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Isolation of Hydrocarbonoclastic Bacteria and Plasmid Analysis for *alk* B Gene Primers

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Authors' contributions

This work was carried out in collaboration between all authors. All the authors designed the study. Authors UOE and SPA wrote the protocol and wrote the first draft of the manuscript. Author UOE managed the analyses of the study. Authors UOE and SPA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Hydrocarbonoclastic microorganisms elaborate a number of hydrocarbons utilising genes that enable them to use crude oil hydrocarbons as carbon sources. These genes could either be located on the plasmid or chromosome. The primary aim of this study was, therefore, to isolate hydrocarbon utilising microbes and profile their plasmid for alkB gene. The physicochemical, microbiological and plasmid analyses were done using standard methods described previously. Plasmid profiling for the alkB gene was carried with four selected bacteria isolates using the universal degenerate primers *Rh* alkB1-F: ATCTGGGCGCGTTGGGATTTGAGCG, *Rh* alkB1-R: CGCATGGTGATCGCTGTGCCGCTGC and *Pp* alkBP-F: TGGCCGGCT ACTCCGATGATCGGAATCTGG, *Pp* alkBP-R: GCGTGGTGATCCGAGTGCCGCTGAAGGTG. Physicochemical analysis revealed anthropogenic influence on the environment as iron and copper levels were higher than permissible international levels. Aerobic counts for bacteria were higher

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than those of fungi with values that ranged from 70 to 92 ($x10^6$) CFU/g for bacteria and 14 to 19 ($x10^3$) CFU/g for fungi. Microbiological and biochemical characterisation revealed that the hydrocarbonoclastic bacterial isolates were *Enterobacter sp, Bacillus sp, Micrococcus sp, Pseudomonas sp, Corynebacterium sp* and *Klebsiella sp* while the fungal isolates were *Penicillium sp, Aspergillus flavus, Fusarium sp, Rhizopus sp and Aspergillus sp*. Molecular characterisation revealed that the selected isolates for plasmid profiling were *Bacillus thuringiensis, Pseudomonas stutzeri, Bacillus cereus* and *Klebsiella pneumoniae*. Plasmid profiling revealed that none of the isolates were positive for the monoxygenase (*alk* B) genes. The findings in this study support earlier findings that indicated that the chromosome could indeed a preferred location for domiciliation of functional genes.

Keywords: Plasmid; alk B1 gene; crude oil; hydrocarbon utilizing isolates; Niger Delta Region of Nigeria.

1. INTRODUCTION

Crude oil is a complex mixture of organic and inorganic compounds, and these include aliphatic and aromatic hydrocarbons [1]. Bacteria are usually the dominant degraders in aquatic ecosystems and can utilise the most recalcitrant petroleum hydrocarbons components [2-3]. This degradation is possible because of the enzymes they elaborate with which they degrade these pollutants or xenobiotics [4,5,6]. One of such enzymes is the alkane monooxygenase (alk) genes [7]. The degradation of many xenobiotics and hydrocarbon compounds such as a polycyclic aromatic hydrocarbon is known to be mediated by plasmid-encoded enzymes [7-9].

Plasmids are mobile genetic elements capable of independent replication from host chromosome. Furthermore, they play prominent roles in prokaryote evolution and can be transferred vertically and horizontally to other prokaryotes [7-8]. There is a proposition that the development of the resistant/degradative population in a polluted site can lead to gene transfers via transposons, plasmids and possibly spontaneous mutants [10].

Their host cell for its survival does not usually require plasmids. Instead, they carry genes that confer a selective/specific advantage on their host, such as resistance to heavy metals, toxic substances, antibiotic or resistance to naturally made antibiotics by other organisms [10-11]. Many bacterial strains have genetic determinants of resistance/degradative abilities to pollutants. These determinants are often found on plasmids, chromosomes and transposons. These degradative abilities occur in high frequencies with much greater quantitative prominence after pollution [12]. Loss of plasmid does not lead to loss of degradative ability [13]. Furthermore, due to their stable inheritance, chromosomes are favoured location for functioning essential genes [14]. The primary aim of this study was, therefore, to carry out plasmid analysis of crude oil degrading isolates for *alk* B1 from *Rhodococcus* species and *Pseudomonas putida*.

2. METHODOLOGY

2.1 Sampling Location and Collection of Samples

The site for sampling was Emereoke II (Ward 5) community of Eastern Obolo Local Government Area of Akwa Ibom State, Nigeria, an oil producing community in the Niger Delta region. It is located on co-ordinates 4°32′0″N and 7°42′0″E. Sediment samples were collected in triplicates and made into composite samples as previously described [15].

2.2 Physicochemical Characterisation

This was carried out as previously described [16-17]. The parameters examined are as reported in Table 1.

2.3 Total Heterotrophic Bacteria and Fungi Counts

These were done as previously described using pour plate method [2,18-20].

2.4 Screening for Hydrocarbon Utilizing Bacteria

This was carried out using the pour plate method [18]. Briefly, 10 g of the homogenised composite benthic and epipellic sediment sample was dissolved in sterile 90ml of distilled water. From this, serial dilution was carried out. Following serial dilution, freshly prepared mineral salt medium (MSM) and plate count agar, crude oil

and filter paper were autoclaved at 121^{o}C at psi for 15 minutes. After autoclaving, the media were dispensed (15 ml/plate) and in triplicates. The filter paper was then soaked with 1ml of the sterile crude oil and carefully used to cover the lid of the plates and sealed with masking tape. The plates were then incubated inverted for 24 to 96 hours. The growth of fungi was inhibited by supplementing the MSM medium with cycloheximide 100 mg/ml and benomyl 50 mg/ml before autoclaving.

2.5 Morphological, Cultural Characterisation and Molecular Characterisation

Resulting pure bacteria isolates were characterised using gram staining, microscopy and biochemical tests. These were done as previously described [21-24]. The fungal isolates were identified as previously described [24]. Molecular characterisation was performed on the isolates. DNA extraction was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps kit Model D6005 (Zymo Research, California, USA) by following manufacturer's instruction carefully. PCR reaction was performed on the extracted isolate DNA using universal degenerate primers 27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3 and 1492R reverse 5'GGTTACCTTGTTACGACTT 3' that amplifies the entire 16s variable region at an annealing temperature of 58. This was done following the method previously described [25]. DNA sequencing was performed by Sanger (dideoxy) sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems [26].

2.6 Isolation of Plasmids and Loading of Gel

Four bacteria isolates were selected and grown overnight in Luria-Bertani (LB) broth and cells were pelleted by centrifugation at 5,000 rpm for 15 min at 4°C. Gently, the supernatant was decanted leaving behind 50-100 ml together with the cell pellet and vortexed to resuspend the cell completely. To the resulting mixture, 300ml of TENS (TENS composition: Tris25mM, EDTA 10mM, NaOH0.1N and SDS 0.5) and the vortexed 3-5 times until the mixtures became sticky. As a matter of caution, the mixtures were then set on ice to prevent co-precipitation of plasmid and chromosomal DNA. During this time, 150 ml of sodium acetate was added and the mixture vortexed completely and after which the mixture was micro-centrifuged to pellet the debris and chromosomal DNA. The supernatant was then transferred to a fresh tube and mixed well with 900 ml of absolute ethanol. This was then spun for 10 minutes to pellet the plasmid DNA (white pellets). The supernatant were then discarded and the resulting white pellet rinsed twice with 1 ml of 70% ethanol and pellet dried. The dried pellet was then re-suspended in 30 ml of distilled water for further use. These were done using methods previously described [27-28].

2.7 PCR Amplification of alk B Genes

Polymerase chain reaction was performed on the extracted plasmid DNA using two alkane monoxygenase gene primers (Rh alk B1-F ATCTGGGCGCGTTGGGATTTGAGCG, Rh alkB1-RCGCATGGTGATCGCTGTGCCGCTGC and Pp alkBP-F TGGCCGGCT ACTCCGATGATCGGAATCTGG, alkBP-R GCGTGGTGATCCGAGTGCCGCTGAAGGTG). Each of the PCR reaction contained 5 µl of 10 × Taq buffer, 2 mM $MgCl_2$,1.5 USuper-Therm DNA Polymerase (Southern Cross), 0.25 mM dNTP's, 0.1 µM of each primer, 1 µl of extracted DNA and Nuclease-Free Water (NFW) up to the final reaction volume of 50 µl. The PCR cycle started with an initial denaturation step at 95°C for 10 min. This was followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C [25,27].

2.8 Gel Electrophoresis

Exactly 10 µL PCR product were loaded in 1.5% agarose gel in TAE buffer (40mM Tris-acetate, 2 mM EDTA [pH 8.3]) and ran at 90 V for 45min. Gels were stained with 0.5 µg/ml of ethidium bromide for 45 min and destained with water for 20 min. Stained gels were examined under ultra-violet (UV) transilluminator in a photo documentation system (Clinix, Model 1500). A suitable DNA ladder (λ DNA-Hind III Digest from New England Biolabs) was used as a molecular weight marker. The electrophoretic profiles were observed visually for clarity. Only major amplicons and consistent minor bands were considered in the analysis [25,27].

2.9 Statistical Analysis

The results of the microbiological and physicochemical analyses were analysed using analysis of variance (ANOVA). The results were presented as mean plus or minus standard deviation (Mean±SD). Mean values with probability values ($p < 0.05$) were considered significant at 95% level of significance. Analysis of variance was done as reported [29].

3. RESULTS

The results of the study are as presented in the tables and figures below. Table 1 shows the result of the physicochemical analysis of the various composite sediment samples. Across the various locations sampled, pH readings were 5.50, 5.60 and 5.30 for the beach marine sediment, sub soil and top soil, respectively. Temperature levels were uniform and ranged from 28.50 to 28.70^{o}C.

The most abundant metal was iron followed by copper with values ranging from 4.49 to 8.62mg/L. Base saturation was the most abundant parameter examined with values ranging from 50.30 to 77.30 mg/L. This was followed closely by nitrate levels which was highest in the sub soil.

Table 2 shows the total aerobic counts for bacteria and fungi. From the results, it can be seen that the counts for bacteria were higher than those of fungi. Aerobic bacteria counts ranged from 70 to 92 (x10^{6}) CFU/g while those of fungi ranged from 14 to 19 (x10^{3}) CFU/g. Generally, the soil samples recorded higher values than the sediment samples.

Table 3 shows the biochemical characterization of the aerobic and hydrocarbonoclastic bacteria isolates. The bacteria isolates were *Enterobacter sp, Bacillus sp, Micrococcus, Pseudomonas, Corynebacterium sp* and *Klebsiella sp.* From these isolates, four were then subjected to plasmid profiling for *alkB* genes. The selected four isolates were *Bacillus species* (19), *Pseudomonas species* (21), *Bacillus species* (22) and *Klebsiella species* (23). Molecular identification identified the isolates as *Bacillus thuringiensis, Pseudomonas stutzeri, Bacillus cereus* and *Klebsiella pneumoniae,* respectively. See Fig. 1 for gel electrophoresis result.

Table 1. Physicochemical analysis of the composite marine soil and sediment sample

Parameters	BMS	BSS	BTS
pH	5.50±0.01	5.60±0.02	5.30±0.01
Temperature ^{o}C	28.50±0.01	28.70±0.10	28.60±0.20
Phosphorus mg/L	23.20±0.10	28.50±0.10	30.50±0.20
EC (ms/cm)	30.50±0.04	11.80±0.07	25.00±1.41
BS (mg/L)	50.30±1.41	77.30±3.31	65.30±2.41
Al^{3+} (mg/L)	0.80±0.01	1.10±0.01	0.90±0.01
Cu (mg/L)	1.01±0.01	0.96±0.00	1.40±0.01
Zn (mg/L)	0.20±0.01	0.12±0.01	0.18±0.00
Fe (mg/L)	8.62±0.10	4.59±1.14	4.49±0.07
Cr (mg/L)	0.010±0.00	0.068±0.01	0.015±0.00
Sulphide(mg/L)	6.00±0.01	11.00±1.14	2.00±0.14
N-nitrate (mg/L)	41.20±0.01	56.40±2.71	35.80±1.71
N-nitrite (mg/L)	0.040±0.10	0.045±0.01	0.065±0.01
Nickel (mg/L)	0.078±0.01	0.069±0.01	0.077±0.01
Cobalt (mg/L)	0.022±0.01	BDL	0.019±0.10
N-Ammonia (mg/L)	0.13±0.01	0.08±0.01	0.09±0.10

Key: BMS= beach marine sediment, BSS = beach marine sub soil and BTM = beach marine top soil and BDL = below detection level

Table 2. Mean total heterotrophic bacteria and fungi counts

	BMS	BSS	BTS
Bacteria (x10^{6}) CFU/g	70	84	92
Fungi (x10^{3}) CFU/g	14	16	19

BMS= Beach marine sediment, Beach Marine sub soil and BTM = Beach marine top soil

Table 3. Biochemical characterization of the aerobic and hydrocarbonoclastic bacteria isolates

Isolate	Shape	Gram	Motility	Citrate	Catalase	Indole	MR	VP	Starch hydrolysis	H_2S Production	Oxidase	Glucose	Lactose	Mannitol	Sucrose	Probable organism
1	Rod	+ve	+	+	+	-	-	+	+	-	-	A	A	A	-	*Bacilli* sp.
2	Cocci	+ve	-	-	+	-	+	-	-	-	+	A	A	A	A	*Micrococcus sp.*
3	Rod	-ve	+	-	+	-	-	-	-	-	+	AG	A	V	A	*Pseudomonas sp.*
4	Rod	+ve	+	+	+	-	-	+	+	-	-	A	A	A	-	*Bacillus sp.*
5	Rod	-ve	+	+	+	-	-	+	-	-	-	A/G	-	A	A	*Enterobacter sp.*
6	Bluish cocci in cluster	+ve	NA	NA	+	NA	NA	NA	NA	NA	+	A	A	A	A	*Corynebacterium sp.*
7`	Moist colonies	-ve		+	+	-	-	+			-		A			*Klebsiella sp.*

Keys: - = negative, + = positive reaction, A= acid, AG = Acid and gas, NA = not applicable, V = variable

Table 4. Characteristics of the hydrocarbonoclastic fungi isolates

Isolates	Colonial morphology	Nature & colour of hyphae	Appearance of sporangiophores	Spores	Probable organism
1	Small round green colour with smooth appearance	Septate& green	Single long conidiophores	Tiny spherical spores scattered around	*Penicillium sp*
2	Yellowish green colonies with smooth appearance	Septate& light yellow	Elongate conidiophores	Small round spores crowded	*Aspergillus flavus*
3	Pink round colour with smooth appearance	Non-septate& white	ND	Small oval spores	*Fusarium sp*
4	Wavy white colony with round shape	Non septate& white	Group of long sporangiophores	Oval scattered around	*Rhizopus sp*
5	Green colonies with smooth appearance with reverse furrow	Septate& light yellow	Elongate conidiophores	Small round spores crowded	*Aspergillus sp*

Key: ND= Not determined

The isolates overlapped and for both aerobic and hydrocarbonoclastic isolates. Aerobic isolates were 1 to 7 while Isolates 1, 3, 4, and 7 were also hydrocarbonoclastic isolates.

Table 4 shows the various fungal hydrocarbonoclastic isolates. From the results, it can be seen that the fungi isolates were *Penicillium* species*, Aspergillus flavus, Fusarium* species*,* and *Rhizopus* species.

Figure one shows the results of the gel electrophoresis for the plasmid profiling of the *alk* B gene following plasmid DNA extraction. As can be seen from the gel picture, there are five lanes. Lane M was loaded with the marker of band size 23,130bp while lanes labelled 19, 21, 22 and 23 were loaded with the selected isolates as thus: as *Bacillus thuringiensis* (19)*, Pseudomonas stutzeri* (21)*, Bacillus cereus* (22) and *Klebsiella pneumoniae* (23)*,* respectively.

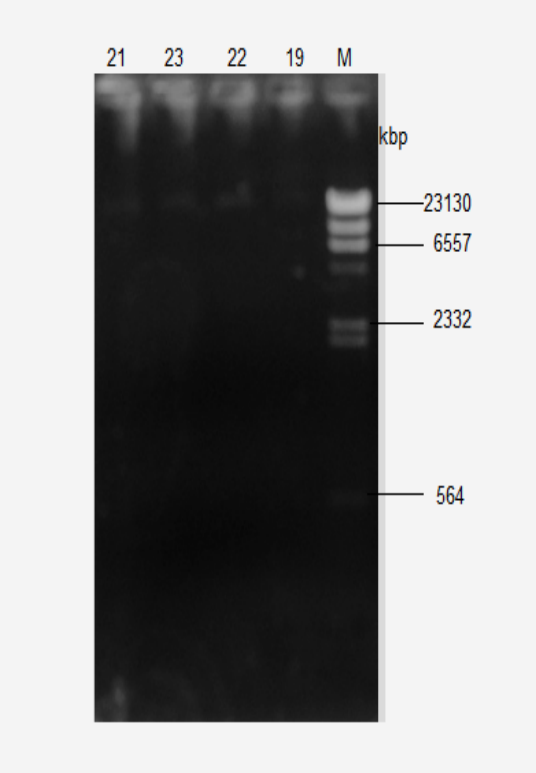

Fig. 1. Gel electrophoresis of the *alk B1* amplified product of the selected isolates.. Molecular identification identified the isolates as *Bacillus thuringiensis* (19)*, Pseudomonas stutzeri* (21)*, Bacillus cereus* (22) and *Klebsiella pneumoniae* (23)*,* respectively

4. DISCUSSION

The results of the physicochemical analysis show significant variation across the sampled locations. The pH values recorded in our study indicate that the samples are mildly acidic and were within range of the maximum permissible limits of 5.1 to 6.5. Our findings for base saturation were also within maximum permissible limits of 60-80% for standard reported previously [30]. Copper and iron levels were higher than the 0.05 and 0.3-1.0 mg/L set by the World Health Organization. However, chromium levels and zinc were less than those of WHO [31]. Nitrate levels reported here were far higher than those reported by Bello and Ukut [32] from soil collected from Cross River Estuary. Nitrate and phosphate levels were highest and this indicates anthropogenic sources.

Bacteria counts across the sampled locations in our study were similar to those reported earlier by Bello and Ukut [32]. In their study, they reported a mean count range of 10 - 79 x 10^6 (CFU/g). As expected our bacteria counts were higher than those of fungi. Lower fungi counts were also reported by these authors and were also within range of fungi counts of 14 - 19 x 10^3 CFU/g.

Hydrocarbonoclastic bacterial isolates were *Klebsiella, Pseudomonas, Bacillus* and *Bacillus species*. While the aerobic isolates were *Enterobacter, Bacillus, Micrococcus, Pseudomonas, Corynebacterium* and *Klebsiella species.* Hydrocarbon degrading isolates were *Aspergillus, Penicillium* and *Rhizopus species* while the aerobic fungal isolates were *Penicillium* sp*, Aspergillus flavus, Fusarium, Rhizopu*s and *Aspergillus species. Bacillus and Aspergillus species* were the most frequent isolates from our study. Similar isolates were also reported by Bello and Ukut [32] who also isolated *Penicillium sp, Aspergillus flavus, Fusarium* sp and *Rhizopus* sp and different bacterial isolates from estuaries soil.

The results of the molecular characterization via Sanger sequencing revealed that the selected hydrocarbonoclastic isolates were *Bacillus thurigiensis serovar thuringiensis, Bacillus cereus, Pseudomonas stutzeri* and *Klebsiella pneumoniae*. These genera were also isolated by Isiodu et al. [33] who isolated *Shewanella haliotis*, *Shewanella sp*, *Vibrio alginolyticus*, *Pseudomonas putida*, *Bacillus cereus*,

B. pumilus and *Shewanella sp* capable of degrading crude oil. They were also similar to species previously reported from oil producing communities [2,19-20].

Certain plasmids play important role in adaptation of natural microbial populations to crude oil and other hydrocarbons pollution [33-34]. In an earlier study, they showed that enzymes responsible for the oxidation of n-octane to octanoic acid or beyond in *Pseudomonas oleovorans* are octane inducible and are coded by genes borne on a plasmid. They also reported that the chromosome also carries genes coding octanol oxidation enzymes that, in contrast, are induced by octanol, not by octane [33]. Furthermore, the degradation of many xenobiotic and hydrocarbon compounds such as polyaromatic hydrocarbon is known to be mediated by plasmid encoded enzymes [7].

Amongst the selected isolates for plasmid profiling, none of the isolates showed the presence of plasmid borne alk B genes from *Rhodococcus* species and *Pseudomonas putida* used in this study. However, in an earlier study, plasmid extraction studies of six selected hydrocarbon utilizing isolates showed that isolates had two plasmids each, following plasmid curing. Furthermore, they observed that loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of their degradative abilities of Chevron Escravos crude oil but only resulted in reduction in their degradation potential [13].

In another study, it was concluded that chromosomes are indeed favoured locations for functioning essential genes, due to their stable inheritance. In addition, they stated that for plasmids to carry such genes depend on the rate at which these genes degrade by chance events in each location [14].

5. CONCLUSION

The physicochemical analysis shows anthropogenic influence on the studied ecosystem. Given the absence of *alk* B1 genes on the plasmid for the selected hydrocarbon degraders, it is safe to speculate that *alk* B gene is chromosomally favoured. However, we recommend further studies be carried out on other catabolite genes of crude oil degrading isolates.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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