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ARTICLE

Diversity of culturable bacteria recovered from Pico Bolívar's glacial and subglacial environments, at 4950 m, in Venezuelan tropical Andes

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Abstract: Even though tropical glaciers are retreating rapidly and many will disappear in the next few years, their microbial diversity remains to be studied in depth. In this paper we report on the biodiversity of the culturable fraction of bacteria colonizing Pico Bolívar's glacier ice and subglacial meltwaters, at ~4950 m in the Venezuelan Andean Mountains. Microbial cells of diverse morphologies and exhibiting uncompromised membranes were present at densities ranging from 1.5×10^4 to 4.7×10^4 cells/mL in glacier ice and from 4.1×10^5 to 9.6×10^5 cells/mL in subglacial meltwater. Of 89 pure isolates recovered from the samples, the majority were eurypsychrophilic or stenopsychrophilic, according to their temperature range of growth. Following analysis of their 16S rDNA nucleotidic sequence, 54 pure isolates were assigned to 23 phylotypes distributed within 4 different phyla or classes: *Beta-* and *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes*. *Actinobacteria* dominated the culturable fraction of glacier ice samples, whereas *Proteobacteria* were dominant in subglacial meltwater samples. Chloramphenicol and ampicillin resistance was exhibited by 73.07% and 65.38%, respectively, of the subglacial isolates, and nearly 35% of them were multiresistant. Considering the fast rate at which tropical glaciers are melting, this study confirms the urgent need to study the microbial communities immured in such environments.

Key words: glacier ice bacteria, tropical Andes, tropical glaciers, psychrophilic bacteria, biodiversity.

Résumé : Malgré le fait que les glaciers tropicaux reculent rapidement et que bon nombre disparaîtront dans les années à venir, on n'a toujours pas étudié leur diversité microbienne en profondeur. Dans le présent article, nous faisons état de la biodiversité de la fraction cultivable de bactéries colonisant les glaces du glacier Pico Bolívar et de ses eaux de fonte sous-glaciaires, en un lieu situé à une altitude de ~4950 m dans les Andes vénézuéliennes. On a observé des cellules microbiennes aux morphologies diverses et présentant des membranes intactes à des densités variant entre $1{,}5 \times 10^4$ et $4{,}7 \times 10^4$ cellules/mL dans la glace de glacier, et variant entre $4{,}1 \times 10^5$ et $9{,}6 \times 10^5$ dans l'eau de fonte sous-glaciaire. La plupart des 89 isolats purs issus des prélèvements étaient eurypsychrophiles ou sténopsychrophiles d'après leur plage de températures de croissance. Suivant une analyse de la séquence nucléotidique de leur ADNr 16S, on a classé 54 isolats purs dans 23 phylotypes distribués dans quatre phylums ou classes distincts, soit les *Beta-* et *Gammaproteobacteria*, les *Actinobacteria* et les *Bacteroidetes*. Les *Actinobacteria* ont dominé la fraction cultivable des prélèvements de glace de glacier, tandis que les *Proteobacteria* étaient dominantes dans les échantillons d'eau de fonte sous-glaciaire. On a noté une résistance au chloramphénicol et à l'ampicilline chez respectivement 73,07 % et 65,38 % des isolats sous-glaciaires, et tout près de 35 % d'entre eux étaient multirésistants. Au vu de la rapidité à laquelle les glaciers tropicaux fondent, cette étude confirme le besoin urgent d'étudier les communautés microbiennes emmurées dans de tels environnements. [Traduit par la Rédaction]

Mots-clés : bactéries de glaces de glacier, Andes tropicales, glaciers tropicaux, bactéries psychrophiles, biodiversité.

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Introduction

Psychrophilic microorganisms (i.e., microbes able to grow actively in permanently cold environments and even at subzero temperatures) have attracted the attention of scientists for more than a century. Since the first expeditions to Antarctica, conducted during the early 20th century, permanently cold regions of the world have been thoroughly explored with the aim of isolating, culturing, identifying, and characterizing this particular kind of extremophile (Miteva 2008; Margesin and Miteva 2011). Among permanently cold microbial habitats, glaciers deserve a special place. Besides enduring low temperatures, glacier-ice-entrapped microorganisms must face several stressful conditions simultaneously, among which are high hydrostatic and osmotic pressures, very low nutrient availability, and elevated doses of cosmic, solar, and earth radiations (Miteva 2008). As a result, some of these microorganisms have evolved several physiological responses that allow them to survive for long periods of time under glacial conditions. Some of these responses include the production of antifreeze proteins and cryoprotectants, the modification of membrane fluidity by means of synthesizing increased amounts of unsaturated fatty acids, the production of carbohydrate-based extracellular polymeric substances (EPS), and the synthesis of psychrophilic enzymes, among others (D'Amico et al. 2006; Siddiqui et al. 2013; De Maayer et al. 2014). Over the years, the discovery of these particular features of psychrophiles has supported the development of numerus biotechnological products and processes, some of which are of great importance in the current world, such as production of cold-active detergents, production of biofuels, and food processing at low temperatures, among many others (Cavicchioli et al. 2011; Nevalainen et al. 2012; Feller 2013).

To date, many reports describe in detail both Arctic and Antarctic glacier ice microbial communities. On the contrary, only a few papers have been published concerning microbes immured in tropical glaciers (Christner et al. 2000; Ball et al. 2014; Balcazar et al. 2015; Fritz et al. 2015). This is striking, considering that compared with high and mid-latitude glaciers, tropical glaciers are peculiar in several aspects (Kaser and Osmaston 2002). These glaciers exist mostly in the Andes of South America, where more than 99% (with respect to surface area) are found. Besides being more affected by climatic perturbations, tropical glaciers are particularly close to major biological ecosystems (like tropical forests and savannas), with substantial vegetation and exposed soils. That is why some authors believe that microorganisms colonizing these neighboring environments are actually transported to the surface of the glaciers by means of airborne particles, which enable the microorganisms to travel long distances (Liu et al. 1998). The aerosolized biological material can be entrapped by glacial ice sheets, and this has been proposed as one of the reasons explaining the high density of culturable bacteria in the ice samples of tropical glaciers (Christner et al. 2000).

Considering that tropical glaciers will disappear in the next few years as a consequence of their rapid meltdown (Urrutia and Vuille 2009; Vuille et al. 2003, 2008; Rabatel et al. 2013), and that they might harbor microbial communities markedly different from those colonizing glaciers of higher latitudes, studying their microbiology is of extreme importance for several reasons: first, glacial ecosystems are considered as massive repositories of a virtually unexplored biologic, physiologic, and genomic diversity (Edwards 2015); second, this largely unexplored biological diversity faces a real risk of extinction owing to the loss of its harboring ecosystem (Griffiths 2012); and, third, rapid meltdown of these glaciers might contribute to the reactivation and release of human, animal, and plant pathogens that have remained contained in glacial ice for centuries and even millennia (Rogers et al. 2004).

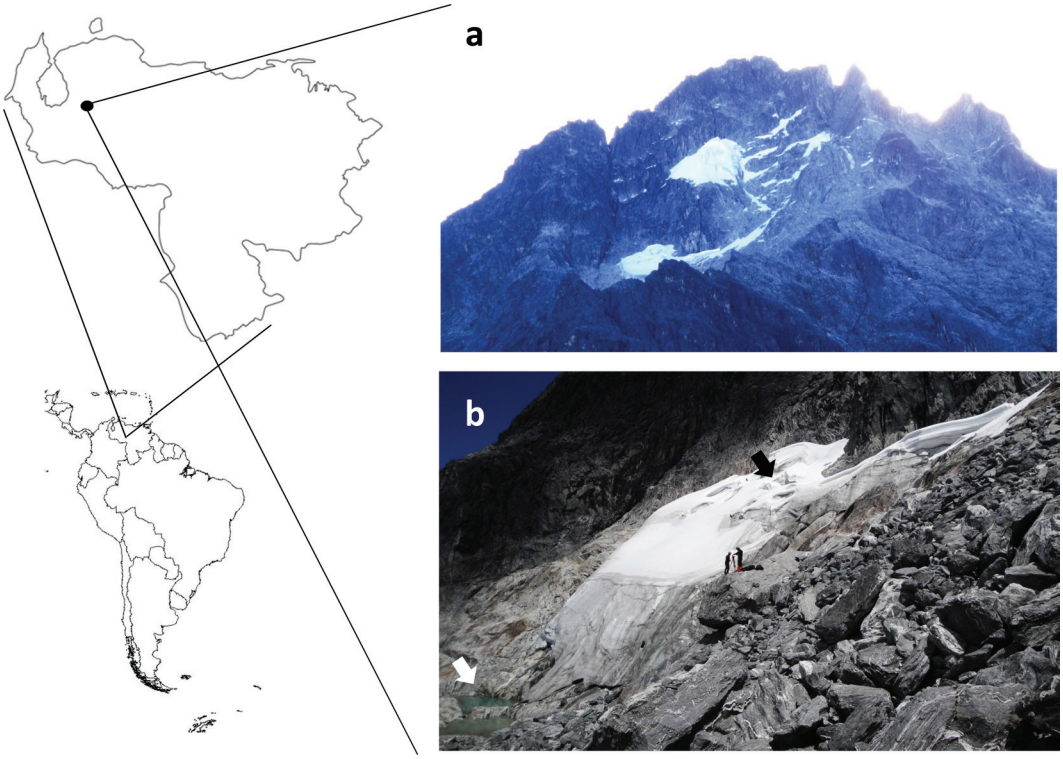
Venezuelan glaciers are among the most rapidly retreating glaciers of the entire Andean region. These glaciers have lost an area of 1.7 km^2 during the past 50 years, which corresponds to approximately 84% of its area of coverage (Schubert and Clapperton 1990; Carrillo and Yépez 2010; Braun and Bezada 2013). With this trend, the glaciers of the Venezuelan Andes will totally disappear in less than 5 years. In this study, we used culture methods to isolate, identify, and further characterize viable bacteria immured in glacial ice and (or) colonizing subglacial meltwater at Pico Bolívar glacier in the Andean region of Venezuela. Additionally, we tested the resistance of a selected group of isolates to different antibiotic classes and metals to gain more information about their possible role as potential donors of genetic resistance determinants in horizontal transfer events. By doing this, we extend our previous observations concerning bacterial communities of tropical glaciers (Ball et al. 2014; Balcazar et al. 2015). Finally, we contribute to preserve a minute, though potentially useful, fraction of this vanishing biodiversity for future research.

Materials and methods

Sampling site

Pico Bolívar's glaciers are currently composed of 2 very small ice or firn patches of no more than 0.1 km^2, located at 8°32′0″N and 71°2′0″W at the base of the highest peak in the Venezuelan Andes (Sierra Nevada National Park, Mérida, Venezuela) (Fig. 1*a*). As can be seen, these patches remain "clinging" to the steep northern face of Pico Bolívar. The glacier's surface is full of holes of different diameters, from dozens of centimetres to several metres, and crevasses (Fig. 1*b*). These are most probably the visible signs of the ongoing melting process. A stream of running water (melted ice) emerges from the glacier snout and accumulates in a small pond (Fig. 1*b*, arrows).

Fig. 1. (*a*) View of the northwestern side of Pico Bolívar with its 2 remaining small ice or firn fields on February 2013. (*b*) Samples were collected at the base of the glacier, near a small pond of glacial meltwater (white arrow). The black arrow points at big holes and crevasses in the glacier's surface.



Sample collection

Two different types of samples were collected at the same location in Pico Bolívar glacier during the end of the dry ("summer") season on 2 March 2014 at 1500 h. The first one corresponded to glacial ice, collected with flame-sterilized instruments directly into sterile Falcon tubes. To achieve this objective, we selected a large block of glacier ice (approximately 4 m high × 5 m wide × 5 m deep), recently detached from the glacier's front edge and located approximately 1 m away from it. During the sampling procedure, we took care in removing the surface layer of ice by thoroughly flaming it, and then collected the ice (discarding the superficial 5 cm) by means of a flame-sterilized ice-screw device (28 cm long) connected by its (open) back end to a sterile 15 mL Falcon tube. At this location, the ice was crystal clear and devoid of any visible trace of debris, gravel, or sediment. The second type of sample corresponded to subglacial meltwater running at the base of this glacier and in contact with the glacier bed. Meltwater samples were collected from the glacier snout by aseptic means into sterile 50 mL Falcon tubes. Five replicate samples were collected at each site. Once collected, ice samples were kept at <4 °C and transported to the laboratory in less than 24 h.

Media and isolation of bacteria

Cultures were started as previously described by Ball et al. (2014). In brief, 0.1 mL of either melted ice or subglacial water was streaked on the surface of the following solid media: R2A (full, 1/50, or 1/100 strength) (Reasoner and Geldreich 1985) or Luria–Bertani broth (LB) (full, 1/50, or 1/100 strength). The plates were sealed with Parafilm and incubated aerobically for up to 3 months at 4, 10, and 30 °C in a humid chamber until colonies became visible. Morphologically different colonies were selected, re-streaked several times, and checked for purity both macro- and microscopically. The purified isolates were stored at −80 °C in 20% glycerol.

Microscopic examination

Microscopic analysis of the melted ice samples was performed by fluorescent microscopy. For this, 50 mL of melted ice and 10 mL of subglacial water were filtered onto a black 0.2 μm Nucleopore track-etched membrane filter (Whatman), followed by staining with propidium iodide and SYTO9 using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, Oregon). Cells were visualized by epifluorescence microscopy (Hobbie et al. 1977) at ×1000 magnification (Olympus BX16). The number of cells in 20 random fields was counted. Controls, comprising filtered ultrapure water, were also checked.

Ice samples for scanning electron microscopy (SEM) were concentrated by filtering 5 mL of melted ice onto a 10-mm-diameter spot of a 0.1 μm pore-size Nucleopore filter (Whatman). The filters were immediately im-

mersed in a fixing solution containing 3% glutaraldehyde and 3% formaldehyde in 0.1 mol/L cacodylate buffer, pH 6.3, (Palacios Prü and Mendoza Briceño 1972) for 6 h at 4 °C. Once fixed, the filters were mounted on a metal stub with the help of conductive adhesive tape and dehydrated in a vacuum chamber for 5 days. Subsequently, the samples were coated with gold using a 11430E SPI sputter coater. SEM analysis was carried out at 12 kV accelerating voltage using a Hitachi S-2500 SEM.

Characterization of isolates

The temperature growth range of the isolates was tested on R2A or LB agar media at 4, 10, 15, 20, 25, 30, and 37 °C by visual inspection of the plates.

Enzymatic activities for amylases, proteases, and β-galactosidases were screened on R2A agar medium supplemented with 2.5 g/L starch, 10 g/L skim milk, or 40 μg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma, St. Louis, Missouri), respectively. All tests were performed at 15 °C.

Antibiotic and metal resistance of isolates

All psychrophilic and psychrotolerant strains were tested for antibiotic resistance as previously reported (Ball et al. 2014). In brief, colonies were streaked onto R2A agar plates containing one of the following antibiotics (up to 100 mg/L): ampicillin, penicillin, streptomycin, nalidixic acid, kanamycin, chloramphenicol, and tetracycline. Plates were incubated at 15 °C and growth of the strains was compared with the control experiment. The following strains were used as positive controls of growth: *Escherichia coli* BL21 (Kan^R, Cam^R), XL1-Blue (Tet^R), and PA601 (Str^R) (derivative of K-12). The negative control used was *E. coli* Hfr H (derivative of K-12).

Metal resistance was tested in a similar way, i.e., by culturing the isolates onto R2A plates containing from 0 to 200 ppm Zn^{2+} (supplied as $ZnCl_2$), Cu^{2+} (supplied as $CuSO_4$), Co^{2+} (supplied as $Co(NO_3)_2{\cdot}6H_2O$), and Ni^{2+} (supplied as $NiSO_4$). The isolates were also streaked on R2A plates supplemented with 10 μmol/L $HgCl_2$ to test for mercury resistance (Møller et al. 2011). Isolates growing on the mercury plates were re-streaked at least 3 times on fresh plates of an appropriate mercury-containing medium to confirm mercury resistance. To confirm experimentally the toxicity of the metals at the concentrations tested, we used *E. coli* Hfr H as negative control.

PCR amplification, sequencing, and analysis of 16S rDNA

The gene-encoding 16S rRNA (16S rDNA) was PCR amplified from some selected isolates using bacterial universal primers fD1 and rD1 (Weisburg et al. 1991). The following cycle conditions were used: 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 3 min (Lane 1991). The PCR products were purified with the Wizard SV PCR clean-up system kit (Promega, Wisconsin, USA) and sequenced at Macrogen Inc. (Seoul, South Korea). The nucleotide sequences were subjected to a Blast search using the BlastN program (Altschul et al. 1997). As reference, we used nucleotide sequences deposited in the GenBank, and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups.

Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011) using the Neighbor-Joining method (Saitou and Nei 1987).

Nucleotide sequence accession numbers

GenBank 16S rRNA gene sequence accession numbers for each of the isolates used in the alignment are given in Table 1.

Results

Isolation of psychrophilic and (or) psychrotolerant bacteria

Initial examination of glacier ice samples showed the presence of a few motile bacterial cells when observed under light microscopy (not shown). SEM revealed the presence of diverse morphologies (including rods, cocci, and small filaments); small cells ("dwarf") and cells showing signs of undergoing division were also present (Fig. 2*a* and supplementary Fig. S1[1]). By using fluorescence techniques, we were able to detect cells with uncompromised membranes, i.e., potentially viable, in these samples (Fig. 2*b*). Abundant cells of different morphologies (possibly both prokaryotes and eukaryotes), with intact plasma membranes, were observed when examining subglacial water collected at the base of the Pico Bolívar's glacier (Figs. 2*c* and 2*d*). The microbial density, as calculated from direct microscopic counts, ranged from 1.5×10^4 to 4.7×10^4 cells/mL in the case of glacial ice, and from 4.1×10^5 to 9.6×10^5 cells/mL in the case of subglacial meltwater. The approximate proportion of live: dead cells was 70:30 and 90:10 in the case of glacial ice and subglacial meltwater, respectively, (supplementary Fig. S2[1]).

Long-time incubations (for up to 3 months) in R2A and LB media at both 15 and 4 °C permitted us to isolate and purify a small number of bacteria from ice samples, i.e., only 1 or 2 colonies per plate for a total of 14 pure isolates in total. On the contrary, and as expected, we were able to isolate and purify a higher number of colonies from subglacial water samples (>350 colonies).

Morphological and physiological characterization of the isolates

Eighty-nine pure isolates (11 isolated from glacier ice and 78 from subglacial meltwater), originating from

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2016-0172.

Table 1. Characteristics of bacterial isolates.

Isolate No.	Growth temperature (°C)[a]						Antibiotic resistance[b]							Metal resistance[c]				Exoenzyme production[d]			Closest relative species	% Identity	GenBank Acc. No.
	4	10	15	20	30	37	Amp	Tet	Cam	Pen	Str	Nal	Kan	Cu	Ni	Co	Zn	Prot.	β-Gal.	Amyl.			
PGV200	++	+++	+++	+++	+++	–	100	0	100	100	100	100	0	0	100	100	200	+	–	–	*Pseudomonas meridiana*	99.42	KJ417589
PGV201	+	++	++	++	–	–	100	0	100	20	0	25	0	50	100	100	50	+	–	–	*Janthinobacterium lividum*	93.37	KJ417605
PGV202	+	na	+++	+++	–	–	100	0	100	20	0	100	0	100	100	100	200	–	–	–	*Janthinobacterium lividum*	99.48	KJ417606
PGV203	+	+++	+++	+++	+++	–	100	0	100	100	100	100	0	0	100	100	200	+	–	–	na	na	na
PGV205	+	++	++	+++	–	–	100	0	100	100	0	25	0	0	50	100	200	+	–	–	*Janthinobacterium lividum*	99.6	KJ417607
PGV206	+	++	+++	++	+++	–	100	0	100	100	0	100	0	50	100	50	200	+	–	–	*Pseudomonas migulae*	99.53	KJ417590
PGV207	+	++	++	++	+++	–	100	0	100	100	0	25	0	0	50	50	200	+	–	–	*Pseudomonas meridiana*	99.4	KJ417608
PGV208	+	+	+++	+++	++	–	100	0	100	100	0	25	25	0	50	0	200	+	–	–	*Pseudomonas brenneri*	99.41	KJ417609
PGV209	+++	++	+++	+++	+++	–	100	0	100	100	0	100	25	0	50	50	200	+	–	–	*Pseudomonas brenneri*	99.32	KJ417610
PGV210	+	+++	+++	+++	–	–	100	0	100	100	0	100	0	50	50	100	200	+	–	–	*Janthinobacterium lividum*	99.54	KJ417611
PGV211	+	+++	++	+	–	–	0	0	0	0	0	25	0	0	50	0	0	–	–	–	*Janthinobacterium lividum*	96.96	KJ417612
PGV212	++	–	+++	–	+++	–	100	0	100	100	0	25	0	0	50	0	200	+	–	–	*Pseudomonas meridiana*	99.9	KJ417613
PGV213	+	+++	++	+++	–	–	0	0	0	0	0	0	0	0	50	0	50	–	–	–	*Janthinobacterium lividum*	97.39	KJ417614
PGV214	+	+++	+++	++	++	–	100	0	100	100	0	25	0	0	50	100	50	–	+	–	na	na	na
PGV216	–	+++	++	+++	–	–	100	0	100	100	0	100	0	50	50	100	200	+	–	–	na	na	na
PGV217	++	+++	+++	+++	+++	–	100	0	100	100	100	25	0	0	50	50	200	+	–	–	*Pseudomonas poae*	98.74	KJ417615
PGV218	+	++	+++	++	+++	–	100	0	100	100	0	25	0	0	50	100	200	+	–	–	*Pseudomonas meridiana*	97.71	KJ417616
PGV220	+	++	+++	++	++	–	100	0	100	100	0	25	0	0	0	50	50	+	–	–	*Pseudomonas frederiksbergensis*	98.58	KJ417617
PGV222	+	+	++	++	+++	–	100	0	100	100	0	25	0	0	50	50	200	+	–	–	*Pseudomonas fluorescens*	98.74	KJ417618
PGV223	+	++	+++	+++	–	–	0	0	0	0	0	0	25	100	100	50	50	–	–	–	*Arthrobacter stackenbrandtii*	97.85	KJ417591
PGV224	+	++	++	+++	–	–	100	0	100	100	0	100	0	50	50	50	200	+	–	–	na	na	na
PGV225	+++	+++	+++	+++	+++	–	100	0	100	100	0	100	0	50	200	100	100	+	–	–	na	na	na
PGV226	+++	+++	+++	++	+++	–	100	20	100	100	100	100	25	0	100	50	200	+	–	–	na	na	na
PGV227	++	++	+++	+++	+++	–	100	0	100	100	0	25	25	0	50	50	200	+	–	–	na	na	na
PGV228	++	+++	+++	+++	+++	–	100	0	100	100	100	100	0	0	50	50	200	+	–	–	*Pseudomonas fluorescens*	99.16	KJ417619
PGV229	+	++	+++	+++	–	–	100	0	100	0	0	25	0	50	50	100	200	–	–	–	*Janthinobacterium lividum*	99.3	KJ417620
PGV230	++	++	+++	+++	+++	–	100	0	100	100	0	100	0	0	50	50	200	+	–	–	na	na	na
PGV231	+	++	+++	++	+++	–	100	0	100	100	0	100	0	0	50	50	200	+	–	–	na	na	na
PGV232	+	++	+++	++	+++	–	100	0	100	100	0	25	0	0	50	0	200	+	–	–	na	na	na
PGV233	++	+++	+++	+++	+++	–	100	0	100	100	100	100	0	0	50	100	100	+	–	–	*Pseudomonas salomonii*	99.66	KJ417592
PGV234	++	+++	+++	+++	++	–	100	0	100	100	0	100	0	100	50	100	200	+	–	–	*Janthinobacterium lividum*	98.97	KJ417621
PGV235	+	–	+++	–	–	–	100	0	100	100	0	100	0	50	50	100	200	+	–	–	*Janthinobacterium lividum*	99.3	KJ417622
PGV237	–	+++	+++	+++	++	–	100	0	100	100	0	100	0	50	50	100	200	+	–	–	*Janthinobacterium lividum*	99.4	KJ417623
PGV238	++	+++	+++	+++	+++	–	100	0	100	100	100	100	0	0	50	50	200	+	–	–	*Pseudomonas fluorescens*	99.33	KJ417624
PGV239	++	+++	+++	+++	++	–	100	0	100	100	0	100	0	100	50	100	200	+	–	–	*Janthinobacterium lividum*	99.35	KJ417625
PGV241	++	+++	+++	+++	–	–	100	0	100	100	0	100	0	100	50	100	200	+	–	–	*Janthinobacterium lividum*	99.44	KJ417626
PGV242	++	+++	+++	+++	–	–	100	0	100	100	25	100	0	50	50	100	200	+	–	–	*Janthinobacterium lividum*	92.38	KJ417627

Table 1 (*continued*).

Isolate No.	Growth temperature (°C)[a]						Antibiotic resistance[b]							Metal resistance[c]				Exoenzyme production[d]			Closest relative species	% Identity	GenBank Acc. No.
	4	10	15	20	30	37	Amp	Tet	Cam	Pen	Str	Nal	Kan	Cu	Ni	Co	Zn	Prot.	β-Gal.	Amyl.			
PGV244	++	+++	+++	+++	++	–	100	0	100	100	0	25	0	50	50	100	0	+	–	–	*Pseudomonas frederiksbergensis*	99.5	KJ533730
PGV246	+	+++	++	+++	–	–	100	0	100	100	100	100	25	100	200	200	200	–	+	+	na	na	na
PGV247	+	+	+++	+++	+++	–	100	0	100	100	0	25	0	50	50	50	0	+	–	–	na	na	na
PGV248	++	+++	+++	+++	++	–	100	0	100	100	0	25	0	100	100	100	200	+	–	–	*Janthinobacterium lividum*	99.65	KJ417628
PGV249	++	+++	+++	++	++	–	100	0	100	100	0	100	25	0	50	50	200	+	–	–	*Pseudomonas brenneri*	99.22	KJ417629
PGV250	na	+	na	na	na	na	100	0	100	100	0	0	0	50	100	0	200	+	–	–	*Pseudomonas frederiksbergensis*	99.17	KJ417630
PGV251	++	+++	+++	+++	++	–	100	0	100	20	0	25	0	100	100	100	200	+	–	–	*Janthinobacterium lividum*	99.11	KJ417631
PGV252	+	+++	+++	++	–	–	100	0	100	20	0	25	0	0	50	100	200	–	–	–	*Janthinobacterium lividum*	99.2	KJ417632
PGV254	++	+++	++	+++	+++	–	0	0	25	20	0	100	0	100	50	50	50	–	+	–	*Arthrobacter citreus*	99.67	KJ417633
PGV255	++	++	+++	+++	+	–	100	0	100	100	0	25	0	50	50	100	200	–	–	–	na	na	na
PGV256	+++	+++	+	+++	–	–	100	0	100	100	100	25	25	100	100	100	200	+	–	–	na	na	na
PGV258	+++	+++	+	+++	–	–	100	0	100	100	100	0	0	200	200	100	200	–	–	–	na	na	na
PGV260	+++	+++	++	+++	–	–	0	0	25	0	0	100	0	50	100	100	200	+	–	–	na	na	na
PGV259	na	+	na	na	na	na	0	0	100	0	0	0	0	100	100	0	50	–	–	–	*Janthinobacterium lividum*	97.78	KJ417634
PGV261	+++	+++	+++	+++	–	–	0	0	0	0	0	0	0	200	0	0	0	+	–	–	*Janthinobacterium lividum*	97.73	KJ417593
PGV263	+++	+++	+++	+++	+++	–	100	0	100	100	0	25	0	100	200	100	200	+	–	–	*Pseudomonas chlororaphis*	98.4	KJ417635
PGV264	–	–	+++	–	++	+++	0	0	0	0	0	0	0	100	100	50	50	–	–	–	na	na	na
PGV265	+	++	+++	++	–	–	20	0	100	0	0	0	0	50	50	0	0	–	–	–	*Pseudomonas syringae*	98.57	KJ417636
PGV266	–	–	++	–	–	–	0	0	25	0	0	0	0	50	100	50	0	–	–	–	*Rhodoferax ferrireducens*	98.24	KJ417637
PGV269	na	+	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	–	–	–	*Undibacterium pigrum*	97.55	KJ417638
PGV273	+	++	+++	++	–	–	100	0	100	0	0	25	0	100	100	0	0	–	–	–	*Duganella zoogloeoides*	98.07	KJ417639
PGV284	++	+++	+++	+++	++	–	100	0	100	100	0	0	0	100	100	100	100	+	–	–	*Pseudomonas frederiksbergensis*	99.81	KJ417594
PGV294	+	+	++	–	–	–	0	0	0	0	0	0	0	50	100	100	50	–	–	–	na	na	na
PGV305	–	+	+	na	–	–	0	0	0	0	0	0	0	100	0	0	0	–	–	–	*Variovorax paradoxus*	98.69	KJ417640
PGV308	+	na	++	na	+++	–	0	0	0	0	0	0	0	0	0	0	0	–	–	–	*Nocardioides kribbensis*	95.25	KJ417641
PGV310	–	++	+++	+++	+++	–	100	0	100	100	0	0	0	0	50	0	0	–	–	–	na	na	na
PGV311	–	+	+	na	+	–	20	20	25	20	25	100	25	0	0	0	0	–	+	–	na	na	na
PGV312	–	–	++	+	–	–	0	0	0	0	0	0	0	50	50	0	0	–	+	–	na	na	na
PGV313	–	–	++	+	–	–	0	0	0	0	0	0	0	50	50	0	0	–	+	–	na	na	na
PGV318	–	+	++	+	+++	+++	0	0	100	0	0	0	0	50	50	50	0	–	–	–	na	na	na
PGV321	–	–	++	–	–	–	0	0	0	0	0	0	0	0	50	0	50	–	+	–	*Subtercola frigoramans*	97.48	KJ417595
PGV323	++	++	++	++	–	–	0	0	0	0	0	0	0	100	100	50	0	–	–	–	*Arthrobacter oryzae*	100	KJ417596
PGV324	–	++	++	++	–	–	0	100	0	20	100	0	0	0	50	0	0	–	–	–	na	na	na
PGV332	+	++	++	+	–	–	0	0	100	0	0	0	0	50	100	0	100	–	–	–	na	na	na
PGV333	–	+	+	na	+	–	100	0	0	0	0	25	0	0	0	200	0	+	+	–	na	na	na
PGV334	–	+	++	na	++	–	100	0	100	100	100	25	0	0	0	0	200	+	+	+	na	na	na

Table 1 (*concluded*).

Isolate No.	Growth temperature (°C)[a]						Antibiotic resistance[b]							Metal resistance[c]				Exoenzyme production[d]			Closest relative species	% Identity	GenBank Acc. No.
	4	10	15	20	30	37	Amp	Tet	Cam	Pen	Str	Nal	Kan	Cu	Ni	Co	Zn	Prot.	β-Gal.	Amyl.			
PGV335	+	+	++	na	–	–	100	0	100	100	25	0	25	200	200	0	200	+	+	–	*Flavobacterium johnsoniae*	99.04	KJ417597
PGV336	+	+	++	na	++	–	100	0	100	100	0	25	0	0	0	0	200	+	–	+	na	na	na
PGV337	++	++	+++	na	+++	–	100	0	100	100	25	100	25	200	200	0	0	–	–	–	*Pseudomonas frederiksbergensis*	98.52	KJ417598
PGV338	+	++	+++	+++	++	–	20	100	100	20	100	0	0	0	0	0	50	–	–	–	na	na	na
PGV344	++	+++	+++	+++	–	–	100	0	0	100	0	0	0	20	100	0	100	–	–	–	*Iodobacter fluviatilis*	98.73	KJ417599
PGV349	+	–	++	–	–	–	0	0	0	0	0	0	0	0	0	0	0	–	–	–	na	na	na
PGV352	++	++	++	++	–	–	0	0	100	0	0	0	0	50	100	50	50	–	–	–	*Janthinobacterium lividum*	97.61	KJ417600
PGV355	++	++	+++	++	–	–	100	0	100	20	0	0	0	100	100	50	0	–	–	–	na	na	na
PGV363	+++	+++	+++	+++	–	–	20	0	100	20	0	25	0	0	0	0	50	–	–	–	*Duganella zoogloeoide*	97.23	KJ417601
PGV373	+	+	++	+	–	–	0	0	0	0	0	0	0	0	50	0	0	–	+	–	na	na	na
PGV374	+	+	++	+	–	–	0	0	0	0	0	0	0	50	50	0	50	–	–	–	na	na	na
PGV375	na	+	na	na	na	na	0	20	0	0	0	100	0	0	200	0	0	–	–	–	na	na	na
PGV376	+	+	++	–	–	–	0	0	0	0	0	0	0	0	0	0	0	–	–	–	*Janthinobacterium agaricidamnosum*	98.41	KJ417602
PGV397	–	+	+	na	–	–	0	0	0	0	0	0	0	0	50	0	0	–	–	–	*Subtercola boreus*	99.42	KJ417603
PGV399	++	++	+++	+	++	–	100	0	100	100	100	0	150	200	100	50	200	–	–	–	na	na	na
PGV500	+++	+++	+++	+++	–	–	0	0	0	0	0	0	0	0	0	0	0	–	–	–	*Janthinobacterium lividum*	97.71	KJ417604

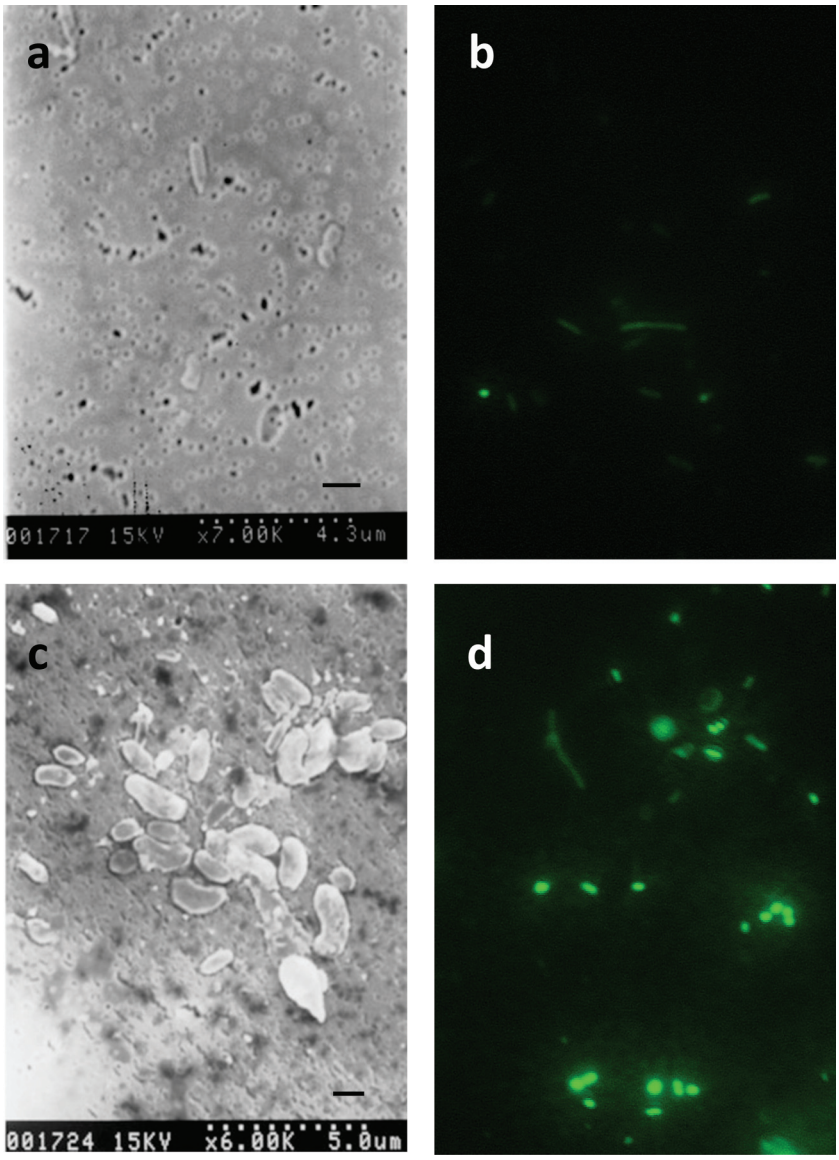
[a]Growth: –, no growth; +, low growth; ++, moderate growth; +++, abundant growth.

[b]Antibiotic resistance: Amp, ampicillin; Cam, chloramphenicol; Kan, kanamycin; Nal, nalidixic acid; Pen, penicillin; Str, streptomycin; Tet, tetracycline. The maximum concentration (in μg/mL) at which the isolates were able to grow is reported.

[c]Metal resistance: the maximum concentration of each metal (expressed in ppm) at which the isolates were able to grow is reported.

[d]Exoenzyme production: Prot., proteases; Amyl., amylases; and β-Gal., β-galactosidases. +, positive for production; –, negative for production.

Fig. 2. Microbial diversity in glacier ice (*a*, *b*) and subglacial water (*c*, *d*) samples. (*a*, *c*) Scanning electron microscopy of bacterial cells. Multiple bacterial cells can be seen, including very small ("dwarf"), rod- and cocci-shaped bacterial cells. The bar represents 1 μm. (*b*, *d*) Epifluorescence microscopy at × 1000 magnification, showing microbial cells with uncompromised membranes (potentially viable). Some filaments can be also observed. [Colour online.]



direct plating, were preserved in 20% glycerol at −80 °C for thorough characterization (Gram staining, colony morphology, pigmentation, growth temperature, antibiotic, and metal resistance) (Table 1). Among these, 34 isolates (38.20%) were eurypsychrophiles, as they grew well at a wide range of temperatures ranging from 4 to 30 °C. Thirty-one isolates (34.83%) grew between 4 and 20 °C but not above this temperature and were thus considered preliminarily as stenopsychrophiles (formerly "true psychrophiles"). Only 2 isolates were capable of growing at 37 °C and were considered as mesophiles.

Among the tested isolates, many were able to synthesize and excrete exo-proteases (54.54%); conversely, only a few isolates produced β-galactosidases (12.5%) or amylases (3.40%) (Table 1). Some pigmented colonies were also observed on the plates. Strikingly, 14 isolates (15.73%) produced high amounts of what seemed to be extracellular polymers (i.e., highly mucoid colonies), particularly when growing at lower temperatures (i.e., 4 and 10 °C).

Antibiotic and metal resistance

The proportion of isolates from subglacial water exhibiting antibiotic resistance was high, with many of them resistant to high doses of different antibiotic classes (i.e., 100 μg/mL) (Table 1). The most frequent antibiotic resistance detected was against chloramphenicol (73.07%), followed by resistance to penicillins (65.38%), nalidixic acid (32.05%), aminoglycosides (15.38%), and tetracycline (2.56%). Multiresistance was frequent among these isolates, with 65.38%, 34.71%, and 8.94% isolates simultaneously resistant to 2, 3, and 4 antibiotic classes, respectively.

Resistance against metals was also high. Among the isolates tested, 57.69% of them grew in the presence of 100 ppm Zn^{2+}, whereas 38.46%, 35.89%, and 23.07% resisted at least 100 ppm Co^{2+}, Ni^{2+}, and Cu^{2+}, respectively (Table 1). Surprisingly, 23.07% of the subglacial water isolates showed simultaneous resistance to up to 3 antibiotic classes and to at least 2 metals. Besides, some of them were able to grow even in the presence of 200 ppm of the tested metals. On the contrary, no isolate grew in the presence of 10 μmol/L $HgCl_2$. The strain used as negative control did not grow at any of the metal concentrations tested.

Phylogenetic analysis of 16S rDNA sequences of isolates

From a subgroup of isolates characterized in the present work, we obtained 54 16S rDNA sequences. Based on a similarity criterion of at least 97% at the 16S rRNA gene sequence level, 53 out of 54 isolates were assigned to 23 phylotypes distributed within 4 different phyla or classes: *Beta-* and *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes* (Table 1). A phylogenetic tree including a representative from each of the 23 phylotypes and their closest relatives is shown in Fig. 3. Actinobacteria were predominant in glacial ice samples (i.e., isolates closely related to *Arthrobacter*, *Subtercola*, and *Nocardioides* spp.), whereas the majority of the isolates identified in subglacial meltwater samples belonged to the *Proteobacteria* and particularly to the *Pseudomonas* and *Janthinobacterium* genera. It should be noted that the closest relative for many isolates was either a well-known psychrophile or had been isolated from cold environments (e.g., *Subtercola boreus*, *Pseudomonas antarctica*). Furthermore, when the 16S rDNA sequences of strains isolated from Pico Bolívar's glacier were compared with those isolated from Pico Humboldt's glacier, located at 6 km approximately, they were closely related (supplementary Fig. S3[1]). Interestingly, these sequences belonged to the same clade and they diverged from sequences belonging to related isolates originating from other glacial environments, which are grouped in different clades.

Discussion

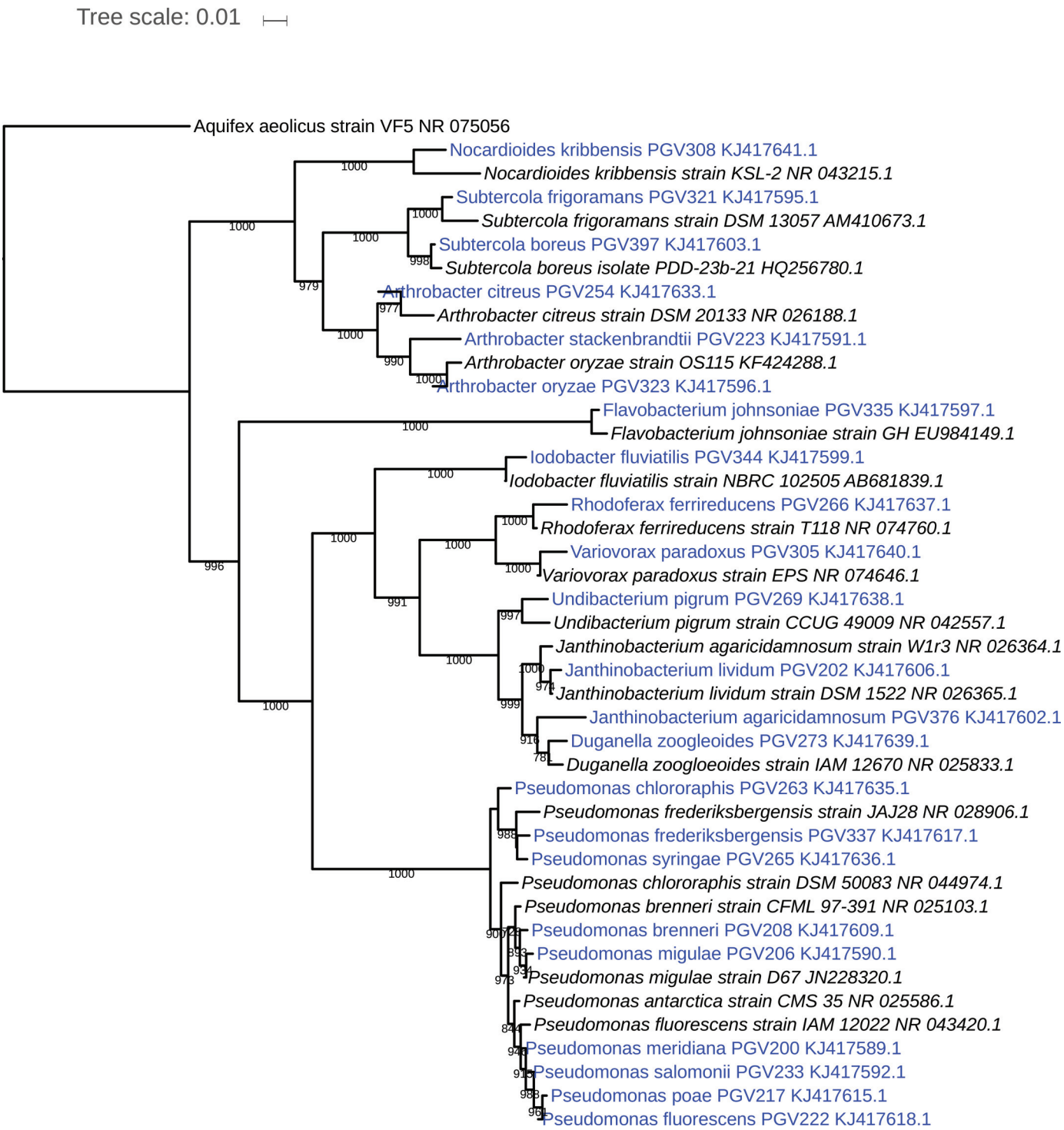
The results presented here support the idea that tropical glacial environments harbor an abundant bacterial community, consistent mainly of eurypsychrophilic (formerly "psychrotolerant") and stenopsychrophilic (formerly "true psychrophilic") microorganisms, many of which face total extinction considering the rapid melting of these environments. From this point of view, and considering important differences with other glacial environments elsewhere, tropical glaciers can be considered as important reservoirs of a still barely known biodiversity that deserves to be explored in more depth before they become extinct.

Using both direct microscopic counts and culture-dependent techniques, we detected bacteria at different cell densities in both glacier ice and subglacial meltwater samples. As expected, the abundance of bacteria in glacial ice samples was low, but cells were morphologically diverse and many exhibited uncompromised membranes. Some cells even showed signs of undergoing division in situ, as observed through electron microscopy of ice samples, which had been carefully thawed and immediately preserved. Consequently a few viable and culturable bacteria were recovered from these samples. These results are consistent with the idea that bacteria can be metabolically active inside chemically concentrated water-filled veins, located in the boundaries around neighboring ice crystals, where organic and inorganic molecules can reach molarities similar to those characteristic of rich laboratory media (Junge et al. 2001; Price and Sowers 2004; Mader et al. 2006).

On the contrary, the microbial communities of subglacial meltwater were more densely populated (i.e., more than 10-fold higher than those from glacial ice samples). Physicochemical differences between these 2 environments might account for this result (Skidmore et al. 2000, 2005; Foght et al. 2004; Cheng and Foght 2007; Kaštovská et al. 2007; Boyd et al. 2011). Glacial ice is permanently frozen and isolated from sediments, and cells immured on it are confined to microscopic veins of liquid water surrounding ice crystals (Junge et al. 2001; Mader et al. 2006); on the contrary, subglacial waters are closely associated with sediments at the glacier bed, whose weathering can provide organic and inorganic nutrients to support more complex microbial communities (Sharp et al. 1999; Tranter et al. 2002; Miteva et al. 2004; Skidmore et al. 2005).

Molecular identification of a subgroup of isolates showed the presence of bacteria closely related with members of the *Actinobacteria* phylum, mainly in glacier ice samples (e.g., *Subtercola*, *Arthrobacter*, *Nocardiodes*). These bacteria are known to be resistant to extreme environments, including cryo-habitats (ice sheets, glaciers, marine ice shelves, sea ice, and permafrost), deep seas, deserts, salt-lakes, and hot springs (Bull 2011). In the particular case of glacier environments, *Actinobacteria* has been shown to be one of the main dominant groups in glacial ice cores drilled from the Greenland ice sheet (Miteva et al. 2004), the Muztag Ata glacier in China (Xiang et al. 2005), the Puruogangri glacier on the Tibetan Plateau (Zhang et al. 2008), and the Nevado Pastoruri glacier in Perú (González-Toril et al. 2015). In fact, in some cases, *Actinobacteria* can be the only culturable bacteria isolated from glacial ice cores (Miteva et al. 2009). This predominance has been related to several aspects, including (*i*) the facility of isolating members of this bacterial taxon; (*ii*) their complex cell wall and extreme robustness, which allow them to withstand cold, desiccated, and nutrient-limited environments for extended periods of time; (*iii*) the production of spores by several members of this class; and (*iv*) their versatility utilizing organic substrates as their sole or principal sources of

Fig. 3. Evolutionary relationships of glacial and sub glacial bacterial isolates of Pico Bolívar's glacier. The evolutionary history was inferred using the Neighbor-Joining method. Bootstraps values for 1000 iterations are shown at nodes for values >500. Reference sequences are labeled in italics, and strains obtained from glacial ice are labeled in roman font. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



carbon and energy (Miteva 2008; Liu et al. 2009; Bull 2011).

The culturable fraction of subglacial meltwater included members of 4 different phyla or classes (i.e., *Beta-* and *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes*), many of which have already been associated with permanently cold environments (Margesin and Miteva 2011). Among these isolates, many *Janthinobacterium-* and *Duganella-*related isolates were particularly frequent, which is in accordance with previous results. Indeed, both

eurypsychrophilic and stenopsychrophilic strains of *Janthinobacterium* species have been isolated from permanently cold environments such as Antarctic soils (Shivaji et al. 1991), Alaskan soils (Schloss et al. 2010), and glacier ice (Lu et al. 2009; Ambrožič Avguštin et al. 2013). On the other hand, these and other *Betaproteobacteria* have been frequently isolated from oligotrophic water systems, such as glacial stream runoff (Battin et al. 2001), polar and alpine environments (Skidmore et al. 2005), and subglacial waters (Foght et al. 2004; Cheng and Foght 2007).

Pseudomonas-related isolates (*Gammaproteobacteria*) were also abundant in subglacial meltwater samples. This was surprising, since *Gammaproteobacteria* have not been detected in significant numbers from nonpolar glacial or subglacial ecosystems either by culturable-dependent or culturable-independent methods (Margesin and Miteva 2011). However, it is well known that several *Pseudomonas* species are well adapted to survive for long periods of time at low temperatures and are frequent colonizers of frozen environments (Christner et al. 2000; Moreno and Rojo 2014). Moreover, several eurypsychrophilic *Pseudomonas* species have been isolated from Antarctic environments, including *P. antarctica*, *P. meridiana*, *P. proteolytica*, and *P. guineae* (Reddy et al. 2004; Bozal et al. 2007). *Pseudomonas* species' tolerance to cold environments is possibly related to the high genetic and physiological adaptability exhibited by members of this genus (Spiers et al. 2000). Interestingly, the majority of the isolates identified in this work were closely related to bacteria isolated from other glacial environments elsewhere.

Of particular interest was the correspondence between the results presented here and those obtained by us when studying basal-ice samples collected at the front edge of another retreating glacier (i.e., Pico Humboldt's glacier) located approximately 6 km from Pico Bolívar glacier (Ball et al. 2014). In both cases, *Proteobacteria* (i.e., *Janthinobacterium* sp. and *Pseudomonas* sp.) accounted for more than 80% of the culturable fraction of bacteria, whereas *Actinobacteria* were less abundant. Furthermore, as already mentioned, the 16S rDNA sequences of strains isolated from these neighbor glaciers were closely related. It could be argued that due to their close proximity (~6 km) and same geological history (Braun and Bezada 2013), both glaciers might have been seeded almost simultaneously with biological material containing the same type of microorganisms. This would explain also why these sequences belong to the same clade and why they diverge from clades that group sequences belonging to related isolates originating from other glacial environments. These similarities might also be due to the fact that members of both bacterial taxa are easily cultured in rich media. Nonetheless, it should also be mentioned that similar observations have been previously highlighted by others, while comparing distant glaciers worldwide (Christner et al. 2003; Foght et al. 2004; Cheng and Foght 2007; Liu et al. 2009). According to some authors, the presence of a selected and narrow group of related, but not identical, microorganisms in glaciers would be the consequence of the serious constraints these environments impose to incoming (tourist) bacteria (e.g., desiccation, freezing, high pressure, and low nutrient and oxygen concentrations), acting by selecting similar phylogenetic groups (Priscu and Christner 2003; Miteva et al. 2004).

Recently, González-Toril et al. (2015) studied the microbial community at another Andean retreating glacier, Pastoruri glacier in the Huascarán National Park (Perú), by using pyrosequencing methods. Even though there are some similarities between their results and those presented here (e.g., *Proteobacteria* was the dominant phylum, followed by *Actinobacteria* and other phyla in the majority of the samples), there are also some important differences. For instance, *Bacteroidetes*, *Firmicutes*, and *Acidobacteria* were also abundant in Pastoruri's glacier samples. However, these differences may result from (*i*) the particular water physicochemistry in the Peruvian glacier' studied sites, due to an ongoing acid rock drainage process in the area, and (*ii*) the different methods employed to assess the microbial community diversity and richness (culturable fraction versus massive pyrosequencing of the 16S rRNA genes).

Among the various physiological adaptations exhibited by psychrophilic bacteria, the production of high levels of exopolysaccharides is of particular relevance (De Maayer et al. 2014). These substances are generally produced under cold conditions by different bacterial species to avoid the deleterious effect of growing ice crystals. Besides, EPS also help psychrophilic bacteria to trap water, metal ions, and nutrients, and to protect exoenzymes from cold denaturation (Nichols et al. 2005). Our results add to these observations by showing that several of the isolates exhibited a highly mucoid aspect when cultured at lower temperatures, which was absent when grown at higher temperatures.

A significant proportion of the isolates, able to produce more than one hydrolytic cold-active enzyme, were isolated from Pico Bolívar's glacial and subglacial meltwater samples. This might indicate the potential role of these bacteria as decomposers of organic matter in glacial environments (Skidmore et al. 2000; Foght et al. 2004). Additionally, this result emphasizes the potential of some of these isolates for the development of new biotechnological products and (or) processes making profit of cold-active enzymes. One such possibility was recently highlighted by us, when we determined the ability of some psychrophilic bacterial isolates colonizing the same tropical glacier environment to act as potential plant-growth promoters at low temperatures (Balcazar et al. 2015). This type of isolate is thought to be of fundamental interest for developing cold-active biofertilizers that are able to (*i*) solubilize inorganic forms of phosphorus due to the production of high amounts of organic

acids by means of cold-active enzymes (i.e., membrane-linked glucose dehydrogenases); (*ii*) synthesize and excrete phytohormones (like indole acetic acid); and (*iii*) inhibit growth of plant pathogens.

The significant proportion of antibiotic- and metal-resistant isolates present in the collected samples was also of particular interest. Multiresistance (i.e., resistance to 3 or more classes of antimicrobial agents) (Schwarz et al. 2010) was exhibited by approximately one third of the tested isolates of subglacial meltwater. Although impressive, this result was not unexpected, since we obtained very similar results when studying Pico Humboldt glacier's isolates (Ball et al. 2014). In fact, resistance to different antibiotic classes and to metals were also frequent among this glacier's isolates (varying from 5% to 65%, depending on the antibiotic tested). Furthermore, more than 45% of the isolates also harbored high-molecular-weight plasmids, supporting the possibility of horizontal gene transfer among them. Due to their remoteness and high altitude, both Andean glaciers have been visited infrequently, making very unlikely their anthropogenic contamination with therapeutic antibiotics and the consequent spread of microbial resistance against these. However, an alternative explanation to these results is possible: according to some authors, elevated frequencies of antibiotic resistance among natural microbial communities colonizing pristine environments might be related to the expression of genes encoding nonspecific multidrug pumps and to the horizontal transfer of genetic resistance markers among members of natural microbial communities (Summers 2002; Hogan and Kolter 2002; Baker-Austin et al. 2006).

It is also important to consider a radically different explanation to these observations. It is well acknowledged that many cryosphere ecosystems are colonized by microorganisms that did not evolve in situ, rather they were transported there via airborne particles from remote areas (Miteva et al. 2009; Bull 2011). In a very recent study, Segawa et al. (2013) observed a widespread distribution of antibiotic resistance genes in glacier environments all around the world, supposedly not affected by human intervention. The authors concluded that this universal distribution of resistance genes might be explained by inoculation of these environments with incoming bacteria, either transported by winds or by migrating birds. According to some authors, the size of this "inoculum" is thought to be more important in low-latitude high-altitude glaciers, which are closer to tropical and subtropical ecosystems and might thus explain the abundance of glacier-ice-borne microorganisms in these ecosystems (Christner et al. 2000). As mentioned before, the close proximity of exceptionally rich ecosystems (like the Amazonian forest, for instance) to these glaciers makes it very probable that many of the immured microbes actually were transported there by wind, the most common way of microbial spreading (Smith et al. 2011).

Whatever the case, this study adds to previous reports dealing with the widespread distribution of antibiotic resistance determinants, particularly those with clinical and agricultural importance, such as chloramphenicol, β-lactams, streptomycin, and tetracycline, in microbes colonizing glacial systems worldwide (Edwards 2015). Considering the accelerated melting of tropical glaciers, the reactivation and release of both active and dormant bacteria, and their potential behavior as donors of these kind of genetic elements in horizontal gene transfer events (the "genome recycling concept") (Rogers et al. 2004), it becomes evident that more efforts should be made to characterize their potential as threats to human, animal, and plant health.

Andean tropical glaciers are retreating at a very fast rate and many will disappear in the next few years (Rabatel et al. 2013). Although the methodology we used to characterize the microbial community of Pico Bolívar's glacier was biased towards culturable species, the results presented here confirm that these glaciers contain abundant microbial communities of poorly known diversities that deserve to be studied before they vanish. Besides their importance from a basic research point of view, it is possible that many of the microbial species immured inside these glaciers might be potentially useful for the development of new biotechnologies, as recently shown by Balcazar et al. (2015). Finally, considering the elevated frequency of multiresistance against therapeutic antibiotics exhibited by tropical glacial borne microorganisms, their reactivation and release could be considered a potential threat to human, plant, and animal health that merit further analysis.

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