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Predominant culturable crude oil-degrading bacteria in the coast of Kuwait

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ABSTRACT

Total of 272 crude oil-degrading bacteria were isolated from seven locations along the coast of Kuwait. The analysis of the 16S rDNA sequences of isolated bacteria revealed the predominance of six bacterial genera: *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Acinetobacter*, *Kocuria* and *Micrococcus*. Investigation of the factors associated with bacterial predominance revealed that, dominant culturable crude oil-degrading bacteria were better crude oil utilizers than the less frequently occurring isolates. Bacterial predominance was also influenced by the ability of bacteria to adapt to the level of organic content available. Predominant culturable bacteria constituted 89.7–54.2% of the total crude oil-degrading bacterial communities. Using 16S-RFLP analyses to assess the diversity of the dominant crude oil-degrading bacterial genera, four phylotypes of *Pseudomonas* sp. and seven phylotypes of *Bacillus* sp. were determined. This suggested high degree of diversity of crude oil-degrading bacterial population at the strain level, but low diversity at the genus level.

1. Introduction

Crude oil is considered a major pollutant of Kuwait marine environment. Hence, efforts are directed to investigate factors affecting its bioremediation (Sorkhoh et al., 1990; Radwan et al., 1995; Readman et al., 1996; Banat et al., 1998; Radwan et al., 2002; Obuekwe et al., 2003; AL-Saleh and Obuekwe, 2005). During the Gulf war the coast of Kuwait witnessed the largest oil spill ever known, with more than 1,500,000 tons of crude oil spill covering some 1500 km^2 of sea surface and polluting more than 500 km of coastline (Reynolds 1993; Sadiq and McCain, 1993). The coastal areas of Kuwait are continuously impacted by crude oil attributed to natural oil seepage, accidental damage to pipelines, accidental spillage from tankers, and oil production from marine wells (Massoud et al., 1996).

Natural removal of crude oil from marine sediments is slow, and oil deposits continue to have long term effects on the benthic community (Blumer 1970; Shriadah 1998). The persistence of a pollutant in the environment is influenced by the nature of the contaminant, the amount of the contaminant present and the interplay between chemical, geological, physical and biological characteristics of the contaminated site. Among the biological factors, the diversity of microbial species and their metabolic capabilities constitute important factors in pollutants removal (Alexander 1999).

Since crude oil is made of a mixture of compounds, and since individual microorganisms metabolize only a limited range of hydrocarbon substrates (Britton 1984), biodegradation of crude oil requires mixture of different bacterial groups or consortia functioning to degrade a wider range of hydrocarbons (Bordenave et al., 2007). Contaminated marine environments are inhabited by a range of selected microorganisms able to tolerate and remediate pollutants that impacted the environment, leading to the dominance of pollutant-tolerant bacteria. Hence, bacterial communities in contaminated sites are typically less diverse than those in non-stressed systems (Harayama et al., 2004). Another characteristic of marine environments is that, the vast majority of bacteria (90–99%) are uncultivable (Rozsak and Colwell, 1987; Amann et al., 1995), hence, the analysis of microbial communities that contribute to *in situ* hydrocarbon biodegradation activities has been a challenge to microbiologists (Rollins and Colwell, 1986; Wilkinson 1988; Sadiq and McCain, 1993). Therefore, the use of rRNA approaches to study changes in the structure of marine microbial communities during oil spills provides a means of detecting non-culturable organisms (Harayama et al., 2004). For, this purpose, 16S rRNA gene (rDNA) clone libraries, fluorescence *in situ* hybridization (FISH) analysis with targeted oligonucleotide probes, and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified rDNA have been used in revealing the large diversity of marine bacteria (Wise et al., 1997; Cottrell and Kirchman, 2000). Molecular approaches were also used to fingerprint and investigate changes in bacterial communities subjected to stress factors (Fredrickson and Balkwill, 1998; Röling et al., 2004). For example, DGGE analyses were used to compare

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bacterial communities in crude oil-treated and untreated sediments. No significant differences were detected and two bacterial genera *Alcanivorax borkumensis* and *Pseudomonas stutzeri* dominated the treated and untreated sediments (Röling et al., 2004). Unfortunately, the effectiveness of petroleum pollutants bioremediation remains a controversial issue since the apparent biodegradative potential of bacteria is the result of the interaction of complex physical, chemical and biological factors (Siron et al., 1995; Alexander 1999).

Identification of major organisms responsible for the biodegradation of pollutants and characterization of their biodegradative potentials is imperative for the comprehension, evaluation, and development of *in situ* bioremediation strategies (Cohen 2002; Harayama et al., 2004). In the current study, the community structure of the crude oil-degrading bacteria isolated along the cost of Kuwait was characterized. Dominant strains were investigated for their potential to mineralize crude oil, ability to grow in nutrient-rich and poor media, and their genetic affiliation.

2. Materials and methods

2.1. Sediment samples

Sediment samples were collected from the upper 10 cm of the intertidal zone of seven locations (M1, Douha; M2, Sulaibikhat; M3, Shuwaikh; M4, Salwa; M5, Shuaiba port; M6, Badullah port; M7, Azzour) along the coast of Kuwait. From each location, five sediment samples were collected randomly within an area covering 20 m^2 of coastal area. Sediment samples from M1 to M7 locations were immediately characterized on-site using multiparameter water quality meter (Horiba U-10, Horiba Europe GmbH, Germany) to determine dissolved oxygen (DO), pH, salinity and conductivity. Dissolved oxygen was determined by dipping the oxygen electrode immediately into sediments. Samples for further analyses were aseptically transferred into sterile plastic containers that were prestored in ice and transported immediately to the lab for microbiological and molecular analyses. Samples belonging to each coastal location were pooled in one sterile plastic container and mixed thoroughly. The organic carbon contents of sediments were determined by the loss-on-ignition method (Shem-Tov et al., 1991). The unused samples were stored at −20 °C.

2.2. Enumeration and isolation of aerobic crude oil-degrading bacteria

The culturable crude oil-degrading bacteria in sediments were counted using standard plate dilution method (AL-Saleh and Obuekwe, 2005). Six portions from each sediment sample were pooled, thoroughly mixed, and passed through a sterile sieve (mesh size, 2 mm) to aid mixing and to remove large particles. Ten grams (wet weight) of sediments were suspended in 40 ml of 50 mM phosphate buffer (pH 7.2) and vortexed for 1 min at low speed. Aliquots of 100 μl of undiluted samples, and 10^{-1}–10^{-6} dilutions were spread on Hutner's minimal agar plates (Cohen-Bazire et al., 1957), incubated under crude oil vapor in a desiccator at 30 °C for up to 21 days. Grown discrete colonies were isolated, purified and stored in 15% glycerol at −70 °C. The potential of isolated bacteria to grow on nutrient-rich and/or poor media was investigated by growing bacteria on undiluted and diluted (1:1000) nutrient broth, solidified with 2% agar, at 30 °C for 24 h. The mean and standard error of the mean of three independent experiments were calculated using SPSS (version 14).

2.3. Mineralization of crude oil by isolated bacteria

Crude oil mineralization by the isolated bacteria was determined by respirometry using the method of AL-Saleh and Obuekwe (2005). The amounts of carbon dioxide evolved were measured using a Micro-oxymax respirometer (Columbus Instruments, Columbus, Ohio, USA). The reaction vessels contained 49 ml of Hutner's minimal medium and 1 ml of washed bacterial inoculum that was previously grown in nutrient broth, washed twice with Hutner's minimal medium and adjusted to 1 OD_{600}. Crude oil was supplied (200 μl) in the reaction vessels that were incubated at 30 °C with shaking at 300 rpm. Amounts of carbon dioxide produced were plotted against time and the rates of carbon dioxide evolution were calculated and expressed as μg min^{-1}. Three independent experiments were conducted. The mean and standard error of the mean were calculated using SPSS (version 14).

2.4. Identification of isolated bacteria and phylogenetic analysis

Genomic DNA was purified from pure bacterial cultures using the Wizard Genomic DNA purification kit as recommended by the manufacturer (Promega, Madison, Wisconsin, USA) and quantified by fluorometry using a model TK 100 fluorometer (Hoefer Scientific Instruments, San Francisco, USA). DNA extracts were stored at −20 °C and used for the amplification of 16S rDNA from extracted DNA using 27F and 1492R primers (Kuske et al., 1997). All reactions were carried out in 25 μl volumes, containing 12.5 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate, 2.5 μl of 10x PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), and 0.5 U of Taq DNA polymerase (Applied Biosystems), and made up to 25 μl with sterile water. PCR was performed in a Thermocycler (Applied Biosystems, Warrington, UK) with the following thermocycling programme: 5 min denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C, and a final extension step of 5 min at 72 °C. PCR products were visualized by electrophoresis in 2% (wt vol^{-1}) agarose gels and with ethidium bromide (0.5 μg ml^{-1}) staining. Amplified DNA was purified using a QIAQUICK PCR cleanup kit (Qiagen, Inc., Valencia, California, USA), and DNA concentrations were determined as described previously. Approximately 10 ng amounts of 16S rDNA were used as template in dye terminator cycle sequencing reactions (Applied Biosystems PRISM dye terminator cycle sequencing kit.). The 16S rDNA sequences obtained were analyzed using the National Center for Biotechnology Information (NCBI; Bethesda, Maryland, USA) BLAST. Sequences were assigned to recognized representatives of the main eubacterial lineages based on the highest score and 99% 16S sequence similarity.

2.5. Random fragment length polymorphism (RFLP) analysis of 16S rDNA

16S rDNA sequences of cultured isolates were amplified as mentioned previously. Following PCR amplification of 16S sequences, 10 μl of PCR products were digested separately with 2.5 U of BstUI (New England Biolabs, Ipswich, Maryland, USA) in 12 μl reaction mixtures as recommended by the manufacturer. BstUI digests were electrophoresed in 2% agarose gels, with TBE buffer. The gels were stained with ethidium bromide and photographed under UV light using InGenius gel documentation system (Syngene, Inc. Frederick, Maryland, USA). DNA fragment sizes were determined using GeneTools analysis software (Syngene, Inc., Frederick, Maryland, USA). The quality of the numerical data was checked by comparing the sum of fragment sizes in each restriction pattern with the original product size (approximately 1499 bp). Cluster analysis of band patterns was carried out using GelComparII software (Applied Maths). Profiles of isolated bacteria were compared based on Dice coefficient with an optimization of 1.0% and a tolerance of 1.0%. Dendrograms were obtained using the hierarchical unweighted pair group method with arithmetic mean