

Cold-active acetogenic bacteria from surficial sediments of perennially ice-covered Lake Fryxell, Antarctica

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Introduction

Acetogenic bacteria are common in anoxic freshwater and marine sediments (Drake, 1992), sewage sludge (Schink & Bomar, 1999), the gastrointestinal tracts of termites (Breznak & Switzer, 1986) and in the bovine rumen (Greening & Leedle, 1989). Acetogens are obligate anaerobes and can grow autotrophically with CO₂ as the sole carbon source and H₂ as the electron donor, forming acetate via the acetyl-CoA pathway by the reaction: $4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$ (Schink & Bomar, 1999). In addition, typical acetogenic bacteria can catabolize C₁ compounds, such as formate and methanol, some methylated and aromatic substrates, certain organic and fatty acids, and several sugars, all of which are converted to acetate (Schink & Bomar, 1999).

While much is known about the ecology and physiology of acetogenic bacteria from temperate ecosystems (Dolfing, 1988; Breznak, 2001; Drake & Küsel, 2003), acetogens from cold environments, especially permanently cold environments, have only been described in recent years (Kotsyurbenko *et al.*, 1995; Simankova *et al.*, 2000). The microbiology of cold anoxic environments has predominantly focused on methanogenesis and sulfidogenesis

Abstract

Cold-active acetogenic bacteria in the permanently cold sediments of Lake Fryxell, Antarctica were investigated using culture-based methods. Two psychrophilic, acetogenic strains were isolated and found to be physiologically and phylogenetically related to *Acetobacterium bakii* and *Acetobacterium tundrae*. However, the Antarctic isolates showed a lower growth temperature range than other species of *Acetobacterium*, with growth occurring from -2.5 to 25 °C and optimally at 19 – 21 °C. Cultures incubated at $+5$ and $+1$ °C grew with generation times of 7 and 9 days, respectively. The rapid growth of these strains at low temperatures suggests that acetogenesis may be an important anaerobic process in the sediments of Lake Fryxell.

(Smith *et al.*, 1993; Kotsyurbenko *et al.*, 1996; Nozhevnikova *et al.*, 1997; Knoblauch *et al.*, 1999; Ravensschlag *et al.*, 2000; Bohn *et al.*, 2002; Purdy *et al.*, 2003; Karr *et al.*, 2005, 2006). However, in contrast to temperate regions where acetogens are often out-competed by methanogens and sulfate-reducing bacteria (SRB), acetogenesis appears to be a significant process in cold anoxic environments (Jones & Simon, 1985; Conrad *et al.*, 1989; Conrad & Wetter, 1990; Nozhevnikova *et al.*, 1994; Kotsyurbenko *et al.*, 2001). This is because the growth rates of acetogenic bacteria often exceed those of methanogens and SRB at cold temperatures (Simankova *et al.*, 2000; Kotsyurbenko *et al.*, 2001; Nozhevnikova *et al.*, 2001; W.M. Sattley and M.T. Madigan, unpublished results).

Cold-active acetogenic bacteria have been isolated from several low-temperature environments, including lake sediments (Nozhevnikova *et al.*, 2001), polluted pond sediments from a paper mill, tundra wetland soils, and low-temperature-digested cattle manure (Kotsyurbenko *et al.*, 1995; Simankova *et al.*, 2000; Nozhevnikova *et al.*, 2001). All of these organisms belong to the genus *Acetobacterium* and have a minimum growth temperature of 1 °C and a maximum growth temperature of 30 or 35 °C. Optimal growth of these species occurs at 20 – 30 °C. In this work, two strains of acetogenic bacteria isolated from surficial sediments of

the permanently ice-covered, meromictic Lake Fryxell, located in the McMurdo Dry Valleys of Antarctica, are described. The new strains show significant metabolic activity in the near-freezing *in situ* temperatures of the lake and represent the first acetogenic isolates capable of growth at subzero temperatures.

Materials and methods

Field study site and sample collection

Sediments were collected from Lake Fryxell during November 2001 and November 2003 as previously described (Karr *et al.*, 2003; Sattley & Madigan, 2006). Surficial sediment samples (within the top 25 cm) were retrieved with an Ekman dredge (benthic grab sampler). A sterile scoop was used to completely fill acid-washed (10% HCl), sterile polyvinyl chloride bottles. Samples were transported at 4 °C in darkness to Crary Laboratory in McMurdo for culture manipulations.

Growth media and culture incubation

Enrichment cultures for acetogenic bacteria were established in a defined growth medium previously used for the enrichment of SRB (Karr *et al.*, 2005). Primary enrichment cultures were established by aseptically placing 0.5 g of Lake Fryxell sediment into completely filled, screw-capped, 17-mL tubes of buffered minimal medium (pH 7.2) containing 20 mM sodium lactate. Solutions of NaHCO₃ and Na₂S · 9H₂O were autoclaved separately and added after cooling to final concentrations of 0.1% and 0.03%, respectively. Resazurin (1.0 mg L⁻¹) was used to confirm anoxic conditions. Trace elements (Sattley & Madigan, 2006) contained only chloride salts when used in sulfate-free control media.

Primary enrichment cultures were incubated in the dark at 4 °C for 3–4 weeks and transferred to fresh tubes of liquid medium when visibly turbid. Purification was carried out using a modified version of the agar shake tube dilution method (Isaksen & Teske, 1996) as follows. Ten-fold dilutions of each enrichment culture were carried out in tubes containing 6 mL of cooled (10 °C), prereduced (0.5 mM sulfide) liquid lactate medium. Each diluted culture was then poured directly into a molten agar deep (50–55 °C) containing 3 mL of 3% sterile agar, resulting in a combined temperature of 25–30 °C. These procedures protected the cold-adapted organisms from heat shock. The agar shake tubes were then gently mixed and placed immediately on ice. A 5 mL overlay of sterile mineral oil was placed over the solidified medium in each tube as an oxygen barrier, and the tubes were incubated in GasPakTM anoxic jars to ensure that they remained anoxic during extended incubations at low temperatures.

Pure cultures were obtained by aseptically transferring colonies that developed in the agar shake dilutions to tubes of liquid medium. Multiple passes through shake-tube dilution series were used to purify the strains. Culture purity was verified by microscopic observations and by the lack of growth in anoxic complex media, including tryptic soy broth and nutrient broth. Pure cultures of the acetogenic strains were routinely maintained at 10 °C in defined medium supplemented with 10 mM morpholinepropanesulfonic acid (MOPS) (Sigma, St Louis) and transferred monthly. To prevent freezing, growth media were supplemented with 2% (final concentration) dimethyl sulfoxide when incubations were carried out at subzero temperatures.

The capacity for autotrophic growth was tested using a H₂/CO₂ (80:20) headspace in anoxic mineral salts media. Cultures were established in 24 mL crimp-top butyl rubber-stoppered tubes containing 8 mL of the sulfide-reduced minimal medium. The headspace of each tube was purged for 2 min with pure H₂, and 3 mL of gaseous CO₂ was then injected with a sterile syringe, leaving the tubes under a slight overpressure. The overpressure helped maintain anoxic conditions at cold temperatures and forced headspace gases into solution. Tubes were incubated at 10 °C on their sides to increase the surface area of the growth medium to the H₂/CO₂ headspace.

Microscopy

Electron micrographs of mid-exponential phase cells were obtained as described by Kimble *et al.* (1995), in which cells were first fixed in 2% glutaraldehyde followed by fixation in OsO₄ (transmission electron microscopy), or via dehydration by ethanol washing followed by critical point drying (scanning electron microscopy).

Molecular analyses

Genomic DNA extractions and PCR amplification of 16S rRNA genes were performed as previously described (Sattley & Madigan, 2006). Controls containing no DNA were included in all PCR reactions, and all 16S rRNA gene amplification products were verified to be of proper size (~1500 bp) using a 1 Kb DNA ladder (Promega Corp, Madison, WI). PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen Sciences, Germantown, MD).

Automated sequencing of 16S rRNA gene products was performed at either Southern Illinois University in Carbondale, IL (strain LS1) or Washington University in St Louis, MO (strain LS2). Sequenced fragments of the 16S rRNA genes of isolated strains were assembled using the CAP3

sequence assembly program (Huang & Madan, 1999), and closely related species were identified using both the BLASTN function of BLAST and the Sequence Match function of the Ribosomal Database Project II. A multiple alignment was assembled, and a phylogenetic tree was constructed using MacVector™ 7.2.2. Details of the tree construction are described in the legend to the phylogenetic tree.

Nucleotide sequence accession numbers

Sequences of the 16S rRNA genes from Antarctic acetogenic strains LS1 and LS2 were deposited into GenBank under accession numbers DQ767879 and DQ767880, respectively. Accession numbers for all organisms used in the phylogenetic analysis are indicated directly on the phylogenetic tree. Cultures of strains LS1 and LS2 are available from the authors.

Results

Enrichment, isolation and morphology

Primary enrichment cultures were established by placing 0.5 g of Lake Fryxell surficial sediments into the sulfide-reduced liquid mineral salts medium containing lactate (20 mM) as carbon and energy source. Replicate enrichments were established at 4, 10 and 18 °C. Cultures became turbid after 2–4 weeks, depending upon incubation temperature. Two strains of morphologically identical acetogenic bacteria, strains LS1 and LS2, were eventually isolated in pure culture.

The isolated strains consisted of lemon-shaped cells with bluntly pointed ends (Fig. 1a and b). Cells averaged 0.6–0.9 µm in width and 0.7–1.0 µm in length. The motile, gram-positive cells existed singly or in pairs (Fig. 1), but nonmotile chains of up to 10 cells were common in older batch cultures. Both strains grew well in the anoxic, lactate-containing medium at 10 °C. Viability

remained high in cultures maintained for several weeks beyond the exponential phase of growth, as evidenced by growth upon transfer of stationary phase cells to fresh liquid growth media.

Phylogeny

Phylogenetic analyses indicated that isolates of this study grouped within the family *Eubacteriaceae*, specifically, within the genus *Acetobacterium* (Fig. 2). The Lake Fryxell strains were very similar to one another but not identical (99.7% identity) and were most closely related to two moderately psychrophilic acetogens, *Acetobacterium bakii*, isolated from a polluted paper-mill waste pond in northern Russia (Kotsyurbenko *et al.*, 1995), and *Acetobacterium tundrae*, isolated from a tundra wetland in northern Russia (Simankova *et al.*, 2000). The Antarctic acetogens were also related to *Acetobacterium paludosum*, isolated from the anoxic sediment of a swamp north of Moscow (Kotsyurbenko *et al.*, 1995). In general, the neighbor-joining distance tree showed the close relationship of the Lake Fryxell acetogens to acetobacteria from other cold environments and their more distant relationship to mesophilic species of *Acetobacterium*, including the type species, *Acetobacterium woodii* (Fig. 2).

Electron donors and culture conditions

The Lake Fryxell acetogens used a variety of carbon sources for growth (Table 1). Growth was supported by several organic acids and sugars and also occurred autotrophically with H₂/CO₂ (80:20); however, autotrophic growth was slow compared with growth on organic substrates. For example, lactate-containing cultures of the LS strains became visibly turbid (OD_{540 nm} > 0.08) after 9–12 days at 18 °C, while cultures grown autotrophically took 1–2 weeks longer to reach similar turbidities.

Strains LS1 and LS2 grew from pH 6.6 to 8.6 in lactate-containing media incubated at 18 °C; optimal growth

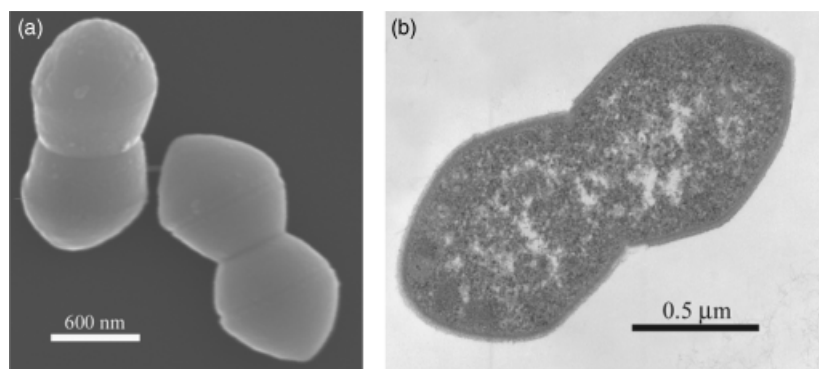


Fig. 1. Scanning (a) and transmission (b) electron micrographs of dividing cells of strain LS1. The gram-positive cell wall of this strain is evident in the thin-section (b).

Fig. 2. Phylogenetic tree generated from 1473 nucleotide positions of the 16S rRNA gene using the Kimura-2 parameter distance algorithm in a heuristic search. Only cultured strains were included in the analysis. GenBank accession numbers are listed in parentheses. Organisms shown are members of the *Eubacteriaceae*, with the exception of the outgroup organism, *Desulfotomaculum ruminis*, an endospore-forming sulfate-reducing bacterium. Tree topology was maintained with all analyses, and high bootstrap values (> 75%) based on 1000 replicates are represented.

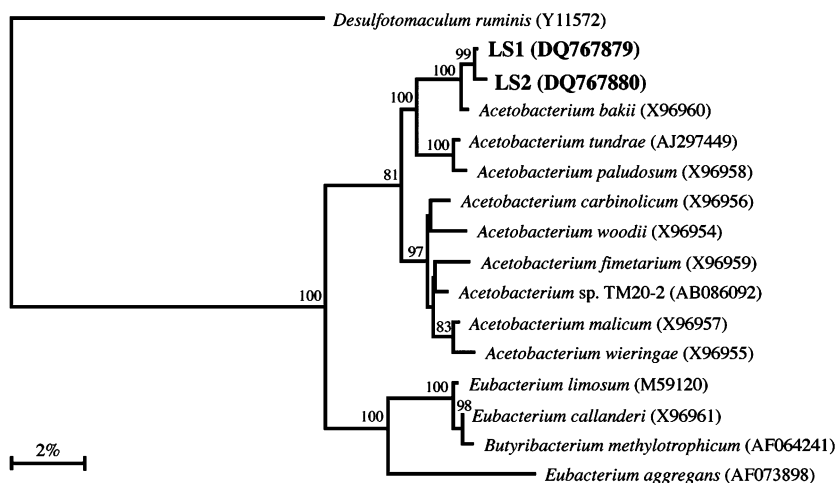


Table 1. Growth-supporting substrates for Lake Fryxell acetogenic strains LS1 and LS2 and other species of *Acetobacterium*

Substrate	Growth					
	Strains LS1/LS2*	<i>A. bakii</i> [†]	<i>A. paludosum</i> [†]	<i>A. fimetarium</i> [†]	<i>A. tundrae</i> [‡]	<i>A. carbinolicum</i> [§]
H ₂ /CO ₂	+	+	+	+	+	+
Formate	+	+	+	+w	+w	+
Sucrose	+w	–	–	–	–	–
Maltose	+	+w	+	–	+	ND
Cellobiose	–	–	+w	–	–	ND
Glucose	+	+w	+	–	++	+
Fructose	+	++	++	++	++	+
Mannose	–	ND	ND	ND	+	ND
Ribose	+	–	–	–	–	ND
Galactose	+	–	–	–	–	ND
Xylose	+	+w	+w	–	+w	ND
Methanol	+	+	++	–	+	+
Ethanol	–	–	–	–	–	+
Propanol	–	–	–	–	–	+
Butanol	–	–	–	–	–	+
Betaine	+w	+	+	+	+	+
Lactate	++	+	+	+	+	+
Pyruvate	++	ND	ND	ND	+	+
Succinate	–	ND	ND	ND	–	ND
Malate	+w	+	+	+	–	–
Citrate	–	ND	ND	ND	–	ND
Vanillate	+w	+w	–	+w	+w	ND

*Time zero OD_{540 nm}, 0.05–0.06; ++, OD_{540 nm} > 0.2; +, OD_{540 nm} 0.1–0.19; +w, OD_{540 nm} 0.06–0.09; –, OD_{540 nm} < 0.06. Strains LS1 and LS2 were incubated at 18 °C for 2.5 weeks in a mineral salts medium containing a single carbon source. Fumarate, trimethylamine, and lactose do not support growth of any *Acetobacterium* species listed. Arabinose, melibiose, raffinose and rhamnose do not support growth of any *Acetobacterium* species listed except for *A. carbinolicum*, where these substrates were not tested. Additional substrates tested but not supporting growth of strains LS1 and LS2 included propionate (10 mM), butyrate (5 mM), benzoate (3 mM), valerate (5 mM), and caproate (3 mM).

[†]Kotsyurbenko *et al.* (1995).

[‡]Simankova *et al.* (2000).

[§]Eichler & Schink (1984); Tanaka & Pfennig (1988).

++, excellent growth; +, good growth; +w, weak growth; –, no growth; ND, no data.

occurred at pH 7.5. The pH of dense MOPS-buffered cultures of strains LS1 and LS2 dropped by about 0.5 U. In addition, both strains were mildly halotolerant, growing in media containing up to 3.6% NaCl (w/v). However,

neither strain required NaCl, nor did the addition of small amounts of NaCl stimulate growth. The most rapid growth of these strains occurred in media devoid of NaCl (data not shown).

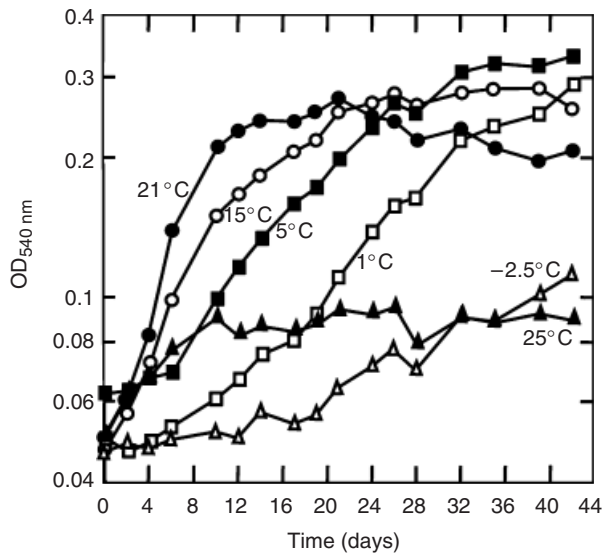


Fig. 3. Growth ($OD_{540\text{nm}}$) vs. temperature of strain LS1 over a 42-day incubation period in lactate-containing media. Strain LS2 showed a nearly identical growth response to temperature, and thus, only results for strain LS1 are shown.

Temperature relationships

Strains LS1 and LS2 both showed a cold-active phenotype. Growth occurred from -2.5 to 25 °C (Fig. 3), which is cooler than the growth temperature range for other bacteria recently isolated from Lake Fryxell, including anoxygenic phototrophic bacteria (Madigan *et al.*, 2000; Jung *et al.*, 2004) and sulfur-oxidizing chemolithotrophic bacteria (Sattley & Madigan, 2006). The optimum temperature for growth of strains LS1 and LS2 was 19 – 21 °C, at which cultures became visibly turbid in about 1 week. A generation time of 60 h was calculated for strain LS1 grown at 21 °C (Fig. 3). At 5 °C, strain LS1 had a generation time of 7 days, with cultures reaching the stationary phase after *c.* 1 month (Fig. 3). Strain LS1 grew at 1 °C with a generation time of 9 days, becoming densely turbid and reaching the stationary phase after about 40 days of growth (Fig. 3). At -2.5 °C, cells of strain LS1 grew steadily after an extended lag phase and continued to grow over a 42-day incubation period (Fig. 3). Growth of strains LS1 and LS2 was weak at the upper temperature limit of 25 °C (Fig. 3).

Discussion

The acetogenic bacteria described here were enriched and isolated from a mineral salts/lactate/sulfate medium designed for the growth of SRB. Enrichment cultures established with Lake Fryxell sediment contained two cell types: the lemon-shaped acetogenic bacteria and rod-shaped organisms that turned out to be SRB. Although mesophilic acetogenic bacteria are often outcompeted

by SRB in enrichment cultures containing sulfate (Widdel, 1988), the Antarctic acetogens maintained approximately equal cell numbers with the SRB after several transfers at low temperatures (≤ 10 °C). Following their isolation in pure culture, the LS strains were shown to be phylogenetic relatives of *Acetobacterium*, specifically, *A. bakii* and *A. tundrae* (Fig. 2). Morphologically, strains LS1 and LS2 resemble *A. bakii*, while cells of *A. tundrae* are more elongated, swollen and rod-shaped than the LS strains (Kotsyurbenko *et al.*, 1995; Simankova *et al.*, 2000).

Strains LS1 and LS2 grew on a variety of organic acids and sugars, with growth also occurring on methanol and betaine, and autotrophically on $H_2 + CO_2$, as is typical for many species of *Acetobacterium* (Schink & Bomar, 1999). However, unlike other species of *Acetobacterium*, strains LS1 and LS2 grew on galactose and ribose, and weakly on sucrose but not on mannose (Table 1), further distinguishing the Lake Fryxell acetogens from other *Acetobacterium* species.

The LS strains grew between pH 6.6 and 8.6, with an optimal pH of 7.5. This is distinct from the more acid-tolerant *A. bakii*, which grows from pH 5.5 to 8.5, and optimally at pH 6.5 (Kotsyurbenko *et al.*, 1995). The pH range of *A. tundrae* is closer to that of the Lake Fryxell isolates but still slightly lower at pH 6.0–8.0, with an optimum of pH 7.0 (Simankova *et al.*, 2000). In addition, the LS strains were able to tolerate up to 3.6% NaCl (w/v); the bottom waters and sediments of Lake Fryxell are mildly saline – about 1% NaCl (Karr *et al.*, 2005) – and so some salt tolerance was to be expected.

Strains LS1 and LS2 grew optimally from 19 to 21 °C. However, being capable of growth at subzero temperatures (-2.5 °C) and having a maximum growth temperature of 25 °C, Antarctic acetogens of this study show the greatest degree of cold tolerance of any described species of *Acetobacterium*. For example, *A. bakii* and *A. tundrae* grow between 1 and 30 °C, with optimal growth occurring at 20 °C (Kotsyurbenko *et al.*, 1995; Simankova *et al.*, 2000). The warmer growth temperature range of *A. bakii* and *A. tundrae* may be due to wider temperature fluctuations in their habitats. By contrast, the capacity to withstand occasional warming would provide no advantage to acetogens inhabiting the constantly cold sediments of Lake Fryxell.

The Lake Fryxell acetogens had a similar growth temperature optimum (~ 20 °C) to the Lake Fryxell SRB with which they were initially enriched. However, subsequent studies of pure cultures have revealed that the acetogens grow significantly faster at colder temperatures than the SRB. For example, at 1 °C, strain LS1 reached maximum turbidity after 42 days (Fig. 3), while cultures of SRB took 3 weeks longer to reach similar turbidities at the same temperature (data not shown; W.M. Sattley & M.T. Madigan,

unpublished results). Conversely, the generation time of Lake Fryxell SRB at 21 °C was only half that of the 60 h generation time of strain LS1. Thus, when enrichments were carried out at cold temperatures, the acetogens could compete successfully with SRB. Although it cannot be assumed that all acetogenic bacteria and SRB in Lake Fryxell have a similar temperature response, the growth results from isolates of this study support the general picture of acetogenesis as a process that competes well with other forms of anaerobic catabolism in cold anoxic environments (Jones & Simon, 1985; Conrad *et al.*, 1989; Conrad & Wetter, 1990; Nozhevnikova *et al.*, 1994; Kotsyurbenko *et al.*, 2001).

SRB have a higher affinity for H₂ than do either methanogens or acetogens and thus tend to dominate sulfate-containing anoxic waters and sediments (Widdel, 1988). Although SRB and low (micromolar) levels of sulfate are present in the cold sediments of Lake Fryxell (Karr *et al.*, 2005; W.M. Sattley & M.T. Madigan, unpublished results), the comparatively rapid growth of the Antarctic acetogenic isolates at low temperatures may help these organisms overcome the bioenergetic advantage that higher H₂ affinities confer on SRB. Moreover, the ability to use a wide variety of sugars, substrates not used by most SRB (Widdel, 1988), may also provide a competitive advantage to acetogens in Lake Fryxell. Collectively, these observations suggest that, in addition to the methanogenesis (Smith *et al.*, 1993; Karr *et al.*, 2006) and sulfate reduction (Karr *et al.*, 2005; Vladimir Samarkin & M.T. Madigan, unpublished results) that occurs in Lake Fryxell, acetogenesis may also be a significant anaerobic process in the sediments of this polar lake. Because acetogens are also autotrophs, they may also provide organic matter to Lake Fryxell, an exclusively microbial ecosystem.

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