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Characterization of polycyclic aromatic hydrocarbons degradation and arsenate reduction by a versatile *Pseudomonas* isolate

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ABSTRACT

A *Pseudomonas* isolate, designated PAHAs-1, was found capable of reducing arsenate and degrading polycyclic aromatic hydrocarbons (PAHs) independently and simultaneously. This isolate completely reduced 1.5 mM arsenate within 48 h and removed approximately 100% and 50% of 60 mg l^{-1} phenanthrene and 20 mg l^{-1} pyrene within 60 h, respectively. Using PAHs as the sole carbon source, however, this isolate showed a slow arsenate reduction rate (4.62 μM h^{-1}). The presence of arsenic affected cell growth and concurrent PAHs removal, depending on PAH species and arsenic concentration. Adding sodium lactate to the medium greatly enhanced the arsenate reduction and pyrene metabolism. The presence of the alpha subunit of the aromatic ring-hydroxylating dioxygenase (*ARHD*) gene, arsenate reductase (*arsC*) and arsenite transporter (*ACR3(2)*) genes supported the dual function of the isolate. The finding of latter two genes indicated that PAHAs-1 possibly reduced arsenate via the known detoxification mechanism. Preliminary data from hydroponic experiment showed that PAHAs-1 degraded the majority of phenanthrene (>60%) and enhanced arsenic uptake by *Pteris vittata* L. (from 246.7 to 1187.4 mg kg^{-1} As in the fronds). The versatile isolate PAHAs-1 may have potentials in improving the bioremediation of PAHs and arsenic co-contamination using the plant-microbe integrated strategy.

1. Introduction

With the rapid urbanization and industrialization in many countries, more attention has been paid to sites co-contaminated by polycyclic aromatic hydrocarbon (PAHs) and heavy metals. This concern is especially obvious in China (Sun et al., 2011a). Among this type of co-contamination, the simultaneous occurrence of PAHs and arsenic in the environment is of great public health concern, since both of them are potentially immunotoxic, mutagenic, and carcinogenic (Haritash and Kaushik, 2009; Camacho et al., 2011). Mixtures of PAHs and arsenic are introduced into the environment via many ways, including combustion of fossil fuels, extensive use of creosote oil and wood preservatives containing chromated copper arsenate (CCA), as well as runoff from hazardous waste sites (Maier et al., 2002; Elgh-Dalgren et al., 2011). In recent years, well-known PAHs and arsenic co-contaminated sites, such as old wood preservation sites, coking and chemical industry sites, mining and metallurgy industry sites, and even urban residential communities, are frequently found in different regions (Priha et al., 2001; Lambert and Lane, 2004; Elgh-Dalgren et al., 2009, 2011; Sun et al., 2011b). Epidemiological studies on interactions of PAHs and arsenic have demonstrated a synergy between them, thereby potentiating their harmful effects on humans and animals (Maier et al., 2002; Lau and Chiu, 2006). Therefore, it is essential to develop feasible and economical strategies to remediate arsenic and PAHs co-contaminated soils.

The remediation of PAHs and arsenic co-contaminated soils is considered a complex problem because of the considerable differences in their chemistry. Thus, different chemical processes and remediation technologies are required for the treatment of the two pollutants (Elgh-Dalgren et al., 2011). Although soil washing of PAHs and arsenic, which uses both single and mixtures of different additives, is the only available method, many limitations exist in this approach, especially because it often generates large amounts of soil leachates with increased toxicity (Elgh-Dalgren et al., 2009).

Bacteria-mediated degradation has been presented as a promising alternative to conventional physico–chemical methods to remove recalcitrant PAHs from contaminated sites. A wide range of aerobic and anaerobic PAHs-degraders have been isolated (Lu et al., 2011). On the other hand, phytoextraction using arsenic hyperaccumulators such as Chinese brake fern (*Pteris vittata* L.) has been

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recognized as an effective and inexpensive technology in the decontamination of arsenic-contaminated sites (Mathews et al., 2010; Sun et al., 2011b). Successful applications of this approach in the remediation of arsenic contamination in hydroponic and pot systems have been well documented (Shelmerdine et al., 2009; Mathews et al., 2010; Yang et al., 2012). We hypothesize that using *P. vittata* in conjunction with PAH-degrading bacteria could represent an ideal solution to the remediation of PAHs and arsenic co-contamination.

Because of the arsenic toxicity, PAH-degraders without arsenic resistance might not survive under PAHs and arsenic co-contaminated condition, making these isolates unfit candidates for remediation of PAHs and arsenic co-contaminated environments. Moreover, arsenate is a known structural analogue to phosphate, which may inhibit arsenate uptake by *P. vittata* (Wang et al., 2002). Therefore, PAH-degraders with the ability of transforming arsenate to arsenite might play a more important role in the remediation of PAHs and arsenic co-contamination in conjunction with *P. vittata*.

Given the lack of reports on such versatile microorganisms, our objectives were to (1) isolate and characterize a bacterial strain capable of simultaneously degrading PAHs and reducing arsenate, and (2) evaluate the potential of remediating PAHs and arsenic co-contamination by the isolate in conjunction with *P. vittata*.

2. Materials and methods

2.1. Chemicals and media

Phenanthrene and pyrene were purchased from Fluka (Germany) with the purity greater than 96%. Each PAH stock solution was 5×10^4 mg l^{-1} in 1,2-dichloroethane. High purity As(III) (As_2O_3) and As(V) (Na_3AsO_4) were purchased from Beijing Chemical Reagents Company (Beijing, China). Stock solutions of 126.3 mM As(III) and 400.0 mM As(V) were prepared by dissolving As_2O_3 and Na_3AsO_4 in 1.0 M sodium hydroxide and distilled water, respectively. All stock solutions were filtered through a 0.22 μm syringe filter (Nuclepore Corp., CA, USA). All other solvents and chemicals used were of analytical reagent grade or better.

A mineral medium adapted from chemically defined medium (CDM) described by Weeger et al. (1999), herein referred to as modified CDM (MCDM), was used for the enrichment and isolation of bacteria. The medium composition was modified by adding equal amount of sodium chloride instead of sodium lactate to the defined medium. Agar (1.5%) was added to make a medium plate. The MCDM medium was also used to determine the PAHs degradation in the presence of individual arsenic compounds and As(V) reduction by the isolate. Evaluations of arsenic resistance and reduction potential of the isolate in the absence of PAHs were conducted using the CDM medium. When required, 2X medium was diluted to 1X with autoclaved distilled water and filter-sterilized As stock solution followed by pH adjustment to 7.2 with NaOH or HCl.

2.2. Strain isolation

Soil samples (0–15 cm in depth) used for bacterial enrichment were collected from an aged coking plant in Shanghai, China. The average background concentrations of all 16 PAHs defined by US Environmental Protection Agency and total arsenic were 51.6 and 400.2 mg kg^{-1} dry weight, respectively. Approximately 10 g of homogenized sample was inoculated into a 250-ml flask containing 100 ml MCDM, supplemented with 0.2% (w/v) yeast extract, 100 mg l^{-1} phenanthrene, 50 mg l^{-1} pyrene, and 2 mM As(V). The flask was shaken at 150 rpm at 30 ± 1 °C for 7 days. Subsequently, 10 ml of the enrichment culture was transferred to fresh medium and this procedure was repeated three times. One aliquot (100 μl) from the culture was plated onto PAHs-coated MCDM agar plates containing 2 mM As(V). Selections of positive PAH-degrading and As(V)-reducing isolates were made by dioxygenase activity examination (Guo et al., 2010) and As(V)-reducing ability analysis using a qualitative $KMnO_4$ method (Salmassi et al., 2002).

2.3. Evaluations of PAHs utilization, arsenic resistance and arsenate reduction

2.3.1. As(III) and As(V) resistance

The minimum inhibitory concentration (MIC) was defined as the lowest arsenic concentration in the medium that completely inhibited the growth of the isolate. MICs of the isolate were determined according to the method described by Weeger et al. (1999). The concentrations of As(III) and As(V) added to the CDM broth varied from 1 to 20 and 1–35 mM, respectively. The sensitivity of the MIC determination was 1 mM.

2.3.2. PAHs degradation and arsenate reduction assays

The time-courses of microbial growth and arsenate reduction by PAHAs-1 in PAHs-free solution were determined in CDM growth media containing 1.5 mM As(V). Cultures of the isolate grown in CDM for 2 days were harvested, washed, and re-suspended in fresh CDM. Each flask, except for the un-inoculated control, was inoculated with a 1 ml aliquot of the cell suspension to give an initial optical density (OD) of about 0.1 at 600 nm.

To examine the effect of arsenic species and dose on PAHs degradation by isolate PAHAs-1, the same amount of inoculum in sterile MCDM was inoculated into 100- ml flasks containing 29 ml of MCDM broth supplemented with mixed PAHs (60 mg l^{-1} phenanthrene and 20 mg l^{-1} pyrene) only or mixed PAHs with different levels of arsenic (0.8 and 2.4 mM for As(III) and 1.5 and 4.5 mM for As(V)). To accelerate the simultaneous As(V) reduction and PAHs removal, additional experiments were conducted in which the cell growth was stimulated by the addition of 200 mg l^{-1} sodium lactate to the MCDM medium. Bacterial growth was monitored by measuring OD_{600}, and pH changes in the solution were monitored by a pH meter. Sterile flasks subject to the same treatments except for inoculation served as abiotic controls. For quality control purposes, unless otherwise indicated, experiments on cultures spiked with PAHs and arsenic mixtures were performed in sextuplicates. All other treatments were performed in triplicates. Cultures were incubated at 150 rpm at 30 ± 1 °C for 60 h in dark. At selected intervals, samples from both the experiment groups and controls were retrieved for analyses.

2.4. Characterization of PAHAs-1 and detection of functional genes

To amplify nearly full-length 16S rRNA gene, universal primers 27F and 1492R (Azhari et al., 2010) were used (Table 1). The forward primer AC114F (Wilson et al., 1999) and reverse primer PAH-RHD_α GN 916R (Cébron et al., 2008) were selected to amplify the gene that encodes the alpha subunit of the aromatic ring-hydroxylating dioxygenase (*ARHD*). The PCR amplification of three arsenite transporter genes (*arsB*, *ACR3(1)*, and *ACR3(2)*), and the cytosolic *arsC* gene was performed using primers and conditions previously described. All PCR reactions were performed in 50 μl reaction volume containing 10 ng of DNA, 0.4 μmol l^{-1} of primers and 25 μl Premix Taq Version 2.0 (Takara, Japan).

PCR products excised from 1.5% agarose gels were purified using the Gel Extraction Kit (SBS Genetech, Shanghai, China) according to the manufacturer's instructions and directly sequenced using the same primers and an ABI 3730xl DNA analyzer (Applied