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Study of plasmid profile and alkane hydroxylase genes in crude-oil degrading bacteria isolated from the Persian Gulf

Y Esmaeili, H Sasan, A Zargarimarandi & M Hassanshahian*

Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

*[E-mail: mshahi@uk.ac.ir]

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Bioremediation, using microorganisms, especially hydrocarbon-degrading bacteria are inexpensive and eco-friendly methods to reduce oil pollutions. Plasmids containing hydrocarbon-degrading genes encode the enzymes necessary for the derivative pathways are important for bioremediation. The aim of the present work was to study the plasmid profile of bacterial strains isolated from crude-oil contaminated sites in the Persian Gulf in previous researches. In this study, plasmids were extracted from 21 strains using alkaline lysis method. Then, alkane hydroxylase gene groups (I, II, III) were detected in extracted plasmids using specific primers by PCR. The results of this study indicated that eight crude oil degrading strains out of 21 had plasmid. The results of PCR amplification confirmed that 50 % of plasmid containing strains had alkane hydroxylase gene group (I), 75 percent of them had alkane hydroxylase gene group (III) and none of them had alkane hydroxylase gene group (II).

[Keywords: Alkane, Gene, Marine environment, Persian Gulf, Plasmid]

Introduction

The marine ecosystem is highly susceptible to crude oil pollution that has many threats and side effects for ecology[1-3]. The decontamination of crude-oil polluted sites is urgent as many petroleum hydrocarbons compounds are supposed to be toxic, mutagenic, or carcinogenic[4,5]. Currently, microorganisms have a crucial role in the environmental pollutants cleanup[6-7]. Notably, the bacteria possessing different enzymes to breakdown the petroleum compounds are attractive choices in bioremediation process[8,9]. The biodegradation of crude-oil in the marine environment is largely performed by diverse bacterial populations, including various *Pseudomonas*[10], *Vibrio*[10], *Acinetobacter*[10], *Rhodococcus* sp.[11], *Bacillus* sp.[12] and *Mycobacterium* species[13-14].

The major component of crude-oil is alkane which is quickly degraded by bacteria through alkane hydroxylase enzyme. In fact, this enzyme provides an oxygen atom derived from molecular oxygen into the alkane substrate that leads to crude-oil bioremediation[15-17]. On the other hand, the genetics of hydrocarbon degrading microorganisms have been investigated in a small number of microbes and their genetic pathways not fully understood. Besides, although many important genes for hydrocarbons degradation are arranged in inducible operon structures with several regulated genes available in the same transcription unit, in some cases, these operons are expressed constitutively[18-19]. It is worthy to note that, in addition to chromosomal DNA, a large number of bacteria also have extra-chromosomal DNA known as plasmids that bear catabolic genes encoding some alkane degrading enzymes. These plasmids are classified into three main groups: a) the plasmids containing alkane degradation genes (as the OCT plasmid)[20], b) the plasmids containing naphthalene and salicylate degradation genes (as the NAH plasmids)[21], and c) the plasmids containing toluene- and xylene-oxidizing genes (as the TOL plasmids)[21]. Additionally, plasmids are highly mobile form of DNA, which could transmit new phenotypes, such as alkane degradation ability to the recipient organisms[22-23].

For instance, Lu Feng *et al.*[24] sequenced *Geobacillus thermodenitrificans* NG80-2 genome that isolated from a deep oil reservoir in Northern China and indicated that NG80-2 consists of a 57,693-bp plasmid that can be highly effective in long-chain alkane degradation pathway[24]. Alkane hydroxylase genes could be categorized into three groups according to the phylogenetic analysis. *AlkB* genes produce alkane hydroxylase group (I), this enzyme can breakdown n-alkanes with short-chain (C_6–C_{12}).

AlkM genes synthesized alkane hydroxylase group (II), this enzyme can degrade n-alkanes with medium-chain (C_8–C_{16}), and some *alkB* genes also encode alkane hydroxylase group (III), this enzyme can transform long-chain n-alkanes (>C_{16})[25].

The aim of the present work was to study the plasmid profiles of bacterial strains isolated from crude-oil contaminated locations in Persian Gulf in previous researches[26-27]. In addition, screening of alkane hydroxylase genes (*alkB*) in plasmid containing strains was also carried out in this study.

Materials and Methods

Bacterial strains

Twenty-one bacterial strains that were isolated from mussels and mangrove forests at Persian Gulf in previous studies were utilized in this research[26-27]. The genus and species of these bacteria with Gene Bank accession number are presented in Table 1.

Chemicals

All chemicals used in this research were purchased from Sigma Co. and include: Glucose, Tris-HCL, Tris-Base, Ethylenediaminetetraacetic acid (EDTA), NaOH, Sodium dodecyl sulfate (SDS), Sodium acetate, chloroform, Isopropanol, Alcohol 70 %, Master mix red , Boric acid, Loading dye, Ethidium bromide (EtBr), Agarose.

Table 1 — Crude-oil degrading bacterial strains used in this research

Crude-oil degrading Bacteria	Strain	Accession Number in Gene Bank
Shewanella algae	BHA1	LK391612
Micrococcus luteus	BHA7	LK391613
Pseudoalteromonas sp.	BHA8	LK391614
Shewanella algae	BHA12	LK391615
Alcanivorax sp.	BHA14	LK391616
Halomonas sp.	BHA16	LK391617
Vibrio alginolyticus	BHA17	LK391618
Alcanivorax dieselolei	BHA25	LK391619
Idiomarina baltica	BHA28	LK391620
Alcanivorax dieselolei	BHA30	LK391621
Alcanivorax sp.	BHA32	LK391622
Shewanella haliotis	BHA35	LK391623
Vibrio azureus	BHA36	LK391624
Vibrio alginolyticus	NW4	LN866606
Vibrio azureus	BP14	LN866607
Pseudomonas salomonii	NP16	LN866608
Vibrio azureus	NP19	LN866609
Idiomarina baltica	BW32	LN866610
Kangiella marina	DP40	LN866611
Marinobacter bryozoorum	DW44	LN866612
Halomonas organivorans	BS 53	LN866613

Buffers and solutions

The buffers and solutions utilized in this study were prepared as follow: Solution I: GTE buffer (50 mM Glucose,10 mM Tris-HCL pH 8.0, 25 mM EDTA pH 8.0, de-ionized water) and solution II (2N NaOH, 10 % SDS, de-ion water) were prepared as lysis buffer[28]. The GTE buffer was used as lysis buffer to disintegrate the bacterial cells to release their contents. The solution II contains SDS as a detergent used for cell lysis and NaOH to break down the cell wall easily, however, it disrupts the hydrogen bonding between the DNA bases and converting the double-stranded DNA (dsDNA) in the cell to single stranded DNA (ssDNA). Solution III (3M sodium acetate, acetic acid, de-ion water, pH 5) was prepared to created acidic condition, so that the ssDNA can re-nature to dsDNA. TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) was prepared to dissolve the pellet of plasmid. TBE buffer (10.8 gr Tris-Base, 5.5 gr Boric acid, 0.93 gr EDTA) was prepared for determining the isolates plasmid DNA and the amplified PCR products by gel electrophoresis[29].

Extraction of plasmid DNA

The Plasmid DNA was extracted by the alkaline lysis protocols explained by Birnboim and Doly[29]. In brief, the bacterial strains were cultured in Nutrient Broth (NB) medium supplemented with 4 % NaCl and incubated at 37 °C with shaking for 72 h. Afterwards 1.5 ml of the culture was centrifuged at 6,000 rpm for 5 min. The cell pellets were suspended with 200 µl of GTE lysis buffer at room temperature for 5 min. Then 400 µl of solution II was added, mixed slowly, and kept for 5 min on ice. Then, 400 µl of solution III was added and mixed gently. After 5 min on ice, the sample was centrifuged at 12,000 rpm for 10 min at 4 °C. Following, the supernatant was removed and extracted once with chloroform. After centrifugation at 12,000 rpm for 5 min, the supernatant was removed and transferred to a new 1.5 mL falcon max 300 µL. Then 2 volume of isopropanol was added to the supernatant and was incubated at -21 °C for 30 min. Precipitates were collected by centrifugation for 5 min at 12,000 rpm, rinsed once with 70 % ethanol, and were dried under vacuum. Finally, the dried pellet was dissolved in 50 µl of TE buffer or de-ionized water. The size of plasmid was determined by agarose gel electrophoresis (0.8 % w/v agarose) for 1 hour at 75 V in 1XTBE buffer. The DNA was stained with ethidium bromide and was visualized under UV light.

The size of the plasmid DNA was determined by comparison with a 1Kb ladder.

Detection of alkane hydroxylase gene (*alkB*) in extracted plasmids

Total plasmid DNA of the bacterial strains was extracted using the alkaline lysis method. The purified plasmid DNA was subsequently screened by PCR to distinguish between three catabolic genes that encode enzymes involved in alkane degradation pathways, *alkB* gene (group I), *alkB* gene (group II) and *alkB* gene (group III). The primers used for identification of these three groups of gene were listed in Table 2[25,30]. A 25 µl PCR mixture containing 12.5 µl master mix red, 0.4 µl each primer, 1 µl plasmid DNA as template DNA and 10.9 µl de-ion water. Target DNA was amplified in a thermocycler (Eppendorf AG 22331, Hamburg, Germany). The PCR program was as follows: initial denaturation of the target DNA was doneat 94 °C for 5 min. Then, 34 cycles of three-step PCR amplification consisting of denaturation at 94 °C for 1 min, primer annealing for primer group (I, II) at 54 °C for 1 min primer annealing for primer group (III) at 49 °C for 1 min and primer extension at 72 °C for 1 min. The samples were incubated at 72 °C for 10 min at the end of the amplification cycles to complete the extension reaction. The PCR products were visualized by gel electrophoresis on agarose gel (1 %) with 1X TBE buffer (Sigma, St. Louis, MO). The gels were stained in a solution of ethidium bromide and were visualized with a UVP UV trans illuminator (UVP Inc., San Gabriel, CA) [25,31]

Results and Disscussion

Plasmid profile of crude-oil degrading marine bacteria

Twenty one crude-oil degrading marine bacterias were isolated from mussels and mangrove forests in the Persian Gulf in previous studies were selected for plasmid extraction and the examination of the occurrence of plasmid in these strains. Out of 21 strains, plasmid was detected only in 8 strains, beingabsent in other strains. The molecular weight of plasmid in these strains was more than 12 kb (Fig. 1). It can be concluded from these results that 38 percent of isolated crude-oil degrading marine bacteria had plasmid. The presence of plasmids in bacteria has been widely reported. The plasmids that bear genes encoding enzymes capable of hydrocarbon degradation are considered important. These plasmids, known as catabolic plasmids can give the organisms harbouring them,the ability to degrade certain compounds.

Many researchers regarded plasmids as important components for hydrocarbon degradation. For example, John and Okpokwasili[32] studied the plasmid profile of autotrophic nitrifying bacteria, *Nitrosomonas*

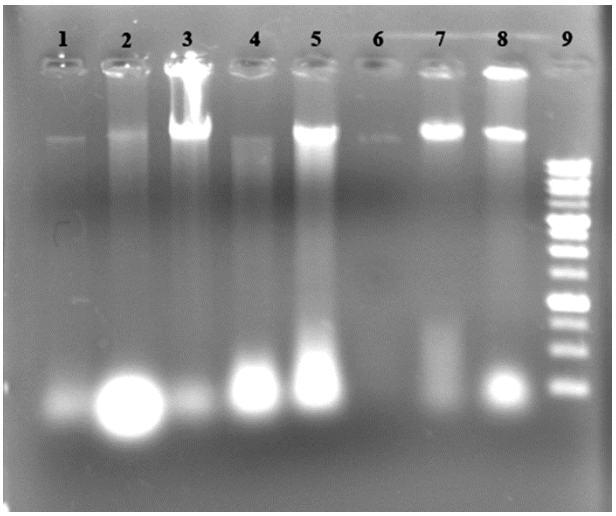

Fig. 1 — Electrophoresis of plasmid extracted by the alkaline lysis method: Lane 1: 19F (*Vibrio*), Lane 2: 16F (*Pesudomonas*), Lane 3: 44F (*Marinobacter*), Lane 4: 8B (*Pesudo alteromonas*), Lane 5: 36B (*Vibrio*), Lane 6: 32B (*Alcanivorax*), Lane 7: 30B (*Alcanivorax*), Lane 8: 25B (*Alcanivorax*), and Lane 9: DNA Ladder 1 kb

Table 2 — Sequences of primers used in this study

Primer	Sequence	Length (bp)	GC content (%)	Annealing temperature
Alk-1F	5'-CATAATAAAGGGCATCA-3'	21	43	57
Alk-1R	5'-GATTTCATTCTCGAAACT-3'	24	38	57
Alk-2F	5'-GAGACAAATCGTCTAAAACGTAA-3'	23	35	49
Alk-2R	5'-TTGTTATTATTCCAACTATGCTC-3'	23	30	49
Alk-3F	5'-TCGAGCACATCCGCGGCCACCA-3'	22	68.2	57
Alk-3R	5'-CCGTAGTGCTCGACGTAGTT-3'	22	55	57

and *Nitrobacter* species, being isolated from mangrove sediment in the Niger Delta of Nigeria. Their results showed that the ability of autotrophs to degrade crude oil was found to be plasmid-mediated through curing experiment and electrophoresis[32].

Isiodu *et al*.[33] isolated 19 hydrocarbon degrading bacteria from oil contaminated marine environment. They studied the plasmid profile of these isolates. Their results showed that 10 strains (47 %) had plasmid[33].

In this research, some crude-oil degrading bacteria isolated in the Persian Gulf were studied for the occurrence of plasmid. The molecular identification of these strains were done and recorded in GeneBank in previous studies. The results of this research are in accordance with the results of previous researchers. The plasmid profile of these marine bacteria confirmed that the frequency of occurrence of plasmid between these strains was 38 %.

Detection of Alkane Hydroxylase Genes (*alkB*) in the Plasmid

The screening of three groups of alkane hydroxylase gene (*alkB*) was performed for plasmid containing bacteria. On the other hand, the presence of *alkB* gene in the plasmid was studied. Figures 2 and 3 illustrate the results of PCR amplification for these genes.These figures confirmed that 4 strains had *alkB* group (I) gene and 6 strains had *alkB* group (III) gene. Alkane hydroxylase group (II) gene was not detected in each plasmid containing strains. Table 3 summarizes the results for the presence of plasmid and detection of *alkB* gene. This table shows that 75 % of bacteria have *alkB* group (III) gene and 50 percent of bacteria have *alkB* group (I) gene. In addition, some strains have two groups of *alkB* genes. It can be concluded that between three *alkB* genes, group (III) has the highest frequency.

The first step in the aerobic degradation of alkanes by bacteria is catalyzed by oxygenases. These enzymes, introduce oxygen atoms derived from molecular oxygen into the alkane substrate, play an important role in oil bioremediation and the cometabolic degradation of compounds. Some researchers detected *alkB* genes in crude-oil degrading bacteria. For instance, Vomberg *et al*.[34] isolated 45 alkane degrading bacteria belonging to 37 bacterial species from soil contaminated with crude-oil in Germany. They investigated the distribution of *alkB* between isolates by PCR-hybridization method. They concluded that in group (III), alkane hydroxylase is predominant between the isolates[34].

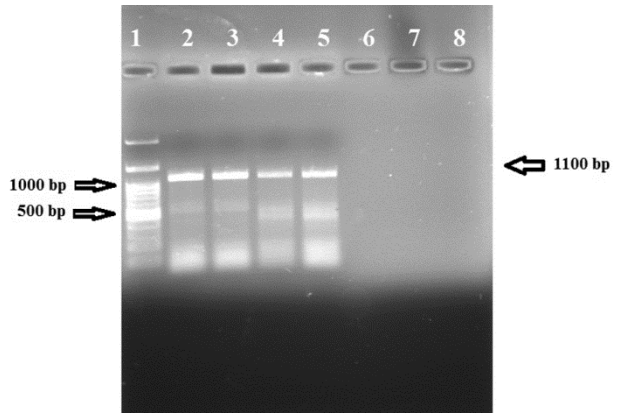

Fig. 2 — Electrophoresis of the PCR products by primer I: Lane 1: DNA Ladder 100 bp, Lane 2: 44F (*Marinobacter*), Lane 3: 8B (*Pesudoalteromonas*), Lane 4: 36B (*Vibrio*), Lane 5: 25B (*Alcanivorax*), Lane 6, 7, 8: Negative control

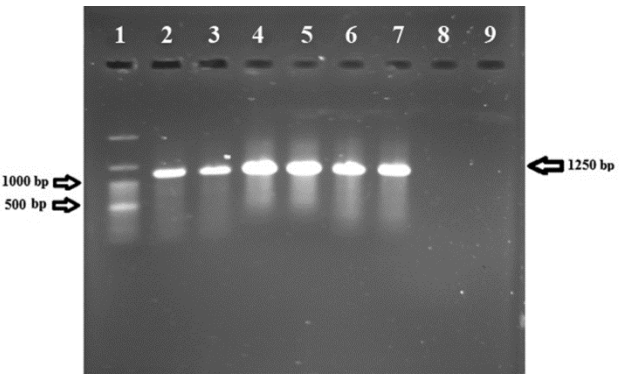

Fig. 3 — Electrophoresis of the PCR products by primer III: Lane 1: DNA Ladder 100 bp, Lane 2: 19F (*Vibrio*), Lane 3: 44F (*Marinobacter*), Lane4: 8B (*Pesudoalteromonas*), Lane 5: 36B (*Vibrio*), Lane 6: 32B (*Alcanivorax*), Lane 7: 25B (*Alcanivorax*), Lane 8, 9: Negative control

Heissblanquet *et al*.[35] demonstrated that there were clear differences in the predominance of the two *alkB* genotypes in freshwater and soil microcosms. However, both types of *alkB* genes increased in the most polluted soils[35]. To date, *alkB* has been found in various environments, such as Alaskan sediments, contaminated soil, cold enviroments, fuel oil-contaminated site, shallow aquifer, bulk soil, land treatment units, Arctic and Antarctic soil, and Sargasso Sea water[36]. The gene *alkB* could possibly be used as a marker to predict the potential of different environments for oil degradation[36].

All studies conducted on the detection of *alkB* genes were manily concerned with chromosome of degrading bacteria and their identification in the selected environment. There were few reports on the detection of *alkB* genes in the plasmids of crude-oil degrading bacteria. The novelty of this research, was

Table 3 — Results of plasmid extraction and detection of alkane hydroxylase genes in crude-oil degrading bacteria

Bacterial Strain	Plasmid	Alk-b gene group (I)	Alk-b gene group (II)	Alk-b gene group (III)
Shewanella algae strain BHA1	Negative	Negative	Negative	Negative
Micrococcus luteus strain BHA7	Negative	Negative	Negative	Negative
Pseudoalteromonas sp. strain BHA 8	Positive	Positive	Negative	Positive
Shewanella algae strain BHA12	Negative	Negative	Negative	Negative
Alcanivorax sp. strain BHA14	Negative	Negative	Negative	Negative
Halomonas sp. strain BHA16	Negative	Negative	Negative	Negative
Vibrio alginolyticus strain BHA17	Negative	Negative	Negative	Negative
Alcanivorax dieselolei strain BHA25	Positive	Positive	Negative	Positive
Idiomarina baltica strain BHA28	Negative	Negative	Negative	Negative
Alcanivorax dieselolei strain BHA30	Positive	Negative	Negative	Negative
Alcanivorax sp. Strain BHA32	Positive	Negative	Negative	Positive
Shewanella haliotis strain BHA35	Negative	Negative	Negative	Negative
Vibrio azureus strain BHA36	Positive	Positive	Negative	Positive
Vibrio alginolyticus strain NW4	Negative	Negative	Negative	Negative
Vibrio azureus strain BP14	Negative	Negative	Negative	Negative
Pseudomonas salomonii strain NP16	Positive	Negative	Negative	Negative
Vibrio azureus strain NP19	Positive	Negative	Negative	Positive
Idiomarina baltica strain BW 32	Negative	Negative	Negative	Negative
Kangiella marina strain DP40	Negative	Negative	Negative	Negative
Marinobacter bryozoorum strain DW44	Positive	Positive	Negative	Positive
Halomonas organivorans strain BS53	Negative	Negative	Negative	Negative

determining three groups of *alkB* gene in the plasmid of crude-oil degrading marine bacteria isolated from the Persin Gulf.Our results were in accordance to prvious studies indicating the prevalence of group (III) alkane hydroxylase gene as compared to the other groups of this gene.

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