Diversity of culturable bacteria recovered from Pico Bolivar’s glacial and subglacial environments, at 4,950 m, in Venezuelan Tropical Andes

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Diversity of culturable bacteria recovered from Pico Bolívar's glacial and subglacial environments, at 4,950 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 ~4.950m in the Venezuelan Andean Mountains. Microbial cells of diverse morphologies and exhibiting uncompromised membranes were present at densities ranging from $1.5 \times 10^4$ and $4.7 \times 10^4$ cells/ml in glacier ice, and from $4.1 \times 10^5$ and $9.6 \times 10^5$ cells/ml in subglacial meltwater. From 89 pure isolates recovered from the samples, the majority were eurypsychrophilic or stenopsychrophilic, according to their temperature range of growth. Following analysis of their 16 rDNA nucleotidic sequence, 54 pure isolates were assigned to 23 phylotypes distributed within four different phyla/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% of the subglacial isolates respectively 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.

Keywords: glacier ice bacteria; Tropical Andes; Tropical Glaciers; psychrophilic bacteria; biodiversity.

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 one 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 extremophiles (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). Through 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. 2013; Balcazar et al. 2015; Fritz et al. 2015). This is striking considering that, compared to high and midLatitude 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 from 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 these neighbor ecosystems contribute with a large number of airborne particles that transport and protect attached microorganisms to the glaciers’ surfaces (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 tropical glacier’s ice samples (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 to 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 due 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 amongst 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 correspond to approximately 84% of its area of coverage (Schubert and Clapperton 1990; Carrillo and Yépez 2010; Braun and Bezada 2013). At this trend, the glaciers of the Venezuelan Andes will totally disappear in less than five 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 Bolivar glacier in the Andean region of Venezuela. Additionally, we tested the resistance of a selected group of isolates to different antibiotic classes and metals, in order 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 tropical glaciers bacterial communities (Ball et al. 2013; Balcázar et al. 2015). Finally, we contribute to preserve a minute fraction –though potentially useful– of this vanishing biodiversity for future research.

Materials and methods

Sampling site

Pico Bolivar’s glaciers are currently composed of two very small ice/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. 1a). As can be seen, these patches remain “clinging” to the steeped northern face of Pico Bolivar. The glacier’s surface is full of holes of different diameters, from dozens of cm to several meters, and crevasses (Fig. 1b). 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. 1b).

Sample collection

Two different types of samples were collected at the same location in Pico Bolivar glacier during the end of the dry (“summer”) season on 02 March 2014 at 15:00 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 4m high × 5m wide × 5m depth), 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 collecting 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, gravels or sediments. The second type of samples corresponded to subglacial meltwater running at the base of this glacier and in contact with the glacier bed. Meltwater samples were collected aseptically in sterile 50 mL Falcon tubes from the glacier snout. Five replicate samples were collected at each site. Once collected, ice samples were kept at < 4°C and transported to the lab in less than 24 h.

**Media and isolation of bacteria**

Cultures were started as previously described (Ball et al. 2013). 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), Luria-Bertani broth (LB) (full, 1/50 or 1/100 strength). The plates were sealed with Parafilm and incubated aerobically at 4, 10 and 30°C in a humid chamber until colonies became visible, for up to 3 months. 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 Nuclepore tracketch membrane filter (Whatman), followed by staining with propidium iodide and SYTO9 using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, Oreg.). 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 ultra pure water were also checked.

Ice samples for scanning electron microscopy were concentrated by filtering 5 ml melted ice onto a 10 mm diameter spot of a 0.1 µm pore-size Nuclepore filter (Whatman). The filters were immediately immersed in a fixing solution containing 3% glutaraldehyde and 3% formaldehyde in 0.1M cacodylate buffer, pH 6.3 (Palacios-Prü and Mendoza-Briceño, 1972) for 6h at 4°C. Once fixed, the filters were mounted on a metal stub with the help of a conductive adhesive tape and dehydrated in a vacuum chamber for five days. Subsequently, the samples were coated with gold using a 11430E SPI sputter
Scanning electron microscopy analysis was carried out at 12 kV accelerating voltage using a Hitachi S-2500 Scanning Electron Microscope.

**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 respectively, by visual inspection of the plates.

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

**Antibiotic and metal resistance of isolates**

All psychrophilic/psychrotolerant strains were tested for antibiotic resistance as previously reported (Ball et al. 2013). In brief, colonies were streaked onto R2A agar plates containing up to 100 mg/L one of the following: ampicillin, penicillin, streptomycin, nalidixic acid, kanamycin, cloramphenicol and tetracycline. Plates were incubated at 15°C and growth of the strains was compared to the control experiment. As positive controls of growth the following strains were used: *E. coli* BL21 (Kan<sup>R</sup>, Cam<sup>R</sup>), XL1-Blue (Tet<sup>R</sup>), PA601 (Str<sup>R</sup>) (derivative of K<sub>12</sub>). The negative control used was *E. coli* Hfr H (derivative of K<sub>12</sub>).

Metal resistance was tested in a similar way, *i.e.* by culturing the isolates onto R2A plates containing from 0 to 200 ppm Zn<sup>2+</sup> (supplied as ZnCl<sub>2</sub>), Cu<sup>2+</sup> (supplied as CuSO<sub>4</sub>), Co<sup>2+</sup> (supplied as Co[NO<sub>3</sub>]<sub>2</sub>·6H<sub>2</sub>O) and Ni<sup>2+</sup> (supplied as NiSO<sub>4</sub>). The isolates were also streaked on R2A plates supplemented with 10 µM HgCl<sub>2</sub> to test for mercury resistance (Møller et al. 2011). Isolates growing on the mercury plates were restreaked on fresh plates of an appropriate mercury containing medium at least three times 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 parentheses after the isolate number 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). Scanning electron microscopy 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 (Figs. 2a and supplementary S1). By using fluorescence techniques, we were able to detect cells with uncompromised membranes, *i.e.* potentially viable, in these samples (Fig. 2b). 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. 2c and 2d). The microbial density, as calculated from direct microscopic counts, ranged from $1.5 \times 10^4$ and $4.7 \times 10^4$ cells/ml in the case of glacial ice, and from $4.1 \times 10^5$ and $9.6 \times 10^5$ cells/ml in the case of subglacial meltwater. The approximate proportion of live/dead cells attained 70:30 and 90:10 in the case of glacial ice and subglacial meltwater respectively (supplementary Fig. S2).

Long-time incubations (for up to 3 months) in R2A and LB media at both 15°C and 4°C permitted us to isolate and purify a small number of bacteria from ice samples, *i.e.* only one or two colonies per plate for a total of fourteen 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 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 two 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 tetracyclines (2.56%). Multiresistance was frequent among these isolates, with 65.38%, 34.71% and 8.94% isolates simultaneously resistant to two, three and four 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$^{++}$, whereas 38.46%, 35.89% and 23.07% resisted at least 100 ppm Co$^{++}$, Ni$^{++}$ and Cu$^{++}$ respectively (Table 1). Surprisingly, 23.07% of the subglacial water isolates showed simultaneous resistance up to three antibiotic classes and -at least- two 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 µM 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 sub-group of isolates characterized in the present work, we obtained 54 16S rDNA sequences. Based on a similarity criterium of at least 97% at the 16S rRNA gene sequence level, 53 out of 54 isolates were assigned to 23 phylotypes distributed within four different phyla/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 Phylum 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 Bolivar’s glacier were compared with those isolated from Pico Humboldt’s glacier, located at 6 km approximately, they were closely related (supplementary Fig. S3). 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 eurypychrophilic (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, bacteria were low-abundant in glacial ice samples, 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, 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 neighbor 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 ten-fold higher than those from glacial ice samples). Physicochemical
differences between these two 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 sub-group 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 Peru (Gonzáles-Torial 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 carbon and energy (Miteva 2008; Liu et al. 2009; Bull 2011).

The culturable fraction of subglacial meltwater included members of four different phyla/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 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 non-polar 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 at 6 km approximately of Pico Bolivar Glacier (Ball et al. 2013). 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 which groups 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, Gonzalez-Toril et al. (2015) studied the microbial community at another Andean retreating glacier, Pastoruri Glacier in the Huascaran National Park (Peru), 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 account for 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 showing that several of the isolates exhibited a highly mucoid aspect when cultured at lower temperatures, that 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 Bolivar’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 possibilities 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 isolates is thought to be of fundamental interest for developing cold-active biofertilizers, 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 ii) 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 three 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. 2013). 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 HGT 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 with the expression of genes encoding non-specific 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 which did not evolve in situ and were rather 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, beta-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- or 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 in order 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 Pico Bolivar’s glacier microbial community 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.

Acknowledgements
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References


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Figure captions

Figure 1. View of the northwestern side of Pico Bolivar with its two remaining small ice/firn fields on February 2013. 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.

Figure 2. Microbial diversity in glacier ice- (a, b) and subglacial water samples (c, d). (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 horizontal black-line represents 1 µm. (b, d) Epifluorescence microscopy at 1000× magnification, showing microbial cells with uncompromised membranes (potentially viable). Some filaments can be also observed.

Figure 3. Evolutionary relationships of Pico Bolivar’s Glacier glacial- and sub-glacial bacterial isolates. The evolutionary history was inferred using the Neighbor-Joining method. Bootstraps values for 1000 iterations are shown for nodes with values >500. Reference sequences are labeled in italics, and strains obtained from glacial ice are labeled in boldface. 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.
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**Table 1:** Characteristics of bacterial isolates

- **Antibiotic resistance:** Amp, Tet, Cam, Pen, Str, Nan, Kan, Cu, Ni, Co, Zn
- **Exoenzyme production:** Prot, β-gal, Amil
- **Metal resistance:** Cu, Ni, Co, Zn
- **Closest relative species/strain % identity**
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**Janthinobacterium agaricidamnosum** 98.41 KJ417602

**Subtercola boreus** 99.42 KJ417603

**Janthinobacterium lividum** 97.71 KJ417604

**Table caption:**

Growth temperature: - (no growth); + (low growth); ++ (moderate growth); +++ (abundant growth). Antibiotic resistance: Ampicillin (Amp); Penicillin (Pen); Streptomycin (Str); Nalidixic acid (Nal); Kanamycin (Kan); Cloramphenicol (Cam); Tetracycline (Tet). The maximum concentration (in µg/ml) at which the isolates were able to grow is reported.

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