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Manuscript Draft

Manuscript Number: ENVPOL-D-16-01549R1

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

Article Type: Research Paper

Keywords: Bioremediation; phenanthrene (PHE); Copper; Sphingobium; PAH-RHDα gene; C23O gene

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First Author: Mengke Song, Ph.D

Order of Authors: Mengke Song, Ph.D; Ying Yang; Longfei Jiang, Ph.D; Qing Hong, Ph.D; Dayi Zhang; Zhenguo Shen, Ph.D; Hua Yin; Chunling Luo, Ph.D

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.

Chunling Luo, Professor

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Baoshan Xing, Ph.D.
Associate Editor
Environmental Pollution

October 12, 2016

Dear Prof. Xing

Thank you very much for the processing and considering our manuscript entitled "Characterisation of the phenanthrene degradation-related genes and degrading ability of a newly isolated copper-tolerant bacterium" (ENVPOL-D-16-01549) to Journal of Environmental Pollution for possible publication after a major revision. We have seriously considered the reviewers' comments and made the responsive correction/modification according to the reviewers' and editor's comments, and the response was seen in the following. The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:

http://www.textcheck.com/certificate/NeUUhK

The present study investigated a newly isolated bacterium exhibiting high PHE biodegradability and copper tolerance, and the potential application in bioremediation of PHE-copper-co-contaminated soil. The genes encoding polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase (PAH-RHDα) and catechol-2,3-dioxygenase (C23O), and the PAH-RHD gene cluster involved in the

PHE degradation by strain PHE-1 were identified. The expression of PAH-RHDα and C23O genes has been reported to be stimulated by copper at a high concentration for the first time. When strain PHE-1 was applied to PHE-contaminated soil, the activity of C23O gene was improved by the planting of ryegrass and the presence of copper. The best performance of PHE degradation and the highest number of C23O gene copies occurred in PHE-copper-co-contaminated soil planted with ryegrass. The findings expand our knowledge on the microbial resource for bioremediation, and will be of interest for a wide range of researchers of environmental microbiology.

I would be very grateful if you could let me know the results of the review process in the near future. Thank you very much for your help. I am looking forward to hearing from you soon.

Responses to reviewers and editor:

Editor:

Comment:

Please see below the referees' comments on your manuscript. As you can see, the reviewers have major concerns about your manuscript, for example, more experiments need to be designed to verify the conclusion that PHE-1 possessed powerful PHE biodegradability, even better than that reported by other researchers; some major reviews on biodegradation of PAHs need to be referred and included; the data discussion needs to be strengthened; and the language in this manuscript needs to be significantly improved.

I concur with the reviewers. Your manuscript is not suitable for publication in its present form. It needs to be carefully revised and likely reviewed again before a final decision can be made on its suitability for publication in Environmental Pollution.

Response:

The authors would like to thank the efforts of the editor on the comments and have tried the best to correct the mistakes and modify the whole manuscript. The language

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Sorry for the mistake, and we have changed P1 to P2 in the revised version, please see Figure 3.

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Best wishes,

Sincerely yours,

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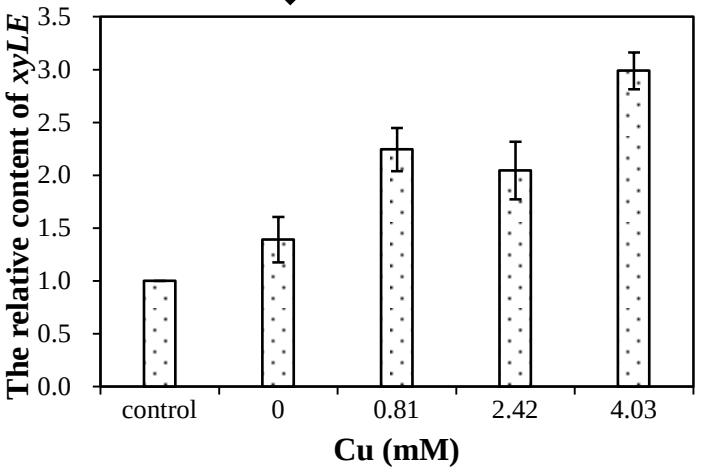
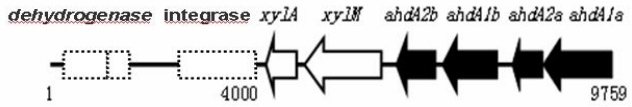
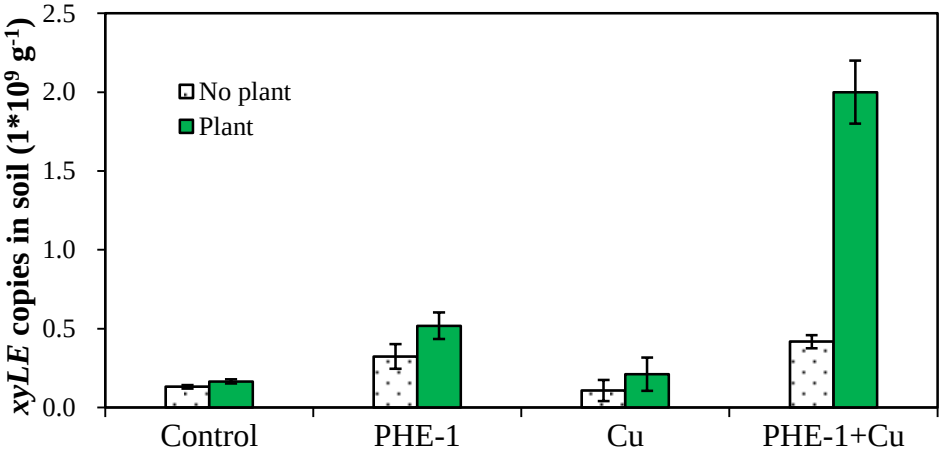
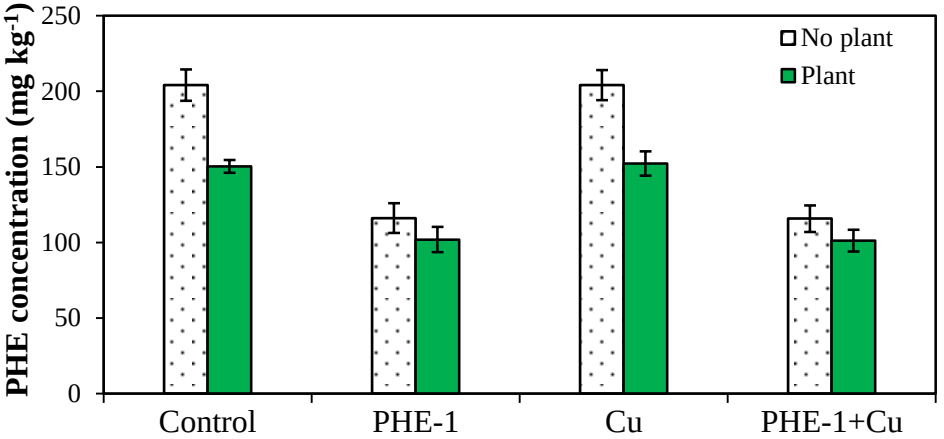
*Graphical Abstract





***ahdA1b* gene cluster of strain PHE-1**





Highlights

- ✧ A newly isolated bacterium possesses high PHE biodegradability and Cu-tolerance.
- ✧ High level Cu was reported to promote PAH-RHDα and C23O genes expression.
- ✧ Structure of PAH-RHD gene cluster has high similarity to other *Sphingobium* strains.
- ✧ Ryegrass and Cu enhanced PHE degradation and abundance of *Sphingobium* PHE-1.

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.

Keywords: Bioremediation; phenanthrene (PHE); Copper; *Sphingobium*; PAH-RHDα gene; C23O gene

Capsule: The PHE biodegradability and expression of PHE degradation genes in a newly isolated bacterium strain were enhanced by high level copper.

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; Thavamani et al., 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., 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 PAHs 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 30ºC. 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} ≈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, and 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: 95ºC for 2 min; 30 cycles at 94ºC for 30 s, 60ºC for 30 s and 72ºC for 60 s; and final extension at 72ºC 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: 95ºC for 1 min, 94ºC for 30 s, 30ºC for 3 min, 70ºC for 5 min and 72ºC 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: 94ºC for 30 s and 70ºC for 5 min. Thirdly, eight cycles of thermal asymmetric PCR were carried out with the following program: one cycle of 94ºC for 30 s, 50ºC for 30 s and 70ºC for 5 min; followed by two cycles of 94ºC for 30 s and 70ºC for 5 min. Fourthly, 1 μL of the products were diluted 1000 times and used as the template for another PCR in a 30-μL mixture containing 15 μL of 2×GC buffer I, 4 μL of dNTP (2.5 mM), 2 μL of SP2 (5 μM), 0.3 μL of LA-Taq and 1 μL of template. Then, the PCR was carried out by the following program: 95ºC for 2 min, followed by 30 cycles of 94ºC for 30 s and 70ºC for 5 min, with final extension at 72ºC for 10 min. The presence of final PCR products was checked by agarose gel electrophoresis, followed by purification, ligation to vector pMD19-T and sequence. After assembly, the 9.1-kb DNA fragment containing the PAH-RHD gene was analysed using the online Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the Blastx programme (http://www.blast.ncbi.nlm.nih.gov).

2.2.3. *Transcriptional analysis of PAH-RHD and C23O genes*

Quantitative reverse transcription PCR (qRT-PCR) was used to analyse the transcriptional levels of PAH-RHD and C23O genes as follows. Total RNA was extracted from each sample with a Bacterial RNA Extraction Kit (CWBIO, Beijing, China) following the manufacturer's instructions. After removing genomic DNA with RNase-Free DNase (Qiagen, Hilden, Germany), cDNA was synthesised from RNA

template using the QuantiTect reverse transcription kit (CWBIO, Beijing, China) according to the manufacturer's instructions. Primers used to amplify PAH-RHD, C23O, and 16S rRNA genes were designed based on the sequence of genomic DNA of *Sphingobium* PHE-1, respectively (Table S2). qRT-PCR was performed on an ABI Prism 7500 real-time PCR detection system using TransStart Top Green qPCR SuperMix as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Melting curves were obtained by slow heating from 60°C to 90°C at 0.1°C/s and continuous monitoring of the fluorescence signal (Singleton et al., 2009). Three replicates were performed for each sample. The quantities of PAH-RHD and C23O gene transcripts for each sample were determined by relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Standard curves for the quantification of PAH-RHD, C23O, and 16S rRNA genes were created by performing qPCR with serial dilutions of the standard plasmid containing the target DNA sequence by the $2^{-\Delta\Delta Ct}$ method.

2.3 Pot experiment with copper–PHE-co-contaminated soil

2.3.1 *Set-up of pot experiment*

Soil without detectable PAHs and copper was collected from an agricultural field in Jiangning District, Nanjing, China. After transferred to the laboratory, the soil was air-dried and sieved through a 2-mm mesh. The physiochemical properties of the soil were as follows: pH 7.10, total organic matter 2.92%, total nitrogen 0.68 g/Kg and total phosphorus 1.03 g/Kg.

To prepare the soils contaminated with 500 mg/Kg PHE, the PHE (purity > 96%; Sigma-Aldrich, Germany) dissolved in methanol was spiked into 5% (w/w) of the total soil. After the evaporation of methanol in a fume-hood, this contaminated soil was thoroughly mixed with the remaining soil (Brinch et al., 2002). For PHE-copper co-contaminated soil, copper (as $CuCO_3$) was added to the PAH-spiked soil at a final concentration of 500 mg/Kg. Next, 1 Kg of contaminated soil was placed in a ceramic pot. Following four dry-wet cycles within 4 weeks, the soil was planted with ryegrass seeds and inoculated with pre-cultivated strain *Sphingobium* PHE-1 at a density of 1.5×10^7 cells/g. In total, the four treatments included: PHE-1 inoculation, ryegrass

planting, ryegrass planting with *Sphingobium* PHE-1 inoculation, and soil without ryegrass or PHE-1. The pots were watered daily with deionised water to maintain the moisture content at approximately 60% of the water-holding capacity of the soils. After 56 days of cultivation in a glasshouse at 20-30°C under natural light, the soils in the pots were collected, mixed, sieved through a 2-mm mesh, and stored at -20°C for DNA extraction and PHE analysis.

2.3.2 *Quantitation of C23O gene in soil*

Microbial genomic DNA was extracted from soil using the FastDNA Spin kit (MoBIO, USA) in accordance with the manufacturer's instruction and then used as template to perform qPCR to quantify C23O gene named *xylE*. The standard curve for absolute quantitation of *xylE* gene was established by SYBR Green fluorescence quantitative PCR with a template obtained by a series of 10-fold dilutions of the plasmid constructed with vector pEASY-T1 and *xylE* fragment.

2.4 PHE extraction and analysis

During strain isolation and cultivation, the PHE was collected by liquid–liquid extraction. Briefly, the liquid culture was mixed with methylene chloride (1:1 v/v) by vigorous shaking and then held for 2 h at room temperature. After drying with anhydrous sodium sulphate, the resulting extract was then concentrated to 1 mL with a gentle stream of N_2 for HPLC analysis (Thavamani et al., 2012c).

PHE in soil samples were collected by ultrasonic extraction. After freeze-drying, 5 g of soil was placed in a glass tube, to which 10 mL of dichloromethane was added. The suspension was ultrasonicated for 30 min with occasional stirring to prevent its adherence to the bottom of the tube. The mixture was then centrifuged at 4000 rpm, and the supernatant was discarded. The above procedure was repeated three times. All the supernatants were pooled and concentrated to ~0.5 mL after solvent exchange to hexane. The soil extracts were purified in a multilayer silica gel/alumina column (8 mm i.d.) filled (from top to bottom) with anhydrous Na_2SO_4 (1 cm), neutral silica gel (3 cm, 3% w/w; deactivated) and neutral alumina (3 cm, 3% w/w; deactivated) via elution with 15 mL of hexane/dichloromethane (1:1, v/v). After concentrating with a

gentle stream of N_2, the residue was dissolved in methane with a final volume of 1.0 mL for HPLC analysis (Chigbo et al., 2013).

PHE was detected on a HPLC (Waters 600) equipped with an ^{18}C reversed-phase column (4.6 mm × 25 cm) and a photo diode-array detector. Methanol:water (90:10, v/v) at a flow rate of 0.8 mL/min was used as the mobile phase. HPLC analysis was performed at a wavelength of 254 nm, and a 20-µL sample or standard PHE compounds were injected into the chromatograph under standardised conditions. An external standard method was used for quantitation in terms of peak areas (Dong et al., 2008).

2.5 Statistical analysis

Statistical analysis was performed using SPSS 17.0. The statistical significance of differences (p-value <0.05) in PHE concentration, abundance of *ahdA1b-1* and *xyLE* genes, and the biomass of dry ryegrass among the different treatments was analysed using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test.

3. Results and discussion

3.1 Characterisation of PHE-degrading bacteria

After incubation in Luria–Bertani medium for 48 h, the colony of the isolated strain was observed to be yellow, with a diameter of 3–4 mm, translucent and glossy, with a smooth surface and neat edges (Figure 1A-a). The images of cells obtained by transmission electronic microscopy showed that the strain was rod-shaped (0.5-0.7 µm × 1.2-1.7 µm) with flagella (Figure 1A-b). The results of 16S rRNA sequence showed its 99% identity with the nucleotide sequences of the *Sphingobium abikonense* strain NBRC 16140 (NR 113839.1), *Sphingobium abikonense* strain IAM 12404 (NR 112079.1) and *Sphingobium lactosutens* strain DS20 (NR 116408.1) (Figure 1B). The strain was therefore classified as a *Sphingobium* strain within the genus *Sphingomonas*, family *Sphingomonadaceae*, class *Alphaproteobacteria*, and named as *Sphingobium* PHE-1.

3.2 Impacts of Cu(II) on PHE degradation by *Sphingobium* PHE-1

Figure S1 showed the PHE removal efficiency in MSM at 24 h with different concentrations of PHE as the sole carbon source. PHE was completely degraded when its initial concentration was less than 600 mg/L. Above this level, the removal efficiency decreased with the increasing initial PHE concentration. The results indicated that *Sphingobium* PHE-1 possesses powerful PHE biodegradability, better than *Sphingobium chlorophenolicum* C3R metabolizing ~60% of the PHE in 2 days with an initial concentration of 300 mg/L in liquid culture similar to this work (Colombo et al., 2011) and a *Sphingobium* strain utilizing more than 200 mg/L PHE within 24 h in liquid culture (Prakash and Lal, 2006). Besides, it was observed that ~ 50% of the added PHE was degraded owing to the inoculation of strain *Sphingobium* PHE-1 in PHE-copper co-contaminated soils .

To study the influence of Cu(II) on PHE degradation by strain *Sphingobium* PHE-1, the removal efficiency of PHE was tested in the presence of different concentrations of Cu(II). Figure S2 showed that PHE was almost completely degraded when Cu(II) was less than 3.22 mM. The removal efficiency maintained 88.2% even when the Cu concentration rose to 4.03 mM and then decreased with the increasing Cu(II). The similar behaviour was also observed for the mixed flora derived from soil suspensions, the PHE degradation ability of which was not significantly affected when the Cu(II) concentration was no more than 0.43 mM. Previous study showed the declining microbial respiration in the presence of Cu(II), and the higher the Cu(II) concentration, the more pronounced the inhibition (Sokhn et al., 2001). The limited impact caused by high level Cu(II) in this work might be explained by the little influence of Cu(II) on the enzymatic activity of dioxygenase related to PAHs degradation. It was reported replacement of the iron at the active site of iron-containing 2,3-dioxygenase with copper weakly affects its activity owing to the stability of the metal complexes (Gopal et al., 2005; Guzik et al., 2013).

Sphingomonas species have long been known for degrading a wide range of PAHs in contaminated soils and are often detected in copper-contaminated media, such as copper-exposed groundwater treatment plants and soils near copper mines (Stolz, 2009). *Sphingobium*, comprising 25 recognised species, is the main subgenus of the

Sphingomonas genus with the capacity of PAHs degradation (Kertesz and Kawasaki, 2010). It was demonstrated that *Sphingobium chlorophenolicum* strain C3R significantly improves the biodegradation rate of PHE in PAHs-contaminated soils in the presence of both cadmium and arsenic (Colombo et al., 2011). Some other *Sphingobium* strains with the ability to degrade PAHs and substituted PAHs were also isolated from a river, a pentachlorophenol-contaminated industrial site and freshwater sediment, a polluted stream and 2,4-dichloroprop-pretreated soils (Kertesz and Kawasaki, 2010). Furthermore, the enzymes involved in the catabolic pathways and the corresponding genes in *Sphingobium* strains have also been well studied (Leys et al., 2004; Pinyakong et al., 2003a; Story et al., 2000).

3.3 The effect of PHE and Cu(II) on C23O gene transcription

In this work, we successfully amplified a C23O gene from strain *Sphingobium* PHE-1 by using the primers designed in accordance with known ones. The phylogenetic information in Figure S3 showed that the *xylE* gene of *Sphingobium* PHE-1 was closely related to the genus *Sphingobium*, sharing 95%, 92% and 92% similarity with the nucleotide sequences of *S.* strain ZP1, *S. yanoikuyae* strain B1 and *S.* strain P2, which was consistent with 16s rRNA results. The copies of *xylE* gene were then analysed by qRT-PCR using the primers designed according to the nucleic sequences of acquired *xylE* gene.

To test the effect of PHE addition on the expression of *xylE* gene and evaluate its change with time, the copy numbers of *xyLE* gene of *Sphingobium* PHE-1 in the presence of PHE were compared to the control (PHE replaced by glucose), as illustrated in Figure 2A. It was clear that the copies of *xyLE* kept increasing in the first 24 h and then decreased from 24 to 48 h. Comparison with the constant copy numbers of *xyLE* gene in the control yielded the inference that *xyLE* gene is induced by PHE. In the treatment with an initial concentration of 100 mg/Kg PHE, the expression of *xyLE* gene peaked when all PHE had been consumed. The same results were also observed in an *ex situ* system, in which *xyLE* gene was initially present at high PAHs concentration, but disappeared with a substantial decrease of PAHs after 1 week (Wikstrom et al., 1996). This is also consistent with the results of Zhao et al. (Zhao et

al., 2011), who described that the expression of C23O gene in PHE-degrader *Pseudomonas sp.* ZP1 increased during the PHE degradation, but dramatically dropped off when PHE ran out.

Figure 2B showed the expression of *xyLE* gene against different concentrations of Cu(II) with 100 mg/L PHE as the sole carbon source after 24 h. An unexpected promotion of *xyLE* expression was observed when Cu(II) was added, compared to the control (no Cu(II)), and its expression level increased with the rise of Cu(II). This indicated that *Sphingobium* PHE-1 would probably resist the damage caused by Cu(II) via encouraging *xyLE* expression for PHE metabolism and catabolism, generating more energy to enhance the expression of genes related to the oxidative stress response, DNA and protein repair, metal transport and other processes (Baker-Austin et al., 2005; Gu et al., 2016). Generally, heavy metals inhibit the microbial degradation of organic compounds via disrupting general enzymes or functional genes responsible for PAHs degradation (Sokhn et al., 2001). The exception involved a metal-tolerant and phenol-degrading strain, for which cadmium was reported to increase its C23O activity (Hupert-Kocurek et al., 2013). It is interesting that a high level of Cu(II) promoted the expression of the C23O gene in *Sphingobium* PHE-1 in the present study, which should be useful for the bioremediation of copper-PAHs co-contaminated soils.

The degradation of PHE by bacteria is driven by enzymes and is dependent on the levels of enzymatic activity. Two key enzymes are involved in the PHE-degradation process: ring-hydroxylating dioxygenase (PAH-RHD) and aromatic ring-cleavage dioxygenase. PAH-RHD controls the incorporation of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiol in the initial step of PAHs metabolism. Thereafter, the dihydroxylated cis-dihydrodiol intermediates are cleaved by dioxygenase *via ortho*-cleavage or *meta*-cleavage pathways. C23O protein acting as a ring-cleavage enzyme in the *meta*-cleavage pathways seems to consist of a superfamily of enzymes (Boldt et al., 1995). C23O genes have been found in strains *Sphingomonas* ZP1 (Zhao et al., 2011), *Pseudomonas stutzeri* AN10, *Pseudomonas putida* G7, and *Pseudomonas putida* NCIB9816 (Habe and Omori, 2003), and their

gene sequences in these bacteria with the ability to degrade different PAHs have been uncovered. Thus, C23O genes are potentially good indicators in monitoring the bacterial subpopulations involved in the ring cleavage of aromatics and the final steps of the degradation of some PAHs. For example, it was reported that C23O genes were successfully used to monitor the subpopulations of PAHs-degrading microbes in different types of soils (Wikstrom et al., 1996).

3.4 Sequence analysis of PAH-RHD gene clusters and the effects of PHE and Cu(II) on its transcription

By amplifying the PAH-RHDα gene from the total DNA of *Sphingobium* PHE-1 with the designed primers (Table S1), a DNA fragment of 1490 bp was obtained. The nucleotide sequence of the fragment exhibits 99% and 97% similarity with the *ahdA1b* operon affiliated to *Sphingomonas* sp. ZP1 (EU082776) and *Sphingomonas* sp. P2 (AB091693), respectively, which is then named as *ahdA1b*-1 (Figure S4).

The effects of PHE and Cu(II) on *ahdA1b-1* transcription were investigated using the primers designed according to the nucleic sequences of *ahdA1b-1*, as illustrated in Figure 4. It was clear that the *ahdA1b-1* gene held a similar expression pattern as *xyLE* gene, rapidly increasing over time before 24 h but slowed down from 24 to 48 h with the exhaustion of PHE in the medium. Meanwhile, the expression of *ahdA1b-1* gene in the control (glucose instead of PHE) was significantly lower than those supplemented with PHE ($p<0.05$) and had tiny fluctuation throughout the experiment. It was proposed that the expression of *ahdA1b-1* gene was induced by PHE and reached a plateau when the PHE was used up. The same results were also obtained by Pinyakong et al., who found the inducible genes encoding terminal oxygenase in *Sphingobium* sp. strain P2 (Pinyakong et al., 2003b). Preliminary study on the response of *Sphingomonas aromaticivorans* strain F199 to various aromatic compounds demonstrated that its aromatic catabolic activity was induced after exposure to naphthalene (Pinyakong et al., 2003b). Additionally, Cu(II) significantly affected the expression of *ahdA1b-1* gene in *Sphingobium* PHE-1, from the higher expressed copy numbers of *ahdA1b-1* gene copies in Cu(II) amended treatments than that in samples without copper, even when the Cu(II) concentration was increased to

4.03 mM (p<0.05). The results indicated that *ahdA1b-1* expression is promoted by Cu(II), consistent with the influence of Cu(II) on the expression of *xyLE* gene. Although previous research showed that the expression of the genes responsible for naphthalene degradation in a *Pseudomonas* strain is enhanced by nickel (Siunova et al., 2007), no work prior to this study reported the transcription of PAHs-degrading genes is promoted by high level of Cu(II).

In the first step of PHE metabolism, the aromatic nucleus is attacked by molecular oxygen forming *cis*-dihydrodiol with the function of PAH-RHD. PAH-RHD is composed of an iron-sulphur flavoprotein reductase, an iron–sulphur ferredoxin and a terminal dioxygenase. The terminal dioxygenase consists of large α and small β subunits, and two conserved regions exist in α subunit (RHDα) (Kauppi et al., 1998). Primers based on these conserved regions have been designed and successfully applied to amplify the target *pahAc*-like, *phnAc*-like and *nagAc*-like genes (Cebron et al., 2008). Besides, genes encoding degrading proteins are frequently found in grouping together in a cluster (Qiu et al., 2013). SEFA-PCR was therefore performed to obtain the flanking DNA sequences of *ahdA1b-1* in this study. A 6.6-kb upstream and a 2.1-kb downstream DNA fragments were amplified with three pairs of primers (Table S1). After cloning and sequencing, it is noted that the 6.6-kb upstream DNA fragment contains a small subunit and a large subunit belonging to the PAH-RHD gene, and the 2.1-kb downstream DNA fragment has a small subunit affiliated to the PAH-RHD gene. The 1490-bp core fragment was assembled with the flanking fragments to generate a 9.1-kb DNA fragment, which shows 96% similarity to the PAHs-degrading gene cluster affiliated to *Sphingomonas* sp. P2 and *Sphingobium yanoikuyae* strain B1. As shown in Figure 3, the analysis of nucleotides and the deduced amino acid sequences of the 9.1-kb DNA fragment using the online ORF Finder and Blastx programme revealed that six consecutive ORFs exhibited 99%, 96%, 93%, 97%, 99% and 96% nucleotide sequence identities with *xylA*, *xylM*, *ahdA2b*, *ahdA1b*, *ahdA2a* and *ahdA1a*, respectively, which are located in the PAHs-degrading gene cluster of *Sphingomonas* sp. P2 (AB091693) and *S. yanoikuyae* strain B1 (EF151283) (referred to as *xylA*, *xylM*, *bphA2b*, *bphA1b*,

bphA2a and *bphA1a*) (Pinyakong et al., 2000, 2003a) . The clusters from *xylA* to *ahdA1a/bphA1a* in the three strains were assembled in the same order and transcriptional direction (Figure 3), indicating the high conservation of the functional genes encoding PAH-RHD in strains affiliated to the *Sphingomonas* genus. It was also reported that the aromatic-degrading genes from the *Sphingobium* sp. B1, *Sphingobium* sp. Q1 and *Novosphingobium aromaticivorans* F199 exhibited high homology (Pinyakong et al., 2003a; Pinyakong et al., 2003b). For example, in *Sphingomonas*, the degrading genes encoding arene cis-dihydrodiol dioxygenase and the enzymes responsible for the conversion of 1,2-dihydroxynaphthalene to salicylate were reported to be similar in sequence (Waigi et al., 2015).

3.5 The application of *Sphingobium* PHE-1 coupled with ryegrass in the remediation of PHE-copper co-contaminated soils

The inoculation of *Sphingobium* PHE-1 significantly improved the growth of ryegrass (Figure 5). Interestingly, the growth of ryegrass was also found to be greatly stimulated by Cu(II), with plants grown in PHE-copper co-contaminated soils having higher biomass than those grown in soils with PHE contamination only (Figure 5), which was consistent with the trends of *ahdA1b-1* and *xyLE* gene transcription in MSM (Figure 2B and Figure 4B). This improvement in ryegrass growth was attributed to the increasing activities of *Sphingobium* PHE-1 in the presence of Cu(II). It was reported that some heavy metal-resistant bacteria could promote the growth of host plants. Examples included a copper-resistant plant growth-promoting bacterial (PGPB) strain, Ax10, which was isolated from a copper mine soil and facilitated *Brassica juncea* growth and Cu(II) uptake (Ma et al., 2009). Besides, the nickel-resistant strain PGPB SRS8 was also found to be capable of stimulating plant growth and nickel accumulation in the crops *Ricinus communis* and *Helianthus annuus* (Ma et al., 2011).

The residual PHE concentrations in soils subjected to different treatments on day 56 were presented in Figure 6. The results clearly illustrated that more PHE was removed from soils inoculated with *Sphingobium* PHE-1, compared to the uninoculated treatments, and ryegrass planting also resulted in lower residual PHE.

459 The best performance in terms of PHE reduction occurred in the treatment with both
460 ryegrass and *Sphingobium* PHE-1. This optimal PHE removal was explained by
461 microbial degradation and, to some extent, by enhanced adsorption to roots and
462 accumulation in ryegrass shoots, as found in our previous study (Wang et al., 2012).
463 Besides, the bioavailability of PAHs, which often limits the biodegradation of these
464 compounds, may be increased with the aid of some components in the root exudation
465 (An et al., 2010; Cerniglia, 1993; Gao et al., 2010).
466 The abundance of *xylE* genes in soils was also studied to monitor the effect of
467 ryegrass on the growth and degrading activities of *Sphingobium* PHE-1, attributing to
468 its higher specificity than *ahdA1b-1* and 16s rRNA genes. A higher abundance of *xylE*
469 gene in soils with ryegrass was found than that in soils without ryegrass (Figure 7,
470 $p<0.05$), which proved that ryegrass could encourage the abundance of *Sphingobium*
471 PHE-1. Additionally, the copy numbers of *xylE* gene in soil was also enhanced by the
472 Cu(II) addition, which was in agreement with the transcription of *xylE* in
473 *Sphingobium* PHE-1 (Figure 2B). The increase of *xylE* abundance in soils planting
474 with ryegrass was attributed to the positive influence of rhizospheric effect on
475 microbes. The fibrous root of ryegrass observed in this work provided microbial
476 attachment sites and enhanced soil aeration, and the vegetation cover created
477 favourable environmental conditions such as temperature and soil moisture. More
478 importantly, the root exudates as the ecological driver in the rhizosphere supply
479 microorganisms with a relatively constant source of nutrients, such as water soluble
480 carbon, nitrogen and phosphorus, especially in poor soils. The rhizosphere therefore
481 improves the microbial growth, activities and the abundance of functional genes
482 (Haritash and Kaushik, 2009). Besides, many secondary plant metabolites in root
483 exudation with similar structure to aromatic hydrocarbons may stimulate the
484 metabolic pathways of PAHs degraders (Martin et al., 2014). It was observed that
485 more microbes, enhanced activities and increased abundance of PAHs-degrading
486 genes in planted versus unplanted soils occurred in PAHs contaminated soils (Haritash
487 and Kaushik, 2009; Thomas and Cebron, 2016).
488 In the present study, the growth of ryegrass was improved by *Sphingobium*

PHE-1. In turn, the ryegrass provided abundant nutrients to *Sphingobium* PHE-1 and promoted its colonisation in the rhizosphere, which enhanced the dissipation of PHE. It was proved that PHE and pyrene (PYR) dissipation with the aid of microbes in soils was improved by planting ryegrass and the levels of PHE and PYR increased with the distance from the root surface (Gao et al., 2013). A similar phenomenon was also observed in another study, in which the growth of ryegrass significantly increased soil peroxidase activities, leading to enhanced dissipation of PHE and PYR in, and additionally, the interactions of ryegrass with the two microbes further improved the dissipation of PHE and PYR (Yu et al., 2011). In the present study, the growth of ryegrass was improved by inoculation with *Sphingobium* PHE-1, further benefiting bacterial growth by rhizospheric effect and improving PHE dissipation as well. The capacity of strain *Sphingobium* PHE-1 to exhibit Cu(II) tolerance and degrade PHE suggests its feasibility in the remediation of PAHs-heavy metal co-contaminated soils and sediments.

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Legends of tables and figures

Fig. 1. A: Colonies (a) and transmission electronic microscopy image (b) of *Sphingobium* PHE-1. B: Phylogenetic tree derived from the 16S rRNA genes of *Sphingobium* PHE-1 and related species by the neighbor-joining method using MEGA 5.1.

Fig. 2. The expression of *xylE* gene at different incubation time (A) or Cu(II) concentration (B). The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 3. Comparison of PAH-RHD cluster structure between *Sphingomonas* sp. strain P2, *Sphingobium* sp. strain PHE-1 and *Sphingobium yanoikuyae* strain B1. The open reading frames (ORFs) are indicated by arrows. The scale is in bases.

Fig. 4. The expression of *ahdA1b-1* gene at different incubation time (A) or Cu(II) concentration (B). The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 5. Biomass of dry ryegrass in different treatments. Control: soil amended with PHE. PHE-1: soil amended with PHE and *Sphingobium* PHE-1. Cu: soil amended with PHE and Cu(II). PHE-1+Cu: soil amended with PHE, Cu(II) and *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–c) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 6. Residual PHE concentration in soils from different treatments. Control: soil amended with PHE. PHE-1: soil amended with PHE and *Sphingobium* PHE-1. Cu: soil amended with PHE and Cu(II). PHE-1+Cu: soil amended with PHE, Cu(II) and *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 7. The abundance of *xylE* gene in soils from different treatments. Control: soil

amended with PHE. PHE-1: soil amended with PHE and *Sphingobium* PHE-1. Cu: soil amended with PHE and Cu(II). PHE-1+Cu: soil amended with PHE, Cu(II) and*Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–e) represent the statistical significance at the 0.05 level with the LSD test

Supporting information

Table S1 Primers used in this study

Table S2 Primers used for transcriptional analysis of PAH-RHD and C23O genes

Fig. S1. Effects of initial PHE concentration on PHE degradation efficiency by *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a-d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. S2. Effects of Cu(II) concentration on the PHE degradation efficiency by *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a-e) represent the statistical significance at the 0.05 level with the LSD test.

Fig. S3. Phylogenetic tree of catechol -2,3- dioxygenase gene (*xyLE*) from *Sphingobium* PHE-1 along with the closest matches in GenBank, constructed with MEGA 5.1 using the neighbor-joining method.

Fig. S4. Phylogenetic tree of aromatic compounds-catabolic gene (*ahdA1b-1*) from *Sphingobium* PHE-1 along with the closest matches in GenBank, constructed with MEGA 5.1 using the neighbor-joining method.

Fig. S5. Gel electrophoresis image of the flanking DNA fragments of *ahdA1b-1* by SEFA-PCR. M: λDNA/*Hind*III marker; 1: *ahdA1b* upstream SEFA-PCR products; 2: *ahdA1b* downstream SEFA-PCR products. The arrows show the bands of target fragments.

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.

Keywords: Bioremediation; phenanthrene (PHE); Copper; *Sphingobium*; PAH-RHDα gene; C23O gene

Capsule: The PHE biodegradability and expression of PHE degradation genes in a newly isolated bacterium strain were enhanced by high level copper.

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; Thavamani et al., 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., 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 PAHs 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 30ºC. 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} ≈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, and 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: 95ºC for 2 min; 30 cycles at 94ºC for 30 s, 60ºC for 30 s and 72ºC for 60 s; and final extension at 72ºC 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: 95ºC for 1 min, 94ºC for 30 s, 30ºC for 3 min, 70ºC for 5 min and 72ºC 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: 94ºC for 30 s and 70ºC for 5 min. Thirdly, eight cycles of thermal asymmetric PCR were carried out with the following program: one cycle of 94ºC for 30 s, 50ºC for 30 s and 70ºC for 5 min; followed by two cycles of 94ºC for 30 s and 70ºC for 5 min. Fourthly, 1 μL of the products were diluted 1000 times and used as the template for another PCR in a 30-μL mixture containing 15 μL of 2×GC buffer I, 4 μL of dNTP (2.5 mM), 2 μL of SP2 (5 μM), 0.3 μL of LA-Taq and 1 μL of template. Then, the PCR was carried out by the following program: 95ºC for 2 min, followed by 30 cycles of 94ºC for 30 s and 70ºC for 5 min, with final extension at 72ºC for 10 min. The presence of final PCR products was checked by agarose gel electrophoresis, followed by purification, ligation to vector pMD19-T and sequence. After assembly, the 9.1-kb DNA fragment containing the PAH-RHD gene was analysed using the online Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the Blastx programme (http://www.blast.ncbi.nlm.nih.gov).

2.2.3. *Transcriptional analysis of PAH-RHD and C23O genes*

Quantitative reverse transcription PCR (qRT-PCR) was used to analyse the transcriptional levels of PAH-RHD and C23O genes as follows. Total RNA was extracted from each sample with a Bacterial RNA Extraction Kit (CWBIO, Beijing, China) following the manufacturer's instructions. After removing genomic DNA with RNase-Free DNase (Qiagen, Hilden, Germany), cDNA was synthesised from RNA

template using the QuantiTect reverse transcription kit (CWBIO, Beijing, China) according to the manufacturer's instructions. Primers used to amplify PAH-RHD, C23O, and 16S rRNA genes were designed based on the sequence of genomic DNA of *Sphingobium* PHE-1, respectively (Table S2). qRT-PCR was performed on an ABI Prism 7500 real-time PCR detection system using TransStart Top Green qPCR SuperMix as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Melting curves were obtained by slow heating from 60°C to 90°C at 0.1°C/s and continuous monitoring of the fluorescence signal (Singleton et al., 2009). Three replicates were performed for each sample. The quantities of PAH-RHD and C23O gene transcripts for each sample were determined by relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Standard curves for the quantification of PAH-RHD, C23O, and 16S rRNA genes were created by performing qPCR with serial dilutions of the standard plasmid containing the target DNA sequence by the $2^{-\Delta\Delta Ct}$ method.

2.3 Pot experiment with copper–PHE-co-contaminated soil

2.3.1 *Set-up of pot experiment*

Soil without detectable PAHs and copper was collected from an agricultural field in Jiangning District, Nanjing, China. After transferred to the laboratory, the soil was air-dried and sieved through a 2-mm mesh. The physiochemical properties of the soil were as follows: pH 7.10, total organic matter 2.92%, total nitrogen 0.68 g/Kg and total phosphorus 1.03 g/Kg.

To prepare the soils contaminated with 500 mg/Kg PHE, the PHE (purity > 96%; Sigma-Aldrich, Germany) dissolved in methanol was spiked into 5% (w/w) of the total soil. After the evaporation of methanol in a fume-hood, this contaminated soil was thoroughly mixed with the remaining soil (Brinch et al., 2002). For PHE-copper co-contaminated soil, copper (as $CuCO_3$) was added to the PAH-spiked soil at a final concentration of 500 mg/Kg. Next, 1 Kg of contaminated soil was placed in a ceramic pot. Following four dry-wet cycles within 4 weeks, the soil was planted with ryegrass seeds and inoculated with pre-cultivated strain *Sphingobium* PHE-1 at a density of 1.5×10^7 cells/g. In total, the four treatments included: PHE-1 inoculation, ryegrass

planting, ryegrass planting with *Sphingobium* PHE-1 inoculation, and soil without ryegrass or PHE-1. The pots were watered daily with deionised water to maintain the moisture content at approximately 60% of the water-holding capacity of the soils. After 56 days of cultivation in a glasshouse at 20-30°C under natural light, the soils in the pots were collected, mixed, sieved through a 2-mm mesh, and stored at -20°C for DNA extraction and PHE analysis.

2.3.2 *Quantitation of C23O gene in soil*

Microbial genomic DNA was extracted from soil using the FastDNA Spin kit (MoBIO, USA) in accordance with the manufacturer's instruction and then used as template to perform qPCR to quantify C23O gene named *xylE*. The standard curve for absolute quantitation of *xylE* gene was established by SYBR Green fluorescence quantitative PCR with a template obtained by a series of 10-fold dilutions of the plasmid constructed with vector pEASY-T1 and *xylE* fragment.

2.4 PHE extraction and analysis

During strain isolation and cultivation, the PHE was collected by liquid–liquid extraction. Briefly, the liquid culture was mixed with methylene chloride (1:1 v/v) by vigorous shaking and then held for 2 h at room temperature. After drying with anhydrous sodium sulphate, the resulting extract was then concentrated to 1 mL with a gentle stream of N_2 for HPLC analysis (Thavamani et al., 2012c).

PHE in soil samples were collected by ultrasonic extraction. After freeze-drying, 5 g of soil was placed in a glass tube, to which 10 mL of dichloromethane was added. The suspension was ultrasonicated for 30 min with occasional stirring to prevent its adherence to the bottom of the tube. The mixture was then centrifuged at 4000 rpm, and the supernatant was discarded. The above procedure was repeated three times. All the supernatants were pooled and concentrated to ~0.5 mL after solvent exchange to hexane. The soil extracts were purified in a multilayer silica gel/alumina column (8 mm i.d.) filled (from top to bottom) with anhydrous Na_2SO_4 (1 cm), neutral silica gel (3 cm, 3% w/w; deactivated) and neutral alumina (3 cm, 3% w/w; deactivated) via elution with 15 mL of hexane/dichloromethane (1:1, v/v). After concentrating with a

gentle stream of N_2, the residue was dissolved in methane with a final volume of 1.0 mL for HPLC analysis (Chigbo et al., 2013).

PHE was detected on a HPLC (Waters 600) equipped with an ^{18}C reversed-phase column (4.6 mm × 25 cm) and a photo diode-array detector. Methanol:water (90:10, v/v) at a flow rate of 0.8 mL/min was used as the mobile phase. HPLC analysis was performed at a wavelength of 254 nm, and a 20-µL sample or standard PHE compounds were injected into the chromatograph under standardised conditions. An external standard method was used for quantitation in terms of peak areas (Dong et al., 2008).

2.5 Statistical analysis

Statistical analysis was performed using SPSS 17.0. The statistical significance of differences (p-value <0.05) in PHE concentration, abundance of *ahdA1b-1* and *xyLE* genes, and the biomass of dry ryegrass among the different treatments was analysed using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test.

3. Results and discussion

3.1 Characterisation of PHE-degrading bacteria

After incubation in Luria–Bertani medium for 48 h, the colony of the isolated strain was observed to be yellow, with a diameter of 3–4 mm, translucent and glossy, with a smooth surface and neat edges (Figure 1A-a). The images of cells obtained by transmission electronic microscopy showed that the strain was rod-shaped (0.5-0.7 µm × 1.2-1.7 µm) with flagella (Figure 1A-b). The results of 16S rRNA sequence showed its 99% identity with the nucleotide sequences of the *Sphingobium abikonense* strain NBRC 16140 (NR 113839.1), *Sphingobium abikonense* strain IAM 12404 (NR 112079.1) and *Sphingobium lactosutens* strain DS20 (NR 116408.1) (Figure 1B). The strain was therefore classified as a *Sphingobium* strain within the genus *Sphingomonas*, family *Sphingomonadaceae*, class *Alphaproteobacteria*, and named as *Sphingobium* PHE-1.

3.2 Impacts of Cu(II) on PHE degradation by *Sphingobium* PHE-1

Figure S1 showed the PHE removal efficiency in MSM at 24 h with different concentrations of PHE as the sole carbon source. PHE was completely degraded when its initial concentration was less than 600 mg/L. Above this level, the removal efficiency decreased with the increasing initial PHE concentration. The results indicated that Sphingobium PHE-1 possesses powerful PHE biodegradability, better than Sphingobium chlorophenolicum C3R metabolizing ~60% of the PHE in 2 days with an initial concentration of 300 mg/L in liquid culture similar to this work (Colombo et al., 2011) and a Sphingobium strain utilizing more than 200 mg/L PHE within 24 h in liquid culture (Prakash and Lal, 2006). Besides, it was observed that ~ 50% of the added PHE was degraded owing to the inoculation of strain Sphingobium PHE-1 in PHE-copper co-contaminated soils .

To study the influence of Cu(II) on PHE degradation by strain *Sphingobium* PHE-1, the removal efficiency of PHE was tested in the presence of different concentrations of Cu(II). Figure S2 showed that PHE was almost completely degraded when Cu(II) was less than 3.22 mM. The removal efficiency maintained 88.2% even when the Cu concentration rose to 4.03 mM and then decreased with the increasing Cu(II). The similar behaviour was also observed for the mixed flora derived from soil suspensions, the PHE degradation ability of which was not significantly affected when the Cu(II) concentration was no more than 0.43 mM. Previous study showed the declining microbial respiration in the presence of Cu(II), and the higher the Cu(II) concentration, the more pronounced the inhibition (Sokhn et al., 2001). The limited impact caused by high level Cu(II) in this work might be explained by the little influence of Cu(II) on the enzymatic activity of dioxygenase related to PAHs degradation. It was reported replacement of the iron at the active site of iron-containing 2,3-dioxygenase with copper weakly affects its activity owing to the stability of the metal complexes (Gopal et al., 2005; Guzik et al., 2013).

Sphingomonas species have long been known for degrading a wide range of PAHs in contaminated soils and are often detected in copper-contaminated media, such as copper-exposed groundwater treatment plants and soils near copper mines (Stolz, 2009). *Sphingobium*, comprising 25 recognised species, is the main subgenus of the

Sphingomonas genus with the capacity of PAHs degradation (Kertesz and Kawasaki, 2010). It was demonstrated that *Sphingobium chlorophenolicum* strain C3R significantly improves the biodegradation rate of PHE in PAHs-contaminated soils in the presence of both cadmium and arsenic (Colombo et al., 2011). Some other *Sphingobium* strains with the ability to degrade PAHs and substituted PAHs were also isolated from a river, a pentachlorophenol-contaminated industrial site and freshwater sediment, a polluted stream and 2,4-dichloroprop-pretreated soils (Kertesz and Kawasaki, 2010). Furthermore, the enzymes involved in the catabolic pathways and the corresponding genes in *Sphingobium* strains have also been well studied (Leys et al., 2004; Pinyakong et al., 2003a; Story et al., 2000).

3.3 The effect of PHE and Cu(II) on C23O gene transcription

In this work, we successfully amplified a C23O gene from strain *Sphingobium* PHE-1 by using the primers designed in accordance with known ones. The phylogenetic information in Figure S3 showed that the *xylE* gene of *Sphingobium* PHE-1 was closely related to the genus *Sphingobium*, sharing 95%, 92% and 92% similarity with the nucleotide sequences of *S.* strain ZP1, *S. yanoikuyae* strain B1 and *S.* strain P2, which was consistent with 16s rRNA results. The copies of *xylE* gene were then analysed by qRT-PCR using the primers designed according to the nucleic sequences of acquired *xylE* gene.

To test the effect of PHE addition on the expression of *xylE* gene and evaluate its change with time, the copy numbers of *xyLE* gene of *Sphingobium* PHE-1 in the presence of PHE were compared to the control (PHE replaced by glucose), as illustrated in Figure 2A. It was clear that the copies of *xyLE* kept increasing in the first 24 h and then decreased from 24 to 48 h. Comparison with the constant copy numbers of *xyLE* gene in the control yielded the inference that *xyLE* gene is induced by PHE. In the treatment with an initial concentration of 100 mg/Kg PHE, the expression of *xyLE* gene peaked when all PHE had been consumed. The same results were also observed in an *ex situ* system, in which *xyLE* gene was initially present at high PAHs concentration, but disappeared with a substantial decrease of PAHs after 1 week (Wikstrom et al., 1996). This is also consistent with the results of Zhao et al. (Zhao et

al., 2011), who described that the expression of C23O gene in PHE-degrader *Pseudomonas sp.* ZP1 increased during the PHE degradation, but dramatically dropped off when PHE ran out.

Figure 2B showed the expression of *xyLE* gene against different concentrations of Cu(II) with 100 mg/L PHE as the sole carbon source after 24 h. An unexpected promotion of *xyLE* expression was observed when Cu(II) was added, compared to the control (no Cu(II)), and its expression level increased with the rise of Cu(II). This indicated that *Sphingobium* PHE-1 would probably resist the damage caused by Cu(II) via encouraging *xyLE* expression for PHE metabolism and catabolism, generating more energy to enhance the expression of genes related to the oxidative stress response, DNA and protein repair, metal transport and other processes (Baker-Austin et al., 2005; Gu et al., 2016). Generally, heavy metals inhibit the microbial degradation of organic compounds via disrupting general enzymes or functional genes responsible for PAHs degradation (Sokhn et al., 2001). The exception involved a metal-tolerant and phenol-degrading strain, for which cadmium was reported to increase its C23O activity (Hupert-Kocurek et al., 2013). It is interesting that a high level of Cu(II) promoted the expression of the C23O gene in *Sphingobium* PHE-1 in the present study, which should be useful for the bioremediation of copper-PAHs co-contaminated soils.

The degradation of PHE by bacteria is driven by enzymes and is dependent on the levels of enzymatic activity. Two key enzymes are involved in the PHE-degradation process: ring-hydroxylating dioxygenase (PAH-RHD) and aromatic ring-cleavage dioxygenase. PAH-RHD controls the incorporation of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiol in the initial step of PAHs metabolism. Thereafter, the dihydroxylated cis-dihydrodiol intermediates are cleaved by dioxygenase *via ortho*-cleavage or *meta*-cleavage pathways. C23O protein acting as a ring-cleavage enzyme in the *meta*-cleavage pathways seems to consist of a superfamily of enzymes (Boldt et al., 1995). C23O genes have been found in strains *Sphingomonas* ZP1 (Zhao et al., 2011), *Pseudomonas stutzeri* AN10, *Pseudomonas putida* G7, and *Pseudomonas putida* NCIB9816 (Habe and Omori, 2003), and their

gene sequences in these bacteria with the ability to degrade different PAHs have been uncovered. Thus, C23O genes are potentially good indicators in monitoring the bacterial subpopulations involved in the ring cleavage of aromatics and the final steps of the degradation of some PAHs. For example, it was reported that C23O genes were successfully used to monitor the subpopulations of PAHs-degrading microbes in different types of soils (Wikstrom et al., 1996).

3.4 Sequence analysis of PAH-RHD gene clusters and the effects of PHE and Cu(II) on its transcription

By amplifying the PAH-RHDα gene from the total DNA of *Sphingobium* PHE-1 with the designed primers (Table S1), a DNA fragment of 1490 bp was obtained. The nucleotide sequence of the fragment exhibits 99% and 97% similarity with the *ahdA1b* operon affiliated to *Sphingomonas* sp. ZP1 (EU082776) and *Sphingomonas* sp. P2 (AB091693), respectively, which is then named as *ahdA1b*-1 (Figure S4).

The effects of PHE and Cu(II) on *ahdA1b-1* transcription were investigated using the primers designed according to the nucleic sequences of *ahdA1b-1*, as illustrated in Figure 4. It was clear that the *ahdA1b-1* gene held a similar expression pattern as *xylE* gene, rapidly increasing over time before 24 h but slowed down from 24 to 48 h with the exhaustion of PHE in the medium. Meanwhile, the expression of *ahdA1b-1* gene in the control (glucose instead of PHE) was significantly lower than those supplemented with PHE ($p<0.05$) and had tiny fluctuation throughout the experiment. It was proposed that the expression of *ahdA1b-1* gene was induced by PHE and reached a plateau when the PHE was used up. The same results were also obtained by Pinyakong et al., who found the inducible genes encoding terminal oxygenase in *Sphingobium* sp. strain P2 (Pinyakong et al., 2003b). Preliminary study on the response of *Sphingomonas aromaticivorans* strain F199 to various aromatic compounds demonstrated that its aromatic catabolic activity was induced after exposure to naphthalene (Pinyakong et al., 2003b). Additionally, Cu(II) significantly affected the expression of *ahdA1b-1* gene in *Sphingobium* PHE-1, from the higher expressed copy numbers of *ahdA1b-1* gene copies in Cu(II) amended treatments than that in samples without copper, even when the Cu(II) concentration was increased to

4.03 mM (p<0.05). The results indicated that *ahdA1b-1* expression is promoted by Cu(II), consistent with the influence of Cu(II) on the expression of *xyLE* gene. Although previous research showed that the expression of the genes responsible for naphthalene degradation in a *Pseudomonas* strain is enhanced by nickel (Siunova et al., 2007), no work prior to this study reported the transcription of PAHs-degrading genes is promoted by high level of Cu(II).

In the first step of PHE metabolism, the aromatic nucleus is attacked by molecular oxygen forming *cis*-dihydrodiol with the function of PAH-RHD. PAH-RHD is composed of an iron-sulphur flavoprotein reductase, an iron–sulphur ferredoxin and a terminal dioxygenase. The terminal dioxygenase consists of large α and small β subunits, and two conserved regions exist in α subunit (RHDα) (Kauppi et al., 1998). Primers based on these conserved regions have been designed and successfully applied to amplify the target *pahAc*-like, *phnAc*-like and *nagAc*-like genes (Cebron et al., 2008). Besides, genes encoding degrading proteins are frequently found in grouping together in a cluster (Qiu et al., 2013). SEFA-PCR was therefore performed to obtain the flanking DNA sequences of *ahdA1b-1* in this study. A 6.6-kb upstream and a 2.1-kb downstream DNA fragments were amplified with three pairs of primers (Table S1). After cloning and sequencing, it is noted that the 6.6-kb upstream DNA fragment contains a small subunit and a large subunit belonging to the PAH-RHD gene, and the 2.1-kb downstream DNA fragment has a small subunit affiliated to the PAH-RHD gene. The 1490-bp core fragment was assembled with the flanking fragments to generate a 9.1-kb DNA fragment, which shows 96% similarity to the PAHs-degrading gene cluster affiliated to *Sphingomonas* sp. P2 and *Sphingobium yanoikuyae* strain B1. As shown in Figure 3, the analysis of nucleotides and the deduced amino acid sequences of the 9.1-kb DNA fragment using the online ORF Finder and Blastx programme revealed that six consecutive ORFs exhibited 99%, 96%, 93%, 97%, 99% and 96% nucleotide sequence identities with *xylA*, *xylM*, *ahdA2b*, *ahdA1b*, *ahdA2a* and *ahdA1a*, respectively, which are located in the PAHs-degrading gene cluster of *Sphingomonas* sp. P2 (AB091693) and *S. yanoikuyae* strain B1 (EF151283) (referred to as *xylA*, *xylM*, *bphA2b*, *bphA1b*,

bphA2a and *bphA1a*) (Pinyakong et al., 2000, 2003a) . The clusters from *xylA* to *ahdA1a/bphA1a* in the three strains were assembled in the same order and transcriptional direction (Figure 3), indicating the high conservation of the functional genes encoding PAH-RHD in strains affiliated to the *Sphingomonas* genus. It was also reported that the aromatic-degrading genes from the *Sphingobium* sp. B1, *Sphingobium* sp. Q1 and *Novosphingobium aromaticivorans* F199 exhibited high homology (Pinyakong et al., 2003a; Pinyakong et al., 2003b). For example, in *Sphingomonas*, the degrading genes encoding arene cis-dihydrodiol dioxygenase and the enzymes responsible for the conversion of 1,2-dihydroxynaphthalene to salicylate were reported to be similar in sequence (Waigi et al., 2015).

3.5 The application of *Sphingobium* PHE-1 coupled with ryegrass in the remediation of PHE-copper co-contaminated soils

The inoculation of *Sphingobium* PHE-1 significantly improved the growth of ryegrass (Figure 5). Interestingly, the growth of ryegrass was also found to be greatly stimulated by Cu(II), with plants grown in PHE-copper co-contaminated soils having higher biomass than those grown in soils with PHE contamination only (Figure 5), which was consistent with the trends of *ahdA1b-1* and *xyLE* gene transcription in MSM (Figure 2B and Figure 4B). This improvement in ryegrass growth was attributed to the increasing activities of *Sphingobium* PHE-1 in the presence of Cu(II). It was reported that some heavy metal-resistant bacteria could promote the growth of host plants. Examples included a copper-resistant plant growth-promoting bacterial (PGPB) strain, Ax10, which was isolated from a copper mine soil and facilitated *Brassica juncea* growth and Cu(II) uptake (Ma et al., 2009). Besides, the nickel-resistant strain PGPB SRS8 was also found to be capable of stimulating plant growth and nickel accumulation in the crops *Ricinus communis* and *Helianthus annuus* (Ma et al., 2011).

The residual PHE concentrations in soils subjected to different treatments on day 56 were presented in Figure 6. The results clearly illustrated that more PHE was removed from soils inoculated with *Sphingobium* PHE-1, compared to the uninoculated treatments, and ryegrass planting also resulted in lower residual PHE.

459 The best performance in terms of PHE reduction occurred in the treatment with both
460 ryegrass and *Sphingobium* PHE-1. This optimal PHE removal was explained by
461 microbial degradation and, to some extent, by enhanced adsorption to roots and
462 accumulation in ryegrass shoots, as found in our previous study (Wang et al., 2012).
463 Besides, the bioavailability of PAHs, which often limits the biodegradation of these
464 compounds, may be increased with the aid of some components in the root exudation
465 (An et al., 2010; Cerniglia, 1993; Gao et al., 2010).

466 The abundance of *xylE* genes in soils was also studied to monitor the effect of
467 ryegrass on the growth and degrading activities of *Sphingobium* PHE-1, attributing to
468 its higher specificity than *ahdA1b-1* and 16s rRNA genes. A higher abundance of *xylE*
469 gene in soils with ryegrass was found than that in soils without ryegrass (Figure 7,
470 $p<0.05$), which proved that ryegrass could encourage the abundance of *Sphingobium*
471 PHE-1. Additionally, the copy numbers of *xylE* gene in soil was also enhanced by the
472 Cu(II) addition, which was in agreement with the transcription of *xylE* in
473 *Sphingobium* PHE-1 (Figure 2B). The increase of *xylE* abundance in soils planting
474 with ryegrass was attributed to the positive influence of rhizospheric effect on
475 microbes. The fibrous root of ryegrass observed in this work provided microbial
476 attachment sites and enhanced soil aeration, and the vegetation cover created
477 favourable environmental conditions such as temperature and soil moisture. More
478 importantly, the root exudates as the ecological driver in the rhizosphere supply
479 microorganisms with a relatively constant source of nutrients, such as water soluble
480 carbon, nitrogen and phosphorus, especially in poor soils. The rhizosphere therefore
481 improves the microbial growth, activities and the abundance of functional genes
482 (Haritash and Kaushik, 2009). Besides, many secondary plant metabolites in root
483 exudation with similar structure to aromatic hydrocarbons may stimulate the
484 metabolic pathways of PAHs degraders (Martin et al., 2014). It was observed that
485 more microbes, enhanced activities and increased abundance of PAHs-degrading
486 genes in planted versus unplanted soils occurred in PAHs contaminated soils (Haritash
487 and Kaushik, 2009; Thomas and Cebron, 2016).

488 In the present study, the growth of ryegrass was improved by *Sphingobium*

PHE-1. In turn, the ryegrass provided abundant nutrients to *Sphingobium* PHE-1 and promoted its colonisation in the rhizosphere, which enhanced the dissipation of PHE. It was proved that PHE and pyrene (PYR) dissipation with the aid of microbes in soils was improved by planting ryegrass and the levels of PHE and PYR increased with the distance from the root surface (Gao et al., 2013). A similar phenomenon was also observed in another study, in which the growth of ryegrass significantly increased soil peroxidase activities, leading to enhanced dissipation of PHE and PYR in, and additionally, the interactions of ryegrass with the two microbes further improved the dissipation of PHE and PYR (Yu et al., 2011). In the present study, the growth of ryegrass was improved by inoculation with *Sphingobium* PHE-1, further benefiting bacterial growth by rhizospheric effect and improving PHE dissipation as well. The capacity of strain *Sphingobium* PHE-1 to exhibit Cu(II) tolerance and degrade PHE suggests its feasibility in the remediation of PAHs-heavy metal co-contaminated soils and sediments.

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Legends of tables and figures

Fig. 1. A: Colonies (a) and transmission electronic microscopy image (b) of *Sphingobium* PHE-1. B: Phylogenetic tree derived from the 16S rRNA genes of *Sphingobium* PHE-1 and related species by the neighbor-joining method using MEGA 5.1.

Fig. 2. The expression of *xylE* gene at different incubation time (A) or Cu(II) concentration (B). The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 3. Comparison of PAH-RHD cluster structure between *Sphingomonas* sp. strain P2, *Sphingobium* sp. strain PHE-1 and *Sphingobium yanoikuyae* strain B1. The open reading frames (ORFs) are indicated by arrows. The scale is in bases.

Fig. 4. The expression of *ahdA1b-1* gene at different incubation time (A) or Cu(II) concentration (B). The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 5. Biomass of dry ryegrass in different treatments. Control: soil amended with PHE. PHE-1: soil amended with PHE and *Sphingobium* PHE-1. Cu: soil amended with PHE and Cu(II). PHE-1+Cu: soil amended with PHE, Cu(II) and *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–c) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 6. Residual PHE concentration in soils from different treatments. Control: soil amended with PHE. PHE-1: soil amended with PHE and *Sphingobium* PHE-1. Cu: soil amended with PHE and Cu(II). PHE-1+Cu: soil amended with PHE, Cu(II) and *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a–d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. 7. The abundance of *xylE* gene in soils from different treatments. Control: soil

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Supporting information

Table S1 Primers used in this study

Table S2 Primers used for transcriptional analysis of PAH-RHD and C23O genes

Fig. S1. Effects of initial PHE concentration on PHE degradation efficiency by *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a-d) represent the statistical significance at the 0.05 level with the LSD test.

Fig. S2. Effects of Cu(II) concentration on the PHE degradation efficiency by *Sphingobium* PHE-1. The values are the averages of three replicates. Error bars are the standard errors of the mean of three replicates. The small letters (a-e) represent the statistical significance at the 0.05 level with the LSD test.

Fig. S3. Phylogenetic tree of catechol -2,3- dioxygenase gene (*xyLE*) from *Sphingobium* PHE-1 along with the closest matches in GenBank, constructed with MEGA 5.1 using the neighbor-joining method.

Fig. S4. Phylogenetic tree of aromatic compounds-catabolic gene (*ahdA1b-1*) from *Sphingobium* PHE-1 along with the closest matches in GenBank, constructed with MEGA 5.1 using the neighbor-joining method.

Fig. S5. Gel electrophoresis image of the flanking DNA fragments of *ahdA1b-1* by SEFA-PCR. M: λDNA/*Hind*III marker; 1: *ahdA1b* upstream SEFA-PCR products; 2: *ahdA1b* downstream SEFA-PCR products. The arrows show the bands of target fragments.

Figure

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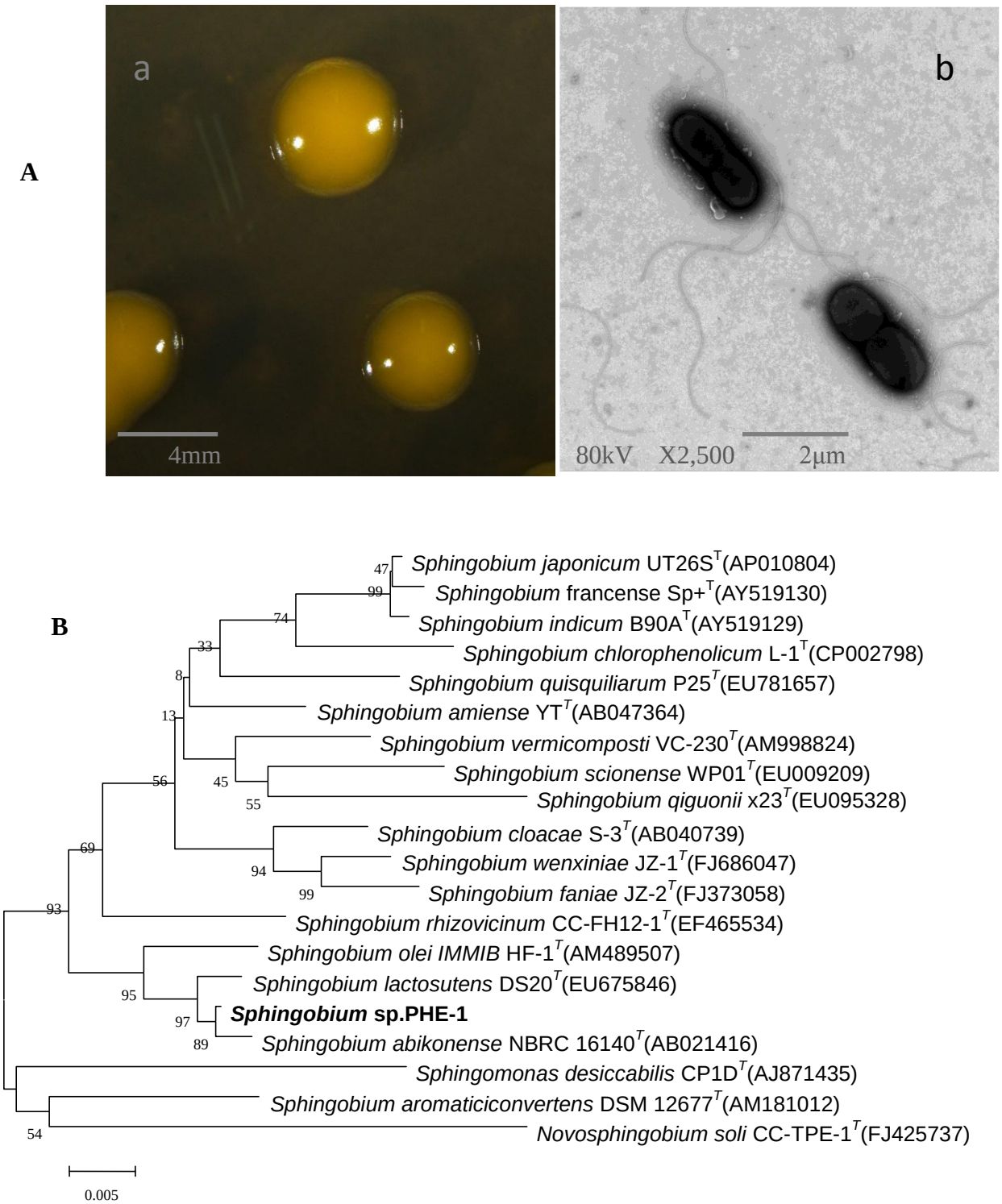
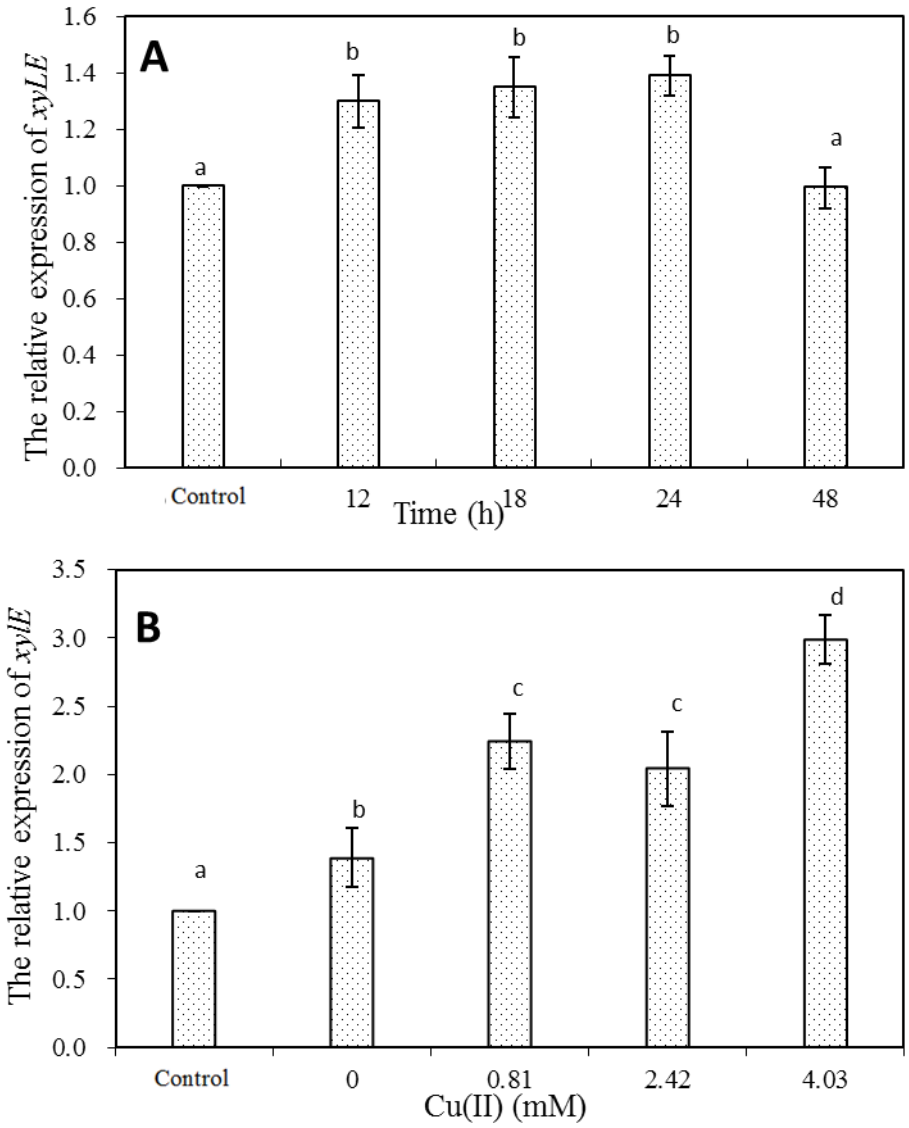


Fig. 1. A: Colonies (a) and transmission electronic microscopy image (b) of *Sphingobium* PHE-1. B: Phylogenetic tree derived from the 16S rRNA genes of *Sphingobium* PHE-1 and related species by the neighbor-joining method using MEGA 5.1.



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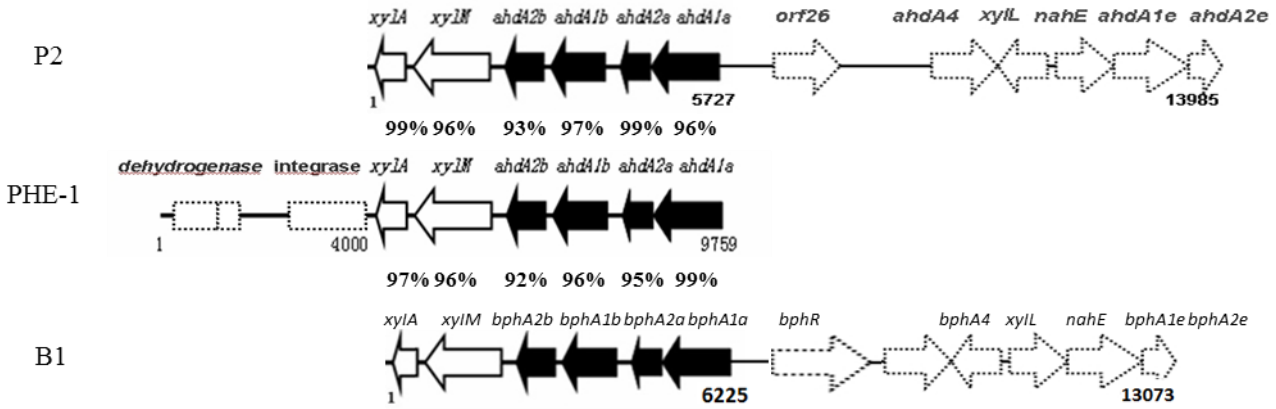

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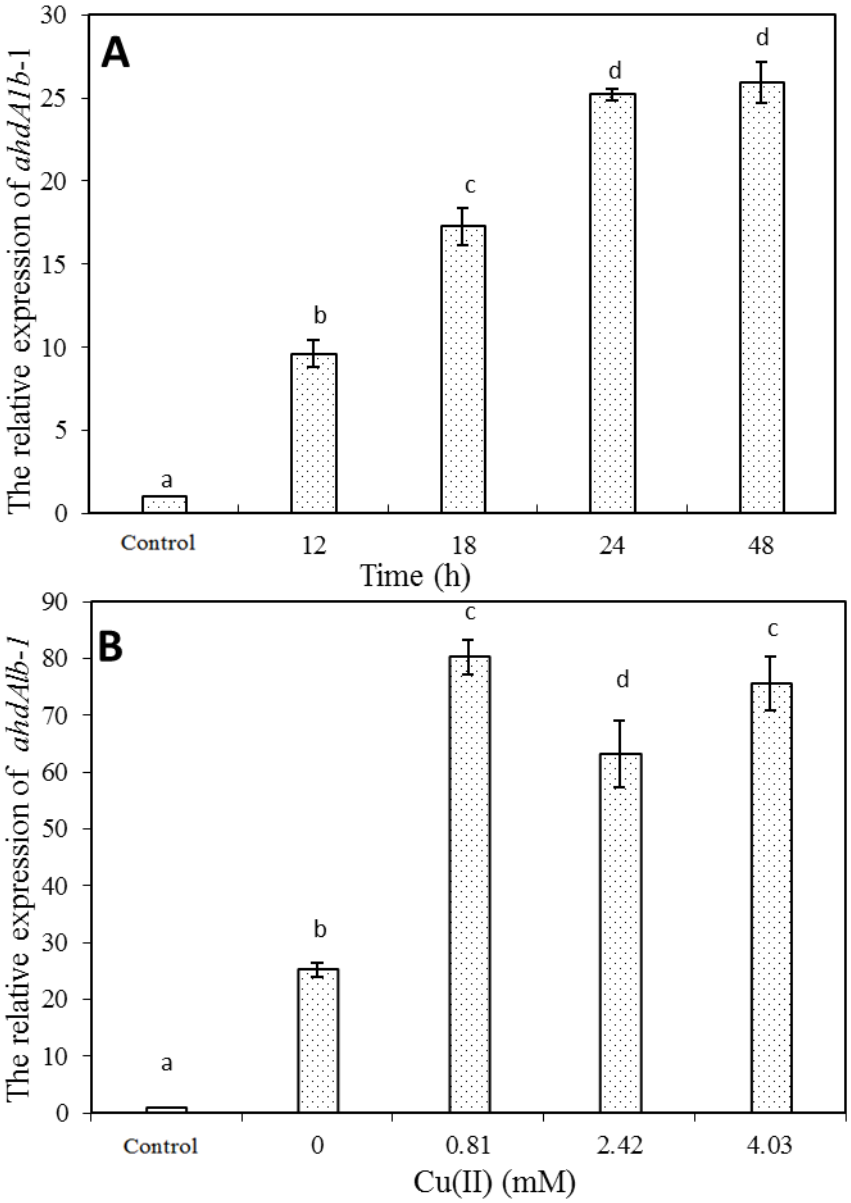

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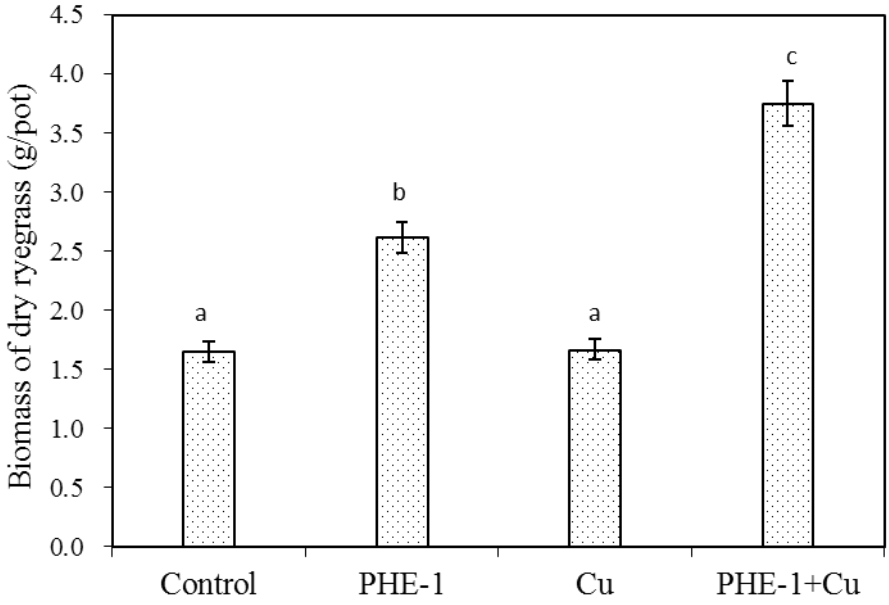

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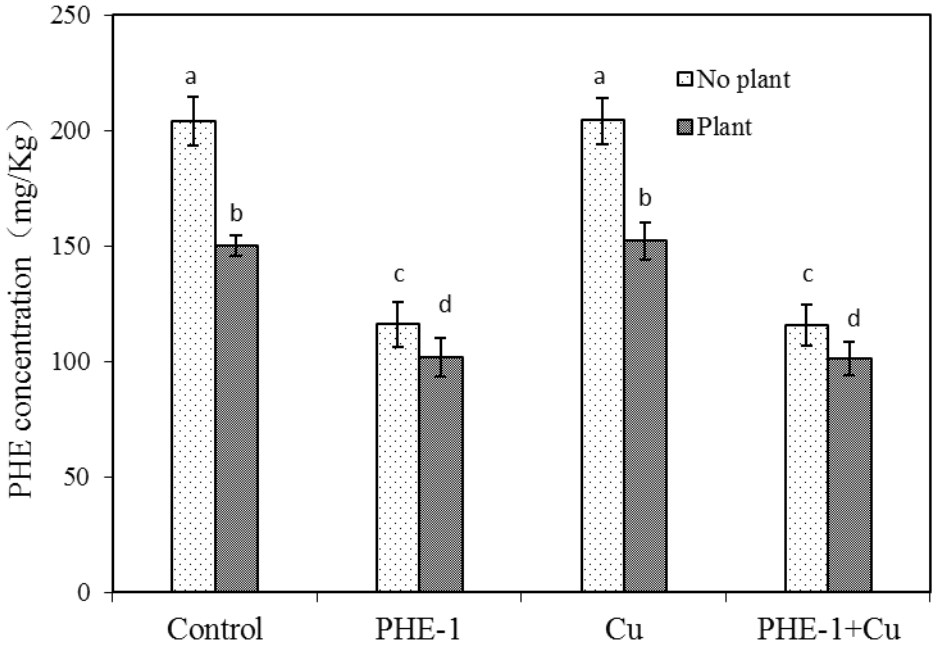

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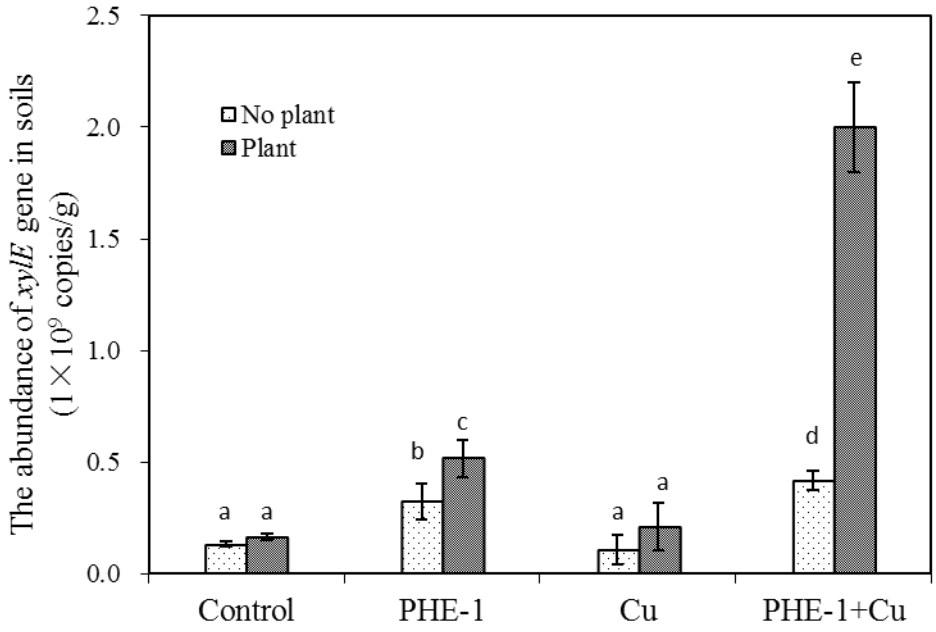

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Supplementary Material

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