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Monitoring survival of phenanthrene-utilizing *Sphingobium* sp. P2 in soil microcosms using green fluorescent protein as a marker

Nattachai Kengpipat[a], **Kenichi Iwata**[b], **Toshio Omori**[c], **Onruthai Pinyakong**[a,d,*]

[a] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Biotechnology Research Center, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[c] Open Research Center, Shibaura Institute of Technology, Tokyo 108-8548, Japan
[d] National Centre of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), Chulalongkorn University, Bangkok 10330, Thailand

*Corresponding author, e-mail: onruthai@gmail.com

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ABSTRACT: Phenanthrene is a polycyclic aromatic hydrocarbon which belongs to a class of toxic environmental pollutants that has been accumulating due to a variety of anthropogenic activities. We attempted to use *Sphingobium* sp. P2, which is capable of utilizing phenanthrene as its sole source of carbon and energy, in bioaugmentation treatments. For successful bioremediation, monitoring the survival and metabolic activity of bioaugmented bacteria are required. This study aimed to use green fluorescent protein as a marker to estimate the survival of *Sphingobium* sp. P2 in bioaugmented soil microcosms. The *gfp* was integrated via Tn*5* transposition into the chromosome of strain P2. The *gfp*-inserted strains were identified by green fluorescence emission under UV light. The *gfp* was stably maintained in strain P2, and the strain still retained the ability to use phenanthrene as a sole carbon and energy source. Soil microcosm experiments revealed that survival of the strain P2-*gfp* and its phenanthrene-degrading capacity may depend on indigenous microorganisms and nutrients in the soils. The strain P2-*gfp* can be used to evaluate the success of bioaugmentation.

KEYWORDS: biodegradation, bioremediation, bioaugmentation, polycyclic aromatic hydrocarbons

INTRODUCTION

Sixteen polycyclic aromatic hydrocarbons (PAHs), including phenanthrene, have been classified as priority pollutants by the U.S. Environmental Protection Agency because of their toxic, mutagenic, and carcinogenic properties[1]. The contamination of soil with PAHs is of primary concern due to the possible effects of these toxic substances on human health[2].

Bioaugmentation is the addition of microbes to contaminated materials and sites in order to remove contaminated compounds from the sites. This technology is useful for cleaning up contaminated sites if the effective pollutant-degrading microorganisms are not naturally present at the sites[3]. However, under natural conditions, bioaugmented microbes must compete with indigenous microorganisms and are negatively impacted by environmental factors such as pH, nutrient availability, and temperature[4]. Therefore, for successful bioaugmentation to occur, assessment of the surviving number of microorganisms that are able to retain a high enough metabolic activity to degrade the pollutant at the particular contaminated site is required.

Sphingobium sp. strain P2 was previously isolated for its ability to degrade phenanthrene[5]. The biochemical and genetic pathways for phenanthrene catabolism in this strain have been studied[6,7]. In order to employ this bacterium as an inoculum for bioremediation at pollutant sites, its survival, persistence, and pollutant-degrading activity in the soil ecosystem need to be assessed.

One promising tool for this purpose is the use of marker genes for discriminating a specific microorganism of interest from indigenous soil microorganisms. For example, the *gfp* gene, encoding the green fluorescent protein (GFP) from the jellyfish, has been used as a marker to estimate the survival of bacteria in the environment[8]. This marker can be used to monitor microorganisms in soil because its expression does not depend on exogenous substrates. Since GFP was originally isolated from a marine eukaryote, it should be absent in naturally occurring soil microorganisms. Moreover, the detection of GFP requires

only irradiation with near UV or blue light. GFP has been used as a marker to detect certain pollutant-degrading bacteria during bioaugmentation treatments of soil and activated sludge[9–13].

The objective of this study was to use green fluorescent protein as a marker to estimate the survival of labelled *Sphingobium* sp. P2 in phenanthrene-contaminated soil microcosms.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and inoculant preparation

Sphingobium sp. strain P2 was grown on carbon-free mineral medium (CFMM) supplemented with 100 ppm phenanthrene or on Luria Bertani (LB) medium at 30 °C, as described previously[6]. When necessary, kanamycin was added at a final concentration of 50 µg/ml. The cultures for the inoculant were cultivated in CFMM supplemented with 100 ppm phenanthrene at 30 °C with shaking for 2 days. The cultures were harvested by centrifugation, and the pellets were washed twice with CFMM and then resuspended in the same medium.

Insertion of the *gfp* gene into *Sphingobium* sp. strain P2

Transposon insertion by biparental mating was performed with donor strain *E. coli* S17-1 harbouring an mTn5*gusA-pgfp21*[14] to generate *Sphingobium* strain P2 possessing *gfp* genes. Conjugation was carried out on 0.45-µm-pore-size sterile membranes placed on 2xYT agar at 30 °C for 20 h with a 1:1 donor-to-recipient ratio. The kanamycin-resistant transconjugants, which could grow on CFMM supplemented with benzoate, were further screened for their ability to display bright green fluorescence and phenanthrene-degrading activity by spraying ethereal phenanthrene onto the agar plate.

Selection and stability of *gfp*-transformed bacteria

Stability of the *gfp* gene in the *gfp*-transformed bacterium was evaluated by subculturing on LB medium without kanamycin. The isolates were incubated by shaking at 30 °C for 24 h after each transfer. After 20 transfers onto nonselective medium, colonies were plated on the LB agar medium. Colonies expressing the *gfp* gene were detected under UV light.

Qualitative assessment of phenanthrene degradation by the transformants

Sphingomonas sp. strain P2 and the *gfp*-transformed strain were compared for their ability to degrade phenanthrene in liquid cultures. Each bacterium was grown in LB broth at 30 °C for 24 h, centrifuged at 4000*g* for 10 min, washed twice with saline buffer (0.85% NaCl), and resuspended in the same buffer to an O.D. of 0.1 at 600 nm. Then, 100 µl of the cell suspension was added to each tube of 5 ml CFMM supplemented with 100 ppm of phenanthrene. The cultures were incubated at 30 °C on a shaker (200 rpm) and were examined for growth of bacteria and remaining phenanthrene after 5 days of cultivation. Growth of bacteria was determined by viable plate counts on LB medium. The remaining phenanthrene was extracted with an equal volume of ethyl acetate and was dehydrated over anhydrous disodium sulphate. The extracts were evaporated to dryness using a rotary evaporator. The residues were dissolved in 5 ml of *n*-hexane and were analysed by gas chromatography, as described below.

Table 1 Characteristics of the soils used in this study.

Characteristics	red soil	black soil
Source	Petchaburi	Bangkok
pH	6.3	6.4
Electric conductivity (dS/m)	0.834	0.292
Organic matter (%)	0.47	4.1
Carbon (%)	0.02	2.38
Nitrogen (%)	0.06	0.21
Phosphorus (ppm)	5	209
Potassium (ppm)	35	380
Calcium (ppm)	681	2471
Magnesium (ppm)	224	316
Texture		
Sand (%)	62	76
Silt (%)	18	11.6
Clay (%)	20	12.4

Soil and soil microcosm experiments

Soil samples were collected from Petchaburi Province (red soil) and Bangkok (black soil), Thailand. They were sieved to a particle size of 1.18 mm. The contamination of phenanthrene was analysed using the methods for analysis of phenanthrene in soil, described below. The characterization of their physical properties and chemical composition was performed at the Agriculture Chemistry Division, Department of Agriculture, Ministry of Agriculture. These soils have different characteristics, as shown in Table 1.

To construct soil microcosms, 2 g aliquots of the soil samples were introduced into 22 ml glass screw cap vials. One set of soils was autoclaved 3 times at 121 °C for 30 min. Phenanthrene dissolved in dimethylsulphoxide was added to the soils to a final concentration of 300 ppm. The treated soils were then

inoculated with 50 µl washed cells of strain P2-*gfp* at 10^7 cells/g dry soil. The soil water content was adjusted to 80% of the water holding capacity. The moisture content was maintained by monitoring the lost weight of the microcosm every week until the end of the experiment. The control set was autoclaved soil with the addition of phenanthrene and without inoculation of P2-*gfp*. Soil microcosms were incubated at 30 °C in the dark. The experiments were performed in triplicate. The cell density of strain P2-*gfp* and the concentration of remaining phenanthrene were determined periodically by extraction from individual microcosms, also described below.

Enumeration of bacteria

Bacteria were extracted from triplicate samples of soil using a saline buffer (0.85% NaCl) and agitation on a shaker (30 min). Extracts were serially diluted in the same buffer and aliquots were spread on LB medium agar plates containing 50 µg/ml of kanamycin and incubated at 30 °C for 2 days. Enumerations were performed under UV light to identify and quantify the CFU of P2-*gfp*.

Monitoring of phenanthrene depletion

Phenanthrene from microcosms (2 g of soil) was extracted with 4 ml of *n*-hexane and 1.5 ml of 15% Tritron X-100 by shaking at 200 rpm for 24 h. Extracts were kept at −20 °C for 24 h to allow soil setting. The extracts were dehydrated over anhydrous disodium sulphate. Phenanthrene concentration in the sample was quantified by gas chromatography (GC). 1 µl of sample was analysed on an Agilent GC model 6890 N equipped with a flame ionization detector (Agilent Technologies) and HP-5 (5% phenyl methyl siloxane) fused-silica capillary column having inside diameter, length and film thickness of 320 µm, 30 m, and 0.25 µm, respectively. The injector and detector temperatures were 280 °C and 250 °C, respectively. The carrier gas was helium and the makeup gas was nitrogen flowing at 60 ml/min. Split ratio was kept at 10:1. Under this condition, the retention time of standard phenanthrene is 13.27 ± 0.5 min. The amount of phenanthrene in each sample was determined by comparing to a standard curve.

RESULTS AND DISCUSSION

Insertion of the *gfp* gene into *Sphingobium* sp. P2 cells

An abundance of kanamycin-resistant transconjugant that exhibited green fluorescence under UV light was obtained. 24 transformants were picked and inoculated on a CFMM plate that was covered by spraying with phenanthrene solution. A clear zone was observed after 3 days of incubation, indicating that all transformants could degrade phenanthrene. Two colonies were chosen for further examination of the *gfp* gene stability on non-selective medium. The results showed that the *gfp* gene was stably maintained in strain P2 after culture for more than 20 generations on non-selective medium. One colony designated P2*gfp* was then further examined to determine its growth and ability to degrade phenanthrene in CFMM liquid medium supplemented with phenanthrene.

The results revealed that the strain P2*gfp* and the wild type of strain P2 could both degrade 100% of the supplemented phenanthrene within 5 days of cultivation and the cells increased from about 6.1 log CFU/ml to 8.3 log CFU/ml. The ability of strain P2*gfp* to exhibit green fluorescence under UV light and the ability to degrade phenanthrene on CFMM plates sprayed with phenanthrene solution were observed and are shown in Figs. 1a and 1b, respectively.

The survival and metabolic activity of strain P2-*gfp* in soil microcosms

The survival of P2-*gfp* cells and phenanthrene degradation ability in two different soils were assessed to evaluate the performance of this bacterium when released into soils. The red soil sample contained very low amount0s of nutrients (organic matter, nitrogen, phosphorus, potassium, and calcium) compared to the black soil sample (Table 1). These two soil samples have no background contamination of phenanthrene.

After incubation, enumeration of the bacteria in soil microcosms revealed that the number of P2-*gfp* cells was maintained around 7 log CFU/g soil in the bioaugmented non-autoclaved microcosms of both soil types and in black soil which was bioaugmented and autoclaved (Figs. 2a and 2c). In non-autoclaved black soil, P2-*gfp* cell number increased to reach 8 log CFU/g soil after 7 days of incubation (Fig. 2c). Interestingly, a decrease in cell number of P2-*gfp* in red soil, which was autoclaved and bioaugmented, was observed (Fig. 2a). Whereas indigenous bacteria in autoclaved soil were found to increase after the initial time of the experiment, our results indicate that autoclaving three times as we did in this study was not enough to sterilize all forms of bacteria (Figs. 2a and 2c).

Regarding the phenanthrene degradation ability in soil microcosms, we found that phenanthrene was degraded, achieving a remaining phenanthrene concentration of 30% of the initial amount after 14 days of incubation in non-autoclaved black soil with bioaug-

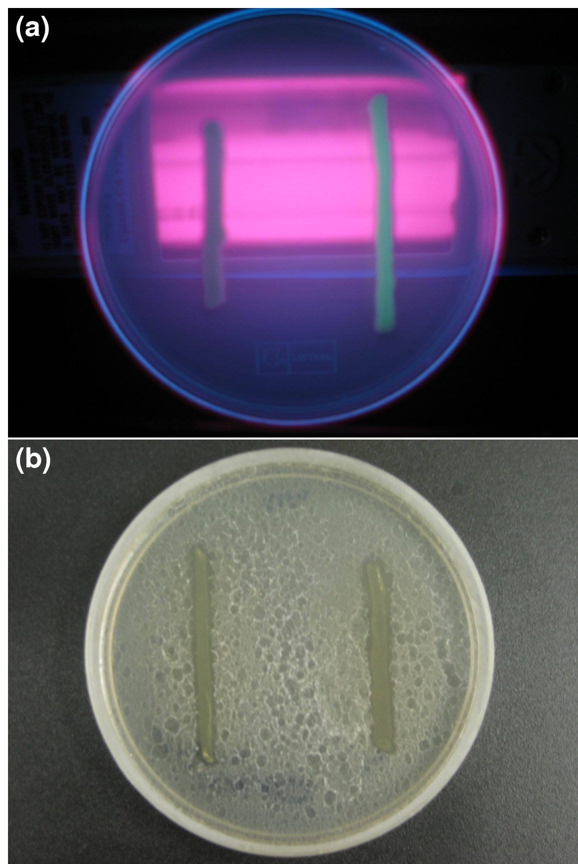

Fig. 1 (a) Activity and appearance of bright green fluorescence and (b) growth and production of a clearing zone on a CFMM plate sprayed with phenanthrene. Left streak: P2; right streak: P2-*gfp*.

mentation treatment (Fig. 2d). On the other hand, only slight degradation of phenanthrene in the non-autoclaved soil samples and in the P2*gfp*-inoculated autoclaved soil microcosms was observed. Meanwhile, there was no phenanthrene degradation in the autoclaved soil microcosms, showing that the remaining indigenous microorganisms have no capacity to degrade phenanthrene. These results indicated that the degradation of phenanthrene in this soil may depend on the function of both the P2*gfp* and some indigenous soil microflora. On the other hand, phenanthrene degradation in red soil microcosms seems to depend on only P2*gfp* activity, which was not so high in this condition (Fig. 2c). This might be due to the limitation of nutrients and catabolic activity of both P2-*gfp* and indigenous microorganisms in this soil. Previous studies have shown that bioaugmentation treatment of PAH-contaminated soil were limited in some cases for various reasons, including the inability

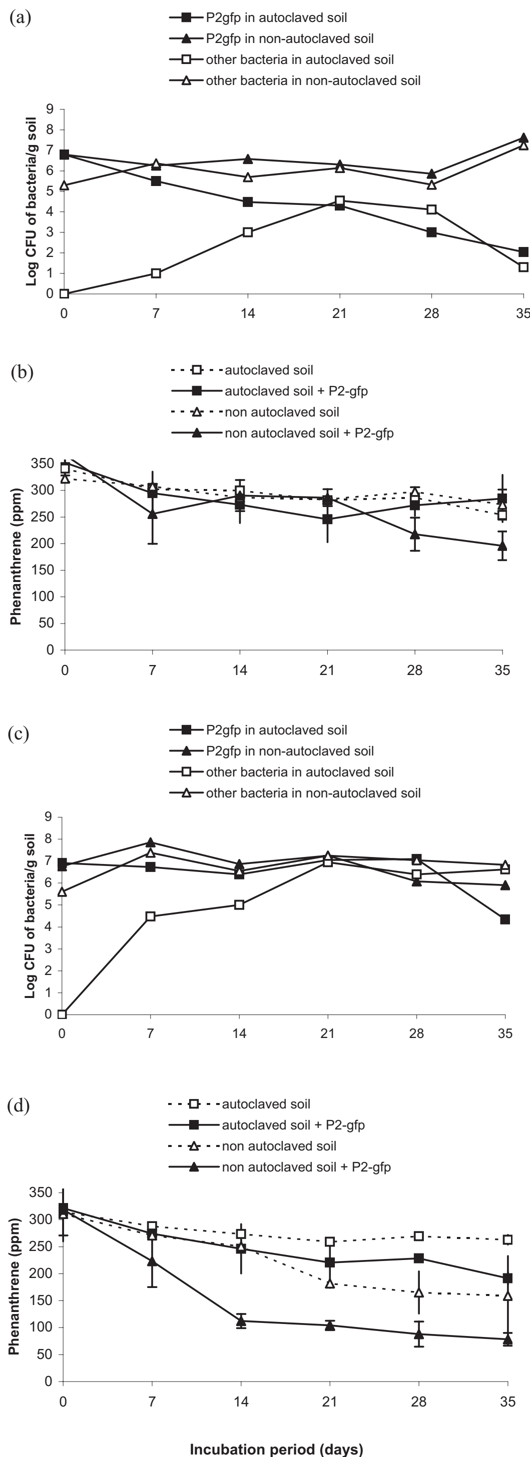

Fig. 2 (a) log CFU of bacteria in red soil microcosms, (b) remaining phenanthrene in red soil microcosms, (c) log CFU of bacteria in black soil microcosms, and (d) remaining phenanthrene in black soil microcosms.

of the inoculants to compete with the indigenous microorganisms, die-off of inoculants, and limited substrate availability[3,15].

In conclusion, phenanthrene-degrading *Sphingobium* sp. strain P2 containing the *gfp* gene was successfully constructed and was used as a bioaugmented bacteria in the preliminary experiment of soil microcosms to assess success of the treatment. The results obtained suggested that bioremediation in soil microcosms was due to several factors and not only a bioaugmented strain but the bacterial populations seem to play an important role in phenanthrene degradation. A more extensive study of the role of indigenous microorganisms in soil and on the relationship among the bacteria involved in the biodegradation is of great interest.

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