Global Journal of Science Frontier Research
Biological Science
Volume 13 Issue 6 Version 1.0 Year 2013
Type : Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4626 & Print ISSN: 0975-5896

Degradation of Crude Oil by Bacteria: A Role for Plasmid-Borne Genes

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GJSFR-C Classification : *FOR Code: 279999p*

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Degradation of Crude Oil by Bacteria: A Role for Plasmid-Borne Genes

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Keywords : plasmid DNA, crude oil, biodegradation, bacterial isolates, transformation, plasmid curing.

I. Introduction

The increase in petroleum exploration and production has brought with it an ever increasing rate of environmental pollution involving both terrestrial and aquatic habitat (Mandri and Lin, 2007). Oil spillage in an oil producing country is inevitable. The impact of these wastes in the Niger Delta ecosystems of Nigeria is an obvious environmental concern particularly with regards to the persistence and ecotoxicity of these wastes (Benka-Coker and Olumagin, 1995). Soil and ground water contamination by crude oil are becoming increasingly sensitive issues in Nigeria, since most of her potable water supply is derived from shallow and unconified aquifers. It is therefore important to assess all remediation options on the basis of their ability to remove organic contaminants successfully. This is because most of these product especially the polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic and carcinogenic (Clemente *et al*., 2001).

Prolonged exposure to high concentration may cause the development of live or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al*., 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment crude oil pollution of soil and surface water have been prevalent in Nigeria and other oil producing countries since the commencement of soil exploration and development petroleum industry (Ifeadi and Nwankwo 1987; Okoh *et al*., 2001). Of the many remediation methods, currently in use or under development, bioremediation is viewed as one of the most promising technologies. The microbial by-product of oil biodegradation becomes part of the natural food chain with much of the degraded hydrocarbon material further metabolized by marine organism or incorporated into soil humus without accumulation to toxic materials in the environment (Shoemaker, 1989; Ijah *et al.*, 2003).

The ability of microorganisms to degrade an organic compound is the result ultimately of the genetic makeup of the organisms. The chemical reactions involved in metabolism are mediated by enzymes. The range of enzymes which a bacterium has is a reflection of the specific genetic information in the cell. Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids. The bacterial chromosome is a single circular, highly folded double-strand of DNA. In addition to chromosomal DNA, a larger number of bacteria also have extra-chromosomal DNA in the form of plasmids (Zylstra and Gibson, 1991).

Many plasmids contain genes which code for the enzymes necessary for the derivative pathways important to bioremediation. Enzymes involved in the degradation of toluene, naphthalene, salicylate, octane etc. have been shown to be plasmid encoded (Barbly and Barbour, 1984; Nelson, 1990).

Plasmids are also important in the development (transformation) of new organisms with enhanced degradative capability. Using molecular biology techniques, it is possible to slice pieces of DNA containing genes for specific degradative pathways into plasmids. These plasmids can then be introduced into a host organisms resulting in a recombinant or genetically engineered microorganisms (GEM) with new degradative capabilities (Brook and Madigan, 1991; Brand *et al.* 1992). These are used to bioremediate contaminated sites mainly as organisms for

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bioaugumentation (McClune *et al.,* 1989; Philips *et al.,* 1989; Focht, 1998). Appropriate environmental factors are essential for the performance of these organisms.

Many bacteria of diverse types have extra chromosomal DNA, suggesting the widespread presence of plasmids in prokaryotic cells. Thus the ability to identify plasmids in bacterial systems is important. Both physical agents and chemical agents like surfactants, DNA intercalating agents, antibiotics and metals may increase the frequency of elimination of plasmids in bacteria (Caro *et al.,* 1984). These agents interfere with DNA replication or affect a particular organelle or enzyme of a bacterial cell (Stanisich, 1984).

The goal of this current study is to determine the role of plasmid- borne genes in the crude oil degrading capabilities some selected crude oil degrading bacterial isolates. The possibility of transforming a control organism (*E. coli* K12 DH1) with any isolated plasmid DNA will also be examined.

II. Materials and Methods

a) Sources/Confirmation of Isolates

Six (6) crude oil degrading bacteria previously isolated from different environmental sources (Akpe, 2011) were used for this study. The isolates used were *Pseudomonasfluorescence* isolated from crude oil contaminated soil, *Klebsiella pneumoniae* isolated from ripe paw-paw fruit, *Serratia marscencens* isolated from oil palm mill effluent, *Pseudomonasaeruginosa* isolated from garden soil, *Bacillussubtilis* isolated from rubber effluent and *Enterobacteraerogenes* isolated from cassava mill effluent.

The identities of these isolates were confirmed using morphological and biochemical characteristics. These tests includes gram staring, catalase test, oxidase test urease test, indole test, citrate utilization test and sugar fermentation tests as described Barrow and Feltham, (1986) and Holt, (1994).

b) Plasmid Curing and Isolation

Plasmid Curing was carried out on the isolates using the methods of Winkler *et al*., (1979) and Lexmi*et al*., (1987). The curing agent used was sodium dodecyl/ sulphate (SDS). Physical evidence for the presence or loss of plasmid(s) in curved and non-curved isolates was obtained by alkaline phosphate method of rapid DNA isolation technique of Dillion (1985) and Birnboim and Doly, (1979). This involved four basic steps – cellharvest, lysisdeproteination and decontamination.

The plasmids were characterized using agarose gel electrophoresis (Sambrook *et al*., 1989). The molecular weights of plasmids were visualized using UV transilluminator (AlphaImager TM 2200) at 302-365nm.

c) Transformation Studies on E. coli K-12 DHI with Isolated Plasmid using Calcium

i. *Chloride Method*

In the transformation of *E. coli* K-12 DH1, the organism (*E. coli* K-12 DH1) was inoculated into fresh nutrient broth in a tube and incubated overnight at 37°C. Half a milliliter (0.5ml) of this overnight culture was taken into 20ml of fresh nutrient broth in a flask attached to a rotating shaker and incubated at 37°C until the cell density is $5x10^7$ cell per ml with an absorbance reading of 0.55 at 550 nm. The culture was chilled in ice for 10 minutes and centrifuged at 3000xg for 15 minutes at 4°C. The cell pellets were collected and re-suspended in 5 ml of ice cold 0.1M $CaCl_2$. This was incubated on ice for 20 minutes and centrifuged at 3000xg for 15 minutes at 4°C. Thereafter the cell pellets were re-suspended in 5ml of ice cold 0.1m calcium chloride and incubated on ice for 20 minutes, this was centrifuged as before at 3000xg for 15 minutes at 4°C. Then the cells were re-suspended in another 5 ml of ice cold calcium chloride. 200µl of these competent cells were dispensed into labeled tubes. 40 µl of DNA extract was added to it and the control were placed on ice for 30 minutes. The tubes were removed from ice and placed in a water bath already set at 42°C for 2 minutes (heat shocking) and this enabled the cell membrane of the bacterium to be permeable to DNA). The tubes were removed from the water bath after two minutes and placed back on ice, then 1ml of nutrient broth was added into each of the tubes and incubated (don't shake). After one hour of incubation 150µl from each of the tubes were plated in already prepared mineral salt agar with crude oil saturated filter paper on the plate cover and incubated at 37°C for 24 – 72 hours. The plates viz-a-vis the control were examined. The *E. coli* K-12 DH1 cells able to grow on this medium were said to be transformed. Transformed cells were used for crude oil degradation studies in a liquid mineral salt medium with crude oil as only source of carbon (Mervat, 2009).

d) Crude oil degradation studies/Quantitative Determination of Biodegraded Crude Oil

The crude oil used was Chevron Escravos crude oil obtained from chevron tank farm at Escravos. The mineral salt medium used was that previously described by Mills *et al.* (1978) and modified by Okpokwasili and Amanchukwu (1988). Crude oil degradation studies were carried out on the control strain -*E. coli* K12 DH1, *E. coli* K12 DH1 transformed with *Klebsiella pneumoniae* plasmidgene, *E. coli* K12 DH1transformed with *Serratia marscencens* plasmid gene, original *Klebsiella pneumoniae* isolate, original *Serratia marscencens* isolate, plasmid-cured *Klebsie-llaaerogenes* isolate, plasmid-cured *Serratia marscencens* isolate, consortium of the six selected isolates and the isolated DNA. Samples were inoculated onto 100ml of liquid mineral salt medium containing 1ml

(1%) crude oil and incubated on a shaker at a temperature of 37°C for 21 days. The residual crude oil recovered at the end of the degradation period was analyzed using spectrophotometer (Optima SP-300). The values obtained were expressed as percentage of the original optical density of the crude oil at the onset of the experiment (Oruansi and Ogugbue, 2003).

III. Results

Result of plasmid extraction showed that two of the six selected isolates, had two plasmids each (lanes 1-6 of Plate 1). The isolates were *Klebsiella pneumoniae* (lane 2) from oil palm mill effluent and *Serratia marscencens* (lane 3) from ripe pawpaw fruit. The plasmids as revealed in Figure 7 were of the same size (lanes 2 and 3). The smallest is 300bp while the largest was found to be above 1.5kbp. The results also showed that the plasmids were successfully cured hence the disappearance of the band post plasmid curing as shown in lanes 8 and 9 of Plate 1.

The transformation experiment using the extracted plasmid DNA and competent *Escherichia coli* K – 12 DH1 cells was successful. The percentage degradation of crude oil at 37°C by *E. coli* K – 12 transformed with the plasmid DNA from *Klebsiella pneumoniae* was 93.03% while that transformed with the plasmid DNA from *Serratia marscencens* degraded 76.97% of the crude oil.

The optical densities of the degraded crude oil by the non-cured and cured isolates were 0.095 and 0.272 for *Klebsiella pneumoniae,* 0.198 and 0.265 for *Serratia marscencens* and 0.053 and 0.175 for *E. coli* K-12 transformed with plasmids from *Klebsiella pneumonia* and *Serratia marscencens* respectively. The optical densities of the controls (competent *E coli* K-12 and DNA extracts) were approximately the same as the original crude oil (0.76) meaning there were no degradations. The lower the optical density value the higher the percentage degraded. This is shown in figure 1. It was observed that the loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of degradative ability. It only resulted in reduction in their degradation potential.

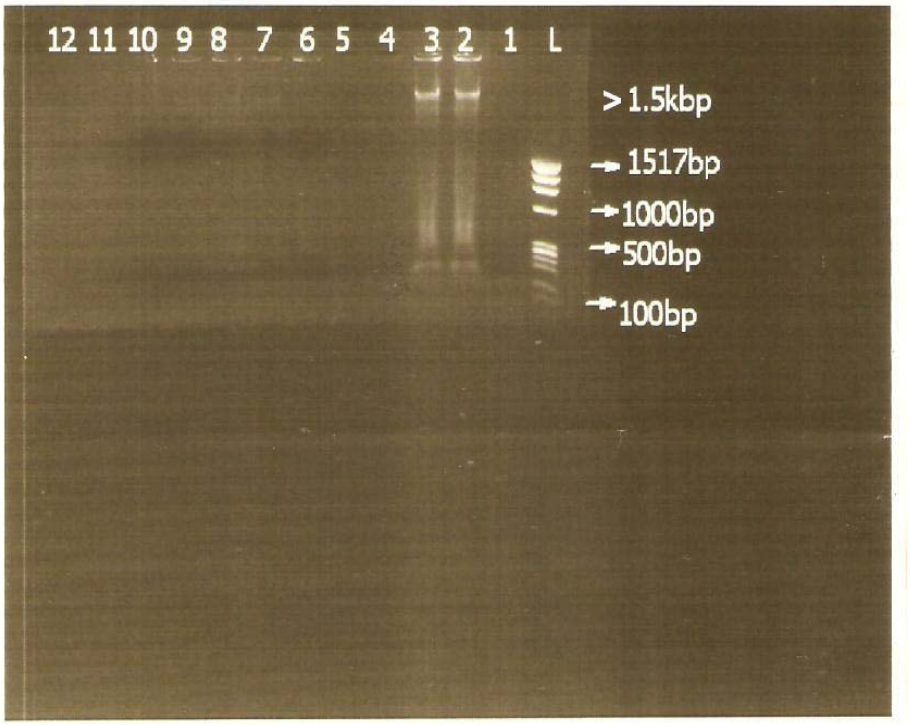

Plate 1: Plasmid profile of cured and non-cured isolates

KEY:

L = 100bp DNA ladder

Lane 1 = Non-cured isolate of *Pseudomonas fluorescens*

Lane 2 = Non-cured isolate of *Klebsiella pneumoniae*

Lane 3 = Non-cured isolate of *Serratia marscescens*

Lane 4 = Non-cured isolate of *Pseudomonas aeruginosa*

Lane 5 = Non-cured isolate of *Bacillus subtilis*

Lane 6 = Non-cured isolate of *Enterobacter aerogenes*
Lane 7 = Cured isolate of *Pseudomonas fluorescens*
Lane 8 = Cured isolate of *Serratia marscescens*
Lane 9 = Cured isolate of *Klebsiella pneumoniae*
Lane 10 = Cured isolate of *Pseudomonas aeruginosa*
Lane 11 = Cured isolate of *Bacillus subtilis*
Lane 12 = Cured isolate of *Enterobacter aerogenes*

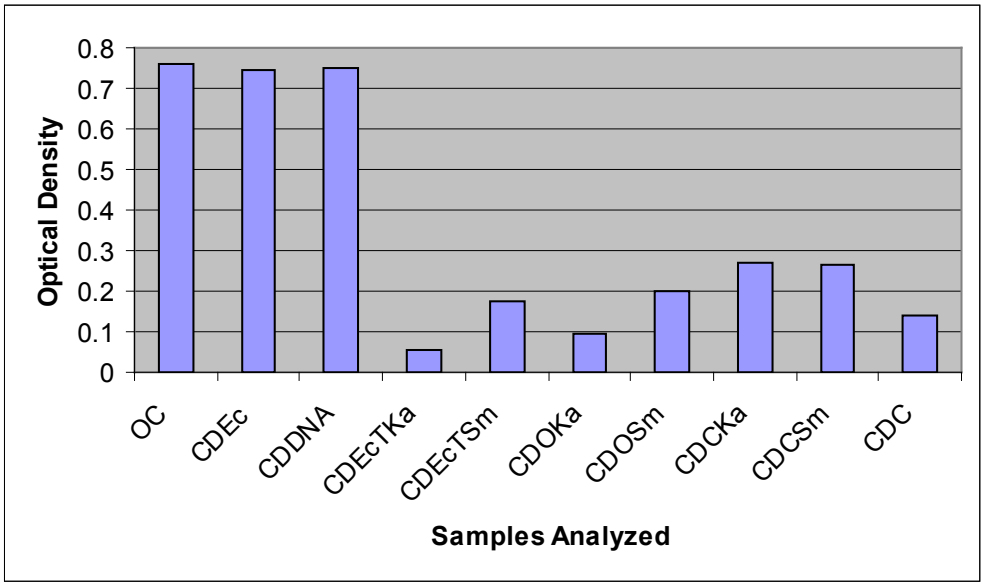

Figure 1: Optical Densities of Original and Degraded Crude Oil at 37°C by Cured and Non-Cured Isolates of *Klebsiella pneumoniae* and *Serratia marscescens* and Original and Transformed *E coli* K12DH1

KEY

OC = Original Crude Oil
CDEc = Crude degraded by *E. coli* K12 DH1
CDDNA = Crude degraded by isolated DNA
CDEcTKa = Crude degraded by *E. coli* transformed with *Klebsiella pneumoniae* plasmidgene
CDEcTSm = Crude degraded by *E. coli* transformed with *Serratia marscescens* plasmid gene
CDOKa = Crude degraded by Original *Klebsiella pneumoniae* isolate
CDOSm = Crude degraded by Original *Serratia marscenscens* isolate
CDCKa = Crude degraded by plasmid-cured *Klebsiella pneumoniae* isolate
CDCSm = Crude oil degraded by plasmid-cured *Serratia marscenscens* isolate
CDC = Crude degraded by consortium of six selected isolates

Note that the lower the OD value, the higher the quantity of crude oil degraded

IV. Discussion

Two of the isolates in this study were found to harbour two plasmids each (Plate 1). This supports the claims and works of Summers and Silver (1978) who reported that in some cases the hydrocarbon degrading capabilities of some bacterial strains are plasmid borne. The presence of plasmids in bacteria has been widely reported. The plasmid that bear genes encoding for enzymes capable of degradation have been a great attraction. These plasmids, known as catabolic plasmids can give the organisms harbouring them the ability to degrade certain compounds. Sayer *et al.* (1990) reported the presence of catabolic plasmids in species of *Pseudomonas, Alcaligenes, Actinobacter, Flavobacterium, Klebsiella, Moraxella* and *Arthrobacter.*

The two plasmids in the two isolates in this study have the same band and their approximate molecular size were 300bp and above 1.5kbp.

These isolates were successfully cured of plasmids using 1% sodium dodecyl sulphate (SDS). The success of this curing as revealed in Plate 1 agrees with the works of Lakshmi *et al.* (1988), Corah *et al.* (2006), Enabulele and Orikpete (2009), Esumeh, *et al.* (2009) and Chu-Hui *et al.* (2011) who independently reported the elimination of plasmids using DNA intercalating agents - SDS, acridine orange, ethidium bromide and other agents. Esumeh *et al.* (2009) for instance isolated *Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa* from ripe pawpaw fruits and *Serratia marscencens* from ripe orange fruits. All these isolates had one plasmid of 23.1 kbp each except *Enterobacter cloacae,* which possessed an additional plasmid of 12.0 kbp. Plasmids obtained in this current study are of smaller molecular weight of 300bp and a little above 1.5kbp, this shows that plasmids borne by bacteria can be of different molecular sizes. These supported the claims by Vahaboglon *et al.* (1996) that microorganisms capable of degrading petroleum hydrocarbon could possess one or more plasmids.

Unlike the findings of Corah *et al.* 2006) whose plasmid-cured isolates lost resistance to tellurite, and Esumeh *et al.* (2009) whose plasmid-cured isolates lost the ability to grow in crude oil medium, the plasmid-cured isolates in this present study retained their ability to degrade crude oil although non-cured isolate recorded higher percentage degradation. However there is a correlation between this present finding and that of Ajayi and Ebeigbe (2009) whose *Staphylococcus aureus* isolate haboured plasmid gene that did not code for antibiotic resistance. This observation suggest that the chromosomal DNA of these organisms also carry genes for crude oil degradation and the ability of the plasmid cured isolates to degrade crude oil was due to the chromosomal genes. The higher or enhanced degradative potential of the non-cured isolates of *Klebsiella pneumoniae* and *Serratia marscencens* suggest that there is a complementary action by the chromosomal and plasmid DNA.

The crude oil degrading potentials of the extracted plasmids were confirmed by using them to transform *E. coli* K12 DH1. The results were successful as the transformed cells became good degraders (Figure 1). Similar findings was reported by Mervat (2009) who successfully transformed *E. coli* K – 12 DH5α with plasmid encoding for methomyl degradation from *Stenotrophomonasmaltophilia* M♀ strains and the transformed cells were able to grow on the contaminant. Also Chu-Hui *et al.* (2011) reported that the isolated ESBLs gene from *E. coli* in their study can be transferred to other organisms.

This study have clearly indicated that the crude oil degrading capability of some microorganisms is as a result of their possession of plasmids and that there was complementary action between the plasmid and chromosomal DNA because bacterial isolates that were cured of their plasmid could not degrade as much crude oil as they degraded when they had the plasmid. It is believed that plasmid isolated can be manipulated to enhance their degradation potentials.

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