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Phylogenetic and Functional Gene Analysis of Crude Oil and Produced Water-Derived Sulphate-Reducing Bacteria

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ABSTRACT: Phylogenetic and functional gene analysis of sulphate-reducing bacteria (SRB) from crude oil and produced water was carried out using molecular methods. The concentration of hydrogen sulphide (H_2S) produced by SRB, as an indication of SRB presence, was determined using spectrophotometric sulphide assay. More H_2S was observed to be produced at 55 ˚C than at 30 ˚C, with sample B found to produce more H_2S at these temperatures than sample A. This implies more SRB contamination in sample B, possibly from subterranean water. DNA was extracted from enrichment after incubation in Postgate's medium at 30 ˚C and 55 ˚C for 28 days and amplified by Polymerase Chain Reaction (PCR) using primers specific for SRB functional genes. PCR products were cloned and sequenced. Sequence analysis of the clones was found to display 99 % homology to SRB of the genera *Desulfovibrio.* Clones were found to be different species of SRB as shown by the distances between them. Phylogenetic analysis was successful and showed affiliation of the clones to related members of sulphate-reducing bacteria and sulphur-oxidizing bacteria.

Keywords: Hydrogen sulphide, *apsA* genes, phylogenetic analysis, sulphate-reducing bacteria, produced water, crude oil.

Introduction

Sulphate-reducing bacteria (SRB) are problematic in the oil industry where they cause crude oil souring by the hydrogen sulphide (H_2S) they produce and the pitting corrosion of iron and steel in pipelines and tanks (Dunsmore *et al*., 2006; Geesey *et al.*, 2000). They accelerate corrosion in several ways; which include formation of concentration and differential aeration cells, by directly oxidising/reducing metallic atoms/ions and production of corrosive metabolic by-products (Videla, 2001). SRB belong to a mixed group of morphologically and nutritionally diverse, anaerobic bacteria, which utilise sulfate as an electron acceptor for the dissimilation of organic compounds and produce sulphide (Dunsmore *et al*., 2002; Lopes *et al.*, 2006). They are ubiquitous and quantitatively important members in many ecosystems, including anoxic pelagic environments (Teske *et al*., 1996), marine sediments (Ravenschlag *et al.*, 2000), and microbial mats (Teske *et al*., 1998; Minz *et al*., 1999). They are also found as symbionts of marine poly and oligochaetes (Cottrell and Cary, 1999; Dubilier *et al.*, 2001).

Dissimilatory sulfite reductase is the central enzyme in the dissimilatory sulfate reduction and catalyses the final six electron transfer to bisulfite to form sulfide. Dissimilatory sulfite reductase gene sequences of the alpha and beta subunits of the DSR gene, dsrAB, have shown to be a promising and specific approach for investigating the diversity of SRB (Wagner *et al*., 1998; Klein *et al*., 2001; Perez-Jimenez *et al*., 2001; Friedrich, 2002). In addition to dissimilatory sulfite reductase, SRB possess Adenosine-5'-phosphosulphate (APS) reductase (Rabus *et al.,* 1999). The genes for APS reductase, *apsBA*, from bacteria and *aprBA* from archaea encode subunits that appear to form a 1:1 alpha beta-heterodimer. Both subunits of the APS reductase are highly conserved, and the APS reductase genes have been proposed as a useful phylogenetic marker (Ben-Dov *et al.,* 2007).

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Crude oil contains hundreds of compounds and the chemical composition varies between geologic formations (Orisakwe *et al*., 2004). Crude oil and its refined products account for over 90% of Nigeria's national income (Moffat and Linden, 2005). The Niger-Delta region produces more than 80% of the country's crude oil (Odokuma and Dickson, 2003; Okpokwasili and Amanchukwu, 1988; Okpokwasili and Odukuma, 1994). Oil spills in Nigeria occur due to: corrosion of pipelines and tankers (accounts for 50% of all spills), sabotage (28%), and oil production operations (21%, with 1% of the spills being accounted for by inadequate or non-functional production equipment. Flocculants, such as aluminium or ferric sulfate can introduce sulfate into previously sulfate-free systems by the injection of fluids such as sea water. For reasons of corrosion control, steps are taken to remove oxygen from injected water and this provides an environment conducive to the growth of the obligately anaerobic SRB (Magot and Ollivier, 2005). For these reasons, it is common for viable planktonic SRB population densities approaching 1 ml^{-1} to be present in injected seawater (Magot and Ollivier, 2005).

The direct methods for studying SRB include the use of antibodies raised against SRB (Lillebaek *et al*., 1995), an immunoassay for the enzyme APS reductase, and the use of 16S rRNA probes (Daly *et al.*, 2000). Several problems are encountered when they are used *in situ* (Amann *et al*., 1995), for example, rRNA fluorescent probes are difficult to use in sediments or industrial wastewater because of high background autofluorescence of inorganic particles (Muyzer *et al.*, 1995). Furthermore, not all known types of dissimilatory sulphate reducers in environmental samples can be unequivocably identified with the RNA probes (Teske *et al*., 1996). In addition, SRB constitute a heterogenous group, thus, it is not feasible to use 16S rRNA gene as a general molecular marker (Adamczyk *et al*., 2003; Castro *et al*., 2000; Klein *et al.*, 2001; Wagner *et al.*, 1998). Molecular microbial ecology studies involve DNA extraction and purification, followed by an enzymatic reaction or DNA hybridization (Amann *et al.,* 1995; Head *et al.,* 1998). Although many extraction protocols and commercial kits are now available (Steffan *et al.,*1988; Zhou *et al.,* 1996; Miller *et al.,* 1999; Martin-Laurent *et al*., 2001; Luna *et al.,* 2006), they suffer from bias (Martin-Laurent *et al.,* 2001; Niemi *et al.,* 2001; Luna *et al.,* 2006) and from the persistence of contaminants in the purified DNA extract (Schneegurt *et al.,* 2003). These contaminants, particularly humic substances, might preclude PCR amplification (Zhou *et al.,* 1996; Miller *et al.,* 1999; Niemi *et al.,* 2001; Braida *et al.,* 2003), interfere with DNA hybridization (Steffan *et al.,* 1988), and increase the background in microarray hybridization (Lemarchand *et al*., 2005).

Analytical techniques targeting 16S rDNA or functional genes include hybridization-based techniques, such as membrane hybridization (Raskin *et al.,* 1995) and fluorescence in situ hybridization (FISH) (Okabe *et al*., 1999) as well as PCR-based techniques, such as denaturing gradient gel electrophoresis (Muyzer *et al.,* 1993) and cloning-sequencing (Zhang and Fang 2001; Zhang *et al*., 2003a). 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 analyse for SRB functional genes to confirm their presence in crude oil and its produce water and to phylogentically analyse them to indicate their affilation with related members as this will assist in the prevention and control strategies.

In this paper, phylogenetic and functional gene (*apsA*) analysis of crude oil and produced water-derived SRB responsible for H_2S production were carried out, to determine their presence and relationship with other SRB and sulphur-oxidising bacteria (SOB).

Materials and Methods

Collection and Enrichment of Samples: Five hundred millilitres each of Bonny Light Crude Oil (Sample A) and Bonny Light produced water (sample B) samples were collected aseptically from Bonny dispatch tank and oil well respectively into sterile sample bottles from Shell Well 9, Awoba flow station in Degema Local Government Area of Rivers State, Nigeria, for laboratory analysis.

Mixed carbon postgate's (MCP) medium was prepared and used for culture enrichment. MCP medium is a modification of Postgate's medium (Postgate, 1984). It contained sodium lactate (60 % w/v solution 1.25 ml/l), sodium acetate (5.0 g/l), sodium propionate (1.0 g/l), sodium butyrate (0.4 g/l) and sodium chloride (25 g/l). The medium was prepared by dissolving the constituents in 1 litre deionised water, and pH 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 redox agents as follows: 1 ml each of 10 % w/v solutions of sodium thioglycollate and ascorbic acid, and the pH adjusted to 7.2 with 1 M NaOH or HCL. The bulk medium was introduced into an anaerobic cabinet (Don Whitley, Yorkshire) where aliquots were dispensed as required into injection bottles (90 ml aliquots in 125 ml bottles, Adelphi Tubes Ltd). All vessels were closed in the anaerobic cabinet before being

sterilized by autoclaving at 121 ºC for 15 mins. All laboratory inoculations into injection bottles were achieved by transfer with sterile hypodermic needles and syringes.

Inoculation: Ten milliliters of sample A was dispensed into 125ml sterile bottles containing sterile 90 ml MCP. Incubation was carried out at 30 ºC and 55 ºC for 28 days. The same procedure was carried out for sample B. Subcultures were later made into fresh MCP medium contained in sealed injection bottles. Ten millilitres of the reference strain *Desulfobacter curvatus* was dispensed anaerobically into 90 ml MCP in 125 ml sterile bottles, cultured under the same condition and used as positive control.

Spectrophotometric Sulphide Assay: This was carried out according to the method described by Truper and Schlegel (1964) and Cline (1969) and relies on the liberation of sulphide and subsequent development of methylene blue from *N,N*-dimethyl-*p*-phenylene diamine sulphate. A standard sodium sulphide solution, concentration of 50 µg/l, was prepared by dissolving a washed, dried, weighed crystal of $Na_2S.9H_2O$ in 100 ml of 10 % w/v zinc acetate solution. The assay reagent, *N,N*-dimethyl-*p*-phenylene diamine sulphate was prepared by adding 500 ml concentrated hydrochloric acid to 500 ml distilled water and cooling before adding 4 g of *N,N*-dimethyl-*p*-phenylene diamine and 6 g of ammonium ferric sulphate. The reagent was wrapped with aluminium foil and kept in the fridge. A solution of 1 % w/v zinc acetate was prepared with which to fix sulphide in test samples. Four and half millilitres each of a range of dilutions of the standard sulphide solution, containing between 1 µg/ml and 10 µg/ml sodium sulphide as calculated from the mass of dissolved salt in 1% zinc acetate solution, were mixed with 0.4 ml of diamine reagent, mixed and allowed to stand for 5 mins, while methylene blue developed and absorbance was measured at 670 nm using Cecil 3000 spectrophotometer. A standard curve was prepared to calculate sulphide concentrations in samples A and B. Four and half millilitres of 1 % zinc acetate and 0.5 ml of the sample were mixed with 0.4 ml of the diamine reagent and allowed to stand for 5 mins before taking the absorbance as stated above. All experiments were carried out in triplicate.

DNA extraction and agarose gel electrophoresis: DNA was extracted from a 28 days culture of samples A and B in MCP at 55 ºC according to the method described by Sambrook *et al.,* (1989). Agarose gel electrophoresis was carried out on the extracted DNA fragments 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 5 µl of each sample with 2.5 µl loading dye. It was then run at 100 V for 40 mins and viewed under UV light.

PCR amplification of *apsA* gene fragments: PCR of amplification of *apsA* gene fragments in crude oil and produced water was carried out using primers specific for SRB functional genes, (*apsA*) responsible for H_2S production (Ben-Dov *et al.*, 2007). The PCR reaction mixture contained a total volume of 50 µl, 25 µl Mastermix, 2 µl of the forward primer, 0.5 µl of the reverse primer and 2 µl DNA template. The standard thermal profile for amplification was as follows: an initial denaturation step (3 mins, 94 ºC) was followed by 34 cycles of denaturation (30 secs, 94 ºC), annealing (55 secs, 45 ºC) and extension (60 secs, 72 ºC). After a terminal extension (7mins, 72 ºC), the samples were kept at 4 ºC before aliquots of the amplicons (5 µl) were analysed by electrophoresis on 1 % agarose gels and visualized after staining with ethidium bromide using a UVP bioimaging system. A no-template control was included during the PCR reaction. PCR products of the samples were then purified and cloned.

Cloning and Sequencing: Purification of PCR products was carried out using the QIAquick PCR Purification Kit Protocol, designed to purify DNA fragments from PCR reactions. The purified PCR products were cloned into the TOPO vector according to manufacturer's instructions (www.invitrogen.com). This was transformed into chemically competent *Escherichia coli* cells. Plasmid DNA was isolated using the QIA prep spin miniprep kit. Analysis of the plasmids for inserts by restriction analysis was carried out by digestion with restriction enzyme, *Eco* R1 endonuclease and separated by electrophoresis on agarose gel. After staining with ethidium bromide, the bands were visualised on a UV transilluminator to select clones containing the appropriately sized insert. The clones with the correct plasmid insert were then used for sequencing. Sample plasmids were sent to MWG-Biotech in Germany after purification, for sequencing. Sequencing was carried out using the same primers for PCR. The primers were freeze-dried using the Edwards Modulyo freeze drier, before they were sent along with samples for sequencing. Analysis of sequences was carried out using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990) by aligning sequences obtained with the closest match found in the GenBank. Distances between clones were calculated to determine whether the clones were the same or different.

Phylogenetic analysis of sequences
Phylogenetic analysis of BLAST results from sequence analysis of samples to indicate the affiliation of the isolates to some members of the SRB and SOB was carried out. Construction of phylogenetic tree was done using the Neighbour-Joining method (Saito and Nei, 1987) with Mega package (Kumar *et al.,* 2004).

Results and Discussion

H_2S Assay: Spectrophotometric sulphide assay was successful to detect H_2S produced by SRB. Volatile sulphide which may escape as gas was precipitated as insoluble zinc sulphide and on addition of *N*,*N*-dimethyl-*p*-phenylene diamine sulphate, methylene blue develops. Assay was carried out in triplicate and standard curve drawn from the results, was used to determine the concentration of H_2S produced by the samples. Figure 1 illustrates the production of H_2S by sample A in MCP enrichment. It showed that at 55 ºC, more H_2S was produced than at 30 ºC at various time-points. The highest H_2S produced at 55 ºC was 0.6 µg/ml and this was at the fourth time-point of the experiment, as compared to a highest concentration of 0.3 µg/ml produced at 30 ºC. The lowest concentration of H_2S produced at 55 ºC was 0.15 µg/ml at the second time-point, while that produced at 30 ºC was 0.13 µg/ml on the first time-point.

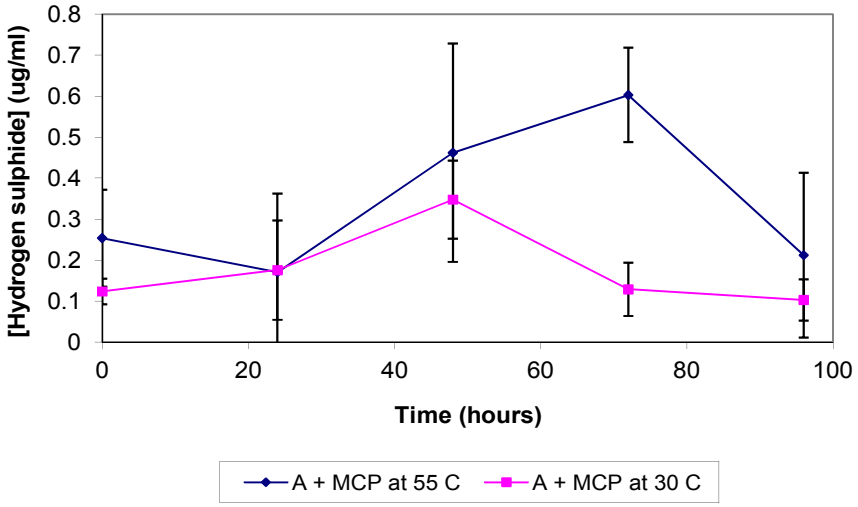

Figure 1: Hydrogen sulphide production by sample A at 30ºC and 55ºC (n = 3)

Figure 2 shows the H_2S production by sample B in MCP enrichment. At 55 ºC, the highest H_2S produced was 30 µg/ml on the fourth time-point while that produced at 30 ºC was 18 µg/ml on the third time-point. The lowest H_2S produced at 55 ºC was 9.0 µg/ml while that at 30 ºC was 7.0 µg/ml. These results showed that sample B produced more H_2S at 55 ºC than at 30 ºC and it produced more H_2S at both temperatures than sample A. These results were similar to those of Bass and Lappin-Scott (1997) who reported marked increase in sulphide production with more available nutrients. There was evidence of growth and presence of sulphidogenic activity in the enrichment medium seen as culture turbidity, blackened particulate material, and sulphide production in the medium. Subculturing from the initial enrichments into further fresh medium was successful. This observation was supported by Postgate (1984) who found that mixed carbon Postgate's medium encourages growth of SRB than any other media for anaerobic sulphidogens. These observations were found to be consistent with earlier findings of Dunsmore (2001), who found that microbial sulphide generation was directly proportional to microbial growth.

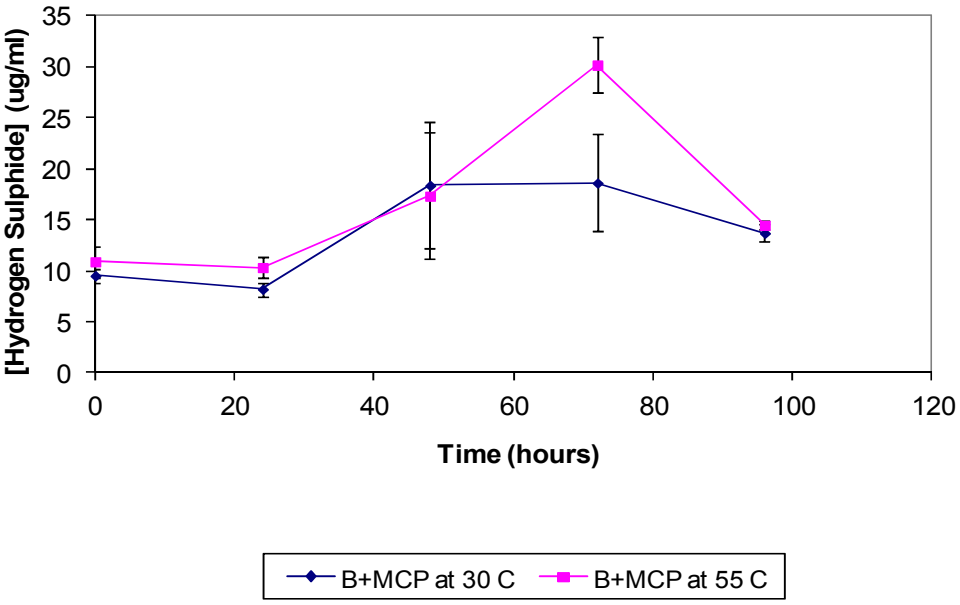

Figure 2: Hydrogen sulphide production by sample B at 30ºC and 55ºC (n =3)

Molecular analysis of functional genes revealed presence of *apsA* genes (Figure 3). This was similar to that observed by Ben-Dov *et al*. (2007) who found APS genes responsible for H_2S production in SRB. Cloning of PCR products following PCR amplification of functional genes was successful (Figure 4).

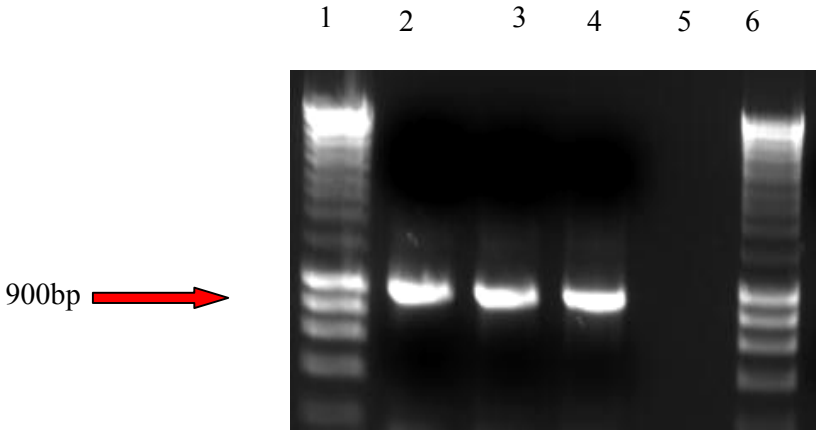

Figure 3: PCR amplification of *apsA* gene fragments. Lane 1 and 6: Hyperladder 1; lane 2: Sample A; lane 3: Sample B; lane 4: Positive control; lane 5: negative control

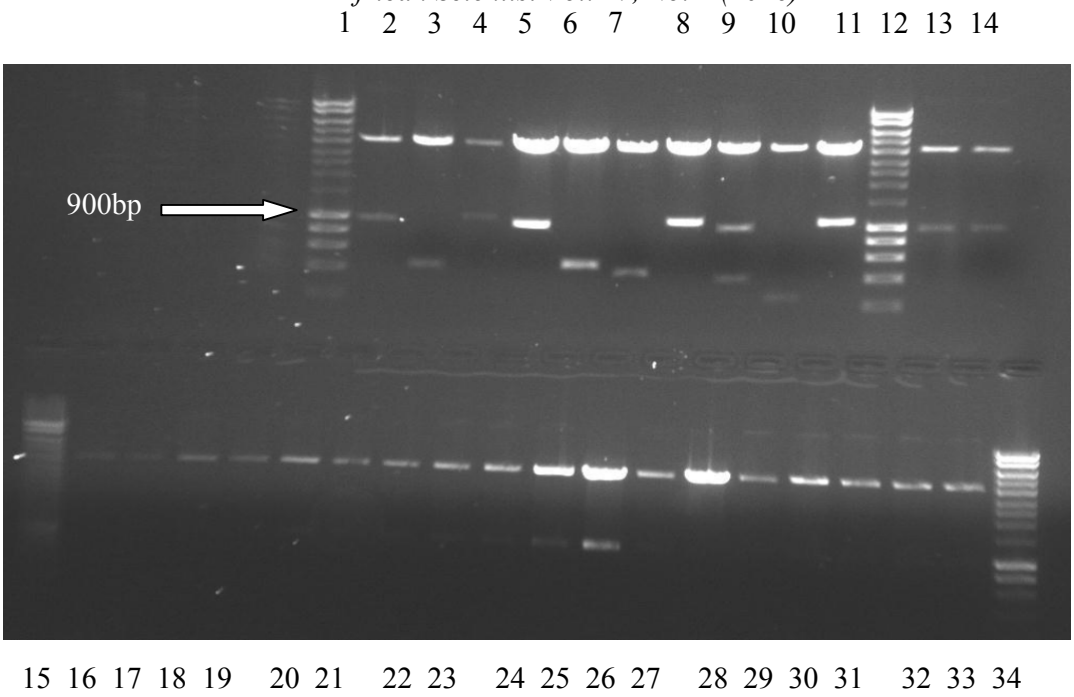

Figure 4: Agarose gel electrophoresis of digestion products, lanes 1, 12, 15, and 34: Hyperladder 1; lanes 2 - 11: Plasmids with inserts for sample A; lanes 13 – 33: Plasmids with inserts for sample B.

Table 1 shows distances between clones of samples which confirmed similarity and differences between the clones and BLAST analysis of clones from both samples showed the detection of SRB with 99% similarity to *Desulfovibrio.* This was consistent with the results of Castro *et al*. (2000) who noted that SRB would be detected in clones by future microbiologists. Phylogenetic analysis of the clones (Figure 5), showed affiliation of the clones to related members of sulphate-reducing bacteria and sulphur-oxidizing bacteria (SOB). Bootstrap values are shown and the scale bar represents 0.05 changes per nucleotide.

Table 1: Clone distances in samples

	A1	**A4**	**B1**	**B2**	**B10**	**B11**	**B12**	**B13**
A1	0.0000	6.0600	0.0021	0.0043	6.0600	0.0053	6.0600	4.1702
A4	6.0600	0.0000	6.0600	6.0600	6.0600	6.0600	6.0600	4.8624
B1	0.0021	6.0600	0.0000	0.0021	6.0600	0.0032	6.0600	4.1148
B2	0.0043	6.0600	0.0021	0.0000	6.0600	0.0053	6.0600	4.1148
B10	6.0600	6.0600	6.0600	6.0600	0.0000	6.0600	0.0037	6.0600
B11	0.0053	6.0600	0.0032	0.0053	6.0600	0.0000	6.0600	4.1744
B12	6.0600	6.0600	6.0600	6.0600	0.0037	6.0600	0.0000	6.0600
B13	4.1702	4.8624	4.1148	4.1148	6.0600	4.1744	6.0600	0.0000

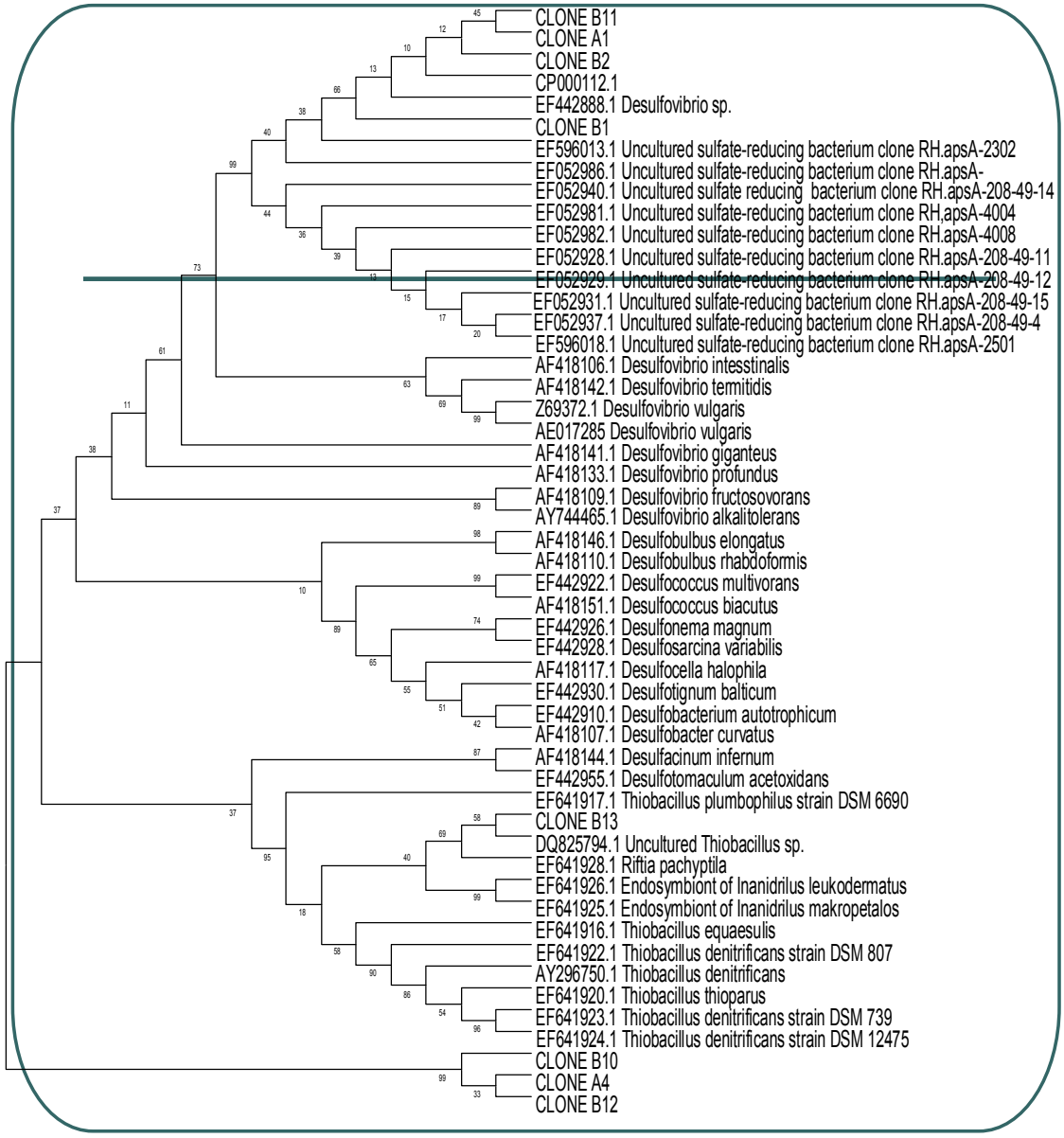

Figure 5: Phylogenetic relationships of *apsA* gene sequences (clones) that were retrieved from samples with other members of related genera of SRB and SOB.

Conclusion

Phylogenetic and functional genes for SRB responsible for H_2S production, reservoir souring, microbially induced corrosion and loss of oil quality have been successfully analysed in this research. Results from this study revealed the presence of SRB, therefore frequent maintenance overhaul for pipelines, flowlines and flowstations, as well as continuous treatment of injected sea water with nitrate are recommended to prevent the activity of SRB in crude oil and produced water.

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References

Adamczyk J, Hesselsoe M, Iversen N, Horn M, Lehner A, Nielsen PH, Schloter M, Roslev P, Wagner M: The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. **69:** 6875 - 6887. 2003.

Altschul SF, Gish W, Miller W, Myers E, Lipman DJ: Basic local alignment search tool. J. Mol Biol. **215:** 403 - 410. 1990.

Amann RI, Ludwig W, Schleifer KH: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. *Rev.* **59:** 143 - 169. 1995.

Bass CJ, Lappin-Scott HM: The bad and the good guys in petroleum microbiology. Schlumberger Oilfield Rev. **9**: 17 - 25. 1997.

Bass CJ, Lappin-Scott HM, Sanders P: Bacteria that sour reservoirs. J. PET. Technol. 1: 31 – 36. 1993.

Ben-Dov E, Brenner A, Kushmaro A: Quantification of sulphate-reducing bacteria in industrial wastewater by Real-time Polymerase Chain Reaction (PCR) using dsrA and apsA genes. Microbial Ecology. **54:** 439 - 451. 2007

Braida MD, Daniels LM, Kitts CL: Removal of PCR inhibitors from soil DNA by chemical flocculation. J. Microbiol. Methods **52:** 389 - 393. 2003.

Castro HF, Williams NH, Ogram A: Phylogeny of sulfate-reducing bacteria. FEMS Microbiol. Ecol. **31**: 1 - 9. 2002.

Cline JD: Spectrophotometric determination of hydrogen sulphide in natural waters. Limnol. Oceanogr. **14:** 454 - 458. 1969.

Cottrell MT, Cary SC: Diversity of dissimilatory sulfite reductase genes of bacteria associated with the deep-sea hydrothermal vent polychaete *Alvinella pompejana*. Appl. Environ. Microbiol. **65:** 1127 - 1132. 1999.

Daly K, Sharp RJ, McCarthy AJ: Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulfate-reducing bacteria. Microbiology **146**: 1693 - 1705. 2000.

Dubilier N, Mulders C, Ferdelman T, de Beer D, Pernthaler A, Klein M: Endosymbiotic sulfate-reducing and sulphide-oxidizing bacteria in an oligochaeta worm. Nature **411**: 298 – 302. 2001.

Dunsmore, B.C: Environmental Microbiology and the Oil Industry. Ph.D. thesis, University of Exeter, UK, 247pp. 2001.

Dunsmore, B.C., Jacobsen, A., Hall-Stoodley, L., Bass, C.J., Lappin-Scott, H.M. and Stoodley, P: The influence of fluid shears on the structural and material properties of sulphate-reducing bacterial biofilms. JIMB. **29**: 347-353. 2002.

Dunsmore, B.C., Youldon, J., Thrasher, D.R. and Vance, I: Effects of nitrate treatment
on mixed species, oil field microbial biofilm. J. Ind. Microbiol. Biotechnol. **33:** 454-462. 2006.

Friedrich MW: Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. J. Bacteriol. **184**: 278 – 289. 2002.

Geesey G, Beech I, Bremer P, Webster BJ, Wells DB: Biocorrosion. In *Biofilms II Process Analysis and Applications* ed. Bryers, J.D. NewYork: Wiley-Liss pp 281 – 325, 2000.

Head IM, Saunders JR, Pickup RW: Microbial evolution, diversity and ecology, a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbiol Ecol. **35:** 1 - 21. 1998.

Klein M, Friedrich M, Roger AJ, Hugenholtz P, Fishbain S, Abicht H, Blackall LL, Stahl DA, Wagner M: Multiple lateral transfers of dissimilatory sulfate reductase genes between major lineages of sulfate-reducing prokaryotes. Appl. Environ. Microbiol. **67:** 6028 – 6035. 2001.

Kumar S, Tomura K, Nei M: MEGA 3: Intergrated software for molecular evolutionarygenetics analysis and sequence alignment. Brief Bioinformatics. **5:** 150 - 163. 2004.

Lemarchand K, Berthiaumea F, Maynarda C, Harelb J, Payment P, Bayardelled P, Massona L, Brousseaua R: Optimatization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J. Microbiol. Methods **63:** 115 - 126. 2005.

Lillebaek R: Application of antisera raised against sulfate-reducing bacteria for indirect immunofluorescent detection of immunoreactive bacteria in sediment from the German Baltic Sea. Appl. Environ. Microbiol. **61**: 3436 - 3442. 1995.

Lopes FA, Morin P, Oliveira R, Melo LF: Interaction of *Desulfovibrio*
desulfuricans with stainless steel surface and its impact on bacterial metabolism. Journal of Applied Microbiology. **101:** 1087 - 1095. 2006.

Luna MG, Dell AA, Danovaro R; DNA extraction procedure: A critical issue for bacterial diversity assessment in marine sediments. Environ. Microbiol. **8:** 308 - 320. 2006.

Magot M, Ollivier B: *Petroleum Microbiology* ASM Press, Washington, DC. 365pp. 2005.

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, Soulas G, Catroux G: DNA extraction from soils; old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. **67:** 2354 - 2359. 2001.

Miller DN, Bryant JE, Madsen EI, Ghiorse WC: Evaluation and optimization of DNA extraction and purification procedures for soil and sediments samples. Appl. Environ. Microbiol **65:** 4715 - 4724. 1999.

Minz D, Flax JL, Green SJ, Muyzer G, Cohen Y, Wagner M: Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. Appl. Environ. Microbiol. **65**: 4666 - 4671. 1999.

Moffat AO, Linden P: Perception and reality. J. Environ. Sci. Technol. **1:** 283 - 297. 2005.

Muyzer G, de Waal EC, Uitterlinden AG: Profiling of complex microbial population by DGGE analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. Appl. Environ. Microbiol. **62:** 2676 - 2680. 1993.

Muyzer G, Ramsing NB, (1995). Molecular methods to study the organization of microbial communities. Water Sci. Technol. **32**: 1 - 9. 1995.

Heiskanen I, Wallenius K, Lindstrom K: Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J. Microbiol. Methods. **45:** 155 - 165. 2001.

Odokuma L.O, Dickson AA: Bioremediation of a crude oil-polluted tropical rain forest soil. Glob. J. Environ. Sci. **2:** 29 - 40. 2003.

Okabe S, Satoh H, Watanabe Y: *In situ* analysis of nitrifying biofilms as determined by the *in situ* hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **65:** 3182 - 3191. 1999.

Okpokwasili GC, Amanchukwu SC: Petroleum hydrocarbon degradationby Candida species. Environ. Int. **14:** 243 - 247. 1988.

Okpokwasili GC, Odokuma LO: Tolerance of *Nitrobacter* to toxicity of some Nigerian crude oils. Bull. Environ. Contam.Toxicol. **52:** 388 - 395. 1994.

Orisakwe OE, Njan AA, Afonne OJ, Akumka DD, Orish VN, Udemezue OO: Investigation into the nephrotoxicity of Nigerian Bonny Light Crude oil in Albino rats. Int. J. Environ. Res. Public Health. **1(2):** 106 - 110. 2004.

Pérez-Jimenez JR, Young LY, Kerkhof LJ: Molecular characterization of sulfate-reducing bacteria in anaerobic hydrocarbon-degrading consortia and pure cultures using the dissimilatory sulfite reductase (dsrAB) genes. FEMS Microbiol. Ecol. **35**: 145 - 150. 2001.

Postgate RJ: The Sulphate-Reducing Bacteria. Cambridge University Press. Cambridge, 208pp, 1984.

Rabus R, Hansen TA, Widdel F: Dissimilatory sulfate- and sulfur-reducing prokaryotes. *In*: Dworkin, M, Schleifer, K.H. and Stackebrandt, E. (Eds.) *The Prokaryotes: An Evolving Electronic Database for the Microbiological Community.* Springer, New York, pp 1–87, 1999.

Raskin L, Amann RI, Poulsen LK, Rittmann BE, Stahl DA: Use of ribosomal RNA-based communities in anaerobic biofilms. Water Sci. Technol. **31:** 261 - 272. 1995.

Ravenschlag K, Sahm K, Knoblauch C, Jorgensen BB, Amann R: Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine Arctic sediments. Appl. Environ. Microbiol. **66**: 3592 - 3602. 2000.

Saito N, Nei M: The neighbour-joining method: A new method for constructing phylogenetic trees. Mol. Biol. Evol. **4:** 406 - 425. 1987.

Sambrook JE, Fritish F, Maniatis T: *Molecular Cloning: A Laboratory Manual.* 2nd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. 1989.

Schneegurt MA, Kulpa CF: The application of molecular techniques in environmental biotechnology for monitoring microbial systems. Biotechnol. Appl. Biochem. **27:** 73 - 79. 1998.

Steffan RJ, Gosoyr J, Bej AK, Atlas RM: Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **57:** 1070 - 1074. 1988.

Teske A, Ramsing NB, Habicht K, Fukui M, Küver J, Jørgensen BB, Cohen Y: Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake (Sinai, Egypt). Appl. Environ. Microbiol. **64:** 2943 - 2951. 1998.

Teske A, Wawer C, Muyzer G, Ramsing NB: Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbol. **62**: 1405 - 1415. 1996.

Truper HG, Schlegel HG; Sulphur metabolism in Thiorhodaceae and quantitative measurements on growing cells of *Chromatium okenii.* Antonie Van Leuwenhoek. **30:** 225 - 238. 1964.

Videla HA: Microbially induced corrosion : an updated overview. Int. Biodeterior. Biodegrad. **48:** 176 - 201. 2001.

Wagner MA, Roger J, Flax JL, Brusseau GA, Stahl DA: Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J. Bacteriol. **180**: 2975 - 2982. 1998.

Zhang T, Fang HHP: Phylogenetic diversity of SRB-rich marine biolfilm. Appl. Microbiol. Biotechnol. **57:** 437 - 440. 2001.

Zhang T, Fang HHP, Ko BCB: Methane producing bacteria in the corrosion biolfilm on mild steel. Appl. Microbiol. Biotechnol. **63:** 101 - 106. 2003a.

Zhou J, Bruns MA Tiedje JM: DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:** 316 - 322. 1996.