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Y. Prabhu · P. S. Phale

Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation

Abstract *Pseudomonas* sp. strain PP2 isolated in our laboratory efficiently metabolizes phenanthrene at 0.3% concentration as the sole source of carbon and energy. The metabolic pathways for the degradation of phenanthrene, benzoate and *p*-hydroxybenzoate were elucidated by identifying metabolites, biotransformation studies, oxygen uptake by whole cells on probable metabolic intermediates, and monitoring enzyme activities in cell-free extracts. The results obtained suggest that phenanthrene degradation is initiated by double hydroxylation resulting in the formation of 3,4-dihydroxyphenanthrene. The diol was finally oxidized to 2-hydroxymuconic semialdehyde. Detection of 1-hydroxy-2-naphthoic acid, α-naphthol, 1,2-dihydroxy naphthalene, and salicylate in the spent medium by thin layer chromatography; the presence of 1,2-dihydroxynaphthalene dioxygenase, salicylaldehyde dehydrogenase and catechol-2,3-dioxygenase activity in the extract; O_2 uptake by cells on α-naphthol, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylate and catechol; and no O_2 uptake on *o*-phthalate and 3,4-dihydroxybenzoate supports the novel route of metabolism of phenanthrene via 1-hydroxy-2-naphthoic acid $\rightarrow$ [α-naphthol] $\rightarrow$ 1,2-dihydroxy naphthalene $\rightarrow$ salicylate $\rightarrow$ catechol. The strain degrades benzoate via catechol and *cis,cis*-muconic acid, and *p*-hydroxybenzoate via 3,4-dihydroxybenzoate and 3-carboxy-*cis,cis*-muconic acid. Interestingly, the culture failed to grow on naphthalene. When grown on either hydrocarbon or dextrose, the culture showed good extracellular biosurfactant production. Growth-dependent changes in the cell surface hydrophobicity, and emulsification activity experiments suggest that: (1) production of biosurfactant was constitutive and growth-associated, (2) production was higher when cells were grown on phenanthrene as compared to dextrose and benzoate, (3) hydrocarbon-grown cells were more hydrophobic and showed higher affinity towards both aromatic and aliphatic hydrocarbons compared to dextrose-grown cells, and (4) mid-log-phase cells were significantly (2-fold) more hydrophobic than stationary phase cells. Based on these results, we hypothesize that growth-associated extracellular biosurfactant production and modulation of cell surface hydrophobicity plays an important role in hydrocarbon assimilation/uptake in *Pseudomonas* sp. strain PP2.

Introduction

Polycyclic aromatic hydrocarbons (PAH) constitute a large and diverse class of organic compounds consisting of three or more fused aromatic rings in various structural configurations. Increase in aromatic rings, structural angularity, and hydrophobicity make PAH recalcitrant to degradation. PAH are widely distributed in the environment and are carcinogenic, mutagenic and toxic (Kanaly and Harayama 2000; Langworthy et al. 2002). Phenanthrene, a three-ring PAH, is an ideal model system to study the various aspects of microbial metabolism and physiology. Various pathways and metabolic diversity involved in phenanthrene biodegradation are well documented (Doddamani and Ninnekar 2000; Iwabuchi and Harayama 1997; Saito et al. 1999, 2000; Samanta et al. 1999). Bacteria degrade phenanthrene by one of two pathways. Both pathways are initiated by double hydroxylation of the phenanthrene ring by a dioxygenase enzyme to yield *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (Gibson and Subramanian 1984), which undergoes enzymatic dehydrogenation to 3,4-dihydroxyphenanthrene. The diol is cleaved and subsequently metabolized to 1-hydroxy-2-naphthoic acid, which is degraded by two distinct routes. In route one, 1-hydroxy-2-naphthoic acid is ring cleaved by a dioxygenase to yield 2'-carboxy benzalpyruvate (Adachi et al. 1999; Iwabuchi and Harayama 1998), which is further metabolized to 3,4-dihydroxybenzoate via 2'-carboxybenzaldehyde and *o*-phthalate. Genes re-

Y. Prabhu · P. S. Phale (✉)
Biotechnology group,
BJM School of Biosciences and Bioengineering,
Indian Institute of Technology Bombay,
400 076 Powai, Mumbai, India
e-mail: pphale@btc.iitb.ac.in
Fax: +91-22-25723480

sponsible for conversion of phenanthrene to *o*-phthalate have been cloned and sequenced from *Nocardioides* sp. strain KP7 (Saito et al. 1999). In route two, 1-hydroxy-2-naphthoic acid is metabolized to 1,2-dihydroxynaphthalene. The diol is ring cleaved by dihydroxynaphthalene dioxygenase to 2'-hydroxybenzalpyruvate, which is metabolized to catechol via salicylaldehyde and salicylic acid. Organisms degrading phenanthrene by route two also have the ability to degrade naphthalene via 1,2-dihydroxynaphthalene, salicylate, and catechol (Kiyohara et al. 1994; Takizawa et al. 1994; Yang et al. 1994). Metabolic diversity in these pathways is further generated by the conversion of 1-hydroxy-2-naphthoic acid to α-naphthol, which is metabolized via either the salicylate or phthalate pathway (Samanta et al. 1999) and degradation of salicylate via the gentisate pathway (Gibson and Subramanian 1984).

The limiting step in the degradation of phenanthrene and other PAH is their insolubility, thus decreasing the efficiency and rate of degradation. This limitation can be overcome either by addition of surface-active compounds surfactant to the growing culture, thus making hydrocarbons more water-soluble and available for the cell to degrade, or by production of its own surfactant by the organism to facilitate uptake. Surfactants are 'amphiphilic' molecules consisting of both hydrophobic and hydrophilic domains giving them the characteristic property of organizing at interfaces of different degrees of polarity such as oil/water or air/water and helping to lower the interfacial energy/tension. Due to these properties, biosurfactants find a wide range of applications in various industries (Banat et al. 2000; Desai and Banat 1997; Georgiou et al. 1992). Several groups have reported improved efficiency and rates of hydrocarbon degradation when cultures were supplemented with biosynthetic or chemically synthesized surfactants (Barkay et al. 1999; Beal and Betts 2000; Gu and Chang 2001; Moran et al. 2000; Sandrin et al. 2000; Schippers et al. 2000). Increased levels of mineralization of phenanthrene by strains of *Pseudomonas* were observed when cultures were co-inoculated with rhamnolipid-producing *Pseudomonas aeruginosa* (Dean et al. 2001). In the case of hexadecane-degrading *P. aeruginosa*, external addition of rhamnolipid leads to efficient degradation of hexadecane (Noordman et al. 2002; Shreve et al. 1995). Some hydrocarbon-degrading microbes respond to these nonsoluble carbon sources by producing surface-active compounds, as well as by changing cell surface properties such as cell surface hydrophobicity (Al-Tahhan et al. 2000; Beal and Betts 2000; Bouchez-Naitali et al. 1999; Goswami and Singh 1991; Kobayashi et al. 1999; Makin and Beveridge 1996; Phale et al. 1995a; Zhang and Miller 1994). In a microbial culture, there are three pools of biosurfactants: an intracellular pool including membrane lipids, an extracellular pool including excreted polysaccharide-protein-lipid complex, and an interposed cell surface pool located on the cell wall and capsule including lipid and lipid-polymer complex. Production of biosurfactant by cells will help to pseudo-solubilize

hydrocarbons and facilitate its uptake (Haferberg et al. 1986; Phale 1994; Rosenberg 1986; Rosenberg and Ron 1999; Zajic and Seffens 1984). Biosurfactants from the cell surface pool may lead to changes in cell surface properties, e.g., hydrophobicity. Besides its role in hydrocarbon uptake, cell surface hydrophobicity plays an important role in bacterial invasion and infection, cell adhesion, biofilm formation, etc. (van Loosdrecht et al. 1987; de Maagd et al. 1989; Makin and Beveridge 1996; Minagi et al. 1986; Mukherjee et al. 1992; Panagoda et al. 2001; Singleton et al. 2001; Stenström 1989; Wibawan and Lammler 1992).

Here, we report faster and more efficient degradation of phenanthrene by a soil isolate *Pseudomonas* sp. strain PP2 via the α-naphthol, 1,2-dihydroxynaphthalene, salicylate, and catechol route. Besides hydrocarbon metabolism, cells were found to produce growth-dependent extracellular biosurfactant and bring about changes in cell surface hydrophobicity. Based on these findings, we hypothesize an interplay between cell surface hydrophobicity and extracellular biosurfactant production, and its role in hydrocarbon assimilation.

Materials and methods

Phenanthrene, salicylate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, catechol, 1,2-dihydroxynaphthalene, and 1-hydroxy-2-naphthoic acid were purchased from Sigma-Aldrich (St. Louis, Mo.). All other chemicals used were of analytical reagent grade and purchased locally.

Bacterial culture and growth conditions

Pseudomonas sp. strain PP2 was isolated in our laboratory from gas station soil (Ahmednagar, India) by an enrichment culture technique using phenanthrene as the sole source of carbon and energy. The culture was grown on 150 ml mineral salt medium (MSM, Phale et al. 1995b) in 500 ml capacity baffled flasks at 30°C on a rotary shaker. The medium was supplemented with 100 mg solid phenanthrene per 150 ml MSM (0.067%) as the sole source of carbon and energy. Other hydrocarbons (benzoate and *p*-hydroxybenzoate) were supplemented at a concentration of 0.1%, w/v.

Metabolite analysis

To isolate intermediate metabolites of the phenanthrene pathway, the culture was grown on phenanthrene until early (30 h) and late (120 h) growth phase. The metabolites were extracted from the acidified spent medium (pH adjusted to 2.0 with HCl after centrifugation) into an equal volume of ethyl acetate. The organic phase was dried over anhydrous sodium sulphate, concentrated, and resolved on 0.5 mm thick silica gel plates by thin layer chromatography (TLC) using hexane:chloroform:acetic acid (10:3:2, v/v/v) as a solvent system. Proper controls (MSM and phenanthrene) were performed to ascertain abiotic reactions. Metabolites were identified by comparing R_f and UV-fluorescence properties with authentic compounds.

Preparation of cell-free extract

Cell-free extracts were prepared by growing cells on phenanthrene (72 h), benzoate (7 h) or dextrose (0.2%, 7 h). Cells were harvested

by centrifugation (12,000 *g* for 10 min) and washed twice with phosphate buffer (50 mM, pH 7.5). Cells (1 g) were suspended in 4 ml ice-cold phosphate buffer and sonicated at 4°C in three cycles of ten pulses each (1 s pulse with 1 s interval, cycle duration 20 s, power output 8 W) using an Ultrasonic processor (model GE130; General Electric, Cleveland, Ohio). The cell homogenate obtained was centrifuged at 19,000 *g* for 15 min. The clear pale yellow-brown supernatant obtained was referred to as cell-free extract and used for monitoring various enzyme activities. Protein estimation was carried out as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Enzyme assays

Catechol-1,2-dioxygenase (Hayaishi et al. 1957), catechol-2,3-dioxygenase (Kojima et al. 1961), gentisate dioxygenase (Harpel and Lipscomb 1990), 1-hydroxy-2-naphthoate dioxygenase (Iwabuchi and Harayama 1998) and 3,4-dihydroxybenzoate dioxygenase (Stanier and Ingraham 1954) were monitored as described. To identify the reaction products, time-dependent spectral changes for catechol-1,2-dioxygenase, catechol-2,3-dioxygenase, 1-hydroxy-2-naphthoate dioxygenase and 3,4-dihydroxy benzoate dioxygenase were monitored every 10 s in the 200–500 nm range using a Shimadzu UV-visible spectrophotometer (model UV-160). Activity of 1,2-dihydroxynaphthalene dioxygenase was monitored (Kuhm et al. 1991) using an oxygraph. The reaction mixture (1 ml) contained 1,2-dihydroxynaphthalene (final concentration 0.1 mM in 10 μl tetrahydrofuran), an appropriate amount of extract and acetate buffer (50 mM, pH 5.5). Enzyme units are defined as nanomoles of substrate disappearing or product appearing, or nanomoles of O_2 consumed per minute. Specific activity is defined as U min^{-1} mg protein^{-1}.

Oxygen uptake studies

Oxygen uptake by whole cells in the presence of various probable metabolic intermediates was carried out as described earlier (Phale et al. 1995b) with minor modifications. Cell suspension was prepared by growing cells until late-log phase on the appropriate hydrocarbon, followed by centrifugation, washing twice with phosphate buffer (50 mM, pH 7.0) and re-suspending 100 mg (wet wt) cells in 1 ml phosphate buffer. In an oxygraph reaction chamber, 2 ml assay mixture contained 4 mg cells, substrate (0.1 mM) and an appropriate amount of phosphate buffer (50 mM, pH 7.0). Oxygen uptake rates were measured at 30°C using an Oxygraph (Hansatech, King's Lynn, Norfolk, UK) fitted with a Clark's type oxygen electrode. The rates are expressed as nmol O_2 consumed min^{-1} (mg wet cells)$^{-1}$. The rates were corrected for endogenous oxygen consumption.

Emulsification assay

Production of extracellular biosurfactant by the culture was analyzed by monitoring the ability of surfactant to stabilize 1-naphthaldehyde-in-water emulsion as described earlier (Phale et al. 1995a). The assay mixture (5 ml) contained 200 μl spent medium, 3.8 ml phosphate buffer (50 mM, pH 7.5), and 1 ml 1-naphthaldehyde-in-water emulsion (100 μl naphthaldehyde in 10 ml double-distilled water followed by 1 min sonication). The mixture was vortexed for 1 min and incubated at room temperature for 5 h. The absorbance due to stability of emulsion was measured at 660 nm. One unit is defined as the amount of biosurfactant required to obtain an increase in absorbance of 1.0 OD unit.

Determination of cell surface hydrophobicity

Affinity of cells towards various aromatic (xylene and benzene) and aliphatic (hexane and heptane) hydrocarbons was measured by the method of Rosenberg et al. (1980). Cells were grown on benzoate, dextrose or phenanthrene, harvested, washed twice with phosphate buffer (50 mM, pH 7.5), and re-suspended in the same buffer so as to obtain a cell suspension with a final OD of 0.300 at 600 nm. The 6 ml assay mixture contained 3 ml cell suspension and 3 ml test hydrocarbon. After 5 min of pre-incubation, the mixture was vortexed for 60 s and incubated for an additional 15 min at room temperature. The OD of the aqueous phase was measured at 600 nm. The cell surface hydrophobicity was expressed as percent cells transferred to hydrocarbon phase by measuring the OD of the aqueous phase before and after mixing and was calculated using following formula:

%Cells transferred to hydrocarbon = 100

$$-\left[\frac{OD_{600} \text{ after mixing}}{OD_{600} \text{ before mixing}} \right] \times 100 \qquad (1)$$

Results

Culture identification and hydrocarbon degradation properties

Using an enrichment culture technique, a phenanthrene-degrading soil bacterium was isolated and identified as *Pseudomonas* on the basis of various physiological and biochemical tests as described in *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974). The bacterium is Gram negative, motile (polar flagella) rod-like, and grows aerobically. Results for various biochemical tests were: oxidase positive, nitrogenase negative, growth on 12–15% NaCl negative, requirement of cysteine and iron salts for growth negative, arginine dihydrolase positive, gelatin liquification positive, citrate utilization positive, O-F test positive, intracellular polyhydroxybutyrate accumulation positive, growth on cetrimide agar positive, requirement of growth factors negative ,and growth at pH 3.6 negative. These results identify the isolate as belonging to the family *Pseudomonadaceae* and the genus *Pseudomonas*. To differentiate it from others, this isolate is designated as *Pseudomonas* sp. strain PP2.

Compared to other degraders, *Pseudomonas* sp. strain PP2 is unique as it metabolizes phenanthrene rapidly up to the highest concentration so far tried (0.3%). The organism takes 72–84 h to reach stationary phase when supplemented with 0.067% phenanthrene. The culture also utilizes salicylate, benzoate and *p*-hydroxybenzoate as sole sources of carbon and energy. However, naphthalene, 1-methylnaphthalene, 1- and 2-naphthoic acid, phthalate isomers (*o*-, *m*- and *p*-), and *m*-hydroxybenzoate did not support any growth.

TLC analysis of phenanthrene metabolites

To identify intermediates of the phenanthrene pathway, TLC analysis of early and late-growth phase spent medium was carried out. R_f and UV-fluorescence properties of standard compounds and identified metabolites are summarized in Table 1. 1-Hydroxy-2-napthoic acid,

Table 1 Thin layer chromatographic (TLC) analysis of phenanthrene metabolites. The biotransformation reaction was carried out by incubating phenanthrene-grown cells with 1-hydroxy-2-naphthoic acid for 3 h at 30°C and metabolites were extracted as described in Materials and methods. The salicylaldehyde dehydrogenase enzyme reaction was carried out on a large scale: 10 ml reaction mixture contained phosphate buffer (100 mM, pH 7.5), an appropriate amount of enzyme, NAD^+ (0.1 mM), and salicylaldehyde (0.1 mM). The reaction mixture was incubated at 30°C for 30 min and products were extracted as described in Materials and methods. 1-OH-2-NA 1-hydroxy-2-naphthoic acid, 1,2-(OH) Naph 1,2-dihydroxynaphthalene

Standards			Metabolites identified from[a]			
Compound	R_f	UV–fluorescence	Phenanthrene culture		Biotransformation of 1-(OH)-2-NA	Enzyme reaction with salicylaldehyde
			Early	Late		
Phenanthrene	0.96	Black, non-fluorescent	++[b]	+	–	–
1-(OH)-2-NA	0.78	Purple blue, fluorescent	++	–	+	–
α–Naphthol	0.68	Yellow/ brown, non-fluorescent	+	+	+	–
1,2-(OH) Naph	0.24	Black, non- fluorescent	+	+	–	–
Salicylate	0.71	Blue, fluorescent	–	++	–	+
Phthalate	0.18	Black, non- fluorescent	–	–	–	–
3,4–Dihydroxy benzoate	0.09	Black, non- fluorescent	–	–	–	–

[a] Compounds were identified by comparing the R_f and UV-fluorescence with those of the authentic compounds, no metabolites were detected in abiotic control experiments
[b] ++ High intensity, + medium intensity, – not detected

Table 2 Oxygen uptake rates by *Pseudomonas* sp. strain PP2 cells on various probable metabolites

Substrate	Oxygen uptake rates (nmol O_2 consumed min^{-1} mg^{-1}) by cells grown on[a]			
	Phenanthrene	Benzoate	*p*–Hydroxybenzoate	Dextrose
1-Hydroxy-2-naphthoic acid	7.5	–[b]	–	tr[c]
α-Naphthol	1.5	–	–	tr
1,2-Dihydroxynaphthalene	3.0	–	–	–
Salicylaldehyde	8.8	–	–	tr
Salicylate	1.8	tr	–	tr
Catechol	28	37.2	tr	tr
Benzoate	–	25.6	tr	tr
p-Hydroxybenzoate	–	tr	19	–
3,4-Dihydroxybenzoate	tr	tr	23.2	tr
2,3-Dihydroxybenzoate	tr	–	–	tr
2,5-Dihydroxybenzoate	tr	–	–	–
o-Phthalate	tr	–	–	–

[a] All values are corrected for the endogenous O_2 uptake
[b] Not determined
[c] Trace (0–0.5 nmol O_2 consumed min^{-1} mg^{-1}) or could not be detected by this method

α-naphthol, 1,2-dihydroxynaphthalene, and salicylate were identified as intermediate products by comparing R_f and UV-fluorescence properties. Early culture extract showed a prominent metabolite spot corresponding to 1-hydroxy-2-napthhoic acid, while the late culture showed a major spot corresponding to salicylate. However, the extract failed to show any spots corresponding to phthalate and 3,4-dihydroxybenzoate. Whole cell biotransformation experiments with phenanthrene-grown cells using 1-hydroxy-2-naphthoic acid as a substrate, yielded a metabolite with R_f and fluorescence properties similar to α-naphthol (Table 1).

Oxygen uptake by whole cells

Oxygen uptake studies were carried out on various probable metabolic intermediates of the pathway. The rates are summarized in Table 2. Phenanthrene-grown cells showed good O_2 uptake on 1-hydroxy-2-naphthoic acid, α-naphthol, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylate and catechol. However, cells failed to respire on phthalate, 3,4-dihydroxybenzoate, 2,3-dihydroxybenzoate, and 2,5-dihydroxybenzoate. Dextrose-grown cells failed to respire on any of these intermediates.

Enzyme activities in cell-free extracts

Enzyme extract prepared from phenanthrene-grown cells showed 1,2-dihydroxynaphthalene dioxygenase, 1-hydroxy-2-naphthoic acid dioxygenase and catechol-2,3-dioxygenase activity (Table 3). The extract also showed NAD^+-dependent conversion of salicylaldehyde to salicylate as analyzed by TLC (Table 1) indicating the presence of the enzyme salicylaldehyde dehydrogenase. The presence of *meta*-cleaving catechol-2,3-dioxygenase activity was confirmed by the appearance of a yellow-

Table 3 Specific activities of various enzymes in the cell-free extracts of *Pseudomonas* sp. strain PP2 on different carbon sources. Spectral changes observed for various enzyme reactions with their respective substrates: catechol-2,3-dioxygenase showed increase at 375 nm as a result of formation of 2-hydroxymuconicsemialdehyde, confirming *meta* cleavage; catechol-1,2-dioxygenase showed increase at 260 nm due to formation of *cis,cis*-muconic acid, confirming *ortho* cleavage; 3,4-dihydroxybenzoate dioxygenase showed decrease at 288 due to disappearance of 3,4-dihydroxybenzoate and formation of 3-carboxy-*cis,cis*-muconic acid, confirming *ortho* cleavage; and 1-hydroxy-2-naphthoate dioxygenase showed increase at 300 nm due to formation of 2'-carboxybenzalpyruvate, confirming *meta* cleavage

Enzyme	Specific activity (U min^{-1} mg protein^{-1})			
	Phenanthrene	Benzoate	*p*-Hydroxybenzoate	Dextrose
Catechol-2,3-dioxygenase	290	nd[a]	nd	nd
1,2-Dihydroxynaphthalene dioxygenase[c]	71	–[b]	–	–
1-Hydroxy-2-naphthoate dioxygenase	100	–	–	nd
Benzoate-1,2-dioxygenase	–	0.5	–	–
Catechol-1,2-dioxygenase	nd	4.5	nd	nd
3,4-Dihydroxybenzoate dioxygenase[c]	nd	nd	1,000	nd
2,5-Dihydroxybenzoate dioxygenase	nd	nd	–	nd

[a] Not detectable
[b] Not determined
[c] Oxygraph assay, nmol O_2 consumed min^{-1} mg protein^{-1}

Table 4 Biosurfactant production by *Pseudomonas* sp. strain PP2 on various carbon sources

Carbon source	Cell mass (g/l)	Total emulsification activity (U/l)	Emulsification activity U/g cell mass
Dextrose	3.0	9,000	3,000
Benzoate	3.0	7,250	2,416
Phenanthrene	1.0	6,250	6,250

colored product with λ_{max} at 375 nm, due to formation of 2'-hydroxymuconicsemialdehyde. The enzyme extract failed to show any activity for 3,4-dihydroxybenzoate dioxygenase. None of these activities could be detected in the extract prepared from dextrose-grown cells (Table 3).

Benzoate and *p*-hydroxybenzoate metabolism

To deduce the benzoate and *p*-hydroxybenzoate pathway, whole cell O_2 uptake studies and key enzyme activities in cell-free extracts were monitored. Benzoate-grown cells showed O_2 uptake on catechol and benzoate; however, cells failed to respire on phenol, *p*-hydroxybenzoate, and 3,4-dihydroxybenzoate (Table 2). Benzoate cell-free extract showed good activity of *ortho*-cleaving catechol-1,2-dioxygenase but failed to show any activity of 3,4-dihydroxybenzoate dioxygenase (Table 3). *p*-Hydroxybenzoate-grown cells showed good O_2 uptake with 3,4-dihydroxybenzoate and *p*-hydroxybenzoate (Table 2), while cell-free extract showed good activity of 3,4-dihydroxybenzoate dioxygenase and no activity of catechol-1,2- and 2,3- dioxygenase. Dextrose-grown cells failed to show O_2 uptake or enzyme activities of these pathways (Table 3).

Growth-dependent extracellular surfactant production

Spent medium of the culture was frothy and viscous, typical of many surfactant-producing microorganisms. The emulsification assay method followed is based on the ability of surfactant to stabilize preformed 1-naphthaldehyde-in-water emulsion (Phale et al 1995a). Preformed naphthaldehyde-in-water emulsion is highly unstable after vortexing leading to phase separation and coalescence. The presence of surface-active compound stabilizes this preformed emulsion and makes it resistant to coalescence. The spent medium of PP2 culture showed good emulsion-stabilization activity when the culture was grown on phenanthrene (6.25 U/ml), benzoate (7 U/ml), or dextrose (9 U/ml) (Table 4, Fig. 1). Quantitatively, surfactant produced per gram of cell mass was 3,000 U/g for dextrose, 2,416 U/g for benzoate, and 6,250 U/g for phenanthrene. Detection of emulsification activity from the dextrose culture suggests that production of surfactant is constitutive. However, a 2-fold increase was observed when grown on insoluble hydrocarbon (phenanthrene) as a carbon source. Other properties observed (data not shown) were: (1) Saturation phenomenon: when a fixed amount of surfactant with varying concentrations of substrate was used in emulsification assay and vice versa. (2) Surfactant showed activity in the range pH 7–10. At acidic pH, no activity could be detected due to rapid coalescence of the emulsion leading to phase separation. (3) No requirement of divalent metal ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, or Fe^{2+} (1 mM) for maximum activity. Addition of metal chelators like EDTA (1 mM and 5 mM) had no inhibitory effect on the emulsification activity. (4) Over a period of time (24 h), the emulsion settles without coalescence or phase separation, indicating that the
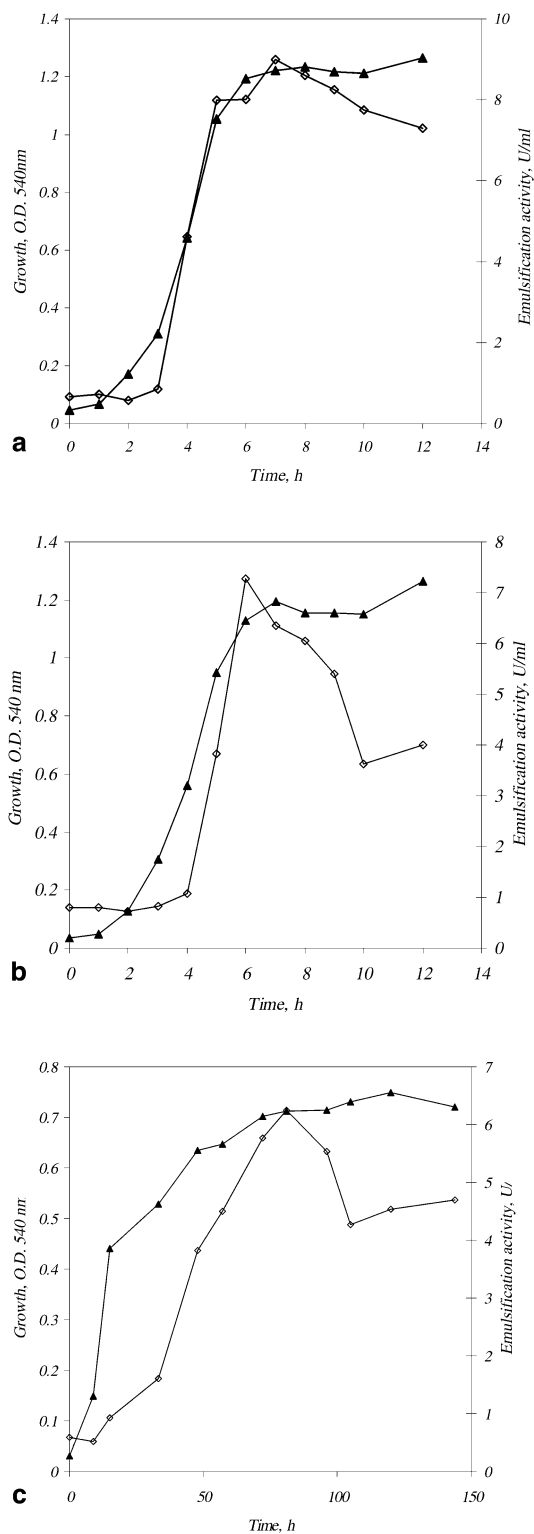
Fig. 1 Growth-associated production of biosurfactant by *Pseudomonas* sp. strain PP2 on **a** dextrose, **b** benzoate and **c** phenanthrene. *Solid triangles* Cell growth monitored at 540 nm, *open diamonds* emulsification activity

emulsion formed is stabilized by the biosurfactant secreted by the culture.

Cell surface properties

The involvement of surfactant in hydrocarbon assimilation/uptake was monitored by correlating biosurfactant production as a function of emulsification activity and growth of *Pseudomonas* sp. strain PP2 on phenanthrene, benzoate, or dextrose. Irrespective of the carbon source, the culture showed a growth-dependent increase in extracellular biosurfactant production (Fig. 1). Activity starts increasing from early-log phase, peaks at the late-log to early-stationary phase transition, and drops during the stationary phase. The profiles of emulsification activity vs growth for hydrocarbon- and dextrose-grown cultures were the same. The growth-dependent changes in cell surface hydrophobicity as measured by analyzing cell affinity towards aromatic (xylene and benzene) and aliphatic (hexane and heptane) hydrocarbons are summarized in Table 5. Irrespective of the carbon source, cells showed higher affinity towards aromatic, compared to aliphatic, hydrocarbons. However, phenanthrene-grown stationary phase cells showed high affinity towards both aromatic and aliphatic hydrocarbons. Log phase phenanthrene- and benzoate-grown cells showed significantly higher affinity for aromatic and aliphatic hydrocarbons compared to dextrose-grown cells. The increase was substantial in the early-log phase (5 h) as compared to late-log-stationary phase (7 h) cells (Table 5). These results suggest that the growth-dependent increase in biosurfactant production and cell surface hydrophobicity probably plays an important role in hydrocarbon assimilation/uptake.

Discussion

Several phenanthrene-degrading microorganisms reported so far mineralize phenanthrene at very low concentrations (100 mg to 1 g/l) either as a sole source of carbon or by co-metabolizing it with other carbon sources. *Pseudomonas* sp. strain PP2, a soil isolate, is unique as it efficiently degrades high concentrations (0.3%) of phenanthrene as the sole carbon source and produces surface-active compound(s). The strain grows fast and attains stationary phase by 80 h (Fig. 1). Metabolism of phenanthrene by two distinct routes, via either phthalate or salicylate, has been well documented (Iwabuchi and Harayama 1997; Saito et al. 2000; Samanta et al. 1999). The sequence involved in the degradation of phenanthrene by strain PP2 was elucidated by identifying metabolites from the spent medium, O_2 uptake studies, and monitoring enzyme activities in cell-free extracts. Identification of 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, α-naphthol, and salicylate as metabolites by TLC analysis (Table 1); oxygen uptake by phenanthrene-grown cells on 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, α-

Table 5 Growth-dependent changes in the cell surface hydrophobicity of *Pseudomonas* sp. strain PP2. Cells were grown on different carbon sources for the time periods indicated. Cell surface hydrophobicity was calculated as described in Materials and methods. Each time point was performed in triplicate in at least two independent experiments. Standard deviation values were in the range of ±2–5%

Hydrocarbon	% Cells transferred to hydrocarbon phase											
	Benzoate					Dextrose					Phenanthrene	
	3 h	5 h	7 h	9 h	12 h	3 h	5 h	7 h	9 h	12 h	Log phase	Stationary phase
Benzene	57	74	59	35	37	48	50	33	30	30	69	58
Xylene	40	74	47	21	20	36	38	21	24	20	51	45
Hexane	33	78	32	13	10	36	36	23	19	10	49	42
Heptane	15	71	28	12	7	18	21	10	8	4	46	38

naphthol, salicylaldehyde, salicylate and catechol (Table 2); and detection of 1,2-dihydroxynaphthalene dioxygenase, salicylaldehyde dehydrogenase, and catechol-2,3-dioxygenase activities in the extracts of phenanthrene-grown *Pseudomonas* sp. strain PP2 cells (Table 3) suggests the probable phenanthrene degradation pathway as: 1-hydroxy-2-naphthoic acid → [α-naphthol] →1,2-dihydroxynaphthalene → salicylate → catechol. This pathway is further supported by: (1) failure to detect phthalate as a metabolite in the spent medium, (2) no growth on phthalate, (3) inability of phenanthrene-grown cells to respire on phthalate and 3,4-dihydroxybenzoate, and (4) absence of 3,4-dihydroxybenzoate dioxygenase activity in phenanthrene-grown cell-free extract. However, p-hydroxybenzoate-grown cells showed very high 3,4-dihydroxybenzoate dioxygenase activity (Table 3). These results ruled out the possibility of a phthalate → 3,4-dihydroxybenzoate route of phenanthrene degradation by *Pseudomonas* sp. strain PP2. Probable involvement of α-naphthol and salicylate in phenanthrene biodegradation by *Brevibacterium* sp. has been reported earlier by Samanta et al. (1999). Here, we report that phenanthrene-grown PP2 cells have the ability to respire on and accumulate α-naphthol in the spent medium, suggesting α-naphthol as a probable intermediate in the pathway This warrants further investigation by purifying and characterizing the enzymes involved in α-naphthol metabolism. Based on these results, we propose the pathway (Fig. 2) involved in the degradation of phenanthrene, as well as benzoate and p-hydroxy benzoate. As reported for several phenanthrene-degrading bacterial strains (Gibson and Subramanian 1984; Iwabuchi and Harayama, 1997; Saito et al. 2000; Samanta et al. 1999), we propose that, in *Pseudomonas* sp. strain PP2, phenanthrene degradation is initiated by double hydroxylation resulting in the formation of *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, which is further converted enzymatically to 3,4-dihydroxyphenanthrene → → 1-hydroxy-2-naphthoic acid. The hydroxynaphthoate thus generated enters the TCA cycle via the 1,2-dihydroxynaphthalene, salicylate and catechol pathway (naphthalene route).

Intriguingly, besides detection of 1,2-dihydroxynaphthalene dioxygenase, salicylaldehyde dehydrogenase, and catechol-2,3-dioxygenase activities in the phenanthrene-grown cell-free extract, we also detected 1-hydroxy-2-
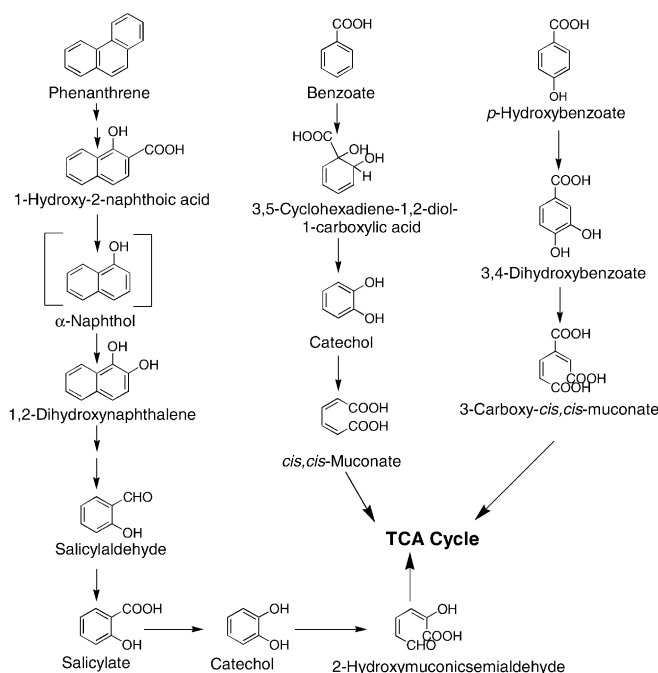

Fig. 2 Proposed metabolic pathway for the degradation of phenanthrene, benzoate and p-hydroxybenzoate by *Pseudomonas* sp. strain PP2. Involvement of α-naphthol as a metabolic intermediate is based on O_2 uptake, growth of organism on α-naphthol, and its accumulation in the spent medium during growth on phenanthrene

naphthoic acid dioxygenase activity. The observed increase in absorbance at 300 nm could be due to the formation of the ring cleaved product, 2'-carboxybenzalpyruvate, as reported earlier by Barnsley (1983) and Adachi et al. (1999). This enzyme is involved in the phthalate → 3,4-dihydroxybenzoate route of phenanthrene metabolism. The results obtained with strain PP2 suggest that the phthalate route is non-operative. Therefore, detection of the ring cleavage product of 1-hydroxy-2-naphthoic acid in the extracts could be a result of relaxed substrate specificity exhibited by 1,2-dihydroxynaphthalene dioxygenase. Naphthalene diol dioxygenase has been reported to accept hydroxylated naphthalene diols, and dihydroxy biphenyls as well as methyl catechols as substrates (Kuhm et al. 1991; Patel and Barnsley 1980; Tsoi et al. 1988). However, so far there

are no reports on the activity of this enzyme with 1-hydroxy-2-naphthoic acid as a substrate. Purification of 1-hydroxy-2-naphthoic acid dioxygenase and the structure of the reaction product have been characterized by Adachi et al. (1999). However, the activity of this enzyme on various dihydroxy and hydroxy substituted naphthalenes has not been investigated or reported so far.

It has been well documented that cultures degrading phenanthrene via the salicylate/catechol route have the ability to grow on naphthalene due to metabolic pathway similarity (Siato et al. 2000; Samanta et al. 1999). Interestingly, naphthalene failed to support growth of PP2 even at very low concentrations (0.025%). Although the culture grew on 0.025% salicylate, higher concentrations (0.05 and 0.1%) failed to support growth, indicating that high concentrations of salicylate are toxic to the cells. The ability of the culture to metabolize phenanthrene, benzoate and p-hydroxybenzoate by completely different *ortho* and *meta* ring cleavage pathways, with selective induction of the corresponding enzymes (Tables 2, 3) suggests that the pathways are probably encoded and regulated by different operons. The enzymes involved in these pathways are inducible, as dextrose-grown cells failed to respire on any of these metabolic intermediates and the enzyme preparation showed no corresponding enzyme activities.

A major limiting factor in hydrocarbon degradation is the lack of bioavailability of the hydrocarbon due to poor solubility, leading to accumulation and the accompanying toxic and carcinogenic effects. In nature, a few smart organisms tackle the hydrocarbon uptake/degradation problem either by direct contact, e.g., attaching themselves to these compounds, or by producing surface-active compounds. Both these mechanisms involve modulation of cellular physiology, which will lead either to changes in cell surface properties like hydrophobicity or to secretion of surface-active compounds into the medium, or a combination of both. These mechanisms are not unique to hydrocarbon degradation but are also found to play a significant role in virulence and invasion of pathogen, biofilm formation etc. (van Loosdrecht et al. 1987; de Maagd et al. 1989; Makin and Beveridge 1996; Minagi et al. 1986; Mukherjee et al. 1992; Panagoda et al. 2001; Singleton et al. 2001; Stenström 1989; Wibawan and Lammler 1992). The spent medium of *Pseudomonas* sp. strain PP2 has the ability to stabilize a 1-naphthaldehyde-in-water emulsion. The emulsion formed was very stable and phase separation or coalescence was not observed, even after prolonged storage. Although the emulsification units/ml produced were higher in dextrose-grown culture, surfactant produced per gram of cell mass was 2-fold higher when grown on phenanthrene (Table 4). These results suggest that, irrespective of the carbon source, the organism produces surfactant, and surfactant production is higher when the carbon source is insoluble. Several hydrocarbon-degrading microbes have been reported to produce biosurfactant in a growth-associated manner (Goswami and Singh 1991; Persson et al. 1988; Phale 1994; Phale et al.

1995a). The emulsification activity in the spent medium of PP2 culture increases with growth and reaches a maximum at late-log-phase to early-stationary-phase (Fig. 1), suggesting that production of biosurfactant is growth-associated and probably plays a role in hydrocarbon uptake. Besides surfactant production, PP2 cells showed carbon source- and growth-dependent changes in cell surface hydrophobicity (Table 5). Early- to mid-log-phase cells showed highest cell surface hydrophobicity compared to stationary phase cells. Phenanthrene-grown stationary phase cells were more hydrophobic compared to cells grown on polar carbon sources like dextrose and benzoate. Involvement of a hydrophobic cell surface in 1-naphthoic acid degradation, and pH-dependent interactions between cell surface and biosurfactant have been reported in *Pseudomonas maltophilia* CSV89 (Phale et al. 1995a). Based on the results obtained, we hypothesize that the biosurfactant produced by *Pseudomonas* sp. strain PP2 cells pseudo-solubilizes phenanthrene in the medium. The cells take up this pseudo-solubilized phenanthrene via interaction with the hydrophobic cell surface. The increased cell surface hydrophobicity will also facilitate the direct contact between cells and the phenanthrene particle. These two mechanisms will help the organism to grow, even at higher concentrations of phenanthrene. This hypothesis is supported by the following facts: (1) growth-associated production of extracellular biosurfactant, (2) cells grown on phenanthrene produce more biosurfactant for effective pseudo-solubilization, and (3) a significant increase in cell surface hydrophobicity in the early log phase helps to associate pseudo-solubilized hydrocarbon to hydrophobic cells as well as probably allowing direct cell contact with phenanthrene particles for efficient uptake.

In conclusion, *Pseudomonas* sp. strain PP2 is unique as it degrades phenanthrene at high concentrations via the 1-hydroxy-2-naphthoic acid → [α-naphthol] → 1,2-dihydroxy naphthalene → salicylate → catechol pathway. Involvement of α–naphthol as a probable intermediate needs further investigation by purifying and characterizing enzymes of α-naphthol metabolism. The culture also degrades benzoate and p-hydroxybenzoate efficiently, and shows diversity with respect to catechol metabolism. Growth-dependent changes in biosurfactant production and cell surface hydrophobicity, suggest that interplay/interaction between these components is probably essential for efficient hydrocarbon assimilation/uptake. Existence of natural metabolic diversity at the lower pathway level can be exploited to construct microbes capable of handling various hydrocarbons efficiently. An added advantage for such microbes will be biosurfactant production to help in efficient assimilation/uptake of hydrocarbons, *Pseudomonas* sp. strain PP2 encompasses both these features.

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