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Isolation and characteristics of a novel biphenyl-degrading bacterial strain, *Dyella ginsengisoli* LA-4

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Abstract

A novel biphenyl-degrading bacterial strain LA-4 was isolated from activated sludge. It was identified as *Dyella ginsengisoli* according to phylogenetic similarity of 16S rRNA gene sequence. This isolate could utilize biphenyl as sole source of carbon and energy, which degraded over 95 mg/L biphenyl within 36 h. The major metabolites formed from biphenyl, such as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) and benzoic acid, were identified by LC-MS. The crude cell extract of strain LA-4 exhibited the activity of 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) and the kinetic parameters K_m was 26.48 μmol/L and V_{max} was 8.12 U/mg protein. A conserved region of the biphenyl dioxygenase gene *bph*A1 of strain LA-4 was amplified by PCR and confirmed by DNA sequencing.

Key words: *Dyella ginsengisoli*; biphenyl; biodegradation; metabolic pathway; biphenyl dioxygenase

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Introduction

Biphenyl is a natural component of coal tar, crude oil, and natural gas. It has been widely used in organic synthesis, food preservatives, heat transfer fluids, and the synthesis of polychlorinated biphenyl (Moody *et al*., 2002; Hawley, 1971; Weaver *et al*., 1979). Although the application of biphenyl has been decreased quickly in recent years, it still remains in the environment, resulting in serious environmental problems.

According to its toxicological properties, biphenyl in the diet has been reported to cause kidney disorders, reduced life span (Ambrose *et al*., 1960) and result in bladder cancer (Boehncke *et al*., 1999) for animal, and also could cause slight eye irritation, hepatotoxicity and toxic effects on the central and peripheral nervous systems (Sandmeyer, 1981).

The use of microorganisms is expected to be an effective tool for bioremediation of polluted environment. Lunt and Evans (1970) and Catelani *et al*. (1970) first reported bacterial isolates that could grow on biphenyl as sole source of carbon and energy. From then on, several biphenyl-degrading bacteria have been isolated, and biphenyl degradation by these microorganisms has been widely studied (Pieper, 2005).

An upper pathway via dioxygenation at the 2,3-position is the major catabolic pathway of biphenyl biodegradation under aerobic conditions (Furukawa *et al*., 2004; Borja *et al*., 2005; Pieper, 2005). The initial step in the aerobic catabolism of biphenyl by most microorganisms occurs via oxidation of biphenyl at 2,3-position to a *cis*-dihydrodiol, followed by dehydrogenation to 2,3-dihydroxybiphenyl. The ring is cleaved to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA, the ring *meta*-cleavage product, a yellow intermediate), which is then hydrolyzed to benzoic acid and 2-hydroxypenta-2,4-dienoate (Erickson and Mondello, 1992; Hayase *et al*., 1990; Peloquin and Greer, 1993; Masai *et al*., 1995; Kikuchi *et al*., 1994; Furukawa and Miyazaki, 1986). The genes encoding the enzymes of the biphenyl upper pathway (termed *bph*) were first cloned from *Pseudomonas pseudoalcaligenes* KF707 and later from *Burkholderia* sp. LB400 (Furukawa and Miyazaki, 1986; Mondello, 1989), which is now reclassified as *Burkholderia xenovorans* strain LB400. To date, four types of *bph* gene cluster have been cloned (Yang *et al*., 2007). But the researche works on biphenyl/PCBs degradation and *bph* genes have been mainly limited to the genus *Pseudomonas*, *Burkholderia*, and *Rhodococcus*. Therefore, it is necessary to isolate novel microorganisms and clone novel *bph* gene to achieve effective bioremediation of the biphenyl/PCBs pollution.

Here we report the isolation of a new biphenyl-degradation strain, *Dyella ginsengisoli* LA-4, and its characterization, including growth on and degradation of biphenyl, the catabolic pathway, 2,3-dihydroxybiphenyl biodegradation by the crude cell extracts, and amplification

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and sequencing of a conserved region of the *bph*A1 gene encoding the α subunit of the biphenyl dioxygenase.

1 Materials and methods

1.1 Chemicals

Biphenyl (> 99% purity) and 2,3-dihydroxybiphenyl (> 98% purity), 4-chlorobiphenyl (> 99% purity) were purchased from Sigma-Aldrich (Shanghai, China). Bovine serum albumin (BSA) was obtained from TaKaRa Bio. Co., Ltd. (Dalian, China). All other chemicals were of the highest purity, commercially available and used without further purification.

1.2 Media and culture conditions

The media used in this study were a Defined Basal Salts Medium (DBSM) (Qu *et al*., 2005), which (pH 6.2) contained (g/L): NaCl 0.5, $K_2HPO_4{\cdot}3H_2O$ 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, and $FeSO_4{\cdot}7H_2O$ 0.01, and a modified DBSM containing 2 g/L of bactotryptone and 500 mg/L biphenyl additionally. Biphenyl and other aromatic compounds were added from an acetone stock solution (acetone does not support the growth of strain LA-4). Solid media contained 20 g/L agar in DBSM. Both media were autoclaved at 121°C for 20 min. The strain was cultivated aerobically at 30°C in both media.

1.3 Isolation and identification of biphenyl-degrading strain

A sludge sample was taken from the wastewater treatment plant of PetroChina Petrochemical Company, China. The fresh sludge sample was inoculated into the DBSM liquid medium supplemented with biphenyl crystals (100 mg/L). After two months of enrichment and screening, the most efficient strain designated as LA-4 was chosen for this study. The morphology was observed by scanning electron microscope (JEM-1200EX, Japan).

Genomic DNA was extracted using a modified technique for extracting DNA from Gram-negative bacteria (Neilan, 1995). The 16S rRNA gene was amplified by PCR. The primers 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1522R (5'-AAGGACGTCATCCAGCCGCA-3') were used. The PCR product was sequenced by TaKaRa Biotechnology Co., Ltd. (Dalian, China), and the sequence was compared with the sequences in the GenBank database using the Blastn Program. The 16S rRNA gene sequence of strain LA-4 and related sequences retrieved from GenBank were aligned using Clustal W, and the aligned sequences were used to construct a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) and Jukes-cantor distance correction matrix method. The branching pattern was checked by 1000 bootstrap replicates.

1.4 Isolation and identification of metabolites during biphenyl biodegradation

At 72, 120, and 312 h of incubation of strain LA-4 in DBSM containing biphenyl, cell suspension was taken aseptically, and the culture supernatant was separated by centrifugation (15000 r/min, 20 min). The pH of the supernatant was measured. After adjusted to pH 2.5, 4.3, 11.9, and 12, the UV-Vis absorbance spectra of the supernatant at each pH was scanned using a scanning spectrophotometer (V-560, Jasco, Japan).

Following adjusting the supernatant to pH 4.0, the metabolites present in the supernatant were extracted from 20 mL supernatant with an equal volume of ethyl acetate for four times. The extracts were dried using anhydrous Na_2SO_4 and an evaporator. The dry samples were dissolved in 1 mL methanol for LC-MS analysis.

1.5 Growth of strain LA-4 on other aromatic compounds

The growth of strain LA-4 on other aromatic compounds (100 mg/L, Table 1) was tested in flasks containing DBSM. The growth of strain LA-4 was determined by optical density (OD) at 660 nm using a scanning spectrophotometer (V-560, Jasco, Japan).

1.6 Analytical methods

Bacterial growth was determined by measuring increase in the bacterial protein content (Yamazoe *et al*., 2004) using the Bradford assays with BSA serving as the standard (Bradford, 1976).

The residual biphenyl was extracted with an equal volume of ethyl acetate. The biphenyl concentration was analyzed using an Agilent 6890 Series Gas Chromatography (Agilent, USA), equipped with a flame ionization detector (FID) and capillary column (30 m × 0.32 mm × 0.25 μm HP-5) (Agilent, USA). The following temperature program was performed: initial column temperature 80°C for 5 min, 5°C/min to 100°C, 15°C/min to 280°C holding for 5 min. The injector and detector temperature were all 280°C. The carrier gas was N_2 at a constant flow rate of 1.0 mL/min. The injection volume was 1 μL using split (50:1).

The identification of the metabolites from biphenyl was performed by LC-MS (Hewlett Packard 1100 MSD system, Agilent, USA) with a Zobax column (150 mm × 2.1 mm i.d., 5 μm, Agilent, USA). The final extract (20 μL) obtained above was injected automatically into the LC-MS system. The flow rate was 1 mL/min and UV absorbance spectra were obtained online at 254 nm. The compounds were eluted using a linear gradient of 50%–95% methanol/water (*V/V*) over 40 min. The mass spectrometry (MS) was equipped with atmospheric

Table 1 Growth of strain LA-4 on related compounds

Compound	Result	Compound	Result
4-Chlorobiphenyl	+	Toluene	+
Benzoic acid	+	Diphenyl amine	+
Catechol	+	Naphthalene	+
Nitrobenzene	–	1,3,5-Triphenylbenzene	+
Aniline	+	4-Aminophenol	+
Phenol	–		

+: utilization of this substrate as a sole source of carbon and energy; –: no utilization of this substrate as a sole source of carbon and energy.

pressure chemical ionization and electro spray ionization interface (APCI-ESI); a capillary voltage was set at 3.5 kV with drying gas temperature at 350°C and a drying gas flow of 8 L/min; nebuliser pressure was 207 kPa; the spectra were acquired in both positive and negative scan modes, over the *m/z* range 50–550 at 1 scan/s.

1.7 Preparation of crude cell extracts

Strain LA-4 cells were harvested from 1 L of modified DBSM by centrifugation (15000 r/min, 20 min at 4°C), and the pellet was washed twice with 50 mmol/L Tris-HCl buffer (pH 8.0). Then the washed pellet was re-suspended in 10 mL of the same buffer and lysed by freezing and thawing followed by sonication (225 W, at 4°C for 30 min, Ultrasonic processor CPX 750, USA). After centrifugation (22000 r/min, 20 min at 4°C), the supernatants were used as crude extracts.

1.8 Activity analysis of 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD)

The 2,3-DHBD activity was assayed by the formation of the yellow *meta*-cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. Enzymatic activity was measured by monitoring the reaction product at 434 nm with a scanning spectrophotometer (V-560, Jasco, Japan). The reaction time was measured from 0 to 50 s each time, and the total volume of the reaction mixture was 2 mL. Activity assays were performed at 20°C in 50 mmol/L Tris-HCl buffer (pH 8.0) containing the 2,3-dihydroxybiphenyl at final concentrations ranging from 125 to 375 μmol/L. The reaction was initiated by the addition of 50 μL of the crude cell extracts. One unit of enzyme activity was defined as the formation of 1 μmol *meta*-cleavage compound per minute at 20°C. The molar extinction coefficient of the product under the above conditions was set as 17900 L/(mol·cm) (Eltis *et al*., 1993). Specific activity of the enzyme was defined as U/mg protein.

1.9 Cloning of a conserved region of *bph*A1 gene from strain LA-4

Primers BPH4-F (5'-AAGGCCGGCGACTTCATGAC-3') and BPH3-R (5'-TGCTCCGCTGCGAACTTCC-3') (Baldwin *et al*., 2003) based on conserved regions of the biphenyl 2,3-dioxygenase gene were used to amplify possible genes encoding biphenyl 2,3-dioxygenase. The PCR reaction was performed with TaKaRa LA PCR^{TM} Kit Ver. 2.1 in a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio, Dalian, China) with the following temperature program: 10 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 63°C, and 2 min at 72°C; and a final extension step of 10 min at 72°C. The fragment of the PCR products, which was about 500 bp, was recovered and purified using TaRaKa Agarose Gel DNA Fragment Purification Kit, and then sequenced by TaKaRa Biotechnology Co., Ltd. The sequence was compared with the sequences in the GenBank database using the Blastn program. The *bph*A1 gene sequence of strain LA-4 and related sequences retrieved from GenBank were aligned using Clustal W, and the aligned sequences were used to construct a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) and Jukes-cantor distance correction matrix method. The branching pattern was checked by 1000 bootstrap replicates.

1.10 Nucleotide sequence accession numbers

The sequences of the 1464 bp 16S rRNA gene, the 473 bp conserved region of *bph*A1 gene from *Dyella ginsengisoli* LA-4 have been deposited in the GenBank database under accession numbers EF191354 and EU391618, respectively.

2 Results

2.1 Isolation and identification of *Dyella ginsengisoli* LA-4

Strain LA-4 was isolated from the activated sludge sample, and it could use biphenyl as sole sources of carbon and energy. It is Gram-negative, non-motile, aerobic long rod (Fig. 1). It formed round yellow colonies on the solid medium containing biphenyl. It was oxidase-positive, catalyase-positive, and nitrification-positive. It hydrolyzed casein and DNA, and it produced acids by glucose fermentation.

The 1465 bp fragment of the 16S rRNA gene of strain

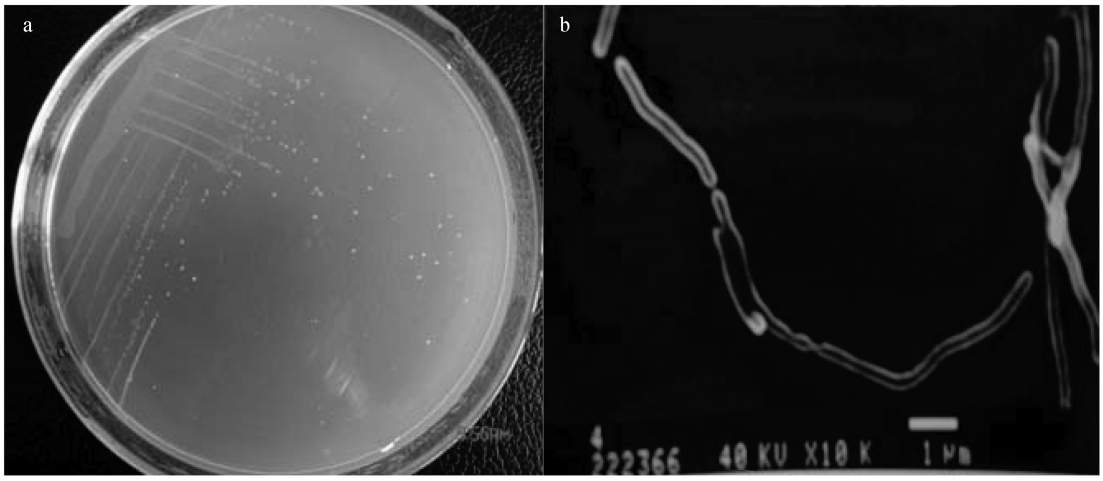

Fig. 1 Colonial morphology of *Dyella ginsengisoli* LA-4 on the plate (a) and the electron microscopic micrograph (b).

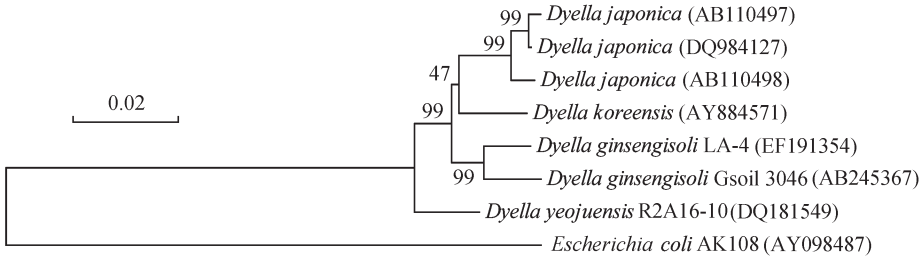

Fig. 2 A phylogenetic tree of *Dyella ginsengisoli* LA-4 and related species. The tree was inferred from a sequence alignment of 16S rRNA genes using the neighbor-joining method. The numbers indicate the bootstrap values, derived from 1 000 samples. Bar indicates 0.02 Jukes-cantor distances. *E. coli* was used as an out-group. The GenBank sequence accession numbers are in parentheses.

LA-4 (accession number: EF191354) showed 98.22% homology to that of *Dyella ginsengisoli* Gsoil 3046 (Fig. 2). Phylogenetically, the genus *Dyella* clusters within the family Xanthomonadaceae of the class Gammaproteobacteria.

2.2 Degradation of biphenyl by strain LA-4

The bacterial protein reached the maximal value at 72 h and over 95% of the biphenyl (100 mg/L) was degraded within 36 h (Fig. 3). During the incubation, the medium turned yellow, but the yellow color disappeared after 6 d. Figure 4 shows the UV-Vis absorbance spectra during the biodegradation of biphenyl. At the beginning of degradation, there was no absorbance peaks in visible area, but when cultures turned yellow, the absorbance peak at 350 nm appeared within 72 h. The maximum absorbance at 350 nm appeared at 120 h and then this peak decreased gradually.

The ability of strain LA-4 to utilize eleven other substrates is listed in Table 1. Evidently, strain LA-4 can degrade a variety of aromatic compounds.

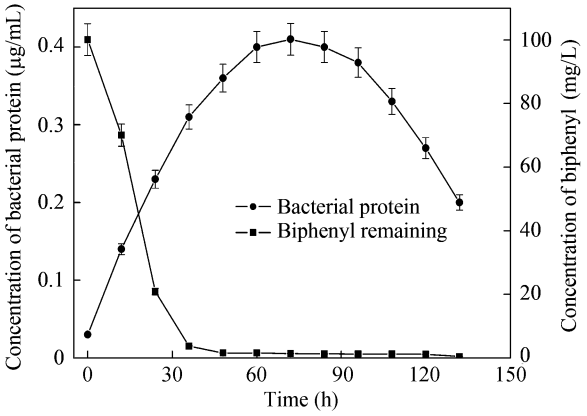

Fig. 3 Time course of the growth of strain LA-4 on biphenyl and corresponding degradation.

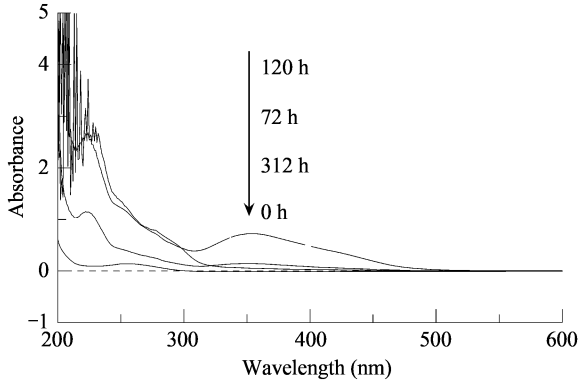

Fig. 4 UV-Vis spectra of the culture supernatant at 0, 72, 120, and 312 h incubation of strain LA-4.

2.3 Isolation and identification of metabolites from biphenyl

The absorbance peak of HOPDA, an important yellow intermediate in biphenyl degradation, could vary at different pH (Catelani *et al*., 1973). Consistently, we observed different absorbance peaks of the culture supernatant at different pH. The maximum absorbance wavelength shifted to 336 nm at pH 2.5, 344 nm at pH 4.3, 432 nm at pH 11.9, and 435 nm at pH 12 (data not shown). The results suggested that HOPDA would be produced during biphenyl degradation by strain LA-4.

The intermediates extracted from biphenyl-growing cell cultures were detected by LC-MS. The results showed a compound that was eluted at 1.806 min, and the APCI-ESI mass showed a molecule ion in positive ion mode $[M+H]^+$

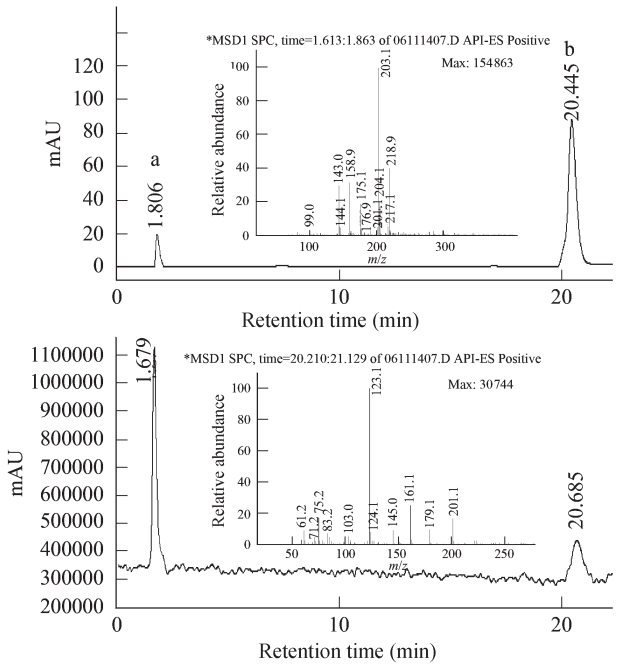

Fig. 5 LC-MS chromatograms of metabolites after 120 h of incubation. (a) a compound that was eluted at 1.806 min and the APCI-ESI mass showed a molecule ion in positive ion mode $[M+H]^+$ at *m/z* 219; (b) a compound that was eluted at 20.445 min and the APCI-ESI mass showed a molecule $[M+H]^+$ at *m/z* 123.

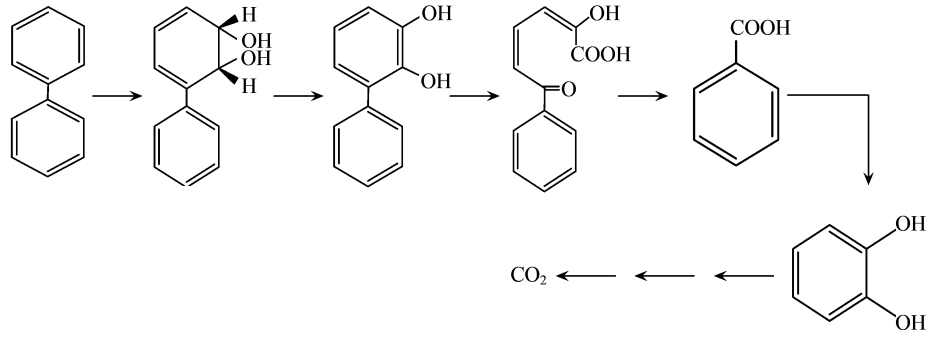

Fig. 6 A proposed metabolic pathway of biphenyl degradation by *Dyella ginsengisoli* LA-4.

at *m/z* 219, consistent with the *meta*-cleavage product of biphenyl (molecule weight 218), a yellow intermediates (Fig. 5a). Another compound was eluted at 20.445 min and the APCI-ESI mass showed a molecule ion $[M+H]^+$ at *m/z* 123, consistent with benzoic acid (molecule weight 122) (Fig. 5b). The same results were obtained in the LC-MS analysis of the supernatant extracts after 72 and 312 h of incubation. Indeed, strain LA-4 grew on benzoic acid and catechol as a sole source of carbon and energy. Therefore, a classical metabolic pathway of biphenyl by strain LA-4 was proposed (Fig. 6), which was consistent with the previous report (Pieper, 2005).

2.4 Kinetics of 2,3-DHBD by crude cell extracts of strain LA-4

Kinetic measurements, performed at low 2,3-dihydroxybiphenyl concentrations to avoid substrate inhibition, indicated that the optimal pH and temperature of the reaction were 8 and 20°C, respectively. All subsequent kinetic measurements were performed under these conditions. A Lineweaver-Burk representation of typical kinetic data was shown in Fig. 7. When the concentrations of 2,3-dihydroxybiphenyl were over 375 μmol/L, the dioxygenase showed strong substrate inhibition. The kinetic constants of 2,3-DHBD by the crude cell extracts of strain LA-4 were K_m = 26.48 μmol/L and V_{max} = 8.12 U/mg protein. The specific activity of the crude extract was 7.37 U/mg protein.

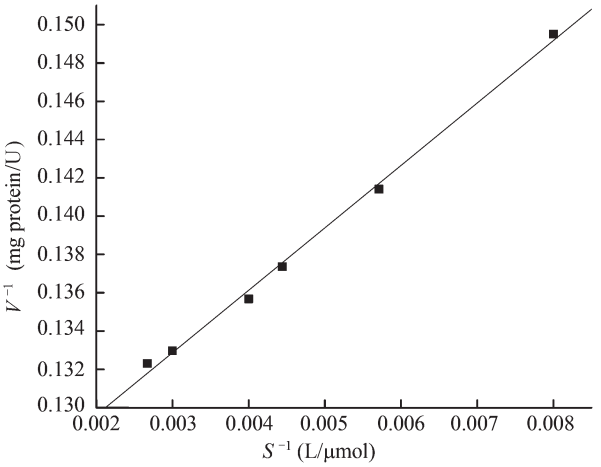

Fig. 7 Lineweaver-Burk plot of 2,3-dihydroxybiphenyl cleavage at pH 8.0 and 20°C. The fitted parameters are K_m = 26.48 μmol/L, V_{max} = 8.12 U/mg protein.

2.5 Cloning of the conserved region of *bph*A1 gene from strain LA-4

A single fragment of 473 bp was amplified by the PCR (Fig. 8). Its sequence was identified by comparison with other *bph*A1 genes. It showed the highest similarity with *bph*A in *Pandoraea pnomenusa* B-356 (80% identity) (accession number: CTU47637) (Sylvestre *et al*., 1996), followed by *Achromobacter georgiopolitanum* KKS102, *Burkholderia xenovorans* LB400 and *Pseudomonas pseudoalcaligenes* KF707 (79% identity).

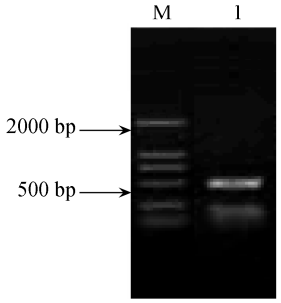

Fig. 8 Agarose gel electrophoresis of the *bph*A1 PCR product amplified from *Dyella ginsengisoli* LA-4. Lane M: DL 2000 DNA marker; lane 1: the PCR product.

3 Discussion

A novel biphenyl-degrading bacterium, strain LA-4, was isolated from activated sludge, and was identified as *Dyella ginsengisoli* according to its physiological and biochemical characteristics and 16S rRNA gene sequence. There was little information on the genus *Dyella* with respect to biodegradation of environmental pollutants. Strain LA-4 could use biphenyl and other 9 aromatic compounds as a sole source of carbon and energy. When compared to *Mycobacterium* sp. PYR-1, which required 4 d to transform 98% of 80 mg/L biphenyl (Moody *et al*., 2002), strain LA-4 could degrade biphenyl more rapidly, transforming over 95% of 100 mg/L biphenyl within 36 h. As such, LA-4 may play a more important role in the degradation of biphenyl and other related pollutants in the environment.

Most bacteria isolates capable of degrading biphenyl have the upper pathway with 2,3-dihydroxybiphenyl and

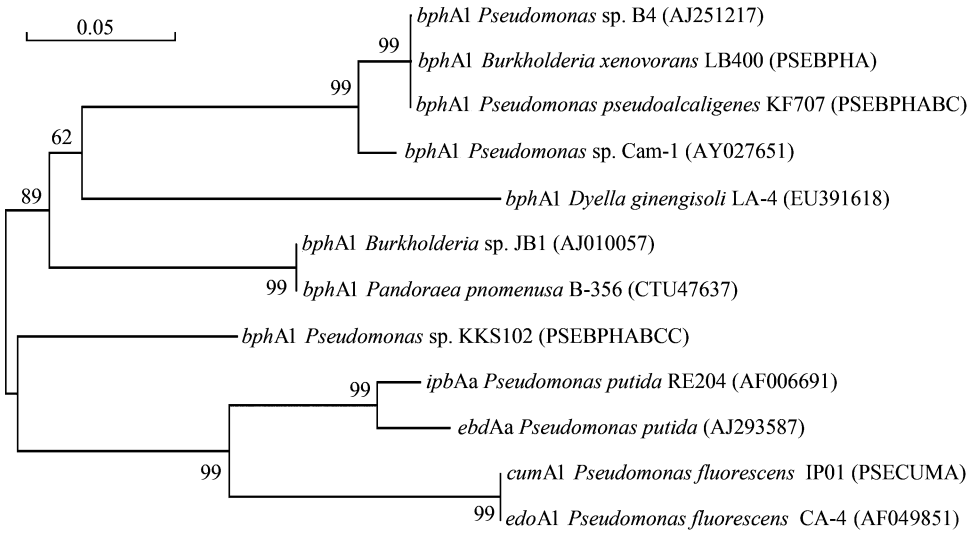

Fig. 9 A phylogenetic tree of the large subunits of aromatic dioxygenase genes. The tree was constructed by the neighbor-joining method. The numbers indicate the bootstrap values, derived from 1000 samples. Bar indicates 0.05 Jukes-cantor distances.

its *meta*-cleavage product (HOPDA) being the key intermediates, the latter of which is then converted to benzoic acid (Furukawa *et al*., 2004; Pieper, 2005; Borja *et al*., 2005). The yellow color of the HOPDA formed often makes it a "signature" intermediate. The maximum absorption wavelength of HOPDA was 435 nm at pH 12, but shifted to 336 nm at pH 2.5. As the pH decreases, the maximum absorption wavelength also decreases (Catelani *et al*., 1973). The characteristic interactions between pH and absorbance spectra observed for strain LA-4 is consistent with that of HOPDA, suggesting that HOPDA is likely an intermediate of biphenyl degradation by strain LA-4. Our LC-MS analysis further confirmed the two key metabolites: HOPDA and benzoic acid.

The cloned 473 bp region of *bph*A1 gene from strain LA-4 is only about 80% identical to the *bph*A gene in *Pandoraea pnomenusa* B-356, *Achromobacter georgiopolitanum* KKS102, *Burkholderia xenovorans* LB400, and *Pseudomonas pseudoalcaligenes* KF707. It forms a separate branch from other known *bph*A genes of other aromatic compound degraders (Fig. 9). Collectively, these results showed that strain LA-4 and the biphenyl-degrading species in other genera, such as *Pseudomonas*, *Burkholderia*, *Rhodococcus*, etc. (Erickson and Mondello, 1992; Hayase *et al*., 1990; Peloquin and Greer, 1993; Masai *et al*., 1995; Kikuchi *et al*., 1994; Furukawa and Miyazaki, 1986), share the same biphenyl biodegradation pathway.

Strain LA-4 showed metabolic versatility toward other aromatic compounds, but it remains to be determined if it contains a multiple dioxygenase systems or a single dioxygenase system possessing broad substrate specificity. Although it could utilize 4-chlorobiphenyl as sole source of carbon and energy, its ability to degrade PCBs is not known. Nonetheless, *Dyella ginsengisoli* LA-4 may be used in the degradation of environmental pollutants in the future.

In summary, *Dyella ginsengisoli* LA-4 showed strong ability to degrade a variety of aromatic compounds. Being a strain of *Dyella*, it expands the biphenyl degradation capability to other taxa. Future studies on its genetics of biphenyl degradation will be useful to better understanding of biphenyl degradation in the environment and to biotechnological applications.

Acknowledgments

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