RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2012.38.036

ScienceAsia 38 (2012): **36–43**

Isolation of novel phenanthrene-degrading bacteria from seawater and the influence of its physical factors on the degradation of phenanthrene

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Received 17 Aug 2011
Accepted 18 Jan 2012

ABSTRACT: This study aimed to isolate marine bacteria that can degrade phenanthrene and to investigate the effects of physical factors on the degradation of phenanthrene by the isolate. Two phenanthrene-degrading bacteria were isolated from seawater using an enrichment method. The isolated strains, designated PHPY and SK, degraded 99% of 500 mg/l phenanthrene in sterilized seawater within 15 and 10 days, respectively. In addition to phenanthrene, strain PHPY degraded anthracene, while strain SK could degrade fluorene. 16S rDNA-based phylogenetic analysis suggested that strains PHPY and SK might represent new genera of *Sphingomonadaceae* and *Rhodobacteraceae*, respectively. Studies on the effect of physical factors indicated that at temperatures of 30 °C and 37 °C, and pH 7.4–9.0, the strain PHPY could effectively grow and degrade phenanthrene. However, this strain could not grow at higher temperature or lower pH. Moreover, in the presence of NaCl at 22.79 g/l and even without added NaCl, strain PHPY was able to grow and degrade phenanthrene. PCR experiments with primers specific for large subunit of aromatic-ring-hydroxylating dioxygenase (*bphA1f*) from *Novosphingobium aromaticivorans* F199 suggested that strain PHPY might possess genes for phenanthrene degradation that are not highly homologous to the genes in strain F199. Isolation of two new marine bacteria capable of degrading PAHs in this study confirmed that bacteria in genera *Sphingomonadaceae* and *Rhodobacteraceae* play an important role in the degradation of PAHs in marine environments.

KEYWORDS: *Sphingomonadaceae*, *Rhodobacteraceae*, PAHs, marine bacteria, bioremediation

INTRODUCTION

Phenanthrene, a polycyclic aromatic hydrocarbon (PAH), is a widely distributed environmental contaminant causing detrimental biological effects [1]. In marine environments, sources of PAHs include the petrochemical industry, domestic and industrial wastewater, and spillage of petroleum products from ships. The presence of these PAHs in marine environments may pose a threat to public health and marine life [2,3]. Microbial degradation represents a potential route to eliminate PAHs from the environment [4]. Marine bacteria capable of degrading PAHs have been isolated from the following genera: *Cycloclasticus*, *Neptumonas*, *Pseudoalteromonas*, *Marinomonas*, *Halomonas*, *Sphingomonas*, and *Burkholderia* [5–8]. Among these bacteria, *Cycloclasticus* is expected to be of great use for PAH biodegradation of oil-polluted seawater [5,9]. Culture-independent studies have also revealed that bacteria in the genus *Cycloclasticus* are the dominant PAH-degrading bacteria after oil spills in marine environments [10,11].

Physical factors such as pH, temperature, and salinity have been shown to play a major role in controlling microbial growth and activity during hydrocarbon biodegradation [12–14], but few studies have examined the effect of the above physical factors on PAH degradation by marine bacteria. In the present study, to further explore PAH degradation in marine environments, we isolated two marine bacteria capable of degrading phenanthrene and investigated the effects of physical factors, pH, temperature, and salinity on bacterial growth and phenanthrene degradation ability by one of the isolated strain. We also found that this isolated marine bacterium does not contain the same catabolic genes as those found in related microorganisms.

MATERIALS AND METHODS

Enrichment culture and isolation of phenanthrene-degrading bacteria

Enrichment of phenanthrene-degrading bacteria was undertaken in cotton-plugged Erlenmeyer flasks containing 100 ml of oil-contaminated seawater supplemented with 100 mg NH_4NO_3, 20 mg K_2HPO_4, 5 mg yeast extract (SW medium), and 50 mg phenanthrene. The cultures were incubated at 30 °C and shaken at 200 rpm. When an orange brown colour was seen in the culture, 2 ml of culture was transferred into 40 ml of sterilized seawater (SW) supplemented with phenanthrene solution in $N'N'$-dimethyl formamide at a final concentration of 500 mg/l. The culture was incubated as above until the colour changed and turbidity was observed. After three enrichments, the culture was plated on SW solid medium, the surface was sprayed with a 2% phenanthrene solution in diethyl ether, and plates were incubated at 30 °C. Colonies with a clearing zone on the plates were then picked and purified by streaking several times onto fresh marine agar (MA, Difco) plates. Purified strains were tested for phenanthrene degradation ability, either by streaking on SW agar plates sprayed with phenanthrene solution or inoculation into 5 ml SW supplemented with 500 mg/l phenanthrene.

PAH degradation experiments

Bacterial inocula were prepared by growing each bacterial strain in 5 ml of ONR7a medium[15] containing 0.33% sodium succinate, or in 5 ml of five-fold diluted marine broth (MB, Difco) on a rotary shaker at 200 rpm and 30 °C for 36 h. Cells were washed and resuspended in 5 ml SW or ONR7a to give an absorbance at 600 nm of 0.3 (10^6 CFU/ml). Phenanthrene was added at 500 mg/l, and the cultures were incubated at 30 °C and shaken at 200 rpm for 18 days. Culture medium containing phenanthrene without added bacterial inoculum was used as a control. Experiments were performed in triplicate. At three-day intervals, culture tubes were taken for measurements of phenanthrene concentration and bacterial growth. Phenanthrene was extracted as described previously[16]. The remaining phenanthrene was detected using gas chromatography with flame ionization detection (Agilent GC model 6890 N), with an HP-5 column equipped with a flame ionization detector under the following conditions: injector temperature, 280 °C; detector temperature, 250 °C; initial column temperature, 80 °C hold for 1 min, then programmed at 80 °C to 160 °C at a rate of 25 °C/min, hold for 3 min and then set to 160 °C to 220 °C at a rate of 3 °C/min, hold for 2 min and then 220 °C to 300 °C at a rate of 40 °C/min, and hold for 7 min. Bacterial growth measurements were performed using the viable plate count method with MA medium.

Substrate specificity of the isolated strains was determined with several PAHs, including phenanthrene, naphthalene, anthracene, acenaphthene, acenaphthylene, fluorene, fluoranthene, and pyrene, at a final concentration of 100 mg/l. Bacterial inocula were prepared as above. The cultures were incubated at 30 °C and shaken at 200 rpm for 15 days. Culture medium containing PAHs without added bacterial inoculum was used in control experiments. The experiments were performed in triplicate. Measurements of remaining PAHs and bacterial growth were performed as above.

Determining the influence of physical factors on phenanthrene degradation

The influence of the physical factors, pH, temperature, and salinity on phenanthrene degradation and growth of strain PHPY was determined. An inoculum of strain PHPY was prepared as described above. To determine the effect of pH, log-phase cells were cultivated in 5 ml SW containing 500 mg/l phenanthrene, in which the pH of the SW was adjusted to 5, 6, 8, or 9 using 1 M HCl or 0.1 N NaOH. Culture was performed at 30 °C with shaking at 200 rpm. Control experiments without the added inoculum and control experiments with added inoculum, but without adjusting the pH (initial pH of SW was 7.4) were conducted. To determine the effect of temperature, log-phase cells were cultivated in 5 ml SW containing 500 mg/l phenanthrene at 37, 45, or 50 °C and shaken at 200 rpm. Control experiments without added inoculum at each temperature, and control experiments with added inoculum which was cultivated at 30 °C were also conducted. To determine the effect of salinity, log-phase cells were cultivated in 5 ml ONR7a containing 500 mg/l phenanthrene and various concentrations of NaCl (0, 40, 22.79, 60, or 80 g/l). Control experiments without added inoculum were conducted. Culture was performed at 30 °C and shaken at 200 rpm. Experiments were performed in triplicate. After a 9-day culture, measurements of the remaining phenanthrene and bacterial growth were performed as described above.

DNA manipulations

Total DNA extraction from bacterial strains was performed as described[17]. Plasmid DNA extraction, PCR product purification, and gel extraction were carried out using Mini Plasmid purification kits, PCR

purification kits, and the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). DNA cloning, Southern hybridization, and other DNA manipulations were carried out as described[18].

16S rDNA sequencing and phylogenetic analysis

16S rRNA genes were amplified using universal primers and the following program: 1 min at 96 °C, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min, using maximal ramp rates throughout, with the final 72 °C segment of the cycle extended to 6 min before cooling to 25 °C. The 16S ribosomal DNA PCR products were cloned into a PCR cloning vector (Qiagen) and sequenced. The nucleotide sequences obtained were analysed with DNASIS-MAC software (version 2.05; Hitachi Software Engineering Co. Ltd., Yokohama, Japan). A homology search was performed using a BLAST program. Multiple sequence alignment was carried out using CLUSTALW version 1.83, and phylogenetic analysis was performed with the PHYLIP package.

Detection of the dioxygenase gene by PCR

To detect the phenanthrene dioxygenase gene in strain PHPY, PCR experiments with primers specific for the *bphA1f* gene of *Novosphingobium aromaticivorans* strain F199 (accession no. AF079317.1) (bphA1FF 5′-TATTTGGGGGACTTCTGCTG-3′ position 478–497 and bphA1FR 5′-TTGGGAGGATCGACGTATTC-3′ position 882–901) were carried out. Amplification was performed with 0.6 μM of the forward and reverse primers in a total volume of 30 μl with 200 μM of each dNTP, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase, and template DNA at 100 ng. PCR was carried out with the program described above except that the annealing temperatures were set at 50 and 45 °C. Total DNA of strain F199 was used in positive control experiments. Primers 341F and 1100R specific for 16S rDNA were used in other control experiments.

Nucleotide sequence accession number

The nucleotide sequences of 16S rRNA genes of strains PHPY and SK described in this report have been deposited in the GenBank databases under Accession Nos. EU179717 and EU179718, respectively.

RESULTS

Isolation and identification of phenanthrene-degrading marine bacteria

Two phenanthrene-degrading bacteria, designated as strains PHPY and SK, were isolated from oil-contaminated seawater collected from Chonburi and Songkhla provinces in Thailand. A clearing zone around colonies of both strains on SW agar plates sprayed with phenanthrene was observed after 3 days of culture. Both strains were characterized based on their 16S rRNA gene sequences.

Analysis of a partial region of the 16S rRNA gene of strain PHPY revealed that the sequence (1060 bp) showed similarity with those of *Sphingopyxis* sp. PR52-21 (EU440978) (99%), *Sphingopyxis* sp. 2PR58-1 (EU440980) (98%), *Sphingomonadaceae* bacterium E4A9 (EF623998) (98%), *Sphingomonas* sp. 2MPII (U90216) (97%), and *Sphingomonas* sp. Pee Shoal (AF361178) (98%). The 16S rDNA sequence of strain PHPY had less than 96% similarity to those of other *Sphingomonas* sp. Although the sequence similarity between the 16S rRNA gene of strain PHPY was 99% with those of *Sphingopyxis* sp. PR52-21, there was no evidence of the identification of the strain PR52-21 in any previous reports. Phylogenic analyses based on 16S rDNA sequences (Fig. 1a) suggest that strain PHPY might belong to a new genus in the family *Sphingomonadaceae*.

Analysis of a partial region of the 16S rRNA gene of strain SK revealed that a sequence (1419 bp) showing similarity with those of *Rhodobacteraceae* bacterium R11 (AF539789) (98%), *Pseudoruegeria* sp. ZXM137 (FJ436725) (98%), *Roseobacter* sp. QDHT-07 (FJ210803) (98%), and *Roseobacter* sp. QDHT-05 (FJ210801) (98%), but had less than 96% similarity with other *Roseobacter* sp. Fig. 1b provides a phylogenic tree based on the 16S rRNA gene sequence. The results obtained and the phylogenetic tree (Fig. 1b) suggested that strain SK is a member of *Rhodobacteraceae* and might constitute a novel genus.

PAH degradation activity

The biodegradation experiment showed that strain PHPY could degrade 95% of 500 mg/l phenanthrene within nine days (Fig. 2). For strain SK, the percentage of phenanthrene remaining was determined once after a 10-day culture, and results showed that this strain could degrade 99% of 500 mg/l phenanthrene by the end of the experiment. The abilities of both strains to degrade other PAHs, including naphthalene, acenaphthene, acenaphthylene, fluorene, fluoranthene, and pyrene, were then investigated. The experiments revealed that strain PHPY could degrade about 30% of 100 mg/l anthracene within 15 days, while strain SK could degrade 23% of 100 mg/l fluorene within 10 days. However, both strains were unable to degrade naphthalene, acenaphthene, acenaphthylene, fluoranthrene, or pyrene. Table 1 showed that strains PHPY and SK have comparable phenanthrene degradating

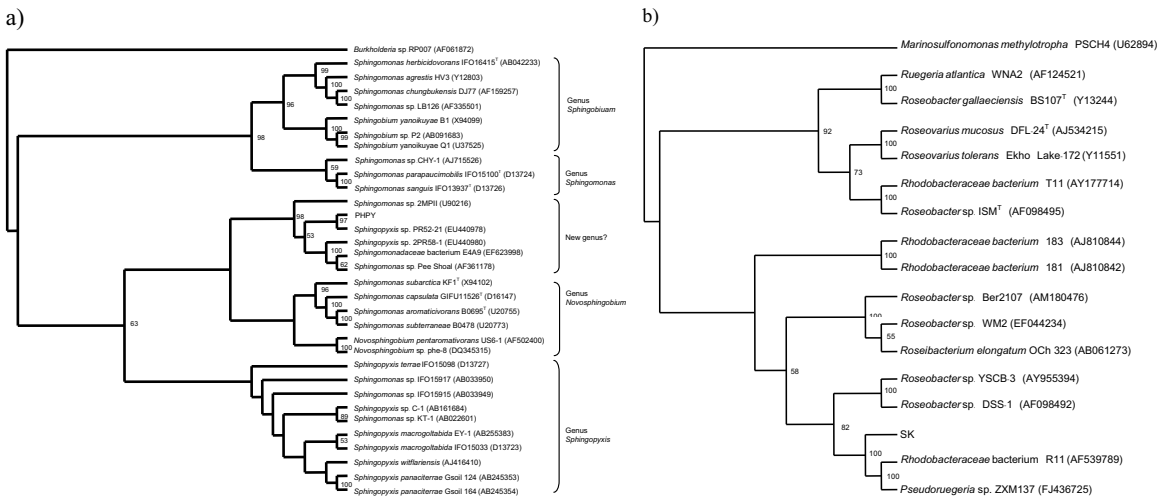

Fig. 1 Phylogenic trees based on 16S rRNA gene sequences showing relationships between (a) strain PHPY and members of *Sphingomonadaceae*, (b) strain SK and members of *Rhodobacteraceae*. The numbers at the nodes indicate the levels of bootstrap support.

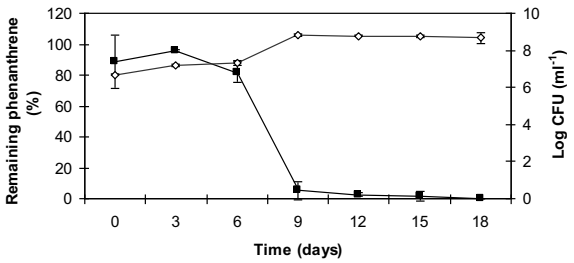

Fig. 2 Time course of the growth of strain PHPY (◇) and remaining phenanthrene (■). Error bars represent standard deviations.

activity to those of some strains reported so far such as *Pseudomonas* sp. ARP26 and ARP28 [19]. Furthermore, they exhibited higher phenanthrene degradation efficacy to those of several strains such as *Sphingomonas* sp. ZP1 [20], *Microbacterium* sp. F10a [21], *Bordetella petrii* B1 [22], *Pseudoxanthomonas mexicana* L2 [22] and *Streptomyces rochei* PAH-13 [23].

Effect of physical factors on the degradation of phenanthrene

We investigated the influence of environmental conditions with differing physical factors including pH, temperature, and salinity, on phenanthrene degradation by strain PHPY. The results indicated that strain PHPY could grow at 30 and 37 °C and degrade about 90–95% of 500 mg/l phenanthrene within nine days (Fig. 3). However, less growth was found at 45 °C, and growth was inhibited at 50 °C. Furthermore, strain PHPY could grow in sterile seawater at pH 6, 7.4, 8, and 9 (Fig. 4). Phenanthrene degradation efficacy of this strain incubated at pHs 7.4–9.0 was more than 95% of the supplemented amount (Fig. 4). No bacterial growth occurred at pH 5. Moreover, strain PHPY could grow in artificial seawater without NaCl and with NaCl at final concentrations of 22.79, 40, and 60 g/l (Fig. 5), although less bacterial growth was found at 80 g/l NaCl. Phenanthrene degradation at zero and 22.79 g/l NaCl was 20.64 and 30.44%, respectively, while less phenanthrene degradation was found at 60 and 80 g/l NaCl (Fig. 5).

Detection of the dioxygenase gene

No PCR products could be detected when genomic DNA of strain PHPY and primers specific for the *bphA1f* gene of *N. aromaticivorans* strain F199 were used (Fig. 6). However, when total DNA from F199 was used, a PCR product of the expected size (424 bp) was obtained. Results from the Southern hybridization experiment with the *bphA1f* probe confirmed that strain PHPY possesses a different dioxygenase gene involved in phenanthrene degradation (data not shown).

DISCUSSION

In this study, two marine bacteria capable of degrading phenanthrene were isolated from different oil-contaminated areas in Thailand. These isolated strains, PHPY and SK, are proposed to be new members of *Sphingomonadaceae* and *Rhodobacteraceae*, respectively. Several reports have shown the important role of bacteria in *Sphingomonadaceae* for PAH

Table 1 Phenanthrene-degrading bacteria and their biodegradation activity.

Bacteria	Biodegradation Efficiency			Substrate specificity*	Reference
	Initial phenanthrene concentration (mg/l)	Phenanthrene degradation (%)	Phenanthrene degradation time (days)		
Strain PHPY	500	95	9	anthracene	This study
Strain SK	500	99	10	fluorene	This study
Pseudomonas sp. ARP26	500	93	7	–	Ref. 19
Pseudomonas sp. ARP28	500	98	7	–	
Sphingomonas paucimobilis ZX4	1000	98.7	14	naphthalene, fluorene	Ref. 24
Arthrobacter sp. Sphe3	400	90	4	anthracene	Ref. 25
Sphingomonas sp. GY2B	100	99.1	2	naphthalene	Ref. 26
Sphingomonas sp. ZP1	250	100	8	naphthalene	Ref. 20
Microbacterium sp. F10a	50	98	7	pyrene	Ref. 21
Janibacter anophelis JY11	500	98.5	5	naphthalene, anthracene, pyrene	Ref. 27
Pseudomonas stutzeri ZP2	250	96	6	naphthalene	Ref. 28
Ochrobactrum sp. PWTJD	1000	99	7	–	Ref. 29
Brevibacillus sp. PDM-3	250	93.9	6	anthracene, fluorene	Ref. 30
Bordetella petrii B1	100	80	7	–	Ref. 22
Pseudoxanthomonas mexicana L2	100	99	9	–	
Streptomyces rochei PAH-13	100	80	15	fluorene, anthracene, pyrene	Ref. 23

* – means not studied.

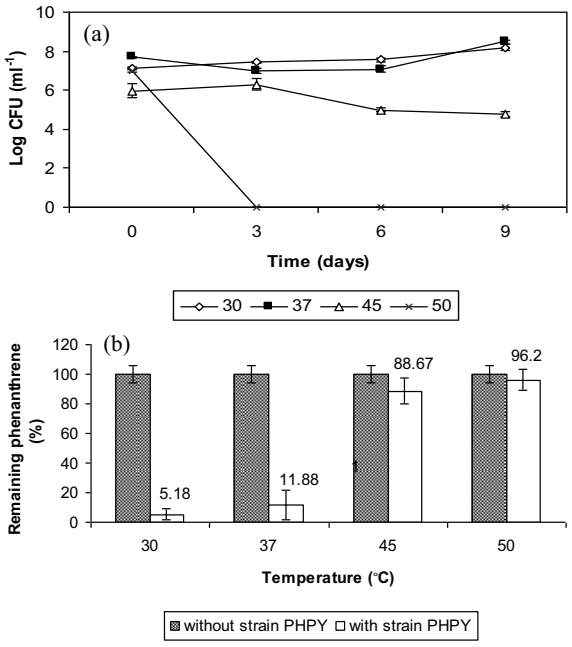

Fig. 3 Effect of temperature on (a) growth and (b) phenanthrene degradation of strain PHPY. Error bars represent standard deviations.

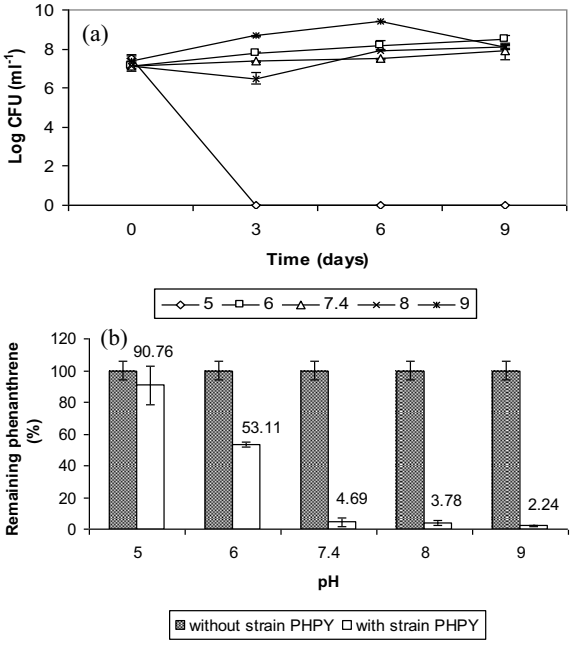

Fig. 4 Effect of pH on (a) growth and (b) phenanthrene degradation of strain PHPY. Error bars represent standard deviations.

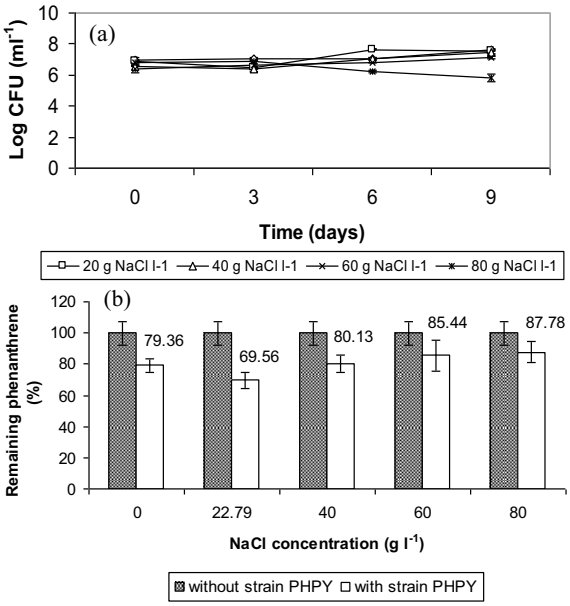

Fig. 5 Effect of salinity on (a) growth and (b) phenanthrene degradation of strain PHPY. Error bars represent standard deviations.

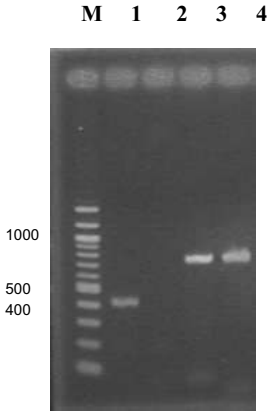

Fig. 6 PCR products based on primers specific for the *bphA1f* gene (1, 2); primers specific for 16S rDNA (3, 4); *Novosphingobium aromaticivorans* F199 (1, 3); PHPY (2, 4), 100-bp DNA ladder (M).

degradation in soils[16,31,32], mangrove sediments[33], and deep subsurface sediments[34]. This study, in accordance with previous reports[35], also found that sphingomonads with PAH degradation ability can be isolated from seawater, indicating that sphingomonads are found in a wide variety of environments and might play a key role in the degradation of PAHs in various environments, including the marine environment.

We further investigated the effect of variations in physical factors (pH, temperature, and salinity) on growth and phenanthrene degradation ability of strain PHPY and found that this strain can grow and degrade phenanthrene at temperatures up to 37 °C and at a wide range of pHs (6–9) and salinity (0–80 g/l NaCl). The results from this study indicate that growth of strain PHPY was inhibited when the bacteria were cultivated at 45 and 50 °C and when the pH of seawater was adjusted to 5. The phenanthrene degradation ability of strain PHPY in the salinity experiments was low compared to that of other physical factors used in this study. This could be due to the use of ONR7a medium instead of seawater, which might have affected the degradation ability of the bacterium because some nutrients or elements present in seawater that enhance phenanthrene degradation may have been absent from ONR7a medium.

Among PAH-degrading sphingomonads, *N. aromaticivorans* strain F199 has been extensively studied in terms of the catabolic genes involved in hydrocarbon degradation[34]. These catabolic genes seem to be unique in phenanthrene-degrading sphingomonads[36,37]. The detection of a dioxygenase gene in strain PHPY using PCR with a primer specific for the *bphA1f* gene encoding the large subunit of the terminal dioxygenase of aromatic-ring-hydroxylating dioxygenase from strain F199 revealed that strain PHPY might possess catabolic genes that are not highly homologous to the genes in strain F199. These might be one of the reasons that why strain PHPY had substrate specificity towards PAHs different from those of strain F199 and other sphingomonads. The capability to degrade a variety of PAHs by several sphingomonads including strain F199 has been reported[37]. However, some of sphingomonads such as *Sphingomonas* sp. A4 showed very low substrate specificity towards PAHs[38]. Furthermore, although most of phenanthrene-degrading bacteria could degrade naphthalene, there was also some phenanthrene-degrading bacteria using different lower biodegradation pathway cannot use naphthalene as the sole carbon source such as *Alcaligenese faecalis* AFK2 and *Nocardioides* sp. KP7[39]. The difference in biodegradation activity of these strains depends on the difference in their catabolic genes.

For strain SK, this is the first report showing that a member of *Rhodobacteraceae* has the ability to degrade PAHs. Previously, *Roseobacter*-related bacteria have been implicated in the degradation of *n*-hexadecane[40], crude oil[41], and *n*-alkane[11]. McKew and colleagues[11] suggested the possible inhibition of *Roseobacter* by some compounds within crude oil, such as PAHs, since *Roseobacter* clones could be

found in 16–23% of the total library from decane, hexadecane, and alkane microcosms, but only 3% of their clones were found in crude oil microcosms and none were found in PAH microcosms. However, the findings in our study indicate that a member of *Rhodobacteraceae* also plays a role in PAH degradation.

In conclusion, isolation of two marine bacteria capable of degrading phenanthrene has expanded our knowledge about marine bacteria with the ability to degrade PAHs.

Acknowledgements: This study was supported by a research grant from Chulalongkorn University to the Bioremediation Research Unit, Department of Microbiology, Faculty of Science and the Thai Government Stimulus Package 2 (TKK2555), under the Project for Establishment of Comprehensive Centre for Innovative food, Health Products and Agriculture. We are grateful to Dr Margaret F. Romine (Pacific Northwest National Laboratory, Richland, WA, USA) for providing *Novosphingobium aromaticivorans* strain F199. We also thank Ms. Thitima Kasemsuk for technical assistance.

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