Biodegradation (2016) 27:1–13
DOI 10.1007/s10532-015-9750-5

ORIGINAL PAPER

Isolation and characterization of heavy polycyclic aromatic hydrocarbon-degrading bacteria adapted to electrokinetic conditions

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Received: 27 February 2015 / Accepted: 18 November 2015 / Published online: 28 November 2015

Abstract Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria capable of growing under electrokinetic conditions were isolated using an adjusted acclimation and enrichment procedure based on soil contaminated with heavy PAHs in the presence of an electric field. Their ability to degrade heavy PAHs under an electric field was individually investigated in artificially contaminated soils. The results showed that strains PB4 (*Pseudomonas fluorescens*) and FB6 (*Kocuria* sp.) were the most efficient heavy PAH degraders under electrokinetic conditions. They were re-inoculated into a polluted soil from an industrial site with a PAH concentration of 184.95 mg kg^{-1}. Compared to the experiments without an electric field, the degradation capability of *Pseudomonas fluorescens* and *Kocuria* sp. was enhanced in the industrially polluted soil under electrokinetic conditions. The degradation extents of total PAHs were increased by 15.4 and 14.0 % in the electrokinetic PB4 and FB6 experiments (PB4 + EK and FB6 + EK) relative to the PB4 and FB6 experiments without electrokinetic conditions (PB4 and FB6), respectively. These results indicated that *P. fluorescens* and *Kocuria* sp. could efficiently degrade heavy PAHs under electrokinetic conditions and have the potential to be used for the electro-bioremediation of PAH-contaminated soil, especially if the soil is contaminated with heavy PAHs.

Keywords Heavy polycyclic aromatic hydrocarbons · Degrading bacteria · Electric field · Industrially polluted soil

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in nature due to numerous polluting anthropogenic activities (Samanta et al. 2002; Kaushik and Haritash 2006). They are recognized as a potential health risk due to their toxicity, mutagenicity and carcinogenicity (IARC 1984). Although PAHs can be removed by photolysis, chemical oxidation, volatilization and adsorption, microbial degradation is the major process for the elimination of PAHs from contaminated soil because it is cost-effective and environmentally acceptable (Straube et al. 2003; Haritash and Kaushik 2009). Recently, bioremediation has been

shown to be effective in soils contaminated with light PAHs (compounds containing three or fewer aromatic rings), and a number of bioremediation programs have had success in the decontamination of light PAH–contaminated sites (Banerjee et al. 1995; Delgado-Balbuena et al. 2013). However, the level of success of bioremediation for heavy PAHs (compounds containing four or more aromatic rings) is lower, especially with mixed heavy PAHs at high concentrations. To enhance the biodegradation of heavy PAHs, electro-bioremediation, a hybrid technology of bioremediation and electrokinetics, has garnered increasing interest (Niqui-Arroyo and Ortega-Calvo 2007; Li et al. 2012; Saichek and Reddy 2003; Suni and Romantschuk 2004).

Contaminant bioavailability and the presence of suitable microorganisms are critical factors in the process of electro-bioremediation. The sorption, sequestration and heterogeneous distribution of pollutants can lead to their persistence in soil (Reid et al. 2000). Harbottle et al. (2009) found that electrokinetics could overcome these factors via contaminant desorption and redistribution on both micro- and macro-scales. Furthermore, the introduction of specialized microorganisms has been considered a valuable tool for increasing the biodegradation extent of PAHs. To date, many specialized PAH-degrading microorganisms have been isolated and applied in PAH removal, including *Pseudomonas* sp. (El-Naas et al. 2009), *Mycobacterium* sp. (Pagnout et al. 2007), *Rhodococcus* sp. (Song et al. 2011), *Kocuria* sp. (Ahmed et al. 2010), *Neptunomonas* sp. (Li and Chen 2009), *Sphingomonas* sp. (Miguel et al. 2009) and *Cycloclasticus* sp. (Kasai et al. 2002). However, changes in soil properties (e.g., pH and moisture content) and electrochemical reactions, which can be rapidly caused by an applied electric field, may have some adverse effects on biodegradation and contaminant behavior (Acar and Alshawabkeh 1993). Liu et al. (1997) reported that the electrolysis of pore fluids occurred at both electrodes, leading to pH changes and the production of toxic reactive oxygen or chlorine species that were considered to be the main reasons for antimicrobial effects of low DC fields near electrodes when a DC field was applied to wet soil via inert electrodes. Because of their stable aromatic structures, the absence of suitable endogenous microbial populations, and incompatible environmental conditions (unsuitable pH, lack of essential nutrients (N, P), lack of oxygen for aerobic degradation, low temperature, and low moisture content), heavy PAHs are more resistant to biodegradation and have therefore been persistent in the environment for many years (Gan et al. 2009; Covino et al. 2010). Thus, in order to enhance the efficiency of electro-bioremediation, it is important to obtain heavy PAH–degrading microorganisms that can adapt to electrokinetic conditions.

Microorganisms are very sensitive to the changes of environment and can rapidly respond when faced with such variability (Andreoni et al. 2004). Microbial communities that have been chronically exposed to PAHs tend to be enriched for species capable of using PAHs as carbon and energy sources (Gallego et al. 2007; González et al. 2011). The process of soil electro-remediation comprises electroosmosis, electromigration, electrophoresis and electrochemical reactions, which can cause changes of soil properties. When an electric field is applied during soil remediation, the microbial activity and community will be directly or indirectly affected by the electric current or changes of soil properties, and microbial composition will shift towards those organisms adapted to the soil conditions in the presence of an electric field (Lear et al. 2004; Wick et al. 2010). Therefore, both soil contaminated with heavy PAHs and an electric field are necessary to obtain heavy PAH–degrading microorganisms that can adapt to electrokinetic conditions.

In this study, an adjusted acclimation and enrichment procedure, based on soil contaminated with heavy PAHs and an electric field, was used to obtain heavy PAH–degrading bacteria adapted to electrokinetic conditions. Efficient heavy PAH degraders were screened by comparing their ability to degrade heavy PAHs in artificially contaminated soil in the presence of an electric field. Investigations were then carried out to further characterize the most efficient heavy PAH–degrading microorganisms identified. Finally, experiments to assess the degradation ability of the microorganisms in industrially polluted soil were conducted, and the results suggested a potential practical application for the isolates in the electro-bioremediation of heavy PAH–contaminated soil. This study offered a method to isolate and investigate those strains capable of efficiently degrading heavy PAHs in the presence of an electric field.

Materials and methods

Soil

Two types of soil were used in this study: a clean soil artificially contaminated with PAHs and a polluted soil from an industrial site with a known historic contamination by heavy PAHs.

The clean soil was collected from the surface horizon (0–10 cm) in National Field Research Station of Shenyang Agroecosystems (Shenyang, China), which was classified as silk loam (USDA Soil Taxonomy). Selected characteristics of the soil are summarized in Table 1. After the removal of debris and plant roots, the soil was air-dried at room temperature and passed through a 2-mm mesh sieve. To prevent the activity of indigenous microorganisms, the soil was sterilized by autoclaving it three times at 103.4 kPa and 121 °C for 30 min (Wolf et al. 1989; Carter et al. 2007). Autoclaving was interspersed with incubation of the soil at 25 °C for 24 h. The artificially contaminated soil was created by adding an acetone solution of individual PAHs to the sterilized soil and then completely evaporating the acetone in a fume hood. During evaporation, the contaminated soil was mixed continuously to ensure homogeneous PAH distribution and allow reproducibility in repeated experiments. The PAH concentration was verified by the analysis of at least four replicates and determined 1 month after spiking.

The industrially polluted soil was a sandy loam (USDA Soil Taxonomy) sampled from the top 10 cm layer of soil surrounding an iron and steel industrial site in Benxi, China (Li et al. 2011). Selected characteristics of the soil are provided in Table 1. The soil used for isolation of the PAH-degrading bacteria was sieved to 2 mm and kept in the dark at 4 °C prior to use. The soil used for the degradation experiments was air-dried, sieved to 2 mm and sterilized three times by autoclaving it at 103.4 kPa and 121 °C for 30 min (Wolf et al. 1989; Carter et al. 2007). Autoclaving was interspersed with incubation of the soil at 25 °C for 24 h. After sterilization, the total concentration of PAHs was 184.95 mg kg^{-1} dry soil (the sum of 11 US Environmental Protection Agency (EPA) priority PAHs). The concentration and percentage of the individual PAHs are listed in Table 2.

Chemicals and medium

A mixture of 16 PAHs standard (US EPA priority PAHs) and individual PAH standards with a purity of 98–99 % were purchased from JK Chemical Ltd., Beijing, China. All solvents and chemicals used were of analytical grade or better.

A mineral salt medium was used for acclimation, enrichment, isolation and screening of the PAH-degrading bacteria adapted to an electric field. The mineral salt liquid medium contained (per liter) 1.0 g $(NH_4)_2SO_4$, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 1.0 g $MgSO_4$, 0.1 g $CaCl_2.H_2O$, 5 mg $FeCl_3{\cdot}6H_2O$, and 1 mg $(NH_4)_6Mo_7O_2{\cdot}4H_2O$. The medium pH was 7.0–7.2. The mineral salt solid medium was prepared by adding 18.0–20.0 g agar into 1000 ml mineral salt liquid medium.

Luria–Bertani (LB) media was used for culture of highly concentrated bacterial suspensions. The LB liquid media contained (per liter) 10 g of tryptone, 5 g of yeast extract, 10 g NaCl, and the solution pH was adjusted to 7.0–7.4 with sodium hydroxide (10 %). LB solid medium was prepared by adding 18.0–20.0 g agar into 1000 ml LB liquid medium.

Table 1 Characteristics of the soils used in the experiments

Analytical determination	Clean soil	Polluted soil
Sand (2–0.05 mm)(%)	23.5	64.9
Silt (0.05–0.002 mm)(%)	57.5	21.9
Clay (<0.002 mm) (%)	19.0	13.3
Organic matter (g kg^{-1})	19.2	17.7
Total nitrogen (g kg^{-1})	1.2	0.73
Total phosphorus (g kg^{-1})	0.21	0.46
pH	6.68	8.40
Cation exchange capacity ($cmol_c$ kg^{-1})	22.52	13.14
Zeta potation (mV)	−44.26	−19.92

Table 2 Concentration and percentage of individual PAH in historically polluted soil after sterilization

Polycyclic aromatic hydrocarbons	rings	Concentration (mg/kg)	Percentage (%)
Fluoranthene (Flu)	4	27.85 ± 0.32	15.1
Pyrene (Pyr)	4	29.40 ± 0.33	15.9
Benzo(a)anthracene (BaA)	4	19.82 ± 0.31	10.7
Chrysene (Chr)	4	20.26 ± 0.16	11.0
Benzo(b)fluoranthene (BbF)	5	24.39 ± 0.36	13.2
Benzo(k)fluoranthene (BkF)	5	10.30 ± 0.25	5.6
Benzo(a)pyrene (BaP)	5	27.78 ± 0.55	15.0
Dibenzo(a,h)anthracene (DahA)	5	1.09 ± 0.02	0.6
Indeno(1,2,3-cd)pyrene (IcdP)	6	14.52 ± 0.12	7.9
Benzo(g,h,i)perylene (BghiP)	6	9.53 ± 0.20	5.2
Total PAHs	–	184.95 ± 1.51	–

Electrokinetic setup

The electrokinetic setup was prepared as reported by Li et al. (2012). It contained a Perspex soil cell (24 cm length × 12 cm width × 10 cm height), two pairs of column-shaped graphite electrodes (15 cm length × 0.5 cm diameter), a control system for the electrode polarity and a DC power supply (Fig. 1). During experimental preparations, the soil was rehydrated to a moisture content of approximately 25 % (w/w) with a sterilized mineral salt liquid medium. The moist soil was then placed into the reactor cell in layers, and each layer was tamped into the cell using an aluminum pestle to minimize the void space. The final density and porosity of artificially contaminated soil were 1.41 g cm^{3-} and 47.42 %, and those of industrially polluted soil were 1.85 g cm^{3-} and 32.91 %, respectively. Two pairs of electrodes were placed in the soil at either end of the soil bed (8 cm height), and a direct electric current (1 V cm^{-1}) was applied to the soil bed. The two electrodes changed polarity every 12 h to decrease the changes in the soil pH and moisture, leading to more rapid contaminant mineralization and an increase of soil enzyme activity (Harbottle et al. 2009). All experiments were conducted at room temperature (25 ± 1 °C), which was controlled by air conditioning. Three parallel sampling lines along the length of the soil bed, each with three equally spaced sampling points, provided a total of nine soil samples per sampling round. Samples (10 mm diameter × 8 cm height) were collected using a metal tubing (10 mm inner diameter). For each sampling round, nine soil samples were thoroughly mixed together to form a composite sample before analysis.

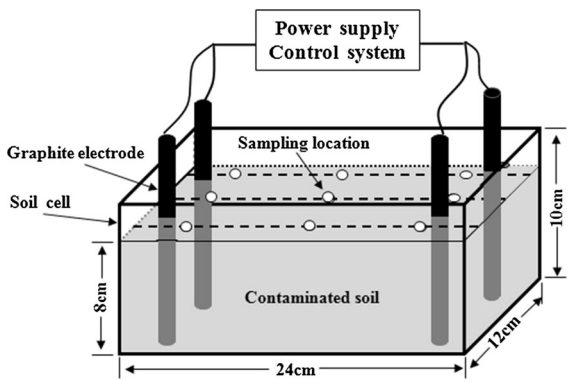

Fig. 1 Schematic representation of the experimental setup

Acclimation, enrichment and isolation of the PAH-degrading bacteria

The industrially polluted soil for isolation of the PAH-degrading bacteria was inoculated with mineral salt liquid medium at 25 ± 1 °C under electrokinetic conditions. Soil moisture was routinely determined by drying the soil samples (20 g) at 105 °C to a constant dry weight and kept at 25 % (w/w) with sterilized mineral salt liquid medium. After 2 months, appropriate dilutions of the enriched sample were spread on a mineral salt solid medium and incubated at 25 °C (Kiyohara et al. 1982; Tian et al. 2008). Before use of the mineral salt solid medium, 185.0 mg l^{-1} (w/v) mixed PAHs extracted from the contaminated soil was dissolved in acetone, and then 0.5 ml of the solution was spread on the surface of the medium as

the sole carbon source. Different strains were identified by their colony characteristics, and their growth was determined by colony counts. Based on the colony characteristics, bacteria that grew well were isolated and subsequently maintained on mineral salt solid medium supplemented with PAHs.

Screening of efficient heavy PAH degraders

To screen the efficient heavy PAH degraders adapted to an electric field, the degradation ability for individual heavy PAHs was studied for the isolated bacteria in soil artificially contaminated with different PAHs. The degradation experiments for each bacterium were performed with (FB2 + EK, PB4 + EK, FB6 + EK and FB7 + EK) and without electrokinetic conditions (FB2, PB4, FB6 and FB7). The negative control (EK) was conducted under electrokinetic conditions. All experiments were performed in triplicate. The isolated bacteria were grown in LB liquid media on a shaker at 28 °C, 160 rpm and collected by centrifugation when the growth reached the exponential phase. After washing twice, they were resuspended in 0.85 % normal saline to obtain a highly concentrated bacterial suspension for the experiments. A clean soil sample was artificially contaminated by individually spiking with one of eight heavy PAHs [including Fluoranthene (Flu), Pyrene (Pyr), Benzo(a)anthracene (BaA), Chrysene (Chr), Benzo(b)fluoranthene (BbF), Benzo(k)fluoranthene (BkF), Benzo(a)pyrene (BaP), Dibenzo(a,h)anthracene (DahA)], each at an initial concentration of 100 mg kg^{-1}. Inoculation was conducted by mixing a bacterial suspension directly into the PAH-spiked soil during soil sample preparation. The initial concentration of bacteria was about 6.32×10^8 colony-forming units (cfu) g^{-1} dry soil. The soil moisture was kept at 25 % using mineral salt liquid medium. Soil samples were taken from the chambers for further analysis of PAH degradation at 60 days.

Identification of efficient heavy PAH degraders

The morphological and physiological characteristics of efficient heavy PAH degraders were identified by the bacterial colonies, gram staining, spore staining and flagellar staining (John 1989). Gram staining was carried out by using a standard Gram reaction procedure (Huker and Conn 1923). Spores were stained with malachite green (Collins and Lyne 1984). A silver-plating stain for flagella was used according to the method of Macnab (1977). The stain results were examined using an optical microscope (Olympus, Japan) and a scanning electron microscope (KYKY-100B).

The molecular characterization of efficient heavy PAH degraders was based on 16S ribosomal RNA (rRNA) gene sequence analysis. Genomic DNA was obtained from efficient heavy PAH degraders using the EasyDNA Kit (Invitrogen). The 16S rRNA from PAH-degrading isolates was amplified by PCR using genomic DNA with primers 27F and 1492R (Zhang et al. 2009). The PCR mixtures (25.0 µl) consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM of each deoxynucleoside triphosphate, 20.0 µM each of forward and reverse primer, 1.0 U of Taq^{TM} DNA polymerase (TaKaRa), and ~2.5 ng of DNA template. DNA amplification was performed in a Peltier Thermal Cycler (Bio-RAD DNA Engine) programmed as follows: 2 min of denaturation at 94 °C; followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; with a final elongation at 72 °C for 10 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen Ltd., Maryland, USA). The purified PCR product was sequenced by BGI Sequencing. The sequences were submitted to the GenBank database to align with published sequences using NCBI BLASTN.

Microbial activity of efficient heavy PAH degraders in industrially polluted soil

The degradation capability of the isolates that most efficiently degraded heavy PAHs under electrokinetic conditions in the artificially contaminated soils was further investigated in an industrially polluted soil from the surroundings of an iron and steel industrial site in Benxi, China. Degradation experiments with each bacterium were performed with (PB4 + EK, FB6 + EK) and without electrokinetic conditions (PB4, FB6). The amount of each bacterium inoculated into the industrially polluted soil was about 1.2×10^7 cfu g^{-1} dry soil during soil sample preparation. The experiments only with electrokinetics were used as a negative control (EK). All experiments were performed in triplicate. Soil samples were removed from the chambers at 0, 10, 20, 30, 40, 50 and 60 days

after the electric field was applied. Counts of efficient heavy PAH degraders, the concentration of individual PAHs and total PAHs were monitored during the experiments.

PAH analysis

Extraction of total PAHs was carried out according to EPA Standard Method 3550C (USEPA 1996). Freeze-dried soil samples (1 g) were thoroughly mixed with Na_2SO_4 and transferred into 100-ml Teflon tubes, which were filled with 30 ml dichloromethane/acetone (1:1, v/v). Each sample was extracted for 40 min in an ultrasonic bath in which the water temperature was below 35 °C. The tubes were then centrifuged for 10 min at 5000 rpm to separate the supernatant from the soil samples. After three cycles of extraction and centrifugation, the extracts were combined and completely evaporated under a gentle stream of nitrogen, then redissolved in 1 ml of acetonitrile. PAHs were analyzed using high performance liquid chromatography (HPLC, Waters) equipped with a variable wavelength fluorescence detector (FLD, waters 2475) and a Waters PAHs Column (250 × 4.6 mm I.d., 5 μm particle size). Prior to injection, the extract was filtered through a 0.22 μm Teflon filter. In all cases, 10.0 μl of sample was injected into the HPLC with an autosampler. The column temperature was 25.0 °C. The gradient elution program was, at a flow rate of 1.0 ml min^{-1}, as follows: for the first 12 min, a mixture of 60 % water and 40 % acetonitrile was used as the solvent; during the next 11 min, acetonitrile in the mixture was linearly increased to 100 % and maintained for 11 min; finally, the solvent composition was returned to the initial conditions over the next 5 min.

The extent of the PAH degradation (D %) was calculated using the formula: D % = 100 % (Ci–Cn) Ci^{-1}, where Ci was the initial concentration of PAH, and Cn was the final concentration of PAH in each treatment.

Soil microbial counts

A modified spray-plate technique based on Kiyohara et al. (1982) and Tian et al. (2008) was used to quantify the PAH-degrading bacteria. For each soil sample, 5 g was combined with 45 ml sterile water and shaken (160 rpm) for 2 h in a 150-ml conical flask. After the mixture settled for 30 min, the upper 1 ml was removed and decimal dilutions were prepared. Stock PAHs solutions were prepared at a concentration of 0.2 mg ml^{-1} acetone for enumeration of the PAH-degrading bacteria. Acetone solutions (0.5 ml) of PAHs were sprayed onto the plates, and 0.1 ml of the diluted soil subsample was added to each plate with PAHs after acetone volatilization. After incubation at 30 °C for 3 weeks in the dark, enumeration of PB4 was calculated by a hand-held long wave (366 nm) UV lamp and FB6 colonies were counted according to the morphological properties and colour of single colonies. Bacterial counts were calculated per gram of air dry soil and log-transformed ($\log_{10}^{\times}$) to improve the normal distribution of the continuous probability distribution of the data.

Statistical analysis

All analytical determinations of the PAH concentrations and bacterial counts were performed in triplicate, and means were calculated. All data obtained in the study are presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS for Windows (Version 11.5). Statistical significance was tested at $P < 0.05$ using Tukey multiple comparisons test.

Nucleotide sequence accession numbers

Using the BankIt sequence submission tool, the 16S rRNA sequences of *Pseudomonas fluorescens* PB4 and *Kocuria.* sp. FB6 were deposited in the GenBank database under the accession numbers KF444431 and KF444432, respectively.

Results

Isolation and screening of PAH-degrading bacteria

Four bacteria (coded FB2, PB4, FB6 and FB7) that grew well were isolated from an enriched sample on the basis of their distinct morphological characteristics. Their ability to degrade individual heavy PAHs (including Flu, Pyr, BaA, Chr, BbF, BkF, BaP and DahA) were then tested in artificially contaminated soil under electrokinetic conditions (Fig. 2). Figure 2 shows that four bacterial strains can degrade heavy PAHs in the presence and absence of an electric field.

However, their ability to degrade PAHs was different under an electric field. The ability of PB4 and FB6 to degrade Flu, Pyr, BaA, Chr, BbF, BkF, BaP and DahA was significantly enhanced, while it decreased for FB2; there was only a slight increase for FB7. The degradation extents of 8 individual heavy PAHs were 19.1–32.9 % in the PB4 + EK experiment, which were 8.8–16.8 and 12.8–18.4 % higher than in FB4 and EK experiments, respectively. Likewise, the degradation extents of eight individual heavy PAHs (25.6–37.3 %) in FB6 + EK experiment were 15.4–28.6 and 21.4–33.0 % higher than in FB6 and EK experiments. While for FB7 + EK experiment, the degradation extents of eight individual heavy PAHs were only 0.3–2.4 and 6.0–13.5 % higher than that for the FB7 and EK experiments. Compared to the FB2 experiment, the degradation extents of 8 individual heavy PAHs decreased 0.6–2.6 % in FB2 + EK experiment. The results showed that strains PB4 and FB6 had a good ability to degrade heavy PAHs in the presence of an electric field. Therefore, PB4 and FB6 were studied in more detail and used for subsequent experiments on the electro-remediation of an industrially polluted soil.

Identification of efficient heavy PAH degraders

Morphological and biochemical observations (Table 3) were used in combination with nearly complete 16S rRNA gene sequence data to identify PB4 and FB6. Neighbor-joining analysis indicated that the closest relatives of strains PB4 and FB6 were

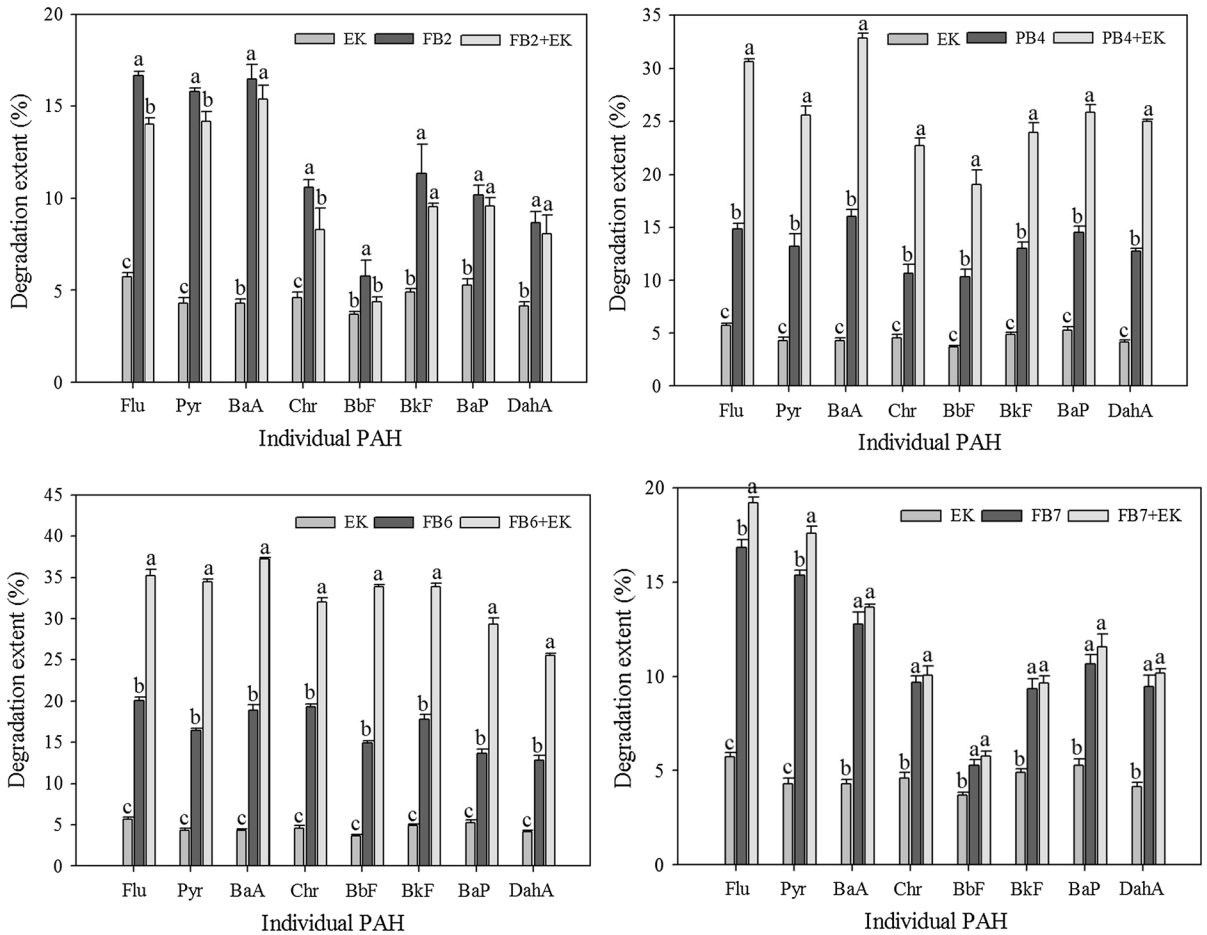

Fig. 2 Ability of isolates to use different individual heavy PAHs in artificially contaminated soil (▭ EK: only in the presence of an electric field; ▬ FB2, FB4, FB6 and FB7: inoculated FB2, FB4, FB6 and FB7, respectively; ▭ FB2+EK, FB4+EK, FB6+EK and FB7+EK: inoculated FB2, FB4, FB6 and FB7 in the presence of an electric field, respectively)

Table 3 Comparison of morphological and physiological characteristics of strains PB4 and FB6

Characteristic	*P. fluorescens*	*P. fluorescens* PB4	*K. rosea*	*K.* sp. FB6
Color of colonies	White, transparent	White, transparent	Pink or orange	Pink
Morphology	Rod-shaped	Rod-shaped	Coccoid	Coccoid
Motility	+	+	−	−
Gram straining	−	−	+	+
Spore formation	−	−	−	−
Catalase activity	+	+	+	+
Oxidase test	+	+	+	+
Nitrate reduction	−	−	+	+
Indole production	−	−	−	−
Methyl red	−	−	+	+
Voges-Proskauer reaction	−	−	−	−
Citrate utilization test	−	−	−	−
Acid production from:				
D-Glucose	+	+	−	−
Sucrose	+	+	−	−

+ Positive reaction; − negative reaction

Pseudomonas fluorescens strain NFL16 (99.83 %) and *Kocuria* sp. TK 815 (99.92 %), respectively. Strains PB4 and FB6 were closely related to *Pseudomonas fluorescens* and *Kocuria* sp.

Activity of efficient heavy PAH degraders in industrially polluted soil

The growth of PB4 and FB6 and the total degradation of PAHs were monitored in an industrially polluted soil (Figs. 3 and 4). As shown in Fig. 3, the growth of PB4 and FB6 in the soil was enhanced under electrokinetic conditions. After 60 days, the counts for strains PB4 and FB6 in the experiments with electrokinetic conditions (PB4 + EK and FB6 + EK) were 47.9 and 30.3 % higher than in non-electrokinetic experiments (PB4 and FB6), respectively. Additionally, the degradation extents of the total PAHs showed the same tendency as the microbial counts (Fig. 4). The degradation extent of the total PAHs increased in degradation experiments under electrokinetic conditions. In the PB4 + EK and FB6 + EK experiments, the degradation extents of the total PAHs reached 27.8 and 29.0 %, respectively, which were 2.23 and 1.93 times as much as those in the PB4 and FB6 experiments after 60 days, respectively

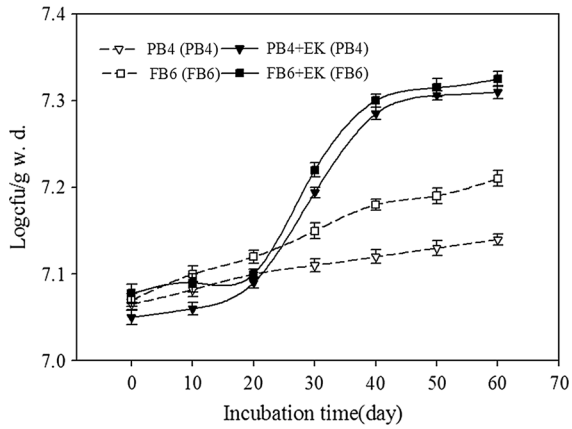

Fig. 3 Growth of PB4 and FB6 for degradation experiments without and with electrokinetic stimulation in an industrially polluted soil (*inverted open triangle* PB4:only inoculated PB4; *inverted filled triangle* PB4+EK:inoculated PB4 and in the presence of an electric field; *open square* FB6:only inoculated FB6 and in the presence of an electric field; *filled square* FB6+EK: inoculated FB6 and in the presence of an electric field)

(Fig. 4). While a longer lag phase was observed under electrokinetic conditions for PB4 and FB6 with respect to microbial growth and PAH degradation, the growth and degradation rates were higher than under non-electrokinetic conditions after 20 days.

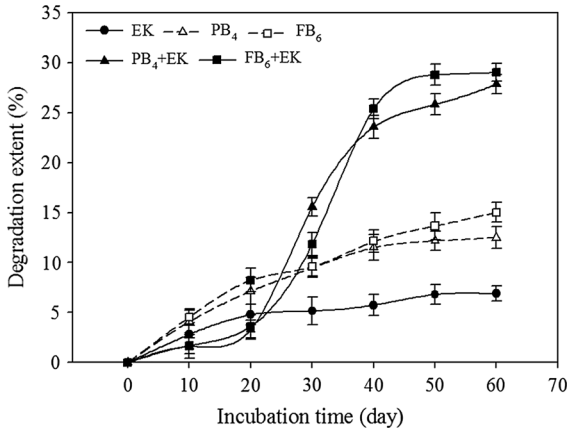

Fig. 4 Degradation extents of total PAHs in control experiments and degradation experiments with and without electrokinetic stimulation in an industrially polluted soil (*filled circle* EK: only in the presence of an electric field; *open triangle* PB4: inoculated PB4; *filled triangle* PB4+EK: inoculated PB4 and in the presence of an electric field; *open square* FB6: only inoculated FB6; *filled square* FB6+EK: inoculated PB4 and in the presence of an electric field)

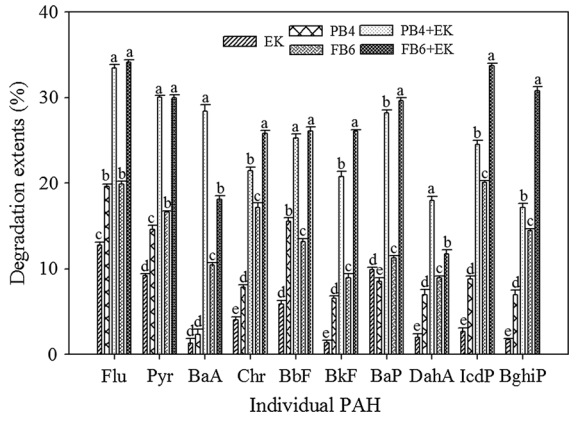

Fig. 5 Degradation extents of individual PAHs in control experiments and degradation experiments with and without electrokinetic stimulation in an industrially polluted soil (▨ EK: only in the presence of an electric field; ▭ PB4: only inoculated PB4; ▭ PB4+EK: inoculated PB4 and in the presence of an electric field; ▨ FB6: only inoculated FB6; ▬ : inoculated PB4 and in the presence of an electric field)

The degradation extents of 10 heavy PAHs were analyzed in five experiments at 60 days, as shown in Fig. 5. The degradation extents of the heavy PAHs were 2.3–19.6 and 9.0–20.1 % in the PB4 and FB6 experiments, which was significantly ($P < 0.05$) higher with 3.8–9.7 and 7.1–17.4 %, respectively, than those observed in the EK experiments except for BaP and BaA. In Fig. 5, it can be seen that the electric field had a different effect on the removal of individual PAHs in the EK experiment. The highest and lowest degradation extents were 12.8 and 1.3 % in EK experiment, respectively. However, the degradation extents were significantly enhanced compared to the PB4, FB6 and EK experiments in the PB4 + EK and FB6 + EK experiments, reaching 17.1–33.5 and 11.8–34.2 %, respectively.

Discussion

Many PAH-degraders have been isolated from PAH-contaminated soils, seawater and sediments (Pinyakong et al. 2012; Al-Thani et al. 2009; Darmawan et al. 2015) and the metabolism of PAH has been well characterized in organisms across the microbial world (Herbes and Schwall 1978; Heitkamp and Cerniglia 1988; Trzesicka-Mlynarz and Ward 1995; Patel et al. 2012). To enhance the removal efficiency of heavy PAHs during soil electro-bioremediation, the degraders should be capable of (i) utilizing heavy PAHs (>3 rings), (ii) degrading PAHs associated with soil particulates, and (iii) adapting to the conditions of an electric field.

PAH contamination exerts selective pressure on microorganisms, resulting in a shift of the microbial community towards those species with enhanced PAH degradation capability (Patel et al. 2012). This study, as in previous work, used soil that was chronically exposed to heavy PAHs for the acclimation, enrichment and isolation of the PAH-degraders. In addition, electric fields were applied during the processes of acclimation, enrichment and isolation of the PAH degraders. In many studies, the enrichment of PAH-degraders was performed in shaken liquid media utilizing PAHs as the carbon source (Juhasz and Naidu 2000a; Willison 2004; Hilyard et al. 2008). However, the strains growing well in liquid media might not show good activity in soil (Bastiaens et al. 2000). Herein, the enrichment was therefore performed in a soil contaminated with heavy PAHs. More importantly, the electric field acted as a selective pressure for isolating those bacteria capable of growing under such conditions. To the best of our knowledge, this was the first time PAH-degraders were enriched in soil contaminated with heavy PAHs under an electric field.

Heavy PAHs (compounds containing four or more aromatic rings) are generally recalcitrant to microbial attack. Until recently, only a few bacterial genera capable of degrading heavy PAHs have been isolated (Juhasz and Naidu 2000b; Dandie et al. 2004; Rentza et al. 2008). In these microorganisms, degradation of heavy PAHs usually occurs in the presence of other PAHs or hydrocarbons. Boonchan et al. (2000) reported that *Stenotrophomonas maltophilia* VUN10,010 could co-metabolize BaP, BaA and DahA when Pyr was also present. Rentza et al. (2008) proved that *Sphingomonas yanoikuyae* JAR02 needed salicylate to stimulate its BaP degradation capability. Also, similar results were observed in *Mycobacterium* sp. PYR-1 and *Mycobacterium* sp. strain 1B (Kelley and Cerniglia 1995; Dandie et al. 2004). In the current research, four bacteria (FB2, PB4, FB6 and FB7) could degrade eight individual heavy PAHs in artificially contaminated soil, differently than the previously mentioned strains (Fig. 2), possibly because the soil organic matter was favorable to heavy PAH degradation.

The effect of an electric field on microbial activity and viability has also been recently investigated (Gan et al. 2009; Luo et al. 2005; Shi et al. 2008; Tiehm et al. 2009). When an electric field is applied, different bacterial responses will occur depending on the current, treatment period, cell type, and medium (Wick et al. 2007). In this study, an electric field enhanced the degradation activity of PB4, FB6 and FB7. The possible mechanism of the impact of an electric field on soil microbial activity stems from changes in soil properties that are crucial for microbial metabolism and effect of electric current on microbial activity. Thrash and Coates (2008) found that substrate utilization increased and microbial metabolism was improved by the direct transfer of electrons from an electrode to bacteria and by the indirect transfer of electrons via the hydrolysis of water. She et al. (2006) suggested that bacterial respiration might have been enhanced by the generation of anodic oxygen by electrolysis. However, the results from the current study showed that the degradation activity of FB2 for heavy PAHs was inhibited by an electric field. Previous investigations demonstrated that stress from the growth conditions reduced total bacteria number and was a reason for cells entering a viable but non-culturable state (Ibekwe and Grieve 2004; Ohtomo and Satio 2001). Kim et al. (2010) reported that soil pH changes induced by electrokinetics reduced microbial cell numbers and diversity, especially of culturable bacteria. Thus, it is of great interest to isolate and investigate those strains capable of efficiently degrading heavy PAHs in the presence of an electric field.

The efficient heavy PAH degraders in this study were identified as *Pseudomonas fluorescens* and *Kocuria* sp. on the basis of their morphological, cultural and physiological characteristics and the phylogenetic position of their 16S rRNA sequences. *Pseudomonas fluorescens* and *Kocuria* sp. commonly occur in hydrocarbon-contaminated soils and have been reported to degrade two to four-ring PAHs such as naphthalene, phenanthrene, pyrene, chrysene and benzo(a)anthracene (Caldini et al. 1995; Bugg et al. 2000; Yuan et al. 2009; Ahmed et al. 2010). Reintroduction of *Pseudomonas fluorescens* and *Kocuria* sp. into an industrially polluted soil led to a significant removal of indigenous PAHs under an electric field (Fig. 4 and Fig. 5). The electric field might have led to a number of simultaneous process and changes within the soil that could have influenced biodegradation, including ionic movement and changes in the moisture and/or dissolved oxygen content, producing the latter via an electrolytic reaction (Virkutyte et al. 2002). These factors limit the natural attenuation of PAHs in industrially polluted soils. In this study, *Pseudomonas fluorescens* and *Kocuria* sp. still maintained good PAH- degrading activity in an industrially polluted soil. Their cell numbers were also significantly higher in the experiments with an electric field than in the non-electrokinetic experiments. Lear et al. (2004) had revealed that the electrokinetic process could increase the number of *Bacillus* and *Arthrobacter*. The results from this study are consistent with previous findings, suggesting that *Pseudomonas fluorescens* and *Kocuria* sp. have the ability to tolerate environmental stress and can be used in electro-bioremediation.

A comparison of results from samples inoculated with *Pseudomonas fluorescens* and *Kocuria* sp. to sterile controls exposed to electrokinetic conditions indicated that only minor losses of PAHs resulted from EK, reaching a maximum (6.9 %) after 20 days (Fig. 4). Recent studies have suggested that electrochemical reactions can be effectively used to mineralize many organics, including apolar and immobile compounds, and these reactions mainly occurred close to the electrodes (Yu and Neretnieks 1997; Rahner et al. 2002; Röhrs et al. 2002; Zheng et al. 2007). The

investigation of Sanromán et al. (2005) showed that the generation of oxidizing agents near the electrodes, such as OH^-, Cl_2, ClO^-, HClO, O_3 at the anode and $\cdot OH$ at the cathode, and their subsequent diffusion within the soil matrix was a possible oxidation pathway of removal of organic pollutants. Because of the low mobility of hydrophobic PAHs, the degradation of PAHs mainly occurred near the electrodes when electric field was applied in soil (Huang et al. 2013). After the PAHs near electrodes were completely degraded, the degradation extents of the total PAHs reached a plateau in EK experiment. These results therefore indirectly indicated that the enhanced PAH degradation ability of *Pseudomonas fluorescens* and *Kocuria* sp. was strongly enhanced by an electric field in an industrially polluted soil. To understand the ability of these particular species to exhibit enhanced PAH degradation under EK conditions, future research should focus on the particular biological functions that set these species apart from others.

Conclusion

Two heavy PAH-degrading bacteria adapted to electrokinetic conditions were isolated from an industrially polluted soil in the presence of an electric field. They were identified as *Pseudomonas fluorescens* and *Kocuria* sp.. Further investigation of PAH biodegradation in an industrially polluted soil demonstrated that *Pseudomonas fluorescens* and *Kocuria* sp. had good ability to degrade heavy PAHs in the presence of an electric field, which indicated that the two strains could be used in the electro-bioremediation of PAH-contaminated soil, especially if the soil is contaminated with heavy PAHs.

Acknowledgments This research was supported by the National Natural Science Foundation of China (21047006), Major Science and Technology Program for Water Pollution Control and Treatment (No. 2013ZX07202-007) and the National High Technology Research and Development Program of China (No. 2013AA06A210). We thank Kerusha Lutchmiah of KWR Watercycle Research Institute for proof reading the final manuscript. We thank two anonymous reviewers for their constructive comments that helped improve this manuscript.

Compliance with ethical standards

Conflict of Interest No conflict of interest declared.

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