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Characterisation of the phenanthrene degradation-related genes and degrading ability of a newly isolated copper-tolerant bacterium☆

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ABSTRACT

A copper-tolerant phenanthrene (PHE)-degrading bacterium, strain *Sphingobium* sp. PHE-1, was newly isolated from the activated sludge in a wastewater treatment plant. Two key genes, *ahdA1b-1* encoding polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase (PAH-RHDα) and *xylE* encoding catechol-2,3-dioxygenase (C23O), involved in the PHE metabolism by strain PHE-1 were identified. The PAH-RHD gene cluster showed 96% identity with the same cluster of *Sphingomonas* sp. P2. Our results indicated the induced transcription of *xylE* and *ahdA1b-1* genes by PHE, simultaneously promoted by Cu(II). For the first time, high concentration of Cu(II) is found to encourage the expression of PAH-RHDα and C23O genes during PHE degradation. Applying *Sphingomonas* PHE-1 in PHE-contaminated soils for bioaugmentation, the abundance of *xylE* gene was increased by the planting of ryegrass and the presence of Cu(II), which, in turn, benefited ryegrass growth. The best performance of PHE degradation and the highest abundance of *xylE* genes occurred in PHE-copper co-contaminated soils planted with ryegrass.

1. Introduction

Soil contamination by organic pollutants and heavy metals is a global environmental issue due to rapid industrialisation and urbanisation. Polycyclic aromatic hydrocarbons (PAHs), among the most widespread organic pollutants in the environment, are of great concern for their persistence, chronic toxicity and accumulation throughout the food web (Gondek et al., 2008; Macek et al., 2000). Different to PAHs, heavy metals including copper are non-degradable, stay stabilized in soils for long-term, accumulate in vegetables, harm microbes by interfering with enzymes and DNA at high concentration, and often co-exist with organic contaminants in various environmental media (Guzik et al., 2010; Sokhn et al., 2001). It is even worse when PAHs and heavy metals co-exist, leaving the higher potential risks to human health and ecosystems. However, the establishment of effective methods to reduce the levels of these pollutants is a major challenge. Bioremediation, the introduction of allochthonous strains (called bioaugmentation) to degrade organic pollutants (Peng et al., 2008), has received increasing attentions because of its high potential for *in situ* or on-site treatments, which is low cost, high safety and no requirements for secondary waste treatment.

The success of biodegradation depends greatly on the characteristics of allochthonous bacteria. Heavy metals can inhibit the biodegradation of organic pollutants by impacting both the physiology and ecology of degrading microorganisms (Ibarrolaza et al., 2009; Sandrin and Maier, 2003; Shen et al., 2006; Thavamani et al., 2012a, 2012b, c). For example, the activity of catechol dioxygenase is inhibited in the presence of some heavy metals (Guzik et al., 2010). Bioaugmentation with bacteria exhibiting heavy metal tolerance and PAHs degrading capability is suggested as a potentially cost-effective strategy for the remediation of PAHs-metal co-contaminated soil (Thavamani et al., 2011). To date, more than 40 species of PAHs-degrading bacteria have been isolated from different environments (Gan et al., 2009; Zhang et al., 2004), e.g. *Acinetobacter calcoaceticus* (Zhao and Wong, 2009), *Sphingomonas* sp. (Gou et al., 2008), *Pseudomonas* sp. (Kazunga and Aitken, 2000), *Mycobacterium* sp. (Dandie et al., 2004; Zeng et al.,

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2010), *Rhodococcus* sp. (Song et al., 2011), *Achromobacter xylosoxidans* (Al-Thani et al., 2009), *Microbacterium* sp. (Sheng et al., 2009). and *Alcaligenes faecalis* (Xiao et al., 2010). However, only PAHs-degrading abilities are revealed for most of these strains and little is known about whether their PAHs degrading performance can be maintained or encouraged in the presence of heavy metals (Wang et al., 2011).

Some key PAH dioxygenase genes in bacteria involved in PAHs metabolism are typically used as indicators, attributing to their substrate-specificity, high conservation, and direct link to the functions of PAHs biodegradation (Baldwin et al., 2003). Microorganisms can adapt to the stress of organic pollutants by regulating the expression of degradation-related genes, and the degradation efficiency depends largely on the activities of enzymes encoded by the functional genes. The initial PAHs dioxygenase (PAH-RHD) and catechol-2,3-oxygenase (C23O) have been identified as the two key PAHs-degrading enzymes. They participate in the initial step of PAHs metabolism via the incorporation of molecular oxygen into the aromatic nucleus and the complete cleavage of the aromatic ring of the intermediate metabolites, respectively. Therefore, identifying the catabolic genes encoding these enzymes would significantly contribute to understanding the mechanism and mediating bacteria involved in the service of improving the degradation efficiency (Mrozik et al., 2003).

The activities of PAHs-degrading bacteria and the functional genes are often promoted in rhizospheric soils due to the root exudates and root deposition (Lin et al., 2006). In turn, the growth of bacteria in the rhizosphere can increase host plant tolerance to abiotic stress by improving nutritional status, inhibiting plant disease, and degrading toxic xenobiotic substances (Peng et al., 2015). Ryegrass is usually selected as the model plant for treating hydrocarbon-contaminated soils for its fibrous root system with a large surface area near the soil surface (Xu et al., 2013). In previous studies, the combination of ryegrass and microorganisms performed well in the biodegradation of soil PAHs, petroleum and pesticides (Rezek et al., 2008; Tang et al., 2010; Xie et al., 2012). However, limited information is available on the influence of ryegrass planting on the microbial degradation of organic pollutants in the co-presence of heavy metals (Sandrin and Maier, 2003).

In the present study, phenanthrene (PHE) was selected as a model PAH given its ubiquity in nature and typical characteristics of PAHs, such as K region and bend structure. This work involved three objectives: (1) to test the Cu(II) tolerance and PHE-degrading ability of bacterial strain isolated from a wastewater treatment plant; (2) to characterise the phylotype and expression of PAH-RHD and C23O genes of the newly isolated strain; and (3) to study its potential in the remediation of PHE-copper co-contaminated soils with ryegrass planting.

2. Materials and methods

2.1. Enrichment, isolation and PHE degradation test of PHE-degrading bacteria

Activated sludge (20 g) from a wastewater treatment plant was added to an Erlenmeyer flask with 200 mL of sterile deionised water and shaken for 30 min at 180 rpm and 30 °C. Five millilitres of this suspension was transferred into 95 mL of mineral salt medium (MSM) with 100 mg/L PHE as the sole carbon source and subsequently incubated on a rotary shaker (180 rpm) for 4 days at 30 °C. The following enrichment cycles were performed by transferring 5 mL of the enrichment culture from the preceding enrichment cycle into fresh MSM supplemented with 100 mg/L PHE every 4 days. After isolating the pure PHE-degrading microorganisms by spreading serially diluted enrichment culture samples onto MSM agar plates containing 100 mg/L PHE, high-performance liquid chromatography (HPLC) analysis was applied to evaluate PHE degradation rate in liquid culture medium. Among all the isolated bacterial strains, one strain was selected for further study because of its high PHE-degrading ability.

The PHE-degrading ability of the isolated strain was tested by incubation in fresh MSM with initial PHE concentration of 0, 300, 500, 600, 700, 800 and 900 mg/L, respectively. The effect of copper on PHE degradation was investigated by incubating the strain in fresh MSM containing 100 mg/L PHE and Cu(II) (as $CuCl_2$) concentration of 0, 0.81, 1.61, 2.42, 3.22, 4.03, 4.84, or 5.64 mM. Culture without inoculum was used as a sterile control to assess the abiotic loss of PHE. The residual PHE was analysed by HPLC immediately after sampling.

For the treatments assessing the transcription of PAH-RHD and C23O genes, the isolated strain was inoculated in the MSM with 100 mg/L PHE and Cu(II) concentration of 0, 0.81, 2.42 and 4.03 mM for 24 h, or with 100 mg/L PHE only for 12, 18, 24 and 48 h, respectively. MSM supplemented with glucose (no PHE) was used as the blank control. For each treatment, 3% (v/v) ($OD_{600} \approx 2.0$) inocula were inoculated initially in triplicates. All the incubations were conducted on a rotary shaker (180 rpm) at 30 °C without light.

2.2. Characteristics of the isolated strain

2.2.1. Identification of isolated strain, PAH-RHD and C23O genes

The isolated strain was identified by 16S rRNA sequencing. After DNA extraction with the PowerSoil kit (MO BIO Laboratories, USA), the 16S rRNA genes were amplified in accordance with the procedures described previously (Song et al., 2015). The purified polymerase chain reaction (PCR) products were ligated into vector pEASY-T1 and transformed into *Escherichia coli* DH5α. Plasmids were extracted and sequenced as described previously (Jiang et al., 2015). Sequence similarity searches and alignments were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (National Center for Biotechnology Information) and Molecular Evolutionary Genetics Analysis (MEGA 5.1). Sharing 99% identity with the nucleotide sequences of *Sphingobium abikonense* NBRC 16140, the isolated strain was named as *Sphingobium* PHE-1.

The PAH-RHD and the C23O genes were amplified using the primers listed in Table S1 with genomic DNA of *Sphingobium* PHE-1 as the template. The primers were designed based on the previously reported PAH-RHDα and C23O genes. DNA amplification was performed by the following PCR program: 95 °C for 2 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and final extension at 72 °C for 10 min. The PCR products were checked by agarose gel electrophoresis (1.2%). The amplicons were further cloned, sequenced and subjected to phylogenetic analysis as mentioned above.

2.2.2. Analysis of the PAH-RHD gene cluster

The PAH-RHD gene cluster of *Sphingobium* PHE-1 was obtained by self-formed adaptor PCR (SEFA-PCR) as described previously (Wang et al., 2007). The primers used in this study were presented in Table S1. SEFA-PCR was conducted by the following procedures. Firstly, a single cycle with primer SP3 and the genomic DNA of *Sphingobium* PHE-1 as template was carried out as follows: 95 °C for 1 min, 94 °C for 30 s, 30 °C for 3 min, 70 °C for 5 min and 72 °C for 5 min. The 30-μL PCR mixture was prepared with 15 μL of 2×GC buffer I, 4 μL of dNTP (2.5 mM), 1 μL of SP3 (5 μM), 0.3 μL of LA-Taq and 1 μL of template (about 50 ng/μL). Secondly, 1 μL of primer SP1 (25 μM) was added to the reaction mixture and 25 cycles of PCR were performed as follows: 94 °C for 30 s and 70 °C for 5 min. Thirdly, eight cycles of thermal asymmetric PCR were carried out with the following program: one cycle of 94 °C for 30 s, 50 °C for

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