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Isolation and phylogenetic classification of culturable psychrophilic prokaryotes from the Collins glacier in the Antarctica

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Abstract

Culturable psychrophilic prokaryotes were obtained of samples of glacier sediment, seaside mud, glacier melted ice and *Deschampsia antarctica* rhizosphere from Collins glacier, Antarctica. The taxonomic classification was done by a culture-dependent molecular approach involving the Amplified Ribosomal DNA Restriction Analysis. Two hundred and sixty colonies were successfully isolated and sub-cultivated under lab-conditions. The analysis showed a bacterial profile dominated by Betaproteobacteria (35.2%) followed by Gammaproteobacteria (18.5%), Alphaproteobacteria (16.6%), Gram-positive with high GC content (13%), Cytophaga-Flavobacterium-Bacteroides (13%) and Gram-positive with low GC content (3.7%). Eleven of the isolates have been reported previously and the others microorganisms remain uncharacterized. The isolated microorganisms here could be a potential source for biotechnological products such as cold-active enzymes and secondary metabolites.

Keywords: Antarctica, ARDRA, culture dependent, extremophile, microbial diversity, psychrophilic.

Introduction

The search for novel biological products such as enzymes, dyes, antibiotics, and others, still stimuli the search of microorganisms in exotic locations. Extreme environments are often rich in microorganisms with high potential to be used in biotechnological applications. For instance, psychrophilic microorganisms as source of cold-active enzymes have received considerable research attention (Garcia Echauri *et al.* 2009; Hinsa-Leasure *et al.* 2010). Although major advances have been made in the last decade, our knowledge on the microbial ecology, their interactions, physiology, metabolism, enzymology and genetics in this fascinating microbial group of extremophilic microorganisms is still limited.

Molecular biology techniques are excellent tools for a rapid identification and the analysis of the microorganism diversity. Culture-independent methods allow an integrative and thorough study of the microbial communities. Whereas, culture-dependent methods are time-consuming and in many cases the appropriate growth-protocols are not available. However, isolation of culturable microorganisms is mandatory for realistic applications and microbiological studies.

In this work, we report the isolation, identification and phylogenetic classification of the culturable psychrophilic prokaryotes in samples collected from the Collins glacier, King George Island, Antarctica.

Materials and methods

Sample collection

Samples from glacier sediment (GS), seaside mud (SM), glacier melted ice (GI) and *Deschampsia antarctica* rhizosphere (DAR) were collected in the Collins glacier at Fildes Peninsula, King George Island, Antarctica (62°10'S, 58°55'W). The samples were stored in sterile polyethylene Falcon tubes (Nalgene Labware) and kept at -20ºC until they were processed.

Isolation of culturable prokaryotes

Solid samples (GS, SM and DAR) of 0.1 g were resuspended in 500 µl of 0.1 M sodium phosphate buffer pH 8.0 (PBS). Whereas, 30 ml of liquid sample (GI) was centrifuged at 11,500 *g* for 30 min and the pellet was resuspended in 500 µl of PBS. Dilutions in the range of $1:1x10^3$ to $1:1x10^6$ were plated on Petri-dishes containing potato dextrose agar (PDA, Difco), Luria-Bertani (LB, Invitrogen), MRS (Difco) or YPG (yeast extract 0.25 g/l, peptone 0.25 g/l, glucose 0.25 g/l, agar 15 g/l) and incubated at 4ºC under aerobic and anaerobic conditions until the apparition of colonies, then they were sub-cultivated in fresh Petri-dishes containing the same culture medium and conditions.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16S rDNA was amplified by colony-PCR using the universal oligonucleotide set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Reysenbach *et al.* 1994) forward and reverse, respectively. Each reaction tube with 50 µl

contained: 1.5 U *Pfu* DNA polymerase (Biotools), 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mM MgSO4, 0.2 mM of each dNTP and 10 pM of each oligonucleotide. The conditions were the following: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C; and finally 8 min at 72°C. The PCR products were subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide to visualize the amplified products. Amplified rDNA was digested at 37ºC for 2 h using the enzymes *Hae*III and *Rsa*I (Invitrogen). The restriction patterns were visualized in 2% agarose gels and the differential selected clones were sequenced in Molecular Cloning Laboratories (MCLAB, San Francisco CA).

Classification of 16S rRNA genes

The ambiguous bases from the 5' and 3' terminal sequences were eliminated, and the resultant sequences were confirmed using BioEdit software (Ibis Therapeutics). Sequences were then compared against the Ribosomal Database Project (Cole *et al.* 2007) and GenBank using BLAST (Altschul *et al.* 1997) against the NCBI non-redundant nucleotide database "nt". The sequences closely related to the 16S rDNA genes were extracted and then aligned against the identified genes to infer the phylogenetic trees by the neighbor-joining method using the MEGA software version 4.0. The bootstrap analysis was performed with 10,000 replicates.

Results

Two hundred and sixty colonies were isolated from the all samples collected from the Collins

glacier and successfully reseeded in the same culture medium used for the first isolation. The distribution of the colonies number by type of culture medium is shown in the Fig. 1. The highest number of colonies was obtained in YPG with 166 colonies (64%), followed by PDA 65 (25%), LB 27 (10%) and MRS 2 (1%). Visually, a large diversity of morphologies (smooth and rough) and colors from white to dark red were observed in the colonies. This suggests the presence of secondary metabolites with potential biotechnological applications.

An example of the 16S ribosomal gene amplification for a set of 11 colonies is shown in the Fig. 2A. In all cases the PCR product was of approximately 1.46 kpb, which corresponds to the 16S ribosomal gene size in *Escherichia coli*. A typical ARDRA pattern for seven colonies in the ARDRA is shown in Fig. 2B. Lanes 1 and 3 showed the same restriction pattern, which strongly suggest that both clones correspond to the same microorganism and this was verified by sequencing. Among the 260 isolated colonies, we observed 54 unique restriction patterns (20.8% of the total), and the restriction pattern corresponding to the clone N25 was found 95 times (36.5%). The major amount of unique colonies was obtained in YPG with 37 (68%), followed by LB 14 (26%), MRS 2 (4%) and PDA 1 (2%).

The 16S DNA sequences were submitted to GenBank with accession numbers from EU636014 to EU636065 (Table 1) and the phylogenetic tree is shown in Fig. 3. Additional features of the isolated bacteria such as the closest relative match, the percentage of identity, culture medium used for isolation, frequency and the clone origin are included in Table 1. BLAST results showed identities in the range of 93.8 to 99.9%. Eleven sequences had an identity above 99%

and the closest relative matches were *Bacillus simplex* (EU236732), *Caulobacter henricii* (AJ227758), *Carnobacterium maltaromaticum* (AY573049), *Janthinobacterium lividum* (Y08846), *Pseudomonas antarctica* (AJ537601), *Pseudomonas boreales* (AJ012712), *Pseudomonas grimontii* (AF268029), *Pseudomonas meridiana* (AJ537602) and *Pseudomonas frederiksbergensis* (AJ249382).

The bacterial strains obtained comprise a wide genetic collection covering 14 genera of six phylogenetic groupings: Gram-positive, Proteobacteria alpha, beta and gamma, and Cytophaga-Flavobacterium-Bacteroides (CFB) (Table 2). In our study, the most abundant group was the Betaproteobacteria with 35.2%, followed by Gammaproteobacteria (18.5%), Alphaproteobacteria (16.6%), Gram-positive with high GC content (13%), CFB (13%) and Gram-positive with low GC content (3.7%).

Discussion

We isolated 260 clones cultivable at 4°C; of which 54 corresponded to unique microorganisms. It is possible that other prokaryotes were present in the samples collected but they could not be isolated in this work. Some colonies showed bright colors due to the presence of pigments, which may help them to survive under low temperatures (Chattopadhyay 2006). The YPG was the most efficient medium to isolate psychrophilic prokaryotes. Similar to the results obtained here, Christner et al. (Christner *et al.* 2003) reported that high nutrient concentration in the culture media did not allow the recovering of psychrophilic prokaryotes. Using the criteria of Drancourt et al. (Drancourt *et al.* 2000) to classify the bacterial divisions/taxonomic groupings,

we found 11 species already characterized for those clones with identities above 99%; whereas16 clones with identities below 97% and 27 clones with 97 to 99% identities potentially corresponds to new genera and new species, respectively. However, to assign them as new microorganisms, further studies such as biochemical characterization are required. In our work, Betaproteobacteria was the most abundant group. Similar results were obtained in the classification of the psychrophilic bacteria isolated from New Zealand glacier (Foght *et al.* 2004), subglacier from Iceland (Gaidos *et al.* 2004) and Canada (Skidmore *et al.* 2005). Firmicutes was the most abundant phylum in almost all the analyzed samples from rhizospheres of both maritime Antarctica vascular plants in Admiralty Bay (Teixeira *et al.* 2010). The main bacterial groups in the sediments fell into 4 major lineages of the gram-negative bacteria: the α, γ and δ subdivision of Proteobacteria in a lake sediment core of Ardley Island, west Antarctica (Li *et al.* 2006). Yergeau et al. (Yergeau *et al.* 2007) studied bacterial communities across a latitudinal gradient in the maritime Antarctica and found that Proteobacteria was the prevalent phylum in their 16S rDNA clone libraries. In the Muztag Ata glacier (China) and Puruogangri glacier (Tibet), the Gram-positive high GC was the main group and Betaproteobacteria was not found (Xiang *et al.* 2005; Zhang *et al.* 2008).

Regarding to the psychrophilic bacteria applications, it has been reported the production of antibiotics such as janthinocins and bacteriocins by *J. lividum* (O'Sullivan *et al.* 1990) and *C. maltaromaticum* (Leisner *et al.* 2007), respectively. *B. simplex* has been used for biodegradation of hydrocarbons (Purswani *et al.* 2008). Vardhan Reddy et al. (Vardhan Reddy *et al.* 2009) analyzed the bacterial diversity and bioprospecting for cold-active enzymes from culturable

bacteria associated with sediment from a melt water stream of Midtre Lovenbreen, an Arctic glacier. They found than half of the isolates were pigmented and 14 strains exhibited amylase, lipase and (or) protease activity.

The isolated microorganisms here could be a potential source for biotechnological products such as cold-active enzymes and secondary metabolites. However, further work is necessary.

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Figure legend

Figure 1. Distribution of the 260 isolated colonies on the different culture media.

Figure 2. A) Typical PCR product for the 16S rDNA amplification for various clones isolated from the seaside sediment samples. M corresponds to molecular markers in bp (100 bp DNA Ladder, Invitrogen Life Technologies). 2B) Representative ARDRA profiles of 16S rDNA fragments amplified from DNA samples digested with *Hae*III and *Rsa*I. M corresponds to molecular markers in bp (100 bp DNA Ladder, Invitrogen Life Technologies).

Figure 3. Phylogenetic tree using the 16S rDNA sequences of the 54 clones with unique ARDRA pattern.

1 Table 1. Taxonomic classification and features of the bacterial isolates
2

Strain	Accession number	Closest relative according to the NCBI	Identity (%)	Culture medium	Frequency	Location
2CA	EU636016	*Cryobacterium psychrophilum* (EF467640)	96.8	LB	3	GS
2CD	EU636015	*Frigoribacterium faeni* (AM410686)	96.7	LB	1	GS
3C2	EU636065	*Sphingomonas echinoides* (AB021370)	97.0	LB	1	GS
3C3	EU636014	*Frigoribacterium faeni.* (AM410686)	96.7	LB	1	GS
3C4	EU636037	*Pseudacinetobacter hongkongensis* (AF543466)	93.7	LB	1	GS
3C6	EU636020	*Humicoccus flavidus* (DQ321750)	96.7	LB	1	GS
3C8	EU636038	*Pseudacinetobacter hongkongensis* (AF543466)	93.8	LB	1	GS
G003	EU636062	*Pedobacter lentus* (EF446146)	97.5	YPG	1	GS
G020	EU636027	*Polaromonas rhizosphaerae* (EF127651)	98.7	YPG	3	GS
G024	EU636026	*Polaromonas jejuensis* (EU030285)	98.8	YPG	1	GS
GA028	EU636061	*Pedobacter lentus* (EF446146)	98.2	YPG	10	GS
GA036	EU636064	*Sphingomonas echinoides* (AB021370)	97.1	YPG	18	GS
GA045	EU636047	*Janthinobacterium agaricidamnosum* (Y08845)	98.2	YPG	2	GS
GA051	EU636048	*Janthinobacterium agaricidamnosum* (Y08845)	98.0	YPG	3	GS
G054	EU636045	*Janthinobacterium agaricidamnosum* (Y08845)	97.7	YPG	2	GS
GA055	EU636025	*Polaromonas jejuensis* (EU030285)	98.1	YPG	1	GS
G057	EU636044	*Janthinobacterium agaricidamnosum* (Y08845)	98.6	YPG	4	GS
GA058	EU636052	*Pseudomonas boreales* (AJ012712)	99.7	YPG	1	GS
G064	EU636019	*Frigoribacterium faeni* (AM410686)	96.4	YPG	6	GS
G076	EU636024	*Polaromonas jejuensis* (EU030285)	97.9	YPG	1	GS
G079	EU636030	*Rhodoferax ferrireducens* (AF435948)	98.6	YPG	4	GS

G081	EU636018	*Labedella kawkjii* (DQ533552)	96.8	YPG	1	GS
GA082	EU636060	*Pedobacter lentus* (EF446146)	98.1	YPG	4	GS
G088	EU636029	*Polaromonas rhizosphaerae* (EF127651)	98.2	YPG	1	GS
G089	EU636043	*Janthinobacterium lividum* (Y08846)	99.4	YPG	3	GS
G091	EU636041	*Aquaspirillum arcticum* (AB074523)	96.7	YPG	2	GS
A02	EU636035	*Devosia yakushimanensis* (AB361068)	97.3	YPG*	1	DAR
CA1	EU636023	*Rhodoferax ferrireducens* (AF435948)	98.3	YPG	2	GI
CC0Q	EU636040	*Herminiimonas fonticola* (AY676462)	97.3	YPG	3	GI
CC9	EU636039	*Janthinobacterium agaricidamnosum* (Y08845)	96.5	YPG	10	GI
CC10	EU636028	*Polaromonas vacuolata* (U14585)	98.7	YPG	1	GI
GA0A	EU636049	*Pseudomonas meridiana* (AJ537602)	99.5	LB	3	GS
GA0F	EU636050	*Pseudomonas meridiana* (AJ537602)	99.7	LB	11	GS
GA0G	EU636051	*Pseudomonas antarctica* (AJ537601)	99.4	LB	12	GS
GA0K	EU636034	*Sejongia marina* (EF554366)	97.9	LB	1	GS
GA0L	EU636046	*Herminiimonas saxobsidens* (AM493906)	96.4	LB	1	GS
L1	EU636013	*Carnobacterium maltaromaticum* (AY573049)	99.8	MRS*	2	SM
L2	HQ226068	*Bacillus simplex* strain Q1 (EU236732)	99.9	MRS*	1	SM
L04	EU636059	*Haematobacter genomospecies* (DQ342319)	95.4	YPG*	2	SM
L10	EU636033	*Flavobacterium segetis* (AY581115)	97.8	YPG*	1	SM
M02	EU636032	*Sejongia marina* (EF554366)	97.9	YPG*	1	GI
N04	EU636031	*Flavobacterium limicola* (AB075230)	95.8	YPG*	16	GS
N14	EU636042	*Janthinobacterium agaricidamnosum* (Y08845)	98.9	YPG*	1	GS
N25	EU636053	*Pseudomonas frederiksbergensis* (AJ249382)	98.6	PDA YPG*	95	GS

N44	EU636057	*Rhodobacter apigmentum* (AF035433)	96.7	YPG*	1	GS
N82	EU636021	*Caulobacter henricii* (AJ227758)	99.3	YPG*	2	GS
N88	EU636017	*Cryobacterium psychrophilum* (EF467640)	97.7	YPG*	1	GS
N92	EU636058	*Rhodobacter ovatus* (AM690348)	96.1	YPG*	1	GS
N97	EU636022	*Brevundimonas subvibrioides* (AJ227784)	98.1	YPG*	2	GS
R02	EU636054	*Pseudomonas frederiksbergensis* (AJ249382)	99.9	LB	1	DAR
R03	EU636055	*Pseudomonas grimontii* (AF268029)	99.7	LB	3	DAR
R13	EU636036	*Devosia euplotis* (AJ548825)	97.4	YPG	9	DAR
R19	EU636063	*Pedobacter aurantiacus* (DQ235228)	98.4	YPG	2	DAR
R25	EU636056	*Pseudomonas frederiksbergensis* (AJ249382)	99.1	YPG	1	DAR

* Anaerobiosis

Table 2. Taxonomic classification according to bacterial division and families

Group	Family	Number of colonies (percentage)	Strain Identification
Gram positive (low GC content)	*Bacillaceae and Carnobacteriaceae*	2 (3.7%)	L1, L2
Gram positive (high GC content)	*Microbacteriaceae, Nakamurellaceae*	7 (13.0%)	2CA, 2CD, 3C3, G064, G081, N88 3C6
Alphaproteobacteria	*Caulobacteraceae, Hyphomicrobiaceae, Rhodobacteraceae and Sphingomonadaceae*	9 (16.6%)	N82, N97, A2, R13, L4, N44, N92, 3C2, GA036
Betaproteobacteria	*Comamondaceae and Oxalobacteraceae*	19 (35.2%)	G020, G024, GA055, G076, G079, G088, CC10, CA1, GA045, GA051, G054, G057, G089, G091, CC9, GA0D, GA0L, N14, CC0Q
Gammaproteobacteria	*Moraxellaceae and Pseudomonadaceae*	10 (18.5%)	3C4, 3C8, GA058, GA0A, GA0F, GA0G, N25, R02, R03, R25
Cytophaga-Flavobacterium-Bacteroides (CFB)	*Flavobacteriaceae and Sphingobacteriaceae*	7 (13.0%)	GA0K, L10, M2, N4, R19, GA028, GA082