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Biodegradation of Polycyclic Aromatic Hydrocarbon Mixtures by Rhodococcus Pyridinivorans FF2 and Pseudomonas Aeruginosa F4b Isolated from Sediments of Lagos Lagoon, Nigeria

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental pollutants that need urgent attention because of their toxicity. Development of microbial inoculants for PAH bioremediation is a potential avenue by which the environmental hazards posed by PAH can be addressed. The goal of the study was to determine if using PAH mixtures, rather than single PAH, as enrichment substrates would yield isolates that have superior PAH degradation abilities and/or are retrieve novel taxa from the PAH-contaminated sediments of Lagos Lagoon, Nigeria. The use of a quaternary mixture of PAH (pyrene, fluoranthene, fluorene, benz[a]anthracene) was successful in yielding two isolates, Pseudomanas aeruginosa strain F4b and Rhodococcus pyridinivorans strain FF2 with capabilities to grow on multiple PAH, and thus potentially useful in bioremediation. In addition to the PAH degraded both isolates could grow on a wide range of other hydrocarbon substrates. The isolates of P. aeruginosa and R. pyridinivorans were identified as possessing PAH ring-hydroxylating dioxygenases of the nahAC and narAa genotypes, respectively. The present study extends our knowledge of PAH biodegradation by P. aeruginosa and is the first report of PAH biodegradation by R. pyridinivorans. The capability of the R. pyridinivorans isolate to effectively degrade a highly toxic PAH, benz[a]anthracene, has particular importance for use in bioremediation.

Introduction

In Nigeria, oil exploration and indiscriminate discharge of municipal and industrial effluents has resulted in a tremendous increase in environmental pollution by many organic compounds including polycyclic aromatic hydrocarbons (PAHs). These compounds, especially the high molecular weight PAHs (HMW-PAHs, composed of four or more rings) are suspected carcinogens and are very hazardous to humans when ingested, inhaled or through other direct contact [1,2]. The concentration of PAHs in the environment is usually directly proportional to the type of human activities inherent in such environment [3]. Thus, the major source of PAHs in coastal sediments is usually through anthropogenic sources such as atmospheric deposition, urban runoffs, municipal and industrial effluents agricultural wastes and oil spillages [4,5].

PAHs may undergo adsorption, volatilization, photolysis, and chemical degradation but microbial degradation is recognized as the main pathway of PAHs removal from a contaminated site [6,7]. PAH degradation depends on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded [8]. Microbial degradation of PAHs in the environment can occur through metabolism or co-metabolism [9]. Bioremediation of hydrocarbon-contaminated sites relies on either the activity of microorganisms already present at such site or the

addition of selected microorganisms that have the suitable enzymatic systems with desired catabolic traits. Researchers over the years has demonstrated the abilities of different group of microorganisms including fungi [10-12], bacteria [7,13,14,15], yeast [16] and algae [17-19] to degrade PAHs by pure cultures or when applied as consortium.

Among the microbes identified as contaminant degraders, the bacteria Rhodococcus and Pseudomonas have been frequently described in the catabolism of a range of substrates such as short-chain, long-chain, and halogenated hydrocarbons, and aromatic compounds, like PAHs, polychlorinated biphenyls and dibenzothiophenes [20-23]. Their well-established cellular resistance and metabolic ability for the degradation of all these compounds are related to their genomic properties, with an uncommon presence of multiple homologs of enzymes participating in major catabolic pathways and also a remarkable capacity for acquiring large linear plasmids which possibly conferred antibiotic resistance [20,24]. Their oxidative metabolism is capable of using several organic compounds as sole carbon and energy sources that fostered several industrial and bioremediation applications [20]. For the genus Rhodococcus the range of PAH serving as substrates includes fluorene, fluoranthene, phenanthrene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene and benzo[a]pyrene [25-28]. Furthermore, metabolic pathway during biodegradation of PAHs by Rhodococcus spp has also been proposed from several previous reports [29,30].

The abilities of microbes to degrade both low molecular weight (LMW)-PAHs and HWM-PAHs has been extensively studied [26,29,31,32]. Bacterial degradation of PAH aerobically begins with the addition of both molecules of molecular oxygen to the aromatic ring by a ring hydroxylating dioxygenase (RHD), [33]. The RHD are multicomponent enzymes that consist of an electron transport chain, containing a ferredoxin and a reductase, and a terminal dioxygenase [34]. The latter is composed of two polypeptides, one of which (alpha subunit, RHDα) is the catalytic component of the enzyme system. The RHDα contain two conserved regions: the [Fe2-S2] Rieske center and the mononuclear iron-containing catalytic domain [35]. The RHDα serve as biomarkers of potential PAH-degraders, and the cognate genes can be PCR-amplified by using different primer sets [26,29,32,36].

In environments polluted by PAH, these compounds are invariably introduced in substances containing mixtures of PAH, such as coal tar and crude oil [37-40]. It's well-established that biodegradation of any given PAH can vary greatly when present as a part of a PAH mixture [39,41-43], as other PAH may exert either positive or negative effects on biotransformation [37,43]. A variety of processes can operate simultaneously at cellular and intercellular (community) levels to contribute to variation of PAH biodegradation in PAH mixtures. It's possible that prolonged exposure to PAH mixtures can select for organisms adapted (e.g., enzyme specificities, regulatory circuits etc.) to catabolize PAH when present as mixtures. Thus, in efforts to identify microbes that effectively degrade PAH mixtures, employing PAH mixtures as enrichment substrates could be a strategy to acquire organisms better suited to function as bioremediation agents.

In this study, we examined the utility of using PAH mixtures, rather than single PAH, as enrichment substrates. We wished to determine if this approach would yield isolates that have superior PAH degradation abilities and retrieve isolates from diverse microbial taxa. We examined sediments of Lagos Lagoon, Nigeria, an important estuary in Africa that is impacted by PAH pollution [44].

Materials and Methods

Chemicals and reagents.

Analytical standards of various PAHs used in this study were obtained from Sigma Aldrich Corporation (St Louis, MO, USA). This includes fluorene (99% purity), pyrene (98% purity), benz[a]anthracene (99% purity) and fluoranthene (98% purity). HPLC grade organic solvents such as dichloromethane and acetone were purchased from Fisher Scientific Co. (Springfield, NJ, USA) while ethyl acetate was procured from Sigma Aldrich. Also purchased from Sigma Aldrich are analytical grades of the following compound; biphenyl, salicylic acid, pthallic acid, dibenzofuran, dibenzothiophene, methylnaphthalene, acenapthene, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid and 2,3-dihydroxybenzoic acid. Stock solutions of the hydrocarbon substrates were prepared by dissolution in acetone.

Sediment Sample Collection, Enrichment and Isolation of PAH-Degrading Bacteria

Sediment samples used for the enrichment technique were collected at three sampling stations (Apapa, Ofin and Eledu) from the Lagos Lagoon as described by Obi et al [44]. The continual enrichment technique adopted for the isolation of PAH-degrading bacteria has been described elsewhere [16]. Briefly, Sediment from each of the three sampling locations was mixed in equal parts to create a composite, and then 1 g of composite was suspended in

90 ml basal mineral salt medium (BMS) containing (g/l) 0.5 g (NH4)2SO4, 1.0 g K2HPO4, 2.79g NaH2PO4, 0.2 MgSO4.7H2O, 0.5g NaCl, 50 mg Ca(NO3)2.4H2O and 1mL of trace elements, pH 7.0 ± 0.2. The medium was supplemented with the following PAHs mixture as sole sources of carbon and energy: pyrene, fluoranthene, benz[a]anthracene and fluorene at concentration of 25 mg l-1 each making a total of 100 mg l-1. The flasks were incubated at 30ºC on an orbital shaker (Excella E10, New Brunswick Scientific Edison, New Jersey, USA) programmed at 150 rpm. After 14 days incubation, an aliquot of the enriched cultures was transferred into a fresh medium and handled as described above. This step was repeated 6 times to attain well-adapted PAH-degrading enriched bacterial consortia.

Bacterial strains in each consortium were isolated by spreading onto MSM-agar plates coated with a thin layer of different PAHs (benz[a]anthracene, pyrene, fluoranthene and fluorene) as described by Kiyohara et al. [45]. Bacterial colonies surrounded by a clear zone were scored positive, and subsequently purified and screened for utilization of PAHs.

Culture Maintenance

Pure cultures were grown overnight in Luria Bertani medium at 30°C, harvested by centrifugation (Eppendorf 5424/5424R Thermo Fisher Scientific, USA) at 6,000 rpm for 20 min. The pellets were resuspended in LB broth and subsequently distributed (1 ml) into 3 ml cryotubes already containing 1 ml glycerol (30% v/v). The mixture was thoroughly homogenized by shaking while avoiding foaming. The cryotubes were all stored at -20°C. For inocula development, isolates were routinely cultured on LB agar for purity check. Overnight cultures on LB broth were harvested by centrifugation, washed twice with MSM and subsequently resuspended in the same medium and adjusted to an OD (λ600 nm) of 1.

Hydrocarbon Degradation Profiling of Isolates

In addition to the PAHs of interest, isolates were tested for their abilities to grow on a range of other organic compounds (Table 1). Flasks containing 50 ml MSM were supplemented with 1% (v/v) petroleum feedstocks such as diesel, crude oil, pyridine and fresh engine oil. All other compounds were supplied at 100 mg l-1. The flasks were inoculated with 200 µl of washed cell suspension and incubated on a rotary shaker at 30ºC for 7 d in the dark.

PAH Biodegradation Studies

Pyrene, fluoranthene, benz[a]anthracene and fluorene were added to sterile 250-ml conical flasks from concentrated stocks dissolved in acetone (100 ul of each). The acetone was dried under a sterile stream of nitrogen gas, and BMS (50 ml) was dispensed into the flask to obtain a final PAH concentration of 100 mg l-1. For the degradation of PAH mixtures, the experiment was handled as described above except that 25 mg l-1 of each the four PAH was added into a single 250 ml conical flask to obtain a final concentration of 100 mg l-1. The flasks were inoculated with 200 µl of washed cell suspension and incubated at 30ºC for 21 to 30 d on a rotary shaker at 180 rpm. Non-inoculated flasks served as negative controls for abiotic loss. Cell density (λ600 nm) and residual substrate concentration

Table 1: Growth substrate range test.

+++= Luxuriant growth (≥ OD600 = 1); ++ = Moderate growth (OD600 = 0.5-0.7), + = Slight growth (OD600 = 0.2-0.4); - = No growth.

		Isolate
Substrate	FF2	F4b
Pyrene	+++	+++
Phenanthrene	++	+
Fluorene	++	+++
Fluoranthene	+++	-
Benz[a]anthracene	+++	-
Methyl Naphthalene	+	-
2,4-Dihydroxybenzoic acid	+++	+++
2,5-Dihydroxybenzoic acid	+ ++	+++
3,5-Dihydroxybenzoic acid	+++	+++
Salicylic acid	++	++
Phthalic acid	++	+++
Vanillic acid	+++	+++
Acenaphthene	++	-
Dibenzofuran	+++	+
2,3-Dihydroxybenzoic acid	+	+++
3-Hydroxybenzoic acid	-	-
Pyridine	-	-
Biphenyl	+	++
Diesel	+++	+++
Crude oil	+++	+++

were determined at each time point as biodegradation indices. All the treatments were established in triplicates.

Analytical Methods

Residual PAH concentration from both experimental and control flasks were analyzed by HPLC (Agilent, Santa Clara, CA) fitted with a programmable UV detector (UVD 170 U Dionex). Separations were performed on C-18 reversed-phased column (54 cm by 35 cm, i.d.; 4 µm particle diam.) by isocratic elution with a mobile phase constituting 90% acetonitrile set at a flow rate of 1 ml min-1. Absorbance of PAHs was monitored at 254 nm. The equipment was connected to a computer equipped with Chromeleon chromatography management software version 6.6. The software was used for quantification of PAHs throughout the experiment. PAH concentrations in the samples were determined against standard curves and the percentages of PAH remaining in the samples were calculated with the aid of deuterated PAH internal standard as a correction factor.

Genomic DNA Extraction

Genomic DNA of the PAH-degrading isolates was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories). The DNA was quantified using a fluorometer (Qubit® 3.0, ThermoFisher Scientific) and dsDNA quantitation done using the broad range protocol according to the manufacturer's instruction.

PCR Amplification and Sequencing of 16s Rrna Genes

PCR Amplification of the 16S rRNA coding region of the genomic DNA was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Akkermans et al., 1996). PCR was prepared in 50 µl reaction mixtures containing 2.5 mM MgCl, 0.2 mM dNTPs, 50 pmol of each primer, 50 ng DNA template, 2.5 U Taq DNA polymerase (Promega) and PCR buffer (Promega, Madison WI). Amplification conditions used had an activation time of 3 min at 94°C, 30 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1min, followed by 7 min of final primer extension at 72°C. Purified PCR products were cloned into the T-easy vector (Promega, Madison WI) and transformed into E. coli cells for propagation. Plasmids were extracted by using a plasmid miniPrep kit (Promega, Madison WI), and sequencing was done by the Sanger sequencing. Taxonomic identification was done by BLAST-N against the NCBI nr database. Multiple sequence alignments were created with MUSCLE and phylogenetic trees were constructed using MEGA 7 by the neighbor-joining algorithm and Kimura 2-parameter distance estimation method with bootstrap analysis for 1,000 replicates [46].

PCR amplification and sequencing of Pah-Rhdα genes.

Degenerate primers were used in PCR to amplify the PAH-RHDα in the isolates. For the Gram-positive bacteria these were Nid forward and Nid rev1 [36] while for Gram-negative bacteria, the primers were AC114F and 1096R [47]. PCR reactions were done in 50-µl reaction mixtures containing 2.5 mM MgCl, 0.2 mM dNTPs, 500 nmol of each primer, 2.5 U Phusion polymerase (New England Biolabs) and PCR buffer (Promega, Madison WI). Amplification conditions were as follows: initial denaturation for 3 min at 94°C, 40 cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec, followed by 7 min of final primer extension at 72°C. PCR reactions were validated by using genomic DNA extracted from Mycobacterium vanbaaleeni PYR1 and Delftia acidovorans Cs1-4 in which PAH metabolism and PAH degradation genes have been characterized. PCR products were resolved on 1.5% agarose gel and the band of expected size was excised, cleaned (Qiagen), cloned into TOPO 11 (ThermoFischer) and transformed into E. coli Top ten cells (ThermoFischer). Templates were prepared from colonies by amplification with the Templiphi system (GE Healthcare) and then submitted to the University of Wisconsin-Madison Biotechnology Center for sequencing. Vector and primer regions were trimmed from the sequences, which were then translated, and the resulting deduced amino acid sequence used for identification via BLASTP against the GenBank database. Multiple sequence alignments were created with MUSCLE and phylogenetic trees were constructed using MEGA 7 by the neighbor-joining algorithm and Kimura 2-parameter distance estimation method with bootstrap analysis for 1,000 replicates [46].

Nucleotide Sequence Deposition

The gene sequences determined in this study have been deposited in the GenBank database under accession numbers KT894768 (strain FF2) and KT894767 (strain F4b) for 16S rRNA genes and under accession numbers KT894771 (strain F4b) and KT894770 (strain FF2) for PAH-RHDα genes.

Results and Discussion

Isolation and Taxonomic Identification of Bacterial Isolates

The enrichment and isolation procedure produced a total of 12 bacterial isolates from growth on different PAHs (pyrene, fluoranthene, fluorene and benz[a]anthracene) and the mixtures of the four PAHs. Two strains (FF2 and F4b) exhibiting broad spectrum PAH utilization were selected for further studies. Strain FF2 showed a zone of clearance when

cultured on minimal salts agar sprayed with a thin film of each of the four test PAH added individually as well as tin the mixture. However, while strain F4b created a zone of clearance when sprayed with flourene, pyrene and the PAH mixture, no clearance was observed with fluoranthene and benz[a]anthracene. These findings indicate that clearance of PAH mixtures on spray plates can occur even if all of the PAH present in the mixture are not metabolized by the bacteria forming the colony. In this case, clearance may occur when surfactants released dissolve all PAH present.

The 16S rRNA gene of strain FF2 had 99% identity across the entire sequence (1476 bp) with those from Rhodococcus sp. strain 2G and Rhodococcus pyridinivorans strain SB3094. Construction of an evolutionary tree for strain FF2 showed that it clustered with three type strains Rhodococcus rhodochrous strain 372, Rhodococcus biphenylivorans strain TG9 and R. pyridinovorans strain PDB9, most closely with the latter (Figure 1). Hence, designation of this isolate as R. pyridinivorans. The description of the type strain R. pyridinovorans sPDB9, included growth on pyridine (the compound on which it was enriched, Yoon et al., [48] as a key chemotaxomonic characteristic. However, R. pyridinivorans strain FF2 did not utilize pyridine as a growth supporting substrate (see below), a discrepancy that likely reflects the fluidity of biodegradation genotypes relative to bacterial phylotypes.

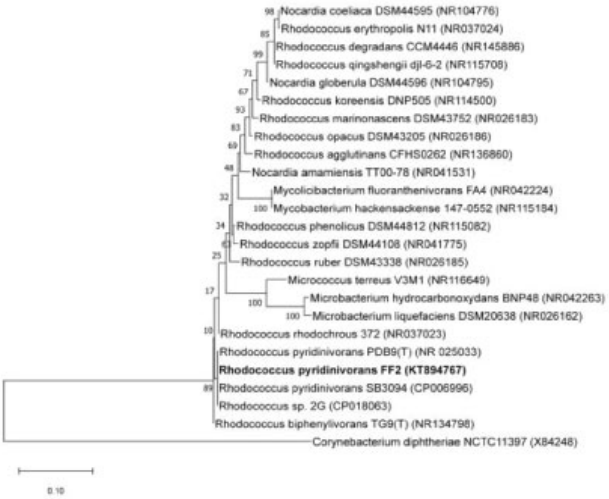

Figure 1: Phylogenetic tree indicating the relationship of the 16S rRNA gene sequence from R. pyridinivorans strain FF2. Bootstrap values at given at the nodes. Corynebacterium diphtheria NCTC11397 was used as the out group. The Genbank accession numbers for each sequence is given in parentheses.

For the strain F4b, construction of an evolutionary tree placed it within the Pseudomonas aeruginosa group (Figure 2). The species P. aeruginosa represents one of two primary lineages within the genus Pseudomonas (Figure

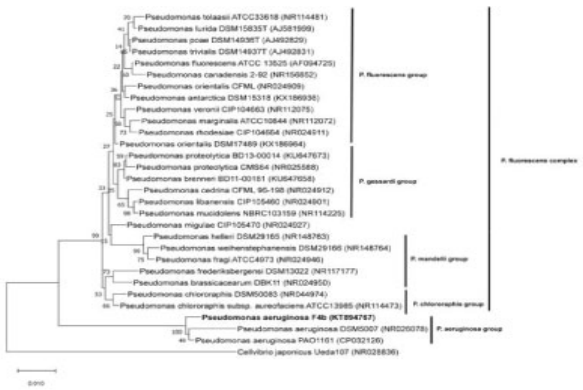

Figure 2: Phylogenetic tree indicating the placement of the 16S rRNA gene sequence from P. aeruginosa strain F4b within the genus Pseudomonas. Bootstrap values at given at the nodes. Cellvibrio japonicus Ueda107 was used as theout group. The Genbank accession numbers for each sequence is given in parentheses.

2). The other Pseudomonas linage is the "Pseudomonas flourescens complex", which is constituted by several groups that collectively contains essentially all other Pseudomonas species except P. aeruginosa [49]. Thus, the clustering of the current isolate with P. aeruginosa type strains strongly supports the species designation for strain F4b.

Biodegradation of individual PAH

Strain FF2 grew with all four of the PAH tested when supplied individually (Table 1). Strain FF2 was able to utilize 82 % of the added benz[a]anthracene after 21 days of incubation, the rapid utilization of this compound is a characteristic that might make strain FF2 potentially useful in the bioremediation of PAH-contaminated sites, as removal of especially hazardous PAH like benz[a]anthracene is a key regulatory goal.

Strain F4b grew with pyrene and fluorene when supplied individually, but not with either benz[a]anthracene or fluoranthene (Table 1). The inability of P. aeruginosa strain F4b to utilize benz[a]anthracene was not unexpected because biodegradation of benz[a]anthracene by species of Pseudomonas are uncommon [50,51]. There are a few reports on biodegradation of pyrene by Pseudomonas species [52,53], but information on the biodegradation of other HMW PAH by pseudomonads is still limited.

Biodegradation of PAH in Mixtures

The abilities of strains FF2 and F4b to degrade mixtures of PAH are shown in (Figures 3 & 4), respectively. Strain FF2 utilized 70 %, 42 %, 35 % and 30 % of fluorene, pyrene, fluoranthene and benz[a]anthracene, respectively (Fig. 1). Strain F4b was able to degrade 10 % benz[a]anthracene, 50 % pyrene, 72 % fluorene and 30 % fluoranthene (Fig. 2)

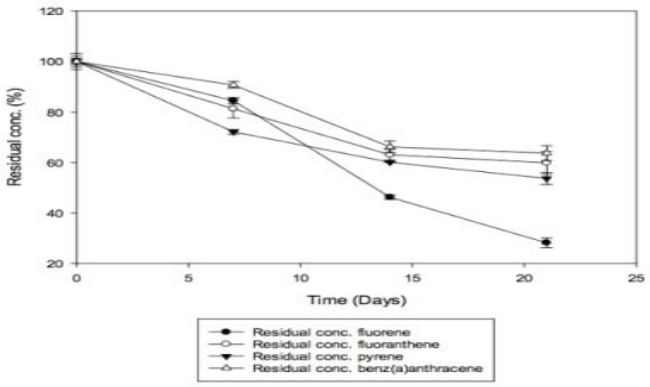

Figure 3: Time course degradation of a mixture of pyrene, fluoranthene, fluorene and benz[a]anthracene degradation by R. pyridinivorans strain FF2

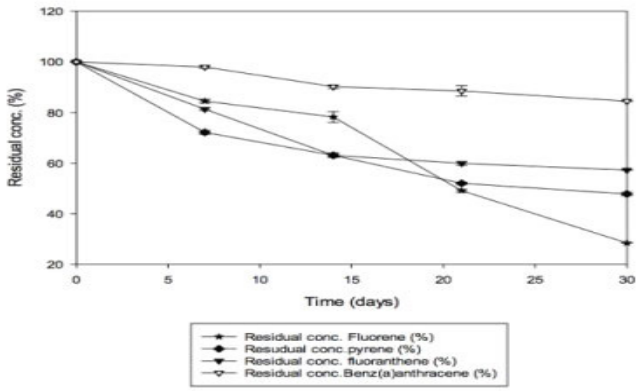

Figure 4: Time course degradation of a mixture of fluoranthene, pyrene, fluorene and benz [a]anthracene by P. aeruginosa strain F4b during biodegradation experiment.

after 21 days of incubation. In the presence of mixed PAH, R. pyridinivorans FF2 exhibited a specific growth rate and mean generation time of 0.072 h-1 and 8.75 h, respectively (Table 2). P. aeruginosa strain F4b exhibited a specific growth rate of 0.0601 h-1 and mean generation time of 7.64 h (Table 1).

Table 2: Growth kinetic parameters of strain FF2 and strain F4b.

T = Mean generation time, μ = Specific growth rate, NA = Not applicable

Substrates	Strain FF2		Strain F4b	
	T (h)	μ (hr-1)	T (h)	μ (hr-1)
Pyrene	6.993	0.09912	7.919	0.08753
Fluorene	9.008	0.07695	8.961	0.07735
Fluoranthene	10.74	0.06455	NA	NA
Benz[a] anthracene	14.46	0.04794	NA	NA
Mixed PAH	8.75	0.07213	7.64	0.07735

The greater efficacy of R. pyridinivorans strain FF2 in PAH biodegradation compared to that of P. aeruginosa strain F4b was consistent with prior reports. For example, Guo and coworkers [6] investigated the degradation of mixed PAHs by Sphingomonas, Rhodococcus, Paracoccus and Mycobacterium strains isolated from mangrove sediments. They observed that all of their isolates degraded completely a mixture of phenanthrene, fluoranthene and pyrene in 14 days. But, the Firmicute isolates (Mycobacterium and Rhodococcus) were more effective in degradation of mixed PAHs than were the Proteobacteria isolates (Sphingomonas and Pseudomonas). Mueller et al. [54] also concluded that biodegradation of PAH mixtures was more effective by Firmicutes than by Proteobacteria.

Growth of strain F4b in the presence of the four PAH resulted in degradation of 10 % of benz[a]anthracene, which was likely cometabolism driven by the growth-supporting PAH, pyrene and fluorene (Figure 2). Juhasz et al. [55] reported cometabolic biodegradation of two HMW PAH, dibenz[a,h] anthracene and benzo[a]pyrene, by a Burkholderia cepacia strain utilizing phenanthrene for growth. Decreases of 41 % and 52 % in dibenz[a,h]anthracene and benzo(a)pyrene, respectively, were observed after 56 days of incubation. But, this organism could not grow on these compounds as a sole source of carbon and energy. Thus, degradation of dibenz[a,h]anthracene and benzo[a]pyrene by this B. cepacia strain was cometabolic, a case similar to the present finding of benz[a]anthracene degradation by P. aeruginosa strain F4b.

Substrate Utilization Specificity of Isolates

The abilities of the isolates to utilize a range of different hydrocarbons as carbon sources is summarized in (Table 1). The two isolates grew well on crude oil, engine oil, 2,5-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid and vanillic acid. Strain FF2 also grew well on benz[a]anthracene, fluoranthene and didenzofuran while it had moderate growth on fluorine, salicylic acid, phthalic acid and acenaphthene. Strain FF2 was not able to utilize 3-hydroxybenzoic acid or pyridine as sole carbon and energy sources. Strain F4b had good growth on fluorene, phthalic acid and 2,3-hydroxybenzoic acid and moderate growth on salicylic acid and biphenyl.

Overall, R. pyridinivorans strain FF2 exhibited a relatively broad spectrum of hydrocarbon utilization. Strong biodegradation capabilities of R. pyridinivorans strain FF2 mirror results reported by other investigators with other Rhododcoccus isolates [20,38,56,57]. Pizzul et al. [58] reported that Rhodococcus wratislaviensis, degraded >

90 % of pyrene after 49 days while Rhodococcus opacus degraded 20 % of pyrene within a 10 day incubation period. The genus Rhodococcus exhibits a diverse range of metabolic activities, including the degradation of various aromatic and aliphatic hydrocarbons (short- and long-chain alkanes), monoaromatics (halogenated and nitro-substituted), heterocyclic compounds and polycyclic aromatic compounds [57].

P. aeruginosa strain F4b did not show growth when cultured in a minimal salts medium supplemented with methyl naphthalene, acenaphthene or pyridine, but grew well on fluorene, phenanthrene, phthalic acid, crude oil, diesel fuel, vanillic acid and other aromatic acids (Table 1). A similar finding was reported by Obayori et al. [53]. The luxuriant growth of P. aeruginosa strain F4b in the presence of crude oil and diesel fuel was important due to the fact that both contain a variety of hydrocarbons making it more likely to support microbial growth in actual contaminated environments [58]. Ilori and Amund [59] reported that P. aeruginosa isolated from a tropical soil polluted with crude petroleum was able to utilize crude oil and a host of other hydrocarbons.

For a microbe to be useful in hydrocarbon bioremediation, utilization of a wide range of hydrocarbons is a desirable characteristic [60] as these pollutants typically occur in the environment in complex mixtures. In this study, the two isolates showed a wide range of substrate utilization when grown in minimal salts medium (MSM) supplemented with different hydrocarbon substrates (Table 1).

Identification of PAH-RHDA genes.

For strain F4b the nahAC primers yielded a PCR product of expected size (ca. 500 bp), which had 100 % identity to nahAC-like genes of several Pseudomonas strains. Construction of a phylogenetic tree placed the PAH-RHDα of strain F4b within the nahAC group of the Gram-negative PAH-RHDα (Figure 5). For strain FF2 the nid primers also gave a product of the expected size (ca. 600 bp), which had 100 % identity to narAa of other Rhodococcus species/strains [61,62]. In the phylogenetic tree, the PAH-RHDα of strain FF2 was placed within narAa group of the Gram-positive PAH-RHDα (Figure 5).

Genes encoding PAH-RHDα are reliable biomarkers of PAH-degrading bacteria and their phylogeny provides a framework to distinguish genotypes based on PAH metabolism. The PAH-RHDα of Gram-negative and Gram-positive bacteria form two distinct lineages, each of which is comprised of several groups (Fig. 5). Within the Gram-

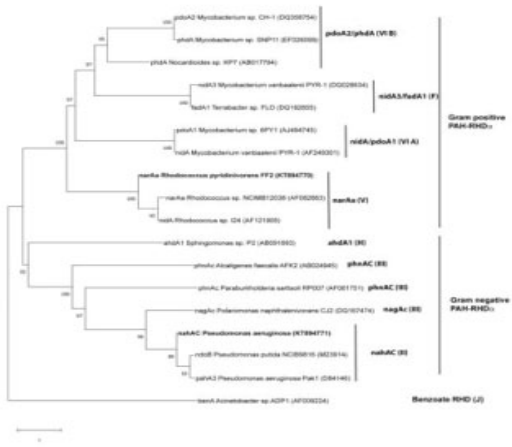

Figure 5: Phylogenetic tree of PAH-RHDα amplified from R. pyridinivorans strain FF2 and from P. aeruginosa strain F4b. The tree illustrates the main groups within the Gram-positive branch (pdoA2/phdA, nidA3/fadA1, nidA/pdoA1, narAa) and Gram-negative branch (nahAC, nagAc, phnAC, phnAC, ahdA1). The Genbank accession numbers for each sequence is given in parentheses following each sequence. The Roman numerals and letters in parentheses following the groups are the clades corresponding to Meynet et al. (2015).

negative branch, the nahAC group is the largest and has been associated with PAH degraders spanning two classes, the gamma- and beta-proteobacteria (Cébron et al., 2008), and is invariably the genotype of PAH-degraders within the genus Pseudomonas [63-66]. Thus, identification of P. aeruginosa strain F4b as an nahAC-type PAH degrader is consistent with this framework. For strain FF2, the classification as narAa-type was also consistent with expectations as it is the genotype commonly associated with PAH degrading strains of Rhodococcus [63-66].

Conclusions

The use of multiple PAH during enrichment was effective for recovering bacteria with capabilities to grow on multiple PAH. The technique was successful in retrieving isolates from both the Proteobacteria and Firmicutes, and was thus capable of recovering isolates from a diverse range of microbial taxa. The present study extends our knowledge of PAH biodegradation by P. aeruginosa and is the first report of PAH biodegradation by R. pyridinivorans. The capability of the R. pyridinivorans isolate to effectively degrade a highly toxic PAH, benz[a]anthracene, has particular importance for use in bioremediation [67,68].

Acknowledgments

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