Phylogenetic Analysis of Sulphidogenic Bacteria Isolated From Nigerian Crude Oil and Its Produce Water

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Abstract: Phylogenetic analysis of sulphidogenic bacterial isolates from Nigerian Bonny light crude oil and its produce water samples was carried out following preliminary detection of the bacterial species. Samples were enriched in mixed carbon postgate's (MCP) medium and incubated at 55 ºC for 28 days. DNA was extracted from isolated culture and amplified by Polymerase Chain Reaction (PCR) using 16S rRNA eubacterial primers with a GC–clamp. Successful amplification was confirmed by ethidium bromide fluorescence in 1 % agarose gelafter agarose gel electrophoresis. PCR-amplified DNA was separated by Denaturing Gradient Gel Electrophoresis (DGGE) analysis, to show the relationship between samples based on their GC content. Following successful DGGE analysis, DGGE bands were excised, purified and sequenced to display sequence homology and affiliation to related genera of sulphide producers. The DGGE products sequencing was successful, displaying 98 % and 99 % similarity and homology to *Petrotogamexicana* (AY125964.1) and *Petrotogaolearia* (AJ311703.1) respectively. Phylogenetic analysis showed a relationship between the sulphidogenic bacteria with other members of the Thermotogales.

Keywords: *Thermotogales*, Sulphidogenic bacteria, crude oil, produced water, reservoir souring, *Petrotoga Mexicana*, *Petrotogaolearia*

Introduction

The largest energy source on the planet is crude oil (Dunsmore, 2001). Crude oil and refined petroleum products are extremely complex mixtures of thousands of organic hydrocarbons and related compounds. Presently, despite the expense involved in the location, extraction and refining of petroleum,it is the largest energy source on the planet. Studies have revealed the presence of bacteria in numerous deep oil fields (Nilsen *et al*., 1996). It has been widely accepted that bacteria contaminate surface production equipment and among these bacteria, great emphasis has been placed on the presence or absence of sulfate-reducing bacteria (SRB) in most sampling programmes, since they are the most prolific members of the oilfield community and of economic importance to oil field operators (Bass, 2000). The activity of sulphidogenic bacteria has created economically significant problems for the oil industry, encouraging corrosion of iron and steel in storage tanks, pipelines and tanks. In oil reservoirs, permeability reductions may be caused by growth of bacteria in oil-bearing rock pore spaces leading to reduced recovery efficiency (Bass *et al.*, 1993; Herbert and Gilbert, 1984; Iverson and Olson, 1984; Herbert, 1987).

Bacterial isolations and identifications using cultural techniques are time-consuming and prohibitive for large-scale studies. However, current developments in molecular analysis have overcome many of these restrictions and the design and employment of carefully evaluated probes and primers is proving to be of value in identifying bacteria in complex microbial communities. Culture-independent methods based on the analysis of 16S rRNA genes have shown that cultivated microorganisms may represent minor components of microbial communities. These techniques have been used for the characterisation of complex microbial assemblages in wide diversity of environments, including the deep terrestrial subsurface (Chandler *et al.*, 1998). They were used to investigate the microbial diversity in wellhead samples from onshore oil fields in California (Orphan *et al.*, 2000, 2003). Bottom-hole temperatures for the sampled petroleum formations ranged from 50 - 125 ºC. 16S rRNA genes from a wide diversity of thermophilic archaea and bacteria were characterised, including *Methanobacterium, Thermococcus, Petrotoga Thermoanaerobacter, Desulfotomaculum,* and *Anaerobaculum* species. Nevertheless, in addition, to these thermophilic microorganisms, a large number of rRNA gene sequences were related to those of mesophilic archaea and bacteria from a very diverse group of genera (e.g. *Marinobacter, Pseudomonas* and *Desulfovibrio*) (Magot and Ollivier, 2005).

The application of molecular biological methods to investigate the occurrence and distribution of bacteria in the environment has the advantage of providing direct information on community structure (Klein *et al*., 2001; Amann*et al.*, 1995; Head *et al.*, 1998). Conventional culture-based methods of analysis take time as a result of slow bacterial growth rates, and do not necessarily provide an accurate assessment of the types and numbers of bacteria present in a sample. Microorganisms are sensitive to fluctuations and

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changes in their environment. Whenever their chemical or physical environment is suddenly altered, there is a lag period during which the microbial community Sulphate-rich seawater commonly injected into the oil reservoir to enhance secondary oil recovery, stimulates the growth of sulphide producers in the reservoir, with the subsequent release of sulphide, causing reservoir souring and increase in the sulphur content of oil and gas, a major concern in the oil industry (Bass *et al.*, 1993). It is therefore necessary to determine the presence of sulphide producers in crude oil and its produce water as often and as quickly as possible, which is better done using molecular methods. This will assist in the prevention and control of the menance they cause.

The purpose of this study therefore was to phylogenetically analysesulphide-producing bacteria detected in crude oil and produce water and also to determine their relationship with other members of the *Thermotogales.*

Materials and Methods

Sample Collection

Five hundred milliliters each of Bonny Light Crude Oil (Sample A) and its produce water (sample B) were collected aseptically with sterile sample bottles from a dispatch tank and an oil well respectively at Shell Well 9, Awoba flow station in Degema Local Government Area of Rivers State, Nigeria.

Preparation of Enrichment Medium

Mixed Carbon Postgate (MCP) medium, a modification of Postgate's medium (Postgate, 1984) was prepared and used for culture enrichment. The medium contained 1.25 ml sodium lactate (60 % w/v solution, 5.0 g sodium acetate, 1.0 g sodium propionate, 0.4 g sodium butyrate and 25 g sodium chloride per litre of deionized water. pH was adjusted to 7.2, prior to autoclaving at 105 ºC for 20 mins to remove dissolved oxygen. The solution was cooled under nitrogen gas followed by addition of 1ml each of 10 % w/v solutions of sodium thioglycollate and ascorbic acid, and the pH adjusted to 7.2 with 1M NaOH or HCL. The bulk medium was introduced into an anaerobic cabinet (Don Whitley, Yorkshire) where aliquots 90 ml were dispensed into 125 ml injection bottles (Adelphi Tubes Ltd). All vessels were closed in the anaerobic cabinet before being sterilized by autoclaving at 121 ºC for 15 mins.

Inoculation

Ten milliliters of sample A was dispensed into 125 ml sterile bottles containing sterile 90 ml MCP medium. Incubation was carried out at 55 ºC for 28 days. The same procedure was carried out for sample B. All laboratory inoculations into injection bottles were achieved by transfer with sterile hypodermic needles and syringes. Subcultures were made after 28 days incubation into fresh MCP medium contained in sealed injection bottles. Reference strain adapts to the new conditions (Chikere and Okpokwasili, 2004; Nweke and Okpokwasili, 2004).

Desulfobactercurvatus(DSM3379) (10 ml) was dispensed anaerobically into 90 ml MCP in 125 ml sterile bottles, incubated under the same condition described above and used as positive control.

Detection of Microbial Hydrogen Sulphide(H_2S) Production

Preliminary indication of microbial H_2S production from the samples was shown by characteristic black ferrous precipitate (Iron sulphide) which remained visible after agitation of the sample. Quantitative measurement of microbially produced H_2S was determined by the methylene blue method (Cline, 1969).

DNA Extraction

DNA was extracted from 28 days culture of samples A and B in MCP medium at 55 ºC (Sambrook*et al.*, 1989). Bacterial suspensions were centrifuged for 10 minutes at 8500 rpm and then resuspended in 0.6 ml CTAB buffer in sterile Eppendorf tube. Phenol:chloroform:isoamyl alcohol (0.5 ml) pH 8, was added and vortex briefly. This was then transferred to sterile screw cap tubes (2 ml) containing 1 g Zirconnia/silica beads, and bead beating was done at maximum speed of 4600 rpm for 40 sec and later put on ice. This was followed by centrifugation at 13000 rpm for 5 mins and the top aqueous layer was transferred to a new sterile Eppendorf tube and 0.5 ml of phenol: chloroform:isoamyl alcohol was added. This was followed by agitation (vortex) and centrifugation at 13000 rpm for 5 mins. The top aqueous layer was then transferred to a new sterile Eppendorf and 1 ml of 30 % polyethylene glycol(PEG) was added, mixed and left at room temperature for two hours, after which centrifugation at 13,000 rpm for 5 mins was carried out and supernatant discarded. Pellets were dried in air and resuspended in 40 µl ultrapure PCR water and stored in a – 20 ºC freezer

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out on the extracted DNA from cultures of samples A, B and reference strain to separate the DNA fragments by their sizes and to visualise the fragments. 1.0 g of agarose was dissolved in 100 ml of 1 x TAE buffer and heated in the microwave for 30 secs, swirling halfway through. It was allowed to cool and 4 µl of ethidium bromide was added and poured into taped tray. Bubbles were removed with the tip of comb and the comb was inserted and allowed to set for 20 mins. The tape and comb were then removed and the hyperladder and samples were loaded after mixing 5µl of each sample with 2.5 µl loading dye. It was then run at 100 V for 40 mins and viewed under UV light.

Polymerase Chain Reaction (PCR) Amplification

PCR amplification was carried out on DNA samples from cultures of sample A and B from MCP enrichments over five days using 16S rRNA eubacterial primers with a GC-clamp (Muyzer*et al.*, 1993). All PCR reactions were performed using PTC-100 Peltier Thermal Cycler. Each 50 µl reaction mixture contained 25 µlMastermix (50 units/ml Taq polymerase, 400 µlmoles DNTPs, 3mM $MgCl_2$) (Promega), 19 µl ultrapure PCR water, 2 µl of forward primer, 2 µl of reverse primer and 2 µl of DNA template. Amplification was carried out under the following PCR conditions: Initial denaturation at 95 ºC for 5 mins, 30 circles at 95 ºC for 1 min, an annealing at 55 ºC for 1 min, elongation at 72 ºC for 2 mins, with a final elongation step of 72 ºC for 5 mins. Successful amplification was confirmed by ethidium bromide fluorescence in 1 % agarose gel.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

PCR amplified DNA was separated by DGGE analysis according to the method of Muyzer*et al.*, 1993, to show relationships between samples based on their GC content. Bands formed in different samples at the same concentration of urea show similar GC content and can be indicative that samples contain the same species.DGGE bands were excised and purified and sequenced.

Sequence Analysis of DGGE Products

Samples were sequenced after purification. Sequencing was carried out using the same primers for PCR. The primers were freeze-dried using the Edwards Modulyo freeze drier. Analysis of sequences was carried out using the Blast search (Altschul*et al.*, 1990) by aligning sequences obtained with the closest match found in the GenBank, after which phylogenetic analysis was carried out for the blast results.

Phylogenetic Analysis of Sequences

Phylogenetic analysis of the blast results was carried out to determine the relationship of the isolates with other sulphide-producing bacteria. Phylogenetic tree was constructed using the Neighbor-Joining method (Saito and Nei, 1987) with the Mega package (Kumar *et al.*, 2004).

Results and Discussion

Microbial Growth and Extraction of DNA

Evidence of growth and presence of sulphidogenic activity in the enrichment medium (culture turbidity, formation of insoluble black precipitate and sulphide production) was seen. Control samples did not form precipitates. Subcultures of the sample enrichment blackened and produced $H_2S$with time. These observations were found to be consistent with the findings of Dunsmore (2001), who observed that microbial sulphide generation and accumulation was proportional to microbial growth (increase in biomass). DNA extraction from growth cultures of samples A and B was successful as confirmed by agarose gel electrophoresis (Figures 1 and 2). The use of polyethylene glycol during the DNA extraction (Zhou *et al.*, 1996; Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001; Luna *et al.*, 2006), prevented poor DNA yield by humic substan*ces* (Jackson *et al.*, 1997; Wilson, 1997; Braida*et al.*, 2003; Schneegur*tet* al., 2003), sometimes present in environmental samples.

PCR and DGGE Analysis

Successful PCR amplification of the 16S rRNA gene was achieved at day 4 in sample A and days 2 and 4 in sample B (Figure 3). Amplified PCR products were successfully compared for the bacterial diversity at each time interval, and within each sample using DGGE analysis. Figure 4 shows successful DGGE profile of the PCR-amplified DNA obtained from samples A and B. Each stained band in the DGGE profile represented a unique DNA fragment. In sample A, few bands were observed at day 4 which might indicate a low diversity of microorganisms as compared to sample B with more bands observed at day 2 than at day 4, indicating greater diversity of microorganisms. This observation of a number of diverse microorganisms in the samples was similar to those of Leu *et al.*, 1998; Orphan, 2000.

Sequence Analysis: Sequence analysis of the excised bands showed presence of sulphide-producing bacteria similar to the genus *Petrotoga*as shown in Table 1. Eubacterial primers with GC-clamp were used in this analysis (Muyzer*et al.*, 1993). Band 11 showed a fairly high homology (98 %) to *Petrotogamexicana*(AY125964.1) with query coverage of 100 %. Members are anaerobic, thermophilic, xylanolytic, motile rod-shaped bacterium with a sheath-like outer structure. They are Gram-negative and reduce elemental sulphur, thiosulphate and sulphite to H_2S. They have optimum growth at temperature of 55ºC and pH of 6.6. It was first detected in oil-producing well in the Gulf of Mexico. Band 13 showed a high homology (99 %) to *Petrotogaolearia*(Accession number: AJ311703.1) with query coverage of 81 %. They are obligately anaerobic, thermophilic, motile rod-shaped bacteria, surrounded by a sheath-like structure, a feature characteristic of *Thermotogales* (Davey *et al.*, 1993). Optimum temperature for growth is 55 ºC and pH 7.2. They reduce elemental sulphur and cystine, but not thiosulphate or sulphate to hydrogen sulphide. These observations were similar to those of Philips and Lappin-Scott, 1997; Davey *et al.*, 1993, who observed bacteria similar to members of the order *Thermotogales* exhibiting a sheath-like outer structure known as a 'toga'.

Phylogenetic Analysis: Phylogenetic analysis of isolates to indicate the affiliation of the isolates to other known lineages of the *Thermotogales*was successful. Bootstrap values are shown and the scale bar represents 0.05 changes per nucleotide. Phylogenetic analysis (Figure 5) showed that the isolates were affiliated with high bootstrap support to the *Petrotoga* lineage of the*Thermotogales,* displaying 98 % and 99 % similarity and homology to *Petrotogamexicana* (AY125964.1) and *Petrotogaolearia* (AJ311703.1) respectively. Studies have shown the occurrence of sulphide-producing bacteria similar to the *Petrotoga*sp in oil fields and reservoirs, in different continents, suggesting a conserved occurrence of the organisms among continents (Bass, 2000).

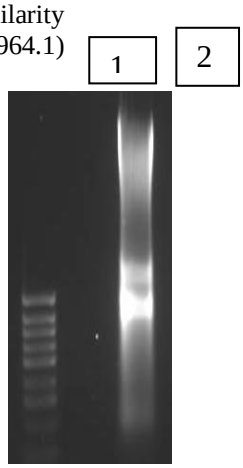

Figure 1: Agarose gel electrophoresis of sample DNA. Lane 1: Hyperladder IV; lane 2: DNA fragment of sample A

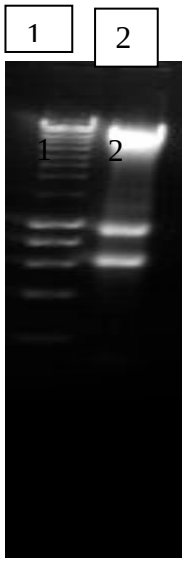

Figure: 2: Agarose gel electrophoresis of sample DNA. Lane 1: Hyperladder I; lane 2: DNA fragment of sample B

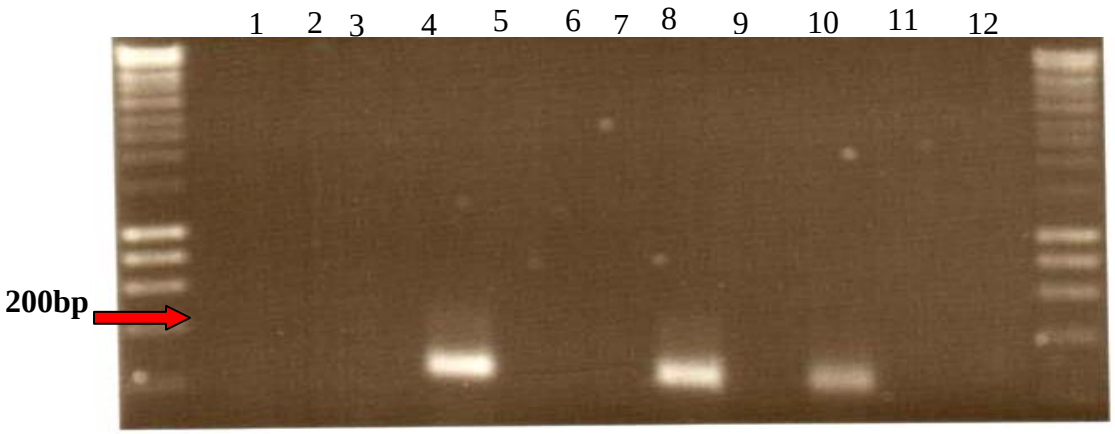

Figure 3: PCR amplification of samples using Muyzer primers. Lanes 1 and 12 Hyperladder I; lane 5: sample A at day 4; lanes 8 and 10 sample B at days 2 and 4

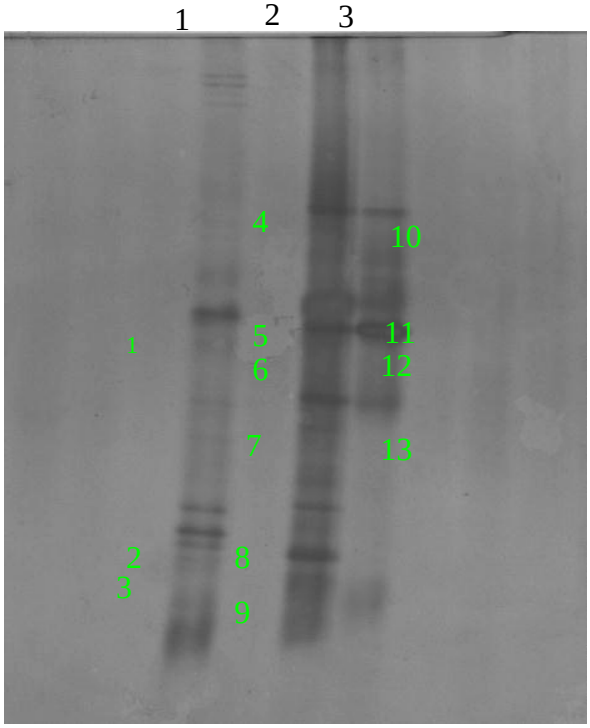

Figure 4: **DGGE analysis of PCR amplified 16S rRNA genes of samples A and B, showing lane 1: Sample A at day 4, lane 2: Sample B at day 2 and lane 3: Sample B at day 4**

Table 1:Sequence analysis

Bands	Homology	GenBank Accession number	Query coverage	Similarity
11	*Petrotogamexicana*16S rRNA gene, partial sequence	AY125964.1	100%	98%
13	*Petrotogaolearia*16S rRNA gene strain SL24T	AJ311703.1	81%	99%

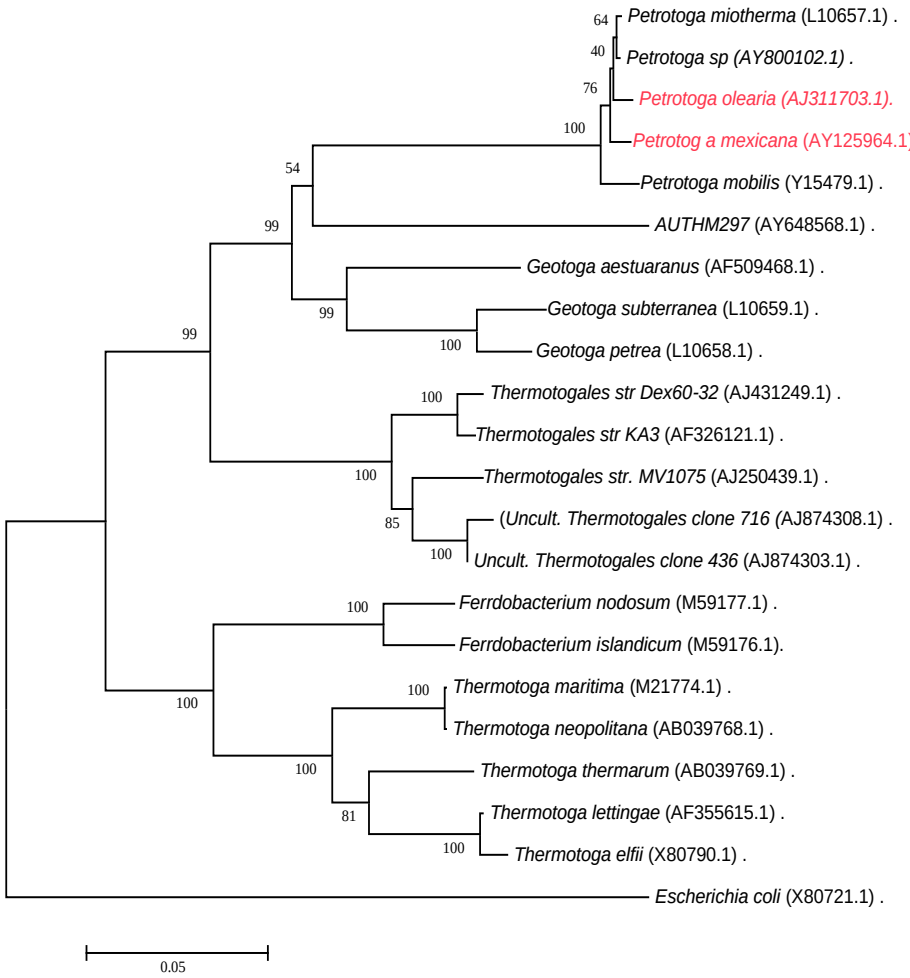

Figure 5: Phylogenetic analysis of isolates showing their affiliation to other known lineages of the Thermotogales.

Conclusion

The presence of sulphide-producing bacteria (sulphidogenic bacteria) responsible for H_2S production, reservoir souring, microbially induced corrosion and loss of oil quality have been detected and phylogenetically analysed in this research, therefore frequent maintenance overhaul for pipelines, flowlines and flowstations, as well as continuous treatment of injected sea water with nitrate as observed by Dunsmore*et al*. (2006), are recommended to prevent the growth of sulphidogenic bacteria.

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