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Cold-Active Chemoorganotrophic Bacteria from Permanently Ice-Covered Lake Hoare, McMurdo Dry Valleys, Antarctica▽

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Eight strains of chemoorganotrophic bacteria were isolated from the water column of Lake Hoare, McMurdo Dry Valleys, Antarctica, using cold enrichment temperatures. The isolates were *Alpha*-, *Beta*-, and *Gammaproteobacteria* and *Actinobacteria* spp. All isolates grew at 0°C, and all but one grew at subzero temperatures characteristic of the water column of Lake Hoare. Growth temperature optima varied among isolates, but the majority showed optima near 15°C, indicative of cold-active phenotypes. One isolate was truly psychrophilic, growing optimally around 10°C and not above 20°C. Half of the isolates grew at 2% salt while the other half did not, and all but one isolate grew at 2 atm of O_2. Our isolates are the first prokaryotes from the water column of Lake Hoare to be characterized phylogenetically and physiologically and show that cold-active species of at least two major phyla of *Bacteria* inhabit Lake Hoare.

Prokaryotes are abundant and active in polar environments (3, 18). Antarctic lakes are particularly interesting in this respect because they are exclusively microbial ecosystems (28, 42). Several permanently ice-covered lakes exist in the McMurdo Dry Valleys, Antarctica. The major Taylor Valley lakes, Hoare, Bonney, and Fryxell, were formed by glacial deepening and have a permanent ice cover that varies from 2 to 6 m thick (10, 28). Among Taylor Valley lakes, the ice cover of Lake Hoare is the most rugged (Fig. 1A). Lake Hoare is also the most oligotrophic and oxic of Taylor Valley lakes; dissolved organic carbon (DOC) levels are below 0.5 mg/liter, salt is present in only trace amounts, and the water column is supersaturated with oxygen to a depth of 24 m (Fig. 1B).

Although cultures of various bacteria have been isolated from Lakes Fryxell and Bonney (2, 14, 37, 45–47) and molecular evidence has been obtained for *Archaea* in Lake Fryxell (15), studies by Mikell et al. (20, 21) and Van Trappen et al. (47) are the only reports of cultured organisms from Lake Hoare. The focus of the Mikell et al. studies (20, 21) was not biodiversity but instead the effect of high levels of dissolved oxygen on Lake Hoare bacteria. The study by Van Trappen et al. (47) focused on bacteria recovered from benthic microbial mats that develop in several of the Taylor Valley lakes. In contrast to these studies, we focus here on planktonic bacteria from Lake Hoare and document the phylogeny and physiology of eight strains of chemoorganotrophic bacteria enriched from different depths. Our results are the first to reveal planktonic bacterial diversity in Lake Hoare and suggest that this constantly cold and oligotrophic lake contains several phylogenetic groups of cold-active bacteria.

Sampling, enrichment, and isolation. Samples were collected from the water column of Lake Hoare through a hole drilled in the ice near the eastern edge of the lake (Global Positioning System coordinates, 77°38′S, 162°53′E) as previously described (16). Lake water was collected with a 5-liter Niskin bottle and immediately transferred to sterile 1-liter polycarbonate bottles; the completely filled bottles were stored in darkness at 4°C until processed.

Enrichment cultures using 10-, 20-, and 22-m Lake Hoare water as inocula were established in 25 ml of liquid medium R2A (30) or in a starch medium prepared in 125-ml Erlenmeyer flasks. The starch medium contained the mineral salts of medium R2A supplemented with the following (per liter): yeast extract, 50 mg; $CaCl_2 \cdot 2H_2O$, 25 mg; NaCl, 0.5 g; NH_4Cl, 0.5 g; and soluble starch, 1 g. Flasks were incubated without shaking in darkness at 2, 10, or 18°C in thermostatically controlled cold boxes. Turbid enrichments were serially diluted with sterile deionized water, plated onto the surface of R2A or starch-based agar, and incubated at the original enrichment temperature. Resultant colonies were picked and restreaked until pure cultures were obtained. Cultures of the Lake Hoare isolates are available from us upon written request.

Physiological studies. Upper and lower temperature limits for growth were determined on plates of medium R2A incubated at −3 to +40°C. For incubations below 0°C, media were supplemented with 1% (vol/vol) sterile dimethyl sulfoxide to prevent the medium from freezing. All plates were wrapped in clear plastic wrap to prevent desiccation and scored for growth by visual inspection. To determine temperature optima, duplicate 10-ml screw-cap tubes containing 3 ml of liquid medium R2A were inoculated with 0.2 ml of exponential-phase cultures and incubated at a temperature series. Optimal growth temperatures are reported as the temperature range that gave the highest cell yields (as measured turbidimetrically [optical density at 540 nm]) in a defined incubation period. Salt tolerance was tested in liquid medium R2A containing either 2% or 5% (wt/vol) NaCl; tubes were inoculated and incubated at 10°C for 21 days and scored for growth against unsupplemented controls. To assess anoxic growth capacity, 3 ml of liquid medium R2A contained in 10-ml tubes was inoculated and incubated in an anoxic jar (Becton Dickinson, Sparks, MD) that was activated and sealed within an anoxic glove box. The tubes were

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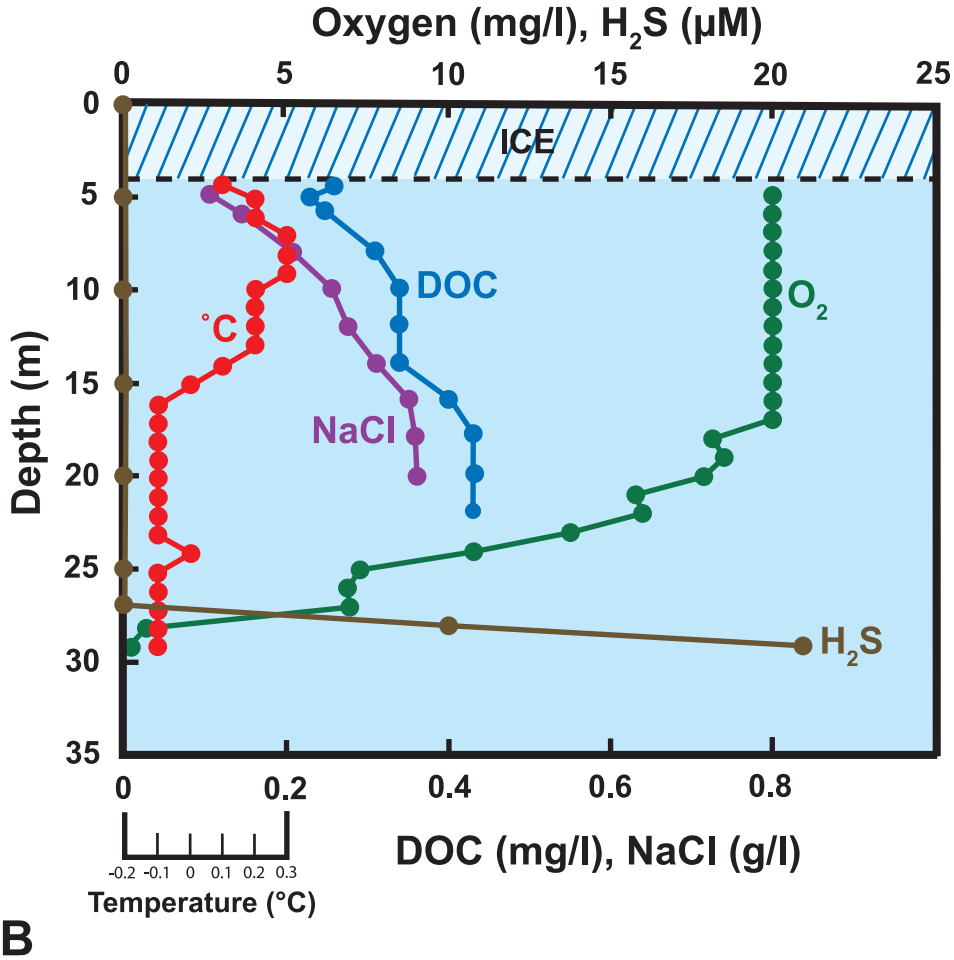

B

TABLE 1. Major properties of cold-active chemoorganotrophs isolated from Lake Hoare

Strain[a]	Enrichment temp (°C)	Gram stain reaction/ morphology	Pigment(s)	Cardinal temp[b] (°C)			Growth with supplement or condition[c]:			Starch hydrolysis[d]
				Max	Min	Opt	2% NaCl	5% NaCl	2 atm O_2	
LH10	18	Negative/curved rods	None	27	−3	15–18	−	−	+	−
LH11	18	Negative/*Vibrio*-like	Yellow	31	−2	14–22	−	−	+	−
LH19	18	Positive/coccoid	Pink/red	33	−2	15–20	+	−	+	+
LH1D	10	Negative/rod	Orange	26	0	15–20	−	−	+	−
LH14	10	Negative/rod	Pink	19	−3	11–15	+	−	−	−
LH15	10	Positive/rod	Yellow/green	40	−2	23–31	+	−	+	+
LH90	2	Negative/rod	None	21	−2	8–11	−	−	+	−
LH197	2	Negative/rod	None	32	−3	10–15	+	+	+	−

[a] Strains LH10, LH1D, LH19, and LH90 were obtained from 10-m Lake Hoare water; strain LH197 was from 20-m water; and strains LH11, LH14, and LH15 were from 22-m water. All isolates except for strain LH197 (enriched on starch) were enriched in medium R2A.
[b] Max, growth temperature maximum; Min, minimum growth temperature; Opt, temperature range that yielded the highest cell density in a defined incubation period in medium R2A.
[c] Medium R2A supplemented with either 2% or 5% (final concentration) NaCl or medium R2A pressurized to 2 atm (200 kPa) with O_2.
[d] As assessed by a zone of clearing around colonies on starch agar treated with Gram's iodine.

scored for growth (optical density at 540 nm) after 8 days at either 15°C or 23°C. Growth at elevated oxygen tensions was assessed in 25-ml crimped-top tubes containing 3 ml medium R2A and pressurized with 99.9% O_2 to 200 kPa.

Phylogenetic analyses. DNA was isolated from 1.5-ml liquid cultures, and small-subunit (SSU) rRNA genes were PCR amplified using universal primers for *Bacteria* (8F, 5′-AGAGTT TGATCCTGGCTCAG-3′, and 1525R, 5′-AAGGAGGTGA TCCAGCC-3′). The PCR product was purified using either the GeneClean Turbo Kit (Q-BIOgene, Albany, NY) or the QIAquick PCR purification kit (QIAGEN Sciences, Valencia, CA) at Southern Illinois University at Carbondale and then sequenced at the Genome Sequencing Center, Washington University, St. Louis, MO. Sequence alignments were made using the ClustalW program of MacVector 7.2 software (Accelrys, San Diego, CA) and confirmed by visual inspection. A phylogenetic distance tree was generated within MacVector using the Jukes-Cantor correction. GenBank accession numbers for the eight Lake Hoare strains and reference organisms used to build the tree are listed on the phylogenetic tree (see Fig. 2).

Enrichment and isolation. Enrichment cultures established from Lake Hoare water and incubated aerobically from 2 to 18°C in medium R2A became visually turbid within 1 to 2 weeks and were subsequently diluted and plated. Medium R2A has been widely used as a culture medium for isolating bacteria inhabiting oligotrophic waters, glaciers, and other Antarctic habitats (1, 4, 22, 45, 47, 49). From the enrichments, pure cultures were eventually obtained by plating and eight strains were chosen for further study based on their robust growth at the enrichment temperature. Seven of the eight strains were enriched and isolated in medium R2A while one was obtained from the starch medium. Table 1 lists the major characteristics of the isolates including enrichment details, Gram stain reaction and morphology, pigmentation, salinity and oxygen tolerances, and cardinal temperatures.

Phylogeny and morphology. The phylogeny and morphology of the eight Lake Hoare strains are shown in Fig. 2. Gram-negative rods dominated; only two of the eight isolates were gram positive. All gram-negative isolates were *Proteobacteria* (Table 1), organisms that are widespread in aquatic environments (11, 17) and Antarctic microbial mats (2, 47). However, only two of our isolates, the gram-positive strain LH19 and the *Gammaproteobacteria* strain LH197, were fairly closely related (>97% SSU sequence identity) to isolates obtained from a Lake Hoare microbial mat (47).

Three gram-negative Lake Hoare isolates were *Betaproteobacteria* (Fig. 2) and were related to cultured relatives from other cold environments. For example, the closest known relative of strain LH14 was the psychrophile *Polaromonas vacuolata*, a bacterium isolated from Antarctic sea ice (12). Strains LH10 and LH90 were related to uncharacterized glacier bacteria, and both showed a more distant relationship to the phototrophic purple bacterium *Rhodoferax antarcticus*, isolated from the water column of Lake Fryxell (14) (Fig. 2).

Lake Hoare strains LH11 and LH1D were *Alphaproteobacteria*, related to species of *Caulobacter* and *Sphingomonas*, respectively (Fig. 2). An uncharacterized bacterium related to *Sphingomonas* has previously been isolated from Ace Lake in the Antarctic Vestfold Hills (47) and the highly oligotrophic Crater Lake in Oregon (25). *Caulobacter* spp. are aquatic bacteria that inhabit seawater, freshwater, and occasionally soil (27). *Caulobacter henricii* was the closest cultured relative of strain LH11. Both *C. henricii* and strain LH11 produced yellow pigments and a stalked morphology in which stalks become

FIG. 1. Lake Hoare geomorphology and geochemistry. (A) Photo of the surface of Lake Hoare looking toward the southeast taken in November 2003. The west side of the Canada Glacier is in the left background; Lake Fryxell lies adjacent to the east edge of the Canada Glacier. The rough surface ice on Lake Hoare forms spikes over 1 m tall, and ice cover thickness varies seasonally from 2 to 6 m. (B) Profiles of temperature, dissolved oxygen, sulfide, NaCl, and DOC in the water column of Lake Hoare. At 0°C, O_2 saturation in water is 14.6 mg/liter. The O_2 meter used here reads to a maximum of 20 mg/liter, but chemical measurements of dissolved oxygen in Lake Hoare show oxygen levels to be over twice saturation in the upper waters (20, 21). DOC and NaCl data were obtained from the McMurdo LTER website (http://www.mcmlter.org/lakes_home.htm) from sampling casts of December 2002 (DOC) and November 2003 (NaCl). All other data were recorded in November 2005.

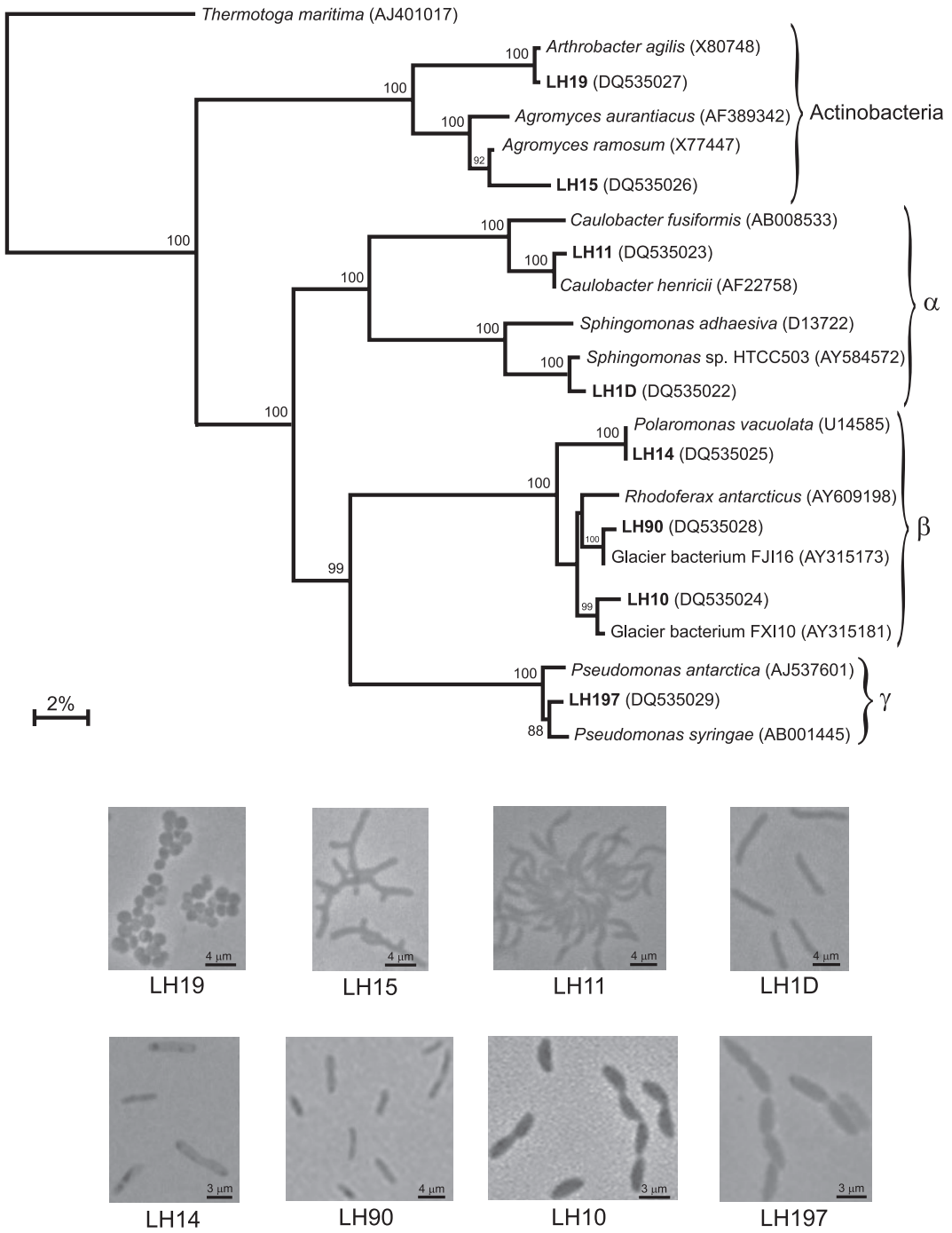

FIG. 2. Phylogeny and morphology of Lake Hoare isolates. (Top) Evolutionary distance tree (using the Jukes-Cantor correction) based on SSU rRNA gene sequencing (1,236 bp) showing the phylogenetic positions of the eight Lake Hoare (LH) strains isolated in this work. Bootstrap values are shown at the nodes and are based on 1,000 replications. Similar trees were also obtained using other distance methods, including Tajima-Nei, Kimura two-parameter, and Tamura-Nei, and also using parsimony methods. GenBank accession numbers are shown in parentheses. (Bottom) Phase-contrast photomicrographs of cells of each Lake Hoare strain.

attached to form cell rosettes (26) (Fig. 2). However, *C. henricii* cannot grow at the low temperatures that supported growth of strain LH11 (26) (Table 1).

The gram-positive Lake Hoare isolates, strains LH19 and LH15, were related to *Actinobacteria* (7, 39). Strain LH19 showed a coccoid morphology and was related to species of *Arthrobacter*, a genus containing cocci and short rods that are common in soil (13) and which have also been detected in a Lake Fryxell microbial mat (47). By contrast, strain LH15 was only a distant relative of species of *Agromyces*, a genus of the *Actinomycetes* (7); the filamentous branching pattern of cells of strain LH15 (Fig. 2) is typical of some actinomycetes (7).

Physiology. All Lake Hoare isolates grew aerobically but not anaerobically on medium R2A (which contains glucose); thus, none were capable of fermenting glucose (Table 1). Anoxic medium R2A supplemented with 10 mM (final concentration) of dimethyl sulfoxide, nitrate, or fumarate also did not support growth. We conclude that our isolates are incapable of these common forms of anaerobic respiration, which suggests that they are obligate aerobes. These results are consistent with the high levels of dissolved oxygen in Lake Hoare (Fig. 1B). Only the gram-positive Lake Hoare isolates hydrolyzed starch (Table 1).

Since all of the Lake Hoare isolates experience constant cold in their natural habitat, their cardinal temperatures were a major focus of our study; the results are shown in Table 1. Minimal growth temperatures for the isolates ranged from 0°C to −3°C, indicating that all can grow at in situ temperatures (Fig. 1B). Strain LH14 showed the greatest cold adaptation and is a psychrophile in the classical sense (23); strain LH14 grew to as low as −3°C and showed a maximum growth temperature under 20°C and an optimum near 10°C. Maximum growth temperatures were as high as 40°C in one strain (LH15), but even in this case, growth was still possible at subzero temperatures (Table 1). Interestingly, no strong correlation was observed between enrichment temperature and growth temperature limits, as isolates from any enrichment temperature grew at subzero temperatures (Table 1). Similar findings have emerged from studies of other Antarctic *Bacteria* and *Archaea* (3). These results indicate that, surprisingly, psychrophily may not be common in prokaryotes from this permanently cold environment.

Because of the extremely low salinity of Lake Hoare relative to other Taylor Valley lakes (W. B. Lyons, May 2006, MCM LTER data sets [http://www.mcmlter.org/lakes_home.htm]) (Fig. 1B), the salt tolerance of the isolates was also of interest, and the results are shown in Table 1. Only one of the eight strains, strain LH197, grew in medium containing 5% (wt/vol) NaCl. However, because strain LH197 also grew in medium lacking NaCl, it is halotolerant, not halophilic. Strains LH14, LH15, and LH19 were less halotolerant but still capable of growth at 2% NaCl. Growth of the remaining strains was inhibited by 2% NaCl (Table 1). All strains except the psychrophilic strain LH14 grew in sealed tubes containing 2 atm O_2 (Table 1).

Several of our Lake Hoare strains were pigmented (Table 1), as was true of strains isolated from microbial mats from several Taylor Valley lakes (31–33, 44, 45, 47). For example, in the mat study by Van Trappen et al. (47) 68% of the strain clusters defined by fatty acid composition contained pigmented strains. Intact cells of our Lake Hoare strains LH14, LH15, and LH19 showed absorbance maxima between 430 and 551 nm (data not shown), well within the range for typical carotenoids (41). Strain LH19 had maxima at 551, 515, and 485 nm, very near that of spirilloxanthin (43), while strain LH15 showed maxima at 485 and 452 nm, close to those of spheroidene (43). By contrast, although yellow or orange in color, strains LH11 and LH1D showed absorbance maxima to the blue of 375 nm (data not shown), outside the absorption range of typical carotenoid pigments (41). The nature of these pigments is unknown. None of our isolates yielded spectral evidence for bacteriochlorophyll *a*.

Concluding remarks. Bacteria in Lake Hoare experience several stress factors, in particular low temperature, high oxygen, and oligotrophy. Interestingly, the cardinal temperatures of our eight isolates were similar to those reported for phototrophic purple bacteria (14, 19), sulfate-reducing bacteria (16), and sulfur chemolithotrophic bacteria (34) isolated from Lake Fryxell, which lies adjacent to Lake Hoare on the eastern side of the Canada Glacier (Fig. 1A). That is, although none of our Lake Hoare isolates showed optimal growth at in situ temperatures, all grew readily at 0°C and all but one grew at subzero temperatures. Therefore, all of our isolates (except for strain LH14 [Table 1]) are psychrotolerant. The observation that most of our isolates (and those from other Taylor Valley lakes [14, 19, 34, 36, 42]) are psychrotolerant rather than psychrophilic may be a reflection of the young age of these lakes compared to other constantly cold microbial habitats, such as marine sediments, where psychrophiles seem to be more common (3).

The very deepest waters of Lake Hoare are anoxic and even slightly sulfidic; however, even at a depth of 25 m, the water is oxygen supersaturated (Fig. 1B) (5, 21). This may help explain why pigmented colonies appeared among our Lake Hoare isolates even though light intensities in the water column of Lake Hoare are extremely low (21, 35). Carotenoids can protect cells from oxidative damage. This was dramatically demonstrated in the study by Mikell et al. (20), where enrichments from Lake Hoare water incubated under hyperbaric oxygen yielded only pigmented colonies. However, besides removal of toxic forms of oxygen, carotenoids may improve the survival of cold-active bacteria in other ways. These include affecting membrane structure (40) or functioning as global regulators in response to cell stress from cold shock (8). All but one of our Lake Hoare isolates grew under hyperbaric oxygen (Table 1), and all pigmented strains remained pigmented at different temperatures and oxygen tensions. This indicates that pigmentation is not subject to control by these major environmental variables.

Levels of NaCl and DOC in Lake Hoare are very low (Fig. 1B). Surprisingly, however, half of our isolates grew in medium containing 2% NaCl, and one grew at 5% NaCl, nearly twice the salinity of seawater. This was unexpected but could be a legacy of the origin of these bacteria (see below). Moreover, the discovery that one of our isolates was a species of *Caulobacter*, a classic oligotrophic bacterium (27), is consistent with the low DOC in Lake Hoare (Fig. 1B). Oligotrophy was also underscored by the isolation in a starch-containing medium of strain LH197, an organism subsequently shown to be unable to catabolize starch (Table 1). This organism was therefore enriched on the 50 μg/ml of carbon present from yeast extract added to the medium as a source of vitamins. Collectively, these observations indicate that at least some Lake Hoare bacteria are oligotrophic, as could be predicted from the extremely low DOC present in the lake (Fig. 1B).

The origin of Taylor Valley lake bacteria is an interesting question that has arisen in previous studies (14, 16, 19, 29, 45–47). Lake Hoare is the youngest Taylor Valley lake, some 1,000 to 3,000 years old (6, 48), and thus the organisms that we characterize herein likely originated from nearby aquatic and terrestrial sources. Our gram-positive isolates probably originated from soil blown onto the surface of the lake from the

surrounding hills (9, 24, 29, 38). In austral summer the dark soil heats up and melts the ice and generates pockets of liquid water. The soil and its associated microflora then travel downward in water-filled cracks through the ice to the water column. Indeed, it has been estimated that the bulk of Lake Hoare sediment has originated in this fashion (38). Although soil could also be the source of the *Proteobacteria* that inhabit the Lake Hoare water column, it is more likely that they originated from glacial meltwater, marine waters (McMurdo Sound is only a few kilometers east of Lake Hoare), or adjacent (and older) Taylor Valley lakes. The more salt-tolerant isolates (Table 1), in particular, could have originated from the latter two sources.

However, regardless of their origin, of major importance to the ecology of the Lake Hoare bacteria that we describe here is their ability to grow at and even below in situ temperatures. We therefore hypothesize that our isolates were from bacterial populations indigenous to the water column of Lake Hoare that function as consumers in this oligotrophic and permanently cold ecosystem.

Nucleotide sequence accession numbers. The GenBank accession numbers for the eight Lake Hoare strains are as follows: LH19, DQ535027; LH15, DQ535026; LH11, DQ535023; LH1D, DQ535022; LH14, DQ535025; LH90, DQ535028; LH10, DQ535024; and LH197, DQ535029.

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