Simultaneous Biodegradation of Polyaromatic Hydrocarbons by a *Stenotrophomonas* sp: Characterization of *nid* Genes and Effect of Surfactants on Degradation

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Abstract A polyaromatic hydrocarbon degrading bacterium was isolated from a petroleum contaminated site and designated as *Stenotrophomonas* sp. strain IITR87. It was found to utilize pyrene, phenanthrene and benzo(a)-pyrene as sole carbon source, but not anthracene, chrysene and fluoranthene. Gas chromatography and mass spectroscopy analysis resulted in identification of pyrene metabolites namely monohydroxypyrene, 4-oxa-pyrene-5-one, dimethoxypyrene and monohydroxyphenanthrene. Southern hybridization using naphthalene dioxygenase gene (*nidA*) as probe against the DNA of strain IITR87 revealed the presence of *nidA* gene. PCR analysis suggests dispersed occurrence of *nid* genes in the genome instead of a cluster as reported in a PAH-degrading *Mycobacterium vanbaalenii* PYR-1. The *nid* genes in strain IITR87, dioxygenase large subunit (*nidA*), naphthalene dioxygenase

small subunit (*nidB*) and aldehyde dehydrogenase gene (*nidD*) showed more than 97 % identity to the reported *nid* genes from *Mycobacterium vanbaalenii* PYR-1. Most significantly, the biodegradation of PAHs was enhanced 25–60 % in the presence of surfactants rhamnolipid and Triton X-100 due to increased solubilization and bioavailability. These results could be useful for the improved biodegradation of high-molecular-weight PAHs in contaminated habitats.

Keywords *Stenotrophomonas* · Dioxygenase · Surfactants · Biodegradation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants having mutagenic and carcinogenic potential [1]. These are highly persistent in the environment and are extremely toxic to human and thus the United States-Environmental Protection Agency (US-EPA) listed 16 PAHs as priority pollutants. In India, for the energy sufficiency, ca. 10 major industries are involved in oil drilling activities and due to which newer sites are contaminated with huge volumes of oily sludge. The environmental fate of PAHs is associated with both abiotic and biotic factors including bio-accumulation, volatilization, chemical oxidation, photo-oxidation, and biotransformation [2, 3]. PAHs are persistent due to their hydrophobicity and ability to absorb into the soil particulates resulting in decreased bioavailability and biodegradation [4]. Biodegradation is found to be a preferred method over incineration and other physico-chemical methods for removal of PAHs from the contaminated sites. Many bacteria have been isolated and characterized for biodegradation of PAHs, varying from three

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to five rings [5–7]. Gram negative bacteria that biodegrade PAHs include *Burkholderia* [8], *Pseudomonas* [7] and *Sphingomonas* [9]. Gram positive bacteria such as *Mycobacterium* [6, 10, 11], *Rhodococcus* sp. were also reported to degrade PAHs [12]. The initial degradation reactions are catalyzed by multi-component dioxygenase to produce dihydrodiols [6, 13]. Pyrene degradation by *Mycobacterium* species is initiated by incorporation of mono or di-oxygens at C-1, C-2, C-4 and C-5 position [13–15]. *M. vanbaalenii* was found to metabolize pyrene into cis-4,5-dihydrodiol, cis-1,2-dihydrodiol [14] and 4,5-dihydroxypyrene were cleaved to form phenanthrene-4,5-dicarboxylic acid, which was further metabolized into 3,4-dihydroxyphenanthrene [14].

Studies on the biochemical, molecular and genetic analysis of pyrene degradation were reported from a *Mycobacterium* sp. strain PYR-1 [16] in detail. It was shown that *nid* genes involved in the degradation of pyrene were arranged in clusters in strain PYR-1 [14]. Later presence of *nid* gene homologues were also reported from *Mycobacterium gilvum* PYR-GCK, *M. sp.* JLS, *Pseudoxanthomonas* sp. and *Diaphorobacter* sp. having high levels of sequence identity among them [17, 18]. Here, we report the isolation and characterization of a *Stenotrophomonas* sp. that is able to metabolize three high molecular weight PAHs. We have shown presence of *nid* genes in the strain ITR87 and also identified the metabolites of pyrene degradation. Moreover, enhanced biodegradation of pyrene, phenanthrene and benzo(a)pyrene in presence of two surfactants was also studied.

Materials and Methods

Chemicals and Reagents

All the polyaromatic hydrocarbons (PAHs) were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents used were of high performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific Pvt. Ltd. and Merck Limited Mumbai, India. Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Supelco, USA. Reagents and enzymes for polymerase chain reactions were purchased from Fermentas Inc. Maryland, USA. DIG labeling and detection kit was procured from Roche Diagnostics GmbH Mannheim, Germany. Restriction enzymes were from New England Biolabs, USA. Plasmid extraction kit was purchased from Genetix Biotech Asia Pvt. Ltd. PCR purification and DNA gel extraction was purchased from Axygen, USA.

Isolation of PAH Degrading Bacterium and Its Growth Conditions

PAH-degrading bacterium was isolated from oily waste contaminated sediments collected from Mathura Oil Refinery, India. The soil sediment slurry was mixed in minimal medium (MSM) and incubated in an orbital shaker at 30 °C with 180 rpm for 2 weeks. The culture was dilution plated onto MSM agar and sprayed with pyrene dissolved in di-ethyl ether. Formation of clearance zone around the colonies was observed after incubation at 30 °C for 1 week. An experiment for degradation of pyrene was performed under aerobic conditions using 0.48 mM concentration as a sole carbon source for the growth in MSM [19]. Growth was monitored at 600 nm (OD₆₀₀) using UV-spectrophotometer and also by enumerating the colonies by colony forming unit (CFU) method. For the identification of bacteria 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR-product of 1.4 kb was cloned into pGEM-T easy vector (Promega) and sequenced. The sequences were compared with data base in Genbank using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/>). Multiple alignments of sequences ClustalW2 [20] and MEGA 5.0 [21] software package used for construction of neighbor-joining phylogenetic tree.

Growth on Pyrene and Other PAHs

The ability of strain ITR87 to grow on and degrade pyrene, phenanthrene and benzo(a)pyrene was studied in 500 mL Erlenmeyer flask in which 200 mL MSM with 100 mg L⁻¹ i.e., 0.49, 0.56 and 0.39 mM concentration of PAHs respectively as source of carbon and incubated with shaking (180 rpm) at 30 °C for 25 days. Growth was monitored at 0, 5, 10, 15 and 25 days by measuring optical density. To evaluate the degradation preference of strain ITR87 for PAHs when present as mixtures, a batch experiments in duplicates were studied. Equimolar concentration, i.e., 100 mg L⁻¹ (0.49, 0.56 and 0.39 mM) of pyrene, phenanthrene, benzo(a)pyrene respectively were added to 150 mL volume flasks containing 20 mL minimal medium and 0.1 OD (1.5 × 10⁻⁸ cfu mL⁻¹) cells grown as an initial inoculums. Flasks without inoculation of strain ITR87 but containing all the other contents in duplicate served as control. At every 5 days of interval for 15 days, the culture was monitored for growth and the contents were extracted (see below) and analyzed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC).

Analysis of Pyrene Metabolites

Strain IITR87 was cultured in 200 mL minimal medium in a 500 mL flasks containing 0.5 mM pyrene as a sole source of carbon. After incubation for 30 days with shaking (180 rpm) at 30 °C the reaction mixture was extracted twice with equal volume of ethyl acetate. The aqueous layer was also acidified to pH 2.5 and further extracted separately. Both acidic and neutral extracts were air dried in chemical fume hood and evaporated residues were dissolved in 400 µL acetonitrile and analyzed by GC/MS. The extracts were derivatized using silylating reagent bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Briefly, 300 µL of dissolved residual sample was mixed with 100 µL of BSTFA with 1 % trimethyl chlorosilane and to this 60 µL of pyridine was added and kept at 70 °C for 1 h in thermo-shaker. GC/MS analyses were performed using Trace GC ultra gas chromatograph connected to a Quantum XLS mass spectrometer (Thermo Scientific, FL, USA) using the conditions described previously [22]. Surfactant mediated biodegradation of phenanthrene, pyrene and benzo(a)pyrene was analyzed by reverse phase-HPLC system. The separation was achieved using C18 column (Thermo Aclaim C18, 4.6 × 250 mm 5 µm particle size). The RP-HPLC consist of Dionex ultimate 3000 UHPLC system (Thermo, USA), equipped with a binary pump and UV detector and analysis was monitored by using Chromeleon software. An aliquot of 20 µL of filtered sample was injected into the system. The column flow rate was maintained at 2 mL min⁻¹ in isocratic mode. The mobile phase consisting of acetonitrile:water (85:15, v/v). Total run time of the analysis is 13 min per sample and the detector (UV) wavelength was set at 254 nm.

PCR Amplification and Cloning of *nid* Genes

For the verification of pyrene degradation genes, primers were designed (Table 1) based on the reported *nidA*, *nidB* and *nidD* gene sequences of *Mycobacterium vanbaalenii* PYR-1 (Gene accession No. AY365117.2). PCR conditions (Mastercycler, Eppendorf, Germany) were set as initial denaturation step at 94 °C for 7 min, followed by 35 cycles for denaturation at 94 °C for 1 min, annealing at 55 °C 1 min, extension at 72 °C for 1 min, and final extension at

72 °C for 7 min. Amplified products were gel purified (Axygen, CA 94587 USA) ligated into pGEM-T Easy vector (Promega Inc. Madison, USA) and transformed into *E. coli* DH5α (New England Biolabs, USA) (Table 2). Cells were spreaded on LB agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 µg mL⁻¹ ampicillin. Ten white clones were randomly selected and inoculated in LB broth containing ampicillin for plasmid DNA isolation (Nucleopore, Genetix Biotech Asia Pvt. Ltd.). The insert DNA was verified by digesting with *EcoRI* restriction enzyme and sequences were determined using Sanger's dideoxy nucleotide sequencing procedure. The occurrence of *nid* genes in strain IITR87 was also verified by Southern hybridization by standard protocols using *nid* gene fragment of 600 bp as probe employing DIG non-radioactive labeling kit (Roche diagnostics GmbH, Mannheim, Germany). The digested genomic DNA was electrophoresed on 0.8 % agarose gel and transferred to a positively charged nylon membrane. Cross hybridizing DNA fragments were detected by nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma-Aldrich, USA) colorimetrically.

Effect of Surfactant on Dissolution and Degradation

Aqueous solutions containing rhamnolipid (Rh) and Triton X-100 (TX) were prepared separately with concentrations ranging from 77 to 1232 and 58 to 928 µM, respectively based on the reported critical micelle concentration (CMC) known as concentration of a surfactants above which micelles form, values for these two surfactants from previous study [23]. Five different concentrations were tested for their dissolution capacity in filtered sterile water (Milli-Q, Millipore). Briefly, in a 30 mL screw cap glass tubes, 10 mL of minimal salt medium (MSM) containing 77, 154, 308, 616 and 1232 µM concentration of rhamnolipid and 58, 116, 232, 464 and 928 µM concentrations of Triton X-100 were added and incubated with shaking for 72 h at 30 °C. The solution, after 72 h was filtered through a Millex-GV 0.45 µm syringe filter (Merck Millipore Ltd, Germany) and pass through solution was extracted with ethyl acetate. The residual PAHs after evaporation of the solvent was recovered by dissolving in HPLC grade

Table 1 PCR primer sequences, gene encoding enzymes

Gene	Size (bp)	Primer sequence (5'–3')	Enzyme code
<i>nidA</i>	1187	F-5'-ATGACCACCGAAACAACCGGA-3' R-5'-TCAAGCACGCCCCGCCGAATGC-3'	Dioxygenase large subunit
<i>nidB</i>	510	F-5'-ATGAACGCGGTTGCGGTCGAT-3' R-5'-CTACAGGACTACCGACAGGTT-3'	Dioxygenase small subunit
<i>nidD</i>	1461	F-5'-ATGATCAGCAACCTGA-3'	Aldehyde dehydrogenase

Table 2 Plasmid and vector used in this study

Strain, plasmid	Characteristic	Reference (s)
<i>Strains</i>		
<i>Stenotrophomonas</i> sp.	Degrading PAHs such as pyrene, phenanthrene	This study
<i>E. coli</i> DH5 α	Host cell	
<i>Plasmid</i>		
pGEM-T Easy	Ap ^r , TA cloning vector	Promega
pPYNA	1.18 kb PCR fragment including <i>nidA</i> gene	This study
pPYNB	0.51 kb PCR fragment including <i>nidB</i> gene	This study
pPYND	1.46 kb PCR fragment including <i>nidD</i> gene	This study

acetonitrile and analyzed using RP-HPLC. Degradation experiments were performed using 1232 and 928 μ M concentration of Rh and TX respectively which showed the maximum dissolution of tested PAHs. Degradation was initiated by the addition of 0.1 OD₆₀₀ IITR87 cells in the presence of 100 mg L⁻¹ PAHs. The culture after incubation of 5, 10 and 15 days, was extracted and analyzed for the degradation of PAHs analyzed by RP-HPLC.

Nucleotide Sequence Submission

The 16S rRNA gene sequence of the strain IITR87 is submitted in GenBank under the accession number KJ933407 and the *nidA*, *nidB* and *nidD* genes carry the accession numbers KM379097, KM379098 and KM387408.

Results

Isolation and Identification of a Pyrene Degrading Bacterium

Strain IITR87 was isolated for its ability to utilize PAHs as sole source of carbon. Initially the bacteria were subjected to substrate clearance assay on minimal agar plate sprayed with pyrene, which formed halo zones after incubation for 5 days at 30 °C (Fig. S1). The 16S rRNA sequence of 1513 bp nucleotides from strain IITR87 using BLAST [24] revealed that the strain belonged to the genus *Stenotrophomonas* and was designated as strain IITR87 based on the neighbor joining tree [21] method of phylogenetic analysis (Fig. S2).

Growth and Characterization of Pyrene Metabolites

The strain IITR87 could efficiently utilize at least three different PAHs as a sole carbon source. Total colony forming units (CFU) were recorded to assess the growth of IITR87 in presence of PAHs. CFU count revealed increased growth of IITR87 in presence of PAHs; the

Fig. 1 Growth analysis of *Stenotrophomonas* sp. strain IITR87 on different PAHs. Strain was cultured in presence of using 100 mg L⁻¹ of each PAH as sole source of carbon in mineral medium for 25 days. Control: Pyrene (open square), Phenanthrene (open triangle), Benzo(a) pyrene (open diamond). Experimental:- Pyrene (filled square), Phenanthrene (filled triangle), Benzo(a)pyrene (filled diamond)

increased growth also coincided with the substrate depletion as analyzed by HPLC. On comparing the growth of IITR87 strain in presence of different PAHs, we observed that IITR87 showed highest growth in presence of phenanthrene (4.5-fold) followed by pyrene (3.4-fold) and benzo(a)pyrene (2.7-fold) as compared to the initial OD after 25 days at 30 °C (Fig. 1). Moreover, in presence of 100 mg L⁻¹ PAHs, highest degradation rate by IITR87 strain was observed in phenanthrene (60 %) followed by pyrene (55 %) and benzo(a)pyrene (19 %). A total of four metabolites two each from acidified and neutral extracts were identified by GC–MS analysis (Fig. 2). 1-2-dimethoxypyrene (Rt = 15.28) and 4-oxa-pyrene-5-one (Rt = 14.22, *m/z* 220) were identified from the acidified extracts of growth medium. 4-oxa-pyrene-5-one is formed from the dehydrogenation of cis-4,5-dihydroxypyrene upon acidification of growth medium. Pyrene metabolites such as monohydroxypyrene (Rt = 13.91, *m/z* 290) and monohydroxyphenanthrene, (Rt = 12.30, *m/z* 266) were identified from the neutral extracts of growth medium. Monohydroxypyrene is formed via initial enzymatic monooxygenation and further non-enzymatic isomerization of pyrene. Monohydroxyphenanthrene, is the

Fig. 2 GC mass spectra of pyrene metabolites obtained from *Stenotrophomonas* sp. strain IITR87 grown in minimal media at 30 °C in presence of pyrene (100 mg L⁻¹) along with their retention

time. **a** neutral and **b** acidic condition pyrene (13.04), monohydroxypyrene (13.91), 4-oxa-pyrene-5-one (14.22), 1,2-dimethoxypyrene (15.28), monohydroxyphenanthrene (12.30)

dehydrogenation product of phenanthrene-4-carboxylate obtained by an unknown dehydrogenase enzyme. These metabolites were also reported previously in *Mycobacterium vanbaalenii* PYR-1 [11, 13, 25] (Fig. S4).

Identification of *nid* Genes

Pyrene degradation genes, essentially the *nid* genes present in the strain IITR87 were cloned and sequenced. Components of aromatic-ring-hydroxylating dioxygenase such as the α -subunit pyrene dioxygenase (*nidA*), β -subunit pyrene dioxygenase (*nidB*) and aldehyde dehydrogenase (*nidD*) that involved are involved in pyrene degradation (Fig. S4). Since *nid* genes have been previously shown to occur in clusters for pyrene degradation, it was also verified for organization of *nid* gene as cluster in the strain IITR87. Based on the *nid* gene cluster information, *nidB* forward

primer and *nidA* reverse primer did not yield an expected product of 1938 bp which is an indication that they are not present together. Similarly, other combinations (*nidDF-nidBR*, *nidDF-nidAR*) by using enzyme, Phusion High fidelity DNA polymerase, New England Biolabs, also failed to produce expected PCR-amplicons implying their probable dispersed arrangement in the genome and not as an operonic gene cluster. Presence of two copies of *nidB* was also verified using the primer pairs covering *nidD* locus and *nidB* locus of the cluster. DNA fragment of 2.0 kb was obtained from strain IITR87 but not a 2.5 kb which was expected when occurrence of 2 copies of *nidB*. The results indicate the occurrence of only one copy of *nidB* unlike the two copies of it reported from *Mycobacterium vanbaalenii* PYR-1. Southern blot analysis showed presence of *nidA* gene in *Stenotrophomonas* sp. strain IITR87 (Fig. S5).

Effect of Surfactants on Dissolution and Degradation of PAHs by Strain IITR87

The aqueous solubility of phenanthrene, pyrene and benzo(a)pyrene in the absence of surfactant was measured to be 0.15, 1.58, and 0.75 mg L⁻¹ respectively by RP-HPLC. Dissolution experiment data showed that solubility of phenanthrene, pyrene and benzo(a)pyrene (0.60, 2.11 and 1.2 mg L⁻¹ respectively) was increased in the presence of 1.23 mM rhamnolipid (biosurfactant) to 4.0-, 1.33- and 1.60-fold. Similarly, solubility of phenanthrene, pyrene and benzo(a)pyrene (2.25, 3.72 and 1.61 mg L⁻¹ respectively) was enhanced in presence of 0.92 mM concentration of Triton X-100 (chemical surfactant) to 15.00, 2.33 and 2.14 fold higher than without surfactants. Thus, chemical surfactant Triton X-100 was found to be a better than the biological surfactant rhamnolipid and led to higher dissolution of phenanthrene, pyrene and benzo(a)pyrene which is nearly 3.75-, 1.76- and 1.34-fold than the solubility in presence of rhamnolipid (Fig. 3a).

In the absence of surfactants, the biodegradation of phenanthrene, pyrene and benzo(a)pyrene was only 15, 19 and 14 % respectively. Addition of 1.23 mM concentration of rhamnolipid showed no effect on the degradation of phenanthrene (Fig. 3b). In the presence of 0.92 mM of Triton X-100, the degradation rate of phenanthrene was

increased from 25 to 60 % in comparison to the other two PAHs (Fig. 3c).

Discussion

The PAH-degrading bacterium designated as IITR87 was isolated from an oily sludge waste and identified as *Stenotrophomonas* sp. based on the 16S rRNA gene sequence. IITR87 was found to degrade three major PAHs as sole carbon sources under aerobic conditions. Few more bacteria have been reported previously that are able to metabolize multiple PAHs [12]. Gene involved in pyrene degradation was identified from strain IITR87 and were found to be homologous to the naphthalene dioxygenase large subunit (*nidA*), small subunit (*nidB*) and aldehyde dehydrogenase (*nidD*). The detailed analysis of the above gene sequences showed more than 98 % identity to the *nid* genes characterized from few other PAH-degrading genes. A multi-component phthalate gene cluster system involved in biodegradation of pyrene has also been described from many *Mycobacterium* species [26]. The genes encoding phthalate biodegradation such as large subunit of phthalate dioxygenase and dihydrodiol dehydrogenase were PCR amplified, cloned and sequenced (results not presented here). These results also found to match the phthalate gene sequences reported from strain PYR-1 [27]. Previous

Fig. 3 Effect of surfactants concentration on the dissolutions of mixed PAHs by Rhamnolipid and Triton X-100 (a). Degradation of mixed PAHs by *Stenotrophomonas* sp. strain IITR87 in presence of rhamnolipid (b) and Triton X-100 (c) at a concentration of 1.23 and 0.92 mM respectively. Phenanthrene (phe), pyrene (pyr), benz(a)pyrene (bap). Values are represented as the mean and standard error of duplicate measurement (n = 2). C Control, *rhl* Rhamnolipid, *tx* Triton X-100

reports [18] suggested that *nid* genes were located on megaplasms in *Pseudoxanthomonas* sp. and *Diaphorobacter* sp. Our efforts to extract megaplasmid from strain IITR87 at least by three protocols failed to yield any megaplasmid suggesting the location of degradative genes on chromosomal DNA.

Pyrene degradation intermediates identified from the isolated bacteria were found to be similar to the reported metabolites from *Mycobacteria* species. One of the intermediate, pyrene dihydrodiol was found in several bacteria such as *Burkholderia* and *Pseudomonas* sp. The formation of this intermediate also confirms the reaction mediated by multi-component dioxygenase that was determining the substrate metabolism similar to *Mycobacterium* sp. strain KMS [28], *M. vanbaalenii* PYR-1 [16] and *Mycobacterium* sp. strain 6PY1 [29].

Previous studies have also reported naphthalene-induce dioxygenases from several gram positive *Mycobacterium* species, including *M. vanbaalenii* PYR-1, *M. frederiksbergense* FAn9T, *Mycobacterium* sp. strain RJGII-135, *M. flavescens* PYR-GCK, [30] *Mycobacterium* sp. strain MCS, *Mycobacterium* sp. strain KMS and *Mycobacterium* sp. strain JLS [5]. We also observed a high sequence similarity of *nid* genes in a gram negative strain like IITR87, suggesting that these genes are quite conserved across diverse bacteria. We also observed that *nid* genes of IITR87 are not clustered and are dispersed within the genome unlike previous reports. The results indicate that *Stenotrophomonas* sp. strain IITR87 evolved independently and exhibited biodegradation traits, similar to other reported *Mycobacterium* species. The goal of our study was to obtain a bacterium with potential to degrade multiple PAHs so that it can be utilized to biodegrade PAHs from oily sludge contaminated sites. We are currently investigating the biodegradation efficiency of this bacterium in an oily sludge samples in a microcosm approach along with many other isolate degrading a range of model PAH compounds.

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