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# Different bacterial groups for biodegradation of three- and four-ring PAHs isolated from a Hong Kong mangrove sediment

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**Abstract**

Mangrove sediments have been found to degrade three- to four-ring PAHs extensively. In the present study, 11 strains from 4 genera *Mycobacterium* (3 strains), *Sphingomonas* (5), *Terrabacter* (2) and *Rhodococcus* (1) were isolated from a single surface sediment sample of a Hong Kong mangrove swamp, among which the *Terrabacter* strains were isolated to grow with fluoranthene for the first time. Although all four genera could degrade three- and four-ring PAHs, their *in situ* activities in natural sediment slurry were found to be different. A cultivable method showed that *Sphingomonas* strains grew rapidly under the induction of three-ring, but not four-ring PAHs, while only *Mycobacterium* degrading strains dominated in the four-ring PAHs spiked slurry. Culture-independent method using a reverse transcriptional PCR showed expressions of *nahAc*-like (mainly found in Gram-negative bacteria) and *nidA*-like (in Gram-positive bacteria) dioxygenase genes parallel with the degradation of three- and four-ring PAHs, respectively. The present study suggested that surface mangrove sediments harbored diverse PAH-degrading bacteria, which showed different importance for biodegradation of three- and four-ring PAHs in the sediment.


*Keywords:* Polycyclic aromatic hydrocarbons; Mangrove; Biodegradation; *Mycobacterium*; *Sphingomonas*; *Terrabacter*

## 1. Introduction

Environmental bacteria are generally considered to be the most important organisms in the natural biodegradation of polycyclic aromatic hydrocarbons (PAHs) [1–3]. A large number of consortia or pure bacterial strains with PAH-degrading ability have been isolated from diverse environments, including polluted and pristine soils, sediments and water bodies [4–8]. Some genera of the PAH-degrading bacteria have been found to reside in specific environments, for instance, *Cycloclasticus*, *Sphingomonas* and *Vibrio* strains were suggested to be common phenanthrene-degraders in marine systems [9–11]. A large group of PAH-degrading *Paenibacillus* sp. strains were isolated from the rhizosphere of salt marsh plants [12]. The distribution of these organisms in specific sites may be advantageous to bioremediation activities.

In natural environments, the low molecular weight (LMW) PAHs (consisting of 2–3 aromatic rings) are relatively easy to be degraded, while the high molecular weight (HMW) PAHs (containing 4 or more aromatic rings) are persistent [7]. Studies suggested that many bacteria prefer LMW PAHs than HMW PAHs in pure culture conditions [13]. In addition, it was recently found that PAHs with different aromatic rings were degraded by different microbial groups even in the same environment [14,15]. The relative abundance and activity of different groups of PAH-degrading microorganisms in the environment could lead to different persistence and accumulation of HMW and LMW PAHs.

Mangroves are unique inter-tidal estuarine wetlands along the coastlines of tropical and subtropical regions, and are subject

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to anthropogenic pollution. The sediments were rich in organic matter, iron and sulfide, and were sinks for many pollutants such as PAHs [16,17]. Ke et al. [18] reported that mangrove sediments had extensive potential to degrade three- and four-ring PAHs. In our previous work, several PAH-degrading consortia were enriched from mangrove sediments with different degrees of PAH contamination [5,18,19]. However, there is little information on the diversity of PAH-degrading microbial communities in mangrove ecosystems.

The present study aims to (1) study the species diversity of cultivable aerobic PAH-degrading bacteria in surface mangrove sediments; (2) compare the microbial groups for the biodegradation of three-ring with four-ring PAHs in the sediment. A combination of culture-dependent methods including the enrichment and most probable number (MPN), and culture-independent methods including the 16S rRNA sequencing and the reverse transcriptional (RT)-PCR determination of PAH ring-hydroxylating dioxygenase was employed to characterize the diversity and activity of the PAH-degrading bacteria in surface mangrove sediments.

## 2. Materials and methods

### 2.1. Isolation and identification of PAH-degrading bacteria

Surface sediment sample was collected at the landward region (close to the discharge point of a public sewer) of the Ho Chung (HC) mangrove swamp in Hong Kong SAR, China during low tides. This swamp covers a mangrove area of 2.37 ha and has been affected by vehicle exhausting deposition, and discharge of industrial, livestock and household waste and wastewater [19]. A relatively high PAH pollution of 11,098 ng total PAHs $g^{-1}$ freeze-dried sediment was recorded in this swamp [17].

Two groups of enrichments, each in triplicate, were carried out by spiking a mixture of three- or four-ring PAHs into fresh HC sediment slurries prepared in the MSM at a 1:1 (w/v) ratio. The mixed three-ring PAH group consisted of a mixture of fluorene and phenanthrene each at an initial concentration of 10 mg $l^{-1}$ (prepared by adding an appropriate volume of 5 mg $ml^{-1}$ stock solution prepared with acetone), while the four-ring PAH group was spiked with pyrene and fluoranthene, each at an initial concentration of 10 mg $l^{-1}$. The flasks were shaken at 30 °C for 4 weeks in the dark.

The $R_2$A-PAH plate was prepared by pouring 1 ml PAH stock solutions dissolved in acetone on the surface of the solidified $R_2$A (Difco, USA) plate. The plate was let stand overnight in fume hood and a film of PAH formed on the top of the plate after the venting of acetone. To form a reasonable good PAH film on the plate, the concentrations of PAH solutions were 5 mg $ml^{-1}$ phenanthrene, 2.5 mg $ml^{-1}$ fluorene, 1 mg $ml^{-1}$ fluoranthene and 1 mg $ml^{-1}$ pyrene.

At the end of weeks 2, 3 and 4, supernatants from the three-ring PAH enrichment group were streaked on $R_2$A plates covered with a film of mixed fluorene and phenanthrene (1 ml of each PAH for one plate), and supernatants from the four-ring PAH enrichment group were streaked on $R_2$A plates covered with a film of mixed fluoranthene and pyrene (1 ml of each PAH for one plate). The plates were incubated at 30 °C in dark and were routinely checked under the light microscope for 2 weeks for detection of clear zone of PAH film surround the colony [20]. The bacterial colony with a clear zone was then picked up from the plate, and purified by repetitive streaking on another set of $R_2$A-PAH plates. Pure degrading strains were cultured in Luria–Bertani (LB) broth and stored at −70 °C.

Total bacterial genomic DNA was extracted with EZNA Bacterial DNA kit (OMEGA, USA). 16S rRNA gene was amplified using the set of primer pair 27F and 1492R. The DNA sequences using the primer 530F were determined directly from the purified PCR product. DNA sequence of the cloned 16S rDNA fragments was compared using BLASTN.

### 2.2. Biodegradation of PAHs by isolated bacterial strains

The ability of the each isolated bacterial strain to degrade PAH compounds was assessed under two conditions, namely on solid agar ($R_2$A plates) and in liquid culture (MSM).

For the biotransformation on solid agar plate, 5 μl fresh culture of the isolates in LB medium was dropped on the $R_2$A plate covered with a film of single PAH of phenanthrene, fluorene, anthracene, fluoranthene or pyrene (prepared as above). The plates were incubated at 30 °C in dark and routinely checked under a light microscope for 1 month. A positive result was recorded if an obvious clear zone was observed around the colony.

The biodegradation in liquid culture was carried out in conical flasks, 1 ml aliquot of the fresh culture of the isolated strains at the late exponential growth phase was added aseptically to 10 ml MSM containing a mixture of five PAHs (fluorene, phenanthrene, anthracene, pyrene and fluoranthene, each at an amount of 100 μg). The MSM containing the same amount of mixed PAHs but without the bacterial inoculation was used as the control to determine any abiotic loss of PAHs. The flasks were shaken on an orbital shaker at a speed of 150 rpm at 30 °C in dark for 7 days. At the end of the experiment, triplicate flasks from each set-up were sacrificed and the residual PAH concentrations were extracted with ethyl acetate and analyzed by GC-FID [21].

To determine if the isolates could use single PAH as the sole carbon and energy source for growth, one colony of the bacterial isolate was inoculated into MSM spiked with a single PAH of naphthalene, fluorene, phenanthrene, anthracene, fluoranthene or pyrene at an initial concentration of 100 mg $l^{-1}$. All acetone in the PAH stocks was let evaporated to make sure no carbon source other than the spiked PAH was present in the medium. The cultures were shaken at 30 °C for 4 weeks and changes of turbidity were checked regularly.

### 2.3. Effect of three-ring and four-ring PAHs on PAH-degrading microorganisms in fresh sediments

Fresh HC sediment slurry was spiked with a mixture of three-ring or four-ring PAHs to compare the diversity and activity of the intrinsic PAH-degrading bacteria in the sediment.

The biodegradation of spiked PAHs, the abundance of PAH-degrading bacteria, and the expression of dioxygenase genes were determined.

Four treatments, each at triplicates, were set up. Group A was spiked with a mixture of three-ring PAHs, fluorene and phenanthrene (50 mg $kg^{-1}$ sediment each); group B was spiked with a mixture of four-ring PAHs, pyrene and fluoranthene (50 mg $kg^{-1}$ sediment each); group C was spiked with a mixture of three- and four-ring PAHs, phenanthrene, pyrene and fluoranthene (50 mg $kg^{-1}$ sediment each); Group D was control (without any PAH contamination). To minimize the effect of acetone used to dissolve PAHs in the stock solution (as a possible carbon source), PAH stocks were spiked to the conical flask and stood overnight for the venting of acetone before the addition of sediment slurry. In each 500 ml flask, 150 g fresh HC surface mangrove sediment was mixed with 150 ml MSM at 1:1 (w/v) ratio. The flasks were shaken on an orbital shaker at a speed of 150 rpm at 30 °C in dark. At 2 h and days 1, 4, 7, 11, 15, 21 and 27, 10 ml slurry was collected, freeze-dried and extracted by a DCM/Methanol mixture and determined by GC-FID [19].

The population sizes of the PAH-degrading bacteria in sediment slurries were determined by a modified most probable number (MPN) method from a technique reported by Kiyohara et al. [20]. The $R_2$A-PAH agar plate was divided into three parts and each part was subdivided into five sections. Ten μl of the serial dilution of sediment slurry was dropped onto each of the sections ($n = 5$). The inoculated $R_2$A-PAH plates were incubated at 30 °C for 2 weeks. After incubation, the colonies developed in the division and surrounded by clearing zones were marked as positive PAH-degraders, while those growing without clearing zones were recorded as non-degraders. The total of heterotrophs was calculated by adding the number of PAH-degraders with the number of non-degraders. The morphology and the growth rate of the colony on the $R_2$A-PAH plate, which were consistent with the isolation results, were used to differentiate the genus of PAH-degrading bacteria.

At each of above sampling time, the total RNA was extracted from the sediment slurry using a FastRNA Pro Soil-Direct Kit (Bio101, USA) and treated with an RNase-free DNase I (Takara, Japan). Two sets of nested PCR were used to determine the expression of *nahAc*-like and *nidA*-like dioxygenase genes in the sediment slurry [22]. In the first round RT-PCR, the primers Nah-for (tgcmvntaycayggytgg)/Nah-rev1 (cccggtarwanccdckrta) were used to determine the *nahAc*-like dioxygenase genes present in the PAH-degrading bacteria belonging to the genera of *Sphingomonas* and *Pseudomonas*, and the primers Nid-for (tccrmtgcccdtaccacgg)/Nid-rev1 (gaasgayarrttsgggaaca) were used to determine the *nidA*-like dioxygenase genes present in the PAH-degrading bacteria belonging to the genera of *Mycobacterium*, *Terrabacter* and *Rhodococcus* [22]. The RT-PCR analyses were carried out using an Access RT-PCR system (Promega, USA) following a standard method. An RT-PCR without reverse transcriptase was performed as a negative control. The PCR condition was: 1 cycle of 45 °C for 45 min, 1 cycle of 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min, and finally 1 cycle of 68 °C for 7 min and stop at 4 °C.

After the RT-PCR amplification, a nested round PCR was performed. About 0.1 μl of the above RT-PCR product was used as the template and the reaction components were: 1× Ex Taq buffer ($Mg^{2+}$ free) (Takara, Japan), 1.25 mM $Mg^{2+}$, 0.25 mM dNTP each, 100 pmol for each primer (Nah-for and Nah-rev2 crggtgycttccagttg for the amplification of the *nahAc*-like dioxygenase, Nid-for and Nid-rev2 gcgsckrkcttccagttcg for the amplification of the *nidA*-like dioxygenase gene) [22], and 1 U Ex Taq. The PCR cycle condition was 94 °C for 3 min, then 30 cycles of 94 °C 45 s and 55 °C 45 s and 72 °C 45 s, and a final extension at 72 °C for 5 min.

## 3. Result

### 3.1. PAH-degrading bacteria isolated from single mangrove sediment

A total of 11 PAH-degrading strains were enriched from a single sample of HC surface sediment. The six Gram-positive strains were isolated with the four-ring PAHs, three belonged to *Mycobacterium*, and two were *Terrabacter* and one was *Rhodococcus* (Table 1). On the other hand, all the five strains enriched with the three-ring PAHs were Gram-negative *Sphingomonas* strains (Table 1).

### 3.2. Biodegradation of PAHs by the isolated strains on agar plate

Among the five investigated PAHs, fluorene and phenanthrene were easily to be degraded as shown by the biodegradation of single PAH on the agar plate (Table 1). Anthracene was more difficult to degrade, only 7 out of 11 strains could transform anthracene and more than 4 days were needed to form a clear zone surrounding the colony. For the two four-ring PAHs, fluoranthene was more degradable than pyrene in terms of the number of degrading strains and the speed of clearance.

*Mycobacterium* sp. strain HH1 degraded all five investigated PAHs, while strains HH2 and HH3 removed four PAHs except anthracene. *Terrabacter* sp. strains HH4 and HH5 preferred fluoranthene over other PAHs, and had relatively poor pyrene degradation. On the contrary, the five Gram-negative strains enriched with three-ring PAHs showed higher ability to degrade three-ring PAHs, and only strains HL1 and HL4 could remove fluoranthene. Strain HL5 was the poorest strain degrading only fluorene.

### 3.3. Growth with single PAH as the sole carbon and energy source

Among the five PAHs investigated, *Mycobacterium* sp. strains HH1 and HH2 could grow with both phenanthrene and pyrene while *Mycobacterium* sp. HH3 and four *Sphingomonas* sp. strains HL1, HL2, HL3 and HL4 grew with phenanthrene (Table 1). The two *Terrabacter* sp. strains HH4 and HH5 could grow with fluoranthene. The remaining two strains, *Rhodococcus* sp. HH6 and *Sphingomonas* sp. strain HL5 could not grow

Table 1
PAH-degrading bacteria isolated from a single sediment sample collected from Ho Chung (HC) mangrove swamp

| Strain no. | Colony morphology | Identify by 16S rRNA sequence, best matched (similarity) | PAH biodegradation[a] | | | | | Growth[b] |
|---|---|---|---|---|---|---|---|---|
| | | | flo | phe | ant | fla | pyr | |
| Isolated with four-ring PAHs | | | | | | | | |
| HH1 | Yellow, convex, 0.5 mm, circular | *Mycobacterium gilvum* AF544636 (99%) | 2+++[c] | 2+++ | 7+ | 2++ | 2++ | phe, pyr |
| HH2 | Yellow, convex, 1.5–2 mm, circular | *M. gilvum* AF544636 (99%) | 3++ | 1+++ | – | 1++ | 1+ | phe, pyr |
| HH3 | Pink, convex, 1.5–2 mm, circular | *Mycobacterium psychrotolerans* AJ534886 (98%) | 3+ | 1+++ | – | 3++ | 8+ | phe |
| HH4 | Pale-white, flat, 2–3 mm, circular | *Terrabacter* sp. DFA1 AB180233 (99%) | 3+ | 2+ | 7+ | 1++ | 14± | fla |
| HH5 | Pale-yellow, flat, 2–3 mm, circular | *Terrabacter* sp. DFA1 AB180233 (98%) | 3+ | 2+ | 6++ | 1++ | 15± | fla |
| HH6 | Pink, convex, 3 mm, irregular | *Rhodococcus ruber* OUCZ91B AY785745 (98%) | 2+ | 2++ | – | 3+ | – | ng |
| Isolated with three-ring PAHs | | | | | | | | |
| HL1 | Pale-white, transparent, 1 mm, circular | *Sphingomonas wittichii* DSM 6014 AB021492 (97%) | 1+ | 1++ | 4+ | 1++ | – | phe |
| HL2 | Yellow-green, convex, 2 mm, circular | *Sphingomonas paucimobilis* 21C AJ698833 (99%) | 1++ | 1++ | 4++ | 3± | – | phe |
| HL3 | White, flat, 2–3 mm, circular | *Sphingomonas* sp. A1–13 AY512602 (97%) | 2+ | 1++ | 4+ | – | – | phe |
| HL4 | Yellow, convex, dry surface, 1.5 mm | *Sphingomonas* sp. A1–13 AY512602 (97%) | 4++ | 2++ | 9++ | 3+ | – | phe |
| HL5 | Yellow, convex, 1 mm, circular | *S. paucimobilis* EPA505 X94100 (99%) | 2+ | – | – | – | – | ng |

[a] Biodegradation on $R_2$A-PAH agar plate.
[b] Growth with single PAH as the sole carbon and energy source; five PAHs investigated were flo: fluorene; phe: phenanthrene; ant: anthracene; fla: fluoranthene; pyr: pyrene. ng: no growth with any of these five PAHs.
[c] Time needed for clear zone formation (day) and abundance of the clear zone surrounding each colony: (−) no clear zone; (±) color formation but without obvious clear zone; (+) a clear zone just around the colony; (++) an obvious clear zone but the radius was smaller than the colony itself; (+++) an obvious clear zone with the radius larger than the colony.

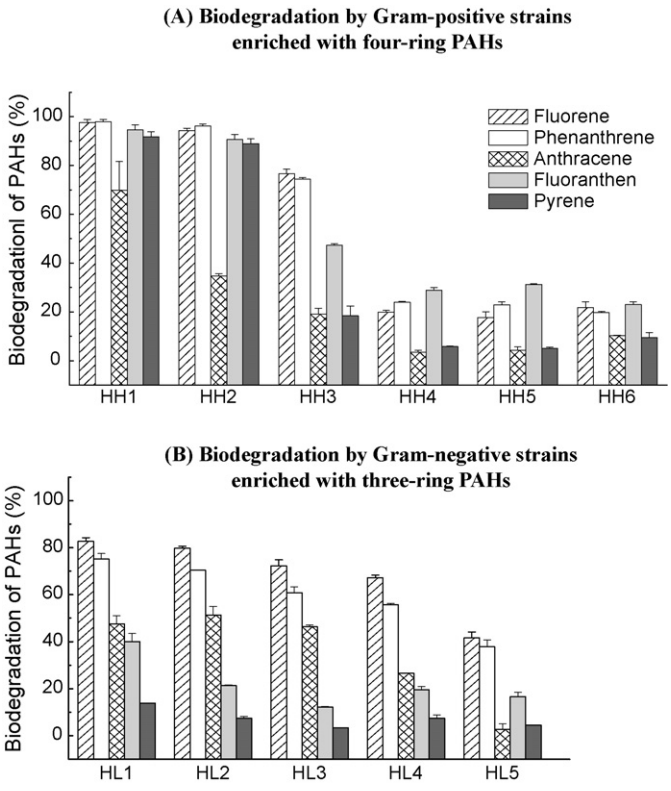


Fig. 1. (A and B) Biodegradation of 5 PAHs in MSM liquid culture by 11 PAH-degrading bacterial strains isolated from HC mangrove sediment (mean and standard deviation of triplicates are shown. Descriptions of the strains were explained in Table 1).

with any of the five PAHs, indicating these two strains degraded PAHs by co-metabolism.

### 3.4. Biodegradation of PAHs by the isolated strains in MSM liquid culture

All isolated bacteria degraded more than 10% of the five determined PAHs in mixture. *Mycobacterium* spp. strains had the highest degrading potential, particularly for four-ring PAHs (Fig. 1). *Terrabacter* and *Rhodococcus* strains were relatively poor in degrading fluorene, phenanthrene, anthracene and pyrene, but interestingly had the highest degradation percentage of fluoranthene than the other four PAHs. *Sphingomonas* spp. strains had higher ability in degrading three-ring than four-ring PAHs, and only strain HL1 showed relatively high fluoranthene degradation.

### 3.5. Abundance of PAH-degrading bacteria in the HC sediment spiked with different PAHs

In the sediment slurry spiked with three-ring PAHs (Group A, phenanthrene and fluorene), *Sphingomonas* spp. strains were the dominant PAH-degrading bacteria from day 4 to day 27, and no PAH-degrading strain from the genus of *Mycobacterium* was detected during this period (Fig. 2(A)). The two three-ring PAHs were degraded rapidly within 1 day and were almost completely removed at day 4 (Fig. 3(A)). On the other hand,

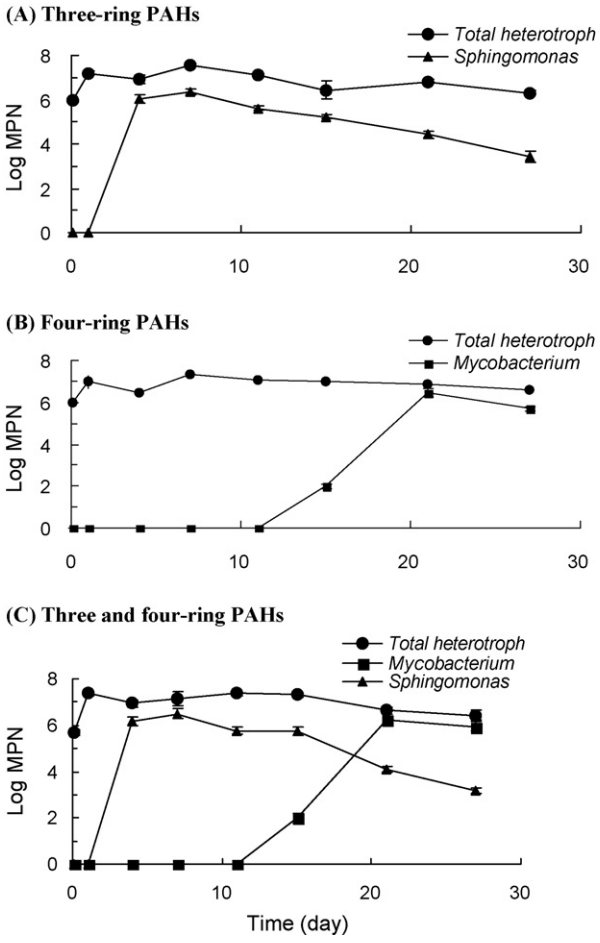


Fig. 2. (A–C) Abundance of PAH-degrading bacteria in HC sediment slurries spiked with different PAHs (means and standard deviations of triplicates are shown).

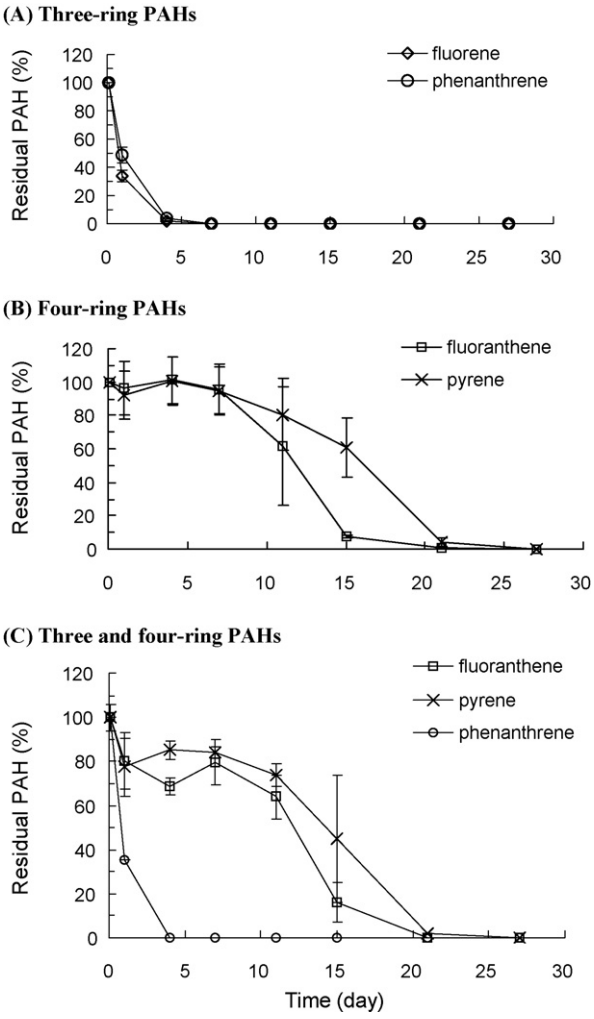


Fig. 3. (A–C) Biodegradation of PAHs in HC sediment slurry spiked with different PAHs (means and standard deviations of triplicates are shown).

in the sediment slurry spiked with pyrene and fluoranthene (four-ring PAHs, Group B), no PAH-degrading bacteria were determined from day 0 to day 11, but the *Mycobacterium* degrading strains became dominant from day 15 onwards (Fig. 2(B)), and the four-ring PAHs were degraded rapidly with the growth of *Mycobacterium* spp. (Fig. 3(B)). For the sediment spiked with a mixture of three- and four-ring PAHs, phenanthrene, fluoranthene and pyrene (Group C), *Sphingomonas* spp. strains were determined from day 4 onwards (Fig. 2(C)), and phenanthrene was rapidly degraded with the growth of *Sphingomonas* strains (Fig. 3(C)). Under this condition, about 20% of fluoranthene and pyrene were degraded at day 1 (Fig. 3(C)), which might be induced by co-metabolism in *Sphingomonas* spp. At day 15, *Mycobacterium* spp. were determined and grew rapidly (Fig. 2(C)) with simultaneous degradation of pyrene and fluoranthene (Fig. 3(C)). In the control group without any PAH, no PAH-degrading bacterium was determined.

### *3.6. Expression of dioxygenase genes in sediment slurry spiked with three- or four-ring PAHs*

No dioxygenase gene was determined in the sediment slurry sampled at day 0 and the samples of the control group (Fig. 4). From day 1 to day 21, expression of the *nahAc*-like dioxygenase gene (Fig. 4, I) was determined in the Group A sediment slurry spiked with three-ring PAHs and in the Group C sediment slurry spiked with a mixture of three- and four-ring PAHs. On the other hand, the *nidA*-like dioxygenase gene (Fig. 4, II) was determined in the Group B sediment slurry spiked with four-ring PAHs from day 11 to day 21 and in the Group C sediment slurry spiked with a mixture of three- and four-ring PAHs (Fig. 4, II).

## 4. Discussion

It is well known that the microbial diversity in soils and sediments are very high—approximately $10^9$ microbial cells from thousands of species can be found from 1 g of soil [23]. However, the ecology of PAH-degrading bacteria, which is critical for bioremediation applications, has seldom been investigated [24,25]. Obviously the diversity of cultivable PAH-degraders is affected by enrichment methods used for isolation, and modifying enrichment procedure would change the final isolation results [26,27].

In our previous studies using traditional enrichment method, microbial consortia consisting of 1–3 cultivable PAH-degrading

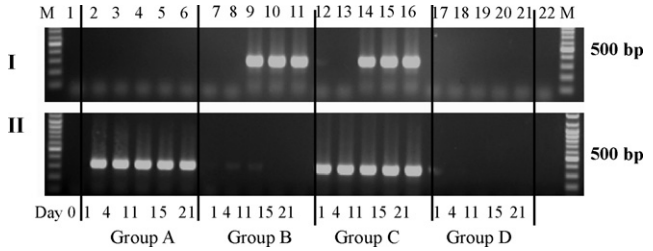


Fig. 4. RT-PCR determination of expression of dioxygenase genes from PAH-degrading bacteria in the sediment slurry spiked with different PAHs (I: expression of dioxygenase gene determined with the *nidA*-like primer set; II: expression of dioxygenase determined with *nahAc*-like primer set; lane M: 100 bp ladder; lane 1: day 0; lanes 2–6: days 1, 4, 11, 15 and 21 of the sediment slurry spiked with fluorene and phenanthrene (Group A in Figs. 2 and 3); lanes 7–11: the same sampling time of the sediment slurry spiked with fluoranthene and pyrene (Group B in Figs. 2 and 3); lanes 12–16: the same sampling time of the sediment slurry spiked with phenanthrene, fluoranthene and pyrene (Group C in Figs. 2 and 3); lanes 17–21: the same sampling time of the sediment slurry without PAH spiking; lane 22: negative control without reverse transcriptase with mixed RNA as a template).

bacterial strains were usually obtained from each sampled mangrove sediment [5,19,21]. Because the present work targeted to study the microbial ecology, two enrichment steps were modified to isolate as many PAH-degrading bacteria as possible from a single sediment sample.

The first change was, rather than transferring the enriched culture for 3–4 times as that in the traditional procedure [5], the study just incubated the slurry without any subculture. In the process, the species number in the slurry was not reduced by transfer and kept available for isolation. For instance, in traditional procedure, rapid growth bacteria like *Sphingomonas* strains have higher opportunity to be transferred and finally dominate the enrichment culture [28]; but in the present study, the slurry from the same flask was streaked on $R_2$A-PAH plates at the end of 1, 2, 3 and 4 weeks, therefore both the rapid and slow growth bacteria including *Sphingomonas* and *Mycobacterium* strains were obtained.

The adoption of $R_2$A-PAH plate was the second critical change to the method. The traditional enrichment procedure relies on the turbidity increase to indicate the growth of PAH-grading bacteria. Then the culture is streaked on technical agar-PAH plates and PAH-degrading colonies are purified [20]. However, when the method was employed to isolate PAH-degrading bacteria from marine environments, two problems were encountered: (1) the technical agar without PAH was found to support the growth of some oligotrophic marine bacteria. Therefore the colonies formed on technical agar-PAH plates were not only growth on PAH, but also on other carbon sources from technical agar; (2) on the other hand, the nutrients in technical agar-PAH plates were so poor that the colonies grew too slow and small to differentiate from each other. In the present study, $R_2$A-PAH plates were selected for identification of PAH-degrading bacteria. Because the $R_2$A medium is good for marine bacterial growth, the colonies on $R_2$A-PAH, grew rapidly and the colony morphology could be easily differentiated under microscopy. It should be noted that the bacterial colonies might use many carbon sources from $R_2$A plates rather than the PAH film on the plate, suggesting that these bacteria might degrade PAH by co-metabolism but not using them as a sole carbon and energy source. All isolated bacteria with a clear zone of PAH around the colony were proved to degrade PAHs in liquid culture (Fig. 2) and in sediment slurry (data not shown), suggesting that formation of clear zone on $R_2$A-PAH plates was a good indicator of PAH-degrading ability.

Although most of the isolated bacteria degraded both three- and four-ring PAHs in pure culture conditions, their performance in aboriginal community of sediment slurry was different. *Sphingomonas* PAH-degrading bacteria grew and removed three-ring PAHs rapidly in natural mangrove sediments, but none was determined in four-ring PAHs group. Sphingomonads strains have been reported to grow with four-ring PAHs [11], but they did not show any importance in four-ring PAHs biodegradation in the determined mangrove sediment. On the other hand, *Mycobacterium* strains were frequently reported as four-ring PAH degraders [1,25,29]. Agreeing with these reports, *Mycobacterium* spp. dominated in the sediment slurry only with four-ring PAHs biodegradation. Although *Mycobacterium* spp. degraded three-ring PAHs extensively in pure culture, they were less competitive than *Sphingomonas* strains in using three-ring PAHs, therefore no *Mycobacterium* spp. were determined in three-ring PAHs group.

A shift from *Sphingomonas* to *Mycobacterium* was observed in the sediment slurry spiked with a mixture of three- and four-ring PAHs (Group C, Fig. 3). The abundance of these two genera and the biodegradation pattern of PAHs were similar to that in the sediment slurry spiked with only three- or four-ring PAHs, groups A and B, respectively, suggesting few interactions between these two genera in the sediment slurry. Greene et al. [30] found a succession of *Pseudomonas* and *Rhodococcus* to *Alcaligenes* spp. in a soil contaminated with a mixture of aromatic hydrocarbons. Such community change would absolutely affect the *in situ* biodegradation of PAHs since the real environment was normally contaminated by mixed PAHs [30].

The RT-PCR could determine the expression of PAH-dioxygenase from *Mycobacterium*, *Terrabacter* and *Rhodococcus* with *nidA*-like primers, and that from *Sphingomonas* and *Pseudomonas* with *nahAc*-like primers [22]. In the present study, results from the RT-PCR were in good agreement with that from the culture-dependent method. The expression of dioxygenase genes was detected earlier than the growth of PAH-degrading bacteria, suggesting that the RT-PCR method was more sensitive than cell growth and PAH biodegradation.

The present study suggested two possible reasons for the different persistence of LMW and HMW PAHs: (1) The PAH-degrading bacteria in polluted environment degrade LMW PAHs faster; (2) LMW and HMW PAHs are degraded by different bacterial groups in the environment, and the abundance and activity of the two bacterial groups affect the biodegradation. The present study suggested that surface mangrove sediments harbored diverse PAH-degrading bacteria, which showed different importance for biodegradation of three- and four-ring PAHs in the sediment. To the best of our knowledge, the present study is the first isolation of fluoranthene-degrading *Terrabacter* strains, although strains from this genus were reported to transform some three-ring PAHs like fluorene and phenanthrene

[31,32]. The metabolism pathway of fluoranthene by *Terrabacter* sp. strain HH4 was determined (submitted). However, strains from this genus were found to be inhibited by pyrene higher than 5 mg $ml^{-1}$ (data not shown), therefore no *Terrabacter* strains was abundant in the three treatments.

Although 11 strains from four genera were obtained in the study, considering that most of the marine sediment bacteria are uncultivable and the limitations of the isolation method, it is reasonable to deduce that the species diversity of PAH-degrading bacteria in mangrove sediment is higher than the present data. In addition, the present study did not reveal any population abundance of PAH-degrading bacteria in natural environments. Studies using culture-independent methods such as 16S rDNA library and stable isotope probing are promising to reveal the ecology of *in situ* PAH-biodegradation [15,33].

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