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Molecular Characterization of Aerobic Heterohophic Bacteria Isolated from Petroleum Hydrocarbon Polluted Brackish Waters of Bodo Creeks, Rivers State Nigeria

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Abstract

Surface water sources in the oil producing Niger Delta region of Nigeria are highly susceptible to pollution by petroleum hydrocarbons and so it is important to understand the microbial diversity of such ecosystems. Water and sediment samples were collected between April-August, 2013 from Bodo creeks and taken to Environmental Microbiology laboratory of University of Portharcourt for analysis. A total of thirty aerobic heterotrophic bacterial strains isolated ranged from 3.0 - 7.0 × 10^4 cfu for surface water and 1.6 - 5.6 × 10^4 cfu for sediment samples of Bodo creek using serial dilution and spread plate technique. Pure cultures of bacteria were obtained on the basis of their morphological characteristics and subjected to biochemical tests and further classified on the basis of 16S rRNA gene sequence analysis. The DNA was isolated from size fractionated samples and the diversity of bacteria in each fraction was studied using PCR amplification of partial 16S rRNA. The sequences were submitted to NCBIGen bank for identification and assigning of accession numbers. The isolated aerobic heterotrophic bacteria belong to the families of Enterobacteriaceae, Bacilliceae, Alcaligenaceae, Pseudomonadaceae, Flavobactericeae and Planococcaceae.

Keywords

Petroleum Hydrocarbon, Brackish Water, Aerobic Heterotrophic Bacteria, Diversity

1. Introduction

Bacteria are said to be ubiquitous with the capacity to colonize any habitat of the planet, having a greater active

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biomass compared to any other group of organism. The main reason for this ubiquitousness is their large surface area to volume ratio which gives them the ability to out-compete other microbes for dissolved compounds. They are metabolically diverse and can obtain energy from oxidizing carbon, parasitism, chemoautotrophy and photoautotrophy. They play a major role in most biogeochemical cycling of nitrogen, carbon, sulphur and oxygen in aquatic ecosystems [1] [2].

Various types of bacteria have been identified in different environments at different conditions [1]. With the exception of cyanobacteria, all other identified groups of bacteria are heterotrophic and do not contribute to primary production in the ecosystem but rather assimilate dissolved organic matter. Most bacteria species from the natural environment are reportedly difficult to identify with phenotypic methods commonly applied in clinical laboratories [3] hence the use of 16SrDNA based identification provides a better alternative to the phenotypic characterization methods [4] [5].

Large amounts of hydrocarbon contaminants are released into the environment as a result of human activities and are said to pose severe, immediate and long term ecological and environmental repercussion since a lot of the hydrocarbon components are toxic and persistent in terrestrial and aquatic environment [6]. Irrespective of these effects, several studies have isolated and identified bacteria species from different surface water sources contaminated with hydrocarbons [7]-[13]. Betrand *et al.* [14] isolated from interface water sediments a strain of halophilicarcheon, *Harloacula villismortis*. Other hydrocarbonoclastic halophilic and halotolerant microorganisms have been isolated from chronically hydrocarbon impacted sites and petroleum industry facilities [15]-[18].

Bodo creek, located in Bodo community of Gokana LGA of Rivers State is characterized by brackish water system and has been a site for oil industry operations since 1950 with varying degrees of petroleum hydrocarbon pollution. The aim of the present study was to isolate and identify aerobic heterotrophic bacteria from brackish waters of hydrocarbon polluted Bodo creeks and to produce their biochemical, physiological and molecular characterization and identification. The research provides updated information on the bacterial diversity of a petroleum hydrocarbon contaminated brackish surface water source which underscores the relevance of the study.

2. Materials and Methods

Sampling Isolation and Growth Conditions

Petroleum hydrocarbon polluted water (measured by total petroleum hydrocarbon using agilent 6890 GC-FID, TPH 769 mg/L) and sediments samples (TPH 1109 mg/kg) were collected from Bodo creeks in Bodo community of Gokana LGA. Ogoniland covers about 1000 km^2 in Rivers state and has been the site for oil industry operations since 1950. The samples were collected from April to August, 2013 under sterile conditions using NiskinPvc water sampler at a distance of 50 metres distance apart and 20 m from the coast at five designated points and transported immediately to Environmental Microbiology Laboratory of the University of Portharcourt for isolation procedures. The bacteria were isolated using serial dilution method as described by Cheesebrough [19], with distilled water and the 10^{-3} dilution was considered and the duplicate of it plated on nutrient agar (oxoid) medium using spread plate technique. The nutrient agar comprises of the following in gm/litre; yeast extract 4.0, tryptone 5.0, glucose 50.0, potassium dihydrogen phosphate 0.55, potassium chloride 0.425, calcium chloride 0.125, magnesium sulphate 0.125, Ferric chloride 0.0025, manganese sulphate 0.0025, bromocresol green 0.022 and Agar 15.0 with a pH 5.5 ± 0.2. Incubation of the resulting inoculated plates was done at about 37˚C which is the mean temperature of the sites where samples were collected for 24 h and counted, the resulting mean from the duplicate was taken as the colony forming unit of the sample. Pure cultures of the resulting morphologically distinct colonies were singly isolated and plated on the same nutrient agar medium and incubated for another 24 h after which the isolates were inoculated in bijou bottles containing the growth medium and sent for molecular characterization.

The physicochemical parameters: electrical conductivity, turbidity, salinity, total organic carbon, phosphate, nitrate, total nitrogen and sulphate content of the samples were analysed as described by [20].

3. Morphological, Biochemical and Physiological Characteristics

Morphology of cells of the isolated strains was examined by light microscopy using Gram stain technique of 24 - 48 h old cultures grown on nutrient agar medium (oxoid) as described by Cheesebrough [19]. The physio-

logical tests carried out were: catalase and oxidase reactions [21], utilization of lactose and glucose formation of gas from glucose, production of indole, MR and VP and Motility test [22] [23]. The tests were read after incubation for 24 - 48 h at room temperature.

3.1. Extraction of DNA Using CTAB Method

Bacterial cells of each isolate grown overnight in 10 mL of nutrient agar broth medium and shaken at room temperature was collected in Eppendorf tube by centrifugation at 14,000 rpm for 2 mins at room temperature. DNA was extracted from the pellet by suspending in 600 µL of 2× CTAB buffer incubated at 65˚C for 2 mins. The DNA pellet was finally resuspended in 100 µL of sterile distilled water. DNA concentration of the samples was measured on spectrophotometer at 260 nm and 280 nm and the genomic purity were determined. The genomic purity ranged between 1.8 - 2.0 for all the DNA samples as described by [24].

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% w/v agarose gels supplemented with ethidium bromide (0.5 µg/mL 0.5× TBE buffer solution as described by [24].

3.2. PCR Analysis of 16S rRNA Gene

PCR analysis was run with a universal primer for bacteria and fungi called 16S. The universal primers used for bacteria were Eub 27F (5^1-3^1. AGA GTT TGA TCC TGG CTC AG) forward primer and Eub 1492R (5^1-3^1. ACG GCT ACC TTG TTA CGA CTT) for reverse primers [25]. The PCR mix comprises of 11 µL of 10× buffer, 0.4 µL of 50 mM $MgCl_2$, 0.5 µL of 2.5 mM dNTPs, 0.5 µL 5 mM Forward primer, 0.5 µL of 5 mM Reverse primer, 0.05 µL of 5 units/µL Taq DNA polymerase; 5 µL template DNA and 5.05 µL of distilled water to make up 10 µL reaction mix.

The PCR profile used is initial denaturation temperature of 94˚C for 3 mins, followed by 30 cycles of 94˚C for 60 sec, 56˚C for 60 sec, and the final extension temperature of 72˚C for 5 mins and then 10˚C hold forever.

The amplicon was further purified before the sequencing using 2 M Sodium Acetate wash techniques. To about 10 µL of the PCR product, we added 1 µL of 2 M NaAct pH 5.2, followed by 20 µL Absolute Ethanol, kept at −20˚C for 1 hr and spinned at 10,000 rpm for 10 mins, then washed with 70% ethanol and air dried. This was again suspended in 5 µL sterile distilled water and kept at 4˚C for sequencing.

The PCR sequence product was also purified using 2 M sodium acetate wash technique before the sequencing was run. To 10 µL of the PCR product was added 1 µL 2 M NaAct pH 5.2, then 20 µL Absolute Ethanol and kept at −20˚C for 1 h and subsequently spinned at 10,000 rpm for 10 mins, then washed with 70% Ethanol and air-dried. This was re-suspended in 5 µL of sterile distilled water and kept at 4˚C for sequencing running.

3.3. Gene Sequencing and Analysis

The Cocktail mix is a combination of 9 µL of Hi di Formamide with 1 µL of purified sequence making a total of 10 µL. The samples were loaded on the ABI machine and the data in form of A, C, T, and G, was released.

Sequence results obtained from above were compared with known sequences in the GenBank using the basic local alignment search tool of the national centre for biotechnology information. Species were identified based on the percentage similarity with the known species sequences in the data base.

4. Results

4.1. Physicochemical Characteristics

Table 1 shows the physiochemical parameters of surface water and sediments samples of Bodo creek. The pH for water and sediments were 7.68 and 7.87 respectively amongst other parameters as contained in the table.

Table 2 shows the mean of the duplicate colony counts of bacteria isolated from the water and sediment samples. Water samples had more colonies compared to sediment as observed though no significant difference was observed between the counts from water and sediments. $t(4) = 2.034$ ($p > 0.05$).

4.2. Physiological and Biochemical Test

All the bacterial strains isolated were aerobic and motile and showed varying responses to series of test carried

Table 1. Physiochemical parameter of Bodo water and sediments samples.

Parameters	Water	Sediments
pH	7.68	7.87
Electrical conductivity	31609 µS/cm	7240 µS/cm
Turbidity	252 NTU	
Salinity	19.67 ppt	17.87 ppt
Total organic carbon	3.06%	2.20%
Total phosphate	40.8 mg/L	11.15 mg/kg
Total Nitrogen	673.2 mg/L	886.30 mg/kg
Nitrate	40.6 mg/L	37.4 mg/kg
Total petroleum hydrocarbon	769 mg/L	1109 mg/kg
Sulphate	30.5 mg/L	28.46 mg/kg

Table 2. Total heterotrophic bacteria count.

Counts in cfu/mL		
Sample	Water	Sediment
A	6.6×10^4	5.6×10^4
B	4.3×10^4	2.8×10^4
C	4.4×10^4	1.6×10^4
D	3.0×10^4	3.8×10^4
E	7.0×10^4	5.6×10^4

out on them. **Table 3** gives a summary of the physiological and biochemical properties differentiating the isolates. Most of the colonies examined were colorless, circular and some were rod like and smallish.

4.3. 16S rRNA Sequencing and Bacterial Identification

Table 4 and **Table 5** shows the molecular identification of the isolates based on 16SrDNA sequences. The PCR amplified fragments had 250 bp in size and generated sequences ranged from 700 to approx. 1500 bp. The 16SrDNA sequence similarity of the isolates to that of previously characterized bacterial species ranged from 86% to 100% for bacteria isolates from water samples and 80% to 99% for bacteria isolates from sediments.

5. Discussion

We studied the diversity of aerobic heterotrophic bacteria present in petroleum hydrocarbon contaminated brackish water and sediment of Bodo creeks. This study became necessary because of the toxic effect of petroleum hydrocarbon components on aquatic life, to establish the existence or otherwise of bacteria and the need for their molecular identification which is rare since the commencement of oil activities in this region to date.

The introduction of 16SrDNA analysis is said to be a useful tool for identifying bacterial species and comparing their biochemical, morphological and physiological properties [23]. Bovin-Jahns *et al.* [26] opined that the analysis of a small subunit of rDNA sequences for bacteria identification isolated from a natural environment is more efficient compared to the use of phenotypic methods.

From the result of our study, the genus *Bacillus* dominated the water and sediment samples among other genera like *Alcaligenes*, *Enterobacter*, *Pseudomonas* and *Escherichia* which are known to be very frequent in sea water and were also isolated from petroleum hydrocarbon polluted aquatic environments in several studies [7] [27].

In bacterial taxonomy, it is common knowledge that two bacteriado not belong to the same species if their 16SrDNA sequence similarity is less than 97% [28]. In our experiments, isolates M1, M22, BMC1 and AU19157 displayed 86%, 91%, 90% and 86% identity to the sequences available in the NCBI GenBank for *Alcaligenes faecalis*, *Bacillus cohnii*, *Bacillus pumilus*, *Bordetella* spp. Similar low identities were observed in

Table 3. Biochemical and physiological analysis of bacteria isolates.

Code	Starch hydrolysis	Lactose	Glucose	Sucrose	Motility	Citrate	Gas production	H_2S production	Slant	Butt	MR	VP	indole	Catalase	Gram stain
B + Cw	+	+	−	+	+	+	+	+	−	−	−	−	−	−	−
B + Cs	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−
Aspink	+	+	+	+	+	+	−	+	+	−	+	−	−	−	+
CS	−	−	+	+	−	−	−	+	+	−	−	−	−	−	+
FW	+	−	+	+	−	+	+	+	+	−	−	−	−	−	+
Kirr	+	+	−	+	+	+	+	−	+	−	+	−	−	+	−
SA2	+	−	+	+	+	−	+	−	+	+	−	−	+	−	+
EW	−	+	+	+	+	+	+	+	+	−	+	−	−	−	−
FS	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−
Jspink	+	+	+	+	+	−	−	+	+	−	+	−	+	−	+
Iwirr	+	+	+	−	+	−	+	+	−	−	+	−	−	−	−
GW	+	+	+	−	+	+	−	+	+	−	+	−	−	−	+
JW	+	+	−	+	+	−	−	−	−	−	−	−	−	+	+
K1W	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+
BW	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
SD1	−	+	−	+	+	−	−	−	−	−	−	−	−	+	+
A2w	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SE1	+	+	−	+	+	−	−	+	−	−	−	−	−	+	−
A2	−	−	−	−	+	+	+	+	−	−	−	+	−	+	+
SW	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+
CW	+	+		−	+	+	−	−	+	+	−	−	+	−	+
SW2	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−
JW	−	−	−	−	+	−	−	+	−	−	−	−	−	+	+
Sh2	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
G1w	−	+	+	−	+	+	+	−	−	−	−	−	−	+	+
SJ1	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+
SG2	−	−	+	+	−	−	+	−	−	−	−	+	+	+	−
SH1	−	+	+	+	+	−	+	+	+	+	−	−	−	+	+
SH2	+	+	−	−	+	+	−	−	−	−	+	+	−	+	
	−	−	+	−	+	−	−	+	+	−	+	−	−	−	−

isolates from sediments. These low sequence similarities of suggest that the novel strains may represent new species which is subject to further detailed molecular and physiological studies.

Our studies further isolated bacterial strains with high percentage of similarity ranging from 99% to 100% for both water and sediments samples. These include; (Y39), PLW-J 4, INA01087, KUDC1737 which were affiliated to *Bacillus pumilus*, *Bacillus pumilus*, *Bacillus pumilus*, *Bacillus aerophilus* to for sediment samples. Those isolated from water include: VKK-20L, LLS-M1-17, 44A and P18F04 which were identified as *Bacillus pumilus*, *Bacillus pumilus*, *Lysinibacillus fusiformis*, *and Bacillus pumilus* respectively. It is however important to note that our experiment did not consider the effect of seasonal variations on the abundance of bacteria isolated though previous studies revealed the effect of seasonal dynamics on the abundance of bacteria strains isolated from a marine environment [29]. Majority of bacteria isolated and identified are mostly Gram-positive which are known to dominate hydrocarbon polluted site, hence this result was expected.

Table 4. Bacterial isolates from petroleum hydrocarbon polluted brackish water of Bodo creek.

Microorganism	Similarity%	Isolate	Accession number
Alcaligenes faecalis	86	M1	KF 056 900.1
Bacillus pumilus	99	VKK-20L	KF 717600.1
Bacillus pumilus	99	MCC1A08154	JX680132.1
Myroides odoratimimus	97	IHBB1337	GU186112.2
Bacillus cohnii	91	M22	KC813164.1
Citrobacter murliniae	98	14s (br26)	KF 254752.1
Bacillus pumilus	99	LLS-M1-17	HQ334985.1
Bacillus pumilus	98	TKLS-C_4-15	HM744710.1
Bordetella sp	86	Au19157	KF601910.1
Lysinibacillus fusiformis	99	44A	KC329831.1
Bacillus pumilus	90	BMC1	KF387715.1
Myroides pelagicus	97	AB176662.1	NR041042
Bacillus pumilus	100	P18F04	JQ833612.1
Enterobacter cloacae	98	P45 B06	JQ832514.1

Table 5. Bacterial isolates from petroleum hydrocarbon polluted brackish sediments from Bodo creek.

Sediments	**Similarity %**	**Isolate**	**Accession number**
Bacillus safensis	99	Y39	KF641818.1
Bacillus pumilus	93	1K-MB13-518F	FJ906741.1
Bacillus pumilus	90	B34	KC492105.1
Bacillus pumilus	99	PLW-J4-4	HQ334984.1
Bacillus licheniformis	91	P85A08	JQ835466.1
Cedecea davisae	91	HME 8588	KC201361.1
Emterpbarter hormaechei	92	P63E06	JQ829491.1
Bacillus pumilus	99	INA01087	KF717600.1
Bacillus pumilus	95	SSR07	JQ833773.1
Bacillus subtilis	85	P95H01	JQ832514.1
Enterobacter hormaechei	81	P61D018	JQ829302.1
Bacillus aerophilus	99	KUDC 1737	KC414716.1
Citrobacter freundii	97	BAB 1679	RF535142.1
Psendomonas aeruginosa	96	R8-550-1	JQ659950.1
Bacillus megaterum	91	P7H12	JQ881645.1
Escherichia sp.	80	GDR 06	HE583396.1

The 16SrDNA analysis clearly showed that the bacteria isolated belonged to the respective genera with which they were identified based on the percentage sequence similarity, though when compared to physiological properties some slight differences with respect to some *Bacillus* strains were revealed. Our isolates labeled as A_2w, AS, B + Cw and B + Cs stained as Gram-negative rod which is rarely found among aerobic rod shaped bacteria [21] whereas the structure of the cell wall of the bacilli as viewed corresponded with that of a Gram-positive which could explain why this unusual occurrence though their high sequence similarity clearly showed same species affiliation.

6. Conclusion

The result of our studies from the characterization of aerobic heterotrophic bacteria from petroleum hydrocarbon

polluted brackish water of Bodo Greeks suggest that other bacteria from air, marine, fresh water and soil ecosystems can occur in this environment depending probably on the extent of their tolerance to salinity and other intrinsic environmental factors in the creek. The molecular characterization of the bacteria isolated and identified provides evidence of existence of other microorganisms and will serve as baseline data for bacteria diversity of the crude oil contaminated Bodo creek. Further studies on different types of microorganisms and their petroleum hydrocarbon biodegradation potential of both the isolated bacteria and other microbes not investigated in this experiment are suggested.

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