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Isolation of marine benzo[a]pyrene-degrading *Ochrobactrum* sp. BAP5 and proteins characterization

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Abstract

A bacterial strain BAP5 with a relatively high degradation ability of benzo[a]pyrene (BaP) was isolated from marine sediments of Xiamen Western Sea, China and identified as *Ochrobactrum* sp. according to 16S rRNA gene sequence as well as Biolog microbial identification system. Strain BAP5 could grow in mineral salt medium with 50 mg/L of BaP and degrade about 20% BaP after 30 d of incubation. *Ochrobactrum* sp. BAP5 was able to utilize other polycyclic aromatic hydrocarbons (PAHs) (such as phenanthrene, pyrene and fluoranthene) as the sole carbon source and energy source, suggesting its potential application in PAHs bioremediation. The profile of total soluble protein from *Ochrobactrum* sp. BAP5 was also investigated. Some over- and special-expressed proteins of strain BAP5 when incubated with the presence of BaP were detected by two-dimensional polyacrylamide gel electrophoresis, and found to be related with PAHs metabolism, DNA translation, and energy production based on peptide fingerprint analysis through matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Key words: benzo[a]pyrene; biodegradation; *Ochrobactrum* sp. BAP5; proteins
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in terrestrial and aquatic areas, which attract a serial concern on human health and environment as they are toxic, mutagenic and carcinogenic. Due to their high hydrophobicity and stability, PAHs are prone to bind onto particles in soils, sediments and atmosphere, which might bioaugment into the food chains and lead to great harm to the environment and human health (Blumer, 1976).

Benzo[a]pyrene (BaP) is one of PAHs with a five-ring structure, usually produced by incomplete combustion of organic carbon-based substances (Cerniglia, 1992). The BaP level in the environment is usually used to represent the basis for the development of environmental assessment and cleanup regulations throughout the world (Juhasz and Naidu, 2000). Chemical oxidation or photolysis might affect the presence of BaP, but microbial degradation has been reported as the more effective process for the recovery of BaP in nature (Vidali, 2001). However, the low substrate bioavailability, the great toxicity of BaP as well as the poor degradation abilities of microorganisms may limit the rate and extent of BaP transformation (Johnsen *et al*., 2005), therefore, more and more efforts have been put on developing strategies for its bioremediation.

Previous studies have reported the biodegradation of BaP by numerous bacterial strains, but most of them were investigated to cometabolize PAHs only when one or more alternative carbon sources were present. *Sphingomonas paucumobilis* EPA505 induced with fluoranthene removed 28% of BaP within 2 d (Ye *et al*., 1996), while *S. yanoikuyae* JAR02 mineralized 3.8% of BaP of the radio-label (Rentz *et al*., 2008). Kanaly *et al*. (2000) reported that a bacterial consortium could degrade 33%–65% of BaP within 16 d of cultivation when diesel fuel was presented in the medium at the same time. However, previous study in isolating microorganisms using BaP as the sole carbon source and energy source is rare.

Biodegradation of PAHs by bacterial strains refers to a series of enzymes, such as dioxygenase and reductase, and some degradation-related proteins are only found when induced by their respective degradative substrates. However, study on the relative enzyme about the BaP metabolism is limited. To our knowledge, even no study on the protein profile of BaP-degrading strain has been performed.

In current works, a bacterial strain capable of utilizing BaP as the sole carbon source was isolated from marine sediments and identified as *Ochrobactrum* sp. Experiments were conducted out to determine the extent of the degradation of BaP and other PAHs, including phenanthrene,

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pyrene and fluoranthene. Through two-dimension electrophoresis (2DE), we compared the protein contents of strain BAP5, respectively, when incubated in the presence and absence of BaP. The differential profiling of protein expression was identified through the peptide fingerprint analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS).

1 Materials and methods

1.1 Isolation of bacterial strain and culture conditions

The strain BAP5 was isolated from the marine sediment in Xiamen City, Fujian Province, China, through a six month enrichment using mineral salt medium (MSM) with an addition of BaP (99%, Sigma, USA) as the sole carbon source at a final concentration of 50 mg/L. The MSM was comprised of (mg/L): $(NH_4)_2SO_4$ 1000, NaH_2PO_4 800, K_2HPO_4 200, $MgSO_4$ 200, $CaCl_2$ 100, $FeCl_3$ 5, $MnSO_4$ 0.2, $(NH_4)_6Mo_7O_{24}$ 1, $ZnSO_4$ 0.1, $CuSO_4$ 0.02, $CoCl_2$ 0.02, and the initial pH of the medium was adjusted to (7.0 ± 0.1). The bacterial cells were reactivated on ZoBell 2216E medium (Oppenheimer and ZoBell, 1952) for 24 h and washed three times by MSM before inoculation. All of cultures were incubated at 25°C on a rotary shaker at 150 r/min.

1.2 Determination of the strain growth and BaP metabolism ability

In order to determine the BaP degradation ability, strain BAP5 was inoculated in 50 mL conical flasks containing 20 mL of MSM with the initial BaP concentration of 50 mg/L for 30 d. The culture samples were collected every six days. BaP was extracted three times using CH_2Cl_2 (1:0.5, *V/V*), frozen, and then quantitatively analyzed using high performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies Corp., USA) with Agilent Hypersil ODS 4.0 × 250 mm (5 μm inside diameter) column, under wavelength 261 nm and methanol to deionized water ratio (9:1, *V/V*) at a flow rate of 1 mL/min. Quantification was based on the internal standard, phenanthrene, and pyrene calibration curves.

The growth of strain BAP5 was determined based on the modified method of 2-(4-indophenol)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT, Smith and Acfeters, 1997) as follows: 50 μL of 5% (*W/V*) INT solution (50 mmol/L Tris-HCl, pH 7.2) was added to 1 mL of cells supernatant ($A_{600\,nm}$, 0.1–0.8). After incubation in the dark for 30 min, the reaction was stopped using 50 μL of 40% (*V/V*) formaldehyde solution. Cells were collected and oxidized-type INT was extracted with acetone-ethanol solution (1:1, *V/V*), and the extraction was spectrophotometerically detected at 540 nm.

1.3 Screening of other PAHs degradation by strain BAP5

A series of experiments similar to that previously described in Section 1.2 was carried out by adding 10 mg/L phenanthrene (Phe), pyrene (Pyr), and fluoranthene (Flu) into the MSM, respectively, to investigate the degradation ability of these PAHs by strain BAP5.

1.4 Identification of BaP-degrading strain BAP5

Cells of strain BAP5 were harvest by incubating with 2216E medium, and total genomic DNA was extracted according to the method introduced by Hu *et al*. (2006). The 16S rRNA gene fragment was amplified by PCR using the set of primers: 16S-F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-C GGCTACCTTGTTACGA CTTC-3'). The PCR conditions (35 cycles of 30 s at 95°C, 30 s at 50°C, and 150 s at 72°C) were performed in a PCR thermal cycler (Mastercycler Gradient, Eppendorf, USA) with Pfu DNA polymerase. The PCR products were then purified and linked to the T-vector according to the method described by Sambrook and Russell (2001). The DNA sequence of the positive clones with 16S rRNA gene fragment was compared with the available database (Genbank) using the BLAST program at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and deposited at GenBank with the accession number (EF093798).

Strain BAP5 was also characterized by Biolog microbial identification system according to the operation manual. Detection of oxidase and catalase were carried out using the *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride and hydrogen peroxide, respectively.

1.5 Preparation of crude extracts for protein analysis and 2DE assay

After 20 d of incubation in the presence and absence of BaP, bacterial cells were harvested and frozen at –80°C prior to protein extraction. After been washed by 100 mmol/L phosphate buffer (pH 7.0) twice, cells were homogenized using ultrasonic crasher at 4°C (interval 3 s, for 10 min). The crude protein extracts were collected by centrifugation for 15 min (4°C, 15000 r/min) to remove cell debris and insoluble materials. Proteins were received after the acetone precipitation, and the protein contents of the supernatants were determined according to Bradford assay (Bradford, 1976).

A total of 100 μg of each protein sample (BaP treatment and control) was resuspended in 125 μL rehydration solution containing 9.5 mol/L urea, 4% CHAPS, 0.3% Bio-Lyte® 4/7 ampholyte (Bio-Rad, USA), 30 mmol/L DTT, and 0.002% (*W/V*) Bromophenol Blue and applied on 7 cm long pH 4–7 ReadyStrip™ IPG Strips (Bio-Rad, USA) for rehydrating about 16 h. Isoelectric focusing process was then performed using the IPGphor (Bio-Rad, USA) at a constant temperature of 20°C with a total voltage of 22700 V as follows: the voltage firstly increased from 0 to 250 V for 30 min in a varied gradient, from 250 to 4000 V for 2 h in a constant gradient, maintained at 4000 V for 5 h, and at last declined to 500 V for 2 h. The strips were then equilibrated with DDT and iodoacetamide, respectively, as described by O' Farrell (1975) and Görg *et al*. (1988, 1995) before PAGE process. Polyacrylamide gels (10%) were applied to separate the proteins in 25–100 kDa range by Mini Protean III Electrophoresis System (Bio-Rad, USA).

Proteins in gels were stained with Coomassie Brilliant Blue R250 (Wilson, 1983). Experiments were repeated three times without a distinct difference in the 2D patterns. Bands and spots detection and gel to gel comparison polypeptide were performed visually.

1.6 In-gel protein digestion and protein identification

Following in-gel protein digestion by methods described by Zhang *et al*. (2006), 0.5 μL of the digested peptide extraction mixed with the matrix R-cyano-4-hydroxycinnamic acid (1:1, *V/V*) was spotted onto a stainless steel MALDI plate. A Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, USA) running in delayed ion extraction reflection mode was used to obtain MALDI mass spectra with the following parameters: accelerating voltage (20000 V); mass range (800–3500 amu); laser power (2100 W). External calibration was done by using a mixture of angiotensin I (1296.19) and ACTH (fragment 18–39) (2465.68) under the same instrumental setting.

The selected peptide masses obtained from MALDI-TOF-MS were submitted to Mascot website (http://www.matrixscience.com) for NCBInr and MSDB databases search. Proteins were examined by the following search parameters: mass error less then 150 ppm, with a maximum of 2 missed cleavages, chemical modifications selected was "methionine oxidation", taxanomy selected was "Eubacteria", and mass values selected was "MH^+" with monoisotopic.

2 Results

2.1 Isolation, identification and characterization of BaP-degrading strain BAP5

Several BaP-degrading bacterial strains were isolated from the microbial consortia established by continuous transferring by MSM with BaP as the sole carbon source and energy source. Stain BAP5, which had the relatively higher degradation ability of BaP than others, was selected and discussed in the following sections. It was identified as *Ochrobactrum* sp. based on the comparison of 16S rRNA gene sequence and biolog characterization. The partial 16S rRNA gene fragment was over 98% similarity to that of *Ochrobactrum tritici* isolate TJ3 (AF508089), and the phylogenetic tree based on a comparison of the sequences is shown in Fig. 1. Biolog characterization provided the corresponding result with a similarity index value (SIM) more than 0.5, suggesting a good match to *Ochrobactrum* (Table 1). *Ochrobactrum* sp. BAP5, a Gram-negative short rod, approximately 1 to 0.4 μm, could use glucose and lactose without producing any acids or gas and utilize cysteine and tryptophan to produce H_2S and indole, respectively.

MSM with the initial BaP concentration of 50 mg/L was established to investigate the biodegradation of BaP by *Ochrobactrum* sp. BAP5. The result showed that about 17% BaP could be degraded by strain BAP5 after 30 d of incubation, although it was in a relatively slow degradative velocity (Fig. 2). It was also observed that the increment of bacterial cell was corresponded to the biodegradation of BaP. *Ochrobactrum* sp. BAP5 also showed a broad activity towards other PAHs compounds (Fig. 3) that strain BAP5 could degrade Phe, Pyr, and Flu, respectively, 40%, 19%, 31% within 30 d of incubation.

Table 1 Biochemical and physiological characteristics of *Ochrobactrum* sp. BAP5

Characteristics test	Result
Oxidase and catalase	+
Production of H_2S, indole	+
Utilization of dextrin, glycogen, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, Gentiobiose, α-D-glucose, *m*-inositol, maltose, D-mannose, D-psicose, L-rhamnose, sucrose, D-trehalose, turanose and glycerol	+
Utilization of α-cyclodextrin, Tween 40, α-D-lactose, lactulose, D-mannitol*, D-melibiose, D-raffinose, β-methyl-D-glucoside, D-sorbitol* and xylitol	–
Utilization of methyl pyruvate, mono-methyl-succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, D,L-lactic acid, α-keto glutaric acid, propionic acid, quinic acid, succinic acid, bromo succinic acid, urocanic acid, inosine and uridine*	+
Utilization of D-galacturonic acid, D-glucuronic acid, glucuronamide, *p*-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid*, α-keto valeric acid, malonic acid, D-saccharic acid, sebacic acid and succinamic acid*	–
Utilization of D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, D,L-carnitine γ-amino butyric acid	+
Utilization of L-alaninamide*, L-phenylalanine, L-pyroglutamic acid and D-serine*	–
Utilization of thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate	–

* The differences between *Ochrobactrum* sp. BAP5 and *Ochrobactrum anthropi* was analyzed by Biolog identification system.

2.2 Comparison and characterization of special proteins

The differential expression profile of proteins in the absence and presence of BaP by 2DE is shown in Fig. 4. Over 200 polypeptides detected from the gels mainly ranged from 25 to 100 kDa and distributed in the pH range 4–7. The presence of BaP led to some modifications of polypeptides patterns, which were mainly observed in acidic parts of gel at 100.1 (5.7–5.8), 66.0 (5.4–5.5), 61.0 (4.8), 42.0

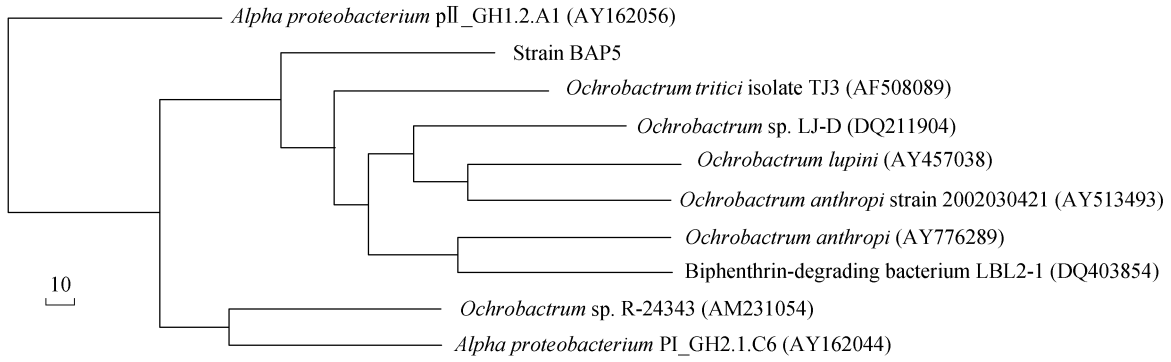

Fig. 1 Phylogenetic tree based on 16S rRNA gene fragment sequence with maximum likelihood method by using program PHYLIP (Version 3.86).

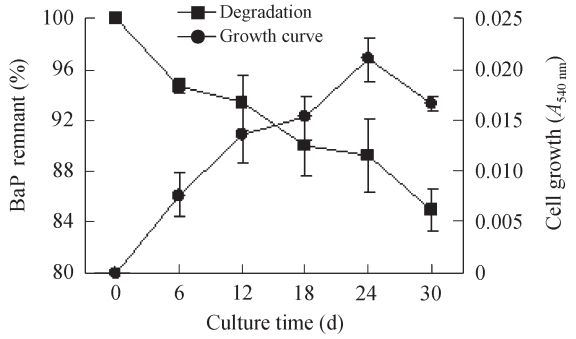

Fig. 2 Degradation of BaP by *Ochrobactrum* sp. BAP5 in the MSM with 50 mg/L BaP. Bars represent standard deviation of the mean of triplicate cultures.

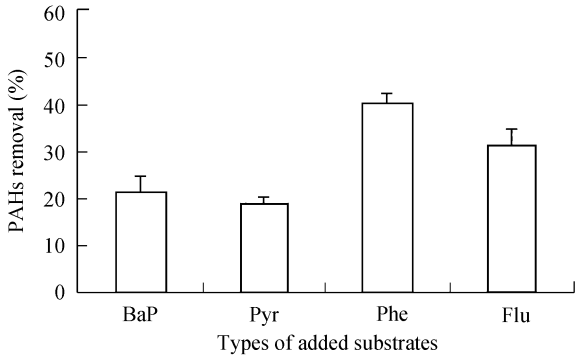

Fig. 3 Degradation of Pyr-pyrene (Pyr), Phe-phenanthrene (Phe), Fluoranthene (Flu) for 30 d of incubation by *Ochrobactrum* sp. BAP5.

(5.1), 25.5 (4.7), 38.0 (6.2–6.4) and 31.0 (5.7), and belonged to the overexpressed or specific-expressed patterns when compared with the control without BaP treatment. They were identified as some relative BaP-degrading enzymes, such as cyclase, oxidoreductase, dehydrogenase, through the peptide fingerprint analysis based on MALDI-TOF-MS (Table 2).

3 Discussion

Marine areas, especially marine sediments, have been considered a reservoir of PAHs with a high level due to a number of accumulating sources, including atmospheric deposition, marine seeps of petroleum hydrocarbons, off-shore production or petroleum transportation, sewage disposal or boating (Juhasz and Naidu, 2000), which lead to the biodiversity of marine microorganisms with PAHs degradation ability. Cultivation using marine sediment was set up for the growth of enrichment culture, and a BaP-degrading bacterial strain BAP5 was isolated. This strain was identified as *Ochrobactrum* based on 16S rRNA gene sequence and physiological characteristics. As a genus of microorganisms for bioremediation, *Ochrobactrum* has a broad substrate specificity towards organic pollutants, such as dimethylformamide (Veeranagouda *et al*., 2006), vinyl chloride (Danko *et al*., 2006) and phenol (El-sayed *et al*., 2003). But relatively limited data has been reported on the PAHs degradation by this genus, especially BaP. In our study, *Ochrobactrum* sp. BAP5 also showed a non-specific PAHs degradation ability, all the PAHs substrates used were degraded by this strain to a certain extent. As reported previously, a series of bacteria, especially *Mycobacterium* sp. and *Pseudomonas* sp., with the ability to oxidize or degrade BaP have been isolated and identified (Schneider *et al*., 1996; Chen and Aitken, 1999), but almost all could cometabolize PAHs only when one or more alternative carbon sources are present. Our results from *Ochrobactrum* sp. BAP5 isolated from marine sediments showed its utilization of BaP as well as Pyr, Phe, Flu as the sole carbon source at the first time.

Our investigation was mainly to study the global response profile of *Ochrobactrum* sp. BAP5 with external exposure of BaP as well as the identification of some proteins, in particular, those overexpressed and specific-expressed ones and MALDI-TOF-MS was applied to identify these induced polypeptides. The results provided the evidences (Fig. 4) that BaP can induce the synthesis of some specific proteins in *Ochrobactrum* sp. BAP5. The specific proteins induced specifically in response to growth with the presence of BaP may play certain important roles for BaP biodegradation, such as inducer of BaP degradation (P4, P6, P7), enzymes related to BaP metabolic pathway (P3, P5), stress transport and energy-related proteins (P1, P4). The P3 polypeptide (61.0 kDa) was found with the relatively high sequence coverage (17%) and similarity of a magnesium-protoporphyrin IX monomethyl ester anaerobic oxidative cyclase. This enzyme, received from *Chlorobium ferrooxidans* DSM 13031, is an important enzyme in the heme biosynthesis pathway and is responsible for the cyclization process of the pyrrole

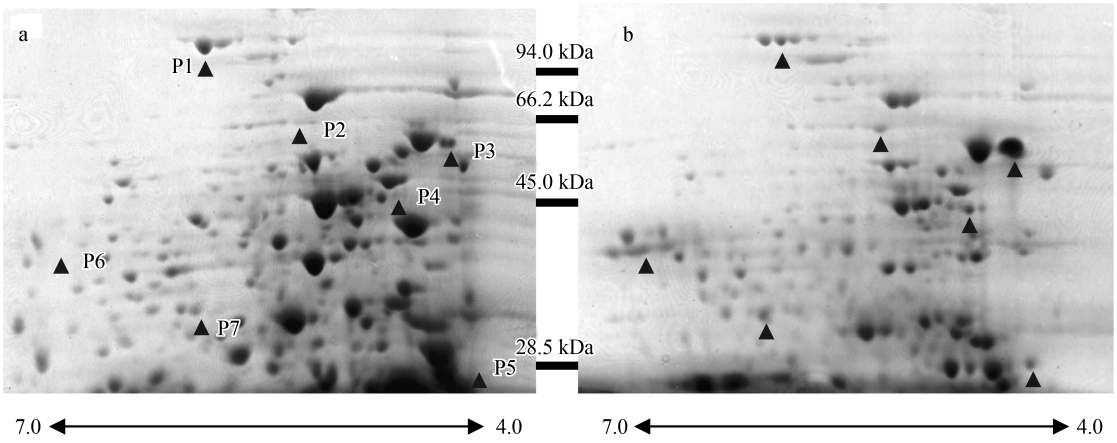

Fig. 4 Coomassie Brilliant Blue-stained 2DE gels showing soluble protein patterns of *Ochrobactrum* sp. BAP5 grown for 20 d in the absence (a) and presence (b) of BaP.

Table 2 List of identified function of proteins of strain BAP5 grown in the presence of BaP

Spot No.	Protein description (species)	Peptides matched	Sequence coverage (%)	Observed migration	
				Mr (kDa)	pI
P1	Transposase Tn3 (Frankia sp. CcI3)	7	6	100.1	5.80
P2	No match			66.0	5.40
P3	Magnesium-protoporphyrin IX monomethyl ester anaerobic oxidative cyclase (*Chlorobium ferrooxidans* DSM 13031)	11	17	61.0	4.80
P4	ABC transporter, ATP-binding component (*Vibrio* sp. MED222)	5	12	42.0	5.10
	Oxidoreductase (*Lactococcus lactis* subsp. *lactis* Il1403)	4	11		
	Hydroxymethylglutaryl-CoA lyase (*Roseobacter* sp. MED193)	4	13		
P5	Putative short-chain dehydrogenase (*Rhizobium leguminosarum* bv. *viciae* 3841)	5	11	31.0	4.70
P6	Thiamine pyrophosphate-dependent dehydrogenase, E1 component alpha subunit (*Sinorhizobium medicae* WSM419)	5	10	38.0	6.20
P7	ANTAR(*Mycobacterium* vanbaalenii PYR-1)	7	29	24.7	5.70
	Acyl-CoA dehydrogenases (*Pseudomonas aeruginosa* C3719)	9	52		

Mr: relative molecular weight; pI: isoelectric point.

rings into the protoporphyrin IX, the key component of the heme. The induction of this protein was obviously overexpressed, indicating that a heme constitutive enzyme is involved in the metabolism of *Ochrobactrum* sp. BAP5 when grown with the presence of BaP. Moreover, the heme is one of the most important prosthetic groups in cytochrome P450 system, which may refer to BaP biodegradation. It has been studied by Khan *et al*. (2001) and Brezna *et al*. (2006) that some specific proteins from *Mycobacterium* sp., including cytochrome P450 monooxygenases and dioxygenases, were induced only when grown within the presence of PAHs. Some other indirect studies have been also reported that the cytochrome P450 system was involved in the metabolism of BaP (Verdin *et al*., 2005; van den Brink *et al*., 1998).

Two-dimensional PAGE combined with peptide fingerprint analysis can be considered as a useful tool to have a protein profile screening of *Ochrobactrum* sp. BAP5 with certain stressed condition, but the lack of its genome sequences and proteomic databases restricted further identification of the target proteins. In addition, as microorganisms might have some mutations together with the increasing genetic differences during the evolvement, the degree of protein similarity may decrease (Kim *et al*., 2004). In our study, not all of the proteins from *Ochrobactrum* sp. BAP5 can be received by 2DE, especially those insoluble or with low copy number, but it can give some insights for the protein composition, and the induced polypeptides are likely to represent the proteins related to BaP-degrading, as reported by Kim *et al*. (2004).

Ochrobactrum sp. BAP5, degrading a wide range of PAHs, belongs to another versatile PAH-degrading bacterial strain with a potential ability for the bioremediation of PAHs in PAHs-contaminated soils and sediments. It is reasonable, according to the differences of protein profiles, to assume that some proteins, viz. enzymes, should play an important role in the BaP metabolic pathways. In fact, the induction of BaP leads to different expression signals for proteins from strain *Ochrobactrum* sp. BAP5, which can make it possible to detect the presence of BaP by screening the characteristics of certain functional protein or a series of proteins.

The current study is to build up a method to begin the proteomic work of those microorganisms, including bacteria and fungi, with the ability of degrading high molecular weight PAHs, and provide some fundamental and important information referring to their metabolic procedure and degradation-related proteins and genes.

Acknowledgments

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