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A Halophilic Bacterium Inhabiting the Warm, $CaCl_2$-Rich Brine of the Perennially Ice-Covered Lake Vanda, McMurdo Dry Valleys, Antarctica

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Lake Vanda is a perennially ice-covered and stratified lake in the McMurdo Dry Valleys, Antarctica. The lake develops a distinct chemocline at about a 50-m depth, where the waters transition from cool, oxic, and fresh to warm, sulfidic, and hypersaline. The bottom water brine is unique, as the highly chaotropic salts $CaCl_2$ and $MgCl_2$ predominate, and $CaCl_2$ levels are the highest of those in any known microbial habitat. Enrichment techniques were used to isolate 15 strains of heterotrophic bacteria from the Lake Vanda brine. Despite direct supplementation of the brine samples with different organic substrates in primary enrichments, the same organism, a relative of the halophilic bacterium *Halomonas* (*Gammaproteobacteria*), was isolated from all depths sampled. The Lake Vanda (VAN) strains were obligate aerobes and showed broad pH, salinity, and temperature ranges for growth, consistent with the physicochemical properties of the brine. VAN strains were halophilic and quite $CaCl_2$ tolerant but did not require $CaCl_2$ for growth. The fact that only VAN strain-like organisms appeared in our enrichments hints that the highly chaotropic nature of the Lake Vanda brine may place unusual physiological constraints on the bacterial community that inhabits it.

The McMurdo Dry Valleys region of Victoria Land, Antarctica, is a polar desert where temperatures and precipitation average −20°C and 10 mm/year, respectively (1). The valleys contain several permanently ice-covered lakes that vary dramatically in their geochemistries. For example, Lake Bonney (Taylor Valley) contains hypersaline deep waters where NaCl and $MgCl_2$ are the predominant salts (~2.2 M and 1.4 M, respectively), but sulfide and methane are virtually absent (2, 3). In contrast, Lake Fryxell, just a few kilometers east of Lake Bonney, has weakly saline deep waters (~0.12 M NaCl) and sediments that are both highly sulfidic and methanogenic (2–5).

Lake Vanda is a meromictic lake with a maximum depth of about 75 m; the lake lies about 30 km northwest of Lake Bonney in the Wright Valley (1, 2, 6). Waters in the upper 50 m of the Lake Vanda water column are cool, oxic, and oligotrophic, whereas at about 50 m waters transition to a warm, hypersaline, and anoxic brine that forms in the bottom 20 m of the lake (2, 6, 7) (Fig. 1). The high transparency of the relatively thin Lake Vanda ice cover allows the deep penetration of solar energy (8, 9). Solar heating warms the water column, with the water temperature gradually warming from 0°C at the surface to about 12°C at a depth of 50 m and then rising steadily thereafter to reach a maximum of nearly 25°C on the bottom (6) (Fig. 1A). Besides supplying heat, this deep influx of solar energy supports a phytoplankton bloom at a 55- to 60-m depth, where the relatively warm temperatures and inorganic nutrients diffusing upward from the brine stimulate photosynthesis (10).

The Lake Vanda brine is weakly acidic (pH ~6) and unique in its substantial content of dissolved $CaCl_2$ and $MgCl_2$ but comparatively low NaCl content (2, 6, 11–13). For example, Lake Vanda water from a depth of 73 m contains nearly 0.6 M dissolved Ca^{2+} and over 0.3 M Mg^{2+} but only about 0.25 M Na^+; Cl^- is the major anion (Fig. 1B; Table 1). The Ca^{2+} content of the Lake Vanda brine is 14-fold higher than that of the deep waters of the east lobe of Lake Bonney, and the Na^+ content is more than 8-fold lower than that of the deep waters of the east lobe of Lake Bonney, the latter of which are cold and of neutral pH (1, 12, 13) (Table 1). Moreover, the Ca^{2+} content of the deepest waters in Lake Vanda exceeds that of all known hypersaline environments, with the exception of Don Juan Pond, an extremely hypersaline playa lake that never freezes and lies southwest of Lake Vanda in a depression in the Wright Valley (14–17) (Table 1). Even the Dead Sea contains lower Ca^{2+} levels than the Lake Vanda brine (Table 1). Unlike the Lake Bonney brine, which is of marine origin (1, 18), the Lake Vanda brine is thought to have originated from periodic dry downs followed by seasonal meltwater inputs from the Wright Glacier (that flow into Lake Vanda from the Onyx River) along with the influx of $CaCl_2$-rich groundwater that pervades the Don Juan Basin (15, 19, 20).

A few bacteria have been reported from Lake Vanda, but in only one study has both a phylogenetic and a physiological picture of a pure culture emerged. In Lake Vanda water taken at depths of between 5 and 30 m, Nagashima et al. (21) described several yeasts and bacteria; the bacteria were orange-pigmented and aerobic Gram-negative rods. A yellow-pigmented Gram-negative coccus was isolated from water taken at 69 m, but surprisingly, the organism was not halophilic (21). Kriss et al. (22) described isolates

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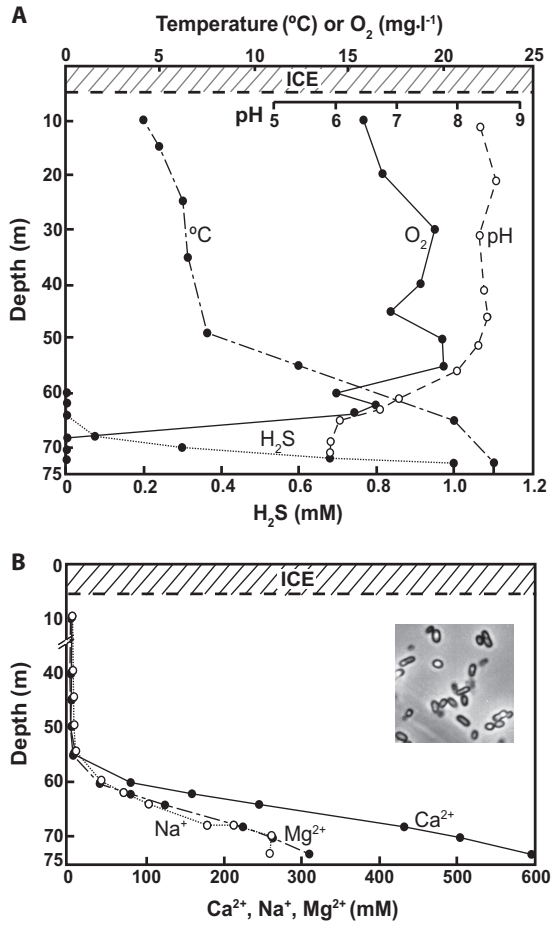

FIG 1 Limnological profile of Lake Vanda. (A) Temperature, pH, and O_2 and H_2S concentrations. (B) Concentrations of major cations. (Inset) Phase-contrast photomicrograph of cells of strain VAN1. Data are from this study and references 25 and 51.

thought to be *Pseudomonas*, *Chromobacterium*, *Bacillus*, and *Mycobacterium* species from Lake Vanda upper waters, but these identifications were made prior to the development of phylogenetic methods and so were based solely on phenotypic criteria.

Using epifluorescence microscopy, Takii et al. (23) identified rod-shaped and coccoid bacteria in Lake Vanda at a depth of 72 m but did not culture isolates from this sample. From water taken below 60 m in Lake Vanda, Bratina et al. (24) isolated several strains of a *Carnobacterium* species. This organism was linked to the reduction of Mn^{4+} to Mn^{2+} in Lake Vanda water from depths of 60 to 65 m, and cultured isolates were shown to reduce Mn^{4+} in agar plate assays (24).

Although some bacteria have been isolated from Lake Vanda, bacteria indigenous to its Ca-rich brine have not been cultured. In this study, we employed enrichment techniques to cultivate bacteria from several depths of the Lake Vanda brine. Our results yielded 15 strains of a previously undetected halophilic and heterotrophic bacterium that appears to be well adapted to the Lake Vanda brine and grows in medium containing the unique mixture of chaotropic salts found in this unusual hypersaline microbial habitat.

MATERIALS AND METHODS

Field site and analytical methods. Water samples were obtained from Lake Vanda on 1 December 2008 from a hole drilled through the ~3-m ice cover, using a gas-powered Jiffy drill, at coordinates 77°32.0292′S/161°33.1674′E. Samples were obtained using an ethanol-wiped and air-dried 5-liter Niskin bottle from depths of 60, 62, 66, 70, and 72 m and were maintained at 4°C during transport from the field site to the Crary Lab in McMurdo Station and later during air shipment (at approximately 1 month after collection) to Carbondale, IL. Temperature and pH were measured in the field, and sulfide was trapped in Zn acetate and assayed as previously described (4). Oxygen (O_2) and ionic data were obtained from the McMurdo Long Term Ecological Research (LTER) Program database (25). In the LTER protocols, O_2 is assayed by Winkler titration, while Ca^{2+}, Na^+, Mg^{2+}, Cl^-, and SO_4^{2-} are quantified by ion chromatography (25). Water activity estimates for the Lake Vanda and Lake Bonney brines (Table 1) were calculated using the Phreeqc (v. 3) program (26) and the database for the specific-ion-interaction model of Pitzer (27).

Enrichment and isolation. Microscopic observations of samples and cultured isolates were performed using a Leica phase-contrast microscope. Enrichment cultures were established in both McMurdo and Carbondale by placing 4 ml of lake water into sterile 10-ml or 17-ml tubes and adding yeast extract to a final concentration of 0.01% (wt/vol). One of the following substrates was also added to some of the enrichments: glucose, fructose, ribose, succinate, pyruvate, or lactate (final concentration, 10 mM in each case) or yeast extract or peptone (final concentration, 0.1% in each case). All enrichments were incubated aerobically (or anaerobically in GasPak anoxic jars) at 20°C. Additionally, to specifically target endospore-forming bacteria, tubes containing Lake Vanda water and organic supplements were pasteurized (80°C for 15 min) before incubation.

Aliquots of turbid enrichment cultures were transferred to tubes of Lake Vanda (VAN) medium, which contained (at the indicated final concentration [in mM]) MOPS (3-morpholinopropanesulfonic acid; Sigma, St. Louis, MO), 10; NaCl, 200; $CaCl_2$, 500; $MgCl_2$, 200; KCl, 10; NH_4Cl, 2; Na_2SO_4, 5; KH_2PO_4, 0.5; 1 ml trace elements (28); and the organic substrate used in the enrichment (final pH, 6.8). To avoid precipitates, the phosphate and trace elements were autoclaved together as a separate so-

TABLE 1 Major cations and anions in some calcium-rich waters and in seawater for comparison

Location	Ion concn (mM)					TDS[a] (%)	a_w[b]	pH
	Ca^{2+}	Mg^{2+}	Na^+	Cl^-	SO_4^{2-}			
Lake Vanda[c]	587	312	262	2,001	6.6	10.9	0.93	5.97
Lake Bonney[c]	41	1,372	2,204	4,980	36	26.2	0.7	6.76
Don Juan Pond[d]	2,850	50	500	5,980	0.1	33.9	0.41	5.58
Dead Sea[d]	430	1,750	1,725	6,177	4.1	31.8	0.67	6.2
Seawater[d]	10	53	470	547	28	3.5	0.98	7.8

[a] TDS, total dissolved solids, calculated from values in the table.
[b] a_w, water activity. Values for Lakes Vanda and Bonney were calculated (26, 27).
[c] Data from water samples from a 72-m depth of Lake Vanda and a 35-m depth of the east lobe of Lake Bonney obtained in November 2006 (25).
[d] Data from references 42 and 44 and this study.

lution and added to the other medium components after cooling. Turbid cultures were subsequently streaked onto VAN medium plates, and colonies were picked and restreaked several times to obtain pure cultures. Liquid cultures originating from isolated colonies obtained in the final round of streak plates were designated Lake Vanda (VAN) strains, and aliquots were preserved at −80°C in growth medium containing 10% (final concentration) dimethyl sulfoxide (DMSO).

Physiology. For most physiological experiments, a modified VAN (M-VAN) medium was used. M-VAN medium contained (at the indicated final concentration [in mM]) MOPS, 10; NaCl, 1,000; $MgCl_2$, 1; KCl, 10; NH_4Cl, 2; Na_2SO_4, 5; KH_2PO_4, 1; 1 ml trace elements (28); and organic additions, as indicated in Tables 2 and 3 and Fig. 3 (final pH, 6.8). For growth studies, select VAN strains were grown in 4 ml of VAN or M-VAN medium in 10-ml screw-cap tubes. For temperature experiments, the tubes were incubated aerobically at different temperatures and optical density measurements were taken over a 72-h period. To test the effect of pH on growth, the media were buffered at pH 5 with MES (morpholinoethanesulfonic acid), pH 6 and 7 with MOPS, and pH 8 with POPSO [piperazine-*N*,*N*′-bis-(2-hydroxypropanesulfonic acid)] (all buffers were purchased from Sigma [St. Louis, MO] and were added to a 10 mM final concentration).

To test for NaCl or $CaCl_2$ tolerances and requirements, M-VAN medium minus NaCl and M-VAN medium, respectively, were used (both media contained 10 mM pyruvate as the carbon source); NaCl or $CaCl_2$ supplements were added from sterile concentrated stock solutions. For these experiments, *Chromohalobacter salexigens* strain DSMZ 3043 (29) was used for comparison with the VAN strains.

Various carbon sources were tested both aerobically and anaerobically as the sole sources of carbon and energy for select VAN isolates. For tests under aerobic conditions, screw-cap tubes were incubated in air for 72 h and vigorously mixed periodically. Turbid cultures were transferred to fresh medium of the same composition and incubated for 72 h before final scoring for growth. To test for anaerobic growth, inoculated tubes were incubated in GasPak jars for 8 days, and the turbidities versus those for controls lacking an electron acceptor or incubated aerobically were scored; all electron acceptors were tested at 10 mM (final concentration). Nitrogen source utilization was tested in M-VAN medium lacking NH_4Cl. Nitrogen sources were added at 10 mM for amino acids and 5 mM for KNO_3 (final concentrations for both). Tubes were incubated for 72 h, and turbid cultures were serially transferred twice before final scoring of growth.

Except for temperature experiments, all cultures were incubated at 30°C and growth was monitored turbidimetrically by comparison with the growth of the uninoculated or no-substrate controls.

Phylogenetic methods. Genomic DNA was extracted from the cultures of the VAN strains using a Wizard genomic purification kit (Promega, Madison, WI) according to the manufacturer's protocol. Amplified 16S rRNA genes were purified using a GeneJET gel extraction kit (Fermentas, Glen Burnie, MD), followed by sequencing (Molecular Cloning Laboratories, San Francisco, CA). Nearly full 16S rRNA gene sequences were analyzed using the BLASTN program (30), aligned by use of the CLUSTAL X program (31), and edited manually. A neighbor-joining phylogenetic tree was constructed using the PHYLIP (v.3.69) package (32), and the SEQBOOT tool was used to calculate bootstrap values on the basis of 1,000 replications.

Nucleotide sequence accession numbers. The GenBank accession numbers of the strains analyzed in this study have been submitted to GenBank, and their accession numbers are provided in Table 2.

RESULTS

Enrichment, isolation, and morphology of VAN strains. Direct phase-contrast microscopy of a Lake Vanda brine sample from a 72-m depth showed mineral debris but no discernible bacterial cells. When 10 ml of the brine was filtered (0.45-μm-pore-size filter), the filter turned black, presumably from iron sulfides. Resuspension of the filtered material in 1 ml of liquid showed abundant mineral debris and a few rod-shaped and coccus-shaped bacterial cells.

TABLE 2 Sample depth, enrichment substrates, and VAN isolates obtained from Lake Vanda deep waters

Depth (m)	Enrichment substrate[a]	Isolate(s) (GenBank accession no.[b])
60	Yeast extract	VAN2 (JX262608)
62	Succinate	VAN7 (JX262612), VAN8 (JX262613)
62	Yeast extract	VAN18 (JX262620)
66	Glucose/yeast extract/lactate	VAN3 (JX262609), VAN4 (JX262610)
66	Glucose	VAN1 (HQ290519), VAN11 (JX262615)
66	Ribose	VAN5 (HQ290520)
66	Lactate	VAN6 (JX262611), VAN12 (JX262616), VAN14 (JX262617)
66	Yeast extract	VAN17 (JX262619)
72	Glucose	VAN9 (JX262614)
72	Peptone	VAN15 (JX262618)

[a] All substrates were added to a final concentration of 10 mM, except for yeast extract and peptone (final concentration, 0.1% each). All enrichments were supplemented with 0.01% yeast extract.
[b] GenBank accession numbers for 16S rRNA gene sequences.

Bacteria were enriched directly in aliquots of Lake Vanda brine taken from depths of 60 to 72 m and supplemented with organic substrates; turbid cultures were subsequently transferred to liquid VAN medium. Several carbon/energy sources, including sugars, organic acids, and complex substrates, were used in the primary enrichments. Positive enrichments typically became turbid within 1 week, and microscopy revealed motile Gram-negative rods of uniform morphology approximately 3 by 1 μm (Fig. 1B, inset). When these primary enrichments were plated, a single colony type formed within 5 days; the colonies were unpigmented, translucent, and entire in their morphology. Extended incubation did not yield additional colonies. From well-isolated colonies, a total of 15 strains were obtained from different depths, and each strain was given the prefix VAN followed by a number. The Lake Vanda enrichment results are summarized in Table 2.

Parallel enrichments were established using heat-shocked water and unheated water incubated anaerobically to select for endospore-forming bacteria and anaerobic bacteria, respectively. None of these enrichments showed detectable growth.

Phylogeny and physiology of VAN strains. Unexpectedly, considering the various geochemistries of the Lake Vanda water samples (Fig. 1) and the organic substrates used for enrichment (Table 2), phylogenetic analyses of the VAN strains showed them to be highly related (>99% 16S rRNA gene sequence homology), indicating that they were all strains of a single species (Fig. 2). The closest relatives of the VAN strains were *Halomonas* species (family *Halomonadaceae*, *Gammaproteobacteria*). Other close relatives included *Chromohalobacter* species, such as *C. salexigens* (Fig. 2). Notably, the VAN strains grouped within a clade of halophiles to the exclusion of a neighboring clade of nonhalophilic *Gammaproteobacteria* that included the genera *Zymobacter* and *Carnimonas* (Fig. 2).

Key physiological properties of selected VAN isolates, including growth temperature and pH optima, ability to use different substrates as the sole sources of carbon and energy, anaerobic growth potential, and growth response to NaCl or $CaCl_2$ chal-

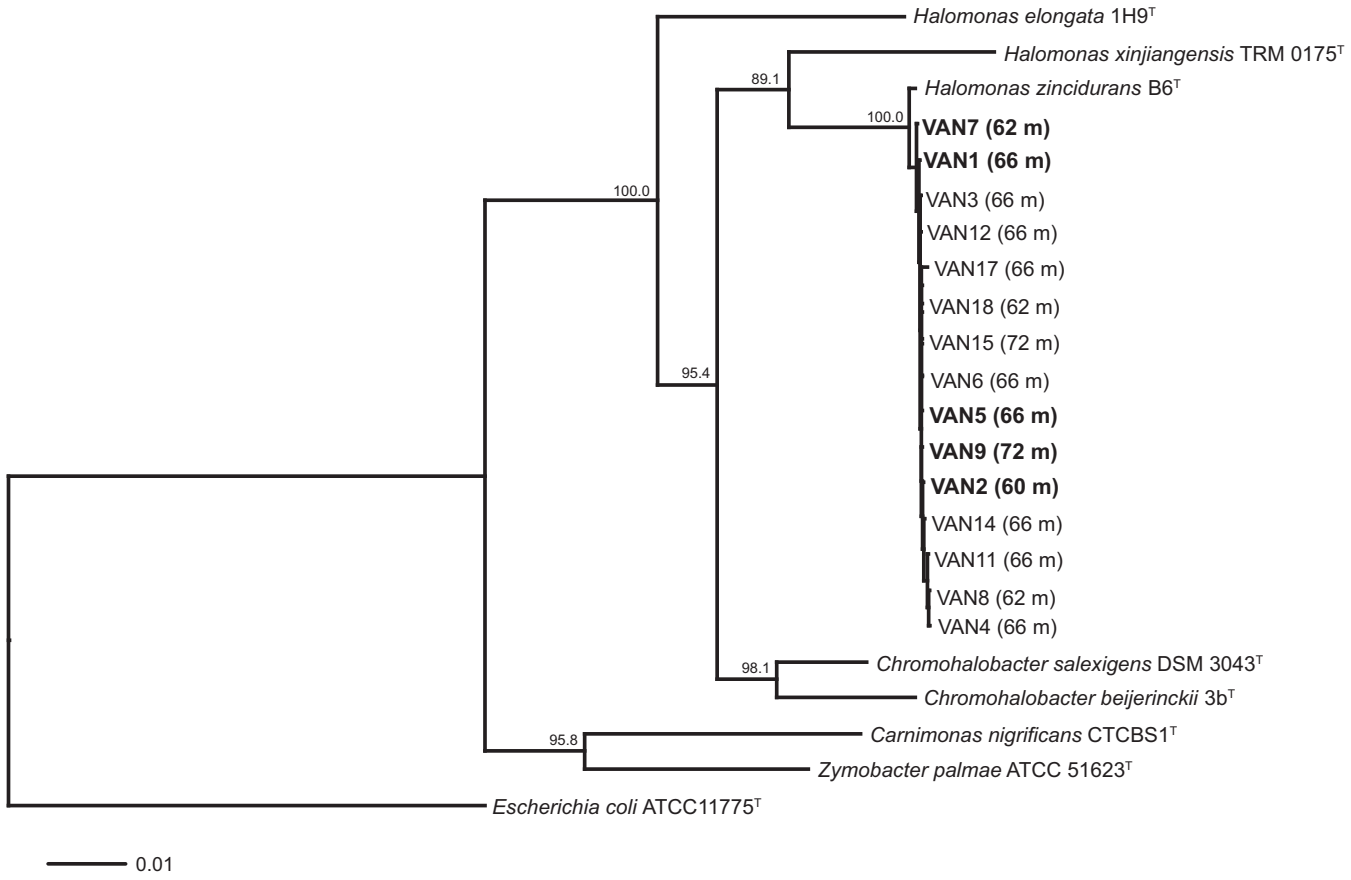

FIG 2 Phylogenetic tree of some *Halomonadaceae*, including all the VAN isolates. The neighbor-joining phylogenetic tree was constructed from 16S rRNA gene sequences using the Jukes-Cantor correction; bootstrap values (based on 1,000 replications) are shown at the nodes. GenBank accession numbers for all VAN isolates are listed in Table 2. Strains shown in bold were used in the salt studies whose results are presented in Table 3.

lenge, were examined. Strains VAN1 and VAN5 were examined for their growth temperatures and pH optima. Strain VAN1 grew best at 30°C and was unable to grow at temperatures below 15°C or above 42°C (Fig. 3); such results clearly identify strain VAN1 to be a mesophile. When cultured in buffered M-VAN medium adjusted to different initial pH values, strain VAN1 showed optimal growth at pH 6 to 7; growth was poor at pH 5 and suboptimal yet still significant at pH 8 (Fig. 3). Similar results were obtained with strain VAN5 (data not shown). These results are consistent with the pH and temperature of the Lake Vanda brine (Fig. 1A). Although quantitative experiments were not done on other VAN strains, each grew well at 30°C and pH 7 during isolation and purification and so likely had temperature and pH optima similar to those determined for strains VAN1 and VAN5.

Both strain VAN1 and strain VAN5 grew aerobically in defined media on glucose, fructose, pyruvate, succinate, or malate as the carbon and energy sources. Complex mixtures, such as peptone or yeast extract, also supported growth, but the highest cell yields were obtained on organic acids. Neither strain grew on sucrose, ribose, lactose, formate, acetate, propionate, or butyrate or on C_1 to C_4 *n*-alcohols. The growth of both strains was strictly aerobic; tests for the fermentation of glucose or fructose or for the anaerobic respiration of succinate or glucose linked to the reduction of nitrite, nitrate, fumarate, thiosulfate, DMSO, or trimethylamine oxide were uniformly negative. In addition to ammonia, strains

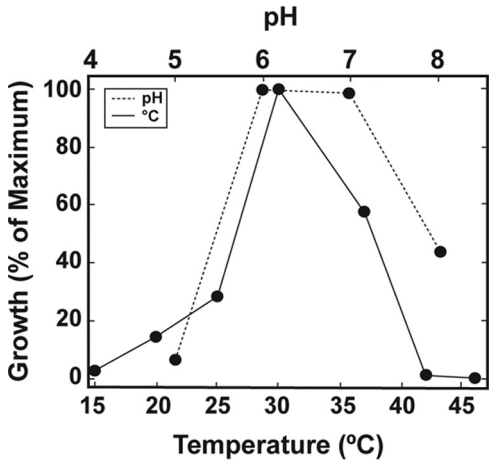

FIG 3 Growth of strain VAN1 as a function of temperature or pH. All experiments were performed in M-VAN medium containing pyruvate as the carbon source and with incubation for 72 h (30°C or other temperatures as indicated). Maximum growth is defined as a change in the optical density at 540 nm of 0.3 (temperature experiment) and 0.22 (pH experiment). Growth is reported as the mean optical density for triplicate cultures for each value.

TABLE 3 Effect of NaCl and $CaCl_2$ on growth of select VAN strains[a]

Ion and ion concn (mM)	Growth[b]					
	VAN1	VAN2	VAN5	VAN7	VAN9	DSMZ 3043[c]
NaCl						
300	−	++	−	++	+	−
400	+	++	−	++	+	−
500	++	+	++	+	++	++
600	+++	++	+++	+++	+++	+++
800	+++	NT[d]	+++	NT	NT	+++
$CaCl_2$						
600	+++	++	+++	++	+++	++
700	+++	++	+++	++	+++	++
800	++	+	++	++	++	+
900	+	−	+	−	−	+
1,000	−	−	+	−	−	+

[a] All NaCl and $CaCl_2$ values are final concentrations (mM) in M-VAN medium. All strains were grown aerobically at 30°C with pyruvate as the substrate. No growth of any strain occurred at a NaCl concentration of 200 mM or lower or at a $CaCl_2$ concentration of 1,100 mM or higher. The growth of all strains at 500 mM $CaCl_2$ was the same as that at 600 mM $CaCl_2$.

[b] Optical densities at 540 nm (OD_{540}) were measured as the mean for triplicate cultures. Growth scoring for NaCl was as follows: +++, a change in the OD_{540} (ΔOD_{540}) of >0.27; ++, ΔOD_{540} of 0.15 to 0.27; +, ΔOD_{540} of 0.03 to 0.14; −, ΔOD_{540} of <0.03. Growth scoring for $CaCl_2$ was as follows: +++, ΔOD_{540} of >0.1; ++, ΔOD_{540} of 0.066 to 0.1; +, ΔOD_{540} of 0.03 to 0.065; −, ΔOD_{540} of <0.03.

[c] *Chromohalobacter salexigens* type strain.

[d] NT, not tested.

VAN1 and VAN5 used glutamine, asparagine, and glutamate as sources of nitrogen (data not shown). Nitrate was not used as a nitrogen source, and growth in M-VAN medium minus ammonia (as a test of aerobic diazotrophic potential) was not observed.

Salt requirements and tolerances. Five VAN strains were selected for testing of their growth response to salt challenges, with at least one strain being obtained from each brine depth sampled (Tables 2 and 3). In M-VAN medium lacking NaCl, no VAN strain grew. In the absence of other salts, the growth of all strains tested required at least 300 mM NaCl, and higher cell yields were typically obtained at higher salinities (Table 3). The halophilic bacterium and close relative of the VAN strains *Chromohalobacter salexigens* (Fig. 2) was used for comparison and required a minimum of 500 mM NaCl for growth, as did strain VAN5 (Table 3). Strain VAN1 grew well in the presence of NaCl up to 1 M, but higher levels of salt progressively slowed growth; no growth was obtained at NaCl concentrations above 2 M (data not shown).

All VAN strains grew in Ca-free M-VAN medium and in M-VAN medium supplemented with 800 mM $CaCl_2$. Only strains VAN1 and VAN5 grew in the presence of 900 mM $CaCl_2$, and strain VAN5 also grew, albeit weakly, in the presence of 1 M $CaCl_2$ (Table 3). Although we originally considered growth in the presence of these high levels of $CaCl_2$ to be remarkable and a sign that the VAN strains possibly possessed unique Ca^{2+} tolerance, parallel experiments performed with *C. salexigens* showed that this halophile also tolerated significant levels of $CaCl_2$ (Table 3).

DISCUSSION

Until now, only a single study has reported both the phylogeny and the physiology of a bacterium isolated from the warm Lake Vanda brine. In contrast, several bacteria have been characterized from cold hypersaline Dry Valley lakes, such as Lake Vida and Lake Bonney. However, none of these isolates are relatives of the VAN strains isolated herein, and in contrast to the mesophilic VAN strains, these isolates are either psychrotolerant or psychrophilic (33–35).

The Gram-negative coccus isolated from Lake Vanda water at a depth of 69 m (21) differs from the VAN strains in at least three major ways. First, the organism was a coccus, and second, it had no salt requirement for growth; that is, it was not halophilic. A third major difference was that the coccus grew at low temperatures. The coccus was isolated on medium containing only 0.5% (~86 mM) NaCl and grew at temperatures as low as 5°C in media lacking NaCl or containing 10% NaCl; the organism can thus be described to be both halotolerant and psychrotolerant (21, 36). In contrast, the VAN strains were rod shaped and halophilic—requiring a minimum of 300 mM NaCl for growth in the absence of $CaCl_2$—and did not grow at temperatures below 15°C. Such a phenotype should effectively exclude VAN strains from inhabiting the upper more fresh and cooler waters of Lake Vanda (Fig. 1).

Bratina et al. (24) described a *Carnobacterium* species from Lake Vanda deep water. However, in contrast to the Gram-negative, halophilic, and aerobic VAN strains, which are *Gammaproteobacteria*, *Carnobacterium* species are facultative aerobes, nonhalophilic, and related to the lactic acid bacteria (Gram-positive bacteria of the phylum *Firmicutes*). The absence of VAN-like bacteria in the study of Bratina et al. (24) can be explained by the fact that their enrichment media contained only 100 mM NaCl. Since this was insufficient to support the growth of VAN strains, the enrichment of such would not have been expected.

From a phylogenetic perspective, the VAN isolates were highly related to each other and grouped closely with species of the genera *Halomonas* and *Chromohalobacter*, with the closest relative being *Halomonas zincidurans*, isolated from sediments in the South Atlantic Ocean (37). In terms of salinity, *Halomonas* species are all halophilic and quite halotolerant (37–39). In contrast, although they were halophilic, the VAN strains were not nearly as halotolerant as other halomonads. For example, while VAN strains grew best in the presence of NaCl at concentrations below 1 M and did not grow in the presence of NaCl at concentrations above 2 M, their close relatives, *H. zincidurans* and *Halomonas*

xinjiangensis, grow in the presence of NaCl at concentrations of 3 M and 4 M, respectively (37, 38). Moreover, the type species of the genus *Halomonas*, *Halomonas elongata*, can grow in the presence of NaCl at saturation (5.5 M) (39).

While *Chromohalobacter* species are obligate aerobes, only some *Halomonas* species (for example, *H. zincidurans* and *H. xinjiangensis*) are obligate aerobes (37, 38); *H. elongata* is facultatively aerobic (39). Considering their habitat in the anoxic Lake Vanda brine, the obligately aerobic nature of the VAN strains is puzzling. However, because the opposing gradients of sulfide and O_2 between depths of 60 and 72 m in Lake Vanda are so steep (Fig. 1A) (2, 6, 20, 24, 25), traces of O_2 sufficient to support growth might remain in the brine all the way to the sediments. Alternatively, it is possible that the VAN strains are indeed facultative and can carry out some unusual fermentation or anaerobic respiration not tested for herein.

As for other major physiological properties, the VAN strains were neutrophilic, mesophilic, and heterotrophic, consistent with the phenotype that one would predict for bacteria indigenous to the Lake Vanda brine. The major carbon and energy sources for the VAN strains were sugars and organic acids. Although the cold Lake Vanda freshwaters contain very low levels of dissolved organic carbon (DOC), DOC levels in the Lake Vanda brine are significantly higher (40). Thus, the organic matter needed to support the growth of the VAN strains should be available in their habitat.

The results of our $CaCl_2$ experiments were surprising for at least two reasons. First, the VAN strains had absolutely no growth requirement for $CaCl_2$, despite the fact that $CaCl_2$ was the dominant salt in their habitat. In contrast, bacteria indigenous to NaCl- or $MgCl_2$-rich brines require substantial levels of NaCl or $MgCl_2$ plus NaCl, respectively, for growth (14, 17, 41, 42). The lack of a Ca requirement by the VAN strains also suggests that if bacteria that require high $CaCl_2$ levels for growth (calciophilic bacteria) exist in nature, they are either absent from the Lake Vanda brine or eluded our enrichments. Second, the fact that significant $CaCl_2$ tolerance was also displayed by *C. salexigens*, a bacterium that inhabits solar salterns where NaCl dominates and $CaCl_2$ is only a trace salt (20, 42), was also surprising and hints that $CaCl_2$ tolerance might be a characteristic trait of some halophilic bacteria.

Measurements of bacterial abundance in the Lake Vanda brine have yielded conflicting results. In the study of Bratina et al. (24), DAPI (4′,6-diamidino-2-phenylindole) measurements of Lake Vanda water from depths of 60 to 65 m revealed less than 5,000 cells/ml; this count is 10- to 100-fold lower than measures of bacterial abundance in the water column of other Dry Valley lakes, including the upper waters of Lake Vanda (25). In contrast, total cell counts determined by employing acridine orange suggest that cell numbers in the Lake Vanda brine are roughly similar to those in other Dry Valley lakes (22, 23). This discrepancy could be due to the abundant precipitates and mineral debris suspended in the Lake Vanda brine; staining of particulate matter with acridine orange is a common problem (43).

If bacterial abundance in the Lake Vanda brine is indeed lower than that in other Dry Valley lakes, it is likely because its microbial community experiences significant salt-induced stresses. Water activity, *per se*, does not seem to be a serious problem in the Lake Vanda brine. Our calculated water activity for the Lake Vanda brine indicates that it is well above the water activities of other Ca-rich brines (Table 1) and the minimum water activity known to support microbial growth (44–46). However, the Lake Vanda brine confers more than just ionic effects because its major salts—$CaCl_2$ and $MgCl_2$—are highly chaotropic (47). Chaotropic solutes create molecular disorder in cells (including the denaturation of macromolecules) by their ability to reduce water-macromolecule interactions. With the exception of $HgCl_2$, $CaCl_2$ is the most chaotropic of all divalent or monovalent salts and $MgCl_2$ is significantly chaotropic as well. In contrast, NaCl is a kosmotropic salt, a solute that promotes rather than destroys the stability of macromolecular systems (47).

Both *Bacteria* and *Archaea* have been detected in brines containing high levels of $MgCl_2$, such as the deep Mediterranean brines (48, 49). The Discovery brine, the most extreme in this regard, contains about 16 times the amount of $MgCl_2$ of the Lake Vanda brine. However, the deep Mediterranean brines are nearly devoid of $CaCl_2$, and most have high levels (3 to 4 M) of NaCl; hence, the chaotropic effects of $MgCl_2$ are offset somewhat by the kosmotropic effects of NaCl (47, 50). Notably, however, the Discovery brine is an exception in this regard, as it contains low levels of NaCl; in terms of chaotropicity and water potential, the characteristics of this highly unusual brine are probably at or very near the limits of those that can support life (45–50).

Although the Lake Vanda brine is not nearly as hypersaline as the deep Mediterranean brines, the Lake Vanda brine, composed of significant levels of two chaotropes coupled with low levels of a kosmotrope (Fig. 1 and Table 1), may also place significant constraints on its microbial community. New studies of the biogeochemistry and microbiology of the Lake Vanda brine are needed to approach this question, and the VAN strains described here should be a good starting point for such studies. Further study of the VAN strains could yield new insight into how halophilic bacteria respond to the challenge from multiple chaotropic salts and may reveal new principles that contribute to the habitability of brines of unusual ionic composition.

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