

1 **Cold-Active Chemoorganotrophic *Bacteria* from the**
2 **Permanently Ice-Covered Lake Hoare, McMurdo Dry**
3 **Valleys, Antarctica**

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20 **Eight strains of chemoorganotrophic *Bacteria* were isolated from the water column of Lake**
21 **Hoare, McMurdo Dry Valleys, Antarctica using cold enrichment temperatures. The isolates**
22 **were species of alpha, beta, and gamma Proteobacteria, and Actinobacteria. All isolates grew**
23 **at 0°C and all but one grew at subzero temperatures characteristic of the water column of**
24 **Lake Hoare. Growth temperature optima varied among isolates but the majority showed**
25 **optima near 15°C, indicative of cold-active phenotypes. One isolate was truly psychrophilic,**
26 **growing optimally around 10°C and not above 20°C. Half of the isolates grew at 2% salt while**
27 **the other half did not, and all but one isolate grew at 2 atm of O₂. Our isolates are the first**
28 **prokaryotes from the water column of Lake Hoare to be characterized phylogenetically and**
29 **physiologically and show that cold-active species of at least two major phyla of *Bacteria***
30 **inhabit Lake Hoare.**

31 **KEY WORDS:** McMurdo Dry Valleys; Taylor Valley; Lake Hoare; Psychrophily;
32 Proteobacteria; Actinobacteria

33 **RUNNING HEAD:** *Bacteria* in Lake Hoare, Antarctica

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35 Prokaryotes are abundant and active in polar environments (3, 18). Antarctic lakes are
36 particularly interesting in this respect because they are exclusively microbial ecosystems
37 (29, 43). Several permanently ice-covered lakes exist in the McMurdo Dry Valleys,
38 Antarctica. The major Taylor Valley lakes, Hoare, Bonney, and Fryxell, were formed by
39 glacial deepening and have a permanent ice cover that varies from 2 to 6 m thick (10, 29).
40 Among Taylor Valley lakes, the ice cover of Lake Hoare is the most rugged (Fig. 1A).
41 Lake Hoare is also the most oligotrophic and oxic of Taylor Valley lakes; dissolved
42 organic carbon (DOC) levels are below 0.5 mg/l, salt is present in only trace amounts,
43 and the water column is supersaturated with oxygen to a depth of 25 m (Fig. 1B).

44 Although cultures of various *Bacteria* have been isolated from Lakes Fryxell and
45 Bonney (2, 14, 38, 46–48), and molecular evidence obtained for *Archaea* in Lake Fryxell
46 (15), studies by Mikell et al. (21, 22) and Van Trappen et al. (48) are the only reports of
47 cultured organisms from Lake Hoare. The focus of the Mikell et al. studies (21, 22) was
48 not biodiversity but instead the effect of high levels of dissolved oxygen on Lake Hoare
49 bacteria. The study by Van Trappen et al. (48) focused on bacteria recovered from
50 benthic microbial mats that develop in several of the Taylor Valley lakes. In contrast to
51 these studies, we focus here on planktonic bacteria from Lake Hoare, and document the
52 phylogeny and physiology of eight strains of chemoorganotrophic bacteria enriched from
53 different depths. Our results are the first to reveal planktonic bacterial diversity in Lake
54 Hoare and suggest that this constantly cold and oligotrophic lake contains several
55 phylogenetic groups of cold-active *Bacteria*.

56 **Sampling, enrichment, and isolation.** Samples were collected from the water column of
57 Lake Hoare through a hole drilled in the ice near the eastern edge of the lake (GPS
58 77°38'S; 162°53'E) as previously described (16). Lake water was collected with a 5-l
59 Niskin bottle and immediately transferred to sterile 1-l polycarbonate bottles; the
60 completely filled bottles were stored in darkness at 4°C until processed.

61 Enrichment cultures using 10, 20, and 22 m Lake Hoare water as inocula were
62 established in 25 ml of liquid medium R2A (31) or in a starch medium prepared in 125-
63 ml Erlenmeyer flasks. The starch medium contained the mineral salts of medium R2A
64 supplemented with (per liter): yeast extract, 50 mg; CaCl₂•2H₂O, 25 mg; NaCl, 0.5 g;
65 NH₄Cl, 0.5 g; and soluble starch, 1 g. Flasks were incubated without shaking in darkness
66 at 2, 10, or 18°C in thermostatically controlled cold boxes. For incubations below 0°C,
67 media were supplemented with 1% (v/v) sterile dimethyl sulfoxide to prevent the medium
68 from freezing. Turbid enrichments were serially diluted with sterile deionized water,
69 plated onto the surface of R2A or starch-based agar, and incubated at the original
70 enrichment temperature. Resultant colonies were picked and re-streaked until pure

71 cultures were obtained. Cultures of the Lake Hoare isolates are available from the authors
72 upon written request.

73 **Physiological studies.** Upper and lower temperature limits for growth were determined
74 on plates of medium R2A incubated at -3 to $+40^{\circ}\text{C}$. All plates were wrapped in clear
75 plastic wrap to prevent desiccation and scored for growth by visual inspection. To
76 determine temperature optima, duplicate 10-ml screw-cap tubes containing 3 ml of liquid
77 medium R2A were inoculated with 0.2 ml of exponential-phase cultures and incubated at
78 a temperature series. Optimal growth temperatures are reported as the temperature range
79 that gave the highest cell yields (as measured turbidimetrically, OD_{540}) in a defined
80 incubation period. Salt tolerance was tested in liquid medium R2A containing either 2%
81 or 5% (w/v) NaCl; tubes were inoculated and incubated at 10°C for 21 days and scored
82 for growth against unsupplemented controls. To assess anoxic growth capacity, 3 ml of
83 liquid medium R2A contained in 10-ml tubes was inoculated and incubated in an anoxic
84 jar (Becton Dickinson, Sparks, MD) that was activated and sealed within an anoxic glove
85 box. The tubes were scored for growth (OD_{540}) after 8 days at either 15° or 23°C . Growth
86 at elevated oxygen tensions was assessed in 25-ml crimp-top tubes containing 3 ml
87 medium R2A and pressurized with 99.9% O_2 to 200 kPa.

88 **Phylogenetic analyses.** DNA was extracted from 1.5 ml liquid cultures and small subunit
89 (SSU) rRNA genes were PCR amplified using universal primers for *Bacteria* (8F 5'-
90 AGAGTTTGATCCTGGCTCAG-3' and 1525R 5'-AAGGAGGTGATCCAGCC-3').
91 The PCR product was purified using either the GeneClean Turbo Kit (Q-BIOgene,
92 Albany, NY) or the QIAquick PCR Purification Kit (Qiagen Sciences, Valencia, CA) at
93 SIUC, and then sequenced at the Genome Sequencing Center, Washington University, St.
94 Louis, MO. Sequence alignments were made using the ClustalW program of MacVector
95 7.2 software (Accelrys, San Diego, CA) and confirmed by visual inspection. A
96 phylogenetic distance tree was generated within MacVector using the Jukes-Cantor

97 correction. Genbank accession numbers for the eight Lake Hoare strains and reference
98 organisms used to build the tree are listed on the phylogenetic tree.

99 **Enrichment and isolation.** Enrichment cultures established from Lake Hoare water and
100 incubated aerobically from 2–18°C in medium R2A became visually turbid within one to
101 two weeks and were subsequently diluted and plated. Medium R2A has been widely used
102 as a culture medium for isolating bacteria inhabiting oligotrophic waters, glaciers, and
103 other Antarctic habitats (1, 4, 23, 46, 48, 50). From the enrichments, pure cultures were
104 eventually obtained by plating and eight strains chosen for further study based on their
105 robust growth at the enrichment temperature. Seven of the eight strains were enriched and
106 isolated in medium R2A while one was obtained from the starch medium. Table 1 lists
107 the major characteristics of the isolates including enrichment details, Gram reaction and
108 morphology, pigmentation, salinity and oxygen tolerances, and cardinal temperatures.

109 **Phylogeny and morphology.** The phylogeny and morphology of the eight Lake Hoare
110 strains is shown in Fig. 2. Gram-negative rods dominated; only 2 of the 8 isolates were
111 Gram-positive. All gram-negative isolates were Proteobacteria (Table 1), organisms that
112 are widespread in aquatic environments (11, 17) and Antarctic microbial mats (2, 48).
113 However, only two of our isolates, the gram-positive strain LH19 and the
114 gammaproteobacterium strain LH197, were fairly closely related (> 97% SSU sequence
115 identity) to isolates obtained from a Lake Hoare microbial mat (48).

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

123 Lake Hoare strains LH11 and LH1D were alphaproteobacteria, related to species
124 of *Caulobacter* and *Sphingomonas*, respectively (Fig. 2). An uncharacterized bacterium
125 related to *Sphingomonas* has previously been isolated from Ace Lake in the Antarctic
126 Vestfold Hills (48) and the highly oligotrophic Crater Lake in Oregon (26). *Caulobacter*
127 is an aquatic bacterium that inhabits seawater, freshwater, and occasionally soil (28).
128 *Caulobacter henricii* was the closest cultured relative of strain LH11. Both *C. henricii*
129 and strain LH11 produced yellow pigments and a stalked morphology in which stalks
130 become attached to form cell rosettes (27, and Fig. 2). However, *C. henricii* cannot grow
131 at the low temperatures that supported growth of strain LH11 (27, and Table 1).

132 The gram-positive Lake Hoare isolates, strains LH19 and LH15, were related to
133 Actinobacteria (7, 40). Strain LH19 showed a coccus morphology and was related to
134 species of *Arthrobacter*, a genus containing cocci and short rods that are common in soil
135 (13) and which have also been detected in a Lake Fryxell microbial mat (48). By contrast,
136 strain LH15 was only a distant relative of species of *Agromyces*, a genus of the
137 Actinomycetes (7); the filamentous branching pattern of cells of strain LH15 (Fig. 2) is
138 typical of some Actinomycetes (7).

139 **Physiology.** All Lake Hoare isolates grew aerobically but not anaerobically on medium
140 R2A (which contains glucose); thus, none were capable of fermenting glucose (Table 1).
141 Anoxic medium R2A supplemented with 10 mM (final) of DMSO, nitrate, or fumarate
142 also did not support growth. We conclude that our isolates are incapable of these
143 common forms of anaerobic respiration, which suggests that they are obligate aerobes.
144 These results are consistent with the high levels of dissolved oxygen in Lake Hoare (Fig.
145 1B). Only the gram-positive Lake Hoare isolates hydrolyzed starch (Table 1).

146 Since all of the Lake Hoare isolates experience constant cold in their natural
147 habitat, their cardinal temperatures were a major focus of our study; the results are shown
148 in Table 1. Minimal growth temperatures for the isolates ranged from 0°C to -3°C,
149 indicating that all can grow at *in situ* temperatures (Fig. 1B). Strain LH14 showed the

150 greatest cold adaptation and is a psychrophile in the classical sense (24); strain LH14
151 grew to as low as -3°C and showed a maximum growth temperature under 20°C and an
152 optimum near 10°C . Maximum growth temperatures were as high as 40°C in one strain
153 (LH15), but even in this case, growth was still possible at subzero temperatures (Table 1).
154 Interestingly, no strong correlation was observed between enrichment temperature and
155 growth temperature limits, as isolates from any enrichment temperature grew at subzero
156 temperatures (Table 1). Similar findings have emerged from studies of other Antarctic
157 *Bacteria* and *Archaea* (3). These results indicate that, surprisingly, psychrophily may not
158 be common in prokaryotes from this permanently cold environment.

159 Because of the extremely low salinity of Lake Hoare relative to other Taylor
160 Valley lakes (19, Fig. 1B), the salt tolerance of the isolates was also of interest, and the
161 results are shown in Table 1. Only one of the eight strains, strain LH197, grew in media
162 containing 5% (w/v) NaCl. However, because strain LH197 also grew in media lacking
163 NaCl, it is halotolerant not halophilic. Strains LH14, LH15, and LH19 were less
164 halotolerant but still capable of growth at 2% NaCl. Growth of the remaining strains was
165 inhibited by 2% NaCl (Table 1). All strains except the psychrophilic strain LH14 grew in
166 sealed tubes containing 2 atm O_2 (Table 1).

167 Several of our Lake Hoare strains were pigmented (Table 1) as was true of strains
168 isolated from microbial mats from several Taylor Valley lakes (32–34, 45, 46, 48). For
169 example, in the mat study of Van Trappen et al. (48) 68% of the strain clusters defined by
170 fatty acid composition contained pigmented strains. Intact cells of our Lake Hoare strains
171 LH14, LH15, and LH19 showed absorbance maxima between 430 and 551 nm (data not
172 shown), well within the range for typical carotenoids (42). Strain LH19 had maxima at
173 551, 515, and 485 nm, very near that of spirilloxanthin (44), while strain LH15 showed
174 maxima at 485 and 452 nm, close to those of spheroidene (44). By contrast, although
175 yellow or orange in color, strains LH11 and LH1D showed absorbance maxima to the
176 blue of 375 nm (data not shown), outside the absorption range of typical carotenoid

177 pigments (42). The nature of these pigments is unknown. None of our isolates yielded
178 spectral evidence for bacteriochlorophyll *a*.

179 **Concluding remarks.** *Bacteria* in Lake Hoare experience several stress factors, in
180 particular, low temperature, high oxygen, and oligotrophy. Interestingly, the cardinal
181 temperatures of our eight isolates were similar to those reported for phototrophic purple
182 bacteria (14, 20), sulfate-reducing bacteria (16), and sulfur chemolithotrophic bacteria
183 (35) isolated from Lake Fryxell, which lies adjacent to Lake Hoare on the eastern side of
184 the Canada Glacier (Fig. 1A). That is, although none of our Lake Hoare isolates showed
185 optimal growth at *in situ* temperatures, all grew readily at 0°C and all but one at subzero
186 temperatures. Therefore, all of our isolates (except for strain LH14, Table 1) are
187 psychrotolerant. The observation that most of our isolates (and those from other Taylor
188 Valley lakes, 14, 20, 35, 37, 43) are psychrotolerant rather than psychrophilic may be a
189 reflection of the young age of these lakes compared to other constantly cold microbial
190 habitats, such as marine sediments, where psychrophiles seem to be more common (3).

191 The very deepest waters of Lake Hoare are anoxic and even slightly sulfidic;
192 however, even at a depth of 25 m, the water is oxygen supersaturated (Fig. 1B, 5, 22).
193 This may help explain why pigmented colonies appeared among our Lake Hoare isolates
194 even though light intensities in the water column of Lake Hoare are extremely low (22,
195 36). Carotenoids can protect cells from oxidative damage. This was dramatically
196 demonstrated in the study of Mikell et al. (21), where enrichments from Lake Hoare
197 water incubated under hyperbaric oxygen yielded only pigmented colonies. However,
198 besides removal of toxic forms of oxygen, carotenoids may improve the survival of cold-
199 active bacteria in other ways. These include affecting membrane structure (41) or
200 functioning as global regulators in response to cell stress from cold shock (8). All but one
201 of our Lake Hoare isolates grew at hyperbaric oxygen (Table 1), and all pigmented
202 strains remained pigmented at different temperatures and oxygen tensions. This indicates
203 that pigmentation is not subject to control by these major environmental variables.

204 Levels of NaCl and DOC in Lake Hoare are very low (Fig. 1B). Surprisingly,
205 however, half of our isolates grew in media containing 2% NaCl, and one grew at 5%
206 NaCl, nearly twice the salinity of marine waters. This was unexpected, but could be a
207 legacy of the origin of these bacteria (see next paragraph). Moreover, the discovery that
208 one of our isolates was a species of *Caulobacter*, a classic oligotrophic bacterium (28), is
209 consistent with the low DOC in Lake Hoare (Fig. 1B). Oligotrophy was also underscored
210 by the isolation in a starch-containing medium of strain LH197, an organism
211 subsequently shown to be unable to catabolize starch (Table 1). This organism was
212 therefore enriched on the 50 µg/ml of carbon present from yeast extract added to the
213 medium as a source of vitamins. Collectively, these observations indicate that at least
214 some Lake Hoare bacteria are oligotrophic, as could be predicted from the extremely low
215 DOC present in the lake (Fig. 1B).

216 The origin of Taylor Valley lake bacteria is an interesting question that has arisen
217 in previous studies (14, 16, 20, 30, 46–48). Lake Hoare is the youngest Taylor Valley
218 Lake, some 1000 to 3000 years old (6, 49), and thus the organisms we characterize herein
219 likely originated from nearby aquatic and terrestrial sources. Our gram-positive isolates
220 probably originated from soil blown onto the surface of the lake from the surrounding
221 hills (9, 25, 30, 39). In austral summer the dark soil heats up and melts the ice and
222 generates pockets of liquid water. The soil and its associated microflora then travel
223 downward in water-filled cracks through the ice to the water column. Indeed, it has been
224 estimated that the bulk of Lake Hoare sediment has originated in this fashion (39).
225 Although soil could also be the source of the Proteobacteria that inhabit the Lake Hoare
226 water column, it is more likely that they originated from glacial melt-water, marine
227 waters (McMurdo Sound is only a few kilometers east of Lake Hoare), or adjacent (and
228 older) Taylor Valley lakes. The more salt tolerant isolates (Table 1), in particular, could
229 have originated from the latter two sources.

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

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ACKNOWLEDGMENTS

237 This work was supported by NSF grant MCB0237576 from the Microbial Observatory
238 Program. We thank Raytheon Polar Services, Petroleum Helicopters Inc., and John
239 Priscu and the McMurdo LTER limno team for logistic support in Antarctica. We thank
240 Matt Sattley for help in sampling Lake Hoare and for the sulfide data from Lake Hoare.

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

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Strain ^a	Enrich. Temp. (°C)	Gram Rx/ Morphology	Pigments	Cardinal Temperatures ^b			Growth With ^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	Yellow	31	-2	14-22	-	-	+	-
LH19	18	Positive/ Coccus	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	+	+	+	-

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274 ^aStrains LH10, LH1D, LH19 and LH90 were obtained from 10 m Lake Hoare water,
275 strain LH197 from 20 m water, and strains LH11, LH14, and LH15 from 22 m water. All
276 isolates except for strain LH197 (enriched on starch) were enriched in medium R2A.

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278 ^bTemperatures in °C. Max, growth temperature maximum; Min, minimum growth
279 temperature; Opt, temperature range that yielded the highest cell density in a defined
280 incubation period in medium R2A.

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282 ^cMedium R2A supplemented with either 2% or 5% (final concentrations) NaCl, or
283 medium R2A pressurized to 2 atm (200 kPa) with O₂.

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285 ^dAs assessed by a zone of clearing around colonies on starch agar treated with Gram's
286 iodine.

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289 **FIGURE LEGENDS**

290 **Figure 1.** Lake Hoare geomorphology and geochemistry. (A) Photo of the surface of
291 Lake Hoare looking toward the southeast taken in November 2003. The west side of the
292 Canada Glacier is in the left background; Lake Fryxell lies adjacent to the east edge of
293 the Canada Glacier. The rough surface ice on Lake Hoare forms spikes over 1 m tall and
294 ice cover thickness varies seasonally from 2–6 m. (B) Profiles of temperature, dissolved
295 oxygen, sulfide, NaCl, and dissolved organic carbon (DOC) in the water column of Lake
296 Hoare. At 0°C, O₂ saturation in water is 14.6 mg/l. The O₂ meter used here reads to a
297 maximum of 20 mg/l but chemical measurements of dissolved oxygen in Lake Hoare
298 show oxygen levels to be over twice saturation in the upper waters (21, 22). DOC and
299 NaCl data obtained from the McMurdo LTER website (19) from sampling casts of
300 December 2002 (DOC) and November 2003 (NaCl). All other data recorded in
301 November 2005.

302 **Figure 2.** Phylogeny and morphology of Lake Hoare isolates. (Top) Evolutionary
303 distance tree (using the Jukes-Cantor correction) based on SSU rRNA gene sequencing
304 (1236 base pairs) showing the phylogenetic positions of the eight Lake Hoare (LH)
305 strains isolated in this work. Bootstrap values are shown at the nodes and are based on
306 1000 replications. Similar trees were also obtained using other distance methods,
307 including Tajima-Nei, Kimura 2-parameter, and Tamura-Nei, and also using parsimony
308 methods. Genbank accession numbers are shown in parentheses. (Bottom) Phase-contrast
309 photomicrographs of cells of each Lake Hoare strain.

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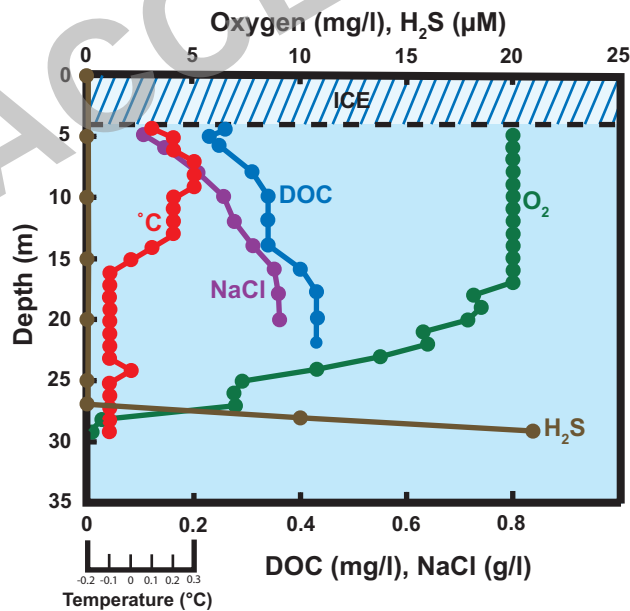
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