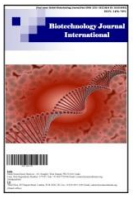

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Molecular Identification of Crude Oil-Degrading Bacteria and Screening for Catechol 2, 3 Dioxygenase (C23O) Gene

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Aims: To identify crude oil-degrading bacteria isolated from polluted soils and waters and screen the presence of catechol 2, 3 dioxygenase (C23O) gene encoding oil-degradation in the strains with the highest degradative activity.
Study Design: Laboratory-experimental design was used in this study.
Place and Duration of Study: Crude oil polluted soils and waters were collected from Awoye, Mese and Oluwa villages in Ondo State, Nigeria and three different flow stations (Agbada-Aluu shell, Obite, and Bonny) in Rivers State, Nigeria.
Methodology: The identities of the isolates were confirmed by extracting their total genomic DNA using standard DNA protocols while a portion of 16S bacterial gene of their DNA was amplified by polymerase chain reaction (PCR) using the primers E9F and U1510R and sequenced using Sanger method. Degenerate primers were used to isolate and identify the gene encoding C23O, responsible for the degradation of oil. Molecular cloning of the gene was done by transforming into *Escherichia coli* DHα. The correct inserts from the selected clones were performed by colony PCR. The isolated gene was sequenced with a Dye terminator sequencing kit and the product was analyzed with Prism DNA sequencer.

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Results: The results obtained from the conserved sequence of the 16S rRNA coupled with the nucleotide sequence revealed ten (10) crude oil-degrading bacteria, with CFfab 14 and CFfab 12 having the highest and lowest degrading activity of 78.92 ± 0.9 Unit/mL/h and 43.89 ± 1.3 Unit/mL/h on day 3 respectively.
Conclusion: The gene C23O responsible for the production of catechol 2, 3 dioxygenase was isolated from strains CFfab 5, CFfab 14 and CFfab 15. The nucleotide base sequence of the gene was determined to be 238 bp. It is expected that in bioremediation, indigenous microorganisms from polluted environments should be screened for the possible existence of this unique gene sequence for effectiveness. Further studies could be conducted on the possibility of cloning this C23O gene into other bacteria for more efficiency and effectiveness in the bioremediation process.

Keywords: Bioremediation; Catechol 2; 3 dioxygenase (C23O); crude oil; degenerate primers; molecular characterization; oil-degrading bacteria.

1. INTRODUCTION

There is a global interest in the microbial biodegradation of pollutants in recent years, as a result of the effect of oil pollution in the environment. Polycyclic aromatic hydrocarbons (PAHs) are of particular concern in environmental pollution because of the toxic, mutagenic and carcinogenic properties they exhibit and the tendency to accumulate in food chains [1]. Since oil is widely used, cases of oil spills and leakages would continue to occur despite all the precautions. Thus, it is essential that there are effective countermeasures to deal with the problem of oil pollution.

Biodegradation is a process whereby organisms gradually degrade complex organic compounds into their smaller units or inorganic form or less/non-toxic compounds. Crude oil is biodegradable and has the capability to decompose within 3 years through natural biological processes into non-toxic carbonaceous oil, water, carbon dioxide or methane in the environment. Saturated oil, especially those of smaller molecular weight are readily biodegraded in marine environments. Aromatic oils with one, two or three aromatic rings are also efficiently biodegraded. However, those hydrocarbons with four or more aromatic rings are quite resistant to biodegradation thus, they are recalcitrant [2]. Oil-degrading microorganisms usually degrade branched alkanes and isoprenoid compounds at much slower rates than straight chain alkanes. Therefore, the ratio of straight chain alkanes to the highly branched biomarker compounds can reflect the extent to which microorganisms can degrade hydrocarbons [3].

The principle of biodegradation is based on the ability to live organisms to utilize crude oil as a source of carbon and energy for their growth. Crude oil components are decomposed by the aerobic biochemical pathway to less toxic products, such as carbon dioxide and water. When crude oil typically gets into the soil they are absorbed by the microorganisms and broken down by enzymes into less toxic compounds, such as methyl catechol, which are further degraded into harmless chemicals by catechol 2, 3 dioxygenase. While catechol 2, 3 dioxygenase is not the only enzyme used by microorganisms to neutralize petroleum-based compounds, it is a key enzyme responsible for the biodegradation of aromatic hydrocarbons [4]. The different types of dioxygenases which catalyze the scission of aromatic hydrocarbons include catechol 1, 2-dioxygenase (C12O), catechol 2, 3-dioxygenase (C23O), protocatechuate 3, 4-dioxygenase and protocatechuate 4, 5-dioxygenase. The cleavage occurs at *the ortho*- or *meta*-position under aerobic conditions [5,6]. The enzyme, catechol 2, 3 dioxygenase plays a major role in the degradation of aromatic rings intradiol and extradiol dioxygenase [7], thus, many researchers are interested in acquiring an in-depth knowledge of the enzymes. The reduction or complete removal of hazardous and toxic organic chemicals from the contaminated environment by microbes is known as bioremediation. This is an efficient and cost-effective approach to cleaning up the environment.

There are many studies focusing on the ability of microbes to degrade aromatic hydrocarbons in both terrestrial and marine environments [8]. This is very necessary so as to provide efficient and effective strains for bioremediation processes in different environments [9]. The microbial degradation strategy to combat crude oil contaminant is considered the most preferred universally because they are eco-friendly and more cost-effective [10]. These enzymes are capable of degrading and utilizing different hydrocarbons as a source of carbon and

energy [2]. In addition, they completely mineralize the contaminant and at very low cost [11].

Mixed microbial communities have been shown to have the most powerful biodegradative potential because the genetic information of more than one single species is required to degrade complex mixture of hydrocarbon present in contaminated areas [12].

According to [13], the diversity and distribution of C23O genes in hydrocarbon-contaminated soil, seawater, groundwater and natural river environments have been reported [6]. However, little is known regarding C23O genes in marine sedimentary environments. For effectiveness, it is imperative that the best approach to bioremediation is to utilize microorganisms or their enzymes, therefore, screening microorganisms for enzymes to be used to clean up polluted sites is a necessary task in bioremediation. It is therefore important that novel organisms, present in oil-contaminated environments with high degradative ability and having catabolic enzymes (C23O) be studied as a means for bioremediation.

The aim of this study is to identify crude oil-degrading bacteria that were previously isolated from polluted environmental samples using molecular techniques and to screen the best strains for the presence of gene (C23O) encoding oil-degradation.

2. MATERIALS AND METHODS

2.1 Culture Media

Enrichment media, Bushnell-Haas broth (BHB) and agar (BHA) (Sigma, Germany) were used in this study. Fifteen (15) grams of bacteriological agar was added to the BHB to make the BHA. Luria Bertani (LB) medium (Sigma, Germany) and the general purpose medium, Nutrient agar (Merck, Germany) were also used in this study.

2.2 Source of Samples

The crude oil-contaminated environmental samples (1 litre of water and 100 g of soil each) were collected from two (2) Niger Delta States in Nigeria. Awoye, Mese and Oluwa villages in Ondo State and three different flow stations (Agbada-Aluu shell, Obite, and Bonny) in Rivers State. The six (6) water samples were collected aseptically into screw-capped containers while six (6) soil samples were collected with a soil auger into sterile cellophane bags. Uncontaminated samples (one each for soil and water) were collected from the same villages and flow stations and used as control.

2.3 Isolation of Crude Oil-Degrading Bacteria from Soil and Water Samples

Two (2) grams of the soil samples and two (2) ml of the water samples were suspended in 100 ml of BHB supplemented at 2% (v/v) with filter sterilized oil in 250 ml Erlenmeyer flasks each. The Erlenmeyer flasks were incubated at 30°C on a Gallenkamp orbital shaker incubator (Germini BV) at 200 rpm for 7 days. After which, the samples were serially diluted and the 1 ml suspension was aseptically transferred from each 10^{-3} dilution into sterile Petri dishes using pour plate technique. The plates were incubated at 30°C for 3 – 7 days [14]. Pure colonies of each isolate were obtained by streak technique on nutrient agar and stored on agar slants at 4°C for further characterization.

2.4 Molecular Identification and Characterization of Crude Oil-Degrading Bacteria DNA Extraction

Total genomic DNA was extracted from 24 h old culture grown in nutrient broth. Modified method of [14] was used for this protocol. The culture (5 ml) was centrifuged at 9,500 g for 10 min and cells pellet was resuspended in 100 µl Tris EDTA (TE) solution (buffer). Lysozyme (100 mg/µl), RNase (10 mg/µl), proteinase K (20 mg/ml) and 10% sodium dodecyl sulphate (SDS) were added and incubated at 60°C for 1 h. Thereafter, 1 ml of phenol:chloroform:isoamyalcohol (25:24:1) mixture was added, centrifuged for 3 min and the water phase was carefully transferred to a new Eppendorf tube. An equal volume of cold isopropanol was added and left for 5 min. It was centrifuged and the DNA pellet was resuspended in 100 µl TE buffer. The concentration of the DNA was measured using the Nanodrop spectrophotometer ND-1000 (Delaware-USA) at 260 nm.

2.5 Amplification of 16S rRNA Gene Using PCR

A region of 16S rRNA bacterial gene was amplified by PCR from the extracted bacterial DNAs using forward primers E9F (5′- GAG TTT GAT CCT GGCTCAG -3′) between 7-26 bases and reverse primers U1510R (5′-

GGTTACCTTGTTACGACTT -3′) between 1490-1512 bases. The reaction mixture was performed in a total volume of 50 µl containing 27.75 µl Milli-Q water, 5 µl reaction buffer (10X), 5 µl dNTPs, 5 µl of each Primer, 0.250 µl Dream Taq Polymerase (Fischer Scientific, Germany), 2 µl Template DNA. DNA amplification was performed in a PCR thermal cycler using the following programme: 5 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, followed by 10 min at 72°C and then held at 4°C. The PCR product was analyzed on a 1% TAE agarose gel.

2.6 Agarose Gel Electrophoresis

Agarose (1%) gel was prepared in Tris-Acetate EDTA (TAE). Ethidium bromide (0.5 µg/ml) was added and mixed thoroughly. The gel was covered with 50X TAE buffer and DNA suspension was transferred into the well. A DNA molecular weight marker was also loaded into a separate well. The gel was run with voltage 80V for 50 mins. Gels were visualized with ultraviolet (UV) light illumination and photographed with a digital imaging system (Alphalmager 2000, Alpha Innotech, San Leandro, CA).

2.7 Measurement of Oil Degradative Activity of Each Isolate

The measurement for the crude oil degradative activity of the ten isolates were thereafter determined with the view to identifying the best strains that would be screened for C23O. The Bushnell-Haas Broth supplemented with 2% (v/v) filter sterilized crude oil was inoculated with each isolate, incubated for 7 days at 30°C during which optical density of each culture medium was quantified with spectrophotometer at 600 nm and the degradative activity (Unit/mL/h) was calculated according to [15] as shown below. The best strains of the isolates with the highest degradative activities were thereafter screened for C23O.

Actual OD reading (AOR) = $\mathrm{OD\ read} \times \mathrm{dilution\ factor}$

Where dilution factor (DF) = $\frac{\mathrm{Total\ Volume}}{\mathrm{volume\ of\ sample\ taken}}$

2.8 Isolation and Amplification of Catechol 2, 3 Di-oxygenase Gene

The catechol 2, 3 di-oxygenase (C23O) gene was amplified using the standard method of polymerase chain reaction. The primers used were DEG F (5'-CGACCTGATC(AT)(CG)CATGACCGA-3') and DEG R (5' T(CT)AGGTCA(GT)(AC)ACGGTCA-3') [16]. The PCR was conducted with a reaction mixture (total volume, 25 µl) containing, 2.5 µl buffer (10X), 2.5 µl dNTPs (0.2 mM), 2.5 µl primer E9F (5 Mm), 2.5 µl U1510R (5 Mm), 1 µl template DNA, 0.125 µl Dream Taq (5U μl^{-1}) and 13.875 µl sterile distilled water. A reaction without DNA was included as a negative control. The thermal profile used for the amplification of the C23O fragment consisted of 5 min of activation of the polymerase at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C and 1 min 25 s at 72°C and final extension for 10 min at 72°C, with a hold temperature of 10°C.

2.9 Purification of the PCR Products

The PCR products were loaded into agarose wells and electrophoresed. The DNA bands were visualized under the UV light. The DNA bands were excised from the gel and purified using the Nucleospin kit according to the manufacturer's instructions. The purified PCR products were stored at -20°C.

2.10 Cloning of the PCR Products

The gene encoding catechol 2, 3 dioxygenase was cloned into a vector pGEM - T Easy after linearizing the vector with the restriction enzyme, EcoR V. The ligation mixture was made up of 5 µl ligation buffer, 1 µl T_4 DNA ligase, 1 µl pGEM – T Easy and PCR products (3 µl). This was incubated at 4°C for 16 hours.

2.11 Transformation of *E. coli* DHα with the Cloned Catechol Gene

Escherichia coli DHα competent cells were transformed with the cloned DNA at 4°C for 10 mins and heat shocked at 42°C for 5 mins. One millilitre of Luria Bertani (LB broth) was added and incubated for 1 h at 37°C. An aliquot (100 µl) of the cells was plated on LB agar plates containing ampicillin (100 µg/ml), Isopropyl-B-D-thiogalactopyrosidase (IPTG) (20 µg/ml) and X-gal (30 µg/ml) for blue/white selection. Agar plates were incubated overnight at 37°C. Some of the grown colonies were then selected for colony PCR.

2.12 Identification of True Transformants

In order to identify the transformants bearing the correct inserts from the selected clones, colony PCR was performed in 96 wells PCR plates

using M13 primers [4]. Each 25 µl reaction mixture contained 0.5U *Taq* polymerase enzyme, 1X PCR buffer, 0.4 pmol primers and 0.2 mM of each dNTP. Template DNA (5 µl) was added to the PCR solution. The cells were first lysed at 95° for 10 mins, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min. The reaction was held at 15ºC. Five microliters of PCR product was analyzed on a 2.5% agarose gel.

2.13 DNA sequencing analysis

The sequence of the 16S rRNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (Applied Biosystems). The nucleotide sequences of the 16S rRNA fragment bacteria obtained after sequencing and their alignments with the genes (16S rRNA) from the other bacteria in the data bank - National Centre for Biotechnology Information (NCBI), were further analyzed.

2.14 Sequence Analysis of the Gene Encoding Oil Degradation

In order to sequence the gene (C23O) of interest, a 16 h culture of *E. coli* harbouring the plasmid of interest, was prepared by inoculating into a 5 ml of LB containing ampicillin (100 mg/ml). Plasmid DNA was prepared from these cultures using QIAprep Spin Miniprep kit (Qiagen). The sequence of the gene encoding oil degradation was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (Applied Biosystems).

2.15 Construction of a Phylogenetic tree

The sequences obtained for the gene (C23O) encoding oil degradation from the best degrading bacteria in this study and those of other bacteria retrieved from NCBI were aligned using ClustalW and the trees were constructed using UPGMA method [17]. The evolutionary analyses were conducted in MEGA7 [18] while the evolutionary distances were computed using the Maximum Composite Likelihood method [19].

2.16 Statistical Analysis

The experiments were carried out in replicates of three. Data were statistically analyzed using SPSS version 20, the mean bacterial counts obtained were statistically analyzed using analysis of variance (ANOVA), and tests of significance carried out by New Duncan's multiple range test at $p < 0.05$ for Table 1. Microsoft Excel Software version 2016 for Data Logging and Processing, MINITAB Software version 17 was used for the statistical analysis subjected to one way analysis of variance (ANOVA) and means were compared using Post hoc comparison result (Tukey test) for Table 2.

3. RESULTS

3.1 Crude Oil-Degrading Bacterial Population of Environmental Samples from Oil-Polluted Sites

Crude oil-degrading bacterial counts ($x10^4$) of oil polluted soil and water collected from Ondo and Rivers States are presented in Table 1. The oil-degrading bacterial loads of the oil polluted soil ranged between 10.37 ± 1.0^{ab} Cfu/g in Awoye and 33.00 ± 1.1^{f} Cfu/g in Obite. The oil-degrading bacterial loads of the Agbada-Aluu water was the highest 26.67 ± 1.0^{f} Cfu/ml) while that of the Awoye polluted water was the lowest (11.67 ± 1.2^{bc} Cfu/ml). The sample collected from unpolluted soil and water (control) had bacterial counts of 9.67 ± 1.2^{a} Cfu/g and 8.67 ± 1.5^{a} Cfu/ml respectively. The bacterial populations of unpolluted soils and water were observed to be lower than those of oil polluted sites.

3.2 Distribution of Oil-Degrading Bacteria in Oil Polluted Soils and Water from Ondo and Rivers State

The distribution of oil-degrading bacteria in oil polluted soils and water from different sites is shown in Table 2. Bacterial isolates 2 and 10 were found only in soil samples while bacterial isolates 6, 13 and 14 were found only in water samples. Bacterial isolates 9 and 11 were found only in Agbada-Aluu soil and Obite water samples respectively. Bacterial isolates 12 and 15 were common in soil and water samples obtained from Agbada-Aluu and Obite respectively. Bacterial isolates 2, 12 and 15 were observed to be widely distributed in the soil and water samples from Ondo and Rivers States. Bacterial isolates 2, 6, 8 and 11 were found to be present in the unpolluted environment.

3.3 PCR Amplification of 16S rRNA Gene

The PCR amplification of genomic DNA targeted to amplify the 16S rRNA gene of the crude oil-

degrading bacterial isolates from environmental samples is presented in Fig. 1.

3.4 Complete Nucleotides BLAST of 16S rRNA Genes

Out of the fifteen (15) samples sent for sequencing, only ten (10) sequences came out good. The complete nucleotides BLAST results indicating 16S rRNA genes identity of the ten (10) crude oil-degrading bacteria isolated from oil polluted soil and water in Ondo and Rivers States as retrieved from the National Centre for Biotechnology Information (NCBI) are presented in Table 3. The percentage of identities ranged between 72% and 96%. The genes of the isolates showed remote homologies to the 16S ribosomal RNA gene of various organisms including *Citrobacter freundii, Pseudomonas pseudoalcaligenes*, uncultured *Citrobacter* spp., uncultured bacterium clone, *Enterobacter* spp., *Klebsiella oxytoca*, *Ewingella americana* and *Bacillus megaterium*.

3.5 Crude Oil Degradative Activity of Each Bacterial Isolate

The degrading tests performed on the bacterial isolates revealed that they were capable of degrading crude oil with variation in their rates of degradation (Table 4). It was observed that the bacteria *CFfab* 2, *CFfab* 3, *CFfab* 5, *CFfab* 10,

Table 1. Crude oil–degrading bacterial population from polluted sites in Ondo and Rivers State

S/no.	Sampling sites	Microbial load of Soil samples ($\times 10^4$ cfu/g)	Microbial load of Water samples ($\times 10^4$ cfu/ml)
1.	Awoye	10.37 ± 1.0^{ab}	11.67 ± 0.9^{b}
2.	Mese	12.33 ± 0.5^{c}	12.67 ± 1.3^{bc}
3.	Oluwa	11.67 ± 1.2^{bc}	14.00 ± 0.5^{c}
4.	Agbada-Aluu	16.33 ± 1.5^{d}	26.67 ± 1.0^{f}
5.	Obite	33.00 ± 1.1^{f}	24.67 ± 1.5^{e}
6.	Bonny	26.00 ± 1.6^{e}	21.67 ± 1.3^{d}
7.	Control	9.67 ± 1.2^{a}	8.67 ± 1.5^{a}

Values are means of triplicates ± SE. Values in the same column carrying the same superscripts are not significantly different according to Duncan's multiple range test at ($p < 0.05$)

Table 2. Distribution of oil-degrading bacteria in oil polluted soils and water from Ondo and Rivers State

Isolates/ Sampling sites	AWS	AWW	MSS	MSW	OLS	OLW	AGS	AGW	OBS	OBW	BNS	BNW	OCS	OCW
1	+	-	-	-	-	+	-	-	+	-	-	-	-	-
2	+	-	+	-	+	-	+	-	+	-	+	-	+	-
3	-	+	+	-	-	-	-	+	-	-	-	-	-	-
4	+	-	-	-	-	-	-	+	-	-	-	-	-	-
5	-	+	+	-	-	+	-	-	-	+	+	+	-	-
6	-	+	-	-	-	+	-	-	-	-	-	+	-	+
7	-	+	-	-	-	-	-	-	-	-	+	-	-	-
8	-	-	-	+	-	-	-	-	-	-	+	-	-	+
9	-	-	-	-	-	-	+	-	-	-	-	-	-	-
10	+	-	+	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	+	-	-	-	-	+	-	-	-	+
12	+	-	+	-	-	+	+	+	-	+	-	+	-	-
13	-	+	-	+	-	-	-	-	-	+	-	-	-	-
14	-	-	-	-	-	-	-	+	-	+	-	-	-	-
15	+	-	-	+	-	+	+	-	+	+	+	-	-	-

Legend: + =Present, - = Absent
AWS = Awoye soil, MSS = Mese soil, OLS = Oluwa soil
AWW = Awoye water, MSW = Mese water, OLW = Oluwa water
AGS = Agbada-Aluu soil, OBS = Obite soil, BNS = Bonny soil
AGW = Agbada-Aluu water, OBW = Obite water, BNW =Bonny water

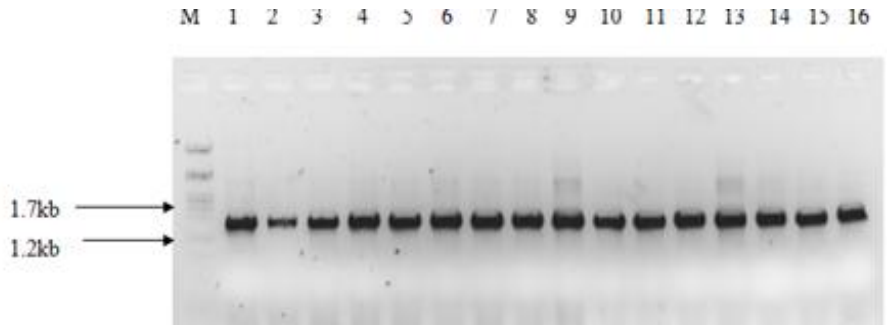

Fig. 1. PCR amplification of genomic DNA targeted to amplify the 16S rRNA gene of the oil-degrading bacterial isolates from polluted soil and water from Ondo and Rivers States

Legend: Lane M 1.5kb Pst ladder/marker
Lanes 1-15 Isolates 1-15
Lane 16 Positive control (E. coli)

Table 3. Complete Nucleotides BLAST results indicating 16S rRNA genes identity of crude oil-degrading bacteria isolated from oil polluted soil and water in Ondo and Rivers States

Bacterial isolate	No. of bases	% Identity	Accession no.	Organism	Description
CFfab 1	1272	96%	dbj|AB548826.1	*Citrobacter freundii* strain: JCM 24061	*Citrobacter freundii* 16S ribosomal RNA, partial sequence
CFfab 2	1141	83%	EU419938.1	*Pseudomonas pseudoalcaligenes* strain RW51	*Pseudomonas pseudoalcaligenes* strain RW51 16S ribosomal RNA gene, partial sequence
CFfab 3	520	95%	gb|GQ417907.1	Uncultured *Citrobacter* spp. clone F4jan.7	Uncultured *Citrobacter* spp. clone F4jan.7 16S ribosomal RNA gene, partial sequence
CFfab 5	1021	92%	gb|DQ816624.1	Uncultured bacterium clone aab20d12	Uncultured bacterium clone aab20d12 16S ribosomal RNA gene, partial sequence
CFfab 6	1248	94%	gb|DQ816402.1	Uncultured bacterium clone aab17f05	Uncultured bacterium clone aab17f05 16S ribosomal RNA gene, partial sequence
CFfab 10	1151	95%	gb|DQ816407.1	Uncultured bacterium clone aab17f10	Uncultured bacterium clone aab17f10 16S ribosomal RNA gene, partial sequence
CFfab 12	1272	93%	emb|FN997633.1	*Enterobacter* spp.	*Enterobacteria* spp. MS39 partial 16S rRNA gene, strain MS39
CFfab 13	441	95%	GU993916.1	*Klebsiella oxytoca* strain ss-11	*Klebsiella oxytoca* strain ss-11 16S ribosomal RNA gene, partial sequence
CFfab 14	707	72%	gb|U29438.1	*Ewingella americana*	EAU29438 *Ewingella americana* 16S rRNA gene, partial sequence
CFfab 15	707	90%	JF939003.1	*Bacillus megaterium* strain IV22	*Bacillus megaterium* strain strain IV22 16S ribosomal RNA gene, partial sequence

CFfab 14 and *CFfab* 15 degraded the crude oil within 48 h of incubation, *CFfab* 1 and *CFfab* 6 degraded the crude oil within 72 h of incubation while *CFfab* 12 and *CFfab* 13 degraded the crude oil within 24 h of incubation. *CFfab* 14 had the highest degrading activity of 78.92 ± 0.9^{Aa}

Unit/mL/h while *CFfab* 12 had the lowest degrading activity of 43.89 ± 1.3^{Db} Unit/mL/h on day 3 i.e 72 hr. *CFfab* 13, which was rated second with degrading activity of 69.33 ± 1.9^{Ba} Unit/mL/h on day 3 was not used for C23O production because of the short base pair length of 441. Based on the above reason, *CFfab* 14, *CFfab* 5 and *CFfab* 15 were thereafter screened for the production of C23O.

3.6 Presence of C23O Gene in Oil-Degrading Bacteria

The degenerative primer pair DEG-F and DEG-R designed to amplify the C23O gene fragment from the best crude oil-degrading bacteria gave positive amplification product of 238 bp as shown in Fig. 2.

3.7 Nucleotide BLAST Results of C23O Genes

The results for nucleotides BLAST of C23O gene from crude oil-degrading bacteria are presented in Table 5. The score for the maximum identity for the alignment is 100% for all the crude oil-degrading bacteria.

3.8 Colony PCR of Inserts

The agarose gel electrophoreses of the colony PCR of inserts using M13 F and M13 R primers are presented in Fig. 3 (A and B).

3.9 Phylogenetic Tree of C23O

The phylogenetic tree of C23O gene obtained from crude oil-degrading bacteria in this study is presented in Fig. 4. In order to show the evolutionary relatedness of the C23O gene obtained in this study with other C23O genes from bacteria deposited in the NCBI, their sequences were aligned using ClustalW while evolutionary pathway was shown by assigning a root to the tree. MEGA7 software package was used to analyze the evolutionary relatedness [18].

Table 4. Oil-degrading ability of bacterial isolates grown on crude oil

Isolates	Time (hours)				
	24	48	72	96	120
CFfab 1	44.75 ± 1.3^{Db}	48.66 ± 1.7^{Dab}	52.19 ± 1.2^{CDa}	37.45 ± 2.2^{Ec}	36.91 ± 1.3^{Cc}
CFfab 2	43.44 ± 1.0^{Dc}	59.60 ± 1.8^{Ca}	54.45 ± 1.9^{BCb}	44.61 ± 1.1^{Dc}	46.41 ± 1.1^{Bc}
CFfab 3	47.63 ± 1.7^{Dc}	58.35 ± 2.2^{Ca}	51.71 ± 2.0^{CDbc}	54.17 ± 1.0^{Bab}	12.74 ± 0.5^{Dd}
CFfab 5	61.59 ± 2.1^{Ba}	62.30 ± 2.4^{Ca}	57.75 ± 1.5^{Ba}	51.23 ± 0.7^{BCb}	39.13 ± 1.6^{Cc}
CFfab 6	35.56 ± 1.4^{Ec}	45.91 ± 2.3^{Db}	48.77 ± 1.0^{Db}	54.51 ± 0.9^{ABa}	55.56 ± 0.6^{Aa}
CFfab 10	33.71 ± 1.5^{Eb}	57.16 ± 1.7^{Ca}	37.18 ± 1.4^{Eb}	5.75 ± 1.1^{Gc}	3.74 ± 1.4^{Fc}
CFfab 12	54.19 ± 2.1^{Ca}	43.89 ± 1.3^{Db}	50.49 ± 1.1^{CDa}	50.15 ± 2.1^{Ca}	15.76 ± 0.7^{Dc}
CFfab 13	71.62 ± 1.8^{Aa}	69.33 ± 1.9^{Ba}	70.23 ± 1.7^{Aa}	58.17 ± 1.5^{Ab}	38.16 ± 0.8^{Cc}
CFfab 14	55.55 ± 1.3^{Cc}	78.92 ± 0.9^{Aa}	68.54 ± 1.9^{Ab}	18.11 ± 0.5^{Fd}	14.25 ± 1.3^{De}
CFfab 15	55.45 ± 1.5^{Cb}	60.14 ± 1.5^{Ca}	53.46 ± .1^{BCDb}	42.24 ± 1.4^{Dc}	7.17 ± 1.0^{Ed}

The mean ± SD with the same lower case alphabets are not significantly different at (p<0.05) along the row
The mean ± SD with the same upper case alphabets are not significantly different at (p<0.05) along the column

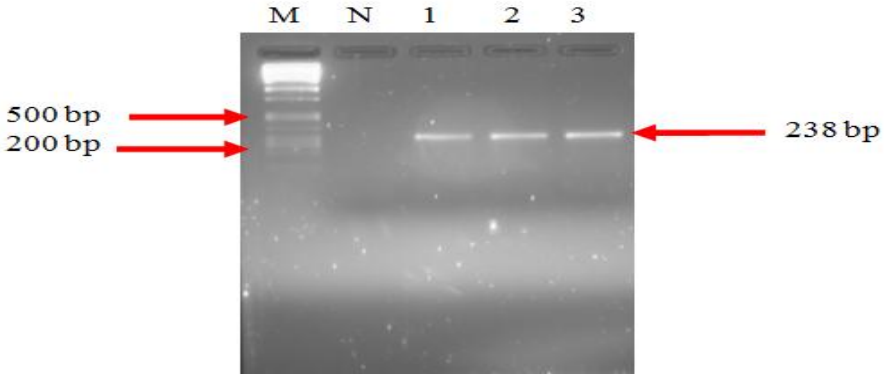

Fig. 2. Amplification of C23O gene in oil-degrading bacteria
Legend: M- Pst I marker; N- Negative; 1- CFfab 5; 2- CFfab 14; 3- CFfab 15

Table 5. Nucleotide BLAST results of C23O genes from oil-degrading bacteria

S/N	% Identity	Accession no	Organism	Description
1	100	DQ133708.1	Uncultured bacterium clone Diox Therm 17-05	Uncultured bacterium clone Diox Therm17-05 catechol 2,3 dioxygenase (C23O) gene, partial cds
2	100	DQ133705.1	Uncultured bacterium clone Diox Therm 17-02	Uncultured bacterium clone Diox Therm17-02 catechol 2,3 dioxygenase (C23O) gene, partial cds
3	100	DQ133704.1	Uncultured bacterium clone Diox Therm 17-01	Uncultured bacterium clone Diox Therm17-01 catechol 2,3 dioxygenase (C23O) gene, partial cds
4	100	DQ133703.1	*Pseudomonas fluorescens* clone Diox Ctrl-02	*Pseudomonas fluorescens* clone Diox Ctrl-02 catechol 2,3 dioxygenase (C23O) gene, partial cds
5	100	DQ133702.1	*Pseudomonas fluorescens* clone Diox Ctrl-01	*Pseudomonas fluorescens* clone Diox Ctrl-01 catechol 2,3 dioxygenase (C23O) gene, partial cds
6	100	AY513289.1	Uncultured bacterium clone 03/1	Uncultured bacterium clone 03/1 catechol 2,3 dioxygenase (C23O) gene, partial cds

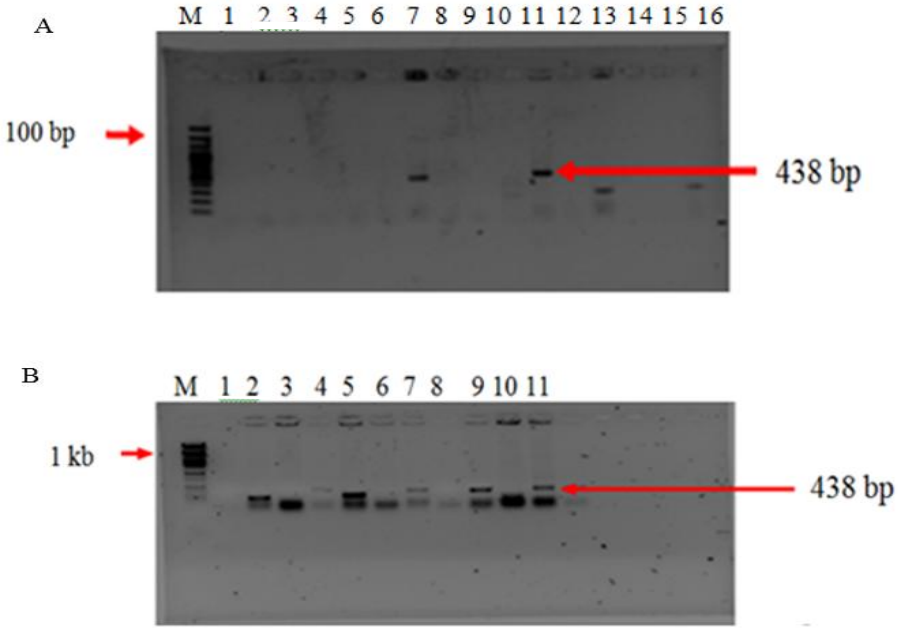

Fig. 3. Colony PCR of inserts with M13 F and M13 R primers

Legend: A: 1 – 16 are clones
M is Molecular weight marker of 100 bp ladder
B: 1 – 11 are clones
M is Molecular weight marker of 1kb ladder

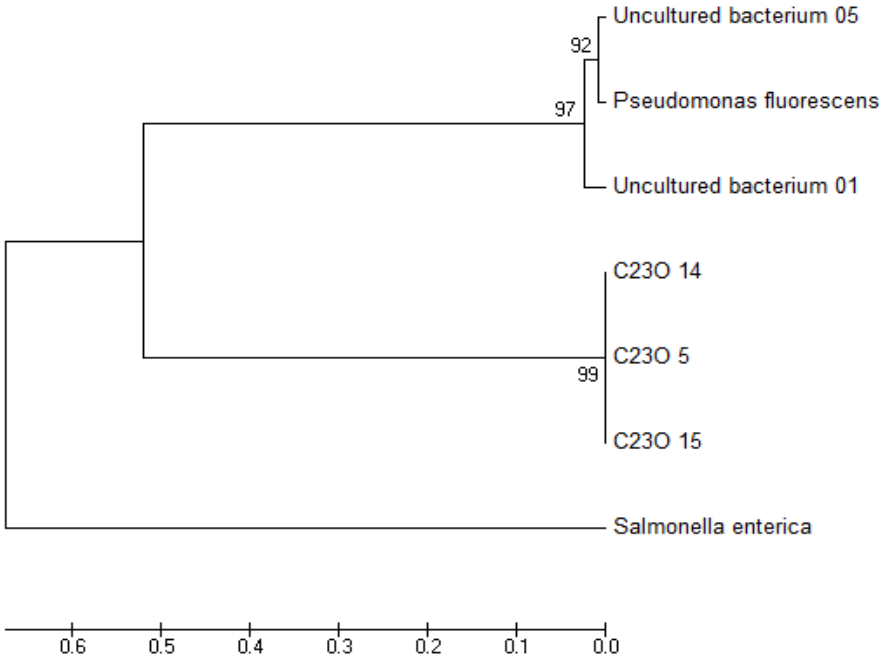

Fig. 4. Phylogenetic tree of C23O gene obtained from crude oil-degrading bacteria

The branch length in the tree represents evolutionary distances and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (300 replicates) are shown next to the branches [20]. It could be observed from the phylogenetic tree that Uncultured bacterium-05 and *P. fluorescence* l form one clade, Uncultured bacterium-01 forms another, while *CFfab* 5, *CFfab* 14 and *CFfab* 15 also form a clade. This clearly shows their evolutionary relatedness. *Salmonella enterica* which does not belong to the group of oil-degrading bacteria was used as an outgroup to root the tree.

4. DISCUSSION

In this study, bacterial isolates obtained from both soil and water confirms the ubiquitous and diverse nature of bacteria and also, their ability to cope with extreme environmental conditions, in this case, crude oil pollution [21]. The high population of oil-degrading bacteria indicates that indigenous bacteria in the polluted soil and water samples have utilized part of the oil for their energy and carbon sources [22]. The unpolluted samples had the least numbers of oil-degrading bacteria in this study. This low population may probably be due to the absence of oil contaminants in the unpolluted soil and water that could stimulate the growth and encourage the multiplication of oil-degrading bacteria in the environment. This finding was confirmed by [23], who reported that the presence of high numbers of certain oil-degrading microorganisms from an environment is an indication that those organisms are the active degraders of that oil. [24] opined that the proportion of microbial populations possessing plasmids containing genes for utilization of oil is increased in such environments.

In this study, the 16S rRNA gene analysis of the oil-degrading bacteria, showed remote homologies (72% - 96%) to the 16S rRNA gene sequences of previously identified bacteria and uncultured bacteria present in environmental samples. These include: *Citrobacter freundii* strain JCM 24061*, Pseudomonas pseudoalcaligenes* strain RW51, Uncultured *Citrobacter* spp. clone F4jan.7, Uncultured bacterium clone aab20d12, Uncultured bacterium clone aab17f10, Uncultured bacterium clone aab17f05, *Enterobacter* spp., *Klebsiella oxytoca* strain ss-11, *Ewingella americana* and *Bacillus megaterium* strain IV22. These oil-degrading bacteria are similar to those reported by [15], who also isolated the genera of *Pseudomonas*, *Bacillus* and *Enterobacter* as oil degraders in their studies. The percent identity scores of ≥97% and ≥99% are used to classify bacteria to genus and species level respectively [25]. In this

study however, the percent identity scores which is less than 97% may be regarded as novel species [26].

The remote identity with Pseudomonas and *Bacillus* genera obtained in this study agrees with the findings of other researchers that these two genera are good biodegraders of hydrocarbons [27,28]. The bacteria identified in this study are Gram-negative with the exception of *Bacillus megaterium*. This agrees with the findings of other researchers that Gram-negative bacteria are higher in hydrocarbon polluted soils [29,30]. According to [31], most Gram-negative hydrocarbon degraders are able to grow on short and medium *n*-alkanes. The presence of *Bacillus megaterium*, belonging to the phylum Firmicutes, in crude oil polluted soil and water has been reported by several authors. They are able to survive in high levels of crude oil which could have eliminated other bacteria due to their genetic make-up [29]. The partial 16S rRNA gene sequence from the bacterial isolates, when compared with the sequences from the database, revealed that they belong to two main taxonomic lineages (*Proteobacteria* and *Firmicutes*). All of them are members of the domain *Eubacteria* with Gammaproteobacteria being the dominant division. This is in agreement with the findings of [27], who isolated Gammaproteobacteria as dominant species of crude oil sludge degrading bacteria and [13], who also isolated from the sediments of Bohai Sea, phylum Proteobacteria comprising mainly Betaproteobacteria and Gammaproteobacteria.

In this study, the oil-degrading bacteria had the capabilities to degrade the crude oil and utilize it for their growth and development.Table 4 presents the degrading activities of the bacteria. All the bacteria, including the bacteria isolated from unpolluted samples, can be said to be active degraders of the crude oil since they were able to utilize the crude oil as their sole source of carbon and energy [32]. This further buttresses the fact that bacteria can cope even in the extreme conditions [21]. The oil-degrading bacteria obtained from the environmental samples are indigenous bacteria, carrying out their metabolic activity, which could be responsible for the bioremediation of the environment [33]. The results showed that the oil-degrading bacteria maximally utilized the crude oil as the major source of carbon and energy. The bacterial cells multiplied within the incubation period mostly between 24 h and 72 h, which brought about the increase in the broth concentration (turbidity) at different rates during incubation period. The increase in the oil degrading activity of the isolates over time until the optimum was obtained means that incubation period affects them during degradation. [34] reported that increase in the concentration of the broth (turbidity) indicates bacterial growth. In this study, the oil-degrading bacteria had the capabilities to degrade the crude oil and utilize it for their growth and development. The wide distribution of *Pseudomonas*, *Enterobacter* and *Bacillus* species as hydrocarbon degraders in this study corroborates the findings of [35], who also found the same species as hydrocarbon degraders.

The degenerative primer pair DEG-F and DEG-R designed to amplify the C23O gene fragment from the best crude oil-degrading bacteria gave positive amplification products of 238 bp as shown in Fig. 2. The fragments showed close identity towards other catechol 2, 3 dioxygenase identified in previous studies. The molecular size of C23O gene obtained in this study corresponds with the result of [36] but differs from that of [7] who obtained a molecular size of 924 bp. These differences could be adduced to the difference in the set of primer pairs. In this study, the gene that endowed the oil-degrading bacteria with the potential of oil degradation was identified as catechol 2, 3-dioxygenase (C23O) from the best crude oil degraders: *CFfab* 5, *CFfab* 14 and *CFfab* 15. Following cloning of C23O gene into pGEM -T easy vector and colony PCR, it was observed that some white colonies that were picked did not contain any inserts; some carried the inserts but were false inserts while some revealed the right inserts (Fig. 3). The enzyme C23O is a key enzyme in the degradation of aromatic rings intradiol and extradiol dioxygenase [7] and in the neutralization of petroleum-based compounds [4], thereby, breaking down the toxic compounds in the crude oil into harmless chemicals. The screening for the C23O gene in bacteria further authenticates their involvement in the degradation of crude oil.

The results for complete nucleotides BLAST of C23O gene from crude oil-degrading bacteria are presented in Table 5. The score for the maximum identity for the alignment is 100% for all the oil-degrading bacteria, indicating their close identity with the bacteria deposited in the NCBI [25]. The bacteria found to possess this gene are uncultured bacterium clone Diox Therm 17-01, uncultured bacterium clone Diox Therm 17-05 and *Pseudomonas fluorescence* Diox Ctrl 01.

The statistical analysis showed that there was significant difference ($p \geq 0.05$) in the hydrocarbon degrading population from polluted sites in Ondo and Rivers States and that the incubation period had significant difference on the degrading activity of the isolates at 95% confidence interval.

5. CONCLUSIONS

This study is aimed at screening oil-degrading bacteria for C23O gene that encodes oil degradation by isolating them from crude oil polluted sites in Nigeria. C23O was obtained from best strains with oil degrading activity which confirmed the catabolic ability of the bacteria. The percent identities (72 – 94) % of the oil-degrading bacteria obtained in this study when compared with the database revealed novel bacteria. It is expected that in bioremediation, indigenous microorganisms from polluted environments are screened for the possible existence of this unique gene sequence for effectiveness. Further studies could be conducted on the possibility of cloning this C23O gene into other bacteria for more efficiency and effectiveness in the bioremediation process.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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