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ORIGINAL PAPER

Anaerobic biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by a facultative anaerobe *Pseudomonas* sp. JP1

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are harmful persistent organic pollutants, while the high-molecular-weight (HMW) PAHs are even more detrimental to the environment and human health. However, microbial anaerobic degradation of HMW PAHs has rarely been reported. One facultative anaerobe *Pseudomonas* sp. JP1 was isolated from Shantou Bay, Shantou, China, which could degrade a variety of HMW PAHs. After 40 days cultivation with strain JP1, anaerobic biodegradation rate of benzo[a]-pyrene (BaP), fluoranthene, and phenanthrene was 30, 47, and 5 %, respectively. Consumption of nitrate as the electron acceptor was confirmed by N-(1-naphthyl) ethylenediamine spectrophotometry. Supplementation of sodium sulfite, maltose, or glycine, and in a salinity of 0–20 ‰ significantly stimulated anaerobic degradation of BaP. Lastly, the anaerobic degradation metabolites of BaP by strain JP1 were investigated using GC/MS, and the degradation pathway was proposed. This study is helpful for further studies on the mechanism of anaerobic biodegradation of PAHs.

Keywords *Pseudomonas* · Facultative anaerobe · PAHs · Anaerobic · Biodegradation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons with two or more fused benzene rings. They are stable in the environment, and have a strong toxicity (Verdin et al. 2004; Rafin et al. 2009). This pollutant tends to persist in the environment partly because of its very low water solubility and its strong adsorption onto the marine sediments. PAHs are removed primarily through volatilization, photodegradation, settlement, bioaccumulation, chemical oxidation and microbial degradation. Among these, microbial degradation is the most thorough, least harmful PAHs removal method (Haritash and Kaushik 2009), which has become a major research direction on pollution remediation. The aerobic degradation of PAHs by microorganisms has been studied (Johnsen et al. 2005; Haritash and Kaushik 2009; Wu et al. 2009; Song et al. 2011; Sun et al. 2014) and their biochemical pathway of degradation has been described. But most of the contaminated sites such as sediments and groundwater become anaerobic due to high pollutant concentration and microbial biodegradation processes. Theoretically, in the anaerobic environment, anaerobe and anaerobic biodegradation of PAHs should play greater roles than the respective aerobic ones.

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In recent years, most of studies on microbial degradation of PAHs have been carried out under aerobic conditions (Luan et al. 2007; Song et al. 2011). However, little attention has been paid to the degradation mechanism under anaerobic conditions, and if any, it was confined to that of low-molecular-weight (LMW) PAHs (Zhang et al. 2000; Coates et al. 2001; Annweiler et al. 2002; Lily and Phelps 2005; Fuchs et al. 2011; Sun et al. 2014). In fact, the anaerobic degradation of HMW PAHs has been demonstrated in soil and marine sediment incubations under various electron-accepting conditions (Coates et al. 1997; Rothermich et al. 2002; Meckenstock et al. 2004; Yan et al. 2014). Anaerobic microbial remediation is an attractive remediation technique for polluted environments.

The facultative anaerobic strain JP1 was isolated from the surface sediments of Shantou (China) offshore, which degrades a variety of HMW PAHs. Anaerobic degradation metabolites of HMW PAHs were identified by GC/MS. The present study will facilitate our understanding of the molecular mechanism underlying HMW PAHs degradation, and promote microbial environmental remediation of PAHs in the future.

Materials and methods

Chemicals

Benzo[a]pyrene (BaP, 96 %), pyrene (Pyr, 98 %), chrysene(99 %), benz[a]anthracene (99 %), fluoranthene (Flu, 99 %), phenanthrene (Phe, 98 %), and anthracene(99 %) were purchased from Sigma–Aldrich (St. Louis, MO). Dichloromethane (HPLC grade) was purchased from Oceanpak (Gothenburg, Sweden). Other chemicals used were all analytical grade.

Culture conditions

Aerobic culture method was carried out as described previously (Song et al. 2011). Anaerobic culture approach was adopted from Li et al. (2010). Briefly, brine bottles filled with tested samples were put into an anaerobic glove box (YQX-II, Shenglan Instrument, Jintan, China), vacuum pumped (66 kpa) three times, refilled with nitrogen gas (99.99 %), followed by another three times of vacuum pumping (66 kpa) and refilling with mixed gases (H_2 10 %, CO_2 5 %, N_2 85 %). Brine bottles were then sealed with butyl rubber plugs and incubated in a rotary shaker (150 r/min) at 25 °C in dark.

Isolation and identification of bacterial strain

The method used for screening anaerobic strain has been described by Li et al. (2010). Strain JP1 was isolated from sediments in Shantou harbor (Shantou, China), which had been contaminated by crude oil and contained high concentration of PAHs. Briefly, 25 ml minimal salts medium (MSM), reducing reagent KNO_3 (15 mM/L, used as the electron acceptor) and 25 µl BaP stock (10 mg/ml in acetone, used as the sole carbon source) were added into a brine bottle in a sterile hood. Acetone was evaporized for 120 min, and fresh sediment (5 g) was then transferred into the bottle. Anaerobic culture was carried out as described above for 7 days. A pure culture of strain JP1 with PAH-degrading capability was obtained by repeated transfer onto 2216E plates (Oppenheimer 1952).

PAHs biodegradation

Strain JP1 was first inoculated under anaerobic conditions in 20 ml 2216E liquid media with 150 r/min shaking for 24–28 h at 25 °C. The bacteria were collected by centrifugation at 6,000 g for 5 min, washed twice with MSM, and resuspended in 1 ml MSM. For aerobic degradation of PAHs, the bacteria (0.5 g/ml) were inoculated into 20 ml MSM containing 10 mg/L PAHs and incubated in a rotary shaker (150 r/min) for 30 days at 25 °C in dark. For anaerobic degradation of PAHs, the bacteria were inoculated into 20 ml MSM containing 15 mM KNO_3 and 10 mg/L PAHs. The anaerobic cultures were purged with mixed gases and incubated in a rotary shaker (150 r/min) for 40 days at 25 °C in dark.

Effects of culture conditions on anaerobic degradation of BaP

To measure the effects of carbon sources on the degradation of BaP, glucose, sucrose, lactose, or maltose (500 mg/L) was added into the media. Aerobic degradation of BaP in the absence of sugar was used as the control. To measure the effect of nitrogen sources on the anaerobic degradation of BaP,

L-cystine, L-glutamic acid, L-asparagine, glycine or KNO_3 (100 mg/L) was put into the media. To measure the effect of electron acceptors on the anaerobic degradation of BaP, KNO_3 (10 mM), sodium thiosulfate (10 mM), ferric citrate (30 mM), sodium nitrite (5 mM), sodium sulfite (10 mM), potassium perchlorate (10 mM), sodium bicarbonate (50 mM), manganese dioxide (10 mM), iron oxide (10 mM), or potassium chlorate (10 mM) was supplemented to the media.

PAH concentration measurement

The residual PAH concentration was measured as per the protocols of Wu et al. (2009) and Song et al. (2011). For Pyr measurement, Phe was used as an internal control; for measurement of other PAHs, Pyr was used. At the end of cultivation, all of the culture was extracted twice with 20 ml CH_2Cl_2 by votexing vigorously. After standing at room temperature for 30 min, the organic phase was collected, filtered, and transferred to a 2 ml chromatography bottle, sealed and stored at −40 °C until used for residual PAH measurement. Reversed-phase HPLC analysis of PAH concentration was performed with an Agilent 1,100 series Liquid Chromatograph (Agilent technologies, Santa Clara, CA) fitted with an Agilent Hypersil ODS 5 μm particle size column (4.0 × 250 mm). The mobile phase consisted of methanol and water (90/10 v/v) with a flow rate of 1 ml/min. Eluted substances was detected at wavelength of 286 (BaP), 251 (Phe) or 241 (Pyr) nm. Different PAHs were identified by comparing their retention time with those of the authentic chemicals.

Measurement of nitrate and nitrite concentration

The nitrite concentration was measured using N-(1-naphthyl) ethylenediamine spectrophotometry (Mahmood et al. 2007). Under acidic conditions (pH = 2), NO_2^- is diazotized with sulfanilic acid amine, reacts with N-1-Naphthyl ethylenediamine dihydrochloride, and generates a magenta azo dye, which can be measured at 543 nm wavelength. The concentration of nitrate was measured by N-(1-naphthyl) ethylenediamine spectrophotometric assay after the reduction of NO_3^- to NO_2^- by cadmium column (Mahmood et al. 2007).

Analysis of PAHs anaerobic metabolites

The method used for the study of PAHs anaerobic metabolites has been described by Luan et al. (2007) and Song et al. (2011). Strain JP1 was grown for 15 days in 200 ml MSM respective containing 10 mg/L PAH (naphthalene, Phe, anthracene, chrysene, Pyr, benz[a]anthracene and BaP; MSM was used as the negative control). After cultivation, the supernatant was collected by centrifugation at 12,000 g for 15 min and extracted with the same volume of CH_2Cl_2 (HPLC grade). The organic phase was collected, concentrated with N_2 (>99.99 %), filtered through a 0.45 μm membrane, and then analyzed by gas chromatography–mass spectrometry (GC/MS) using a Shimadzu GC–MS-QP5050A system (Shimadzu, Milan, Italy). A HP-5MS fused silica column (30 m by 0.25 mm by 0.25 μm) was used with helium as the carrier gas (1.0 ml/min). The oven temperature program was as follows: 100 °C for 1 min, then linearly increased at a rate of 15 °C/min up to 160 °C, 160–300 °C at a ramp of 5 °C/min, and maintained at 300 °C for 2 min (Luan et al. 2007). The spilt flow was 43 ml/min. The injector temperature was at 250 °C. The mass spectra were taken at 70 eV. Mass range was between m/z 30–450. A library search was carried out by using Wiley7, NIST147, NIST05s, NIST05, and Nist27.

Statistical analysis

All data were presented as mean ± SEM ($n = 3$). One-way ANOVA followed by Tukey's test was used to compare the difference with the software SASv9.3 (SAS Institute, Cary, NC). The significance level was set at $P < 0.05$.

Results

Identification of strain JP1

The strain JP1 was a gram-negative, there was no plasmid in this strain. The 16S rDNA gene sequence was determined using the method described by Dandie et al. (2004). The sequence was analyzed using the Blastn program in NCBI databank. The bacterium shows 99 % similarity with *Pseudomonas stutzeri* standard strain in sequence, and the phylogenetic tree based on a comparison of the sequences is shown in

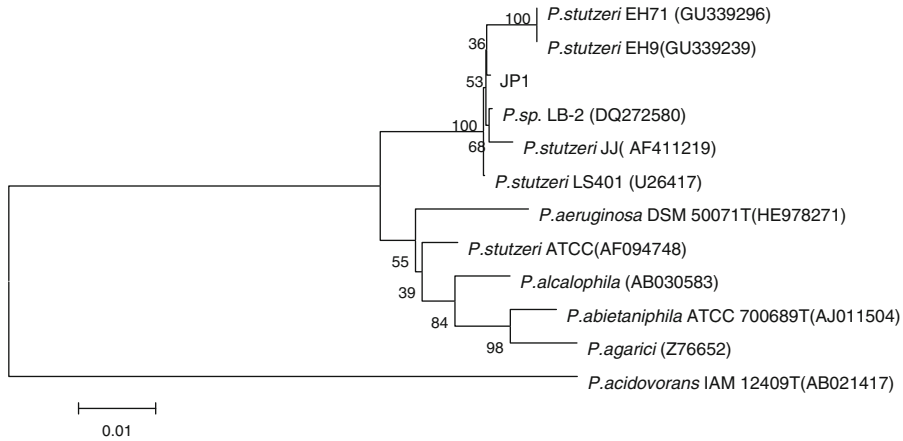

Fig. 1 Phylogenetic tree based on 16S Rdna sequence showing the positions of the strain JP1

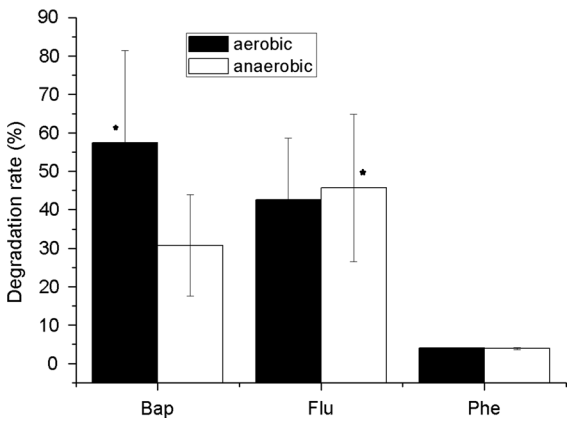

Fig. 2 Biodegradation of PAHs by strain JP1 under aerobic/anaerobic conditions. Bap, Flu and Phe were cultivated with JP1 for 40 days under aerobic/anaerobic conditions. The residual PAH concentration was measured using reverse phase HPLC

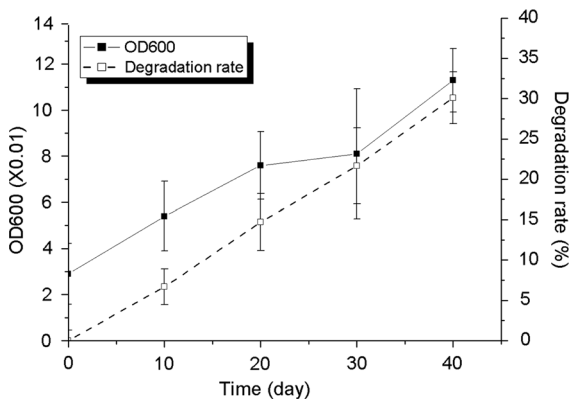

Fig. 3 Growth of strain JP1 and its anaerobic degradation of BaP. Bap was cultivated with JP1 for 40 days under anaerobic conditions, residual BaP and the growth of JP1 were measured at different time points

Fig. 1, it showed maximum identity to the DNA sequence of *P. stutzeri* EH71 (GU339296 and *P. stutzeri* EH9(GU339239). Therefore, the strain JP1 was identified as *Pseudomonas.*

Aerobic and anaerobic biodegradation of PAHs by strain JP1

Under aerobic conditions, after 40 days cultivation at 25 °C, biodegradation rate of, BaP, Flu and Phe was 57, 42 and 4 %, respectively. Under anaerobic conditions, biodegradation rate of BaP, Flu and Phe was 30, 47 and 5 %, respectively, at day 40 (Fig. 2). Anaerobic degradation rate of BaP is significantly lower than the aerobic degradation rate, but there are no significantly differences between anaerobic and aerobic degradation of Flu and Phe.

Anaerobic degradation of BaP and denitrification

Anaerobic growth of strain JP1 was maintained for 40 days, and degradation of BaP were measured at different time points (Fig. 3). Strain JP1 proliferated very slowly under anaerobic condition, and reached stable growth period after 14 days. However, degradation of BaP proceeded steadily until reaching about 30 % at day 40. At the same time, nitrate concentration decreased from 7.5 to 2.4 mM, while nitrite

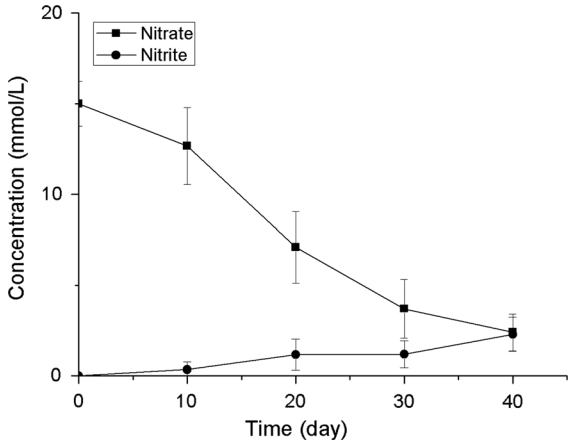

Fig. 4 Measurement of denitrification by strain JP1. Bap was cultivated with JP1 for 40 days under anaerobic conditions, residual nitrate and nitrite measured at different time points using N-(1-naphthyl) ethylenediamine spectrophotometry

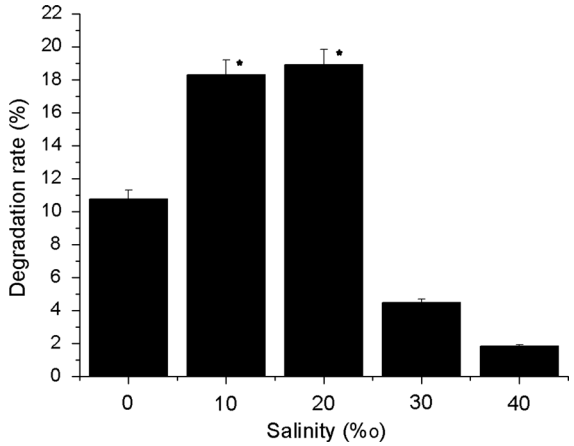

Fig. 6 Effects of salinity on biodegradation of BaP by strain JP1. Bap was cultivated with JP1 for 40 days under anaerobic conditions with different salinities. Residual BaP concentration was measured at day 40

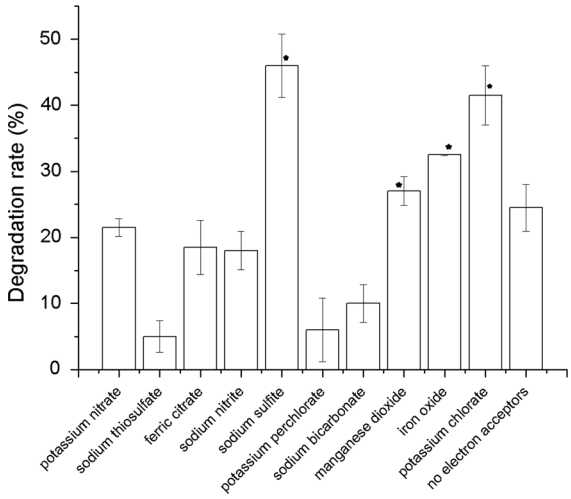

Fig. 5 Effects of electron acceptors on biodegradation of BaP by strain JP1. Bap was cultivated with JP1 for 40 days under anaerobic conditions with different electron acceptors. Residual BaP concentration was measured at day 40

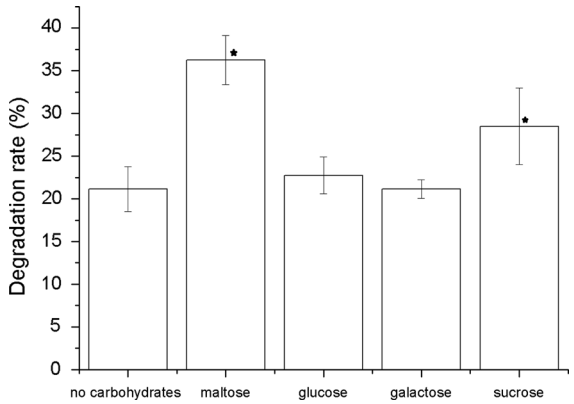

Fig. 7 Effects of carbohydrates on anaerobic biodegradation of BaP by strain JP1. Bap was cultivated with JP1 for 40 days under anaerobic conditions with carbohydrates. Residual BaP concentration was measured at day 40

concentration increased from 0 to 2.3 mM after 40 days (Fig. 4). However, the amount of consumed nitrate was not equal to the amount of generated nitrite, indicating that nitrate was denitrificated to an intermediate metabolite before being further reduced to nitrite.

Effects of culture conditions on the anaerobic degradation of BaP by strain JP1

Electron acceptors were tested for their effects on anaerobic BaP degradation. Sodium sulfite, iron oxide, manganese dioxide, potassium chlorate showed positive effects, while sodium thiosulfate, potassium

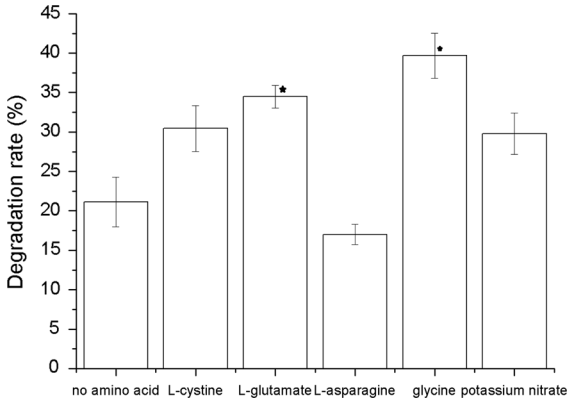

Fig. 8 Effects of amino acid on anaerobic biodegradation of BaP by strain JP1. Bap was cultivated with JP1 for 40 days under anaerobic conditions with different nitrogen sources. Residual BaP concentration was measured at day 40

perchlorate, sodium bicarbonate had inhibitory effects on the degradation of BaP (Fig. 5).

We then tested the effects of salinity, which showed that when salt concentration was 10–20 ‰, a condition similar to the original environment of Shantou harbor, strain JP1 had the strongest ability of BaP degradation (Fig. 6). Interestingly, strain JP1demostrated a relatively strong degradation capacity even when salt concentration was 0 ‰, suggesting a good application prospect of strain JP1 in offshore and coastal environment pollution control.

Effect of carbohydrates (500 mg/L) was investigated. Anaerobic biodegradation rate of BaP was 42.29 % in the absence of carbohydrates, which changed to 72.50, 45.49, 40.31 and 57.00 % when maltose, glucose, galactose and sucrose was added, respectively (Fig. 7).

Lastly, we determined the effect of nitrogen sources, showing that the degradation rate of BaP was 21.15 % without addition of amino acid, which changed to 30.45, 34.49, 17, 39.68 and 29.79 % when L-cystine, L-glutamic acid, L-asparagine, glycine and KNO_3 was supplemented, respectively (Fig. 8).

GC/MS detection of PAHs metabolites

To understand better the molecular mechanism underlying PAH anaerobic degradation, we identified the degradation metabolites using GC/MS with BaP as an example. Two MS spectra were detected, corresponding to GC retention time of 29.3 (Fig. S1 a) and 23.6 min (Fig. S1 b). The 29.3 min spectra match 1, 12-dimethyl-Benz[a]anthracene ($C_{20}H_{16}$, CAS:313-74-6), 7,8,9,10-Tetrahydrobenzo[a]pyrene (CAS:17750-93-5) and 5-ethylchrysene ($C_{20}H_{16}$, CAS:54986-62-8). 1,12-dimethyl-Benz[a]anthracene and 5-ethylchrysene can be considered as the intermediate metabolites of benz[a]anthracene (CAS:56-55-3) and chrysene (CAS:218-01-9), respectively, which were matched by the 23.6 min spectra, and confirmed by GC/MS analysis of standard benz[a]anthracene and chrysene (Fig. S2).

Due to the unavailability of 7,8,9,10-tetrahydrobenzo[a]pyrene, we used pyrene instead in culture media to investigate further the degradation pathway. GC/MS analysis identified the anaerobic metabolites of pyrene as 4-methyl-phenanthrene (CAS:832-64-4, $C_{15}H_{12}$), 4,5-dimethylphenanthrene (CAS:3674-69-9, $C_{16}H_{14}$), and phenanthrene (CAS:85-01-8) detected at the retention time of 14.2 (Fig. S3 a), 16.3 (Fig S3 b), and 11.8 min (Fig. S4 a), respectively, which was confirmed by phenanthrene standard mass spectrogram (Fig. S4 b).

Similarly, the metabolites of anaerobic degradation of benz[a]anthracene were investigated by GC/MS. The retention time of 13.8 min matched 2-methylphenanthrene (CAS: 2531-84-2, $C_{15}H_{12}$) (Fig. S5 a). 2-methylanthracene (CAS:613-12-7, $C_{15}H_{12}$) and 1-methylanthracene (CAS:610-48-0, $C_{15}H_{12}$) were matched to the retention time of 13.9 min (Fig. S5 b). 2,3-dimethylphenanthrene (CAS:3674-65-5, $C_{16}H_{14}$) was detected at both retention times of 16.4 and 16.3 min (Fig. S5 c, d).

Anaerobic degradation of chrysene detected alpha-methylstilbene (CAS: 779-51-1, $C_{15}H_{14}$) at the retention time of 12.3 min (Fig. S6 a), 2-methyl-Phenanthrene (CAS: 2531-84-2, $C_{15}H_{12}$) at 13.7 min (Fig. S6 b) (also detected at 14.2 min), 1-methylphenanthrene (CAS: 832-69-9, $C_{15}H_{12}$) at 14.2 min (Fig. S6 c) (also detected at 13.7 min), 1-ethyl-2-methyl-phenanthrene (CAS: 61983-53-7, $C_{17}H_{16}$) at 19.9 min (Fig. S6 d).

Finally, the metabolite of anaerobic degradation of phenanthrene matched 1, 2, 3, 4-tetrahydro-4-methyl-4-phenanthrenol (CAS: 77536-58-4, $C_{15}H_{16}O$) at the retention time of 12.3 min (Fig. S7), and anaerobic degradation of anthracene found 9, 10- anthraquinone (CAS:84-65-1, $C_{14}H_8O_2$) and 1-Anthraquinonecarboxylic acid (CAS: 602-69-7) at 15.1 min (Fig. S8).

Discussion

Strain JP1 isolated from Shantou offshore with the capability of degrading PAHs belongs to the

Pseudomonas family, which is a facultative anaerobe using nitrate as electron acceptor for denitrification in the absence of oxygen. Similar results have been reported by Song et al. (2007) showing that strain *P. stutzeri* T7 is capable of degrading 1,2,4-trichlorobenzene, and by Shi et al. (2004) documenting a strain of *P. stutzeri* UP1 with the ability to degrade dibenzothiophene (DBT) to water-soluble sulfide.

When oxygen was exhausted, nitrate, sulfate, manganese (IV), iron ion or carbon dioxide can be used as alternative electron acceptors. When nitrate acts as the electron acceptor, it underwents a complete denitrification following NO_3^-–NO_2^-–NO^-–N_2O^-–N_2, which requires nitrate reductase, nitrite reductase, NO, and N_2O reductase (Schreiber et al. 2007). The alpha-subunit of nitrate reductase was detected in strain JP1, showing that nitrate reductase was involved in the denitrification process. However, since the amount of consumed nitrate was not equal to the amount of generated nitrite, that nitrate was probably denitrificated to an intermediate metabolite before being further reduced to nitrite.

Large amount of energy can be theoretically obtained during the process of denitrification of NO_3^- to N_2 (Thauer et al. 1977), which is much higher than that when other substances (sulfate, manganese, iron) were used as electron acceptors. However, in reality, Strohm et al. 2007) found that a lot less energy than calculated was generated when he studied the denitrification of *Paracoccus denitrificans* and *P. stutzeri* with different concentrations of formic acid ester as the electron donor and different concentrations of nitrate as the electron acceptor. Consistently, in this study, strain JP1 grew slowly in the presence of KNO_3 as the electron acceptor, suggesting that less amount of energy was obtained.

Presently, microorganism anaerobic denitrification research primarily used mixed bacteria. For example, (Rockne and Strand 2001) found that when NO_3^- was eliminated, PAHs ceased to be degraded, and the degradation was resumed when NO_3^- was retrieved, indicating a coupling relation between PAHs degradation and denitrification process. Formation of N_2O was also detected in the process of denitrification. Nieman et al. (2001) reported that Nap, Phe, Pyr and other PAHs were completely oxidized to CO_2 by mixed bacteria through denitrification. There have been very few studies on the degradation of PAHs by single strain bacteria in denitrifying conditions, if any, they are limited to degradation of LMW PAHs (Nap, Phe). Hence, strain JP1 is invaluable for study the pathways and mechanisms of PAHs degradation under anaerobic conditions.

As a first attempt, we investigated the degradation pathway of BaP by strain JP1 under anaerobic conditions using GC/MS. The results enable us to propose a potential pathway via which the facultative anaerobic bacteria *Pseudomonas* sp. JP1 anaerobically degrade BaP (Fig. 9). The first opening of BaP ring generates 1,12-dimethyl-Benz[a]anthracene, 7,8,9,10-Tetrahydrobenzo[a]pyrene, and 5-ethylchrysene, which subsequently generate benz[a]anthracene, pyrene and chrysene, respectively. Pyrene was further being degraded into 4,5-dimethylphenanthrene, and 4- methylphenanthrene, and phenanthrene by demethylation, which can be degraded into 1,2,3,4-tetrahydro-4-methyl-4-phenanthrenol in the anaerobic environment.

When chrysene is generated, it can be degraded into, 1-ethyl-2-methyl-phenanthrene, and then further into 1-methylphenanthrene or 2-methyl-phenanthrene, which is continued to be degraded to alpha-methylstilbene, or likely to generate phenanthrene by demethylation. When 1,12-dimethyl-benz[a]anthracene is generated, it can be turned into benz[a]anthracene by demethylation. Either one or both can be detected at the peak, since the GC/MS mass spectra of benz[a]anthracene and chrysene are almost the same. They could not be distinguished even compared with the standard control (Fig. S2). Benz[a]anthracene can also be anaerobically degraded by strain JP1, generating 2,3-dimethylphenanthrene, and then a methyl is removed to generate 2-methylphenanthrene, or 2-methylanthracene/1-methylanthracene. They may continue to be demethylated to generate Phe and anthracene, respectively.

Conclusions

Pseudomonas sp. JP1 isolated from Shantou offshore. It is able to degrade phenanthrene, benzo[a]pyrene (BaP) and fluoranthene, and can hardly degrade pyrene in aerobic or anaerobic conditions. Sodium sulfite, iron oxide, manganese dioxide, potassium chlorate; maltose; glycine, and a salinity of 20 % significantly stimulated anaerobic degradation of BaP. Lastly, the anaerobic degradation metabolites of BaP by strain JP1 were investigated using GC/MS, and the anaerobic degradation

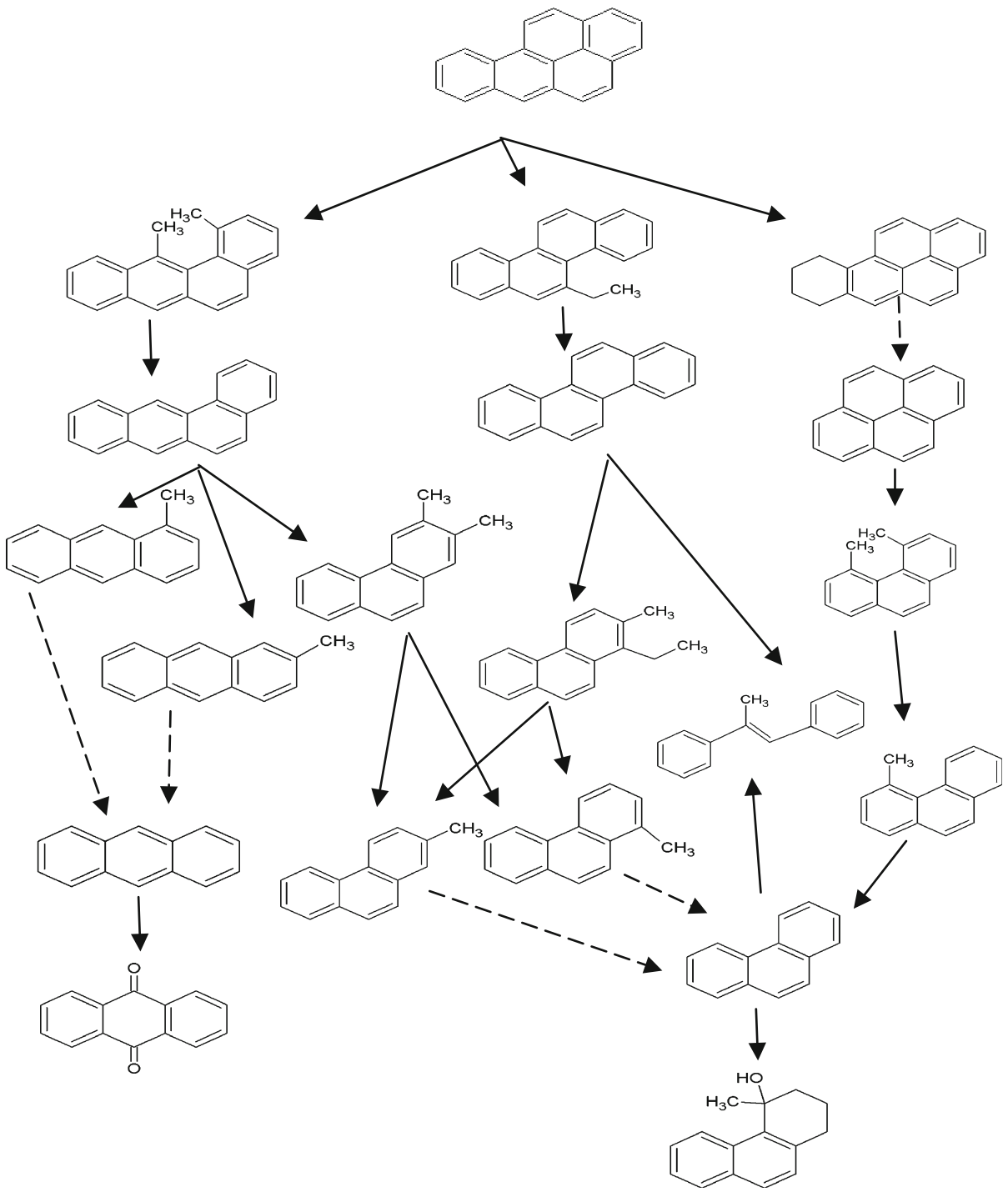

Fig. 9 Proposed pathways for anaerobic degradation of PAHs by *Pseudomonas* JP1

pathway was proposed: BaP to 1,12-dimethyl-Benz[a]anthracene/7,8,9,10-Tetrahydrobenzo[a]pyrene/5-ethylchrysene, chrysene/Benz[a]anthracene, and to three-ring PAHs, two-ring PAHs. This study is helpful for further studies on the mechanism of anaerobic biodegradation of PAHs.

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