JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Comparative Protein and Metabolite Profiling Revealed a Metabolic Network in Response to Multiple Environmental Contaminants in *Mycobacterium aromativorans* JS19b1^{T}†

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ABSTRACT: *Mycobacterium aromativorans* JS19b1^{T} was isolated from a petroleum-contaminated site that was acclimated almost 100 years. In the present study, metabolism of several polycyclic aromatic hydrocarbons (PAHs) and structural analogues in JS19b1^{T} was studied. The proteomic profiles were compared when JS19b1^{T} was cultured in nutrient broth and glucose-, phenanthrene-, and phthalate-supplemented mineral media. Proteomic analysis showed notable characteristics of this species, for instance, the existence of enzymes for degradation of multiple classes of chemicals including biphenyl, phenanthrene, dibenzothiophene, and organophosphorus pesticides. Phenanthrene degradation enzymes were detected only in phenanthrene-fed cells, suggesting a very tight regulation of the enzymes. Detection of the other enzymes under various treatment conditions indicated that their regulation may be through very complex mechanisms. In comparison with common major metabolites, PAH transformations produced various types of potentially toxic intermediates, including epoxide, quinone, phenols, aldehydes, and phthalates. In a bioenergy production aspect, PAH transformation does not seem to provide substrates for glycolysis and pentose phosphate pathways. This study signifies the potential of protein profiling for studies of relatively uncharacterized bacteria for biodegradation of environmental pollutants.

KEYWORDS: *Mycobacterium aromativorans*, proteomics, PAH, metabolism, biodegradation, bioremediation, biotransformation

INTRODUCTION

Mycobacterium bacteria are widespread and diverse in the environment. Some *Mycobacterium* spp. are among the most formidable human pathogens, whereas some are most promising xenobiotic chemical degraders. Extensive research has been done with the pathogenic species such as *Mycobacterium tuberculosis*. However, limited proteomic work has been done with nonpathogenic, but environmentaly significant, saprophytic species. Proteomic studies can offer insights into the evolutionary difference between the pathogenic and saprophytic species, which may give further understanding to the prevention of infection and environmental remediation at the protein level.

Mycobacterium is a well-known genus capable of mineralizing polycyclic aromatic hydrocarbons (PAHs) including phenanthrene, fluoranthene, pyrene, and benzo[*a*]pyrene.[1−5] *Mycobacterium* species usually incorporate mono- or dioxygens at several different sites on PAHs. Putative genes encoding degradation enzymes have been reported for some *Mycobacterium* species.[4,6,7] Recently, Kim et al.[8] have classified PAH-cleaving enzymes and other proteins in response to pyrene, pyrene-4,5-quinone, phenanthrene, anthracene, and fluoranthene in *Mycobacterium vanbaalenii* PYR-1. The authors separated more than a thousand proteins using two-dimensional gel electrophoresis (2DE) and identified several PAH-induced proteins. These proteins included a catalase-peroxidase, a putative monooxygenase, a dioxygenase small subunit, a small subunit of naphthalene-inducible dioxygenase, and aldehyde dehydrogenase presumably involved in the PAH metabolic pathways.

We have isolated 19 PAH-degrading bacterial isolates from a highly PAH-contaminated soil in Hilo, HI.[9] One of them, JS19b1^{T}, is capable of efficiently degrading phenanthrene and pyrene and potentially degrading the pesticides temephos, diazinon, and pirimiphos-methyl. This strain was characterized, named *Mycobacterium aromativorans* JS19b1^{T} (ATCC BAA-1378), and deposited in the American Type Culture Collection.[10] The objective of this study was to study protein responses after *M. aromativorans* JS19b1^{T} was exposed to different environmental contaminants. To profile proteins in JS19b1^{T} during the metabolism of environmental contaminants, we took a proteomic approach using one-dimensional SDS−polyacrylamide (1D SDS-PAGE) and liquid chromatography−tandem nanoelectrospray mass spectrometry (GeLC-MS/MS).

MATERIALS AND METHODS

Chemicals. Phenanthrene, pyrene, biphenyl, 2,4-dichlorophenoxyacetic acid (2,4-D), dibenzothiophene, and their metabolites were

† Part of the Symposium on Pesticide Toxicology in Honor of Professor John Casida. Q.X.L. was a postdoctoral fellow with Dr. Casida 1991−1995.

Special Issue: Casida Symposium

Received: August 3, 2010
Revised: October 1, 2010
Accepted: October 4, 2010
Published: October 20, 2010

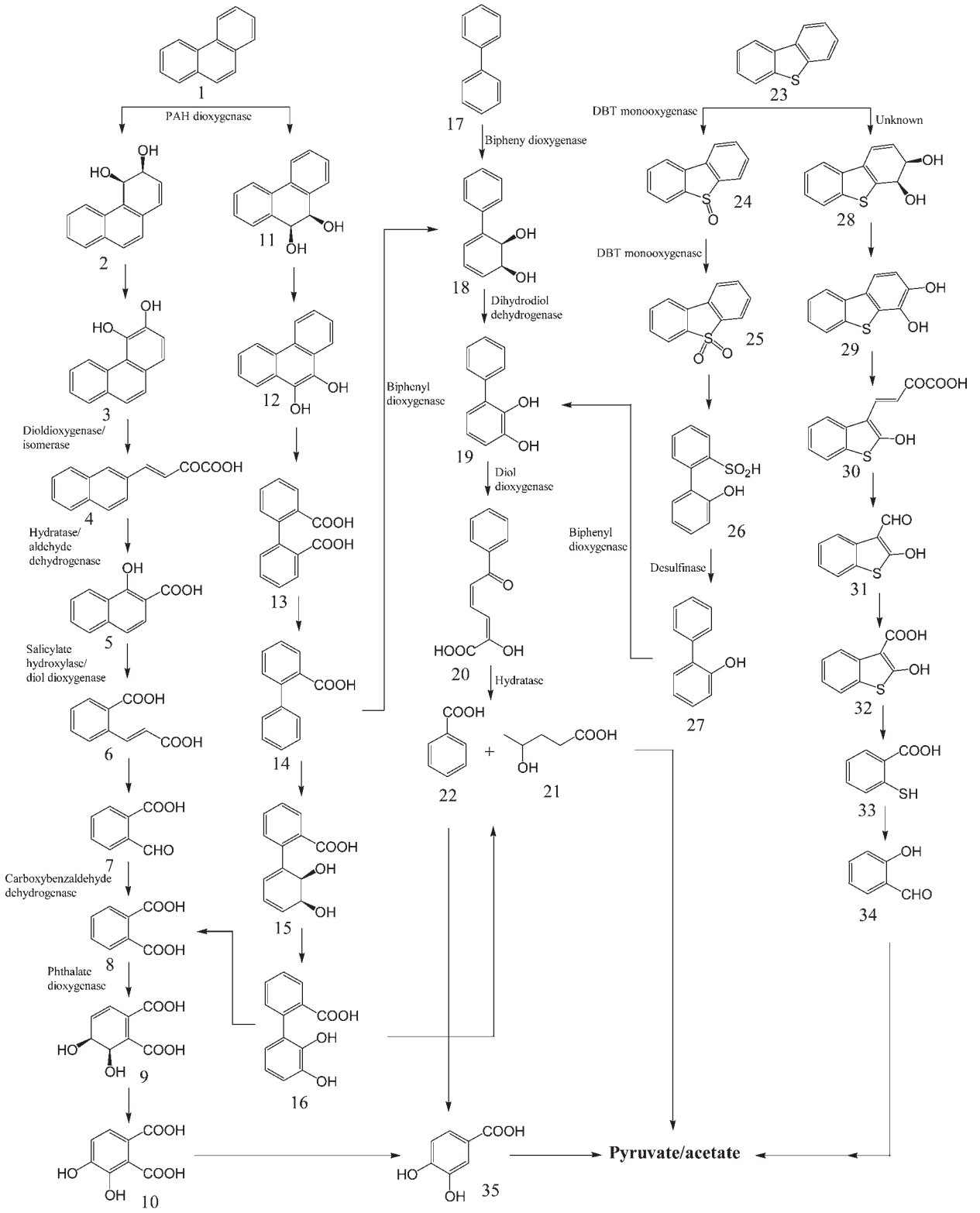

Figure 1. Proposed metabolic map of phenanthrene, biphenyl, and dibenzothiophene in *Mycobacterium aromativorans* JS19b1^T.

purchased from Sigma-Aldrich (Milwaukee, WI), Fisher Scientific (Morris Plains, NJ), or TCI America (Portland, OR). Metabolites that were not commercially available were synthesized according to published methods.[11] Ethyl acetate and other solvents were of the highest grade commercially available. The carboxylic and phenolic metabolite standards were derivatized to the corresponding methyl esters or ethers with diazomethane that was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a diazomethane generator (Aldrich).

Growth of Bacterium and Extraction of Metabolites. JS19b1^T was grown in a mineral medium[12] supplemented with

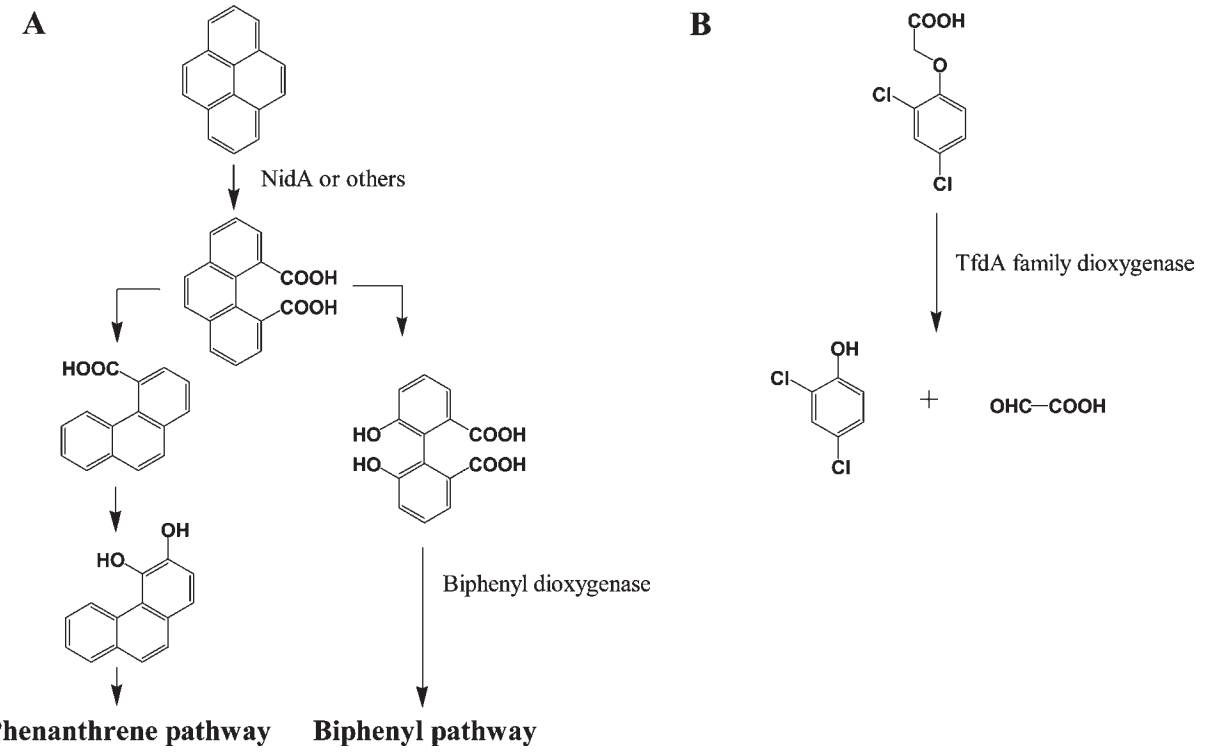

Figure 2. Proposed metabolic maps of (A) pyrene and (B) 2,4-D in *Mycobacterium aromativorans* JS19b1^T.

phenanthrene, biphenyl, or dibenzothiophene (each 300 mg/1.5 L) as a sole source of carbon and energy at 28 °C and 150 rpm (C24 rotary shaker, New Brunswick Scientific, New Brunswick, NJ). After a 14 day incubation, the culture medium was filtered through glass wool and centrifuged at 6000*g* for 10 min. The supernatant was adjusted to pH 2−3 with 6 N HCl and was extracted with ethyl acetate (3 × 500 mL). The extracts were combined and then extracted with aqueous sodium hydroxide (3 × 500 mL, 10 mM). The remaining organic phase was dried over anhydrous sodium sulfate and concentrated to 5 mL of ethyl acetate (neutral fraction). The aqueous layer was acidified to pH 2−3 and then extracted with ethyl acetate (3 × 500 mL, acidic fraction).

Metabolites in the neutral fraction after derivatization or without derivatization were analyzed by gas chromatography−mass spectrometry (GC-MS). For the detection of diols or *cis*-dihydrodiols in the extracts, ethyl acetate was evaporated, and the residue was reconstituted in acetone (10 mL) containing *n*-butylboronic acid (50 mg). After refluxing for 30 min, the mixture was concentrated to 1 mL followed by GC-MS analysis. Metabolites in the acidic fraction were derivatized with diazomethane.

GC-MS anaysis was performed on a Varian QP-5000 gas chromatograph 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) and helium as carrier gas at a rate of 2 mL/min. The column temperature was held at 120 °C for 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 set at 270 and 280 °C, respectively. The mass spectrometer was operated in electron impact (EI) mode (70 eV).

1D SDS-PAGE. 1D SDS-PAGE, acetamidation, in-gel tryptic digestion, and digested peptide extraction were performed according to the procedure previously described.[13]

Nanoelectrospray LC-MS/MS Analysis. LC-MS/MS analyses were carried out on an Ultimate nanoelectrospray LC system interfaced to an esquireHCTplus ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The nano LC column was a C18 PepMap100 (3 μm film thickness, 75 μm i.d. × 15 cm, Dionex, Sunnylvale, CA). The mobile phase (A = 0.1% formic acid; B = 0.1% formic acid in acetonitrile) started at 95% A and 5% B for 5 min, was changed to 40% A and 60% B in 88 min, 5% A and 95% B in 10 min, 95% A and 5% B in 15 min, and finally was held at 95% A and 5% B for 20 min. The flow rate was 200 μL/min. Peptide spectra were recorded over a mass range of m/z 300−2500 whereas MS/MS spectra were recorded in information-dependent data acquisition over a mass range of m/z 50−1600. One peptide spectrum was recorded followed by two MS/MS spectra; the accumulation time was 1 s for peptide spectra and 2 s for MS/MS spectra. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Doubly or triply charged ions were selected for product ion spectra. MS/MS spectra were interpreted by Mascot (Matrix Science Ltd., London, U.K.) search via Biotools 2.2 software (Bruker Daltonics).

Analysis of Peptide Sequences. Peptide mass fingerprint (PMF) searches based on peptide masses measured were performed using the Swiss-Prot or MSDB databases with the Mascot server. PMF used the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although matches that contained any missed cleavages were not found. Mass tolerance of 1.0 Da was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match populations and were reported as $10 \times \log_{10}(p)$, where p is the absolute probability. Scores in Mascot larger than the MOWSE score at $p = 0.05$ were considered to be significant, meaning that the probability of the match being a random event is <0.05 when scores in Mascot were larger than the MOWSE score at $p = 0.05$. The MS/MS spectra of all positively identified peptides were manually confirmed. Only proteins identified in triplicate analyses, with each peptide containing two tryptic termini, were accepted.

Table 1. Proteins and Their Putative Functions in *M. aromativorans* JS19b1^T in Response to Nutrient Broth, Glucose, Phenanthrene, and Phthalate as Substrates

					no. of peptide hits[c]			
ID[a]	ACC no.[b]	taxanomy	protein	role	NB	Glu	PHE	PHT
1	Q84EU7	*Mycobacterium* sp. 6PY1	PAH dioxygenase, a unit, type 2	PAH metabolism	0	0	2	0
2	Q84EU6	*Mycobacterium* sp. 6PY1	PAH dioxygenase, b unit, type 2	PAH metabolism	0	0	3	0
3	Q6H2J9/Q9LC87	*Mycobacterium vanbaalenii*/ *Nocardioides* sp. KP7	PAH extradiol dioxygenase, PhdF	PAH metabolism	0	0	4	0
4	Q51948	*Pseudomonas* sp.	2-hydroxy-2*H*-benzo[*h*]chromene-2-carboxylate isomerase	PAH metabolism	0	0	1	0
5	Q6H2J7	*M. vanbaalenii*	hydratase-aldolase, PhdG	PAH metabolism	0	0	2	0
6	Q9ZHH7	*Burkholderia* sp. RP007	aldehyde dehydrogenase	PAH metabolism	0	0	1	0
7	Q8FUA4	*Corynebacterium efficiens*	salicylate hydroxylase	PAH metabolism	0	0	1	0
8	BAA23265	*Nocardioides* sp. KP7	2-carboxybenzaldehyde dehydrogenase	PAH metabolism	0	0	2	0
9	Q6UP12	*M. vanbaalenii*	phthalate dioxygenase, b unit	PAH metabolism	0	0	1	2
10	O69354	*Rhodococcus erythropolis*	histidine kinase, receptor-like, bphS	biphenyl metabolism	0	0	4	0
11	Q46373	*Comamonas testosteroni*	biphenyl dioxygenase, b unit	biphenyl metabolism	0	2	1	0
12	P08694	*Pesudomonas pseudoalcaligenes*	biphenyldihydrodiol dehydrogenase	biphenyl metabolism	0	0	1	2
13	O69350	*R. erythropolis*	2,3-dihydroxybiphenyl 1,2-dioxygenase, bphC6	biphenyl metabolism	0	1	0	0
14	O69359	*R. erythropolis*	2,3-dihydroxybiphenyl 1,2-dioxygenase, bphC1	biphenyl metabolism	0	0	1	0
15	G70605	*Mycobacterium tuberculosis*	2-hydroxy-6-phenylhexa-2,4-dienoic acid hydrolase, bphD	biphenyl metabolism	1	0	1	0
16	O05151	*Rhodococcus* sp. RHA1	4-hydroxy-2-oxovalerate aldolase	biphenyl metabolism	1	0	4	1
17	P54998	*Rhodococcus* sp. IGTS8	dibenzothiophene desulfurization enzyme C	dibenzothiophene metabolism	0	1	2	2
18	Q8GIB8	*Mycobacterium* sp. G3	HBPS desulfinase	dibenzothiophene metabolism	0	0	2	1
19	Q5YVK3	*Nocardia farcinica*	4-carboxymethylenebut-2-en-4-olide lactonohydrolase (hydroxylase)	protocatechuate metabolism	0	0	2	0
20	O86605	*Streptomyces coelicolor*	3-oxoadipate enol-lactone hydrolase/ 4-carboxymuconolactone decarboxylase	protocatechuate metabolism	0	0	2	0
21	Q73XZ3	*Mycobacterium paratuberculosis*	hypothetical, carboxymuconolactone decarboxylase (CMD) motif	protocatechuate metabolism	2	0	0	0
22	P67755	*M. tuberculosis*	dioxygenase, tfdA dioxygenase family, Rv0097/MT0106	2,4-D metabolism	2	1	0	0
23	P64303	*M. tuberculosis*	haloalkane dehalogenase 2	haloalkane metabolism	1	0	0	0
24	Q50642	*M. tuberculosis*	haloalkane dehalogenase 3	haloalkane metabolism	1	0	0	0
25	Q53586	*Streptomyces avermitilis*	4-hydroxyphenylpyruvate dioxygenase	aromatic amino acid metabolism	0	0	1	0
26	P65053	*M. tuberculosis*	hypothetical, Rv2910c, contain aromatic ring-hydroxylation dioxygenase, b unit	aromatic compound metabolism	0	0	2	0
27	O53311	*M. tuberculosis*	dioxygenase, Rv3161c	aromatic compound metabolism	1	0	0	0
28	O65936	*M. tuberculosis*	aromatic ring monooxygenase	aromatic compound metabolism	0	0	3	0
29	BAA23268	*Nocardioides* sp. KP7	cytochrome P450	aromatic compound metabolism	0	0	1	0
30	Q8VV90	*Terrabacter* sp. DBF63	hydroxylase (phenol hydroxylase-like)	aromatic compound metabolism	1	0	0	0
31	P95277	*M. tuberculosis*	phenolhydroxylase reductase, electron transfer protein, Rv1937	aromatic compound metabolism	0	0	1	0

[a] Identification numbers. [b] Accession numbers. [c] Number of peptide hits: number of peptides detected and that matched, by amino acid sequence, with the proteins in the databases. NB, nutrient broth; Glu, glucose; PHE, phenanthrene; PHT, phthalate.

Profiles of proteins detected in the treatment samples were compared with those in the appropriate control samples. Detection of a protein in the treatment sample but not in the control is referred to as up-expression/production of the protein, whereas the absence of a protein in the treatment sample but its presence in the control is referred to as down-expression/production of the protein.

RESULTS AND DISCUSSION

Metabolic Network. The metabolism of various chemicals by JS19b1^T is highly complex and diverse. Common metabolites derived from different substrates indicate the network and versatility of these enzyme systems (Figures 1 and 2). The

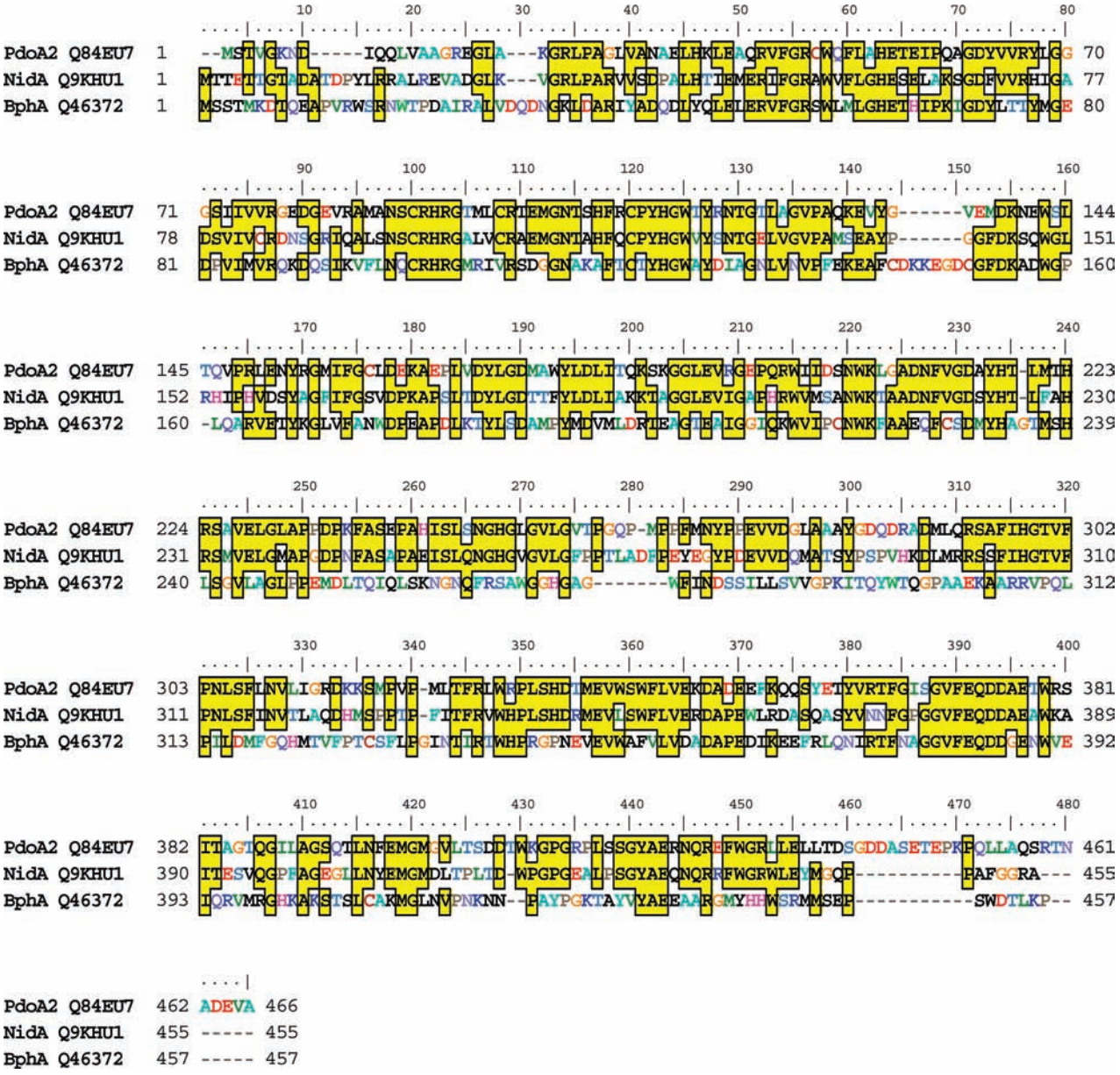

Figure 3. Amino acid sequence alignment of the PAH dioxygenase, PdoA2 from *Mycobacterium* sp. 6PY1, NidA from *Mycobacterium vanbaalenii* PYR-1, and the biphenyl dioxygenase (BphA) from *Comamonas testosteroni*.

metabolic network in JS19b1^T, derived for various substrates, was constructed by extensive analysis of specific metabolites. For example, both phthalate and benzoate (**8** and **22**, Figure 1) were observed in replacement culture using diphenic acid as the substrate (**13**, Figure 1). Diphenic acid was transformed through two different pathways: (a) decarboxylation of one carboxyl group followed by dioxygenation, ring disclosure, and then to phthalate (from **13** to **14**, **15**, and **16** and then to **8**, Figure 1) and (b) decarboxylation of both carboxyl groups, dioxygenation, ring-opening, and then to benzoate (from **13** to **14**, **18**, **19**, and **20** and then to **22**, Figure 1). If biphenyl metabolic enzymes have broad specificities,[14] they may participate in part of the phenanthrene metabolism pathways. Induction of proteomes being relevant to biphenyl metabolism in phenanthrene-fed cells supported the above-stated possibility. Biphenyldihydrodiol dehydrogenase, biphenyldiol dioxygenase, hydratase, and 4-hydroxy-2-oxovalerate aldolase (P08694, O69359, G70605, and O05151, respectively, Table 1) are probably involved in phenanthrene metabolism. The metabolic network is likely connected with dibenzothiophene metabolism, specifically from 2-hydroxybiphenyl to biphenyl-2,3-diol (i.e., **27** to **19**, Figure 1). It was unclear whether the transformation of 2-hydroxybiphenyl to its diol was catalyzed by biphenyl dioxygenase or not. In addition to major dioxygenation, aromatic ring hydroxylating dioxygenases can catalyze desaturation and monooxygenation.[15] However, it does not exclude the possible involvement of other enzymes of 2-hydroxybiphenyl transformation. Cytochrome P450, aromatic ring monooxygenase, and phenol hydroxylase (BAA23268, O65936, and Q8VV90, respectively, Table 1) may be also responsible for the transformation of 2-hydroxybiphenyl to its diol, which is a junction of dibenzothiophene and biphenyl metabolism.

Enzymes with broad specificity may participate in the metabolic network. Salicylate 1-hydroxylase (Q8FUA4, Table 1) can be an example. Unlike the isofunctional 4-hydroxylase, 1-hydroxylase catalyzes decarboxylation and consecutive hydroxylation to produce catechol with broad substrate specificity.[16] Detailed analysis of metabolites showed that catechol is transformed from salicylate and naphthalene-1,2-diol from 1-hydroxy-2-naphthoic acid; the two reactions may be catalyzed by the same enzyme. This enzyme may also catalyze the transformation of 2-mercaptobenzoic acid (**33**, Figure 1) to 2-mercaptophenol (**34**, Figure 1). Pyrene degradation apparently leads to phenanthrene pathways through phenanthrene 4-carboxylic acid and biphenyl pathways via 6,6′-dihydroxybiphenyl-2,2′-dicarboxylic acid (DHBPDC) (Figure 2). Although the replacement culture was not done with DHBPDC, its structural similarity with diphenic acid suggests that this metabolite may be decomposed by the same or a similar set of enzymes. General metabolic characteristics of JS19b1^T include potential uses of the same or similar enzyme(s) to produce common metabolites and integration of metabolic wetwork through enzymes with broad substrate specificity.

Diverse and Versatile Enzyme Systems. Except for 2,4-D, there was a structural resemblance in the substrates. If relaxed substrate specificities of dioxygenases related to aromatic compound metabolism[17] are assumed, a very limited number of enzymes may initate the metabolism. However, the proteomic analyses revealed that specific enzymes are involved in the degradation of specific classes of chemicals (Figure 1 and Table 1). Phenanthrene dioxygenation was initiated at 1,2-, 3,4-, and 9,10-C positions. Although the relaxed regiospecificities of dioxygenase were reported,[15] it is unclear whether a single dioxygenase performs all of the dioxygenation reactions or not. Multiple dioxygenases in some *Mycobacterium* spp. have been identified.[17−20] The PAH dioxygenase units PdoA2 (Q84EU7) and PdoB2 (Q84EU6) from *Mycobacterium* sp. 6PY1 were found in JS19b1^T (Table 1). Dioxygenases in the genus *Mycobacterium* can be classified into two groups, highly homologous nidA/nidB and heterologous pdoA/pdoB. The first group of dioxygenases was found in all PAH-degrading *Mycobacterium* spp., whereas the second group usually has very low homologies with the first group and are highly diverse in the amino acid sequences. It is attributed to low concetrantions, post-translational modifications, peptide fragmentation, cell growth phases analyzed, and other possible factors. In addition, transcripts of nidA and nidB were found in the cells grown with nutrient broth, glucose, and phthalate. These findings suggested that post-transcriptional regulation of dioxygenase may occur in JS19b1^T.

In comparison with phenanthrene metabolism-related proteins, enzymes associated with biphenyl catabolism were found in the cells grown in different substrates (Table 1). Amino acid sequence similarities between biphenyl dioxygenase and PAH dioxygenase were 30−45% (Figure 3). The subsequent biphenyl metabolism enzymes are proposed in each of the biphenyl metabolism steps (Figure 1). These results showed that JS19b1^T has a complete set of biphenyl metabolism enzymes that have low similarities to those of phenanthrene metabolism.

Dibenzothiophene first underwent sulfur monooxygenation or dioxygenation on the peripheral ring (Figure 1). Previous genetic research on dibenzothiophene-metabolizing enzymes focused on desulfurization (from **23** to **27** in Figure 1).[21] Limited research effort has been made on the peripheral ring dioxygenation of dibenzothiophene.[22] Identical enzymes (i.e., pdoA2 and pdoB2) may be involved in the ring dioxygenation of both phenanthrene and dibenzothiophene due to their structural similarities. Oxidation of dibenzothiophene sulfoxide to sulfone is known to be catalyzed by the FMN-containing monooxygenase, SoxC (P54998), which is not related to dioxygenases. The detection of desulfinase (Table 1, Q8GIB8) supported the desulfurization pathways in JS19b1^T, in which desulfinase shares no similarities with the enzymes responsible for phenanthrene metabolism.

The incorporation of dioxygen is catalyzed by aromatic ring hydroxylating dioxygenases consisting of a catalytic subunit and a structural subunit. Several structurally unrelated dioxygenases exist in microorganisms. The TfdA family dioxygenases are one example (P67755 in Table 1). Their major role includes dioxygenolytic breakdown of ether, sulfide, and sulfone bonds in specific chemicals, including 2,4-D.[23] The detected 2,4-D metabolites suggested that the TfdA family dioxygenase be responsible for 2,4-D initial degradation (Figure 2).

Multiple PAH/biphenyl diol dioxygenases (Q6H2J9, Q9LC87, O69350, and O69359) and hydratase/aldolase (Q6H2J7 and G70605) were detected in JS19b1^T (Table 1). In summary, JS19b1^T has dioxygenases capable of degrading phenanthrene, biphenyl, dibenzothiophene, and 2,4-D. The protein profiles suggested that degradation enzyme are responsive to the substrates.

Substrate Effects. It appears that metabolic enzymes of phenanthrene, relative to biphenyl and dibenzothiophene, were more strictly regulated. For example, none of the phenanthrene metabolic enzymes (ID 1−9, Table 1) were observed in glucose- or nutrient broth-grown cells, which suggest substrate inducible regulations of these proteins. Salicylate is known as a transcriptional activator of PAH or biphenyl metabolic genes in Gram-negative bacteria (e.g., *Pseudomonas* sp. and *Burkholderia* sp.).[24] Expression of metabolic enzymes in the low portion of the metabolism suggests production of salicylate from PAH or biphenyl. The accumulated salicylate can activate the metabolic genes. However, the regulatory mechanism of the similar genes in Gram-positive bacteria has not yet been studied. It is reported that the metabolism of PAHs by *Rhodococcus* species, a Gram-positive bacterium, was not affected by coexistent substrates (e.g., glucose), and the transcription of aromatic ring hydroxylating dioxygenase was constitutive.[25] However, our proteomic analyses and those from another research group[8] suggested that the induction of PAH dioxygenases is very chemical-specific. The fact that none of the enzymes responsible for the degradation of phenanthrene and its metabolites (from **2** to **7** in Figure 1) was detected in phthalate-grown cells suggests that the parent PAHs or metabolites above phthalate may induce the degradation enzymes. However, further studies are needed to pinpoint the inducer for the metabolic genes.

Possible Gene Exchange. PAH metabolism-related proteins in JS19b1^T are dominantly related to those from Gram-positive bacteria. However, some proteins (ID 4, 6, 11, and 12 in Table 1) were homologous with those found in Gram-negative bacteria, which have very low amino acid sequence homologies with the corresponding proteins in Gram-positive bacteria (e.g., *Mycobacterium* and *Rhodococcus*) (Figure 3). In addition to these proteins being similar to those from taxonomically distant bacteria, several enzymes, especially those involved in biphenyl and dibenzothiophene metabolism, were from other genera. Low similarities of these proteins to those in other *Mycobacterium* species may suggest that horizontal gene transfer between

JS19b1^T and other bacteria might occur during adaptation in the soil.

Diverse PAH Metabolism. *M. aromativorans* JS19b1^T can utilize a very diverse set of chemicals as a sole carbon and energy source, including PAHs, aromatic heterocycles, biphenyl, and 2,4-D. According to the proteomic and metabolic analyses, catabolism of PAHs and other environmental contaminants in JS19b1^T can be summarized to be associated with (a) diverse initial degradtion enzymes, (b) integration of different metabolic pathways through specific enzymes, (c) strictly regulated specific enzymes, and (d) possible gene exchange between JS19b1^T and other bacteria within the community.

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Author Contributions

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Funding Sources

This work was supported in part by U.S. EPA Award 989512-01-1 and USDA-TSTAR Grants 0034135-9576, 2001-34135-11295, and 2002-34135-12724.

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