Polar Biol (2017) 40:1319–1331
DOI 10.1007/s00300-016-2036-5

ORIGINAL PAPER

Bacterial community structures and ice recrystallization inhibition activity of bacteria isolated from the phyllosphere of the Antarctic vascular plant *Deschampsia antarctica*

Fernanda P. Cid[1,3] · Nitza G. Inostroza[3] · Steffen P. Graether[4] · León A. Bravo[2,3] · Milko A. Jorquera[3]

Received: 24 August 2016 / Revised: 12 September 2016 / Accepted: 12 September 2016 / Published online: 22 September 2016

Abstract Despite the recognized interest in Antarctic bacteria, the relationship between bacteria and Antarctic plants has scarcely been studied. Studies have demonstrated that bacteria in the phyllosphere may contribute to plant growth, but their role in native plants, such as Antarctic vascular plants living in hostile environments, is still unknown. Here we explore the bacterial community structure associated with the phyllosphere of *Deschampsia antarctica*, and evaluate the presence of ice recrystallization inhibition (IRI) activity in crude protein extracts from phyllosphere culturable bacteria. Denaturing gradient gel electrophoresis analysis (16S rRNA genes) showed significant differences in the total bacterial community of eight sampled plants; however, members of *Pseudomonadales* (*Pseudomonas* and *Psychrobacter*) and *Rhizobiales* (*Agrobacterium* and *Aurantimonas*) orders were dominant in all of the analyzed samples. Use of enterobacterial repetitive intergenic consensus polymerase chain reaction technique also revealed a high (>76 %) genetic diversity in 265 isolates from the phyllosphere. With respect to IRI activity, 32 isolates (21 %) showed IRI activity in crude protein extracts from cold-acclimated bacterial cultures, and 5 isolates (3 %) showed IRI activity in crude protein extracts from nonacclimated cultures.

Keywords Antifreeze proteins · Antarctic bacteria · Ice-binding proteins · *Deschampsia antarctica* · Phyllosphere · Ice recrystallization inhibition

✉ Milko A. Jorquera
milko.jorquera@ufrontera.cl

[1] Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Temuco, Chile

[2] Departamento de Ciencias Agronómicas y Recursos Naturales, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Temuco, Chile

[3] Center of Plant, Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Casilla 54D, Temuco, Chile

[4] Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Introduction

The phyllosphere is defined as the aerial part of plant leaves circumscribed by the epidermal cell wall (Hirano and Christen 2000; Borges and Lopes 2008). The phyllosphere is governed by diverse biotic (plant species, plant phenological stages, etc.) and abiotic factors [ultraviolet radiation, temperature, dehydration, humidity, etc.], which can radically change within hours, days, and even seasons, thus the phyllosphere has been categorized as an extreme environment itself (Yang et al. 2001; Lindow and Brandl 2003). The microorganisms that colonize this habitat are known as epiphytes, and bacteria are considered to be the dominant microbial group (Bulgarelli et al. 2013).

In this context, diverse approaches [phospholipid fatty acid, denaturing gradient gel electrophoresis (DGGE), community-level physiological profile, etc.] have reported the occurrence of *Firmicutes* (*Bacillus*) and *Proteobacteria* (*Pseudomonas*) phyla as the dominant bacterial groups in the phyllosphere of vegetables, such as spinach, celery, rape, broccoli, and cauliflower (Zhang et al. 2010). By using high-throughput DNA sequencing (454-pyrosequencing), members of phyla *Proteobacteria* (*Pseudomonas*, *Massilia*, and *Pantoea*), *Firmicutes* (*Bacillus*), *Bacteroidetes* (*Flavobacterium*), and *Actinobacteria* (*Arthrobacter*) were found to be the most represented in the

phyllosphere of field-grown lettuce plants (Rastogi et al. 2012). Nevertheless, our knowledge of the role of and ecological relationship between epiphytic bacterial populations and their plant host is still very limited since the majority of research studies have focused on the rhizosphere followed by the endosphere of plants (Berg et al. 2014). Raja et al. (2008) described that an epiphyte of the *Methylobacterium* strain utilizes methanol produced by cell metabolism of the host plant as an energy source, whereas the plant is favored by phytohormones (auxins) produced by the strain, which stimulate plant growth. Phyllosphere bacteria have also been found to fulfill nitrogen requirements in host plants that have lost contact with the ground, such as the perennial flowering plant *Tillandsia* (Bromeliaceae) (Brighigna et al. 1992). Cyanobacteria and gammaproteobacteria have also been suggested to be the most active diazotrophs in the phyllosphere of the tropical rainforest (*Carludovica drudei*, *Grias cauliflora*, and *Costus laevis*) (Fürnkranz et al. 2008).

Currently, Antarctic bacteria are of interest as producers of biotechnological bioactive compounds (enzymes, antibiotics, pigments, etc.) (Rojas et al. 2009); however, bacteria colonizing the phyllosphere of vascular Antarctic plants have not been investigated in detail so far. In this context, ice-binding proteins (IBPs) produced by bacteria living in cold environments could be potentially applied to diverse biotechnological fields, such as medicine (cryopreservation of cells and cryosurgery), food industry (improving ice-cream and frozen food quality), and agriculture (inhibition of ice nucleation and frost damage in plants) (Cid et al. 2016). IBPs, such as ice-nucleating proteins (INPs) and antifreeze proteins (AFPs), regulate the formation and growth of ice crystals (Davies 2014), and have been found in diverse microorganisms that survive and proliferate under freezing temperatures (Kawahara et al. 2004). AFPs act by binding to ice crystals to bring about thermal hysteresis (TH) and ice recrystallization inhibition (IRI). In the first case, TH is a noncolligative effect defined by a difference of the equilibrium freezing and melting points of a solution (Raymond and DeVries 1977), whereas IRI prevents generation of large ice crystals by boundary migration of smaller ice crystals (Yu et al. 2010). Few studies have purified and characterized AFPs from bacteria. Sun et al. (1995) isolated a *Pseudomonas putida* GR12-2 from the rhizosphere of Canadian High Arctic plants, which secretes an extracellular AFP. Inoculation of spring and winter canola with these AFP-producing strains resulted in an increase in root elongation length at 5 °C (Sun et al. 1995). Recent studies have identified AFPs secreted by epiphytic bacteria from Antarctic moss (Raymond 2015; Davies 2016). However, AFP-producing bacteria in the phyllosphere and their contribution to adaptation and/or protection of vascular plants against freezing temperatures have not been investigated so far.

In the light of these findings, the objective of this study is to explore the bacterial community structure associated with the phyllosphere of *Deschampsia antarctica*, and to look for IRI activity in protein crude extracts from culturable bacteria isolated from its phyllosphere.

Materials and methods

Plant collection

The selection of sampling sites and sample numbers were subject to (1) logistics provided by the Chilean Antarctic Institute (INACH), (2) abundance of plants, and (3) weather conditions. Eight whole plants were collected during February 2014 in the South Shetland Islands as follows: The first three plants (plants 1, 2, and 3) were taken from a rocky area at Gemelos Hill (62°11′45.73″S; 58°59′39.95″W) at height of 40 m above sea level. The plants were separated by no more than 2 m from each other. No more plants were taken from this site given the low amount of plants present at the sampling site. Plants 4, 5, and 6 were taken from Collins Glacier (62°10′9.00″S; 58°51′21.06″W) in areas with different characteristics. Plant 4 was taken from a bird breeding area, 15 m above sea level. Plant 5 was taken from an area highly populated by mosses, 30 m above sea level. Plant 6 was taken from a rocky place, 45 m above sea level. Only one plant (plant 7) was taken from Ardley Peninsula (62°12′36.78″S; 58°56′59.25″W), 10 m above sea level. No more plants were taken from this sandy area also populated by mosses, given the low amount of plants found at this site. Another plant (plant 8) was taken from Hanna Point (62°39′15.64″S; 60°36′51.82″W). This is an area highly covered by *D. antarctica* plants, but only one plant was taken given the bad weather conditions when samples were collected. A map showing these sampling sites is included in Fig. 1. We would like to emphasize that the number of samples and locations were defined at the time according to available resources (time, plant coverage, etc.) and changing climate conditions.

The plants were transported in coolers and processed at the laboratory of the Prof. Julio Escudero Scientific Base of the Chilean Antarctic Institute (INACH), King George Island.

Bacterial community structures

Bacterial community composition was assessed by DGGE as described by Kawai et al. (2002). First, 1 g of *D. antarctica* leaves was cut (aerial parts), gently washed,

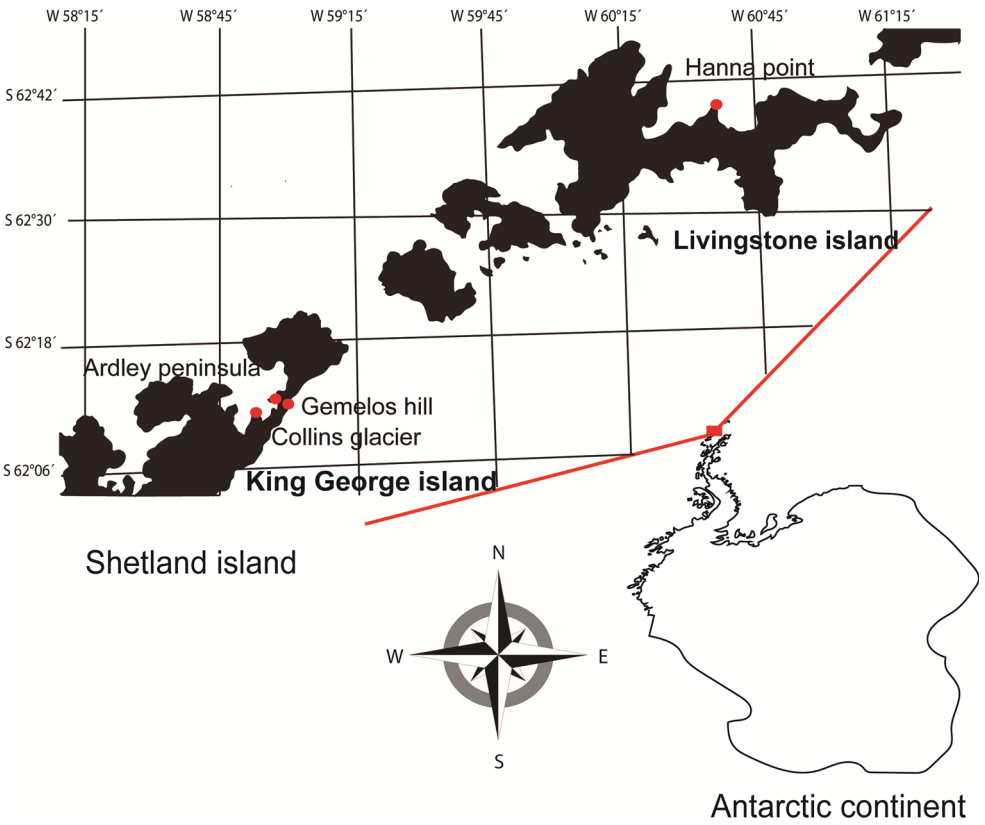

Fig. 1 Map of Antarctic Peninsula. In detail, sampling sites of *D. antarctica* plants (*red circles*) on South Shetland Islands. Plants 1, 2, and 3 were taken from Gemelos Hill (62°11′45.73″S; 58°59′39.95″W). Plants 4, 5, and 6 were taken from Collins Glacier (62°10′9.00″S; 58°51′21.06″W). Plant 7 was taken from Ardley Peninsula (62°12′36.78″S; 58°56′59.25″W). Plant 8 was taken from Hanna Point (62°39′15.64″S; 60°36′51.82″W). (Color figure online)

then vortexed for 10 min in 10 ml sterile saline solution (0.85 % NaCl). Leaves were removed, and the recovered liquid was centrifuged at 15,700×*g* for 10 min to collect detached bacterial cells. To obtain 1 g of leaf, it was necessary to use almost all of the leaves on a single plant. Therefore, the DGGE banding profile was obtained from total DNA extract from a composited sample of several leaves of every single plant sampled. Bacterial cells were suspended in 50 µl sterile distilled water, and this suspension was subsequently frozen in liquid nitrogen and thawed at room temperature three times to break the cells. The samples were then centrifuged at 15,700×*g* for 40 min, and the supernatant (∼40 µL) was used as template DNA in the PCR reaction.

The bacterial 16S rRNA genes (regions V6–V8) were amplified by touchdown PCR with primer set EUBf933–GC/EUBr1387 as described by Iwamoto et al. (2000). The conditions for the PCR reaction were as follows: hot start was performed at 95 °C for 10 min, the annealing was initially set at 65 °C and then decreased by 0.5 °C every cycle and held at 55 °C for 1 min, followed by extension at 72 °C for 3 min. Ten additional annealing cycles were then carried out at 55 °C, followed by denaturation at 94 °C for 1 min and extension at 72 °C for 3 min. The final extension step was done for 7 min at 72 °C. The DGGE runs were performed using the DCode™ universal mutation detection system (Bio-Rad Laboratories Inc., USA). Twenty-microliter aliquots of PCR product were loaded onto a 6 % (w/v) polyacrylamide gel with a 40–65 % denaturing gradient (7 M urea and 40 % formamide). The electrophoresis was run for 12 h at 100 V. The gel was stained with SYBR Gold (Invitrogen™, Thermo Fisher Scientific Inc., USA) for 30 min and photographed on a UV transilluminator (GelDoc-It®TS2 Imager, UVP). Clustering of DGGE banding profiles using a dendrogram was carried out with Phoretix 1D Pro gel analysis software (TotalLab Ltd., UK; http://totallab.com/). Based on the matrix obtained from the Phoretix 1D analysis, differences between bacterial communities were calculated by similarity profile analysis (SIMPROF test) with Bray–Curtis similarity index, 5 % significance level, and <0.1 stress values (Clarke 1993; Clarke et al. 2008), and visualized by nonmetric multidimensional scaling (NMDS) analysis using Primer 6 software (Primer-E Ltd., UK; http://www.primer-e.com/).

A total of 19 representative bands from DGGE gels were carefully excised, purified, reamplified by touchdown PCR, and run again to avoid inclusion of more than one band, to be sent to Macrogen, Inc. (Korea) for sequencing (Fig. 2a). The sequences obtained in this study were compared with those present in the GenBank database (National Center for Biotechnology Information; NCBI) by using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify bacterial groups. More bands were sequenced, however only

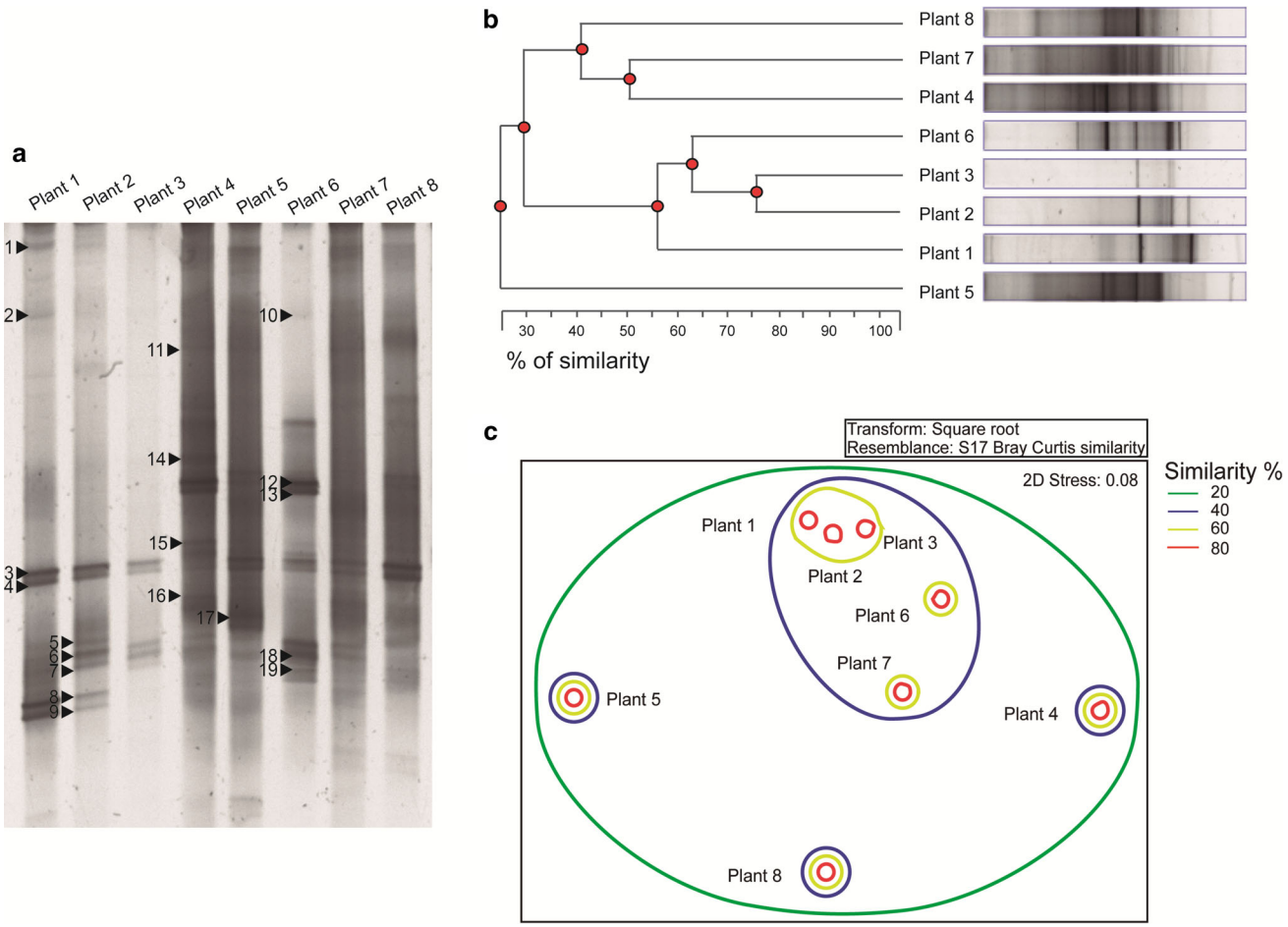

Fig. 2 **a** Denaturing gradient gel electrophoresis (DGGE) banding profile of bacterial communities present in eight plants of the *D. antarctica* phyllosphere. *Arrows* indicate excised and sequenced bands. Analysis of similarity percentages of dendrogram **b** and nonmetric multidimensional scaling **c** of DGGE profiles (16S rRNA gene) from bacterial communities from eight plants of the *D. antarctica* phyllosphere

those having higher similarity % (≥90) are included in Table 1.

Isolation of culturable bacteria

Isolation of bacteria from the *D. antarctica* phyllosphere was carried out by a plating method using four culture media: NM-1 [0.5 g l^{-1} D-glucose, 0.5 g l^{-1} polypeptone, 0.5 g l^{-1} sodium glutamate, 0.5 g l^{-1} yeast extract, 0.44 g l^{-1} KH_2PO_4, 0.1 g l^{-1} $(NH_4)_2SO_4$, 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$, 15 g l^{-1} agar, and 1 ml vitamin solution containing 1 g l^{-1} nicotinamide, 1 g l^{-1} thiamine hydrochloride, 0.05 g l^{-1} biotin, 0.5 g l^{-1} 4-aminobenzoic acid, 0.01 g l^{-1} vitamin B12, 0.5 g l^{-1} D-pantothenic acid hemicalcium salt, 0.5 g l^{-1} pyridoxamine dihydrochloride, 0.5 g l^{-1} folic acid (Nakamura et al. 1995)], R2A (Oxoid Ltd., Thermo Fisher Scientific Inc., UK), *Pseudomonas* (Oxoid Ltd.) and *Actinomyces* (Oxoid Ltd.) agar medium. One gram of *D. antarctica* leaves in 45 ml sterile saline solution (0.85 % NaCl) was vortexed for 10 min to detach the adhered bacteria. Then, dilutions of bacterial suspensions were spread on culture media agar plates in triplicate. The plates were incubated at 13 °C until colony-forming units (cfu) were observed on agar. The value of cfu per cm^2 was also calculated by scanning the leaf's surface area with ImageJ free software (http://imagej.nih.gov/ij/). After 1 week of incubation, 267 single colonies showing diverse phenotypes (color, brightness, form, elevation, and margin; Smibert and Krieg 1994) were transferred to fresh media, purified by streaking on agar, and stored in 3:7 glycerol:LB (10 g l^{-1} D-glucose, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 5 g l^{-1} NaCl) broth at −20 °C for further analysis.

Selection of isolates by genotyping

The enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) technique was used to differentiate colonies with similar phenotype but different

Table 1 Phylogenetic assignment of representative DGGE bands

Band	Taxonomic group[a]	Closest relatives or cloned sequences (accession no.)[b]	Identity (%)	Accession no.
1	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. HC7-3, 16S rRNA gene, partial sequence from Arctic cyanobacterial mats (JF313021)	98.8	KU645377
2	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. TP-Snow-C84, 16S rRNA gene, partial sequence from a culturable bacterium from Tibetan snowpack (KC987014)	97.6	KU645378
3	Proteobacteria, Alphaproteobacteria, Rhizobiales	Uncultured endophytic bacterium clone str. CEA10_5 from *Typha angustifolia* roots (HM142813)	98	KU645379
4	Proteobacteria, Alphaproteobacteria, Rhizobiales	*Agrobacterium* sp. BE516, partial 16S RNA gene, isolated from root of *Miscanthus sacchariflorus* (JQ764998)	99.8	KU645380
5	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas migulae* strain SHZ1.3, partial 16S rRNA gene, isolated from Arctic lake sediments (KP236597)	97.3	KU645381
6	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. RC7.6, partial 16S rRNA gene, isolated from *Caesalpinia spinosa* rhizosphere (KP267840)	98.8	KU645382
7	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. St29 DNA complete genome, isolated from *Solanum tuberosum* rhizosphere (AP014628)	100	KU645383
8	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. str. UMAB-64, partial 16S rRNA gene, isolated from Antarctic soil (KF263636)	96	KU645384
9	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. HA72, partial 16S rRNA gene, isolated from subarctic Alaska grasslands (KF011656)	96.1	KU645385
10	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. 39A_2, partial 16S rRNA gene, an Antarctic bacterium (KC912628)	97.8	KU645386
11	Proteobacteria, Gammaproteobacteria, Pseudomonadales	Uncultured *Pseudomonas* sp. clone BBS8w52, partial 16S rRNA gene, obtained from beech and spruce litter (AY682157)	91.6	KU645387
12	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Psychrobacter* sp. MVS1-N6, partial 16S rRNA gene, isolated from Antarctic soil (KR023910)	97	KU645388
13	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Psychrobacter* sp. SCS3-N7, partial 16S rRNA gene, isolated from Antarctic soil (KR023909)	95.9	KU645389
14	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. NSJ-3, partial 16S rRNA gene, partial sequence from a cotton endophytic bacterium (FJ941082)	90.3	KU645390
15	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. B7E, partial 16S rRNA gene. Bacterium isolated from Antarctica (KC433647)	97.7	KU645391
16	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas frederiksbergensis* strain BDR1P1B2, partial 16S rRNA gene isolated from *Zingiber* sp. rhizosphere soil (KJ567114)	99.8	KU645392
17	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. HX32, partial 16S rRNA gene, isolated from alpine grassland soil (EF601817)	95.9	KU645393
18	Proteobacteria, Gammaproteobacteria, Pseudomonadales	*Pseudomonas* sp. RaSa70e, partial 16S rRNA gene, endophyte of *Arabidopsis thaliana* (LN830927)	99.8	KU645394
19	Proteobacteria, Alphaproteobacteria, Rhizobiales	*Aurantimonas* sp. str. FB13, from Arctic and Antarctic soil (AM933504)	93	KU645395

[a] Phylogenetic assignment based on nonredundant GenBank database from NCBI (http://www.ncbi.nlm.nih.gov) or Ribosomal Database Project (http://rdp.cme.msu.edu/classifier) prediction. Ribosomal database gives the phylum as well as the lowest predictable phylogenetic rank

[b] Based on partial sequencing of the 16S rRNA gene and comparison with those present in GenBank by using Blastn and Megablast

Table 2 Bacterial counts (cfu g^{-1} of leaf), ERIC-PCR genotyping, and ice recrystallization inhibition (IRI) activity of culturable bacteria isolated from the *Deschampsia antarctica* phyllosphere

	Growth medium				
	NM1[a]	R2A	PA	AA	Total
Colony-forming units (cfu × 10^6 g^{-1} of leaf)	6.54 ± 0.75[b]	6.65 ± 0.77	4.70 ± 0.63	3.57 ± 0.33	
Total number of isolates	89	81	49	46	265
Isolates with different genotypes by ERIC-PCR	78 (88 %)[c]	74 (91 %)	37 (76 %)	38 (83 %)	227 (85 %)
IRI[c] positives after first screening	23 (29 %)	n.d.	8 (22 %)	1 (3 %)	32 (21 %)
IRI positives without cold acclimatization	2 (3 %)	n.d.	2 (5 %)	1 (3 %)	5 (3 %)

n.d. Not determined

[a] *NM1* Culture medium NM-1 agar, *R2A* Reasoners 2A agar, *PA Pseudomonas* agar, and *AA Actinomyces* agar

[b] The values represent the average ($n = 3$) and standard errors

[c] Percentages are relative to the total number of isolates obtained in each culture medium

genotype, following the protocol described by Houf et al. (2002). Briefly, crude DNA extracts from samples were obtained after boiling bacterial cell suspension followed by a quick spin down to discard cell debris. Supernatant at concentration of 50 ng DNA μl^{-1} was used in the PCR mixture. DNA fragments were amplified by PCR using the primer set ERIC motifs 1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′). The PCR conditions were as follows: hot start at 94 °C for 5 min, followed by 40 cycles of denaturing at temperature of 94 °C for 1 min and annealing at temperature of 25 °C for 1 min, and extension at temperature of 72 °C for 2 min. The final extension step was set at 72 °C for 7 min. PCR products were then run on a 2 % agarose gel at 100 V for 1 h and stained with ethidium bromide. Electrophoretic gels were photographed, and the images were analyzed using Phoretix 1D Pro gel analysis software. The isolates that showed different banding profiles were considered genetically different and used for further analysis.

Ice recrystallization inhibition (IRI) detection

Detection of IRI was based on the behavior of ice at subzero temperatures. Ice recrystallization and the consequent ice crystal growth is visually different with higher transparency, whereas in IRI, ice crystals remain at small size, being visually more turbid (Gilbert et al. 2004; Budke et al. 2009). Given these differences in turbidity, ice recrystallization inhibition effect was measured in this work by a spectrophotometric method. The details of IRI detection theory are given in Fig. 4a. Absorbance differences were determined using a microtiter plate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Inc., USA). First, the most sensitive absorbance to detect IRI under our conditions was determined by wavelength (λ) scanning from 300 to 650 nm using type III AFP (A/F Protein, Inc., USA) and bovine serum albumin (BSA) as positive and negative protein control, respectively. Detection of IRI was assayed with both pure proteins at final concentration of 0.04 mg ml^{-1} in 30 % sucrose solution according to the protocol described by Gilbert et al. (2004). The microtiter plate was frozen at −80 °C for 15 min and incubated for 2 days at −6 °C before reading the absorbance in a spectrophotometer. Microtiter plate wells with 30 % sucrose solution were used as blanks. The detection of IRI was assayed with different total protein concentrations ranging from 0.01 to 2 mg ml^{-1}.

Screening for IRI in isolates

Based on our results of IRI detection with different wavelengths and protein concentrations, the screening of IRI detection by spectrophotometry was standardized at λ of 500 nm using crude extracts with protein concentrations from 0.01 to 2 mg ml^{-1}. Antarctic bacteria isolates were grown in LB broth at 15 °C for 1 week, then cold acclimatized at 4 °C for 1 week. The cultures were then centrifuged at 5000×*g* for 10 min, and soluble proteins from the pellets were extracted by using B-PER bacterial protein extraction reagent (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. The protein concentrations in crude extracts were determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., USA), and IRI detection was carried out as described above. Type III AFP and BSA were used in each microtiter plate reading to verify that IRI detection was working correctly. Crude protein extracts from *Escherichia coli* JM109 were used as negative control for IRI screening. *E. coli* JM109 was chosen because its genome information in the

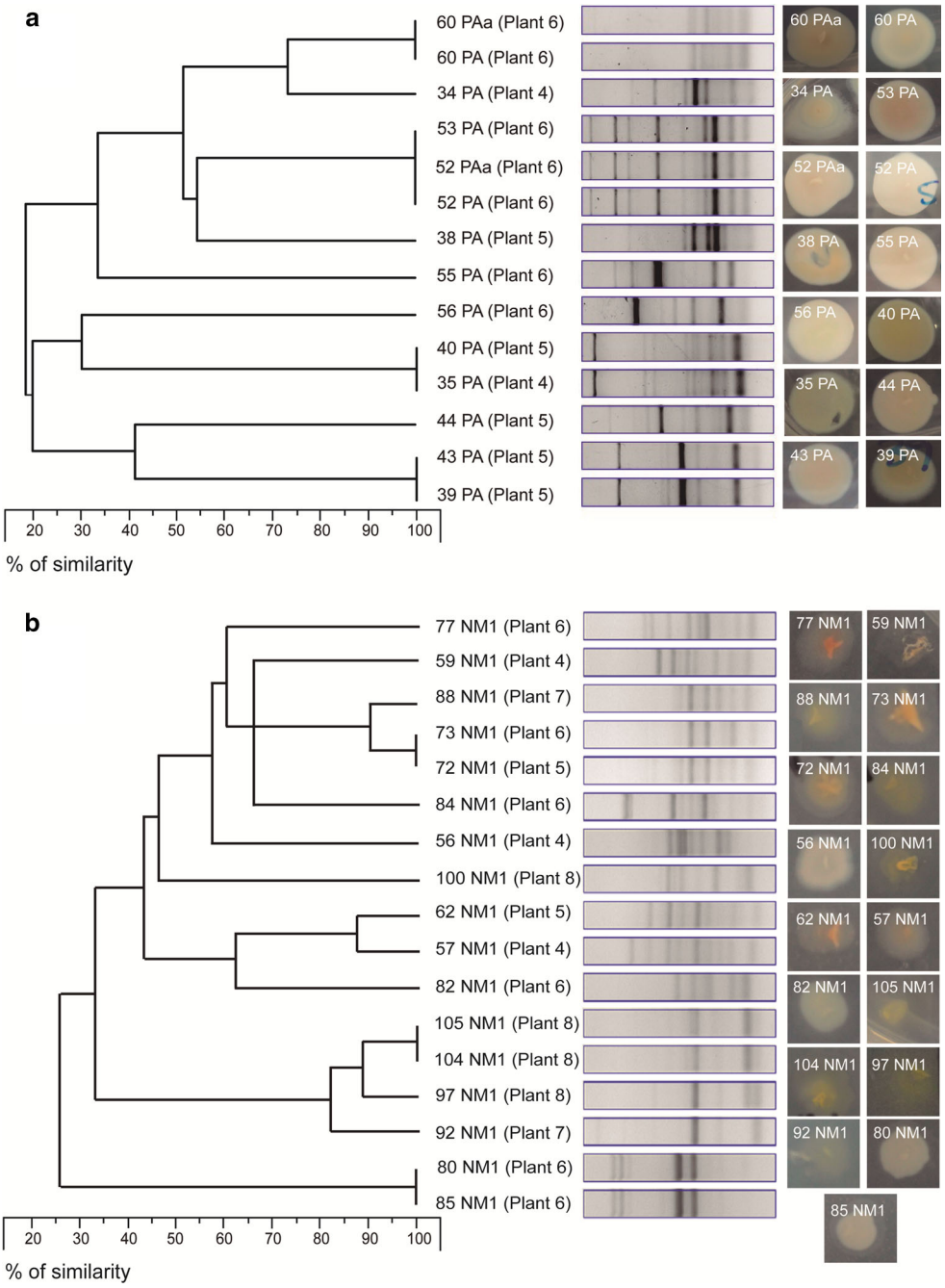

Fig. 3 Dendrogram of ERIC-PCR genotyping of culturable bacteria from the *D. antarctica* phyllosphere. **a** Isolates grown in *Pseudomonas* agar (PA) plates and **b** grown in NM1 culture medium

GenBank database did not include any genetic traits associated with IBPs. This was also confirmed in our laboratory, where fresh LB cultures were exposed to −20 °C for 24 h. Before and after freezing, serial dilutions (from 10^{-1} to 10^{-7}) of *E. coli* JM109 culture plated on LB agar plates did not grow. Isolates from the *D. antarctica* phyllosphere were also included in this assay to compare strain growth after freezing exposure.

Samples with statistically higher absorbance (see below) compared with *E. coli* JM109 were considered as active isolates for IRI. These IRI-active isolates were repeatedly screened to confirm IRI activity, and those isolates which always showed higher absorbance compared with *E. coli* JM109 were selected for additional assays for IRI detection without cold acclimation.

Statistical analysis

Absorbance data were analyzed by Dunnett multiple comparisons (Dunnett 1955). Means and standard errors

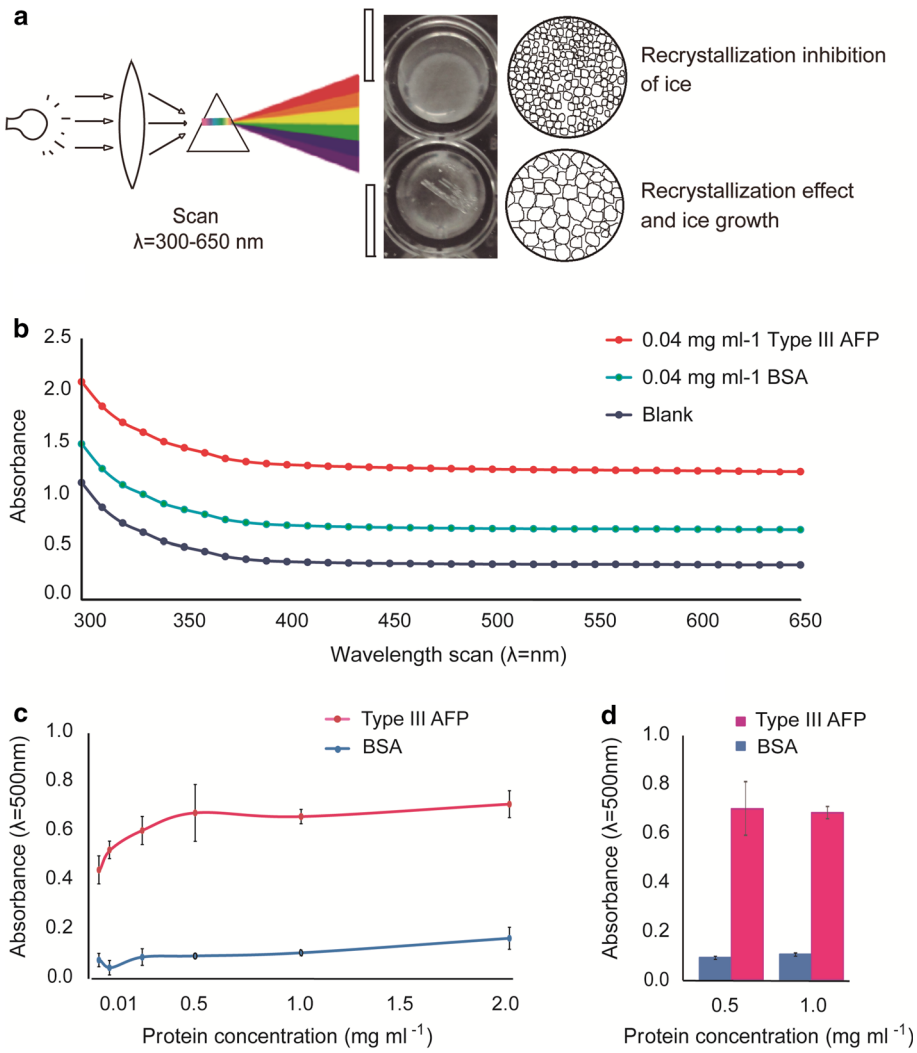

Fig. 4 Standardization of detection of ice recrystallization inhibition (IRI) in culturable bacteria of the *Deschampsia antarctica* phyllosphere. **a** Background theory, **b** absorbance at different wavelengths (from 300 to 650 nm), **c** and **d** absorbance at 500 nm at different BSA and AFP protein concentrations (from 0.01 to 2.0 mg ml^{-1}). AFP: antifreeze protein in 30 % sucrose solution; BSA: bovine serum albumin in 30 % sucrose solution; Blank: 30 % sucrose solution

were calculated in quadruplicate, and difference at $p \leq 0.05$ was considered significant. Statistical analyses were conducted using IBM SPSS 21 software.

Results

Bacterial community structure

The results obtained from the DGGE analysis and visualized with a dendrogram showed that the bacterial community in the *D. antarctica* phyllosphere was mainly distributed in two clusters (Fig. 2b). One of the clusters showed higher similarity (>57 %) among plants collected at Gemelos Hill (plants 1, 2, and 3), sharing some bands with plant 6 collected at Collins Glacier, whereas the second cluster with lower similarity (>40 %) was obtained from plants taken from Collins Glacier (plant 4), Ardley Peninsula (plant 7), and Hanna Point (plant 8). The most dissimilar bacterial community structure was observed for plant 5, collected from Collins Glacier. Differences between bacterial communities were also visualized by nonmetric multidimensional scaling (NMDS) analysis (Fig. 2c), where only 40 % similarity was found among plants collected from different sampling sites (plants 1, 2, 3, 6, and 7) and only 20 % similarity for all samples studied.

Despite this, significant differences between bacterial communities were observed among collected plants: sequencing of representative dominant bands on DGGE gels revealed that most members belonged to the *Pseudomonadales* order, followed by less abundant bands represented by the *Rhizobiales* order (Table 1). Coincidently, the closest relative sequences deposited in the GenBank database are associated with bacteria from Arctic and Antarctic ecosystems, while others were also reported to be related to plant rhizosphere habitats. The sequences obtained in this study were deposited in GenBank database under accession numbers from KU645377 to KU645395.

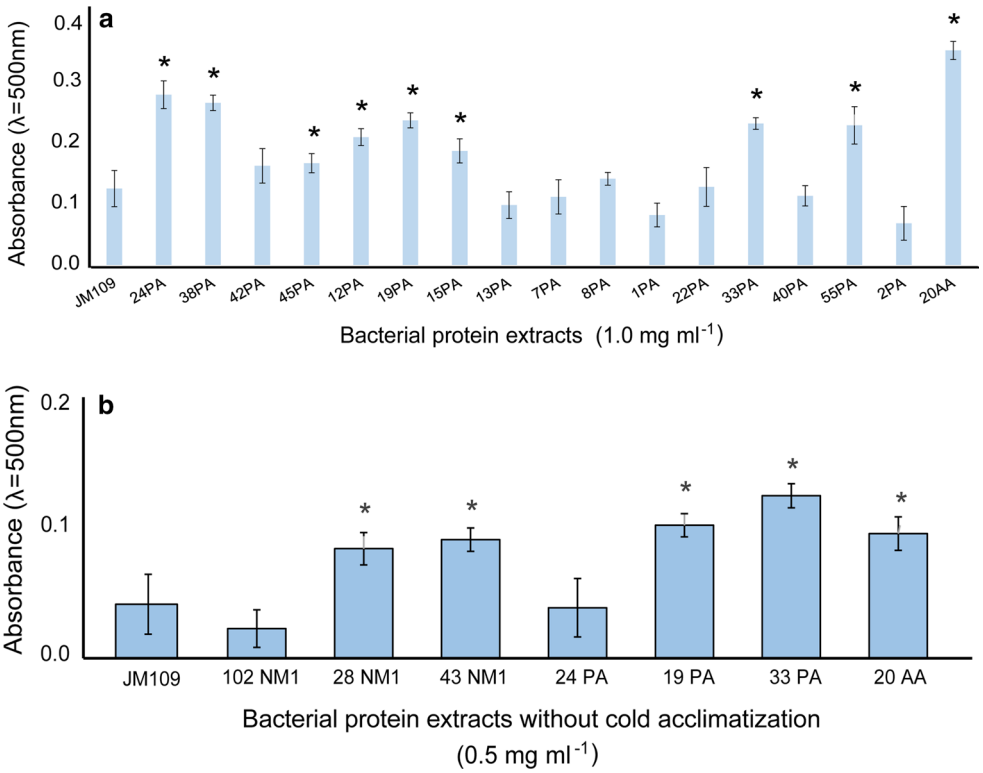

Fig. 5 Ice recrystallization inhibition in the phyllosphere: **a** Screening of ice recrystallization inhibition (IRI) activity in crude protein extract (1 mg ml^{-1}) from bacteria isolated from the *D. antarctica* phyllosphere using *Pseudomonas* and *Actinomyces* agar plates. **b** Detection of IRI activity in crude protein extract (0.5 mg ml^{-1}) from bacteria grown without cold temperature acclimatization. *Asterisks* denote significant ($p \leq 0.05$, Dunnett's test) differences in comparison with crude protein extract of *E. coli* JM109 (negative control)

Isolation and genotyping of culturable bacteria

As shown in Table 2, bacterial counts in the *D. antarctica* phyllosphere revealed bacterial loads ranging from 3.6×10^6 to 6.7×10^6 cfu g^{-1} of leaf, equivalent to 3.6×10^4 to 6.7×10^4 cfu cm^{-2}. A total of 265 isolates were obtained with high genetic variability (from 76 to 91 %) as revealed by ERIC-PCR genotyping (Table 2; Fig. 3). It is noteworthy that the same genotypes were found in different plants; however, this occurred in bacterial isolated from plants sampled at the same site, such as plants 4, 5, and 6, all obtained from Collins Glacier (Fig. 3).

Detection and screening for IRI in isolates

During standardization of the protocol for the detection of IRI activity, our results showed differences in absorbance between the positive (type III AFP) and negative (BSA) controls at the different tested wavelengths (from 300 to 650 nm) (Fig. 4b). These differences in absorbance between controls were observed at 500 nm using different protein concentrations (from 0.01 to 2.0 mg ml^{-1}) (Fig. 4c). The screening for IRI activity was carried out at 500 nm, given that this was the wavelength in the middle of the plateau region, as presented in Fig. 4b.

Compared with crude protein extract from *E. coli* JM109, the screening for IRI activity showed significantly higher absorbance ($p \leq 0.05$) for protein extracts obtained from 32 isolates; therefore, 21 % of isolates were considered as IRI active. The screening was repeated to confirm IRI activity in positive isolates. These data are presented in Fig. 5a and summarized in Table 2. However, when IRI activity was screened without cold acclimation, to explore whether IRI was induced by low temperature or was a constitutive phenotype of selected isolates, only five (3 %) isolates (strains 28 NM1, 43 NM1, 19 PA, 33 PA, and 20 AA) showed significantly higher absorbance ($p \leq 0.05$) compared with *E. coli* JM109 protein extract (Fig. 5b).

Discussion

The profiles obtained by DGGE (16S rRNA gene) analysis revealed differences in bacterial communities in the *D. antarctica* phyllosphere, which varied among the different plants and collection sites. Variations of bacterial communities in the phyllosphere between different vegetables (spinach, celery, rape, broccoli, and cauliflower) have previously been described by Yang et al. (2001). High variability was found in bacterial community composition in forest, among *Pinus* trees, as described by pyrosequencing (16S rRNA gene) (Redford et al. 2010). High diversity of bacterial composition was also detected in the phyllosphere of the leaf canopy of a tropical Atlantic forest (Lambais et al. 2006). One of the more intriguing findings

of this study is that the microbial communities varied substantially between different locations, but were more similar between plants at the Gemelos Hill location, sharing higher similitudes with plant 6 sampled from a rocky place at Collins Glacier. Some theories postulate that environments select their colonizers, meaning that local conditions regulate the abundance, composition, and activity of the inhabitants (Perfumo and Marchant 2010). In a controlled greenhouse experiment, the spatial distribution of *Arabidopsis thaliana* plants was found to affect the dispersion of bacteria on plants, showing similar bacterial community structure in closer plants (Maignien et al. 2014). Our results agree with this finding; however, more studies are required for confirmation. In this sense, considering that the majority of the Antarctic Continent is covered by ice, less than 1 % of the land, mostly in the Antarctic Peninsula and coastal areas, is available for plant colonization (Alberdi et al. 2002). Oceanic winds, rain, hailstones, and snow could be the most important mechanisms for dispersion of microorganisms in this area; For example, Santl-Temkiv et al. (2013) demonstrated that hailstone samples were dominated mostly by plant-surface bacteria, thus those authors hypothesized that adaptations to life in the phyllosphere favor survival of airborne bacteria, affecting long-distance transport and the spatial distribution of bacteria on Earth. In other ecosystems such as desert, dispersion of bacteria is a natural phenomenon conducted by bacteria attached to dust soil particles, contributing to the diversity of downwind ecosystems (Yamaguchi et al. 2012). Another mechanism for bacterial transport is local fauna such as skuas and gulls, to whose nests this plant has often been found to be attached (Gerighausen et al. 2003). In addition, differences in bacterial communities in the phyllosphere could also be dependent on the location and morphology of the leaves, phenological status of plants, and amount of carbon-containing exudates released by leaf plants and used by bacteria as a nutrient source (Wilson and Lindow 1994; Yang et al. 2001).

Despite differences in bacterial community composition in the phyllosphere, the sequencing of dominant DGGE bands revealed the presence of members of *Pseudomonadales* (*Pseudomonas* and *Psychrobacter*) and *Rhizobiales* (*Agrobacterium* and *Aurantimonas*) orders. *Pseudomonadales* is a large taxonomic bacterial group that is metabolically versatile and collectively exhibits a high diversity of activities such as nutrient cycling, degradation of xenobiotic organic compounds, and growth promotion and pathogen protection of plants (Redford et al. 2010; Loeschcke and Thies 2015). By using molecular approaches (DGGE and 454-pyrosequencing), *Pseudomonadales* have been reported as a dominant inhabitant in the phyllosphere of different plants such as spinach, celery, rape, broccoli, cauliflower, and *Arabidopsis thaliana* (Bodenhausen et al. 2013). *Pseudomonadales* are widely described as a common inhabitant of soil; therefore, we cannot discard the soil as a source of phyllosphere colonizers (mentioned above). *Rhizobiales* is also a metabolically versatile soil bacterial group characterized by conversion of atmospheric nitrogen to ammonia, to the benefit of the host plant. *Rhizobiales* are commonly found in the phyllosphere of rice (*Oryza* spp.) and perennial herbaceous plants (*Typha angustifolia*) by metaproteogenomic analysis and 16S rDNA gene sequencing (Li et al. 2011; Knief et al. 2012; Banik et al. 2015). Both bacterial groups are also frequently isolated from Antarctic and Arctic soils (Goh and Tan 2012; Park and Kim 2015). It is noteworthy that the sequencing of dominant DGGE bands also revealed the presence of *Psychrobacter*. *Psychrobacter arcticus* is a cold-adapted bacteria used as a model for psychrophilic "cold-shock" proteins, proposed as key for life at subzero temperatures (Kuhn 2012).

In samples from the *D. antarctica* phyllosphere, our counts ranged from 3.6×10^6 to 6.7×10^6 cfu g^{-1} of leaf, equivalent to 3.6×10^4 to 6.7×10^4 cfu cm^{-2}. The bacterial loads found in the *D. antarctica* phyllosphere seem to be similar to some plants from other climate zones. Variable bacterial loads have been reported for different plant species, such as olive plants [from 1.7×10^4 to 3.4×10^5 cfu cm^{-2} (Ercolani 1991), Mediterranean perennial species (from 1.3×10^4 to 1.4×10^7 cfu g^{-1} (Yadav et al. 2004)], evergreen subtropical forest trees [from 7.41×10^2 to 3.02×10^5 cfu g^{-1} (Yadav et al. 2013)], and common beans [from 10^6 to 10^7 cfu cm^{-2} (Hirano and Upper 1989)]. Other authors have suggested that differences in bacterial counts could be explained mostly by the distance of leaves to the soil, as a direct source of epiphytic bacteria, and environmental factors, such as relative humidity, especially in water-saturated soil conditions (Kinkel et al. 2000).

Similar to DGGE analysis, ERIC-PCR genotyping also suggests high genetic variability (from 76 to 91 %) among the culturable bacteria from *D. antarctica*. In addition, this result helped us to differentiate colonies with similar phenotype but different genotype. Interestingly, the same genotypes determined by ERIC-PCR were found in plants sampled from the same location, but not among plants from different sampling sites. The latter result could be explained by the fact that vegetative reproduction is the most frequent and reliable means of dispersion in *D. antarctica* plants (Smith 2003). Other authors have postulated that the occurrence of bacteria with the same genotype in different plants could be attributed to migration of bacteria from neighboring plants and/or plant debris transported by wind, insects, and other animal sources (Whipps et al. 2008; Vokou et al. 2012). In addition, numerous phyllosphere bacteria have been associated with

seeds, with the ability to grow on seedlings and leaves as the plant grows (Hirano and Christen 2000), thus seeds could also be considered vectors for specific bacterial genotype transference in this type of ecosystem; however this statement has not been evaluated in extreme environments such as Antarctica.

With regards to screening for IRI activity, a total of 32 (21 %) crude protein extracts from phyllosphere isolates showed significantly higher absorbance compared with protein extract from *E. coli* JM109. Ice recrystallization inhibition has been shown to be a functional and cost-effective mechanism for preliminary detection of AFP activity within a solution (Capicciotti et al. 2013). Moreover, the percentage obtained of active IRI isolates (21 %) is higher than that previously described by Gilbert et al. (2004) (10 %) for putative AFP-producing isolates from an Antarctic lake. However, the presence of IRI activity in culturable bacteria does not mean that these are the dominant group in the bacterial community structures in the phyllosphere of *D. antarctica*, and such activity does not ensure that bacterial IRI activity is expressed on leaf surfaces. In addition, as reported in other studies, the IRI effect has mostly been related to freezing tolerance, as it maintains ice crystals at a small, likely harmless size (Do et al. 2014). A different theory points out that proteins secreted into the surrounding environment have IRI activity that favors formation of water channels (water pockets) through which bacteria could obtain nutrients and divide (Raymond 2011; Raymond and Kim 2012). In this context, it has been proposed that the repeated occurrence of the DUF3494 sequence (coding a protein of unknown function) in the genome of psychrophilic organisms (bacteria, archaea, and eukaryotes) is correlated with IRI activity, and that it can be transferred between prokaryotes and eukaryotes by horizontal gene transfer (Raymond 2014). The discovery of epiphytic bacteria coding the DUF3494 domain in metagenomic analysis conducted in Antarctic nonvascular plants (mosses) suggests that this activity might favor a commensal relationship between the moss and epiphytic bacteria, where the latter receives sustenance and the former freezing protection, nevertheless this statement has not been empirically proven (Raymond 2015).

Detection of IRI activity in crude protein extracts from bacteria grown without cold acclimatization was also observed (Fig. 5b). The constitutive expression of AFP by bacteria could provide an immediate benefit during sudden harsh local conditions, such as freezing; however, constitutive protein expression represents a permanent metabolic cost for cells, as discussed by Geisel (2011).

Finally, further studies are required to elucidate factors influencing the diversity and abundance of bacteria in the phyllosphere of Antarctic vascular plants and their activity under freezing temperature, such as production of ice-binding proteins. This information could be pivotal for our understanding on Antarctic ecosystems and the physiological response of Antarctic vascular plants to tolerate freezing temperature and climate change (Cavieres et al. 2016).

Acknowledgments We thank the reviewers for their contributions that improved the quality of this work. This study was financed by international cooperation project Conicyt-USA code USA2013-0010. F.P.C. thanks the Antarctic Chilean Institute for a doctor scholarship (code DT_01-13) and for the permits given by them to collect plants in Antarctic protected areas no. 125, 126, and 150, Conicyt for a doctor scholarship (no. 21140534), and La Frontera University for a scholarship.

References

Alberdi M, Bravo LA, Gutiérrez A et al (2002) Ecophysiology of Antarctic vascular plants. Physiol Plant 115:479–486

Banik A, Mukhopadhaya S, Dangar T (2015) Characterization of N2-fixing plant growth promoting endophytic and epiphytic bacterial community of Indian cultivated and wild rice (Oryza spp.) genotypes. Planta 243:799–812

Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. Front Microbiol 5:1–7

Bodenhausen N, Horton M, Bergelson J (2013) Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One 8:1–9

Borges LE, Lopes F (2008) Phylloepiphytic interaction between bacteria and different plant species in a tropical agricultural system. Can J Microbiol 54:918–931

Brighigna L, Montaini P, Favilli F, Cabarez A (1992) Role of the nitrogen-fixing bacterial microflora in the epiphytism of Tillandsia (Bromeliaceae). Am J Bot 79:723–727

Budke C, Heggemann C, Koch M et al (2009) Ice recrystallization kinetics in the presence of synthetic antifreeze glycoprotein analogues using the framework of LSW theory. J Phys Chem 113:2865–2873

Bulgarelli D, Schlaeppi K, Spaepen S et al (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838

Capicciotti CJ, Doshi M, Ben RN (2013) Ice recrystallization inhibitors: from biological antifreezes to small molecules. In: Wilson P (ed) Recent developments in the study of recrystallization. InTech, Rijeka-Croatia, pp 177–224

Cavieres L, Sáez P, Sanhueza C et al (2016) Ecophysiological traits of Antarctic vascular plants: their importance in the responses to climate change. Plant Ecol 217:343–358

Cid FP, Rilling JI, Graether SP et al (2016) Properties and biotechnological applications of ice binding proteins in bacteria. FEMS Microbiol Ecol 363:1–12

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143

Clarke RK, Somerfield PJ, Gorley RN (2008) Testing of null hypotheses in exploratory community analyses: similarity profiles and biota-environment linkage. J Exp Mar Biol Ecol 366:56–69

Davies PL (2014) Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth. Trends Biochem Sci 39:548–555

Davies PL (2016) Antarctic moss is home to many epiphytic bacteria that secrete antifreeze proteins. Environ Microbiol Rep 8:1–2

Do H, Kim S-J, Kim H, Lee J (2014) Structure-based characterization and antifreeze properties of a hyperactive ice-binding protein from the Antarctic bacterium Flavobacterium frigoris PS1. Biol Crystallogr 70:1061–1073

Dunnett CW (1955) A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 50:1096–1121

Ercolani G (1991) Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microb Ecol 21:35–48

Fürnkranz M, Wanek W, Richter A et al (2008) Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. ISME J 2:561–570

Geisel N (2011) Constitutive versus responsive gene expression strategies for growth in changing environments. PLoS One 6: e27033

Gerighausen U, Brautigam K, Mustafa O, Hans-Ulrich P (2003) Expansion of vascular plants on an Antarctic island a consequence of climate change? In: Huiskes AHL, Gieskes WWC, Rozema J et al (eds) Antarctic biology in a global context. Blackhuys, Leiden, pp 79–83

Gilbert JA, Hill PJ, Dodd CER, Laybourn-Parry J (2004) Demonstration of antifreeze protein activity in Antarctic lake bacteria. Microbiology 150:171–180

Goh YS, Tan IKP (2012) Polyhydroxyalkanoate production by Antarctic soil bacteria isolated from Casey station and Signy island. Microbiol Res 167:211–219

Hirano SS, Christen DU (2000) Bacteria in the leaf ecosystem with emphasis on Pseudomonas syringae-a pathogen, ice nucleus, and epiphyte. Microbiol Mol Biol Rev 64:624–653

Hirano S, Upper C (1989) Diel variation in population size and ice nucleation activity of Pseudomonas syringae on snap bean leaflets. Appl Environ Microbiol 55:623–630

Houf K, Zutter L, Van Hoof J, Vandamme P (2002) Assessment of the genetic diversity among arcobacters isolated from poultry products by using two PCR-based typing methods. Appl Environ Microbiol 68:2172–2178

Iwamoto T, Tani K, Nakamura K et al (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol Ecol 32:129–141

Kawahara H, Nakano Y, Omiya K et al (2004) Production of two types of ice crystal-controlling proteins in Antarctic bacterium. J Biosci Bioeng 98:220–223

Kawai M, Matsutera E, Kanda H et al (2002) 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 68:699–704

Kinkel L, Wilson M, Lindow S (2000) Plant species and plant incubation conditions influence variability in epiphytic bacterial population size. Microb Ecol 39:1–11

Knief C, Delmotte N, Chaffron S et al (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 6:1378–1390

Kuhn E (2012) Toward understanding life under subzero conditions: the significance of exploring psychrophilic "cold-shock" proteins. Astrobiology 12:1078–1086

Lambais MR, Crowley DE, Cury JC et al (2006) Bacterial diversity in tree canopies of the Atlantic forest. Science 312:1917

Li YH, Liu QF, Liu Y et al (2011) Endophytic bacterial diversity in roots of Typha angustifolia L. in the constructed Beijing Cuihu Wetland (China). Res Microbiol 162:124–131

Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883

Loeschcke A, Thies S (2015) Pseudomonas putida—a versatile host for the production of natural products. Appl Microbiol Biotechnol 99:6197–6214

Maignien L, DeForce EA, Chafee ME et al (2014) Ecological succession and stochastic variation in the assembly of Arabidopsis thaliana phyllosphere communities. MBio 5:1–10

Nakamura K, Hiraishi A, Yoshimi Y et al (1995) Microlunatus phosphovorus gen. nov., sp. nov., a new gram-positive polyphosphate-accumulating bacterium isolated from activated sludge. Int J Syst Bacteriol 45:17–22

Park H, Kim D (2015) Isolation and characterization of humic substances-degrading bacteria from the subarctic Alaska grasslands. J Basic Microbiol 55:54–61

Perfumo A, Marchant R (2010) Global transport of thermophilic bacteria in atmospheric dust. Environ Microbiol Rep 2:333–339

Raja P, Balachandar D, Sundaram SP (2008) PCR fingerprinting for identification and discrimination of plant-associated facultative methylobacteria. Indian J Biotechnol 7:508–514

Rastogi G, Sbodio A, Tech JJ et al (2012) Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J 6:1812–1822

Raymond J (2011) Algal ice-binding proteins change the structure of sea ice. Proc Natl Acad Sci 108:E198

Raymond J (2014) The ice-binding proteins of a snow alga, Chloromonas brevispina: probable acquisition by horizontal gene transfer. Extremophiles 18:987–994

Raymond J (2015) Dependence on epiphytic bacteria for freezing protection in an Antarctic moss, Bryum argenteum. Environ Microbiol Rep 8:14–19

Raymond JA, DeVries AL (1977) Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc Natl Acad Sci USA 74:2589–2593

Raymond J, Kim H (2012) Possible role of horizontal gene transfer in the colonization of sea ice by algae. PLoS One 7:e35968

Redford AJ, Bowers RM, Knight R et al (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environ Microbiol 12:2885–2893

Rojas JL, Martín J, Tormo JR et al (2009) Bacterial diversity from benthic mats of Antarctic lakes as a source of new bioactive metabolites. Mar Genom 2:33–41

Santl-Temkiv T, Finster K, Dittmar T et al (2013) Hailstones: a window into the microbial and chemical inventory of a storm cloud. PLoS One 8:e53550

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhard P, Murray RGE, Wood WA, Kreig NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington D.C., pp 607–654

Smith R (2003) The enigma of Colobanthus quitensis and Deschampsia antarctica in Antarctica. In: Huiskes A, Gieskes W, Rozema J et al (eds) Antarctic biology in a global context. Blackhuys, Leiden, pp 234–239

Sun X, Griffith M, Pasternak JJ, Glick BR (1995) Low temperature growth, freezing survival, and production of antifreeze protein by the plant growth promoting rhizobacterium Pseudomonas putida GR12-2. Can J Microbiol 41:776–784

Vokou D, Vareli K, Zarali E et al (2012) Exploring biodiversity in the bacterial community of the Mediterranean phyllosphere and its relationship with airborne bacteria. Microb Ecol 64:714–724

Whipps JM, Hand P, Pink D, Bending GD (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. J Appl Microbiol 105:1744–1755

Wilson M, Lindow SE (1994) Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl Environ Microbiol 60:4468–4477

Yadav R, Halley J, Karamanoli K et al (2004) Bacterial populations on the leaves of Mediterranean plants: quantitative features and testing of distribution models. Environ Exp Bot 52:63–77

Yadav RKP, Shrestha S, Pokhrel CP, Jha PK (2013) Phyllosphere bacterial population of woody species in subtropical forest at Shivapuri-Nagarjun National Park, Nepal Himalaya. Int J Life Sci Med Res 3:210–219

Yamaguchi N, Ichijo T, Sakotani A et al (2012) Global dispersion of bacterial cells on Asian dust. Sci Rep 2:1–6

Yang C, Crowley DE, Borneman J, Keen N (2001) Microbial phyllosphere populations are more complex than previously realized. Proc Natl Acad Sci 98:3889–3894

Yu SO, Brown A, Middleton AJ et al (2010) Ice restructuring inhibition activities in antifreeze proteins with distinct differences in thermal hysteresis. Cryobiology 61:327–334

Zhang B, Zhihui B, Hoefel D et al (2010) Microbial diversity within the phyllosphere of different vegetable species. In: Méndez-Villas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex, Badajoz, pp 1067–1077