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Detection, expression and quantitation of the biodegradative genes in Antarctic microorganisms using PCR

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Abstract In this study, 28 hydrocarbon-degrading bacterial isolates from oil-contaminated Antarctic soils were screened for the presence of biodegradative genes such as alkane hydroxylase (*alks*), the ISPα subunit of naphthalene dioxygenase (*ndoB*), catechol 2,3-dioxygenase (*C23DO*) and toluene/biphenyl dioxygenase (*todC1/bphA1*) by using polymerase chain reaction (PCR) methods. All naphthalene degrading bacterial isolates exhibited the presence of a 648 bp amplicon that shared 97% identity to a known *ndoB* sequence from *Pseudomonas putida*. Twenty-two out of the twenty-eight isolates screened were positive for one, two or all three different regions of the *C23DO* gene. For alkane hydroxylase, all 6 *Rhodococcus* isolates were PCR-positive for a 194 bp and a 552 bp segment of the *alkB* gene, but exhibited variable results with primers located at different segments of this gene. Three *Pseudomonas* spp. 4/101, 19/1, 30/3 amplified 552 bp segment of *alkB*. Only two *Pseudomonas* sp. 7/156 and 4/101 amplified a segment of *alkB* exhibiting 89–94% nucleotide sequence identity with the existing sequence of *alkB* in the GenBank sequence database. Transcripts of three genes, *alkB2*, *C23DO* and *ndoB*, that were amplified by DNA-PCR in three different bacterial isolates also exhibited positive amplification by reverse transcriptase PCR (RT-PCR) method confirming that these genes are functional. A competitive PCR (cPCR) method was developed for a quantitative estimation of *ndoB* from pure cultures of the naphthalene-degrading *Pseudomonas* sp. 30/2. A minimum of $1x10^7$ copies of the *ndoB* gene was detected based on the comparison of the intensities of the competitor and target DNA bands. It is expected that the identification and characterization of the biodegradative genes will provide a better understanding of the catabolic pathways in Antarctic psychrotolerant bacteria, and thereby help support bioremediation strategies for oil-contaminated Antarctic soils.

Keywords Antarctica · Biodegradation · cPCR · PCR · RT-PCR

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Introduction

Antarctica is one of the last pristine environments on Earth today (Cowan and Tow 2004; Ebinghaus 2008). Human activity over the years has led to hydrocarbon contamination of the soils near active and abandoned research stations (Aislabie et al. 1999; Martin-Laurent et al. 2003; Saul et al. 2005). Fuel storage and refueling of aircrafts and other vehicles has led to localized fuel spills on land. Jet fuels, commonly used in the Antarctic, are complex mixtures of hydrocarbon that partition in the environment according to their physical properties. They may evaporate, be absorbed into the soil, or dissolve in water thereby becoming harmful to the marine ecosystem or be degraded by chemical or biological processes (Chandler and Brockman 1996). Bioremediation of the Antarctic soils has been proposed to clean up the spills (Aislabie et al. 1998, 2004, 2006; Kerry 1993; Mesarch et al. 2000). However, the Antarctic Treaty does not support the introduction of foreign organisms (Eltis and Bolin 1996) on this continent, and the physical removal of contaminated soil is not monetarily feasible. Therefore, use of indigenous biodegradative bacteria seems be the preferred means to best achieve this objective. These bacteria would already be well adapted to the cold, dry, alkaline, low nutrient soil conditions (Campbell et al. 1998).

Jet fuel is mostly composed of alkanes of the which the ~81% is in C_8–C_{17} range, and the remainder of the fuel is aromatics like substituted benzenes and naphthalenes along with low levels of highly volatile chemicals like benzene, toluene and xylene (Zeiger and Smith 1998). Based on the removal of JP4 and JP8 Jet fuel from soil, it was shown that higher molecular weight hydrocarbons were removed significantly faster by the activity of the biodegradative microbial population than in soil treated with chemicals (2% $HgCl_2$) to kill microorganisms (Dean-Ross et al. 1992; Dean-Ross 1993). Historically, research has focused on the catabolic pathways and genetics of hydrocarbon-degrading mesophilic bacteria. But with increasing numbers of investigations of hydrocarbon-contaminated polar soils, degradation of hydrocarbons by psychrotolerant bacteria has been documented and certain biodegradative genes characterized (Baraniecki et al. 2002; Ensminger et al. 1999; Laramee et al. 2000; Panicker et al. 2001). There are more than 1,301 known microbial biocatalytic reactions and 190 biodegradation pathways listed in The University of Minnesota Biocatalysis/Biodegradation Database (http://umbbd.msi.umn.edu/) (Ellis et al. 2006).

The alkane degradation pathway located on the OCT-plasmid, was first genetically characterized in *Pseudomonas oleovorans* (Kok et al. 1989; van Beilen et al. 1994). Since then the alkane hydroxylase gene (*alkB*) has been cloned and sequenced in several other bacteria such as *Acinetobacter* sp., *Rhodococcus* sp., *Mycobacterium* sp., *Pseudomonas putida* P1, *P. aureofaciens* and *P. fluorescens*. *alkB* gene and encoded protein AlkB were found to be diverse in nucleotide as well as amino acid sequence (Ratajczak et al. 1998; Sotsky et al. 1994; van Beilen et al. 2002; Vomberg and Klinner 2000; Wasmund et al. 2009; Whyte et al. 2002a, b, 1998). Aromatic hydrocarbons such as benzene, toluene, xylene and naphthalene that constitute Jet fuel are degraded by microorganisms via catechol-like intermediates to less toxic compounds (Cerniglia 1984; Head et al. 2006). These biodegradative pathways make use of either ortho- or meta-cleavage catechol dioxygenases that have been characterized in several bacteria (Eltis and Bolin 1996; Head et al. 2006), which may possibly be present in many of the Antarctic psychrotolerant bacteria.

Naphthalene degradation has also been well studied in many biodegradative bacteria, in which the first step in the degradation pathway is carried out by naphthalene dioxygenase, which is encoded by the *ndoB* gene (Kurkela et al. 1988; Ferrero et al. 2002). This gene has been identified in mesophilic as well as in psychrotolerant bacteria from hydrocarbon contaminated soils using polymerase chain reaction (PCR) or Southern blot DNA-DNA hybridization methods (Herrick et al. 1997; Stapleton and Sayler 1998; Whyte et al. 1996; Wilson et al. 2003).

Our aim was to identify biodegradative genes encoding alkane hydroxylase (*alks*), naphthalene dioxygenase (*ndoB*), catechol 2,3-dioxygenase (*C23DO*) and toluene/biphenyl dioxygenase (*todC1/bphA1*) using PCR in Gram-positive and Gram-negative bacteria isolated from oil-contaminated Antarctic soils and also to assess active transcription of these genes. This would provide a better understanding of the nature and distribution of biodegradative genes in Antarctic soils that are contaminated with Jet fuel, as well as a comparison of gene

sequences among mesophiles and psychrotolerant bacteria. We also developed a competitive PCR (cPCR) assay, which utilizes an internal control to quantify the *ndoB* of a naphthalene-degrading bacterium isolated from this environment.

Materials and methods

Bacterial strains

The hydrocarbon-degrading bacterial strains used in this study were isolated from hydrocarbon-contaminated soils near Scott Base, the former Vanda Station and Marble Point, Antarctica (Table 1) and characterized as described previously (Bej et al. 2000; Panicker et al. 2001). *P. putida* ATCC 17484 and *P. putida* mt2 were maintained on Nutrient Agar (Difco, Detroit, MI) at 26°C.

PCR amplification

PCR was used to detect the presence of genes involved in alkane, naphthalene and catechol degradation in the various isolates. Genomic DNA was purified from by the method described by Ausubel et al. (1987). The oligonucleotide primer sets designed from the gene sequence coding for different regions of alkane hydroxylase (*alk*), an ISPα subunit of naphthalene dioxygenase (*ndoB*), competitor *ndoB* (*cndoB*), catechol 2,3-dioxygenase (*C23DO*) and toluene/biphenyl dioxygenase (*todC1/bphA1*) are

Table 1 Bacterial strains isolated from oil-contaminated Antarctic soils used in this study

Strain I.D.	Organism	Location	Isolation temperature (°C)	Substrate
5/1	*Rhodococcus*	Scott Base	16	Alkanes
5/103	*Rhodococcus*	Scott Base	4	Alkanes
7/1	*Rhodococcus*	Scott Base	16	Alkanes
8/11	*Rhodococcus*	Scott Base	16	Alkanes
8/103	*Rhodococcus*	Scott Base	4	Alkanes
22/3	*Rhodococcus*	Marble point	16	Alkanes
7/156	*Pseudomonas*	Scott Base	4	JP5 jet fuel
Ant 5	*Pseudomonas*	Scott Base	16	Naphthalene
Ant 6	*Pseudomonas*	Scott Base	4	Naphthalene
Ant 9	*Pseudomonas*	Scott Base	28	Naphthalene
6/170	*Pseudomonas*	Scott Base	4	Naphthalene
8/48	*Pseudomonas*	Scott Base	16	Naphthalene
30/1	*Pseudomonas*	Vanda station	16	Naphthalene
30/2	*Pseudomonas*	Vanda station	16	Naphthalene
Ant 18	*Sphingomonas*	Scott Base	16	1-methyl naphthalene
Ant 20	*Sphingomonas*	Scott Base	4	1-methyl naphthalene
Ant 10	*Pseudomonas*	Scott Base	28	*m*-toluene, *m*- xylene
Ant 11	*Pseudomonas*	Scott Base	16	*m*-toluene, *m*- xylene
Ant 29	*Sphingomonas*	Scott Base	4	*m*-toluate
Ant 30	*Pseudomonas*	Scott Base	4	Toluene
7/22	*Pseudomonas*	Scott Base	16	*m*-toluene, *m*- xylene
4/101	*Pseudomonas*	Scott Base	4	Sodium salicylate
19/1	*Pseudomonas*	Marble point	16	JP8 jet fuel
30/3	*Pseudomonas*	Vanda station	16	JP8 jet fuel
35/1	*Sphingomonas*	Vanda station	16	JP8 jet fuel
8/44	*Sphingomonas*	Scott Base	16	Sodium salicylate
8/51	*Pseudomonas*	Scott Base	16	*m*-toluene
31/1	*Coryneform*	Vanda station	16	JP8 jet fuel

listed in Table 2. All oligonucleotide primers were custom synthesized based on sequences of respective biodegradative genes from mesophilic bacteria (Table 2). *P. putida* ATCC 17484 and *P. putida* mt2 were used as positive controls for *ndoB* and *C23DO* amplification, respectively. Each PCR amplification was performed in a 25 µl reaction volume consisting of 1 µg of purified genomic DNA; 200 µM of each of the dNTPs; 1 µM of each of the oligonucleotide primer and 2.0 U AmpliTaq (Perkin Elmer, Norwalk, CT) DNA polymerase; and 1× PCR reaction buffer [10× buffer consisted of 300 mM Tris–Cl (pH 9.0), 75 mM $(NH_4)_2SO_4$ and 2.0 mM $MgCl_2$]. All PCR amplifications were performed in a GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT) thermocycler using the following temperature cycling parameters: initial denaturation at 94°C for 2 min followed by a total of 30 cycles of amplification in which each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 2 min. After amplification, final extension of the incompletely synthesized DNA was carried out at 72°C for 7 min. For those isolates that showed a negative result, annealing temperatures of 45, 50 and 55°C were also attempted. For amplification of the *C23DO* gene an annealing temperature of 55°C was used. The PCR fragments were analyzed by agarose gel electrophoresis (1% wt/vol). The gel was stained with ethidium bromide and visualized under a Photoprep I UV transilluminator (Fotodyne, Inc., Hartland, WI).

Cloning and nucleotide sequence analysis

The PCR amplified DNA fragments from the representative groups of different primer sets were cloned in a pCR4-Topo™ plasmid vector using the Topo TA™ cloning kit (Invitrogen, Inc., Carlsbad, CA). After transformation, randomly selected white colonies were grown in (Ausubel et al. 1987) and plasmid DNA extracted using the Qiagen™ mini-prep columns (Qiagen, Valencia, CA). The purified DNA from each putative clone was then treated with *Eco*RI restriction endonuclease (New England Biolab, Beverly, MA) and the correct molecular weight cloned DNA fragment was determined by agarose gel electrophoresis. The nucleotide sequence of the cloned gene fragments were analyzed by using M13 forward or reverse primers and an ABI Prism automated DNA sequencer (Perkin Elmer, Norwalk, CT). The nucleotide and deduced amino acid sequences were then compared with the respective GenBank (National Institute for Health, Bethesda, MD) sequence database using the BLAST program.

Reverse transcriptase PCR amplification

Total RNA was isolated from 1.5 ml of the saturated cultures of *Pseudomonas* sp. 4/101, 7/156, Ant6 and *Rhodococcus* sp. 5/1, respectively, using NucleoSpin RNA II kit (Clontech, Mountain View, CA) following manufacturer's protocol. The purified total RNA was used for One-step RT-PCR using TITANIUM™ One-Step RTPCR kit (Clontech, Mountain View, CA) following manufacturer's protocol. The amplified product was observed on 1% (w/v) agarose gel.

Competitive PCR

In competitive PCR, the competitor DNA strand (*cndoB*) was constructed by the amplification of a 501 bp segment of the genomic DNA of *Pseudomonas* sp. 30/2 (Celi et al. 1993) using L-ndoB and R-cndoB primers (Table 2). The amplified DNA fragment was cloned on pCR4™ plasmid using the Topo TA™ cloning kit (Invitrogen, Carlsbad, CA). The correct DNA insert was confirmed by restriction endonuclease treatment and DNA sequence analyses (Ausubel et al. 1987). The inserted *cndoB* DNA segment on the pCR4™ plasmid was then used as the template for cPCR amplification. DNA concentration of the competitor DNA was determined by using a spectrophotometer (Lambda 2, Perkin Elmer, Norwalk, CT) at 260 nm wavelength. The PCR conditions and concentration of *cndoB*, which were necessary to perform competitive PCR were optimized using twofold dilutions of the plasmid ranging from 452 pg to 4.52 fg and a constant amount of target DNA. The *ndoB* gene copy number was determined using the size and the concentration of the template DNA with the appropriate unit conversion (Mesarch et al. 2000). The annealing temperature was set for 60°C with the PCR conditions as stated above. The PCR products were separated on 1.2% (w/v) agarose gels. The mean intensities

Table 2 Oligonucleotide gene-specific primers used for PCR amplification of biodegradative genes

Gene/primer name	Primer sequence (5′–3′)[a]	Origin	Reference
Alkane hydroxylase (*alk*)			
L-*alkB*	gtatcgtgaacccaactaccgctcaat	*Pseudomonas oleovorans* ATCC 29347	Kok et al. (1989)
R-*alkB*	ggtggaacaccactagatagagacg		
L-*alkB*870G	tggccggctactccgatgatcggaatctgg	*P. oleovorans* ATCC 29347	Van Beilen et al. (2002)
R-*alkB*870G	cgcgtggtgatccgagtgccgctgaaggtg		
L-TS2S	aayagagctcaygarytrggtcayaag	*P. oleovorans GPo1* and *Acinetobacter* sp. ADP1	Phillips et al. (2000)
L-TS2Smod	aayagagctcaygaritiggicayaar		
L-TS2Smod2	aayagagctcaygarititicayaar		
R-deg1RE	gtragictrgtrgtrcgcttaaggtg		
R-deg1RE2	gtrtcrctrgtrgtrcgcttaaggtg		
RH L-*alkB1*	atctgggcgcgttgggatttgagcg	*Rhodococcus* sp. strain Q15	Whyte et al. (1998)
RH R-*alkB1*	cgcatggtgatcgctgtgccgctgc		
RH L-*alkB2*	actctggcgcagtcgttttacggcc	*Rhodococcus* sp. strain Q15	Whyte et al. (1998)
RH R-*alkB2*	cccactgggcaggttgggcgcaccg		
RH L-*alkB*194	cacagytggaacagygatcrc	*Rhodococcus* sp. strain Q15 degenerate primer to region common to *alkb1* and *alkb2*	This study
RH R-*alkB*194	tccatcacyttkcgccacag		
(Ac) *alkM*-F	cctgtctcatttggcgctcgttcctacagg	*Acinetobacter* sp. ADP-1	Ratajczak et al. (1998)
(Ac) *alkM*-R	ccaaagtggcggaatcatagcaggc		
Aromatic ring hydroxylating dioxygenases (ARHDs)			
Naphthalene dioxygenase (*ndoB*)			
L-*ndoB*	cactcatgatagcctgattcctgaccccggcg	*Pseudomonas putida* ATCC 17484	Kurkela et al. (1988)
R-*ndoB*	ccgtcccacaacacacccatgccgctgccg		
R-*cndoB*	ccgtcccacaacacacccatgccgctgccgg-ccttccagttggcc	*Pseudomonas* sp. 30/2	This study
Catechol 2,3 dioxygenase (*C23DO*)			
L-*cat*238	cgacctgatctccatgaccga	Degenerate primer from conserved region of C23DO gene in *Pseudomonas* sp.	Mesarch et al. (2000)
R-*cat*238	tcaggtcagcacggtca		
*xylE*b-F	gtgcagctgcgtgtactggacatgagcaag	*Pseudomonas putida* ATCC 33015	Nakai et al. (1983)
*xylE*b-R	gcccagctggtcggtggtccaggtcaccgg		
cat2,3 1a-F	aggtgctcggtttctacctggccg	*Pseudomonas putida* ATCC 33015	Laramee et al. (2000)
cat2,3 6a-R	acggtcatgaatcgttcgttgag		
Toluene dioxygenase			
*todC*1-F	cgggtgggcttacgacaccgccggcaatct	*Pseudomonas putida* F1	Zylstra and Gibson (1989)
*todC*1-R	tcgagccgcgctccacgctacccagacgtt		
Biphenyl dioxygenase			
*bphA*1-F	tcacctgcagctatcacggctgg	*Pseudomonas pseudoalcaligenes* KF707	Furukawa and Arimura (1987)
*bphA*1-R	ggatctccacccagttctcgccatcgtcctg		

[a] y = c or t; r = a or g; i = 2′-deoxyinosine; k = g or t

of the target and competitor band were visualized and compared by the Kodak 1D software (Scientific Imaging Systems, New Haven, CT). The concentration of the target gene was determined by plotting a standard curve using Microsoft Excel™ software. The experiment was repeated three times.

Results

PCR amplification of *alk* genes

PCR amplification of *Pseudomonas* sp. 7/156 and 4/101 exhibited an 870 bp PCR amplified DNA fragment at 55°C annealing temperature with the alkB870G oligonucleotide primers (Table 3). The nucleotide sequence analysis of the amplified DNA confirmed the presence of *alkB* (Accession number AY034587). The deduced amino acid sequence of the *alkB* gene from these strains exhibited 94, 93 and 89% homologies with that of *P. putida* P1 (Smits et al. 1999), *P. aureofaciens* RWTH 529 (Vomberg and Klinner 2000) and *P. oleovorans* (Kok et al. 1989), respectively. The four Gram-negative strains i.e. *Pseudomonas* sp. 19/1, 30/3 and *Sphingomonas* sp. 35/1, 8/44 isolated from JP8 Jet fuel were PCR-positive for *alkB*. All other Gram negative strains showed no amplification for *alkB* genes (Table 3).

Among Gram positive strains, *Coryneform* 31/1 isolated from JP8 Jet fuel was PCR-positive for *alkB2*. *Rhodococcus* sp. 5/1, 5/103 and 7/1 exhibited amplification of an 870 bp DNA segment with alkB870G primers (Table 3). Similar results were described for biodegradative *Pseudomonas* sp. and *Rhodococcus* sp., which were isolated from Canadian high Arctic environment (Whyte et al. 1999a).

The Gram-positive *Rhodococcus* isolates were PCR positive for *alkB1* or *alkB2* gene segments when amplified with RH-alkB1, RH-alkB2 or RH-alkB194 primers (Table 3). The RH-alkB194 primer set was designed to amplify a region common to the *alkB1* and *alkB2* in *Rhodococcus* isolates. All *Rhodococcus* isolates were PCR positive for the 194 bp region of RH-*alkB* primers (6 out of 6 isolates). Also, they were PCR positive for the the 552 bp *alkB2* (6 out of 6 isolates), whereas only 2 out of 6 isolates were positive for the 629 bp gene fragment of *alkB1*. However, *Rhodococcus* 7/1, which is phylogenetically similar to the *R. erythropolis* and *Rhodococcus* strain Q15, exhibited positive PCR amplification of both *alkB1* and *alkB2* gene segments (Bej et al. 2000). The specificity of the amplified PCR products was confirmed by nucleotide sequence analysis using Sanger di-deoxy chain termination method. Among the Gram negative strains, 13 out of 16 *Pseudomonas* spp. and 3 out of 5 *Sphingomonas* spp. were PCR-negative for *alkB1*, *alkB2* and *alkB*194, which were designed from a Gram positive strain *Rhodococcus* sp. Q15 (Table 3). These results suggest that these primers sets were specific to *alkB* sequences of *Rhodococcus* spp. (Whyte et al. 2002a).

Using the degenerate primers TS2S/deg1RE, only two of the isolates, *Rhodococcus* 5/1 and 5/103, exhibited a 550 bp DNA fragment, while the others amplified multiple non-specific fragments in the range of 600–900 bp fragments. The deduced amino acid sequence from the 550 bp fragment from a representative strain (*Rhodococcus* 5/1) exhibited 87% nucleotide sequence identity to the *alkB* gene. Previous studies by Smits et al. (2002) and van Beilen et al. (2002) showed that although majority of the isolates amplified a 550 bp fragment, only a few of them had sequences similar to the *alkB* gene. All other primer sets targeting the alkane-degradation genes such as *alk*M (496 bp) and *alkB* (546 bp) tested in this study exhibited PCR-negative results (Table 3).

PCR amplification of ARHDs

Amplification of the ndoB gene

The L-ndoB and R-ndoB oligonucleotide primers amplified a 648 bp DNA fragment from all isolates that utilized naphthalene as a sole carbon source (Table 3). Three *Pseudomonas* spp. isolated on different sources i.e. JP8 jet fuel, *m*-toluene and sodium salicylate also were PCR-positive for *ndoB*. The *Sphingomonas* strains Ant 18 and Ant 20 utilizing 1-methyl naphthalene were negative for the *ndoB*. One *Sphingomonas* strain 35/1 utilizing JP8 jet fuel was positive for the *ndoB* (Table 3). A 648 bp amplified region of the *ndoB* from a representative strain *Pseudomonas* sp. 30/2 was sequenced (Accession no. AY034588). The Blast N and Blast P (Genbank) analysis revealed 82% nucleotide and 97% amino acid sequence identity with the known *ndoB* sequences from *P. putida*. Among Gram positive strains, all *Rhodococcus* sp. were PCR negative except *Coryneform* strain 31/1 utilizing JP8 jet fuel which showed positive amplification for the *ndoB* gene (Table 3).

Amplification of the catechol 2,3 dioxygenase gene

The *cat238* primers developed by Mesarch et al. (2000) (Table 2) were designed to amplify the I.2.A

Table 3 Results of PCR amplification using different primer sets for biodegradative genes like alkane hydroxylase, naphthalene dioxygenase and catechol 2,3 dioxygenase

Strain I.D.[a]	Organism	alkB (546 bp)	Rh-alkB1 (629 bp)	Rh-alkB2 (552 bp)	Rh-alkB194 (194 bp)	alkB870G (870 bp)	TS2S/deg1RE (550 bp)	(Ac) alkM (496 bp)	ndoB (642 bp)	cndoB (501 bp)	C23DO (238 bp)	XylEb (834 bp)	Cat2,31a (405-408 bp)	todC1 (560 bp)	bphA1 (830 bp)
5/1	*Rhodococcus*	−	−	+	+	+	+	−	−	−	−	−	−	−	−
5/103	*Rhodococcus*	−	−	+	+	+	+	−	−	−	−	+	+	−	−
7/1	*Rhodococcus*	−	+	+	+	+	−	−	−	−	−	+	+	−	−
8/11	*Rhodococcus*	−	+	+	+	−	−	−	−	−	−	+	+	−	−
8/103	*Rhodococcus*	−	−	+	+	−	−	−	−	−	−	−	+	−	−
22/3	*Rhodococcus*	−	−	+	+	−	−	−	−	−	−	+	−	−	−
7/156	*Pseudomonas*	−	−	−	−	+	−	−	+	+	−	+	+	−	−
Ant 5	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
Ant 6	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
Ant 9	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
6/170	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
8/48	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
30/1	*Pseudomonas*	−	−	−	−	−	−	−	+	+	−	+	+	−	−
30/2	*Pseudomonas*	−	−	−	−	−	−	−	+	+	+	+	+	−	−
Ant 18	*Sphingomonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ant 20	*Sphingomonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ant 10	*Pseudomonas*	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Ant 11	*Pseudomonas*	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Ant 29	*Sphingomonas*	−	−	−	−	−	−	−	−	−	−	+	+	−	−
Ant 30	*Pseudomonas*	−	−	−	−	−	−	−	−	−	−	+	−	−	−
7/22	*Pseudomonas*	−	−	−	−	−	−	−	−	−	+	−	+	−	−
4/101	*Pseudomonas*	−	−	+	−	+	−	−	+	+	−	+	−	−	−
19/1	*Pseudomonas*	−	−	+	−	−	−	−	+	+	−	+	−	−	−
30/3	*Pseudomonas*	−	−	+	−	?	−	−	−	−	+	+	+	−	−
35/1	*Sphingomonas*	−	−	−	+	−	−	−	+	+	−	−	−	−	+
8/44	*Sphingomonas*	−	+	+	+	+	−	−	−	−	−	−	−	−	−
8/51	*Pseudomonas*	−	−	−	−	−	−	−	+	+	+	+	+	−	−
31/1	*Coryneform*	−	−	+	−	−	−	−	+	+	−	−	−	−	+
P. putida	mt2[b]	−	−	−	−	−	−	ND	−	−	+	ND	ND	ND	ND
P. putida	ATCC 17484[c]	−	−	−	−	−	−	ND	+	+	+	ND	ND	ND	ND

[a] The strain designation as described in Table 1

[b] *P. putida* mt2 was used as positive control for amplification of C23DO

[c] *P. putida* ATCC 17484 was used as positive control for amplification of *ndoB*

ND not determined

subfamily of C23DO genes, which are involved in biodegradation of wide variety of aromatic compounds. The primers were tested on all isolates that were able to use toluene, xylene or naphthalene as sole carbon source. Three isolates, which were *m*-toluene, *m*-xylene or JP8 jet fuel degraders: Ant 10, Ant 11, 7/22, 30/3 and 8/51 amplified a 238 bp band under the PCR conditions used (Table 3). Nucleotide and amino acid sequences derived (Accession number AY034589) were 95 and 100% similar to the known sequences of *xylE*, which codes for *C23DO* in the mesophilic *P. putida* strain and only one isolate, *Pseudomonas* sp. 30/2, out of the eight naphthalene degraders showed a positive amplification with *C23DO* primers (Table 3). We designed and tested two other primers *xylE* and *cat2*, *3* (Laramee et al. 2000; Nakai et al. 1983) (Table 2). The naphthalene degrader *Pseudomonas* spp. as well as others utilizing JP8 jet fuel and one out of five *Sphingomonas* sp. i.e. Ant 29 showed positive amplification for both the *xylE* and *cat2*,*3* genes (Table 3), indicating the presence of naphthalene degradation pathway. Interestingly, 5 out of 6 *Rhodococcus* spp. also were PCR-positive for either *xylE* or *cat2*,*3* or both because the primers for both the genes were designed from *Pseudomonas putida* ATCC 33015 (Tables 2, 3).

Amplification of the toluene/biphenyl dioxygenase gene

None of the isolates tested in this study showed amplification for the *todC1* (Table 3). And only two strains i.e. *Sphingomonas* 35/1 and *Coryneform* 31/1 amplified an 830 bp band for the *bphA*1 gene.

Reverse transcriptase PCR amplification

The reverse transcriptase PCR for three genes i.e. *alkB2*, *C23DO* and *ndoB* from three different strains i.e. *Rhodococcus* sp. 5/1, *Pseudomonas* sp.7/156 and *Pseudomonas* sp. Ant 6, which showed positive amplification for these genes in conventional PCR, also exhibited amplification of the cDNA of the transcript of these biodegradative genes (Fig 1). This suggests that these biodegradative genes are in fact

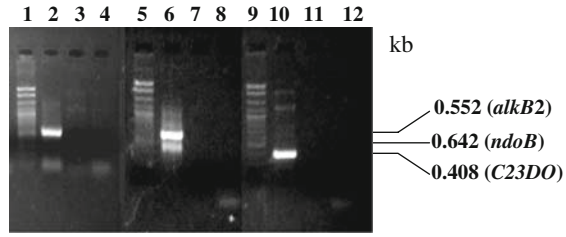

Fig. 1 A photopositive of a representative agarose gel of the RT-PCR amplified gene fragments. *Lane 1*: 100-bp size standard (Clone-Sizer, Norgen Biotek); *Lane 2*: 0.552 kb *alkB2* from *Rhodococcus* sp. 5/1; *Lane 3*: negative control (no DNA).; *Lane 4*: PCR with RNA from 5/1; *Lane 5*: 100-bp size standard; *Lane 6*: 0.642 kb *ndoB* from *Pseudomonas* sp.7/156; *Lane 7*: negative control (no DNA).; *Lane 8*: PCR with RNA from *Pseudomonas* sp.7/156; *Lane 9*: 100-bp size standard; *Lane 10*: 0.408 kb *C23DO* from *Pseudomonas* sp. Ant 6; *Lane 11*: negative control (no DNA); *Lane 12*: PCR with RNA from *Pseudomonas* sp. Ant 6

are functional and contribute to the biodegradation of the targeted compounds.

Competitive PCR

Due to the conserved nature of the *ndoB* gene in psychrotolerant as well as in mesophilic bacteria, a quantitative analysis of this gene by cPCR was developed in this study. First, the cPCR reaction was optimized with equal concentrations of the 648 bp target and the 501 bp competitor DNA fragments of the *ndoB* gene to ensure equal levels of amplified products results from the cPCR. Presence of DNA bands of equal intensity suggested that the PCR conditions used to amplify both the target and competitor gene were adequate. Next, twofold dilutions of the competitor template were co-amplified with constant amounts of genomic DNA from *Pseudomonas* sp. 30/2 to determine the target gene concentration (Fig. 2). The mean intensities of the competitor and the target DNA exhibited a good correlation ($R^2 = 0.96$) with the concentration of the targeted DNA being 48.8 pg/µl (SD ± 0.06) (Fig. 3), which corresponds to 1×10^7 copies of the template DNA. These results were consistent among three individual experiments conducted at different times. The results suggest that cPCR using the *ndoB* gene can potentially be applied in the Antarctic soils to quantify relatively low concentrations of targeted *ndoB* gene.

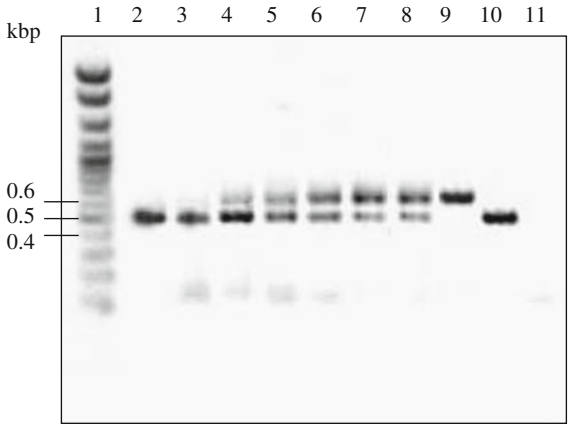

Fig. 2 A photonegative of a representative agarose gel showing the PCR amplified *cndoB* and *ndoB* gene fragments from *Pseudomonas* sp. 30/2. *Lane 1*: 100-bp size standard (Clone-Sizer, Norgen Biotek); *Lane 2*: 452 pg *cndoB*; *Lane 3*: 226 pg *cndoB*; *Lane 4*: 113 pg *cndoB*; *Lane 5*: 56.5 pg *cndoB*; *Lane 6*: 45.2 pg *cndoB*; *Lane 7*: 28.2 pg *cndoB*; *Lane 8*: 14.52 pg *cndoB*; *Lane 9*: *ndoB* (positive control); *Lane 10*: *cnodB*; *Lane 11*: negative control (no DNA). The amount of target DNA used for cPCR was the same in all the reactions

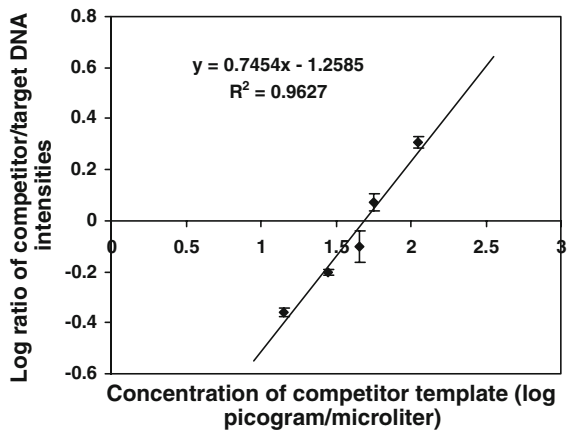

Fig. 3 Standard curve to calculate the gene copy number of the target *ndoB* gene using competitive PCR. The points plotted correspond to the concentrations of *cndoB* listed in *lanes 4*, *5*, *6*, *7* and *8* of Fig. 1. Regression analysis performed on the results exhibited an $R^2 = 0.96$. The experiment was repeated three times with consistent results

Discussion

Oil-spills in pristine Antarctic continent are a threat to the indigenous flora and fauna. In order to implement bioremediation using indigenous hydrocarbon-degrading microorganisms, it is necessary to understand the physiology, community structure as well as the genetics behind the catabolic pathways. In this study we have used conventional PCR to identify the occurrence and distribution and expression of the biodegradative genes in microorganisms isolated from Antarctic oil-contaminated soils. With regard to naphthalene degradation, *ndoB* has been found to be highly conserved in *Pseudomonas* B17, B18 and other psychrotolerant and mesophilic bacteria (Whyte et al. 1996, 1997). It has been reported that, although the biodegradative pseudomonads are not the only species that degrade naphthalene in soil, they are prevalent in oil contaminated environments (Aislabie et al. 2000; Eriksson et al. 2003; Whyte et al. 2002a). Similarly in our study, *Pseudomonas* spp. with *ndoB* was commonly isolated from hydrocarbon-contaminated Antarctic soils. Psychrotolerant bacteria with *ndoB* gene sequence homology to that in mesophiles have been documented (Whyte et al. 1996). Our results also suggest that there is a considerable similarity in the catabolic pathway involved in naphthalene degradation between mesophilic and psychrotolerant bacteria.

The conserved nature of *ndoB* in all these isolates proved helpful in the design and application of the cPCR method. The assay conditions tested in this study can potentially be used as the first step for the enumeration of the *ndoB* gene-copy number in oil-contaminated Antarctic soils. Quantification of the *ndoB* from total genomic DNA extracted from contaminated soils could provide an assessment of the population dynamics of culturable as well as non-culturable naphthalene degrading microorganisms. cPCR has been widely used in the medical diagnostics but has also found its application in environmental biotechnology. The use of cPCR with 16s rRNA gene or the biodegradative genes such as ammonia monooxygenase (*amoA*), atrazine (*atzC*), cadmium (*cadA*) as targets have been used in quantification of non-culturable bacteria as well as to assess population density in both pristine and contaminated soil environments (Ka et al. 2001; Kurkela et al. 1988; Martin-Laurent et al. 2003; Oger et al. 2001). Mesarch et al. (2000) has demonstrated the usefulness of the cPCR for C23DO gene present in contaminated soil samples. Therefore, the development of the cPCR would help in situ analysis of the distribution and quantitation of naphthalene and C23DO degrading microorganisms in pristine oil-contaminated soils in Antarctica. Due to the diverse

nucleotide sequences of *alkB*, selection of a single set of primer to establish a comprehensive detection of this gene in total microbial biomass by using cPCR was not possible. However, the cPCR protocol targeting alkane degradation genes present in *Rhodococcus* spp. can be applied in polar soils (Whyte et al. 2002a).

Catechol 2,3-dioxygenase was chosen as a target for PCR amplification because of its broad-specificity for a number of hydrocarbons like benzene, xylene, toluene and naphthalene, which are present in Jet fuel. The amino acid residues deduced from the 238 bp conserved region of the C23DO gene exhibited 100% sequence identity to that found in mesophilic bacteria suggesting that most likely they have similar degradative pathways. However, a number of bacterial isolates listed in Table 3 were PCR negative for this gene. But, the PCR amplification using the *xylE*b-F and *xylE*B-R; and *cat2*, *3* 1a-F and *cat2*, *3*a-R, targeting different segments of *C23DO* gene successfully amplified in most of the bacteria. In addition, 4 out of 5 *Sphingomonas* spp. did not amplify for any of the three primer sets. The results suggest the presence of a different pathway for the degradation of catechol, such as the ortho-cleavage dioxygenase or a different gene altogether is present in these isolates. Alternatively, there is need for designing of new primers from genes specific for the Sphingomonas isolates. Therefore, the presence of the *ndoB* gene but absence of the *C23DO* gene in the same organism may suggests that the psychrotolerant bacteria possess a modified or an alternative naphthalene degradation pathway.

The *alkB* gene has been reported to have diverse sequences in different genera, especially among Gram-negative and Gram-positive isolates (Smits et al. 1999). The *alkB* sequence is also thought to be dependent on the chain-length of the alkane and/or types of pathways utilized by the alkane-degrading bacterium (Heider et al. 1999; Rehm and Reiff 1981; Vomberg and Klinner 2000). Therefore, not all microorganisms in our study isolated from JP5 Jet fuel contaminated soils exhibited amplification of the *alkB* gene using a single set of primers. However, the majority of the rhodococcal isolates exhibited positive amplification for *alkB2*. This could be due to the bacteria being isolated from Antarctic soils typically contaminated with C_8–C_{20} *n*-alkanes that include C_{10}–C_{16} *n*-alkanes, which is the proposed substrate range for *alkB2* (Bej et al. 2000; Chandler and Brockman 1996; van Beilen and Funhoff 2007). This in contrast to the study by Whyte et al. (2002a) in which the *alkB1* was predominantly identified in rhodococcal population in diesel fuels contaminated soils in Antarctic and Arctic environments. Thus, it is apparent that the distribution of different *alkB* genes in rhodococcal population correlates with the types of alkanes present in the soil.

Among other Gram negative isolates, 13 out of 16 *Pseudomonas* spp. and 3 out of 5 *Sphingomonas* spp. were PCR-negative for *alkB1*, *alkB2* and *alkB*194 which were designed from a Gram positive strain *Rhodococcus* sp. Q15 (Table 3) suggesting that these primers sets are more successful in amplifying *alkB* sequences of *Rhodococcus* spp. (Whyte et al. 2002a). This is probably due to the presence of a gene with low homology to known *alkB* sequences or a novel gene altogether. In fact, genes with low homology to known *alkB* have been detected in several microorganisms that were isolated from alkane-contaminated soils (Smits et al. 2002; van Beilen et al. 2002). The functionality of a few of these genes has been investigated to prove that they play a role in alkane degradation, but still others remain ambiguous (Smits et al. 2002). Another reason we were unable to detect short-chain alkane degrading microorganisms from Antarctic soils could be due to decreased volatilization of the short chain alkanes ($<C_{10}$) at cold temperatures. This results in increased solubility of alkanes in the aqueous phase, which becomes toxic to the microorganisms (Whyte et al. 1999b). Thus, further investigation is necessary to understand if the alkane degrading genes in psychrotolerant bacteria evolved independent of *alkB* gene found in mesophiles. Recently, Kuhn et al. (2009) described the predominant presence of a novel *alkM* gene in the sediments of a pristine site near contaminated sediments in the Admiralty Bay, King George Island, Peninsula Antarctica. However, this novel *alkM* gene was described only in *Acinetobacter* sp. In our study, none of the contaminated soil samples exhibited the presence of *Acinetobacter* sp. therefore the test for this gene was irrelevant. In another study by Lo Giudice et al. (2009), 253 biodegradative indigenous bacterial isolates from the diesel fuel-enriched marine surface water from the Victoria Land coast of Antarctica showed the presence of *Rhodococcus*, *Sphingomonas*, *Pseudomonas* and *Corynebacterium*

similar to the isolates in our study from Antarctic Dry Valleys soils. This suggests that they are found in both continental spill sites and coastal waters of the Antarctic continent (Atlas 1981; Lo Giudice et al. 2009; Michaud et al. 2004a, b).

Almost all isolates did not show positive amplification for the *todC1* and *bphA1* gene. The *todC1* gene, located on the chromosome of *P. putida* F1, encodes for the α-subunit of terminal dioxygenase, one of the three components of toluene dioxygenase, the first enzyme involved in the toluene degradation pathway (Baraniecki et al. 2002). The *xylE* and *cat2, 3* genes, located on the TOL plasmid of *P.putida* ATCC 33015, encode for catechol 2,3 dioxygenase, a key enzyme involved in the lower degradation pathway of aromatic compounds, such as toluene and xylene (Laramee et al. 2000; Nakai et al. 1983). Though these organisms did not show amplification for the first gene of the pathway i.e. *todC1* but presence of the enzyme C23DO from the lower degradation pathway of toluene or xylene suggests the occurrence of this pathway in these organisms. The *bphA* gene, located on the chromosome of the polychlorinated biphenyl-degrading strain *P. pseudoalcaligenes* KF707, encodes for biphenyl dioxygenase, a multi-component enzyme responsible for the catalysis of the initial oxidation of biphenyl and chlorobiphenyls (Furukawa and Arimura 1987). Appropriate primers targeting the specific genes for the complete pathway are yet to be determined. In our study, we also tested the functionality of three genes i.e. *alkB*, *ndoB* and *C23DO* by RT-PCR. Positive amplification of the cDNA from the transcripts of these genes suggests that in fact these genes are transcriptionally active thereby the microbial isolates bearing these genes are capable of biodegradation of the targeted PAH compounds.

It is apparent from studies by our laboratory as well as others that degradation of hydrocarbon compounds can be achieved by microorganisms that have been isolated from hydrocarbon-contaminated Antarctic soils. In this study, we further confirmed the presence, nature and functionality of the degradative genetic elements. Therefore, bioremediation of the Jet fuel contaminated Antarctic soils may be possible because of these hydrocarbon-degrading pathways present in the indigenous microbial populations. Moreover, the growth, survival and adaptation of some of these biodegradative psychrotolerant microorganisms have already been elucidated (Bej et al. 2000; Aislabie et al. 2000; Panicker et al. 2001; Whyte et al. 1999b). Thus, as our understanding of the workings of the indigenous psychrotolerant bacteria increases, we can harness the biodegradative capabilities of the hardy indigenous microbial populations to degrade a wide range of pollutants.

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Conflict of interest The authors declare that they have no conflict of interest.

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