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

Chioma Blaise Chikere, Emmanuel Oliver Fenibo

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-RHDe-GP) amongst bacteria isolated from two disparate locations (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-RHDe-GP genes 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-RHDe-GP genes 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.

© 2018 The Authors. Published by Elsevier B.V. on behalf of African Institute of Mathematical Sciences / Next Einstein Initiative. This is an open access article under the CC BY license. (http://creativecommons.org/licenses/by/4.0/)

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.
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 (α3β3) 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-RHDA 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 Commonastestosterone strain GZ39 [29], nag genes ofRalstonia sp. strain U2 [30] and phn ofBurkholderia sp. strain RP007 (Lu et al., 2011). Some examples of Gram-positive RHD genes previously identified include nad genes of Mycobacteria spp. and Diaphorobacter spp., pdo and dfn/fln genes of Terrabacter spp., nar genes ofRhodococcus 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-RHDA-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 Tombiai 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 (Σ 16 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 isotoninc 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 × 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. Filterate 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 a 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-RDox-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-RDox-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’CGGCGCGCAAAYTGTNGG3’); PAH-RHD-GPr (5’GGGAAACACGCTGCRTGDAATRAA3’) and nahAcF (5’TGGCGATGAAAGACTTITCC3’); nahAcR (5’AACCTACCGTAAACCGAGTC3’). 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 120V for 20 minutes. The DNAs moved towards the anode as a result of its negative charges. Afterward, the electric field was turned off
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 × 10^7 CFU/g for THB, 1.9 × 10^6 CFU/g against 3.6 × 10^3 CFU/g for HUB and 4.6 × 10^5 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 10^3 respectively. This implies that hydrocarbon degrading bacterial community exists in the midst of improved biodiversity (Afrmman 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>
<thead>
<tr>
<th>Physicochemical parameter</th>
<th>Unit</th>
<th>PSB sample</th>
<th>PSN sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td></td>
<td>Grainy</td>
<td>Smooth</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
<td>Dark</td>
<td>Light brown</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>4.5</td>
<td>6.86</td>
</tr>
<tr>
<td>Total petroleum hydrocarbons (TPHs)</td>
<td>mg/kg</td>
<td>858 (C₄)</td>
<td>2,067.72</td>
</tr>
<tr>
<td>Polyaromatic hydrocarbons (PAHs)</td>
<td>ppm</td>
<td>Total: 5.84</td>
<td>Σ16PAH: 193</td>
</tr>
<tr>
<td>Bacterial counts THB</td>
<td>CFU/g</td>
<td>7.1 × 10^9</td>
<td>2.2 × 10^7</td>
</tr>
<tr>
<td>HUB</td>
<td>CFU/g</td>
<td>1.9 × 10^6</td>
<td>3.6 × 10^3</td>
</tr>
<tr>
<td>PDB</td>
<td>CFU/g</td>
<td>4.6 × 10^5</td>
<td>4.9 × 10^4</td>
</tr>
</tbody>
</table>
Table 2
Molecular identification and PCR amplification of functional genes of PAH-degrading bacteria from soil collected at artisinal refining sites in the Niger Delta.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Accession no.</th>
<th>Closest related strain</th>
<th>Similarity (%)</th>
<th>nahAc</th>
<th>PAH-RHΔx-GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCC_01</td>
<td>KX754444</td>
<td>Enterobacter xiangfangensis strain 9A</td>
<td>100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FCC_02</td>
<td>KX754445</td>
<td>Shewanella halialai strain 0315</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FCC_03</td>
<td>KX754446</td>
<td>Pseudomonas denitrificans strain Y-16</td>
<td>100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FCC_04</td>
<td>KX754447</td>
<td>*Burkholderia terrestris strain R-233,321</td>
<td>99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>NA</td>
<td>*Firmicute bacteria</td>
<td>90</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Isolate from Ngia Ama (PSN)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Accession no.</th>
<th>Closest related strain</th>
<th>Similarity (%)</th>
<th>nahAc</th>
<th>PAH-RHΔx-GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCC_06</td>
<td>KX754448</td>
<td>Acinetobacter calcoaceticus strain N7</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_09</td>
<td>KX754449</td>
<td>Acinetobacter pittii strain AP_882</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_11</td>
<td>KX754450</td>
<td>Pseudomonas nitroreducens strain VITWW2</td>
<td>99</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_12</td>
<td>KX754451</td>
<td>Pseudomonas stotitidis strain IND2</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_13</td>
<td>KX754452</td>
<td>Stenotrophomonas maltophilia strain C_</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_15</td>
<td>KX754453</td>
<td>Pseudomonas aeruginosa SJTD-1</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B16</td>
<td>NA</td>
<td>Blast not conducted</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FCC_37</td>
<td>KX754454</td>
<td>Pseudomonas fluorescens strain KRST 01</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_38</td>
<td>KX754455</td>
<td>Pseudomonas aeruginosa SJTD-1</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_39</td>
<td>KX754456</td>
<td>Pseudomonas aeruginosa SJTD-1</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_40</td>
<td>KX754457</td>
<td>Pseudomonas fluorescens strain KRST 01</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_41</td>
<td>KX754458</td>
<td>Exiguobacterium alcaliphilum strain 12/1</td>
<td>99</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FCC_42</td>
<td>KX754459</td>
<td>Pseudomonas fluorescens strain KRST 01</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B43</td>
<td>MF375210</td>
<td>Acinetobacter baumannii strain HNHB02</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B44</td>
<td>MF375211</td>
<td>Acinetobacter calcoaceticus strain N7</td>
<td>100</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B45</td>
<td>NA</td>
<td>Blast not conducted</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Detected; - Undetected; *New Caballersona terrestris; †Sequence bp was too short hence identified with biochemical characterisation as Bacillus; ‡Plasmids of isolates from Ngia Ama were not amplified.
C: chromosomes; P: plasmids.

![Image of agarose gel electrophoresis](image_url)

Fig. 1. Agarose gel electrophoresis of 16S band (1500bp) 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 (Caballersona) 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 Exiguobacterium) 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 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 onset 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
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 fluorescens. 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-GP genes in all the 21 bacterial isolates and 5 plasmids from Bomu isolates. The gel images of the amplified nahAc and PAH-RHDα-GP genes 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 (Shewanella halotis 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-GP gene 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 500bp molecular ladder.*
in all the plasmids of isolates from Bomu study site. The sizes of the PCR products for nahAc and PAH-RHΔc-GP are 487 bp and 292 bp respectively.

The positive test to the dioxygenase activity assay and the amplification of nahAc and PAH-RHΔc-GP genes 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-RHΔc-GN genes (Cebron et al., 2008) akin to Pseudomonas sp. strain G7 and NCIB9816 [11]. Identification of PAH-RHΔc-GN genes have been applied in PAH-degrading genera such as Pseudomonas, Burkholderia, Comamonas andRalstonia/Polaromonas due to their possession of PAH functional gene markers nahAc, phnAc, pahAc and nagAc respectively (Cebron et al., 2008). Same way PAH-RHΔc-GP gene alleles (nahAa, phdA/pddA2, nidA/pdA1, nidA3/pdA1) 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-dioxynase 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 genes 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 Pseudomonas stutzeri N3. Jurelevecius 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 Vergeau 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-RHΔc genes copy number to 16S RNA 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-RHΔc genes copy number similar to that of Cebron et al. (2008) with a PAH-degrading cell mass of 4.6 × 10^7 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-RHΔc-GP gene copies g⁻¹ soil [18] and 3.9 × 10^5 to 1.0 × 10^7 PAH-RHΔc–GN gene copies g⁻¹ soil (Cebron et al., 2008) even if we set our detection limit around 10^5 and 10^3 cells g⁻¹ of soil according to Nicolaïsen et al. [32].

The distribution of nahAc and PAH-RHΔc–GP genes 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 Pseudomonas putida 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-RHΔc–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-RHΔc–GP gene only in the plasmid suggested that it could possibly be plasmid bound [13]. It is also pertinent to suggest that the distribution of PAH-RHΔc–GP genes 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-RHΔc–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-RHΔc–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-RHΔc–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-RHDo-GP is purely plasmid borne or it is influenced by age if selective pressure increases 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.

References


