meta-Cleavage of hydroxynaphthoic acids in the degradation of phenanthrene by *Sphingobium* sp. strain PNB

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Polycyclic aromatic hydrocarbons (PAHs) comprise a group of priority organic pollutants that are toxic and/or carcinogenic. Phenanthrene, the simplest PAH among recognized priority pollutants, is commonly used as a model compound for the study of PAH biodegradation. *Sphingobium* sp. strain PNB, capable of degrading phenanthrene as a sole carbon and energy source, was isolated from a municipal waste-contaminated soil sample. A combination of chromatographic and spectrometric analyses, together with oxygen uptake and enzyme activity studies, suggested the presence of phenanthrene degradation pathways in this strain. Identification of metabolites suggested that initial dioxygenation of phenanthrene took place at both 3,4- and 1,2-carbon positions; *meta*-cleavage of resultant diols led to the formation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively. The hydroxynaphthoic acids, in turn, were metabolized by a *meta*-cleavage pathway(s), leading to the formation of 2,2-dicarboxychromene and 2-hydroxychromene-2-glyoxylic acid, respectively. These metabolites were subsequently transformed to catechol via salicylic acid, which further proceeds towards the tricarboxylic acid cycle leading to complete mineralization of the compound phenanthrene. The present study establishes the metabolism of hydroxynaphthoic acids by a *meta*-cleavage pathway in the degradation of phenanthrene, expanding our current understanding of microbial degradation of PAHs.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of hydrophobic organic compounds consisting of two or more fused benzene rings in linear, angular or cluster arrangements (Habe & Omori, 2003). On the basis of their abundance, toxicity and intrinsic chemical stability to various transformation processes, 16 PAHs have been identified by the US Environmental Protection Agency as priority pollutants (Keith & Telliard, 1979). Phenanthrene is one such pollutant and is often used as a model substrate for metabolic studies of carcinogenic PAHs (Bücker *et al.*, 1979; Moody *et al.*, 2001).

Several phenanthrene degradation pathways have been documented, indicating metabolic diversity among different bacterial species (Peng *et al.*, 2008; Seo *et al.*, 2009; Mallick *et al.*, 2011; Ghosal *et al.*, 2010). The majority of studies report dioxygenation at the 3,4-carbon positions to

form phenanthrene-3,4-dihydrodiol. This is subsequently metabolized to 1-hydroxy-2-naphthoic acid, and further degraded through one of two distinct pathways. In the first route, direct ring cleavage of 1-hydroxy-2-naphthoic acid leads to metabolism through the tricarboxylic acid (TCA) cycle intermediates via *o*-phthalic acid and protocatechuic acid (Kiyohara *et al.*, 1976; Kiyohara & Nagao, 1978; Barnsley, 1983; Ghosh & Mishra, 1983; Houghton & Shanley, 1994; Iwabuchi & Harayama, 1997). In the other route, oxidative decarboxylation of 1-hydroxy-2-naphthoic acid leads to the formation of 1,2-dihydroxynaphthalene, which is metabolized by the well-known naphthalene degradation pathway via salicylic acid (Evans *et al.*, 1965; Gibson & Subramanian, 1984). In the lower pathway of metabolism of phenanthrene, salicylic acid is degraded via the formation of either catechol or gentisic acid, followed by ring fission leading to TCA cycle intermediates (Houghton & Shanley, 1994).

Apart from dioxygenation at the 3,4-position, there are reports of initial dioxygenation at the 1,2-position of phenanthrene leading to the formation of 2-hydroxy-1-naphthoic acid as one of the metabolic intermediates. Recent studies revealed that degradation of phenanthrene was initiated by dioxygenation at both 3,4- and 1,2-positions and was subsequently metabolized to

Abbreviations: ESI, electrospray ionization; PAHs, polycyclic aromatic hydrocarbons; TCA, tricarboxylic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Sphingobium* sp. strain PNB is HM367594.

Four supplementary figures are available with the online version of this paper.

1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively (Balashova *et al.*, 1999; Pinyakong *et al.*, 2000; Keum *et al.*, 2006; Seo *et al.*, 2006, 2007). These two intermediates were proposed to be transformed and converged into naphthalene-1,2-diol, which was further metabolized via either salicylic acid (Pinyakong *et al.*, 2000) or phthalic acid (Keum *et al.*, 2006) or both (Seo *et al.*, 2006, 2007). Dioxygenation exclusively at the 1,2-position of phenanthrene, forming phenanthrene *cis*-1,2-dihydrodiol, has also been reported, where the dihydrodiol is subsequently metabolized to 2-hydroxy-1-naphthoic acid, which was further degraded by a *meta*-cleavage dioxygenase, ultimately leading to TCA cycle intermediates via salicylic acid and catechol (Mallick *et al.*, 2007; Ghosal *et al.*, 2010).

Bacterial species belonging to the genus *Sphingomonas*, class alphaproteobacteria, have been reported for their versatile ability to degrade diverse aromatic compounds. Recently, the genus *Sphingomonas* was reclassified into four separate genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi *et al.*, 2001; Maruyama *et al.*, 2006). Species belonging to these genera have been documented to metabolize compounds such as biphenyl, (substituted) naphthalene(s), fluorene, (substituted) phenanthrene(s), pyrene, (chlorinated) diphenylether(s), (chlorinated) furan(s), (chlorinated) dibenzop-dioxin(s), carbazole, polyethylene glycols, chlorinated phenols, as well as different herbicides and pesticides (Pinyakong *et al.*, 2003; Basta *et al.*, 2004; Stolz, 2009). The ability to degrade diverse environmentally harmful aromatic compounds may be linked to their unique but complex genetic organization, comprising multiple dioxygenases and representatives of various pathway genes, scattered within several gene clusters, with an intricate regulatory system (Romine *et al.*, 1999; Stolz, 2009). Further characterization of PAH-assimilating pathways in *Sphingomonadaceae* may shed new insight into the microbial degradation of PAHs.

In the present study, we report novel intermediates in the phenanthrene degradation pathways of *Sphingobium* sp. strain PNB. The intermediates, 2,2-dicarboxychromene and 2-hydroxychromene-2-glyoxylic acid, the respective breakdown products of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, were detected in the degradation process. Furthermore, metabolism of the hydroxy-naphthoic acids suggesting involvement of a *meta*-cleavage dioxygenase(s) has been described, expanding current understanding of phenanthrene degradation pathways.

METHODS

Chemicals. Phenanthrene, 2-hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid, salicylic acid, catechol, 1,2-dihydroxynaphthalene, phthalic acid, 1-naphthol, 2-naphthol, protocatechuic acid, *o*-phenanthroline and deferroxamine mesylate were purchased from Sigma-Aldrich. Unless stated otherwise, all other chemicals and reagents used in this study were of analytical grade and were used without further purification.

Enrichment and isolation of bacteria. Enrichment of cultures was started by inoculating a 1 g municipal waste-contaminated soil sample (Dhapa, Kolkata, India) in liquid mineral salt medium (MSM; Mallick *et al.*, 2007) supplemented with solid phenanthrene (1 g l^{-1}) as the sole carbon and energy source, and incubated at 28°C on a rotary shaker (180 r.p.m.). When growth was observed, the enrichment process was repeated with several transfers under the same conditions and the enriched cultures were subsequently purified by plating on nutrient agar (2%, w/v) medium. Each type of colony was isolated based on a distinct morphology and pigmentation pattern and was then allowed to grow individually in MSM supplemented with phenanthrene, as mentioned above. Finally, one strain capable of growing in the presence of phenanthrene as the sole carbon source (*Sphingobium* sp. strain PNB) was selected for further analysis.

Characterization of strain PNB. Morphological features of *Sphingobium* sp. strain PNB were studied by phase-contrast microscopy (Olympus CX40). Conventional biochemical tests were performed using standard methods (Holtz *et al.*, 1994; Smibert & Krieg, 1994; Yabuuchi & Kosako, 2005). The 16S rRNA gene was amplified using universal bacterial-specific primers f27 and r1492 (Johnson, 1994) and was sequenced according to the manufacturer's specifications for *Taq* DNA polymerase-initiated cycle sequencing reactions using fluorescently labelled dideoxynucleotide terminators with an ABI PRISM 377 automated sequencer (Perkin-Elmer Applied Biosystems). Levels of 16S rRNA gene sequence similarity were determined using BLAST version 2.2.12 of the National Center for Biotechnology Information (Altschul *et al.*, 1990). Fatty acid methyl ester analysis was carried out at the Institute of Microbial Technology, Chandigarh, India. The bacterial culture was harvested by centrifugation and the pellet was subjected to saponification, methylation and extraction, using the methods described by Miller (1982) and Kuykendall *et al.* (1988). Analysis was done using the Sherlock Microbial Identification System (MIDI).

Cultivation of bacteria. Cells were grown in 100 ml Erlenmeyer flask containing 25 ml MSM supplemented with solid phenanthrene (1 g l^{-1}) or possible metabolites ($0.2\text{--}1.0 \text{ g l}^{-1}$) of the phenanthrene degradation pathway(s) individually as the sole carbon source, by incubating them at 28°C on a rotary shaker (180 r.p.m.) for different periods of time. Resting cell incubations, individually with phenanthrene and pathway intermediates (concentrations of $0.2\text{--}1 \text{ g l}^{-1}$), were done as described previously (Mallick *et al.*, 2007). Unless stated otherwise, each experimental set was performed in triplicate.

Isolation of metabolites. After incubation, the spent broth and resting cell culture were centrifuged (8000 g, 10 min) and the pH of the supernatants was altered to 1.5–2.0 with 6 M hydrochloric acid and extracted three times with an equal volume of ethyl acetate. The combined organic layer was re-extracted with aqueous sodium hydroxide (10 mM). The organic phase was dried over anhydrous sodium sulfate and evaporated under reduced pressure (neutral fraction). The pH of the aqueous sodium hydroxide extract was altered to 1.5–2.0 as above and then extracted with ethyl acetate (acidic fraction). The extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. A portion of the evaporated neutral fraction was dissolved in acetone (10 ml) and refluxed for 30 min after the addition of 50 mg *n*-butylboronic acid (Keum *et al.*, 2006). After refluxing, the derivatized mixture was concentrated for further analysis. Metabolites in the acidic fractions were derivatized with boron trifluoride-methanol solution (Merck) as needed prior to analysis.

Oxygen uptake. Oxygen uptake measurements by whole cells in the presence of phenanthrene and various probable metabolic intermediates of phenanthrene were carried out at 28°C with a YSI model

5300A biological oxygen monitor (Yellow Springs Instrument) equipped with Clark-type polarographic oxygen electrodes (YSI model 5331A) and sample chambers fitted within a YSI model 5301B standard bath. The sample size was 2.0 ml, and the reaction mixture contained 500 µl cell suspension (25 mg cells, wet weight), substrate (0.5 ml) and 1 ml phosphate buffer (50 mM, pH 7.0). The reaction was initiated by injecting a suitable amount of the assay substrate and oxygen uptake was monitored for 5 min. Phenanthrene (0.5 ml) was added as a saturated aqueous solution, $\sim 1.3 \text{ mg l}^{-1}$ (Cerniglia, 1992), and aqueous solutions of the possible phenanthrene degradation pathway intermediates were added so as to give a final concentration of 0.1 mM. 1,2-Dihydroxynaphthalene (0.1 mM) was added as a solution in tetrahydrofuran. Respiration upon addition of an equivalent amount of tetrahydrofuran as substrate was included as a control. The oxygen uptake rate was expressed as $\text{nmoles min}^{-1} (\text{mg protein})^{-1}$. Rates were corrected for endogenous oxygen consumption.

Preparation of cell-free extracts. Suspensions of cells grown individually on phenanthrene, 2-hydroxy-1-naphthoic acid and 1-hydroxy-2-naphthoic acid were prepared as described previously (Mallick *et al.*, 2007) and were loaded into a pre-cooled French press (Constant Cell Disruption System, One Shot model) fitted with a 8.0 ml cell followed by lysis at 30 000 p.s.i. ($\approx 207 \text{ MPa}$) for two cycles. Cell-free extracts were obtained by centrifuging the cell lysates at 20 000 g for 20 min at 4 °C. The supernatant was used as cell-free enzymes for further studies. Protein was measured by using the Lowry method with BSA as the standard (Lowry *et al.*, 1951).

Enzyme assays. The enzymic transformations of various substrates were carried out by recording cell-free extract-catalysed changes in UV-visible spectra on a Cary 100 Bio UV-visible spectrophotometer (Varian Australia) using 1 cm path length quartz cuvettes. Data were analysed with the Varian Cary Win UV Scan application software. Time-dependent spectral changes of various possible metabolites of the phenanthrene degradation pathway(s) were tested individually with cell-free extracts to monitor the activities of catechol 1,2-dioxygenase (Hayaishi *et al.*, 1957), catechol 2,3-dioxygenase (Kojima *et al.*, 1961), salicylaldehyde dehydrogenase (Eaton & Chapman, 1992), 1-hydroxy-2-naphthoate *ortho*-cleavage dioxygenase (Kiyohara *et al.*, 1976; Iwabuchi & Harayama, 1997), 1,2-dihydroxynaphthalene dioxygenase (Davies & Evans, 1964) as well as 1-hydroxy-2-naphthoate and 2-hydroxy-1-naphthoate *meta*-cleavage dioxygenases (Mallick *et al.*, 2007; Ghosal *et al.*, 2010).

Chemical analyses. Ethyl acetate-extracted metabolites present in the neutral fraction and acidic fraction were analysed by using a Thermo Scientific model TraceGC Ultra column (Thermo Fischer Scientific) with a model PolarisQ mass spectrometer equipped with a 30 m $\times$ 0.25 mm (0.25 µm film thickness) DB-5MS capillary column. The ion source was kept at 230 °C and both the inlet temperature and the transfer line temperature were kept at 280 °C. The temperature programme comprised 2 min hold at 70 °C, increase to 200 °C at 10 °C min^{-1} , followed by hold for 1 min at 200 °C, further increase to 325 °C at 5 °C min^{-1} , and 15 min hold at 325 °C. The injection volume was 1 µl, and the carrier gas was helium (1 ml min^{-1}). The mass spectrometer was operated at an electron ionization energy of 70 eV. In addition, metabolites were resolved by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C₁₈ reversed-phase column or analytical Inertsil ODS-3 column (MetaChem Technologies) attached to a model SIL-20A autosampler. The biodegraded products were eluted using a programmed gradient solvent system at a flow rate of 1.0 ml min^{-1} and detected at 254 nm along with diode array analysis. The mobile phase was a 45 min linear gradient from 50 % (v/v) to 95 % (v/v) aqueous methanol with hold at 95 % (v/v) aqueous methanol for 10 min followed by 95 % (v/v) to

50 % (v/v) aqueous methanol over 5 min. However, in the C₁₈ reversed-phase column, the mobile phase aqueous solution contained 1 % (v/v) acetic acid. Metabolites were identified by comparing their retention times and UV-visible absorbance spectra with those of the authentic compounds analysed under the same set of conditions. For further characterization, HPLC-resolved metabolites obtained from the organic extracts were subjected to ¹H- and ¹³C-NMR spectral analyses, as described previously (Mallick *et al.*, 2007). The intensity of each chemical shift was determined relative to tetramethylsilane as an internal standard, while deuterated water was used to dissolve each sample.

Cloning and screening of catabolic genes/operons involved in degradation. A genomic library of *Sphingobium* sp. strain PNB was prepared in pEpiFOS-5 Fosmid vector according to the manufacturer's protocol (Epicentre). Total DNA from the strain was randomly sheared to approximately 40 kb fragments and the ends were blunted using an End-repair Enzyme Mix (EpiFOS Fosmid Library Production kit; Epicentre) and ligated into pEpiFOS. The ligated DNA was packaged with MaxPlax Lambda Packaging Extracts to form the fosmid library. The library was transduced into *Escherichia coli* LE392MP and spread on Luria-Bertani agar plates containing 12.5 µg chloramphenicol ml^{-1} followed by replica-plating. Colonies harbouring ring-hydroxylating dioxygenase activity were screened by incubating the plates in an indole-saturated chamber while those harbouring catechol 2,3-dioxygenase activity were screened by spraying catechol solution (0.1 M) over the colonies.

RESULTS

Isolation and characterization of *Sphingobium* sp. strain PNB

Using an enrichment culture technique, a phenanthrene-degrading bacterium was isolated from a municipal waste-contaminated soil sample. Cells were Gram-negative rods and formed golden yellow colonies on Luria-Bertani agar plates. The 16S rRNA gene sequence (1451 bp) of the isolate was determined and has been deposited in GenBank/EMBL/DDBJ with accession no. HM367594. The phylogenetic position of strain PNB among closely related strains in GenBank was analysed based on levels of 16S rRNA gene sequence similarity. Strain PNB showed the highest 16S rRNA gene sequence similarity to *Sphingobium fuliginis* TKP^T (99.79 %) followed by *Sphingobium chlorophenicum* ATCC 33790^T (96.63 %). However, comparison of the major fatty acid methyl esters of strain PNB via the TSBA (version 4.0) database revealed a strong correlation with *Novosphingobium capsulatum* (with which it exhibited a similarity index of 0.573) but substantial differences from *Sphingobium fuliginis* TKP^T. Biochemical analyses (Holtz *et al.*, 1994; Smibert & Krieg, 1994; Yabuuchi & Kosako, 2005) of the novel strain showed that it was able to utilize maltose and xylose but not adipate, inositol, malonate, mannitol, oxalate or sorbitol and was positive for catalase, oxidase, urease and β-galactosidase. On the basis of 16S rRNA gene sequence similarity as well as morphological, nutritional and biochemical characteristics, strain PNB was presumed to belong to the family *Sphingomonadaceae* and genus *Sphingobium*.

Utilization of phenanthrene

Strain PNB was able to utilize phenanthrene as the sole source of carbon and energy, while the optimal temperature for growth in MSM (at pH 7.0) was 28 °C in the presence of 1 g phenanthrene l⁻¹ under shaking culture conditions. Utilization of phenanthrene by *Sphingobium* sp. strain PNB was confirmed by its removal from phenanthrene-MSM, with a corresponding increase in bacterial biomass (see Supplementary Fig. S1, available with the online version of this paper). Besides phenanthrene, strain PNB utilized 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid and salicylic acid individually as sole sources of carbon and energy. However, 1-naphthol, 2-naphthol, *o*-phthalic acid and protocatechuic acid did not support growth. Strain PNB was also capable of utilizing anthracene, naphthalene and biphenyl singly as carbon and energy sources.

Oxygen uptake by whole cells

Oxygen uptake studies with whole cells were carried out with various probable metabolic intermediates of the phenanthrene degradation pathway, as reported previously (Mallick *et al.*, 2007; Ghosal *et al.*, 2010). The results are summarized in Table 1. In the presence of phenanthrene, 2-hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid, salicylate or catechol, cells grown on phenanthrene showed depletion of molecular oxygen due to oxygenase activities. Moreover, cells grown on either 2-hydroxy-1-naphthoic acid or 1-hydroxy-2-naphthoic acid showed depletion of oxygen on 2-hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid, salicylate and catechol. However, cells grown on salicylate depleted molecular oxygen on salicylate and catechol only. The above results suggested the involvement of three inducible operons in strain PNB (the upper one inducible with phenanthrene, followed by

the middle operon, inducible with either of the hydroxynaphthoic acids, and finally the lower one, inducible with salicylate) for degradation of phenanthrene. On the other hand, cells grown on succinate as a sole carbon source failed to deplete molecular oxygen on all of these compounds, indicating the inducible nature of the phenanthrene degradation pathway. None of the above cultures showed any oxygen uptake activity with 1,2-dihydroxynaphthalene, protocatechuic acid or *o*-phthalic acid.

Isolation and identification of phenanthrene metabolites

HPLC (using a C₁₈ reversed-phase column) of phenanthrene-degraded products obtained from resting cell incubation (48 h) with phenanthrene showed a number of well-resolved peaks (Fig. 1). Among them, peaks I–III correspond to salicylic acid, catechol and salicylaldehyde, while peaks V–VII correspond to 2-hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid and unspent phenanthrene, respectively. These compounds were identified by comparing the retention times and UV-visible spectra (obtained from diode array analysis) with those of the authentic compounds analysed under identical conditions. Peaks IV and VIII could not be identified.

Nine metabolites were identified by GC-MS from the organic extracts of the spent culture (up to 72 h) and resting cell incubation (48 h) with phenanthrene (Table 2). Among the metabolites, only two (IX and X) were found in the neutral extracts while the remaining seven were detected from the acidic fractions. Analysis of *n*-butylboronate derivatives of the neutral extract revealed derivatives of *cis*-1,2-phenanthrenedi-hydrodiol and *cis*-3,4-phenanthrenedi-hydrodiol, similar to the MS data reported previously (Krivobok *et al.*, 2003; Keum *et al.*, 2006;

Table 1. Oxygen uptake rates with various compounds by resting-cell suspensions of *Sphingobium* sp. strain PNB grown on different substrates

All values are corrected for endogenous O₂ uptake. ND, Not detected; Tr, trace [<1 nmol O₂ consumed min⁻¹ (mg protein)⁻¹].

Substrate	Oxygen uptake rate (nmoles O ₂ consumed min ⁻¹ mg ⁻¹) by cells grown on:				
	Phenanthrene	2-Hydroxy-1-naphthoic acid	1-Hydroxy-2-naphthoic acid	Salicylic acid	Succinate
Phenanthrene	29.2	ND	ND	ND	ND
2-Hydroxy-1-naphthoic acid	42.1	35.5	33.3	Tr	ND
1-Hydroxy-2-naphthoic acid	37.7	33.8	29.3	ND	ND
2-Naphthol	ND	ND	ND	ND	ND
1-Naphthol	ND	ND	ND	ND	ND
1,2-Dihydroxynaphthalene	Tr	ND	Tr	ND	ND
Salicylic acid	44.0	39.6	37.2	40.6	ND
Catechol	46.5	40.8	39.6	43.7	ND
<i>o</i> -Phthalic acid	ND	ND	ND	ND	ND
Protocatechuate	ND	ND	ND	ND	ND

Fig. 1. HPLC profile of phenanthrene and its metabolic intermediates in the culture supernatant during resting-cell transformation of strain PNB incubated with phenanthrene for 48 h.

Schuler *et al.*, 2009). Among the other metabolites, peaks I, II and III detected in the acidic fraction were identified as salicylic acid, catechol and salicylaldehyde, respectively. 7,8-Benzocoumarin (XI) and 5,6-benzocoumarin (XII) were identified by matching their mass fragmentation pattern with the data reported by Pinyakong *et al.* (2000). Metabolites V and VI, with molecular ions (M^+) at m/z 202, were identified as methyl esters of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively, based on retention times and fragmentation patterns of the methyl esters of the authentic hydroxynaphthoic acids.

Metabolism of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid

Transformation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid was depicted spectrophotometrically based on the cell-free extract of strain PNB (Supplementary Fig. S2). The reaction mixture turned slightly yellowish when 1-hydroxy-2-naphthoic acid was incubated with the crude cell-free extract of strain PNB grown on phenanthrene, with an increase in absorbance around 290, 330 and 343 nm, indicating ring cleavage of 1-hydroxy-2-naphthoic acid (Supplementary Fig. S2a). Likewise, an increase in absorbance around 285, 297 and 334 nm was observed when 2-hydroxy-1-naphthoic acid was incubated, indicating ring cleavage of 2-hydroxy-1-naphthoic acid (Supplementary Fig. S2b). However, no change in spectral pattern was observed when 1,2-dihydroxynaphthalene was incubated under similar conditions, even in the presence of ferrous or ferric ions. This observation was further supported by the fact that phenanthrene-grown cells of *Sphingobium* sp. strain PNB showed oxygen uptake in the presence of 1,2-dihydroxynaphthalene and that the bacterium did not utilize the compound as a sole carbon source. The above facts essentially rule out the possibility of transformation of either of the hydroxynaphthoic acids to 1,2-dihydroxynaphthalene.

It is important to mention here that the ring cleavage of hydroxynaphthoic acids may have occurred due to the presence of a single ring-cleavage dioxygenase, which is supported by the data on oxygen uptake with both the hydroxynaphthoic acids (Table 1). Alternatively, the cell-free extract may contain two different hydroxynaphthoic

Table 2. GC-MS data for the metabolites of phenanthrene obtained from the organic extracts of the culture and resting cell incubation of *Sphingobium* sp. strain PNB

Metabolite	Retention time (min)	m/z of major ion peaks (%)*	Suggested structure†
I	9.73	138 (M^+ , 52), 120 (100), 92 (61), 64 (24)	Salicylic acid
II	8.94	110 (M^+ , 100), 92 (12), 81 (13), 64 (35), 63 (15)	Catechol
III	7.65	122 (M^+ , 100), 121 (91), 104 (17), 93 (28), 76 (25), 65 (43)	Salicylaldehyde
V	16.32	202 (M^+ , 66), 171 (33), 170 (100), 143 (21), 142 (55), 115 (34), 114 (48), 91 (10), 79 (16)	2-Hydroxy-1-naphthoic acid, methyl ester‡
VI	15.67	202 (M^+ , 47), 171 (28), 170 (100), 143 (19), 142 (53), 115 (30), 114 (42), 91 (9), 79 (14)	1-Hydroxy-2-naphthoic acid, methyl ester‡
IX	15.85	212 (M^+ , 56), 194 (44), 181 (24), 168 (84), 166 (100), 165 (90), 152 (38), 151 (13), 82 (17)	<i>cis</i> -3,4-Phenanthrenedihydrodiol
X	16.15	212 (M^+ , 52), 194 (47), 181 (20), 168 (55), 166 (100), 165 (91), 152 (36), 140 (23), 82 (16)	<i>cis</i> -1,2-Phenanthrenedihydrodiol
XI	18.21	196 (M^+ , 62), 168 (100), 139 (79), 70 (12), 63 (16)	7,8-Benzocoumarin
XII	18.43	196 (M^+ , 48), 168 (100), 139 (85), 70 (14), 63 (18)	5,6-Benzocoumarin

*Ion abundance percentages are shown in parentheses.

†Identification was based on the match of mass spectra (fragmentation and peak intensity) and GC retention times with those of authentic samples, other than metabolites IX–XII, which were identified based on the comparison of mass fragmentation patterns of previously identified metabolites.

‡Analyses were performed after methylation of organic extract with boron-trifluoride-methanol.

acid-specific ring-cleavage dioxygenases where both the hydroxynaphthoic acids are common inducers of two different operons. Nevertheless, it was observed that the ring-cleavage dioxygenase(s) catalysing the transformation of both 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid possessed dissociable ferric iron at the catalytic centre, given that an increase in ring-cleavage activity was noted when the cell-free extract was supplemented with 1 mM FeCl_3 (data not shown). This was further verified by the fact that upon treatment of the cell-free extract for 30 min with deferoxamine mesylate (0.2 mM), a ferric chelating reagent, the resultant cell-free extract preparation showed neither 1-hydroxy-2-naphthoic acid ring-cleavage activity nor 2-hydroxy-1-naphthoic acid ring-cleavage activity. The ring-cleavage activities were restored upon further treatment with FeCl_3 (1 mM) for 30 min. The above indicate the presence of *meta*-cleavage dioxygenase activity towards hydroxynaphthoic acids in the cell-free extracts of phenanthrene-grown culture of strain PNB. Although dissociable ferrous ions were previously reported to be present in the catalytic centre of ring-cleavage dioxygenase catalysing *ortho*-cleavage of 1-hydroxy-2-naphthoic acid (Iwabuchi & Harayama, 1997), EDTA, a ferrous chelating reagent, had no impact on the ring-cleavage activity towards hydroxynaphthoic acids.

HPLC (using an Inertsil ODS-3 column) of the cell-free transformation of both the hydroxynaphthoic acids showed that they had one major and one minor peak each (Fig. 2). The HPLC-diode array profiles of these products were highly similar to the spectrophotometric profiles obtained during enzymic transformations of the hydroxynaphthoic acids (Supplementary Fig. S2). To characterize the ring-cleavage products, the major unknown metabolites (1H2NA-II and 2H1NA-I, Fig. 2) were further isolated by preparative HPLC. ^1H -NMR spectral data of freshly prepared solutions of the HPLC-isolated products in deuterated water (4 mg ml^{-1}) are shown in Fig. 3. Based on ^1H -NMR data and the profiles of 2D NMR analysis (COSY), the ring-cleavage metabolites of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid are consistent with the proposed structure of 2,2-dicarboxychromene and 2-hydroxychromene-2-glyoxylic acid, respectively. The ^1H -NMR data for 1H2NA-II and 2H1NA-I were consistent with typical *cis*-orientated olefinic hydrogens, as evidenced by coupling constants in the range 7.0–8.2 Hz, indicating the absence of *trans*-orientated olefinic hydrogens in 1H2NA-II and 2H1NA-I. Moreover, neither the UV-visible spectra (Fig. 2, insets) nor the ^1H -NMR data of the isolated product (Fig. 3) correspond to the *ortho* ring-cleavage product of 1-hydroxy-2-naphthoic acid and the *meta*-cleavage product of 1,2-dihydroxynaphthalene, reported by Eaton & Chapman (1992) and Adachi *et al.* (1999). To verify the cyclic ring-cleavage products (compounds XIX and XX) as possible intermediates in the assimilation of phenanthrene, these compounds were individually subjected to resting cell incubation with phenanthrene-grown cells and subsequently the cultures

Fig. 2. HPLC profiles of products formed during transformation of 1-hydroxy-2-naphthoic acid (a) and 2-hydroxy-1-naphthoic acid (b) by cell-free extract of phenanthrene-grown cells of strain PNB incubated for 3 h in a total volume of 5 ml containing 250 nmol substrate and $200 \mu\text{g protein ml}^{-1}$. Insets, UV-visible spectra of peaks obtained with diode array analysis.

were analysed by HPLC using an Inertsil ODS-3 column; the results suggested the metabolism of both the cyclic ring-cleavage products furnishing salicylaldehyde (compound III), salicylic acid (compound I) and catechol (compound II) (Supplementary Fig. S3), similar to the lower pathway intermediates detected in the degradation of phenanthrene. However, no such metabolites were detected from parallel resting cell incubation of phenanthrene-grown cells without the addition of cyclic ring-cleavage products as substrates. The ^1H -NMR data thus indicated the formation of cyclic ring-cleavage intermediates arising from both 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid. This is also supported from their lower retention time in HPLC analysis (Fig. 2) compared with that of *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid, the *meta*-cleavage product from 2-hydroxy-1-naphthoic acid with *trans*-orientated olefinic hydrogens with coupling constants of 15.6 Hz, as reported previously

2,2-Dicarboxychromene	2-Hydroxychromene-2-glyoxylic acid
¹ H	
a 8.25 (d), J = 8.28	9.13 (d), J = 9.81
b 7.54 (t), J = 7.39, 7.01	7.87 (t), J = 8.57, 8.0
c 7.60 (t), J = 7.91, 7.69	7.71 (t), J = 8.10, 7.92
d 7.85 (d), J = 8.11	8.16 (d), J = 8.94
e 7.78 (d), J = 8.67	8.22 (d), J = 9.70
f 7.37 (d), J = 8.55	7.48 (d), J = 9.76

Fig. 3. Characterization of 2,2-dicarboxychromene and 2-hydroxychromene-2-glyoxylic acid, the ring-cleavage products (1H2NA-II and 2H1NA-I) of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively, by NMR analysis in D₂O. Chemical shift (p.p.m.); coupling constant, J (Hz); d, doublet; t, triplet.

(Mallick *et al.*, 2007). Direct probe electrospray ionization (ESI)-high-resolution mass spectral analysis in the negative ion mode, for both the HPLC-isolated metabolites, 1H2NA-II and 2H1NA-I, showed a molecular mass of 220, with the major ions at m/z 219 $[M-H]^-$ and 175 $[M-45]^-$, respectively. However, direct probe ESI-mass spectral analysis of the total organic extracts of cell-free enzyme-mediated transformed products of 1-hydroxy-2-naphthoic acid showed the presence of metabolites with molecular masses of 220, 238 and 256, indicating the presence of both monohydrated and dihydrated derivatives of 2,2-dicarboxychromene. Similar analysis of the transformed products of 2-hydroxy-1-naphthoic acid showed metabolites with molecular masses of 220 and 238, indicating the presence of monohydrated derivatives of 2-hydroxychromene-2-glyoxylic acid.

In addition, cell-free extract obtained from phenanthrene-grown cells showed salicylaldehyde dehydrogenase and catechol dioxygenase activities. Supplementary Fig. S4(a) shows cell-free-mediated NAD⁺-dependent conversion of salicylaldehyde to salicylic acid, consistent with data from Eaton & Chapman (1992). The presence of both *meta*-cleavage and *ortho*-cleavage catechol dioxygenase activities was confirmed during the transformation of catechol (Supplementary Fig. S4b). The appearance of a yellow product with $\lambda_{\max}$ at 374 nm (Supplementary Fig. S4b), due to the formation of 2-hydroxymuconaldehyde acid (Kojima *et al.*, 1961), indicates the *meta*-cleavage of catechol by catechol 2,3-dioxygenase. On the other hand, the observed shift of $\lambda_{\max}$ from 275 nm towards 260 nm indicates the *ortho*-cleavage of catechol by catechol 1,2-dioxygenase (Ngai *et al.*, 1990) yielding *cis,cis*-muconic acid (Supplementary Fig. S4b). However, none of these activities could be detected in the cell-free extract obtained from succinate-grown cells, indicating the inducible nature

of these catabolic enzymes. Based on the above analyses, the proposed metabolic pathways involved in the degradation of phenanthrene by strain PNB is elucidated in Fig. 4.

Apart from the formation of a yellow product during transformation of catechol (Kojima *et al.*, 1961), a phenanthrene-induced culture of *Sphingobium* sp. strain PNB was also capable of transforming indole to indigo (a blue dye), a characteristic of various mixed-function oxygenases (Ensley *et al.*, 1983; Gillam & Guengerich, 2001). These biochemical characteristics were employed for plate-based colorimetric screening of a fosmid library constructed from the genomic DNA of strain PNB. Several clones were detected showing either aromatic ring-hydroxylating dioxygenase activity or catechol 2,3-dioxygenase activity. However, neither of the clones showed the presence of both of the classes of dioxygenase activities nor the presence of hydroxynaphthoic acid ring-cleavage dioxygenase activity, indicating that the genes involved in the degradation of phenanthrene in strain PNB are scattered in distantly localized gene clusters as reported in other sphingomonads (Stolz, 2009), in contrast with the gene organization of similar degradative genes in strains belonging to other proteobacterial classes (Mallick *et al.*, 2011). Further genetic characterization of the phenanthrene degradation pathway to delineate its complex regulatory mechanism in *Sphingobium* sp. strain PNB is under way.

DISCUSSION

Strain PNB, a phenanthrene-degrading bacterium, was isolated from a municipal waste-contaminated soil sample and characterized as representing a *Sphingobium* species based on levels of 16S rRNA gene sequence similarity and the results of biochemical analyses. It was shown that strain PNB utilizes phenanthrene in MSM as the sole source of carbon and energy, as established from its growth yield and complete substrate (phenanthrene) removal.

Based on the identification of metabolites by HPLC, GC-MS and NMR, oxygen uptake and UV-visible spectral studies, metabolic pathways of degradation of phenanthrene by strain PNB have been proposed (Fig. 4). Characterization of the initial oxidation and ring-fission products in the degradation of phenanthrene indicated multiple routes of enzyme attack. Strain PNB initiates its attack on phenanthrene by dioxygenation at both 3,4- and 1,2-carbon positions to yield *cis*-3,4-phenanthrenediol and *cis*-1,2-phenanthrenediol, respectively, followed by *meta*-cleavage of the corresponding dihydroxyphenanthrenes to form 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid. Dioxygenation of phenanthrene at multiple positions was ascertained by the identification of compounds IX and X (Fig. 4) based on their mass fragmentation patterns and from comparisons of the major ion peaks with those reported for *cis*-phenanthrenediols (Moody *et al.*, 2001; Mallick *et al.*, 2007). Apart from the above, identification of

Fig. 4. Proposed pathway for the degradation of phenanthrene by *Sphingobium* sp. strain PNB. The transient catechol derivatives and several possible intermediates, which have not been detected by GC-MS analysis, are shown in square brackets. Compounds XVII and XVIII have been characterized by NMR analyses. Filled arrows lead to mineralization; open arrows lead to a dead-end metabolite. Chemical designations: I, salicylic acid; II, catechol; III, salicylaldehyde; V, 2-hydroxy-1-naphthoic acid; VI, 1-hydroxy-2-naphthoic acid; VII, phenanthrene; IX, *cis*-3,4-phenanthrenediol; X, *cis*-1,2-phenanthrenediol; XI, 7,8-benzocoumarin; XII, 5,6-benzocoumarin; XIII, 3,4-dihydroxyphenanthrene; XIV, 1,2-dihydroxyphenanthrene; XV, *cis*-2-oxo-4-(1'-hydroxynaphthyl)-but-3-enoic acid; XVI, *cis*-2-oxo-4-(2'-hydroxynaphthyl)-but-3-enoic acid; XVII, 2-carboxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoic acid; XVIII, 2-oxo-3-hydroxy-5-(2'-oxo-3,5-cyclohexadienyl)-penta-3,5-dienoic acid; XIX, 2,2-dicarboxychromene; XX, 2-hydroxychromene-2-glyoxylic acid; XXI, *trans*-2-hydroxy-2-carboxy-4-(2'-hydroxyphenyl)-but-3-enoic acid; XXII, *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid; XXIII, 2,4-dihydroxy-2-carboxy-4-(2'-hydroxyphenyl)-butanoic acid; XXIV, 2,3-dioxo-5-hydroxy-5-(2'-hydroxyphenyl)-pentanoic acid.

7,8-benzocoumarin (compound XI) and 5,6-benzocoumarin (compound XII) further justified the dioxygenation at 3,4- and 1,2-carbon positions of phenanthrene and subsequent *meta*-cleavage of the respective dihydroxyphenanthrenes (Fig. 4), as supported by earlier reports of phenanthrene metabolism (Pinyakong *et al.*, 2000; Keum

et al., 2006; Seo *et al.*, 2007; Mallick *et al.*, 2007; Ghosal *et al.*, 2010). The above results suggest that metabolism of phenanthrene in strain PNB follows two upper degradation pathways signifying the presence of either diverse ring-hydroxylating dioxygenase systems or a dioxygenase system with broad specificity (Parales, 2003; Seo *et al.*, 2007).

Hydroxynaphthoic acids have been considered as the most crucial metabolic intermediates in the degradation of phenanthrene (Keum *et al.*, 2006; Mallick *et al.*, 2007; Ghosal *et al.*, 2010). During phenanthrene degradation, the two well-documented pathways of degradation of hydroxynaphthoic acid are oxidative decarboxylation of hydroxynaphthoic acids to 1,2-dihydroxynaphthalene followed by *meta*- or *ortho*-cleavage, and direct *ortho* ring-cleavage of 1-hydroxy-2-naphthoic acid yielding 2-carboxybenzalpyruvate (Iwabuchi & Harayama, 1997; Keum *et al.*, 2006; Seo *et al.*, 2006, 2007). However, in our previous studies on the degradation of phenanthrene, direct *meta*-cleavage of 2-hydroxy-1-naphthoic acid, a third possible pathway, was proposed in both Gram-positive and Gram-negative bacteria (Mallick *et al.*, 2007; Ghosal *et al.*, 2010). In the present study, this *meta*-cleavage pathway has been shown to apply also to 1-hydroxy-2-naphthoic acid. Upon transformation of both 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid by cell-free extract of phenanthrene-grown cells, the spectral changes in the UV-visible region were consistent with ring-cleavage activity. In addition, *meta*-cleavage of hydroxynaphthoic acids (Fig. 4) has been substantiated by the characterization of 2,2-dicarboxychromene (compound XIX) and 2-hydroxychromene-2-glyoxylic acid (compound XX) by NMR analysis as the transformed products of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively. Moreover, it was observed that the *meta*-cleavage enzyme(s) catalysing hydroxynaphthoic acids possesses dissociable ferric ion as the cofactor. On the other hand, ferrous ion was observed as the cofactor for *ortho*-cleavage enzymes, 1-hydroxy-2-naphthoate dioxygenase and gentisate dioxygenase (Harpel & Lipscomb, 1990; Adachi *et al.*, 1999).

In a previous study on the degradation of phenanthrene by *Staphylococcus* sp. strain PN/Y, *meta*-cleavage of 2-hydroxy-1-naphthoic acid was reported via *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid, leading to the formation of salicylic acid (Mallick *et al.*, 2007). Based on the structural relationship, it is speculated that 2-hydroxychromene-2-glyoxylic acid, characterized by NMR analysis in this study, may be the possible precursors of *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid (compound XXII, Fig. 4). However, in the metabolism of 2-hydroxy-1-naphthoic acid, apart from 2-hydroxychromene-2-glyoxylic acid, ESI-MS data indicated the presence of a metabolite with molecular mass of 238, possibly a hydrated derivative of *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid (compound XXIV, 2,3-dioxo-5-hydroxy-5-(2'-hydroxyphenyl)-pentanoic acid, Fig. 4). This hydrated derivative is believed to be transformed to salicylaldehyde by the action of aldolase present in the cell-free extract. On the other hand, in the metabolism of 1-hydroxy-2-naphthoic acid, apart from 2,2-dicarboxychromene, ESI-MS data indicated the presence of metabolites with molecular masses of 238 and 256. These are possibly the monohydrated and dihydrated products of 2,2-dicarboxychromene, which may be the hydrolysed product

of 2,2-dicarboxychromene (compound XXI), 2-hydroxy-2-carboxy-4-(2'-hydroxyphenyl)-but-3-enoic acid (a possible *trans*-product) and the hydrated derivative of the latter compound, 2,4-dihydroxy-2-carboxy-4-(2'-hydroxyphenyl)-butanoic acid (compound XXIII), respectively (Fig. 4). Note that 2,4-dihydroxy-2-carboxy-4-(2'-hydroxyphenyl)-butanoic acid is possibly the substrate for aldolase yielding salicylaldehyde.

Preliminary investigations on the screening of the fosmid library indicated that the phenanthrene catabolic operons in strain PNB are likely to be scattered into several gene clusters, as with other sphingomonads (Stolz, 2009). Thus, further cloning and characterization of catabolic genes and purification of key enzymes will allow us to better understand the mechanistic details of this unique third possible pathway of hydroxynaphthoic acid metabolism, providing new insight into the bacterial degradation of phenanthrene in particular and of PAHs in general.

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