

Analysis of Antarctic proteobacteria by PCR fingerprinting and screening for antimicrobial secondary metabolites

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ABSTRACT. Fifty-seven proteobacterium species were successfully isolated from soils of Barrientos Island of the Antarctic using 11 different isolation media. Analysis of 16S rDNA sequencing of these isolates showed that they belonged to eight different genera, namely *Bradyrhizobium*, *Sphingomonas*, *Methylobacterium*, *Caulobacter*, *Paracoccus*, *Ralstonia*, *Rhizobium*, and *Staphylococcus*. All isolates were studied for capability of producing antimicrobial and antifungal secondary metabolites using high-throughput screening models. Approximately 23 (13/57) and 2% (1/57) of isolates inhibited growth

of *Candida albicans* ATCC 10231^T and *Staphylococcus aureus* ATCC 51650^T, respectively. These results indicated that proteobacterium species isolates from Antarctic could serve as potential source of useful bioactive metabolites. Enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting produced nine clusters and 13 single isolates, with a high D value of 0.9248. RAPD fingerprinting produced six clusters and 13 single isolates, with a relatively low D value of 0.7776. ERIC-PCR analysis proved to have better discrimination capability than RAPD analysis and generated better clustering for all proteobacterium species isolates. We conclude that ERIC-PCR is a robust, reliable and rapid molecular typing method for discriminating different genera of proteobacteria.

Key words: Diversity; Proteobacteria; High-throughput screening; 16S rRNA; ERIC-PCR; RAPD

INTRODUCTION

Proteobacteria are a major phylum of bacteria, which are all Gram-negative bacteria. This bacterial taxonomy has been frequently found in Antarctic soils (Aislabie et al., 2008). Soil is an intensively exploited ecological niche that harbors inhabitants, which could produce many useful bioactive natural products, like antibiotic and antifungal compounds (Thakur et al., 2007). Lately the development of antimicrobial resistance among pathogenic bacteria and fungus has become a major health concern worldwide (Dias de Oliveira et al., 2005). The rise of resistant pathogenic bacteria and fungus is driving the need for new antimicrobial drugs, which continue to be of the utmost importance in screening programs globally. For many years, the drugs used successfully to treat various diseases were bacterium-derived compounds (Aislabie et al., 2008). Recently, due to the possible frequent genetic exchange between species (Bredholt et al., 2008), the chances of novel discovery of bioactive molecules from various well-known bacteria seem to have fallen dramatically. For example, the isolation of highly bioactive *Streptomyces* from different environments still produces similar metabolites or compounds.

The increase in duplicate findings has led to a serious rise in demand for new useful molecular structures in pharmacology industries. Many reports have shown that careful exploration of poorly explored areas and new habitats of the world like the Antarctic, Australia, China, and Jordan could provide novel microorganisms and useful products (Moncheva et al., 2002; Saadoun and Gharaibeh, 2003). Therefore, the exploration of new habitats with poorly explored areas and unusual environments has become critical for the discovery of novel bacteria and metabolites (Lam, 2007; Newman and Cragg, 2007; Thakur et al., 2007).

Poorly explored areas like the Antarctic present significant potential for the discovery of novel microorganisms like bacteria and biological active metabolites (Moncheva et al., 2002; Marinelli et al., 2004; Tindall, 2004; Taton et al., 2006; Bull and Stach, 2007). Barrientos Island is located at 62°24'S, 59°47'W, at the north entrance to the English Strait between Greenwich and Robert Islands. This island is occupied by various breeders like chinstrap penguins (*Pygoscelis antarctica*), gentoo penguins (*Pygoscelis papua*), southern giant petrels (*Macronectes giganteus*), kelp gulls (*Larus dominicanus*), and skuas (*Catharacta* spp). The

whole centre of the island is covered by a tremendously extensive moss carpet. Lichens *Xanthoria* spp, *Caloplaca* spp and other crustose lichen species are present.

The novelty and effectiveness of antimicrobial screening models are important in order to increase the chances of discovering novel metabolites. Therefore, high-throughput screening models were made available to detect antimicrobial activities (Hong et al., 2009). Molecular-based techniques like 16S rRNA analysis were used for molecular identification of isolates, whereas PCR fingerprinting methods like enterobacterial repetitive intergenic consensus (ERIC)-PCR and RAPD were examined for their discriminatory capability in differentiating different genera of proteobacteria. All molecular techniques stated above were used to study the genetic relatedness of bioactive proteobacterium isolates.

The aims of this study were: i) to recognize the distribution of proteobacteria from soils of Barrientos Island using a culturable method; ii) to identify the isolates using 16S rRNA analysis and high-throughput screening models to determine the bioactivity of secondary metabolites; iii) to use ERIC-PCR and RAPD to create a molecular profile of proteobacteria from different genera; iv) to study genetic relatedness of bioactive proteobacteria using 16S rRNA, ERIC-PCR and RAPD.

MATERIAL AND METHODS

Environmental sampling

Soil samples of different locations were collected from Barrientos Island during the XI Ecuadorian Antarctic Expedition to Research Station "Pedro Vicente Maldonado", Greenwich Island, and South Shetlands Islands of 2007. The soil samples of the upper 20-cm layer (after removing the top 2-3 cm) were collected from 5 different sites that harbor various interesting fauna and flora activities. Soils were sampled into sterile plastic bags using an aseptic metal trowel, and kept in the dark for transport to Malaysia. Soils were subsequently stored at -20° and -80°C.

Isolation of proteobacteria

Soil samples were taken out from -20°C and air dried for 7 days. Then, mortar and pestle were used to grind the samples into smaller particles to increase the efficiency of bacterial isolation. Phenol (1.5%, 30 min at 30°C) was used for pretreatment of soil samples according to Pisano et al. (1986). The pretreated soil samples were diluted 1:10 (v/v) with sterile ¼ Ringer solution and serial dilution up to 10⁻⁴. One hundred microliters of the 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ suspensions were spread in triplicate onto isolation media.

Dilutions of soil suspensions were spread onto 12 different isolation media, namely ISP media 2-4, 5-7 (Shirling and Gottlieb, 1966), IM2 (Gause modified medium 1; Ivantiskaya et al., 1978), IM3 (rafinose-histidine medium; Williams et al., 1984), SCA (starch casein agar; Küster and Williams, 1964), HVA (humic acid-vitamin agar; Hayakawa and Ohara, 1987), SA (*Streptomyces* agar; Atlas, 1993), and AIA (Actinomycetes isolation agar; Atlas, 1993). All the media were supplemented with 50 mg/L cycloheximide, 50 mg/L nystatin and 20 mg/L nalidixic acid (Williams and Davies, 1965) and incubated at 28°C for 1-4 weeks. Purified cultures were maintained on ISP medium 2 (Shirling and Gottlieb, 1966) at room temperature and as glycerol suspensions (20%, v/v) at -20°C.

Molecular characterization of proteobacterium species isolates

DNA extraction from pure cultures and PCR amplification

Genomic DNA of proteobacteria was extracted as described by Hong et al. (2009). The DNA yield and quality were assessed by 0.8% (w/v) agarose gel electrophoresis followed by DNA quantification using a Biophotometer (Eppendorf, Germany). Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9. PCR amplification was performed using the primer pair EuBac27F-Eubac1492R (Lane, 1991) and an Eppendorf Mastercycler (Eppendorf) was used to run PCR. The PCR mixture consisted of 20-200 ng bacterial genomic DNA, 2.0 μ L 10X PCR buffer with 20 mM $MgCl_2$, 2.0 μ L 10 mM dNTPs, 1 U Taq polymerase (Intron Biotechnology, South Korea), 200 pM of each primer (EuBac27F and Eubac1492R), and sterile ultrapure water was added to a final volume of 20 μ L. The cycling parameters were 5 min at 95°C for pre-denaturation, 35 cycles each of 50 s at 95°C for denaturation, 60 s at 52°C for annealing, 90 s at 72°C for extension and a final extension of 72°C for 10 min. The PCR amplification products were resolved by electrophoresis on 1.5% agarose gel (Promega, USA), which was stained with ethidium bromide (0.5 μ g/mL) and viewed under the Alpha Imager gel documentation system (Alpha Innotech, USA).

Molecular cloning, sequencing and phylogenetic analysis

PCR products were purified using a GeneAll Expin Gel SV purification kit (GeneAll, South Korea) and ligated to pDrive plasmid vector (Qiagen, Germany) and the ligation products transformed into competent *Escherichia coli* JM109. The insertion of the gene of interest was verified through blue-white colony selection followed by colony-PCR. Purified plasmid DNA was extracted using the Eppendorf FastPlasmid™ Mini kit and served as templates for PCR to confirm the insertion of the gene of interest. Plasmid DNA was sequenced with an ABI PRISM® 3100 DNA sequencer (Applied Biosystems).

The 16S rRNA gene sequences were aligned manually with sequences from the most closely related genera retrieved from the GenBank/EMBL/DDBJ databases using the CLUSTAL-X software (Thompson et al., 1997). The calculations of level of sequence similarity were performed using EzTaxon server 2.1 (Chun et al., 2007). A phylogenetic tree was inferred using neighbor joining algorithms (Saitou and Nei, 1987) using MEGA version 4.0 (Tamura et al., 2007). The stability of the resultant tree topologies was evaluated by bootstrap analysis (Felsenstein, 1985). Pairwise distances between sequences were generated using Kimura's 2-parameter model (Kimura, 1980).

ERIC-PCR analysis of proteobacteria

The proteobacterium species isolates were cultured and purified on ISP 2 medium. Genomic DNA was obtained and PCR were carried out as described by Versalovic et al. (1991) using the primer set ERIC-1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). The cycling parameters were 4 min at 95°C for pre-denaturation, 35 cycles each of 1 min at 94°C for denaturation, 1 min at 52°C for annealing, 3 min at 65°C for extension, and a final extension at 65°C for 8 min. Negative controls (no added DNA) were in-

cluded in all sets of reactions. The PCR amplification products were resolved by electrophoresis on 1.5% agarose gel (Promega), which was stained with 0.5 µg/mL ethidium bromide and viewed under the Alpha Imager gel documentation system (Alpha Innotech). The ERIC-PCR assays were repeated three times to determine the reproducibility of the banding patterns generated. The ERIC-PCR profiles were compared on the basis of the presence or absence of each DNA band and a data matrix was constructed. Strain diversity was calculated using the Dice's coefficient and UPGMA (unweighted pair-group arithmetic average) cluster analysis.

RAPD analysis of proteobacteria

A total of 6 arbitrary primers (OPO 06, 07, 08, 09, 10, and 11) were tested to discriminate representative isolates of proteobacteria. Only one primer, namely OPO 10 (5'-TCAGAGCGCC-3'), was selected for RAPD analysis as it provided reproducible and discriminatory banding patterns. The PCR mixture consisted of 20-200 ng bacterial genomic DNA, 2.0 µL 10X PCR buffer with 20 mM MgCl₂, 2.0 µL 10 mM dNTPs, 1 U Taq polymerase (Intron Biotechnology), 200 pM primer OPO 10, and sterile ultrapure water was added to a final volume of 20 µL. The cycling parameters were 4 min at 95°C for pre-denaturation, 40 cycles each of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 1 min at 72°C for extension, and a final extension at 72°C for 7 min. Negative controls (no added DNA) were included in all sets of reactions. The PCR amplification products were resolved by electrophoresis as mentioned above. RAPD was repeated three times to determine the reproducibility of the banding patterns generated. Strain diversity was calculated using the Dice's coefficient and UPGMA cluster analysis.

Calculation of discrimination indices

The discriminatory power of each typing method was determined by calculating the discriminatory index using the Simpson's index of diversity, a method described by Hunter and Gaston (1988). The following is the formula for calculating the discrimination index:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^S n_j(n_j - 1)$$

where D is a numerical index of discrimination; N is the total of strains in the sample population; S is the total number of types described, and n_j is the number of strains belonging to the j th type.

Calculating the discrimination value D allows comparison of the discriminatory powers of typing methods.

Preparation of crude extracts for secondary metabolite screening

The fermentation medium used was FM3 (Garcia et al., 1999), FM2 (Yu et al., 2008) and FM17 (Chen et al., 2006). All media were autoclaved at 121°C for 20 min. Each of the 57 isolates were transferred to a test tube (30 × 200 mm), which contained 20 mL of the relevant fermentation medium, and cultured at 200 rpm, at an angle of 45°, for 7-10 days at 28°C.

Screening for antimicrobial metabolites

The method used for screening of antimicrobial metabolites was modified from Hong and Xiao (2006). *Candida albicans* ATCC 10231^T was cultured overnight at 30°C at 200 rpm in YPD medium (2% glucose; 2% tryptone; 1% yeast extract; pH 5.0–5.5) whereas *Staphylococcus aureus* ATCC 51650^T, methicillin-resistant *S. aureus* (MRSA) ATCC BAA-44^T and *Pseudomonas aeruginosa* ATCC 10145^T were cultured overnight at 37°C and at 200 rpm in nutrient broth. The resultant cultures were diluted with their respective media to 0.8–1.2 × 10⁶ CFU/mL, and 100 µL aliquots of this inoculum were transferred to individual wells in a 96-well plate. An aliquot of 1-mL culture fermentation broth (FM3, FM2, FM17) from each isolates was centrifuged at 10,000 rpm and at 4°C. The fermentation broth supernatant (100 µL) was taken from each of the isolates and added to each of the wells. The two culture media were used as negative controls, and fluconazole as the positive controls for the anti-*C. albicans* while cefoperazone/tazobactam were used as the positive controls for anti-*S. aureus*, anti-MRSA and anti-*P. aeruginosa* assays. The 96-well plates were shaken at 200 rpm for 24 h at 37°C for all assays except for anti-*C. albicans* assay that was shaken at 30°C. After incubation, all plates were assayed with a microplate spectrophotometer (Multiskan Mk3, Finland) at 570 nm. The absorbance readings from each well were used to record bioactivities as “+”, “++”, or “+++” for higher or equal to 4–6, 6–8 and 8 µg/mL of standard, respectively. Serial dilutions of the antibiotics (128, 64, 32, 16, 14, 12, 10, 8, 6, 4, 2, and 1 µg/mL) were used to generate standard curves.

RESULTS

Isolation and identification of proteobacteria

A total of 57 proteobacterium species isolates were successfully isolated from 3 soil samples collected from Barrientos Island. Soil samples labeled 445, 446 and 453 contributed to 19, 12 and 68%, respectively, to the total number of isolates. Isolates were identified using PCR and sequencing of 16S rRNA gene sequences. Fifty-seven isolates belonged to 8 different genera in phylum proteobacteria, namely genera *Bradyrhizobium*, *Sphingomonas*, *Methylobacterium*, *Caulobacter*, *Paracoccus*, *Ralstonia*, *Rhizobium*, and *Staphylococcus* with 27, 16, 9, 1, 1, 1, 1, and 1 isolates, respectively. From a total of 12 types of isolation media used, 11 isolation media successfully isolated proteobacteria, namely media SCA, AIA, HVA, ISP 3, SA, IM2, ISP 7, raffinose-histidine agar, ISP 4, ISP 6, and ISP 2 with 11, 7, 7, 7, 7, 5, 5, 3, 2, 2, and 1 isolates, respectively. This result indicated that SCA was the most suitable medium to isolate proteobacteria from Barrientos Island.

Taxonomy diversity of bioactive proteobacteria

The antimicrobial capability of secondary metabolites of 57 proteobacterium species isolates were determined using 4 high throughput screening models, namely against *C. albicans* ATCC 10231^T, *S. aureus* ATCC 51650^T, MRSA ATCC BAA-44^T, and *P. aeruginosa* ATCC 10145^T. After the screening, a total of 14 isolates produced bioactive metabolites and proceeded to cloning and sequencing of the 16S rRNA gene sequence. Bioactive proteobacte-

rium isolates were classified into 4 genera in 4 families (Table 1). The genera *Bradyrhizobium*, *Paracoccus* and *Sphingomonas* contributed 9, 1 and 1 isolates active against the pathogen *C. albicans*. While 3 isolates from the genus *Methylobacterium* showed bioactivity against *S. aureus* (1 isolate) and *C. albicans* (2 isolates). None of the isolates of proteobacteria showed any bioactivity against *P. aeruginosa* or MRSA (Table 1).

Table 1. Identification of different antimicrobial secondary metabolites from proteobacteria isolated from soils of Barrientos Island, Antarctic.

Phylum	Genera	Families	Number of identified representative strains			
			Anti- <i>S. aureus</i>	Anti-MRSA	Anti- <i>C. albicans</i>	Anti- <i>P. aeruginosa</i>
Proteobacteria	<i>Bradyrhizobium</i>	<i>Bradyrhizobiaceae</i>	0	0	9	0
	<i>Methylobacterium</i>	<i>Methylobacteriaceae</i>	1	0	2	0
	<i>Paracoccus</i>	<i>Rhodobacteraceae</i>	0	0	1	0
	<i>Sphingomonas</i>	<i>Sphingomonadaceae</i>	0	0	1	0

Sequence alignment and phylogenetic analysis of bioactive proteobacteria

Analysis of the 16S rRNA gene sequences were performed using the Basic Local Alignment Search Tool (BLAST) under EzTaxon server 2.1 (Chun et al., 2007). The 16S rRNA sequences of type strains were retrieved from DDBJ/EMBL/GenBank databases and aligned with isolate sequences obtained in this study. Sequence alignment showed that 8 isolates (BR42, BR62, BR65, BR82, BR88, BR96, BR100, and BR105) belonged to a member of the genus *Bradyrhizobium* with 99.3-99.7% identity to *B. pachyrhizi* PAC48^T (Table 2). Only 1 isolate (BR45) exhibited 99.2% similarity to *B. lablabi* CCBAU 23086^T. The sequences of isolates MB63 and MB104 exhibited 100 and 99.7% similarity to *Methylobacterium jeotgali* S2R03-9^T while isolate MB20 demonstrated 98.7% identity to *M. platani* PMB02^T. The sequences of isolates PC101 and SM14 demonstrated 97.1 and 99.9% identity to sequences of type strain *Paracoccus chinensis* KS-11^T and *Sphingomonas melonis* DAPP-PG 224^T, respectively. Proteobacteria that exhibited high taxonomical diversity were those that demonstrated activity against *C. albicans*. A total of 13 isolates from 4 different genera were positive in producing secondary metabolites against *C. albicans* (Table 1).

Table 2. Bioactive isolates obtained in this study.

Isolate number	Closest relative	Accession No.	Sequence identity (%)
BR45	<i>Bradyrhizobium lablabi</i> CCBAU 23086 ^T	GU433448	99.2
BR42	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.6
BR62	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.7
BR65	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.7
BR82	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.5
BR88	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.7
BR96	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.7
BR100	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.7
BR105	<i>Bradyrhizobium pachyrhizi</i> PAC48 ^T	AY624135	99.3
MB63	<i>Methylobacterium jeotgali</i> S2R03-9 ^T	DQ471331	100.0
MB104	<i>Methylobacterium jeotgali</i> S2R03-9 ^T	DQ471331	99.7
MB20	<i>Methylobacterium platani</i> PMB02 ^T	EF426729	98.7
PC101	<i>Paracoccus chinensis</i> KS-11 ^T	EU660389	97.1
SM14	<i>Sphingomonas melonis</i> DAPP-PG 224 ^T	AB055863	99.9

The phylogenetic tree of the 16S rRNA gene sequence (Figure 1) signified that isolates BR42, BR45, BR62, BR65, BR82, BR88, BR96, BR100, and BR105 were grouped in the *Bradyrhizobium* clade together with type strain *B. lablabi* CCBAU 23086^T, *B. pachyrhizi* PAC48^T and *B. jicamae* PAC68^T supported by a high bootstrap value of 100% (Figure 1). Isolate MB20 formed a distinct clade with type strain *M. platani* PMB02^T supported by a bootstrap of 70%, while isolate MB63 and MB104 clustered with type strain *M. jeotgali* S2R03-9^T and formed a subclade with 100% bootstrap support. The positions of the *Paracoccus* clade exhibited that isolate PC101 grouped with *P. chinensis* KS-11^T at a low bootstrap value of 38%, while isolate SM14 clustered together with *S. melonis* DAPP-PG 224^T with bootstrap support of 99%. All the bioactive proteobacteria from the different genera (*Bradyrhizobium*, *Methylobacterium*, *Paracoccus*, and *Sphingomonas*) formed relatively distinct phyletic lines with their respective type strain from the same genera.

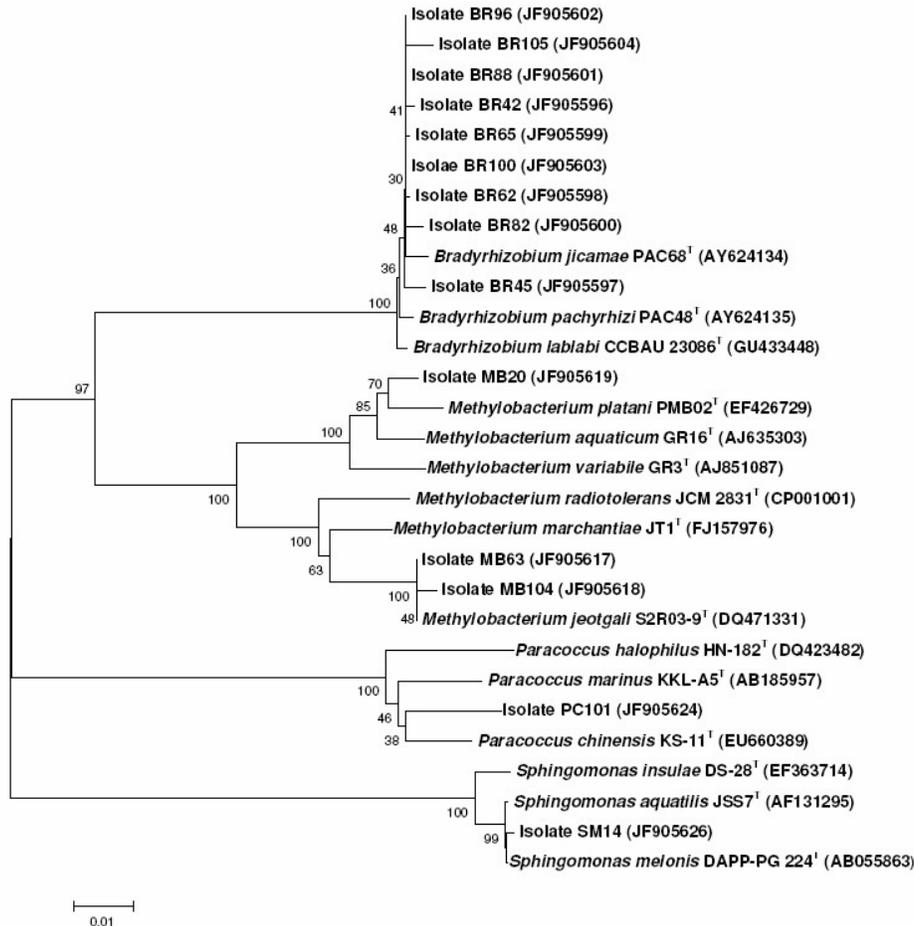


Figure 1. Neighbor-joining tree based on almost complete 16S rRNA gene sequences showing relationships between 14 bioactive isolates of proteobacteria and between them the type strains of the highest 16S rRNA sequence similarity. Numbers at nodes indicate bootstrap values based on 1000 replicates. Bar = 1% sequence divergence.

ERIC-PCR analysis of proteobacteria

ERIC-PCR fingerprinting of a total of 57 isolates exhibited specific patterns corresponding to particular genotypes with amplified bands ranging in size from <250 to 10,000 bp. A dendrogram (Figure 2) generated from ERIC-PCR analysis demonstrated high discriminatory capability by producing genus-specific clusters that were able to discriminate all of the different genera in this study. A total of 57 proteobacterium species isolates were grouped into 9 clusters (A, H, I, J, K, O, P, Q, and T) and 13 single isolates (B, C, D, E, F, G, L, M, N, R, S, U, and V) at a similarity level of 65% with a high D value of 0.9248 (Figure 2). ERIC-PCR demonstrated good discriminatory capability as all the clusters formed (9 clusters) comprised a single type of genus only (Figure 2).

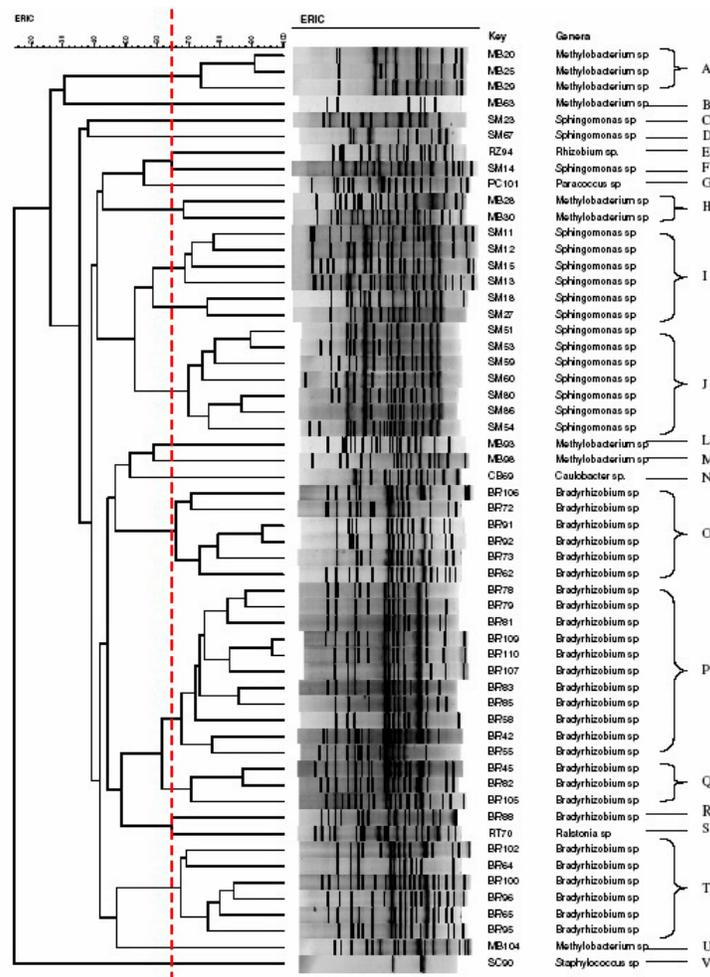


Figure 2. Dendrogram generated from enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting of 57 isolates of proteobacteria. A total of 9 clusters (A, H, I, J, K, O, P, Q, and T) and 13 single isolates (B, C, D, E, F, G, L, M, N, R, S, U, and V) were observed at similarity level of 65%.

The dendrogram in Figure 3 was derived from ERIC-PCR analysis of 14 bioactive proteobacteria. This dendrogram comprised 4 main clusters. The first and second clusters were isolates MB63 and MB20 from genera of *Methylobacterium*. The third cluster consisted of isolate MB104 (*Methylobacterium*), SM14 (*Sphingomonas*) and PC101 (*Paracoccus*). The fourth cluster comprised 9 isolates (BR42, BR88, BR45, BR82, BR105, BR100, BR96, BR65, and BR62) from the genus *Bradyrhizobium*.

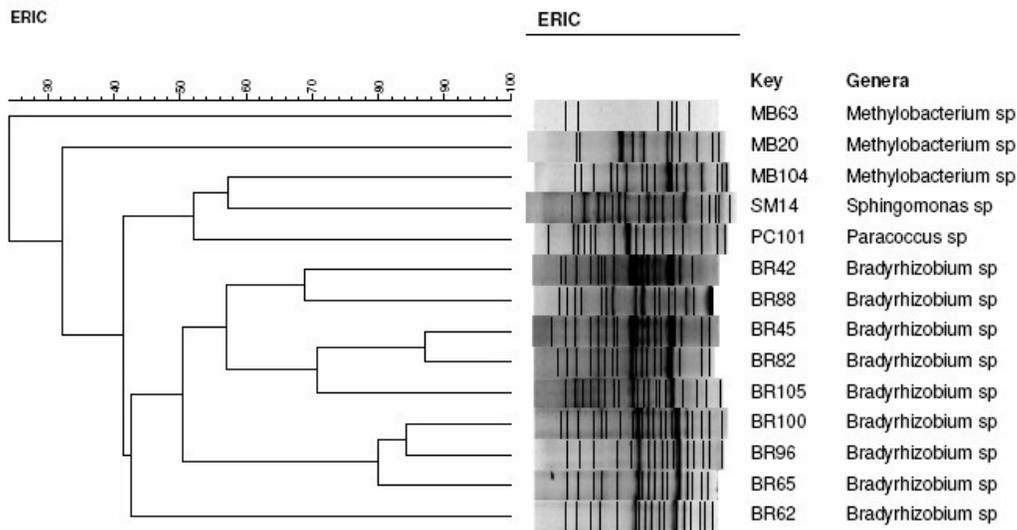


Figure 3. Dendrogram generated from enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting of 14 isolates of bioactive proteobacteria.

RAPD analysis of proteobacteria

RAPD results from arbitrary primer OPO 10 showed specific patterns corresponding to particular genotypes with amplified DNA fragments ranging from <250 to 10,000 bp. Figure 4 shows a dendrogram generated from the analysis of 57 proteobacterium species isolates and exhibited low discriminatory capability by producing 6 clusters (d, e, h, i, j, o) and 13 single isolates (a, b, c, f, g, k, l, m, n, p, q, r, s) with a D value of 0.7776 at a similarity level of 65%. From the 6 clusters formed, 5 clusters (d, h, i, j, o) comprised a single genus only, whereas cluster 'e' comprised isolates from 2 different genera, namely *Ralstonia* and *Rhizobium*.

RAPD pattern analysis of 14 bioactive proteobacterium species isolates was performed and is illustrated in Figure 5. This dendrogram comprised 5 main clusters. The first cluster comprised 8 isolates from the genus *Bradyrhizobium*, namely BR105, BR45, BR42, BR65, BR82, BR96, BR100, and BR62. Isolates BR88 (*Bradyrhizobium*) and MB104 (*Methylobacterium*) formed the second cluster. The third cluster consisted of the single isolate MB63 from the genus *Methylobacterium* while the fourth cluster contained isolate MB20 and SM14 from genera *Methylobacterium* and *Sphingomonas*. The last cluster comprised 1 isolate (PC101) from the genus *Paracoccus*.

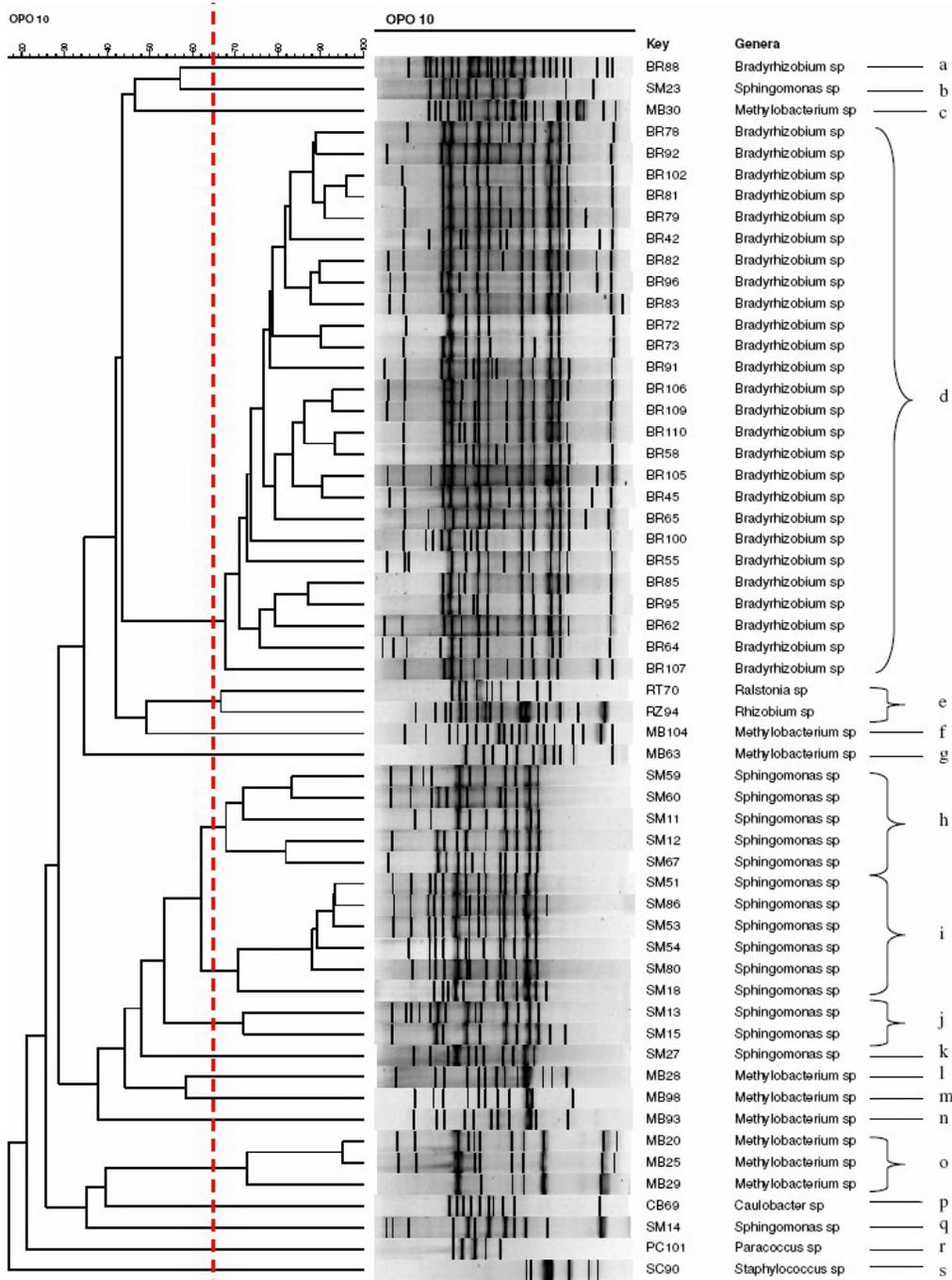


Figure 4. Dendrogram generated from RAPD fingerprinting of 57 isolates of proteobacteria. A total of 6 clusters (d, e, h, i, j, o) and 13 single isolates (a, b, c, f, g, k, l, m, n, p, q, r, s) were observed at a similarity level of 65%.

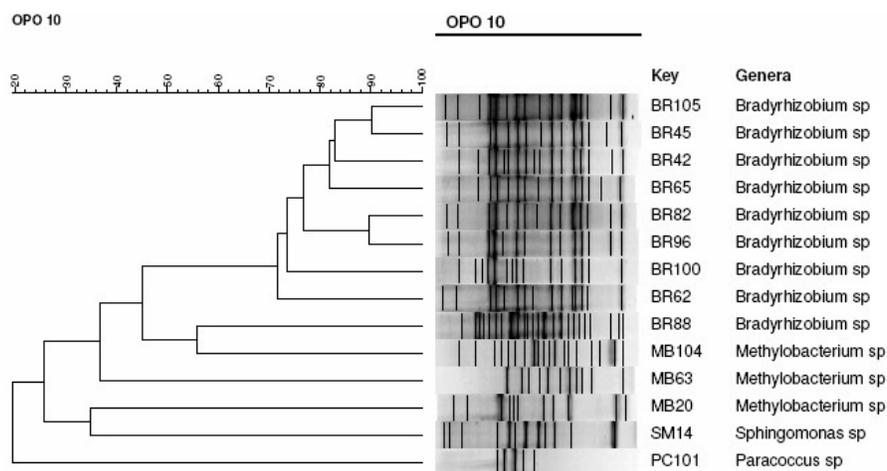


Figure 5. Dendrogram generated from RAPD fingerprinting of 14 isolates of bioactive proteobacteria.

DISCUSSION

Lately, due to frequent genetic exchange between species of bacteria, the chances of discovering new bioactive compounds from bacteria are low. This phenomenon has resulted in similar or identical finding of compounds from well-known bacteria even though they were isolated from different environments (Bredholt et al., 2008). The reduction of new metabolites discovered has forced the search into new habitats. Poorly explored areas or unusual environments have become critical for the discovery of novel compounds and bacteria (Saadoun and Gharaibeh, 2003; Lam, 2007; Newman and Cragg, 2007; Thakur et al., 2007). Therefore, soil samples taken from the Antarctic, one of the coldest and most poorly explored regions of the world, present good potential for the discovery of novel bioactive metabolites and compounds from microorganisms like bacteria (Marinelli et al., 2004; Tindall, 2004; Taton et al., 2006).

This study was conducted to investigate the diversity and antimicrobial bioactivity of proteobacteria by performing isolation, molecular identification, molecular characterization, and bioactive secondary metabolite screening using 4 high-throughput screening models. A total of 57 isolates of proteobacteria were successfully isolated from 11 different media and they belonged to 8 different genera. Soil sample '453' exhibited the highest diversity and density of the proteobacterium population among all soil samples used in this study, with 68% (39/57) of isolates from 8 different genera of proteobacteria successfully isolated from this soil sample. The wide spread of various genera of proteobacteria in the Antarctic showed that their distribution is highly endemic, especially in soil. This observation is in agreement with Aislabie et al. (2008), which reported that the diversity of bacteria, including proteobacteria, in the Ross Sea region of the Antarctic is highly widespread. This information will benefit the bio-prospecting of bacteria from sampling soil from the Antarctic region.

A total of 25% (14/57) of proteobacterium species isolates belonged to 4 genera (*Bradyrhizobium*, *Methylobacterium*, *Sphingomonas*, and *Paracoccus*) and 4 families exhibited antimicrobial properties. Four genera (*Caulobacter*, *Rhizobium*, *Ralstonia*, and *Staphy-*

lococcus) did not exhibit any bioactivity against 4 different screening models used. From the 4 screening models used, 2 obtained positive results with 13 isolates (23%) active against *C. albicans* and 1 isolate (2%) against *S. aureus*. The most bioactive genus was *Bradyrhizobium*, which contributed with 9 bioactive isolates or 64% (9/14) of the total bioactive isolates in this study. This showed that the production of antibiotics or toxins from Antarctic bacteria could be a mechanism to confer them a competitive survival capability (Vincent, 2000).

Recently, more global findings have shown that novel bacteria from various extreme or poorly explored environments are capable of producing valuable sources of new bioactive metabolites and compounds (Bredholt et al., 2008; Rojas et al., 2009). Therefore, it is important to determine the taxonomic relationships and metabolic diversity of bacteria. In this study, 16S rRNA sequence analysis showed that 1 bioactive isolate (PC101) was highly likely to be assigned to new species as they are separated from their respective type strains of phylogenetic neighbors by sequence similarities well below those found between closely related species in the same genera. The 16S rDNA sequence similarity of PC101 to the type strain *P. chinensis* KS-11 was lower than 97.1% (Table 2). This value is low as compared to the similarity of type strain *P. chinensis* KS-11 to its closest related species *P. niistensis* NII-0918, which was as high as 99.1% (data not shown). The irregular combination of selection pressures could lead to evolution that produces novel chemical adaptation and formation of novel strains (Ellis-Evans and Walton, 1990; Vincent, 2000).

PCR fingerprinting methods like ERIC-PCR and RAPD were used to study the relatedness between proteobacterium species isolates from different genera. ERIC-PCR uses oligonucleotides targeting short repetitive sequences dispersed throughout various bacterial genomes. ERIC sequences are dispersed throughout the genome of enterobacteriaceae in different orientations thus enabling their location in bacterial genomes, which allows discrimination at genus and species level based on their electrophoretic amplification DNA fragments (Yoke-Kqueen et al., 2008; Nimnoi et al., 2010). The ERIC-PCR pattern obtained in this study showed good resolution and generated a high discriminatory pattern with 100% (9/9) genus-specific clusters that deciphered isolates of different genera from each other completely. The D value of ERIC-PCR fingerprinting was the highest in this study, with 0.9248 at a 65% similarity level (Figure 2).

RAPD analysis has achieved excellent results for genomic typing of various microorganisms (de Oliveira et al., 2002; Learn-Han et al., 2008; Nimnoi et al., 2010). Therefore, arbitrary primer OPO 10 was used to fingerprint proteobacterium species isolates in this study. However, our results indicated that RAPD exhibited rather weak discriminatory capability as compared to ERIC-PCR. RAPD analysis produced 83% (5/6) genus-specific cluster with a low D value of 0.7776 at a similarity level of 65% (Figure 4). This result is significantly lower compared to ERIC-PCR, thus demonstrating that ERIC-PCR is much more discriminatory than RAPD for fingerprinting of Antarctic proteobacteria in this study.

The consistencies and differences of 14 bioactive proteobacterium species isolates were evaluated using 3 molecular-based techniques including 16S rRNA analysis (Figure 1), ERIC-PCR (Figure 3) and RAPD (Figure 5). Cluster 3 in the ERIC-PCR dendrogram (Figure 3) consists of isolates from the genera *Methylobacterium* (MB104), *Sphingomonas* (SM14) and *Paracoccus* (PC101) that are also located at the neighboring location in the genetic evolutions 16S rRNA tree (Figure 1). However, this result was different in the RAPD dendrogram (Figure 5) as the position of isolate PC101 was placed in a different position from the others. Many isolates in the fourth cluster generated by the ERIC-PCR pattern were consistent with 16S rRNA tree analysis. In this

cluster (Figure 3), the position of BR42 with BR88, BR45 with BR82, and BR100 with BR62 were closely grouped together and formed a subclade within this cluster. This observation was similar in the 16S rRNA tree that revealed that these different sets of isolates were closely clade together as well (Figure 1). However, RAPD analysis in Figure 5 generated different results with no similar clade formed as compared to ERIC-PCR and 16S rRNA tree analysis.

In conclusion, this study demonstrated that proteobacteria have been successfully isolated from the soils of Barrientos Island using a culturable method and that they are widely distributed. The screening for bioactive secondary metabolites showed that 25% of proteobacterium species isolates were highly bioactive. These results indicate that proteobacterium species isolates from the Antarctic could serve as a potential source of useful bioactive metabolites for various antimicrobial screening models. Molecular characterization methods like analysis of 16S rRNA sequences is a powerful tool in determining higher taxonomic relationships of proteobacteria. ERIC-PCR analysis showed better discriminatory capability than RAPD with the best clustering and highest D value of 0.9248. Therefore, in this study ERIC-PCR was a robust, reliable and rapid molecular typing method in discriminating different proteobacterium species isolates.

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