Biodegradation of Polycyclic Aromatic Hydrocarbons by *Novosphingobium pentaromativorans* US6-1

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**Abstract**

*Novosphingobium pentaromativorans* US6-1, a marine bacterium isolated from muddy sediments of Ulsan Bay, Republic of Korea, was previously shown to be capable of degrading multiple polycyclic aromatic hydrocarbons (PAHs). In order to gain insight into the characteristics of PAHs degradation, a proteome analysis of *N. pentaromativorans* US6-1 exposed to phenanthrene, pyrene, and benzo[a]pyrene was conducted. Several enzymes associated with PAHs degradation were identified, including 4-hydroxybenzoate 3-monoxygenase, salicylaldehyde dehydrogenase, and PAH ring-hydroxylating dioxygenase alpha subunit. Reverse transcription and real-time quantitative PCR was used to compare RHDα and 4-hydroxybenzoate 3-monoxygenase gene expression, and showed that the genes involved in the production of these two enzymes were upregulated to varying degrees after exposing the bacterium to PAHs. These results suggested that *N. pentaromativorans* US6-1 degraded PAHs via the metabolic route initiated by ring-hydroxylating dioxygenase, and further degradation occurred via the p-phenathlate pathway or salicylate pathway. Both pathways subsequently entered the tricarboxylic acid (TCA) cycle, and were mineralized to CO2.

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**Introduction**

Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons with two or more fused benzene rings, and originate from natural as well as anthropogenic sources. They are widely distributed environmental contaminants that have detrimental biological effects, including toxicity, mutagenicity, and carcinogenicity [1,2]. PAHs in the environment are degraded via volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and adsorption to soil particles; however, the principal removal processes are thought to be microbial transformation and degradation [3–4]. In several microorganisms, metabolic pathways have been identified that lead to complete mineralization of aromatic hydrocarbons or partial transformation to dead end intermediates. As bioremediation efforts aggressively attempt to employ microorganisms to biostimulate or bioaugment degradation of aromatic hydrocarbons on a practical scale [5], the enzymes involved in the degradation of PAHs should be fully characterized as part of a risk assessment of this process.

PAHs are thermodynamically stable and resistant to microbial degradation due to their aromatic nature, which includes stabilization by multiple rings and low aqueous solubility. Despite these properties, a variety of bacterial species can degrade PAHs. Most research on the enzymes involved in PAH metabolism and genetic regulation is focused on *Pseudomonas* and *Sphingomonas* species [6–11], but *Myxobacterium*, *Rhodococcus*, *Nocardioidea*, and *Novosphingobium* species can also mineralize PAHs [12,13]. Proposed pathways for pyrene biodgradation by mycobacteria have been reported [14], including the complete pathway to the tricarboxylic acid (TCA) cycle proposed for *Mycobacterium vanbaalenii* PYR-1, but little is known about the genetic and enzymatic processes involved in the PAHs degradative pathways used by *Novosphingobium* species.

*Novosphingobium pentaromativorans* US6-1, is a marine bacterium that was isolated from muddy sediments of Ulsan Bay, Republic of Korea. Prior research has shown that it can degrade or transform phenanthrene, pyrene, chrysene, benzo[a]anthracene, benzo[h]fluoranthene and benzo[a]pyrene. Because of its metabolic versatility, this bacterium was proposed to be a potential candidate for the bioremediation of PAH-contaminated areas [15]. In the current study, a proteomic analysis of cells exposed to phenanthrene, pyrene, and benzo[a]pyrene was conducted using one-dimensional gel electrophoresis in combination with liquid chromatography-tandem mass spectrometry to identify the proteins involved in the degradation. These analyses documented protein composition profiles in response to exposure to three different PAHs, and identified specific proteins that characterize the physiological state of *Novosphingobium. pentaromativorans* US6-1. A culture-independent method using reverse transcription and real-time quantitative PCR showed that gene expression of these different proteins was important in the biodegradation of PAHs. The results presented here comprise the foundation of a protein index for *N. pentaromativorans* US6-1, and provide fundamental information on PAH degradation as well as other metabolic characteristics in environmental strains of *Novosphingobium* sp.
Materials and Methods

1 Bacterial strain and culture conditions

Novosphingobium pentaromativorans US6-1 used in this study was provided by Dr. Kim Sangjin (Korean Ocean Research and Development Institute). All chemicals were purchased from Sigma-Aldrich unless otherwise specified, and were of high-performance liquid chromatography grade. The PAH compounds (phenanthrene, pyrene, and benzo[a]pyrene) were of >97.0% purity, and stock solutions of each PAH were prepared in cyclohexane at a concentration of 5 mg mL\(^{-1}\). Novosphingobium pentaromativorans US6-1 was cultured in Zobell 2216E broth (5 g L\(^{-1}\) peptone, 1 g L\(^{-1}\) yeast extract, 0.01 g L\(^{-1}\) FePO\(_4\), at pH 7.6–7.8) for 36 h at 25°C until exponential growth was achieved. Cells were collected via centrifugation and washed twice with MM2 medium [18 mM (NH\(_4\))\(_2\)SO\(_4\), 1 mM FeSO\(_4\)·7H\(_2\)O, 100 µL 1 M KH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\) buffer solution and 10% MCD (mixed-cyclodextrin), per liter of aged sea water, at pH 7.2]. The cyclohexane was allowed to volatilize by storing it in the fume hood for 2–3 days at room temperature, then the bacterial inocula were added to 100 mL MM2 with 10 ppm phenanthrene, pyrene, and benzo[a]pyrene, at a final optical density of approximately OD value 0.3 (measured spectrophotometrically at 600 nm). The cultures were incubated at 30°C for 30 days. In order to determine any abiotic loss of PAHs, triplicate samples of PAHs but without the bacterial inoculation was used as the control to determine any abiotic loss of PAHs. Triplicate samples were collected from the experimental system every 12 h for two days.

2 Analysis of PAH biodegradation

The residual PAHs were extracted using the liquid-liquid extraction method, followed by GC-MS determination. Fluoranthene was used as an internal standard to adjust the percent extracted. These samples were treated with equivalent dichloromethane for 15 min in an ultrasonic bath, filtered through anhydrous sodium sulfate, and concentrated by rotary evaporation to 1 mL under a gentle stream of nitrogen. PAH analysis was performed with a CP3800 gas chromatograph with a split/splitless injection port. The GC was equipped with a CP5860 silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) and helium carrier gas. The injection temperature was 300°C with an injection volume of 1 µL (splitless). The temperature program was as follows: isothermal at 100°C for 1 min, with 20°C/min to 200°C, 10°C/min to 320°C, and a final isothermal cycle at 320°C for 3 min. Quantification of PAH concentration was achieved with a GC/HRMS using a Varian 1200 (Palo Alto, USA) in the EI positive ion setting, with an electron energy of 70 eV. Transfer line temperature and ion source temperature were maintained at 250°C and 200°C, respectively, and the mass spectra obtained were compared to the NIST library.

3 Measurement of electron transport system activity (ETSA)

The measurement procedure for bacterial ETSA followed established methods [16]: 0.5 mL of culture was combined with 1 mL of 2-p-iodophenyl-3-p-nitrophenyle-5-phenyl tetra-zolium chloride, and allowed to interact for 30 min at room temperature (25°C) in the dark, then 100 µL of formalin was added to stop the reaction. The mixture was centrifuged (10,000×g for 5 min) in a Hitachi SCR20BC centrifuge, the supernatant was removed, and 1 mL methanol was added to the reaction tube. After the mixture was homogenized, centrifugation for 5 min at 10,000×g with a micro-12 centrifuge was repeated, and the reaction tube was placed in an ice bath. Absorbance was measured at 495 nm using a spectrophotometer. The ETSA in µg O\(_2\)/mL/min was calculated using the following formula:

\[
\text{ETSA} = \frac{(Ab/15.9) \times V \times 32/2 \times 1}{S \times t}
\]

where Ab is absorbance; V is final reaction volume (mL) (methanol volume); S is the sample amount (mL); t is the incubation time (min); 32/2 is a constant (relative molecular weight of oxygen); and 15.9 is the molar absorbance of formazan.

4 Extraction of total proteins

After incubation for 24, 36, or 48 h, the medium dosed with phenanthrene, pyrene, or benzo[a]pyrene was carefully removed by centrifugation at 4,000×g for 5 min at room temperature. Cells were washed twice with 30 mL of PBS (pH 7.6) and pelleted by centrifugation under the same conditions. For protein extraction, the cell pellets were resuspended in 2 mL lysis (7 M urea, 2 M thiourea, 4% w/v 3-[3-cholamidopropyl]dimethylammonio]-1-pro-panesulfonate [CHAPS], 60 mM dithiothreitol, 2% v/v Pharmalyte 3–10, and a protease inhibitor cocktail) and sonicated on ice for 10 min using a 500 ms/s pulse at 40 W. The sonicated cell suspensions were then centrifuged at 10,000×g for 30 min at 4°C. Protein concentration was estimated using the 2-D Quant Kit (Amersham, USA).

5 Electrophoresis and protein identification

SDS-PAGE was carried out using the tris-glycine-SDS buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS) on the SE600 electrophoresis apparatus (GE Healthcare, USA), at 24 mA/gel, until the dye front reached the bottom edge of the gel. A protein MW marker (Takara, China) was used in the gel, which was stained with Coomassie Brilliant Blue G-250 (GE Healthcare, USA), and scanned using an Amersham Biosciences image scanner (GE Healthcare, USA).

Three specific bands differently expressed under phenanthrene, pyrene, and benzo[a]pyrene were excised from the gel and used for in-gel tryptic digestion [17]. The gel pieces were destained and washed, and, after dithiothreitol reduction and iodoacetamide alkylation, the proteins were digested with trypsin for 20 h at 37°C. The resulting tryptic peptides were extracted from the gel pieces with 60% acetonitrile, 0.1% trifluoroacetic acid, and 100% acetonitrile. Extracts were dried completely for nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. After resuspension in 20 µL 0.1% formic acid, the peptide samples were injected onto a Zorbax 300SB C18 peptide trap (Agilent Technologies, USA), and desalted with 0.2% formic acid at a flow rate of 10 µL min\(^{-1}\) for 20 min. Peptides were eluted from the trap and separated on a reverse phase C18 column (0.15 mm×150 mm, Column Technology), with a linear gradient of 4% to 50% mobile phase B (0.1% formic acid-84% acetonitrile) in mobile phase A (0.1% formic acid), over 30 min at 65 µL min\(^{-1}\). LC-MS/MS measurements were made with a linear trap quadrupole (LTQ) mass spectrometer (Thermo Finnigan, USA) equipped with a microspray source. The LTQ mass spectrometer operated in the data-dependent mode with the following parameters: spray voltage (3.4 kV), spray temperature 170°C, full scan m/z range (400–1800). The twenty most intense ions in every full scan were automatically selected for MS/MS.

6 Extraction of total RNA

At each of the aforementioned sampling times, total RNA was extracted from 100 mL of bacterial culture using Trizol (Invitro-
gen, USA), according to the manufacturer’s instructions. Residual DNA was digested for 2 h at 37 °C in a total volume of 200 μL using 100 U of DNase I in 40 mM Tris-HCl (pH 7.5) containing 6 mM MgCl₂. DNase I was removed using standard phenolization and precipitation procedures [18]. The RNA pellet was dissolved in 200 μL RNase-free H₂O and stored at −20 °C. Extract concentrations were measured spectrophotometrically and were adjusted for optimal reverse transcription (RT)-PCR amplification.

7 RT-PCR and real-time quantitative PCR

For reverse transcription PCR (RT-PCR) of the isolated RNA, amplification reactions contained the following: 2 μg of RNA template, 1 μL random hexamer primer (150 ng/μL), and 12 μL of nuclease-free water. Reactions were denatured for 5 min at 65 °C, then 2 μL dNTP (10 mM), 4 μL 5× reaction buffer, 1 μL (20 U) RNase inhibitor, and 1 μL (25 U) RevertAid Reverse Transcriptase were added to obtain a total reaction volume of 20 μL. After centrifugation, the reaction was incubated for 5 min at 25 °C followed by 60 min at 42 °C, then terminated by heating the samples at 70 °C for 5 min. RT-PCR analyses were completed using a Rotor-Gene 6000 (Corbett Research, Australia).

The sequences of the studied proteins, PAH ring-hydroxylation dioxygenase alpha subunit (RHDx) and 4-hydroxybenzoate 3-monoxygenase, were obtained from the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/) [19]. Primer Premier’s software (Premier Biosoft, Canada) was used to design a set of oligonucleotides from which three sets of specific primers (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard. The relative quantification of genes (Table 1) were selected. The 16S ribosomal RNA (rRNA) gene was used as an internal standard.

### Table 1. Characteristics of PCR primer sets used in this study.

| Primer      | Target gene | Sequence 5'-3' | Amplicon size (bp) | Reference |
|-------------|-------------|----------------|--------------------|-----------|
| PAH-RHDx   | RHDx        | GGA AAG GCT TGT GGG TGT CG  | 252                | gi|494073592 |
| PAH-RDx Re | RHDx        | GTG GCA TCA TCG CAT CGT GT  | 252                | gi|494073592 |
| HBMO Fw    | 4-hydroxybenzoate 3-monoxygenase | GCG TGT GCC GCC TGG TTA TCA | 157                | gi|494072487 |
| HBMO Re    | 4-hydroxybenzoate 3-monoxygenase | ACC CGA GTT CGT CCC AGA TGC | 157                | gi|494072487 |
| 968R       | 16S rRNA    | AAC GGG AAG AAC CTT AC      | 433                | Felske A [20] |
| 1401F      | 16S rRNA    | CGG TGT GTA CAA GAC CC      | 433                | Felske A [20] |

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degraded 20–30% of benzo(a)pyrene in the presence of a pyrene substrate after 63 days of incubation time [23], and consortium BL co-metabolized 44.07% of 10 ppm benzo(a)pyrene after an incubation time of 14 days [24]. PAHs biodegradation pathways in bacteria are induced by the presence of either the parent compound or one of the pathway intermediates. In our study, *N. pentaromativorans* US6-1 degraded phenanthrene, pyrene, and benzo(a)pyrene after 24 h, 36 h, and 48 h, respectively, suggesting that the degradation pathways were active in US6-1 during all treatments.

### 2 Comparison of the overall proteome profile in *Novosphingobium pentaromativorans* US6-1

In order to characterize the molecular characteristics involved in the biodegradation of PAHs, the proteome profiles of *N. pentaromativorans* US6-1 induced by phenanthrene, pyrene, and benzo(a)pyrene were compared. The total protein profile of strain US6-1 (separated by SDS-PAGE) is shown in Fig. 2. Many enzymes involved in PAH degradation are likely to be expressed constitutively with so-called housekeeping proteins, and are not necessarily induced; therefore, the proteome profiles and band intensities were similar between the PAH-induced groups and the control. However, three specific bands were expressed differently after exposure to the three different PAH compounds. Compared with the control, Band 1 was upregulated only in the benzo[a]pyrene induced culture, Band 2 was intensified in both the pyrene and benzo[a]pyrene induced cultures, and Band 3 was expressed in the cultures treated with each of the three PAH compounds. As indicated by MW markers, the approximate MWs of Bands 1 and 2 were 29 KDa and 50 KDa, respectively, while that of Band 3 was about 200 KDa.

These three differential protein bands (Bands 1, 2, and 3) were excised and identified (Table 2). Unique proteins were found, but the peptide counts and percent cover were low; thus, it is possibly that although there were peptides in the bands, the integrated proteins were broken during the sample treatment. In Band 1, site-specific DNA-methyltransferase (adenine-specific), 3-oxoacyl-(acyl-carrier-protein) reductase, 4-hydroxybenzoate 3-monooxygenase, and homoserine kinase were identified. The site-specific DNA-methyltransferase (adenine-specific) is a type of transferase enzyme that transfers a methyl group from an S-adenosyl methionine to adenine to form N6-methyladenine. DNA methylation plays a key role in DNA stability, and is linked to the regulation of cell growth and gene expression [25]. Benzo[a]pyrene is a moderately potent carcinogen, and its reactive intermediates can covalently bind to cellular macromolecules (i.e., DNA) leading to mutations, thus, it is reasonable to hypothesize that this DNA-methyltransferase may be related to self-protection against mutation of the microorganism.
Table 2: List of protein functions identified from Novosphingobium pentaromativorans US6-1 grown in the presence of PAHs.

| Position | Protein Name | Accession No. | Species |
|----------|--------------|---------------|---------|
| Band 1   | site-specific DNA-methyltransferase (adenine-specific) | gi|48556136| Sphingomonas wittichii RW1 |
| Band 1   | 3-oxoacyl-[acyl-carrier-protein] reductase | gi|56552118| Sphingomonas wittichii RW1 |
| Band 1   | 4-hydroxybenzoate 3-monoxygenase | gi|494072487| Novosphingobium pentaromativorans US6-1 |
| Band 1   | homoserine kinase | gi|56552496| Sphingomonas wittichii RW1 |
| Band 2   | acyl-CoA dehydrogenase-like protein | gi|103488374| Sphingopyxis alaskensis RWK2256 |
| Band 2   | thiol-disulfide interchange protein DsbC | gi|494068955| Novosphingobium pentaromativorans US6-1 |
| Band 2   | salicylaldehyde dehydrogenase | gi|359402486| Novosphingobium pentaromativorans US6-1 |
| Band 2   | SufBD | gi|87198224| Novosphingobium aromaticivorans DSM 12444 |
| Band 3   | PAH ring-hydroxylating dioxygenase alpha subunit | gi|494073952| Novosphingobium pentaromativorans US6-1 |
| Band 3   | 4-oxalocrotonate decarboxylase | gi|48556243| Sphingomonas wittichii RW1 |
| Band 3   | dihydrolipoamide dehydrogenase | gi|48550590| Sphingomonas wittichii RW1 |
| Band 3   | hypothetical protein Swit_4997 | gi|48550950| Sphingomonas wittichii RW1 |
| Band 3   | hypothetical protein SKAS8_01115 | gi|94495050| Sphingomonas sp. SKAS8 |

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The catalysis of 4-hydroxybenzoate into proto-catechuate by 4-hydroxybenzoate 3-monoxygenase involves the proto-catechuate branch of the 3-oxoadipate pathway. Following ortho cleavage of proto-catechuate by protocatechuate 3, 4-dioxygenase and five additional enzymes convert the ring cleavage product to intermediates of the tricarboxylic acid (TCA) cycle. Many aromatic compounds under aerobic conditions are degraded by bacteria via the salicylate or proto-catechuate branch of the 3-oxoadipate pathway. The 3-oxoadipate pathway is biochemically conserved, and the structural genes encoding enzymes of this pathway are similar in widely differing bacterial species [26-27].

It is noteworthy that the pyridine-nucleotide-dependent reduction of a 3-oxoacyl-[acyl-carrier-protein] of the 3-oxoacyl-[acyl-carrier-protein] reductase, which catalyzes the first reductive step in de novo fatty acid biosynthesis, was identified in Band 1. This enzyme participates in fatty acid biosynthesis and polyunsaturated fatty acid biosynthesis. The composition of fatty acids can affect the fluidity of the cell membrane, and their metabolites also regulate the transcription of a variety of genes [28]. Furthermore, it has been suggested that 3-oxoacyl-[acyl-carrier-protein] reductase may play a role in the absorption of PAHs by changing the fluidity of the cytomembrane. Homoserine kinase also was found in Band 1, which can catalyze ATP and L-homoserine to ADP and O-phospho-L-homoserine, and plays a role in glycine, serine, and threonine metabolism [29].

In Band 2, the detection of salicylaldehyde dehydrogenase may indicate the catalysis of salicylaldehyde into salicylate. This enzyme is involved in the catechol branch of the 3-oxoadipate pathway; therefore, N. pentaromativorans US6-1 may degrade PAH via the salicylate pathway by forming catechol, and then mineralizing this to CO₂ via the TCA cycle [30]. In addition, three proteins whose functions have not been confirmed were found.

In Band 3, PAH RHDz, 4-oxalocrotonate decarboxylase, and dihydrolipoamide dehydrogenase were identified, and two unknown proteins were also found. The PAH RHDz is a component of the RHD enzyme system comprised of three components, in which an iron-sulfur flavoprotein reductase and an iron-sulfur ferredoxin transfer electrons from NAD(P)H to a terminal dioxygenase. The terminal dioxygenase is composed of large α and small β subunits. The alpha subunit (RHDα) contains two conserved regions: the [Fe2-S2] Rieske centre and the mononuclear iron-containing catalytic domain. Because of the hydrophobic nature and chemical resistance of PAHs, the initial step in their metabolism by RHDα is a difficult reaction, and is critical to the whole degradation process. In aerobic conditions, this initial step commonly occurs via the incorporation of molecular oxygen into the aromatic nucleus by a multicomponent RHD enzyme system, forming cis-dihydriodiol [31,32]. The 4-oxalocrotonate decarboxylase, which decomposes the 4-oxalocrotonate to 2-oxopent-4-enoate and CO₂, participates in the metabolic pathways of some benzenoids: benzoate, toluene, xylene and fluorene degradation [33].

Dihydrolipoamide dehydrogenase was also identified in Band 3, which was a catalytic component of the pyruvate dehydrogenase complex. It is a flavoprotein enzyme that degrades lipoamide, and produces dihydrolipoamide and acetyl-CoA, a key intermediate of carbon metabolism [34]. Another three proteins were detected in Band 3, but their functions were not confirmed.

The proteins involved in the self-protection, gene expression, metabolism of the carbon source and amino acid, and fatty acid biosynthesis that were differentially expressed between the cells growing on PAHs and the control indicate that growth on PAHs triggers a global change in cell physiology, and involves many cellular functions [35]. Since PAHs are found in planar, angular, or cluster arrangements, there are multiple sites of enzymatic attack, leading to diverse pathways and complex biochemical reactions that require diverse enzymes [36]. One example is the pyrene degradation pathway in M. vanbaalenii PYR-1 described by Kim et al [14]. In this pathway, degradation was initiated by the RHD to produce pyrene cis-1,2-dihydriodiol and pyrene cis-4,5-dihydriodiol, and the complete pyrene degradation pathway involved 27 enzymes and 25 steps. The proteins involved include 14 proteins responsible for the degradation of pyrene to phthalate, six proteins responsible for the degradation of phthalate to protocatechuate, seven proteins responsible for the lower pathway from protocatechuate to acetyl coenzyme A (acytI-CoA) and succinyl-CoA, and three proteins responsible for the transformation of pyrene to 1,2-dimethoxyxynaphtene. Madhumita [37] reported that the initial dioxygenation of phenanthrene occurred at both 3,
4- and 1, 2-carbon positions in Sphingobium sp. strain PNB. The further meta-cleavage of resultant diols led to the formation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid, respectively. These metabolites were subsequently transformed to catechol via salicylic acid, which is followed by 3-oxoadipate enol-lactone and then the TCA cycle, leading to complete mineralization. The 3-oxoadipate pathway is biochemically conserved, and the structural genes encoding enzymes of this pathway are similar among a variety of bacterial species [27]. Kim [38] reported that N. pentaromativorans US6-1 utilized two different extradiol pathways to degrade PAHs, one of which involves the enzyme salicylaldehyde dehydrogenase. Salicylaldehyde dehydrogenase was also identified in the Plasmid NSU_pLA1134 of N. pentaromativorans US6-1. Notably, PAH RHD, 4-hydroxybenzoate 3-monooxygenase, and salicylaldehyde dehydrogenase were also found in the current study. This suggests that N. pentaromativorans US6-1 degraded PAHs via the metabolic route initiated by RHD; further degradation occurred via either o-phthalate pathway or salicylate pathway, and via ortho- or meta-cleavage to converge at the common intermediate 3-oxoadipate enol-lactone, resulting in TCA cycle intermediates, which finally were mineralized to CO₂.

3 Relative gene expression following induction by three PAH compounds

The catabolic pathways for PAHs are complex, and differ among bacterial strains and under different environmental conditions. A substrate can occasionally be transformed into several intermediates under specific conditions. The recently isolated Novosphingobium is able to degrade a large spectrum of aromatic hydrocarbons, ranging from monocyclic to polycyclic hydrocarbons [39–41]. However, the genes for PAH degradation by Novosphingobium are little investigated and their characteristics are still obscure [42].

In order to verify the results of protein identification and gain further insight to the mechanism of PAH mineralization in N. pentaromativorans US6-1, RHD, and 4-hydroxybenzoate 3-monooxygenase, gene expression under induction by different PAHs in N. pentaromativorans US6-1 was investigated using Real-Time RT-PCR, and products were verified by gel electrophoresis. As shown in the Fig. 3, there were three bands coincident with the target fragments, 400–500 bp for 16S rRNA gene (433 bp), 200–300 bp for RHD gene (252 bp), and 100–200 bp for 4-hydroxybenzoate 3-monooxygenase (HBMO) gene (157 bp).

As determined by real-time PCR analysis, levels of the RHD gene in N. pentaromativorans US6-1 were variably upregulated in PAH-induced cultures compared with the control group. As shown in Fig. 4(A), we found that the gene expression of RHD increased by approximately 17-fold compared with the control group 12 h or 24 h following induction by phenanthrene. This was consistent with the intensity of Band 3 in the SDS-PAGE pattern, and might be associated with the rapid degradation rate of phenanthrene. The gene expression of RHD was upregulated and was highest (about 9-fold higher than the control) after 24 h, then decreased by 2.5-fold after induction by pyrene. This decline of RHD after 24 h of pyrene induction may be related to inhibition by downstream reactions. However, when incubated in benzo[a]pyrene, its expression increased steadily to nearly 10-fold after 48 h of induction. This may be related to the slow and low degradation rate of benzo[a]pyrene, but indicated that the degradation of benzo[a]pyrene in US6-1 was initiated after 48 h incubation. Since benzo[a]pyrene is highly hydrophobic, mutagenic, and resistant to biodegradation, a longer time period is needed for degradation, and more genes and proteins are required [21,36].
reasonable to assume that exposure to various PAHs results in the
initiation of different signals for the induction of proteins in *N. pentaromativorans* US6-1. Our hypothesis was based on the
knowledge that *N. pentaromativorans* US6-1 has been exposed to a
mixture of PAHs [15]. Over evolutionary time scales, such
conditions would be expected to select for US6-1 with versatile
metabolic capabilities. To conserve energy for vital growth-related
processes, the enzymes needed to degrade a particular compound
are not normally synthesized unless the compound is present in the
medium. Therefore, the precise cellular response to a particular
PAH could be specific to that form, thus enabling the identification
of gene expression and the proteome characteristics associated
with exposure.

Conclusions

In conclusion, this investigation showed that *N. pentaromativorans*
US6-1 is capable of degrading three- to five-ring PAHs, including
phenanthrene, pyrene, and benzo[a]pyrene. When incubated with
PAHs, multiple cellular functions and a global change in cell
physiology were observed in response to PAH stress. *Novosphingobium pentaromativorans* US6-1 degraded PAHs via the
metabolic route initiated by RHD, and further degradation
occurred via either the o-phthalate pathway or the salicylate
pathway [38]; both pathways subsequently entered the 3-
oxoacetate pathway and TCA cycle, followed by mineralization
to CO₂. This investigation provides a basis for understanding the
metabolic versatility of this bacterium in the degradation of other
PAHs; however, cloning and characterization of catabolic genes
and identification of key enzymes will enable the full character-
ization of PAHs metabolism of *N. pentaromativorans* US6-1.

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Author Contributions

Conceived and designed the experiments: YT TZ. Performed the
experiments: YL YT. Analyzed the data: YL WZ YT. Contributed
reagents/materials/analysis tools: YT TZ. Wrote the paper: YL.

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