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ORIGINAL PAPER

Comparison of the substrate specificity of two ring-hydroxylating dioxygenases from *Sphingomonas* sp. VKM B-2434 to polycyclic aromatic hydrocarbons

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Abstract The genes of two ring-hydroxylating dioxygenases (RHDs) of *Sphingomonas* sp. VKM B-2434 were cloned and expressed in *Escherichia coli*. The relative values of the RHD specificity constants were estimated for six polycyclic aromatic hydrocarbons (PAHs) based on the kinetics of PAH mixture conversion by the recombinant strains. The substrate specificity profiles of the enzymes were found to be very different. Dioxygenase ArhA was the most specific to acenaphthylene and showed a low specificity to fluoranthene. Dioxygenase PhnA was the most specific to anthracene and phenanthrene and showed a considerable specificity to fluoranthene. Knockout derivatives of *Sphingomonas* sp. VKM B-2434 lacking ArhA, PhnA, and both dioxygenases were constructed. PAH degradation by the single-knockout mutants was in agreement with the substrate specificity of the RHD remaining intact. Double-knockout mutant lacking both enzymes was unable to oxidize PAHs. A mutant form of dioxygenase ArhA with altered substrate specificity was described.

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Introduction

Aerobic bacterial degradation of PAHs begins with hydroxylation of substrate catalyzed by RHDs (Gibson and Parales 2000). Many RHDs from PAH-utilizing bacterial strains have been cloned and characterized, and recently several functionally active RHDs have been cloned using metagenomic DNA (Singleton et al. 2012; Martin et al. 2013).

These enzymes show a broad substrate specificity. For example, naphthalene dioxygenase from the strain *Pseudomonas* sp. NCIB 9816 catalyzes conversion of more than 50 various aromatic substrates including PAHs (Resnick et al. 1996). The enzyme from the strain *Sphingomonas* sp. CHY-1 oxidizes various PAHs containing 2–5 aromatic rings (Jouanneau et al. 2006). Dioxygenase of the strain *Sphingomonas* sp. A4 capable of growth on acenaphthene and acenaphthylene catalyzes, apart from these two substrates, the oxidation of naphthalene, phenanthrene, anthracene, and fluoranthene (Pinyakong et al. 2004). RHD from the strain *Sphingomonas* sp. LH128 oxidizes various PAHs and heterocyclic compounds at different rates (Schuler et al. 2009). The strain *Mycobacterium* sp. 6PY1 has two RHDs, responsible one for phenanthrene biodegradation and the other for

pyrene biodegradation, which are, however, not quite specific (Krivobok et al. 2003). Two RHDs of the strain *Mycobacterium vanbaalenii* PYR-1 show the highest activity to pyrene and fluoranthene, respectively; both of them also oxidize phenanthrene and other PAHs (Kweon et al. 2010). Thus, the specificity of PAH conversion by RHDs is characterized by the following properties: (1) the same RHD catalyzes oxidation of several PAHs; (2) the activity of an RHD differs with respect to different PAHs; and (3) different RHDs vary in substrate specificity.

Reliable description of RHD specificity is needed to investigate structure-specificity relationships and design of enzymes with required properties. While the selectivity of product formation by PAH-oxidizing RHDs is characterized by the ratios of products formed (Parales 2003; Kim et al. 2006), there is no reliable quantitative method to describe the RHD substrate specificity.

In this paper, we apply the earlier reported method for the analysis of PAH mixture biodegradation (Baboshin and Golovleva 2011) to recombinant *E. coli* strains carrying RHD genes from *Sphingomonas* sp. VKM B-2434, estimate the relative values of RHD specificity constants for a series of PAHs, and confirm the results by gene knockouts.

Materials and methods

Reagents

PAHs were of high purity grade (>98 %; purchased from Sigma-Aldrich, Fluka, and Merck). Restriction endonucleases were from Fermentas (Lithuania), T4 DNA ligase was from Sibenzyme (Russia), and Taq DNA polymerase was from Evrogen (Russia). All other chemicals used were of analytical reagent grade and were purchased locally. Solvents were distilled before use.

Strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. Mineral medium contained (g/L): NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$·$7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, two drops of saturated solution, pH adjusted to 7.5 by addition of 50 % KOH; the medium was filtered and sterilized by autoclaving. PAH-containing medium was prepared by addition of PAH solution (100 µL) to mineral medium (95 mL); PAH solution was obtained by dissolving acenaphthylene, acenaphthene, phenanthrene, anthracene, fluoranthene, and pyrene in methanol at a concentration of 200 µmol/L for each compound. LB medium was used as a rich medium for growth of *E. coli* strains and *Sphingomonas* sp. VKM B-2434. Cultures were cultivated on a shaker or in Petri dishes containing agarized LB medium. Appropriate antibiotics were added to the medium for clone selection and cultivation of plasmid-mediated resistant strains. Antibiotics were used at the following concentrations (mg/L): for *E. coli*—ampicillin, 100; tetracycline, 10; kanamycin, 20; gentamycin, 5; for *Sphingomonas* sp. VKM B-2434—rifampicin, 25; tetracycline, 20; kanamycin, 20; gentamycin, 5.

DNA manipulations and molecular techniques

Total DNA was isolated from the *Sphingomonas* sp. VKM B-2434 culture by chloroform extraction (Chen and Kuo 1993). Standard PCR, inverse PCR, and gene cloning were carried out using standard protocols (Sambrook and Russell 2001).

Cloning of RHD genes

In order to express dioxygenase ArhA, the *ahdA4* reductase gene, *bphA3* ferredoxin gene, and *arhA1A2* dioxygenase genes were subsequently cloned into pUC18 vector using EcoRI–KpnI, KpnI–XbaI, and XbaI–PstI sites, respectively, to yield pUC-A plasmid. In order to express dioxygenase PhnA, the *ahdA4* reductase gene, *bphA3* ferredoxin gene, *phnA1* dioxygenase large subunit gene, and *phnA2* dioxygenase small subunit gene were ligated into pUC18 vector using EcoRI–KpnI, KpnI–XbaI, XbaI–PstI, and PstI–HindIII sites, respectively, to create pUC-P plasmid.

Generation of RHD knockout mutants and complementation

For the generation of an RHD knockout mutant, a DNA fragment containing the gene of the RHD catalytic subunit (*arhA1* or *phnA1*) was amplified using a pair of primers ARH_f/ARH_r or PHN_f/PHN_r (Table 2) and cloned into pEX18Tc vector. A

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description or genotype	Reference or source
Strains		
Sphingomonas sp.VKM B-2434 Rif^R	A strain capable of PAH conversion	Baboshin et al. (2008)
E. coli DH5α	F^- endA1 glnV44 thi-1 *recA1 relA1 gyrA96 deoR nupG* Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*(r_K^- m_K^+) λ–	Invitrogen
E. coli S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	Simon et al. (1983)
Plasmids		
pGEM-T Easy	TA-cloning vector, Amp^R	Promega
pUC18	Cloning vector, Amp^R	Yanisch-Perron et al. (1985)
pEX18Tc	Gene replacement vector, Tc^R	Hoang et al. (1998)
pSP329	Broad-host range plasmid, Tc^R	Ivashina et al. (2010)
pSP858	Source of gentamycin resistance cassette	Hoang et al. (1998)
pUC4 K	Source of kanamycin resistance cassette	Pharmacia Biotech
pUC-A	pUC18 carrying *ahdA4, bphA3,* and *arhA1A2* from *Sphingomonas* sp. VKM B-2434	This study
pUC-P	pUC18 carrying *ahdA4, bphA3,* and *phnA1A2* from *Sphingomonas* sp. VKM B-2434	This study
pEX-A	pEX18Tc carrying a DNA fragment containing *arhA1*, disrupted with gentamycin resistance cassette	This study
pEX-P	pEX18Tc carrying a DNA fragment containing *phnA1*, disrupted with kanamycin resistance cassette	This study
pSP-A	pSP329 carrying *bphA3* and *arhA1A2*	This study
pSP-P	pSP329 carrying *bphA3* and *phnA1*	This study

gentamycin resistance cassette from pSP858 or kanamycin resistance cassette from pUC4K was subsequently subcloned into the *arhA1* or *phnA1* gene, using the unique restriction site SacI or XhoI, to yield a plasmid pEX-A or pEX-P, respectively. The pEX18Tc derivative was then mobilized into *Sphingomonas* sp. VKM B-2434 Rif^R from *E. coli* S17-1. The allelic exchange was confirmed by PCR using primers flanking the site of insertion.

For complementation of knockout mutations, KpnI–PstI DNA fragments from the pUC-A and pUC-P were subcloned into pSP329 to yield pSP-A and pSP-P, respectively. The plasmids were mobilized into knockout mutants of *Sphingomonas* sp. VKM B-2434 Rif^R from *E. coli* S17-1.

Site-directed mutagenesis of the *arhA1* gene

Site-directed mutagenesis was performed by the megaprimer method (Sambrook and Russell 2001); pUC-A plasmid was used as a template. Megaprimer (343 residues) was amplified using mutagenic primer arh_mut and a reverse primer arh_m complementary to the sequence containing unique BglII restriction site (Table 2). The generated megaprimer together with the primer bph_f (Table 2) was used in the second round of PCR. The PCR product was treated with BglII and KpnI restriction enzymes and used to replace the corresponding fragment of pUC-A plasmid. The resulting plasmid was verified by sequencing.

Conversion of PAH mixture by a recombinant *E. coli* strain

The recombinant plasmids pUC-A and pUC-P were transformed into *E. coli* DH5α. Bacteria were grown in LB medium at 37 °C to an optical density of 0.6 at 540 nm. IPTG was added at a final concentration of 200 μM and further cultivated on a shaker at 30 °C for 4 h. After cooling the culture (24 mL) on ice, biomass was harvested by centrifugation, washed with ice-cold

Table 2 Primers used in this study

Primer	Primer sequence[a]	Amplified genes
ahd_f	NNNGAATTC**N**GTGCGCTCGATTGCGAT[b]	Gene of reductase (*ahdA4*)
ahd_r	NNNGGTACCTCAGCCCGCCTGCTTGA	
bph_f	NNNGGTACCTTCCATTAACCGCCAG	Gene of ferredoxin (*bphA3*)
bph_r	NNNTCTAGATCAGGCGCTCCCTTCC	
arh_f	NNNTCTAGACCGATTGTTCTTCCTTG	Genes of large and small subunits of dioxygenase ArhA (*arhA1A2*)
arh_r	NNNCTGCAGGCGCTTACAGGAAC	
phn1_f	NNNTCTAGAATAA*GGAGG*TAAATAATGAGCGGCGAC[c]	Gene of large subunit of dioxygenase PhnA (*phnA1*)
phn1_r	TTGCTGCAGCTCTGGCGATCATTCGG	
phn2_f	AAACTGCAGATAA*GGAGG*TAAATAATGTCGACCGAAC[c]	Gene of small subunit of dioxygenase PhnA (*phnA2*)
phn2_r	CTGAAGCTTAATCGCGCATTTCTAAC	
ARH_f	TTGTCGACGCGCACCACAATC	Gene *arhA1* flanked by a 1-kb 5′-genomic sequence and a 0.7-kb 3′-genomic sequence
ARH_r	TTGAATTCATCGGTATTGATGATGACG	
PHN_f	TTGGTACCCCAGGCTGACCAGGTAAG	Gene *phnA1* flanked by a 0.8-kb 5′-genomic sequence and a 1.1-kb 3′-genomic sequence
PHN_r	TTAAAGCTTCTGCGCATCGTCGGC	
arh_mut	GGTGATGG*GA*TATCAC*G*T*C*GGGTG[d]	Part of *arhA1* gene
arh_m	GCTGCCAGATCTTCAACG	

[a] Inserted restriction sites underlined

[b] An arbitrary nucleotide (**N**) was included in the primer to clone the *ahdA4* gene in frame with the *lacZα* gene

[c] RBS sequences italicized

[d] Nucleotide substitutions italicized

mineral medium, and resuspended in the mineral medium (48 mL). The biomass suspension (5 mL) was added into a flask with the PAH-containing medium (95 mL); the flask was tightly closed with a glass cap and incubated on a shaker at 30 °C for a certain period of time (0.5–4 h). Incubation was stopped by adding 1 mL of 5 N sulfuric acid solution. After the incubation, 20 μg of fluorene in 1 mL of ethyl acetate (internal standard) and 5 N potassium hydroxide solutions (1 mL) were introduced into the flask. The liquid was twice extracted with ethyl acetate, (30 + 10) mL. The extracts were evaporated in a rotor evaporator at 38 °C under vacuum (~100 mbar), with preliminary addition of 100 μL of dimethylformamide into each extract. Ethyl acetate vaporized and dimethylformamide remained. Thus, extracted hydrocarbons (including the internal standard) were dissolved in a small volume of dimethylformamide at concentrations sufficient for gas chromatography. Hydrocarbon concentrations in the extracts were determined by gas chromatography in a Kristall-2000M chromatograph (Chromatec, Russia) with the flame-ionizing detector and HP-5 column (30 m × 0.32 mm × 0.25 μm). The evaporator and detector temperatures were 300 and 330 °C, respectively. The column temperature increased from 120 to 197.5 °C during the analysis at a rate of 2.5 °C/min. Hydrocarbon mass in a sample was calculated by the ratio of the hydrocarbon peak area to the peak area of internal standard (fluorene).

PAH conversion by *Sphingomonas* sp. VKM B-2434 and its mutans under growth conditions

A certain volume (0.1–1 mL) of PAH solution in acetone (50 g/L) was added to 100 mL of mineral medium supplemented with vitamin B_{12} (1 μg/L); PAH concentrations in the medium (0.2 μmol/L) were much less than the aqueous solubility of all PAHs used. The medium was inoculated by 1 mL of a culture grown to the stationary phase in LB medium, and further cultivated on a shaker at 30 °C. After incubation, the contents of a flask were twice extracted with ethylacetate (30 + 10 mL). The volume of the extracts was brought up to 40 mL with ethylacetate, and PAH concentration in the extract was measured by gas chromatography.

Conversion of indole to indigo and acenaphthene to 1-acenaphthenol

Conversion of triptophan-derived indole to indigo was observed visually (blue coloring) during the culture growth in liquid LB medium. Oxidation of acenaphthene to 1-acenaphthenol was detected by gas chromatography by measuring the concentration of both the substrate and product.

Calculation of the relative values of specificity constants

Data on the conversion of a PAH mixture by a recombinant strain were processed as follows. For each component of the mixture, we plotted its concentration logarithm versus the logarithm of the concentration of an arbitrarily chosen component (e.g., acenaphthylene). If all PAHs are converted in the same common active site in accordance with the principles of Michaelis kinetics, the slope of each double-logarithmic plot is proportional to the specificity constant (first-order rate constant) of the respective reaction (Baboshin and Golovleva 2011). Thus, we obtain a series of relative specificity constants of the RHDs with respect to a series of PAHs. The result was expressed as proportions of the relative specificity values in the sum of such values for all PAHs in the mixture:

$$k_j = \frac{\alpha_j}{\sum_{i=1}^{n} \alpha_i} \quad (1)$$

where k_j is the relative specificity constant for substrate j, α_j is the slope of the double-logarithmic plot for substrate j, $\sum_{i=1}^{n}\alpha_i$ is the sum of the slopes of the double-logarithmic plots for all n substrates in the mixture.

Phylogenetic analysis

Search for nucleotide and amino acid sequences was performed using BLAST software. The sequences were aligned using T-Coffee (Notredame et al. 2000). The phylogenetic tree was constructed by the neighbor-joining method using TREECON (Van de Peer and De Wachter 1997).

Nucleotide sequence accession numbers

The sequences of the *arhA1A2* and *phnA1A2* genes of *Sphingomonas* sp. VKM B-2434 were deposited in GenBank under accession numbers KF734000 and KF734001, respectively.

Results

Cloning and sequencing of RHD genes

The PCR amplification of *nahAc*-like genes of *Sphingomonas* sp. VKM B-2434 was performed using the degenerate primers Nah-for and Nah-rev1 (Zhou et al. 2006). PCR products were cloned into pGEM-T Easy and sequenced. Sequencing revealed the presence of two different RHD genes in the genome. One of the RHDs shows a 99 % protein sequence identity with dioxygenase ArhA from *Sphingomonas* sp. A4 (Pinyakong et al. 2004). Another RHD shows a 99 % protein sequence identity with dioxygenase PhnA from *Sphingomonas* sp. EPA505 (accession no. CAT03469 and CAT03470) and a 98 % protein sequence identity with dioxygenase PhnA from *Sphingomonas* sp. CHY-1 (Jouanneau et al. 2006). Genes closely related to the genes of reductase AhdA4 and ferredoxin BphA3 from *Sphingomonas* sp. EPA505 were revealed using the PCR with primers corresponding to the conservative sequences of the respective genes. Based on the sequences of inverse PCR products, primers for full-size gene amplification were designed (Table 2). Genes of dioxygenases ArhA and PhnA were ligated into pUC18 vector to yield plasmids pUC-A and pUC-P, respectively. Phylogenetic analysis of deduced amino acid sequences of the ArhA and PhnA catalytic subunits was carried out (Fig. 1).

PAH conversion by the recombinant *E. coli* strains carrying wild-type RHD genes

Both *E. coli* carrying pUC-A plasmid and *E. coli* carrying pUC-P plasmid showed an ability to oxidize indole to indigo and acenaphthene to 1-acenaphthenol. If any component of the dioxygenase system was absent, a recombinant strain was unable to produce indigo.

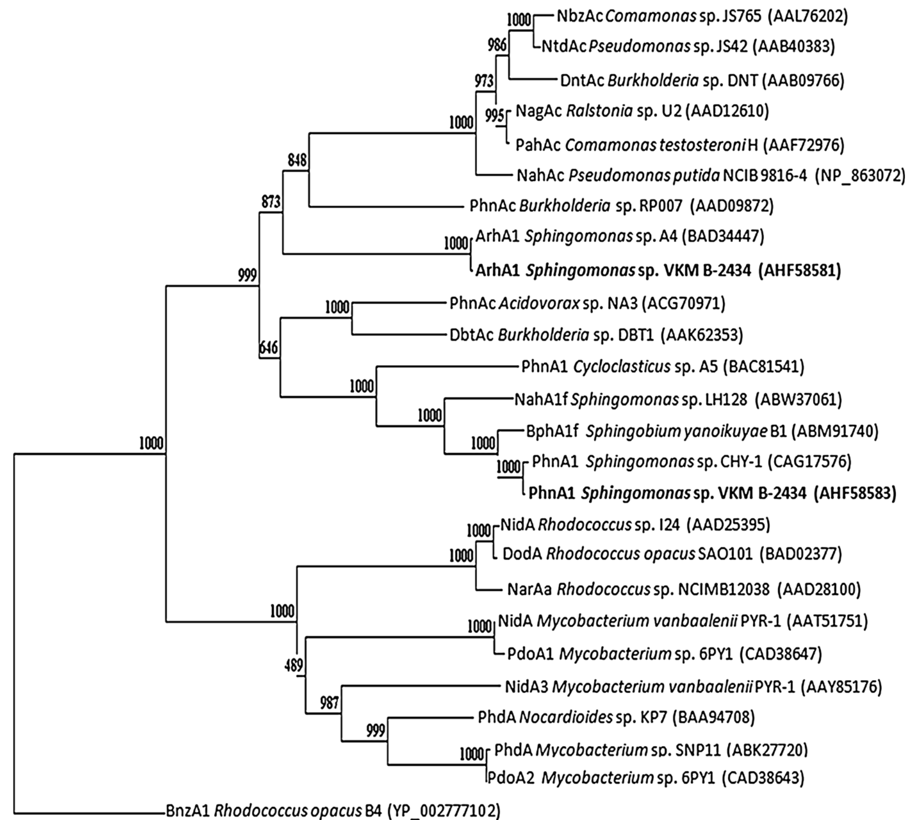

Fig. 1 Positions of *Sphingomonas* sp. VKM B-2434 RHDs on the phylogenetic tree of PAH-oxidizing RHD α-subunits. Accession numbers are shown in *brackets*. Bootstrap values are indicated at *branch nodes*

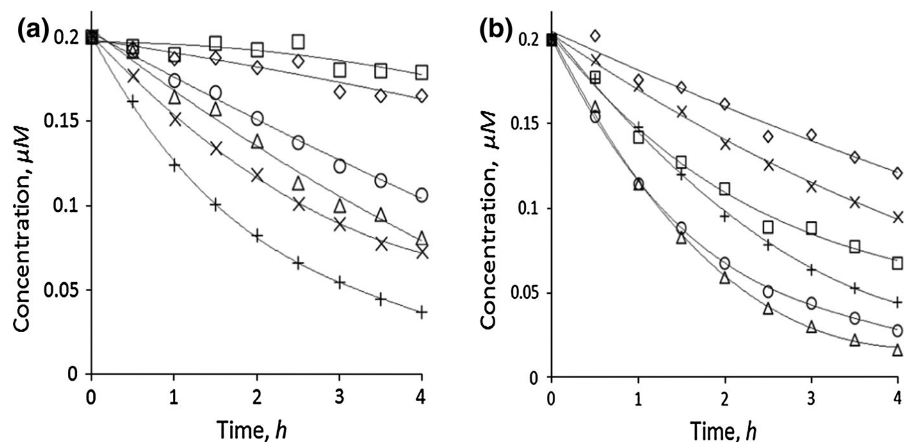

Fig. 2 Conversion of acenaphthylene (+), acenaphthene (×), phenanthrene (○), anthracene (Δ), fluoranthene (□), and pyrene (◇) in a mixture by *E. coli* carrying genes of dioxygenase ArhA (**a**) and by *E.coli* carrying genes of dioxygenase PhnA (**b**)

Both *E. coli* strains carrying pUC-A and pUC-P converted a mixture of six PAHs (Fig. 2). The conversion curves were plotted in double-logarithmic coordinates and fitted by straight lines (Fig. 3). The line slopes were taken as an estimate of the relative values of the specificity constants of dioxygenases ArhA and PhnA (Fig. 4). In a control experiment with *E. coli* carrying pUC18 plasmid, no PAH conversion was observed.

Characteristics of knockout derivatives of *Sphingomonas* sp. VKM B-2434 lacking RHDs

Knockout of the *arhA1* gene encoding the catalytic subunit of dioxygenase ArhA resulted in the inability of *Sphingomonas* sp. VKM B-2434 to grow on acenaphthene, while the rate of growth on anthracene, phenanthrene, and fluoranthene remained about the

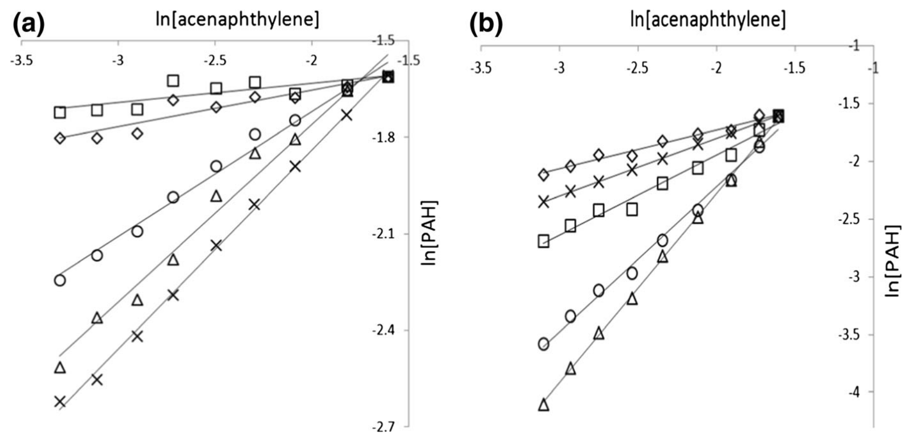

Fig. 3 A double-logarithmic plot for PAH mixture conversion by *E. coli* carrying genes of dioxygenase ArhA (**a**) and by *E. coli* carrying genes of dioxygenase PhnA (**b**). For each component of the mixture, the logarithm of its concentration versus the logarithm of the concentration of acenaphthylene was plotted. The plot for acenaphthylene is not shown since its slope is trivially equal to 1. The *symbols* are the same as in Fig. 2

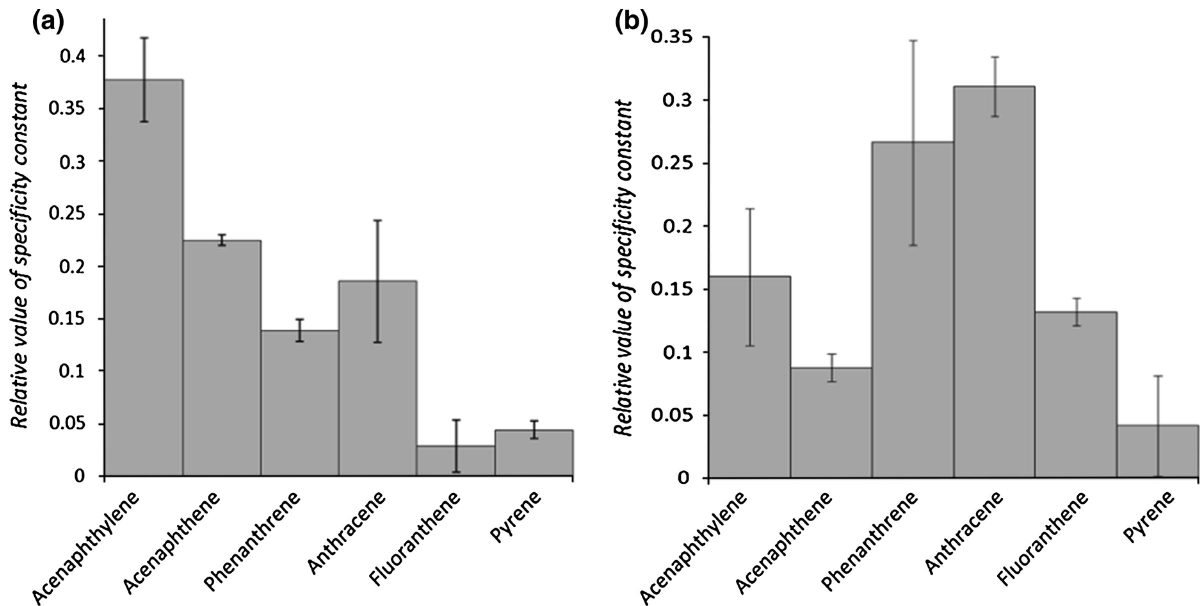

Fig. 4 The substrate specificity profiles of dioxygenases ArhA (**a**) and PhnA (**b**). The relative values of specificity constants were estimated based on the line slopes of the double-logarithmic plot (Fig. 3). Averages are based on three independent experiments. Confidence intervals correspond to a confidence probability of 0.95

same. Knockout of the *phnA1* gene encoding the catalytic subunit of dioxygenase PhnA resulted in the inability of *Sphingomonas* sp. VKM B-2434 to grow on fluoranthene, the rate of growth on anthracene and phenanthrene decreased dramatically, while the rate of growth on acenaphthene remained about the same. Double-knockout mutant lacking both RHDs was completely unable to oxidize PAHs. PAH conversion by knockout strains under growth conditions as compared with wild-type *Sphingomonas* sp. VKM B-2434 is shown in Fig. 5.

Introduction of pSP-A plasmid containing wild type allele of the *arhA1* gene restored the ability of the double knockout mutant to grow on acenaphthene (but

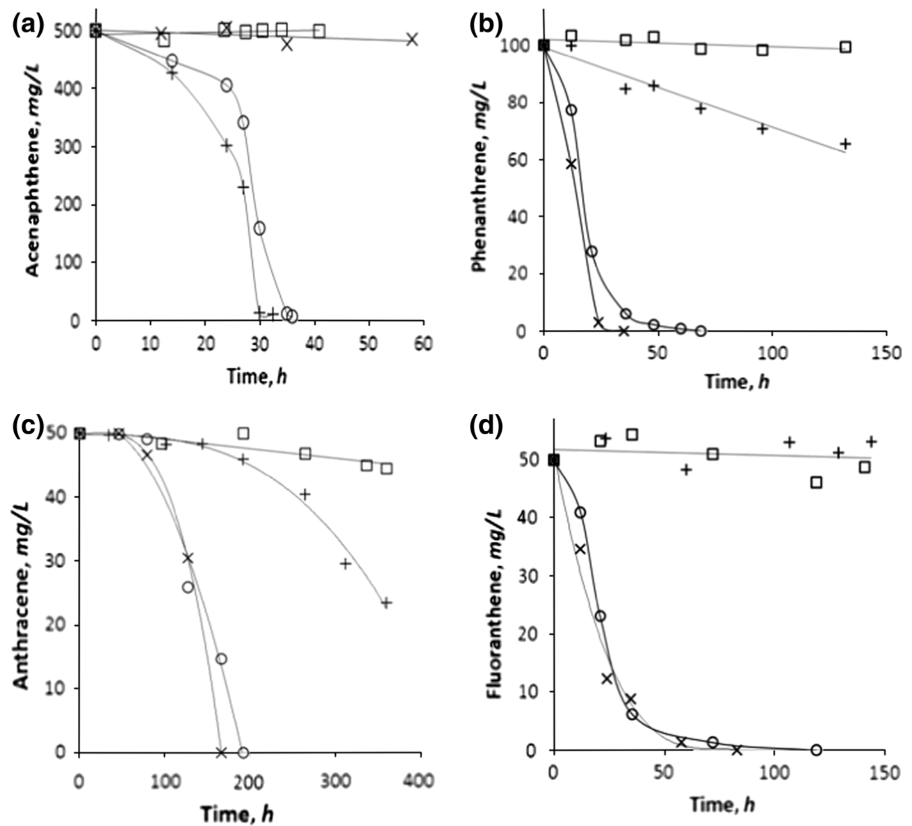

Fig. 5 Conversion of acenaphthene (**a**), phenanthrene (**b**), anthracene (**c**), and fluoranthene (**d**) by wild-type *Sphingomonas* sp. VKM B-2434 (○), by the mutant with the disrupted *arhA1* gene (×), by the mutant with the disrupted *phnA1* gene (+), and by the mutant with the disrupted *phnA1* and *arhA1* genes (□)

not on fluoranthene); introduction of pSP-P plasmid containing wild type allele of the *phnA1* gene restored the ability of the double knockout mutant to grow on fluoranthene (but not on acenaphthene); data not shown.

PAH conversion by a recombinant *E. coli* strain carrying the mutant *arhA1* gene

In an attempt to alter the substrate specificity of dioxygenase ArhA two active site residues, Ala-202 and Ile-205, were replaced with Gly and Val residues, respectively. One more substitution (Phe73Leu) was introduced accidentally during the PCR. Thus, a triple mutant (Phe73Leu, Ala202Gly, Ile205Val) of ArhA1 was obtained. PAH mixture conversion by *E. coli* carrying the mutant dioxygenase ArhA was studied (Fig. 6). The specificity of the mutant dioxygenase to PAHs (Fig. 6c) was found to differ from that of the wild-type enzyme (Fig. 4a), namely the ability to convert phenanthrene and anthracene was lost. The mutant enzyme, in contrast to the wild-type dioxygenase, was unable to oxidize indole to indigo, but conversion of acenaphthene to 1-acenaphthenol remained.

Discussion

The existence of two RHDs with different substrate specificities in *Sphingomonas* sp. VKM B-2434 strain predicted earlier based on the kinetics of PAH mixture conversion (Baboshin and Golovleva 2011) was confirmed in this work by molecular methods. Substrate specificities of dioxygenases ArhA and PhnA were found to be broad but distinctly different (Fig. 4). Of the compounds tested, ArhA is the most specific to acenaphthylene. PhnA is the most specific to anthracene and phenanthrene, and, unlike ArhA, has a considerable specificity to fluoranthene. While *Sphingomonas* sp. VKM B-2434 is able to oxidize a variety of PAHs, the metabolism of acenaphthylene/acenaphthene and fluoranthene is the most efficient in terms of energy utilization (Baboshin and Golovleva

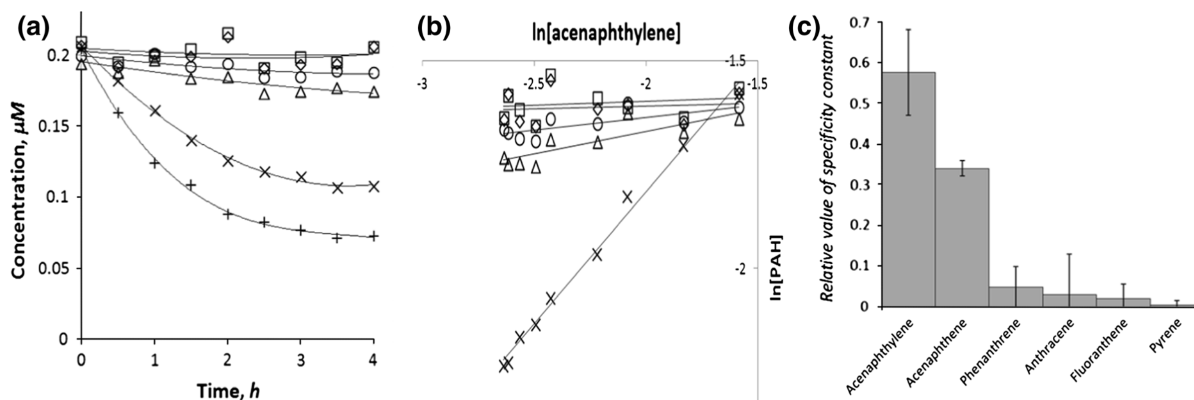

Fig. 6 PAH mixture conversion by *E. coli* carrying the mutant (Phe73Leu, Ala202Gly, Ile205Val) of dioxygenase ArhA (**a**), a double-logarithmic plot (**b**), and a substrate specificity profile (**c**). Designations are the same as in Figs. 2, 3, and 4

2011a). Thus, the strain can be assumed to specialize in acenaphthylene/acenaphthene and fluoranthene consumption, which appears to be natural since the common metabolic pathway via naphthalene-1,8-dicarboxylic acid is used for the degradation of these substrates. The fluoranthene molecule is much larger than the acenaphthylene or acenaphthene molecule, and this is why these molecules are unlikely to be efficiently converted by the same enzyme. Dioxygenases ArhA and PhnA presumably serve to oxidize acenaphthylene/acenaphthene and fluoranthene, respectively. These considerations are supported by the results of gene knockouts.

At the same time, both RHDs are active against medium-molecular-weight PAHs, phenanthrene and anthracene. The strain is incapable of complete mineralization of these PAHs but is able to cleave one aromatic ring in them and to use them as a sole source of energy (Baboshin et al. 2008); the ability is due to the relaxed specificity of the RHDs.

Inability of the double-knockout mutant lacking both investigated enzymes to convert PAHs is evidence that dioxygenases ArhA and PhnA represent all PAH-oxidizing RHDs of *Sphingomonas* sp. VKM B-2434. Although a number of RHD genes are present in sphingomonad genome (Pinyakong et al. 2003), a single enzyme is usually involved in the initial oxidation of PAH molecules (Pinyakong et al. 2004; Demaneche et al. 2004; Yu et al. 2007). To our knowledge, *Sphingomonas* sp. VKM B-2434 is the first characterized sphingomonad strain carrying two such enzymes.

The used method of the double-logarithmic plot enables simple and accurate comparison of RHD specificities to PAHs. The traditional approach of substrate specificity estimation is of little value since the data on the kinetic properties of RHDs are very scarce. To our knowledge, steady-state-kinetic parameters for an RHD with respect to naphthalene were reported in the only paper by Jouanneau et al. (2006), and there are no data on the kinetics of high-molecular-weight PAH conversion; this is probably due to the difficulty of obtaining RHD enzyme preparations and performing the kinetic studies. RHD activities with respect to different substrates were usually assessed by comparing the rates of individual PAH conversion by a recombinant strain or (rarely) by a purified enzyme (Kasai et al. 2003; Krivobok et al. 2003; Jouanneau et al. 2006; Schuler et al. 2009; Kweon et al. 2010; Martin et al. 2013; Singleton et al. 2012). Such an analysis allows only tentative conclusions concerning the specificities of the RHDs due to the differences in Michaelis constants for various PAHs and possibly different impacts of various PAHs and products of their conversion on RHD activities. The system of competing reactions occurring in a common active site is free from these drawbacks since both the catalytic activity and substrate affinity are taken into account and all the competing reactions are equally influenced by any factor.

A series of papers were dedicated to the estimation of the kinetic parameters of PAH conversion by pure bacterial strains (Stringfellow and Aitken 1995;

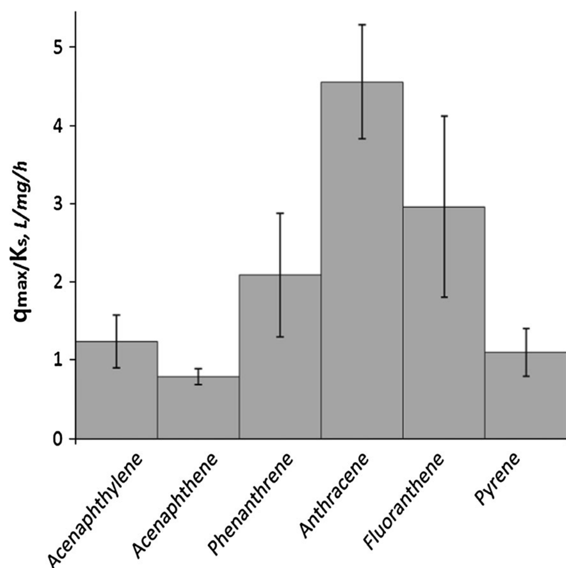

Fig. 7 Specific affinities for PAH conversion by *Sphingomonas* sp. EPA505, a strain carrying an RHD with 99 % identity to dioxygenase PhnA. Confidence intervals correspond to a confidence probability of 0.95. The kinetic parameters were estimated by Dimitriou-Christidis et al. (2007) from the curves of individual PAH conversion

Dimitriou-Christidis et al. 2007; Desai et al. 2008). Surprisingly, similar experiments with RHD-carrying recombinant strains were not conducted. The kinetics of PAH conversion by a recombinant strain *E. coli* carrying an RHD can be interpreted more reliably than the kinetics related to a PAH-degrading strain because *E. coli* does not contain any other enzymes of PAH metabolism. Particularly, the ability of the recombinant strain to oxidize PAHs shows that PAHs are transferred into bacterial cells by way of simple diffusion. Furthermore, the rate of PAH conversion by bacteria is likely to be determined by the reaction in the active site of the RHD and does not depend on the stage of substrate diffusion into the cell. This follows from the fact that PAH mixture conversion by a microbial culture usually satisfies the multisubstrate model, according to which all components of the mixture compete for a single active site in accordance with the principles of Michaelis kinetics (Stringfellow and Aitken 1995; Guha et al. 1999; Lotfabad and Gray 2002; Knightes and Peters 2006; Dimitriou-Christidis and Autenrieth 2007; Desai et al. 2008). The linearity of the double-logarithmic plot in our experiments with the recombinant strains indicates that PAH conversion satisfies the multisubstrate model (Baboshin and Golovleva 2011). Therefore, the kinetic parameters of PAH conversion by a recombinant strain are likely to correspond to the recombinant RHD present in the strain.

The specificity profile of dioxygenase PhnA (Fig. 4b) is similar to the result of kinetic investigation of PAH conversion by *Sphingomonas* sp. EPA505, the strain carrying almost the same dioxygenase (Fig. 7). This observation could be an argument that RHD specificity is the same in the sphingomonad and *E. coli* cell. On the other hand, there is a discrepancy between our present results and our results on the kinetics of PAH conversion by *Sphingomonas* sp. VKM B-2434 reported earlier (Baboshin and Golovleva 2011). In the previous experiments with the biomass of *Sphingomonas* sp. VKM B-2434 the specificity to fluoranthene was 3.6 times higher than that to phenanthrene; in contrast, in the present experiments with the recombinant strains, the specificity to phenanthrene was much higher than that to fluoranthene. Consequently, we can assume that some factors in the sphingomonad cell modify the RHD specificity.

One can hope that the method of describing the RHD substrate specificity in combination with molecular modelling and site-directed mutagenesis will allow obtaining RHDs with desired substrate specificity.

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