Pleiotropic and Epistatic Behavior of a Ring-Hydroxylating Oxygenase System in the Polycyclic Aromatic Hydrocarbon Metabolic Network from *Mycobacterium vanbaalenii* PYR-1

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Despite the considerable knowledge of bacterial high-molecular-weight (HMW) polycyclic aromatic hydrocarbon (PAH) metabolism, the key enzyme(s) and its pleiotropic and epistatic behavior(s) responsible for low-molecular-weight (LMW) PAHs in HMW PAH-metabolic networks remain poorly understood. In this study, a phenotype-based strategy, coupled with a spray plate method, selected a *Mycobacterium vanbaalenii* PYR-1 mutant (6G11) that degrades HMW PAHs but not LMW PAHs. Sequence analysis determined that the mutant was defective in *pdoA2*, encoding an aromatic ring-hydroxylating oxygenase (RHO). A series of metabolic comparisons using high-performance liquid chromatography (HPLC) analysis revealed that the mutant had a lower rate of degradation of fluorene, anthracene, and pyrene. Unlike the wild type, the mutant did not produce a color change in culture media containing fluorene, phenanthrene, and fluoranthene. An *Escherichia coli* expression experiment confirmed the ability of the Pdo system to oxidize biphenyl, the LMW PAHs naphthalene, phenanthrene, anthracene, and fluorene, and the HMW PAHs pyrene, fluoranthene, and benzo[*a*]pyrene, with the highest enzymatic activity directed toward three-ring PAHs. Structure analysis and PAH substrate docking simulations of the Pdo substrate-binding pocket rationalized the experimentally observed metabolic versatility on a molecular scale. Using information obtained in this study and from previous work, we constructed an RHO-centric functional map, allowing pleiotropic and epistatic enzymatic explanation of PAH metabolism. Taking the findings together, the Pdo system is an RHO system with the pleiotropic responsibility of LMW PAH-centric hydroxylation, and its epistatic functional contribution is also crucial for the metabolic quality and quantity of the PAH-MN.

Mycobacterium vanbaalenii PYR-1 was originally isolated from oil-contaminated estuarine sediment in Redfish Bay, Texas, in 1986 (1–4). It was the first bacterium shown to degrade pyrene, a high-molecular-weight (HMW) polycyclic aromatic hydrocarbon (PAH) with four fused benzene rings. This bacterium also degrades other HMW PAHs (fluoranthene, benzo[*a*]pyrene, benzo[*a*]anthracene, and 7,12-dimethylbenzo[*a*]anthracene) and low-molecular-weight (LMW) PAHs (naphthalene, fluorene, phenanthrene, and anthracene), primarily via dioxygenation to isomeric *cis*-dihydrodiols (2, 5–15). Because of its versatile PAH degradation ability, *M. vanbaalenii* PYR-1 has been extensively studied as a model at both the laboratory and field scales (16, 17). These efforts have produced considerable information on its metabolic, biochemical, physiological, and molecular characteristics, which are also found in other aromatic-compound-degrading bacteria (16–22).

A global PAH metabolic network (MN) in *M. vanbaalenii* PYR-1 has been proposed on the basis of genomic, proteomic, metabolic, and biochemical information (23). The PAH-MN describes the biochemical pathways for the biodegradation of 10 PAHs with 183 metabolites and 224 chemical reactions, providing systematic insight into the structure, behavior, and evolution of bacterial PAH metabolism. The scale-free, funnel-like structure of the PAH-MN is intimately related to its behavior and evolution. PAH substrates are degraded by interactions of a set of functional modules, termed ring-cleavage processes (RCPs), side chain processes (SCPs), and central aromatic processes (CAPs). The activation of thermodynamically stable benzene rings and ring-cleavage

reaction of the corresponding dihydroxylated intermediates occur in the RCP, side chain removal to produce biological metabolic precursors occurs in the SCP, and the metabolic connection of protocatechuate to the tricarboxylic acid (TCA) cycle occurs in the CAP. Proteomic experiments have proved that *M. vanbaalenii* PYR-1 regulates the PAH-degrading genes according to the functional modules (13, 14, 23–25). The enzymes responsible for SCP and CAP modules are regulated relatively loosely, whereas RCP enzymes are regulated by the substrates. This function-dependent regulation has been considered to be an evolutionary and metabolic endeavor to enhance functional compatibility among the functional modules (23). The well-organized combination of the functional modules to obtain nutritional benefits from PAHs allows PAH metabolism with more productivity and less toxicity: that is, generating more productive biological precursors (pyruvate and acetyl coenzyme A) and fewer toxic intermediates (such as *o*-quinones) (3, 23, 26, 27). The determination of the catabolic potential of

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about 200 PAH-degrading enzymes encoded in an ~150-kb catabolic gene cluster in *M. vanbaalenii* PYR-1 indicated the exceptional metabolic diversity for HMW PAHs (28). The epistatic interaction (functional combination of two or more paralogous enzymes for an enzyme reaction step) and pleiotropic activity (functional contribution of an enzyme for two or more different substrates) of a limited set of enzymes are widespread phenomena in the PAH-MN and require sophisticated management.

Considerable attention has been devoted in recent years to the identification and annotation of the ring-hydroxylating oxygenase (RHO) enzymes responsible for ring hydroxylation of HMW PAHs, the first step of the RCP module in the PAH-MN, which mainly controls the pathway and rate of degradation (25, 29–31). These endeavors have allowed evidence-based annotation of RHOs for the hydroxylation of HMW PAHs and also a better understanding of the effects of genetic perturbation and responses at a network level (25). A type V RHO (32), like the phenanthrene dioxygenase of *Nocardioideis* sp. KP7, consists of an oxygenase, a [3Fe-4S]-type ferredoxin, and a glutathione-type reductase. The *nidA* genetic perturbation model in *M. vanbaalenii* PYR-1 (25) has proved that two type V RHOs, NidAB and NidA3B3, not only have pyrene- and fluoranthene-hydroxylating responsibilities, respectively, but also have pleiotropic hydroxylating activity for LMW PAH substrates (25). The functional robustness of the PAH-MN with respect to the loss of function due to Nid perturbation depends mainly on the epistatic functional redundancy derived from the constitutively expressed NidA3B3 system, which overlaps considerably in substrate specificity with the NidAB system (31). These observations suggest that the epistatic interaction and pleiotropic activity of the RHO systems may be more important and complex than those of other enzymes in the PAH-MN, although the enzymes are usually substrate regulated. Furthermore, since LMW PAHs are generally more acceptable in the substrate-binding pocket than HMW PAHs, more RHO enzymes could function for hydroxylation of LMW PAHs. Therefore, the functional diversity and complexity of RHO systems for hydroxylation of LMW PAHs should be higher than those for HMW PAHs. Despite the exciting extension of knowledge of PAH metabolism, the epistatic interaction and pleiotropic activity of PAH-degrading enzymes, including RHO enzymes (30), are still poorly understood at the level of the metabolic network. Considering their extremely high functional redundancy with respect to LMW PAHs and, consequently, the increased epistatic and pleiotropic complexity of RHOs, it is challenging to annotate RHO enzymes into RCP functional modules and to understand the functional relationship of regulation/pleiotropy/epistasis of RHOs responsible for LMW PAHs in the PAH-MN.

The PAH-MN of *M. vanbaalenii* PYR-1 represents the metabolic feedback of successful functional interactions—epistatic and pleiotropic combinations—among enzymes involved in the functional modules at the network level. In this study, a phenotype-based approach, coupled with a spray plate method, was used to select a mutant unable to degrade 3-ring PAHs. Using this transposon mutant defective in *pdoA2*, an aromatic ring-hydroxylating oxygenase (RHO) (33), we generated top-down metabolic perturbation evidence of mutant 6G11 and bottom-up, enzyme-centric data of the type V Pdo system. Finally, we systematically integrated the two different types of information into an RHO-centric functional map, which provides an enzymatic overview of the pleiotropic and epistatic behavior of RHO systems in the PAH-MN.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>M. vanbaalenii</i> PYR-1	Mineralizes PAHs, such as fluoranthene, pyrene, and phenanthrene	4
<i>M. vanbaalenii</i> 6G11	Tn mutant of <i>M. vanbaalenii</i> PYR-1, defective in <i>pdoA2</i> gene; Km ^r	This study
<i>E. coli</i> TOP10	F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> <i>galU</i> <i>galK</i> Δ(<i>ara-leu</i>)7697 <i>rpsL</i> (StrR) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> BL21(DE3)	F [−] <i>ompT</i> <i>hsdS_B</i> (<i>r_B[−]</i> <i>m_B[−]</i>) <i>gal dcm</i> (DE3)	Novagen
Plasmids		
pCR2.1 TOPO	TA cloning vector; Ap ^r	Invitrogen
pET-17b	Expression vector; T7 promoter; Ap ^r	Novagen
pBRCD	pBBR1MCS-5 containing <i>phdCD</i> ; Gm ^r	33
pSKU09	pET9a containing <i>pdoA2B2</i> from <i>Mycobacterium</i> sp. strain 6PY1; Ap ^r	33
pCEC55	pCR2.1 TOPO containing 1.98-kb PCR product of <i>pdoA2B2</i> genes from <i>M. vanbaalenii</i> PYR-1; Ap ^r	This study
pCEC551	pET-17b carrying <i>pdoA2B2</i> ; Ap ^r	This study

^a Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. Cells of *M. vanbaalenii* PYR-1 and its mutant were cultured at 30°C in Luria-Bertani (LB) medium or Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) (Remel, Lenexa, KS) (Table 1). Agar (1.5%) was used to solidify media, and kanamycin (25 to 50 μg/ml) was added when needed. For PAH degradation experiments, a slightly modified supplemented minimal medium (SMM) was used (34).

Pyrene and phenanthrene were from Chem Service (West Chester, PA). Fluorene, anthracene, fluoranthene, benzo[*a*]pyrene, and kanamycin were from Sigma-Aldrich (St. Louis, MO). PAHs were dissolved in *N,N*-dimethylformamide (DMF) at various concentrations between 100 mM and 1 M and directly added to culture flasks. Acetone-dissolved PAHs (1% [wt/vol]) were used to select mutant candidates by the spray plate method (35). Cell growth was determined by measuring optical density at 600 nm (OD₆₀₀) with a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT). Kinetic data are means of the results from triplicate samples from three independent experiments.

Selection of a transposon mutant with impaired ability to degrade LMW PAHs. Previously, a library consisting of over 4,000 Tn5-based transposon insertion mutants of *M. vanbaalenii* PYR-1 had been constructed and stored in 10% glycerol-containing 96-well plates at −70°C (25). These mutants were replicated on the surfaces of 150-mm-diameter 7H9 agar plates, using a plate replicator. After 7 to 10 days of incubation, the plates were sprayed with fluorene, phenanthrene, or anthracene and further incubated for 2 to 3 days to recognize clear zones around colonies or color changes of colonies under the PAH film. The transposon integration site was determined by plasmid rescue cloning according to the recommendations of the manufacturer (Epicenter Biotechnologies, Madison, WI) and as described previously (23). Sequences were determined at the University of Arkansas for Medical Sciences (Little Rock, AR).

Construction and biotransformation experiment of *pdoA2B2* ring-hydroxylating oxygenase expression system. For protein expression of the Pdo system in *Escherichia coli*, the pET-17b expression system (Novagen, Madison, WI) was used. A DNA fragment containing *pdoA2B2* was

amplified from *M. vanbaalenii* PYR-1 genomic DNA using primers E-Pdo2-F-NdeI (5'-CATATGTCCTATTGTCGGTAAGAACGACATT-3') and E-Pdo2-R-HindIII (5'-AAGCTTAGAAGAAGTTAGCCAGATTGTGGG-3'). The underlined sequences are the NdeI and HindIII sites, respectively. The 1.98-kb PCR product was initially cloned into a pGEM-T Easy vector system (Promega, Madison, WI) to give pCEC55 and was subjected to DNA sequencing to confirm that PCR amplification did not introduce mutations. The insertion of plasmid pCEC55 was isolated by digestion with NdeI and HindIII and ligated between the NdeI and HindIII sites of pET-17b, resulting in plasmid pCEC551. Two plasmids, pCEC551, containing the *pdoA2B2* genes (Mvan_0546/0547), and pBRCD, expressing the *phdCD* genes of *Nocardioides* sp. strain KP7 (33), were transformed into *E. coli* strain BL21(DE3) (Table 1). *In vivo* assays of the recombinant Pdo system from *M. vanbaalenii* PYR-1 were performed as previously described (30, 31) in which the PdoA2B2 (pSKU09 and pBRCD) of *Mycobacterium* sp. 6PY1 was used as a positive control (33).

Analytical methods for identification of PAH degradation. For the PAH metabolism study of *M. vanbaalenii* PYR-1 and *pdoA2* mutant strain 6G11, cultures were sampled (1.5 ml) over time and directly extracted three times with equal volumes of ethyl acetate. Residual water in the extracted fraction was removed by addition of sodium sulfate. The solvent was evaporated *in vacuo*, and the residues were dissolved in acetonitrile. Sequentially, a 10- μ l filtrate was subjected to high-performance liquid chromatography (HPLC) analysis, using an 1100 series HPLC system (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of two gradients, first for 10 min with 10% to 30% acetonitrile in water with constant 0.1% formic acid and then for 30 min with 30% to 100% acetonitrile in water with constant 0.1% formic acid, both at a flow rate of 0.3 ml min⁻¹. The gradients were followed by the use of 100% acetonitrile for 10 min at a flow rate of 0.5 ml min⁻¹. The diode-array detector signal was monitored at 240, 254, 270, 280, 290, 300, 330, and 348 nm with a reference wavelength of 360 nm. In the *in vivo* analysis of the Pdo system, the oxygenated metabolites were identified on the basis of the previous metabolic information derived from *M. vanbaalenii* PYR-1 culture studies and the two Nid systems, including UV spectrum, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) data (3, 8, 10, 12, 14, 30, 31).

In silico analysis. The homology model of the large oxygenase subunit (PdoA2) from the Pdo system was generated by homology modeling, using the Swiss-Model server (36), with the naphthalene dioxygenase (NDO) (Protein Data Bank [PDB] accession no. 2B24; 56.28% amino acid sequence identity) from *Rhodococcus* sp. strain NCIMB 12038 as a template (37). The root mean square deviation (RMSD; C α) of the PdoA2 model (433 amino acids) superimposed on the template structure (PDB accession no. 2B24; 434 amino acids) was 0.62 Å, in which 433 amino acid residues were aligned with 56.3% sequence identity, which is high enough accuracy for further ligand docking experiments (38). PROCHECK (European Bioinformatics Institute, Cambridge, United Kingdom) was used to check the structural model. Superpose (version 1.0) was used for structural superposition and RMSD calculation. The volumes of active sites were measured using CASTp (39). For computing the orientation of PAHs relative to the active site of the Pdo system, two automatic docking programs, PatchDock (40) and GEMDOCK (41), were used with default docking settings. PyMOL (0.99RC6; <http://www.pymol.org/>) was used to visualize the three-dimensional (3-D) structures (42).

RESULTS

Isolation of a *pdoA2* mutant. By the use of the previously prepared Tn mutant library of *M. vanbaalenii* PYR-1 (25), two rounds of screens, using a spray plate test with diverse PAHs to visualize bacterial degradation activity, were conducted for isolation of mutants defective in the degradation of PAHs with three rings but not with four rings. In the first round, pyrene and fluoranthene were used to test the *M. vanbaalenii* Tn mutant library to confirm the ability to degrade HMW PAHs. In the second round,

FIG 1 Selection of an *M. vanbaalenii* PYR-1 Tn5 mutant with an insertion in the *pdoA2* gene. (a) Mutant candidates on 7H9 agar plate sprayed with fluorene. The arrows indicate the 6G11 mutant, which was from the 96-well plate, number 6, line G, and row 11, producing no clear zone with fluorene. (b) Diagram showing the Tn5 chromosomal insertion in the *pdoA2* gene. KAN, kanamycin resistance.

the Tn mutant library was again screened for mutants with reduced metabolic activity on LMW PAHs. In the spray plate test with fluorene, a mutant, 6G11, which appeared not to degrade fluorene, was identified (Fig. 1a). Sequence analysis of the 6G11 mutant determined that the position of the transposon insertion was in the middle of the *pdoA2* gene, one of the 21 RHO genes of *M. vanbaalenii* PYR-1 (Fig. 1b).

Spray plate test using PAHs of mutant 6G11. The PAH degradation activity of mutant 6G11, which had no Pdo hydroxylation activity, was further tested by additional spray plate tests using different PAHs. Mutant 6G11 produced a clear zone around the colony with phenanthrene, pyrene, and fluoranthene but did not produce a clear zone with either fluorene or anthracene (see Fig. S1 in the supplemental material). In addition, although mutant 6G11 produced clear zones with phenanthrene and fluoranthene, it lacked the yellowish brown color, an indication of ring fission, produced by the wild-type *M. vanbaalenii* PYR-1 (see Fig. S1).

PAH metabolic comparison between the wild-type strain and mutant 6G11. On the basis of the spray plate screening test results, we compared, using spectrophotometric and analytical chemistry methods, PAH degradation by *pdoA2* mutant 6G11 with that by the wild-type *M. vanbaalenii* PYR-1 strain, using fluorene, anthracene, phenanthrene, pyrene, and fluoranthene as PAH substrates. The overall growth rates of the wild-type strain and the mutant were almost the same in SMM containing 1% sorbitol, regardless of whether they were supplemented with PAHs or not (Fig. 2). This indicated that the mutation did not affect the basic phenotype of the wild-type strain associated with growth.

HPLC analysis of the culture medium revealed differences in PAH metabolism between *M. vanbaalenii* PYR-1 and mutant 6G11 (Fig. 3). Whereas there were no significant differences in the rates of phenanthrene and fluoranthene degradation (Fig. 3a), the degradation rates of fluorene, anthracene, and pyrene were lower for the mutant than for the wild type (Fig. 3b). Among those three PAHs, the rate of anthracene degradation was most significantly

FIG 2 Growth of wild-type *M. vanbaalenii* PYR-1 (solid line) and the *pdoA2* 6G11 mutant (dotted line). SMM broth contains 1% sorbitol supplemented with 200 μ M fluorene, anthracene, phenanthrene, pyrene, or fluoranthene. The kinetic values were calculated from the average values of all growth conditions, each of which had triplicate independent cultures.

reduced. Whereas the wild type degraded over 85% of the anthracene within 2 days, the mutant degraded only 30% of the initial amount of anthracene within the same period. A decrease in the rate of fluorene degradation was also detected. The HPLC results for anthracene and fluorene were consistent with the PAH spray plate test results. However, showing inconsistency with the results of the PAH spray plate test, a slight difference was found in the rates of pyrene degradation revealed by HPLC. Whereas the culture of *M. vanbaalenii* PYR-1 had degraded over 40% of the pyrene at day 2, mutant 6G11 had degraded around 28% of the same amount of pyrene at the same time.

Consistent with the results of the PAH spray plate test, we detected a difference in color development during incubation

with PAHs. Culture flasks of the 6G11 mutant, incubated with fluorene, phenanthrene, or fluoranthene, produced no color, unlike wild-type *M. vanbaalenii* PYR-1 (see Fig. S2 in the supplemental material). This indicates a pleiotropic contribution of the Pdo system to the degradation of PAHs.

In vivo PAH hydroxylation by the recombinant Pdo system.

To test its pleiotropic aromatic-ring-hydroxylating ability, the Pdo system was reconstituted in *E. coli* BL21(DE3), using the pET17b expression vector. We detected no enzyme activity of the recombinant Pdo system without addition of a cognate electron transport chain (ETC) partner. Enzyme activity of the Pdo system was reconstituted when it was combined with the type V ETC, PhdCD (33). Using this active Pdo system, the substrate specificity of the Pdo enzyme was investigated, using biphenyl, naphthalene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, and benzo[*a*]pyrene. The relative product yields of the recombinant Pdo system for the PAHs were compared with each other on the basis of the metabolite profiles and HPLC peak areas.

Consistent with the PAH spray plate test and metabolic experiments, the Pdo system was able to oxidize a wide range of PAHs, with from two to five rings. However, it showed an apparent substrate preference for LMW PAHs with two or three rings. Naphthalene, fluorene, anthracene, and phenanthrene were oxidized over 90% (Fig. 4). In the case of biphenyl, the Pdo system showed conversion of ~50%. On the other hand, the HMW PAHs with four or five rings, including pyrene and benzo[*a*]pyrene, showed relatively low conversion of less than 40%, with the exception of fluoranthene, whose conversion was over 80%.

FIG 3 (a) Single-PAH degradation by cultures of *M. vanbaalenii* PYR-1 (filled symbols) and the 6G11 mutant (open symbols). (b) Comparisons are shown for individual PAHs only if they were different ($0.01 < P < 0.07$). Cells were grown in SMM supplemented with 1% sorbitol containing 200 μ M fluorene, anthracene, phenanthrene, pyrene, or fluoranthene. conc., concentration.

FIG 4 Pleiotropic function of the Pdo system with respect to diverse PAH substrates. Chemical structures of the tested PAH substrates and the corresponding metabolite(s), together with their conversion rates, are presented. The regiospecific information for fluorene and fluoranthene was based on binding modes from the docking simulation. The thickness of the arrow indicates differences in transformation efficiency.

In the biotransformation analysis, the Pdo system converted biphenyl and naphthalene to only one metabolite each, with UV spectra similar to those of authentic biphenyl *cis*-2,3-dihydrodiol and naphthalene *cis*-1,2-dihydrodiol, respectively (11, 31). When the Pdo system was incubated with fluorene, three possible metabolites were found at 11.46 min (55%), 13.55 min (31%), and 16.71 min (24%). The major metabolite at 11.46 min, which accounted for ~55% of the total, was likely a fluorene dihydrodiol whose position of substitution could not be determined. In the analysis of anthracene, two possible metabolites were found at 11.70 min (79%) and 16.50 min (21%). The principal metabolite, with a retention time of 11.70 min, had a UV spectrum with $\lambda_{\max}$ of 250, 290, and 300 nm, similar to that of anthracene *cis*-1,2-dihydrodiol (10). In the case of phenanthrene, the *cis*-3,4- and *cis*-9,10-dihydrodiols, eluting at 11.46 and 16.53 min, respectively, were identified as the same as those obtained with NidAB (31). However, unlike NidAB, which formed a 75:25 mixture ratio for phenanthrene *cis*-3,4-dihydrodiol and *cis*-9,10-dihydrodiol, respectively (27), the Pdo system produced more than 99% phenanthrene *cis*-3,4-dihydrodiol. In the case of pyrene, the Pdo system produced only pyrene *cis*-4,5-dihydrodiol. In the conversion of fluoranthene, three possible metabolites were identified at 15.24 min (78%), 16.43 min (6%), and 17.79 min (16%), but none

of these metabolites had a UV spectrum similar to that of fluoranthene *cis*-2,3-dihydrodiol (14). In the benzo[a]pyrene experiment, the Pdo system produced traces of benzo[a]pyrene *cis*-7,8-dihydrodiol (43) at 15.29 min and an unidentified metabolite at 16.49 min.

Topological features of the substrate-binding pocket of the Pdo system. To structurally explore the substrate specificity of the Pdo system, the substrate-binding pocket of the Pdo system was elaborated by structural comparison with other well-organized RHO information, including data from two type V Nid systems from *M. vanbaalenii* PYR-1, and by docking simulation using selected PAH substrates. The root-mean-square deviation (RMSD) ($C\alpha$) of the Pdo system showed high structural similarity to the type V RHOs, the two Nid systems (Table 2), whereas the angular dioxygenase CARDO (carbazole 1,9a-dioxygenase) from *Janthinobacterium* sp. strain J3 (44) had the lowest structural similarity to the Pdo system. Compared with the active sites, the topology and volume of the substrate-binding site and the substrate diversity of the Pdo system showed high structural and functional similarity to those of the type V NidA from *M. vanbaalenii* (31) and the type III PhnI from *Sphingomonas* sp. strain CHY-1 (45, 46), which have substrate preferences for HMW PAHs (Table 2). This common structural feature of the Pdo system indicates it has a

TABLE 2 Structural comparison of the active site of the Pdo system with those of other RHOs with known structures

No.	RHO ^a	Type ^b	Active-site vol (Å ³) ^c	RMSD (Cα) with the following RHO ^d							The best substrate/substrate diversity ^e	PDB accession no.	Source or reference(s)
				1	2	3	4	5	6	7			
1	Pdo	V	469	0							Phenanthrene/1–5-ring compounds		This study
2	NidA	V	607	0.20	0						Pyrene/1–5-ring compounds		31
3	NidA3	V	725	0.29	0.29	0					Fluoranthene/1–5-ring compounds		31
4	BPDO	IV	300	1.68	1.68	1.69	0				Biphenyl/PCBs	1ULJ	53
5	NDO	IIIαβ	414	1.62	1.66	1.64	2.29	0			Naphthalene/1–3-ring compounds	1O7G	54, 55
6	PhnI	IIIαβ	295	1.71	1.69	1.71	2.52	1.29	0		Naphthalene/1–5-ring compounds	2CKF	45, 46
7	CARDO	IIIα	352	2.86	2.96	2.85	2.74	2.87	2.88	0	Carbazole/2–4-ring compounds	1WW9	44, 56

^a BPDO, biphenyl 2,3-dioxygenase; CARDO, carbazole 1,9-dioxygenase.

^b Based on the RHO classification by Kweon et al. (32).

^c The active-site volumes were measured using CASTp (39).

^d RMSD corresponding to the RHO. The RHO is referred to by the number shown in the first column.

^e For more detailed substrate specificity data for each RHO, please refer to the references. PCB, polychlorinated biphenyl.

relatively large active-site cavity. The three aromatic amino acids, Phe-Phe-Phe, which play important roles in binding aromatic substrates via hydrophobic interactions in the substrate-binding pockets of RHO enzyme systems (31), are perfectly conserved in the Pdo system (Phe-212, Phe-365, and Phe-371) (Fig. 5a).

Ligand docking simulations, in conjunction with the structural features of the substrate-binding pocket, further support the observed metabolic properties of the Pdo system. Figure 5b shows the binding modes of phenanthrene, anthracene, fluorene, and fluoranthene from the docking simulation of the Pdo system. The bound PAH substrates in the active site of the Pdo system were positioned in almost the same place next to the iron, within ~5 Å (Fig. 5b). The predicted binding modes of the selected PAH substrates were consistent with the experimentally known regiospecificity of the substrates, in terms of hydroxylation. In the phenanthrene-binding simulation, the best binding mode was oriented in the active site with carbons 3 and 4 of the first ring closest to the mononuclear iron, consistent with the major product observed in the biotransformation assay, phenanthrene *cis*-3,4-dihydrodiol. In the case of the three-ring PAHs, fluorene and anthracene, the

best binding modes were in positions that would produce fluorene *cis*-3,4-dihydrodiol and anthracene *cis*-1,2-dihydrodiol, respectively. In the fluoranthene-docking simulation, the orientation of the substrate with the highest binding affinity (Fig. 5b) suggests that fluoranthene would be hydroxylated at positions C-7,8. Substrate orientation leading to hydroxylation on carbons 2 and 3 of fluoranthene was not predicted, in accordance with the biotransformation result of the enzyme.

Construction of an RHO-centric functional map of the PAH-MN. On the basis of the data obtained in this study and from previous work (16, 17, 25, 29–32, 47), we organized RHO-related information (Fig. 6) and constructed an RHO-centric functional map of the PAH-MN (Fig. 7) with pleiotropic and epistatic numerical scores of RHO enzymes via three steps: step 1, calculation of the relative functional activity (RFA, on a scale of 0 to 10) of each RHO enzyme (Fig. 6); step 2, reconstruction of an RHO-centric functional map by annotation of RHO enzymes with RFA into the PAH-MN, on the basis of the regiospecificity of each enzyme toward each PAH substrate (Fig. 7a); and step 3, validation of the RHO-centric functional map by the experimental met-

FIG 5 Spatially conserved aromatic amino acids in the substrate-binding pockets of three type V RHO systems, the Pdo system and two Nid systems, in *M. vanbaalenii* PYR-1 (a) and surface plot of the substrate-binding pocket of the Pdo system with the PAH substrates bound with the highest binding affinity (b). The mononuclear Fe²⁺ and spatially conserved aromatic amino acids are represented as a red ball and a stick model, respectively.

FIG 6 Relative functional activity (RFA) of the RHO members in fluorene, anthracene, phenanthrene, fluoranthene, and pyrene degradation on the basis of their ETC compatibility, substrate specificity, and protein abundance. RHO enzymes were classified according to Kweon's RHO scheme (32). The ETC compatibility of an RHO enzyme was calculated from its RHO classification. Information on protein abundance for RHO enzymes was retrieved from the proteome database of *M. vanbaalenii* PYR-1 (23). The substrate preference of each RHO enzyme was determined on the basis of the percent conversion rate of each PAH substrate by each enzyme. Please refer to Table S1 in the supplemental material for numerical scores.

abolic data and an RHO-centric functional map of the 6G11 mutant with no Pdo enzyme activity (Fig. 7b).

In step 1, for a digitized functional map of RHO enzymes, three factors—protein abundance level, ETC compatibility, and substrate specificity—of RHO enzymes, including the Pdo system, were quantified using a 10-point rating scale to calculate the RFA of each RHO enzyme with respect to each PAH substrate. The relative functional activity (RFA) was calculated by the equation below:

$$\text{RFA} = \frac{\text{ETC compatibility} \times \text{substrate specificity}}{\text{protein abundance level}} \quad (1)$$

Here, ETC compatibility of an RHO enzyme was calculated on the basis of Kweon's RHO classification (41), providing the relationship and compatibility between the two functional classes (an oxygenase and an ETC); since only one type V ETC exists in the genome of strain PYR-1, the RHO enzymes belonging to type V were awarded 10 points for ETC compatibility, and the others were awarded 1 point. The information on the protein abundance for RHO enzymes was retrieved from the proteome database of *M. vanbaalenii* PYR-1, which was developed through analysis by reversed-phase nano-liquid chromatography-tandem mass spectrometry (RP nano-LC-MS/MS) (23). The constitutively expressed and >2-fold-overexpressed RHO enzymes were awarded 5 and 10 points, respectively. For the scoring of substrate specific-

ity, the percentage of hydroxylation of each PAH substrate to the corresponding dihydrodiol(s) by each RHO enzyme was converted into a 10-point rating scale. The Pdo system presented the highest RFA for fluorene, anthracene, and phenanthrene hydroxylation (Fig. 6; see also Table S1 in the supplemental material), indicating its functional importance as a main RHO system, consistent with the metabolic patterns of the PAH substrates in the wild type and mutant.

In step 2, on the basis of the RFA value of each RHO enzyme, the product regioselectivity of each enzyme toward each PAH substrate was used to assign the RHO enzymes to each degradation route of the RHO-centric functional map (Fig. 7a). For example, in the phenanthrene degradation pathways, the three RHO enzymes function in the initial dioxygenation, and the Pdo system is a main RHO system that contributes about 42.9% (RFA, 10) of the total RHO functional activity (sum of the RFAs, 23.3). The three RHO enzymes epistatically interact in the two productive pathways, C-3,4 and C-9,10, while only the NidA3B3 system functions for the nonproductive C-1,2 route, with minor activity. The Pdo system contributes not only to the productive C-3,4 route, with its RFA of ~9.9 (~42.5% of the total RFA), but also to the C-9,10 route, with its RFA of ~1 (~0.4% of the total RFA).

In step 3, initially, an RHO-centric functional map of the 6G11 mutant with no Pdo enzyme activity (Fig. 7b) was reconstructed, on the basis of that of the wild-type strain, and then confirmed by experimental metabolic data from the 6G11 mutant. The observed metabolic perturbation impacts of mutant 6G11 were seen in the functional map. For example, in fluoranthene metabolism in *M. vanbaalenii* PYR-1, the Pdo system could function in the C-1,2, C-7,8, and C-8,9 dioxygenation pathways but not in the C-2,3 dioxygenation pathway. The C-7,8 route leads to the color-producing acenaphthene or acenaphthylene pathways in the PAH-MN (14, 23, 48). The metabolic features of mutant 6G11 incubated with fluoranthene, such as no color change and no significant metabolic discrepancy from wild-type *M. vanbaalenii* PYR-1, could be explained well by comparing the RHO-centric functional maps of the wild type and the mutant.

DISCUSSION

In this study, a phenotype-based (or forward genetics) strategy, coupled with a spray plate method, provided a unique way to screen for a mutant able to degrade 4-ring PAHs but not 3-ring PAHs in an unbiased, global manner independent of previous assumptions about gene function (25). The use of two rounds of PAH spray plate tests on >4,000 transposon mutants was a time-saving and efficient approach to screen for defectiveness of the type V RHO system with responsibility for LMW PAH hydroxylation. Several layers of experimental evidence in this study were used to evaluate the strength of the systematic combination. The bottom-up, enzyme-centric data of the type V Pdo system were compatible with the top-down metabolic observations on mutant 6G11. This report presents an effort to systematically integrate the two different types of information into an RHO-centric functional map (Fig. 7) which provides information on their pleiotropic activity and epistatic interaction in the PAH-MN. Pleiotropy and epistasis of PAH-degrading enzymes are important for functional enzyme annotation and for connecting enzyme functional dynamics to the corresponding metabolic feedback in the PAH-MN.

Type V Pdo system in the PAH-MN. As the RHO-centric

FIG 7 Schematic representation of relative contributions of the RHO enzymes to the pathways of fluorene, anthracene, phenanthrene, pyrene, and fluoranthene degradation in *M. vanbaalenii* PYR-1 (a) and the 6G11 mutant (b). The arrows indicate degradation pathways, and differences in transformation efficiency are represented by arrow thickness. Colored circles indicate RHO enzymes, with the sizes being proportional to the degrees of functional contribution. RCP, ring-cleavage process; SCP, side chain process; CAP, central aromatic process.

functional map (Fig. 7) reveals, of 21 RHO genes in the *M. vanbaalenii* PYR-1 genome (17, 28), about 10 RHO systems respond dynamically to PAH substrates (23). Among them, the type V RHO systems are mostly active for PAH hydroxylation in the

PAH-MN, due mainly to their type V ETC requirement (23, 32). In Kweon's RHO classification reflecting functional interactions between oxygenase components and ETCs (32), the Pdo system is a type V RHO system and is functionally compatible

(b)

M. Vanbaalenii mutant 6G11

FIG 7 continued

with type V ETC components. Due to their substrate-dependent regulation and differential substrate preferences, type V RHO systems apparently have their own pleiotropic and epistatic roles in dioxygenation of PAH substrates in the PAH-MN. As shown in the RHO-centric functional map, the two Nid systems function mainly for hydroxylation of HMW PAHs, but

the Pdo system functions for LMW PAH-centric hydroxylation. The PAHs that induce the Pdo system and the preferred PAH substrates almost overlap, suggesting that the critical pleiotropic and epistatic functional responsibility is under the control of the channel management of the PAH-MN toward more productivity and less toxicity (3, 23, 26, 27). The product

regiospecificity of the Pdo system also supports its productive pleiotropic and epistatic functional contribution to the PAH-MN.

Structural features of the Pdo system linked to substrate and product specificity. As revealed in this study, the Pdo system was able to oxidize a wide range of PAHs with two to five rings. The Pdo system showed a relatively low product regiospecificity for LMW PAH substrates. This regiospecificity of RHO enzymes for PAH substrates has been observed previously in the two Nid systems (29–31). The Nid systems show relatively low regiospecific oxidation of small substrates, producing several isomeric dihydrodiols from each, but the best substrates, pyrene and fluoranthene, are regiospecifically oxidized only to pyrene *cis*-4,5-dihydrodiol, by the NidAB system, and to fluoranthene *cis*-2,3-dihydrodiol, by NidA3B3, respectively. Therefore, the Pdo and Nid systems share low regiospecificity for LMW PAHs. Unlike the two Nid systems with regiospecific hydroxylation ability with respect to their best substrates (pyrene for NidAB and fluoranthene for NidA3B3), the Pdo system showed relaxed regiospecificity for even the best substrates, fluorene, anthracene and phenanthrene. There was no clear relationship between the degree of regiospecific oxidation and the conversion rate that depended on the size of the PAH substrate (31). As previously proposed for the Nid systems (31), the regiospecificity of the Pdo system is most likely due to substrate mobility in the active site. The structural features of the substrate-binding pocket of the Pdo system dictate its regiospecificity for diverse PAH substrates, strongly supporting the idea of the existence of multiple substrate-binding modes caused by substrate mobility in the active site. The Pdo system substrate-binding pocket satisfies structural and functional requirements for accepting and hydroxylating both LMW and HMW PAHs. The Pdo active site may be relatively large, similar to those of the RHO systems, with a broad specificity for HMW PAHs. The three aromatic amino acids (Phe-Phe-Phe) which keep aromatic substrates within the reactive distance from the iron atom, allowing oxygen to attack the neighboring carbons of the substrate, are spatially conserved in the substrate-binding pocket of the Pdo system.

Functional responsibility of the Pdo system in phenanthrene metabolism. The Pdo system of *Mycobacterium* sp. 6PY1 has been annotated as a competent RHO system responsible for phenanthrene 3,4 dioxygenation (33). Surprisingly, in *M. vanbaalenii* PYR-1, the Pdo system is also extensively involved in the hydroxylation of a broad range of other PAHs, even HMW PAHs. Together with the bottom-up enzymatic evidence, the top-down metabolic observations from mutant 6G11, showing substantial decreases in degradation of fluorene, anthracene, and pyrene and the different color changes of the culture media containing fluorene, phenanthrene, and fluoranthene, prove the critical pleiotropic and epistatic functional responsibility of the Pdo system in the PAH-MN. Comparison of the two RHO-centric functional maps of the wild type and mutant (Fig. 7) clarifies the apparent metabolic discrepancy caused by losing the hydroxylating activity of the Pdo system. The deep pleiotropic and epistatic perturbation of the Pdo system, which affected the PAH-MN, attests to its functional importance for more-productive and less-toxic PAH metabolism (3, 23, 26, 27).

In phenanthrene degradation, identification of isomers of phenanthrene *cis*-dihydrodiols and coexpression of several RHOs have been ascribed to the epistatic functional combination of several RHO enzymes, including the Pdo system, in the initial dioxy-

genation steps in the PAH-MN (10, 23, 47, 49). Although the Pdo system has been considered a major RHO system for the initial hydroxylation of phenanthrene in the RCP module in the PAH-MN (30), there was no direct evidence for its pleiotropic and epistatic function for phenanthrene degradation (23). Several lines of enzymatic and metabolic evidence support the proposed regiospecific responsibility—major C-3,4 dioxygenation and minor C-9,10 dioxygenation without C-1,2 dioxygenation—for phenanthrene hydroxylation in the PAH-MN. First, the PdoA2 system shows tight phenanthrene-dependent regulation (23) and an apparent substrate preference for phenanthrene, with a regiospecificity of C-3,4 (>99%) and C-9,10 (~1%) dioxygenation. Second, functional perturbation of the type V gene for PdoA2 resulted in the disappearance of the distinct yellowish coloration shown by the wild-type strain during phenanthrene degradation, although there were no differences in the degradation rates. The lack of a distinct difference between *M. vanbaalenii* PYR-1 and the 6G11 mutant in phenanthrene metabolism is better explained by epistatic functional redundancy donated by other RHO systems rather than by a minor role for PdoA2. Our previous *nidA* genetic perturbation model study (25) provided evidence for the vertical pleiotropic function of the two HMW PAH RHO systems, NidAB and NidA3B3, which are able to initially dioxygenate phenanthrene. The type V NidA3B3 system is induced by phenanthrene and shows a high conversion ability for it, with relaxed regiospecificity, producing three *cis*-dihydrodiols (3,4-, 9,10-, and 1,2-), with conversion of 53%, 26%, and 21%, respectively (30, 31). Considering that in the PAH-MN, the C-3,4 dioxygenation route is the main productive pathway via 2-hydroxy-1-naphthoic acid, whose direct ring cleavage produces yellowish intermediates (10, 23, 47), the absence of yellow coloration in the 6G11 mutant during phenanthrene degradation indicates that there was a significant change in terms of metabolic quantity and quality of the phenanthrene degradation in the mutant (Fig. 7). However, judging on the basis of the regiospecificity of the two Nid systems, the C-3,4 dioxygenation route still could be a major pathway for phenanthrene degradation, although the allowed quantity was detectably decreased. In the PAH-MN, the C-1,2 dioxygenation route producing dead-end products, such as 1-methoxy-2-hydroxyphenanthrene, 2-methoxy-1-hydroxyphenanthrene, and 1,2-dimethoxyphenanthrene, is not productive (23). Taking the whole picture into account, the epistatic functional combination of the Pdo system and the two Nid systems seems to be important for phenanthrene degradation in terms of metabolic quality (Fig. 7). The Pdo system with responsibility for initial dioxygenation at positions C-3,4 and C-9,10 of phenanthrene could productively contribute to phenanthrene degradation but not to the relaxed pleiotropic dioxygenation at C-1,2 by NidA3B3 that results in dead-end metabolites. The pleiotropic function of the Pdo system with respect to the 4-ring pyrene could contribute to the productive C-4,5 route, which is connected with the phenanthrene pathway in the PAH-MN, indicating its direct and indirect functional impacts on the quantity and quality of pyrene metabolism.

Functional responsibility of the Pdo system in fluorene metabolism. In *M. vanbaalenii* PYR-1, two major degradation pathways for fluorene have been proposed: the monooxygenation route, linked to angular dioxygenation of 9-fluorenone leading to 1,1a-dihydroxy-1-hydro-9-fluorenone, and the C-3,4 dioxygenation route (23). Together with identification of 1-indanone, a reference intermediate of the 3,4 dioxygenation route (50), the

appearance of yellow culture fluid in the wild-type strain but no coloration in the 6G11 mutant in the presence of fluorene indicates that the Pdo system initiates the C-3,4 dioxygenation route related to the yellow coloration (see Fig. S2 in the supplemental material). The relaxed regiospecificity of the Pdo system also suggests that its pleiotropic dioxygenation of fluorene could lead to another metabolic route(s), such as a fluorene C-1,2 dioxygenation route. On the other hand, since the Pdo system shows no angular dioxygenation activity, another RHO system(s) should be involved in the angular carbon dioxygenation followed by C-9 monooxygenation of fluorene. A plausible RHO system, Mvan_0543/44, with high sequence similarity to an angular dioxygenase, DbfA1A2 (51), was expressed when *M. vanbaalenii* PYR-1 was treated with fluorene and fluoranthene (Fig. 6 and 7) (23). The bell-shaped curve for yellow coloration formation suggests that the C-3,4 dioxygenation route operating via the colored ring-cleavage intermediate is also a productive pathway, like the monooxygenation pathway operating via 9-fluorenol that is linked to subsequent angular dioxygenation. The Pdo system could function in the initial C-3,4 dioxygenation step of the RCP of fluorene, together with the NidA3B3 system, which has relatively low functional redundancy. Since the 3-ring fluorene degradation pathway is a part of the dioxygenation routes (C-1,2 and C-2,3) of the 4-ring fluoranthene operating via 9-fluorenol, which is a C-9 monooxygenation product of fluorene (14, 23), the pleiotropic and epistatic hydroxylation by the Pdo system has a crucial influence on fluoranthene metabolism (Fig. 7).

Functional responsibility of the Pdo system in anthracene metabolism. The 6G11 mutant showed a significant functional perturbation of anthracene degradation, resulting in the most decreased degradation rate. This metabolic observation indicates that the Pdo system is a major RHO system for the oxidation step(s) in the RCP modules for anthracene degradation and that the PAH-MN has a relatively low epistatic functional redundancy for the initial oxidation step(s). In *M. vanbaalenii* PYR-1, anthracene is degraded via multiple routes (C-1,2-, C-2,3-, and C-9,10 dioxygenation) of enzymatic attack, which seem to be not connected to any of the other PAHs, until its degradation pathway reaches phthalic acid, a main hub intermediate (10, 23). *M. vanbaalenii* PYR-1 seems to use the C-1,2 dioxygenation route as a major degradation pathway for anthracene, similarly to other degraders (52). Therefore, as shown in the RHO-centric functional map, the Pdo system is a main RHO system to oxidize anthracene to its *cis*-1,2-dihydrodiol, with minor epistatic functional assistance by the NidA3B3 system. Considering their low regiospecificity (Fig. 4), these two RHO systems could also be involved in the other anthracene pathway(s), such as a C-2,3 dioxygenation route (30, 31).

Functional roles of the Pdo system in metabolism of HMW PAHs. Although it has pleiotropic oxygenation ability for diverse PAHs, the Pdo system has an apparent substrate preference for LMW PAHs with three rings, such as fluorene, anthracene, and phenanthrene (Fig. 7), in agreement with its structural properties of active-site and substrate-specific regulation. The Pdo system has a direct pleiotropic responsibility for LMW PAH metabolism. In the PAH-MN, connecting the pathways of LMW and HMW PAHs, the functional pleiotropy of the Pdo system with respect to the LMW PAHs also influences the degradation of HMW PAHs. Owing to its direct dioxygenation ability with respect to HMW PAHs, such as fluoranthene, pyrene, and benzo[*a*]pyrene, the

epistasis and pleiotropy of the Pdo system also could play a functionally important role in HMW PAH metabolism, which requires at least four oxygenation steps for complete degradation.

The Pdo system in fluoranthene metabolism. In *M. vanbaalenii* PYR-1, at least four metabolic routes are initiated by both mono- and dioxygenation reactions for fluoranthene degradation (14, 23, 48). As determined on the basis of its oxygenation ability with respect to fluoranthene and fluorene, the epistatic and pleiotropic contribution of the Pdo system to fluoranthene degradation is crucial for determining the metabolic quantity and quality. During degradation of fluoranthene, color changes from colorless via bright orange to bright yellow occur, indicating the transient accumulation of catechol-like compounds and their meta-ring fission products (14, 23, 48). The bell-shaped curve for coloration suggests that the pathway(s) operating via the color-producing intermediate(s) is productive, with no dead-end products. During the degradation of PAHs, coloration is a rapid and convenient colorimetric indicator for the degradation rate. The lack of coloration of the 6G11 mutant during fluoranthene degradation is a special concern in the functional annotation of RHO systems for the hydroxylation of fluoranthene. The lack of fluoranthene C-2,3 dioxygenation by the Pdo system, combined with the fluoranthene C-7,8 or C-8,9 dioxygenation routes leading into the acenaphthylene or acenaphthene pathway (14, 23, 48) and the production of a dark yellow metabolite(s), suggests that the Pdo system contributes to fluoranthene dioxygenation at either the C-7,8 positions or the C-8,9 positions.

The Pdo system in pyrene and benzo[*a*]pyrene metabolism. The 6G11 mutant showed a degradation rate for pyrene lower than that seen with wild-type *M. vanbaalenii* PYR-1. The clear metabolic perturbation in the mutant indicates the functional involvement of the Pdo system in pyrene degradation. Previously, an RCP enzyme, the type V RHO NidAB, has been annotated as an RHO enzyme that guides the degradation of pyrene exclusively into the pyrene C-4,5 dioxygenation route, the only productive way for pyrene to be channeled into the TCA cycle (25). Considering its regiospecific dioxygenation ability to produce pyrene *cis*-4,5-dihydrodiol, the Pdo system could contribute directly to the productive pathway in the PAH-MN. Therefore, loss of epistatic assistance of the Pdo system for the productive pathway may be related to the observed metabolic discrepancy. In addition, the functional perturbation of the Pdo system with respect to phenanthrene could indirectly affect pyrene degradation, which is connected to the phenanthrene pathway in the PAH-MN. The Pdo system productively contributes to pyrene degradation in metabolic quantity and quality. Although the Pdo system is not up-regulated by benzo[*a*]pyrene, the Pdo system could contribute to oxidation of five-ring PAHs if it is induced by other PAHs, as in the PAH mixtures.

Concluding remarks. In conclusion, using a *pdoA2* genetic perturbation model, we have functionally reannotated the type V Pdo system and constructed an RHO-centric functional map. We have filled the enzymatic gap between HMW PAH metabolism and central aromatic metabolism (phthalate and protocatechuate pathway) and provided the direct pleiotropic and epistatic functional evidence for RHO enzymes in the PAH-MN. Analysis showed that the metabolic quality and quantity of the PAH-MN depend mainly on the pleiotropic activity and epistatic interaction of RHO and that the functional diversity and complexity of RHO

systems depend on the complexity of PAH substrates in the PAH-MN.

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