THE DOMINANCE OF THE GENERA *Myroides* AND *Proteus* WITHIN HYDROCARBON UTILIZING POPULATIONS IN SOME CRUDE OIL-IMPACTED SITES.

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Abstract: Hydrocarbon utilizing bacteria in environmental samples collected from crude oil-impacted sites at Eleme, Rivers State, and five water samples from effluent discharge points at five different flow-stations in Delta State, all in the Niger Delta were isolated using Bushnell-Haas agar. Bacterial genera tentatively characterized were *Acinetobacter, Alcaligenes, Bacillus, Edwardsiella, Enterobacter, Flavobacterium, Klebsiella, Micrococcus, Proteus, Pseudomonas* and *Staphylococcus.* A portion of the 16S ribosomal ribonucleic acid (16S rRNA) gene of the genomic DNA extracted from each bacterial isolate was amplified (ca.550bp) with polymerase chain reaction (PCR) using the universal primer set 27F: GAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACT to amplify the DNA between positions 27 and 1492 of bacterial 16S rRNA genes- Sequence analysis revealed the presence of distinct known hydrocarbon degrading bacteria like *Myroides odoratus,* diverse strains of *Bacteroides propionifaciens* sp. nov, *Myroides pelagicus, Myroides odoratus* strain BVC 52, *Proteus penneri* strain YAK6, *Proteus vulgaris, Proteus vidgaris* strain knp3, *Proteus vulgaris* strain E14, *Myroides odoratimimus* strain LWD09, *Proteus penneri* strain YCY 34, *Myroides Odoratimimus* strain CM9, *Myroides Odoratimimus* strain YCT1, *Alcaligenes* sp. CRRI 27, *Zooglea ramigera, Alcaligenes* sp. XW3, *Bacteroides caccae, Proteus penneri* strain FFL8 and *Alcaligenes* sp. ICT-6. Sequences of *Myroides* and *Proteus* species were the dominant populations within the hydrocarbon utilizers and have been previously associated with hydrocarbon degradation.

Keywords: GenBank; Hydrocarbon utilizing bacteria; Niger Delta; Polymerase chain reaction; 16S rRNA gene.

Introduction

Crude oil pollution is widespread in the environment and at present is a serious ecological problem facing the Niger Delta region of Nigeria. Over 80% of the country's oil-derived revenue comes from this zone and its surrounding offshore area (Chikere *et al.*, 2012a). Within the Delta, the numerous tank farms, flow stations, pipelines, tankers and loading jetties provide a constant threat of oil pollution. The terrestrial and aquatic ecosystems in this area are generally the main recipients of colossal oil spills which over the years have caused serious damage to soil fertility and loss of the indigenous aquatic heritage (UNEP, 2011). According to a DPR spill data report, 72% of cases of oil spill in Nigeria in 2005 were attributed to sabotage occasioned by bunkering, artisanal refining and pipeline

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Nigerian Journal of Microbiology 2015, 29: 3089-3095
Published online at www.nsmjournal.org

vandalism (DPR, 2006). Biodegradation by microbes is the key cost effective and eco-friendly removal process of hydrocarbons in the soil. It is controlled by hydrocarbon physicochemistry, environmental conditions, pollutant bioavailability and the presence of catabolically active microbes (Stroud *et al.*, 2007; Chronopoulou *et al.*, 2014; Kigigha *et al.*, 2014).

Similar to the microbially mediated breakdown of natural organic matter, biodegradation mediated by indigenous microbial communities is the ultimate fate of the majority of oil hydrocarbons that enter the environment (Kostka *et al.*, 2011). Hydrocarbon-degrading microorganisms are ubiquitous in the environment and biodegradation has been shown to be successful in naturally remediating oil contamination associated with several spills that impacted terrestrial ecosystems (Chikere *et al.*, 2012b). Even less information is available on which members of the microbial community are active in degrading hydrocarbons, and the impacts of various environmental parameters in controlling the activities of indigenous hydrocarbon-degrading microorganisms have not been specifically addressed. Thus, the theoretical basis to understand and predict the dynamics of hydrocarbon-degrading microorganisms *in situ* is lacking (Chikere *et al.*, 2011a; 2011b). Although technologies for oil drilling have advanced rapidly in recent decades, strategies to respond to oil spills and to assess environmental impacts of oil contamination have lagged behind (UNEP, 2011). An understanding of the impacts of oil on indigenous microbial communities and identification of oil-degrading microbial groups using both culture-dependent and molecular approaches are prerequisite for directing the management and cleanup of oil-contaminated ecosystems (Kostka *et al.*, 2011; Korenblum *et al.*, 2012). Thus, the objectives of the present study were (i) to identify and elucidate the ecophysiology of predominant oil-degrading taxa that may serve as model organisms and microbial indicators of contamination and (ii) to characterize *in situ* indigenous bacterial communities in these oil installation facilities using both culture dependent and culture independent techniques.

Materials and methods

Four soil samples labeled A, B, C, and D and sediment samples labeled E, F, G, and H were collected from crude oil impacted sites at Eleme, Rivers State. Five water samples labeled J, K, L, M, and N were obtained from the discharge points of five different flow-stations in Delta State, Nigeria. The soil samples were collected using the composite method with soil auger at 0-30cm depth, from four different points at the contaminated site, and kept in labeled polythene bags. The sediment samples were collected using Eckman sediment grab and were stored in clean-labeled polythene bags. The water samples were collected using water sampler, kept in sterile 500ml plastic cans, and taken to the laboratory for analyses within 6 hours. Ten-fold serial dilution was conducted for all samples and 0.1 ml aliquot was plated out on Bushnell Haas agar using either Okono medium crude oil or anthracene crystals as carbon source for the isolation of putative hydrocarbon utilizing bacteria. Petri dishes were incubated at 30°C for 2 weeks. Bacterial colonies were purified by subculturing and tentatively characterized using a battery of biochemical tests. Genomic DNA was extracted from the pure cultures with

Zymo Research Quick-gDNA™ MiniPrep kit and subjected to PCR using a Gene AMP PCR system 9700 thermal cycler with the universal primer set 27F: GAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACT to amplify the DNA between positions 27 and 1492 of bacterial 16S rRNA genes (numbered according to the *Escherichia coli* rRNA). Denaturation was done at 95°C for 30s while primer annealing was at 50°C for 30s. Initial and final primer extensions were done at 72°C for 2min and 10min respectively. The samples were held at 4°C till further analysis commenced. The PCR products (*ca.*550bp) were cleaned up and sequenced using an ABI Big Dye Version 3.1 kit then they were analyzed on the ABI 3500XL genetic analyzer. Electropherograms were inspected with Chromas Lite 2.01 while sequence identification was performed using GenBank's BLAST algorithm.

Results and discussion

The identities of the hydrocarbon utilizing bacteria as determined using biochemical tests and DNA sequencing after PCR-amplification of the 16S rRNA gene are presented in Table1.

It was observed that about 80% of the identities assigned to the isolates using the phenotypic and culture dependent methods did not match with the GenBank identities of the same isolates as determined using the BLAST algorithm. Most of the species tentatively identified as *Flavobacterium* spp. affiliated with sequences of a new genus *Myroides* which was originally under the genus *Flavobacterium* (Cho *et al.*, 2011). The most consistent result from the study is the fact that all the sequences obtained affiliated with known hydrocarbon degrading bacteria showing more than 90% maximum identity with their closest GenBank relatives. This investigation probably is the first to describe the presence of *Myroides* spp. in oil-inundated sites in the Niger Delta. A number of studies have focused on *Myroides* strains as causative agents in various infections (Yagci *et al.*, 2000; Green *et al.*, 2001; Kallman *et al.*, 2006; Thomas *et al.*, 2007; Bachmeyer *et al.*, 2008; Douce *et al.*, 2008). Attention has also been given to strains belonging to *Myroides* because novel types of metalloenzymes have been reported from various species of the genus (Mammeri *et al.*, 2002; Chen *et al.*, 2009) and biosurfactant compounds have been isolated from strains of *M. pelagicus* (Maneerat *et al.*, 2005; 2006). These reports clearly indicate the ubiquity of members of the genus *Myroides* in nature and their potential as a source of biotechnologically useful products, yet the diversity within the genus remains to be fully unravelled and explored. Strains of *Myroides pelagicus* (94% max. id) (tentatively identified phenotypically as *Flavobacterium* spp. in this investigation) were previously shown by Maneerat *et al.* (2006) to grow on weathered crude oil and also emulsify it. They also demonstrated that the biosurfactant able to emulsify crude oil was excreted in the culture supernatant and was identified as being a mixture of L-ornithine lipids. In addition, *M. odoratos*, *M. odoratimimus* and *M. pelagicus* were shown to produce surface active compounds identified as cholic acid, deoxycholic acid and their glycine conjugates in marine broth 2216 (Difco). Biosurfactants and bioemulsifiers are very useful in hydrocarbon degradation as they help

the degraders to reduce the surface tension of water in the aqueous phase and also make the hydrophobic substrate bioavailable by forming microemulsions that enhance hydrocarbon uptake (Satpute *et al.*, 2010; Das and Chandran, 2011; Das *et al.*, 2014; Wang and Shao, 2014; Singh and Sedhuraman, 2015). In the present study, we report the characterization of novel *Myroides* species isolated during a culture-dependent and independent study of bacterial diversity associated with environments habouring crude oil facilities in the Niger Delta. It is worthy of note to report here that the hydrocarbon utilizing bacteria indigenous to the oil-rich Niger Delta may have the natural propensity to degrade crude oil hydrocarbons and possibly play pivotal role during bioremediation of impacted sites. There is need for further investigations to locate specific functional genes and activities in these extant hydrocarbon utilizing bacteria to enhance their applications in bioremediation of crude oil-polluted areas in the Niger Delta.

Table 1. GenBank sequence identification of hydrocarbon utilizing bacteria from oil installation sites.

Isolate	Tentative phenotypic ID	Closest GenBank relative
5K	*Flavobacterium* sp.	*Myroides odoratus*
5D1	*Pseudomonas* sp.	*Bacteroides propionifaciens*
5D2	*Flavobacterium* sp.	*Myroides pelagicus*
5D0	*Flavobacterium* sp.	*Myroides odoratus* strain BVC 52
5E2	*Proteus* sp.	*Proteus penneri* strain YAK6
5E1	*Proteus* sp.	*Proteus vulgaris*
5K2	*Proteus* sp.	*Proteus vulgaris* strain knp3
5K3	*Proteus* sp.	*Proteus vulgaris* strain E14
5VPT	*Flavobacterium* sp.	*Myroides odoratimimus* strain LWD09
6A1	*Edwardsiella* sp.	*Proteus penneri* strain YCY34
6C2	*Flavobacterium* sp.	*Myroides odoratimimus* strain CMP
6C1	*Flavobacterium* sp.	*Myroides odoratimimus* strain YCT1
6B1	*Pseudomonas* sp.	*Alcaligenes* sp. CRRI27
6G2	*Acinetobcter* sp.	*Zooglea ramigera*
7H2	*Proteus* sp.	*Alcaligenes* sp. XW3
7J3	*Proteus* sp.	*Bacteroides caccae*
6C3	*Pseudomonas* sp.	*Proteus penneri* strain FFL8
7N1	*Pseudomonas* sp.	*Alcaligenes* sp. ICT6

Conclusion

This study demonstrated that there are diversities of hydrocarbon utilizing bacteria in the oil-rich Niger Delta Nigeria. Based on their 16S rRNA sequences, most of the hydrocarbons utilizing bacteria obtained were related to different bacterial genera with predominance of *Myroides* and *Proteus*. In addition, the relevance of these hydrocarbon-utilizing bacteria cultivated by direct plating from each contaminated sample was confirmed by comparing the 16S rRNA gene

sequences of these isolates to the phenotypic identities assigned to them. Using this polyphasic approach, specific microbial populations were reliably identified (Atlas, 2005; Chikere, 2013). It is established that in the majority of the natural ecosystems, the study of their microbial communities, their densities, and their diversity is difficult (Zhou *et al.*, 2015). Because more than 90% of microbes are unculturable due to their diversity, organization in consortia, dynamic and specific cultivation characters, molecular techniques must be used to identify the actual players (microbes) in bioattenuation of hydrocarbon contaminants (Chikere *et al.*, 2011a). These techniques promote the identification, phylogenetic diversity analyses, and the study of the metabolic diversity of the microbial communities present in oil-contaminated sites. It is expected that this method will increase the current knowledge of the bacterial diversity in polluted environments in order to predict the potential for biodegradation in these sites. Such novel cultures need to be preserved for reference, microbially enhanced oil recovery and bioremediation purposes since species of most of these genera produce bioemulsifiers and other value-added microbial products which may be beneficial to the petroleum industry [Cho *et al.*, 2011; Ollivier and Magot, 2005; Gao *et al.*, 2015).

Conflict of interest:

Authors declare no conflict of interest.

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