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ORIGINAL PAPER

Isolation and genetic identification of PAH degrading bacteria from a microbial consortium

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Abstract Polycyclic aromatic hydrocarbons (PAH; naphthalene, anthracene and phenanthrene) degrading microbial consortium C2PL05 was obtained from a sandy soil chronically exposed to petroleum products, collected from a petrochemical complex in Puertollano (Ciudad Real, Spain). The consortium C2PL05 was highly efficient degrading completely naphthalene, phenanthrene and anthracene in around 18 days of cultivation. The toxicity (Microtox^{TM} method) generated by the PAH and by the intermediate metabolites was reduced to levels close to non-toxic in almost 40 days of cultivation. The identified bacteria from the contaminated soil belonged to γ-proteobacteria and could be include in *Enterobacter* and *Pseudomonas* genus. DGGE analysis revealed uncultured *Stenotrophomonas* ribotypes as a possible PAH degrader in the microbial consortium. The present work shows the potential use of these microorganisms and the total consortium for the bioremediation of PAH polluted areas since the biodegradation of these chemicals takes place along with a significant decrease in toxicity.

Keywords Biodegradation · Polycyclic aromatic hydrocarbons · Toxicity · Soil · *Pseudomonas* · *Enterobacter* · *Stenotrophomonas*

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Introduction

Polycyclic aromatic hydrocarbons (PAH) are a class of fused-ring aromatic compounds that are ubiquitous environmental pollutants. These compounds are formed by incomplete combustion of pyrolysis of organic matter. Localized accumulations can occur as

a result of human activities, such as industrial processes involving combustion of fossil fuels, wood and organic wastes; or as a result of natural events like forest fires. The PAH have low bioavailability and high hydrophobicity which increase as molecular size increase, making difficult its treatment and depletion. In addition, PAH have an important impact on human health as a consequence of their mutagenic and carcinogenic properties (IARC 1983).

Microorganisms can play an important role in the degradation of PAH in both terrestrial and aquatic ecosystems and they have a potential role in biorestoration of polluted sites. Enhancement of this phenomenon is the basis of bioremediation technologies although other mechanisms such as volatilization, leaching, adsorption, and photodegradation may also be effective (Bossert et al. 1984).

Biodegradation of PAH by microorganisms has been the subject of scientific study for many years (see references in Aitken and Long 2004). Phenotypic characteristics such as colony and cell morphology, biochemical and serological characteristics, protein and fatty acid pattern profiles have been applied traditionally in the microbiology systematic. On the other hand, modern taxonomy is using sequencing technologies of molecular markers such as 16S rRNA. These technologies allow the identification of colonies isolated from microbial consortia and the establishment of phylogenetic relationships between them (e.g., Viñas et al. 2005). It is widely accepted that only a small part of the microorganisms community present in any environmental sample can be cultivated in general laboratory media (Amman et al. 1995). Recently, new developments in culture-independent methods have increased the understanding of the members of microbial consortia (e.g., Viñas et al. 2005). Nevertheless, from the applied point of view, cultivated microorganisms are interesting and necessary for the further extraction and purification of the enzymes involved in PAH degradation. In addition, in some cases, bioaugmentation (inoculation of allochthonous microorganisms) is the best option to clean the polluted area when the autochthonous microbial populations do not have the appropriate metabolic ability to do it (Edgehill 1999).

Most studies providing estimates of biotransformation and biodegradation of PAH and related compounds have been carried out on isolated microorganisms while natural microbial consortia studies are scarce (Hesham et al. 2006). In general, microbial consortia are more efficient in depleting PAH from contaminated sites than isolated microorganisms, mainly because there will be major presence of different enzymatic activities and synergistic promotion of PAH, increasing biodegradation capabilities (Viñas et al. 2002; Jacques et al. 2008). It is important to establish previous laboratory evaluations as the characterization of the microbial consortium to determine its capacity to remove the pollutant, increasing the bioremediation efficiency (Sabaté et al. 2004).

On the other hand, most of the works are focused on isolation bacteria or hydrocarbon-degrading consortium without taking notice of the toxicity evolution during the biodegradation process. It is well known that not always biodegradation means reduction of the toxicity as a consequence of formation of toxic intermediate metabolites (e.g., Delille et al. 2002). The lack of studies regarding toxic metabolites during biodegradation processes highlights the importance of further research in this subject (Pelletelier et al. 2004).

The aim of the present study was to enrich a microbial consortium isolated from a polluted soil with its PAH (naphthalene, phenanthrene, and anthracene) degrading microorganisms originally present. The other goal was to identify both cultured and uncultured microorganism and to establish preliminary phylogenetic relationships between the microorganisms isolated from the consortium C2PL05. In order to meet these objectives, we report the results of the kinetic degradation capacity of the microbial consortium and the toxicity evolution during the biodegradation process.

Materials and methods

Chemicals

Naphthalene, phenanthrene, and anthracene (all >99% purity) were purchased from Sigma–Aldrich (Steinheim, Germany) and Fluka (Steinheim, Germany). Reagent grade dichloromethane and *n*-hexane were supplied by Scharlau Chemie (Barcelona, Spain). BMTM (Basal Mineral Medium with Trace Metals), BHB (Bushnell-Haas Broth) and LB (Luria-Bertani) media were purchased from Panreac (Barcelona, Spain). All chemicals used for toxicity assays were at least reagent grade.

Physicochemical characterization of soils

Soil samples containing unknown petroleum products were collected randomly from different areas of a petrochemical complex in Puertollano (Ciudad Real, Spain) for further characterization and study.

Inorganic nutrients were determined colourmetrically using a automated chemistry analyzer (Skalar, San^{++} System. Cambridge, UK). Concentration of nitrate, nitrite, ammonium and total phosphorous were measured according to the methods described by Hansen and Koroleff (1999). The pH was measured in a 1:2.5 (w/v) soil:water slurry with a GLP 21 micro pHmeter (Crison. Barcelona, Spain). Conductivity was determined with a GLP 31 conductimeter (Crison) in a 1:5 (w/v) soil:water slurry. The water holding capacity (WHC) is the maximum quantity of water retained by the soil against gravity and was measured following the method described by Wilke (2005).

Hydrocarbons from soils were extracted as follows: About 100 g of each soil sample were kept at 110°C until constant weight. The dried soils were accurately weighted and placed in a 250 ml Soxhlet apparatus for 8 h with dichloromethane as extraction agent. Finally, the solvent was removed in a Laborota 4000 rotary evaporator (Heidolph Instruments. Schwabach, Germany) and the residual oil was weighted for mass balance.

Extracted soils were sieved through a stainless steel mesh and the fraction smaller than 2 mm was collected for further particle size distribution analysis using low angle laser light scattering in a Mastersizer 2000 (Malvern Instruments Ltd. Worcestershire, UK) equipped with a Hydro 2000MU accessory. Approximately 500 mg of extracted soil were dispersed in 800 ml of deionized water in the sample cell and the suspension was circulated through the laser diffraction equipment.

Total organic carbon from soil samples was measured through elemental analysis in an Elementar Vario EL system (Varian. Palo Alto, CA, USA) equipped with a thermal conductivity detector. Carbonate from soils was removed by an acid treatment with 4 M HCl at 60°C for 4 h and then samples were dried at 60°C for 16 h prior to analysis.

Enumeration of heterotrophic and hydrocarbon degrading microbial populations

Heterotrophic and PAH-degrading population were enumerated using a miniaturized most probable number technique (MPN) in 96-well microtiter plates with eight replicate wells per dilution (Wrenn and Venosa 1996). Total heterotrophic microbial population was enumerated in Luria Bertani (LB) medium with glucose and PHA-degraders were counted in BMTM medium with a mixture of phenanthrene (0.5 g l^{-1}), anthracene and naphthalene in hexane (each at a final concentration of 0.05 g l^{-1}).

Toxicity

The soil toxicity was estimated in a soil/water slurry (w/v) through the EC_{50} parameter using the Microtox^{TM} test with the luminescent bacterium *Vibrio fischeri*, following the protocol developed by Microbics Corporation (1992). Assays were performed at 15°C after 15 min of contact period with *V. fischeri*. The EC_{50} of the soil lixiviate (%) is the concentration of the soil lixiviate (w/v) at which the light emission of *V. fischeri* decreases 50%. For soils, aqueous soil washings (lixiviate) was prepared with 2 g of each soil (<2 mm) added to 3 ml of 2% NaCl solution. Samples were incubated in an orbital shaker during 10 min and centrifuged for 2 min at 13,000 rpm.

Toxicity was also monitored during the PAH kinetic degradation through a short screening of the samples with the Microtox^{TM} method. The samples were estimated assuming that samples just with *V. fischeri* were 0% of inhibition and samples solely with NaCl (2%) were 100% of inhibition (no emission of luminescence).

Consortium PAH degradation

The consortium C2PL05 was obtained from a soil sample of the petrochemical complex in Puertollano (Spain). Triplicate in miniaturized microcosms were cultivated with BHB broth (50 ml), 1% of Tween-80 as surfactant and phenanthrene (0.5 g l^{-1}), anthracene and naphthalene (0.05 g l^{-1}) as only carbon source. Replicates were incubated in an Innova 40

orbital shaker (New Brunswick Scientific. Edison, NJ, USA) at 200 rpm, 25°C under dark conditions and refreshed every 15 days, adding nutrients from the BHB broth and PAH.

Monitoring the biodegradation PAH evolution were performed in triplicate in miniaturized microcosms with BHB broth (250 ml), 1% of Tween-80, anthracene and naphthalene (1 g l^{-1}), and phenanthrene (10 g l^{-1}). Before to inoculate the consortium C2PL05 (2.5 ml with $7.4 \times 10^5 \pm 5.6\ 10^4$ cells ml^{-1}), samples were placed in an orbital shaker overnight to solubilise most of the PAH added. Samples were withdrawn at different time intervals for further PAH concentration and cell density monitoring. Three more replicates were performed without inoculating the consortium (BHB, Tween-80 and PAH) as a control experiment in order to measure PAH depletion due to abiotic processes.

Preliminary data (not shown) confirmed that the content of PAH (naphthalene, phenanthrene, and anthracene) at precipitate and supernatant phases in aliquots were not significantly different (*t*-test, $t_{17} = 2.0$, $n = 18$, $P = 0.06$; $t_{17} = 1.7$, $n = 18$, $P = 0.1$, respectively) from whole flask. Analyses were carried out on sampling hours 0, 12, 24, 48, 72 and 96 h.

Concentration of naphthalene, phenanthrene, and anthracene in the culture media were monitored by HPLC analysis using a reversed-phase C18 column (Luna C18(2), 7.5 cm × 4.6 mm I.D., 3 µm particle size. Phenomenex, Torrance, CA, USA) with its corresponding guard column. The HPLC system was a ProStar 230 (Varian. Palo Alto, CA, USA) equipped with a quaternary pump and photodiode array UV/Vis and fluorescence detectors. About 10 µl of filtered cultivation media were injected and then eluted from the column at a flow rate of 0.8 ml/min using an acetonitrile:water gradient program as follows: isocratic 60:40 (0–2 min), gradient to 75:25 (2–14 min), isocratic 75:25 (14–15 min), gradient to 100:0 (15–16 min). The content of each individual PAH were calculated through an external standard technique from a standard curve of peak area versus concentration.

Bacterial growth was monitored by changes in the absorbance of the culture media at 600 nm using a Cary-500 NIR/UV/Vis spectrophotometer (Varian).

DNA extraction from cultured bacteria and phylogenetic analysis

Eighteen colonies of *Bacteria* were isolated from the polluted soil and transferred onto LB agar plate in order to increase microbial biomass for DNA extraction. Before DNA extraction, we confirmed that the colonies were PAH-degraders by growing them with PAH as only carbon and energy sources and measuring the cellular density evolution.

In order to perform the molecular identification of the PAH-degrader isolated cultures (DIC), their total DNA was extracted from cells cultured on LB glucose agar plates using Microbial DNA isolation kit (MoBio Laboratories. Solano Beach, CA, USA). Amplification of the 16S rRNA coding region of the DNA was performed as described by Viñas et al. (2005). Primers 16F27 and 16R 1488 were used in the reactions (Lane 1991). The cleaned PCR products (MoBio Laboratories) were sequenced using the same amplification primers. The ABI Prism™ Dye Terminator Cycle Sequencing Ready reaction kit (PE Applied Biosystems) was used and the following settings were applied: BIGDye Terminator V3.1 Cycle Sequencing kit (PE Applied Biosystems) was used as follows: denaturation for 3 min at 94°C, 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

Sequences were edited and assembled using BioEdit software version 4.8.7 (Hall 1999) to check for reading errors and when possible, resolving ambiguities. No position was excluded from the matrix data for further analyses.

BLAST search (Madden et al. 1996, available at URL http://www.ncbi.nlm.nih.gov/BLAST/) was used to find nearly identical sequences for the 16S rRNA sequences determined. Phylogenetic analyses of the sequence data were performed with maximum parsimony (MP) using PAUP 4.0b10 (Swofford 2003). A heuristic search of 100 random taxon addition replicates was conducted with TBR branch-swapping and the MulTrees option in effect. Non-parametric bootstrap (Felsenstein 1985) was used to assess robustness of clades, running 1,000 pseudoreplicates with the same settings as in the heuristic search. Only clades that received bootstrap support above 70% were considered as strongly supported,

following Hillis and Bull (1993). Sequences of *Methylococcus capsulatus* were used as out-group according to previous phylogenetic affiliations (Anzai et al. 2000).

Denaturing gradient gel electrophoresis from microbial consortium

Non culture-dependent molecular techniques as denaturing gradient gel electrophoresis (DGGE) was performed to know the total biodiversity of the microbial consortium C2PL05 at initial and final time (40 days in between) of the biodegradation PAH evolution experiment. Total DNA was extracted from 500 µl of this consortium using Microbial DNA isolation kit (MoBio Laboratories. Solano Beach, CA, USA). Suitable yields of high- molecular-weight DNA (5–20 µg g of soil^{-1}) were obtained. The V3 to V5 variable regions of the 16S rRNA gene were amplified using/with the primers set 341F-GC and 907R (GC clamp: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3′) (Muyzer et al. 1995).

The amplification reaction was performed according to the Taq DNA polymerase protocol (Promega, Madison, Wis). The PCR conditions were as follow: 5 min of initial denaturation at 94°C; 30 cycles at 94°C for 1 min, and 52°C for 1 min (annealing), and 72°C for 2 min; followed by 10 min of final primer extension. DGGE analysis was carried out using a D-Code Universal Detection System instrument (Bio-Rad) and a Model 475 gradient former according to the manufacturer's instructions (Bio-Rad). About 6% polyacryamide (37.5:1 acrylamide: bisacrylamide) gels with a 30–60% urea-formamide denaturant gradient were used in 1× TAE buffer, pH 7.4 at 200 V for 4 h at 60°C. Gels were stained with ethidium bromide and visualized under UV illumination. DNA bands were excised and reamplificated for sequencing.

Results

Soil physical, chemical and biological characteristics

The average particle size was within the range of sands (50–2,000 µm; Table 1) with very low content in silt, and virtually absence of clays. The average soil pH was neutral-basic, the WHC was lower than 50% and PAH-degrading population represented approximately a 10% of the heterotrophic microbial population (9.7×10^4 cells g^{-1}). The average conductivity was 74 µS cm^{-1} and the inorganic nutrients were lower than 1 mg Kg^{-1} (Table 1). The range of soil lixiviate toxicity was from EC_{50} 1.0 to 28.8 g/100 ml^{-1} (average ± standard error; 14.4 ± 8.0 g/100 ml^{-1}). The consortium C2PL05 was obtained from the soil sample that showed the lowest EC_{50}.

Table 1 The average (±SE) physical, chemical, and biological soil characteristics

Property	Unit	Average	SE
Average particle size (in volume)	µm^3	291	6
Composition (sand/silt/clay)	%v	99.10/0.90/00	0.36/0.36/00
pH	–	7.7	0.1
Conductivity	µS cm^{-1}	74	22
WHC	%w	33	7
$(NO_3)^-$	µg kg^{-1}	40	37
$(NO_2)^-$	µg kg^{-1}	11.7	0.1
$(NH_4)^+$	µg kg^{-1}	155	125
Phosphate	µg kg^{-1}	47	6
Total carbon	%w	9.6	2.1
TOC (acid treatment)	%w	5.1	0.4
MPN (heterotrophic) × 10^4	cells g^{-1}	9.7	1.2
MPN (PAH) × 10^3	cells g^{-1}	9.3	1.9
Toxicity EC_{50} soil lixiviate	g/100 ml	14.4	8.0
Extractable hydrocarbons[a]	%w	9.2	1.8

[a] According to the procedure described in section "Materials and methods"

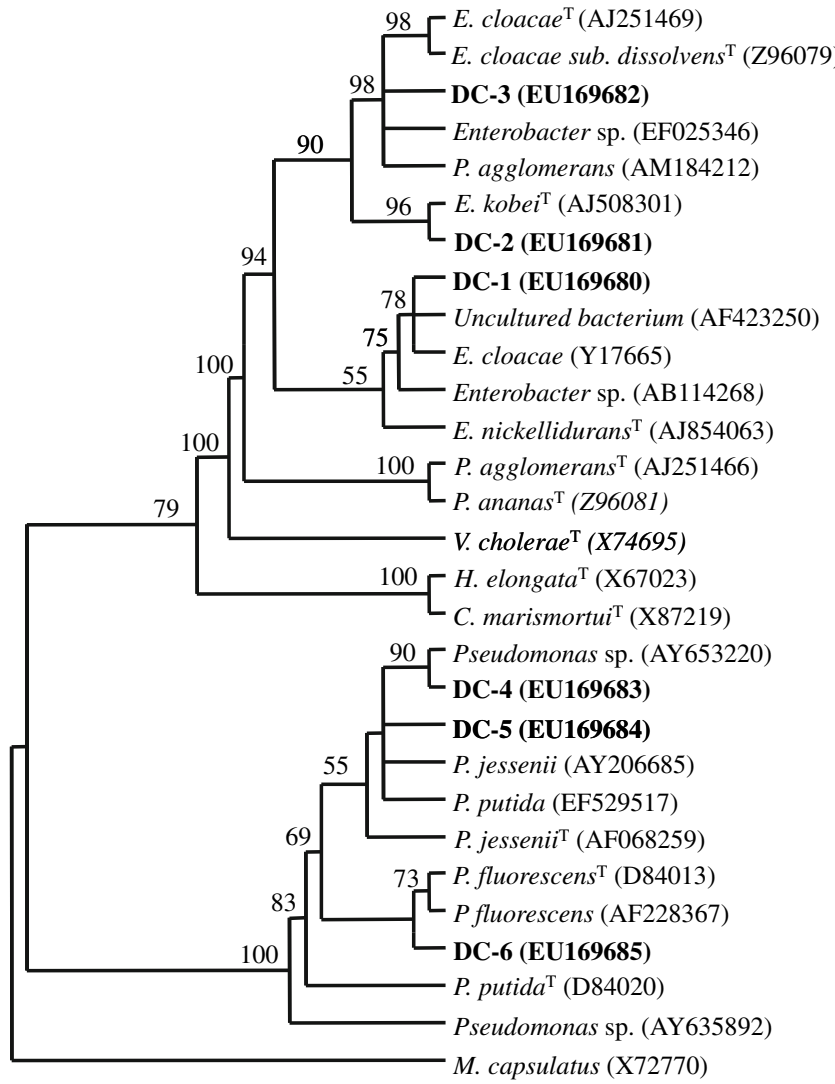

Fig. 1 Parsimonic consensus tree showing the phylogenetic relationship of PAH degrader cultures isolated from polluted soil (tree length = 782 steps, CI = 0.655, RI = 0.885). The 16S rRNA aligned matrix contained 1,478 unambiguous nucleotide position characters with 19.34% of parsimony-informative characters. ^{T} = type strain

Finally, 50% of the dry soil total carbon was organic and 9.2% of dry soil weigh were hydrocarbons (Table 1).

Molecular characterization of microorganisms from polluted soil

Eighteen strains were isolated, sequenced and grouped in six operational taxonomic unit (OTU) based on 100% identity (DIC1–DIC6). All of them were γ-proteobacteria and were located in two strongly support clades. One of these groups includes *Enterobacter* sp. lat clade, which gathers three PAH-degrading strains (Fig. 1). DIC-1 is located in a weakly supported clade with *E. nickellidurans* type strain and it was 99% similar to *Enterobacter cloacae*. DIC-2 was closely related to *E. kobe*i type strain in a clade very well supported and DIC-3 belongs to the same group than *E. cloacae* type strain and it was 99% similar to *Pantoaea agglomerans* and *Enterobacter* sp.

The other clade includes strain of *Pseudomonas* sp. DIC-4 was 99% similar to *Pseudomonas* sp. and DIC-5 is similar to *Pseudomonas putida* in 99%. Both of them were related to *Pseudomonas jessenii* type strain and DIC-5 was nested to *Pseudomonas fluorescens* type strain.

The DGGE microbial consortium showed a similar pattern although one of the ribotype showed an unclear definition at final time of the biodegradation

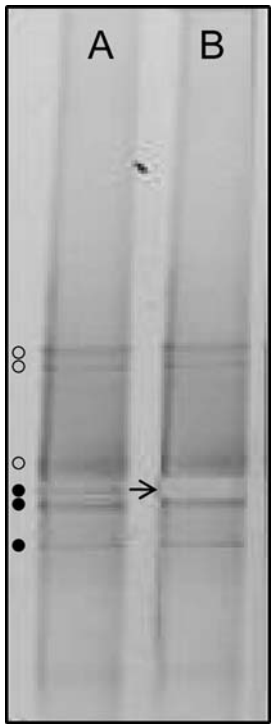

Fig. 2 DGGE fingerprint pattern based on composition difference of 16S rDNA fragments of bacteria show the community structure of the PAH enriched consortium. *A*, initial and *B*, final time of the biodegradation PAH evolution experiment. (○) *Pseudomonas*, (●) *Stenotrophomonas*. The *arrow* indicates an uncertain ribotype

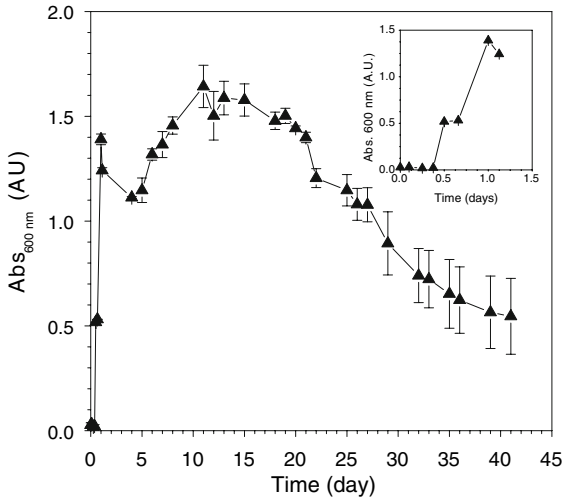

Fig. 3 Cell density (A_{600}) of the C2PL05 consortium during biodegradation experiments

PAH experiment (Fig. 2). Three ribotypes belong to *Pseudomonas* genus after BLAST search, one of these were identified as *P. putida* revealed a 98% match in terms of homology. The other three bands were identified as *Stenotrophomonas*, one of these as *S. maltophila* with a 100% homology.

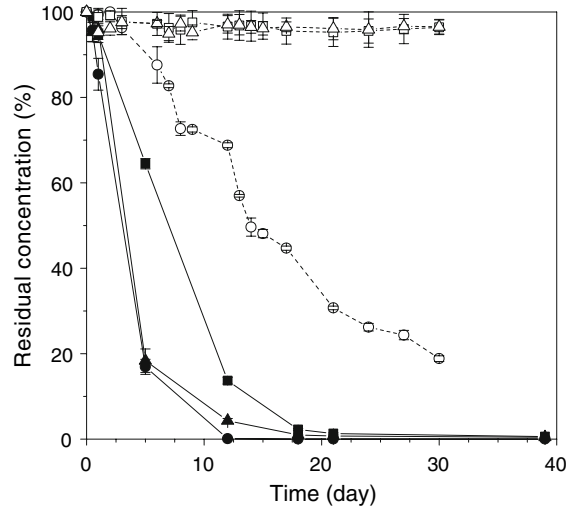

Fig. 4 Degradation of naphthalene (■), phenanthrene (●) and anthracene (◆) by the C2PL05 consortium and degradation of naphthalene (□), phenanthrene (○) and anthracene (◇) during control experiments

Cell growth, biodegradation of PAH and toxicity

The growth curve of the consortium C2PL05 during the PAH biodegradation experiments (Fig. 3) showed, approximately, a 10 h latent phase. The curve showed two different local maxima at day 1 and 10 and subsequently, an important progressive decrement took place during the death phase.

The kinetic plots for the depletion of the three PAH in the medium during the cultivation of the consortium are shown in Fig. 4. It is observed that PAH degrading efficiency of the consortium was evident from the results showed. Naphthalene was completely removed (>99.9%) from the broth in <12 days, and phenanthrene (>98%) and anthracene (>99%) in 18 days. However, phenanthrene showed a slower depletion from the broth along the experiment. The control experiment showed (Fig. 4) that both phenanthrene and anthracene did not show a significant depletion in absence of the microbial consortium while naphthalene showed an important removal at the same conditions. However, the naphthalene depletion rate was significantly slower under abiotic conditions than that observed in presence of the consortium (biotic conditions).

Table 2 Average (±95% confidence interval) apparent first-order biodegradation rate constant (k)

Compound	k (h^{-1})
Naphthalene	$2.7\ 10^{-2} \pm 1\ 10^{-3}$
Phenenthrene	$1.07\ 10^{-2} \pm 4\ 10^{-4}$
Anthracene	$9.8\ 10^{-3} \pm 3\ 10^{-4}$

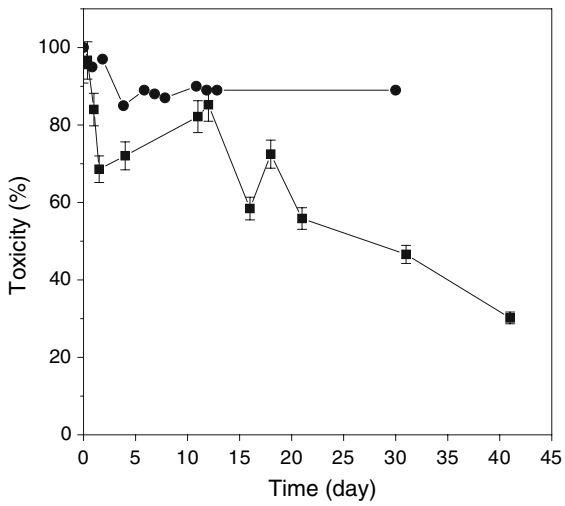

Fig. 5 Toxicity of the cultivation media during the degradation of naphthalene, phenanthrene and anthracene by the C2PL05 consortium (■) and in a control experiment without inoculation (●)

In order to evaluate the degradation rate of naphthalene, phenanthrene and anthracene, the PAH concentration decay versus time was fitted to a first-order kinetics for each substrate. In the case of naphthalene, the abiotic losses were considered in order to calculate the corrected kinetic constant accounting solely for the biological contribution to the degradation. Table 2 shows that the apparent first order kinetic constant for biodegradation follows the order: naphthalene > phenanthrene > anthracene.

The toxicity screening during the PAH degradation process (Fig. 5) showed a complex behaviour, specially at times corresponding to the exponential growth phase, although an overall decreasing trend was observed. Toxicity was still high (65%) when the three PAH were depleted, therefore, it was almost completely eliminated after 40 days of cultivation. Simultaneously to PAH biodegradation, pH suffered an important change, increasing almost one unit from 7.0 to 8.2 during the first 18 days. Once the consortium reached the dead phase the pH kept constant forwards (data not shown).

Discussion

Chemical and physical soil characteristics (abiotic factors) are important to determine the microbial population diversity. The average soil pH was within the optimum pH range (pH 7.4–7.8) for biodegradation processes (Dibble and Bartha 1979). However, soil humidity was very low compared with the optimum range of 40–60% for sandy soils reported by Menn et al. (2000). The soil humidity is important for the biodegradation and the optimum level depends on the properties of each soil, the kind of pollutant and whether the process is aerobic or anaerobic.

Soil nutrient concentration was low for biodegradation processes (Chaineau et al. 2005). Thus, the addition of inorganic nutrients shows a positive effect increasing PAH degrading rates and populations (Chaineau et al. 2005). In general, the soil poor conditions could explain the low heterotrophic bacteria abundance compared with other soil hydrocarbon contaminated (6.5×10^7 cells g^{-1}; Margesin and Schinner 2001). However, soils subjected to a constant contamination can yield a natural selection of autochthonous pollutant-biodegrading microbial population (Kästner 2000). Consequently, microbial consortium obtained from the polluted soil was efficient in using hydrocarbons as carbon and energy sources as nutrients were added.

The biomass growth pattern, especially up to 10 days of cultivation, shows two apparent exponential phases. These can be attributed to a sequential microbial succession corresponding to different strains belonging to the consortium. During the PAH degradation process, pH increased about one unit during the medium into alkaline (pH ≈ 8.2). Apparently, this important change in the abiotic conditions did not negatively affect the microbial consortium because reduction of toxicity and PAH consumption continued. Previous works (e.g., Hambrick et al. 1980) has shown that soil bacteria which degrade PAH prefer alkaline than acid conditions and generally, PAH degradation tends to increase in alkaline conditions.

The degradation of both three-ring hydrocarbons must not be associated to any abiotic process since

any degradation of phenanthrene and anthracene was observed from the control experiments. In the case of naphthalene, some degree of depletion was measured during controls, yielding an apparent first-order rate constant of $2.7\ 10^{-3} \pm 7\ 10^{-5}\ h^{-1}$. This abiotic naphthalene removal was likely due to volatilisation caused by a significant vapour pressure of that compound (>500 and >10,000 times higher than that for phenanthrene and anthracene, respectively). However, the rate of naphthalene depletion during biodegradation experiments was much higher than that measured in the control experiments, proving that degradation of naphthalene was mainly biologically driven. In agreement with our results (depletion order: naphthalene > phenanthrene > anthracene), several studies on PAH degradation by bacteria (e.g., Warmer and Peters 2005) reported a trend showing an inverse relationship between biodegradation rate and number of aromatic rings and molecular weight (e.g., Warmer and Peters 2005). Some abiotic physicochemical processes, such as volatilisation, affecting bioavailability (Dimitriou-Christidis et al. 2007) must account for the significant differences on the degradation rates of both three-ring PAH by the consortium. However, the kinetic plots show that no inhibition occurred during the degradation of PAH mixtures but rather degradation of all three compounds took place simultaneously. The addition of a surfactant such as Tween-80 produce micelle aggregates providing an additional hydrophobic area in the central region of micelles enhancing the aqueous solubility of PAH (Li and Chen 2008). Even with the presence of Tween-80, the amount of PAH added to the cultures exceeded their aqueous solubility. Therefore, a fraction of the initial amount of PAH, especially phenanthrene and anthracene, remained as a precipitate that were progressively solubilised as the PAH were degraded. The above increase in the solubility is determinant for the bioavailability of these compounds whose solubility follows the same order than the degradation rate above described. In fact, solubility of anthracene in the cultivation medium at 25°C was, approximately, 10 times lower than that of phenanthrene.

Most studies (e.g., Grant et al. 2006) report a depletion of target components without considering the toxicity during the processes. The increment of the toxicity concomitant to the decrease in PAH concentration can be partially explained by the PAH low aqueous solubility. The PAH concentration during the experiment exceeds their aqueous solubility. However, the PAH solution process was fast and enough to keep a constant concentration in solution and a rising substrate consumption by the growing population. In addition, according to Grifoll et al. (1995) PAH biodegradation can show a temporary increase in toxicity as a consequence of the potential accumulation of intermediate products (dead-end products) or toxins. Toxicity was still present when the three PAH inoculated were totally depleted, although continued decreasing, reaching values considered almost non toxic (Fig. 5). These results, suggest that intermediate or cometabolism products, although still toxic they can be finally degraded by the microbial population present.

Related to these microorganisms, the molecular identification revealed the presence of different non human pathogens γ-proteobacteria. Some of them belong to *Enterobacter* genus. The enteric bacteria in this group are mainly regarded as inhabitants of animal guts and the ability of this group to degrade PAH compounds seems to be an unusual feature. Some authors reported utilization of aromatic compounds by enterobacteria (e.g., Gupta et al. 2006) and recently, Toledo et al. (2006) noted that one *Enterobacter* strain is able to degrade naphthalene but not phenantrene, fluoranthene or pyrene. Our results show that three isolated cultures from petroleum polluted soils were included in *Enterobacter* sp. lat clade (Fig. 1) and all of them metabolize the three PAH analyzed. The DIC-2 strain forms a statistically strongly support clade with *E. kobei* type strain. The DIC-1 is 99% similar to *E. cloacae*, however, this relationship should be consider carefully due to *E. cloacae* is not a monophyletic group (Fig. 1). DIC-3 is a strain phylogenetic related to *E. cloacae* type strain although with a 99% similarity to *P. agglomerans*. This strain downloaded to GenBank could be a taxonomical mistake since *Pantoea* type clade is sister group to Enterobacter clade (Fig. 1). Several authors have underlined that *Enterobacter* is not a monophyletic group because members of *Enterobacter* are scattered among other enteric genera (Kampfer et al. 2005) so that more sequences and molecular markers are needed in order to infer an encompassing phylogeny of this genus.

The metabolism of PAH has been genetically studied in *Pseudomonas* complex. Some of them

showed a high efficiency to degrade naphthalene and phenanthrene in extreme environmental conditions (Ma et al. 2006). Even *P. putida* and *P. fluorescens* behave as high molecular weight PAH degrader on culture (e.g., Kanaly and Harayama 2000). Three of the isolated cultures from 2PL05 petroleum contaminated soil belong to *Pseudomonas sensu stricto* (Anzai et al. 2000). Two of them, DIC-4 and DIC-5, are located in the same clade that *P. jessenii* type strain (Fig. 1) although this relationship is not statistically strongly supported. *P. jessenii* is able to use aromatic compounds as carbon source but there are a few references about *P. jessenii* as PAH degrader (Singleton et al. 2005). DIC-6 is genetically related to *P. fluorescens*, which is able to used PAH as carbon and energy source (e.g., Bugg et al. 2000).

DGGE analysis in the microbial consortium revealed six bands, three bands corresponding to *Pseudomonas* and three to *Stenotrophomonas* without important variation in bacteria populations over a period of 40 days, although at final time, one of the ribotype is unclear (Fig. 2). *Enterobacter* strains are not identified in any DGGE bands, possibly due to the low representation of these bacteria at the moment of analysis (data not showed). In this context, Dowd et al. (2008) and Cui et al. (2008) did not identify some cultured strain by DGGE, as this technique only display the rDNA fragments obtained from the predominant species present in the community. The low biodiversity observed in the microbial consortium is typical of aged system where the pollutants or restrictive conditions could be an important factor to select specific bacteria populations (Viñas et al. 2005; Sun et al. 2009).

In conclusion, we have presented evidence of an efficient PAH (phenanthrene, anthracene, naphthalene) degradation capability and toxicity elimination by the microbial consortium C2PL05. The results show the potential use of the isolated consortium for the bioremediation of PAH polluted areas since the biodegradation of these chemicals takes place along with a significant decrease in toxicity. *Pseudomonas* and *Enterobacter* strains were identified as PAH degrading microorganisms from a highly oil-polluted soil and *Stenotrophomonas* ribotypes are a uncultured microorganism present in the consortium with potential ability to transform PAH. The contribution of each of these microorganisms in the system, both with regard to the mineralization of PAH in their effect on the overall toxicity, should be analyzed to improve the in situ applications.

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