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Characterization of bacterial consortia from diesel-contaminated Antarctic soils: Towards the design of tailored formulas for bioaugmentation

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ABSTRACT

In Antarctica, the environmental conditions and the restrictions imposed by the Antarctic Treaty prevent inoculation with foreign bacteria. Therefore, our aim was to investigate native bacterial consortia which might serve to design bacterial formulas suitable for soil bioremediation processes at cold temperatures. Two bacterial consortia, M10 and J13, were isolated from diesel contaminated Antarctic soils. Their ability to use hydrocarbons was evaluated *in vitro* and by the detection of three catabolic genes (*alk*B, *nah*Ac, *xyl*E). Both consortia showed similar 16S rRNA gene profiles, suggesting the presence of the same phylotypes. Total 16S rDNA was cloned from M10 grown on diesel. Sixty clones were screened, grouped by restriction profiles of PCR-amplified inserts and sequenced. T-RFLP (Terminal-Restriction-Fragment-Length-Polymorphism) of clones showed that all phylotypes from the entire consortia were recovered. A culture-dependent approach was used to isolate M10 components able to utilise aliphatic and aromatic hydrocarbons. *Pseudomonas*, *Stenotrophomonas*, *Pedobacter* and *Brevundimonas* genera were detected. The combination of dependent and independent culture methods allowed elucidating the taxonomic composition of these native bacterial consortia. Further work will assess whether combining the isolates obtained as a defined mixed culture can enhance bioremediation of contaminated soils.

1. Introduction

Contamination of soil with hydrocarbons is a topic of concern in Antarctica. The in-out transportation of people and equipment is a difficult, expensive and dangerous task and the refuelling of storage tanks in the stations entails a high risk of spills (Aislabie et al., 2004). Consequently, some terrestrial areas near human settlements or affected by the growing tourist activity have high levels of hydrocarbon contamination. However, bioremediation (which is considered the most environmentally friendly process to reduce organic contaminants) represents a challenge in Antarctica, as this continent is remote, isolated and extremely cold. In addition, since the introduction of alien living organisms is prohibited by the Protocol on Environmental Protection to the Antarctic Treaty Consultative Parties (1991), bioaugmentation approaches in Antarctica entail several constraints. The aforementioned normative prevents inoculation with non-indigenous bacteria, making necessary the isolation and characterisation of native microorganisms for their use in bioremediation strategies.

In bioremediation of polluted soils, the addition of nutrients to balance the C:N:P ratio (biostimulation) and, under certain circumstances, the addition of microorganisms adapted to degrade the pollutant (bioaugmentation) may be useful to enhance biodegradation (Thompson et al., 2005). However, the establishment and growth of the inoculated microorganisms in the soil matrix is a difficult process, as their survival depends on many factors. The stress caused by switching from laboratory to field conditions, the size of the inoculum in relation to the soil biomass, the competition with the natural microbiota and predation may become limiting factors (Bouchez et al., 2000). These disadvantages make bioaugmentation a controversial strategy. It has been suggested that appropriate feasibility studies should be performed and new competent bacterial formula should be tailored for any particular site requiring a bioremediation process (Tyagi et al., 2011).

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The initial search for the bacterial constituents of a formula has to be based on the metabolic potential for biodegradation of the soil contaminant and on the ability of the selected bacteria to thrive under the environmental conditions prevailing in the contaminated site. In this sense, as fuels are complex mixtures of hydrocarbons with different chemical structures, a microbial consortium isolated from the contaminated site (or from other similar site) is expected to be more efficient in removing the contaminants than a single isolate, due to the advantage of metabolic diversity and cooperation (Thomassin-Lacroix et al., 2001). However, consortia directly obtained from environmental samples are difficult to scale-up in a reproducible manner and this may hamper repeated use in field trials (Foght et al., 1998). This situation can be overcome using a bacterial formula comprised of selected native strains preserved as pure cultures.

To obtain efficient bacterial formulas for bioremediation, first it is necessary to select consortia with cooperative metabolism for the biodegradation of the pollutant and then, to elucidate their community structure and to isolate the detected components. After individual characterization of the isolates, the minimal combination of them giving equal or even better degradation of the pollutant than the original consortium should be found (Barreiros et al., 2003). In this way, it may be possible to individually culture the different components of the bacterial formula at large scale. This would provide enough quantity of standardised inocula that could be sent to the stations during summer expeditions and subsequently stored overwinter, to be used in the field immediately after an acute event of contamination.

In the attempt to design bioremediation strategies to face a possible fuel spill in the Argentine Antarctic Stations, we have previously obtained two bacterial consortia. These consortia were isolated from chronically contaminated soils from Carlini (J13) and Marambio (M10) stations. Previous studies using these consortia as a "black box" also ended in controversial results. On the one hand, studies performed in small flasks containing soil acutely contaminated with phenanthrene showed a positive effect associated to the presence of M10 when inoculation was combined with fish meal biostimulation (Ruberto et al., 2006). On the other hand, neither M10 nor J13 enhanced the biodegradation activity of the natural microbiota when inoculated in *on site* land plots containing soil chronically contaminated with diesel fuel (Vázquez et al., 2009). In order to shed light on the reasons of these results and to describe the taxonomic composition of the hydrocarbon-degrading consortia we carried on the present work, whose aims were: (1) to assess the degradation capacity of both consortia, (2) to characterise and contrast their bacterial populations according to 16S rRNA genes denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) profiles observed, (3) to identify the dominant components of these consortia by cloned sequences analysis; and (4) to isolate and characterise those dominant components suitable for a further design of microbial formulas, which may be successful in the biodegradation of diesel fuel. The possibility of combining different isolates according to the needs of each particular case of bioremediation provides a good alternative for an efficient, reproducible and controlled preparation of inocula for bioaugmentation, while meeting the requirement of no introduction of alien organisms in Antarctica.

2. Materials and methods

2.1. Isolation and cultivation of microorganisms

Psychrotolerant bacterial consortia J13 and M10 were isolated from hydrocarbon-contaminated soils from the surroundings of diesel fuel storage tanks at the Argentinean Antarctic Stations of Carlini (Jubany) [62°14′S, 58°40′W, (J13)] and Marambio [64°14′S, 56°37′W, (M10)]. Enrichments were obtained after performing four sequential cultures in saline basal medium (SBM) containing a mixture of the polycyclic aromatic hydrocarbons (PAHs) phenanthrene (5 g l^{-1}), anthracene (0.5 g l^{-1}), fluorene (0.5 g l^{-1}), and dibenzothiophene (0.5 g l^{-1}) as sole carbon and energy sources, as described previously (Ruberto et al., 2006). After the last enrichment culture, biomass was concentrated by centrifugation, resuspended in fresh media with 30% v/v glycerol and kept at −70 °C in 2 ml aliquots as stock.

M10 components were obtained from an 8-day culture of M10 in SBM containing 1% v/v diesel oil, spread onto agar plates prepared with sterile SBM plus 16 g l^{-1} agar, mixed by vigorous shaking with 1% v/v final concentration of sterile diesel fuel enriched with 10% w/v of phenanthrene. Thirty colonies were randomly isolated and purified by re-streaking in the same media and pure isolates were cryopreserved.

2.2. Growth of consortia and isolates on hydrocarbons

The capability of M10 and J13 consortia to grow on different hydrocarbons as sole energy and carbon sources was tested in 100 ml Erlenmeyer flasks with 20 ml SBM containing either naphthalene, phenanthrene, pyrene (0.1% w/v), octane, dodecane, hexane, toluene, diesel fuel, J-P1 fuel, or crude oil (1% v/v). Incubation was performed at 15 °C and 250 rpm for 15 days. Growth of isolates obtained from M10 consortium on the same single hydrocarbons was tested in 2 ml glass tubes inoculated at a density of *c.* 1×10^6 CFU ml^{-1} and incubated at 16 °C for 30 days. In both cases, a culture turbidity with an OD_{600} higher than 0.04 was considered as positive growth.

2.3. Genomic DNA extraction

Biomass of consortia for DNA extraction was obtained from 30 ml of 1-day (T0) and 8-day (Tf) cultures of 300 μl of cryopreserved stocks on SBM with 1% v/v of either diesel fuel (GO) or phenanthrene (PAH) as carbon source and incubated at 15 °C and 250 rpm. For DNA extraction from isolates, biomass was obtained from 5 ml of 3-day cultures in half-strength nutrient broth with 1% v/v diesel-phenanthrene, incubated at the same conditions. Cells were harvested by centrifugation, washed with saline solution (0.9% w/v NaCl) and resuspended in 500 μl of extraction buffer. DNA was extracted and checked according to Nogales et al. (2007) but with different times for lysozyme (20 min) and proteinase K (60 min) incubations.

2.4. Detection of genes involved in hydrocarbon degradation

The presence of genes involved in hydrocarbon degradation was examined in total genomic DNA from consortia using molecular methods. For dot-blot hybridization, total DNA extracts from both consortia at Tf were blotted onto nylon membranes and hybridised with probes for different catabolic genes (*Pseudomonas*-like alpha subunit naphthalene dioxygenase [*nah*Ac], cathecol 2,3-dioxygenase [*nah*H], cathecol 1,2-dioxygenase [*cat*A] and alkane monooxygenase [*alk*B]), according to Vázquez et al. (2009). Probes were obtained with genomic DNA from *Pseudomonas putida* GPo1 for *alkB* and from *Pseudomonas stutzeri* AN10 for the other three genes. Signal intensities in dots obtained with the different gene probes were quantified using the GeneTools analysis program (SynGene), and were expressed as values relative to the signal obtained for a 16S rDNA control probe.

For southern-blot hybridisation, PCR amplification of *alk*B, *nah*H and *nah*Ac genes was performed using as templates DNA extracts obtained from both consortia at Tf. For amplification of *nah*H and *nah*Ac, reactions were performed with two kinds of primers, one more specific set designed for amplification of sequences from *Pseudomonas*, *Rhodococcus* and related bacteria, and a second set of highly-degenerated primers (Wilson et al., 1999; Junca and Pieper, 2003), in order to cover a broader range of different sequences (see Table 1). New primers were designed from conserved regions found in multiple alignments of sequences from *Pseudomonas* and *Rhodococcus* (*alk*B), *Pseudomonas, Sphingomonas* and *Bacillus subtilis* (*nah*H), *Pseudomonas, Burkholderia and Ralstonia* (*nah*Ac forward primer F and reverse primer RP) or *Rhodococcus*-like sequences (*nah*Ac reverse primer RR, used in conjunction with forward primer F in PCR reactions). The RR reverse primer was used to detect *Rhodococcus*-like sequences, as we had previously found this genus in the Antarctic soil from which J13 consortium was obtained and we have already described its relevance for biodegradation (Ruberto et al., 2005).

PCR mixtures (50 μl) contained 1x reaction buffer, 1.5 mmol l^{-1} $MgCl_2$, 100 μmol l^{-1} dNTPs, 0.2 μmol l^{-1} each primer, approx. 20 ng of DNA and 1.5 U of Taq DNA polymerase (GE Healthcare). PCRs were carried out with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at the corresponding annealing temperature (Table 1) and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. PCR products were observed in agarose gels stained with ethidium bromide. Positive reactions were hybridised with probes for the same genes obtained from genomic DNA of reference strains, which were used also as positive controls: *P. putida* GPo1 ATCC 29347 for *alkB* (866 bp), and *P. putida* ATCC 17484 for *nahAc* (795 bp) and *nahH* (925 bp). Southern-blot hybridization with probes for *Pseudomonas*-like *nah*Ac, *nah*H and *alk*B genes was performed according to Sambrook and Russell (2001), using enhanced chemiluminescence direct labelling (ECL direct nucleic acid labelling and detection system; GE Healthcare).

2.5. Community structure of consortia inferred through 16S rDNA electrophoretic profiling

The structure of both, M10 and J13 consortia at T0 and Tf was analysed by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) of amplified 16S rRNA genes (16S rDNA). For DGGE profiling, the total genomic DNA from both consortia at T0 and Tf were subjected to PCR amplification of the V3 region of the 16S rDNA using primers 357F-GC and 518R, and representative DGGE bands were excised from the gel and sequenced (Ferrero et al., 2010). T-RFLP profiling and analysis was performed as described previously (Vázquez et al., 2009). Bacterial 16S rDNAs from both consortia at T0 and Tf were amplified from total genomic DNA by PCR and digested with *Alu*I and *Cfo*I restriction enzymes, and two replicate T-RFLP profiles were obtained from each digested DNA. Data from the two restriction reactions and from the two terminal fragments (T-RFs) of the amplified 16S rDNA (5′- and 3′-end) were combined to obtain a matrix with a single description for each sample. Comparison of 16S rDNA T-RFLP profiles from both consortia in different culture conditions was performed using the software PAST—Paleontological Statistics v.1.29 (Hammer et al., 2001).

2.6. Identification of M10 components by cloning and sequencing

A polyphasic approach was used to assess the taxonomic composition of M10 consortium (see Fig. S1, Supplementary data). Total 16S rDNA from M10 grown on diesel fuel (Tf-GO) was amplified as for T-RFLP analysis, with universal primers 27f and 1492r. Freshly amplified PCR products were cloned into *E. coli* DH5α-T1® with the TOPO TA cloning kit (Invitrogen) following the manufacturers' instructions. Sixty clones were screened for the expected inserts by PCR amplification using vector primers. Products with the expected size were digested with the restriction enzyme *Alu*I and the fragments obtained were separated by electrophoresis in 4% w/v NuSieve agarose gels. Clones with identical restriction pattern were grouped and the inserts in at least one clone from each group were sequenced, as described in Aguiló-Ferretjans et al. (2008). The same representative clones were also subjected to T-RFLP with the enzyme *Alu*I, with the aim of correlating the peaks obtained in the T-RFLP profile of the whole consortium with those of individual clones.

Isolates obtained from M10 consortium were identified on the basis of their phenotypic and molecular traits. Briefly, morphology and Gram stain affinity were assessed following standard procedures (Mac Faddin, 2000), and biochemical features were assayed with the commercial API® NE system (Biomerieux), incubated at 20 °C. For molecular identification, isolates were grouped according to their ribosomal RNA intergenic spacer analysis (RISA) profiles. The intergenic transcribed spacers (ITS) were amplified as indicated above using primers 1387f (5′-GCCTTGTACACWWCCGCCC-3′) and 23Sr (5′-GGGTTBCCCCATTCRG-3′), digested with *Hpa*II restriction enzyme and visualised after separation in 2% agarose gels. At least one representative from each different profile observed was selected for 16S rDNA amplification and sequencing.

Table 1
Oligonucleotides used for PCR amplification and probe generation for southern blotting of genes involved in hydrocarbon degradation.

Name	Sequence (5′-3′)	PCR annealing temperature (°C)	Expected length (bp)	Reference
Alkane monooxygenase *alk*B				
*alk*B-F	TGGCCGGCTACTCCGATGATCGGAA	54	866	This study
*alk*B-R	T TGGTGATCCGAGTGCCGCTGMA			
Cathecol 2,3- dioxygenase *nah*H				
*nah*H-F	AAGGCCCACGACGTGGCBTT	50	326	This study
*nah*H-R	ACGGTCATGAATCGTTCGTT			
C23O—F	AGGTGWCGTSATGAAMAAAGG TYAGGTSAKMACGGTCAKGAA	50	934	Junca and Pieper, 2003
C23O-R				
Alpha subunit naphthalene dioxygenase *nah*Ac				
*nah*Ac-F	GTGATCAAGGCNAACTGGAA	54	344	This study
*nah*Ac-RP	TTCGGGAAAACGGTGCMGTT			
*nah*Ac-RR	TTCGGGAACACGGTGCCATG			
Ac114-F	CTGGCWWTTYCTCACYCAR	50	483	Wilson et al.1999
Ac596-R	CRGGTGYCTTCCAGTTG			

2.7. Sequence analysis

The 16S rDNA sequences from DGGE bands, clones and isolates were edited with the BioEdit software (Hall, 1999) and then compared against the databases with FASTA program (Pearson and Lipman, 1988) and with homologous sequences from type strains using the platforms RDP Release 10 (Cole et al., 2009) and leBIBI (Devulder et al., 2003). To choose the proper reference type strain sequences for alignments, the List of Prokaryotic names with Standing in Nomenclature (LPSN) website was consulted (http://www.bacterio.cict.fr, access March, 2010). The clustering analysis was conducted in MEGA5 (Tamura et al., 2007) and involved 44 nucleotide sequences aligned using the ("INFERence of RNA ALignment") secondary-structure based aligner from the RDP Release 10. All positions with less than 5% site coverage were eliminated. There were a total of 506 positions in the final dataset. The tree was inferred with the Neighbour-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood algorithm. The robustness of the tree was assessed by bootstrap (1000 replicates).

The DNA sequences obtained from clones, isolates and DGGE bands were deposited under GenBank ID: JF724048–JF724064, JF724067–JF724076 and AM183967–AM183972, respectively.

3. Results

3.1. Growth of M10 and J13 consortia on hydrocarbons

M10 and J13 consortia were able to grow in liquid culture using both single aliphatic and aromatic hydrocarbons and complex mixtures as sole carbon sources (Table 2). The genetic potential for the catabolism of hydrocarbons in these consortia was analysed by dot-blot hybridisation. Based on previous evidence of the presence of *Pseudomonas* in both consortia (see below), we used probes for *Pseudomonas*-like key catabolic genes of aliphatic and aromatic hydrocarbon degradation pathways (*alk*B, *nah*Ac, *nah*H, *cat*A), obtaining positive dot-blot hybridisation signals (Table 3). The presence of catabolic genes was also detected by southern-blot hybridisation after PCR amplification with primers specific for *Pseudomonas*-like sequences and also with degenerated primers (Table 1), to increase the possibility of targeting distinct sequences. Positive amplification products with the expected sizes were obtained with all the primers used and then confirmed by southern-blot, with two exceptions. The *nah*H gene in sample J13-Tf-PAH gave. Firstly, neither amplification band nor positive signal after southern-blotting, despite having been detected in total genomic DNA from the same sample by dot-blot hybridisation. On another hand, amplification with *nah*Ac-F and *nah*Ac-RR primers yielded the expected products, but also products of non-expected sizes were amplified. None of them gave a positive hybridisation signal after southern-blot.

Table 2
Growth of M10 and J13 consortia on single and complex mixtures of hydrocarbons as sole carbon sources in liquid culture inoculated at a density of *c.* 1×10^6 CFU ml^{-1}. Culture turbidity (compared to the non-inoculated control) was considered as positive growth, with the maximum turbidity observed corresponding to *c.* 5×10^8 CFU ml^{-1}.

Hydrocarbon	M10	J13
Octane	++	nd
Dodecane	+++	+++
Hexane	+++	+++
Toluene	+++	+++
Naphthalene	+++	+++
Phenanthrene	+++	+++
Pyrene	+	nd
Diesel oil	+++	+++
JP1	+++	+++
Crude oil	++	+

nd: not determined.
+: Culture turbidity relative to the maximum turbidity observed (considered as +++).

Table 3
Detection of catabolic genes in M10 and J13 total genomic DNA from 8-day cultures with diesel fuel (GO) or phenanthrene (PAH) by dot-blot hybridization.

Target gene	M10-GO	M10-PAH	J13-GO	J13-PAH
*nah*Ac	+++	+++	++	+++
*nah*H	+++	++	+	++
*cat*A	++	++	+	++
*alk*B	++	++	+	++

+: Signal intensities in dots, relative to the signal obtained for a 16S rDNA control probe (considered as +++).

3.2. Culture-independent analysis of the composition of M10 and J13 consortia

The composition of M10 and J13 consortia at T0 and Tf growing on diesel fuel and phenanthrene as model aromatic hydrocarbon was first analysed by DGGE profiling of 16S rDNA. DGGE profiles obtained from these consortia differed one from each other, but were highly similar when different culture times and culture conditions were compared for the same consortium. Seven characteristic bands were excised, cloned and sequenced. Four bands obtained from both consortia were affiliated to genus *Pseudomonas*, belonging to *Pseudomonas fluorescens* group, and shared 99–100% identity with the homologue sequences from the type strains of *P. brenneri* (AF268968), *Pseudomonas migulae* (AF074383), *P. panacis* (AY787208), *P. proteolytica* (AJ537603), *P. umsongensis* (AF468450) and *P. marincola* (AB301071). Two sequences corresponding to bands obtained only from M10 affiliated with *Pseudomonas chlororaphis* group, sharing 99–100% identity with the sequence from the type strain of *Pseudomonas fragi* (AF094733). The last sequence affiliated with the genus *Stenotrophomonas*, with 98% identity with *Stenotrophomonas rhizophila* (AJ293463).

In order to achieve a more in-depth and semiquantitative comparison of the composition of the consortia, we compared T-RFLP profiles for 16S rDNA from M10 and J13. Both T-RFLP profiles were very similar, suggesting the presence of common phylotypes. The comparison of all profiles according to the presence/absence and relative abundance of the 5′- and 3′-end T-RFs obtained with enzymes *Alu*I and *Cfo*I is shown in Fig. S2 (Supplementary data). Profiles from samples at the start of cultures (T0) clustered together at similarity values over 80%, except for M10-T0-GO; this sample showed several low-abundance peaks in T-RFLP profiles. Likewise, with the exception of J13-Tf-PAH, profiles from samples taken at the end of cultures (Tf) clustered together (over 70% similarity), and separated from those at T0 (67% similarity). These two groups shared over 60% similarity upon comparing their community structure at start and end times of cultures on diesel fuel or aromatic hydrocarbons. From this comparison, it was clear that both consortia shared a similar composition initially and that they changed their composition over the time of culture but in such a way that both remained very similar at the end. These results were not observed with DGGE profiling, which showed no remarkable changes in composition during incubation. Although both consortia proved to be highly similar in their population structure, the taxonomic diversity of M10 was greater than that of J13, and at the end of cultures all phylotypes revealed by T-RFLP in J13 profiles were also found in M10 profiles (Fig. 1). Therefore, M10-

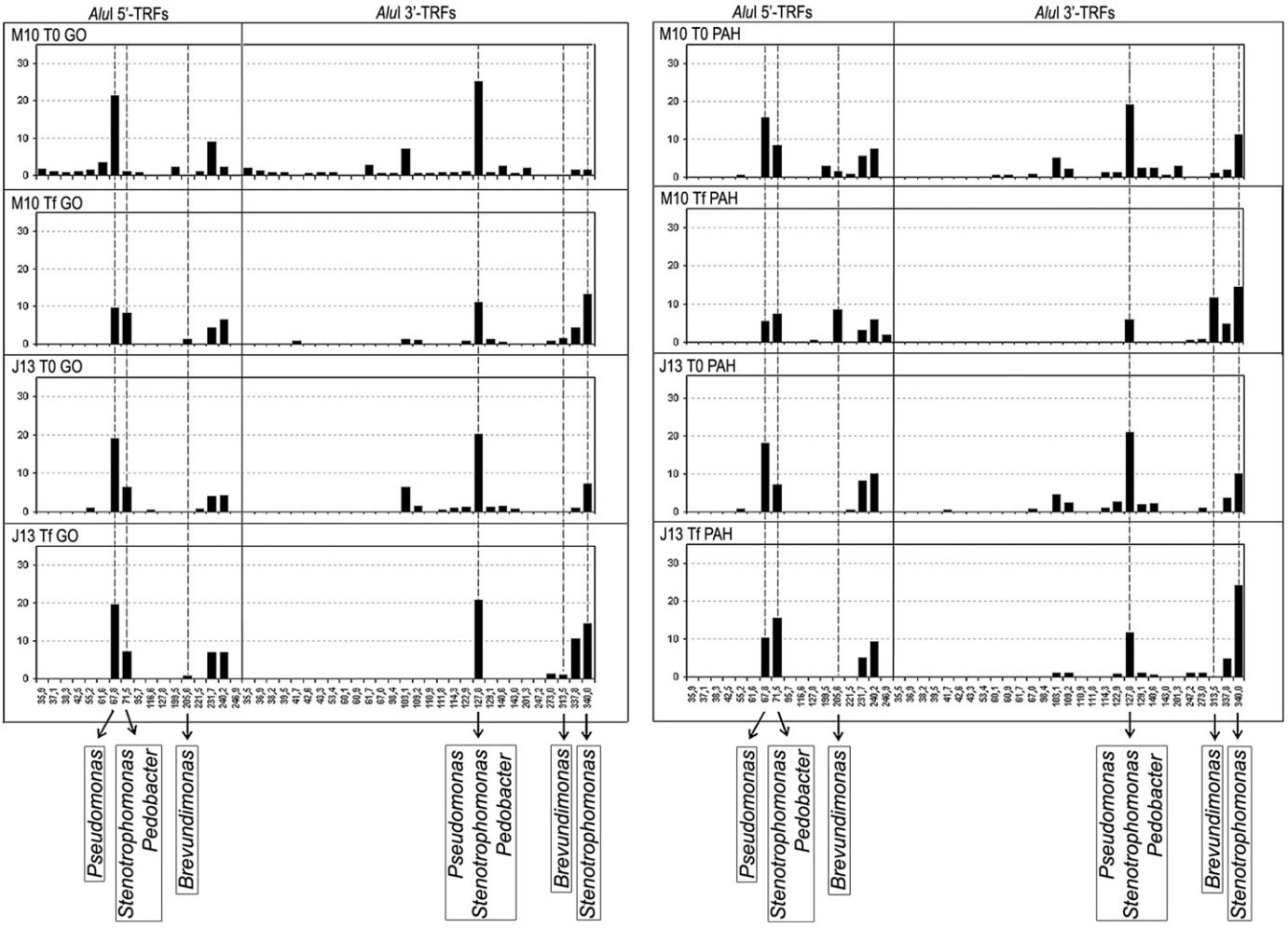

Fig. 1. Identification of the main T-RFs on T-RFLP profiles obtained from M10 and J13 consortia at 1-day (T0) and 8-days (Tf) of culture on diesel fuel (GO) and on a mixture of aromatic hydrocarbons (PAH) as sole carbon and energy sources. The correspondence of peaks with the bacterial genera that originate them was established by comparison of the sizes of these T-RFs and those obtained from the clones recovered from a total 16S rDNA library of an 8-day culture of M10 consortium grown on diesel fuel.

Tf-GO was selected for further characterisation by cloning and sequencing of amplified 16S rDNA and isolation of individual strains in pure culture.

Sixty clones were grouped according to identical restriction profiles after restriction fragment length polymorphism (RFLP) analysis with enzymes *Alu*I and *Cfo*I, and at least one representative of each group was sequenced, summarizing a total of 17 sequences. Seven cloned sequences affiliated with different species of genus *Pseudomonas* (Fig. 2), most of them from the *P. fluorescens* group, in agreement with the results of DGGE band sequencing. Five cloned sequences were related to *S. rhizophila*, also detected in DGGE band sequence. Two sequences were related to *Brevundimonas* spp., and one was affiliated to *Pedobacter*, two genera not detected after DGGE band sequencing. Clones were used for inferring the identity of the main T-RFs observed in profiles from the consortia. For this purpose, the sizes of the T-RFs corresponding to sequenced clones were experimentally determined and then compared with the peaks observed in the profiles from consortia. Almost all the main T-RFs in profiles from M10 consortia were recovered (Fig. 1). As the composition of M10 and J13 proved to be very similar, the most abundant components in both consortia at T0 and Tf, grown either on diesel fuel or on aromatic hydrocarbons, were identified. Upon relating the T-RFLP profiles with clone identification, it was observed that in both consortia (M10 and J13) the populations of *Pseudomonas* decreased their abundance or remained equally abundant from the start to the end times of culture on diesel fuel, while *Stenotrophomonas* and *Brevundimonas* became more abundant, though the last one constituted a minor component. When grown on aromatic hydrocarbons, the behaviour of *Pseudomonas* and *Stenotrophomonas* in M10 was similar to their behaviour on diesel fuel cultures, but the population of *Brevundimonas* in M10 was clearly more abundant. In J13 growing in PAH, *Pseudomonas* became less abundant and *Stenotrophomonas* populations increased considerably, while *Brevundimonas* was not detected. The proportion of each component in M10 was estimated from their abundance in the analysed group of clones and thus, the generic composition of the community in M10-Tf-GO culture was estimated to be: *Pseudomonas* (51%), *Stenotrophomonas* (43%), *Brevundimonas* (4%) and *Pedobacter* (2%).

3.3. Culture-dependent analysis of M10 bacterial composition

Thirty-nine isolates were obtained from M10-Tf-GO culture, as described in methodology section. The isolates were grouped according to identical RISA profiles and ten isolates (at least one from each group) were sequenced. Upon comparison of the obtained sequences against homologue sequences from type strains present in the databases, seven isolates were identified as

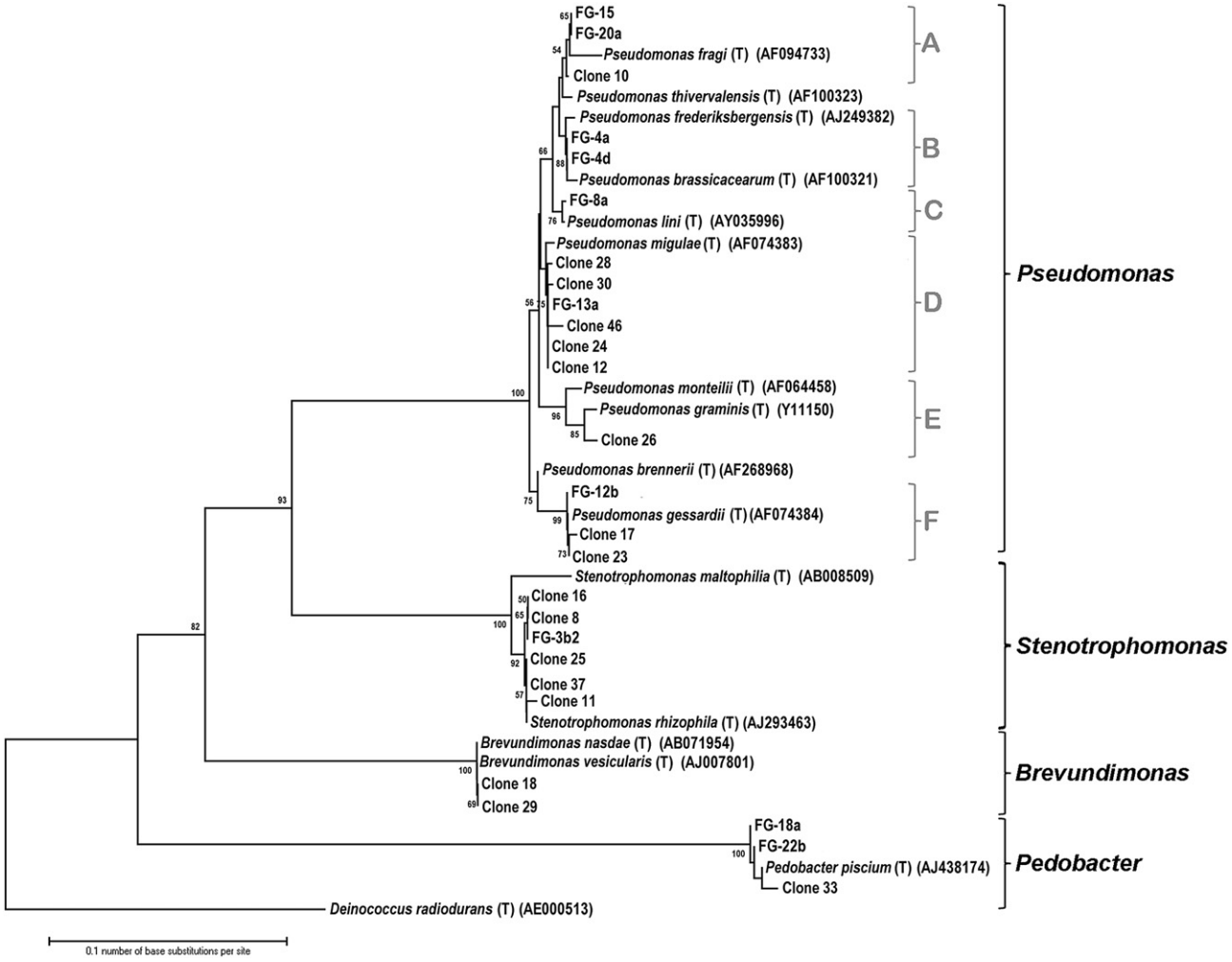

Fig. 2. Rooted phylogenetic tree showing affiliation of isolates and clones to closest related type strain 16S rRNA gene sequences. The tree was inferred using the Neighbour-Joining method and the evolutionary distances were estimated by the Maximum Composite Likelihood algorithm and expressed as the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (only values > 50% are shown). Sequence of the 16S rRNA gene of the type strain of *Deinococcus radiodurans* (AE000513) was used as outgroup.

Pseudomonas spp., one as *Stenotrophomonas* sp. and two as *Pedobacter* spp. (Fig. 2). *Brevundimonas*, found as a low-abundant representative by cloning, was not isolated. Within *Pseudomonas*, six different groups were defined by sequence analysis, named A to F, all belonging to the *P. fluorescens* lineage (Mulet et al., 2010). Groups A (species subgroup of *P. fragi*), D (*P. mandelii* species subgroup), and F (*P. gessardi* species subgroup) contained sequences from both, isolates and clones. Group E (group of *P. lutea*) was found only by cloning, but was not isolated in culture, while groups B and C (group of *P. fluorescens*) were not recovered by cloning. The morphological and physiological characteristics (assessed by standard procedures and the API system) observed for the 39 isolates were in accordance with the taxonomic affiliation assigned by molecular methods.

The ability of the 39 isolates to grow on single aliphatic and aromatic hydrocarbons was tested in liquid culture as was previously described for the whole consortia. The taxonomic affiliation and hydrocarbon degradation profiles of the isolates are shown in Table 4. All isolates were able to utilise at least three of the six hydrocarbons tested, confirming the degradation capacity observed for M10 consortium. Isolates sharing identical RISA pattern showed different capacity to grow on the hydrocarbons tested. In general, all isolates (*Pseudomonas*, *Stenotrophomonas* and *Pedobacter*) grew well on dodecane. In addition, many *Pseudomonas* isolates also grew well on octane and hexane. The ability to use aromatic hydrocarbons corresponded mainly to isolates from the group A of *Pseudomonas*, which grew well on naphthalene, and to several *Pseudomonas* and *Pedobacter* isolates, which showed good growth on toluene.

4. Discussion

In the Antarctic Peninsula, soil indigenous microbiota is composed of a variety of microorganisms, adapted to survive under fluctuating temperatures. During summer, soil surface temperatures can reach up to 20 °C in sunny days (Ruberto et al., 2010), while at night or in cold days temperature drops and soil can even freeze. Such fluctuations in soil surface temperature select psychrotolerant bacteria rather than true psychrophiles, and these communities are able to grow and, in the case of hydrocarbon degraders, to metabolise hydrocarbons at the natural thermal range. These populations are the more important ones for bioremediation processes because they can stay active all the time as far as the soil is not frozen. On the other hand, if true

Table 4
Taxonomic affiliation and growth in liquid culture with single hydrocarbons as sole carbon source (after 8-day culture on diesel fuel) of the isolates obtained from M10 consortium. Isolates whose 16S rDNA was sequenced are typed in bold. Culture turbidity (compared to the non-inoculated control) was considered as positive growth, with the maximum turbidity observed corresponding to *c.* 5×10^8 CFU ml^{-1}.

Identification at genus level	Closest relative, type strain (%id)	Isolate	Hydrocarbon					
			P	N	T	O	D	H
Pseudomonas sp. (A)	*Pseudomonas fragi* T (AF094733) (99.0%)	**FG-15**	+	−	+	+	++	−
		FG-1	+	+	+	+	++	+
		FG-3b1	+	+	+	++	++	++
		FG-11	+	+	+	+	++	+
		FG-14a	+	−	−	−	++	++
		FG-16	+	+	+	+	++	+
		FG-17	+	−	+	+	++	−
		FG-20b	+	+	+	++	++	+
		FG-24b	+	+	++	+	++	++
		FG-25	+	−	+	+	++	+
		FG-20a	+	−	+	+	++	+
Pseudomonas sp. (B)	*Pseudomonas frederiksbergensis* T (AJ249382) (99.7%)	**FG-4a**	+	+	++	++	++	+
		FG-4d	+	+	+	++	++	+
Pseudomonas sp. (C)	*Pseudomonas lini* T (AY035996) (99.4%)	**FG-8a**	+	+	+	+	++	+
Pseudomonas sp. (D)	*Pseudomonas migulae* T (AF074383) (99.4%)	**FG-13a**	−	+	−	++	++	++
		FG-2	+	+	+	+	++	+
		FG-7	−	+	−	−	++	−
		FG-12a	+	+	+	+	++	++
		FG-13b	−	−	++	−	++	++
		FG-22a	+	+	+	+	++	+
		FG-24a	+	+	+	++	++	++
Pseudomonas sp. (F)	*Pseudomonas gessardii* T (AF074384) (99.2%)	**FG-12b**	+	−	+	++	++	++
		FG-4B	+	++	+	+	++	++
		FG-6	−	++	+	+	++	+
		FG-8B	−	+	+	+	++	+
		FG-10a	+	+	+	+	++	+
		FG-10b	+	++	+	+	++	++
		FG-10c	−	+	+	+	++	−
		FG-14b	+	−	−	++	++	++
		FG-21	+	++	+	+	+	+
Stenotrophomonas sp.	*Stenotrophomonas rhizophila* T (AJ293463) (99.6%)	**FG-3b2**	+	+	+	+	++	+
		FG-3a	+	+	+	+	++	+
		FG-4c	+	+	+	+	++	+
		FG-5	−	−	+	+	++	++
		FG-9	+	+	+	+	++	+
Pedobacter sp.	*Pedobacter piscium* T (AJ438174) (99.8%)	**FG-22b**	+	+	+	+	++	+
		FG-23	+	+	++	+	++	++
		FG-18a	+	+	++	+	++	+
		FG-18b	+	−	+	+	++	+

P, pyrene; N, naphthalene; T, toluene; O, octane; D, dodecane; H, hexane.

psychrophiles were used for bioaugmentation, they could stop degrading hydrocarbons and even die when soil temperature and UV radiation levels exceed their survival limits. Therefore, even when both consortia were isolated from Antarctica (where the average temperature is around the freezing point), laboratory cultures for enrichments and isolation were carried out at a constant temperature of 15 °C. Under this condition, the selection of psychrotolerant bacteria is favoured and growth is faster than at lower temperatures, without changes in the characteristics of the microorganisms.

The community structures of M10 and J13 grown either on diesel fuel or on aromatic hydrocarbons proved to be similar by more than 70%, even when they were isolated from different geographic locations. After a 4-step enrichment process performed for isolation, selected populations in J13 were stable irrespective of the type of hydrocarbons supplied, as the T-RFLP profiles from 8-day cultures on diesel fuel or PAHs were very similar. Also, the populations present in M10 after 8 days of culture were closely related in type and number to those found in J13 under the same culture condition. This stability in the structure of a selected consortium was also observed by Song et al. (2002) working with denitrifying consortia, and by Lafortune et al. (2009) after enrichment cultures on pyrene. Nevertheless, it is possible that the uniform selection pressure applied in our experiment to both consortia led to the recovery of similar type of bacteria, with the proper metabolic profile to outgrow other populations present in the soil. This may be an explanation for the almost identical T-RFLP profiles, which can be indicative of the same composition, at least at genus level.

M10 consortium comprised several populations belonging to at least 4 bacterial genera. Of them, *Pseudomonas* was the most frequent, followed by *Stenotrophomonas* and two low-abundant genera, *Pedobacter* and *Brevundimonas*. Other studies reported similar (Deppe et al., 2005) or even larger (Kanaly et al., 2000; Viñas et al., 2005; Lafortune et al., 2009) bacterial diversity in crude oil and PAHs-degrading consortia. On considering culture-independent approaches, it should be noted that DGGE only detected the two main components of these consortia, as the sequences obtained from the bands corresponded to 16S rDNA from populations belonging to *Pseudomonas* and *Stenotrophomonas* genera. In contrast, T-RFLP analysis detected several populations that were then confirmed by cloning and sequencing, allowing the identification of the four representative genera present in M10 at the end of diesel cultures. As the composition of both consortia at the end of cultures on diesel fuel and on PAH was, as mentioned above, very similar, the sequence-aided T-RFLP approach also

helped in the description of the generic composition of consortium J13. With the culture-dependent approach, three of the four genera found to be present in M10 were recovered, as none *Brevundimonas* was cultured. Regarding the performance of fingerprinting methods to estimate the structure of a bacterial community, we conclude that their success depends not only on the inherent advantages and disadvantages of each method but also on the kind of community that is being studied.

Due to the frequent exposure to hydrocarbons, in the vicinities of Antarctic stations the native microbiota of the affected areas is naturally adapted to degrade diesel fuel components (Thomassin-Lacroix et al., 2002). Nevertheless, when previously pristine areas are impacted with a contaminant, it may be useful to help degradation by the addition of specialised bacteria that can degrade the pollutants or still improve the degradation capacity of the indigenous community by horizontal transfer of mobile elements and plasmids carrying catabolic pathways (Sarand et al., 2000). On these bases, the isolation of consortia from those adapted bacterial communities and its use in bioaugmentation can be considered a suitable practice to treat Antarctic soils and to enhance the degradation ability of the impacted native microbiota, shortly after a pollution acute event takes place. In this sense, the broad spectrum of hydrocarbons that supported growth of M10 and J13 consortia in liquid culture confirmed them as a good source of bacteria for using in bioaugmentation, to help the recovery of acutely contaminated soils. Accordingly, the isolates obtained from M10 showed the ability to grow on aliphatic, monoaromatic and polyaromatic hydrocarbons. When the degradation capability was evaluated at the genetic level, the positive detection of *alk*B *Pseudomonas*-like genes confirmed the potential of M10 and J13 consortia to degrade aliphatic hydrocarbons. The presence of *nah*H and *nah*Ac *Pseudomonas*-like genes was also detected in both consortia, indicating their potential for degradation of the main aromatic hydrocarbons present in the complex mixtures which are more frequently contaminating Antarctic soils.

Both consortia were isolated from soils that had a long history of contamination with diesel fuel, which is mostly composed of aliphatic hydrocarbons, with a low proportion of aromatics (Rayner et al., 2007). The diesel blends used in Antarctica are mostly composed of C9-C14 aliphatic hydrocarbons and thus, due to the sandy nature of soils in the Antarctic Peninsula, most ingredients evaporate, percolate, suffer abiotic degradation or are easily degraded by the adapted microbiota in soils with some history of exposure to hydrocarbons. Nevertheless, many aromatic compounds remain in small amounts (Curtosi et al., 2007). These compounds are recalcitrant, particularly in anaerobic conditions, which are very common in the soils of the Antarctic Peninsula that are exposed to heavy seasonal rains and snowmelt. The likelihood of recovering populations able to grow on aromatic hydrocarbons might be low if the successive enrichment cultures are performed on diesel fuel. In contrast, on using PAHs as sole carbon sources in the enrichment cultures (as it was done for isolation of M10 and J13 in this work), even the least abundant populations able to degrade those compounds can be selected and recovered. Since aliphatic hydrocarbons are the dominant sources of carbon and energy in contaminated soils, most PAH-degrading populations in the microbiota of those soils have the metabolic pathways to degrade aliphatics as well, as shown by the types of hydrocarbons that M10 isolates were able to use. The strategy of obtaining a consortium from a soil polluted with a complex mixture of contaminants by using the compounds present in lower proportion as carbon sources in the enrichment media, led to the recovery of those populations able to degrade a broad spectrum of hydrocarbons, including the more recalcitrant polyaromatics.

We isolated 39 strains from M10 consortium, which have in average a good ability to degrade the main groups of hydrocarbons present in that complex mixture. It seems that by selecting the isolates with the broader hydrocarbon degradation profiles or better degradation efficiencies for a particular bioremediation goal, and by combining them in the right proportions to preserve the generic composition of the original consortia, it could be possible to design a defined bacterial formula with similar or even better hydrocarbon-degradation performance than M10. A similar approach was reported for molinate degradation (Barreiros et al., 2003) and for PAHs degradation in mangrove ecosystems (Huijie et al., 2011), with successful results. In addition, to enhance the remediation of the aliphatic compounds, the use of *Rhodococcus* strains previously isolated from Antarctic contaminated soils (Ruberto et al., 2005) in conjunction with the selected isolates from M10 consortium should also be considered.

5. Conclusion

The information obtained about the composition and degradation capability of two bacterial Antarctic consortia and the isolation in pure culture of psychrotolerant bacteria able to utilise a broad spectrum of hydrocarbons will be of help in the further design of tailored bacterial formulas for bioaugmentation, which could eventually be immobilised on a proper carrier to extend their lifetime in the soil under treatment. It is worth mentioning that this is a fundamental step in the ongoing optimization of *in situ* and *on site* bioremediation processes of Antarctic soils suffering acute contamination with hydrocarbons near Antarctic Stations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2012.11.002.

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