Biodegradation (2009) 20:593–601
DOI 10.1007/s10532-009-9247-1

ORIGINAL PAPER

Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil

Salvador Lladó · Nuria Jiménez · Marc Viñas · Anna Maria Solanas

Received: 15 September 2008 / Accepted: 6 January 2009 / Published online: 21 January 2009

Abstract A previous bioremediation survey on a creosote-contaminated soil showed that aeration and optimal humidity promoted depletion of three-ringed polycyclic aromatic hydrocarbons (PAHs), but residual concentrations of four-ringed benzo(a)anthracene (B(a)A) and chrysene (Chry) remained. In order to explain the lack of further degradation of heavier PAHs such as four-ringed PAHs and to analyze the microbial population responsible for PAH biodegradation, a chemical and microbial molecular approach was used. Using a slurry incubation strategy, soil in liquid mineral medium with and without additional B(a)A and Chry was found to contain a powerful PAH-degrading microbial community that eliminated 89% and 53% of the added B(a)A and Chry, respectively. It is hypothesized that the lack of PAH bioavailability hampered their further biodegradation in the unspiked soil. According to the results of the culture-dependent and independent techniques *Mycobacterium parmense*, *Pseudomonas mexicana*, and Sphingobacterials group could control B(a)A and Chry degradation in combination with several microorganisms with secondary metabolic activity.

S. Lladó · N. Jiménez · A. M. Solanas (✉)
Department of Microbiology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain
e-mail: asolanas@ub.edu

M. Viñas
GIRO Technological Centre, Rambla Pompeu Fabra, 1, E-08100 Mollet del Valles, Spain

Keywords Polycyclic aromatic hydrocarbons · Biodegradation · Bioavailability · Bioremediation · Pyrene · Chrysene · 16SrRNA · DGGE

Introduction

A major concern in the bioremediation of PAH-contaminated soils is the biodegradation of high-molecular-weight PAHs (HMW-PAHs), which have recalcitrant properties and mutagenic or carcinogenic effects (Farmer et al. 2003). While low-molecular-weight PAHs, composed of two and three aromatic rings, can be biodegraded under favorable conditions (Wilson and Jones 1993), PAHs with four or more rings offer greater resistance to microbial degradation (Alexander 1999; Sabaté et al. 2006) and may persist at residual concentrations that frequently exceed regulatory limits.

In a previous study we described the bioremediation of a real creosote-contaminated soil. The treatment lasted 200 days and was based on a range of biostimulation and bioaugmentation strategies (Viñas et al. 2005a, b). Given that the addition of nutrients seems to be a universal practice, with one exception all treatments were based on the addition of nitrogen and phosphorus at a C:N:P ratio of 300:10:1 Other biostimulation agents studied were a rhamnolipid produced by strain AT10 of *Pseudomonas aeruginosa*, used as a biosurfactant (Abalos et al.

2004), ferric octoate, used as an iron source, and glucose, an easily assimilable substrate. In addition, the inoculation of a PAH-degrading consortium was assayed as a bioaugmentation strategy. The results showed that two- and three-ringed PAHs were fully removed in all treatments. The one exception was anthracene, which was 84% depleted. Fluoranthene and pyrene were 92% and 87% depleted, respectively. The extent of degradation (3–4 ringed PAH except benzo(a)anthracene (B(a)A) and crysene (Chry)) was the same in all treatments. Unexpectedly, B(a)A and Chry, which also have four benzene rings, were more degraded (72% and 62%, respectively) in the aerated treatment without nutrient amendments than in the fertilized treatment (43% and 39%, respectively). In addition, nutrient addition caused an important shift in the bacterial community. We hypothesised that nutrient addition enriched a fast-growing microbial population that degraded more easily biodegradable PAHs, while in the absence of nutrients, a slow-growing microbial population that was specialized in the degradation of more recalcitrant PAHs prevailed.

Here we describe an experimental strategy based on the incubation of the bioremediated soil in slurry with liquid mineral medium The soil, which can be considered as an aged soil in which the more easily biodegradable PAHs are depleted, was incubated with and without additional B(a)A and Chry. Our aim was to analyse the microbial population involved in the degradation of high-molecular-weight PAHs (HMW-PAHs) in the creosote-contaminated soil, and to attempt to explain the lack of further degradation of these two PAHs in soil.

Materials and methods

Chemicals

Benzo(a)anthracene, chrysene, and o-terphenyl were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Solvents were purchased from Scharlab S.L., Barcelona. Solvents and other chemicals and reagents were of the highest purity available. 16 EPA PAH standard solution (10 ng μl^{-1} in cyclohexane) was purchased from Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). PAH standards for quantification in GC-FID.

Soil material

We combined a sample of creosote-contaminated soil that had been previously bioremediated with aeration and an optimal humidity. The concentrations of the most predominant PAHs in the contaminated soil at the beginning and at the end of the bioremediation process are shown in Table 1.

PAH spiked slurries

In the present study two sets of slurries (spiked and unspiked) were incubated in conditions of horizontal agitation at 25°C in 250 ml flasks covered with sterile aluminium foil, and protected from light, for 30 days. The slurries contained 100 mg of aged soil (Table 1) resuspended in 50 ml of 10 times-diluted mineral medium BMTM (Hareland et al. 1975). The diluted BMTM contained (per liter): $K_2HPO_4 \cdot 3H_2O$, 0.425 g; $NaH_2PO_4 \cdot H_2O$, 0.10 g; NH_4Cl, 0.20 g; $MgSO_4 \cdot 7H_2O$, 0.020 g; $FeSO_4 \cdot 7H_2O$, 0.0012 g; $MnSO_4 \cdot H_2O$, 0.0003 g; $ZnSO_4 \cdot 7H_2O$, 0.0003 g; $CoCl_2 \cdot 6H_2O$, 0.0001 g; nitrilotriacetic acid, 0.012 g. The medium was sterilized by autoclaving at 121°C for 20 min.

The spiked slurries contained 2 mg of B(a)A and 1 mg of Chry. To avoid toxicity, B(a)A and Chry

Table 1 PAH concentration of soil submitted to a bioremediation

PAH compound	Initial concentration ($\mu g \cdot g^{-1}$ of soil)	Final concentration ($\mu g \cdot g^{-1}$ of soil)
Phenanthrene	465.5	15.8
Anthracene	114.3	13.6
3-Methyl-phenanthrene	71.5	n.d.[a]
2-Metyhylphenanthrene	77.7	n.d.
Methylanthracene	25.1	n.d.
9-Methyl-pehanthrene or 4-Cyclopentaphenanthrene	131.1	4.4
1-Methylphenanthrene	40.0	n.d.
Fluoranthene	693.1	54.7
Pyrene	386.9	52.8
Benzo(a)anthracene	108.3	32.1
Chrysene	144.4	58.1
Total	2257.9	231.5

[a] Not detected

were firstly added as a dichloromethane suspension (1 ml) to sterilized empty Erlenmeyer flasks. Dichloromethane was allowed to evaporate under sterile conditions into a laminar-flow cabinet. After that, 50 ml of sterile mineral medium was amended and an additional ultrasonic-bath step of spiked flasks was applied to detach PAH crystals from glass. Finally, 100 mg of aged soil was amended to all treatments. Each treatment was carried out in triplicate for chemical and microbial analysis. Abiotic controls with the sterilized soil were incubated in the same conditions.

PAH analysis

The samples were liquid–liquid extracted with 5 × 10 ml of dichloromethane. Orthoterphenyl dissolved in acetone was added to each slurry, as a surrogate internal standard. The total organic extracts (TOE) obtained were dried over Na_2SO_4 and concentrated in a rotary evaporator to dryness To obtain the total petroleum hydrocarbon (TPH) fraction, TOE was resuspended in dichloromethane and cleaned up by column chromatography using the EPA 3611 method (US Environmental Agency). Both TPH and PAH concentrations were analyzed by gas chromatography with flame-ionization detection (GC-FID) using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) fitted with a DB-5 (30 m × 25 mm i.d. × 0.25 μm film) capillary column (J&W Scientific Products Gmbh, Köln, Germany). The column temperature was held at 50°C for 1 min, ramped to 320°C at 7°C min^{-1} and held for 10 min.

Monitoring of heterotrophic and hydrocarbon-degrading microbial populations

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtiter plates, with eight replicate wells per dilution (Wrenn and Venosa 1996). Total heterotrophs were counted in tryptone soy broth and aromatic hydrocarbon-degraders were counted in mineral medium (Wrenn and Venosa 1996) containing a mixture of phenanthrene (0.5 $g \cdot l^{-1}$), fluorene, anthracene, and dibenzothiophene (each at a final concentration of 0.05 $g \cdot l^{-1}$). Aged soil was used as the starting point (day 0). MPN Plates were incubated at room temperature (25°C ± 2°C) for 30 days. Positive wells were detected by turbidity (heterotrophs) and the presence of coloration (brownish/yellow) for PAH degraders.

DNA extraction

Samples for DNA extraction were collected from slurry cultures and the highest positive dilutions from microtiter plates, placed in sterile Eppendorff tubes and stored at −20°C prior to analysis. To ascertain the repeatability of the DNA extraction process and PCR protocols, a set of replicates was analyzed by denaturing gradient gel electrophoresis (DGGE). This showed a high degree of repeatability of the sampling and molecular protocols (DNA extraction). Thus, a 20 ml sample of each slurry culture was centrifuged at 14,000*g* for 10 min and the pellets were extracted by a bead beating protocol using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA, USA), following the manufacturer's instructions. A further purification step with Clean DNA Wizard kit (Promega, WI, USA) was necessary to avoid PCR inhibition. To obtain DNA from microtiter plates, a composite sample of 1.6 ml containing 200 μl of each replicate ($n = 8$) belonging to the last dilution with eight positives was centrifuged and treated as described above.

Polymerase chain reaction

The V3–V5 hypervariable regions of the 16S rRNA gene were amplified using primers F341-GC and R907 (Yu and Morrison 2004). The primer F341-GC included a GC clamp at the 5′ end (5′-CGCCCGCCGC GC CCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′). All PCR reactions were performed on a Mastercycler personal thermocycler (Eppendorff, Hamburg, Germany). Fifty microliters of PCR mixture contained 2.5 U Takara Ex Taq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 100 ng of template DNA quantified by means of Low DNA Mass Ladder (Gibco BRL, Rockville, MD). After 9 min of initial denaturation at 95°C, a touchdown thermal profile protocol was performed and the annealing temperature was decreased by 1°C per cycle from 65 to 55°C, at which temperature 20 additional cycles were carried out.

Amplification was carried out with 1 min of denaturation at 94°C, 1 min of primer annealing and 1.5 min of primer extension at 72°C, followed by 10 min of final primer extension.

Denaturing gradient gel electrophoresis

Approximately 400 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel, 0.75 mm thick (to obtain better resolution), with denaturing gradients ranging from 50% to 70% (100% denaturant contains 7 M urea and 40% formamide). Low DNA Mass Ladder (Gibco BRL, Rockville, MD, USA) was used for quantification. DGGE was performed in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a DGGE-2001 System (CBS Scientific Company, Del Mar, CA, USA) at 100 V and 60°C for 16 h.

The gels were stained for 45 min in 1× TAE buffer containing SybrGold (Molecular probes, Inc., Eugene, OR, USA), then scanned using a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, CA, USA) in DNA stain gel mode for SybrGold at medium sample intensity. Images of DGGE gels were digitalized and DGGE bands were processed using Quantity-one version 4.1 image analysis software (Bio-Rad Laboratories) and corrected manually.

Sequencing and phylogenetic analysis of DGGE bands

Predominant DGGE bands were excised with a sterile razor blade, under blue light using a Visi-Blue Converter Plate (UVP, Upland, CA, USA), resuspended in 50 µl sterilized MilliQ water and stored at 4°C overnight. An aliquot of supernatant (2 µl) was used to reamplify the DGGE bands with primers F341, without the GC clamp, and R907, under the same conditions. The reamplified bands were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) was used for sequencing cleaned PCR amplicons.

Reamplified bands that were impossible to sequence with this method were cloned into the pGEM-T Easy vector (Promega, WI, USA), and transformed into competent *E. coli* DH5α. The recombinant clones were amplified with primers SP6 and T7, cleaned up and sequenced using primers R907 and F341, as explained above.

Sequences were edited and assembled using version 4.8.7 of the BioEdit software (Hall 1999), inspected for the presence of ambiguous base assignments and subjected to the Check Chimera program of the Ribosomal Database Project (Maidak et al. 2000). The sequences were then submitted to BLAST and RDP search using alignment tool comparison software.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the Statgraphics Plus package (version 5.1; Statistical Graphics Corp., Manguistics Inc., United States). Duncan's multiple-range test of means, with a significance level of 0.05, was applied to the results to determine their statistical significance.

Nucleotide accession numbers

The 35 nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers EU512950 to EU512984.

Results and discussion

Degradation of B(a)anthracene and chrysene in the soil slurries

After 30 days of slurry incubation, the microbial population present in the bioremediated soil was able to degrade 89% and 53% of the added benzo(a)anthracene and chrysene (1639 and 947 µg/flask, respectively), but did not affect the residual concentration of these two PAHs in the slurry of the bioremediated soil (7.7 and 9.1 µg/flask, respectively, Fig. 1). This finding indicates that the aged soil contained a potent B(a)A and Chry-degrading community. The low concentrations of BaA and Chry in the slurries (0.064 mg/l and 0.116 mg/l, respectively), which were close to detection limit (0.1 mg/l), hindered peak integration. Indeed, after 30 days' incubation the concentrations were even higher than starting values but at the same low range below 1 mg/ml (0.55 ± 0.11 for BaA and 0.46 ± 0.001 for Chry).

As the incubation was carried out with agitation, the lack of degradation of B(a)A and Chry in the

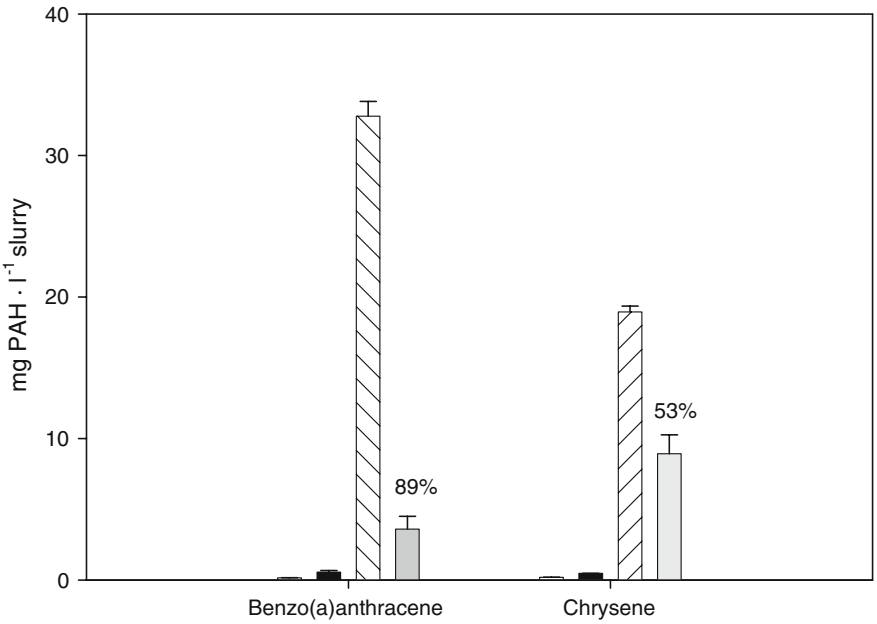

Fig. 1 Benzo(a)anthracene and chrysene concentration at 0 and 30 days, in creosote-contaminated soil with and without supplementation of these two PAHs. White bars represent aged soil; Black bars represent unspiked aged soil after 30 days; Dashed bars represent spiked aged soil at starting point. Gray bars represent spiked aged soil after 30 days of incubation. Percentages show the biodegradation of BaA and Chry after 30 days

unspiked microcosms reflects a high level of sequestration, with the subsequent lack of bioavailability to the microbial population. The residual concentrations of B(a)A and Chry in the soil after application of biostimulation were 32.1 and 58.1 μg/g soil, respectively (Table 1), both above the majority of standard legal limits. Since toxicity and bioavailability correlate strongly, (Alexander 1995), sequestration of PAHs should be considered when establishing of the concentrations below which a soil is to be deemed non-decontaminated.

Monitoring of heterotrophic and hydrocarbon-degrading microbial populations

Low molecular-weight PAHs, namely phenanthrene, fluorene, anthracene, and dibenzothiophene, which are normally used to enumerate the PAH-degraders (Wrenn and Venosa 1996), were replaced by B(a)A and Chry. Nevertheless, no growth was observed, possibly due to the excessively low solubility of these substrates or to the absence of other PAHs, which may be required for cometabolism. We thus decided to use the traditional PAH mixture. The hetrotrophic population and PAH degraders increased up to 3 and 2 magnitude orders, respectively, after 30 days of incubation, reaching populations of 10^5 and 10^4 MPN/ml, respectively. Surprisingly, no significant differences were found between heterotrophic and PAH degraders in either set of slurries ($P > 0.05$) (Table 2). While in the spiked slurries a total of 2586 μg/flask of B(a)A and Chry was depleted, in the non-spiked slurries (from which the residual B(a)A and Chry were not removed) a maximum of 23.1 μg/flask of a mixture of PAHs (Table 1) was consumed. This result has two possible explanations.

Table 2 MPN of heterotrophic and PAH-degrading population at 0 day and at 30 days of slurry incubation with and without supplementation of B(a)anthracene and chrysene

	Heterotrophs (MPN/ml)	PAH degraders (MPN/ml)
Soil 0 day	$5.89 \pm 3.12 \cdot 10^3$ (A)[a]	$7.78 \pm 4.41 \cdot 10^2$ (A)
Soil 30 days	$4.74 \pm 1.50 \cdot 10^5$ (B)	$3.39 \pm 0.71 \cdot 10^4$ (B)
Soil + BaA + Chry	$9.02 \pm 2.12 \cdot 10^5$ (B)	$4.31 \pm 4.00 \cdot 10^4$ (B)

Data are presented as the mean value ± SD (n = 3)

[a] Different letters in brackets in the same column (hetrotrophs or PAH degraders) indicate significant differences between treatments ($P < 0.05$)

Firstly, the depletion of B(a)A and Chry in the spiked slurries may have been due to co-metabolic oxidation rather than the use of these compounds as growth substrates. Alternatively, the possible obligate B(a)A and Chry-degraders present in the spiked slurries may not have been detected by the medium used.

DGGE analysis

To analyze the microbial population initially present in the soil and its response to the presence of high amounts of B(a)A and Chry, two DGGE analyses were carried out. In one we measured the total DNA in the slurries with and without the spiked PAHs. In the other we measured DNA from the more diluted wells of the microtiters used to enumerate the PAH degraders in both types of slurry.

Total DNA

At the end of the experiment, four additional bands (B2, B5, B6, B7) appeared in the total DNA profile of the spiked slurry in comparison to the unspiked one (lane 2 and 3, Fig. 2). However, after purification, band 5 turned out to be six different co-eluting sequences (B5a, B5b, B5c, B5d, B5e, B5f) and band B8 contained two sequences (B8a and B8b). No signal was detected in the lane corresponding to the aged soil (lane 1, Fig. 2).

Band B2 corresponded to *Sphingobacterials*. Bands in B5 corresponded mainly to Sphingomonaceae (Table 3), except B5d, which corresponded to an uncultured Burkolderiaceae and was coincident with Band B17. Band B6 was a chimera and Band B7 corresponded to *Azohydromonas australica* (Group Burkholderials), a nitrogen-fixing bacterium (previously *Alcaligenes latus*) (Xie and Yokota 2005). Given that the soil was not fertilized, this nitrogen fixing bacterium would have a role in the supply of nitrogen. Band B8a presented high similarity (99%) to *Methylibium petroleiphilum,* which has been described as a methyl *tert*-butyl ether-degrading methylotroph bacterium that can also use several monoaromatic hydrocarbons.

Some bands were shared by the two profiles (B9 = B21, B10 = B22, B11 = B23, B12 = B24), and these belonged to *Skermanella* sp. These strictly aerobic bacteria are isolated from airborne particulate matter enriched in PAHs (Weon et al. 2007), but their capacity to degrade hydrocarbons has not been reported.

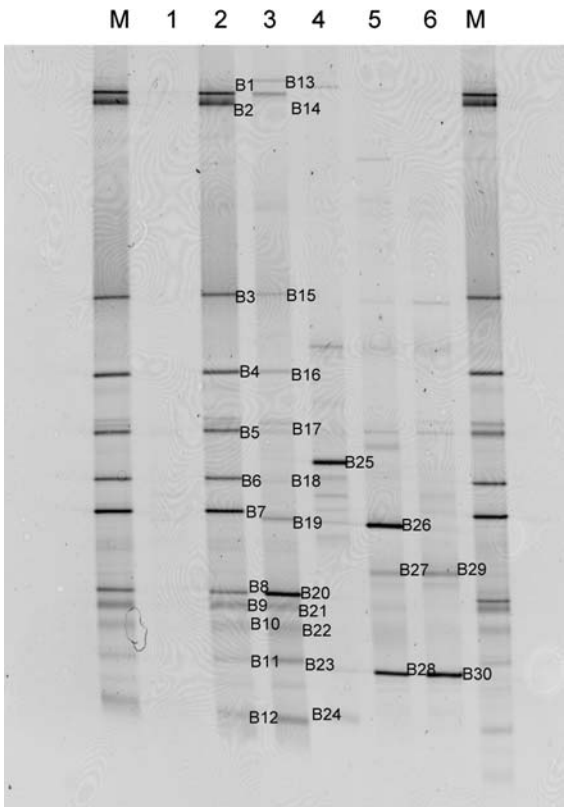

Fig. 2 Denaturing gradient gel electrophoresis profiles of PCR-amplified 16S rRNA gene fragments (V3–V5 regions) of total community DNA. Lane 1: aged soil; Lane 2: Soil + BaA + Chry (slurry 30 days): Lane 3: Soil (slurry 30 days). Lane 4: PAH-degraders MPN plates starting aged soil. Lane 5: PAH degraders (MPN Plates) spiked slurry (30 days). Lane 6: PAH degraders (MPN plates) unspiked slurry (30 day). Lane M: Marker which contains the same DNA sample as Lane 2

DGGE from the wells of the microtiters used to enumerate the PAH degraders

The DGGE profiles of the PAH-degrading populations obtained from microtiter plates from the slurries with and without additional B(a)A and Chry corresponded to lane 5 and lane 6 respectively (Fig. 2). The appearance of many fewer bands than those corresponding to the total DNA it would be indicative of a strong selectivity caused by the liquid mineral medium used in the enumeration of PAH degraders.

Band B26 corresponded to an uncultured Comamonadaceae and Band B27 corresponded to

Table 3 Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Length (bp)	Accession no.	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group[b]
B1 = B14	542	EU512950	Uncultured Bacteroidetes bacterium (AY758564)	98.2%	*Sphingobacteriales* (CFB group)
B2	579	EU512951	Uncultured Bacteroidetes bacterium (AY921801)	96%	*Sphingobacteriales* (CFB group)
B3 = B15	446	EU512952	Uncultured Arizona's soil bacterium (AF507716)	94.6%	*Sphingobacteriales* (CFB group)
B4 = B16	565	EU512953	Uncultured tallgrass prairie soil bacterium (AY957901)	96.8%	Unclassified *proteobacterium* (β)
B5a	436	EU512954	*Sphingomonadaceae* bacterium KF16 (AB269802)	97.9%	*Sphingomonadaceae* (α)
B5b	462	EU512955	Uncultured tallgrass prairie soil bacterium clone FFCH2236 (EU134542)	94.4%	Unclassified *proteobacterium* (δ)
B5c	460	EU512956	Unidentified Rizosphere's Maize bacterium clone 39c (AY669100)	96.2%	Unclassified *Proteobacterium* (β)
B5d = B17	460	EU512957	Uncultured *Burkholderiaceae* bacterium clone GASP-WC2S2_B05 (EF074975)	97.8%	*Burkholderiaceae* (β)
B5e	435	EU512958	Uncultured *proteobacterium* clone Amb_16S_1274 (AF505720)	93.4%	Unclassified *proteobacterium* (γ)
B5f	436	EU512959	Uncultured *Sphingomonas* sp. clone AUVE_04G07 from Australian Vertisoil (EF651167)	95.4%	*Sphingomonadaceae* (α)
B7	586	EU512960	*Azohydromonas australica* (AB188124)	99%	*Alcaligenaceae* (β)
B8a	434	EU512961	*Methylibium petroleiphilum* PM1 (CP000555)	98.9%	*Burkholderiaceae* (β)
B8b = B20	403	EU512962	Uncultured *alpha proteobacterium* from TCE-contaminated site (AY133099)	99.8%	*Rhodospirillaceae* (α)
B9 = B21	371	EU512963	*Skermanella* sp. (DQ672568)	100%	*Rhodospirillaceae* (α)
B10 = B22	402	EU512964	*Skermanella* sp. (DQ672568)	100%	*Rhodospirillaceae* (α)
B11 = B23	449	EU512965	*Skermanella* sp. (DQ672568)	99.8%	*Rhodospirillaceae* (α)
B12 = B24	547	EU512966	*Skermanella* clone (EF651077)	99.5%	*Rhodospirillaceae* (α)
B13	480	EU512967	Uncultured bacterium from California's grassland (EF516948)	97.9%	*Sphingobacteriales* (CFB group)
B14 = B1	473	EU512968	Uncultured Bacteroidetes bacterium (AY758564)	99.6%	*Sphingobacteriales* (CFB group)
B15 = B3	437	EU512969	Uncultured Arizona's soil bacterium (AF507716)	94%	CFB group
B16 = B4	482	EU512970	Uncultured tallgrass prairie soil bacterium (AY957901)	96.5%	Unclassified *proteobacterium* (β)
B17 = B5d	477	EU512971	Uncultured *Burkholderiaceae* bacterium clone GASP-WC2S2_B05 (EF074975)	96.2%	*Burkholderiaceae* (β)
B18	403	EU512972	Uncultured bacterium clone 18 (DQ413077)	97.5%	*Hyphomicrobiaceae*(α)
B19	383	EU512973	*Nitrosospira* sp. Nsp2 (AY123802)	96.9%	*Nitrosomonadaceae* (β)
B20 = B8b	484	EU512974	Uncultured *alpha proteobacterium* from TCE-contaminated site (AY133099)	99.6%	*Rhodospirillaceae* (α)
B21 = B9	456	EU512975	*Skermanella* sp. (DQ672568)	97.1%	*Rhodospirillaceae* (α)
B22 = B10	546	EU512976	*Skermanella* sp. (DQ672568)	99.6%	*Rhodospirillaceae* (α)
B23 = B11	407	EU512977	*Skermanella* sp. (DQ672568)	98.8%	*Rhodospirillaceae* (α)
B24 = B12	454	EU512978	*Skermanella* clone (EF651077)	99.5%	*Rhodospirillaceae* (α)
B25	542	EU512979	*Sphingobium herbicidivorans* strain FL (EF065102)	100%	*Sphingomonadaceae* (α)

Table 3 continued

Band	Length (bp)	Accession no.	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group[b]
B26	569	EU512980	Uncultured *beta proteobacterium* clone IRD18H05 from two temperate rivers (AY947979)	99.1%	*Comamonadaceae* (β)
B27 = B29	355	EU512981	*Pseudoxanthomonas mexicana* (EU119264)	96.9%	*Xanthomonadaceae* (γ)
B28 = B30	555	EU512982	*Mycobacterium parmense* (AF466821)	99.6%	*Mycobacteriaceae*
B29 = B27	576	EU512983	*Pseudoxanthomonas mexicana* (EU119264)	97.9%	*Xanthomonadaceae* (γ)
B30 = B30	549	EU512984	*Mycobacterium parmense* (AF466821)	99.6%	*Mycobacteriaceae*

[a] Sequences were matched with the closest relative from the Genbank database

[b] Sequences were matched with the closest relative from the Ribosomal Database Project (Maidak et al. 2000). α, β, and γ represent Alpha and Gammaproteobacteria, respectively

Pseudoxanthomonas mexicana isolated from an anaerobic digester (Thierry et al. 2004). Band B28 corresponded to *Mycobacterium parmense* (99.6% similarity). Although this species was isolated from a cervical lymph node (Fanti et al. 2004), several species of *Mycobacterium* that are difficult to classify have a high capacity to degrade PAHs (Turenne et al. 2001; Walter et al. 1991, Miller et al. 2007). Taking into account that Bands B29 and B30 were coincident with Bands B27 and B28 we conclude that both microorganisms could play a role in the biodegradation of the residual PAHs of the bioremediated soil. The microorganism corresponding to the uncultured *Comamonadaceae*, which was only present in the spiked slurry (Band B26), may have a role in the biodegradation of Ba(a)A and Chry. Surprisingly, none of these bands appeared in the total community DNA. This absence is difficult to explain, but it could be attributable to the fact that the mixed cultures in the soil had a more complex microbial population than the microtiters, which may have affected the PCR amplification.

We conclude that *Mycobacterium parmense* and *Pseudomonas mexicana* may contribute to the degradation of both 3- and 4-ringed PAHs, in which Sphingobacterials of the CFB group could also have a role. This is consistent with previous results obtained with the same creosote-contaminated soil (Viñas et al. 2005a, b). In connection with other identified microorganisms, supply of nitrogen could be attributed to *Azohydromonas australica*, and other secondary metabolic activities could be attributed to Skermanella sp. and *Methylibium petroleiphilum.*

The use of the PAH-spiked slurry approach coupled with molecular ecology may help us to understand biodegradation and microbial aspects encountered in aged hydrocarbon-polluted environments.

Acknowledgments This study was financially supported by the Spanish Ministry of Science and Technology (CTM2007-61097/TECNO) and by the Reference Network in Biotechnology (XERBA) of the Autonomous Government of Catalonia. The authors declare that the experiments discussed in this paper were performed in compliance with current Spanish and European legislation.

References

Abalos A, Viñas M, Sabaté J, Manresa MA, Solanas AM (2004) Enhanced biodegradation of casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 15:249–260. doi:10.1023/B:BIOD.0000042915.28757.fb

Alexander M (1995) How toxic are toxic chemicals in soil? Environ Sci Technol 29:2713–2717. doi:10.1021/es00011a003

Alexander M (1999) Biodegradation and bioremediation. Academic Press, San Diego

Fanti F, Tortoli E, Hall L, Roberts GD, Kroppenstedt RM, Dodi I, Conti S, Polonelli L, Chezzi C (2004) *Mycobacterium parmense* sp. nov. Int J Syst Evol Microbiol 54:1123–1127. doi:10.1099/ijs.0.02760-0

Farmer PB, Singh R, Kaur B, Sram RJ, Binkova B, Kalina I, Popov TA, Garte S, Taioli E, Gabelova A, Cebulska-Wasilewska A (2003) Molecular epidemiology studies of carcinogenic environmental pollutants: effects of polycyclic aromatic hydrocarbons (PAHs) in environmental pollution on exogenous and oxidative DNA damage. Mutat Res 544:397–402. doi:10.1016/j.mrrev.2003.09.002

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hareland W, Crawford RL, Chapman PJ, Dagley S (1975) Metabolic function and propierties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J Bacteriol 121:272–285

Maidak BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res 28:173–174. doi:10.1093/nar/28.1.173

Miller CD, Child R, Hughes JE, Benscai M, Der JP, Sims RC, Anderson AJ (2007) Diversity of soil mycobacterium isolates from three sites that degrade polycyclic aromatic hydrocarbobns. J Appl Microbiol 102:1612–1624. doi:10.1111/j.1365-2672.2006.03202.x

Sabaté J, Viñas M, Solanas AM (2006) Bioavailability assessment and environmental fate of PAHs in biostimulated creosote-contaminated soil. Chemosphere 63:1648–1659. doi:10.1016/j.chemosphere.2005.10.020

Thierry S, Macarie H, Iizuka T, Geissdoerfer W, Assih EA, Spanevello M, Verhe F, Thomas P, Fudou R, Monroy O, Labat M, Ouattara AS (2004) *Pseudoxanthomonas mexicana* sp. nov. and *Pseudoxanthomonas japonensis* sp. nov., isolated from diverse environments, and emended descriptions of the genus *Pseudoxanthomonas* Finkmann et al. 2000 and of its type species. Int J Syst Evol Microbiol 54:2245–2255. doi:10.1099/ijs.0.02810-0

Turenne CY, Tschetter L, Wolfe J, Kabani A (2001) Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. J Clin Microbiol 39:3638–3648. doi:10.1128/JCM.39.10.3638-3648.2001

Viñas M, Sabaté J, Solanas AM (2005a) Culture-dependent and -independent approaches establish the complexity of a PAH-degrading microbial consortium. Can J Microbiol 51:897–909. doi:10.1139/w05-090

Viñas M, Sabaté J, Espuny MJ, Solanas AM (2005b) Bacterial community dynamics and PAHs degradation during bioremediation of heavily creosote-contaminated soil. Appl Environ Microbiol 71:7008–7018. doi:10.1128/AEM.71.11.7008-7018.2005

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671–676. doi:10.1007/BF00167921

Weon HY, Kim BY, Hong SB, Joa HJ, Nam SS, Lee KH, Kwon SW (2007) *Skermanella aerolata* p. nov., isolated from air, emended description of the genus *Skermanella*. Int J Syst Evol Microbiol 57:1539–1542. doi:10.1099/ijs.0.64676-0

Wilson SC, Jones KC (1993) Bioremediation of PAHs contaminated soils. Environ Pollut 81:229–249. doi:10.1016/0269-7491(93)90206-4 (Review)

Wrenn BA, Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon-degrading bacteria by a most-probable-number procedure. Can J Microbiol 42:252–258

Xie CH, Yokota A (2005) Reclassification of *Alcaligenes latus* strains IAM 12599T and IAM 12664 and *Pseudomonas saccharophila* as *Azohydromonas lata* gen. nov., comb. Nov., *Azohydromonas australica* sp. nov. and *Pelomonas saccharopila* gen. nov., comb. Nov., respectively. Int J Syst Evol Microbiol 55:2419–2425. doi:10.1099/ijs.0.63733-0

Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 70:4800–4806. doi:10.1128/AEM.70.8.4800-4806.2004