A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil

Luz, A. P.; Pellizari, V. H.; Whyte, L. G.; Greer, C. W.

Publisher's version / la version de l'éditeur:
Canadian Journal of Microbiology, 50, 5, pp. 323-333

Web page / page Web
http://dx.doi.org/10.1139/W04-008
http://nparc.cisti-icist.nrc-cnrc.gc.ca/npsi/ctrl?action=rtdoc&an=3539991&lang=en
http://nparc.cisti-icist.nrc-cnrc.gc.ca/npsi/ctrl?action=rtdoc&an=3539991&lang=fr

A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil

A.P. Luz, V.H. Pellizari, L.G. Whyte, and C.W. Greer

Abstract: Total community DNA from 29 noncontaminated soils and soils impacted by petroleum hydrocarbons and chloro-organics from Antarctica and Brazil were screened for the presence of nine catabolic genes, encoding alkane monooxygenase or aromatic dioxygenases, from known bacterial biodegradation pathways. Specific primers and probes targeting alkane monooxygenase genes were derived from *Pseudomonas putida* ATCC 29347 (Pp *alkB*), *Rhodococcus* sp. strain Q15 (Rh *alkB1*, Rh *alkB2*), and *Acinetobacter* sp. ADP-1 (Ac *alkM*). In addition, primers and probes detecting aromatic dioxygenase genes were derived from *P. putida* ATCC 17484 (*ndoB*), *P. putida* F1 (*todC1*), *P. putida* ATCC 33015 (*xylE* and *cat23*), and *P. pseudoalcaligenes* KF707 (*bphA*). The primers and probes were used to analyze total community DNA extracts by using PCR and hybridization analysis. All the catabolic genes, except the Ac *alkM*, were detected in contaminated and control soils from both geographic regions, with a higher frequency in the Antarctic soils. The alkane monooxygenase genes, Rh *alkB1* and Rh *alkB2*, were the most frequently detected *alk* genes in both regions, while Pp *alkB* was not detected in Brazil soils. Genes encoding the aromatic dioxygenases toluene dioxygenase (*todC1*) and biphenyl dioxygenase (*bphA*) were the most frequently detected in Antarctica, and *todC1* and catechol-2,3-dioxygenase (*cat23*) were the most frequent in Brazil soils. Hybridization analysis confirmed the PCR results, indicating that the probes used had a high degree of homology to the genes detected in the soil extracts and were effective in detecting biodegradative potential in the indigenous microbial population.

Key words: catabolic genes, anthropogenic compounds, petroleum hydrocarbons, alkane monooxygenases, aromatic dioxygenases.

Résumé : L'ADN total de communautés de 29 sols non contaminés et contaminés avec des hydrocarbures de pétrole et des organochlorés de l'Antarctique et du Brésil, ont été criblés pour la présence de neuf génes cataboliques, codant des monooxygénases d'alcanes ou des dioxygénases d'aromatiques, faisant partie de voies de biodégradation bactériennes connues. Des amorces et des sondes ciblant les génes de monooxygénases d'alcanes ont été dérivées de *Pseudomonas putida* ATCC 29347 (Pp *alkB*), *Rhodococcus* sp. souche Q15 (Rh *alkB1*, Rh *alkB2*) et *Acinetobacter* sp. ADP-1 (Ac *alkM*). De plus, les amorces et les sondes détectant les génes de dioxygénases d'aromatiques ont été dérivées de *P. putida* ATCC 17484 (*ndoB*), *P. putida* F1 (*todC1*), *P. putida* ATCC 33015 (*xylE* et *cat23*) et *P. pseudoalcaligenes* KF707 (*bphA*). Les amorces et les sondes ont été utilisées pour analyser les extraits totaux d'ADN des communautés à l'aide d'analyses par PCR et par hybridation. Tous les génes cataboliques, sauf le géne Ac *alkM*, ont été détectés dans les sols contaminés et témoins des deux régions géographiques, avec une plus haute fréquence retrouvée dans les sols de l'Antarctique. Les génes de monooxygénases d'alcanes Rh *alkB1* et Rh *alkB2* étaient les génes *alk* les plus fréquemment détectés dans les deux régions, alors que le géne Pp *alkB* n'a pas été détecté dans les sols de Brésil. Les génes codant des dioxygénases d'aromatiques, la toluéne dioxygénase (*todC1*) et la biphényle dioxygénase (*bphA*), étaient les plus fréquemment détectés en Antarctique; le géne *todC1* et la catéchol-2,3-dioxygénase (*cat23*) étaient les plus fréquents dans les sols du Brésil. Les analyses d'hybridation ont confirmé les résultats de PCR, ce qui indique que les sondes utilisées avaient un haut degré d'homologie avec les génes détectés dans les extraits de sol, et ont pu détecter efficacement le potentiel de biodégradation dans les populations microbiennes indiégnes.

Received 14 August 2003. Revision received 12 January 2004. Accepted 15 January 2004. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 27 May 2004.

A.P. Luz[1] and V.H. Pellizari. Biomedical Sciences Institute, Department of Microbiology, University of São Paulo, Lineu Prestes Avenue, São Paulo, SP 05508-900, Brazil.
L.G. Whyte. Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, QC H4P 2R2, Canada; and Department of Natural Resource Sciences, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada.
C.W. Greer. Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, QC H4P 2R2, Canada.

[1]Corresponding author (e-mail: apluz@usp.br).

 doi: 10.1139/W04-008

Mots clés : génes cataboliques, composés anthropiques, hydrocarbures du pétrole, monooxygénases d'alcanes, dioxygénases d'aromatiques.

[Traduit par la Rédaction]

Introduction

Bioremediation, a natural degradation process, has provided a cost-effective alternative to eliminate in situ organic pollutants from the environment, since autochthonous microbial communities have an adaptive response and in many cases can acquire the ability to metabolize such compounds. Petroleum hydrocarbons and polychlorinated compounds are widespread pollutants in the environment and possess known or suspected toxic, mutagenic, and carcinogenic properties. The biodegradation of these compounds has been intensively studied, and many bacterial strains have been isolated for their ability to degrade and use them as sources of carbon and energy (Hamann et al. 1999; Harwood and Parales 1996).

Numerous molecular biology techniques are now available to study microbial community ecology, by detecting and quantifying target microorganisms or target genes in environmental samples. Specific gene probes and (or) specific oligonucleotide primers can be designed, based on existing DNA sequences, encoding different enzymes involved in degradation pathways. These molecular methods are advantageous because uncultivated microorganisms, which may represent up to 99% of the naturally occurring cells in the environment, can be analyzed rapidly and with increased detection sensitivity (Leahy and Colwell 1990; Holben 1992; Greer et al. 1993; Gelsomino et al. 1999; Meyer et al. 1999). Furthermore, these same molecular approaches can help us assess the endemic and the cosmopolitan distribution of different catabolic genes in natural communities. Microbial diversity and biogeographic studies can help identify whether microorganisms or specific genotypic characteristics are widely distributed or if they tend to be endemic only to specific areas (Staley 1999; Staley and Gosink 1999).

Monooxygenases and dioxygenases are key enzymes in the biodegradation of a wide range of environmental pollutants, and the genes encoding these enzymes have been characterized in *Pseudomonas putida* (formerly *P. oleovorans*, see van Beilen et al. 2001) ATCC 29347 (Pp *alkB*), *Rhodococcus* sp. strain Q15 (Rh *alkB1*, Rh *alkB2*), *Acinetobacter* sp. ADP-1 (Ac *alkM*), *P. putida* ATCC 17484 (*ndoB*), *P. putida* F1 (*todC1*), *P. putida* ATCC 33015 (*xylE*, *cat23*), and *P. pseudoalcaligenes* KF707 (*bphA*). Pp *alkB*, located on the OCT plasmid, a member of the IncP-2 family of plasmids, encodes alkane hydroxylase, a membrane-bound monooxygenase that is the first enzyme of the short-chain alkane (C_6–C_{12}) degradation pathway (Kok et al. 1989; van Beilen et al. 1994). Recently, highly similar multiple alkane hydroxylase systems have been isolated and characterized in *Rhodococcus erythropolis* NRRL B-16531 and *Rhodococcus* sp. strain Q15 (Whyte et al. 2002*a*); this genus and other closely related high %G+C actinomycetes are considered important members of hydrocarbon-degrading microbial populations in contaminated soils (Warhurst and Fewson 1994). Both organisms are psychrotrophs, degrade a wide range of n-alkanes (C_8–C_{32}), and contain at least four alkane monooxygenase homologues (Rh *alkB1*, Rh *alkB2*, Rh *alkB3*, and Rh *alkB4*); when the rhodococcal *alkB2* was cloned into *P. fluorescens* KOB2Δ1, the recombinant strain was able to mineralize and grow on C_{12}–C_{16} n-alkanes (Whyte et al. 2002*a*). Ac *alkM* encodes alkane monooxygenase, one of the five essential genes encoding alkane utilization by *Acinetobacter* sp. ADP-1, required for degradation of C_{12}–C_{18} n-alkanes (Ratajczak et al. 1998). The natural diversity of the alkane hydroxylase system has recently been presented by van Beilen et al. (2003). The *ndoB* gene, located on the NAH7 plasmid, is one of three genes that encode naphthalene dioxygenase, the first enzyme in the *P. putida* ATCC 17484 naphthalene degradation pathway (Kurkela et al. 1988). The *todC1* gene, located on the chromosome of *P. putida* F1, encodes for the α-subunit of terminal dioxygenase, one of three components of toluene dioxygenase, the first enzyme involved in the toluene degradation pathway (Zylstra and Gibson 1989). The *xylE* and *cat23* 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 (Nakai et al. 1983; Moon 1995; Okuta et al. 1998; Laramée et al. 2000). 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 et al. 1987; Asturias et al. 1995).

Several studies have been conducted in cold environments in Alaska, the Arctic (Lindstrom et al. 1991; Button et al. 1992; Pritchard et al. 1992; Master and Mohn 1998; Whyte et al. 1999*a*), and the Antarctic (Pietr 1986; Delille et al. 1997; Mac Cormack and Fraile 1997; Aislabie et al. 2000; Bej et al. 2000; Delille 2000) to specifically examine petroleum hydrocarbon and polychlorinated compound degradation in different ecosystems, but relatively little genotypic information is available for Antarctic soils. For bioremediation of oil-contaminated Antarctic soils, indigenous microbial populations that degrade alkanes and aromatic compounds are necessary, since the Antarctic Treaty prohibits the introduction of foreign organisms (Aislabie et al. 1998). In the same manner, little is known about the genotypic diversity of soil microbial communities in tropical and subtropical areas (Pellizari et al. 1996; Fulthorpe et al. 1998) and its potential for bioremediation.

The objective of this work was to compare the total bacterial community DNA extracted from contaminated and control soils from Antarctica and Brazil for the presence of four genotypes of *alk* genes and five different aromatic dioxygenase genes involved in hydrocarbon and chloro-organic metabolism, using PCR and hybridization analysis. The use of these methods will provide basic knowledge on the distribution of these degradative genotypes in cold and tropical

ecosystems, which would help in predicting biodegradation potential under distinctly different environmental conditions.

Materials and methods

Soils

Twenty-nine soil samples were collected from areas that were impacted and not impacted by anthropogenic activities in two distinct geographic regions, Antarctica and Brazil. In Antarctica, 16 soil samples were obtained during the summers of 1998 and 1999, at several sites near the Brazilian Antarctic Station Comandante Ferraz (EACF), from areas surrounding the diesel fuel storage tanks and from other stations and noncontaminated sites distributed along Admiralty Bay and Maxwell Bay, on King George Island, South Shetland Islands, Antarctic Peninsula. The Brazilian Antarctic station (lat 62°05′S, long 058°23.5′W) was established in the summer of 1984 and is presently a medium-sized research station with a summer population of about 50 people, with power being supplied by diesel generators. The station has a 380 t Antarctic grade diesel oil tank, which is replenished every summer by ship. The petroleum derivative most frequently used in the Antarctic is Diesel Fuel Arctic (DFA), which contains mainly semi-volatile aromatic hydrocarbons, such as naphthalene, other aromatics, alkanes, and their corresponding alkyl-substituted derivatives (Bícego et al. 1996; Secirm/Proantar/CNPq/Brazil 2000). Both alkane and aromatic hydrocarbons are spilled on the land because of field operations, involving fuel storage and vehicle refueling activity. In all cases, control soils with similar soil characteristics to the corresponding contaminated soils were collected from regions ~0.2 to 1 km from the corresponding contaminated sites.

In Brazil, 10 samples were obtained during the winter of 1998 from Pilões landfill in Cubatão, São Paulo State (lat 23°54′19″S, long 46°28′00″W), an area where industrial residues were disposed, including polychlorinated biphenyls (PCBs) (Aroclor 1254, 0.089 mg/kg), pentacholorophenol (0.51–28 mg/kg), hexachlorobenzene (6.0–28 mg/kg), benzene (0.09 mg/kg), *o*-xylene (0.028 mg/kg), total petroleum hydrocarbon (25 000 mg/kg), and 4,4′-dichloro-diphenyltrichloroethane (DDT) (0.024 mg/kg) (Moura 2000). (The concentrations indicated are averages for all soils sampled in the landfill area.) Control samples were collected from noncontaminated locations within 200 m outside of the landfill area. Three samples were obtained from an experimental area contaminated with benzene, toluene, ethylbenzene, xylene (BTEX) in Florianópolis, Santa Catarina State (lat 27°35′S, long 48°34′W), during the winter of 1999.

Composite samples from each sampling site were collected just below the surface to a depth of approximately 10 cm with sterile spatulas, sealed in sterile Whirlpak® bags, transported on ice to the laboratory, and stored at –20 °C until they were analyzed.

Total community DNA extraction and purification

The total community DNA of all soil samples was extracted in Montréal, Quebec, using an adapted protocol (Ausubel et al. 1990; Fortin et al. 1998). Prior to lysis treatment, 4.5 mL of distilled water was added to 10 g of soil samples. A 500-μL aliquot of 250 mmol/L Tris–HCl (pH 8.0) and 50 mg of lysozyme were added, and the samples were incubated for 30 min at 30 °C, followed by 30 min at 37 °C with mixing by inversion every 10 min. Proteinase K was added to a final concentration of 20 mg/mL, and samples were incubated for 1 h at 37 °C. The lysis treatment was completed with the addition of 500 μL of 20% SDS and incubated for 30 min at 85 °C. Samples were centrifuged (13 600*g*) for 10 min at room temperature. Supernatants were treated with a one-half volume of 7.5 mol/L ammonium acetate, incubated on ice for 15 min to precipitate proteins and humic acids, and centrifuged for 5 min at 4 °C (13 600*g*). The DNA was precipitated with one volume of isopropanol at –20 °C, overnight. Samples were centrifuged at 4 °C for 20 min (15 800*g*). Pellets were washed with 70% cold ethanol and dried by speed vacuum for approximately 5 min. Each DNA sample was resuspended in 200 μL of Tris–EDTA (pH 8.0) and stored at –20 °C.

To obtain high-quality PCR-amplifiable DNA, all samples were purified using polyvinylpolypyrrolidone (PVPP) spin columns (Berthelet et al. 1996). To assess DNA average molecular size and quality, the soil DNA extracts were run on 0.7% (*m*/*v*) agarose gels, (Sambrook et al. 1989) with a 1-kb ladder as reference. The DNA yields were soil dependent and ranged from 2.5 to 15.7 μg/g dry soil and from 2 to 7.5 μg/g dry soil for the Brazilian and Antarctic soils, respectively. Approximately 100 ng of DNA (quantified using DyNA Quant 200, Hoefer) was used as template for PCR amplification. To determine whether there were any PCR inhibitory compounds in the DNA preparations, universal 16S rDNA eubacterial primers (Coates et al. 1998) were used as a positive PCR amplification control (Table 1).

Detection of catabolic genes by PCR and hybridization of PCR fragments

The purified DNA extracts from 29 soils were screened by PCR to detect the following catabolic genes that encode enzymes involved in a variety of known bacterial biodegradative pathways: *alkB* and *alkM* (n-alkanes), *ndoB* (naphthalene), *todC1* (toluene), *xylE* (xylene), *cat23* (catechol), and *bphA1* (biphenyls). The oligonucleotide primers used in this study were derived from the published sequences of these catabolic genes and designed to specifically detect DNA sequences from within the coding region of these genes, using the reference strains listed in Table 1 as positive controls. The construction and specificity of the primer and probe sets have been described previously (i.e., *alkB* and *alkM* (Whyte et al. 2002*b*); *ndoB*, *todC1*, *xylE* (Whyte et al. 1996)). PCR was carried out, essentially as described by Whyte et al. (1996), for 30 cycles (each) of 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, and 1 min of extension at 72 °C, and a final extension of 3 min at 72 °C, using a Perkin Elmer–Cetus DNA thermal cycler 480 (Perkin Elmer–Cetus, Mississauga, Ont., Canada). Amplification products were analyzed on 1.2% agarose gels, followed by ethidium bromide staining to verify if the amplification was successful (Sambrook et al. 1989).

To verify that the correct PCR fragments had been amplified, they were transferred from the agarose gel onto nylon membranes (Boehringer Mannheim, Mannheim, Germany) using a LKB 2016 VacuGene vacuum blotting system (Pharmacia Biotech, Baie d'Urfé, Quebec), as described be-

Table 1. Oligonucleotide sequences of primers and probes and reference strains used in this study.

Catabolic gene	Sequence (5′ end to 3′ end)	PCR fragment size (bp)	Reference strain (GenBank accession No.)	Reference
Alkane monooxygenase				
Pp *alkB*-F	TGGCCGGCTACTCCGATGATCGGAATCTGG	870	*Pseudomonas putida* ATCC 29347 (X65936)	van Beilen et al. 2001
Pp *alkB*-R	CGCGTGGTGATCCGAGTGCCGCTGAAGGTG	870	*Pseudomonas putida* ATCC 29347 (X65936)	van Beilen et al. 2001
Rh *alkB1*-F2	ATCTGGGCGCGTTGGGATTTGAGCG	642	*Rhodococcus* sp. Q15 (AF388181)	Whyte et al. 2002*a*
Rh *alkB1*-R1	CGCATGGTGATCGCTGTGCCGCTGC	642	*Rhodococcus* sp. Q15 (AF388181)	Whyte et al. 2002*a*
Rh *alkB2*-F1	ACTCTGGCGCAGTCGTTTTACGGCC	552	*Rhodococcus* sp. Q15 (AF388182)	Whyte et al. 2002*a*
Rh *alkB2*-R1	CCCACTGGGCAGGTTGGGCGCACCG	552	*Rhodococcus* sp. Q15 (AF388182)	Whyte et al. 2002*a*
Ac *alkM*-F	CCTGTCTCATTTGGCGCTCGTTCCTACAGG	496	*Acinetobacter* sp. ADP-1 (AJ002316)	Ratajczak et al. 1998
Ac *alkM*-R	CCAAAGTGGCGGAATCATAGCAGGC	496	*Acinetobacter* sp. ADP-1 (AJ002316)	Ratajczak et al. 1998
Naphthalene dioxygenase				
ndoB-F	CACTCATGATAGCCTGATTCCTGCCCCCGGCG	642	*Pseudomonas putida* ATCC 17484 (M23914)	Kurkela et al. 1988
ndoB-R	CCGTCCCACAACACACCCATGCCGCTGCCG	642	*Pseudomonas putida* ATCC 17484 (M23914)	Kurkela et al. 1988
Toluene dioxygenase				
todC1-F	CGGGTGGGCTTACGACACCGCCGGCAATCT	560	*Pseudomonas putida* F1 (J04996)	Zylstra and Gibson 1989
todC1-R	TCGAGCCGCGCTCCACGCTACCCAGACGTT	560	*Pseudomonas putida* F1 (J04996)	Zylstra and Gibson 1989
Catechol-2,3-dioxygenase				
xylE-F	GTGCAGCTGCGTGTACTGGACATGAGCAAG	834	*Pseudomonas putida* ATCC 33015 (M65205)	Nakai et al. 1983
xylE-R	GCCCAGCTGGTCGGTGGTCCAGGTCACCGG	834	*Pseudomonas putida* ATCC 33015 (M65205)	Nakai et al. 1983
cat23 1a-F	AGGTGCTCGGTTTCTACCTGGCCG	405–408	*Pseudomonas putida* ATCC 33015 (M64747)	Laramée et al. 2000
cat23 6a-R	ACGGTCATGAATCGTTCGTTGAG	405–408	*Pseudomonas putida* ATCC 33015 (M64747)	Laramée et al. 2000
Biphenyl dioxygenase				
bphA1-F	TCACCTGCAGCTATCACGGCTGG	830	*Pseudomonas pseudoalcaligenes* KF707 (M83673)	Furukawa et al. 1987
bphA1-R	GGATCTCCACCCAGTTCTCGCCATCGTCCTG	830	*Pseudomonas pseudoalcaligenes* KF707 (M83673)	Furukawa et al. 1987
16S rDNA				
8-F	AGAGTTTGATCCTGGTCCAG	511	Eubacteria domain	Coates et al. 1998
519-R	GTATTACCGCGGCTGCTGG	511	Eubacteria domain	Coates et al. 1998

low. The membranes were pre-wetted with distilled water. Depurination (0.25 mol/L HCl), denaturation (0.5 mol/L NaOH, 0.5 mol/L NaCl), and neutralization (1 mol/L Tris (pH 7.5), 1.5 mol/L NaCl) solutions were applied to the surface of the gel, in this sequence, for 20–30 min each under a vacuum of 55 mbar. Subsequently, the gel was submerged for 2 h in the final solution of 20× SSC (3 mol/L NaCl, 0.2 mol/L sodium citrate, pH 7.0) to complete the transfer of the DNA fragments to the nylon membranes. DNA was fixed to the membranes by UV cross-linking, twice employing the auto cross-link function of a Stratalinker UV-cross-linker. The membranes were kept in sealed plastic bags at –20 °C until the hybridization was carried out. The membranes were prehybridized for 2 h in a prehybridization solution at 65 °C. Hybridization was performed overnight at 65 °C in 10–20 mL fresh hybridization solution, including 10–20 ng/mL digoxigenin (DIG)-labeled DNA probe, followed by high- stringency washes, as described in the DIG System User's Guide (Boehringer Mannheim). Detection was carried out by the chemiluminescent method, with the alkaline phosphatase substrate 25 mmol/L disodium-3-(4-methoxyspiro1,2- dioxetane-3,2′-(5′-chloro)tricyclodecan-4-yl)phenylphosphate (CSPD). Probed membranes were sealed in plastic blotting bags and exposed to Kodak X-Omat AR films at room temperature.

Gene probes for this study were prepared by PCR using primers usually 23–30 nucleotides (nt) in length (Table 1), derived from the published sequences of the specific catabolic genes (Greer et al. 1993). Each set of primers (10–20 ng/mL) was added to a 50 μL PCR reaction. The PCR was performed using a Perkin Elmer–Cetus DNA thermal cycler 480 and was carried out as described by Whyte et al. (1996), with a modification of 1 min annealing at 70 °C. This high annealing temperature was used to minimize nonspecific amplification. The PCR products were visualized by electrophoresis in a 1.2% agarose gel and then purified using the QIAquick PCR purification kit (QIAGEN Inc., Montréal, Que.). The probes were labeled with the DIG nonradioactive nucleic acid labeling and detection system, using the DIG Oligonucleotide 3′-end labeling kit (Boehringer Mannheim).

Results

Crude DNA extracts were recovered from 16 soil samples from the Antarctic and 13 soil samples from Brazil. All DNA extracts from the Antarctic soils, following PVPP column treatment, were pure enough to be used as templates for direct PCR amplification. All DNA extracts amplified a conserved region of 511 base pairs of the 16S rDNA gene with eubacterial primers 8F and 519R, indicating that DNA had been successfully extracted from the soils and confirming the absence of any PCR inhibitory compounds in the DNA preparations. The PCR and hybridization detection limits for Rh *alkB1*, Pp *alkB*, and Ac *alkM* were determined in a previous study (Whyte et al. 2002*b*) using the same soil DNA extraction, PCR amplification and hybridization protocols used in this study; similar detection limits (~10^4 cells/g soil by PCR and a further ~10 to 100-fold increase in sensitivity by Southern hybridization) were found in the three different reference bacteria (i.e., *Acinetobacter*, *Pseudomonas*, *Rhodococcus*), indicating that the techniques used had similar soil DNA extraction and PCR amplification efficiencies.

Antarctic soils

PCR and hybridization analysis of DNA extracts from the 16 Antarctic soil samples (Table 2) resulted in the amplification of DNA fragments of comparable size to that of the positive controls for three of the screened alkane monooxygenase genes (Pp *alkB*, Rh *alkB1*, Rh *alkB2*). Overall, the rhodococcal *alkB1* (94%) and *alkB2* (62%) genes were more frequently detected among the samples than the Pp *alkB* (44%) gene. Rh *alkB1* was detected in all the contaminated and control soils. Strong hybridization signals indicated that the PCR amplification products had relatively strong homology to Rh *alkB1*. The rhodococcal *alkB2* genotype was also detected in many of the contaminated (64%) and control soils (60%). However, in some cases, the PCR bands did not hybridize to the Rh *alkB2* gene probe indicating that these fragments had relatively less homology to the Rh *alkB2*, considering the high stringency conditions used for hybridization analysis, or that these fragments were spurious PCR amplification products. The Pp *alkB* genotype was detected more frequently in the contaminated soils around the diesel fuel storage tanks at EACF (55%) than in the control soils (20%). *Acinetobacter* sp. ADP-1 *alkM* was not detected by PCR or hybridization analysis in any of the Antarctic soils and therefore has not been included in Table 2.

PCR with specific primers for the naphthalene dioxygenase gene (*ndoB*) produced a DNA fragment of 642 bp for all soils sampled around the diesel fuel storage tanks and adjacent areas near the EACF. These PCR fragments hybridized to the *ndoB* probe as strongly as the *P. putida ndoB* PCR fragment, indicating a high homology with the *ndoB* gene. The *ndoB* genotype was detected in 69% of the Antarctic soils. The *xylE* PCR amplification products were confirmed by hybridization to the *xylE* gene probe in 55% of the contaminated soils and in 60% of the control soils. The *cat23* genotype was detected in 44% of the contaminated soils around the EACF, while it was not detected in any of the control soils. PCR amplification and gene probe hybridization using the *todC1* gene resulted in strong signals for 95% of the Antarctic soils, the most common aromatic degradation pathway genotype detected in these soils. The *bphA1* genotype was detected by PCR in 81% of the Antarctic soils, producing the expected 830-bp DNA fragment with a comparable size to that of *P. pseudoalcaligenes* KF707, which was also verified by hybridization to the *bphA1* gene probe (Fig. 1).

Brazilian soils

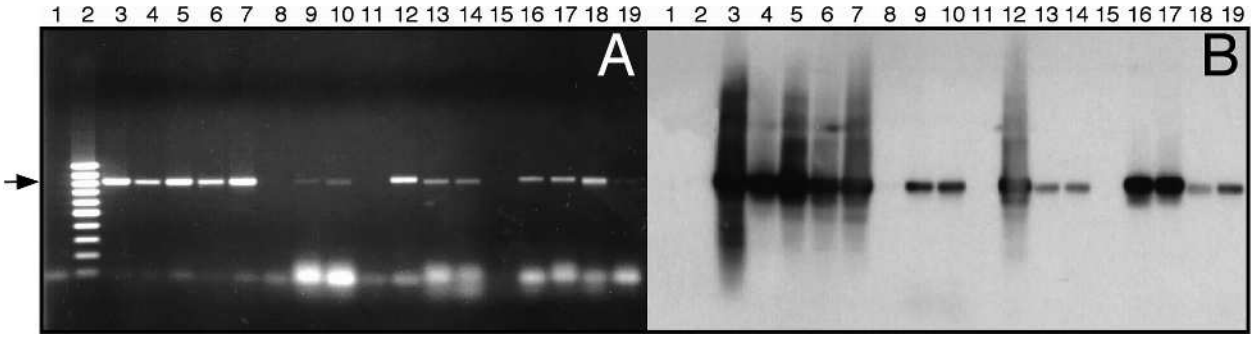
PCR and hybridization analysis of DNA extracts from the 13 Brazilian soil samples (Table 3) revealed that the Rh *alkB1* (46%) and Rh *alkB2* (62%) genes were the most frequently detected among the alkane monooxygenase genes. Although in many cases the Rh *alkB2* gene was not detected by PCR, the hybridization signals indicated that the weak PCR amplification products had relatively strong homology to the Rh *alkB2* gene probe. Rh *alkB1* was not detected among the control soils. Neither Pp *alkB* nor Ac *alkM* were

Table 2. PCR and hybridization (hyb) analyses of total community DNA from Antarctic soils.

		Pp *alkB*		Rh *alkB1*		Rh *alkB2*		*ndoB*		*todC1*		*xylE*		*cat23*		*bphA*	
Antarctic soil	Location	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb
Contaminated																	
1A	Admiralty Bay	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+
1B	Admiralty Bay	+	–	+	+	+	+	+	+	+	+	+	–	w	+	+	+
2A	Admiralty Bay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2B	Admiralty Bay	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4A	Admiralty Bay	+	+	+	+	+	+	+	+	+	+	w	–	+	+	+	+
4B	Admiralty Bay	+	+	+	+	+	+	+	+	w	+	w	+	+	+	–	–
#5	Admiralty Bay	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+
FLD02	Maxwell Bay	–	–	+	+	–	–	–	–	+	+	+	+	–	–	+	+
FLD04	Maxwell Bay	–	+	–	–	w	–	–	–	+	+	–	–	–	–	+	+
FLD10	Maxwell Bay	–	–	+	+	w	–	–	w	–	–	–	w	–	–	+	+
FLD12	Maxwell Bay	–	–	+	+	w	–	–	–	w	+	–	–	–	–	+	+
Control																	
3A	Admiralty Bay	–	w	+	+	+	+	w	+	+	+	+	+	–	–	–	–
3B	Admiralty Bay	–	–	+	+	+	+	w	+	+	+	+	+	–	–	+	+
FLD06	Admiralty Bay	–	–	w	+	–	–	–	–	–	+	–	–	–	–	–	–
FER17	Admiralty Bay	–	–	+	+	+	+	–	+	+	+	–	+	–	–	+	+
FER18	Admiralty Bay	–	+	+	+	w	–	–	+	+	+	–	–	–	–	+	+
Total (n = 16)*		7 (44%)		15 (94%)		10 (62%)		11 (69%)		15 (95%)		9 (56%)		7 (44%)		13 (81%)	

Note: w, weak signal for PCR and (or) hybridization; –, no signal; +, positive signal.
*The number and (percentage) of samples yielding PCR products hybridizing to known catabolic gene PCR products.

Fig. 1. Detection of *bphA* by PCR and hybridization analysis in Antarctic soils. (A) Agarose gel electrophoresis (1.2%) showing the expected 830-bp (arrow) *bphA* fragment obtained by PCR analysis of DNA extracts from the Antarctic soils. (B) Hybridization analysis of *bphA* PCR fragments shown in Fig. 1A, transferred to a nylon membrane and probed with the 830-bp *bphA* gene probe derived from *Pseudomonas pseudoalcaligenes* KF707. Lanes: 1, H_2O (negative control); 2, 100-bp ladder; 3, *P. pseudoalcaligenes* KF707 (*bphA*+); 4, 1A; 5, 1B; 6, 2A; 7, 2B; 8, 3A; 9, 3B; 10, 4A; 11, 4B; 12, 5; 13, FLD02; 14, FLD04; 15, FLD06; 16, FLD10; 17, FLD12; 18, FER17; 19, FER18.



detected in contaminated or control soils and therefore have not been included in Table 3.

The aromatic dioxygenase genes *ndoB* (8%) and *xylE* (23%) were detected less frequently in the Brazilian contaminated and control soil samples than in the Antarctic soils. The *ndoB* gene probe had a strong hybridization signal for only one contaminated soil, while the *xylE* genotype was not detected in any of the control soils. The strong hybridization signals to the *cat23* gene probe revealed that 54% of the samples were probe positive for the *cat23* genotype, although most of the PCR fragments were barely detectable in the agarose gel. Among the aromatic dioxygenase genes, *todC1* (77%) (Fig. 2) was also the most commonly detected catabolic gene between contaminated and control soils from Brazil. Only three contaminated soils did not hybridize with this probe. The *bphA1* genotype was detected by PCR in many of the contaminated and control sites in Brazil, but only 23% of the PCR fragments hybridized to the *bphA1* gene probe, indicating that the amplified fragment had relatively low homology to *bphA1*.

Discussion

In the present investigation, the presence and distribution of nine catabolic genes were assessed in the bacterial communities in Antarctic and Brazilian soils. The molecular approach used in this study successfully detected distinct monooxygenase and dioxygenase genes in total microbial DNA extracts from contaminated and control soils from two distinct geographic regions. In most cases, hybridization analysis of the PCR fragments, using high stringency conditions, indicated that the amplified PCR fragments possessed a high level of homology to the corresponding target catabolic genes studied. In those cases where the amplified PCR fragments did not hybridize to the reference gene probes, there might have been a low degree of homology between the probe and the PCR fragment or the primers could have amplified an unrelated DNA fragment. During PCR analysis, preferential amplification of target genes can occur, but chimeras can also form during the amplification process, especially when working with mixtures of DNA from complex environments, such as those found in soils (Muyzer and Ramsing 1995). In some cases, PCR amplification fragments were not visually detected by ethidium bromide staining but were detected by subsequent hybridization analysis because of the greater sensitivity of the hybridization technique. Also, the number of target copies in the community DNA extract could have been relatively low so that amplification did not produce enough material to allow visualization on the agarose gel.

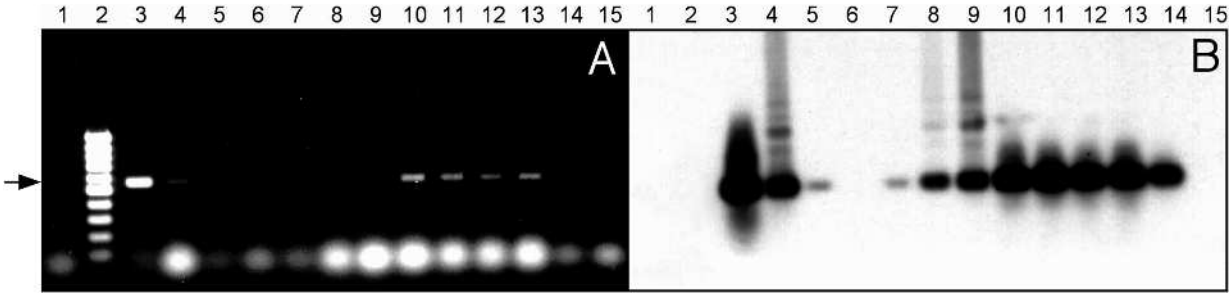
PCR and hybridization analysis indicated that rhodococcal *alkB1* and *alkB2* were the most common alkane-degradative genotypes encountered in both the Antarctic and Brazilian soils: the rhodococcal *alkB1* and *alkB2* were detected in most of the contaminated and many control soils, while Pp *alkB* was generally detected in the contaminated soils but less often in control soils in Antarctica. Surprisingly, Pp *alkB* was not detected in any of the DNA extracts of Brazilian soils. We were not able to detect the *alkM* gene for *Acinetobacter* sp. strain ADP-1 in any of the soils from either geographic region. The failure to detect Ac *alkM* probably indicates that the *alkM* primers were too specific, amplifying only *A. calcoaceticus* ADP-1 *alkM* and not other *Acinetobacter alkM* homologues. In a very recent similar study (Margesin et al. 2003), *alkM* genotypes were detected in contaminated alpine soils, using *alkM* universal primers, but not with the *A. calcoaceticus* ADP-1 *alkM* primer set used in this study. Overall, these results suggest that the *Rhodococcus* spp., which are ubiquitous and known to degrade hydrocarbons (Warhurst and Fewson 1994), may play a predominant role in alkane degradation in contaminated soils in both regions, while polar soils may become enriched with alkane-degradative pseudomonads following contamination events. It is possible that other closely related high %G+C, mycolic-acid-containing actinomycetes (*Mycobacterium tuberculosis*, *Prauserella rugosa*) were also detected by the primer and probe sets, as *alkB* homologues found in these organisms possess significant full-length DNA sequence identity (65%–70%) to the Rh *alkB* genes (van Beilen et al. 2002; Whyte et al. 2002*a*). The absence of Pp *alkB* and *A. calcoaceticus* ADP-1 *alkM* genes in Brazilian soils could be related to the molecular weight of the n-alkanes found in these soils, since high temperatures and sunlight irradiation in tropical and subtropical areas may in-

Table 3. PCR and hybridization (hyb) analyses of total community DNA from Brazilian soils.

		Rh *alkB1*		Rh *alkB2*		*ndoB*		*todC1*		*xylE*		*cat23*		*bphA*	
Brazil soil	Location*	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb	PCR	hyb
Contaminated															
ST1	CSP	–	–	–	+	w	w	+	+	–	–	w	+	+	+
ST2	CSP	+	+	+	+	–	w	–	+	–	+	+	+	+	+
ST3	CSP	w	+	w	+	–	–	–	–	–	w	–	–	–	–
ST4	CSP	w	+	w	+	–	–	–	–	–	+	–	w	–	–
ST5	CSP	+	w	–	–	–	–	w	+	–	–	w	+	w	–
ST6	CSP	w	+	w	+	–	–	w	+	–	–	+	+	–	–
ST7	CSP	w	+	–	w	–	–	+	+	–	–	–	–	+	w
ST10	CSP	–	–	–	+	w	w	+	+	–	–	w	+	+	+
SP2-06	FSC	–	–	–	–			–	+	–	–	–	–	–	–
SP2-80	FSC	w	+	–	+	–	–	–	–	–	+	–	–	w	–
Control															
ST8	CSP	w	–	–	+	–	–	+	+	–	–	–	–	+	–
ST9	CSP	w	w	–	w	w	w	+	+	–	–	w	+	+	w
PF1	FSC	w	–	–	w	–	–	w	+	–	–	w	+	w	–
Total (n = 13)[†]		6 (46%)		8 (62%)		1 (8%)		10 (77%)		3 (23%)		7 (54%)		3 (23%)	

Note: w, weak signal for PCR and (or) hybridization; –, no signal; +, positive signal.
*CSP: Cubatão, São Paulo; FSC: Florianopólis, Santa Catarina.
[†]The number and (percentage) of samples yielding PCR products hybridizing to known catabolic gene PCR products.

Fig. 2. Detection of *todC1* by PCR and hybridization analysis in Brazilian soils. (A) Agarose gel electrophoresis (1.2%) showing the expected 560-bp (arrow) *todC1* fragment obtained by PCR analysis of DNA extracts from the Brazilian soils. (B) Hybridization analysis of *todC1* PCR fragments shown in Fig. 2A, transferred to a nylon membrane and probed with the 560-bp *todC1* gene probe derived from *Pseudomonas putida* F1. Lanes: 1, H_2O (negative control); 2, 100-bp ladder; 3, *P. putida* F1 (*todC1*+); 4, ST1; 5, ST2; 6, ST3; 7, ST4; 8, ST5; 9, ST6; 10, ST7; 11, ST8; 12, ST9; 13, ST10; 14, PF1; 15, SP2-06.



crease the rate of short-chain alkane volatilization (Nicodem et al. 1998; Maki et al. 2001; Wang and Fingas 2003), leaving only the long-chain alkanes for microbial catabolism; *P. putida* is capable of catabolizing C_5–C_{12} n-alkanes, *Acinetobacter* sp. strain ADP-1 catabolizes C_{12}–C_{18} n-alkanes, while *Rhodococcus* sp. strain Q15 possesses a broader alkane substrate range and is capable of degrading C_8–C_{32} n-alkanes. On the other hand, little is known about the diversity of genes responsible for the degradation of n-alkanes. The type of alkane hydroxylase may not only be dependent on the alkane chain length but also on the type of degradation, which could suggest that those communities may contain other alkane degradative systems, which are not related to the *alk* genotypes tested for and, consequently, not detected. Apparently, the alkane hydroxylase genes seem to be quite divergent in different genera (Smits et al. 1999). *Rhodococcus* spp. are known to mineralize the medium, and longer chain alkanes and have been isolated from cold and temperate climates (Whyte et al. 1996, 1998, 1999*b*; Aislabie et al. 1998; Bej et al. 2000).

Bacteria that aerobically degrade aromatic hydrocarbons use dioxygenases to activate and cleave the aromatic ring, and therefore, the corresponding genes are excellent targets for detecting and enumerating potential pollutant biodegraders in complex environments, such as petroleum-contaminated soils (Mesarch et al. 2000). This study revealed that catabolic gene systems involved in the biodegradative pathways for aromatic and chlorinated organic compounds, mostly described in mesophilic microorganisms, also occur in microbial populations in very cold climates, such as the Antarctic. PCR and hybridization analyses revealed that the bacterial communities in both Antarctic and subtropical Brazilian soils possessed genes with homology to the *ndoB*, *todC1*, *xylE*, *cat23*, and *bphA1* genes. A similar geographical distribution of these genes was found in Brazil, Puerto Rico, and the USA (Pellizari et al. 1996), and in the Canadian Arctic (Whyte et al. 1996, 1997, 1999*a*). The most common genotypes found in contaminated and control soils from the Antarctic were *todC1* and *bphA1*. In Brazil, the most common aromatic genotypes were also *todC1* and *cat23*, which are involved in the degradation of toluene and catechol and probably are common chemicals and (or) metabolites found in these soils. A high level of genotypic diversity within a population suggests that the ability to catabolize a specific compound is associated with natural adaptation and that the horizontal transfer of novel genes between geographically distant but related strains is a common event (Fulthorpe et al. 1998).

Among the nine different catabolic genes examined, seven were detected in nonimpacted areas from the Antarctic and three were detected in the control Brazilian soils. The presence of this genotypic diversity in uncontaminated ecosystems could be related with biogenic and (or) geochemical sources of hydrocarbon compounds in these areas and not of anthropogenic origin. This result is especially intriguing in the Antarctic soils, since the Antarctic ecosystem is still considered as one of the last remaining control areas on the Earth (Bícego et al. 1996; Delille 2000).

Most of the examined genotypes were consistently detected in the samples from both geographic regions but with a higher frequency in the Antarctic soils, indicating one of the following: (*i*) Brazilian soil samples had a low number of copies of the target genes (lower detection limit) or a lower population level having these genotypes, (*ii*) the presence of fewer homologous genes in the Brazilian bacterial communities, or (*iii*) the presence of novel or different catabolic gene systems involved in organic pollutant degradation pathways in this geographical area. Since the high diversity of degradative pathways is known, the Brazilian soils may have distinct catabolic genes, which could contribute to the metabolism of organic compounds in tropical and subtropical areas.

The PCR primers and DNA probes used in this study were more effective in the analysis of the Antarctic soils, which is somewhat surprising, since all of these primer sets and probes were developed from bacterial degradation pathways from temperate regions. The results also indicated that there was a higher degree of catabolic genotypic diversity and distribution among the microbial population in oil-contaminated soils than among the control soils for both geographic areas. Even though the presence of these genotypes in relatively control areas was also high, it suggests a selective enrichment of the hydrocarbon-degrading organisms in the contaminated soils, a characteristic commonly observed in sites previously contaminated with pollutants (Leahy and Colwell 1990; Whyte et al. 1999*b*). Bacterial populations capable of degrading toluene, xylene, naphthalene, and biphenyl and (or) chlorobiphenyls can easily be obtained from healthy soils either directly or by selective enrichment, and they appear to be ubiquitous and probably use this catabolic versa-

tility to adapt relatively easily when an organic pollutant is first introduced into the environment (Williams and Sayers 1994).

In summary, bacterial communities from contaminated environments possess a highly advantageous genotypic flexibility, which is important for their adaptation and evolution while facing continuous changes in these ecosystems. A strategic DNA detection method for degradative genes, such as PCR and hybridization analyses, can be utilized to determine both the biodegradative potential and predominant catabolic pathways in an environment, as was demonstrated here for Rh *alkB1* and *alkB2* and for *todC1* and *bphA1*. The primers and probes used in this study were successful in detecting genotypic diversity and catabolic gene distribution in natural environments. This work revealed that there is an evident dispersion of different catabolic genes involved in petroleum hydrocarbon and chloro-organics' degradation in the two locations examined, Antarctica and Brazil. Additional studies would be required to determine whether and to what extent these genotypes could be induced or activated and involved in the biodegradation of organic contaminants. Studies of catabolic diversity and genetic flexibility will be important elements in our understanding of natural bioremediation processes.

Acknowledgements

This work was supported by a doctoral fellowship from CAPES Foundation (Foundation for the Coordination of Higher Education and Graduate Training) and by the Brazilian Antarctic Program (PROANTAR/Secirm/CNPq), Brazil. The authors are grateful to Diane Labbé and Nathalie Fortin for their technical assistance (BRI/NRCC).

References

Aislabie, J., McLeod, M., and Fraser, R. 1998. Potential for biodegradation of hydrocarbons in soil from the Ross Dependency, Antarctica. Appl. Microbiol. Biotechnol. **49**(2): 210–214.

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

Asturias, J.A., Diaz, E., and Timmis, K.N. 1995. The evolutionary relationship of biphenyl dioxygenase from Gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from Gram-negative bacteria. Gene, **156**: 11–18.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.G., Seidman, J.G., Smith, J.A., and Struhl, K. 1990. Current protocols in molecular biology. Vol.1. Massachusetts General Hospital, Harvard Medical School, Cambridge, Mass.

Bej, A.K., Saul, D., and Aislabie, J. 2000. Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica. Polar Biol. **23**: 100–105.

Berthelet, M., Whyte, L.G., and Greer, C.W. 1996. Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. FEMS Microbiol. Lett. **138**: 17–22.

Bícego, M.C., Weber, R.R., and Ito, R.G. 1996. Aromatic hydrocarbons on surface waters of Admiralty Bay, King George Island, Antarctica. Mar. Pollut. Bull. **32**(7): 549–553.

Button, D.K., Robertson, B.R., McIntosh, D., and Jüttner, F. 1992. Interactions between marine bacteria and dissolved-phase and beached hydrocarbons after the Exxon Valdez oil spill. Appl. Environ. Microbiol. **58**(1): 243–251.

Coates, J.D., Ellis, D.J., Blunt-Harris, E.L., Gaw, C.V., Roden, E.E., and Lovley, D.R. 1998. Recovery of humic-reducing bacteria from a diversity of environments. Appl. Environ. Microbiol. **64**(4): 1504–1509.

Delille, D. 2000. Response of Antarctic soil bacterial assemblages to contamination by diesel fuel and crude oil. Microb. Ecol. **40**: 159–168.

Delille, D., Bassères, A., and Dessommes, A. 1997. Seasonal variation of bacteria in sea ice contaminated by diesel fuel and dispersed crude oil. Microbiol. Ecol. **33**: 97–105.

Fortin, N., Fulthorpe, R.R., Grant Allen, D., and Greer, C.W. 1998. Molecular analysis of bacterial isolates and total community DNA from kraft pulp mill effluent treatment systems. Can. J. Microbiol. **44**: 537–546.

Fulthorpe, R.R., Rhodes, A.N., and Tiedje, J.M. 1998. High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. Appl. Environ. Microbiol. **64**(5): 1620–1627.

Furukawa, K., Arimura, N., and Miyazaki, T. 1987. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *P. pseudoalcaligenes*. J. Bacteriol. **169**: 427–429.

Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G., and van Elsas, J.D. 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J. Microbiol. Methods, **38**: 1–15.

Greer, C.W., Masson, L., Comeau, Y., Brousseau, R., and Samson, R. 1993. Application of molecular biology techniques for isolating and monitoring pollutant-degrading bacteria. Water Pollut. Res. J. Can. **28**(2): 275–287.

Hamann, C., Hegemann, J., and Hildebrandt, A. 1999. Detection of polycyclic aromatic hydrocarbon degradation genes in different soil bacteria by polymerase chain reaction and DNA hybridization. FEMS Microbiol. Lett. **173**: 255–263.

Harwood, C.S., and Parales, R.E. 1996. The β-ketoadipate pathway and the biology of self-identity. Annu. Rev. Microbiol. **50**: 553–590.

Holben, W.E. 1992. Isolation and purification of bacterial DNA from soil. *In* Methods of soil analysis. Soil Science Society of America, Madison, Wis., USA.

Kok, M., Oldenhuis, R., van der Linden, M.P., Raatjes, P., Kingma, J., van Lelyveld, P.H., and Witholt, B. 1989. The *Pseudomonas oleovorans* alkane hydroxylase gene. J. Biol. Chem. **264**: 5435–5441.

Kurkela, S., Lehvasaiho, H., Palva, E.T., and Teeri, T.H. 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene, **73**(2): 355–362.

Laramée, L., Lawrence, J.R., and Greer, C.W. 2000. Molecular analysis and development of 16S rRNA oligonucleotide probes to characterize a diclofop-methyl-degrading biofilm consortium. Can. J. Microbiol. **46**(2): 133–142.

Leahy, J.G., and Colwell, R.R. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54**(3): 305–315.

Lindstrom, J.E., Prince, R.C., Clark, J.C., Grossman, M.J., Yeager, T.R., Braddock, J.F., and Brown, E.J. 1991. Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. Appl. Environ. Microbiol. **57**(9): 2514–2522.

Mac Cormack, W.P., and Fraile, E.R. 1997. Characterization of a hydrocarbon degrading psychrotrophic Antarctic bacterium. Antarct. Sci. **9**(2): 150–155.

Maki, H., Sasaki, T., and Harayama, S. 2001. Photo-oxidation of

biodegraded crude oil and toxicity of the photo-oxidized products. Chemosphere, **44**: 1145–1151.

Margesin, R., Labbé, D., Schinner, F., Greer, C.W., and Whyte, L.G. 2003. Characterization of hydrocarbon-degradative microbial populations in contaminated and pristine alpine soils. Appl. Environ. Microbiol. **69**: 3085–3092.

Master, E.R., and Mohn, W.W. 1998. Psychrotolerant bacteria isolated from Arctic soil that degrade polychlorinated biphenyls at low temperatures. Appl. Environ. Microbiol. **64**(12): 4823–4829.

Mesarch, M.B., Nakatsu, C.H., and Nies, L. 2000. Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. Appl. Environ. Microbiol. **66**(2): 678–683.

Meyer, S., Moser, R., Neef, A., Stahl, U., and Kämpfer, P. 1999. Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology (Reading, UK), **145**: 1731–1741.

Moon, J. 1995. Cloning and sequencing of the catechol-2,3-dioxygenase gene of *Alcaligenes* sp. KF711. J. Biochem. Biophys. Res. Commun. **208**(3): 943–949.

Moura, M. (*Editor*). 2000. Valuation of risks in polluted areas. (In Portugese.) Revista Pesquisa/Fapesp, **51** (March 2000): 40–43.

Muyzer, G., and Ramsing, N.B. 1995. Molecular methods to study the organization of microbial communities. Water Sci. Technol. **32**(8): 1–9.

Nakai, C., Kagamiyama, H., Nazaki, M., Nakazawa, T., Inouye, S., Ebina, Y., and Nakazawa, A. 1983. Complete nucleotide sequence of the metapyrocatechase gene. J. Biol. Chem. **258**(5): 2923–2928.

Nicodem, D.E., Guedes, C.L.B., and Correa, R.J. 1998. Photochemistry of petroleum. I. Systematic study of a brazilian intermediate crude oil. Mar. Chem. **63**: 93–104.

Okuta, A., Ohnishi, K., and Harayama, S. 1998. PCR isolation of catechol 2,3-dioxygenase gene fragments from environmental samples and their assembly into functional genes. Gene, **212**: 221–228.

Pellizari, V.P., Bezborodnikov, S., Quensen, J.F., III, and Tiedje, J.M. 1996. Evaluation of strains isolated by growth on naphthalene and biphenyl for hybridization of genes to dioxygenase probes and polychlorinated biphenyl-degrading ability. Appl. Environ. Microbiol. **62**(6): 2053–2058.

Pietr, J. 1986. The physiological groups of microorganisms in different soils at Admiralty Bay region (King George Island, South Shetland Islands, Antarctica). Polar Res. **7**(4): 395–406.

Pritchard, P.H., Mueller, J.G., Rogers, J.C., Kremer, F.V., and Glaser, J.A. 1992. Oil spill bioremediation: experiences, lessons and results from the Exxon Valdez oil spill in Alaska. Biodegradation, **3**: 315–335.

Ratajczak, A., Geibdörfer, W., and Hillen, W. 1998. Alkane hydroxylase from *Acinetobacter* sp. strain ADP1 is encoded by *alkM* and belongs to a new family of bacterial integral-membrane hydrocarbon hydroxylases. Appl. Environ. Microbiol. **64**: 1175–1179.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Secirm/Proantar/CNPq/Brazil 2000. Brazil in Antarctica: exchange information (1999/2000). Brasilia-DF, Brazil.

Smits, T.H.M., Röthlisberger, M., Witholt, B., and van Beilen, J.B. 1999. Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. Environ. Microbiol. **1**(4): 307–317.

Staley, J.T. 1999. Bacterial biodiversity: a time for place. ASM News, **65**(10): 681–687.

Staley, J.T., and Gosink, J.J. 1999. Poles apart: biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol. **53**: 189–215.

van Beilen, J.B., Wubbolts, M.G., and Witholt, B. 1994. Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation, **5**: 161–174.

van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Röthlisberger, M., and Witholt, B. 2001. Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk*-genes. Microbiology-UK, **147**: 1621–1630.

van Beilen, J.B., Smits, T.H.M., Whyte, L.G., Schorcht, S., Röthlisberger, M., Plaggemeier, T., Engesser, K.-H., and Witholt, B. 2002. Alkane hydroxylase homologues in gram-positive strains. Environ. Microbiol. **4**: 676–682.

van Beilen, J.B., Li, Z., Duetz, W.A., Smits, T.H.M., and Witholt, B. 2003. Diversity of the alkane hydroxylase system in the environment. Oil Gas Sci. Technol. **58**: 427–440.

Wang, Z., and Fingas, M.F. 2003. Development of oil hydrocarbon fingerprinting and identification techniques. Mar. Pollut. Bull. **47**: 423–452.

Warhurst, A.M., and Fewson, C.A. 1994. Biotransformations catalyzed by the genus *Rhodococcus*. Crit. Rev. Biotechnol. **14**: 29–73.

Whyte, L.G., Greer, C.W., and Inniss, W.E. 1996. Assessment of the biodegradation potential of psychrotrophic microorganisms. Can. J. Microbiol. **42**: 99–106.

Whyte, L.G., Bourbonnière, L., and Greer, C.W. 1997. Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. Appl. Environ. Microbiol. **63**(9): 3719–3723.

Whyte, L.G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W.E., and Greer, C.W. 1998. Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl. Environ. Microbiol. **64**(7): 2578–2584.

Whyte, L.G., Bourbonnière, L., Bellerose, C., and Greer, C.W. 1999*a*. Bioremediation assessment of hydrocarbon-contaminated soils from the high Arctic. Biorem. J. **3**(1): 69–79.

Whyte, L.G., Slagman, S.J., Pietrantonio, F., Bourbonnière, L., Koval, S.F., Lawrence, J.R., Inniss, W.E., and Greer, C.W. 1999*b*. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. Appl. Environ. Microbiol. **65**(7): 2961–2968.

Whyte, L.G., Smits, T.H.M., Labbé, D., Witholt, B., Greer, C.W., and van Beilen, J.B. 2002*a*. Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. Appl. Environ. Microbiol. **68**(12): 5933–5942.

Whyte, L.G., Schultz, A., van Beilen, J.B., Luz, A.P., Pellizari, V., Labbé, D., and Greer, C.W. 2002*b*. Prevalence of alkane monooxygenase genes in arctic and antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol. Ecol. **41**: 141–150.

Williams, P.A., and Sayers, J.R. 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation, **5**: 195–217.

Zylstra, G.J., and Gibson, D.T. 1989. Toluene degradation by *Pseudomonas putida* F1. J. Biol. Chem. **264**: 14 940 – 14 946.