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Construction of PAH-degrading mixed microbial consortia by induced selection in soil

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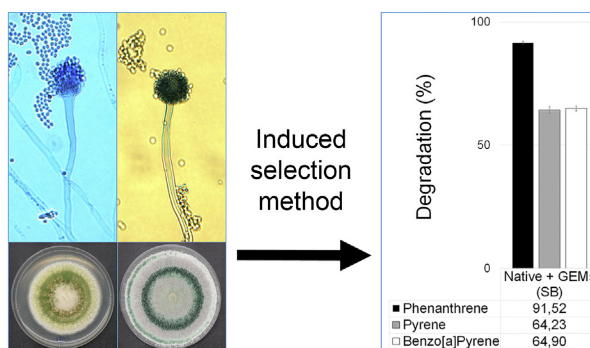
HIGHLIGHTS

- Use of induced selection method to construct PAH-degrading microbial consortia.
- Native and GEM strains showed long-term survival in PAH-polluted soil.
- Fungal GEM strains improved the degradation of HMW-PAHs in bio-stimulated soils.
- Resulting consortia presented high potential for PAH degradation in soil.

GRAPHICAL ABSTRACT



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ABSTRACT

Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soils through the biostimulation and bioaugmentation processes can be a strategy for the clean-up of oil spills and environmental accidents. In this work, an induced microbial selection method using PAH-polluted soils was successfully used to construct two microbial consortia exhibiting high degradation levels of low and high molecular weight PAHs. Six fungal and seven bacterial native strains were used to construct mixed consortia with the ability to tolerate high amounts of phenanthrene (Phe), pyrene (Pyr) and benzo(a)pyrene (BaP) and utilize these compounds as a sole carbon source. In addition, we used two engineered PAH-degrading fungal strains producing heterologous ligninolytic enzymes. After a previous selection using microbial antagonism tests, the selection was performed in microcosm systems and monitored using PCR-DGGE, CO_2 evolution and PAH quantitation. The resulting consortia (i.e., C1 and C2) were able to degrade up to 92% of Phe, 64% of Pyr and 65% of BaP out of 1000 mg kg^{-1} of a mixture of Phe, Pyr and BaP (1:1:1) after a two-week incubation. The results indicate that constructed microbial consortia have high potential for soil bioremediation by bioaugmentation and biostimulation and

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may be effective for the treatment of sites polluted with PAHs due to their elevated tolerance to aromatic compounds, their capacity to utilize them as energy source.

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1. Introduction

Polycyclic aromatic hydrocarbons are an important group of organic pollutants containing two or more fused aromatic rings. They are mainly produced as the result of thermal decomposition, incomplete combustion and pyrolysis of diverse organic molecules (Mrozik and Piotrowska-Seget, 2010). Although several natural and anthropogenic sources contribute to the release of PAHs into the environment, petrochemical activities in particular and their related residues exert a strong negative impact on the environment and account for the majority of PAHs and other hydrocarbons released into soils and water bodies. PAHs are considered persistent pollutants with potential harmful effects on the flora and fauna of affected habitats, resulting in the absorption and accumulation of several toxic products and active intermediaries of their metabolism in diverse organisms. Because of their persistence and potential cytotoxic, carcinogenic, genotoxic and mutagenic effects, the removal of PAHs and other hydrocarbon compounds from contaminated soils is an environmental priority (US-EPA, 2008).

There are a variety of mechanisms by which PAHs are removed naturally from the environment, including several forms of oxidation, adsorption, volatilization, bioaccumulation and biodegradation (Ke et al., 2002; Kong and Ferry, 2003). Although chemical, physicochemical and thermal technologies are available for the remediation of impacted soils, microbial degradation is considered the main natural degradation form of PAHs in soils (Juhasz and Naidu, 2000). More than 50 genera of bacteria and fungi contain species capable of degrading low molecular weight (LMW) or high molecular weight (HMW) PAHs under aerobic or anaerobic conditions (Cerniglia and Sutherland, 2010; Seo et al., 2009). However, microorganisms in soils rarely occur as individual organisms; instead, they live in complex ecological communities with different levels of interactions, which could largely influence PAH degradation efficiency. The use of a microbial consortium as a soil inoculum may be advantageous to efficiently degrade mixtures of PAHs because no single microbial species possesses the ability to metabolize both LMW and HMW PAHs and also because a microbial consortium may show an improved adaptation, survival and permanence in contaminated soils. Thus, the objective of this study was to construct tolerant PAH-degrading microbial consortia by using an induced microbial selection method with PAH-spiked soils, in order to improve their potential for soil bioremediation. We successfully used this method to construct two mixed microbial consortia exhibiting high tolerance and high degradation levels of LMW and HMW PAHs.

2. Materials and methods

2.1. Native degrading microorganisms

Five fungal (*Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, and *Aspergillus fumigatus* H19) and eight bacterial (*Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10, and *Stenotrophomonas maltophilia* B14) native strains were preselected for the construction of the consortia. These strains were previously isolated from heavy crude oil-contaminated soils using culture media with Maya crude oil as sole carbon source, and identified by sequencing their 16s rRNA and ITS genes with identity and similarity values of at least 99% (Zafra et al., 2014). The selection of these strains was made based on their high PAH tolerance levels (up to 6000 mg of mixture of PAHs l^{-1}), ability to use PAHs as a sole carbon source and reported ability to metabolize PAHs (Zafra et al., 2014; Zafra et al., 2015a). Microorganisms were maintained aerobically at 30 °C in potato dextrose agar (PDA) plates containing 0.1% Maya crude oil (fungi) or liquid basal saline medium (g l^{-1}: NaCl, 0.3; $(NH_4)_2SO_4$, 0.6; K_2HPO_4, 0.75; KH_2PO_4; 0.25; $MgSO_4 \cdot 7H_2O$, 0.15; KNO_3, 0.6; yeast extract, 0.125) using 0.1% Maya crude oil as a sole carbon source (bacteria).

2.2. Degrading genetically engineered microorganisms (GEMs)

In addition to native degrading microorganisms, two genetically modified strains of *Aspergillus niger* expressing the lignin peroxidase (LiP^{+5} strain) and manganese peroxidase (MnP^{+7} strain) genes from *Phanerochaete chrysosporium* were used in the construction of the consortia (Cortés-Espinosa and Absalón, 2013). LiP^{+5} and MnP^{+7} strains were maintained on PDA plates as described above for native microorganisms.

2.3. Evaluation of microbial antagonism

In vitro evaluation of fungi to fungi, bacteria to bacteria, fungi to bacteria and bacteria to fungi antagonisms was carried out using Toyama's medium plates (g l^{-1}: $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 3; $CaCl_2 \cdot 2H_2O$, 0.5; NaCl, 0.1; $FeSO_4$, 0.001; glucose, 10, pH 6.5) (Wunder et al., 1994) with and without 2% Maya heavy crude oil (API gravity of 25.1; 31.6% saturated hydrocarbons with predominance of C12-C25 hydrocarbons, 42.5% aromatic hydrocarbons with predominance of C27-C35 hydrocarbons, 10.2% resins and 15.7% asphaltenes (Fulema et al., 2008; Garcia and Grimalt, 2004). For fungi to fungi antagonisms, 1×10^4 spores from each strain were spotted onto an agar plate and incubated at 30 °C until inhibition halos or antagonist effects between fungal colonies were observed. Antagonisms from bacteria to bacteria were evaluated by streaking 1×10^4 CFU of each strain in a grid pattern, allowing direct contact between strains. Fungi to bacteria antagonisms were evaluated by centrally spotting 10^4 spores of a fungal strain (antagonist) and incubating at 30 °C. After observing sporulation (indicative of secondary metabolism), 1×10^4 CFU of each bacterial strain were radially streaked aside fungal colonies and incubated until bacterial growth was observed or inhibited by the fungi. Bacteria to fungi antagonisms were evaluated by spotting 1×10^4 CFU of each bacterial strain (antagonists) in the periphery of the plates and centrally inoculating 1×10^4 spores of a fungal strain at the same time.

2.4. Microbial selection of degrading consortia in solid state culture

The construction of two microbial consortia able to degrade PAHs in contaminated soils was performed by microbial self-selection assays in sterile soil using LMW and HMW-PAHs as selective factors. Assays were performed in microcosm solid culture

systems using sterile sugarcane bagasse (34.34% carbon, 0.18% nitrogen, 0.00343% phosphorous), corn stover (35.7% carbon, 0.465% nitrogen, 0.000031% phosphorous) or wheat straw (37.85% carbon, 0.555% nitrogen, and 0.000187% phosphorous) as fungal growth support. First, 0.35 g dry weight of agroindustrial waste was placed in sterile 50 ml serological flasks and autoclaved for 20 min 2 times at 121 °C with intermediate incubations at 25 °C for 24 h. The sterile material was moistened with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulphate, 0.5; potassium chloride, 0.5; ferrous sulphate, 0.01; pH 7.3) (Atlas, 2004) to reach 30% moisture content, inoculated with 2×10^6 spores g^{-1} of only native fungal strains (C1) or native plus GEM degrading fungal strains (C2), hermetically sealed with sterile rubber caps and aluminium seal, and then incubated for 5 d at 30 °C with aeration every 48 h. Then, flask were open and pre-inoculated agroindustrial wastes were mixed with 6.65 g of sterile sandy loam soil (2.4% organic matter, 1.4% total organic carbon, 0.063% nitrogen, 0.0023% phosphorous and pH of 8.41) that was previously spiked with 1000 mg kg^{-1} of a mixture of Phe, Pyr and BaP (1:1:1 ratio), the soil was autoclaved in the same way as the agroindustrial waste and inoculated with 2×10^6 CFU g^{-1} of each bacterial strain and sealed hermetically again. This combination resulted in a soil:agroindustrial waste proportion of 95:5. Inoculated agroindustrial waste/contaminated soil mixtures were incubated at 30 °C for 14 d. Headspace samples (1 ml) were taken from each flask every 48 h for CO_2 measurements, and then headspaces were flushed for 10 min with sterile and moistened air to preserve aerobic conditions and avoid carbon dioxide accumulation. Systems with non-contaminated soil were inoculated to determine the effect of PAHs on the growth of microorganisms. Abiotic controls consisting in non-inoculated microcosms systems (PAH-spiked sterile sandy loam + sterile agroindustrial waste) were included to assure that the disappearance of PAHs was caused by biodegradation and to compensate for adsorption losses. All treatments were sampled every 48 h for CO_2 production and at day 14 for PAH quantitation and DNA extraction for PCR-DGGE analyses. All assays were carried out in triplicate.

2.5. PCR-DGGE monitoring

Genomic DNA was isolated from 0.25 g IDM (soil plus agroindustrial waste) using the PowerSoil DNA isolation Kit (MoBio, USA). The RNA polymerase β subunit (*rpoB*) bacterial gene was amplified using primers for *rpoB*1698f (5′- CGC CCC CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C AAC ATC GGT TTG ATC AAC-3′) and *rpoB*2041r (5′-CGT TGC ATG TTG GTA CCC AT-3′) (Dahllof et al., 2000). Amplification conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 51 °C for 1 min, 72 °C for 45 s and a final extension at 72 °C for 10 m. Fungal 18s rRNA was amplified using primers for CG-18sF (5′-CGC CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAT TCC AGC TCC AAT AGC GTA-3′) and 18sR (5′-TCG GCA TAG TTT ATG GTT AAG A-3′). Two-step touchdown amplification consisted of an initial denaturation at 95 °C for 5 min, 10 cycles of 95 °C for 40 s, 67 °C for 30 s (with a decrease of 1 °C per cycle), 72 °C for 30 s and 25 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 5 min. PCR products were verified on 1% agarose gels before DGGE analysis. DGGE was performed in a CBS Denaturing Gradient Gel Electrophoresis System (DGGE-1001; C.B.S. Scientific, USA). PCR products (10 μl) were subjected to electrophoresis for 14 h at 80 V through 8% polyacrylamide gels with a formamide/urea denaturant gradient of 40%–100% at 60 °C. After electrophoresis, gels were silver stained according to Sanguinetti et al. (1994) and photographed.

2.6. Heterotrophic activity and PAH analysis

CO_2 production in microcosms was measured using an Agilent 6890 series gas chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. CO_2 was reported as milligrams of CO_2 per g of initial dry matter (IDM). Residual PAHs were extracted from 1 g of IDM (soil plus agroindustrial waste) with the addition of 25 ml of a dichloromethane-acetone solution (7:3 ratio) using an Anton Paar Multiwave 3000 SOLV apparatus for 20 min, according to the EPA 3546 method. The resulting extracts were evaporated, suspended in 2 ml of acetonitrile and analysed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column with an UV absorbance detector set at 245–360 nm under isocratic conditions in acetonitrile:water (90:10) and a flow rate of 1 ml/min.

2.7. Statistical analysis

Data were analysed with an analysis of variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), and differences with a p value < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Microbial antagonism between PAH-degrading strains

Microbial antagonism tests showed inhibitory effects of *Pseudomonas aeruginosa* strains towards fungi, in particular *P. aeruginosa* B7, which inhibited the growth of all of the evaluated fungal strains (Table 1, Fig. 1A and B), whereas strain B6 only showed an apparent inhibitory effect towards *Rhizomucor variabilis* H9. This inhibition could be explained by the reported production of several

Table 1
Inhibitory activity by antagonism tests between bacteria to fungi and fungi to bacteria strains on Toyama's medium in plate.

	Bacteria to fungi							Fungi to bacteria						
	H6	H7	H9	H15	H19	LiP^{+5}	MnP^{+7}	H6	H7	H9	H15	H19	LiP^{+5}	MnP^{+7}
B1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B6	–	–	±	–	–	–	–	–	–	–	–	–	–	–
B7	+	+	+	+	+	+	+	–	–	–	–	–	–	–
B8	–	–	–	–	–	–	–	+	+	–	–	+	–	–
B10	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B14	–	–	–	–	–	–	–	–	–	–	–	–	–	–

–, No inhibition; ±, Partial inhibition; +, Inhibition.
Tested strains were: *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenotrophomonas maltophilia* B14, *Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, *Aspergillus fumigatus* H19, *Aspergillus niger* LiP^{+5} and *Aspergillus niger* MnP^{+7}.

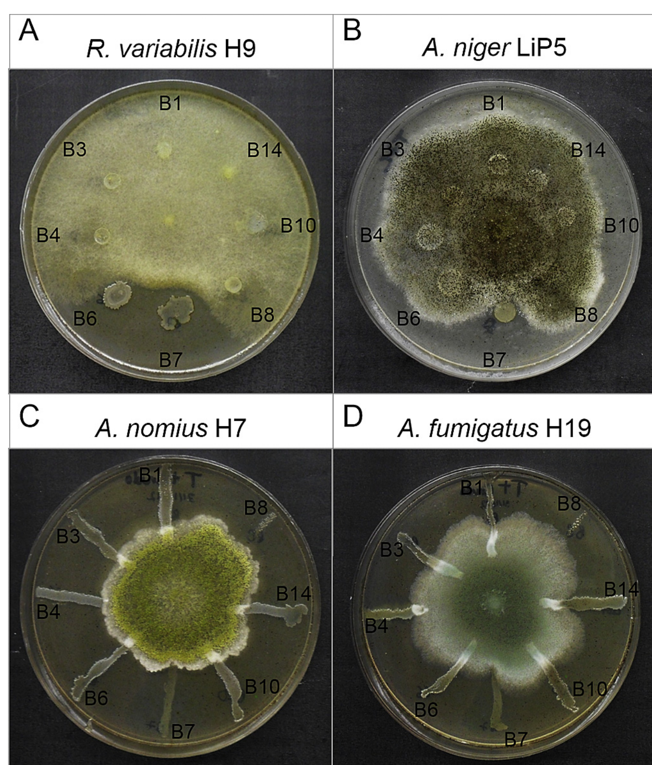

Fig. 1. Growth inhibitory effects from bacteria to fungi (A, B) and fungi to bacteria (C, D) in Toyama's medium plates. Tested bacterial strains were *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenotrophomonas maltophilia* B14.

antimicrobial compounds by PAH-degrading bacteria, which may include wide spectrum antifungals such as indole-3-acetic acid (Mordukhova et al., 2000: 279–85) and phenazine-1-carboxamide (Kumar et al., 2005; Naik and Sakthivel, 2006).

The remaining bacterial strains did not show any antagonistic effect towards fungal strains. An antagonistic effect of *Aspergillus flavus* H6, *Aspergillus nomius* H7 *and Aspergillus fumigatus* H19 towards *Streptomyces* sp. B8 was also observed (Fig. 1C and D). On the other hand, no inhibitory effects were observed between bacteria or between fungi, except for a slight inhibition between *A. niger* strains and *T. asperellum* H15 (Table 2).

The addition of 2% crude oil to Toyama's medium plates did not alter the inhibitory effects observed between strains compared to the condition with no added hydrocarbon. The results indicated that evaluated PAH-degrading strains, with the exception of

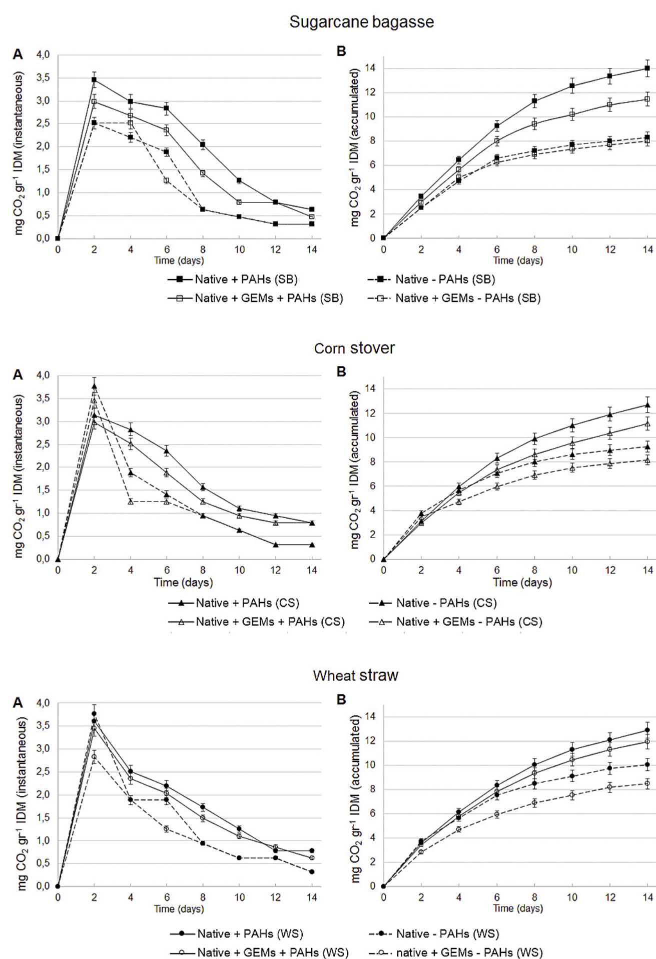

Fig. 2. Instantaneous (A) and accumulated (B) CO_2 production during microbial selection in a soil contaminated with 1000 mg of PAHs kg^{-1} soil, using different agro-industrial wastes as texturizers. (SB): sugarcane bagasse; (CS): corn stover; (WS): wheat straw.

P. aeruginosa B7 and *Streptomyces* sp. B8, were suitable to construct PAH-degrading mixed consortia with no major antagonistic effects between them.

3.2. Effect of PAHs on the microbial growth in solid culture

Common methods for consortia construction often consist of mixing microorganisms, in approximately equal ratios, that were able to transform the compound of interest in prior applications.

Table 2
Inhibitory activity by antagonism tests between Bacteria to bacteria and fungi to fungi on Toyama's medium in plate.

	Bacteria to bacteria									Fungi to fungi						
	B1	B3	B4	B6	B7	B8	B10	B14		H6	H7	H9	H15	H19	LiP^{+5}	MnP^{+7}
B1	–	–	–	–	–	–	–	–	H6	–	–	–	–	–	–	–
B3	–	–	–	–	–	–	–	–	H7	–	–	–	–	–	–	–
B4	–	–	–	–	–	–	–	–	H9	–	–	–	–	–	–	–
B6	–	–	–	–	–	–	–	–	H15	–	–	–	–	–	±	±
B7	–	–	–	–	–	–	–	–	H19	–	–	–	–	–	–	–
B8	–	–	–	–	–	–	–	–	LiP^{+5}	–	–	–	–	–	–	–
B10	–	–	–	–	–	–	–	–	MnP^{+7}	–	–	–	–	–	–	–
B14	–	–	–	–	–	–	–	–								

–, No inhibition; ±, Partial inhibition; +, Inhibition.
Tested strains were: *Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *Bacillus cereus* B4, *Pseudomonas aeruginosa* B6, *Pseudomonas aeruginosa* B7, *Streptomyces* sp. B8, *Klebsiella* sp. B10 and *Stenotrophomonas maltophilia* B14, *Aspergillus flavus* H6, *Aspergillus nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, *Aspergillus fumigatus* H19, *Aspergillus niger* LiP^{+5} and *Aspergillus niger* MnP^{+7}.

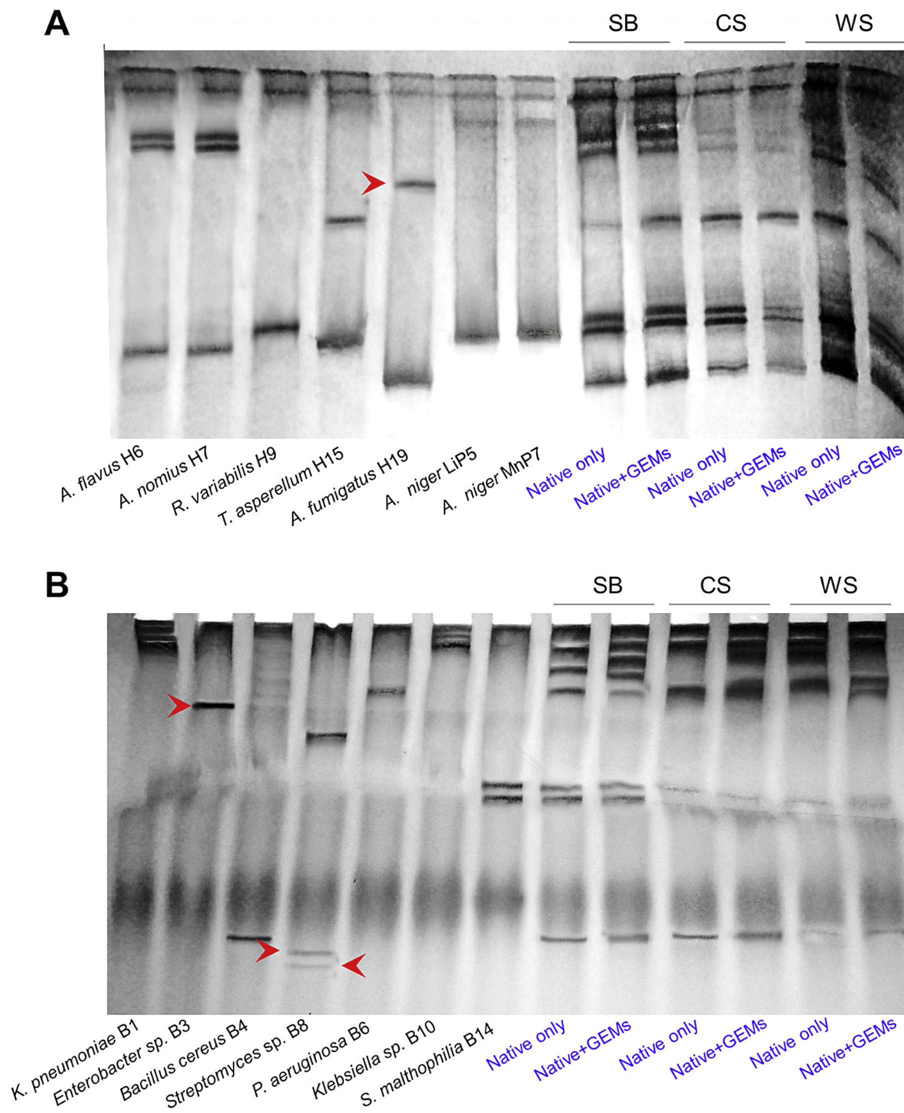

Fig. 3. Survival of inoculated strains in PAH-spiked soils after 14 days. (A) Fungal 18s rRNA and (B) bacterial RNA polymerase β subunit gene (*rpoB*) profiles are shown. Letters at the top of the gel indicate treatments; SB: sugarcane bagasse. CS: corn stover. WS: wheat straw. Red arrows indicate bands disappearing after 14 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although straightforward, this type of selection does not take into account the possible inhibitory effects occurring between inoculated microorganisms nor the survival of each of the strains when applied to the environmental matrix. Simply put, our experiments consisted of an induced natural selection in which the microorganisms better adapted to the new pollution conditions gradually displaced and replaced those organisms less well adapted. Pollution of soils with PAHs may lead to a toxic, but at the same time enriching, effect on soil microbial populations (Gadd, 2007; Gloer, 2007). Toxic effects are effective in the selection of more adapted strains, and enrichment occurs when microorganisms are able to use the pollutant as a source of carbon and energy. As we used sterile soils and inoculated only PAH-degrading/tolerant microorganisms, this selection is expected to have an effect favouring the survival of the most adapted strains for PAH degradation. As observed in Fig. 2, the presence of PAHs in inoculated soil microcosms led to a rapid initial increase in CO_2 levels, producing significantly higher amounts of CO_2 by day 14 than non-contaminated microcosms systems particularly in systems using sugarcane bagasse as texturizer (14 vs 8.3 mg CO_2 gr^{-1} IDM for those inoculated only with native strains, $p = 0.012$ and 11.5 vs 8 mg CO_2 gr^{-1} IDM for those inoculated also with native + GEMs strains, $p = 0.031$). This evidences the utilization of PAHs as a carbon source by the inoculated strains, which is in accordance with previous reports using the same strains (Zafra et al., 2014; Zafra et al., 2015b). It is interesting to observe that microcosms inoculated only with native organisms produced more CO_2 than those inoculated with native plus GEM strains. This could indicate a competition between GEM and native organisms for adaptation in soil, leading to a decrease in CO_2 levels. Systems using sugarcane bagasse for texturizing produced more CO_2 than corn stover- and wheat straw-amended soils, indicating a better texturizing role and possibly providing an alternative carbon source for microbial growth. In fact, sugarcane bagasse and corn stover are known to have a variety of extractable sugars and glucans that can be readily used by microorganisms, especially fungi, during the first stages of growth (Templeton et al., 2010).

3.3. Inoculum survival in PAH-contaminated soils

PCR-DGGE monitoring showed that most of the inoculated fungal strains survived the selection process and prevailed until day

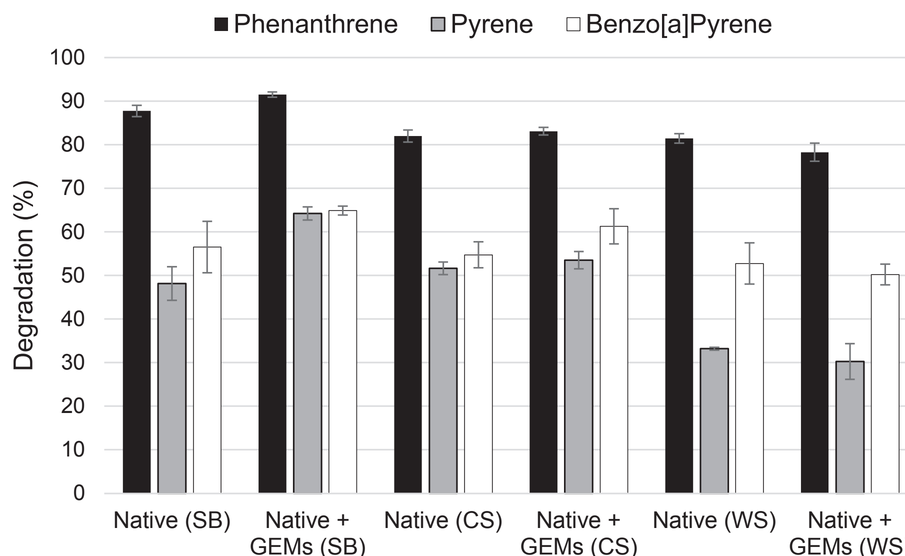

Fig. 4. PAH degradation in solid culture during consortia selection in a soil contaminated with 1000 mg kg^{-1} PAHs after 14 days. SB: sugarcane bagasse; CS: corn stover; WS: wheat straw.

14, except for *Aspergillus fumigatus* H19, which was not detected in any of the microcosms systems (Fig. 3A). Because the H19 strain was found to have moderate tolerance levels to PAHs (Zafra et al., 2014), its disappearance was probably due to a poor adaptation to contaminated soil. *Rhizomucor variabilis* H9 did not survive in the presence of GEM strains and wheat straw, and similarly, GEM strains *Aspergillus niger* LiP^{+5} and MnP^{+7} survived until day 14 when grown with sugarcane bagasse and corn stover but not wheat straw (Fig. 3A), suggesting an important role for agroindustrial waste on the survival of some strains. This was also corroborated when taking into account that wheat straw-amended systems presented the lowest CO_2 production levels (Fig. 2); in addition, wheat straw is known to possess less extractable carbohydrates than sugarcane bagasse and corn stover (Zhang et al., 2011). Attempts of fungal isolation after 14 days were consistent with DGGE results (data not shown). On the other hand, *Enterobacter* sp. B3 and *Streptomyces* sp. B8 DGGE profiles disappeared at day 14 in all microcosm systems. Previous antagonism tests showed inhibition of the B8 strain by several fungal strains, but not inhibition of the B3 strain. As Fig. 3B shows, the remaining five bacterial strains survived until day 14, independently of the presence/absence of fungal GEM strains. Metagenomic data of the bioremediation of a soil contaminated with a mixture of PAHs in microcosms (using a similar set-up as the used in the present study) and inoculated with a consortium composed by native strains H6, H7, H9, H15, B1, B4, B6, B19 and B14 showed a predominance of bacteria over fungal organisms after 30 days of treatment (Zafra et al., 2016). Within bacteria, sequences from *Stenothrophomonas maltophilia* and *Bacillus cereus* were the most abundant after 30 days of treatment, while *Klebsiella* populations showed a tendency to decrease (Zafra et al., 2016). Except for *Rhizomucor variabilis* H9, the microbial species comprising the degrading consortium remained stable across time, with a higher proportion of *Bacillus cereus*, *Klebsiella pneumoniae* and *Aspergillus flavus*.

Taking into account the results from isolation and DGGE, four fungal (*A. flavus* H6, *A. nomius* H7, *R. variabilis* H9, and *T. asperellum* H15) and five bacterial (*K. pneumoniae* B1, *B. cereus* B4, *P. aeruginosa* B6, and *Klebsiella* sp. B10 *S. maltophilia* B14) strains were selected to compose a first degrading microbial consortium, denominated “C1”. A second consortium, denominated “C2”, was constructed using the same strains as C1 plus the *Aspergillus niger* LiP^{+5} and MnP^{+7} GEM strains.

3.4. Degradation of PAHs in soil during consortia selection

While microcosm systems inoculated with only native microorganisms exhibited high degradation levels of PAHs, the addition of GEM strains improved the degradation, particularly of HMW PAHs, in soils biostimulated with sugarcane bagasse and corn stover (Fig. 4). Biostimulation with sugarcane bagasse led to high levels of degradation of Phe, Pyr and BaP during the selection of the C1 consortium (Native), achieving 88%, 48% and 57% degradation out of 333 mg kg^{-1} respectively, after 14 days; however, the highest degradation rates were observed during the selection of the C2 consortium (Native+GEMs), with degradation rates of 92% for Phe, 64% for Pyr and 65% for BaP (Fig. 4). This tendency was also observed when corn stover was used as a texturizing and alternative carbon source, where GEM-inoculated microcosms degraded 83% of Phe, 54% of Pyr and 61% of BaP out of 333 mg kg^{-1}. As the only difference between treatments was the presence of the GEM strains, changes on degradation rates can be directly attributed to them. The use of GEMs in bioremediation has been shown to be an effective way to improve the degradation rates of pollutants (Layton et al., 2012; Urgun-Demirtas et al., 2006). Previous studies with LiP^{+5} and MnP^{+7} strains showed their ability to degrade LMW PAHs in soil (Cortés-Espinosa and Absalón, 2013; Cortes-Espinosa et al., 2011), as both lignin and manganese peroxidases from *P. chrysosporium* are ligninolytic enzymes that are highly effective for the initial oxidation of PAHs (Singh, 2006; Wang et al., 2009). Our results corroborated the involvement of these enzymes in the degradation of both LMW and HMW PAHs in soil. Other enzymes, such as fungal laccases, peroxydases and dioxygenases, were probably involved in PAH oxidation by the consortia, as we reported previously for *Trichoderma asperellum* (Zafra and Cortes-Espinosa, 2015; Zafra et al., 2015a).

Although Phe was degraded at a higher rate than Pyr and BaP, these PAHs were degraded to a higher extent than previous reports using mixed microbial consortia (Jacques et al., 2008; Kim and Lee, 2007). This was likely due not only to the action of fungal enzymes but also to important bacterial mechanisms that are responsible for

PAH oxidation. In fact, most of the inoculated bacteria possessed hydrocarbon-degrading abilities. *Stenotrophomonas maltophilia* is one of the few bacterial organisms able to degrade and use both LMW and HMW PAHs as a sole carbon source (Boonchan et al., 1998; Juhasz et al., 2000), *Pseudomonas aeruginosa* is known to produce PAH-oxidative enzymes and secrete rhamnolipids (Zhao et al., 2011), and *Bacillus cereus* is able to degrade both LMW and HMW PAHs (Mohandass et al., 2012; Tuleva et al., 2005) as well as *Klebsiella pneumoniae* (Ping et al., 2014).

4. Conclusion

In conclusion, we constructed two PAH-degrading microbial consortia. One of them included two GEM strains with remarkably high tolerance levels and high degradation rates of LMW and HMW PAHs. Our results indicate that constructed microbial consortia present a high potential for soil bioremediation by bioaugmentation and biostimulation, and may be effective for the treatment of sites polluted with PAHs and other high molecular weight hydrocarbons due to their elevated tolerance to aromatic compounds, their capacity to utilize them as an energy source and the induced selection process. Further studies testing the degradation capabilities of both consortia in field soils are necessary to better comprehend their bioremediation potential.

Conflict of interest

None declared.

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