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Biodiversity of cultivable psychrotrophic marine bacteria isolated from Terra Nova Bay (Ross Sea, Antarctica)

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Abstract

A set of 146 Antarctic marine isolates from the Ross Sea was characterized by a combination of molecular techniques in order to determine the degree of inter- and intraspecific variability. Isolates were analyzed by amplified rDNA restriction analysis (ARDRA) using the tetrameric enzyme *Alu*I, resulting in 52 different groups, corresponding to at least 52 different bacterial species, indicating a high degree of interspecific variability. The phylogenetic position of bacteria belonging to some ARDRA groups was obtained by sequencing of 16S rDNA. Random amplified polymorphic DNA (RAPD) analysis, carried out on the largest ARDRA groups, revealed a high intraspecific genetic variability, too. The analysis of plasmid content revealed the existence of horizontal gene transfer between strains belonging to the same and to different species. A comparison of the whole body of morphological, physiological and biochemical data was finally carried out.

Keywords: Antarctic marine bacteria; Biodiversity; 16S rDNA; Amplified rDNA restriction analysis; Random amplified polymorphic DNA analysis

1. Introduction

Antarctica offers some unique opportunities for research concerning microbial evolution [1] and it is one of the less-characterized environments available to researchers of microbial diversity and ecology [2]. Prokaryotes dominate many Antarctic ecosystems and colonize a variety of niches of Antarctica where they live under extreme conditions. As the dominant biomass component of Antarctic ecosystems, microbial communities control many processes within the marine environment, like primary production, turnover of biogenic elements, degradation of the organic matter and also mineralization of xenobiotics and pollutants [3–5]. Moreover, they are essential components of the Antarctic pelagic marine food webs [6,7].

During the last years many microbiologists focused their attention on the identification of Antarctic bacterial isolates [8–12]. Approximately 30 new species of Antarctic bacteria have been described up to day and many of them have not been found elsewhere in the world [1,13], suggesting a remarkable endemism of the Antarctic microflora [1]. Such novelty and biodiversity can potentially affect many industrial and scientific applications. For example, the availability of novel Antarctic species could be exploited in biotechnological applications thanks to their production of cold-adapted enzymes [14–18] and polyunsaturated fatty acids [2,8–10,19,20]. Moreover, the study of Antarctic extremophiles might contribute to the investigation of the origin of life, since it has been proposed that they might have been probably among the first inhabitants of the Earth [13].

A preliminary step in microbiological research is the identification of isolates. For a long time identification was based essentially on a wide range of biochemical and physiological tests that require cultivation of the bac-

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terial strains, a time consuming step even when the isolated strains are easy to grow. Moreover, this approach is often subjective in the interpretation, the results not consistent with existing literature about type strains [21] and is not applicable to the uncultivable strains that represent the larger part of natural bacterial communities. On the contrary, molecular approaches are more sensitive and reliable and they presently dominate modern taxonomic studies as a consequence of technological progress [22]. An approach using a combination of both methods is needed to obtain objective information about the community composition, and also to evaluate its ecological and physiological function [22].

A bacterial population consisting of 146 psychrotrophic strains, isolated from two fixed marine coastal stations of Antarctica (Terra Nova Bay, Ross Sea), was previously characterized by using several morphological, physiological and biochemical parameters [23]. These analyses showed a considerable diversity in the structure and nutritional requirements of the population. The majority of the isolates were non-fermentative Gram-negative rods, assigned to the genera *Pseudomonas*/*Alcaligenes* and *Flavobacterium*/*Cytophaga*. The bacterial communities from the two sampling stations differed in their nutritional capabilities, although many isolates were found in both habitats. On the other hand, the assignment of bacterial strains to a given taxon was tentative and based solely on phenotypic tests.

In this work we investigated the diversity of this psychrotrophic [24] Antarctic marine bacterial population by a combination of molecular techniques [25] as previously applied successfully in the study of microbial communities from different environments [26–32]. According to the strategy adopted (i) the 16S rDNA of each isolate was amplified by polymerase chain reaction (PCR); (ii) the isolates were grouped into clusters by the analysis of restriction patterns of the PCR-amplified 16S rDNA (ARDRA: amplified rDNA restriction analysis) [33–35] using the tetrameric restriction enzyme *Alu*I, which often generates species-specific patterns [26–31]; (iii) the degree of intraspecific biodiversity was checked by the random amplified polymorphic DNA (RAPD) technique [35–36]; (iv) analysis of plasmid content [37,38] was carried out to investigate plasmid-mediated lateral gene transfer (LGT); (v) the phylogenetic position of some ARDRA groups was determined by 16S rDNA sequence analysis [39].

2. Materials and methods

2.1. Bacterial strains and media

Seawater samples were collected during the V Oceanographic Cruise in the 1989/90 Antarctic summer from two fixed marine coastal stations in Terra Nova Bay (Ross Sea, Antarctica) [23]. The co-ordinates of the two stations are as follows: Mergellina 74°41′33″S–164°07′15″E; Santa Maria Novella 74°43′S–164°16′E. Sampling depths were 0, 10, and 25 m for Mergellina station and 0, 10, 25, 50, 100 and 200 m for Santa Maria Novella. The water temperature at the sampling time ranged between 0.91°C and 1.96°C for Mergellina station and between −0.85°C and +2.76°C for Santa Maria Novella.

All strains belong to the *Collection of bacterial strains isolated from Ross Sea* (*Antarctica*) which is kept at the Section of Messina of the Antarctic National Museum 'Felice Ippolito'. They are maintained both on marine agar slopes at 4°C and in frozen glycerol cultures at −80°C.

Strains were isolated on marine agar 2216 medium (Difco) and incubated for 21 days at 4°C. A total of 146 bacterial isolates were collected. All media used in this study were sterilized at 121°C for 20 min and plates were incubated at 4°C for 21 days, unless otherwise indicated. All strains investigated in this study were routinely grown on marine agar 2216 (Difco) plates.

2.2. PCR amplification of 16S rDNA

For the amplification of 16S rDNA a single colony of each strain from a marine agar plate was picked-up with a sterile toothpick, resuspended in 20 μl of sterile distilled water and lysed by heating at 95°C for 10 min. Cell lysates were rapidly cooled in ice, briefly centrifuged in a microcentrifuge and directly used for PCR amplification.

Amplification of 16S rDNA was carried out as described previously [25,29], using primers P0 (5′-GAGAGTTTGATCCTGGCTCAG) and P6 (5′-CTACGGCTACCTTGTTACGA) that were designed from the conserved bacterial sequences at the 5′ and 3′ ends of the 16S rRNA gene (positions 27f and 1495r, respectively, on *Escherichia coli* rDNA) and allowed the amplification of nearly the entire gene [29].

2.3. Restriction analysis of amplified 16S rDNA (ARDRA)

A 5-μl aliquot of each PCR mixture containing approximately 1.5 μg of amplified 16S rDNA was digested with 3 U of the restriction enzyme *Alu*I (Gibco BRL) in a total volume of 20 μl at 37°C for 3 h. The enzyme was inactivated by heating at 65°C for 15 min, and the reaction products were analyzed by agarose (2.5%, w/v) gel electrophoresis in TAE buffer containing 1 μg ml^{-1} of ethidium bromide.

2.4. Sequencing 16S rDNA

The amplified 16S rDNA was purified from agarose (1.2%, w/v) gel as follows. A small agarose slice containing the band of interest [observed under long-wavelength (312-nm) UV light] was excised from the gel and purified using the QIAquick gel extraction kit (Qiagen) according to the

Table 1
Psychrotolerant bacterial isolates whose 16S rDNA sequence has been determined

Isolate	ARDRA group	GenBank accession number	Genus	Taxonomy
F46	3	AY451334	*Pseudoalteromonas*	γ-Proteobacteria
G24	32	AY451336	*Pseudoalteromonas*	
D70	22	AY309072	*Psychrobacter*	
C1	12	AY316679	*Psychrobacter*	
D40	21	AY309073	*Roseobacter*	α-Proteobacteria
D39	20	AY451333	*Paracoccus*	
E28	13	AY316680	*Arthrobacter*	Gram-positive high GC
G75	40	AY451331	*Arthrobacter*	
D27	27	AY451328	*Arthrobacter*	
E60	36	AY316681	*Rhodococcus*	
B8	47	AY444156	*Janibacter*	
D3	18	AY451329	*Planococcus*	Firmicutes

supplier's instructions. Automated 16S rDNA sequencing was carried out by cycle sequencing using the dye terminator method [40]. The 16S rDNA sequences obtained were submitted to the GenBank where they were assigned the accession numbers listed in Table 1.

2.5. Analysis of sequence data

The 16S rDNA nucleotide sequences obtained were aligned with the most similar ones in the Ribosomal Database Project (RDP) [41] by using RDP utilities. The alignment was checked manually and analyzed by the neighbor-joining method according to the models of Jukes and Cantor using TREECON 2.2 [42].

2.6. RAPD fingerprinting

Random amplification of DNA fragments was carried out in a final reaction volume of 25 μl of Platinum buffer (Gibco BRL) containing 3 mM $MgCl_2$, 2 μl of lysed cell suspension, 500 ng of primer AP12 (5′-CGGCCCCTGC-3′), which has been shown to give reproducible and reliable results [28], each dNTP at a final concentration of 200 mM, and 0.625 U of Platinum *Taq* polymerase (Gibco BRL). The reaction mixtures were incubated in a thermal cycler (model 9600; Applied Biosystems) at 94°C for 2 min. They were then subjected to 45 cycles consisting of 95°C for 30 s, 36°C for 1 min, and 72°C for 2 min; finally the reactions were incubated at 75°C for 10 min and then at 60°C for 10 min. Reaction products were analyzed by agarose (2%, w/v) gel electrophoresis in TAE buffer containing 1 μg ml^{-1} of ethidium bromide.

2.7. Phenotypic characterization

The following morphological and biochemical tests were performed as previously reported [23]: Gram stain, cell morphology, presence of flagella and endospores, motility, presence of pigment, oxidase and catalase tests, oxidative or fermentative acid production from carbohydrates, hydrolysis of agar, Tween 80 and chitin, susceptibility to penicillin G (10 μg), polimixine B (30 μg) and vibriostatic agent O/129 (10 μg). API 20E and API 20B (BioMerieux) strips were used for detection of metabolic properties.

2.8. Analysis of plasmid content

Plasmid molecules were extracted from 3-ml bacterial cultures grown in marine broth using the commercial kit QIAprep-spin Plasmid Miniprep (Qiagen) according to the supplier's instructions. Additionally, the method described by Le Gouill and Déry [43] was also used. The presence of plasmid molecules was checked by agarose (0,8%, w/v) gel electrophoresis in TAE buffer containing 1 μg ml^{-1} of ethidium bromide.

3. Results

3.1. Analysis of 16S rDNA of bacterial isolates

When the 16S rDNA of each of the 146 isolates was amplified by PCR, an amplification fragment of about 1500 bp was observed (not shown). ARDRA [32–34] was carried out on the amplified DNA of each sample with the enzyme *Alu*I. Each pattern was compared with all the others, and this allowed 52 ARDRA profiles, or haplotypes, to be recognized. According to other previous reports [25–31], these haplotypes corresponded to at least 52 bacterial species. Fig. 1 shows an example of the ARDRA patterns obtained.

Group 36 was the most-represented haplotype consisting of sixteen isolates (Fig. 2) while most of the other haplotypes were represented by only few isolates.

We determined the nucleotide sequence of 16S rDNAs from the 12 strains listed in Table 1 and belonging to twelve different ARDRA groups; together, these groups represent about 50% of the entire population. The twelve

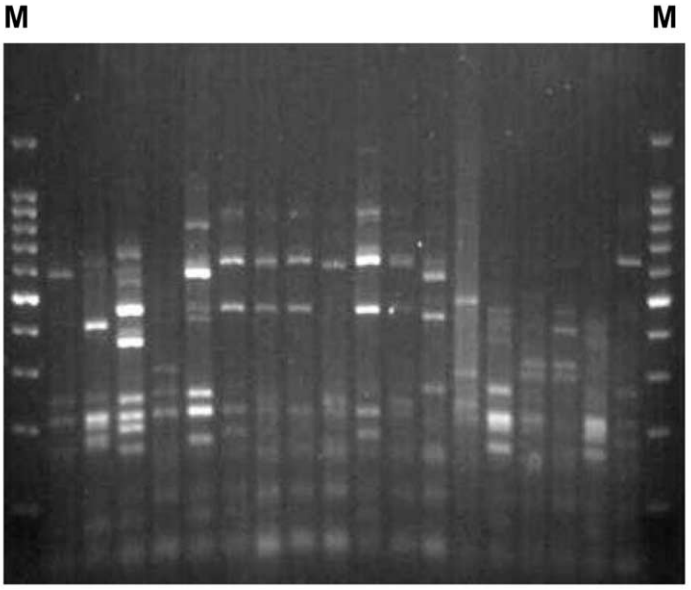

Fig. 1. Agarose gel electrophoresis of amplified 16S rDNA digested with endonuclease *Alu*I showing an example of the molecular haplotypes detected. M: Ladder 1 kb Plus (Invitrogen-Life Technologies, Carlsbad, CA, USA).

sequences were aligned to the most similar ones available in database [41]. Finally, the alignments were used to construct the phylogenetic trees shown in Fig. 3.

The phylogenetic analysis revealed that the twelve haplotypes were representative of eight bacterial genera:

1. Bacteria showing haplotype 13, 27 and 40 were placed within the genus *Arthrobacter*, each in close relation to a different species, consistently with the different ARDRA profiles they exhibited.
2. Isolates of haplotype 21 joined a cluster of α-proteobacteria, which includes different bacterial genera, such as *Roseobacter*, *Octadecabacter*, *Methylarcula*, *Ketogulonigenium*, and many bacteria isolated from Antarctic and Arctic seas. According to the sequence of isolate

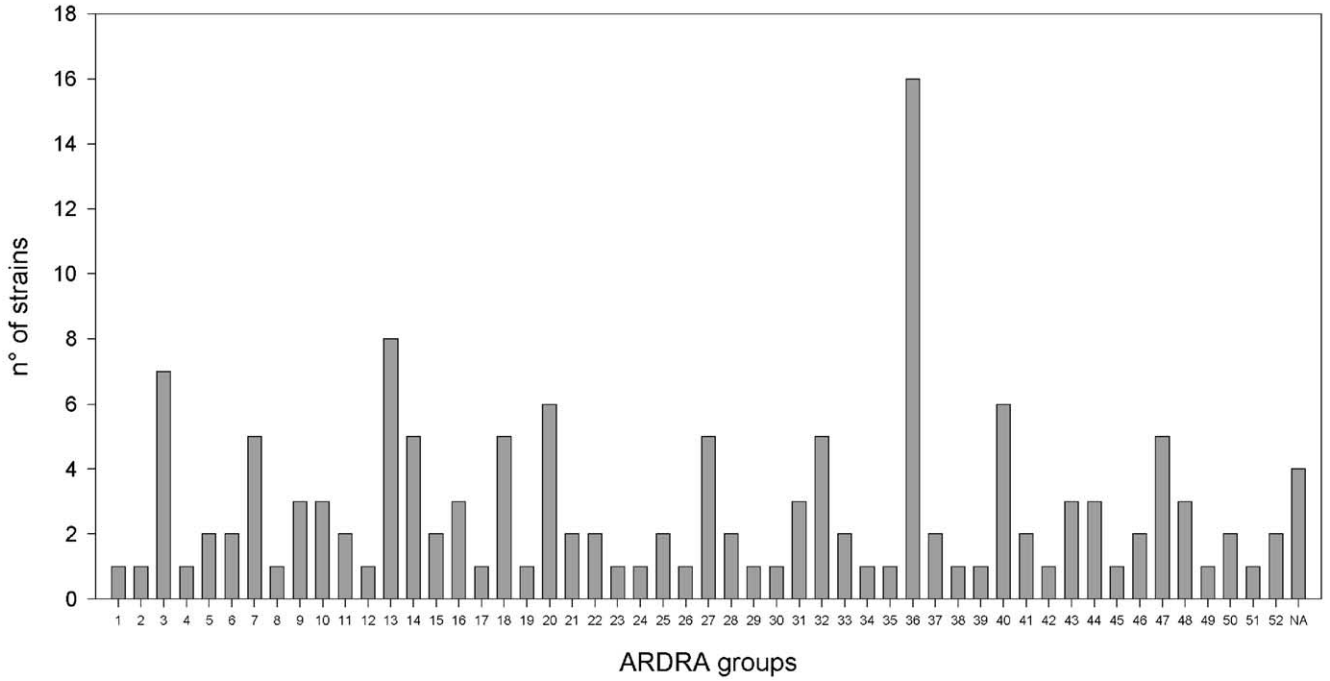

Fig. 2. Distribution of the 52 haplotypes obtained by restriction analysis of 16S rDNA with the endonuclease *Alu*I from 146 bacteria isolated from Ross Sea, NA, not assigned.

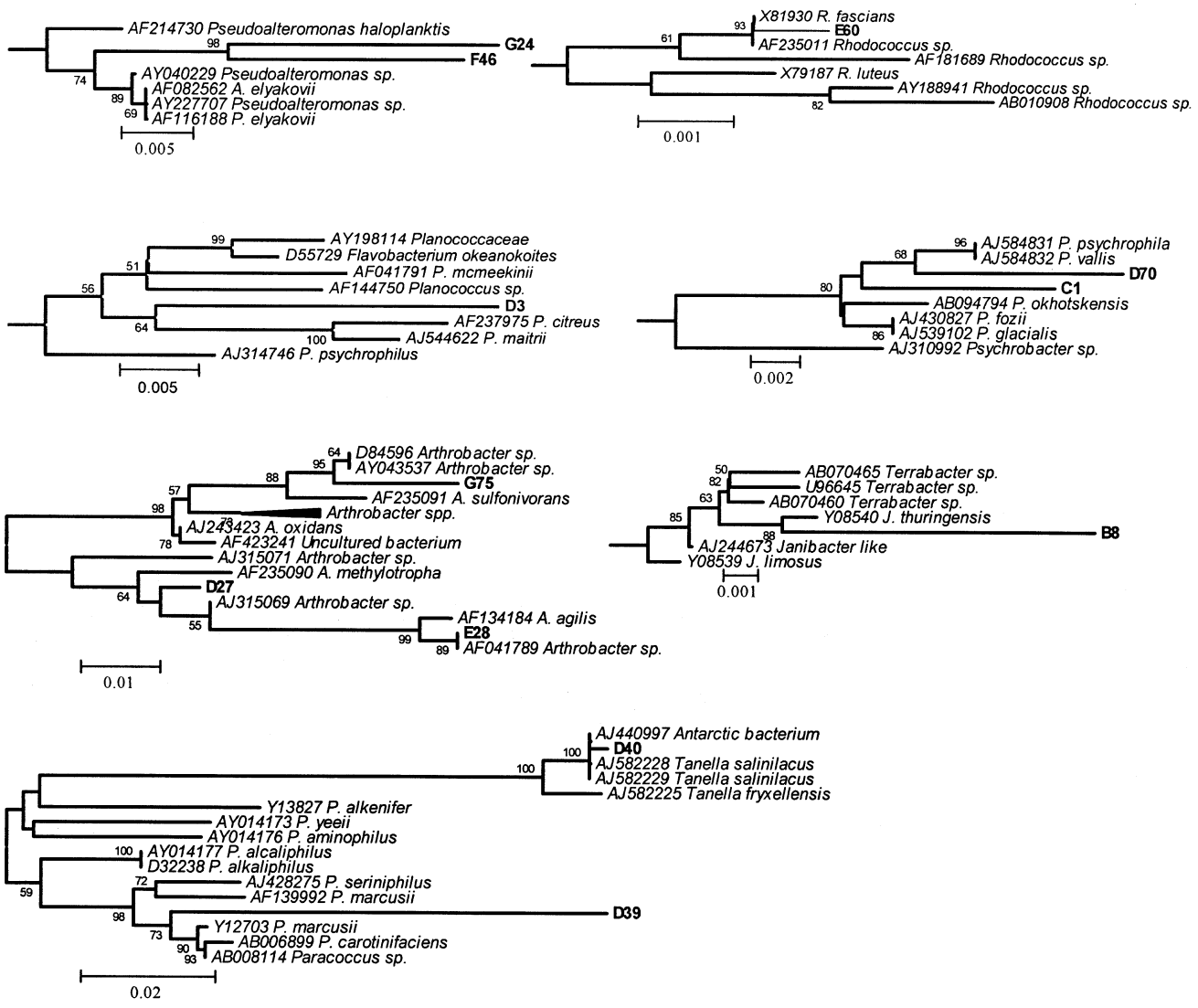

Fig. 3. Phylogenetic trees showing the relationships among the 16S rDNA sequences of isolates belonging to five ARDRA groups and reference organisms from different bacterial taxa. Scale bars represent the Jukes–Cantor distance. Bootstrap values are indicated at the nodes. The accession number of each sequence is also given.

D40, the closest organism to this ARDRA group is the Antarctic bacterium R-8904.

3. Isolates belonging to haplotypes 12 and 22 were clustered in the genus *Psychrobacter*, but most likely they belong to different species, according to the fact that they shared less than 97% of sequence similarity.
4. Strains from ARDRA groups 3 and 32 were placed in the *Pseudoalteromonas* genus. They can be assigned to different species according to the 16S rDNA sequence analysis as well as to their different ARDRA patterns.
5. Isolates belonging to haplotypes 18, 20, and 47 joined three different genera: *Planococcus*, *Paracoccus* and *Janibacter*, respectively.
6. The last (and main) haplotype (36) was placed within the genus *Rhodococcus*, with strain E60 very close to *Rhodococcus fascians* strains (99% similarity).

3.2. RAPD fingerprinting

To study the degree of intraspecific genetic variability, strains of five ARDRA groups (3, 13, 20, 36 and 40) were subjected to RAPD fingerprinting [35,36]. For this purpose, DNAs from lysed cell suspensions were amplified with the 10-mer oligonucleotide AP12 as described in Section 2. As expected, the amplification patterns of strains belonging to different haplotypes were very different (Fig. 4). A high degree of genetic variability was found between the sixteen isolates belonging to the main ARDRA group [36], which were assigned to the genus *Rhodococcus*. Indeed, these isolates showed 16 different RAPD profiles (Fig. 4), suggesting that each of them could be classified as a different strain. The other haplotypes exhibited a lower degree of genetic diversity, like in the case of ARDRA

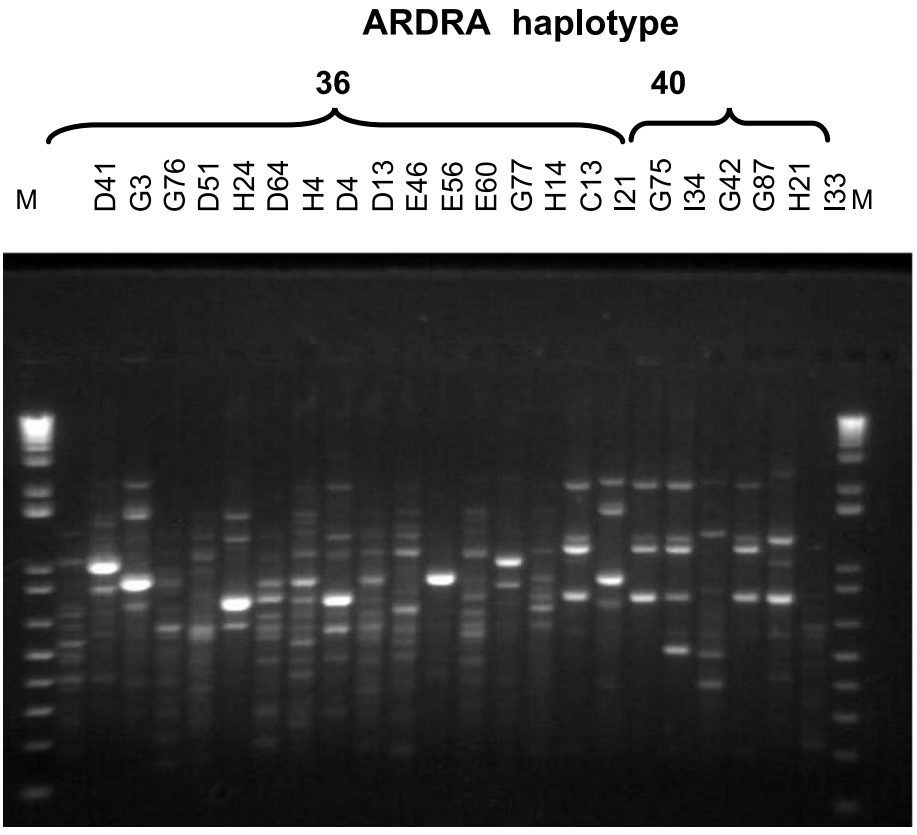

Fig. 4. Example of RAPD patterns of total DNA with primer AP12 from Antarctic isolates.

haplotype 3, where six out of seven strains shared the same RAPD pattern.

3.3. Phenotypic characterization of bacterial isolates

To verify if the identification of the Antarctic isolates according to the sequence of the 16S rDNA was consistent with their phenotype the phenotypic properties of the 12 previously identified isolates were analyzed as described in Section 2 (Table 2). As stated before, according to the 16S DNA sequence data, each ARDRA group might be assigned to a different bacterial species belonging to the same or to different genera. The 12 strains showed different phenotypic properties as anticipated. Moreover, few differences were detected within strains belonging to the same ARDRA group (not shown), except for isolates of group 36 (*Rhodococcus*) (see below and reference [23]). The phenotypic variability of isolates from group 36 is not surprising; in fact RAPD analysis suggested that all the available isolates of this species might be different bacterial strains.

3.4. Analysis of plasmid content

To test the relevance of plasmid-mediated LGT on the biodiversity of the bacterial isolates, the DNA extracted from each strain using two different techniques, as described in Section 2, was analyzed for the presence of plasmid molecules by agarose gel electrophoresis. Results enabled the detection of visible plasmids of different size in 21 isolates belonging to 15 different ARDRA groups. Overall, only one plasmid was found in each isolate, with the exception of strains G29, G30, and D67, belonging to three different ARDRA groups, which showed the presence of other plasmid molecules (not shown).

4. Discussion

In this paper we report the molecular characterization of an aerobic microbial population consisting of 146 psychrotrophic bacterial strains isolated from Ross Sea (Antarctica) by using a combination of PCR-based techniques. Analysis of *Alu*I restriction patterns of 16S rDNA allowed to group the 146 strains into 52 different groups (haplotypes) corresponding to at least 52 different species. Most of ARDRA groups were composed by only one or two isolates. This finding revealed a high degree of interspecific genetic diversity suggesting that several bacterial species can adapt to the extreme environmental conditions of Antarctica.

As suggested by RAPD analysis of the most-representative ARDRA groups, this interspecific genetic variability is parallel to a high degree of biodiversity within single spe-

Table 2
Phenotypic properties of bacterial isolates belonging to twelve ARDRA groups

Phenotypic character	ARDRA group											
	3	12	13	18	20	21	22	27	32	36	40	47
Gram reaction	−	−	−	−	−	−	−	−	−	+	−	−
Cell morphology	r	c	r	r	r	r	c	c	r	r	r	r
Motility	+	−	−	−	−	−	−	−	−	−	−	−
Polar flagellum	+	−	−	−	−	−	−	−	−	−	−	−
Endospores	−	−	−	−	−	−	−	−	−	−	−	−
Utilization as a carbon source												
Glucose	+	−	−	−	−	+	+	−	−	−	+	+
Arabinose	−	−	−	−	+	−	−	−	−	+	−	−
Mannose	−	−	−	−	−	+	−	−	−	−	+	−
Mannitol	−	−	+	−	+	+	−	−	−	+	+	−
Maltose	+	−	−	−	−	+	−	−	−	+	+	−
Malate	−	−	+	−	+	+	+	−	−	+	+	−
Citrate	−	−	+	−	−	−	−	−	−	+	+	−
N-Acetyl-glucosamine, caprate, adipate, fenil-acetate	−	−	−	−	−	−	−	−	−	−	−	−
Biochemical test												
Urease	+	−	+	−	−	−	−	−	−	+	−	−
β-Galactosidase	−	−	−	−	−	+	−	+	−	−	+	−
Oxydase	+	−	−	−	−	−	+	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	+	−	−	−	−	−	−	−	−	−	−
Lysisine decarboxylase, ornithine decarboxylase, indole formation, Voges-Proskauer, H_2S formation, arginine dihydrolase	−	−	−	−	−	−	−	−	−	−	−	−
Macromolecule hydrolysis												
Aesculin	−	−	−	−	+	−	−	−	+	−	−	−
Gelatin	+	−	−	−	−	−	−	−	+	−	−	−
Tween 80	+	nd	+	−	−	nd	−	−	+	+	+	+
Chitin, agar,	−	−	−	−	−	−	−	−	−	−	−	−
Acid produced from												
Glucose	−	−	−	+	+	+	−	+	−	−	−	+
Maltose	−	−	−	−	−	−	+	−	−	−	−	−
Mannose	−	−	−	−	−	−	+	−	−	−	−	−
Sucrose, arabinose, mannitol, fructose, starch, rhamnose, galactose, sorbitol, glycerol, inositol, melibiose, amygdalin	−	−	−	−	−	−	−	−	−	−	−	−

nd: not determined; r or c: rod-shaped or coccoid cells.

cies, which might be due to the presence of many biovars. This is the case for strains belonging to ARDRA group 36, which were previously divided in two different subgroups according to phenotypic tests [23]. The RAPD analysis revealed the existence of even more biodiversity. It is unlikely that such genetic diversity derives from plasmid-mediated horizontal gene transfer as only one of the 16 strains of this haplotype possessed plasmid molecules. The analysis of 16S rDNA sequence of strain E60 assigned this ARDRA group to the genus *Rhodococcus*, suggesting that the group consists of one of the most common bacterial genera within the Antarctic bacterial communities. These data are also in agreement with previous phenotypic tests [5] that assigned several strains belonging to this same ARDRA group to the genus *Rhodococcus*. Part of the biodiversity detected might be attributed to the presence of plasmid molecules in a fraction of bacterial isolates (14%), a percentage similar to that previously reported by Kobori (20%) [38]. In this context, the presence of comigrating plasmid molecules in different isolates belonging to the same and/or to different species (haplotypes) supported the idea of a LGT occurring at different taxonomic levels.

The other isolates were assigned to the genera *Psychrobacter*, *Pseudoalteromonas*, *Arthrobacter* and to a group of α-proteobacteria that includes the genera *Methylarcula* and *Ketogulonigenium*, which have been recently characterized from a taxonomic point of view [44,45].

A high biodiversity, both interspecific and intraspecific, is shown within the marine Antarctic bacterial community and emphasizes its adaptivity to extreme environmental conditions. Certainly, increase in the number of rDNA sequences in the Antarctic database and further use of biomolecular techniques will improve the knowledge about the microbiota from Antarctica and their role in this environment.

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