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Phylogenetic analysis of Oil–degrading Bacteria Associated with Polluted Sites in River State, Nigeria.

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ABSTRACT

There is much concern about the effect of oil pollution in the environment which has led to a global interest in the microbial biodegradation of pollutants in the recent years. In order to reduce or eliminate the effect of oil spillage on the environment and living organisms using biological process, a study was carried out to isolate oil-degrading bacteria associated with oil-polluted lands in River State, Nigeria. Crude oil was supplied to the enrichment culture as a sole carbon and energy source. The degradative ability of the isolates was determined by optical density. A total of 10 oil-degrading bacterial strains were isolated from soil and water samples polluted with oil in River state. All isolates were characterized by molecular method. Phylogenetic analyses of 16S rRNA gene sequences were examined in order to determine the evolutionary relationships of the isolates. Six species belong to the gamma subdivisions of Proteobacter, one specie belong to the division Firmicutes (Gram +ve bacteria) while three species could not be assigned to any known phylum. The 16S rRNA analysis revealed that the isolates belonged to the genera Citrobacter, Enterobacter, Klebsiella, Aeromonas, Ewingella and Pseudomonas. The percentages of Proteobacteria and Firmicutes in soil were greater than those in the water samples. The application of molecular methods for rapid detection of diverse strains of oil-degrading bacteria for bioremediation process is of great relevance in promoting a sustainable development of our environment with low environmental impact.

Keywords: 16S rRNA, phylogenetic analysis, oil-degrading bacteria, molecular techniques, bacteria

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INTRODUCTION

Nigeria, the largest country in Africa with total area of 923,768sq km is located between latitude 4º and 14º north and longitude 2.45º and 14.30º east. The oil industries in Nigeria are primarily located in the Niger Delta which is a coastal area, situated in the South-east of Nigeria. Subsistence farming and fishing are the mainstay of the people, out of which, 75 per cent rely on natural endowments for a living. However, the growth of the oil industry coupled with a population explosion and lack of environmental regulations, has led to substantial damage to Nigeria's environment especially in the Niger Delta region. This in turn, has caused a significant tension between the people and the multinational oil companies operating in the area (Agbogidi *et al*., 2005a).

The greatest environmental problem connected with crude oil exploration in Nigeria is oil spill both on-shore and off-shore (Mandri and Lin, 2007). Crude oil, because of its characteristics, is one of the most important pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem, resulting in the contamination of drinking water, poisoning and killing of aquatic life, thereby, placing hardship on those who make

their living by fishing. Oil pollution produces ecological problems of great dimension. It affects soil fertility adversely, causes alterations in soil physicochemical and microbiological properties (Agbogidi *et al*., 2005b).

Physical, chemical and biological techniques are the various methods employed to deal with large scale oil spill but the most promising of them, is bioremediation. Bioremediation is defined as the use of organisms or their enzymes to provide an effective alternative (Singh *et al*., 2001) or to return the environment altered by contaminants to its original condition (Okon and Trejo-Hernandez, 2006). Microorganisms are known to attack and digest the oil. This report deals with the isolation of oil-degrading bacteria. They are capable of using organic substances, natural or synthetic, as sources of nutrients and energy hence, exhibiting remarkable range of degradative capabilities (Dua *et al*., (2002). Oil-degrading microorganisms are ubiquitous in the environment, particularly in the oil-polluted sites. Both fungi and bacteria have been found to be useful in bioremediation process, even though many researches have been on bacteria in the recent times. Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade many different contaminants (Wackett, 2003). The oil-degrading populations are widely distributed in the lands and water bodies. In a research carried out by Ojo (2006), oil-utilizers detected include *Bacillus megaterium*, *Pseudomonas putida*, *Micrococcus luteus*, *B. brevis*, *B. pumilis* and *Enterobacter aerogenes*. The traditional method of identification of bacteria includes isolation, morphological and biochemical characterization. This forms the basis of classification for bacterial genera (Cowan and Steel, 1993) and has been used for many decades, however, with its limitations (Wahler and Reymond, 2001). Identification and the ecological spread of environmental bacteria are often complex to analyze using conventional methods. As a result, there are some microorganisms that are still uncultured (Kamagata and Tamaki, 2005). Only about 1% bacteria can be detected from many environments when traditional culture-based approaches are used (Kamagata and Tamaki, 2005). As a result of this, new strategies and approaches are being implemented for the rapid, sensitive and specific detection of microorganisms in the environment. The molecular analyses have been found to be more appropriate than the traditional approaches because these involve culture-independent analyses such as genotyping the 16S rRNA genes, fluorescence in situ hybridization (FISH) (Zwirglmaier *et al*., 2005), the use of genetic probes, polymerase chain reaction (PCR) and metagenomics (Josephine *et al*., 1991). Molecular identification involving rRNA enables identity of novel sequences and diversity to be discovered. It also reveals information about metabolic life style of microorganisms which are not amenable to pure cultures (Hugenholtz *et al*., 1998). Maslow *et al*. (1993) reported that 16S rRNA analysis for characterization of bacteria is excellent because the technique depends on the examination of genetic diversity of isolates.

In this study, the indigenous degrading bacteria from oil polluted sites in River State, Nigeria, were isolated and identified using molecular techniques, namely DNA isolation, PCR amplification of 16S rRNA, sequencing and phylogenetic analysis of partial 16S rRNA fragments.

MATERIALS AND METHODS

2.1 Sampling sites and collection of samples

Six oil-contaminated environmental samples were collected from three different flow stations in River State, Nigeria as follows: Agbada-Aluu in Ikwerre Local Government Area; Obite in Ogba/Egbema Local Government Area and Bonny in Bonny Local Government Area. The water samples were collected aseptically into screw-capped containers while the soil samples were collected into sterile cellophane bags.

2.2 Growth medium

The Bushnell-Haas Broth (BHB)used for the enrichment culture contained 0.20g Magnesium sulphate, 0.02g Calcium chloride, 1.00g Monopotasium phosphate, 1.00g Dipotasium phosphate, 1.00g Ammonium nitrate, 0.05g Ferric chloride in 1L distilled water. The BHB was supplemented with 2% (v/v), petrol, diesel, paraffin and crude oil which were filter-sterilized by a membrane filter (0.22μm).

2.3 Measurement of Degradative activity of the isolates

The BHB supplemented with various 2% (v/v) oil (petrol, diesel, paraffin and crude oil) were separately inoculated with each isolate incubated for 7 days at 30^{o}C during which the optical density of each cultured medium was quantified with spectrophotometer at 600nm and the degradative activity (Unit/mL/hr) was calculated (Boboye et al., 2010).

2.4 Bacterial DNA preparation
Total genomic DNA was extracted from bacterial pellet of 24hr old culture by using a lysozyme/proteinase K/SDS treatment followed by standard phenol/chloroform extractions (Wang et al., 2007). The concentration of the DNA was measured using a Spectrophotometer (Nanodrop). The DNA extracts and a standard marker- Lambda (λ) PstI molecular size marker (kbp) were electrophoresed on a 1% agarose gel.

2.5 Polymerase chain reaction (PCR)
The DNAs were used for PCR amplification with the Gene Amp® PCR System (Applied Biosystems, USA). All treatments were carried out in triplicates. The 16S rRNA genes were amplified by polymerase chain reaction using the primers E9F (5'-GAG TTT GAT CCT GGC TCA-3') (Farrelly *et al*., 1995 and U151OR (5'-GGT TAC CTT GTT ACG T -3') (Reysenbach and Pace, 1995) targeting 7-26 and 1490-1512 nucleotides respectively. The PCR thermal cycling programme used was as follows: .initial denaturation at 95^{o}C for 5min; 30 cycles of denaturation, annealing and extension at 94^{o}C, 52^{o}C and 72^{o}C for 30s, 30s and 1min 25s respectively, followed by a final extension at 72^{o}C for 10min and kept at a hold temperature of 4^{o}C. The total volume of PCR mixtures contained 10 X reaction buffer (2.5µl), dNTPs (2.5µl), primer (forward and reverse) (2.5µl each), Taq Polymerase (Dream) (0.125µl) and DNA template (1µl) of approximately 10ng. Positive and negative controls were included in the PCR reactions.

2.6 Gel Electrophoresis:
The PCR products were analyzed on a 1% TAE agarose gel and stained with ethidium bromide at 80 V for 45-60 min. The bands were thereafter, visualized using ultraviolet (UV) light transillumination and photographed with a digital imaging system (Alphaimager 2000, Alpha Innotech, San Leandro, CA).

2.7 Purification of PCR amplified 16SrRNA gene:
With the aid of a clean scapel, the PCR product band of 16SrRNA gene that was observed on the gel, was cut under long wavelength (365nm) UV light with minimal exposure time. 200mg of the cutting gel was then transferred to a clean DNase-free 1.5 ml microcentrifuge tube and a commercial PCR DNA and Gel Band Purification kit (GE Healthcare illustra^{TM} GFX^{TM}) was used to purify the PCR products according to manufacturer's instructions.

2.8 Sequence analysis:
The 16S rRNA gene products (forward and reverse strands) of 10 bacterial isolates were sequenced. The clean PCR products were subjected to cycle sequencing in both direction using universal primers. The sequence was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (Applied Biosystems). 16S rRNA sequences obtained were compared to those stored in the NCBI (www.ncbi.nlm.nih.gov) database using the basic local alignment search tool (BLAST) algorithm (Morgulis *et al*., 2008).

2.9 Phylogenetic Analysis
The bacterial 16S rRNA gene sequences obtained from this research work coupled with the sequences retrieved from the database were aligned using the software ClustalX 2.0.5 while the trees were constructed using Neighbour joining method. The analysis was performed with the software BioEdit while the output trees were prepared using the software Treeview 1.66 (Page, 1996).

2.10 Statistical Analysis
Numerical data obtained for the degradative activity were subjected to Analysis of Variance (ANOVA).using SPSS. Duncan's New Multiple Range Test was used to separate the means.

RESULTS

3.1 Oil-degrading bacterial isolates
A total of 15 bacterial strains (*CFfab 1- CFfab 15*) were isolated from samples collected from oil-polluted sites. All the strains were able to grow on oil (petrol, diesel and crude oil). *CFfab 14* was observed to demonstrate the greatest ability for petrol, diesel and crude oil.

3.2 Degradative ability of the isolates

Figures (1-3) show the ability of the bacterial isolates to biodegrade petrol, diesel and crude oil. The degradative activity of the fifteen isolates showed that all the isolates are capable of degrading petrol, diesel and crude oil, although the rates of degradation varied. The statistical analysis showed that given similar conditions, there was significant difference in the degradative activity of the isolates on the three types of oil at 95% confidence interval and that the incubation period had significant difference on the degrading activity of the isolates at 95% confidence interval. All the isolates degraded the hydrocarbons, suggesting similarities in the gene that encodes degradation in their genetic make-up.

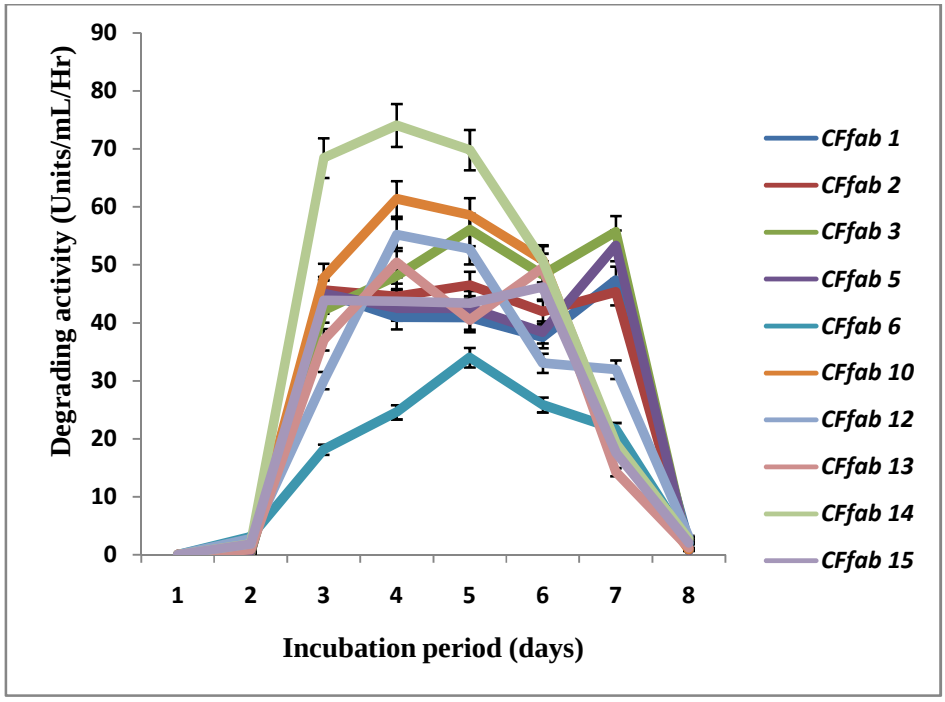

Figure 1: Oil-degrading activity of bacteria grown on petrol

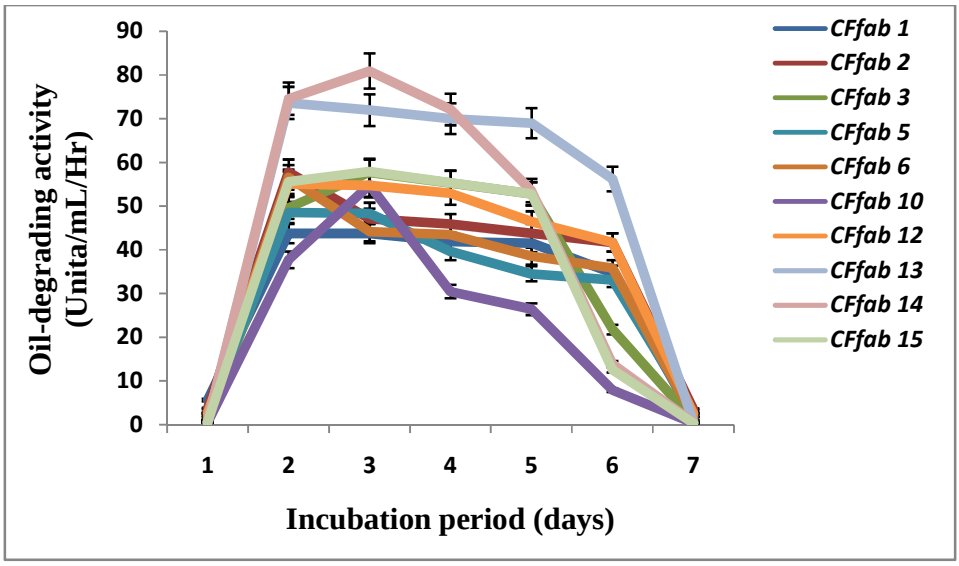

Figure 2: Oil-degrading activity of bacteria grown on diesel

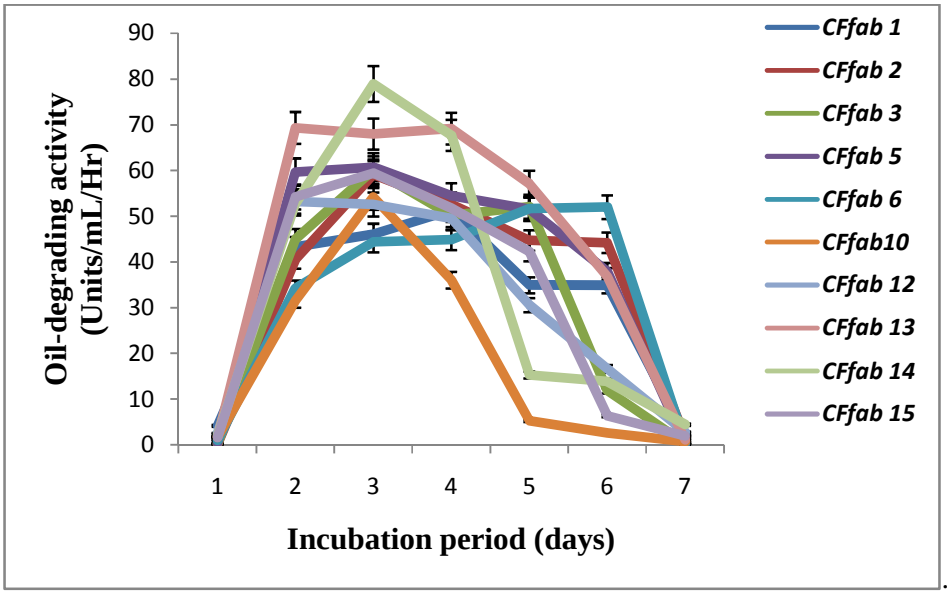

Figure 3: Oil-degrading activity of bacteria grown on crude oil

Table 1: Complete nucleotides blast of genes from oil-degrading bacteria isolated from oil polluted soil and water

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

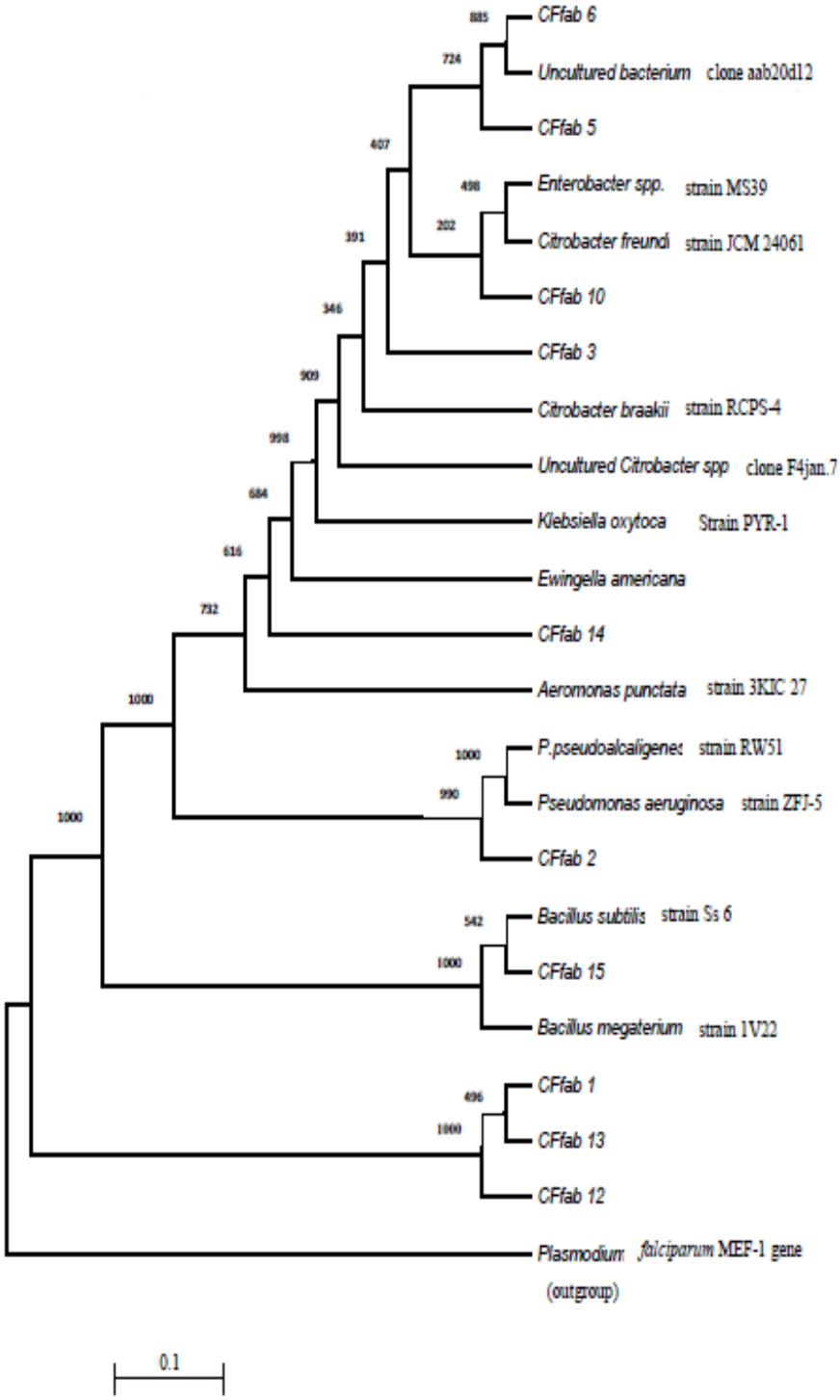

Figure 4 : Phylogenetic tree based on partial 16S rRNA gene sequences of oil- degrading bacteria.

3.3 PCR Amplification of 16S rRNA

The universal primers (E9F and U1510R) successfully amplified the 16S rRNA gene from all the bacteria isolated from the environmental samples. The size of the rRNA gene products obtained for all the fifteen isolates were the same as there were no conspicuous variations noticeable amongst them. However, the size of the PCR amplified 16S

rRNA gene product of all isolated bacteria investigated in this study was approximately 1.5 kb to the relative DNA size marker. Gel purification kit was used to purify the amplified 16SrRNA gene products.

3.4 Alignments of the nucleotide sequences from the Genbank

The 16S rRNA sequences of the bacteria obtained after sequencing were edited and aligned with the genes (16S rRNA) from the other bacteria on the NCBI data bank, using ClustalX2 and BioEdit program. The complete nucleotides blast of genes from the oil-degrading bacteria isolated from oil polluted soil and water are presented in table 1. Alignments of these sequences showed significant similarity (≤ 96%) to previously identified bacteria and uncultured bacteria present in environmental samples.

3.5 Phylogenetic Analysis

The partial 16S rRNA gene sequence from the 10 bacterial isolates when compared with the sequences from the database revealed that they belong to three taxonomic lineages (Figure 4). Six species (*CFfab 2*, *CFfab 3*, *CFfab 5*, *CFfab 6*, *CFfab 10* and *CFfab 14*) belong to the gamma subdivisions of *Proteobacteria*, one specie (*CFfab 15*) belong to the division Firmicutes (Gram positive bacteria), three of the isolates (*CFab 1*, *CFfa 12* and *CFfab 13*) could not be assigned to any known phylum. For instance *CFfab 12* is only 95% similar to the closest sequence in GeneBank (Accession No. gbDQ816407.1),

which is derived from an uncultured bacteria detected from environmental sample. All of them are members of the domain *Eubacteria* with *gamma Proteobacteria* (60%) being the dominant division. *Plasmodium falciparum* which does not belong to the domain, *Eubacteria* was used as an outgroup to root the tree. Sequences from four isolates had a similarity equal or higher than 95% with other 16S rRNA sequences from the database, two had less than 90% similarity and the remaining were between 90 and 94%. The 16S rRNA analysis revealed that all isolates had similarities with the genera *Citrobacter, Enterobacter, Klebsiella, Aeromonas, Ewingella, Pseudomonas,* and uncultured *Bacterium* clone. The percentages of *Proteobacteria* and *Firmicutes* in soil were greater than those in the water samples.

DISCUSSION

The preliminary test for the degrading capabilities of these bacteria on different oil (petrol, diesel and crude oil) revealed that the bacteria isolated from the soil and water samples were able to multiply within the days of study, indicating that they were able to utilize the oil for their growth and development, hence the concomitant increase in the concentration of the BH broth (turbidity). This gradual increase in the concentration of the broth indicates bacterial growth (Aneja, 2007), hence degradation of oil, mostly between days 1 and 3 for both diesel and crude oil; 1 and 4 for petrol; and gradual decline in the concentration of the broth suggests decrease in the bacterial population and that the oil has been degraded, mostly between days 4 and 7. The presence of oil-degrading bacteria in the polluted soil and water is a clear indication that the indigenous bacteria were carrying out their metabolic activity using the added oil in the growth medium as the sole source of carbon and energy. It is evident from this study that bacteria isolated from oil polluted sites were capable of multiplying in the enrichment medium supplemented with 2% crude oil. The activities of these bacteria could be responsible for the bioremediation of the environment which is in agreement with the findings of Ojo (2006).

The amplification fragments were about 1.5kb each. The results obtained from the conserved sequence of the 16S rRNA coupled with the nucleotide sequence, revealed that the isolated bacteria are closely related to *Citrobacter freundii*, *Pseudomonas pseudoalcaligenes*, *Enterobacter* spp., Uncultured *Citrobacter*, Uncultured bacterium clone, *Klebsiella oxytoca*, *Ewingella americana*, and *Bacillus megaterium.* The high percentage similarities that the bacterial isolates have with the blast mean that they are closely related to each other. Some of the bacteria obtained in this study have been isolated by other researchers such as Ojo, (2006) and Boboye *et al*., (2010) by means of traditional techniques. *Ewingella americana* which was found to exhibit the highest degradative activity in the three oils has not been reported to be involved in oil degradation. It is suggested therefore, that these indigenous bacteria are potential degraders of oil in contaminated sites and could be employed during *in situ* bioremediation or bioaugumentation, which is in agreement with the suggestions of Ghazali *et al*. (2004) and Das and Mukherjee (2006).

In constructing the phylogenic tree, the tree was rooted by assigning a root to the tree to show the evolutionary pathway. The bootstrap values were calculated using the software, this value provides a measure of the reliability of

the phylogenetic tree (Hall 2001). Galtier and Gouy distance based method was used in the construction of the tree (Galtier and Gouy**,** 1995). The scale bar of the trees represents a 0.1% difference in nucleotide sequences. The tree showed that the isolates clustered with their respective matches from the database. Bootstrap values of 1000 (maximum) were displayed, indicating that the higher the bootstrap value, the more reliable the phylogenetic analysis.

The molecular analyses method of identifying bacteria to gene level have been found to be more reliable than the traditional approaches because the technique depends on the examination of genetic diversity of isolates. Molecular data generated in this study have shown to be more suited to phylogenetic studies than phenotypic data. This is because all organisms are similar at the molecular level as they all use the same 20 amino acids in their proteins and the same nucleotides in their DNA.

CONCLUSION

Most bacteria obtained from this study are common to other soil environments, but may have adapted to the oil polluted habitat. Bacteria such as *Ewingella americana* and *Klebsiella oxytoca* with high degradative ability which have not been implicated for oil-degradation, were obtained in this study using molecular techniques. The ability of the strains to degrade oil is clear evidence that their genome harbors the relevant degrading gene. The application of molecular methods for rapid detection of diverse strains of oil-degrading bacteria for bioremediation process is of great relevance in promoting a sustainable development of our environment with low environmental impact. A phylogenetic description of oil-degrading bacteria associated with oil polluted sites helps to understand the evolutionary relationships of these bacteria. These oil-degraders are potential organisms for intrinsic bioremediation. The results obtained in this research work can be used as a starting point for in-depth studies, particularly in the area of identifying the gene responsible for degradation of oil from these bacteria. Confirmation on the degradation of oil can father be investigated using Gas Chromatography Mass Spectrometry (GC-MS).

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