Occurrence and Diversity of *Legionellaceae* in Polar Lakes of the Antarctic Peninsula

Fábio R. S. Carvalho · Fernando R. Nastasi · Rosa C. Gamba · Annette S. Foronda · Vivian H. Pellizari

Abstract Legionellaceae is a family of Gram-negative, mesophilic, and facultative intracellular parasitic bacteria that inhabits freshwater environments. In this article, the *Legionella* population of water samples from the North and South Lake, located close to the Brazilian Scientific Station on King George Island, Keller Peninsula, Antarctica has been characterized. Culture onto selective medium and a independent-culture method were applied to the samples. In our attempt to isolate *Legionella* species from Antarctic lakes, we were able to obtain one *L. pneumophila* colony by an amoebic coculture procedure followed by plate culture onto a selective medium. In addition, results obtained from phylogenetic inference showed the presence of noncharacterized specimens of *Legionella* spp. These findings indicated the presence of legionellae in Antarctica and suggest that these bacteria can adapt to extreme conditions and open new possibilities for understanding the survival strategies of mesophilic *Legionellaceae* living in Antarctic environments. Furthermore, the isolation of these symbiotic bacteria in Antarctic lakes will allow future studies on cold-resistant mechanisms of legionellae in polar environments.

Introduction

Legionella species might exist as different forms in the environment: for example, free-living planktonic cells, intracellular parasites of free-living protozoa, inhabitants of mixed-community biofilms, and viable but nonculturable bacteria [16, 20]. Legionellae concentration in aquatic environments is usually low [9]. However, this microorganism is widespread in freshwater habitats and its ubiquity is probably due to the bacteria's capacity to survive in a wide range of environmental conditions (i.e., temperature, pH, and salinity [6, 9, 23]). Furthermore, the ability of *Legionella pneumophila* to survive and grow in a cold environment and the mechanisms of bacterial resistance at a lower temperature has been reported [19, 24]. Recently, molecular characterization of a *Legionella* population confirmed the occurrence of this bacterium as a member of the microbial community of the aquatic environment, even at low temperatures [26].

In the current study, both culturing and culture-independent methods were used to isolate and characterize the *Legionella* species in freshwater lakes of Keller Peninsula, Antarctica. The purpose of this study was to study the occurrence and genetic diversity of Legionellaceae bacteria within the microbiota of Antarctic lakes.

Materials and Methods

Sampling and Concentration of Water

Water samples were collected from two lakes on the Keller Peninsula of King George Island, Antarctica. The lakes are located at S 62°5′1.8″/W 58°23′36.16″ (North Lake) and S 62°5′9.0″/W 58°23′39.75″ (South Lake), near the Brazilian Scientific Station "Comandante Ferraz" (BSSCF). Both lakes are pristine and used for the water supply of BSSCF. The temperature of water at the time of sampling measured 0°C in both lakes. The samples were collected on

F. R. S. Carvalho · F. R. Nastasi · R. C. Gamba · A. S. Foronda · V. H. Pellizari (✉)
Laboratory of Environmental Microbiology, Department of Microbiology, Institute of Biomedical Science, São Paulo, SP, Brazil
e-mail: vivianp@usp.br

December 2004 at the beginning of the Antarctic summer. The samples, 5 L each, were collected in sterile plastic flasks and filtered through a sterile polycarbonate membrane (47 mm in diameter, pore size of 0.45 μm, Millipore, Ireland) at the BSSCF laboratory. Each membrane filter was aseptically removed from the support grid and transferred to a sterile 50-mL polypropylene centrifuge tube with 10 mL of the original water sample. The cells were released from the membrane by vortexing the tubes for 5 min. Aliquots of concentrated water samples were kept frozen, at −20°C, during transport to Brazil for subsequent molecular analyses.

Cultivation of Environmental Isolates

Concentrated water samples (0.1 mL) were inoculated in triplicate on buffered charcoal yeast extract agar supplemented with glycine, vancomycin, and polymyxin B (αBCYE-GVP) as previously described [6]. The culture procedure of environmental samples was done at BSSCF within 2 h after environmental sampling and concentration procedures, and the plates were incubated for 10 days in a humidified atmosphere at 37°C. Presumptive legionellae colonies were subcultured onto αBCYE-GVP and αBCYE agar in the absence of cysteine. Colonies that grew on αBCYE-GVP but were unable to grow on αBCYE without cysteine were considered putative *Legionella* strains and were checked for autofluorescence under ultraviolet light (365 nm), Gram stained, and subcultured again onto a new selective medium. As this method failed to subculture any strains, we enriched *Legionella* environmental isolates obtained from the primary plating culture method with an amoebic coculture, as described by Moffat and Tompkins [17]. Axenic cultures of *Acanthamoeba castellanii* ATCC 30011 strains (American Type Culture Collection, Rockville, MD) were used in the amoebic coculture procedure. Briefly, *A. castellanii* trophozoites were grown in a 25-cm^2 cell culture flask (Costar, Cambridge, MA, USA) containing Neff's amoebic medium (120.0 mg NaCl, 3.0 mg $MgCl_2$. $6H_2O$, 3.0 mg $CaCl_2$, 3.0 mg $FeSO_4$, 142.0 mg Na_2HPO_4, 136.0 mg KH_2PO_4, 10.0 g proteose peptone, 18.0 g glucose, 1.0 L distilled water, pH 6.8). Amoebas were collected for the coculture procedure by vigorous agitation of the media when cells had formed a confluent layer on the bottom of the flask (1.0×10^6 cells/mL). Amoebas were harvested and pelleted by centrifugation at $1000g$ for 10 min. The supernatant was removed, and the amoebas were resuspended in 20 mL of PAS buffer (Page's amoebic saline; 120 mg NaCl, 4 mg $MgSO_4$ · $7H_2O$, 4 mg $CaCl_2$ · $2H_2O$, 142 mg Na_2HPO_4, 136 mg KH_2PO_4, 1.0 L distilled water). The washing procedure was repeated and the amoebas were resuspended in 10 mL of PAS, at a final concentration of 2.0×10^5 cells/mL.

Finally, aliquots of 1 mL of amoebic suspension were transferred to into 24-well cell culture microplates (Costar, Cambridge, MA, USA) and allowed to adhere to the wells for 1 h at 37°C before inoculating putative legionellae colonies. An aliquot of 100 μL of each amoebic suspension was cultured in triplicate onto sheep blood agar before amoebic coculture procedures in order to check for the absence of exogenous contamination, and *L. pneumophila* ATCC 33152 strains was used as a positive control. In the amoebic coculture procedure, 100 μL of each suspension was inoculated in duplicate on both selective (αBCYE-GVP) and nonselective (αBCYE without cysteine) media agar plates by the spread plate technique. Plates were incubated at 37°C and observed for 10 days to determine the presence of intracellular bacteria.

DNA Isolation from Cultures and Environmental Samples

Genomic DNA from both legionellae putative colonies and concentrated water samples (2 mL) was extracted with the QIAamp DNA kit (Qiagen, Hilden), eluted in 50 μL of AE buffer (supplied in the kit) and stored at −20°C. MilliQ ultrapure water was used as a negative control. The concentration of DNA was determined by electrophoresis on 0.8% agarose gels.

PCR Amplification and Sequencing of Environmental Isolates

Preliminary identification of isolates was based on the nearly complete 16S rRNA and specific defect of organelle trafficking A (*dot*A) gene sequences. Reagents concentration and thermal cycling conditions used in the polymerase chain reactions (PCRs) followed previous descriptions [3, 4, 11, 13, 18], as shown in Table 1. Oligonucleotide primers targeting regions of each of the genes were used to amplify 430- to 1525-bp products encompassing regions of variation (Table 1). A previous comprehensive new database was carried out in order to check their specificity and, consequently, to prevent false-positive results. PCR products to be sequenced were purified with the PureLink PCR Purification Kit (Invitrogen Life Technologies, California, USA). The sequencing reaction was done according to the MegaBACE 1000 protocol (Amersham Biosciences, Piscataway, NJ). Both strands of each amplicon were sequenced. Lane tracking and base calling were done with Cimarron 3.12 software.

Molecular Cloning Library and Sequencing

The PCR products (430 pb) generated from genomic DNA extracted from freshwater samples were subsequently

Table 1 Oligonucleotide primers used for amplification and sequencing of genes used in this study

Primer designation	Sequence (5′-3′)	Gene	Amplicon (bp)	Proposed functions for each primer sets	Refs.
LEG-225[a]	aagattagcctgcgtccgat	16S rRNA[b]	654	PCR[c]	[18]
LEG-858[a]	gtcaacttatcgcgtttgct				
LEG-448b[d]	gagggttgataggttaagagc	16S rRNA[b]	430	PCR, cloning, sequencing[c]	[18]
LEG-858[d]	gtcaacttatcgcgtttgct				
DL1	ttgatttggtgaaactcaatgg	*dot*A	434	PCR, sequencing[e]	[11]
DL2	caatcaaaatcctggtgcttc				
27F	agagtttgatcmtggctcag	16S rRNA	1500	PCR, sequencing[e]	[3, 13]
1525R	aaggaggtgwtccarcc				
338F	actcctacgggaggcagcag	16S rRNA	760	PCR, sequencing[e]	[4]
1100R	agggttgcgctcgttg				

[a] Primers used in the first step of a seminested PCR reaction [18]

[b] Specific fragment of 16S rRNA gene applied to the study of Legionelleae diversity

[c] Molecular procedures applied to both environmental isolates (PCR and sequencing reactions) and genomic DNA extracted from freshwater samples (PCR, cloning, and sequencing reactions)

[d] Primers used in the second step of a seminested PCR [18]

[e] Molecular procedures applied to environmental isolates only

cloned into pGEM-T® Vector System I (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Plasmid DNA from the selected colonies was extracted as described previously [2]. Cloned inserts were reamplified with primers LEG-448 and LEG-858. The purity and concentration of each PCR product was verified on 1% agarose gels.

Phylogenetic Analyses

The 16S rRNA gene sequences from both environmental isolates and clones were analyzed and concatenated with the BioEdit Sequence Alignment Editor version 5.0.9 [10]. The nucleotide sequences obtained were compared to the GenBank data library with BLAST software [1]. The possible chimerical sequences were checked using the CHECK-CHIMERA tool [15]. Phylogenetic and molecular evolutionary analyses from 16S rRNA and *dot*A gene sequences were done with MEGA software version 3.1 [12], and phylogenetic trees were constructed with the neighbor-joining (NJ) method without gaps.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences from the cloning library chosen for this study were deposited in the GenBank database under accession Nos. DQ404565 to DQ404588. Partial 16S rRNA gene sequences from environmental strains were deposited under accession Nos. DQ646380 and DQ646381. Sequences from 16S rRNA gene obtained with eubacterial universal primers were deposited under accession Nos. DQ646382 and DQ646383, whereas *dot*A

gene sequences from environmental strains were deposited under accession Nos. DQ646378 and DQ646379.

Results

Preliminary Isolation of *Legionella* spp. from Water Samples

A single suspected *Legionella* colony was observed on a selective culture medium inoculated with water samples from North Lake and grew poorly when subcultured after coculturing with *A. castellanii*. In addition, a single colony was observed on the αBCYE-GVP agar medium when duplicates where plated. Neither isolate was autofluorescent under ultraviolet light. This colony failed to grow on αBCYE agar without L-cysteine, suggesting that the *Legionella* specimen was isolated from freshwater of North Lake.

PCR from Isolates and Genomic DNA Extracted from Environmental Samples

The PCR amplification followed by sequencing of the 16S rRNA gene from an environmental isolate DNA template by using both eubacterial universal primers (27F/1525R and 338F/1100R) and primer sets that target the *Legionella* 16S rRNA gene (LEG-448/ LEG-858 internal primers) showed the presence of *Legionella* and *Yersinia* bacteria in the single colony obtained by the coculture amoebic procedure. For this reason, a virulence-related *dot*A gene from the same DNA template was amplified and sequenced in

order to identify both *Legionella* and *Yersinia* species. Phylogenetic relationships of putative *Legionella* colonies obtained in this study, designated Strain-03 and Strain-04, and *L. pneumophila*, inferred from nucleotide sequences of partial *dot*A and 16S rRNA genes, are shown in Figs. 1 and 2, respectively. Sequence identity analyses to both partial *dot*A and 16S rRNA genes from Strain-03 and Strain-04 nucleotide sequences showed the same homology of 98.8% to *dot*A (GenBank accession No. AF095231) and 16S rRNA (GenBank accession No. AE017354) gene sequences of *L. pneumophila* subsp. *pneumophila* strain Philadelphia 1. In contrast, 16S rRNA gene sequences obtained by amplifying and sequencing the same DNA template with eubacterial primers from Strain-03 (1472-bp fragment length) and Strain-04 (1474-bp fragment length) showed 99.8% and 97.8% identities with *Y. kristensenii* and *Y. aldovae*, respectively.

Molecular Cloning Library of 16S rRNA Gene of Antarctic Lakes

LEG-specific primers (LEG-448 and LEG-858) were used to construct cloning libraries of 16S rRNA gene sequences. The inserts containing a fragment of 16S rRNA gene (430 bp) from 24 clones—15 clones from South Lake (LS) and 9 clones from North Lake (LN)—were sequenced in both strands. The primer sequences on both sides of the sequence were removed, and 417 bp from each clone sequence were considered for alignment. Analyses of clone sequences obtained from the 16S rRNA gene matched the GenBank sequence database of *L. pneumophila*, *L. lytica*, *L. jeonii*, *L. erythra*, and *Legionella*-like amoebal pathogens (strain LLAP-10). Phylogenetic analyses revealed that clone sequences from both North Lake and South Lake

samples clustered into three main groups (Fig. 2). One of these groups consisted of the following clones: LNcl04, LScl03, LScl04, LScl07, LScl11, LScl19, LScl21, and LScl22 (similarity above 98% and bootstrap values of 54–91%), which formed a common lineage with *L. pneumophila*. Clones LNcl01, LNcl02, LNcl03, LNcl09, LNcl10, and LScl16, grouped together with an unidentified *Legionella* specimen designed LLAP-10 (similarities above 95%). Finally, the third main group was represented by clones LNcl06, LNcl07, LScl01, LScl02, LScl05, LScl06, LScl10, and LScl18, which grouped together with *L. lytica*. Other clones of legionellae belonged to *L. jeonii*, which showed the closest relation to the clone LNcl11 (similarity 94.4%), whereas clone LScl14 was related to *L. erythra* (similarity 94.4%).

Discussion

In this study, the importance of natural hosts in the process of resistance and the development of *Legionella* bacteria at low temperatures was shown when the *L. pneumophila* strain was first obtained by the amoebic coculture procedure. Although the interaction between *Y. aldovae* and free-living protozoa has not been described in the literature, the presence of the Enterobacteriaceae family genera has been reported in extreme temperature conditions from polar waters [8]. These symbiotic bacteria could inhibit growth or mask the presence of Legionellaceae colonies on selective agar media [14]. Moreover, sequencing analysis of the *dot*A gene was consistent with results previously obtained by using internal primers of the 16S rRNA gene. In this specific case, the choice of the *dot*A gene for confirming the *L. pneumophila* isolates was based on the fact
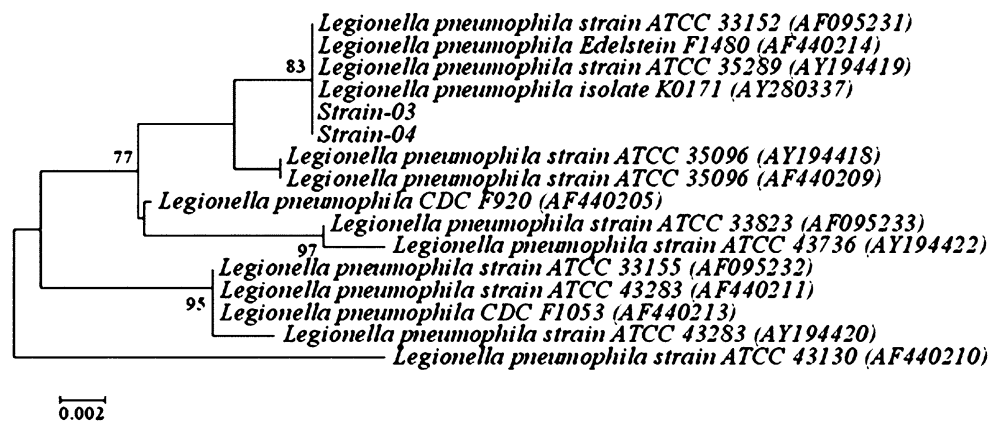
Fig. 1 Phylogenetic relationships of *L. pneumophila* inferred from partial *dot*A gene sequences (428-bp) of environmental strains obtained in this study (designed Strain-03 and Strain-04) and GenBank database. A midpoint rooting option was applied to root the tree due to the absence of a reliable outgroup. The bootstrap values presenting corresponding branches were evaluated from 1000 replications. Values below 75% are not indicated. The scale bar represents 0.2 substitutions per 100 nucleotides

Fig. 2 Bootstrap consensus tree of partial 16S rRNA gene (420-bp) generated by LEG-specific primers (LEG-448 and LEG-858). Comparative analysis were done between putative *Legionella* strains, designated as Strain-03 and Strain-04, and clone sequences from DNA extracted directly from North Lake water, clones designated as LN, and South Lake water, clones designated as LS. The neighbor-joining (NJ) method was used as distance matrix analysis and Tamura–Nei correction was used to compensate for different evolutionary rates in both NJ phylogenetic trees. The 16S rRNA gene sequence of *Coxiella burnetii* was used as outgroup to root tree. Branches supported by values higher than 50% in the bootstrap analysis (1000 replications) are indicated in nodes. The bars represent 2% estimated sequence divergence
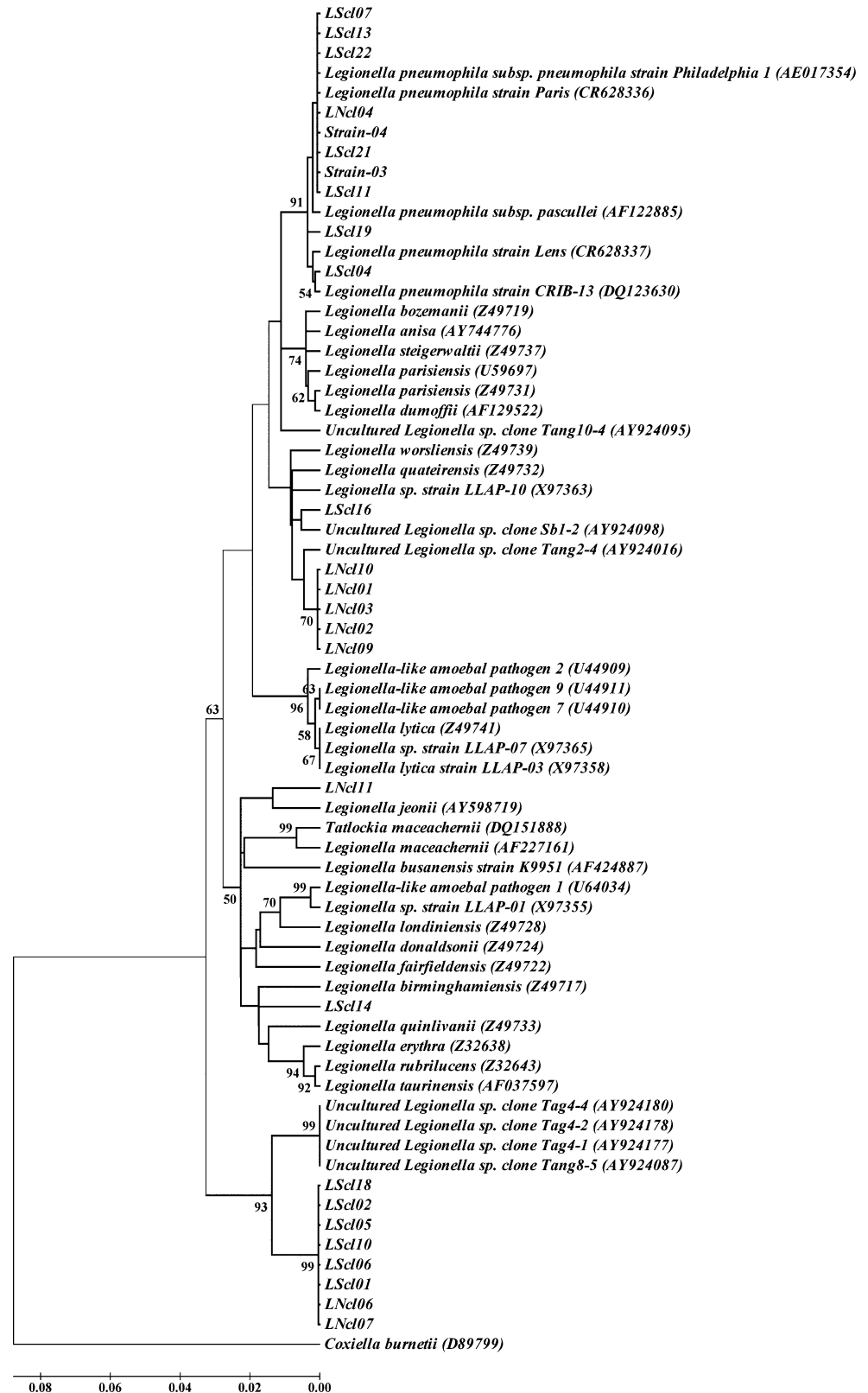
that this gene is a component of the pathogenicity island, which contains the 24 *dot/icm* genes on 2 unlinked 22-kb regions on the *L. pneumophila* genome [11]. This gene has also been related to adaptation and intracellular survival of this bacterium in different hosts, such as amoeba, ciliates, and other free-living protozoans [25].

The amplification of *Yersinia* specimen 16S rRNA gene sequence by eubacterial universal primers could be explained by different DNA concentrations between the *Legionella* and *Yersinia* strains within a mixture culture. The PCR products containing both *Legionella* and *Yersinia* amplicons allowed for identifying *Y. aldovae* or *Y. kristensenii*, which could be present at a higher DNA concentration, by preferentially amplifying the PCR reaction. Consequently, the sequencing reaction of the V3 region to the 16S rRNA gene allowed only the *Yersinia* specimen to be identified when eubacterial universal primers were used. In contrast, when an internal fragment of the 16S rRNA gene that encoded to *Legionella* species was amplified and sequenced with LEG-448 and LEG-858 primers, *L. pneumophila* was likely to be present at lower levels. Preferential amplification is more likely in mixed-target PCR reactions where competition between multiple targets for primer annealing could result in the numerically dominant sequences being preferentially amplified [7]. An additional hypothesis could be related to the specificity of primers used: LEG-448 and LEG-858 primers were designed from the V3 region of the 16S rRNA gene molecule for specific detection of *Legionella* strains, whereas the universal primers were designed to bind to nearly all 16S rRNA gene sequences of the eubacteria. Therefore, results obtained could be mainly influenced not only by different DNA concentrations but also by the specificity of primers used.

The concentration of metallic ions, mainly Al, Fe, and Cu, in frozen lakes and the metal requirements for growth of Legionellaceae could represent a potential environmental precondition for survival of these microorganisms in Antarctic aquatic systems. In the present study, water samples were collected at the beginning of the Antarctic summer, when the surface ice begins to shrink and the air and water temperatures are considerably lower. Cowan and Tow [5] showed that during summer, peripheral moats (melting of the peripheral ice cover) allow the inflow of water from the melting streams, resulting in steep salt gradients in the water column. In addition, Schaefer et al. [22] reported the chemical composition and the concentration of metallic ions, such as iron (0.050 mg/mL, North Lake, and 0.06 mg/mL, South Lake) and zinc (0.525 mg/mL, North Lake, and 0.853 mg/mL, South Lake), in water from the same lakes sampled in this study.

In conclusion, Antarctic lakes could provide a buffered and protected aquatic environment for Legionellae, where the population of these bacteria is phylogenetically diverse. Sequencing results of 16S rRNA gene partial fragments showed that most of Legionellae clone sequences were related to the presence of protozoa hosts and that the population of *Legionella* bacteria in both lakes is phylogenetically diverse. The symbiotic association of *Legionella* species and amoebas could allow for the mechanisms of survival and resistance of the bacteria at low-temperatures environments. Moreover, our results indicated the presence of uncommon and protozoonotic *Legionella* species in a polar environment. Although the process of obtaining viable Legionellae under extreme conditions (i.e., low temperatures) would be difficult and time-consuming, it could explain certain factors that are not well understood, such as adaptation to the cold and changes in the cellular structure that could have occurred in an evolutionary stage.

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