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Isolation and substrate screening of polycyclic aromatic hydrocarbon degrading bacteria from soil with long history of contamination

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ABSTRACT

Microbial degradation is a promising soil remediation strategy for polycyclic aromatic hydrocarbons (PAHs) frequently polluting some post-industrial environments. Thirteen PAH-degrading bacterial strains were isolated from bare or ryegrass-vegetated aged-contaminated soil, based on their potential for phenanthrene biodegradation. 16S rRNA gene phylogeny showed that all isolates were affiliated with three closely related taxonomic subgroups within the *Pseudomonas* genus. Two of these subgroups were exclusively retrieved from planted soil. Genes encoding PAH-ring hydroxylating dioxygenases were detected in all strains and matched known sequences in other *Pseudomonas* strains from polluted environments. Genes for protocatechuate-3,4-dioxygenases (*pca*H) and catechol-2,3-dioxygenases were also detected in all strains, whereas the catechol-1,2-dioxygenase gene was absent. The presence of *pca*H genes, the utilization of protocatechuate, the strong inhibitory effect of salicylate and the detection of phthalate during phenanthrene biodegradation suggest that these isolates preferentially catabolize PAHs *via* the protocatechuate pathway. Metabolic profiling was further performed for three representative isolates on a large range of 61 organic substrates. Although closely related phylogenetically, they were able to use different sets of labile carbon compounds (e.g. sugars, amino acids), PAHs and their metabolites, and released different degradation products from phenanthrene. These contrasted metabolic capabilities might reflect differential adaptation to their respective environment.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common organic pollutants consisting of two or more fused aromatic rings produced during the incomplete combustion of organic matter. They are found notably in waste residues from coal-fired power plants and coking and steelwork industries. They are ubiquitous in environments from industrialized areas, including soils. Owing to their high persistence and their toxic, mutagenic and carcinogenic properties, PAHs represent a significant environmental and public health risk. Among them, phenanthrene (PHE) is a highly reactive three-ring compound frequently found in polluted sites. Phenanthrene is the smallest PAH featuring a sterically hindered bay-region and a K-region in the molecule, and is therefore often used as a model compound to investigate PAH metabolism by microbial communities. During the last decade, different strategies have been considered to remediate PAH-contaminated soils, including physical, chemical and biological techniques (Biache et al., 2008; Gan et al., 2009; Usman et al., 2012). Microbial degradation represents a promising route for PAH depollution to achieve complete degradation at a lower cost, with greater safety and less soil disturbance compared to physicochemical treatments. PAH-degrading soil microorganisms cover a wide phylogenetic diversity, and bacteria belonging to *Alpha-*, *Beta-* and *Gamma-proteobacteria*, *Firmicutes* and *Actinobacteria* with this metabolic property have been described (Cerniglia, 1993). Bacterial pathways for aerobic PAH catabolism have been characterized. They involve initial breakdown of the pollutants through the action of PAH-ring hydroxylating dioxygenases (PAH-RHD) (Peng et al., 2008), leading

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to common central mono-aromatic intermediates (such as catechol or protocatechuate) which are further converted to metabolites that can be used for microbial growth (Fuchs et al., 2011). Two distinct routes for aerobic bacterial catabolism of phenanthrene are well documented, *via* either salicylate or phthalate and protocatechuate (Habe and Omori, 2003; Peng et al., 2008); they involve enzymes such as catechol dioxygenases or protocatechuate dioxygenases.

Plant-assisted PAH dissipation (rhizo-remediation) is another promising line of research. However, contradictory impacts of plants or exudates on overall microbial degradation of PAHs have been reported, ranging from a stimulatory effect to no-change or even inhibition (Cébron et al., 2011; Phillips et al., 2012; Liu et al., 2013). Indeed, plant root exudates can modify the abundance, activity and diversity of PAH-degrading microorganisms (Corgié et al., 2003; Kirk et al., 2005; Cébron et al., 2009, 2011; Louvel et al., 2011) thus leading to diminished PAH removal. Therefore, further studies are required to better characterize the PAH-degrading bacterial populations and their metabolic potential. The aims of the present study were (i) to isolate phenanthrene-degrading bacteria from an aged PAH-contaminated soil and document the effect of vegetation on this cultivable community; (ii) evaluate the degradation potential of the new isolates towards other organic compounds and (iii) gain insights into the metabolic pathways these isolates use to degrade PAHs.

2. Material and methods

2.1. Sample source

A soil sample from heap of a former coking plant site (Neuves-Maisons, France) was dried, sieved to 2 mm and stored in the dark until experimental set-up. Soil characteristics were detailed elsewhere (Cébron et al., 2009). Briefly, soil texture was 12.1% clay, 22.1% silt, and 65.8% sand, pH was about 7.0–7.5, C/N ratio was about 23.0–25.1 and organic carbon content was 62.6 ± 3.2 g kg^{-1} soil dry weight. This soil is mostly contaminated with aged PAHs (1260 mg Σ16PAHs kg^{-1}) and to a lesser extent with trace metals. Fluoranthene, pyrene and benzo-b-fluoranthene are the dominant PAHs in this soil, and phenanthrene concentration is about 90 mg kg^{-1}. Eight ryegrass seeds (*Lolium multiflorum*, Italian Ryegrass, Podium variety, LG seeds, France) were sown in triplicate pots containing 40 g of soil freshly recontaminated with phenanthrene (250 mg kg^{-1}). Seeds were allowed to germinate for 3 days at room temperature in the dark before transfer to a growth chamber (22°C/18 °C day/night, 80% relative humidity, c.a. 250 μmol photons m^{-2} s^{-1}, 16 h photoperiod). Triplicate control pots without ryegrass were prepared in the same way. Soil water content was adjusted to 80% of its water-holding capacity with sterile distilled water by weighing the pots. After six days, bulk soil samples were pooled from triplicate microcosms and immediately used for inoculation.

2.2. Enrichment, isolation and culture of phenanthrene-degrading bacteria

Two enrichment cultures were prepared in 250 ml glass flasks containing 50 ml of Buschnel-Hass broth (BH; Sigma–Aldrich) and 1 g of soil, either unplanted or ryegrass-vegetated. Immediately before the experiment, 0.5 ml of 100 mg ml^{-1} phenanthrene in acetone was added to each flask (final concentration 1 mg ml^{-1}). Flasks were left open for 10 min in a sterile hood to ensure acetone evaporation. Cultures were agitated in the dark at 24 °C, 150 rpm. After 8 days, 0.5 ml of each suspension was transferred to new 50 ml BH with phenanthrene flasks for 6 days. The surface of BH agar plates was sprayed with 500 μl of 10 mg ml^{-1} phenanthrene in acetone. After acetone evaporation, plates were inoculated with 200 μl of ten-time dilutions of bacterial enrichment cultures, and incubated in the dark at 24 °C. Colonies showing a clearing halo were purified twice by streaking on Buschnell-Hass solid medium sprayed with phenanthrene as above. For long-term storage, strains were stored in 10% dimethyl sulfoxide at −80 °C. Liquid cultures of phenanthrene-degrading isolates were performed in 60 ml glass tubes containing 15 ml of BH supplemented with 3.3 mg ml^{-1} phenanthrene, and agitated in the dark at 24 °C. After two weeks, cultures were centrifuged for 15 min at 4500 g. Pellets and supernatants were frozen separately at −20 °C until used.

2.3. Genomic DNA extraction

Genomic DNA from phenanthrene-degrading isolates was extracted from cell pellets resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), supplemented with 0.1% SDS and 0.2 mg ml^{-1} proteinase K (Euromedex), and incubated for 30 min at 37 °C. After phenol/chloroform extraction, DNA was recovered from the aqueous phase by isopropanol precipitation, rinsed with 70% ethanol, dried for 5 min in a vacuum concentrator (Centrivap Jouan RC1010, ThermoScientific) and dissolved in 100 μl PCR-grade water (Gibco, Life Technologies).

2.4. Gene amplification, sequencing and sequence analysis

The genes coding for 16S rRNA, PAH-ring hydroxylating dioxygenase (PAH-RHDα), protocatechuate-3,4-dioxygenase (*pca*H), catechol-1,2-dioxygenase (C12O) and catechol-2,3-dioxygenase (C23O) were amplified by PCR using Taq DNA polymerase (Fermentas). Details are given in Table Supp1. Amplification products were visualized on 1% (w/v) agarose gel. For 16S rDNA and PAH-RHDα, amplicons were purified using the E.Z.N.A. Cycle Pure kit (Omega Bio-Tek). 16S rDNA amplicons were sequenced in both directions using the primers 27F and 1492R. PAH-RHDα amplicons were sequenced in one direction using the primer PAH-RHDα GN F. Sequences were edited and assembled in SeqTrace (Stucky, 2012). BLASTN search (Madden, 2002) and SINA alignment online (Pruesse et al., 2012) were used to retrieve closely related 16S rRNA sequences in the reference nr nucleotide database. All 16S rRNA sequences were aligned using the L–INS–i algorithm in MAFFT (Katoh, 2002). The alignment was manually edited in MEGA6 (Tamura et al., 2013), and a final version comprising 1333 ungapped positions was used to infer a maximum-likelihood phylogenetic tree. For PAH-RHDα sequences, BLASTN and BLASTX searches were used to determine the best blast hit in the nr nucleotide and protein collections, respectively. 16S rRNA and PAH-RHDα sequences were deposited in the GenBank database (KT318812-KT318824 and KT318825-KT318836, respectively).

2.5. Metabolic profiling with Biolog™ Ecoplates

Isolates were grown shaking in LB broth (Sigma–Aldrich) overnight at 24 °C. Cells were centrifuged for 10 min at 4500 g and washed three times in sterile 0.85% NaCl solution. Suspensions were adjusted to optical densities of 0.15 at 600 nm. Biolog™ EcoPlates (Awel Innovations, France) were filled with 100 μl of bacterial suspension in each well, and incubated in the dark at 24 °C in a sealed plastic bag containing wet cotton to avoid desiccation. The absorbance at 595 nm was monitored in a Xenius spectrophotometer (SAFAS, Monaco) over 6 days at t = 0, 16.5, 23, 47, 72 and 144 h. Absorbance values obtained for the blank well were subtracted to all other values. Substrate utilization was defined by $A_{595} > 0.25$ after 144 h of incubation (Garland, 1997). The final