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Degradation pathways of phenanthrene by *Sinorhizobium* sp. C4

Abstract *Sinorhizobium* sp. C4 was isolated from a poly-cyclic aromatic hydrocarbon (PAH)-contaminated site in Hilo, HI, USA. This isolate can utilize phenanthrene as a sole carbon source. Sixteen metabolites of phenanthrene were isolated and identified, and the metabolic map was proposed. Degradation of phenanthrene was initiated by dioxygenation on 1,2- and 3,4-C, where the 3,4-dioxygenation was dominant. Subsequent accumulation of 5,6- and 7,8-benzocoumarins confirmed dioxygenation on multiple positions and extradiol cleavage of corresponding diols. The products were further transformed to 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid then to naphthalene-1,2-diol. In addition to the typical degradation pathways, intradiol cleavage of phenanthrene-3,4-diol was proposed based on the observation of naphthalene-1,2-dicarboxylic acid. Degradation of naphthalene-1,2-diol proceeded through intradiol cleavage to produce *trans*-2-carboxycinnamic acid. Phthalic acid, 4,5-dihydroxyphthalic acid, and protocatechuic acid were identified as probable metabolites of *trans*-2-carboxycinnamic acid, but no trace salicylic acid or its metabolites were found. This is the first detailed study of PAH metabolism by a *Sinorhizobium* species. The results give a new insight into microbial degradation of PAHs.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants which originated from petro-leum industry, fossil fuel combustion, and waste inciner-ation. PAHs pose a serious risk to the natural environment and humans because they have various types of toxicities (Hakura et al. 1998). Various microbial remediation and phytoremediation methods have been studied (Parrish et al. 2004; Pradhan et al. 1998). A number of bacterial species are able to utilize two- to five-ring PAHs as sole sources of carbon and energy (Johnsen et al. 1996; Stringfellow and Aitken 1995; Walter et al. 1991).

Bacteria in the class of *Rhizobiaceae* are found in con-taminated environments where various toxic chemicals are present (Ahmad et al. 1997; Bodour et al. 2003). Among numerous rhizobial microorganisms, several bacterial species in the genus of *Agrobacterium*, *Rhizobium*, and *Sinorhizobium* are able to utilize PAHs, polychlorinated biphenyls (PCBs), or aromatic heterocycles (Aitken et al. 1998; Damaj and Ahmad 1996; Frassinetti et al. 1998). Recent biochemical studies showed that several putative genes, which are usually found in other PAH-degrading bacteria, are also present in *Sinorhizobium meliloti* (Ahmad et al. 1997; Galibert et al. 2001). Because of their possible symbiosis with plant, rhizobial bacteria have been studied in relation to phytoremediation (Johnson et al. 2004; Suominen et al. 2000).

In comparison with other bacteria (e.g., namely, *Pseu-domonas* and *Mycobacterium* species), however, research-es on metabolic pathways of PAHs or metabolic enzymes are very limited for *Sinorhizobium* species or their close-ly related rhizobial bacteria. A recent screening with *Sinorhizobium* sp. C4, isolated from PAH-contaminated soil, showed that this strain utilized phenanthrene as a sole carbon source (Seo et al., unpublished data). The objective of this study was to isolate and identify metabolites of phenanthrene by this strain. Novel metabolism pathways of phenanthrene by strain C4 are found in this study, in ad-dition to those previously reported. Several new metabolic pathways are confirmed with authentic standards. To our knowledge, this is the first study of PAH metabolism by symbiotic bacteria.

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Materials and methods

Chemicals

Phenanthrene (>98 % purity) was purchased from Sigma-Aldrich (Milwaukee, WI). Several metabolites were purchased from Sigma-Aldrich, Fisher Scientific (Morris Plains, NJ), or TCI America (Portland, OR). These are 1-hydroxy-2-naphthaldehyde, 2-hydroxy-1-naphthaldehyde, 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, naphthalene-1,2-dicarboxylic acid anhydride, naphthalene-1,2-diol, 2-carboxycinnamic acid, phthalic acid, 2-formylbenzoic acid, salicylic acid, protocatechuic acid, gentisic acid, and catechol. Ethyl acetate and other solvents were the highest grade commercially available. Synthetic methods of metabolites, which are not commercially available, were described elsewhere (Keum et al. 2005). The carboxyl and phenolic metabolite standards were derivatized to corresponding methyl esters or ethers with diazomethane that was prepared from N-methyl-N-nitroso-p-toluenesulfonamide in a diazomethane generator (Aldrich).

Isolation and identification of the PAH-degrading strain

Soil contaminated by PAHs was collected from Hilo, HI, USA (Seo et al., unpublished data). Concentrations of PAHs, including phenanthrene, pyrene, anthracene, and benzo[a]pyrene ranged from 0.6 to 29.9 ppm each. Several metabolites of the abovementioned PAHs were also detected. Those were acenaphthenone, phthalic acid, gentisic acid, protocatechuic acid, o-hydroxynaphthoic acids, diphenic acid, naphthalene-1,2-dicarboxylic acid, 9-fluorenone-1-carboxylic acid, and phenanthrene-4,5-dicarboxylic acid. Strain C4 was isolated from a soil enrichment culture, established in a mineral medium (MM) with solid phenanthrene (50 mg/50 ml of MM) using the soil as inoculums. Strain C4 was identified as *Sinorhizobium* sp. (AY 943388) through comparison of 16S rRNA gene sequence with those in the National Center for Biotechnology Information basic local alignment search tool (NCBI BLAST) database. The closest neighbor was *Sinorhizobium* sp. HF6 (AB 195269) with 99% identity.

Utilization of phenanthrene

The bacterial cells were pregrown in MM supplied with phenanthrene to optical density of 0.3 at 580 nm. A 200-μl aliquot of each phenanthrene stock solution (1 mg/ml) was spiked into a sterilized culture tube. The MM (4 ml) and pregrown cell (1 ml) were added after evaporation of solvent. After incubation for 0, 1, 2, 5, and 7 days with shaking at 150 rpm, 28°C, pH values of the culture media were adjusted to 2.3 with 6 N hydrochloric acid and extracted with 4 ml of ethyl acetate. The concentration of phenanthrene was quantified with a gas chromatograph–flame ionization detector (GC-FID). Cultures inoculated with heat-sterilized cells were used as controls. All metabolism experiments were done in triplicate.

Growth of bacterium and extraction of metabolites

Strain C4 was grown in mineral media (Bastiaens et al. 2000) with phenanthrene (500 mg/1.5 l) as a sole source of carbon and energy at 28°C and 150 rpm (C24 Rotary shaker, New Brunswick Scientific, NJ). After the incubation for 4, 7, and 14 days, culture media were filtered through glass wool and centrifuged (6,000×g, 10 min), and the pH of supernatant was adjusted to 2.3 with 6 N hydrochloric acid and extracted three times with ethyl acetate (500 ml). The combined organic layer was extracted three times with aqueous sodium hydroxide (500 ml, 10 mM). The remaining organic phase was dried over anhydrous sodium sulfate and concentrated to 5 ml of ethyl acetate (neutral fraction). Aqueous layer was acidified to pH 2.3 and extracted with ethyl acetate (500 ml, three times, acidic fraction).

Gas chromatography–mass spectrometry (GC-MS) analysis of neutral fraction was done with/without derivatization. For the detection of diol or *cis*-dihydrodiols, ethyl acetate was removed, and the residue was dissolved in acetone (10 ml) with *n*-butylboronic acid (50 mg). After refluxing for 30 min, the mixture was concentrated to 1 ml and analyzed by GC-MS. Metabolites in acidic fraction were derivatized with diazomethane.

Analytical methods

Gas chromatography–mass spectrometry analysis was performed with a Varian QP-5000 GC with a Saturn-2000 mass spectrometer (Varian Inc., Palo Alto, CA) equipped with a ZB-1 column (60 m, 0.25 μm film thickness, Phenomenex Inc., Torrance, CA). Helium was the carrier gas at a rate of 2 ml/min. The column temperature was held at 120°C (2 min), programmed to 280°C at a rate of 2°C/min, and held at 280°C for 10 min. Injector and analyzer temperatures were 270 and 280°C, respectively. The mass spectrometer was operated in electron impact (EI) mode (70 eV).
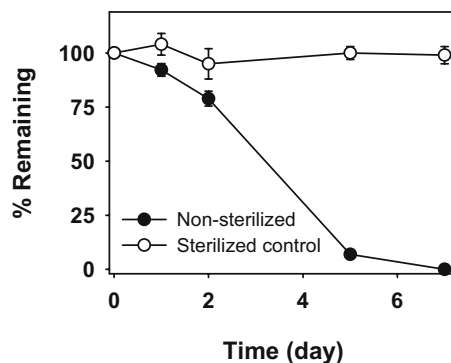

Fig. 1 Degradation of phenanthrene by *Sinorhizobium* sp. C4. Data were averages of three replicates±standard deviations

Table 1 GC retention times and mass spectral data of phenanthrene metabolites by *Sinorhizobium* sp. C4

ID	Chemical name	Retention time (min)	Molecular ion and fragmentation pattern
P1	*cis*-1,2-Phenanthrenedihydrodiol (nBu)	53.66	278 (M$^+$), 221, 194, 178, 164
P2	*cis*-3,4-Phenanthrenedihydrodiol (nBu)	50.78	278 (M$^+$), 221, 194, 178, 163
P3	3,4-Phenanthrenediol (nBu)	58.08	276 (M$^+$), 220, 178, 165, 152
P4	5,6-Benzocoumarin	41.01	196 (M$^+$), 168, 139
P5	7,8-Benzocoumarin	39.83	196 (M$^+$), 168, 139
P6	1-Hydroxy-2-naphthaldehyde (Me)	25.93	186 (M$^+$), 168, 157, 140, 115
P7	2-Hydroxy-1-naphthaldehyde (Me)	31.57	186 (M$^+$), 168, 157, 140, 115
P8	1-Hydroxy-2-naphthoic acid (Me)	28.53	202 (M$^+$), 170, 142, 114
P9	2-Hydroxy-1-naphthoic acid (Me)	29.15	202 (M$^+$), 170, 142, 114
P10	Naphthalene-1,2-dicarboxylic acid (dMe)	39.53	244 (M$^+$), 213, 198, 185, 170, 126
P11	Naphthalene-1,2-diol (nBu)	33.40	226 (M$^+$), 170, 114
P12	2-Carboxycinnamic acid (dMe)	28.47	220 (M$^+$), 189, 173, 161, 145
P13	2-Formylbenzoic acid (Me)	12.63	164 (M$^+$), 133, 105
P14	Phthalic acid (dMe)	15.39	194 (M$^+$), 163, 133
P15	4,5-Dihydroxyphthalic acid (dMe)	31.14	226 (M$^+$), 195, 167, 152, 135, 108
P16	Protocatechuic acid (Me)	21.83	168 (M$^+$), 137, 109

All the metabolites except P1, P2 and P3 were identified by comparison of their GC retention times and mass spectra with those of the standards *nBu* Butylboronate ester, *dMe* dimethyl ester, *Me* methyl ester

Results

Sinorhizobium sp. C4 can utilize phenanthrene as a sole source of carbon and energy. A short lag period was observed at the initial incubation period (<2 days), but the concentration of phenanthrene rapidly decreased below the detection limit after 7 days of incubation (Fig. 1). Sixteen metabolites, ranging from *cis*-phenanthrenedihydrodiol to protocatechuic acid, in the culture supernatant were iden-

tified by comparison of their GC retention times (RTs) and mass spectra with those of authentic standards (Table 1).

Four metabolites (P1–P3 and P11) were identified in the neutral fraction (Figs. 2 and 4). Among several common dihydrodiols from phenanthrene via bacterial metabolism, two *cis*-dihydrodiols (P1 and P2) were found at 50.78 and 53.66 min (Fig. 2). The GC RT and
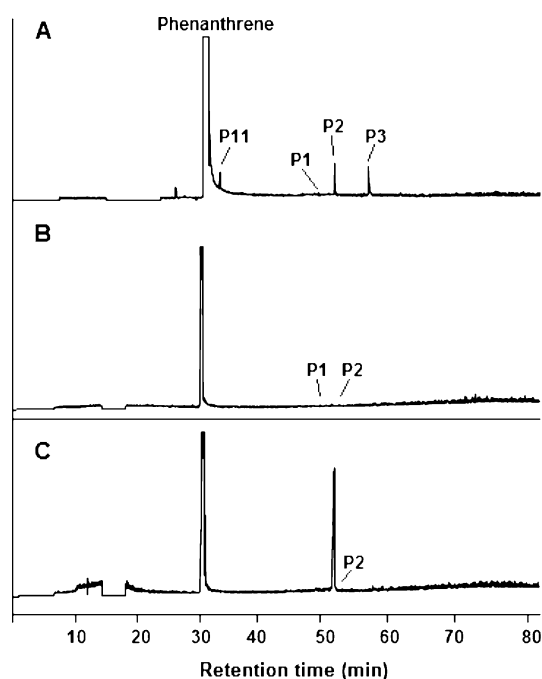

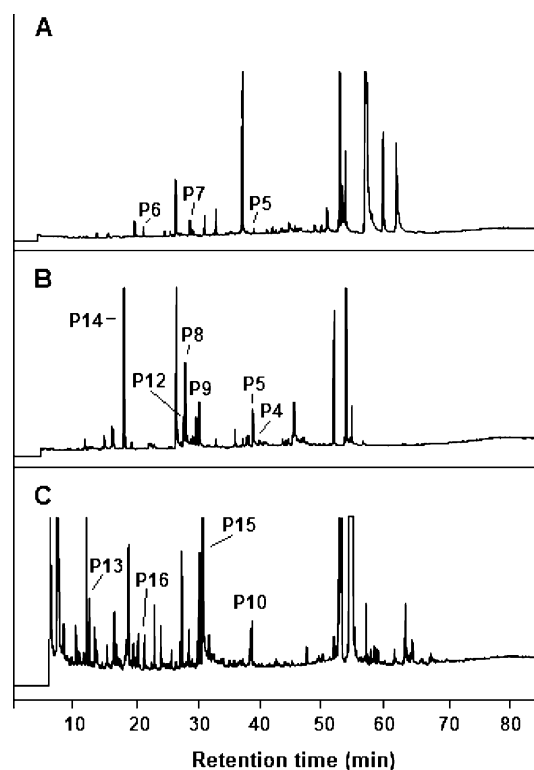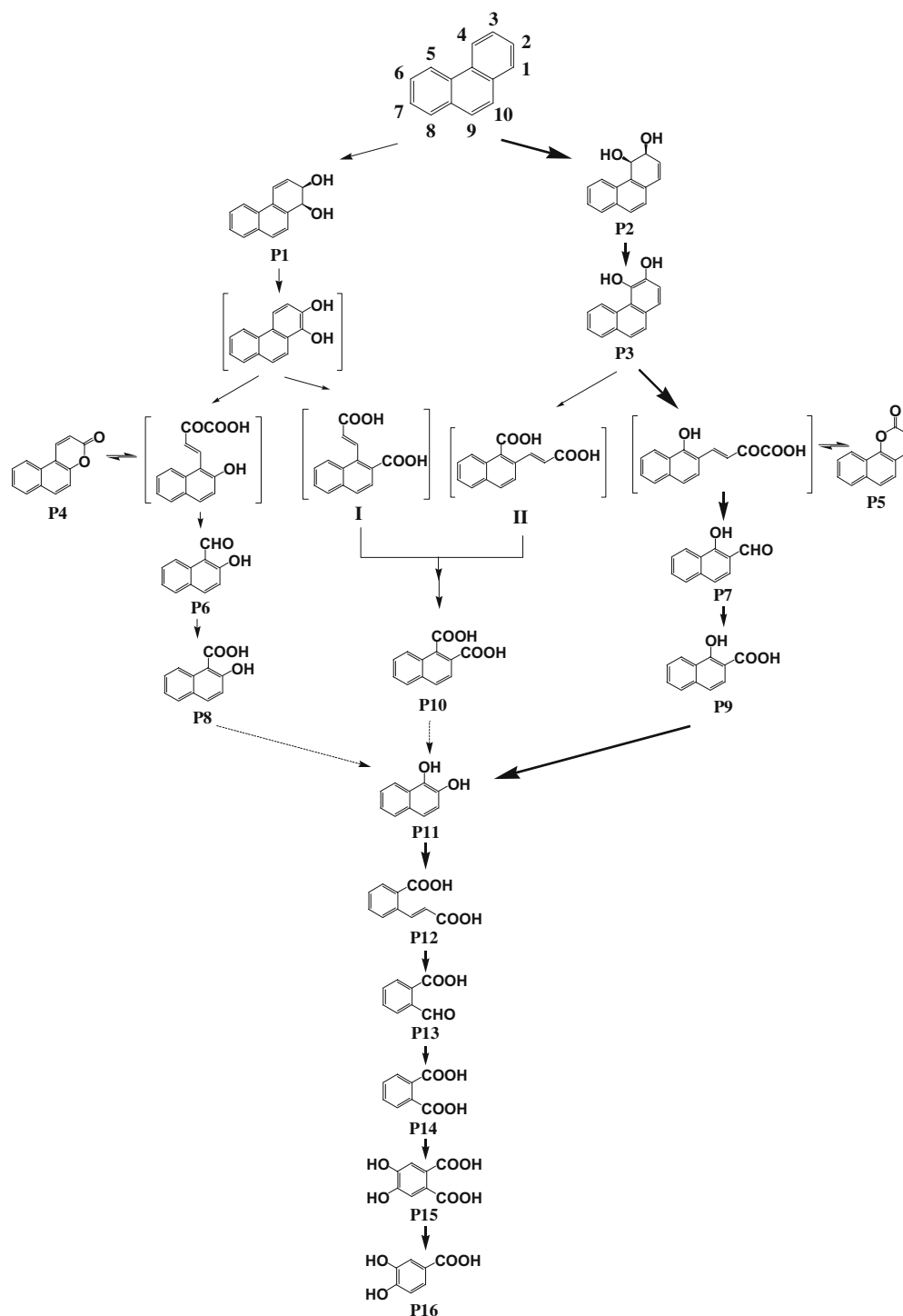**Fig. 2** GC-MS chromatograms of the extracts of the neutral fractions after 3 (**a**), 7 (**b**), and 14 days (**c**) of incubation

Fig. 3 GC-MS chromatograms of the extracts of the acidic fractions after 3 (**a**), 7 (**b**), and 14 days (**c**) of incubation

mass spectrum of P1 were identical to those of the standard *cis*-1,2-phenanthrenedihydrodiol *n*-butylboronate. The mass spectrum of P2 was identical with that of *cis*-3,4-phenanthrenedihydrodiol described by Krivobok et al. (2003). It is noted that the mass spectra of P1 and P2 are very similar with that of the standard *cis*-9,10-dihydrodiol *n*-butylboronate but have different GC RTs (RT of *cis*-9,10-dihydrodiol *n*-butylboronate, 51.11 min). Metabolite P3 (RT 58.08 min) gave a mass spectrum

consistent with that of standard 9,10-phenanthrenediol *n*-butylboronate (RT 58.45 min). The concentrations of P2, P7, and P9 were approximately four to five times higher than those of P1, P6, and P8, respectively, which suggests that P3 is 3,4-phenanthrenediol *n*-butylboronate and undergoes *meta*-cleavage to yield P7 rather than P6. P11 was identified as 1,2-naphthalenediol *n*-butylboronate. Its concentration reached a maximum at 3 days followed by a rapid decrease.

Twelve metabolites (P4–10, P12–16) were identified from acidic fractions (Figs. 3 and 4). The GC chromatograms of the acidic fractions showed that the upper metabolites (from P4 and P5 to P8, P9, and P10) of phenanthrene were abundant after 4 and 7 days of incubation; however, the lower metabolites became abundant after 14 days of incubation. Two benzocoumarins (P5 and P4; RTs 39.83 and 41.01 min, respectively) were detected and identified as 7,8-and 5,6-benzocoumarin, respectively. Both metabolites, however, completely disappeared at 14 days. Metabolites P6 and P7 were methyl ethers of 1-hydroxy-2-naphthaldehyde and 2-hydroxy-1-naphthaldehyde, respectively. Both *o*-hydroxynaphthaldehydes are known intermediates in phenanthrene metabolism (Pinyakong et al. 2000). The concentration of methyl 1-hydroxy-2-naphthoate (P8) reached a maximum after 7 days of incubation and then slowly decreased. P9 was methyl 2-hydroxy-1-naphthoate. Naphthalene-1,2-dicarboxylic acid was detected as its dimethyl ester at 39.53 min (P10). The concentration of P10 was negligible at the initial incubation period (3 days) but rapidly increased during the entire experiment. The mass spectrum and RT of P12 were consistent with *trans*-2-carboxycinnamic acid dimethyl ester. P12 was not detectable after 4 days of incubation, but its level increased up to 7% of total identifiable metabolites after 7 days of incubation. Metabolites P13–P16 were identified as methyl (or dimethyl) esters of 2-formylbenzoic acid, phthalic acid, 4,5-dihydroxyphthalic acid, and protocatechuic acid, respectively. The concentrations of these metabolites rapidly increased till 14 days of incubation, the amount of which accounted for approximately 40% of the total identifiable metabolites.

Discussion

Metabolic pathways of phenanthrene by strain C4 were constructed based on the metabolites isolated and identified with the authentic standards (Fig. 4). Bacterial degradation of PAHs is usually initiated by mono/dioxygenation (Kim et al. 2005). Naphthalene 1,2-dioxygenase or equivalent enzymes catalyze the incorporation of oxygen molecules on several different carbons of PAHs (Kribovok et al. 2003; Rehmann et al. 1998) to produce *cis*-dihydrodiols. Although genes or proteins with similar activities were not reported for *Sinorhizobium* species, a full genome analysis of *S. meliloti* showed the presence of many putative dioxygenases (Galibert et al. 2001), which may be relevant to metabolism of aromatic compounds. In addition, Damaj and Ahmad (1996) reported that several *Rhizobium* species have genes relevant to biphenyl dioxygenase and enzymes of subsequent reactions.

In this study, the detection of two dihydrodiols (P1 and P2) and one diol (P3) suggests an involvement of PAH dioxygenase. The presence of the benzocoumarins also supports the enzymatic dioxygenation of phenanthrene because 5,6- and 7,8-benzocoumarin (P4 and P5) are well-known metabolites of phenanthrene, which are produced from 1,2- and 3,4-dihydrodiols, respectively (Pinyakong et al. 2000). In the metabolic study of dibenzothiophene by

S. meliloti strain Orange 1, Frassinetti et al. (1998) could detect benzothienopyren-2-one and some associated intermediates, of which the structures are close to PAH metabolites. These results support that at least some *Sinorhizobium* species have PAH dioxygenase and subsequent metabolic enzymes.

Concentrations of 2-hydroxy-1-naphthaldehyde (P6) and its carboxylic acid analog (P8) were much lower than that of 1-hydroxy-2-naphthoic acid (P9). The concentrations of their precursors, including 1,2-phenanthrenedihydrodiol (P1) and 5,6-benzocoumarin (P4), were also very low. These results suggest that 1,2-dioxygenation and its subsequent reaction play a minor role in phenanthrene degradation by strain C4. These products, however, were not dead-end metabolites because their concentrations decreased rapidly after 7 days.

We proposed that naphthalene-1,2-dicarboxylic acid (P10) be produced from an intradiol cleavage of 3,4- or 1,2-phenanthrenediol and consecutive metabolisms, which is the first example of an intradiol cleavage of PAH diols by *Sinorhizobium* species as Gram-negative bacteria. PAH diols are usually decomposed by extradiol cleavage to produce *o*-hydroxyaryl α-oxobut-3-enoic acid (Kim et al. 2005; Pinyakong et al. 2000). Several extradiol dioxygenases, which may catalyze the corresponding reactions, are reported (Saito et al. 2000; Stingley et al. 2004). Although various simple catechols can be metabolized through either intradiol or extradiol cleavage by the action of catechol-1,2-dioxygenase or catechol-2,3-dioxygenase (Maltseva et al. 1994), the intradiol cleavage of high-molecular-weight PAH diols are usually limited to K-region diols (Rehmann et al. 1998; Vila et al. 2001). Some Gram-positive bacteria produce intradiol cleavage products from non-K region diol of anthracene and phenanthrene (Dean-Ross et al. 2001; Kim et al. 2005). However, intradiol cleavage has not been reported in Gram-negative bacteria. 1-Carboxyvinyl-2-naphthoic acid and 2-carboxyvinyl-1-naphthoic acid (intermediates I and II, respectively, Fig. 4) are proposed as precursors of naphthalene-1,2-dicarboxylic acid (P10). Although these metabolites were not detected in the cultures in this study, Dean-Ross et al. (2001) reported the conversion of 2-carboxyvinyl-3-naphthoic acid to naphthalene-2,3-dicarboxylic acid. van
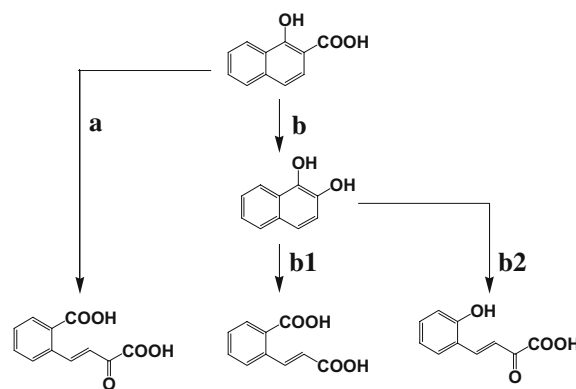

Fig. 5 Proposed bacterial degradation pathways of 1-hydroxy-2-naphthoic acid

Herwijnen et al. (2003) described a mass spectrum of an unknown metabolite of phenanthrene, of which the mass spectrum was well coincided with that of standard 2-carboxyvinyl-1-naphthoic acid synthesized in this study.

Two metabolic pathways are commonly reported for the degradation of 1-hydroxy-2-naphthoic acid (P9) catalyzed by different enzymes (Fig. 5). These are (a) dioxygenation and subsequent *ortho*-ring cleavage to produce 2-carboxy-benzalpyruvate (pathway a in Fig. 5, Adachi et al. 1999) and (b) decarboxylation and hydroxylation to produce naphthalene-1,2-diol (P11) (pathway b, Balashova et al. 2001) that is further degraded through extradiol cleavage (pathway b2; Eaton and Chapman 1992). In this study, naphthalene-1,2-diol (P11) was found without any trace of 2-carboxybenzalpyruvate and 2-hydoxyl benzalpyruvate. This result suggests that strain C4 degrades 1-hydroxy-2-naphthoic acid solely by pathways b and b1 (Fig. 5). 2-Carboxycinnamic acid (P12) was reported in the biodegradation of naphthalene by *Bacillus thermoleovorans* (Annweiler et al. 2000) and phenanthrene by *Mycobacterium* sp. strain AP1 (Vila et al. 2001), but its metabolic origin and degradation pathway were not proposed. Detection of 2-carboxycinnamic acid (P12) in this study has clarified the transformation from naphthalene-1,2-diol (P11) to P12.

Rhizobial bacteria can utilize various aromatic acids (Vela et al. 2002). Salicylate and phthalate are among the preferred growth substrates. A recent genetic study showed that some *Sinorhizobium* species have many genes encoding simple aromatic acid assimilation systems (Galibert et al. 2001). 2-Formylbenzoic acid (P13) was rapidly transformed to phthalate (P14) by strain C4. Accumulation of 4,5-dihydroxyphthalate (P15) and protocatechuic acid (P16) indicates degradation of phthalate (P14) through a common phthalate degradation pathway (Keyser et al. 1976). However, no metabolites associated with salicylate metabolism (e.g., hydroxybenzalpyruvate, salicyladehyde, salicylic acid, and gentisic acid) were detected in the culture media. These results showed that this strain metabolized naphthalene-1,2-diol (P11) to protocatechuic acid through a single unbranched pathway (Fig. 4).

In summary, metabolism of phenanthrene by *Sinorhizobium* sp. C4 was initiated by incorporation of an oxygen molecule on 1,2- or 3,4-C positions. Phenanthrenediols were transformed to *o*-hydroxynaphthoates or naphthalene-1,2-dicarboxylic acid by extradiol or intradiol cleavages. Subsequent metabolisms of these acids produced naphthalene-1,2-diol, which was further degraded to protocatechuic acid. The detailed metabolite profiles indicate diversified PAH metabolic enzymes in this strain.

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