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Distribution of PAH-ring hydroxylating dioxygenase genes in bacteria isolated from two illegal oil refining sites in the Niger Delta, Nigeria

Chioma Blaise Chikere[a,*], Emmanuel Oliver Fenibo[b]

[a] Environmental Microbiology and Bioremediation Division, Department of Microbiology, Faculty of Science, University of Port Harcourt, P.M.B. 5323, East-West Road, Choba, Port Harcourt, Rivers State, Nigeria
[b] World Bank Africa Centre of Excellence, Oilfield Chemical Research, University of Port Harcourt, Choba, Rivers State, Nigeria

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ABSTRACT

Polyaromatic hydrocarbons (PAHs) are commonly found pollutants in the Niger Delta. They are comparatively persistent in the environment. Also, they distort healthy microbial interaction and pose high risk to human health owing to their toxic, mutagenic and carcinogenic properties. This research investigated the distribution of naphthalene dioxygenase gene (*nahAc*) and PAH-ring hydroxylating dioxygenase alpha genes (PAH-RHDα-GP) amongst bacteria isolated from two disparate locations (Bomu in Gokana LGA and Ngia Ama in Degema LGA, in Rivers State, Nigeria) used as illegal modular refining sites. Spread plate method was used to isolate PAH-degrading bacteria followed by identification, characterisation and phylogenetic analysis. Polymerase chain reaction was used to detect the *nahAc* and PAH-RHDα-GPgene sequences (from chromosomal DNA and plasmid) of the α-subunit of PAH-ring hydroxylating dioxygenase, using specific primer sets. Genera of *Enterobacter, Shewanella, Burkholderia, Pseudomonas, Bacillus, Acinetobacter, Exiguobacterium* and *Stenotrophomonas* were isolated from the two study sites. *nahAc* genes were found in the genomes of both Gram-negative and Gram-positive bacteria while PAH-RHDα-GPgenes were detected in all the plasmids recovered from Bomu-isolates. These findings inferred that the catabolic potential detected in the PAH-degrading bacterial community could be contributing to the *in-situ* biodegradation of PAHs.

Introduction

The Niger Delta Region of Nigeria is well endowed with oil and gas resources; and also very rich in biodiversity [9]. Aggressive exploitation and exploration of these petroleum hydrocarbons had led to environmental pollution resulting from routine oil operations, accidents, sabotage and illegal artisanal oil refining activities. Impact of hydrocarbon pollution in the region includes changes in soil physicochemical features, distortion of healthy microbial interaction and ecological services.

* Corresponding author.
E-mail address: chioma.chikere@uniport.edu.ng (C.B. Chikere).

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Hydrocarbon contamination had also caused biodiversity depletion, ground water contamination, bioaccumulation and biomagnification in environmental receptors, alteration of the natural habitat including life threatening diseases like cancer [22].

Polyaromatic hydrocarbons (PAHs) are crude oil components with two or more aromatic rings arranged in linear, angular or in clustered forms [13,48]. They are hydrophobic, recalcitrant and persistent due to their inert chemical nature [17], have low bioavailability and bioaccessibility [26], slow diffusion kinetics and high octanol-water partition coefficient ratio [31]. Biodegradation of PAHs is carried out *via* metabolic and co-metabolic pathways [23]. During aerobic metabolism, PAHs by enzymatic action changes to *cis*-dihydrodiols, the latter is transformed into dihydroxy-compounds. [1]. Catechol, gentisate, protocatechuate and phthalates are unique PAHs degradation intermediates that are subject to ring cleavage (*ortho* or *meta)* pathways [23]. The ensuing metabolites are then channeled into the tricarboxylic acid cycle (TCA).

Aerobic biodegradation of PAHs by bacteria involves dioxygenases which incorporate both atoms of oxygen molecule into the substrates. Dioxygenases are metalloenzymes with non-heme iron at its active site and categorised into ring-hydroxylating dioxygenases (RHDs) and ring cleaving dioxygenases (RCDs) [15]. The ring-hydroxylating dioxygenase acts on two adjacent carbons of a substrate while the ring-cleaving dioxygenase catalyses the opening of ring of catecholic compounds. Ring hydroxylating dioxygenases (RHDs) utilizes NAD(P)H to transfer one or two specific electrons to the iron sulphur protein (ISP) site for di-oxygen activation [19]. The oxygenase component (in most cases) is hexameric ($\alpha_3\beta_3$) where each alpha subunit contains a Rieske-type (2Fe-2S) cluster and a mononuclear iron-centre (where dioxygenase reaction takes place) [3].

Polyaromatic hydrocarbon ring hydroxylating dioxygenases (PAH-RHDα genes) are functional genes that code for the ring hydroxylating dioxygenase (RHD) enzymes responsible for the catalysis of PAH biodegradation under aerobic condition [39]. They are widely distributed among various taxa of Gram-negative and Gram-positive bacteria [33] and have been identified using polymerase chain reaction (PCR). Gram-negative RHD genes include the classical nah-like genes of *Pseudomonas* sp. strain G7 [13], *phd* genes of *Comamonastestosterone* strain GZ39 [29], *nag* genes of *Ralstonia* sp. strain U2 [30] and *phn* of *Burkholderia* sp. strain RP007 (Lu *et al*., 2011). Some examples of Gram-positive RHD genes previously identified include *nid* genes of *Mycobacteria* spp. and *Diaphorobacter* spp., *pdo* and *dfn*/*fln* genes of *Terrabacter* spp., *nar* genes of *Rhodococcus* sp. strain NCIMB12038 [43].

Detection of one or more of these catabolic genes from Gram-negative bacteria and/or Gram-positive bacteria by polymerase chain reaction (PCR) are subject to confirmation by clone library analysis; and when confirmed they serve as biomarkers for PAH degradation and detoxification [46]. Previous studies had demonstrated the correlation of PAH-RHD genes and PAHs contamination level (Cebron *et al*., 2010; [35]); investigated functional genes diversity in PAH-degrading bacteria (Lu *et al*., 2011) and had also been explored to understand the mechanisms of PAH degradation [39].

This study was carried out to detect broad range catabolic genes in Gram-positive bacteria and *nahAc* of Gram-negative bacteria from sites polluted by artisanal oil refining activities in the Niger Delta. Thus, the present investigation aimed at exploring the distribution of *nahAc* and PAH-RHDα-GP genes in bacteria isolated from crude oil polluted soils as a monitoring index to PAH degradation potentials.

Material and methods

Site description

The soil samples for this study were collected from an island (Ngia Ama) along Sombrero River. Ngia Ama (4.7947° N, 6.6831° E) is within the territory of Tombiain Degema Local Government Area of Rivers State where illegal refining of crude oil took place. The pollution (from artisanal oil refining activities) in this island was six months old with respect to the time of sampling. Ngia Ama is surrounded by creeks and mangroves with moderate low lands. The second sample was collected from Bomu (4.6340° N, 7. 3559°E) in Gokana Local Government Area of Rivers State close to Bodo-Bonny bridge, a site known well for oil bunkering and illegal oil refining, dating back to 2007.

Sample collection and physicochemical analysis

Four samples of soil were collected at each site of Bomu (PSB) and Ngia Ama (PSN) with the aid of a sterile hand trowel at a depth of 0–30 cm. These four sub-samples were combined and then thoroughly homogenized to obtain a uniform composite soil for each site, packed in a sterile polythene bags, transported to the laboratory within 6 h, preserved at 4 °C [25]. Forty eight hours later, the soil samples were analysed for pH, total petroleum hydrocarbons (TPHs) and polyaromatic hydrocarbon concentration (Σ16 PAHs) according to Sarkar *et al*. [36].

Isolation of PAHs-degrading bacteria

One gram of soil sample was added to 9 ml of Bushnell Haas (BH) broth supplemented with 1% mixed-PAH solution (0.3 g/l). An aliquot from 10^{-3} and 10^{-4} dilutions were spread on PAH-supplemented BH agar plate with 0.05 g/ml of nystatin (to suppress fungi growth) in triplicates and incubated at 30 °C for 7 days. Colonies with different morphologies were individually selected and sub-cultured by repeated streaking on the same media [27].

DNA extraction

DNA extraction was carried out on axenic cultures from PSN and PSB samples using Zymo Research (ZR) Fungi/Bacteria DNA MiniPrep™ (California, USA) supplied by Inqaba Biotec, South Africa following the manufacturer's instructions. In summary, 100 µl of 24 h old bacterial cells (re-suspended in 200 µl of isotonic buffer) was dispensed into a ZR BashingBead™ lysis tube. Lysis solution of 750 µl was added to the tube, secured in a bead beater with a 2 ml tube holder assembly and processed at 4 minutes. Zymo Research (ZR) BashingBead™ lysis tube was centrifuged at 10,000 x g for 1 minute. Supernatant (400 µl) was transferred to a Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuged at 7,000 × g for another 1 minute. Filtrate from the Collection Tube was added with 1,200 µl of DNA Binding Buffer and mixed to ensure homogeneity. A volume of 800 µl of the mixture was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000 × g for 1 minute. This step was repeated after discarding the flow through from the Collection Tube. A volume of 200 µl of DNA Pre-wash Buffer was transferred to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuged at 10,000 × g for 1 minute. The Zymo-Spin™ IIC was added with 500 µl of DNA Wash Buffer and spun for 1 minute at 10,000 × g. Finally, the Zymo-Spin™ IIC was transferred to a clean 1.5 ml micro-centrifuge and 100 µl of DNA Elution Buffer was directly added to the column matrix tube and centrifuged at 10,000 × g for 30 s. The DNA purity was ascertained with a NanoDrop ND-1000 UV–Vis Spectrophotometer (Waltham, USA). The genomic DNA was stored at −20 °C until the PCR was performed [34].

Plasmid extraction

Pure bacterial isolates were inoculated into Luria Bertani (LB) broth for 24 h. From the turbid broth, 600 µl were dispensed into 1.5 ml micro-centrifuge tube followed by addition of 100 µl of 7X Lysis Buffer and mixed through flicking of the tube up and down for 6 times. The homogenous mixture was added with 350 µl of cold Neutralization Buffer followed by thorough mixing and spun for 4 minutes at 11,000 × g. Supernatant of 900 µl was transferred into a Zymo-Spin™ IIN Column in a collection tube and spun for 30 s at 15,000 × g. The column was placed back to the same collection tube after discarding the flow through. About 200 µl of Endo-Wash Buffer were dispensed into the column and spun at 15,000 × g for 30 s. Plasmid wash buffer of 400 µl was added into the column, spun at 10,000 × g for 1 minute followed by transferring the column into 1.5 micro-centrifuge tube. About 30 µl of DNA elution buffer was added into the column matrix and left to stand for 1 minute at room temperature. The column and its content were spun for 30 seconds at 12,000 × g to elute the plasmids. Nanodrop was used to ascertain the purity of the plasmid using the DNA elution buffer as blank [34].

PCR amplification of the 16S rRNA genes

The 16S rRNA regions of the isolates were amplified using the 27F and 1492R primers on a thermal cycler for 35 cycles at a final volume of 50 µl. The PCR mix contained X2 Dream taq Master mix (DNTPs, taq polymerase and MgCl), primers (at a concentration of 0.4 M) and extracted DNA (template). The thermocycling parameters followed thus: initial denaturation at 95 °C for 5 minutes; denaturation at 95 °C for 30 s; annealing at 52 °C for 30 seconds; extension at 72 °C for 30 seconds with 35 cycles and final extension at 72 °C for 5 minutes before holding and cooling to 4 °C [14].

Dioxygenase activity assay and amplification of nahAc *and PAH-RHDα-GP genes by PCR*

PAH-degrading isolates were pre-grown on nutrient agar plates, indole crystals were sprinkled on the cell-grown plates and incubated for two days (Thenmozhi *et al* 2012). The genomic DNAs and plasmids from PSN and PSB samples were subjected to PCR analyses for the amplification of PAH-RHDα-GP and *nahAc* genes using specific primer sets for detection of functional genes in Gram-negative and Gram-positive bacteria. Primers: PAH-RHD-GPf (5′CGGCGCCGACAAYTTYGTNGG3′); PAH-RHD-GPr (5′GGGGAACACGGTGCCRTGDATRAA3′) and nahAcF (5′TGGCGATGAAGAACTTTTCC3′); nahAcR (5′AACGTACGCTGAACCGAGTC3′). The reaction mixture consisted of 5 µl, 0.2 µl each of the forward and reverse primers, 0.5 µl of cell lysate and 4.1 µl of distilled water in an Eppendorf tube (200 µl). The initial denaturation temperature was 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 55 °C for 1 minute and elongation at 72 °C for 1 minute. The final elongation was at 72 °C for 7 minutes. It was then cooled to 4 °C [14].

Agarose gel electrophoresis

The procedure for the agarose gel electrophoresis was as follows: 1% agarose solution was prepared using a microwave. The hot solution was cooled at about 60 °C, 0.5 µl of ethidium bromide was added and the solution was poured into a gel rack. The comb was introduced at one end side of the gel. The comb was removed carefully and the gel was formed. The latter is put in an electric field. TAE buffer (a combination of Tris base, acetic acid and EDTA at a pH of 8) was added to the electric field until the gel is covered.Molecular weight markers (DNA ladder) including the samples were mixed with TBE (tris borate EDTA) solution and loaded on the gel afterward the electric field was put on with a voltage of 120 V for 20 minutes. The DNAs moved towards the anode as a result of its negative charges. Afterward, the electric field was turned off

Table 1
Soil physicochemical characteristics and bacterial count of Bomu (PSB) and Ngia Ama (PSN).

Physicochemical parameter	Unit	PSB sample	PSN sample
Texture		Grainy	Smooth
Colour		Dark	Light brown
pH	–	4.5	6.86
Total petroleum hydrocarbons (TPHs)	mg/kg	858 (C_9)	2,067.72
Polyaromatic hydrocarbons (PAHs)	ppm	Total: 5.84	Σ16PAH: 193
Bacterial counts THB	CFU/g	7.1×10^9	2.2×10^7
HUB	CFU/g	1.9×10^6	3.6×10^5
PDB	CFU/g	4.6×10^5	4.9×10^4

when the blue dye bands had crossed two-third of the gel. Later on the gel was washed in deionized water and illuminated with a ultraviolet lamp to view the formed DNA bands [8,44].

Small subunit (SSU) ribosomal (16S rRNA) Gene Sequencing and Phylogenetic Analyses

The PCRamplicons of the isolates from PSB and PSN samples were sequenced using a 3500 genetic analyzer. Bioedit algorithm was used to edit the resulting 16S rRNA sequences. BlastN was used to download similar sequences from the database of National Center for Biotechnology Information (NCBI) to determine similarity between sequences and percentage similarities between matches [28]. ClustalW was employed to align the sequences. Neighbor-joining evolutionary tree [40] was generated using MEGA 6. The tree was bootstrapped 1000 times [10]. The evolutionary distance was computed by Jukes-Cantor method [20] which is in the units of the number of base substitutions per site.

Nucleotide sequence accession numbers

The draft nucleotide sequences described in this study have been submitted to GenBank under accession numbers KX754444 to KX7554459.

Results and discussion

Physicochemical and microbiological analysis

The values of the physicochemical (pH, total petroleum hydrocarbon and polyaromatic hydrocarbon) and bacterial count of the sample PSB and PSN are all shown in Table 1. All the physicochemical characteristics of PSB are appreciably lower in concentration and in value when compared to sample PSN (4.5 against 6.86 for pH, 858 ppm against 2,067 ppm for total petroleum hydrocarbons and 5.84 ppm against 193 ppm for polyaromatic hydrocarbons). Bacterial count of total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB) and PAH-degrading bacteria (PDB) of sample the PSB are higher than the PSN sample (7.1×10^9 CFU/g against 2.2×10^7CFU/g for THB, 1.9×10^6 CFU/g against 3.6×10^5 CFU/g for HUB and 4.6×10^6 CFU/g against 4.9×10^4 CFU/g for PDB).

The low pH (4.5) reflects low physiological activity for bacterial biodegradation in Bomu study site. At this pH, fungi metabolic activity is usually more pronounced than bacteria (Sihag *et al*., 2014). The low concentration of total petroleum hydrocarbons (TPHs) and PAHs might be responsible for the high count of total heterotrophic bacteria (THBs) (10^9 CFU/g) due to reduced toxicity. However, hydrocarbon utilizing bacteria (HUB) count and polyaromatic hydrocarbon degrading bacteria (PDB) count fell below the value of the THB in the order of 10^4 and 10^3respectively. This implies that hydrocarbon degrading bacterial community exists in the midst of improved biodiversity (Alrumman *et al*., 2015). Comparatively, the values of the physicochemical characteristics of Ngia Ama (PSN) are higher than that of the PSB. The pH (6.8) value suggests optimal bacteria metabolism [7]. The HUBs and PDBs threshold counts indicate that a good number of the THBs had developed competence to degrade PAHs (Petrovic *et al*., 2008).

Small subunit (SSU) ribosomal gene sequencing and phylogenetic analyses

The megablast search for 16S rRNA sequence similarity gave an exact match from the National Centre for Biotechnology Information (NCBI) database. The least percentage similarity was shown to be 99% with respect to other genera. The computed evolutionary distances were in harmony with the 16S rRNA phylogenetic placement of the isolates within the genera and revealed a high similarity to the genus than other genera within. Table 2 shows the phylogenetic identity of PDB. The gel electrophoresis and the phylogenetic trees of bacterial isolates from Ngia Ama and Bomu are illustrated in Figs. 1, 2 and 3 respectively. The results are based on comparison of SSU rRNA gene sequences of the isolates to the sequence that shows the highest sequence similarity to the isolate.

Table 2
Molecular identification and PCR amplification of functional genes of PAH-degrading bacteria from soil collected at artisanal refining sites in the Niger Delta.

Isolate	Accession no.	Closest related strain	Similarity (%)	*nahAc*		PAH-RHDα-GP	
	Isolates from	Bomu (PSB)		C	P	C	P
FCC_01	KX754444	*Enterobacter xiangfangensis* strain 9A	100	+ +		–	+
FCC_02	KX754445	*Shewanellahaliotis* strain 0315	100	– –		–	+
FCC_03	KX754446	*Pseudomonasdenitrificans* strain Y-16	100	+ +		–	+
FCC_04	KX754447	**Burkholderiaterrestris* strain R-233,321	99	–	–	–	+
B5	NA	^Firmicute bacteria	90	+ +		–	+
	@Isolate from	Ngia Ama (PSN)					
FCC_06	KX754448	*Acinetobacter calcoaceticus strain N7*	100	+	–		
FCC_09	KX754449	*Acinetobacter pittii strain AP_882*	100	+	–		
FCC_11	KX754450	*Pseudomonasnitroreducens* strain VITWW2	99	+	–		
FCC_12	KX754451	*Pseudomonasotitidis* strain IND2	100	+	–		
FCC_13	KX754452	*Stenotrophomonasmaltophilia* strain C_	100	+	–		
FCC_15	KX754453	*Pseudomonasaeruginosa* SJTD-1	100	+	–		
B16	NA	Blast not conducted	NA	–	–		
FCC_37	KX754454	*Pseudomonasfluorescens* strain KRST 01	100	+	–		
FCC_38	KX754455	*Pseudomonasaeruginosa* SJTD-1	100	+	–		
FCC_39	KX754456	*Pseudomonasaeruginosa* SJTD-1	100	+	–		
FCC_40	KX754457	*Pseudomonasfluorescens* strain KRST 01	100	+	–		
FCC_41	KX754458	*Exiguobacterium alkaliphilum strain 12/1*	99	+	–		
FCC_42	KX754459	*Pseudomonasfluorescens* strain KRST 01	100	+	–		
B43	MF375210	*Acinetobacterbaumannii* strain HNHB02	100	+	–		
B44	MF375211	*Acinetobactercalcoaceticus* strain N7	100	+	–		
B45	NA	Blast not conducted	NA	–	–		

+ Detected; - Undetected; *Now *Caballeronia terrestris;* ^Sequence bp was two short hence identified with biochemical characterisation as *Bacillus*; @ Plasmids of isolates from Ngia Ama were not amplified.
C: chromosomes; P: plasmids.

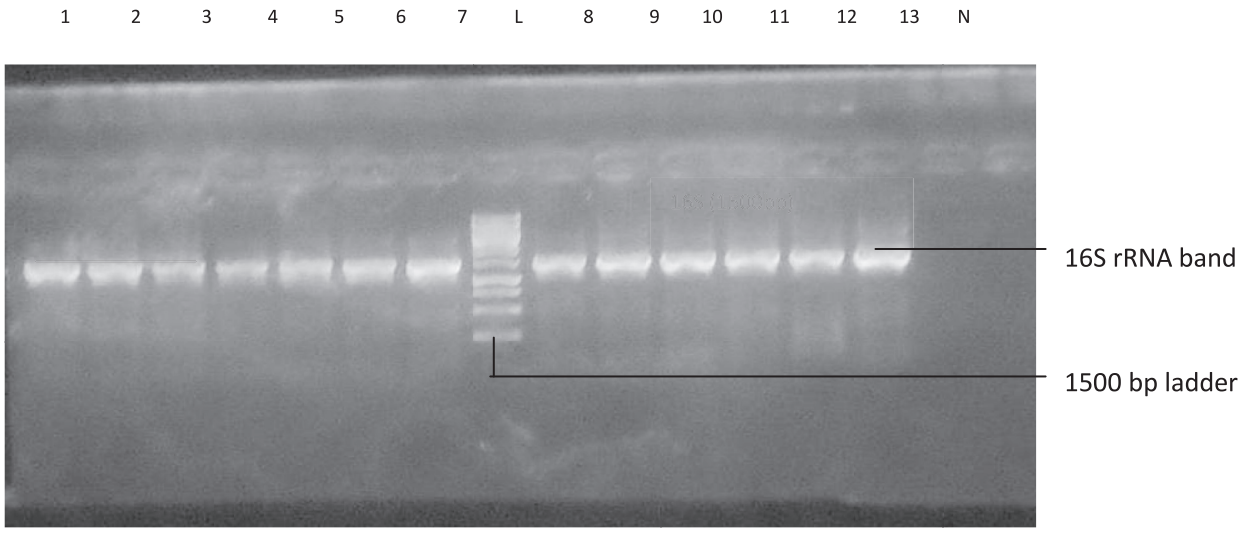

Fig. 1. Agarose gel electrophoresis of 16S band (1500 bp) of some selected isolates.
*L: represents 1 kb ladder, lane 1–12 represent the 16S gene bands of the isolates while N represented the negative control.

Five bacterial genera, identified as PAH-degrading bacteria spread across *gamma*-protobacteria (*Enterobacter, Shewanella* and *Pseudomonas*), *beta*-protobacteria (*Caballeronia*) and a Firmicute, were found in Bomu polluted (PSB) site. At Ngia Ama (PSN) site 14 isolates were identified as 4 genera (8 *Pseudomonas*, 3 *Acinetobacter*, 1 *Stenotrophomonas* and 1 *Exigubacterium*) all belonging to *gamma*-protobacteria (and are all Gram-negative). Distribution of PAH-degrading bacteria in these two sites shows diversity shift (at Ngia Ama site due to fresh hydrocarbon pollution) and resilience (at Bomu site due to hydrocarbon attenuation) [7,37]. Studies carried out in the Niger Delta had shown that soil bacteria are much more diverse as reflected in

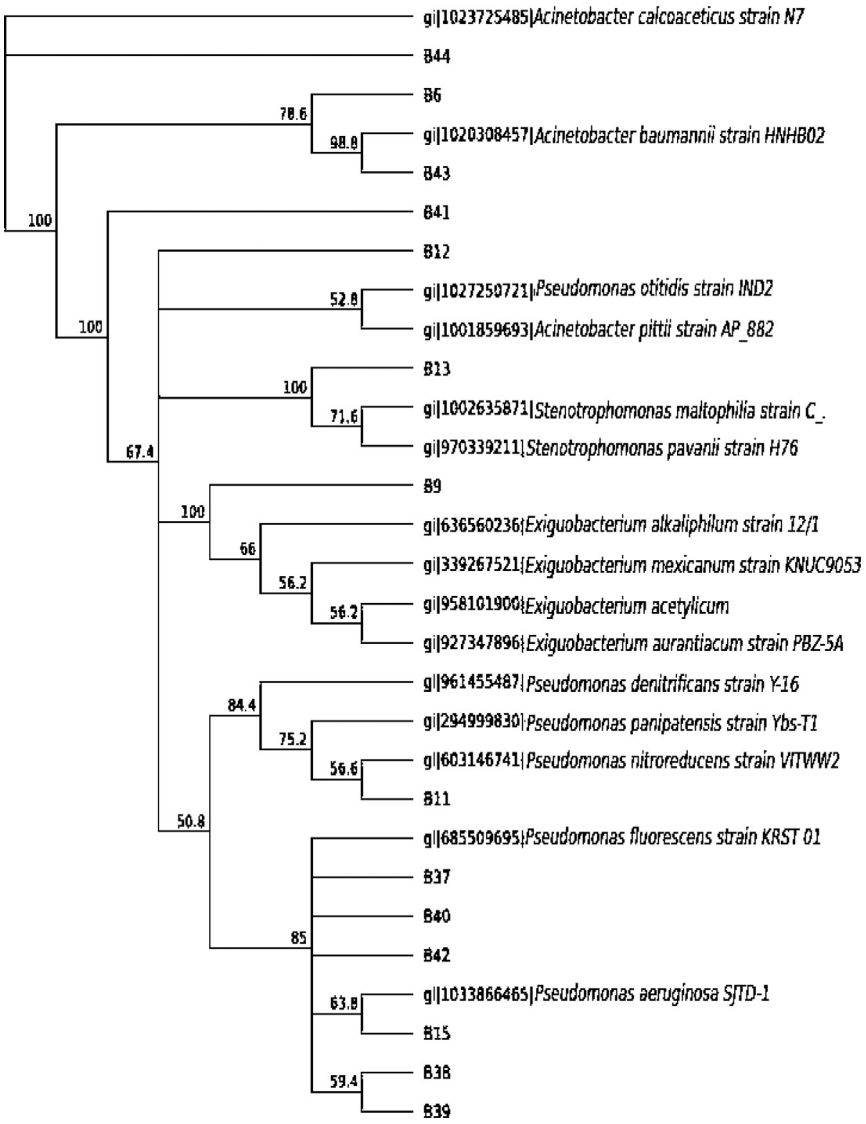

Fig. 2. Phylogenetic analysis of 16S rRNA gene of isolates from Ngia Ama (PSN) and other related bacterial species by neighbor-joining method. Numbers at the nodes indicate bootstrap support (%) based on 1000 replicates.

the Bomu site ([5,6]; Esedafe *et al*., 2015;Stanley *et al*., 2015). The improving microbial diversity at Bomu site is an indication that the toxic effect is reduced and as the attenuation trend continues more genera will be found at the site.

The diversity shift experienced at Ngia Ama corroborates the study carried out by Fuentes *et al*. (2015). They ascribed the *gamma*-protobacteria bloom to the on-set of hydrocarbon impact to the ecological concept: "conditionally rare taxa" which means rareness is a temporary state conditioned by environmental stressors. Possible reason for the *gamma* shift is due to extermination of the sensitive community members leaving behind the *gamma*-protobacteria that can tolerate the toxic effect of the hydrocarbon pollutants. Van Hamme *et al.* [41] had noted that Gram-negative bacteria has membrane architecture that counter intercalation of hydrocarbons that could cause changes in protein conformation and fluidity with the ultimate consequences of altering membrane-bound enzyme activities and disruption of the barrier and energy transduction roles. They also possess porins which help in the selective uptake of substances by the cell and extrusion of others which may be harmful (Eze *et al*., 2013). Further, they display physiological responses that makes them insensitive to toxic effects, access insoluble hydrocarbons, or transfer large substances into the cytoplasm through biochemical mechanisms such as alteration of cell surface hydrophobicity, gaining of protection from hydrophilic lipopolysaccharide constituents and or using of repair mechanisms to compensate for losses in membrane as a result of lipophilic compound intercalation (Lazaroaie, 2010; [41]).

Phylogenetic trees were used to infer the evolutionary history of the characterised PAH-degrading bacteria (Figs. 2 and 3) originating from Bomu and Ngia Ama polluted sites respectively. In tune with the result of the molecular characterization

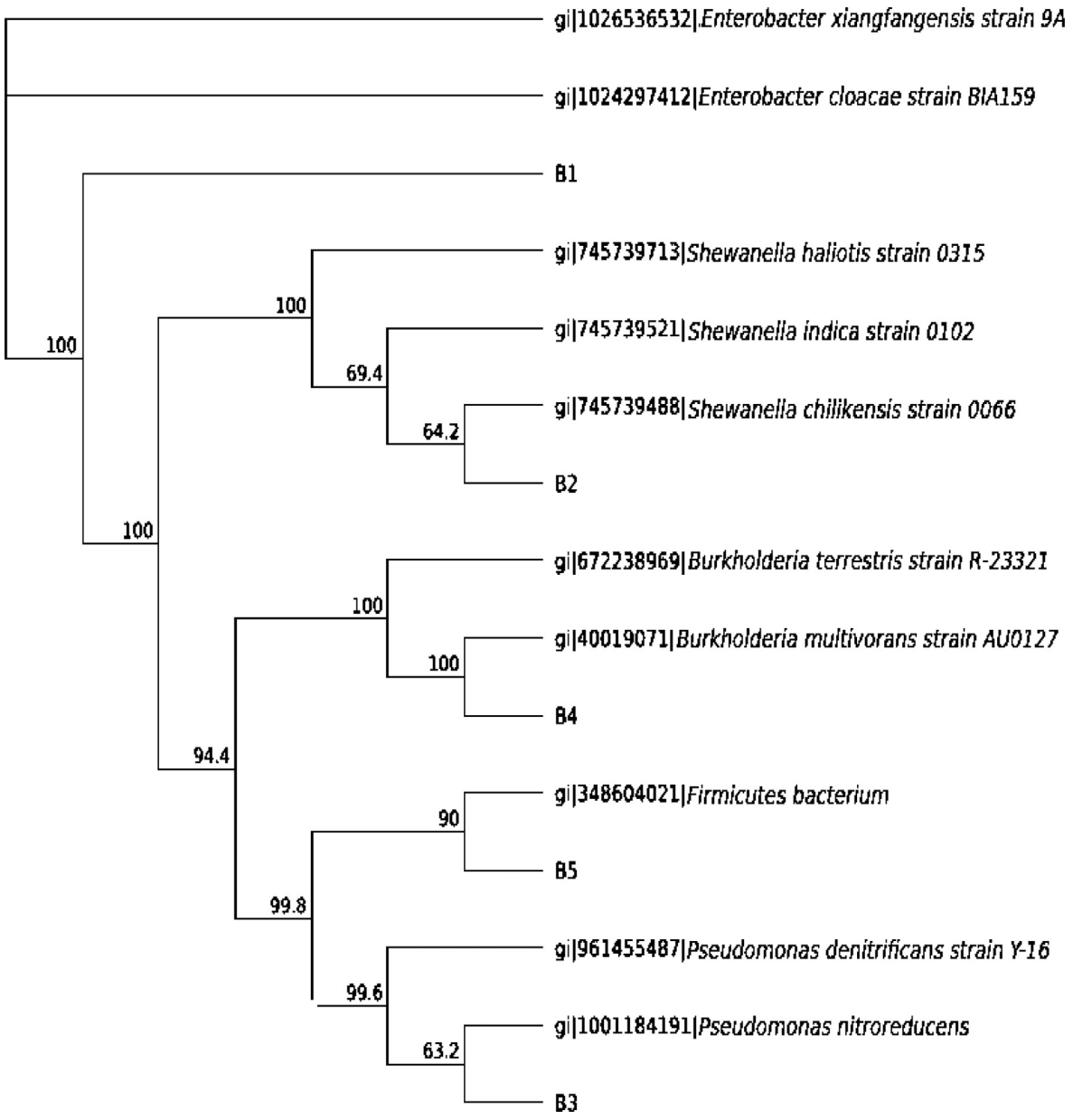

Fig. 3. Phylogenetic analysis of 16S rRNA gene of isolates from Bomu (PSB) and other related bacterial species by neighbor-joining method. Numbers at the nodes indicate bootstrap support (%) based on 1000 replicates.

two or more isolates share same phylogenetic identity in the illustrated tree (Fig. 3) despite their morphological differences-the criteria use for their isolation. For instance isolates B37, B40 and B42 share same identity as *Pseudomonas fluorescence*. This phenotypic innovation [17] observed in the chemically challenged habitat could be as a response mechanism to environmental stress and/or for niche adaptation [38,45].

Dioxygenase activity assay and amplification of nahAc *and PAH-RHDα-GP genes*

The colour of the indole plate changes to purple (Fig. 4) which confirms dioxygenase activity which in turn confirms functional gene expression in the PAH-degrading bacteria. Polymerase chain reaction (PCR) amplification was used to detect *nahAc* and PAH-RHDα-GPgenes in all the 21 bacterial isolates and 5 plasmids from Bomu isolates. The gel images of the amplified *nahAc* and PAH-RHDα-GPgenes are illustrated in Figs. 5 and 6. As shown in Table 2, *nahAc* gene was not detected in the chromosomes and plasmids of isolate B2 (*Shewanellahaliotis* strain FCC_02) and B4 (*Caballeronia terrestris*FCC_04). Also, *nahAc* gene was not detected in the chromosomal DNA of isolate B16 and B45. The PAH-RHDα-GPgene was detected

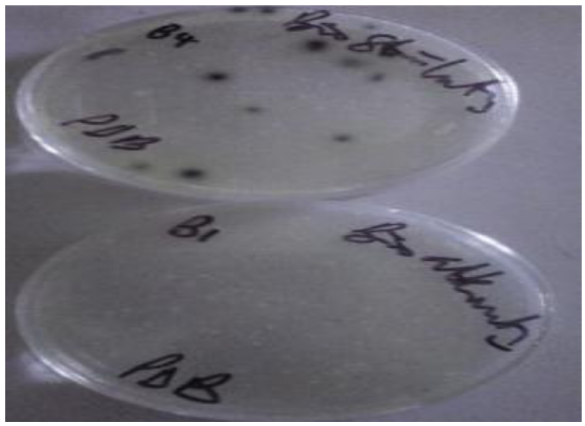

Fig. 4. Indigo production screening for tentative PAH degraders identification using indole as a substrate.

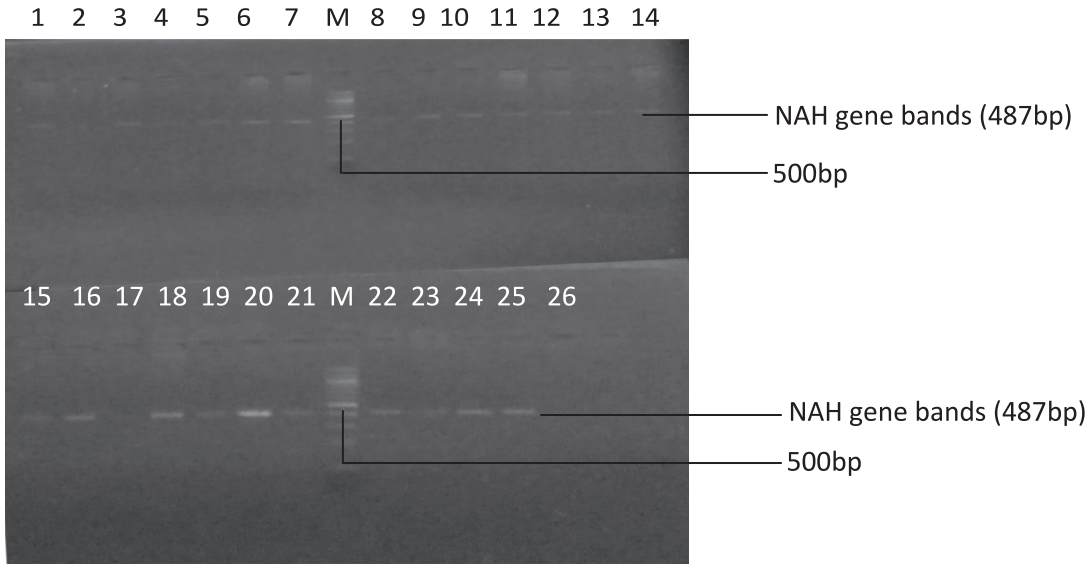

Fig. 5. Agarose gel electrophoresis of *NAH* gene from both plasmid and chromosomal DNA of isolates.
*Lanes 1 –7 and 8–26 represent the bands from Isolates while lane M represents the 500 bp molecular ladder.

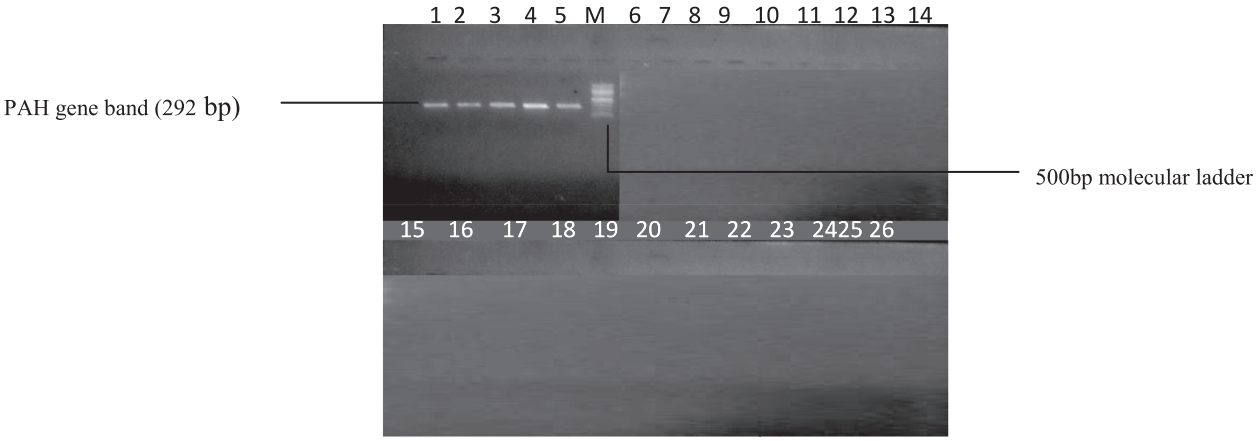

Fig. 6. Agarose gel electrophoresis of the PAH gene from both plasmid and chromosomal DNA of isolates
*Isolates 1–5 are showing PAH gene of band size of ≈292. Lane M represent 500 bp molecular ladder.

in all the plasmids of isolates from Bomu study site. The sizes of the PCR products for *nahAc* and PAH-RHDα-GPare 487 bp and 292 bp respectively.

The positive test to the dioxygenase activity assay and the amplification of *nahAc* and PAH-RHDα-GPgenes indicate the potential ability of the isolated genera to degrade PAHs. The later induces the expressions of these functional genes [17] to activate the initial attack of PAHs degradation to form *cis*-dihydrodiols [13]. *nahAc* is a subset of PAH-RHDα-GN genes (Cebron *et al*., 2008) akin to *Pseudomonasputida* strain G7 and NCIB9816 [11]. Identification of PAH-RHDα-GN genes have been applied in PAH-degrading genera such as *Pseudomonas, Burkholderia, Comamonas* and *Ralstonia*/*Polaromonas* due to their possession of PAH functional gene markers- *nahAc, phnAc, pahAc* and *nagAc* respectively (Cebron *et al*., 2008). Same way PAH-RHDα-GP gene alleles (*narAa, phdA*/pdoA2, *nidA*/*pdoA1, nidA3*/*fadA1*) have been used to identify high molecular weight PAH-degrading genera such as *Rhodococcus, Mycobacterium, Nocardioides* and *Terrabacter* genera (Cebron *et al*., 2008). Though most Gram-positive bacteria have at least two PAH-dioxygenase genes (Zhou *et al*., 2006) the most common among them are *nidA* and *nidA3* of *Mycobacterium* [13]. The phylogenetic analyses conducted by Wu *et al*. [42] showed that all PAH-RHD gene sequences of Gram-negative bacteria were closely related to the *nahAc* gene for *Pseudomonas* sp. 9816–4, while the PAH-RHD gene sequences of Gram-positive bacteria showed high sequence similarity to the *nidA* gene from *Mycobacterium* spp.

Isaac *et al*. [16] studied *nahAc* gene expression alongside with naphthalene dioxygenase activity based on indigo oxidation by PAH-degrading *Pseudomonas* spp. In addition to complete degradation of naphthalene, they found that phenanthrene greatly induced *Pseudomonas* strain P6 to express *nahAc* while pyrene induces *nahAc* in *Pseudomonsstutzeri* N3. Jurelevicius *et al*. [21] used PCR amplification and cloning methods and found classical nah-like genes in Antarctic soil even in pristine soil but in very low number. Detection of PAH-degrading bacteria in pristine soil is a common success in the Niger Delta due to the pervasive nature of hydrocarbon pollution [7]. The genera identified (in this study) to have possessed the PAH-RHD genes fell between two genera- proteobacteria and Firmicutes as it was found in Zafra *et al*. [49]. Just as it was shown in the study of Zafra *et al*. [49] proteobacteria dominated the hydrocarbon stressed habitat of Bomu. Our result also showed that *gamma*-proteobacteria dominated in both sites which is in conformity with Wu *et al*. [42] study.

The culture-dependent method used in this study showed a snap shot (Fig. 3) of Gram-negative bacteria within the class of *gamma*-proteobacteria. They exclusively dominated the freshly polluted site (Ngia Ama) with *r*-strategist predominance. Leys *et al*. [24] had suggested that, following contamination by PAHs, the initial degradation could be mainly performed by Gram-negative r-strategists. This hypothesis supported the outcome of our study and those of others like Cebron *et al*. (2008), Liu *et al*. [25] and Yergeau *et al*. [47]. This *gamma* bloom indicates the selection of the community members able to degrade hydrocarbons components or their degradation products [12]. PAH degradation, in the presence of biodegraders, can be indicated by the ratio of PAH-RHDα genes copy number to 16S rRNA genes copy number (Cebron *et al*., 2008). The latter authors by using real-time PCR was able to get a ratio of 0.01 which positively correlated with the PAH-bacterial biodegradation potential. Johnsen *et al*. [18] detected PAH-RHD genes copy number similar to that of Cebron *et al*. (2008) with a PAH-degrading cell mass of 4.6×10^3 to 3.6×10^5 CFU/g which is lower than our result (4.9×10^4 to 4.6×10^5). So by extrapolation, our study can detect 4.4×10^4 to 4.7×10^7 PAH-RHDα-GP gene copies g^{-1} soil [18] and 3.9×10^5 to 1.0×10^7 PAH-RHDα-GN gene copies g^{-1} soil (Cebron *et al*., 2008) even if we set our detection limit around 10^4 and 10^5 cells g^{-1} of soil according to Nicolaisen *et al*. [32].

The distribution of *nahAc* and PAH-RHDα-GPgenes among the PAH-degrading bacteria suggests the influence of horizontal gene transfer (HGT) as genes conserved for Gram-negative bacteria are found in Gram-positive bacteria. A Firmicute bacteria (*Bacillus*?) was found to harbor *nahAc* gene which is a conserved gene for Gram-negative bacteria and designed with high sequence identity from *Pseudomonasputida* G7 [2]. Also.

Both petri dishes had growths of PAH degrading bacteria. The top petri dish was sprinkled with indole and produced purple colour after 24 hours while the down plate served as control without indole.

PAH-RHDα-GP which is a conserved gene for Gram-positive bacteria was found in the plasmids of Gram-negative bacteria. Van Hamme *et al*. [41] noted that the presence of transferred genes in both chromosomes and plasmid in a given organism, as observed in this study with reference to *nahAc*, could be as a result of recombination and reintegration. The most common mechanisms of horizontal gene transfer (HGT) are transformation, transduction, conjugation [4]. The presence of PAH-RHDα-GPgene only in the plasmid suggested that it could possibly be plasmid bound [13]. It is also pertinent to suggest that the distribution of PAH-RHDα-GPgenes is influenced by age given that selective pressure is behind its lateral distribution. These assumptions call for furthering studies using culture independent approaches.

Conclusion

In this study, we identified the distribution of the polyaromatic ring hydroxylating dioxygenase genes (*nahAc* and PAH-RHDα-GP) amongst bacteria isolates obtained from two sites in the Niger Delta used for artisanal refining activities. The isolates were characterized by culture dependent molecular approach while the PAH-RHD genes were detected using PCR technique. The *nahAc* and PAH-RHDα-GP genes were found in both Gram positive and Gram negative bacterial isolates. The *nahAc* genes showed to be plasmid and chromosome bound while PAH-RHDα-GP was plasmid bound. The trend of the functional genes distribution inferred horizontal gene transfer in the hydrocarbon stressed habitat. Also, the presence of these PAH-catabolic genes in the study area is a proof of *insitu* biodegradation of polyaromatic hydrocarbons. Further studies

are needed to decipher if PAH-RHDα-GP is purely plasmid borne or it is influenced by age if selective pressure influences its lateral transfer as found in this study.

Conflict of interest

Authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2018.e00003.

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