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RESEARCH ARTICLE

Abundance and diversity of functional genes involved in the degradation of aromatic hydrocarbons in Antarctic soils and sediments around Syowa Station

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Abstract Hydrocarbon catabolic genes were investigated in soils and sediments in nine different locations around Syowa Station, Antarctica, using conventional PCR, real-time PCR, cloning, and sequencing analysis. Polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase (PAH-RHD)-coding genes from both Gram-positive and Gram-negative bacteria were observed. Clone libraries of Gram-positive RHD genes were related to (i) *nidA3* of *Mycobacterium* sp. py146, (ii) *pdo*A of *Terrabacter* sp. HH4, (iii) *nidA* of *Diaphorobacter* sp. KOTLB, and (iv) *pdo*A2 of *Mycobacterium* sp. CH-2, with 95–99 % similarity. Clone libraries of Gram-negative RHD genes were related to the following: (i) naphthalene dioxygenase of *Burkholderia glathei*, (ii) *phnAc* of *Burkholderia sartisoli*, and (iii) RHD alpha subunit of uncultured bacterium, with 41–46 % similarity. Interestingly, the diversity of the Gram-positive RHD genes found around this area was higher than those of the Gram-negative RHD genes. Real-time PCR showed different abundance of dioxygenase genes between locations. Moreover, the PCR-denaturing gradient gel electrophoresis (DGGE) profile demonstrated diverse bacterial populations, according to their location. Forty dominant fragments in the DGGE profiles were excised and sequenced. All of the sequences belonged to ten bacterial phyla: Proteobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Firmicutes, Chloroflexi, Gemmatimonadetes, Cyanobacteria, Chlorobium, and Acidobacteria. In addition, the bacterial genus *Sphingomonas*, which has been suggested to be one of the major PAH degraders in the environment, was observed in some locations. The results demonstrated that indigenous bacteria have the potential ability to degrade PAHs and provided information to support the conclusion that bioremediation processes can occur in the Antarctic soils and sediments studied here.

Keywords Antarctica · Syowa · PAHs · PCR-DGGE · Real-time PCR · Dioxygenase · Diversity

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Introduction

Antarctica is the most pristine and least populated continent in the world. However, scientific operations have led to an accumulation of hydrocarbon compounds in the Antarctic environment. Hydrocarbons, in the form of fuel oils, are used by Antarctica research stations for transportation and scientific operations (Aislabie et al. 2004).

Syowa Station is a scientific research station established by Japan in 1957. It is located on East Ongul Island (69° 00′ S, 39° 35′ E) in Antarctica. With an increase in constructions and human activities, the area around the station may have become

contaminated with hydrocarbon compounds, such as polycyclic aromatic hydrocarbons (PAHs). PAHs are the components of petroleum products that are highly persistent in the environment and have toxic, mutagenic, and carcinogenic effects on organisms (Yergeau et al. 2009).

Bioremediation by microorganisms is one of the technologies for the cleanup of the Antarctic environment because it is quite safe and harmless (Aislabie and Foght 2010). The biodegradation of PAHs in some Antarctic areas, such as at Jubany Station, has been reported to be using culture-dependent methods (Ruberto et al. 2006). That study indicated that Antarctic microbial communities are capable of degrading PAHs and that the bioremediation process is possible in Antarctic environments. The genera *Pseudomonas* and *Sphingomonas* have been identified as PAH degraders in Antarctic soils (Ma et al. 2006; Panicker et al. 2010). Further understanding of the diversity of the natural microbial community and specific metabolic genes can help assess the biodegradation potential of environments (Fernández-Luqueño et al. 2011). However, only culture-dependent approaches may not be enough to estimate microbial diversity in the environment since the majority of microorganisms are difficult to be cultivated due to the lack of appropriate conditions and media (Amann et al. 1995). Therefore, molecular methods relying in phylogenetically informative genes, such as denaturing gradient gel electrophoresis (DGGE) and 16S ribosomal RNA (rRNA) gene-based clone libraries, have been applied to identify indigenous genera (Jurelevicius et al. 2012b; Powell et al. 2003; Das and Kazy 2014). In addition, the biodegradation potential in environments can also be estimated using culture-independent methods targeting specific metabolic genes. Ring-hydroxylating dioxygenases are key enzymes that catalyze the first step of PAH degradation pathways (Peng et al. 2008). The genes encoding for the α-subunit of terminal dioxygenase have been widely used as target genes to describe the PAH-degrading potential in different environments (Jurelevicius et al. 2012a; Marcos et al. 2009). Moreover, extradiol dioxygenases which are enzymes that are essential for breaking down the common intermediates in the aerobic bacterial degradation of aromatic compounds are also suitable to be used as markers to detect a broad range of aromatic-degrading bacteria (Suenaga et al. 2009). Many studies have investigated extradiol dioxygenase genes in polluted environments (Sipila et al. 2006; Junca and Pieper 2004).

Knowledge of the PAH biodegradation potential in Antarctic soils is essential for the management of soils for bioremediation. This study aims to investigate the dominant bacteria and the abundance as well as the diversity of genes involved in the degradation of PAHs and hydrocarbons in Antarctic soil and sediment samples around the Syowa Station in order to assess the PAH biodegradation potential in Antarctic soils and sediments. This could provide information to support bioremediation strategies for PAH-contaminated Antarctic soils and sediments.

Materials and methods

Soil sampling and PAH analysis

Surface soil and sediment samples were collected aseptically at a depth of 0–10 cm from nine locations near Syowa Station in Antarctica during the 51st Japanese Antarctic Research Expedition (JARE-51) from November 2009 to March 2010. A map with the sampling sites is provided in Electronic supplementary material (ESM) Fig. S1, and the description of sampling locations (1–9) is shown in Table 1. Samples were kept frozen until processing.

The extraction of PAHs from the soil and sediment samples was conducted with the Soxhlet extraction procedures as outlined in the US Environmental Protection Agency (EPA) test method 3540 (US EPA 1996), with slight modifications in the extraction solvent, time, and extraction volume (Lau et al. 2010). Briefly, 10 g of each soil and sediment sample was dried at an ambient temperature of approximately 30 °C, crushed in a porcelain mortar, and sieved through a ten-mesh stainless sieve. Then, each sample was put into a Whatman extraction thimble that had been pre-extracted with dichloromethane (DCM). The sample was extracted with 250 ml of DCM in a Soxhlet extractor (Buchi, Switzerland) for 24 h, further reduced to 10 ml using a rotary evaporator (Buchi Rotavapor; Switzerland), transferred into a 4-ml glass vial, and kept at 4 °C for analysis. Pyrene at 100 mg l^{-1} was used as an internal control (US EPA test method 3500) (US EPA 2007). Analysis of PAHs was slightly modified from the US EPA test method 8310 (US EPA 1986) using high-performance liquid chromatography (HPLC), where the extractant was analyzed at 30 °C by reverse phase HPLC using a mixture of acetonitrile:water (70:30, *v*/*v*) as the mobile phase at a flow rate of 1 ml min^{-1}. A gas chromatography mass spectrometer was then used (Agilent 7890A GC/7000B Triple Quadrupole MS), and data analysis was conducted using the mass spectra database (the NIST 11 mass spectral database; SIS Inc., USA). The detection limits of 16 PAHs were ranged from 0.02 to 1.8 μg l^{-1} (equivalent to 2 μg kg^{-1} of the soil or sediment samples).

Genomic DNA extraction and PCR-DGGE analysis

DNA was extracted in triplicate from the soil and sediment samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and purified by gel electrophoresis to remove some PCR inhibitors such as humic acids. DNA was recovered by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan), following the

Table 1 Site description

Number	Date of collection	Site name	Condition of the site
1	January 4, 2010	Syowa site	Land terrestrial, disturbed site (location of the main station)
2	January 4, 2010	Syowa site, hill in front of a Syowa building	Land terrestrial, disturbed site (location of the main station)
3	January 11, 2010	Lake 1, East Ongul Island	Freshwater lake, pristine site
4	January 11, 2010	Lake Midori, East Ongul Island	Freshwater lake, pristine site
5	January 20, 2010	Funazuko Lake	Saltwater lake, 55 ppt (salinity), pristine site
6	January 20, 2010	Oyako Ike Lake	Freshwater lake, disturbed site (location of the small station)
7	January 21, 2010	Oku Ika Lake	Freshwater lake, pristine site
8	January 21, 2010	Skavenese beach	Land terrestrial, pristine site
9	January 28, 2010	Kai-no-hama beach, East Ongul	Land terrestrial, pristine site

manufacturer's instructions. The amounts of DNA obtained per gram of the nine soil and sediment samples were 4.60, 2.36, 5.64, 3.55, 10.47, 10.64, 2.81, 5.12, and 1.67 μg, respectively.

PCR was performed in 30-μl volumes containing 50 ng of template DNA, GoTaq® Green Master Mix (Promega, Mannheim, Germany), 20 pmol of each forward and reverse primer, and 30 μl of nuclease-free water. All PCR primers used in this study are listed in Table 2. For the amplification of the bacterial 16S rRNA gene, primers 341F with a GC clamp and 520R were used. The PCR cycling conditions were set up as previously described (Muangchinda et al. 2013).

For the amplification of the *Sphingomonas* 16S rRNA gene, primers Sphingo108f with a GC clamp and Sphingo420r were used. The PCR cycling conditions were set up as previously described (Leys et al. 2004). PCR products were run on a 2 % agarose gel, stained with ethidium bromide, and visualized under UV light.

DGGE was performed using a Bio-Rad DCode system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Muangchinda et al. 2013). The denaturing gradient ranged from 30 to 70 %. The gels were run at a constant voltage of 130 V for 4.5 h at 60 °C. Then, the gels were stained with ethidium bromide for 15 min and photographed.

Bands from the DGGE gels were cut and placed in deionized water at 4 °C overnight and then re-amplified using primers without the GC clamp. PCR products to be sequenced were purified, ligated to a pGEM-T Easy Vector (Promega, Mannheim, Germany), and transformed into *Escherichia coli* JM109 competent cells. The clones with inserts were sequenced using 1st BASE DNA Sequencing Services (First BASE Laboratories Sdn Bhd Malaysia). Sequences were aligned against reference sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic trees were constructed in MEGA5.1 using the aligned sequences by the neighbor-joining method and using Kimura two-parameter distances and 1000 bootstrap repeats.

Detection of dioxygenase genes

PCR amplification was carried out in a 30-μl reaction mixture containing 50 ng of soil DNA as templates. The primer sets, target genes, and product lengths are described in ESM Table S1. The PCR products were purified and cloned using the method described above. Positive inserts were analyzed using restriction fragment length polymorphisms (RFLPs) to group plasmids that had the same patterns of DNA restriction fragments. The restriction enzymes (*Hin*FI and *Rsa*I) were

Table 2 Identities of amino acid sequences from the cloned PCR products obtained via PCR with the polycyclic aromatic hydrocarbon-RHDα-GN and PAH-RHDα-GP primer DNAs from the soil and sediment samples

Clone libraries	Group of clones	Enzyme family	Closest bacteria and corresponding amino acid sequence accession number	% amino acid sequence identity
PAH-RHDα-GN libraries	i	Naphthalene dioxygenase	*Burkholderia glathei* (AAN74945)	46
	ii	*phn*Ac	*Burkholderia sartisoli* (AAD09872)	45
	iii	RHD alpha subunit	Uncultured bacterium (CCG47700)	41
PAH-RHDα-GP libraries	i	*nid*A3	*Mycobacterium* sp. py146 (ADH94647)	95
	ii	*pdo*A	*Terrabacter* sp. HH4 (AAZ38356)	98
	iii	*nid*A	*Diaphorobacter* sp. KOTLB (ACL13523)	97
	iv	*pdo*A2	*Mycobacterium* sp. CH-2 (AAZ78216)	99

used to digest the plasmids. One clone from each pattern was randomly selected for sequencing and analyzed with the BLASTx program. The Shannon–Weaver index of diversity (*H*) was calculated to determine the diversity of the dioxygenase genes. The phylogenetic tree of the deduced amino acid sequences was constructed using the MEGA5.1 program with the neighbor-joining method and 1000 bootstrap repeats.

Real-time PCR assays

Real-time PCR was performed on a MiniOpticon Real-Time PCR detector with MJ Opticon Monitor Analysis Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for the determination of the gene copy numbers of 16S rRNA genes and dioxygenase genes. Reaction mixtures were performed in a 0.2-ml thin-wall, clear PCR strip tubes with 25-μl reaction volumes containing Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.3 μM of primers, and 2 μl of template DNA (50 ng). The amplification conditions were set up as previously described (Muangchinda et al. 2013). Purified 16S rRNA, PAH-RHDα-GP, and PAH-RHDα-GN gene plasmids of three randomly selected clones (one each from a bacterial clone, a GP clone, and a GN clone) served as standards. The limit of quantification (LOQ) for quantitative PCR (qPCR) was ten copies per reaction. Serial dilutions of the bacterial, PAH-RHDα-GP, and PAH-RHDα-GN standards were set up above the LOQ in the range of 10^2–10^{10} gene copies per reaction, and r^2 values of the standard curve of 16S rRNA, PAH-RHDα-GP, and PAH-RHDα-GN genes were 0.98, 0.99, and 0.99, respectively. The data were used to create standard curves correlating the threshold cycle (Ct) numbers with the gene copy numbers. The real-time PCR efficiencies for the primers 968F/1401R, PAH-RHDα-GP, and PAH-RHDα-GN were 81.1, 105.4, and 99.1 %, respectively.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were deposited in the GenBank database under the following accession numbers: the 16S rRNA genes, KJ001006 to KJ001054, and the dioxygenase genes, KJ001055 to KJ001062

Results

PAH contamination

From the PAH extraction and analysis methods described above, contamination by particular PAHs was not detected in any of the samples from the nine locations.

Detection of hydrocarbon catabolic genes

Nine primer pairs were used to detect hydrocarbon catabolic genes in the soil and sediment samples, and the PCR products were detected when using only four primer pairs including GPF/GPR, GNF/GNR, xylE-F/xylE-R, and bphC-F/bphC-R. Ring-hydroxylating dioxygenase genes for PAH degradation were detected in the soil and sediment samples collected near the main building of the Syowa site and at Lake Midori, East Ongul Island (locations 1, 2, and 4), using the GPF/GPR primers and GNF/GNR primers that are specific for the PAH-RHDα genes from Gram-positive and Gram-negative bacteria, respectively. Extradiol dioxygenase genes were detected in several soil and sediment samples using xylE-F/xylE-R primers and bphC-F/bphC-R primers. The PCR products of the xylE-F/xylE-R primers were observed in the samples from locations 1, 2, 3, 4, 8, and 9. The PCR products of the bphC-F/bphC-R primers were observed in the samples from locations 1, 2, 3, 5, and 7. These PCR products from each primer were cloned, with the exception of the PCR products from the bphC primers due to the low intensity of those bands. A total of 60 clones of each primer were compared according to their patterns of digestion by both RFLP restriction enzymes. Clone libraries from the PAH-RHDα-GP primer showed four groups of Gram-positive, ring-hydroxylating dioxygenase genes with 95–99 % similarity: (i) *nidA3* of *Mycobacterium* sp. py146 (38 % of the total 60 clones), (ii) *pdoA* of *Terrabacter* sp. HH4 (28 %), (iii) *nidA* of *Diaphorobacter* sp. KOTLB (10 %), and (iv) *pdoA* of *Mycobacterium* sp. CH-2 (7 %). The PAH-RHDα-GN primer gave three groups of Gram-negative ring-hydroxylating dioxygenase genes with 41–46 % similarity (Fig. 1): (i) naphthalene dioxygenase of *Burkholderia glathei* (70 % of the total 60 clones), (ii) *phnAc* of *Burkholderia sartisoli* (23 %), and (iii) the RHD alpha subunit of an uncultured bacterium (7 %) (Table 2). The phylogenetic tree based on the deduced amino acid sequences generated from the PAH-RHDα-GP libraries, the PAH-RHDα-GN libraries, and the closely related PAH-RHDα from reference strains is shown in Fig. 2. Clone libraries of the xylE primer showed one group, and representatives cloned from this primer were related to *xylE* of *Sphingomonas* sp. LH128, with 88 % similarity.

The Shannon–Weaver diversity index (*H*), calculated from the number and frequency of the PAH-RHDα sequences in RFLP analysis, showed that the diversity of the PAH-RHDα-GP genes was higher than that of the PAH-RHDα-GN genes (Table 3). The index varied from 0.33 to 0.99 in the PAH-RHDα-GN library and from 0.69 to 1.22 in the PAH-RHDα-GP library. In addition, the highest diversity of the PAH-RHDα-GN genes was observed at location 1 (the Syowa site), and the highest diversity of the PAH-RHDα-GP genes was observed at location 4 (Lake Midori, East Ongul Island) (Table 4).

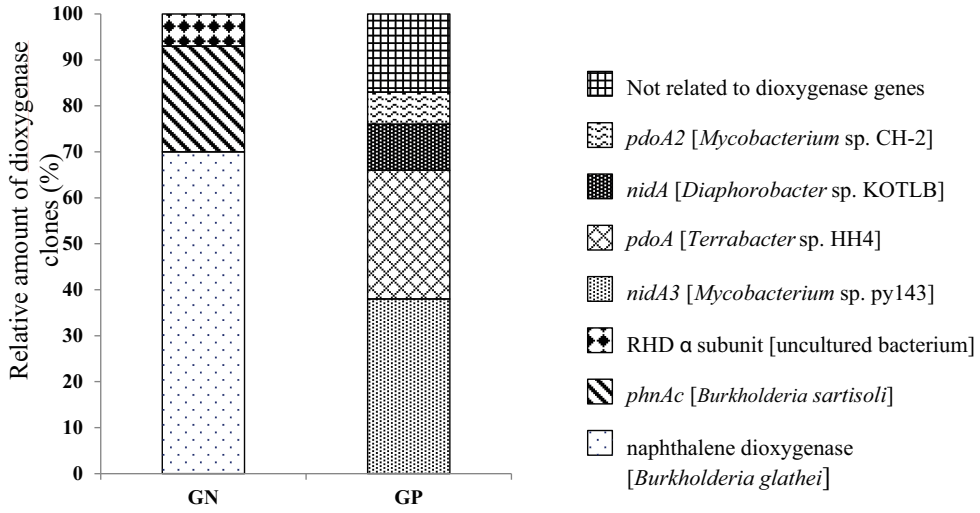

Fig. 1 Relative amount of dioxygenase clones from clone libraries of the PAH-RHDα-GP gene and the PAH-RHDα-GN gene

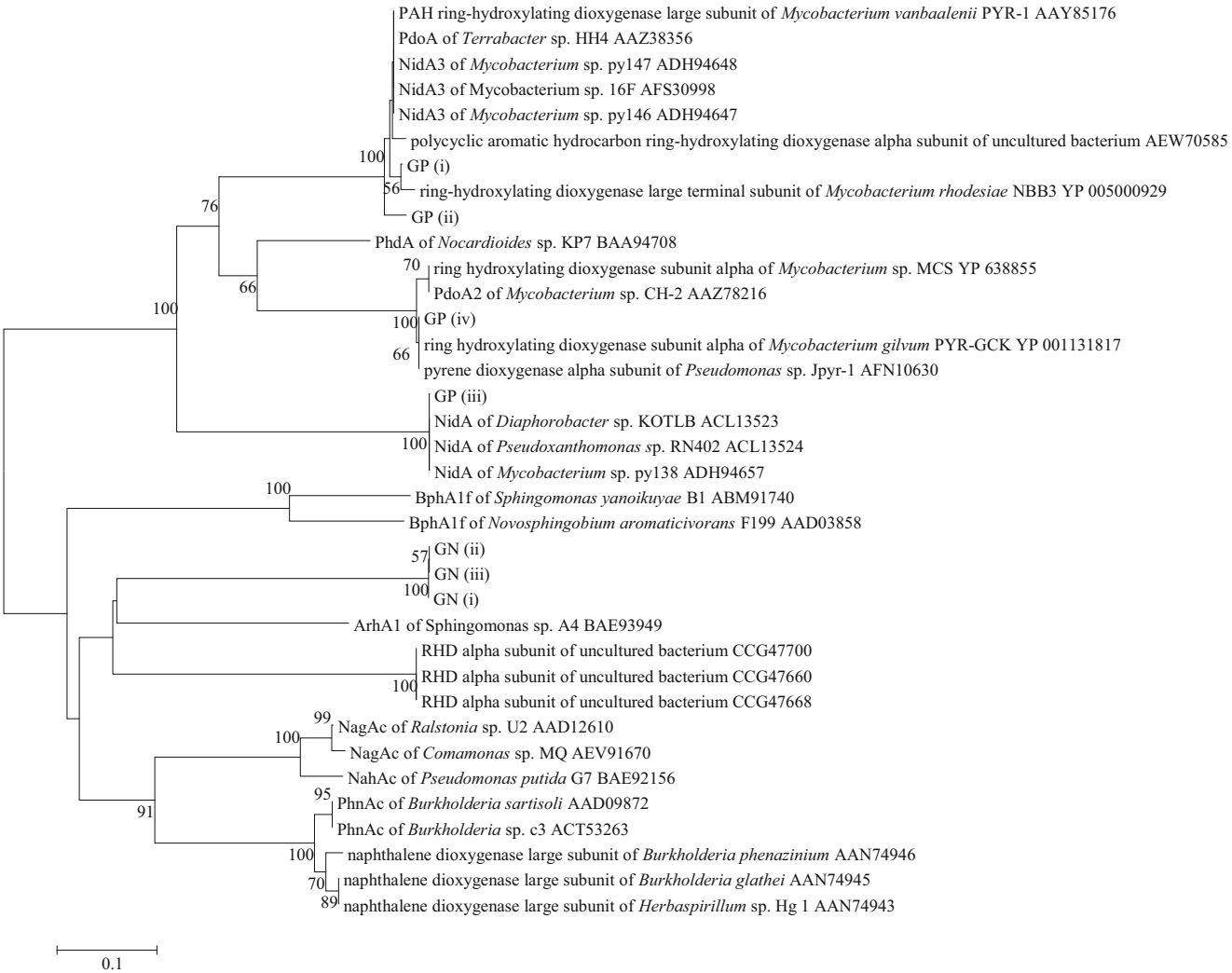

Fig. 2 Phylogenetic tree based on the deduced amino acid sequences generated from PAH-RHDα-GP libraries, PAH-RHDα-GN libraries, and the closely related PAH-RHDα from reference strains. Bootstrap analyses were performed using 1000 repetitions. (GP (i), GP (ii), GP (iii), and GP (iv)—group of clone libraries from the PAH-RHDα-GP primer; GN (i), GN (ii), and GN (iii)—group of clone libraries from the PAH-RHDα-GN primer based on RFLP analysis)

Table 3 Coverage and Shannon–Weiner and Richness indices based on the number and frequency of PAH-RHDα sequences in RFLP found in the soil and sediment samples

Clone libraries	Number of clones	Coverage (%)	Shannon–Weiner	Enzyme family	Richness		
					Location 1	Location 2	Location 4
PAH-RHDα-GN libraries	60	100	0.77	Naphthalene dioxygenase	10	18	14
				*phn*Ac	7	2	5
				RHD alpha subunit	3	0	1
PAH-RHDα-GP libraries	60	83.3	1.18	*nid*A3	10	10	3
				*pdo*A	8	3	6
				*nid*A	0	0	6
				*pdo*A2	0	3	1

Real-time PCR quantification

Real-time PCR was used to quantify the PAH-RHDα genes targeted in the soil and sediment samples. This study used the primer sets GPF/GPR and GNF/GNR to detect and quantify PAH-RHDα genes and the primer set 968F/1401R to detect and quantify 16S rRNA genes. The 16S rRNA gene copy number ranged from 4.89 to 6.47 log copy numbers μg^{-1} DNA. The PAH-RHDα-GP genes copy number and the PAH-RHDα-GN genes were high in the samples from locations 1, 2, and 4 (2.10 to 3.14 log copy numbers μg^{-1} DNA for PAH-RHDα-GP genes and 3.57 to 4.88 log copy numbers μg^{-1} DNA for PAH-RHDα-GN genes), while in another locations, the copy number of two genes were below a quantification limit (Fig. 3). However, the number of copies estimated by qPCR assays should be regarded as an underestimation, as the qPCR assays were not corrected for inhibition.

DGGE fingerprinting

DGGE analysis performed from amplification with the universal primers from the nine soil and sediment locations revealed a diverse bacterial fingerprint for all of the samples (Fig. 4a). A total of 40 bands were cloned and sequenced, and the results of their closest relatives are shown in Table 5. The phylogenetic tree of 16S rDNA sequences from the dominant DGGE bands and those representatives of known PAH-degrading bacteria is shown in ESM Fig. S2. Based on similarity matching, all of the sequences belonged to ten taxonomic bacterial groups: Proteobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Firmicutes, Chloroflexi, Gemmatimonadetes, Cyanobacteria, Chlorobium, and Acidobacteria.

Sphingomonas was detected in the samples from locations 1, 2, 4, and 6 (Fig. 4b). The sequences of dominant DGGE bands were identical to those of *Sphingomonas*, *Porphyrobacter*, and *Methylobacter* (Table 5).

Table 4 Coverage and Shannon–Weiner and Pielou indices based on the number and frequency of PAH-RHDα sequences found in the soil and sediment samples

Sample location	Number of clones	Coverage (%)	Shannon–Weiner
PAH-RHDα-GN libraries			
Location 1	20	100	0.99
Location 2	20	100	0.33
Location 4	20	100	0.75
PAH-RHDα-GP libraries			
Location 1	20	90	0.69
Location 2	20	80	0.92
Location 4	20	80	1.22

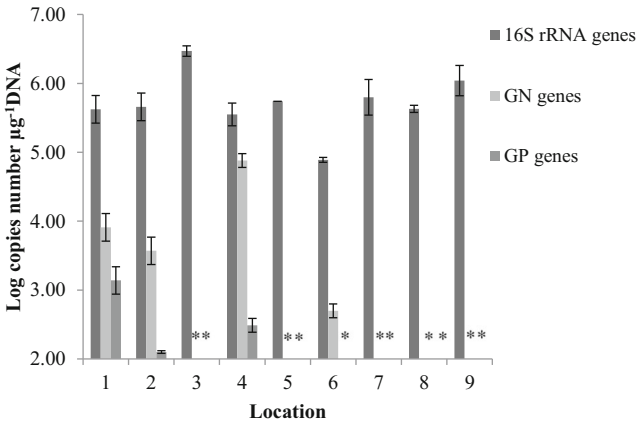

Fig. 3 16S rRNA, PAH-RHDα-GP, and PAH-RHDα-GN gene copy numbers, by real-time PCR, in the soil and sediment samples from locations 1–9. *Asterisk* indicates the sample's copy number was below a quantification limit. (The limit of quantification (LOQ) was ten copies per reaction, and the standard curve was set up above an LOQ in the range of 10^2–10^{10} gene copies per reaction). The number of copies estimated by qPCR assays should be regarded as an underestimation, as the qPCR assays were not corrected for inhibition

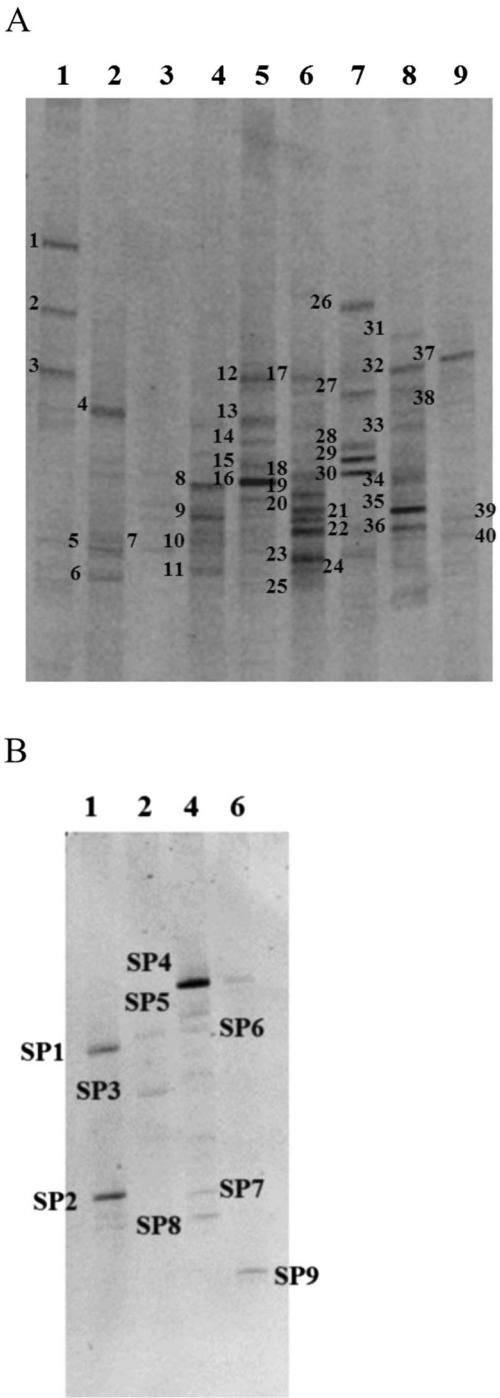

Fig. 4 DGGE profiles (30 to 70 % denaturant) of total bacterial communities in the soil and sediment samples from locations 1–9 (**a**) and *Sphingomonas* communities in the soil and sediment samples from locations 1, 2, 4, and 6 (**b**)

Discussion

Although Antarctica is considered to be the most isolated continent of the world, human activities have increasingly influenced this environment, and these activities are the main sources of contamination by PAHs and other xenobiotic compounds (Aislabie et al. 2004). It is known that an adaptive response of indigenous microorganisms is induced by pollutants, leading to the enrichment in hydrocarbon-degrading bacteria (Ruberto et al. 2003). The presence of indigenous microorganisms with the ability to degrade toxic compounds is a key to bioremediating contaminated areas, especially in Antarctica, where a bioaugmentation treatment may not be possible. However, degraders in polar environments may have difficulty growing and catabolic activity can be inhibited because many factors limit their biological processes, such as low temperature, low levels of phosphorous and nitrogen, UV radiation, and low humidity (Ma et al. 2006; Delille and Coulon 2008). In this study, we attempted to assess the diversity of indigenous bacteria and hydrocarbon catabolic genes in Antarctic areas around the Syowa Station by using culture-independent methods. Bacterial community profiles and the presence of genes involved in hydrocarbon degradation have assumed special relevance to assess the biodegradation potential in this environment.

In this paper, the soil and sediment samples were taken from the nine locations surrounding the Syowa Station and PAH contamination levels were measured. PAHs were not detected in any samples. This result indicates that there was no PAHs or they were below the limit of detection (2 μg kg^{-1} of the soil or sediment samples).

In the present study, hydrocarbon catabolic genes were detected in the soil and sediment samples. The PAH-RHDα related to PAH dioxygenases in Gram-positive and Gram-negative bacteria was observed in the samples from locations 1, 2, and 4. It is noted that locations 1 and 2 are near the main building of the Syowa site, where there are active areas that may have been affected by human activities. Jurelevicius et al. (2012a) studied the PAH-RHDα-coding genes from Gram-positive and Gram-negative bacteria in diesel oil-contaminated and pristine soil samples obtained from King George Island, Maritime Antarctica. All of the contaminated soil samples contained PAH-RHDα sequences from both Gram-positive and Gram-negative bacteria, but in pristine soil samples, they observed only a few sequences related to the PAH-RHDα of Gram-negative bacteria. Thus, the presence of PAH-RHDα-coding genes appears to be affected by the level of anthropogenic impact in the environment. We have suggested that the soil and sediment samples of our study that are not contaminated or have very low levels of PAHs may have a very low number of PAH-degrading bacteria, below the detection limit of the technique used. Therefore, the real-time PCR methodology was developed to detect and quantify the PAH-RHDα-GP and PAH-RHDα-GN genes in this study. Interestingly, real-time PCR showed positive results in the samples from locations 1, 2, 4, and 6. It showed that the real-time PCR technique can be useful to estimate the biodegradation potential in environments. Many studies have designed and used highly specific PCR primers for PAH-

Table 5 Identification of 16S rDNA sequences obtained from DGGE bands

Strains	Location	Closest relative	Accession no.	% similarity	Taxonomic group
1	1	Uncultured Acidobacteriaceae bacterium	HE860751	100	Acidobacteria
2	1	*Gordonia* sp.	JN942134	100	Actinobacteria
3	1	Uncultured actinobacterium	EF220124	98	Actinobacteria
4	2	Uncultured Verrucomicrobia bacterium	EF220937	98	Verrucomicrobia
5	2	*Sphingomonas* sp.	JQ608327	100	α-Proteobacteria
6	2	Uncultured *Gemmatimonas* sp.	AJ577105	99	Gemmatimonadetes
7	3	*Marinobacter* sp.	AB513677	95	γ-Proteobacteria
8	4	*Porphyrobacter* sp.	HQ588835	100	α-Proteobacteria
9	4	*Burkholderia kururiensis*	JN714982	99	β-Proteobacteria
10	4	Uncultured cyanobacterium	DQ181677	100	Cyanobacteria
11	4	Uncultured Verrucomicrobiaceae bacterium	EU809868	96	Verrucomicrobia
12	5	*Pseudomonas* sp.	JX155655	100	γ-Proteobacteria
13	5	Uncultured cyanobacterium	HQ821710	100	Cyanobacteria
14	5	*Marinobacter* sp.	DQ768634	97	γ-Proteobacteria
15	5	Uncultured *Dethiobacter* sp.	GU000350	98	Firmicutes
16	5	Uncultured cyanobacterium	KC687147	100	Cyanobacteria
17	6	Uncultured *Chlorobium* sp.	GU326306	95	Chlorobi
18	6	Uncultured *Bellilinea* sp.	GU556338	98	Chloroflexi
19	6	*Porphyrobacter* sp.	HQ588835	100	α-Proteobacteria
20	6	Uncultured Chloroflexi bacterium	GQ366672	98	Chloroflexi
21	6	Uncultured *Methylobacter* sp.	JQ793373	99	γ-Proteobacteria
22	6	Uncultured Chloroflexi bacterium	GU236058	100	Chloroflexi
23	6	Uncultured *Nitratireductor* sp.	JQ791836	100	α-Proteobacteria
24	6	Uncultured *Flavobacterium* sp.	GU556350	97	Bacteroidetes
25	6	*Mycobacterium* sp.	JQ419515	99	Actinobacteria
26	7	Uncultured *Sporichthya* sp.	GU000292	98	Actinobacteria
27	7	*Algoriphagus* sp.	FJ196000	100	Bacteroidetes
28	7	Uncultured Firmicutes bacterium	JN868216	100	Firmicutes
29	7	Uncultured *Dethiobacter* sp.	GU000253	100	Firmicutes
30	7	Uncultured Firmicutes bacterium	AY211667	99	Firmicutes
31	8	*Rubritalea sabuli*	AB353310	95	Verrucomicrobia
32	8	Uncultured Desulfovibrionales bacterium	EF416877	95	Deltaproteobacteria
33	8	*Flavobacterium* sp.	FR772077	99	Bacteroidetes
34	8	*Hyphomonas* sp.	JX844517	100	α-Proteobacteria
35	8	*Algoriphagus* sp.	FR691439	99	Bacteroidetes
36	8	*Loktanella* sp.	KC160936	100	α-Proteobacteria
37	9	*Lysinibacillus fusiformis*	JN166076	100	Firmicutes
38	9	Uncultured Sphingobacteriales bacterium	GU047432	99	Bacteroidetes
39	9	Uncultured Verrucomicrobia bacterium	EF219818	98	Verrucomicrobia
40	9	*Marinobacter hydrocarbonoclasticus*	JF973388	94	γ-Proteobacteria
SP1	1	Uncultured Sphingomonadaceae bacterium	HM438363	99	α-Proteobacteria
SP2	1	*Sphingomonas* sp.	EU423303	99	α-Proteobacteria
SP3	2	*Sphingomonas* sp.	GU479690	100	α-Proteobacteria
SP4	4	*Porphyrobacter* sp.	FR772128	99	α-Proteobacteria
SP5	4	*Porphyrobacter sanguineus*	AB062105	99	α-Proteobacteria
SP6	4	*Sphingomonas* sp.	JX949373	100	α-Proteobacteria
SP7	4	Uncultured *Sphingomonas* sp.	HM438635	98	α-Proteobacteria
SP8	4	*Sphingomonas* sp.	JX949372	100	α-Proteobacteria
SP6	6	Uncultured *Methylobacter* sp.	JQ038192	99	γ-Proteobacteria

RHDα genes (Cébron et al. 2008; DeBruyn et al. 2007), and some studies used real-time PCR to investigate polar ecosystems (Yergeau et al. 2007; Abell and Bowman 2005).

PAH-RHDα-GN genes related to naphthalene dioxygenase from *B. glathei* were found, and they comprised 70 % of the PAH-RHDα-GN library. The sequences contained only an identity of 46 %; therefore, these clones are most likely to encode for novel dioxygenases involved in the degradation of PAHs and may be important for degrading PAHs in Antarctic soils and sediments. Moreover, sequences associated with the *phnAc* from *B. sartisoli* (45 % of identity) were found for 23 % of the PAH-RHDα-GN library. *B. sartisoli* has been isolated from a PAH-contaminated soil sample in a previous report. This strain has been described as a versatile degrader of low molecular weight PAHs and is able to grow on PAHs such as naphthalene, anthracene, and phenanthrene as a carbon and energy source (Laurie and Lloyd-Jones 1999). In contrast, in some previous studies, the sequences obtained from these primers showed high identity with those reported so far. Jurelevicius et al. (2012a) used the same primer sets for the detection of PAH-RHDα-coding genes from Gram-negative bacteria in contaminated soil samples from King George Island in Antarctica. The sequences obtained shared high identity with NagAc from *Polaromonas naphthalenivorans* CJ2 (97 %), PhnAc from *Acidovorax* sp. NA3 (95 %), and *Burkholderia* sp. Eh1-1 (96 %). Therefore, this information together with our results suggests that in different regions of the Antarctica, hydrocarbon catabolic gene variants were different.

Sequences associated with *nidA3* from *Mycobacterium* sp. py143 and *pdo*A from *Terrabacter* sp. HH4 comprised 38 and 28 % of the PAH-RHDα-GP library, respectively. NidA3 from *Mycobacterium* has been correlated with the transformation of aromatic hydrocarbon compounds, such as biphenyl, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene (Kweon et al. 2010). Jurelevicius et al. (2012a) showed that the PdoA from *Terrabacter* sp. HH4 was obtained in a diesel oil-contaminated soil sample that was collected adjacent to the Brazilian Antarctic Station Comandante Ferraz. *Terrabacter* sp. HH4 can use fluoranthene for growth (Zhou et al. 2006). Moreover, sequences associated with *nidA* from *Diaphorobacter* sp. KOTLB (a pyrene-degrading strain) (Klankeo et al. 2009) and *pdoA2* from *Mycobacterium* sp. CH-2 (a pyrene-degrading strain) (Churchill et al. 2008) were also found in the PAH-RHDα-GP library. The presence of Gram-positive bacteria that have the ability to degrade different PAH compounds in Antarctic soils could represent an important tool for bioremediation processes.

In addition, extradiol dioxygenase gene variants were detected in several soil and sediment samples using primers xylE-F/xylE-R and bphC-F/bphC-R, which are specific for the *xylE* and *bphC* genes, respectively. The *bphC* and *xylE* genes encode 2,3-dihydroxybiphenyl-1,2-dioxygenase and catechol 2,3-dioxygenase, respectively, catalyzing the ring cleavage reaction in the PAH degradation pathway. Cunliffe et al. (2006) monitored PAH metabolism of *Sphingobium yanoikuyae* B1 by measuring *bphC* and *xylE* gene expression using qPCR. The finding of a hydrocarbon-degrading gene is a good indicator of the biodegradation potential of the indigenous bacterial population in the environment. Nevertheless, further studies should be performed to analyze the PAH-RHD gene expression in Antarctic soils and sediments.

A possible reason for the presence of functional genes related to the degradation of aromatic hydrocarbons in the samples where PAHs could not be detected in this study might be due to low PAH levels, either natural or anthropogenic, which are readily degraded by indigenous bacteria and thus become undetectable. In some reports, they showed the detection of genes for the degradation of aromatic compounds in the uncontaminated areas and explained the possible reason that it could be related to the presence of naturally occurring PAHs (Flocco et al. 2009; Margesin et al. 2003).

As reported previously, different bacterial genera have been identified in Antarctic soils as PAH degraders, such as *Pseudomonas*, *Rhodococcus*, and *Sphingomonas* (Panicker et al. 2010). In this study, the *Sphingomonas* community, as analyzed by PCR-DGGE and PCR products, could be detected in the samples from locations 1, 2, 4, and 6. The bacterial genus *Sphingomonas* has been isolated from oil-contaminated soil samples collected from Scott Base, Antarctica, and it has the ability to degrade various PAHs, such as naphthalene, phenanthrene, and fluorene (Aislabie et al. 2000). As shown in Table 5, some of the bands were closely related to *Sphingomonas* such as *Porphyrobacter* sp. Hiraishi et al. (2002) reported that *Porphyrobacter sanguineus* was able to grow on dibenzofuran and biphenyl as a carbon source. Furthermore, and interestingly, some of the dominant bands in the DGGE profiles of total bacteria that had similarity to *Sphingomonas* sp. and *Porphyrobacter* sp. were found in the samples from locations 2, 4, and 6.

Many different bacterial genera that are able to degrade PAHs and have previously been isolated from the environment belong to the genera *Alcaligenes*, *Vibrio*, *Mycobacterium*, *Comamonas*, *Arthrobacter*, *Burkholderia*, and *Flavobacterium* (Zhang et al. 2011). In this study, dominant bands in the total bacterium DGGE profiles of each sample were sequenced, which showed the presence of bacterial genera that are commonly related to biodegradation. For example, sequences from *Burkholderia* sp., *Pseudomonas* sp., *Mycobacterium* sp., and *Flavobacterium* sp. were found as the dominant DGGE bands in the samples from locations 4, 5, 6, and 8. Furthermore, the dominant band of location 1 was identical to the sequences from *Gordonia* sp. The genus *Gordonia* has also been reported to utilize a variety of aliphatic, aromatic hydrocarbons or other pollutants in the

environment (Arensköter et al. 2004). The dominant band of locations 3 and 5 was identical to the sequences from *Marinobacter* sp., and the dominant band of location 9 was identical to the sequences from *Marinobacter hydrocarbonoclasticus*. *Marinobacter* is a widely distributed bacterium that has been isolated from the Antarctic environment (Liu et al. 2012). Some *Marinobacter* strains, such as *M. hydrocarbonoclasticus*, were isolated from petroleum hydrocarbon-contaminated sediments, and it has the ability to degrade several aliphatic components of crude oil and aromatic compounds, such as phenanthrene (Gauthier et al. 1992).

Conclusions

In this study, the presence of indigenous bacteria related to genera that contain known hydrocarbon degraders and the presence of hydrocarbon catabolic genes around the Syowa Station in Antarctica were first revealed. These results imply that these environments have the potential ability to degrade hydrocarbons, and this information will be useful for the bioremediation of hydrocarbon contamination in Antarctic soils and sediments. It would also be appropriate to carry out mineralization assays with radiolabelled PAHs to confirm in situ degradative activity.

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References

Abell GCJ, Bowman JP (2005) Ecological and biogeographic relationships of class Flavobacteria in the Southern Ocean. FEMS Microbiol Ecol 51:265–277

Aislabie J, Foght JM (2010) Response of polar soil bacterial communities to fuel spills. In: Bej AK, Aislabie J, Atlas RM (eds) The ecology, biodiversity and bioremediation potential of microorganisms in extremely cold environments, Taylor & Francis, Florida, pp 215–230

Aislabie J, Foght J, Saul D (2000) Aromatic hydrocarbon-degrading bacteria from soil near Scott Base, Antarctica. Polar Biol 23:183–188

Aislabie JM, Balks MR, Foght JM, Waterhouse EJ (2004) Hydrocarbon spills on Antarctic soils: effects and management. Environ Sci Technol 38:1265–1274

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Mol Biol Rev 59:143–149

Arensköter M, Broker D, Steinbüchel A (2004) Biology of metabolically diverse genus *Gordonia*. Appl Environ Microbiol 70:3195–3204

Cébron A, Norini MP, Beguiristain T, Leyval C (2008) Real-time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHDalpha) genes from Gram positive and Gram negative bacteria in soil and sediment samples. J Microbiol Meth 73:148–159

Churchill PF, Morgan AC, Kitchens E (2008) Characterization of a pyrene-degrading Mycobacterium sp. strain CH-2. J Environ Sci Health B 43:698–706

Cunliffe M, Kawasaki A, Fellows E, Kertesz MA (2006) Effect of inoculum pretreatment on survival, activity and catabolic gene expression of *Sphingobium yanoikuyae* B1 in aged polycyclic aromatic hydrocarbon-contaminated soil. FEMS Microbiol Ecol 58:364–372

Das R, Kazy SK (2014) Microbial diversity, community composition and metabolic potential in hydrocarbon contaminated oily sludge: prospects for in situ bioremediation. Environ Sci Pollut Res. doi:10.1007/s1135601426402

DeBruyn JM, Chewning CS, Sayler GS (2007) Comparative quantitative prevalence of Mycobacteria and functionally abundant *nidA*, *nahAc* and *nagAc* dioxygenase genes in coal tar contaminated sediments. Environ Sci Technol 41:5426–5432

Delille D, Coulon F (2008) Comparative mesocosm study of biostimulation efficiency in two different oil-amended sub-Antarctic soils. Microb Ecol 56:243–252

Fernández-Luqueño F, Valenzuela-Encinas C, Marsch R, Martínez-Suárez C, Vázquez-Núñez E, Dendooven L (2011) Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: a review. Environ Sci Pollut Res 18:12–30

Flocco CG, Gomes NCM, Cormack WM, Smalla K (2009) Occurrence and diversity of naphthalene dioxygenase genes in soil microbial communities from the Maritime Antarctic. Environ Microbiol 11:700–714

Gauthier MJ, Lafay B, Christen T, Fernandez L, Acquaviva M, Bonin PC, Betrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int J Syst Bacteriol 43:568–576

Hiraishi A, Yonemitsu Y, Matsushita M, Shin YK, Kuraishi H, Kawahara K (2002) Characterization of *Porphyrobacter sanguineus* sp. nov., an aerobic bacteriochlorophyll-containing bacterium capable of degrading biphenyl and dibenzofuran. Arch Microbiol 178:45–52

Junca H, Pieper DH (2004) Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. Environ Microbiol 6:95–110

Jurelevicius D, Alvarez VM, Peixoto R, Rosado AS, Seldin L (2012a) Bacterial polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenases (PAH-RHD) encoding genes in different soils from King George Bay, Antarctic Peninsula. Appl Soil Ecol 55:1–9

Jurelevicius D, Cotta SR, Peixoto R, Rosado AS, Seldin L (2012b) Distribution of alkane-degrading bacterial communities in soils from King George Island, Maritime Antarctic. Eur J Soil Biol 51:37–44

Klankeo P, Nopcharoenkul W, Pinyakong O (2009) Two novel pyrene-degrading *Diaphorobacter* sp. and *Pseudomonas* sp. isolated from soil. J Biosci Bioeng 108:488–495

Kweon O, Kim SJ, Freeman JP, Song J, Baek S, Cerniglia CE (2010) Substrate specificity and structural characteristics of the novel Rieske non heme iron aromatic ring-hydroxylating oxygenases NidAB and NidA3B3 from *Mycobacterium vanbaalenii* PYR-1. mBio 1:1–11

Lau EV, Gan S, Ng HK (2010) Extraction techniques for polyaromatic hydrocarbons in soils. Int J Anal Chem. doi:10.1155/2010/398381

Laurie AD, Lloyd-Jones G (1999) The phn genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. J Bacteriol 181:531–540

Leys NM, Ryngaert A, Bastiaens L, Verstraete W, Springael D (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strain in soils contaminated with polycyclic aromatic hydrocarbons. Appl Environ Microbiol 70:1944–1955

Liu C, Chen CX, Zhang XY, Yu Y, Liu A, Li GW, Chen XL, Chen B, Zhou BC, Zhang YZ (2012) *Marinobacter antarcticus* sp. nov., a

halotolerant bacterium isolated from Antarctic intertidal sandy sediment. Int J Syst Evol Microbiol 62:1838–1844

Ma Y, Wang L, Shao Z (2006) *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. Environ Microbiol 8:455–465

Marcos MS, Lozada M, Dionisi HM (2009) Aromatic hydrocarbon degradation genes from chronically polluted Subantarctic marine sediments. Lett Appl Microbiol 49:602–608

Margesin R, Labbé D, Schinner F, Greer CW, Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. Appl Environ Microbiol 69:3085–3092

Muangchinda C, Pansri R, Wongwongsee W, Pinyakong O (2013) Assessment of polycyclic aromatic hydrocarbon biodegradation potential in mangrove sediment from Don Hoi Lot, Samut Songkram Province, Thailand. J Appl Microbiol 114:1311–1324

Panicker G, Mojib N, Aislabie J, Bej AK (2010) Detection, expression and quantitation of the biodegradative genes in Antarctic microorganisms using PCR. Antonie Van Leeuwenhoek 97:275–287

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32:927–955

Powell SM, Bowman JP, Snape I, Stark JS (2003) Microbial community variation in pristine and polluted nearshore Antarctic sediments. FEMS Microbiol Ecol 45:135–145

Ruberto L, Vazquez SC, Mac Cormack WP (2003) Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int Biodeterior Biodegrad 52:115–125

Ruberto LAM, Vazquez SC, Curtosi A, Mestre MC, Pelletier E, Mac Cormack WP (2006) Phenanthrene biodegradation in soils using an Antarctic bacterial consortium. Biorem J 10:191–201

Sipila TP, Riisio H, Yrjala K (2006) Novel upper meta-pathway extradiol dioxygenase gene diversity in polluted soil. FEMS Microbiol Ecol 58:134–144

Suenaga H, Mizuta S, Miyazaki K (2009) The molecular basis for adaptive evolution in novel extradiol dioxygenases retrieved from the metagenome. FEMS Microbiol Ecol 69:472–480

US EPA Method 3500C (2007) Organic extraction and sample preparation. Revision 3. February, 2007

US EPA Method 3540C (1996) Soxhlet extraction. Revision 3. December, 1996

US EPA Method 8310 (1986) Polyaromatic hydrocarbons. September, 1986

Yergeau E, Arbour M, Brousseau R, Juck D, Lawrence JR, Masson L, Whyte LG, Greer CW (2009) Microarray and real-time PCR analyses of the responses of high-arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. Appl Environ Microbiol 75:6258–6267

Yergeau E, Bokhorst S, Huiskes AHL, Boschker HTS, Aerts R, Kowalchuk GA (2007) Size and structure of bacterial, fungal and nematode communities along an Antarctic environmental gradient. FEMS Microbiol Ecol 59:436–451

Zhang S, Wan R, Wang Q, Xie S (2011) Identification of anthracene degraders in leachate-contaminated aquifer using stable isotope probing. Int Biodeterior Biodegrad 65:1224–1228

Zhou HW, Guo CL, Wong YS, Tam NFY (2006) Genetic diversity of dioxygenase genes in PAH-degrading bacteria isolated from mangrove sediments. FEMS Microbiol Lett 262:148–157