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***Mycobacterium aromativorans* JS19b1^T Degrades Phenanthrene through C-1,2, C-3,4 and C-9,10 Dioxygenation Pathways**

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Abstract

Mycobacterium aromativorans strain JS19b1^T can utilize phenanthrene as a sole source of carbon and energy. Strain JS19b1^T degrades phenanthrene through highly branched metabolic pathways, including dioxygenation on C-1,2, C-3,4 and C-9,10 positions and ring opening by both *ortho*- and *meta*-cleavage. The presence of novel metabolic pathways was confirmed by replacement cultivation using synthetic metabolite standards. The metabolites were isolated and identified by gas chromatography-mass spectrometry. Both *ortho* and *meta*-cleavage products of 1,2- and 3,4-dihydroxyphenanthrene were detected. Two *ortho*-cleavage products, 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid and 2-[(*E*)-2-carboxyvinyl]-1-naphthoic acid were further metabolized to naphthalene-1,2-dicarboxylic acid and then to 1,2-dihydroxynaphthalene, which can also be produced from the *meta*-cleavage products hydroxynaphthoic acids. These results suggest that part of the branched pathways is merged into 1,2-dihydroxynaphthalene. The concentrations of the products from C-9,10 dioxygenation were higher than those from other pathways. C-9,10 dioxygenation of phenanthrene produced phthalic acid through decarboxylation and mono-/dioxygenation. The diverse phenanthrene metabolic pathways in JS19b1^T give a new insight of the bacterial degradation of polycyclic aromatic hydrocarbons.

Keywords

Bioremediation; Biodegradation; *Mycobacterium aromativorans*; Phenanthrene; Dioxygenation; Monooxygenation

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants and are produced from petroleum industry, fossil fuel combustion and waste incineration. PAHs and some of their degradation products pose a serious toxicological risk to the environment and human, since they have mutagenic and carcinogenic effects (Atsushi *et al.*, 1998; Yousef *et al.*, 2002). The U.S. Environmental Protection Agency (EPA) listed 16 PAHs as priority pollutants. Various

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bioremediation methods of PAHs have been studied (Pradhan *et al.*, 1998; Daane *et al.*, 2001; Parrish *et al.*, 2004). Anthracene, phenanthrene, and pyrene have been used as model PAHs to study various aspects of their degradation by microorganisms, including bioavailability, metabolism, genetics, and compatibility with other remediation methods (Friedrich *et al.*, 2000; Pinyakong *et al.*, 2000; Dean-Ross *et al.*, 2001; Piskonen and Merja, 2004; Stingley *et al.*, 2004).

A variety of bacterial species are able to utilize two or three-ring PAHs as sole sources of carbon and energy (Stringfellow and Aitken, 1995; Johnsen *et al.*, 1996). Various bacteria in the genera of *Arthrobacter*, *Burkholderia*, *Cycloclasticus*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas*, and *Staphylococcus* (White and Bursey, 1966; Walter *et al.*, 1991; Boldrin *et al.*, 1993; Geiselbrecht *et al.*, 1998; Mallick *et al.*, 2007; Seo *et al.*, 2007a; Tittabutr *et al.*, 2011) were reported to degrade three- or five-ring PAHs. The initial reactions of PAHs degradation are often catalyzed by multi-component dioxygenases to produce dihydrodiols (Moody *et al.*, 2003 and 2004; Kim *et al.*, 2005; Mallick *et al.*, 2007). In addition to the formation of *cis*-dihydrodiol, PAH dioxygenase from *Pseudomonas* species catalyzes several different reactions, including desaturation and monooxygenation (Gibson *et al.*, 1995; Balashova *et al.*, 2001). *Mycobacterium* species usually incorporate mono- or di-oxygens at several different sites of specific PAHs (Moody *et al.*, 2001 and 2003; Kim *et al.*, 2007). For example, *M. vanbaalenii* can metabolize phenanthrene to *cis*-3,4-dihydrodiol and *cis/trans*-9,10-dihydrodiols (Moody *et al.*, 2001). 3,4-Dihydroxyphenanthrene, an oxidation product of corresponding *cis*-dihydrodiol, is degraded to 4-(1-hydroxy-2-naphthyl)-2-oxobut-3-enoic acid by the action of extradiol dioxygenase (Pinyakong *et al.*, 2000; Moody *et al.*, 2004; Seo *et al.*, 2006, 2007b) and further metabolized to 1-hydroxy-2-naphthoic acid (Seo *et al.*, 2006, 2007b). In comparison with 9,10-analogue, ring cleavage of 1,2- or 3,4-diol occurred dominantly through *meta*-cleavage catalyzed by the above-mentioned extradiol dioxygenase. Genes of dioxygenase have been reported from some *Mycobacterium* species (Garnier *et al.*, 2003; Krivobok *et al.*, 2003; Stingley *et al.*, 2004). Diphenic acid is an *ortho*-cleavage product of 9,10-dihydroxyphenanthrene catalyzed by intradiol dioxygenase (Moody *et al.*, 2001; Kim *et al.*, 2005; Zeinali *et al.*, 2008). Although the possible presence of intradiol cleavage enzyme was expected from the study with anthracene (Dean-Ross *et al.*, 2001) and the *ortho*-cleavage of 3,4-dihydroxyphenanthrene was recently proposed by Kim *et al.* (2005), such a pathway and the corresponding metabolites have been confirmed. Diphenic acid is one of the most dominant metabolite of phenanthrene by *Mycobacterium* (Vila *et al.*, 2001). To our knowledge, diphenic acid breakdown has not well been described in the literature. 1-Hydroxy-2-naphthoic acid is metabolized either to 1,2-dihydroxynaphthalene (Balashova *et al.*, 2001) or 2-carboxybenzalpyruate (Adachi *et al.*, 1999). Although both salicylic acid and phthalic acid could be produced from phenanthrene, the latter is more commonly found as the final metabolite in *Mycobacterium* species than the former (Moody *et al.*, 2001; Kim *et al.*, 2005).

A previous study with *Mycobacterium aromativorans* JS19b1^T revealed that this strain can rapidly degrade various PAHs such as phenanthrene and pyrene (Seo *et al.*, 2007a). Protein profiles in response to phenanthrene in strain JS19b1^T have been recently studied (Seo *et al.*, 2011). In the present study, we described the isolation and identification of metabolites of phenanthrene by strain JS19b1^T.

2. Materials and methods

2.1. Chemicals

Standards of phenanthrene and its metabolites were purchased from Sigma-Aldrich (Milwaukee, WI), Fisher Scientific (Morris Plains, NJ), or TCI America (Portland, OR). Ethyl acetate and other solvents were the highest grade commercially available. Metabolites,

which were not commercially available, were from our previous studies (Keum *et al.*, 2005; Seo *et al.*, 2007a,b; Tittabutr *et al.*, 2011) (Table 1).

2.2. Bacterial strain and growth conditions

Mycobacterium aromativorans strain JS19b1^T was deposited in the American Type Culture Collection (Manassas, VA, USA) (ATCC BAA-1378) (Seo *et al.*, 2007a; Hennessee *et al.*, 2009). The bacterial cultures were grown in mineral medium (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). Degradation study was also done with 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid according to the same procedures. Control experiments were done with autoclaved cells according to the same procedures. All experiments were done in triplicate.

2.3. Extraction, derivatization and analysis of metabolites

After incubation for 4, 7, 14, and 21 days, the cultures were filtered through glass wool and centrifuged ($6,000 \times g$, 10 min). The metabolites of phenanthrene were extracted, derivatized and analyzed according to the methods previously described (Seo *et al.*, 2006 and 2007b). Briefly, the supernatant was extracted with ethyl acetate after it was acidified to pH 2–3 with 6 N HCl. The organic layer was extracted with 10 mM NaOH solution. The remaining organic phase was dried over anhydrous sodium sulfate and concentrated to 5 ml (neutral fraction). The aqueous NaOH extract was acidified to pH 2–3 and then extracted with ethyl acetate (acidic fraction) (Seo *et al.*, 2006 and 2007b).

Metabolites in the neutral fraction after derivatization or no derivatization were analyzed with a gas chromatograph-mass spectrometer (GC-MS). After removal of ethyl acetate from the neutral fraction, the residue was dissolved in acetone (10 ml) followed by addition of 50 mg of *n*-butylboronic acid. After refluxing for 30 min, the mixture was concentrated to 1 ml and analyzed by GC-MS. Diol and *cis*-dihydrodiols in the residue were derivatized with diazomethane and reanalyzed by GC-MS. Metabolites in the acidic fraction were also derivatized with diazomethane and detected with GC-MS (Seo *et al.*, 2006 and 2007b).

The metabolites were analyzed on a Varian QP-5000 gas chromatograph - Saturn-2000 mass spectrometer (Varian, Inc., Palo Alto, CA), equipped with a ZB-1 column (60 m, 0.25 μ m film thickness, Phenomenex Inc., Torrance, CA) (Seo *et al.*, 2007b). The carrier gas was helium at a rate of 2 ml min⁻¹. The column temperature started from 120 °C (2 min), increased to 280 °C at a rate of 2 °C min⁻¹, and was held at 280 °C for 10 min. Injector and analyzer temperature were 270 and 280 °C, respectively. The mass spectrometer was at electron impact mode (70 eV).

3. Results

3.1. Degradation of phenanthrene

Phenanthrene was rapidly metabolized through multiple pathways. Twenty six metabolites, covering *cis*-dihydrodiols of phenanthrene to protocatechuic acid, were isolated and identified in this study.

Two metabolites in the neutral fraction were identified as *n*-butylboronate esters of *cis*-9,10-dihydro-9,10-dihydroxyphenanthrene (P2) and *cis*-1,2-dihydro-1,2-dihydroxyphenanthrene (P1) at a retention time (R_f) of 51.11 and 53.67 min, respectively (Fig. 1B). Mass spectra of both dihydrodiols [m/z 278 (M^+), 221, 194, 178, 165] were nearly similar with those of synthetic standards (Table 1) and those reported in the literature (Krivobok *et al.*, 2003). The concentration of P1 reached maximum at 7-days incubation and gradually decreased (Fig.

2E). P2 had a maximum concentration at 14-days incubation (Fig. 2E). At 3 days of incubation, three peaks (R_t = 58.08, 58.44, and 61.06 min) with similar mass spectra (m/z 276 (M^+), 233, 220, 178, 163) were detected in the neutral fraction. The peak at 58.44 min (P5) observed in the neutral extract at a trace level in 7-days incubation (Fig. 1B) was identified as 9,10-dihydroxyphenanthrene *n*-butylboronate in comparison with the synthetic standard (Table 1). Other two peaks at 58.08 (P4) and 61.06 min (P3) were tentatively identified as *n*-butylboronates of 3,4- and 1,2-dihydroxyphenanthrene, respectively. However, P3 was not observed in the extract of the 7-days incubation cultures. P4 and P5 had the highest peak in the 14-days incubation sample (Fig. 2F). In the day-3 incubation samples, 4-(1-hydroxy-2-naphthyl)-2-oxobut-3-enoic acid [as its methoxy-methyl ether/ester derivative; R_t = 53.87 min; m/z 270 (M^+), 255, 239, 211, 196, 168] (P9) at a trace level was detected but not detected after a week. 1,2-Dihydroxynaphthalene [DHN; *n*-butyl boronate; R_t = 32.40 min (P15); m/z 226 (M^+ , 39), 196 (6), 183 (6), 170 (100), 152 (2)] was detected at 7-days incubation (Fig. 1B). Catechol (P23) was also detected as its *n*-butylboronate derivatives [R_t = 10.63 min; m/z 176 (M^+ , 46), 120 (100), 92 (7)] during the entire experiment (Fig. 1B and 2F).

In the acidic fraction, 5,6-benzocoumarin and 7,8-benzocoumarin were detected at R_t of 41.01 (P10) and 42.06 min (P6) [m/z 196 (M^+), 168, 139] (Table 1). P6 was detected in only the 14-days sample. An additional benzocoumarin-like compound was found at 38.67 min (P16), of which its R_t and MS were similar with the synthetic 3,4-benzocoumarin (Table 1). Mass spectra of peaks at 49.09 min (P7) and 50.79 min (P8) [m/z 270 (M^+), 239, 223, 211, 196, 180, 168, 152] (Fig. 1A) were similar with those of unidentified metabolites of van Herwijnen *et al.* (2003). The mass spectrum and R_t of peak P7 were consistent with synthetic 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid (CVNA1) dimethyl ester (Table 1). P8 was tentatively identified as 2-[(*E*)-2-carboxyvinyl]-1-naphthoic acid dimethyl ester (CVNA2) based on the GC-MS data. P8 peaked at 14-days incubation (Fig. 2A). However, a trace amount of P7 was detected throughout the experiment. Although the fully-methylated derivatives of 4-(1-hydroxynaphthalen-2-yl)-2-oxobut-3-enoic acid (NOBA1) (P9) and 4-(2-hydroxynaphthalen-1-yl)-2-oxobut-3-enoic acid (NOBA2), common bacterial metabolites of phenanthrene, have the same molecular weights, the mass spectra and R_t of NOBA1 (Fig. 3B) and NOBA2 (Fig. 3C) were different from the peaks P7 and P8 (Fig. 3D and 3E, respectively). For example, the m/z 239 ion was usually either a base peak (NOBA1) or the second dominant ion (NOBA2) in the NOBA mass spectra. In case of peaks P7 and P8, however, an ion with m/z 211 was the base peak (Fig. 3D and E), which indicates the loss of methoxycarbonyl group [M^+ -59 (COOMe)]. Fragment ion at m/z 152 revealed an additional loss of methoxycarbonyl group, suggesting that at least two carboxylic acid moieties are present in these metabolites. The concentration of diphenic acid [dimethyl ester; R_t = 36.18 min (P11); m/z 270 (M^+ , 0), 239 (8), 211 (100), 196 (8), 180 (8), 152 (11)] reached a maximum at 14 days and then slowly decreased (Fig. 2C). Biphenyl-2-carboxylic acid [methyl ester; R_t = 25.17 min (P14); m/z 212 (M^+ , 89), 197 (32), 181 (26), 168 (100), 152 (28), 140 (92)] was first detected on 7 days and then continuously decreased (Fig. 2D). The concentration of 1-hydroxy-2-naphthoic acid [methyl ester; R_t = 29.15 min (P13); m/z 202 (M^+ , 65), 170 (100), 142 (15), 114 (67)] peaked at the 7-days incubation (Fig. 2A). Naphthalene-1,2-dicarboxylic acid [NDA; dimethyl ester; R_t = 39.53 min (P12); m/z 244 (M^+ , 52), 229 (2), 213 (100), 198 (2), 185 (4), 170 (8), 154 (7), 126 (13)] was gradually increased up to the 21-days sample (Fig. 2A).

8-Hydroxy-3,4-benzocoumarin [methyl ether; m/z 226 (M^+), 211, 195, 183, 168] was detected at 42.71 min (P17) in the 21-days acidic fraction, but not observed in the 3-, 7- and 14- days acidic fractions. The mass spectrum and R_t of peak P17 were consistent with synthetic 8-hydroxy-3,4-benzocoumarin methyl ester (Table 1), which was reported as a fluorene metabolite by *Pseudomonas* species (Grifoll *et al.*, 1994). The concentration of

trans-2-carboxycinnamic acid (P19) [CCA; dimethyl ester; R_t = 28.47 min; m/z 220 (M^+ , 5), 173 (15), 161 (100), 118 (17), 102 (14)] was continuously increased after 7-days incubation (Fig. 1A and 2D). 2-Formylbenzoic acid (P20) [methyl ester; R_t = 12.62 min; m/z 164 (M^+ , 2), 149 (22), 133 (45), 105 (100), 92 (58)] was first detected in the 7-days sample and then continuously increased during the experiments (Fig. 2D). Metabolite P22 (R_t = 15.39 min) was identified as phthalic acid dimethyl ester having a molecular ion (M^+) at m/z 194 and base ion at m/z 163. P22 had a maximal peak height at 14- days incubation (Fig. 1A and 2C). The degradation products of phthalic acid [e.g., 3,4- and 4,5-dihydroxyphthalic acid (P24 and P25, respectively), protocatechuic acid (P26)] were not detected at the initial period (< 3 days) but their concentrations were increased after 3 days (Fig. 2B and C). Mass spectra and R_t of peak P24 and P25 were consistent with synthetic 3,4-dihydroxyphthalic acid [dimethyl ester; R_t = 29.93 min] and 4,5-dihydroxyphthalic acid [dimethyl ester; R_t = 31.14 min], respectively (Table 1 and Fig. 1A). Protocatechuic acid [dimethyl ester; R_t = 21.18 min (P26); m/z 182 (M^+ , 61), 151 (100), 123 (18), 108 (10)] was first detected in the 7-days samples (Fig. 1A and 2B). Interestingly, salicylic acid [methyl ester; R_t = 8.22 min (P21); m/z 152 (M^+ , 66), 120 (98), 92 (100)], which is not common PAH metabolites by *Mycobacterium* species, was detected in all samples (Fig. 1A and 2A). Its concentration reached maximum on 14 days (Fig. 2A). A trace level of coumarin [R_t = 15.01 min (P18); m/z 146 (M^+ , 60), 118 (100)] was detected in the 7-days acidic fraction (Fig. 1A).

3.2. Degradation of selected metabolites

To clarify the degradation pathways, metabolisms of CVNA1 (P7) was studied with the corresponding standard. CVNA1, an *ortho*-cleavage product of 1,2-dihydroxyphenanthrene (P3), was rapidly transformed to other metabolites. In the acidic fraction, NDA dimethyl ester (P12) was the most abundant product (Fig. 4A). Phthalic acid (P22) and 1-hydroxy-2-naphthoic acid (P13) were also detected in the acidic fraction (Fig. 4A). Naphthalene-1,2-diol (P15) was detected in the neutral fraction as a sole product (Fig. 4B).

4. Discussion

The results show that *Mycobacterium aromativorans* strain JS19b1^T rapidly metabolizes phenanthrene through multiple routes as proposed in Fig. 5. Dioxygenation on C-1,2, C-3,4, and C-9,10 of phenanthrene was confirmed using the synthetic standards and GC/MS data comparison with literature, where the 9,10-dioxygenation (K-region oxidation) was the most dominant reaction (Fig. 5). The presence of ring fission and/or rearrangement products [e.g., diphenic acid (P11), 7,8-benzocoumarin (P10), and 5,6-benzocoumarin (P6)] also supported the dioxygenation on multiple positions, which was first reported for *Mycobacterium vanbaalenii* strain PYR-1 (Moody *et al.*, 2001; Kim *et al.*, 2005). Although fungi generally do not degrade PAHs as rapidly as bacteria, a wide variety of fungi can degrade PAHs ranging from low to high molecular weight (Cerniglia, 1997). The initial step of bacterial degradation of PAHs is often catalyzed by dioxygenases to produce dihydrodiols, whereas that of fungal degradation of PAHs is often catalyzed by intracellular cytochrome P-450 monooxygenase to produce arene oxide and extracellular lignin peroxidase, manganese peroxidase and laccase to produce PAH-quinones (Cerniglia, 1992; Haritash *et al.*, 2009; Ting *et al.*, 2011).

Dihydroxyphenanthrenes (P3 and P4), oxidation products of dihydrodiols are further metabolized to 5,6-benzocoumarin (P6) and 7,8-benzocoumarin (P10) *via meta*-cleavage. 3,4-Benzocoumarin (P16) is from diphenic acid (P11) through *ortho*-cleavage. Although several diol isomers are produced from a specific PAH, only a single type of ring cleavage mechanism is reported for each isomer (Pinyakong *et al.*, 2000; Moody *et al.*, 2001). A Gram-positive bacteria species, however, can metabolize 1,2-dihydroxyanthracene through both pathways (Dean-Ross *et al.*, 2001). In the case of *Mycobacterium* species, *ortho*-ring

cleavage was commonly observed during the transformation of 9,10-dihydroxyphenanthrene and 4,5-dihydroxypyrene (Moody *et al.*, 2001; Vila *et al.*, 2001). Kim *et al.* (2005) reported naphthalene-1,2-dicarboxylic acid (NDA) as a possible *ortho*-cleavage product of 3,4-dihydroxyphenanthrene. The intermediate, however, was not reported and the possibility of such an uncommon enzymatic reaction is still vague. NDA (P12) may be a degradation product of two novel intermediates, CVNA1 (P7) and CVNA2 (P8), which are *ortho*-cleavage products of 1,2- and 3,4-dihydroxyphenanthrene (P3 and P4), respectively. Detection of NDA (P12) and CVNAs (P7 and P8) in the present study supports the presence of an *ortho*-cleavage pathway of 1,2- and 3,4-dihydroxyphenanthrene. In addition, strain JS19b1^T can metabolize CVNA1 (P7) to NDA (P12), 1-hydroxy-2-naphthoic acid (P13) and then DHN (P15). This pathway could be elucidated from the replacement culture supplemented with CVNA1 (P7) as a substrate. This result suggests that the decarboxylation and hydroxylation pathway occurs in JS19b1^T, which is closely resembled with that in *Arthrobacter* sp. P1-1 (Seo *et al.*, 2006).

The presence of CVNAs (P7 and P8) and NDA (P12) may pose several points: (a) potential presence of intra-diol dioxygenase for *ortho*-cleavage of diols, (b) metabolic fate and related enzymes of cleavage products, and (c) the role of CVNAs and NDAs in PAH metabolism. Metabolites identified and kinetic profiles of the metabolites elucidated in the present study are some evidences for these points.

Some intra-diol dioxygenases (e.g., catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase) are known to have somewhat broad substrate specificity (Haroune *et al.*, 2002). The structural variation, however, is limited small substituents, namely alkyl, alkoxy, and halogen (Maltseva *et al.*, 1994; Briganti *et al.*, 1997; Murakami *et al.*, 1997). Transformations of *ortho*-substituted aryl oxobutenoate (e.g., NOBAs, hydroxybenzalpyruvate (HBPA) and carboxybenzalpyruvate (CBPA)) are catalyzed by hydratase-aldolase (Eaton and Chapman, 1992; Iwabuchi and Harayama, 1998a). The presence of hydratase-aldolase in strain JS19b1^T was detected in the previous study (Seo *et al.*, 2011). Although CVNA1 (P7) and CVNA2 (P8) have some structural resemblance with NOBA/HBPA and CBPA, both intermediates do not contain an α -keto-acid group, a common substituent of the substrate of hydroxyaryl-oxobutenoate hydratase-aldolase (Eaton and Chapman, 1992). Those results suggest that novel enzyme(s), different from common hydratase-aldolase, may involve transformations of CVNAs.

As in other *Mycobacterium* species (Moody *et al.*, 2001; Vila *et al.*, 2001), 9,10-dioxygenation and subsequent transformations were more dominant than 1,2- or 3,4-dioxygenation pathways. Although the degradation rate was slow, diphenic acid (P11) can be metabolized to biphenyl-2-carboxylic acid (P14). Decarboxylation of hydroxyaryl carboxylic acid by bacteria is usually accompanied by hydroxylation to produce dihydroxyaryl compound (Balashova *et al.*, 2001). However, 1-hydroxynaphthalene was reported as a phenanthrene metabolite, which suggests the presence of decarboxylation without hydroxylation (Kim *et al.*, 2005). Biphenyl-2-carboxylic acid (P14) may be a product from the same reaction mechanism. 3,4-Benzocoumarin (P16), which was found in the culture, was considered as a lactonization product of the possible intermediate 2'-hydroxybiphenyl-2-carboxylic acid. Those results indicate that diphenic acid (P11) is transformed to biphenyl-2-carboxylic acid (P14), 2'-hydroxybiphenyl-2-carboxylic acid and then phthalic acid (P22) (Fig. 5).

Two common metabolic pathways for 1-hydroxy-2-naphthoic acid are (a) formation of 2-carboxybenzalpyruvate under the catalysis of 1-hydroxy-2-naphthoate dioxygenase (Adachi *et al.*, 1999) and (b) DHN (P15) under the catalysis of salicylate-1-hydroxylase (Balashova *et al.*, 2001). In the present study, DHN was found, but no trace of 2-carboxybenzalpyruvate.

A number of Gram-positive bacteria in the genera of *Nocardioides* and *Mycobacterium* are known to have proteins or genes of 1-hydroxy-2-naphthoic acid dioxygenase (Iwabuchi and Harayama, 1998b; Stingley *et al.*, 2004). However, salicylate-1-hydroxylase is not well documented in these bacteria. The presence of DHN (P15) suggests that salicylate-1-hydroxylase, which was detected from protein profiling in the previous study (Seo *et al.*, 2011), may play an important role in the transformation of 1-hydroxy-2-naphthoic acid (P13) in JS19b1^T. This suggestion can also be supported by the detection of salicylic acid (P21) and catechol (P23). Salicylic acid, a building block of siderophore and mycobactins, can be produced from primary metabolism (De Voss *et al.*, 1999). The presence of coumarin (P18) suggests that salicylic acid may be transformed from DHN (P15).

Among the final metabolites, *trans*-2-carboxycinnamic acid (CCA) (P19) may be an *ortho*-cleavage product of DHN (P15). Bacterial metabolism of DHN occurred through two different pathways: (a) a more common *meta*-cleavage to produce 2-hydroxybenzalpyruate, which is a precursor of salicylic acid (P21) and (b) a less common *ortho*-cleavage to form CCA (P19) (Annweiler *et al.*, 2000; Vila *et al.*, 2001). Salicylic acid (P21), found in the present study, may be transformed from DHN (P15) via *meta*-cleavage.

In summary, strain JS19b1^T metabolizes phenanthrene through very complex pathways, including intra- and extra-diol cleavages. The branched pathways, which start with C-1,2 and C-3,4 dioxygenation, are merged at the formation of naphthalene-1,2-diol, and then to salicylate (P21) and phthalate (P22). The 9,10-dioxygenation pathway is also merged to form phthalate (P22).

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Highlights

- *Mycobacterium aromativorans* JS19b1^T utilizes PAHs for growth.
- JS19b1^T degrades phenanthrene via multi-site dioxygenation and *ortho-/meta*-cleavage.
- Part of the branched pathways is merged into 1,2-dihydroxynaphthalene.
- Products from C-9,10 dioxygenation dominate.
- The 9,10-dioxygenation pathway is merged to form phthalate.

Fig. 1.
GC-MS chromatograms of acidic (A) and neutral (B) fractions of phenanthrene after 7 days of incubation, which phenanthrene was the sole carbon source.

Fig. 2. Kinetics of phenanthrene metabolites in acidic (A, B, C, and D) and neutral (E and F) fractions. Relative standard errors for all data points ranged from 4% to 15%.

Fig. 3. Mass spectra of the synthetic standards [CVNA1 (A), NOBA1 (B), and NOBA2 (C)] and extractants [CVNA1 (D) and CVNA2 (E)] from the culture supernatant of *M. aromativorans* strain JS19b1^T treated with phenanthrene.

Fig. 4.
GC-MS chromatograms of acidic (A) and neutral (B) fractions of culture supernatants of which CVNA1 was the sole carbon source.

Fig. 5.
Proposed metabolic pathway of phenanthrene by *Mycobacterium aromaticorans* strain JS19b1^T. Thick arrows represent major pathways as determined by the metabolite concentrations.

Table 1

Synthesized phenanthrene metabolite standards

ID	Chemical name ^a	R _f (min)	MS fragmentation pattern (relative intensity) ^b	Reference
P2	<i>cis</i> -9,10-Dihydro-9,10-dihydroxyphenanthrene (nBuB)	51.11	278 (M ⁺ , 100), 221 (29), 194 (70), 178 (43), 165 (39)	Shing <i>et al.</i> , 1994
P5	9,10-Dihydroxyphenanthrene (nBuB)	57.63	276 (M ⁺ , 100), 233 (10), 220 (51), 178 (1), 163 (14)	Shing <i>et al.</i> , 1994
P6	5,6-Benzocoumarin	41.79	196 (M ⁺ , 88), 168 (100), 139 (56)	Keum <i>et al.</i> , 2005
P7	1-[(<i>B</i>)-2-Carboxyvinyl]-2-naphthoic acid (diMe)	49.09	270 (M ⁺ , 12), 255 (1), 239 (5), 223 (8), 211 (100), 196 (8), 180 (23), 168 (13), 152 (22)	Keum <i>et al.</i> , 2005
P9	4-(1-Hydroxynaphthalen-2-yl)-2-oxobut-3-enoic acid (diMe)	54.78	270 (M ⁺ , 6), 255 (2), 239 (100), 211 (53), 196 (57), 168 (34)	Keum <i>et al.</i> , 2005
	4-(2-Hydroxynaphthalene-1-yl)-2-oxobut-3-enoic acid (diMe)	56.66	270 (M ⁺ , 55), 239 (97), 211 (61), 196 (100), 168 (82)	Keum <i>et al.</i> , 2005
P10	7,8-Benzocoumarin	39.53	196 (M ⁺ , 72), 168 (100), 139 (46)	Keum <i>et al.</i> , 2005
P16	3,4-Benzocoumarin	37.42	196 (M ⁺ , 100), 168 (86), 139 (60)	Kenner <i>et al.</i> , 1957
P17	8-Hydroxy-3,4-benzocoumarin (Me)	41.14	226 (M ⁺ , 100), 211 (1), 195 (77), 183 (4), 168 (19)	Venturello and Chiusoli, 1972
P24	3,4-Dihydroxyphthalic acid (diMe)	29.92	226 (M ⁺ , 18), 195 (15), 167 (100), 152 (14), 135 (22)	White and Bursey, 1966
P25	4,5-Dihydroxyphthalic acid (diMe)	31.13	226 (M ⁺ , 11), 195 (13), 167 (100), 152 (12), 135 (16)	Anderson and Koch, 1978

^aDerivatives of: Me, methyl ester; diMe, dimethyl ester; nBuB, n-butyboronic acid

^bValues in parenthesis represent *m/z* of molecular ion (M⁺) and relative abundance.