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Role of Unstable Phenanthrene-Degrading *Pseudomonas species* in Natural Attenuation of Phenanthrene-Contaminated Site

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An unstable yet efficient phenanthrene-degrading bacterium strain Ph-3 was isolated from a petroleum-contaminated site at the Mathura Oil Refinery, India. The strain was identified as *Pseudomonas* sp. using a polyphasic approach. An analysis of the intermediates and assays of the degradative enzymes from a crude extract of phenanthrene-grown cells showed a novel and previously unreported pattern of 1, 2-dihydroxy naphthalene and salicylic acid production. While strain Ph-3 lost its phenanthrene-degrading potential during successive transfers on a rich medium, it maintained this trait in oligotrophic soil conditions under the stress of the pollutant and degraded phenanthrene efficiently in soil microcosms. Although the maintenance and *in vitro* study of unstable phenotypes are difficult and such strains are often missed during isolation, purification, and screening, these bacteria constitute a substantial fraction of the microbial community at contaminated sites and play an important role in pollutant degradation during biostimulation or monitored natural attenuation.

Keywords: Phenanthrene, microcosm, bioremediation

Introduction

Phenanthrene is a member of the polycyclic aromatic hydrocarbon (PAH) group, a class of hydrophobic organic compounds with mutagenic, genotoxic, and carcinogenic properties and toxic to aquatic organisms [14, 15, 20]. Several phenanthrene-degrading bacteria have already been isolated from PAH-contaminated sites [11, 29, 33], and the metabolic pathways involved in the degradation of phenanthrene have also been documented [5, 16, 21]. In the case of most phenanthrene-degrading bacteria, phenanthrene is first converted to 1-hydroxy-2-naphthoic acid through a common upper pathway, and then further degraded through either a phthalate or salicylate pathway (Figs. 1, 5). However, phenanthrene can also be degraded through 1-naphthol, which is then further degraded through either a salicylate or phthalate pathway [21] or only through a salicylate pathway [16].

The bioremediation of pollutant-contaminated sites using a bioaugmentation approach has not received much attention due to several factors, including the variable physicochemical conditions of contaminated sites and non-compatibility of exogenous organisms(s) with native populations [3, 7, 24]. In contrast, stimulating native degraders by providing suitable physicochemical conditions (biostimulation) is a more viable approach and the current practice for the bioremediation of pollutant-contaminated sites all over the world [3, 9, 19]. Biostimulation is also superior to bioaugmentation in several aspects, including cost, labor, feasibility, and practicality. Thus, to design better biostimulation strategies for a better degradation response during biostimulation, knowledge about the physicochemical condition of the sites and factors affecting the growth of the degrading microbes is imperative.

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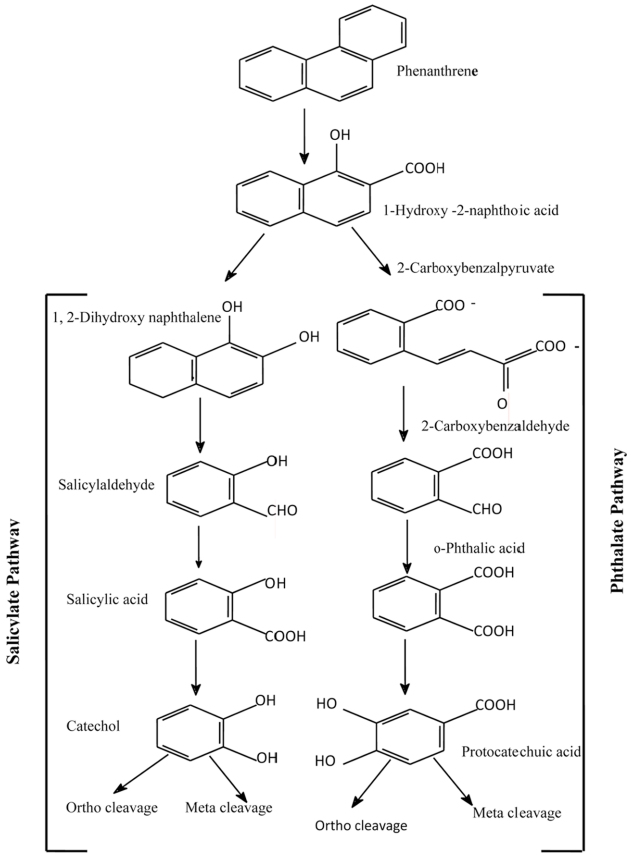

Fig. 1. Bacterial phenanthrene degradation pathway. Phenanthrene is first converted into 1-hydroxy 2-naphthoic acid by a common upper pathway, which is then degraded by either a phthalate pathway or a salicylate pathway.

Degrading microbes are generally plasmid-coated with genes for pollutant degradation, antibiotic resistance, and heavy metal tolerance to provide selective advantages in adverse environmental conditions [10, 31]. However, sustaining these plasmids is a metabolic burden for the microorganisms, as the host machinery is used for replication, thereby reducing the metabolic activities of the cells. In a natural system, bacteria maintain plasmids to cope with adverse environmental conditions, yet lose them under favorable conditions, such as the laboratory, to relieve the extra metabolic burden [23, 27]. Consequently, plasmid-curing organisms exhibit unstable degradative traits, creating a challenge as regards reproducible results and maintaining the organisms in culture collections with intact features.

While several studies on phenanthrene degradation have already been conducted, almost all have focused on stable strains (i.e. the strains do not lose their phenanthrene-degrading potential after transfer to a rich medium) and promoted the concept of bio-augmentation [13, 26, 32]. In contrast, the present study used soil microcosms and an unstable phenanthrene-degrading strain of *Pseudomonas*, and demonstrated that unstable strains are equally efficient in oligotrophic and pollutant-rich contaminated soil. Thus, similar to stable strains, unstable bacteria can also play an important role in the cleanup of contaminated sites by the process of natural attenuation and biostimulation. Furthermore, findings on a novel pattern of intermediate formation will help monitor the intermediates during the bacterial degradation of phenanthrene and add a new link to the ongoing chain of phenanthrene degradation studies.

Materials and Methods

Isolation and maintenance of cultures

The phenanthrene-degrading strains were isolated by enrichment using phenanthrene as the sole source of carbon and energy, as described by Samanta *et al.* [21]. The enrichment was carried out in 100 ml of MSM supplemented with 0.1% (w/v) crystals of phenanthrene and inoculated with 10% (10 g) petroleum-contaminated soil. The flasks were incubated at 30°C and 200 rpm in the dark. After three sub-cultures, the isolates were screened on MSM agar plates sprayed with a 5% ethereal solution of phenanthrene [8]. The phenanthrene-degrading colonies were picked and purified by several re-streakings on nutrient agar plates. The strains were maintained on MSM plates containing phenanthrene and also at -80°C with glycerol. For the taxonomical characterization, the 16S rRNA gene sequencing and phylogenetic analysis were carried out as described by Prakash and Lal [17], the fatty acid methyl ester (FAME) analysis was conducted according to Sasser [22], and the biochemical and physiological tests were conducted according to Prakash *et al.* [18].

Study of degradation and pathway

The degradation studies were conducted in 25 ml of MSM supplemented with 0.1% (w/v) phenanthrene. The medium was inoculated with 1% (v/v) of the over-night grown inoculum (O.D. 0.5 at 600 nm) and incubated at 30°C and 200 rpm in the dark. The experiments were carried out in triplicate with a control (MSM with phenanthrene, yet no bacteria). The entire contents of the flasks were harvested at different time intervals, extracted, and analyzed as

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previously described by Samanta *et al.* (1999). The simultaneous growth of strain Ph-3 on phenanthrene was monitored based on the colony-forming units (CFUs). To study the degradation pathway of phenanthrene with strain Ph-3, the intermediates formed in the culture extract as a consequence of phenanthrene degradation were analyzed by TLC, GC, and GCMS [21]. The growth of strain Ph-3 was also checked in a mineral salt medium supplemented with 2 mM of each intermediate (1-hydroxy-2-naphthoic acid, salicylic acid, catechol, 2-carboxy benzaldehyde, and o-phthalic acid) as the source of carbon and energy.

For the enzyme assays, cell lysates were prepared from phenanthrene (0.1% w/v) and dextrose (0.2% w/v) grown cells, as described by Balashova *et al.* (2001). The total protein content was estimated by Lowry's method using BSA (Bovine Serum Albumin) as the standard. The assays for 1-hydroxy-2-naphthoate dioxygenase [5], 1-hydroxy-2-naphthoate hydroxylase [1], salicylate hydroxylase [30], catechol 1, 2-oxygenase [4], catechol 2, 3-dioxygenase [2], and 2-carboxybenzaldehyde dehydrogenase [6] were all carried out using a Specord-50 UV/Visible spectrophotometer (Analytik Jena, Germany) at 30°C.

Microcosm study

For the microcosm study, soil was taken from an agricultural field, dried in the shade, and powdered by passing through a 2.0 mm sieve. The soil analysis was carried at the Soil Testing Division of the Indian Agricultural Research Institute (IARI), New Delhi, India. The natural rifamycin-resistant property of strain Ph-3 was used to determine the survival of the organism in soil. 150 μg/ml of rifamycin was used for the selection of strain Ph-3 from the selected soil, as this concentration completely inhibited the background growth of all other organisms. The moisture for optimum growth was standardized by adding equal numbers (1.5×10^8 /g of soil) of cells of strain Ph-3 at different moisture levels (10, 20, 30, 40 and 50%) in sterile soil and the survival of the cells was monitored based on the CFUs.

The microcosms were prepared in 100 ml conical flasks with 30 g of phenanthrene-spiked (0.1% w/w) soil. A total of four sets were used: non-sterile phenanthrene-spiked soil with and without strain Ph-3 and sterile phenanthrene-spiked soil with and without strain Ph-3. Un-inoculated sets were treated as the control. Thus, except for the control, the microcosms were all inoculated with 7.5 ml of a phenanthrene-grown culture broth (1.8×10^7 cell/ml) of strain Ph-3 to obtain a 25% final moisture content (moisture for optimum growth). In the case of the control, an equal volume of sterile double distilled water was added. The soil and the inoculum were mixed using a sterile spatula for homogenous distribution of the bacterium. The moisture loss by evaporation was monitored (by weighing the flask with the soil and inoculum) and adjusted by the addition of sterile water from outside. The flask was kept at 30°C in a static condition in the dark. At different time intervals, complete samples were extracted with two volumes of ethyl acetate and the residual phenanthrene detected by GC, as described above. The simultaneous survival of the organism was monitored based on the CFUs on an LB-agar plate with 150 μg/ml of rifamycin.

The GenBank accession number for the 16S rRNA gene sequences of strain Ph-3 is AY792969 and the culture collection number is MTCC 7602.

Results

Isolation and characterization of organism

A total of five isolates were selected on the basis of luxuriant growth, the dissolution of phenanthrene crystals, and the formation of a clear degradation zone around the bacterial colonies on an MSM agar plate sprayed with phenanthrene (Fig. 2). Among the five isolates, strain Ph-3 was then selected for further study on the basis of its efficient phenanthrene degradation potential. During the growth of strain Ph-3 in an MSM broth containing phenanthrene, the color of the culture broth turned from whitish to pinkish red and the phenanthrene crystals disappeared from the medium. An initial inoculum of 0.3×10^6 cells/ml of strain Ph-3 was able to utilize close to 68% (680 μg/ml out of 1000 μg/ml) of the phenanthrene from the culture broth after just 72 h of incubation, plus the cell count of strain Ph-3 increased from 0.3×10^6 to 16×10^6 cells/ml (Fig. 3). The rate of phenanthrene degradation was dependent on the number of cells of strain Ph-3. It was also observed that strain Ph-3 lost its phenanthrene-degrading potential after 3-4 transfers on rich medium plates. Therefore, the strain was maintained on an MSM agar sprayed with phenanthrene to study the degradation and pathway involved in the degradation of phenanthrene.

Taxonomical characterization and growth study

Strain Ph-3 showed a 99.7% 16S rRNA gene sequence

similarity and formed a monophyletic clad with *Pseudomonas aeruginosa* LMG 1242^T (Z76651) with high bootstrap (100%) confidence levels in a phylogenetic tree (Fig. 4). A

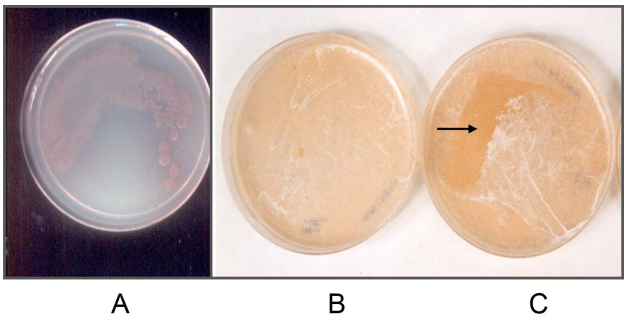

Fig. 2. Photographs of plates.
(A) colony morphology of strain Ph-3 on LB-agar after 72 hours of incubation, (B) un-inoculated control, and (C) degradation of phenanthrene and formation of clear zone of phenanthrene degradation (point of arrow) by strain Ph-3 on MSM agar plates sprayed with phenanthrene.

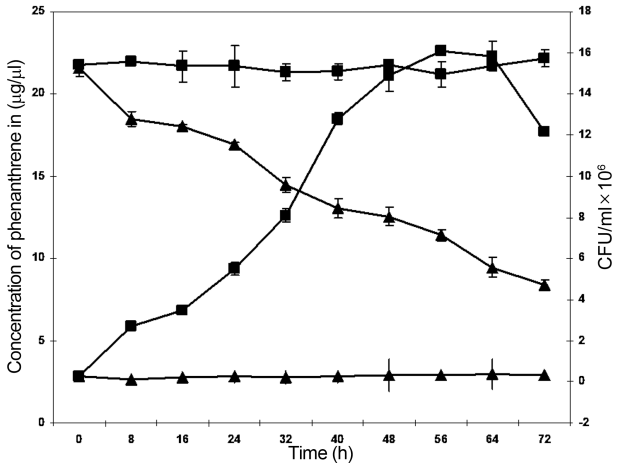

Fig. 3. Growth of Ph-3 (■; lower) with concomitant degradation of phenanthrene(▲; upper).
No growth of Ph-3 (▲; lower) and no degradation of phenanthrene (■; upper) were detected in the case of the controls. Bar indicates the standard deviation and the recovery of the extraction procedure was close to 88%.

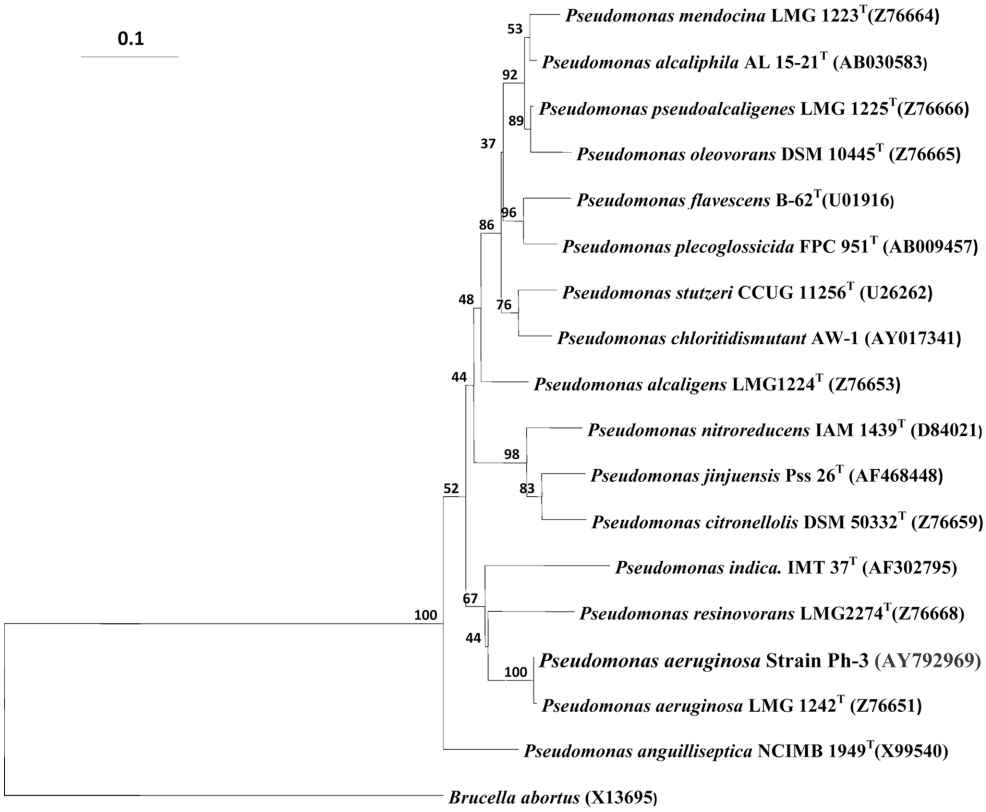

Fig. 4. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing relationship of strain Ph-3 with *Pseudomonas aeruginosa* LMG 1242^T.
The tree was constructed by the neighbor-joining method and rooted using *Brucella abortus* as the outgroup. The numbers at the nodes represent the bootstrap values (based on 100 resamplings). Scale bar indicates 0.1-nucleotide substitutions per nucleotide position. The GenBank accession number for the 16S rRNA gene sequence of each reference species is shown in parenthesis

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Table 1. Comparison of morphological and biochemical features of strain Ph-3 with reference strain of *Pseudomonas aeruginosa*.

S.N.	Features	Strain Ph-3[a]	*Pseudomonas aeruginosa*[b]	FAME
1	Gram reaction	-	-	10:0 (0.2%)
2	Cell size	0.5×1.5 um	0.5×1.0 to 0.5×1.5	12:0 (3.2%)
3	Fluorescence	+	+	14:0 (0.7%)
4	Motality	+	+	16:0 (26.5%)
5	Oxidase	+	+	18:0 (0.5%)
6	Catalase	+	+	12:0 2-OH (4.7%)
7	Nitrate reduction	+	+	12:1 3-OH (0.3%)
8	Gelatin hydrolysis	+	+	10:0 3-OH (3.9%)
9	Arginine dihydrolase	+	+	17:1 w8c (0.3%)
10	Growth at 41°C	+	+	18:1w7c (37.1%)
11	Indigo from indole	+	-	19 cyclo w8c (1.2%)
12	Phenanthrene degradation	+	-	17:0 cyclo (1.2%)
				11-methyl 18:1 w7c (0.4%)

[a]Data from this study and [b]data taken from Bergey's Manual of Systematic Bacteriology 1984.
Resistant to oxytetracyclin, penicillin, erythromycin, ampicillin, chlortetracycline, tetracycline, chloramphenicol, nalidixic acid, amoxicillin, rifampicin, vancomycin, and novoviocin, yet showed sensitivity to gentamycin and neomycin. Urease positive, yet amylase negative. Produced acid from lactose, maltose, glucose, inositol, mannitol, dextrin, arabinose, D-fructose, trehalose, sorbitol, xylose, and glactos.

similarity search using the fatty acid profile of strain Ph-3 in the TSBA505 library of MIDI also revealed a 0.901 similarity index value (SIM) to the fatty acid profile of *Pseudomonas aeruginosa*. In addition, strain Ph-3 was found to be a Gram negative, non-spore forming, motile rod (0.5×1.5 μm) and showed positive tests for oxidase, catalase, nitrate-reductase, gelatinase, urease, and arginine dihydrolase, yet tested negative for amylase. It was also able to grow at pH 4-11, temperatures 4-42°C, and salt 6% (Table 1).

Pathway of degradation

Strain Ph-3 grew on 2 mM of 1-hydroxy-2-naphthoic acid, salicylic acid, and catechol. No intermediates were detected by TLC during the early phase of growth of strain Ph-3. However, the sample harvested after 48 h of growth showed a distinct spot of 1-hydroxy-2-naphthoic acid and faint spots of 1, 2-dihydroxy naphthalene and salicylic acid (Fig. 5), and these results were also confirmed by a GC analysis of the culture extract (Fig. 6). In addition to 1-hydroxy-2-naphthoic acid, clear peaks for 1, 2-dihydroxy naphthalene and salicylic acid were also detected (Figs. 5 and 6). However, unlike 1-hydroxy 2-naphthoic acid, salicylic acid and 1, 2-dihydroxy naphthalene were not accumulated in the culture medium and were quickly degraded after their formation. The results of the GC analysis also indicted that the peaks for salicylic acid and 1, 2-dihydroxy naphthalene gradually decreased and finally disappeared in the late phase of growth (Fig. 6). The GC-MS analysis of the culture extract and a similarity search based on the

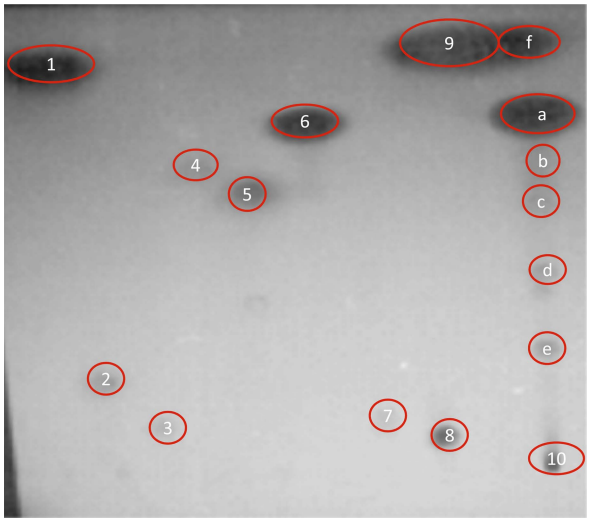

Fig. 5. Image of thin layer chromatography (TLC) plate showing spots for standards of different intermediates in phenanthrene degradation pathway and respective representatives in culture extract.
1, Phenanthrene; 2, catechol; 3, gentisic acid; 4, salicylic acid; 5, 1-naphthol; 6, 1-H2N; 7, o-phthalic acid; 8, protocatechuic acid, 9, control; 10, culture extract from phenanthrene-grown strain Ph-3. Spot f is the leftover phenanthrene, while spots a, b, and c represent 1-H2N, salicylic acid, and 1-naphthol, respectively, spot d is an unknown intermediate, and spot e represents catechol. Due to the low intermediate concentrations in the culture extract, the spots are not very prominent, yet their peaks are visible in a GC chromatogram.

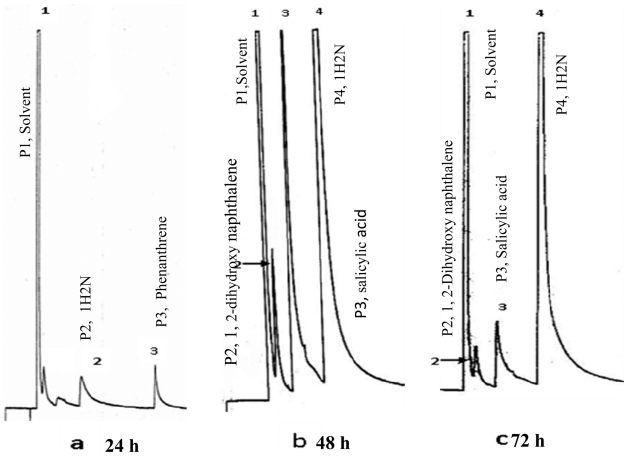

Fig. 6. Chromatogram of GC indicates pattern of metabolite formation during phenanthrene degradation by strain Ph-3. P1, P2, P3, and P4 indicate the peak numbers (1, 2, 3, and 4) in the different chromatograms, while 24 hours, 48 hours, and 72 hours are the sampling times. While 1, 2-dihydroxy naphthalene and salicylic acid exhibited degradation (72 hours) after formation, 1H2N was continuously accumulated in the culture broth without degradation.

fragmentation pattern of putative intermediates using the NIST (National Institute of Standards and Technology) library revealed the presence of a fragmentation pattern of 1-hydroxy-2-naphthoic acid with a characteristic peak of a molecular-ion [M+] at m/z 188 with another prominent daughter or fragment ion at m/z 170, 114, 85, and 57. The mass spectrum of the putative 1-hydroxy-2-naphthoic acid was found to be similar to the fragmentation pattern of authentic 1-hydroxy 2-naphthoic acid obtained under similar operating conditions (data not shown).

The total protein content in the crude extract of the phenanthrene- and dextrose-grown cells was 8.0 and 7.5 mg/ml, respectively. The enzyme activities (values in parentheses) are expressed as the nmol min/mg of protein. The crude extract of the phenanthrene-grown cells only exhibited the activity of 1-hydroxy-2-naphthoate-hydroxylase (253U), salicylate-hydroxylase (158U), and catechol 2, 3-dioxygenase (180U). No activities of 1-hydroxy-2-naphthoate dioxygenase and 2-carboxybenzaldehyde dehydrogenase were detected. Meanwhile, the crude extract of the dextrose-grown cells did not show any activity of the abovementioned enzymes.

Microcosm study

The soil used for the microcosm study was an alkaline (pH 8.4) sandy loam with a 55% water-holding capacity. The electrical conductivity of the soil was 0.38 ds/m (measure of total soluble salt), the organic carbon content 0.43% (measure of total available nitrogen), and the total available phosphorus and potash was 1202 and 207 kg ha^{-1} respectively. Strain Ph-3 survived best with a moisture content between 25-30% (v/w).

The results of the microcosm study showed that the cell count of strain Ph-3 increased from 10^5 to 10^7 during 8.0 days of incubation in both the sterilized and non-sterilized soil amended with phenanthrene (Table 2). However, after 8.0 days, the cell number of strain Ph-3 decreased, and the cell death was faster in the unsterilized than in the sterilized soil. The cell number of strain Ph-3 in the phenanthrene-unamended soil (soil with strain Ph-3, yet no phenanthrene) was found to decrease continuously, and no colonies of strain Ph-3 were detected in the controls (soil with phenanthrene, yet no strain Ph-3). The microcosm data showed complete degradation of phenanthrene after 15 days of incubation in the sterile soil and about 95% degradation in the unsterilized soil inoculated with strain Ph-3. Meanwhile, no degradation of phenanthrene was observed in the control (soil with phenanthrene, yet no bacteria), even after 30 days of incubation (Table 2).

Table 2. Phenanthrene degradation by strain Ph-3 in soil microcosms with concomitant increase in Ph-3 cell count.

Sampling Days	[a]Non-sterile (p+b)		[b]Sterile (p+b)		[c]Non-sterile (b)		[d]Non-sterile (p)	
	cfu/g	[e]amt (mg)	cfu/g	[e]amt (mg)	cfu/g	[e]amt (mg)	cfu/g	[e]amt (mg)
0-day	2.0×10^5	26.7 (± 1.43)	2.0×10^5	26.7 (± 2.26)	2.0×10^5	0.0	0.0	25.93 (± 1.73)
8-days	2.1×10^7	3.8 (± 0.35)	1.6×10^7	3.2 (± 0.13)	6.0×10^4	0.0	0.0	25.40 (± 1.90)
15-days	3.2×10^5	1.4 (± 0.14)	4.0×10^6	ND	6.0×10^4	0.0	0.0	25.96 (± 1.35)
30-days	8.0×10^4	ND	3.2×10^6	ND	3.6×10^4	0.0	0.0	27.46 (± 1.95)

Values in parentheses indicate standard deviation. Recovery of extraction procedure was 83-90%
[a]Non-sterile soil with phenanthrene + bacterium (strain Ph-3), [b]Sterile soil with phenanthrene + bacterium (strain Ph-3), [c]Non-sterile soil with only bacterium (strain Ph-3), [d]Non-sterile soil with only phenanthrene (Control), [e]amount of residual phenanthrene/30 g of soil, ND, not detected

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Discussion

The results of this study confirmed the high catabolic potential of strain Ph-3 towards phenanthrene in a liquid culture broth, solid mineral medium, and soil microcosm. The degradation potential of strain Ph-3 was two, three, and six-fold higher than that of *Burkholderia cocovenenans* [29], *Bacillus sphaericus* [33], and *Pseudomonas putida* [20] respectively, isolated from similar-type habitats. However, the rate of phenanthrene degradation was similar to that of *Sphingomonas* sp., isolated by Liu *et al.* [11] from a crude oil-contaminated site in China. The high catabolic potential of strain Ph-3 towards phenanthrene has special ecological significance as it is desirable for bioaugmentation in order to maintain a high population density in the introduced environment.

The TLC, GC, and GC-MS analyses of the culture extract, growth study on different intermediates, and assays of the enzymes activities all indicated that strain Ph-3 utilized a salicylate pathway to degrade phenanthrene and the pathway enzymes were induced. While the formation and accumulation of 1-hydroxy 2-naphthoic acid [25, 28] and salicylic acid [12, 14] in a culture broth have already been reported, the fast degradation of 1, 2-dihydroxy naphthalene and salicylic acid right after their formation during the degradation of phenanthrene by strain Ph-3 (Fig. 3) would appear to be unique to this organism and will help in more accurate monitoring of these intermediates in future research.

Phenanthrene degradation with a concomitant increase in the cell count of strain Ph-3 in the phenanthrene-amended soil indicated survival and a metabolically active state for the inoculated cells under the defined set of microcosm conditions. The rapid increase in the cell count of strain Ph-3 in the phenanthrene-amended soil was mainly due to the high catabolic potential of strain Ph-3 towards phenanthrene, as it provided a selective advantage to the organism for its easy establishment in the introduced environment [25]. Unlike the batch culture, the phenanthrene was completely removed from the soil microcosms, as the accumulation of metabolites and excretory products in the batch culture experiments reduced the activity of the cells. The microcosm data showed an efficient phenanthrene degrading potential, good survival, and metabolically active state of the inoculated cells in the soil. Thus, strain Ph-3 not only performed well under laboratory conditions, but was equally effective in soil, making it useful for bioaugmentation.

This study also found that the phenanthrene-degrading traits of strain Ph-3 were unstable, as they were lost after 2-3 successive transfers onto rich medium plates (LB, TSA, NA), yet retained on MSM and fortified in soil with phenanthrene. Strain Ph-3 degraded more than 90% of the added phenanthrene in the microcosm. Thus, based on these results, it was concluded that the oligotrophic conditions of the soil and the stress of the pollutant at the contaminated site helped the bacterium to maintain its traits and induced its efficient utilization. Therefore, it is hypothesized that metabolically versatile organisms like *Pseudomonas* acquire their traits via the processes of horizontal gene transfer in order to establish themselves at a contaminated site, and then flourish using the pollutant as the source of carbon and energy and thereby assist in the cleanup of the pollutant by the processes of natural attenuation. Although unstable traits are not good for laboratory studies, as they create difficulties in maintaining the organisms and ignore the previous *in vitro* studies of most strains involved in natural degradation, the present soil microcosm study indicated such strains perform equally well in a natural habitat in terms of pollutant degradation. Therefore, the concept of environmental cleanup using natural attenuation, biostimulation, or monitored natural attenuation [9, 19] based on a guild of natural microorganisms (stable and unstable both) is more appropriate in terms of cost, labor, and energy than emphasizing a single organism in the practice of bioaugmentation [13, 26, 32].

In conclusion, the taxonomic study results indicated that strain Ph-3 belongs to the genus *Pseudomonas* (Gammaproteobacteria) and is closely related to *Pseudomonas aeruginosa*. Further taxonomic delineation requires more experiments and is beyond the scope of this study. The pathway data and degradation kinetics indicated that strain Ph-3 has a high catabolic potential towards phenanthrene. A new pattern of 1, 2-dihydroxy naphthalene and salicylic acid production was exhibited during the bacterial degradation of phenanthrene. Although the bacterium performed well in the laboratory and in soil microcosms, a large-scale field experiment is still required.

Acknowledgments

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