PHENANTHRENE BIODEGRADATION BY *Pseudomonas xanthomarina* ISOLATED FROM AN AGED CONTAMINATED SOIL

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Abstract

The bacterial community of a soil contaminated by a mothball chemical industry mainly consisted of *Betaproteobacteria* and *Gammaproteobacteria* (with *Achromobacter* spp. and *Pseudomonas* spp., respectively, as the most representative genera). Upon addition of phenanthrene, species of these two genera were found in the enrichment cultures as well as among the isolated strains. The isolated bacteria were tested for phenanthrene degradation, but only *Pseudomonas xanthomarina,* either as single culture or in a consortium, showed this capability. Here we show that in aged contaminated soils some members of the bacterial community are active in biodegradation processes, can be easily isolated, and may result appropriated for bioremediation uses.

1. Introduction

Since the second half of the 20th century, the contamination of soils by polycyclic aromatic hydrocarbons (PAHs) is a research topic of great interest due to the increasing industrialization of the society, the inadequate waste management policy in most countries, and the negative health effects of these compounds. Nowadays PAHs are widespread pollutants present in soils, waters and air. Sixteen PAHs are considered priority pollutants due to their carcinogenic, mutagenic and teratogenic properties [1]. These widespread pollutants can affect negatively ecosystem functions. For instance, microbial activity has been shown to be drastically altered by PAHs both in terrestrial [2, 3] and aquatic ecosystems [4, 5].

To avoid dispersion and negative environmental and health consequences, the elimination of PAHs residues from contaminated soils becomes crucial. Although PAHs may undergo adsorption, volatilization, photolysis, and chemical degradation,

microbial degradation appears to be the process primarily responsible for the removal of PAHs in a multiphase soil system [6]. The highly aromatic and condensed structure of PAHs leads to very low water solubility, becoming recalcitrant to their biotransformation [7].

In bioremediation strategies appreciable biodegradation efficiency can be reached with a consortium of microorganisms [8, 9]. However, a successful bioremediation seems to rely, among other factors, on the selectivity and specialisation of the added microorganisms. In this sense, recent studies pointed out the need of using microbes pre-selected from their own environment to get an effective biological treatment [10-14].

The aim of this work was: (1) to analyze the bacterial community of an aged contaminated soil characterized by the presence of phenanthrene (PHE) and other two-, three- and four-ring PAHs; (2) to assess the effect of a further PHE amendment on the soil bacterial community; and (3) to isolate bacteria from the enrichment cultures and test for their ability to degrade PHE, in a search for strains appropriated for bioremediation uses.

2. Material and methods

2.1 Chemicals

Phenanthrene (CAS Number 85-01-B) was obtained from Sigma Aldrich (France). Mineral salts were from VWR (France). HPLC-grade acetonitrile, methanol, and chloroform were purchased from Merck (Germany). All reagents were of analytical grade.

2.2. Soil

The soil studied (SLO) was collected from an area located at Llaneras (Oviedo, Northern Spain) severely affected by contamination of a mothball chemical industry and improper waste disposal practices, including fuel oil and lubricants spills. A surface sample (0-20 cm depth) was collected in amber glass containers and stored at 4ºC in the dark until further physico-chemical and microbial assays for isolation of potential PAH-degrading microorganisms were carried out. The sample was a sandy soil with 2% clay, 9% silt, 89% sand, 2.6% organic matter, pH 9.2, and 22.7% calcium carbonate. The quantification of the 16 major EPA PAHs in the soil sample was performed in a previous paper [15] and the concentrations of PAHs are given in Figure 1. Among the 16 PAHs examined PHE and anthracene were present at the highest concentrations followed by naphthalene and fluoranthene (25.3, 24.7, 17.1 and 12.5% of the total PAHs content, respectively). In general, in this soil lower molecular weight PAHs were more abundant than higher molecular weight PAHs and those with 2- and 3-rings accounted for more than 70% of the total PAHs content.

2.3. Characterisation of the soil bacterial community and isolated strains

A suspension containing 0.3 g of soil and 15 ml of sterilized water was maintained under shaking for 1 h and 100 µl of this suspension were inoculated in Petri dishes with two different culture media, TSA and MSB, and incubated at 28ºC for 30 days. Colonies of bacteria morphologically different were isolated following standard microbiological protocols.

Molecular techniques were used for the detection of bacterial communities and the identification of isolated bacteria. DNA was extracted using a Nucleospin Food DNA Extraction Kit (Macherey-Nagel, Germany). The 16S ribosomal RNA gene (16S rRNA)

was used for the identification of bacteria. The 16S rRNA gene was amplified by PCR using the primers 27F (5´-AGA GTT TGA TCC TGG CTC AG) and 1522R (5´-AAG GAG GTG ATC CAG CCG CA). Positive and negative controls were included in all amplification experiments. PCR reactions were performed in 50 µL volumes, containing 1-2 µL of template DNA, 5 µL of 10X PCR buffer Biotaq (Bioline, USA), 1.5 µL of 50 mM $MgCl_2$ (Bioline), 1 µL of 10 mM deoxyribonucleoside triphosphate mixture (dNTPs) (Invitrogen, Carlsbad, California, USA), 0.5 µL of 50 µM of each primer and 0.25 µL of *Taq* DNA polymerase enzyme (Bioline, USA), made up to 50 µL with nuclease-free water (Sigma-Aldrich, USA). PCR thermal conditions were as follow: 95ºC for 60 s; 35 cycles of 95ºC for 15 s, 55ºC for 15 s, 72ºC for 120 s; and a final extension cycle at 72ºC for 10 min. Forward and reverse strands of the amplified DNA fragment were sequenced using an ABI 3700 sequencer (Applied Biosystems, USA).

Sequence chromatograms were edited using the software Chromas, version 2.01 (Technelysium, Australia). The identification of phylogenetic neighbours was carried out by submitting the sequences to BLAST [18] and by using the GenBank database and the EzTaxon-e database [19].

The accession numbers of partial sequences of 16S rRNA bacteria isolated from the contaminated soil, the sequences obtained from the soil 16S rRNA clone libraries, and from the enrichment cultures are listed in Tables 1-3.

2.4. Enrichment and isolation of phenanthrene-degrading bacteria

Tests were performed in triplicate in 20-ml glass flasks closed with Teflon-coated stoppers and sealed with aluminium caps in which fresh contaminated soil (0.3 g) was

113 added to 15 ml of a Mineral Salt Basal medium (MSB) [16] containing a PHE final
114 concentration of 20 mg l^{-1}, which corresponded to 1000 mg kg^{-1} of soil. In general, PHE
115 contamination in soils does not exceed 200-300 mg kg^{-1}, but we have used
116 concentrations up to 5-times higher, since it is common to use an elevated
117 concentration for enrichment to ensure that PHE-degraders were selected.

118 Three experimental sets were performed: set A, containing MSB medium plus soil;
119 set B, which contained PHE in addition to MSB medium and soil; set C, PHE, MSB
120 medium and sterile (autoclaved) soil. Eighteen replicates for each set were incubated in
121 darkness at 28ºC under shaking conditions and were sampled after 0, 3, 7, 14, 30 and
122 45 days (three replicates/sampling day). PHE losses were determined by high-
123 performance liquid chromatography (HPLC) using liquid-liquid hexane extracts of the
124 aqueous solutions. One mL aliquot of the supernatant was placed in 5-mL glass vials,
125 adding 1 mL of HPLC-grade hexane and sealing the vials, which were shaken for 1 min
126 using a Vortex. After 90 min of leaving the vials to stand, PHE was completely
127 recovered in the separated hexane phase and analysed by HPLC. Analysis of all
128 samples was in triplicate, and controls without soil and with autoclaved soil were also
129 performed to monitor any possible interference or loss by adsorption to soil and/or glass
130 during the experiment. PHE samples were analyzed by HPLC, using a Shimadzu HPLC
131 chromatograph equipped with a column Kromasil 100-5-C18, reverse phase, 150 mm x
132 0.40 mm i.d. (Teknokroma, Spain), and coupled to a fluorescence detector (Shimadzu,
133 RF-10A), with excitation and emission wavelengths at 250 nm and 365 nm,
134 respectively. Details of the method were reported by Morillo et al. [17].

135 After incubation of the enriched cultures, aliquots of the cultures were transferred
136 into fresh liquid medium MSB with the same PHE concentration as the sole carbon
137 source. Microbial populations enriched in PHE-biodegraders were obtained from 8 to 24

week-old subcultures. Strains isolation was performed on solid medium prepared in Petri dishes following standard microbiological protocols. Bacteria collected from the contaminated soil were isolated on MSB medium plus PHE.

The isolated strains were also tested for their individual ability to biodegrade PHE in a liquid MSB-PHE medium. The methodology was similar to that previously described for enrichment experiments but without soil and using a lower PHE concentration (1 mg L^{-1}). Samplings at different times along seven days (168 hours) were performed for estimating PHE biodegradation.

Consortia of the different strains isolated from the untreated soil, and from the soil enriched with PHE were also tested. Two consortia were formed, consortium 1 included *Pseudomonas brassicacearum*, *Achromobacter insolitus* and *Advenella kashmirensis*, and consortium 2 containing *Pseudomonas xanthomarina*, *Achromobacter spanius* and *Advenella kashmirensis*.

3. Results

A rapid PHE loss in MSB medium once inoculated with the contaminated soil was observed (Figure 2) and 100% of PHE concentration initially applied disappeared from the aqueous phase in seven days, while the loss was about 60% in the control experiment carried out with sterilized soil. This indicates that PHE losses in this latter case were probably due to adsorption by soil colloids, or precipitation, in addition to adsorption to the glass container, as proposed by Qian et al. [20]. Therefore, about 40% of the loss of PHE observed in the experiments using the contaminated soil was due to

161 microbial degradation, indicating the presence in the contaminated soil of specific and
162 highly efficient PHE-degraders.

163

164 3.1. Composition of the contaminated soil microbial community

165 The phylogenetic information obtained from the 16S rRNA gene library revealed that the
166 indigenous bacterial community from the contaminated soil consisted of *Proteobacteria*
167 and *Actinobacteria*, which comprised 95.7 and 4.3 % of the total soil bacteria,
168 respectively. In addition, *Proteobacteria* were constituted by 17.4% of
169 *Alphaproteobacteria*, 34.8% of *Betaproteobacteria*, and 43.5% of *Gammaproteobacteria*
170 (Table 1). Soil bacteria belonging to *Betaproteobacteria* and *Gammaproteobacteria*
171 accounted for over 78%.

172 The closest genera identification of bacterial sequences, retrieved from 16S rRNA
173 clone libraries included *Achromobacter*, *Massilia*, *Pseudorhodoferax, Diaphorobacter*
174 and *Parapusillimonas* within *Betaproteobacteria*; *Pseudomonas* and
175 *Pseudoxanthomonas* within *Gammaproteobacteria*; *Brevundimonas, Sphingomonas*
176 and *Rhizobium* within *Alphaproteobacteria*, and *Gaiella* within *Actinobacteria*.

177 As the most relevant for this study, the *Gammaproteobacteria* sequences were
178 affiliated to *Pseudomonas* spp. (six sequences out of 23), and *Pseudoxanthomonas*
179 (four sequences). Eight sequences corresponded to *Betaproteobacteria*, from which
180 three were affiliated to *Achromobacter*.

181

182 3.2. Isolation and characterization of cultivable bacterial strains

Bacteria were grown in plates with MSB medium, alone or with PHE, and the isolates, of varied colony morphology, were identified. The closest taxonomic affiliations of bacterial sequences obtained from soil isolates are shown in Table 2. Analysis of 16S rRNA gene sequences showed that three isolates belonged to *Betaproteobacteria* (*Achromobacter spanius*, *Achromobacter insolitus* and *Advenella kashmirensis*), and three to *Gammaproteobacteria* (two *Pseudomonas xanthomarina,* and one *Pseudomonas brassicacearum*). The cultivable soil bacteria belonging to *Betaproteobacteria* and *Gammaproteobacteria* accounted for 100%. All these strains were used for further degradation tests.

3.3. 16S rRNA clone libraries of enriched cultures

The soil sample was enriched with PHE as an additional source of carbon and energy. The closest taxonomic affiliations of bacterial sequences obtained from 16S rRNA clone libraries from enrichment cultures are shown in Table 3. The enrichment enabled us to obtain five clones of *Advenella*, five of *Achromobacter*, and one clone of *Pseudomonas*.

3.4. Biodegradation of phenanthrene by isolated strains and consortia

Studies of the factors controlling PHE biodegradation and particularly on the individual ability of different strains of *Achromobacter*, *Pseudomonas* and *Advenella* to degrade PHE showed that only two strains of *P. xanthomarina* had the capability to fully degrade PHE, process accomplished in 168 hours with a residual 0.20% of PHE, which denotes an almost complete biodegradation (Fig. 3). Both *P. xanthomarina* strains shared 99.93% identity of 16S rRNA gene sequence level.

From the two consortia formed, only consortium 2 including *P. xanthomarina* was able to degrade PHE, at rates similar to those *P. xanthomarina* alone (Fig 3.).

4. Discussion

An indigenous microbial community involved in the degradation of PAHs already exists in contaminated soils [4, 9, 11-14, 21-25] and the microorganisms have adapted and acclimatized to PAHs degradation. Several authors reported a wide variety of bacteria capable of metabolizing PAHs including the genera *Acidovorax*, *Acinetobacter, Aeromonas*, *Alcaligenes, Arthrobacter, Beijerinckia*, *Brevibacterium*, *Flavobacterium, Micrococcus*, *Mycobacterium, Mycoplana*, *Nocardia, Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Streptomyces, Vibrio,* etc. [13, 25-27].

The abundance of *Pseudomonas* strains in the studied soil, as shown in Tables 1-3 denotes the ability of members of this genus to growth in soils contaminated by PAHs. *Pseudomonas* and *Achromobacter* are specialised PAH-degraders [21, 26, 28]. We isolated two strains of *P. xanthomarina* involved in PHE degradation as showed the biomineralization assays (Fig. 3). The fast disappearance of PHE upon incubation indicates that this bacterium should be active in the degradation of PHE in the contaminated soil. However, the two *Achromobacter* strains (Table 3) were unable to degrade PHE, which suggests that they benefit from the utilization of PHE degradation compounds.

Pseudomonas xanthomarina type strain is phylogenetically closely related to the *Pseudomonas stutzeri* cluster. Mulet et al. [29] reported that *P. xanthomarina* was the only species included within a *P. stutzeri* consensus phylogenetic branch. *Pseudomonas xanthomarina* shares with *P. stutzeri* the basic phenotypic traits of the

231 species, and could be considered a genomovar of *P. stutzeri*. However, Romanenko et
232 al. [30] based on a few additional tests (inability to hydrolyse starch, ability to grow at
233 4ºC and in the presence of 8% NaCl, and fatty acid composition) justified the erection of
234 *P. xanthomarina* as a novel species. In any case, it appears that *P. stutzeri* and *P.*
235 *xanthomarina* share similar mechanisms for PAHs degradation.

236 Ortega-Calvo et al. [31] reported that a *P. stutzeri* strain degraded naphthalene,
237 anthracene and PHE. Zhao et al. [22] isolated a *P. stutzeri* strain from soil samples
238 contaminated with PAH-containing waste from an oil refinery field in Shanghai, China.
239 The strain utilized naphthalene and PHE as its sole carbon source and the authors
240 suggested that their strain of *P. stutzeri* can endure high concentrations of PHE and
241 have great potential in bioremediation application.

242 Mulet et al. [23] collected 343 isolates from seven different sand samples taken
243 from beaches contaminated by the *Prestige* oil tanker accident in Galicia in
244 northwestern Spain, from which 86 were affiliated to *Pseudomonas* on the basis of 16S
245 rRNA gene RFLP profiles and partial sequencing of the 16S rRNA gene. The highest
246 number of isolates (43) was affiliated with the species *P. stutzeri*. Most of the *P. stutzeri*
247 isolates (62%) were obtained by direct plating or after enrichment on mineral medium
248 with the growth substrate naphthalene, in a similar way as we isolated the two *P.*
249 *xanthomarina* strains with PHE.

250 Mixed populations with different enzymatic capacities are required to degrade PAHs
251 [31]. However, commensalism phenomenon existing between bacteria appeared to be
252 the reason for the activation of other bacteria that were not able to degrade PHE. Some
253 intermediary metabolites might be produced by degraders and the accumulated
254 metabolites can then serve as carbon sources for other bacteria, allowing extensive
255 mineralization of the substrate by complex bacterial population. Likely this is the

process occurring for bacteria other than the *P. xanthomarina* strains in the contaminated soil studied.

The inoculation of competent bacteria (bioaugmetation) has been successfully used in the removal of several pollutants over the last few years [32-35], which suggests that bioremediation could be a cost-effective clean-up technology to treat contaminated soils and sediments. Interestingly, in the present work the whole microbial community (Fig. 2) and a few of the isolated strains (Fig. 3) showed a remarkable PHE degradation capability in laboratory conditions. However, this capability should also be tested in decontamination on site or at pilot scale, studying also their effect on the rest of PAHs present in the contaminated soil.

To summarize, in a soil contaminated with PAHs, the addition of further amounts of PHE sparked the metabolism of the indigenous bacterial community, indicating the presence of a PAH-degrading community in the soil already active and involved in PHE degradation. *Pseudomonas xanthomarina* is the bacterium responsible for the fast PHE degradation found in our experimental work. This bacterium has not previously been reported to degrade PAHs, although this capability is common in the phylogenetically closely related P. *stutzeri*. Our data show that in aged contaminated soils some members of the bacterial community actives in biodegradation processes, can be easily isolated and may result appropriated for optimizing engineered bioremediation of PAHs contaminated soil.

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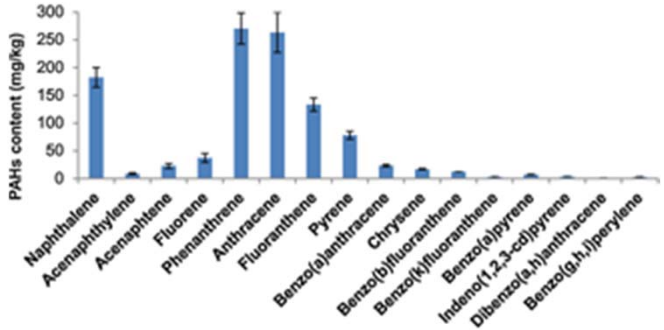
Figure legends

Figure 1. Levels of 16 EPA polycyclic aromatic hydrocarbons (mg kg^{-1} dry weight basis) in the contaminated soil [15].

Figure 2. Phenanthrene degradation in the presence of sterilized (SLO^{*}) and non-sterilized soil (SLO) in MSB medium.

Figure 3. Degradation of phenanthrene (PHE) by *Pseudomonas xanthomarina* (PX) alone (MSB + PHE + PX) and in consortium 2 (MSB + PHE + CONSORTIUM) *versus* control (MSB + PHE).



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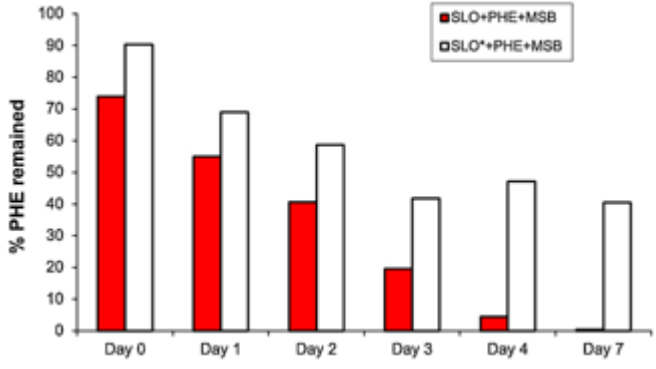

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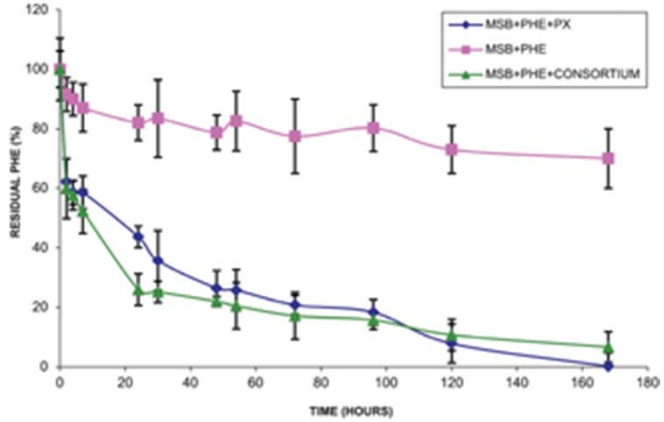

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