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ORIGINAL PAPER

Bacteria recovered from a high-altitude, tropical glacier in Venezuelan Andes

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Abstract Glacial-ice microorganisms are intensively studied world-wide for a number of reasons, including their psychrophilic lifestyle, their usefulness in biotechnology procedures and their relationship with the search of life outside our planet. However, because of the difficulties for accessing and working at altitudes of >5.000 m above sea level, tropical glaciers have received much less attention than their arctic and antarctic counterparts. In the present work we isolated and characterized a total of forty-five pure isolates originating from direct plating of melted ice collected at the base of a rapidly-retreating, small glacier located at around 4.900 m.a.s.l. in Mount Humboldt (Sierra Nevada National Park, Mérida State, Venezuela). Initial examination of melted ice showed the presence of abundant- ($>10^6$ cells ml^{-1}), morphologically diverse- and active bacterial cells, many of which were very small ("dwarf cells"). The majority of the isolates were psychrophilic or psychrotolerant and many produced and excreted cold-active extracellular enzymes (proteases and amylases). The antibiotic tests showed an elevated percentage of isolates resistant to high doses (100 μg/ml) of different antibiotics including ampicillin, penicillin, nalidixic acid, streptomycin, chloramphenicol, kanamycin and tetracycline. Multiresistance was also observed, with 22.22 % of the strains simultaneously resistant up to five of the antibiotics tested. Metal resistance against Ni^{++}, Zn^{++} and Cu^{++} was also detected. In accordance with these results, plasmids of low and high molecular weight were detected in 47 % of the isolates. Twenty-two partial 16S rDNA sequences analyzed allowed grouping the isolates within five different phyla/classes: Alpha-, Beta- and Gamma-proteobacteria, Actinobacteria and Flavobacteria. This is the first report concerning South American Andean glacial ice microorganisms.

Keywords Bacteria · Psychrophiles · Tropical glaciers · Ice-bacteria · Glacier ice · Andes Mountains

Introduction

Psychrophilic microorganisms are intensively studied all over the world. Among the reasons explaining such an enthusiasm we can mention (1) their value as tools for the development of new biotechnologies, (2) their importance as models for seeking and understanding life outside our planet, (3) the interest in depicting the molecular mechanisms allowing the possibility of life at sub-zero temperatures and (4) the possibility of understanding how life arose and evolved in Earth.

Many cold regions of the world have been explored for isolating, culturing and identifying psychrophiles (for an excellent review on this matter see Margesin and Miteva 2011). Of particular interest in this field is the study of glacier ice environments (Miteva 2008). Indeed, in addition to the aforementioned reasons, studying glacial-ice microorganisms adds two more levels of interest: first,

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glacier ice microorganisms endure, in both an active metabolic state and in anabiosis (dormancy), the stressful conditions characteristics of such an environment (*i.e.* high hydrostatic and osmotic pressures, very low nutrient availability, sub-zero temperatures, elevated doses of cosmic, solar and earth radiation). Additionally, the chronological-repository nature of glaciers allow them to store biological materials characteristic of different geologic ages, allowing a comparison of samples belonging to different geological times.

High-elevation-, tropical glaciers had received less attention than their arctic and antarctic counterparts, and no reports have been published to date concerning Andean glacial ice microorganisms. The difficulties for accessing and working at altitudes of >5.000 m above sea level (m.a.s.l.) are among the reasons explaining this absence of information. However, tropical glaciers are rapidly retreating as a consequence of temperature increases and reduced cloud cover (Urrutia and Vuille 2009; Vuille et al. 2003, 2008). According to a recent report, the glaciers of the Andes Mountains have retreated at an unprecedented rate in the past three decades, with more ice lost than at any other time in the last 400 years (Rabatel et al. 2013).

Particularly fast is the retreat of the Venezuelan glaciers, at present restricted to some small icecaps on the northwest slopes of Mount Humboldt and Mount Bolívar, at the Sierra Nevada of Mérida. Since 1952, these glaciers have lost an area of 1.7 km^2 with a retreat rate of 30.3 m/yr, which correspond to 83.74 % of its area of coverage (Carrillo and Yépez 2010; Braun and Bezada 2013). At this trend, the glaciers of the Venezuelan Andes could totally disappear in <5 years.

In the present work, we report the isolation, characterization and identification of psychrophilic bacteria from glacial ice samples collected at Humboldt Glacier, Mount Humboldt, in the Andean region of Venezuela.

Materials and methods

Sampling site

Humboldt Glacier (a.k.a. Sinigüis Glacier), located at 8°33′05″N and 70°59′54″W at the base of the Mount Humboldt (Sierra Nevada National Park Mérida, Venezuela) is a small ice cap of approximately 0.1 km^2, at around 4.900 m.a.s.l. Ice samples were taken at the glacier front, on the north face of the peak (Fig. 1).

Sample collection

Ice samples were collected directly into sterilized plastic bags, by gently fracturing the ice with flame-sterilized instruments. The ice was collected from well below the surface of the glacier, near its base. Once collected, ice samples were kept at <4 °C and transported to the lab.

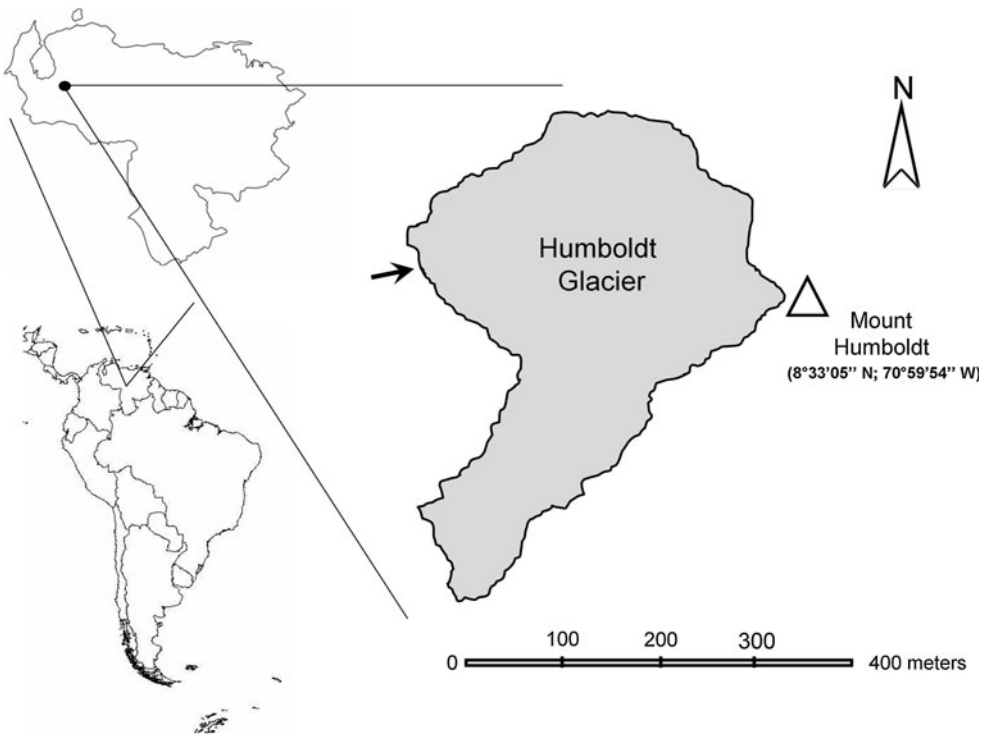

Fig. 1 Ice sample collection site at Humboldt Glacier (Mount Humboldt, Sierra Nevada, Mérida State, Venezuela). The *arrow* points at the exact location of sampling

Media and isolation of bacteria

Cultures were started by inoculating 0.1 ml of melted ice onto the following agarized media: Reasoner's 2A agar (R2A) at full-, 1/50- or 1/100 strength (Reasoner and Geldreich 1985); Luria-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 for up to 3 months until colonies became visible. Morphologically different colonies were selected, restreaked several times and checked for purity both macro- and microscopically.

Alternatively, an enrichment strategy was followed. For this, five milliliters of glacial meltwater were inoculated into 50 mL of R2A (full- or 1/50 strength) or LB (full- or 1/50 strength) broth and incubated aerobically at 4 °C until the liquid medium became turbid. Then 100-µL aliquots of serial dilutions were plated onto the aforementioned agarized media. The plates were incubated aerobically at 4 °C for several days until colonies became visible. Colonies of visibly different morphology and color were picked and purified by restreaking on the same isolation medium. The purified isolates were stored at −80 °C in a 20 % glycerol solution.

Microscopic examination

Microscopic analysis of the melted ice samples was performed by conventional light microscopy. The number of live- and dead cells were estimated by staining with propidium iodide and SYTO9 using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, Oreg.) following instructions of the manufacturer. For this, the melted ice was filtered onto a black 0.2 µm Isopore membrane filter (Millipore) and visualized by epifluorescence microscopy at 1,000 × magnification (Olympus Eclipse 80). The number of cells in 20 random fields was counted. Controls, comprising filtered ultra pure water were 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 mounted on a metal stub with the help of an adhesive tape and coated with gold using a SPI 11430E sputter coater. 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. In addition, the optimum temperature for growth was determined in R2A or LB broth at temperatures ranging from 0 to 30 °C with an increment of 5 °C. Inocula were prepared by harvesting cells grown on agar plates into physiological saline. The cells were centrifuged at 10,000×*g* for 2 min and washed twice in saline and thereafter resuspended by vortexing. The optical density of the final cell suspension was measured at 600 nm (OD_{600}) using a microplate reader (BioTek ELX800) and adjusted to a uniform cell density. Hundred microliters of the inoculum was added to 9 ml of the medium. Growth of the isolates at different temperatures was then determined by measuring OD_{600} of the cultures on a microplate reader at different time intervals. R2A broth was used for the growth experiment as this would enable a uniform comparison of growth data since all cultures grew on this medium.

Enzymatic activities for amylases, proteases and β-galactosidases were screened on R2A agarized medium supplemented with starch 2.5 g/l 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.

Antibiotic and metal resistance of isolates

All psychrophilic/psychrotrophic strains were tested for antibiotic resistance, by streaking colonies onto R2A agar plates containing 25 or 100 mg/l one of the following: ampicillin (Amp), penicillin (Pen), streptomycin (Str), nalydixic acid (Nal), kanamycin (Kan), cloramphenicol (Cam) and tetracycline (Tet). 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^R, Cam^R), XL1-Blue (Tet^R), PA601 (Str^R) (derivative of K-12). The negative control used was *E. coli* Hfr H (derivative of K-12).

Heavy-metal resistance was tested in a similar way, by culturing the strains onto R2A agar plates containing 50, 100 and 200 ppm $ZnCl_2$, $CuSO_4$ and $NiSO_4$. The isolates were also streaked on R2A plates supplemented with 10 mM $HgCl_2$ to test for mercury resistance. Isolates growing on the mercury plates were restreaked on fresh plates of an appropriate mercury containing medium at least three times to confirm purity and mercury resistance. To confirm experimentally the toxicity of the metals at the concentrations tested, we used *E. coli* Hfr H as negative control.

Plasmid DNA extraction and analysis

All selected strains were screened for the presence of plasmids. For this, mini preps were made following the modified alkaline-lysis protocol described by Kotchoni

et al. (2003) using cultures grown to stationary phase in LB or R2A broth. Alternatively, the Qiagen Spin miniprep kit (Qiagen) and the boiling miniprep protocol (Sambrook et al. 1989) were used to extract and purify plasmidic DNA. Plasmid preparations were subjected to electrophoresis in 1 % agarose gels at 5 V/cm. The plasmid bands were visualized on a UV box following ethidium bromide staining of the gels.

PCR amplification, sequencing and analysis of 16S rDNA

The gene-encoding 16S rRNA (16S rDNA) was PCR-amplified from selected strains using bacterial universal primers fD1 and rD1 (Weisburg et al. 1991) as described in Pérez et al. (2007), using whole cells as source of template DNA (=colony PCR). 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 compared to sequences deposited in the GenBank using the BlastN program (Altschul et al. 1997), 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). The evolutionary distances were computed using the Jukes-Cantor method (1969).

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: PGV021 (KF020666), PGV022 (KF020667), PGV024 (KF020669), PGV035 (KF020670), PGV036 (KF020671), PGV059 (KF020672), PGV063 (KF020673), PGV065 (KF020674), PGV070b (KF020675), PGV072 (KF020676), PGV084 (KF020677), PGV087 (KF020678), PGV088 (KF020679), PGV089 (KF020680), PGV090 (KF020681), PGV058 (KF020682), PGV056 (KF020683), PGV102 (KF020684), PGV094 (KF020685), PGV096 (KF020686), PGV097 (KF020687) and PGV101 (KF020688).

Results

Isolation of psychrophilic bacteria

Our major goal in the present work was to isolate a maximum number of psychrophilic/psychrotrophic bacterial strains from melted ice collected at the front edge of Humblodt Glacier (~4.900 m.a.s.l.) (Fig. 1). At this location, basal ice was fully covered with finely crushed pellets, possibly originated by the friction and fracture of rocks during glacier's downward movement. Initial examination of melted samples showed the presence of abundant- ($>10^6$ cells ml^{-1}), morphologically diverse- and active bacterial cells when observed under light-microscopy. Total counts of viable bacteria attained in some cases $\sim 10^7$ cells/ml and was significantly enriched in very small, "dwarf" cells, in which rod-shaped cells and filaments were dominant, as shown by scanning electron microscopy (Fig. 2).

Inoculation of agarized media with melted ice resulted in the appearance of visible colonies after several days, weeks and even months of incubation at the different temperatures. The number of colonies increased with time until the end of the incubation period (3 months). Several mesophilic bacteria (*i.e.* exhibiting growth at 25-30 °C but not at lower temperatures) and some pigmented yeasts were also isolated, but we did not study them further. In total, we were able to isolate and purify a total of 120 bacterial isolates exhibiting growth at both 15 and 4 °C; however, inspection based on morphological-, physiological and biochemical criteria confirmed that some of them were of clonal origin. Once differentiated on the basis of different phenotypic properties (morphological, physiological and biochemical) (see below), forty-five psychrophilic isolates were subsequently stored at −80 °C in 20 % glycerol.

Morphological and physiological characterization of the isolates

A total of forty-five pure isolates originating from direct plating were characterized according to their colony- and cellular phenotypes (Gram staining, morphology, pigmentation, and growth temperature) (Table 1). All forty-five isolates were psychrophiles or psychrotrophs as they grew well at temperatures ranging from 4 to 20 °C (see below). In some cases, the maximal temperature for growth was 20 °C. No isolate was able of growing at 37 °C, while some still grew at 30 °C.

In order to further characterize the isolates from a physiological point of view, the kinetics of growth was determined for each one at 4, 15 and 30 °C. The results obtained allowed us to group the isolates in three categories: the first one corresponds to strains exhibiting growth rates in the following order 30 °C > 15 °C > 4 °C (Fig S1); in the second group the order of growth rates was 15 °C > 30 °C > 4 °C; and in the third group, growth at 30 °C was not observed and the growth rate order was 15 °C > 4 °C (Fig S2). Nevertheless, even though growth of the isolates at 4 °C was slower as compared to cultures incubated at 15 or 30 °C, in all cases the final OD_{600} reached similar- or higher values than those attained at 30

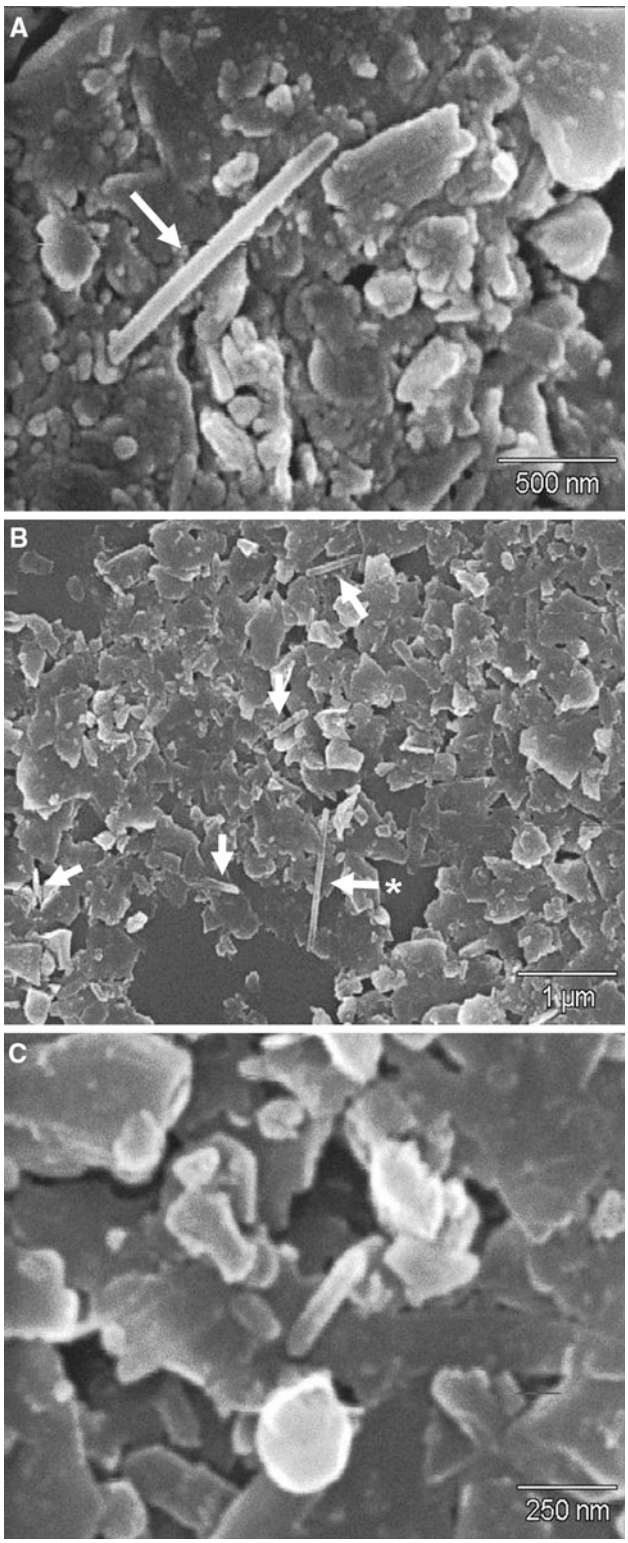

Fig. 2 Scanning electron microscopy of bacterial cells found in glacier ice samples. **A** and **B** *arrows* point toward filament like structures and multiple bacterial cells (rod shaped). **C** very small ("dwarf") bacterial cells rod- and cocci-shaped

or 15 °C. Therefore we considered our strains as *psychrotolerant* (group I) or *psychrophilic* (groups II and III) (see Discussion section).

The results also showed that 86.66 % of all the isolates synthesized pigments (mainly yellowish). Sixteen isolates synthesized and excreted high amounts of what seemed to be extracellular polymers (*i.e.* highly mucoid colonies). The production of such polymers or pigments was more evident at certain temperatures of growth (i.e. <15 °C) (Fig S3). Other isolates exhibited a particular gelatinous or rubbery appearance.

Twenty-nine isolates (64.44 %) synthesized and excreted useful cold-active enzymes, including those with proteolytic and amylolytic activities (Table 2); on the other hand, twenty-one isolates (46.66 %) synthesized cold-active β-galactosidases.

The antibiotic tests showed an elevated percentage of resistant strains to high doses of different antibiotics (i.e. 100 µg/ml) (Fig. 3a; Table 2). Ampicillin-resistance was the most frequent among these strains (64.44 %), followed by nalidixic-acid-, penicillin- and chloramphenicol-resistances (57.77 % each). Resistance to streptomycin and kanamycin reached similar levels (24.44 and 22.22 % respectively). On the contrary, only 4.44 % of the tested strains were resistant to tetracycline. Multiresistance was also observed, with 22.22 % of the strains simultaneously resistant up to five of the antibiotics tested at elevated doses (>100 µg/ml) (Fig. 3b).

In accordance with this, plasmids of low and high molecular weight were detected in 46.66 % of the isolates. In many cases, more than one different method of plasmid isolation and purification had to be employed to reveal the presence of such genetic elements.

Heavy-metal resistance

Among the bacterial strains isolated, no one was able to grow in the presence of 10 µM $HgCl_2$. On the contrary 66.66 % grew in the presence of, at least, 100 ppm Ni^{++}, 71.11 % grew in the presence of at least 100 ppm Zn^{++} and 71.11 % resisted at least 50 ppm Cu^{++} (Table 2). Some of the strains were able to grow even in the presence of 200 ppm of the tested heavy metals. The strain used as negative control did not grow at any of the metal concentrations tested.

Phylogenetic analysis of 16S rDNA sequences of isolates

Among the psychrophilic bacterial isolates, 22 different partial 16S rDNA sequences were obtained and further

Table 1 Characteristics of bacterial isolates

Isolate	Origin	Growth temp. range	Colony description	Gram staining	Growth kinetics (group)	Closest relative species/strain (% identity)
1. PGV020	LB	4–20	Pale yellow, opaque	Neg	II	
2. PGV021	LB	4–20	Pale yelow, opaque	Pos	nd	*Arthrobacter stackebrandtii CCM2783 (96.73 %)*
3. PGV022	LB	4–20	Pale yellow,opaque	Pos	I	*Arthrobacter stackebrandtii CCM2783 (97.55 %)*
4. PGV024	LB 1/50	4–30	Pale yellow	Neg	III	*Pseudomonas fragi ATCC4973 (98.52 %)*
5. PGV035	LB 1/100	4–20	White, glossy	Neg	nd	*Pseudomonas psychrophila E3 (99.26 %)*
6. PGV036	LB 1/100	4–30	White, glossy	Neg	II	*Pseudomonas psychrophila E3 (99.77 %)*
7. PGV038	LB	4–30	Pale yellow, opaque	Pos	I	*NI*
8. PGV040	R2A	4–20	Pale yellow	Neg	nd	*NI*
9. PGV041	R2A	4–20	Pale yellow, opaque	Neg	I	*NI*
10. PGV056	R2A	4–20	White, mucoid	Neg	nd	*Pseudomonas brenneri CFML 97-391 (99.61 %)*
11. PGV057	R2A	4–20	Pale yellow	Pos	nd	*NI*
12. PGV058	R2A	4–30	Purple, opaque	Neg	I	*Janthinobacterium lividum DSM1522 (99.51 %)*
13. PGV059	R2A	4–30	Pale yellow, mucoid	Neg	I	*Pseudomonas proteolytica CMS64 (99.32 %)*
14. PGV063	LB 1/50	4–30	Pale yellow, mucoid	Neg	II	*Pseudomonas proteolytica CMS64 (99.06 %)*
15. PGV065	LB 1/50	4–30	Pale yellow, mucoid	Neg	II	*Pseudomonas brenneri CFML 97-391(99.66 %)*
16. PGV070a	R2A	4–30	Pale yellow	Neg	nd	*NI*
17. PGV070b	R2A	4–30	Pale yellow, mucoid	Neg	nd	*Pseudomonas brenneri CFML 97-391 (99.26 %)*
18. PGV072	R2A 1/50	4–30	White	Neg	I	*Pseudomonas psychrophilaE3 (99.30 %)*
19. PGV073	R2A 1/50	4–30	Pale yellow, mucoid	Neg	I	*NI*
20. PGV074	R2A 1/50	4–20	Pale yellow	Pos	I	*NI*
21. PGV076	R2A 1/50	4–30	Pale yellow, mucoid	Neg	I	*NI*
22. PGV077	LB 1/50	4–30	Pale yellow, mucoid	Pos	II	*NI*
23. PGV078	LB 1/50	4–30	Pale yellow, mucoid	Neg	nd	*NI*
24. PGV079	R2A	4–20	Pale yellow	Pos	nd	*NI*
25. PGV082	R2A	4–30	Pale yellow	Pos	III	*NI*
26. PGV083	R2A 1/50	4–30	Pale yellow	Neg	I	*NI*
27. PGV084	R2A	4–30	White, mucoid	Neg	II	*Janthinobacterium lividum DSM 1522 (99.36 %)*
28. PGV085	R2A	4–30	Pale yellow, mucoid	Neg	II	*NI*
29. PGV086	R2A	4–30	Yellow	Pos	III	*NI*
30. PGV087	R2A 1/50	4–30	Yellow	Neg	I	*Pseudomonas putida F1 (99.76 %)*
31. PGV088	R2A 1/50	4–30	Pale yellow	Neg	I	*Janthinobacterium lividum DSM1522(99.37 %)*
32. PGV089	R2A 1/50	4–30	Yellow, mucoid	Neg	I	*Pseudomonas brenneri CFML 97-391(99.44 %)*
33. PGV090	R2A 1/50	4–30	Pale yellow, mucoid	Neg	I	*Pseudomonas putida F1 (99.74 %)*
34. PGV091	R2A 1/50	4–30	Yellow	Neg	I	*NI*
35. PGV092	R2A 1/50	4–30	Pale yellow	Neg	I	*NI*
36. PGV094	R2A 1/50	4–30	Pale yellow, mucoid	Neg	I	*Pseudomonas brenneri CFML 97-391 (99.51 %)*
37. PGV095	R2A 1/50	4–30	Pale yellow, mucoid	Neg	I	*NI*
38. PGV096	LB 1/100	4–20	Pale yellow	Pos	nd	*Arthrobacter stackebrandtii CCM2783 (97.52 %)*
39. PGV097	LB 1/100	4–20	White	Neg	nd	*Rhizobium huautlense SO2 (97.08 %)*
40. PGV098	LB 1/100	4–20	Pale yellow,opaque	Neg	III	*NI*
41. PGV099	R2A	4–30	Yellow, iridiscent	Neg	II	*NI*
42. PGV100	R2A	4–20	Yellow	Neg	nd	*NI*
43. PGV101	R2A	4–30	Yellow	Neg	nd	*Flavobacterium frigidimaris KUC-1 (97.88 %)*
44. PGV102	R2A 1/50	4–30	Yellow, mucoid	Neg	II	*Pseudomonas brenneri CFML 97-391(99.57 %)*
45. PGV104	R2A 1/50	4–30	Dark yellow	Neg	III	*NI*

Table 2 Antibiotic resistance, heavy-metal resistance and enzymatic activities of bacterial isolates

Isolate	Antibiotic resistance[a]							Heavy metal resistance[b]			Enzymatic activity at 15 °C on			Plasmid presence
	Ap	Kn	Cm	Na	St	Pn	Tc	Ni	Zn	Cu	Xgal	Skim milk	Starch	
1. PGV020		25		100				100	50	50	+			
2. PGV021		25		100				50			+			+
3. PGV022				100				50	100	50	+			
4. PGV024	100		100	100		100		100		50		+		+
5. PGV035	100			100		100		50		50				+
6. PGV036	100		100	100		100		50	200	100		+		+
7. PGV038		25		100				100	200	50	+	+		
8. PGV040				100				50	50		+			+
9. PGV041			100	25				100	100	50	+	+		
10. PGV056	100	150	100	100	25	100	25	50	50	50				
11. PGV057			25	100				50	100		+			
12. PGV058	100		100	25	25	100		200	200	50		+		
13. PGV059	100		100	25	25	100		50	200			+		+
14. PGV063	100	25	100	100		100		100	200			+		+
15. PGV065	100	100	100	25	25	100		50	200			+		
16. PGV070[a]			25	100				50	100		+			
17. PGV070b	100		100	25		100		50	200	50		+		+
18. PGV072	100		100	25		100		100	200	50		+		
19. PGV073	100	25	100	100	100	100		100	200	50		+		
20. PGV074				100				100	100	50	+			
21. PGV076	100	25	100	100	100	100		100	200	50		+		+
22. PGV077	100		100	25	100	100		100	200	50		+		
23. PGV078	100		100	100	100	100		100	200	50		+		
24. PGV079			25	100				50	100	100	+			+
25. PGV082				100		20		200	200	50	+	+		
26. PGV083	100		25	100				100			+			+
27. PGV084	100		100	25	25	100		50	200	50		+		+
28. PGV085	100		100	25	25	100		200	200	100		+		+
29. PGV086			25	100				100			+	+		
30. PGV087	100	100	100	25	100	100		200	200	200	+		+	+
31. PGV088	100		100	100	100	100		100	200	50		+		+
32. PGV089	100	100	100	25	25	100		200	200	100	+	+	+	
33. PGV090	100	100	100	100	100	100	20	200	200	100	+			+
34. PGV091	100		25	100		100		100						+
35. PGV092				100				100		50	+			
36. PGV094	100		100	25	25	100		200	200	100	+	+	+	
37. PGV095	100	100	100	25	100	100		200	200	100	+	+		
38. PGV096		25		100				100	100	50	+	+		
39. PGV097	100				100			50						
40. PGV098		25	25	100				100				+		
41. PGV099	100	100	100	25	100	100		200	200	100		+	+	+
42. PGV100			25	25				50	100		+			+
43. PGV101	100	100	100	25	25	20		100	100	50		+	+	+
44. PGV102	100	100	100	25	25	100		200	200	50		+	+	
45. PGV104	100	100	100	25	100	100		100	200	50		+	+	+

[a] The number shown represents the maximum concentration (in μg/ml) of the particular antibiotic at which the isolate was able to grow

[b] The number reported represents the maximum concentration (in p.p.m.) of the particular metal at which the isolate was able to grow

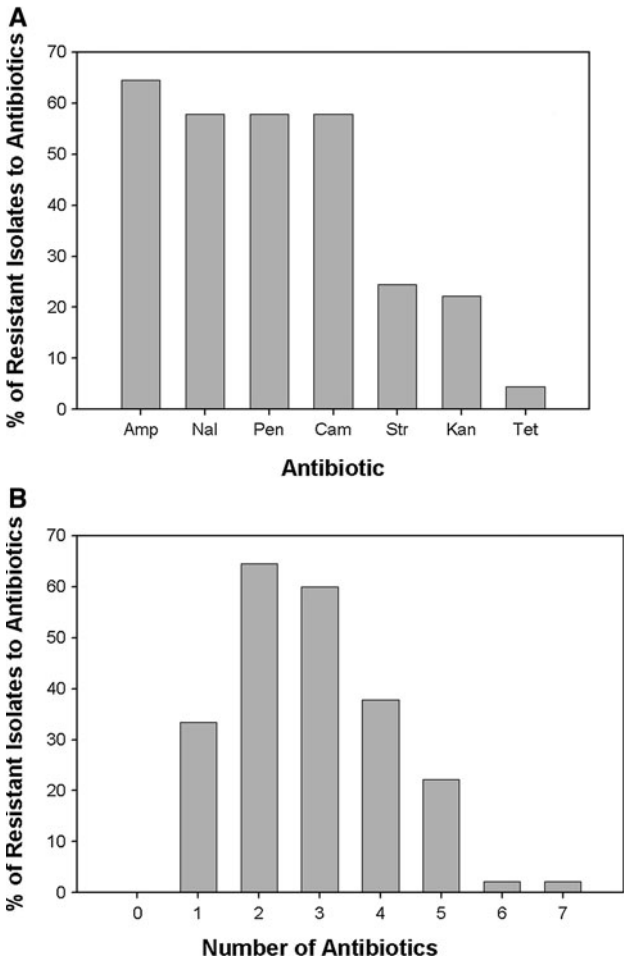

Fig. 3 Levels of antibiotic resistance among bacterial isolates from glacier ice samples. **A** Global levels of antibiotic resistance among bacterial isolates from Humboldt glacier's ice ($n = 45$). The proportion of isolates resistant to ampicillin (Amp), nalidixic acid (Nal), penicillin (Pen), cloramphenicol (Cam), streptomycin (Str), kanamycin (Kan) and tetracycline (Tet) is shown. **B** Multiresistance to antibiotics among glacier-ice bacterial isolates. The proportion of strains resistant to 0, 1, 2, 3, 4, 5, 6 and 7 antibiotics simultaneously is shown

analyzed. These were distributed within five different phyla/classes: Alpha-, Beta and Gammaproteobacteria, Actinobacteria and Flavobacteria. A phylogenetic tree with these strains and their closest relatives is shown in Fig. 4. As can be seen, the majority of the strains belonged to the phylum Proteobacteria and particularly to the *Pseudomonas* genus. It should be noted that the closest relative for nineteen isolates was either a psychrophile or a psychrotroph.

Discussion

Tropical glaciers are of particular interest to study because the distinct characteristics of tropical climates make glacier-climate interactions different from the ones typical of mid- and high-latitudes (Kaser 1999). Nevertheless, these glaciers have not been comprehensively studied, mainly because of their remoteness and limited accessibility. Moreover, no results concerning microbial diversity of tropical glaciers in South America have been published to date. Therefore, this is the first report dealing with psychrophilic/psychrotolerant bacteria immured in glacier ice in a tropical-, South American glacier.

As shown in the present report, ice samples collected from the forefront of Humboldt Glacier were rich in metabolically-active and bacteria, which exhibited high titers. Considering the characteristic low densities of bacterial populations recovered from glacial ice, these results may seem contradictory at first glance. However, both of them are in agreement with previous reports showing that bacterial communities at glacier's bases might have originated both from the atmosphere, reaching the glacier ice by deposition and subsequent burial, and from the underlying soil (Miteva et al. 2004).

The culturable fraction of the bacterial community was diverse, including members of five different phyla/classes (Alpha-, Beta and Gammaproteobacteria, Actinobacteria and Flavobacteria), many of which have already been associated with permanently cold environments (Margesin and Miteva 2011).

Even though some of the selected bacterial isolates exhibited higher growth rates at 30 °C than those recorded at 15 and 4 °C (group I) (Figs S1-S3), a noticeable drop of OD_{600} was observed at the highest temperature, soon after the cultures reached the stationary phase. Hence, most of the isolates tested were psychrophilic according to the updated definition of Margesin (2009). In this author's opinion, the true nature of psychrophily is related to the organism's ability to yield higher amounts of biomass rather than exhibiting higher growth rates at lower temperatures (15 °C or below), when compared with temperatures above 20 °C (see Table 1). Indeed, although the growth rate of some of our isolates was slow at 4 or 15 °C, their cellular yield, estimated indirectly as OD_{600} of the cultures at the stationary phase, was higher at these temperatures as compared with higher ones. Therefore, we decided to consider the strains belonging to groups II and III as *psychrophilic* whereas those belonging to group I were *psychrotolerant*.

The psychrophilic bacterial strains isolated from Humboldt Glacier's ice exhibited several of the phenotypic characteristics associated with long-term resistance to freezing conditions: among these, an abundant production of exopolymers and pigments involved in protection against ice-mediated cell injury and UV damage (D'Amico et al. 2006). Moreover, electron microscopy images of our samples also showed a predominance of very small ("dwarf") cells, a phenotype considered to be a

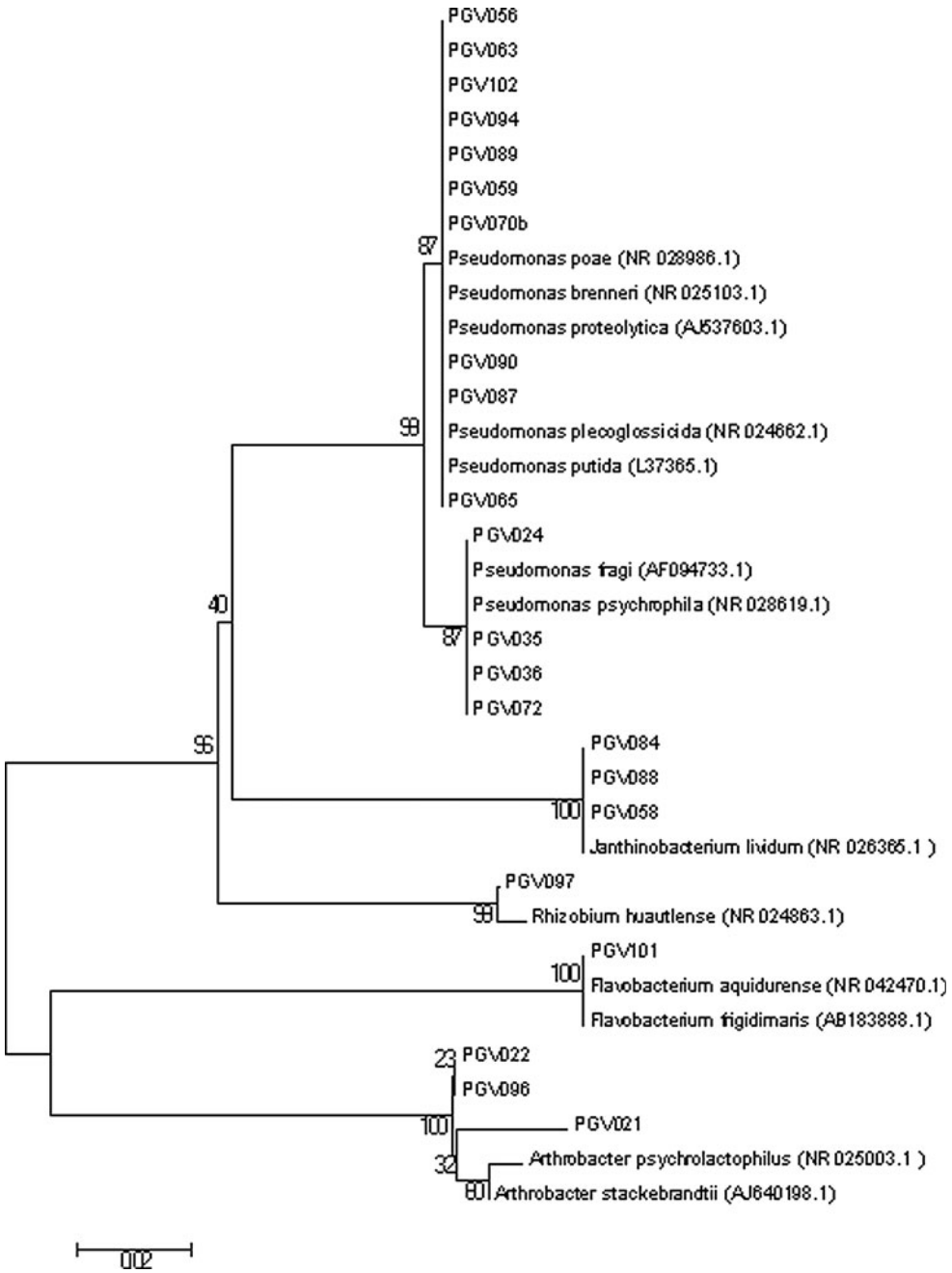

Fig. 4 Evolutionary relationships of Glacier Humboldt's ice bacterial isolates. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.40839611 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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

physiological response to cold temperatures and related to condensation of the cytoplasm, loss of permeability, decrease in RNA content, reduction of metabolic activity, and inability to divide, all leading to the so-called viable but not culturable state (McDougald et al. 1998).

One of our major goals was to examine the levels of antibiotic sensitivity and resistance of microorganisms in the presumed absence of current antibiotic use, *i.e.* by screening organisms trapped in the Humboldt Glacier's ice presumably for several thousand years. Therefore, the significant proportion of antibiotic- and heavy metal resistant strains, which reached in some cases more than 70 % of the culturable fraction of psychrophilic bacteria, was of particular interest. We also showed that multiresistance against up to seven antibiotics and three heavy metals was exhibited by some of these bacterial strains. These results confirm that both antibiotic- and heavy metal resistance are common traits exhibited by bacteria thriving in pristine environments, not influenced by humans, and that these traits are possibly related to a reduced cell wall permeability, expression of genes encoding multidrug pumps and/or horizontal genetic transfer among members of natural communities (Summers 2002; Hogan and Kolter 2002; Baker-Austin et al. 2006). They also confirm that resistance

to multiple antibiotics, combined with the presence of mobile genetic elements, is common among natural communities of psychrophilic bacteria and vastly predate the use of commercial antibiotics (Petrova et al. 2009).

Even though we did not measure total antibiotic- or heavy metal concentrations in Humboldt Glacier's ice we believe that, considering its remoteness, their levels must be very low if not absent at all. However, this does not preclude the possibility of finding this kind of resistant microorganisms for two main reasons: in the first place, it is widely acknowledged that the majority of the glacier's ice-borne microorganisms were transported there by air from remote areas and therefore did not evolve in situ; on the other hand, a correlation between total or bioavailable heavy metals, with percent heavy-metal resistance is not always apparent, as recently shown by Møller et al. (2011).

On the other hand, the aforementioned observations should be also considered in the light of the presence of plasmids in several of the psychrophilic isolates tested. Indeed, it is well known that multiple genes encoding for metal and antibiotic resistance are frequently found on the same plasmids and/or transposons, conferring co-resistance (Summers 2002). These results are in agreement with the idea that microbial exposure to one toxicant could result in an indirect selection for bacteria with resistance to multiple, chemically unrelated toxicants/antibiotics, possibly as a result of horizontal transfer of mobile genetic elements (Tshape 1994; Baker-Austin et al. 2006).

As a final point it is important to mention that, considering (1) the accelerated melting of tropical glaciers together with the release and reactivation of both active- or dormant-bacteria and (2) the potential behavior of these bacteria as donors in horizontal gene transfer events (the "genome recycling concept") (Rogers et al. 2004), it becomes evident that more effort should be made in order to characterize the genetic determinants conferring such resistances.

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