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Full Length Research Paper

Isolation and molecular identification of polyaromatic hydrocarbons- utilizing bacteria from crude petroleum oil samples

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Polyaromatic hydrocarbons (PAHs) are worldwide contaminants that can reach toxic concentrations that are detrimental to the environment and human health. In this study, three different isolates (*Klebsiella oxytoca, Klebsiella pneumonia* and *Acinetobacter* sp.) from crude petroleum oil samples were found to be PAHs utilizers. They are able to utilize four different PAHs, phenanthrene, fluoranthene, pyrene, and benzene. *K. oxytoca* is the most efficient utilizer of the PAHs rather than *K. pneumonia* and *Acinetobacter* sp. It can utilize almost 83% of benzene after 48 h of shaking, if supplemented with nitrogen source. The isolates have been molecularly identified by partial sequencing of the 16S rDNA gene (approximately 900 bp) and the results demonstrated a high degree of homology to *K. oxytoca* (up to 99% similarity), *K. pneumonia* (from 97 to 99% similarity) and *Acinetobacter* sp (up to 99% similarity).

Key words: *Acinetobacter, Klebsiella*, PAHs, 16S rDNA.

INTRODUCTION

Recently, ecosystems have been changed by the growing influence of human activity. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by contamination. During the previous years, the frequency and risk of oil pollution has lead to extensive research. Most of the petroleum goes in the ecosystem via leak of coastal oil refineries. This fact increased the interest of scientists to investigate the oil distribution and its fate in the environment. Approximately, five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995). Shipping accidents, for example, have a serious impact on the surrounding environment. The consequences include serious, widespread and long-term damage to marine ecosystems, terrestrial life, human health and natural resources. Conventional remediation methods include physical removal of contaminated material. These methods also use chemicals, especially shoreline cleaners, which are often organic solvents with or without surfactants (Riser-Roberts, 1992). Polyaromatic hydrocarbons (PAHs) are worldwide contaminants that can reach toxic concentrations that are detrimental to the environment and human health (Samanta et al., 2002). These compounds persist in the environment and, due to their hydrophobicity, become associated with particulate matter, such as clays and humics that are deposited in soils and sediment. PAHs are lipophilic and have the potential to biomagnify through the food chain (Kanaly and Harayama, 2000).

Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are

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biodegradable (Cybulski et al., 2003; Carvalho and Fonseca, 2004). Many microorganisms, such as *Pseudomonas aeruginosa*, *Pseudomonas putida, Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis Bacillus laterospor, Klebsiella spp. and Acinetobacter spp.* excrete emulsifiers that increase the surface area of the substrate. These microorganisms also modify their cell surface to increase the affinity for hydrophobic substrates and, thus facilitate their absorption (Atlas, 1991; Rosenberg and Ron, 1996; Chang et al., 2011; Cybulski et al., 2003; Carvalho and Fonseca, 2004).

The rate-limiting step in the microbial degradation of petroleum hydrocarbon pollutants in open systems, such as lakes, oceans, and wastelands, is generally a utilizable source of nitrogen. Because petroleum contains only traces of nitrogen, the required nitrogen must come from the surrounding environment (Atlas, 1991; Rosenberg and Ron, 1996). Bacterial 16S ribosomal RNA (rRNA) genes contain nine “hypervariable regions” (V1–V9) that demonstrates considerable sequence diversity among different bacteria. Species-specific sequences within a given hypervariable region constitute useful targets for diagnostic assays and other scientific investigations **(**Chakraborty et al., 2007). Isolation and characterization of strains belonging to *Acinetobacter* and *Klebsiella* that can grow on crude oil were reported by many researchers. In most of the cases, these bacterial species have been identified throughout sequencing of the 16S rDNA or 16S–23S intergenic spacer regions (Rainey et al*.,* 1994*;* Maslunka et al*.,* 2008; Guo et al., 2010; Nemec et al*.,* 2011).

The aim of this study is to isolate, characterize and molecularly identify the most dominant bacteria in crude oil samples obtained from a petroleum refinery plant in Saudi Arabia, which can efficiently utilize some important polyaromatic hydrocarbons, phenanthrene, fluoranthene, pyrene, and benzene. This step is a preliminary one for a study of how these bacteria utilize/degrade hydrocarbons and accordingly can be used in hydrocarbon leak clean-up.

MATERIALS AND METHODS

Bacterial isolation, counting and purification

Bacteria were isolated on January 2011 from crude petroleum oil samples, Saudi Arabia. One ml of the sample was suspended in NB (nutrient broth) throughout serial dilutions then incubated at 30°C under shaking conditions (200 rpm) for 24 h. 0.1 ml of the bacterial suspension was inoculated by plating into NA (nutrient agar) plates for 24 h. Colonies were counted then picked up and purified by streaking on NA plates.

Phenotyping and growth on different polyaromatic hydrocarbons

Pure cultures were examined for gram reaction and sporulation ability, after incubation in NA plates at 30°C for 24 and 48 h, respectively. The ability to utilize polyaromatic hydrocarbons (PAHs), phenanthrene, fluoranthene, pyrene, and benzene, which were kindly provided for a petroleum oil refinery plant, Taif, Saudi Arabia was investigated with and without Bushnell-Hass (BH) minimal medium (Sigma co.) supplemented with 1% yeast extract and 1.5% NaCl (Hilyard et al., 2008). Stock solution (50 mg/ml) of each hydrocarbon was prepared in acetone (Hilyard et al., 2008). Sterile Petri-dishes were inoculated with 0.1ml of each hydrocarbon, separately, and around 20 ml of Agar BH medium. Another set of Petri-dishes containing the separate hydrocarbons were inoculated with washed agar with no BH medium. Both sets were allowed to be solidified and streaked with pure bacterial cultures. Growth was checked out after 2 days at 30°C.

Measuring of total hydrocarbons

For measuring of the total hydrocarbons, bacterial cells were allowed to grow in each hydrocarbon, phenanthrene, fluoranthene, pyrene, and benzene (7 mg/100ml), supplemented with BH medium under shaking conditions (200 rpm) for 2 days at 30°C. Cultures were then centrifuged and supernatants were treated according to Parsons et al. (1984). Total hydrocarbons were quantified using Flurometer 450, USA. Flasks were set in duplicates.

DNA extraction, purification and amplification

DNA was extracted and purified using Gene Jet Genomic DNA Extraction and Purification (Fermentas Life Sciences). Polymerase Chain Reaction (PCR) was performed using a thermocycler (Mastercycler 5333) Eppendorf AG- Germany. PCR was carried out using F8 primer (5´-AGA GTT TGA TCC TGG CTC-3´) and R1525 primer (5´-AAG GAG GTG ATC CAG CC-3´) according to Beumer and Robinson, 2005. Primers were manufactured by Eurofins MWG operon, Ebersberg; Germany. The PCR reaction was performed in a total volume of 50 µl by using 20 µl of 2.5x PCR master mix (5 prime GmbH, Hamburg, Germany), 3 µl from forward and reverse primers (10 pmol/ µl from each), 23 µl nuclease free water and 1 µl DNA (equivalent to 10 ng). The following temperature protocol was used: initial denaturizing step of 5 min at 95°C, 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 48°C, and extension for 60 s at 72°C (Beumer and Robinson, 2005).

Gel electrophoresis

Ten microlitre (10 µl) of the PCR product, mixed with loading buffer, were loaded on a 1% w/v agarose gel and electrophoresed with 1X TEA (Tris EDTA Acetate) buffer. DNA was visualized by UV transillumination after staining with ethidium bromide (0.5 µg/ml). The molecular sizes of the amplified DNA fragments were estimated using DNA ladder of 100 bp.

DNA sequencing

PCR products were purified to remove excess primers using QIA quick PCR purification reagents (Qiagen, USA) and then sequenced with the BigDye Terminater cycle sequencing kit (Applied Biosystems, Foster City, CA) in ABI Prism 3700 sequencer (Perkin Elmer, Applied Biosystems, USA). Sequences were deposited in the GenBank and the accession numbers are listed in Table 1.

Data analysis and phylogenetics

After obtaining the sequences, homology search was performed

Table 1. Accession numbers of the newly isolated bacteria.

Bacterium	Accession number
Klebsiella oxytoca	JN001159
Klebsiella pneumoniae	JN001160
Acinetobacter sp	JN001161

Table 2. Three days bacterial growth in plates of PAHs supplemented with BH medium.

Bacterium	Polyaromatic Hydrocarbons			
	Ph	Fl	Py	Bn
Klebsiella oxytoca	++	++	++	++
Klebsiella pneumoniae	+	+	+	+
Acinetobacter sp	+	+	+	+

Weak growth, +; good growth, ++; Ph, phenanthrene; Fl, fluoranthene; Py, pyrene; Bn, Benzene.

Table 3. Total hydrocarbon content remained after 2 days PAHs utilization by *Klebsiella oxytoca.*

Total hydrocarbon content (mg/100 ml)				
Control (no cells)	Phenanthrene	Fluoranthene	Pyrene	Benzene
7	2	2.1	2.9	1.2

against DDBJ (DNA Data Bank of Japan), using Blast program to find the sequences producing significant alignment with the obtained sequences. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2. Multisequence alignment and molecular phylogeny were performed using ClustalW (a distance-based analysis program at http://www.ddbj.nig.ac.jp/) program (Saitou and Nei, 1987). The tree topology was evaluated using the neighbor-joining method based on 1000 resamplings (Saitou and Nei, 1987; Chun and Bae, 2000).

RESULTS

Our recently isolated strains were picked up among 30 bacterial colonies found in some heavy and light crude petroleum oil samples, KSA. All of them are gram negative and non sporulating bacteria. Interestingly, none of them is able to grow in the presence of PAHs without BH medium supplemented with yeast extract. Growth was detected only in the presence of the nitrogen source. The promising growth was detected for *Klebsiella oxytoca* which showed significant appearance after 2 days incubation with the 4 separated PAHs, phenanthrene, fluoranthene, pyrene, and benzene (Table 2). *K. oxytoca* was further tested to quantitatively measure its ability to utilize phenanthrene, fluoranthene, pyrene, and benzene, under shaking conditions within 2 days. As shown in Table 3, *K. oxytoca* is able to utilize almost 83% of Benzene after 48 h, followed by phnanthrene 71.4%, then fluoranthene 70%, and finally Pyrene 58.6%.

The 16S rDNA amplified sequences (Figure 1) have been subjected to purification and sequencing using the same primers, F8 and R1525, used in the PCR reaction. Based on the partial 16S rDNA gene sequences (about 1000 bp), strains belonging to the same species have similarity percentages ranging from 97 to 99. On the other hand, those from different species have dramatically lower percentages of similarity among each other. For instance, *K. oxytoca* Taif has 99% similarity with *K. oxytoca* NFSt18 and has only 81% similarity with *Acinetobacter* BCaL1 (Table 4). In the same time, *Klebsiella pneumonia* Taif sequences have similarity percentages ranging from 97 to 99% with strains belonging to the same species. On the other side, *K. pneumonia* has only 87 or 83 % of similarity with *Acinetobacter* species (Table 4).

As shown in Figure 2, the tree topology is divided into 3 main clusters, *K. oxytoca, K. pneumonia* and *Acinetobacter. K. oxytoca* Taif is closely related to *K. oxytoca* C1, although it has 99% similarity with both *K. oxytoca* C1 and *K. oxytoca* NFSt18 (Table 4). The same can be said for *K. pneumonia* Taif which showed high

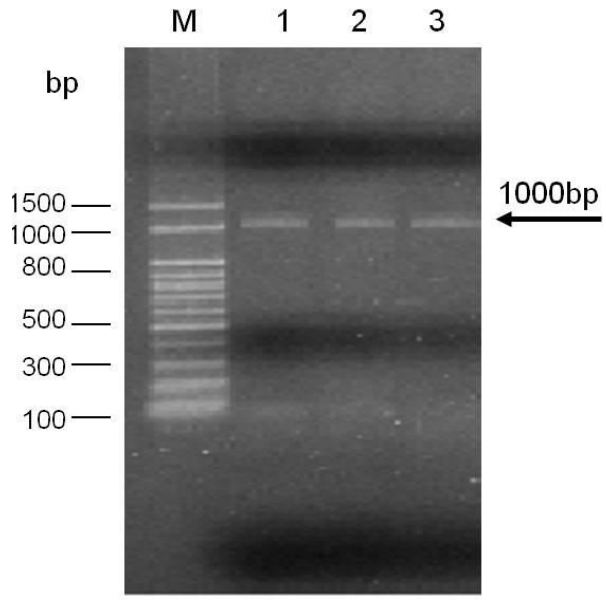

Figure 1. Gel electrophoresis of approx. 1000 bp of the 16S rRNA gene. M, 100 bp genetic marker; lanes 1, 2 and 3 are for *Klebsiella oxytoca, K. pneumonia and Acinetobacter sp*., respectively.

Table 4. Similarity matrix of Taif strains 16Sr DNA and other homologous sequences.

	K. oxy NFSt18	K. oxy C1	K. pneu Taif	K. pneu ECU-21	K. pneu K30	K. pneu 4	Acin sp. Taif	Acin sp. TDB-2008a	Acin sp. N12	Acin sp. BCaL1	Acin sp. SY76
K. oxy Taif	99	99	86	87	85	85	85	85	85	81	85
K. oxy NFSt18		99	86	87	85	85	84	85	84	81	85
K. oxy C1			86	86	87	85	85	85	85	81	85
K. pneu Taif				99	97	99	83	83	83	83	87
K. pneu ECU-21					97	99	83	83	83	83	87
K. pneu K30						97	83	83	83	83	87
K. pneu 4							83	83	83	83	87
Acin sp. Taif								99	99	99	99
Acin sp. TDB-2008a									99	99	99
Acin sp. N12										99	99
Acin sp. BCaL1											99

relatedness to *K. pneumonia* 4 than *K. pneumonia* ECU-21. Finally, *Acinetobacter* sp. Taif has been clustered in the same group with other different *Acinetobacter* species (Figure 2) and showed 99% similarity with all of them (Table 4).

DISCUSSION

Polyaromatic hydrocarbons (PAHs) are common ubiquitous compounds found worldwide in soils and sediments as a result of both natural and anthropogenic production (Chung et al., 2001). In this study, we have characterized 3 different bacterial strains, *K. oxytoca* Taif, *K. pneumonia* Taif, and *Acinetobacter sp*. Taif, that are able to grow on plates containing enriched PAHs, phenanthrene, fluoranthene, pyrene, and benzene. In 2010, Coelho et al. have demonstrated that *Klebsiella* species comprise 31% of the total representative isolates in the surface microlayer of an estuarine system that are able to degrade PAH. Recently, a new *Klebsiella* strain, *Klebsiella aquatic sp*. nov., has been reported to be a

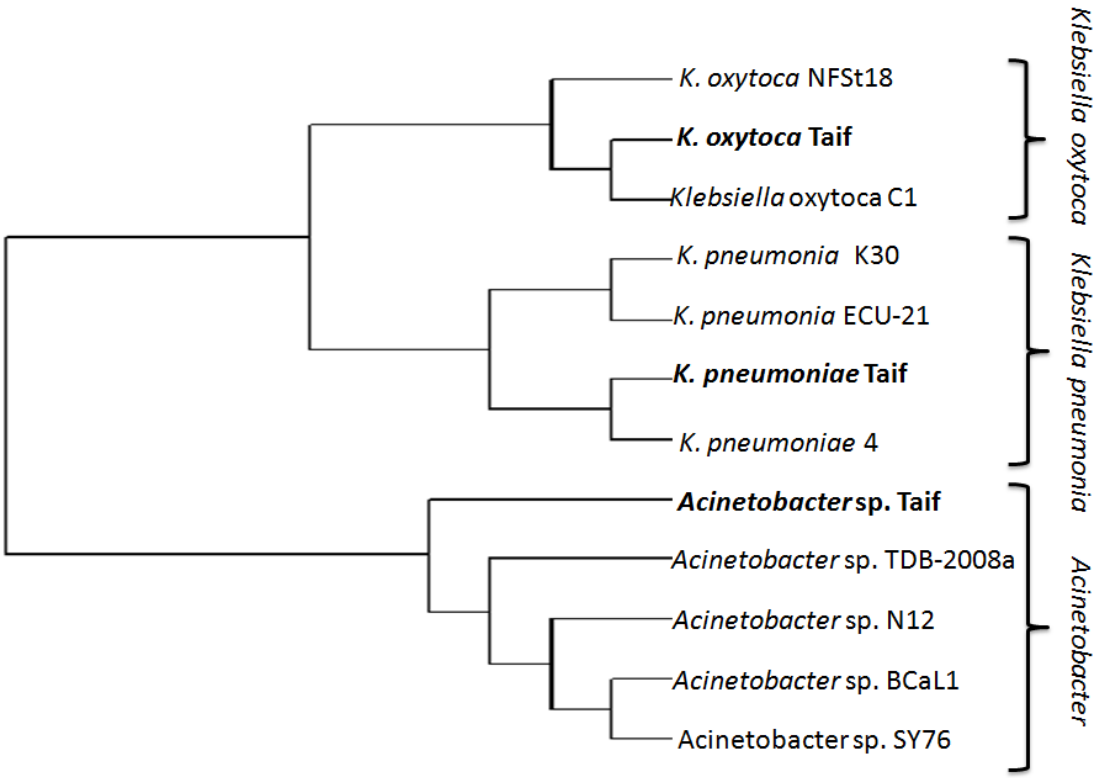

Figure 2. Phylogenetic tree based on partial sequences (approximately 1000 bp) of the 16S rRNA gene. The tree constructed by neighbor-joining method using ClustalW software. Target strains are in bold.

novel PAH degrading bacterium from wastewater (Chunyang et al., 2010). Moreover, *K. oxytoca* C302 has been previously reported to be a degrader of benzoate and catechol (Jun-Ho et al., 2002). *K. oxytoca* is a gram-negative and rod-shaped bacterium that is closely related to *K. pneumoniae*, from which it is distinguished by being indole-positive. In a previous study, some microbial strains belonging to *K. oxtoca* have been isolated from oil-polluted soils. Interestingly, these strains are capable of degrading dibenzothiophene (DBT) (Chang et al., 2011). These results reflect on the common existence of such bacteria in petroleum derivatives. Moreover, some of them not only can tolerate these severe environments, but also can degrade some of its hard components (Chang et al., 2011). In parallel to our findings, Rodrigues et al., 2009 have isolated 16S rDNA- identified *Klebsiella* strains which are closely related to *K. pneumonia* and *Klebsiella ornithinolytica*. These isolates demonstrated a substantial degree of catabolic plasticity for hydrocarbon degradation.

Acinetobacter which is able to utilize PAH in the current study, was also found among the PAH degrading isolates retrieved from surface microlayer in an estuarine system (Coelho et al., 2010). Many environmental strains of *Acinetobacter* with hydrocarbon degrading capacities have been isolated in terrestrial marine environment (Karolien et al., 2004; Rodrigues et al., 2009). Khongkhaem et al. (2011) demonstrated that even phenol can be utilized by an immobilized strain belonging to *Acinetobacter sp*. However, bacteria of this genus are known to be involved in biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes.

In conclusion, *Klebsiella* and *Acinetobacter* species could be an important part of the oil-degrading microbial community. Bacterial capability to degrade different hydrocarbons has a wide range of efficiencies (Rodrigues et al., 2009). It depends on many factors such as the environmental conditions, carbon chain type, hydrocarbon complexity etc., (Atlas, 1981). The presence of plasmids affects the hydrocarbon metabolism dramatically. For instance, a strain belonging to *K. oxytoca* has the ability to degrade phenol throughout the expression of a plasmid- coding phenol hydroxylase gene (Heesche-Wagner et al., 1999). However, enrichment with BH minimal medium supplemented with 1% yeast extract as a nitrogen source enhanced the growth greatly. In parallel to our results, previous researches (Rosenberg and Ron, 1996; Rosenberg et al., 1998) have emphasized that the rate-limiting step in the microbial degradation of petroleum hydrocarbon is generally a utilizable source of nitrogen. This explains the significant growth of the strains under test if yeast extract was added to the minimal medium which contains the PAHs.

Coelho et al. (2010) have reached a similar conclusion that in estuarine environment contaminated with PAHs, bacterial abundance depends in part of the quantity and quality of the organic matter pool used as a substrate for growth.

Our results demonstrated that the partial sequences of the 16S rDNA have successfully differentiated between and precisely identified the three recently isolated strains, crude petroleum oil isolates, which showed less than 100% of similarity with other homologous sequences in the GenBank. Previous studies have indicated that this 5´ hypervariant region (Goto et al., 2000; Mohamed et al., 2007; Mohamed and Badawy, 2011) is a strong discriminative tool for bacterial grouping and differentiation. Moreover, in a previous study *Acinetobacter sp* which has been successfully grown on crude oil was molecularly identified using only the first 5´ end (approx 821 bp) of the 16S rDNA (Koren et al., 2003). Besides, Chamkha et al. (2011) emphasized that this gene is a strong chronometer for identification of a *K. oxytoca* strain BSC5 which has been recently isolated from Sercina oil field, Tunisia. This strain was able to grow on crude oil without a need to a nitrogen source.

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