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Degradation of phenanthrene on plant leaves by phyllosphere bacteria

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The activity of phyllosphere bacteria in the degradation of phenanthrene was investigated as a mechanism for the removal of atmospheric phenanthrene after its deposition on plant leaves. Initially, leaf samples of six plant species were collected from two roadsides in Bangkok to determine the presence of phenanthrene-degrading bacteria. The numbers of phenanthrene-degrading phyllosphere bacteria were varied and ranged from 3.5×10^4 to 1.95×10^7 CFU/g, in which the highest number was found from *Ixora* sp. Further studies were carried out in the laboratory by spraying phenanthrene on *Ixora* sp. leaves and then monitoring the amount of deposited phenanthrene and number of phenanthrene-degrading bacteria after incubation. The results showed that the amount of phenanthrene was significantly reduced on leaves containing phenanthrene-degrading bacteria. These were detected along with a rapid increase in the number of bacteria on leaves. The results indicated that many phyllosphere bacteria could utilize phenanthrene to support their growth and thereby reduce the amount of deposited phenanthrene on leaf surfaces. Several phenanthrene-degrading bacteria were later isolated from the leaves and identified with a high 16S rDNA sequence similarity to the genera *Pseudomonas*, *Microbacterium*, *Rhizobium*, and *Deinococcus*.

Key Words——air pollutants; biodegradation; phenanthrene; phyllosphere bacteria

Introduction

In the last decade, Thailand has seen a massive increase in development and in industrialization. Along with this tremendous growth came an accumulation of urban air pollutants such as polycyclic aromatic hydrocarbons (PAHs), some of which compounds are probable human carcinogens (Norramit et al., 2005). Recent reports showed that Bangkok traffic police officers and street vendors, who spend most of the day outdoor, were exposed to a high amount of genotoxic PAHs in ambient air (Ruchirawat et al., 2002, 2005). High amounts of PAHs in the urban atmosphere were also reported from Birmingham, UK (Harrison et al., 1996), Hong Kong (Lee et al., 2001), and Munich, Germany (Schauer et al., 2003). In addition, these pollutants were found to deposit on the surface of building stones in urban areas (Ortega-Calvo and Saiz-Jimenez, 1997). It is thus important to remove these pollutants from the atmosphere as well as to reduce their accumulation on ground surfaces.

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The interest in using phyllosphere bacteria to degrade the deposited atmospheric PAHs was motivated by the assumptions that plant leaves are always exposed to polluted air and some of their phyllosphere bacteria may be able to utilize PAHs as a carbon source. In fact, high amounts of PAHs were reported on plants growing along roadsides, for example the amounts of total PAHs in orange jasmine leaves, *Murraya paniculata* (L.) Jack, collected from Bangkok roadsides ranged from 63.99 to 82.46 mg/kg leaves (Karnchanasest and Satavibul, 2005). The accumulation of PAHs on plant leaves was found to correlate with the PAH atmospheric concentrations (Bohme et al., 1999; Karnchanasest and Satavibul, 2005; Librando et al., 2002). PAHs are deposited on leaves as gaseous or particle-bound compounds and they may reach the plant surface by both dry and wet deposition (Bakker et al., 2001). The major factor that affects the concentration of PAHs in plant tissue is plant species, which is possibly due to plant-specific morphological and chemical constitutions (Bakker et al., 2001; Bohme et al., 1999; Kipopoulou et al., 1999; Librando et al., 2002).

Microbial communities on plant leaves are diverse, of which bacteria are the major populations with the numbers averaging up to 10^7 cells/cm^2 of leaf surface (Lindow and Leveau, 2002). Phyllosphere microorganisms are able to decompose diverse plant polymers and have been reported to degrade pollutants; for example, microorganisms colonizing spruce needles are responsible for the decrease of trichloroacetic acid in air samples (Forczek et al., 2004). Moreover, recent reports showed that natural phyllosphere bacteria are able to degrade monoaromatic hydrocarbons such as toluene, phenol, ethylbenzene, and xylene (Darlington et al., 2001; De Kempeneer et al., 2004; Sandhu et al., 2007). In Thailand, Charoenchang et al. (2003) found a potent consortium of phenanthrene-, fluoranthene- and pyrene-degrading microorganisms on dried rain tree leaves. Meanwhile, PAH-degrading activities of phyllosphere bacteria on fresh leaves have never been studied.

The objectives of this study were to investigate the presence of phenanthrene-degrading bacteria on various ornamental plants and then to determine whether these phyllosphere bacteria could degrade the deposited phenanthrene on leaf surfaces. Phenanthrene was used in the study as a model PAH because it is one of markers for vehicle exhausts in urban air (Harrison et al., 1996). In addition, phenanthrene-degrading bacteria have been used as indicators for the degradation of deposited atmospheric PAHs in a polluted urban environment (Ortega-Calvo and Saiz-Jimenez, 1997). The study also investigated the effects of bioaugmentation by inoculating *Sphingomonas* sp. P2, a PAH-degrading bacterial isolate (Supaka et al., 2001), onto plant leaves. Finally, the study confirmed the activity and identity of phenanthrene-degrading phyllosphere bacteria by characterizing several bacteria isolated from plant leaves. The degradation of deposited phenanthrene may be a potential approach to reduce the accumulation of this pollutant in the atmosphere and thereby improve air quality.

Materials and Methods

Leaf samples. Six ornamental plants, including *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp. and *Hymenocallis littoralis* Salisb., were screened for the presence of phenanthrene-degrading bacteria on their leaves. These plant species were selected as they are commonly grown along the streets of Bangkok, Thailand. The study was carried out during summer 2005. Leaf samples were collected from several mature plants of the same species on two roadsides, namely Paholyothin and Phayathai Roads, in the urban Bangkok area.

Leaf characterization. The collected leaves were characterized by area, wet weight, moisture and wax content, and number of phenanthrene-degrading bacteria. Moisture content was determined by weighing 4 g of a leaf sample in a tarred crucible and drying the sample for 8 h at 105°C in an oven. Samples were cooled in a desiccator and weighed. The percentage of moisture was calculated as follows: % moisture= (weight of sample−weight of dry sample)/weight of sample×100.

Wax content was determined by extracting a 4 g leaf sample with 20 ml hexane in a microwave extractor (Milestone ETHOS SEL). The extract was filtered through the GF/C filter into a pre-weighed drying round-bottom flask before drying by rotary evaporator. The round-bottom flask was reweighed, and the percentage of wax was calculated as follows: % wax = (reweighed flask−preweighed flask)/weight of sample×100. In this study, wax is defined as all those dis-

solved in hexane.

The number of phenanthrene-degrading bacteria was determined by the spread plate technique. To extract phyllosphere bacteria from leaves, 4 g of freshly picked leaves was immersed in 40 ml potassium phosphate buffer (pH 7.0) and shaken for 30 min. The extracted solution was subjected to tenfold serial dilutions and 100 µl of each dilution was spread on mineral salts (MS) agar (Focht, 1994). Phenanthrene was supplied as vapor by adding phenanthrene crystals to the lid of a Petri dish. The plates were incubated at room temperature for 2 wks.

Phenanthrene biodegradation experiment. Phenanthrene-degrading activities of the phyllosphere bacteria were determined by comparing the amounts of remaining phenanthrene on treated plant leaves. The experiments consisted of three treatments: non-augmentation, bioaugmentation and control (without phyllosphere bacteria). Each experiment was done in triplicate. *Ixora* sp. was selected as a model plant in this experiment. All plants were purchased in one batch from a florist in Bangkok. They were around 2 months old and contained less than 2 ppm of initial phenanthrene on their leaves. This study further amended the high amount of phenanthrene on the leaves to facilitate its monitoring after treatment.

At the beginning, only leaves removed from *Ixora* sp. were used to avoid the effects of plant metabolisms on phenanthrene removal. The experiment was carried out in 500 ml beakers containing 16 g of leaves, in which each leaf was randomly picked from the plant and cut into small pieces. The surface of control leaves were partial sterilized by spraying with 8 ml 70% ethanol. The amount of liquid was enough to cover all those leaves. In non-augmented treatment, the leaves were sprayed with an equal volume of deionized water. This treatment was used to determine the activities of indigenous phyllosphere bacteria. Bioaugmentation was performed by spraying the leaves with an equal volume of 10^7 CFU/ml *Sphingomonas* sp. P2 inoculum prepared according to Supaka et al. (2001). All the treated leaves were left to dry overnight. On the following day, 3 ml of 10 mg/ml phenanthrene dissolved in 20% dichloromethane in hexane was sprayed on the leaves and the deposition of phenanthrene on leaf surface was allowed by covering the beaker with foil paper for 24 h. After that, the foil paper was removed and leaves were randomly sampled to analyze for the amount of deposited phenanthrene. The degradation of phenanthrene was determined from the difference between the initial amount of phenanthrene (100% baseline) and the remaining phenanthrene afterward. The number of phenanthrene-degrading bacteria on the leaves was also monitored.

The whole plants of *Ixora* sp. were later examined to confirm the phenanthrene-degrading activities of the phyllosphere bacteria. Each plant was approximately 45 cm tall and had a leaf area of 50 cm^2. They were grown in pots, in which the potting soil was covered with aluminum foil before use. Non-augmentation, bioaugmentation and control treatment were performed by spraying 70% ethanol, water, or bacterial inoculum on the plant foliage until runoff (i.e. 30 ml of each solution was applied). After the leaves were dried, 10 ml of 10 mg/ml phenanthrene was sprayed on the foliage and the plants were enclosed in plastic bags for 24 h to allow the deposition of phenanthrene on their leaves. After removed of the bags, leaves were sampled randomly from each treatment to determine the amount of deposited phenanthrene and the number of phenanthrene-degrading bacteria. The percent of phenanthrene degradation was determined as in the previous experiment.

Phenanthrene extraction and analysis. Leaves were initially extracted by the microwave extraction technique and hexane similar to the method used for determination of wax content. Then, phenanthrene was isolated from the extracted sample by silica gel columns according to Karnchanasest and Satayavibul (2005). GC analysis of the concentrated sample was then performed with a Hewlett-Packard 6890 equipped with a FID detector and a HP-5 fused-silica capillary column. Concentration of phenanthrene was determined by external standardization. The amount of phenanthrene recovered by this extraction technique was approximately 85% and the values were consistent throughout the experiment.

Phenanthrene-degrading bacteria characterization. The four most abundant bacteria, based on their distinct colony morphology, were isolated from leaves of *Ixora* sp. purchased from a florist. The bacteria were cultivated in liquid MS medium supplemented with 100 ppm phenanthrene and shaken at 200 rpm. The activity of these bacteria was later measured by incubating bacterial cells ($OD_{600}=0.1$) with 100 ppm phenanthrene in MS medium. Phenanthrene degradation was monitored by extracting phenanthrene from the liquid

medium. The total amount of phenathrene remaining was determined by GC analysis.

To identify the bacteria, 16S rDNA sequence analyses were performed by amplifying the 16S rRNA genes using the eubacterial primer set 27f and 1492r (Akkermans et al., 1996). The amplified PCR products of some isolates were used directly for DNA sequencing and those of some isolates were cloned into pGEM-T easy vector (Promega) before sequencing. Partial 16S rDNA sequences of each isolate were analyzed with DNASIS-Mac software (version 2.05; Hitachi Software Engineering Co., Ltd., Yokohama, Japan). A homology search was done over GenBank databases by using a BLAST program. The 16S rRNA sequences for the PAH-degrading isolates have been deposited in GenBank under accession numbers EF193386, EF193387, EF193388, and EF193389 for the isolates KLY, Y1, W2, and P3, respectively.

Results and Discussion

Presence of phenanthrene-degrading bacteria on leaves

Six ornamental plants including *Wrightia religiosa* Benth. ex Kurz, *Pereskia grandiflora* Haw., *Hibiscus rosa-sinensis* L., *Excoecaria cochinchinensis* Lour. var. *cochinchinensis*, *Ixora* sp., and *Hymenocallis littoralis* Salisb. were collected from two roadsides in Bangkok. When compared between species, these plants had different leaf characteristics in terms of arrangement, texture, area, weight, wax content, and number of phenanthrene-degrading bacteria (Table 1). Meanwhile, the moisture content in all species was not much different (70–90%) and the amount of phenanthrene from every collected leaf was under the detection limit (1.83 ppm).

The number of phenanthrene-degrading bacteria on the collected leaves ranged from 4.4×10^4 to 1.2×10^7 CFU/g (Table 1). The results suggested that ambient levels of phenanthrene as well as other PAHs were probably sufficient to maintain phenanthrene-degradation traits in the phyllosphere. When comparing between plant species, *Ixora* sp. had the highest concentration of phyllosphere bacteria. To date, the relationship between plant species, phyllosphere bacteria and organic air pollutants is not known. Many reports show that plant tissues with high lipid content accumulate significantly more PAHs (Bakker et al., 2001; Simonich and Hites, 1994). The high wax content (0.7%) of *Ixora* sp. may be one of factors that promote the colonization of phenanthrene-degrading bacteria. At the same time, various chemical and structural features of leaves can affect the colonization of phyllosphere bacteria (Yadav et al., 2005). More studies are therefore required to elucidate the plant-bacterial-pollutant interactions.

Table 1. Characteristics of plant leaves used in the study.[a]

Plant species	Common name	Leaf arrangement	Leaf texture	Leaf area (cm^2/leaf)	Leaf weight (g/leaf)	Moisture content (%)	Wax content (%)	Number of phenanthrene-degrading bacteria (CFU/g)
Ixora sp.	Ixora	Opposite	Medium, glossy	43	0.8	71	0.72	1.19×10^7
Wrightia religiosa Benth. ex Kurz	Water jasmine	Opposite	Light, smooth	14	0.2	74	0.92	1.01×10^6
Hibiscus rosa-sinensis L.	Hibiscus	Alternate	Heavy, glossy	37	0.8	78	0.43	6.20×10^6
Pereskia grandiflora Haw.	Rose cactus	Whorl	Rough, hairy	42	1.0	90	0.32	1.19×10^6
Hymenocallis littoralis Salisb.	Spider lily	Opposite	Heavy, smooth	160	8.0	91	0.27	1.14×10^5
Excoecaria cochinchinensis	Chinese croton	Whorl	Rough, hairy	26	0.4	85	0.42	4.40×10^4

[a] Each value was averaged from leaves collected at two roadsides.

Activity of phenanthrene-degrading bacteria on leaves

Since *Ixora* sp. contained a high number of phenanthrene-degrading bacteria, the species was selected as a model plant in this study. *Ixora* sp. is an evergreen ornamental plant with largish pointed leaves and clusters of flowers at the end of the branches in various colors. It is mostly planted as hedge and can grow in a wide range of climates. This experiment was conducted to confirm that phyllosphere bacteria on *Ixora* sp. had the potential to degrade phenanthrene after its deposition on the leaves. The differences in phenanthrene degradation when using the whole plants or only the leaves were also examined. This was necessary, since it was believed that the amount of phenanthrene may be decreased by plant activities such as translocation and metabolisms.

The first experiment studied the leaves removed from *Ixora* sp. to exclude other plant activities. Phenanthrene concentration remained almost constant in control treatment throughout the study (Fig. 1). On the other hand, the amount of phenanthrene decreased rapidly in bioaugmentation treatment and there was only 23% of remaining phenanthrene on the leaves after 48 h. In the non-augmented leaves, phenanthrene was gradually decreased to 62% at the end of study. The results indicated that the decrease of deposited phenanthrene was mainly due to bacterial activities, in which the inoculated *Sphingomonas* sp. P2 degraded phenanthrene more rapidly than the indigenous bacteria. In this experiment, the number of phenanthrene-degrading bacteria in augmented leaves was significantly increased from 1.1×10^8 to 4.2×10^8 CFU/g after 48 h, which was higher than for the non-augmented and control leaves at all time points (Fig. 1). Bacteria colonization in the non-augmented leaves was increased slightly and there was 1.1×10^8 CFU/g at the end of study. The results indicated that deposited phenanthrene was used by the augmented as well as indigenous phyllosphere bacteria to support their growth.

In the second experiment, phenanthrene on leaves of the augmented plants was rapidly decreased to 23% of the initial concentration after the first 48 h and then remained almost constant afterward (Fig. 1). In non-augmented plants, there was high amount of phenanthrene after 24 h but only 15% of the initial phenanthrene remained on the leaves after 72 h. The results confirmed that indigenous phyllosphere bacteria were able to degrade phenanthrene but at slower

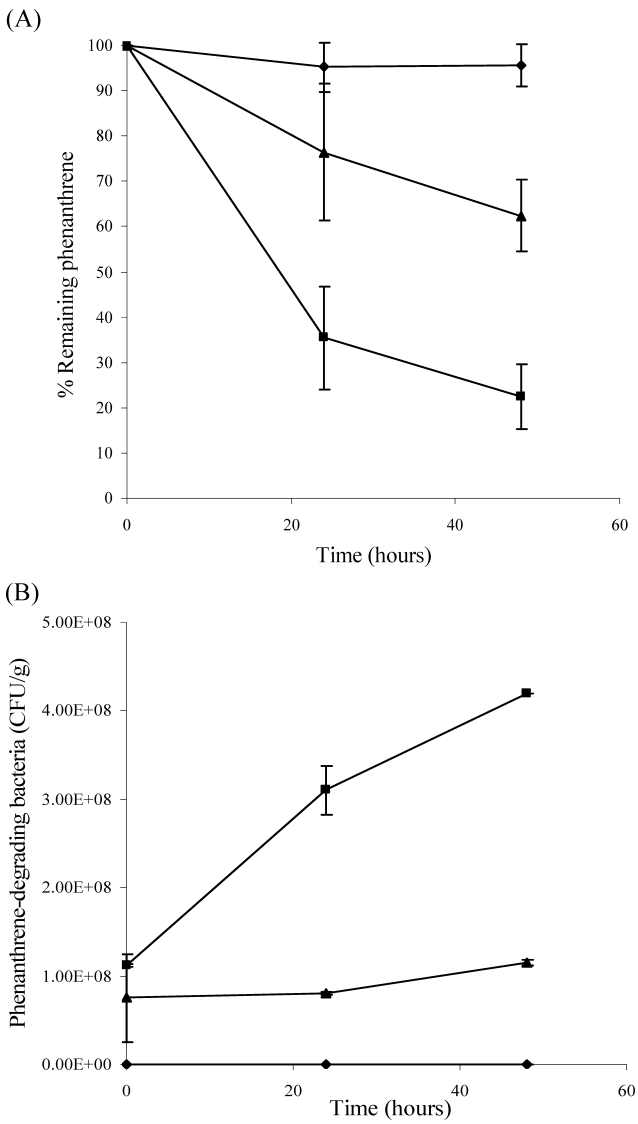

Fig. 1. Relationship between % remaining phenanthrene and number of phenanthrene-degrading bacteria on *Ixora* sp. leaves that were removed from the plants before biodegradation treatments.

There were three treatments including augmentation with *Sphingomonas* sp. P2 (■), non-augmentation (▲), and control without phyllosphere bacteria (◆).

rate than the augmented bacteria. This is similar to a study by De Kempeneer et al. (2004) who found that uninoculated *Azalea indica* can reduce the amount of airborne toluene but the addition of toluene-degrading bacteria significantly enhances toluene removal rate. The removal of phenanthrene was slowest in the control treatment; however, the amount of remaining phenanthrene was similar to that for other treatment at the end of the study. When compared with the first experiment on removed leaves, the results indicated that

plant activities were responsible for the decrease of deposited phenanthrene but at a slower rate than phyllosphere bacterial activities.

Similar to the first experiment, the number of phenanthrene-degrading bacteria in augmented plants was increased gradually and significantly higher than for the non-augmented and control plants at all time points (Fig. 2). A slight decrease in number of phenanthrene-degrading bacteria on the augmented plant was found after 48 h. In contrast, there was a continuous increase in bacteria colonization in the non-augmented species and the number of phenanthrene-degrading bacteria was doubled within 48 h. These results confirmed the ability of indigenous phyllosphere bacteria to utilize phenanthrene as a carbon source. Therefore, the addition of phenanthrene-degrading bacteria may not be necessary for the removal of deposited phenanthrene, if plant species containing high number of phenanthrene-degrading phyllosphere bacteria are grown at polluted sites. Sandhu et al. (2007) also suggested that natural phyllosphere bacteria have a potential role in natural attenuation of volatile organic compounds (VOCs). They defined the technology as phylloremediation, which may be applied for both indoor and outdoor airborne pollutants.

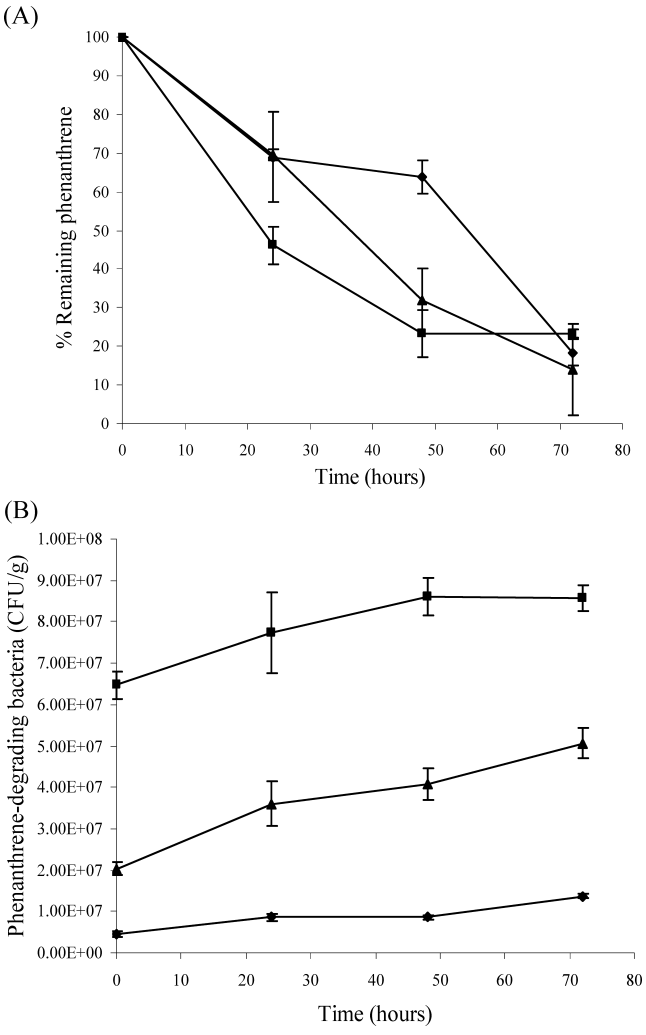

Fig. 2. Relationship between % remaining phenanthrene and number of phenanthrene-degrading bacteria on *Ixora* sp. leaves when using the whole plants for biodegradation treatments.

There were three treatments including augmentation with *Sphingomonas* sp. P2 (■), non-augmentation (▲), and control without phyllosphere bacteria (◆).

Characterization of phenanthrene-degrading phyllosphere bacteria

Four bacteria designated KLY, Y1, W2, and P3 were isolated from *Ixora* sp. leaves and investigated for their phenanthrene-degrading activity. These bacteria represented the most abundant phenanthrene-degrading populations found on *Ixora* sp. leaves during the biodegradation experiment. After a 4-day incubation, less than 2% of 100 ppm phenanthrene remained in the medium containing each isolate (Table 2). The activities of these isolates were comparable to that of *Sphingomonas* sp. P2, which rapidly degraded 100 ppm phenanthrene to undetectable amounts within 72 h (Supaka et al., 2001). The bacteria were further identified by partial sequencing of their 16S rRNA gene. Isolates KLY, Y1, W2, and P3 were identified with a high 16S rDNA sequence similarity to the genera *Pseudomonas*, *Microbacterium*, *Rhizobium*, and *Deinococcus* respectively (Table 2). These genera have also been isolated from other plants but none of them have shown the ability to degrade phenanthrene or other PAHs.

Various species of *Pseudomonas* are thought to play an important role in PAH biodegradation (Ma et al., 2006). On the other hand, there were a few studies showing PAH-degrading activities of bacteria from genus *Microbacterium* (Daane et al., 2001; Gauthier et al., 2003) and *Rhizobium* (Andreoni et al., 2004; Bodour et al., 2003). Genus *Deinococcus* has not been reported to have PAH-degrading activity. Only one study revealed recently the detection of *Deinococcus* during PAH biodegradation experiment by fluorescence in-situ hybridization (FISH) technique (Chang et al., 2006). The restriction of isolated bacterial genera involving in phenanthrene degradation might be due to the variations of methods and sources of samples

Table 2. Phenanthrene-degrading activity of the bacterial isolates from *Ixora* sp. and sequence similarity between these isolates and their closest relatives.

Isolates	% Remaining phenanthrene[a]	Closest strains	Sequence similarity[b]	Accession no. of the closest strains	Source of the closest strains
KLY	1.9%	*Pseudomonas oleovorans*	99% (428 bp)	DQ122200	Potatoes
		Pseudomonas oleovorans	99% (428 bp)	AY623816	Soil
Y1	1%	*Microbacterium foliorum*	99% (408 bp)	AJ249780	Grass
		Microbacterium sp.	99% (408 bp)	DQ401245	Sweet peppers
W2	0.4%	*Rhizobium gallicum*	99% (425 bp)	AY166844	Legumes
		Rhizobium etli	99% (425 bp)	DQ196417	Legumes
		Rhizobium sp.	99% (425 bp)	AY904776	Legumes
P3	0.1%	*Deinococcus* sp.	99% (1,290 bp)	DQ003317	Soil
		Deinococcus grandis	98% (1,290 bp)	Y11329	Soil

[a] Phenanthrene-degrading activity was determined from the % remaining phenanthrene in liquid medium after incubating the isolates with 100 ppm phenanthrene for 4 days.

[b] Length of nucleotide sequences used for comparison is in parenthesis.

used for bacterial isolation. Since these isolates may be useful for bioaugmentation of PAH contaminated areas, further studies should be carried out to determine their abilities to degrade other PAHs.

The results indicated that several phyllosphere bacteria were able to utilize phenanthrene as a sole carbon source and thereby reduce the amount of deposited phenanthrene. In addition, plant leaves may be used as sources for isolating novel PAH-degrading bacteria. As plant leaves are sinks of outdoor air pollutants, this bacterial mechanism could lower the amount of atmospheric phenanthrene by preventing its recirculation into the air. Such a procedure may be carried out by planting *Ixora* sp. or other plants with a high number of phenanthrene-degrading bacteria in polluted areas. Nevertheless, further investigations are required to demonstrate the effectiveness of these phyllosphere bacteria in degrading other airborne PAHs as well as VOCs. More plant species should also examine to elucidate the relationship between phyllosphere bacteria, their host plants, and various air pollutants. The knowledge will help us to develop a sustainable technology for contaminated air clean-up.

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