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Bioprospecting glacial ice for plant growth promoting bacteria

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ABSTRACT

Glaciers harbor a wide diversity of microorganisms, metabolically versatile, highly tolerant to multiple environmental stresses and potentially useful for biotechnological purposes. Among these, we hypothesized the presence of bacteria able to exhibit well-known plant growth promoting traits (PGP). These kinds of bacteria have been employed for the development of commercial biofertilizers; unfortunately, these biotechnological products have proven ineffective in colder climates, like the ones prevailing in mountainous ecosystems. In the present work, we prospected glacial ice collected from two small tropical glaciers, located above 4.900 m in the Venezuelan Andes, for cold-active PGP bacteria. The initial screening strategy allowed us to detect the best inorganic-P solubilizers at low temperatures, from a subsample of 50 bacterial isolates. Solubilization of tricalcium phosphate, aluminum- and iron-phosphate, occurred in liquid cultures at low temperatures and was dependent on medium acidification by gluconic acid production, when bacteria were supplied with an appropriate source of carbon. Besides, the isolates were psychrophilic and in some cases exhibited a broad range of growth-temperatures, from 4 °C to 30 °C. Additional PGP abilities, including phytohormone- and HCN production, siderophore excretion and inhibition of phytopathogens, were confirmed *in vitro*. Nucleotidic sequence analysis of 16S rRNA genes allowed us to place the isolates within the *Pseudomonas* genus. Our results support the possible use of these strains to develop cold-active biofertilizers to be used in mountainous agriculture.

1. Introduction

Glaciers represent long-term reservoirs of a vast microbial diversity with an unexpected metabolic potential (Barker et al., 2006; Miteva, 2008). Bioprospection of such environments started almost two decades ago and has been mainly oriented toward the detection of microorganisms able to produce cold-active enzymes with potential biotechnological applications (Feller and Gerday, 2003; Cavicchioli et al., 2011). However, not only the cold-active biomolecules (*e.g.* enzymes and secondary metabolites) – produced and excreted by psychrophilic microorganisms – are attractive form a biotechnological point of view: the whole cells can also be considered as very useful biotools for the development of efficient products. This is the case of biofertilizers.

Biofertilizers are preparations containing living microorganisms that help crop plants to uptake nutrients, such as N and P, stimulating therefore their growth and development (Glick, 2012). Some biofertilizers may also include microorganisms that release phytostimulators and/or inhibit plant pathogens (Andrews and Harris, 2000). Numerous commercial biofertilizers have been developed and are currently marketed worldwide (Glick, 2012); however, their use in colder climates – like the ones prevailing in mountainous ecosystems – has proven ineffective (Trivedi et al., 2012). This occurs mainly because low temperatures impose serious constraints to the metabolic activity of microorganisms: for instance, a 10 °C drop in the temperature induces a two- to four-fold decrease in enzyme activity (Feller and Gerday, 2003). Therefore, in order to develop proficient cold-active (CA) biofertilizers, it is imperative to search for microorganisms well adapted to low temperatures (=psychrotolerant or psychrophilic).

During the past 15 years, the search for this type of microorganisms has been conducted on natural soils collected in mountainous regions (including alpine and sub-alpine environments, mainly in the Indian Himalayan Region) (Trivedi et al., 2012; Yarzábal, 2014). Considering that, besides their metabolic diversity, glacial-ice

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microorganisms endure low temperatures and nutrient availability, we hypothesized that it should be possible to isolate psychrophilic plant-growth promoting (PGP) microorganisms from glacier ice, capable of readily and efficiently promoting plant-growth in cold climates and in the presence of multiple environmental stresses.

In the present work, we report the thorough characterization of a sub-sample of bacteria isolated from glacial ice samples, collected at two rapidly-retreating, small glaciers located at >4.900 m above the sea level in the Andean region of Venezuela: Pico Bolivar and Pico Humboldt glaciers (Braun and Bezada, 2013). Furthermore, we show that – besides their psychrophilic lifestyle – these bacteria exhibit several direct- and indirect PGP abilities and, therefore, represent excellent candidates for the development of cold-active, efficient biofertilizers aimed at contributing toward the development of mountainous agriculture in the Andean region.

2. Materials and methods

2.1. Sampling site and bacterial isolation

Bacteria were isolated from glacial ice samples as previously described (Ball et al., 2014). Colonies were restreaked several times to obtain a clonal population of each isolate. More than 450 pure isolates were stored at −80 °C in a 20% glycerol solution.

2.2. Screening of cold-active, P-solubilizing bacteria (CA-PSB)

As a starting point to screen this collection of glacial ice bacteria, and in order to prove our working hypothesis, we tested the ability of a sub-sample of 50 isolates to solubilize tricalcium phosphate in NBRIP medium (Nautiyal, 1999). In brief, strains preserved at −80 °C in 20% glycerol were reactivated on R2A medium (Reasoner and Geldreich, 1985) at 15 °C. When the colonies became visible, they were spotted on the surface of NBRIP medium and incubated at 15 °C for several days. Simultaneously, the acidification ability of each isolate was determined in NBRIP broth containing a pH indicator (Bromophenol Blue) for three days at 15 °C (Mehta and Nautiyal, 2001). At the end of the incubation period the final OD600 were subtracted from the initial values. Strains showing the highest P-solubilization efficiency, *i.e.* those exhibiting the highest halo diameter/colony diameter ratio (Nguyen et al., 1992) and the highest acidifying capacity were selected for further studies.

2.3. Characterization of CA-PSB

Morphological characteristics of both the colonies and bacterial cells were recorded for each selected isolate. Also, some standard tests were conducted, including Gram staining, catalase test, oxidative/fermentative test and fluorescence test among others.

The temperature growth range of the selected CA-PSB isolates was preliminary determined by streaking colonies onto R2A plates, followed by incubation at 4, 10, 15, 20, 30 and 37 °C, and visual inspection of the plates. In addition, in order to distinguish between psychrophilic and psychrotolerant isolates, the kinetics of growth of each PSB isolate in R2B (*i.e.* R2A medium without agar) was monitored at 4 °C, 15 °C and 30 °C. For this, inocula were prepared by harvesting cells grown on agar plates into physiological saline. The cells were centrifuged at 10,000 × *g* for 2 min, washed twice in saline and thereafter resuspended by vortexing. The optical density of the final cell suspensions was measured at 600 nm (OD600) and adjusted to a uniform cell density. One 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 OD600 of 200 μl aliquots of each culture on a microplate reader at different time intervals using a microplate reader (BioTek ELX800).

2.4. Qualitative and quantitative estimation of mineral phosphate solubilization

The P-solubilizing efficiencies of the CA-PSB isolates were determined by growing them in a double layer agarized medium (NBRIP), containing insoluble $Ca_3(PO_4)_2$ (tricalcium phosphate, TCP) only on the top layer, and by calculating the ratio between the diameter of the halo of solubilization and the diameter of the colony (×100)) (Nguyen et al., 1992). The P-solubilizing abilities of the strains were also tested in agarized NBRIP-medium containing $FePO_4$ (FeP) or $AlPO_4$ (AlP) instead of TCP. Also, in a different series of experiments, glucose was replaced by lactose, maltose, xylose, galactose, sacharose or glycerol.

Quantitative estimation of mineral phosphate solubilization was carried out at 15 °C. One ml bacterial suspensions (~2×10^7 cells/ml) were inoculated in 50 ml of NBRIP broth (containing either TCP, FeP or AlP at 1 g/l) in Erlenmeyer flasks (50 ml), and incubated for 12 days with periodic agitation.

At different time intervals, aliquots of 2 ml were collected aseptically, centrifuged at 12,000 × *g* for 10 min and the supernatant was stored at -20 °C until analysis. The phosphorus content in culture supernatants was estimated by the vanado-molybdate method (Murphy and Riley, 1962). All experiments were performed in triplicate.

2.5. Qualitative measurement of indoleacetic acid, siderophore and HCN production

Indoleacetic acid (IAA) production by CA-PSB was determined by performing the rapid *in situ* bioassay developed by Bric et al. (1991). In brief, CA-PSB suspensions were spotted on the surface of R2A medium, supplemented with 5 mM tryptophan, and allowed to grow at 15 °C for two days until the colonies became visible. The plate was then overlaid with an 82 mm diameter nitrocellulose membrane and incubated two more days. The membrane was removed from the plate and overlayed with a filter paper (Whatman no. 2) saturated with Salkowski's reagent (2% 0.5 M FeCl in 35% perchloric acid). The reaction was allowed to proceed at room temperature, 22 °C approximately, until characteristic pink to red spots developed on the membrane.

Siderophore- and HCN production by the isolates were estimated qualitatively at two different incubation temperatures (*i.e.* 15 and 30 °C). Siderophore production was detected by the Chrome Azurol-S (CAS) assay (Schwyn and Neilands, 1987) in 100 mm Petri dishes. HCN production was inferred by the qualitative method of Bakker and Schippers (1987). The change in the color of the filter paper previously soaked in 0.2% sodium carbonate prepared in 0.5% picric acid, from yellow to dark brown was rated visually depending on the intensity of the color change.

2.6. Assessment of in vitro anti-phytopathogenic activity

The anti-phytopathogenic ability of the CA-PSB was evaluated against *Pythium ultimum*, *Fusarium oxysporum* and *Phytophtora infestans* using dual culture assays. In brief, a seven day old 5 mm diameter mycelia agar disc of each fungus or oomycete was placed at the center of an agar plate (either potato dextrose agar or carrot agar). A suspension of an actively grown culture (24 h) of each CA-PSB isolate was streaked toward the periphery of plates, at least 3 cm away from the tested pathogen. Plates were incubated at 20 °C (for *P. infestans*) and 25 °C (for *P. ultimum* and *F. oxysporum*), and observations were recorded by visual inspection every 24 h after inoculation up to 8 days. *Pseudomonas fluorescens* CHA0 strain, very

well known for its biocontrol activities (Haas et al., 2002), was included as an internal reference in these assays.

2.7. Gluconic acid quantification

The D-gluconic acid concentration was determined using an enzymatic D-gluconic acid detection kit (Megazyme, product K-GATE) as described by the manufacturer.

2.8. PCR amplification, sequencing and analysis of 16S rDNA

The gene-encoding 16S rRNA (16S rDNA) was PCR-amplified from CA-PSB 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 (Jukes and Cantor, 1969).

2.9. 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: PGV024 (KF020669), PGV035 (KF020670), PGV045 (KM114891), PGV085 (KM114892), PGV094 (KF020685), PGV228 (KJ417619), PGV233 (KJ417592) and PGV284 (KJ417594).

3. Results

3.1. Initial screening of cold-active P-solubilizing bacteria

Our major goal in the present work was to identify cold-active PGPB from ice samples collected at two tropical glaciers in the Venezuelan Andes (>4.900 m.a.s.l.). The preliminary screening of bacterial strains, preserved in 20% glycerol stocks at −80 °C and included the Venezuelan Collection of Psychrophilic Bacteria (at present under construction in our laboratory), on agarized NBRIP medium allowed us to detect up to 13, 22 and 22 strains able to solubilize TCP after 24 h incubation at 4 °C, 15 °C and 30 °C, respectively (Supplementary Fig. S1). With increasing incubation times, the size of the halos increased concomitantly and allowed us to detect a few more PSB. Acidification screening tests, conducted in modified NBRIP liquid medium, revealed that the majority of these halo-producing strains also acidified the liquid medium supernatant when grown at 15 °C for several days (not shown). Based on these results, we decided to keep the best P-solubilizers (*i.e.* nine strains showing both the greatest solubilization halos and acidification capacities) for further studies (Fig. 1).

3.2. Characterization of CA-PSB isolates

The selected CA-PSB isolates were either psychrophiles or psychrotolerants as they grew well at temperatures ranging from 4 to 30 °C (Table 1 and Supplementary Fig. S2). In some cases, the maximal temperature for growth was 20 °C (*e.g.* isolate PGV094). No isolate was able of growing at 37 °C, while the majority grew poorly at 30 °C. Nevertheless, this qualitative test showed that growth was maximal at temperatures within 10 °C and 20 °C.

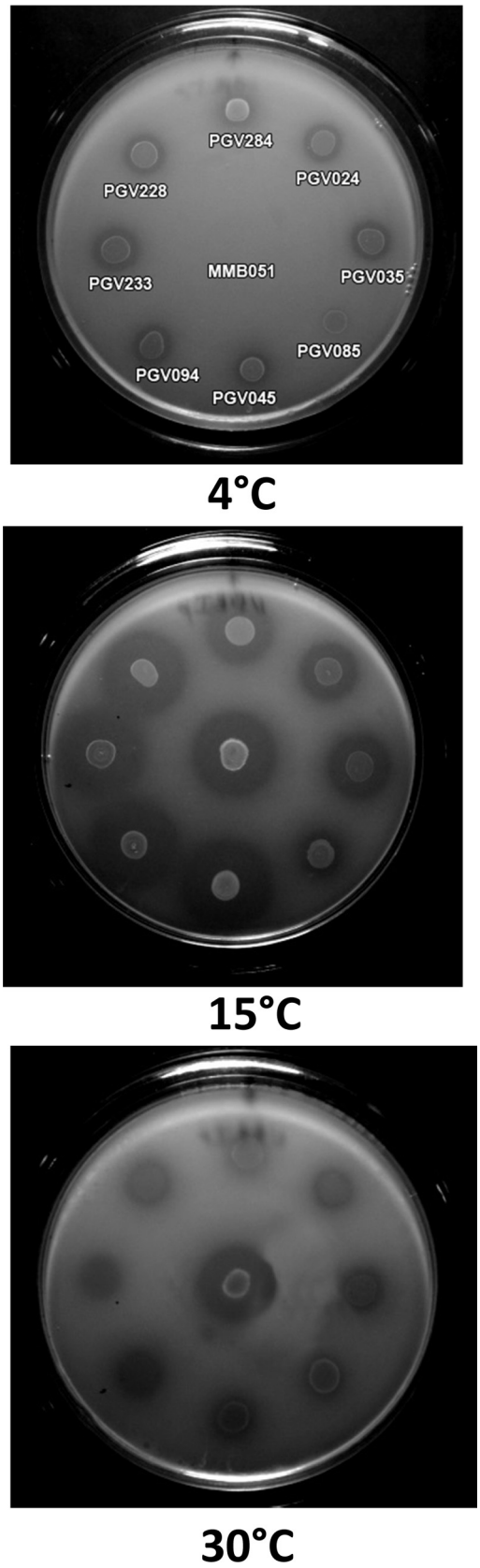

Fig. 1. *Bacterially-mediated calcium phosphate solubilization at different temperatures.* Isolates showing the largest solubilization halos in preliminary tests were incubated for 48 h in NBRIP medium at 4 °C, 15 °C and 30 °C, respectively. From top and clockwise: isolates PGV284, PGV024, PGV035, PGV085, PGV045, PGV094, PGV233 and PGV228. Strain MMB051, included here for comparison purposes (center), is a psychrotolerant PSB unable to grow at 4 °C.

Table 1
Characteristics of bacterial isolates.

Isolate	GenBank Acces No	Gram staining	Colony description	Growth temp range	Growth kinetics (group)	Closest relative species (% identity)
PGV024	KF020669	Neg	Pale yellow	4–30	II	*Pseudomonas fragi* (98.52)
PGV035	KF020670	Neg	White, glossy	4–30	II	*Pseudomonas psychrophila* (99.26)
PGV045	KM114891	Neg	White, glossy	4–30	I	*Pseudomonas orientalis* (99.79)
PGV085	KM114892	Neg	Pale yellow, mucoid	4–20	I	*Pseudomonas brenneri* (99.0)
PGV090	KF020681	Neg	Pale yellow, mucoid	4–20	I	*Pseudomonas putida* (99.74)
PGV094	KF020685	Neg	Pale yellow, mucoid	4–20	I	*Pseudomonas brenneri* (99.51)
PGV228	KJ417619	Neg	White, glossy	4–30	I	*Pseudomonas fluorecens* (99.16)
PGV233	KJ417592	Neg	White, glossy	4–30	I	*Pseudomonas antarctica* (99.0)
PGV284	KJ417594	Neg	White, opaque	4–30	I	*Pseudomonas fredericksbergensis* (99.0)

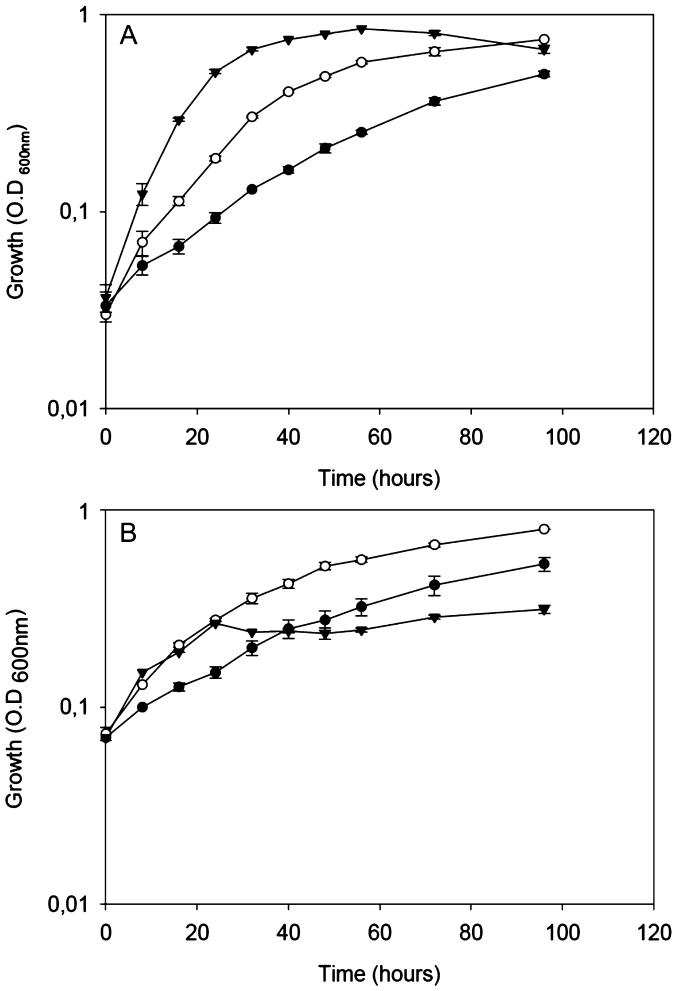

Fig. 2. *Growth kinetics of cold-active PSB isolates*. Isolates PGV045 (A) and PGV024 (B) were grown in R2B medium at different temperatures and the OD_{600} was plotted against time in semi-log graphs. Closed triangles (30 °C); open circles (15 °C); closed circles (10 °C).

In order to further characterize the isolates from a physiological point of view, the kinetics of growth was determined for each isolate at 10, 15 and 30 °C. The results obtained allowed us to group the isolates in two categories: the first one (Group I) corresponds to isolates exhibiting growth rates in the following order 30 °C > 15 °C > 10 °C (Fig. 2A and Table 1); in the second group (Group II), even though the order of growth rates was almost the same at the initial stages of growth, cultures incubated at 30 °C stopped growing very soon (Fig. 2B and Table 1). Strikingly, even though growth of the isolates at 10 °C in both groups was slower as compared to cultures incubated at 15 or 30 °C, in all cases the final OD600 reached similar- or higher values than those attained at 30 or 15 °C. Therefore, in a preliminary classification, we considered our isolates as psychrotolerant (Group I) and psychrophilic (Group II) (however see Discussion section).

3.3. Mineral phosphate solubilization assays

Qualitative estimation of the P-solubilization abilities of the nine selected isolates, conducted in the presence of TCP in agarized NBRIP, revealed that five of them exhibited the greatest P-solubilization efficiencies at 15 °C (namely strains PGV045, PGV085, PGV094, PGV228 and PGV233) and that the P-solubilizing efficiencies were maximal at 15 °C, as compared with 4 °C and 30 °C, respectively (Fig. 3). Besides, the P-solubilizing ability of the isolates depended strongly on the nature of the carbon source supplied to the cells. Addition of glucose to the medium resulted in maximum solubilization efficiencies (Table 2), followed by sacharose and galactose. In some cases, the presence of glycerol also permitted the solubilization of TCP, even though at a lower level, as compared with the rest of the sugars tested. However, no solubilization halos were observed in the presence of FeP- and AlP-supplemented NBRIP plates.

Quantitative estimation of P-solubilized in liquid cultures showed that all the isolates were able to release P from TCP after 12 days incubation at 15 °C (Table 3). In accordance with these results, a significant acidification of culture supernatants was detected in all cases (Table 3). Furthermore, all strains were positive for gluconic acid production from glucose, as confirmed by an enzymatic test (Table 3). According to these results, isolate PGV024 was the best solubilizer, followed by isolates PGV045, PGV284 and PGV035.

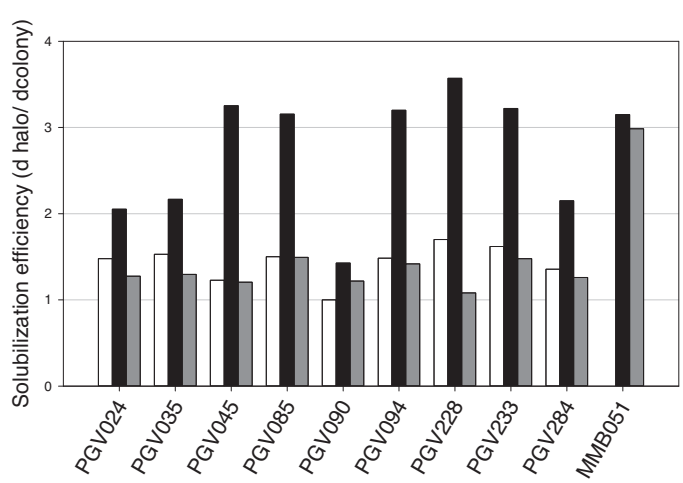

Fig. 3. *Solubilization efficiencies of cold-active isolates against TCP at different temperatures.* Suspensions of each bacterial isolate was spotted on the surface of NBRIP medium, incubated at 4 °C (white bars), 15 °C (black bars) and 30 °C (gray bars) for 48 h and the diameter of the bacterial colonies and the surrounding halos recorded. The solubilization efficiency of each isolate was calculated as proposed by Nguyen et al. (1992).

Table 2
Solubilization efficiencies in the presence of different C-sources at 15 °C.

Isolate	Glucose	Saccharose	Galactose	Glicerol	Xylose	Maltose	Lactose
PGV024	2.63	2.63	2.24	1.43	1.00	1.61	n.g.
PGV035	2.91	2.64	2.21	1.34	2.83	1.92	n.g.
PGV045	3.61	3.47	2.17	1.33	1.00	1.16	n.g.
PGV085	3.73	3.41	2.27	1.58	2.34	1.16	n.g.
PGV090	1.84	1.72	1.00	1.10	1.00	1.28	1.00
PGV094	3.40	2.98	2.33	1.38	2.22	1.23	1.00
PGV228	3.20	2.72	2.00	2.10	1.00	1.28	n.g.
PGV233	3.47	2.92	2.09	1.46	1.00	1.16	n.g.
PGV284	3.57	3.41	2.23	1.27	1.00	1.00	n.g.

Solubilization efficiencies (S.E.) are expressed ad the ratio between the diameter of the halo and the diameter of the colony (Nguyen et al. 1992). An S.E. = 1.00 means that there was no solubilization halo surrounding the colony. n.g.: No growth of the respective isolate.

P-solubilization from significantly less soluble substrates, *i.e.* FeP and AlP, was also monitored. As can be seen in Table 3, even though the amount of P solubilized was significantly smaller than that observed in the presence of TCP, isolate PGV035 was indeed able to solubilize up to 5.4 μg/ml P from AlP and 1.1 μg/ml P from FeP. Strikingly, acidification of the culture supernatants in the presence of FeP and AlP was more pronounced in these cultures as compared to TCP containing medium.

3.4. Plant-growth promotion traits of CA-PSB isolates

In order to monitor some other PGP abilities of the selected CA-PSB isolates, several tests were performed. Besides being able to readily solubilize TCP, the majority of the isolates produced IAA in tryptophane-supplemented R2A medium, as revealed by the production of a pink-colored product (Table 4 and Supplementary Fig. S3). Production of siderophores and HCN by some isolates was also detected (Table 4). In accordance with these results, some isolates inhibited growth of one, two or three of the tested phytopathogens on dual culture assays (Fig. 4).

3.5. Phylogenetic analysis of 16S rDNA sequences of isolates

Nucleotide sequence analysis of 16S rRNA genes revealed that all CA-PSB isolates were closely related to several species of the *Pseudomonas* genus (Table 1). A phylogenetic tree including these strains and their closest relatives is shown in Fig. 5. It should be noted that the closest relative for the majority of the isolates was either a well-known psychrophile or a psychrotolerant. The molecular identification received further support from classical microbiology and microscopy techniques: for example, all the isolates shared the following characteristics: cells were Gram negative rods, isolates were positive for the oxidative test, negative for the fermentative test and positive for the catalase test. Furthermore, six isolates were fluorescent (Supplementary Fig. S6).

4. Discussion

In this study we demonstrate -for the first time- that a subpopulation of culturable bacteria immured in glacial ice exhibit PGP abilities *in vitro*. For example they were able to (a) readily and efficiently solubilize mineral different inorganic phosphates at low temperatures and in the presence of different carbon sources; (b) produce and excrete phytohormone-like compounds (IAA), siderophores and HCN; and (c) inhibit growth of a well-known phytopathogen model organism in a dual culture assay. Therefore, we confirmed our working hypothesis and showed that some of these bacterial isolates, characteristics of glacial ice environments, possess an enormous -but yet uncharacterized- biotechnological potential and, thus, are good candidates for the development of cold active biofertilizers.

Molecular identification of this group of isolates showed that they were closely related to previously characterized psychrophilic or psychrotolerant species of the *Pseudomonas* genus. This was not completely unexpected since members of this genus are ecologically successful bacteria, extremely versatile in their metabolism, and able not only to colonize a myriad of environments but to withstand different kinds of stress, which explain their presence across a range of cold environments (Moreno and Rojo, 2014).

Table 3
Solubilization of inorganic *P* in liquid cultures.

Isolate	μg/ml *P* (CaP)	Final pH	Gluconic acid (g/l)	μg/ml *P* (AlP)	Final pH	μg/ml *P* (FeP)	Final pH
PGV024	154.7 ± 9.3	3.84 ± 0.025	1.49	3.8 ± 0.1	2.68 ± 0.006	0.9 ± 0.03	2.6 ± 0.006
PGV035	132.1 ± 8.7	3.94 ± 0.125	1.44	5.4 ± 0.8	2.61 ± 0.173	1.1 ± 0.04	2.59 ± 0.01
PGV045	142.7 ± 21.7	3.68 ± 0.114	1.67	1.9 ± 0.1	2.58 ± 0.029	0.6 ± 0.1	2.92 ± 0.006
PGV085	122.6 ± 8.3	3.89 ± 0.096	1.49	2.2 ± 0.4	2.57 ± 0.055	0.5 ± 0.01	2.92 ± 0.035
PGV094	122.5 ± 22.8	3.78 ± 0.197	1.45	2.2 ± 0.2	2.55 ± 0.040	0.5 ± 0.05	2.88 ± 0.01
PGV228	98.4 ± 12.8	4.01 ± 0.1	1.27	2.1 ± 0.3	2.53 ± 0.044	0.6 ± 0.07	3.04 ± 0.012
PGV233	116.1 ± 9.3	4.00 ± 0.07	1.46	2.8 ± 0.2	2.85 ± 0.323	0.5 ± 0.5	2.90 ± 0.178
PGV284	132.9 ± 6.1	4.02 ± 0.147	1.15	2.5 ± 0.1	3.05 ± 0.387	0.8 ± 0.06	3.03 ± 0.015
Control	47.9 ± 22.1	6.00 ± 0	0	2.8 ± 0.1	5.9 ± 0.173	0	6.00 ± 0

The phosphorus content in culture supernatants was estimated by the vanado-molybdate method and is expressed as the mean value of three replicas (±SD). Production.

Table 4
Production of exo-enzymes and other products.

Isolate	Proteases	Amylases	β-Galatosidases	Siderophores	IAA	HCN	Fluorescence	Phytopathogen inhibition		
								P. ultimum	*F. oxysporum*	*P. infestans*
PGV024	+	–	–	–	+	–	–	–	–	–
PGV035	+	–	–	–	++	–	–	+++	–	+++
PGV045	+	–	–	+	+++	–	+++	++	++	+++
PGV085	+	–	–	+	–	+	+++	–	++	–
PGV090	+	–	–	+	–	–	–	–	–	–
PGV094	+	+	–	+	–	–	+++	+++	+++	++
PGV228	+	–	–	+	+	–	++	++	++	++
PGV233	+	–	–	+	–	+	++	++	++	++
PGV284	+	–	–	+	+	–	++	++	++	+++

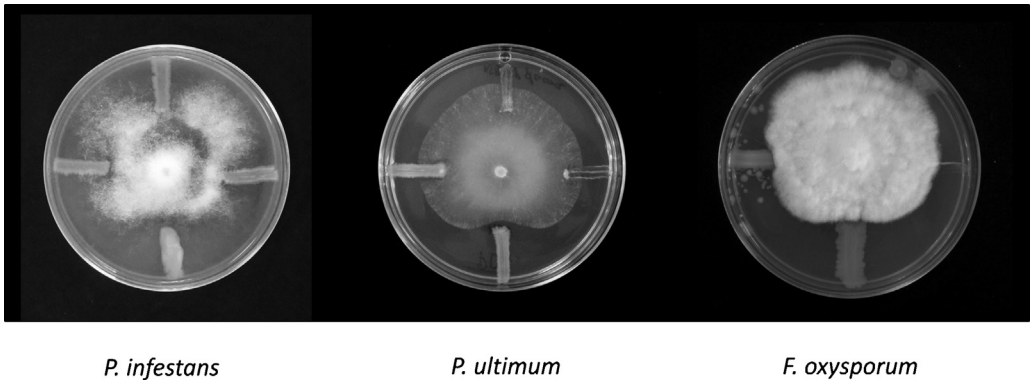

Fig. 4. *Inhibition of phytopathogens by cold-active, P-solubilizing isolates. In vitro* inhibition by CA-PSB of mycelial growth of *P. infestans*, *P. ultimum* and *F. oxysporum* was tested in dual culture assays. Cultures were performed in carrot agar at 20 °C (A) and PDA at 26 °C (B and C). The results were recorded after incubation for 2 day co-cultures (*P. infestans* and *P. ultimum*) and 8 day co-cultures (*F. oxysporum*). From top and clockwise: isolates PGV024, PGV284, PGV094 and PGV085.

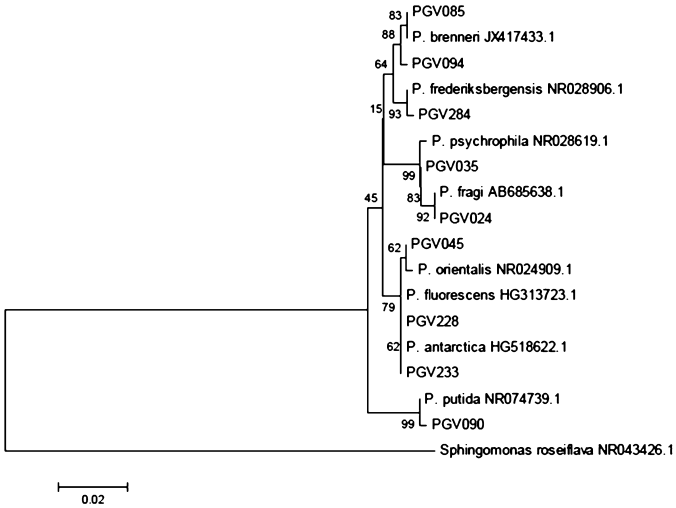

Fig. 5. *Evolutionary relationships of cold-active P-solubilizing isolates inferred using the neighbor-joining method.* The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5.

Even though the preliminary tests suggested that some of our isolates were psychrotolerant, we believe that all of them were – actually – psychrophilic. Indeed, according to the most recent definition proposed by Margesin (2009), the true nature of psychrophily is related to an 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. In fact, although the growth rate of some of our isolates was lower at 10 °C or 15 °C, their cellular yield, estimated indirectly as OD_{600} of the cultures at the stationary phase of growth, was higher at these temperatures. This is a very important feature to have in mind when focusing on the development of potential biofertilizers to be used in cold climates (Trivedi et al., 2012).

Among the PGP traits analyzed here, inorganic-P solubilization is of outstanding importance. Indeed, after N, P is the second most frequently limiting macronutrient for plant growth; although its total amount in the soil may be high, it is often present in unavailable forms like Fe- or Al-phosphates (Schachtman et al., 1998). This emphasizes the beneficial role played by some rhizospheric microorganisms in mobilizing P from these sparingly soluble pools and supplying plant roots with soluble P. As we show here, inorganic-P solubilization mediated by *Pseudomonas* spp. isolates was effective in a wide range of temperatures; strikingly, the solubilization efficiency was maximal at 15 °C. Furthermore, this was shown to depend on the availability of different sugars, with glucose permitting the highest solubilization efficiency. This was not surprising considering that Pseudomonads are well known for oxidizing glucose through a direct oxidation pathway (Lessie and Phibbs, 1984), an alternative aldose-utilization pathway, which is related to the abundant production and excretion of organic acids, mainly gluconic- and keto-gluconic acids (Goldstein, 2007; Rodríguez and Fraga, 1999). The strong acidification of the cell surroundings ends with the efficient solubilization of inorganic phosphates (Rodríguez and Fraga, 1999). In agreement with these findings, release of gluconic acid and acidification of the culture supernatants was also observed for all the tested isolates.

However, we also noticed that other sugars, both substrates and non-substrates of the direct oxidation pathway, permitted P-solubilization at low temperatures. This is a very important finding since microbia colonizing the rhizosphere utilize root exudates as their major nutrient source (Philippot et al., 2013). Indeed, besides including aminoacids, organic acids, and other macromolecules, these exudates are rich in sugars like glucose, sucrose, arabinose, ribose and fructose (Kamilova et al., 2006). It is therefore important to have shown that psychrophilic *Pseudomonas* spp. isolates may use a variety of sugars as C-sources for P-solubilization, a finding that may be relevant when testing the potential biofertilizer use of these strains under field conditions.

Another well documented PGPB characteristic, frequently related to soil fertility and plant nutrition optimization, is the production of bacterial phytohormones like IAA (Glick, 2012). This growth regulator directly affects root primary growth, side root formation and root hairs, having thus a profound physiological effect in plants (Glick, 1995). As shown in the current work, almost all the isolates tested produced IAA which is in accordance to previous reports (Vessey, 2003) and adds to the list of Pseudomonads' positive effects on plant growth.

In addition to their direct PGP abilities, *Pseudomonas* spp. act frequently as biocontrol agents inhibiting plant pathogens, since they excrete hydrolytic enzymes able to degrade cell walls, iron-chelating siderophores, several cyclic lipodepsipeptides (LDP) and a great variety of antibiotics such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin, pyrrolnitrin, and hydrogen cyanide (Raaijmakers et al., 2002). Several of these compounds have strong antagonistic effects against phytopathogenic fungi and have been shown to reduce the need of applying agrochemicals to increase crop yields (Compant et al., 2005). Several of the psychrophilic

Pseudomonas spp. strains tested here were indeed able to produce and excrete some of the aforementioned metabolites and their combined action probably resulted in inhibition of the phytopathogens tested. These results further support the potential use of these isolates as biocontrolers, besides of biofertilizers. We are currently conducting a series of experiments to test the beneficial effect of the PGPB isolates described here on plant growth and development at low temperatures.

5. Conclusions

Psychrophilic bacteria exhibiting remarkable plant-growth promoting traits are immured in tropical glacial ice. As we show here for the first time, these bacteria account for an important fraction of culturable isolates recovered from these extreme environments, which represent a vast reservoir of microbial diversity that deserves to be thoroughly explored and adequately preserved. The potential use of these microbes for the development of environmentally friendly biotechnology products – like cold-active biofertilizers – is, without any doubt, a reality.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2015.05.001

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