that presumably accumulated when the cryoconite hole contained liquid water. The frozen core was transported to the

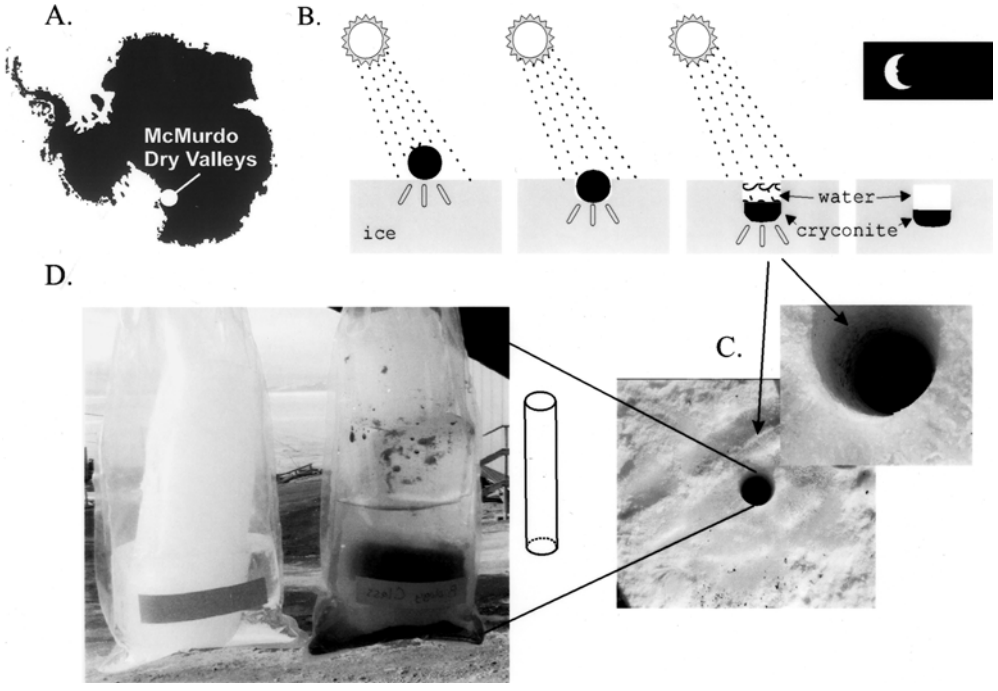

Fig. 1A–D Cryoconite hole location, development, and sampling. **A** The McMurdo Dry Valleys are largely ice-free areas on the Antarctic continent from which particulates are blown onto the surface of the adjacent Canada glacier. **B** In summer, these are warmed by continuous solar irradiation and melt into the ice forming water-filled cryoconite holes (**C**). The particulates disintegrate and biomass accumulates as cryoconite sediment. The surface water may refreeze during cold periods, isolating the community from further external input, and the entire cryoconite hole community is then frozen in the absence of sunlight during the following winter (*upper right*). The cryoconite hole investigated was completely frozen when a core sample was removed in January 2001. **D** A comparison of this cryoconite hole core (*right*) with a core retrieved from the adjacent glacial ice. As shown, the bottom 5 cm of the cryoconite hole core contained dense sediment

Crary Laboratory at the McMurdo research station and was allowed to thaw at 4 °C, which took ~48 h. Melt water (~200 ml) was decanted and filtered through a 0.2-μm filter (Millipore, Bedford, Mass.) at 4 °C, and the particulates so collected were resuspended in 5 ml of phosphate-buffered saline (PBS). The remaining sediment was refrozen, shipped, and stored at −15 °C for investigation at the Ohio State University.

Bacterial isolation

Samples (100 μl) of dilutions of the PBS suspension of filtered particulates and of resuspended sediment were spread on agar-solidified tryptose blood, 1% nutrient broth, 1% tryptic soy broth (Difco, Detroit, Mich.), and R2 medium (Reasoner and Geldreich 1985). Identically inoculated plates were incubated aerobically at 4 °C, 15 °C, and 22 °C. Regardless of the incubation temperature, the highest number of colonies grew on R2 medium, with maximum numbers observed equating to the cryoconite sediment containing 8.6×10^4 cfu/ml.

DNA isolation, amplification, and sequencing

Genomic DNA preparations were isolated from cells that formed a single colony. From these DNA preparations, 16S rDNA molecules that corresponded to nucleotides 27–1492 of the *Escherichia coli* 16S rDNA were polymerase chain reaction (PCR) amplified and sequenced as previously described (Christner et al. 2001). Ultraclean soil DNA extraction kits (Mo Bio Labs, California) were used as recommended by the manufacturer to isolate DNA directly from samples of the cryoconite sediment. When these DNA preparations were used as the template, rDNA amplicons of the appropriate size were generated in PCRs primed using the universal forward 515F and reverse 1492R primers (Reysenbach and Pace 1995), using the *Cyanobacteria*-specific 106F and 781R primers (Nübel et al. 1997), and using the Eukarya-specific 515F and 1195R primers (Reysenbach and Pace 1995). Individual molecules were cloned and sequenced from these amplified DNA populations, as previously described (Christner et al. 2001). Amplicons of the appropriated sizes were not generated in PCRs primed with the Archaea-specific primer pairs 21F with 1525R, 348F with 1525R, and 23F with 927R (Lane 1991; Barns et al. 1994; Reysenbach and Pace 1995) or in PCRs primed with the alga-specific primers 21F with 1798R (Huss et al. 1999).

Sequence analyses and tree construction

The 16S and 18S rDNA sequences obtained were imported into the ARB software environment (Strunk et al. 1998) and compared and aligned, based on secondary structures, with the most similar rDNA sequences available in the Ribosomal Database Project (Maidak et al. 2001) and in GenBank (Benson et al. 2000). Maximum likelihood trees were generated for the bacterial, cyanobacterial, and eukaryal sequences by using fastDNAml (Olsen et al. 1994) with nucleotide masks of 889, 627, and 763 unambiguously aligned positions, respectively.

Results

Bacterial isolates

Approximately 20-fold more bacterial colonies grew on plates incubated initially at 4 °C and 15 °C than at 22 °C, but most of these isolates, on further investigation, were subsequently able to grow at 22 °C. However, four isolates, designated CS57, CS112, CS117, and

CS910, were identified that were incapable of growth at 22 °C, and in the case of CS112, spontaneous cell lysis occurred when growing cultures were shifted from 15 °C to 22 °C. Based on their 16S rDNA sequences, CS57, CS112, and CS910 are *Cytophagales*, with their most closely related, previously documented relatives being *Flavobacterium* species. CS117 belongs to the Actinobacteria lineage and has a 16S rDNA sequence

Table 1 Bacterial isolates from cryoconite sediment

Isolate designation	GenBank accession number	Sequence alignment		Nearest phylogenetic neighbor (GenBank accession number)
		n[a]	% identity[b]	
CD12	AF479324	1430	97.1	*Pseudomonas saccharophila* (AB021407)
			96.5	*Matsuebacter chitosanotabidus* (AB006851)
CD89	AF479326	1442	97.4	*Janthinobacterium lividum* (Y08846)
			97.2	*Pseudomonas mephitica* (AB021388)
CS57	AY124340	1422	95.6	Soil clone (AF423292)
			93.3	*Flavobacterium ferrugineum* (M28237)
CD14	AF479331	1426	95.4	*Haloanella gallinarum* (AB035150)
			94.3	*Chryseobacterium balustinum* (M58771)
CS910	AY124339	1430	96.0	*Flavobacterium sp.* from sea ice (U85888)
			95.8	*Flavobacterium hydatis* (M58764)
CS112	AY124338	1436	96.3	*Flavobacterium succinicans* (D12673)
			96.3	*Flavobacterium hydatis* (M58764)
CD1	AF479325	1440	96.4	*Arthrobacter sp.* from sea ice (AF041789)
			96.0	*Arthrobacter agilis* (X80748)
CD7	AF479339	1420	98.8	Glacial iceisolate G200-A1 (AF479340)
			98.7	*Arthrobacter sp.* (AB039736)
CS117	AY124341	1444	99.5	*Cryobacterium psychrophilum* (AJ297438)
			97.8	*Clavibacter michiganensis* (U30254)

[a] The length of16S rDNA in nucleotides used for alignment and phylogenetic analyses

[b] The percent identity of the experimental sequence with the 16S rDNA sequence of the two nearest known phylogenetic neighbors

Fig. 2 Phylogenetic analysis of the bacterial 16S rDNA sequences amplified from isolates and from bulk DNA isolated from cryoconite sediment. The maximum likelihood tree shown was constructed using fast DNAml (Olsen et al. 1994) and is based on 16S rDNA sequences corresponding to nucleotides 515–1492 of the *Escherichia coli* 16S rDNA. The scale bar indicates 0.1 fixed substitutions per nucleotide position. GenBank accession numbers are listed in parentheses. Bacterial isolates are indicated by *asterisks*, and amplified, cloned sequences are identified as such by the plasmid prefix "p"

Table 2 Analysis of16S rDNA molecules amplified, cloned, and sequenced from cryoconite sediment

DNA clone designation	GenBank accession number	Sequence alignment		Nearest phylogenetic neighbor (GenBank accession number)
		n[a]	% identity[b]	
pCS6	AY124347	938	97.4	Lake Bonneyclone (AF173825)
			91.8	Freshwater lake clone (AJ290043)
pCS11	AY124351	962	94.8	Mammoth Hot Springs clone (AF445725)
			94.2	*Pseudoxanthomonas* sp. (AB039330)
pCS2	AY124343	964	93.0	sludge cloneS6 (AF234751)
			91.0	Lake Bonney clone (AF173824)
pCS5	AY124346	943	84.1	*Flexibacter ruber* (M58788)
			83.1	*Flectobacillus sp*. (AJ011917)
pCS3	AY124344	958	90.3	Obsidian pool clone (AF018188)
			90.2	Thermal soil clone (AF391976)
pCS12	AY124352	972	90.5	pCS4[c]
			85.9	*Gemmimonas aurantiaca* (AB072735)
pCS4	AY124345	965	90.5	pCS12[c]
			87.9	*Gemmimonas aurantiaca* (AB072735)
pCS9	AY124349	963	95.1	Freshwater ake clone (AJ290012)
			94.8	*Prosthecobacter sp*. FC2 (U60013)
pCS10	AY124350	968	98.1	*Nocardioides sp*. OS4 (U61298)
			97.4	Rice paddy isolate (AJ229240)
pCS7	AY124348	961	95.0	*Georgenia muralis* (AJ308598)
			94.3	*Cellulomonas gelida* (AF282627)
pCS1	AY124342	961	96.5	Industrial gas filter clone (AJ318140)
			96.0	CS117[c]
pCSC9	AY124359	627	99.7; 99.0	pCSC28[c]; pCSC22[c]
			99.0	Lake Bonney clone-80 (AF076162)
pCSC28	AY124357	627	99.7; 99.0	pCSC9[c]; pCSC22[c]
			99.0	Lake Bonney clone-80 (AF076162)
pCSC22	AY124356	627	99.7	Lake Bonney clone-80 (AF076162)
			99.5	Lake Bonney clone-47 (AF076163)
pCSC1	AY124353	627	90.9	Lake Bonney clone-75 (AF076164)
			90.8	Lake Bonney clone-46 (AF076165)
pCSC4	AY124358	627	99.3	Lake Bonney clone-76 (AF076158)
			99.0	pCSC17[c]
pCSC17	AY124355	627	99.0; 98.9	pCSC4[c]; pCSC14[c]
			98.7	Lake Bonney clone-76 (AF076158)
pCSC14	AY124354	627	99.5	Lake Bonney clone-7 (AF076161)
			99.3	Lake Bonney clone-76 (AF076158)

[a] The length of 16S rDNA in nucleotides used for alignment and phylogenetic analyses
[b] The percent identity of the experimental sequence with the 16S rDNA sequence of the two nearest known phylogenetic neighbors
[c] Isolate or clone from this study

that is 99.5% identical to that of the psychrophile *Cryobacterium psychrophilium*. Genomic DNA was also prepared from several other isolates that formed visibly different colonies. Based on the sequences of the 16S rDNA molecules amplified from these DNAs, these isolates were also members of the *Cytophagales* or *Actinobacteria* lineages or were *β-Proteobacteria* (Table 1; Fig. 2).

Phylogenetic analysis of the 16S and 18S rDNA sequences amplified and cloned directly from cryoconite sediment

DNA preparations obtained directly from cryoconite sediment were used as the template in many PCRs, but only bacterial rDNA molecules were amplified from these preparations in reactions primed by the universal primers 515F with 1492R (Table 2; Fig. 2). Based on their sequences, these originated from members of the *Acidobacterium*, *Actinobacteria*, *Cytophagales*, *Gemmimonas*, *Planctomycetes*, α- and *γ-Proteobacteria*, and *Verrucomicrobia* bacterial lineages. When the primers used were 106F and 781R, all the 16S rDNA molecules amplified, cloned, and sequenced originated from *Cyanobacteria* (Table 2; Fig. 3). These molecules had sequences that were most similar either to each other or to the sequences of rDNA molecules previously amplified from microbial aggregates in the permanent ice that covers Lake Bonney in the McMurdo Dry Valley region of Antarctica (Priscu et al. 1998). Based on their predicted secondary structures, the few nucleotide differences present in the amplified cyanobacterial rDNAs with >98.8% identity (pCSC4, pCSC9, pCSC14, pCSC17, pCSC22, and pCSC28) were located in single-stranded loops or did not constitute differences likely to destabilize conserved double-stranded RNA regions.

The sequences of the small subunit rDNA molecules that were amplified and cloned from the cryoconite DNA preparations using Eukarya-specific primers demonstrated that the cryoconite hole also contained Eukarya most similar to previously described nematode (*Plectus aquatilis*), tardigrade (*Macrobiotus hufelandi*), rotifer (*Philodina acuticornis*), truffle (*Choiromyces meandriformis*), green algal (*Pleurastrium insigne*), and ciliate (*Spathidium* sp.) species (Table 3; Fig. 4).

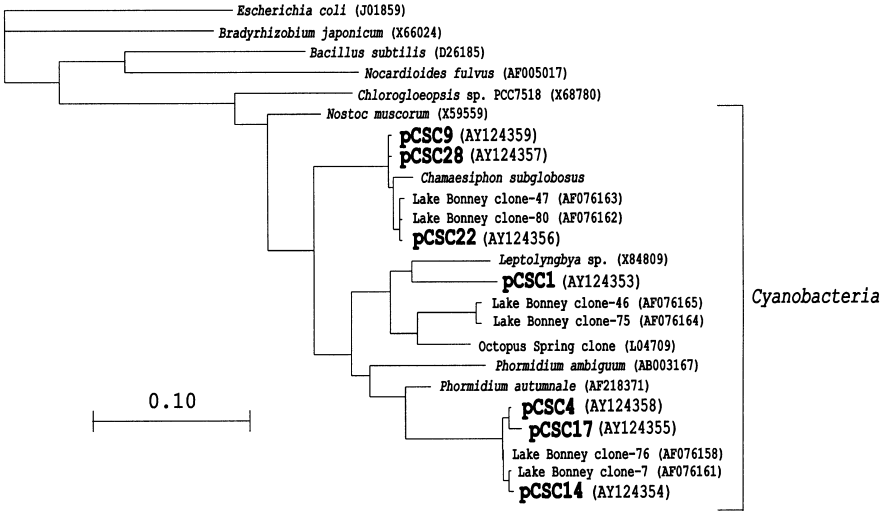

Fig. 3 Phylogenetic analysis of *Cyanobacteria*-derived 16S rDNA sequences. The DNA molecules amplified, cloned, sequenced, and used to construct the maximum likelihood tree shown by fastDNAml (Olsen et al. 1994) corresponded to the region from nucleotides 106–781 of the *E. coli* 16S rDNA. The scale bar indicates 0.1 fixed substitutions per nucleotide position. GenBank accession numbers are listed in *parentheses*. The 16S rRNA sequence for *Chamaesiphon subglobosus* is not listed in GenBank and was obtained from the ARB database (Strunk et al. 1998). The sequences obtained are indicated by *bold* typeface and, as DNA clones (see Fig. 2), have the prefix "p"

Discussion

Based on their isolation and the ease of bacterial 16S rDNA amplification from the cryoconite DNA preparations, bacteria were abundant in this Antarctic ecosystem, and, noticeably, the nearest phylogenetic relatives of several of these isolates were species previously recovered from similar environments, namely, from a Lake Fryxell microbial mat (Brambilla et al. 2001), sea ice (Bowman et al. 1997; Junge et al. 1998), and melted glacial ice (Christner et al. 2000). Photosynthetic CO_2 fixation and microbial N_2 fixation are thought to provide the nutrient foundation for the development of cryoconite hole communities. Only one alga-derived 18S rDNA molecule (pCSE1) was cloned from the population of molecules amplified using Eukarya-specific primers, but seven different 16S rDNA molecules were cloned from the population of molecules amplified by using *Cyanobacteria*-specific primers. These sequences originated from species most similar to previously documented members of the cyanobacterial

Table 3 Analysis of18S rDNA molecules amplified, cloned, and sequenced from cryoconite sediment

DNA clone designation	GenBank accession number	Sequence alignment		Nearest phylogenetic neighbor (GenBank accession number)
		n[a]	% identity[b]	
pCSE19	AY124362	842	99.5	*Plectus aquatilis* (AF036602)
			98.2	*Plectus acuminatus* (AF037628)
pCSE8	AY124369	854	96.2	*Macrobiotus hufelandi* (X81442)
			95.8	pCSE4[c]
pCSE4	AY124361	840	95.8	pCSE8[c]
			93.5	*Macrobiotus hufelandi* (X81442)
pCSE35	AY124364	847	98.9; 99.8	pCSE21[c]; pCSE2[c]
			98.3	*Philodina acuticornis* (U41281)
pCSE21	AY124367	847	99.4	pCSE2[c]
			99.1	*Philodina acuticornis* (U41281)
pCSE2	AY124368	847	99.4	pCSE21[c]
			99.0	*Philodina acuticornis* (U41281)
pCSE14	AY124370	759	97.8	*Choiromyces meandriformis* (AF054904)
			97.0	*Hydnotrya tulasnei* (U53379)
pCSE1	AY124360	836	99.0	*Pleurastrum insigne* (Z28972)
			99.0	*Chloromonas perforata* (U70794)
pCSE9	AY124363	734	96.9	pCSE3[c]
			96.6	*Spathidium* sp. (Z22931)
pCSE3	AY124365	737	98.1	*Spathidium* sp. (Z22931)
			97.8	pCSE28[c]
pCSE28	AY124366	737	98.1	*Spathidium* sp. (Z22931)
			97.8	pCSE3[c]

[a] The length of 16S rDNA in nucleotides used for alignment and phylogenetic analyses
[b] The percent identity of the experimental sequence with the 16S rDNA sequence of the two nearest known phylogenetic neighbors
[c] Isolate or clone from this study

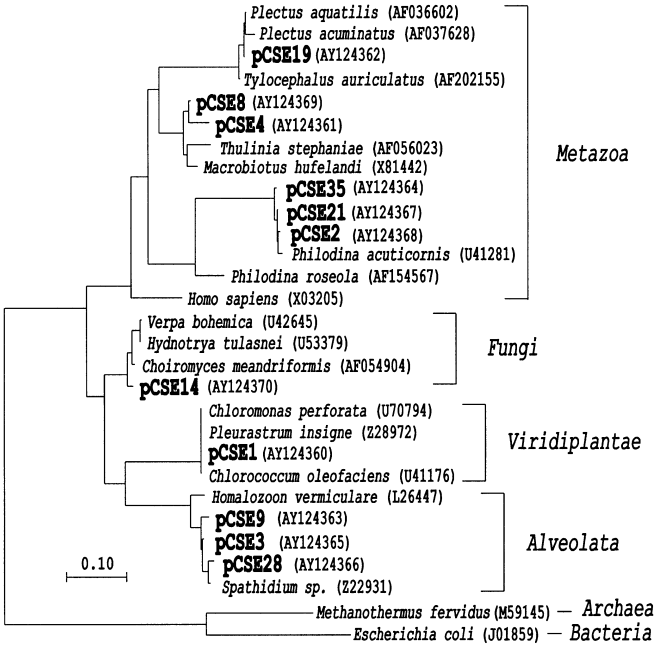

Fig. 4 Phylogenetic analysis of amplified eukaryal 18S rDNA sequences. The DNA molecules amplified, cloned, sequenced, and used to construct the maximum likelihood tree shown by fastDNAml (Olsen et al. 1994) corresponded to the region from nucleotides 515–1195 of the *E. coli* 16S rDNA. The scale bar indicates 0.1 fixed substitutions per nucleotide position. GenBank accession numbers are listed in *parentheses*. The sequences obtained are indicated by *bold* typeface and, as DNA clones (see Fig. 2), have the prefix "p"

genera *Chamaesiphon*, *Leptolyngbya*, and *Phormidium* (Fig. 3). Phylogenetic analyses revealed that the nearest previously described relatives of some of the *Cyanobacteria* in the cryoconite hole community were from microbial aggregates in the permanent ice covering Lake Bonney (Priscu et al. 1998) or from microbial mats also located in the Antarctic Dry Valley region (Gordon et al. 2000).

Cryoconite holes are one of the few environments in the Antarctic Dry Valleys considered inhabitable by metazoan life (Wharton et al. 1985), and this was supported by our amplification and sequencing of 18S rDNA molecules that originated from *Plectus*, *Macrobiotus*, and *Philodina* species (Table 3). Such rotifer, tardigrade, and nematode species have the ability to differentiate under adverse conditions into metabolically dormant forms and, as such, could possibly also survive within a cryoconite sediment that is completely frozen (Sømme 1996).

Every cryoconite hole formed is, in essence, unique and therefore may support a novel and discrete ecosystem. However, based on the results obtained, the cryoconite hole investigated was inhabited primarily by species very similar to species previously documented in other Antarctic Dry Valley environments. It seems most likely, therefore, that particulates blown onto the glacier from adjacent locations were responsible for seeding this cryoconite hole. Cryoconite hole communities are completely frozen during the Antarctic winter, but with summer warming and glacial melting, the members of these communities that survive might serve in reverse to ensure the reseeding of surrounding environments. The concept that cryoconite holes could serve as biological refuges through extended periods of subzero temperatures warrants more detailed investigation. The information gained could be directly relevant to discussions of the likelihood of life surviving a global freezing "Snowball Earth" event (Hoffman et al. 1998; Kirschvink et al. 2000).

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